CIMT
Cancer Immunotherapy

CIMT 2014 Abstracts

12th Annual Meeting

» Next Waves in Cancer Immunotherapy «

MAY 6 – 8, 2014

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12th Annual Meeting

May 6 - 8, 2014

Rheingoldhalle Congress Center

Mainz, Germany

CIMT

Cancer Immunotherapy

Abstract List (001 - 021)
Therapeutic Vaccination

No.:	*Short talk:*	*Title:*
001	-	Dendritic cell vaccination as adjuvant therapy after radical lymph node dissection prolongs overall survival in melanoma patients with regional lymph node metastasis
002	-	IVAC MUTANOME - individualized vaccines for treatment of malignant melanoma - first feasibility assessment of on demand vaccine manufacturing
003	-	Developing an oncolytic virus “armed” to deliver antibodies to tumours
004	-	Cancer vaccine development for hepatocellular carcinoma - HEPAVAC
005	-	Alphavirus-based vaccines encoding nonstructural proteins of Hepatitis C virus induce robust and protective T cell responses
006	-	Development of a unique anti-AML immune therapy consisting of cord blood hematopoietic cell transplantation and cord blood stem cell-derived dendritic cell (CBDC) vaccination
007	-	Optimization of peptide-based vaccines using advanced technology
008	-	A transcriptomic approach to generate better designer DC for clinical application
009	-	Depleting the suppressors for the benefit of immunotherapy against cervical cancer
010	-	Temozolomide promotes an increase in the proportion of regulatory T cells over effector T cells in patients with glioma
011	-	Is the IMA950 multi-peptide vaccine applicable for grade II and III glioma patients?
012	-	Towards a personalized immunotherapy by vaccination with exosome-loaded dendritic cells
013	-	Integrated computational pipeline for epitope characterization for use in personalized genomic vaccination in solid tumors
014	-	Vaccination of melanoma patient with native peptide promotes the early onset development of a dominant T cell repertoire
015	-	Vaccination against the large T antigen as a model for immunotherapy of virally induced cancer
016	-	ASET study: Predictive parameters and immunological data of patients with locally recurrent or metastatic renal cell carcinoma (RCC) treated with the cell-based therapeutic cancer vaccine MGN1601
017	-	The interaction of Antigen-Antibody complexes with complement functional human whole blood reveals promising features for a novel therapeutic long peptide conjugate vaccine
018	-	Targeting lymph node medullary macrophage with immunologically stealth nanogel vaccine induces enhanced anti-tumor T cell response
019	-	The Mutanome Engineered RNA Immuno-Therapy (MERIT) consortium
020	-	Patients treated with oncolytic adenovirus Ad5/3-hTERT-E2F-GMCSF induce immunological responses against the tumor
021	-	Development and characterization of TNF-alpha armed oncolytic adenovirus for treatment of solid tumors

Abstract List (022 - 041)
Therapeutic Vaccination

No.:	*Short talk:*	*Title:*
022	-	Photochemical Internalisation (PCI) - a new adjuvant technology for stimulating cytotoxic T-cell responses
023	-	MultiPro: Development of multipeptide-based immunotherapy for prostate cancer patients independent of their HLA type
024	-	Oncolytic measles virus for tumor vaccination
025	*Yes*	Vaccination with long NY-ESO79-108 peptide in combination with CpG leads to robust activation of CD4 and CD8 T cell responses in stage III and IV melanoma patients
026	-	Expanded γδT-APC - Vehicle for cancer immunotherapy
027	-	MicOryx - a phase I/IIa clinical trial evaluating vaccination of MSI-H colorectal cancer patients with frameshift peptide antigens
028	-	Sequential intra-nodal immunotherapy with low-dose rituximab and dendritic cells in irradiated lymph nodes induces systemic anti-tumor immunity and regression of disseminated follicular lymphoma
029	-	Mining sequencing data to identify targets for cancer immunotherapy
030	-	Self-adjuvanted RNActive® vaccine induces innate immune response at the site of injection that leads to a potent adaptive immunity in both mice and humans
031	-	CpG conjugation to model tumour antigen ovalbumin leads to enhanced $CD8^+$ T-cell proliferation
032	*Yes*	Characterization of the IFNα response upon systemic administration of targeted mRNA vaccine delivery
033	-	Development of a high throughput protein production system for the efficient generation, screening and production of chimeric virus-like particles as cancer vaccines
034	*Yes*	Targeting of antigen into $XCR1^+$ dendritic cells combined with a non-viral boost regime induces massive $CD8^+$ T cell cytotoxicity capable of eradicating established tumors
035	-	The GAPVAC consortium: Translating the concept of actively personalized cancer immunotherapy into the clinic
036	-	iVacALL: Personalized peptide-vaccination for pediatric acute lymphoblastic leukemia patients based on patient-individual tumor-specific variants determined by whole exome and transcriptome sequencing
037	-	Targeting c-MET and RIG-I with 5‘ppp-modified siRNA for immunotherapy of hepatocellular carcinoma (HCC)
038	-	Cripto-1 encoding DNA vaccine elicits tumor protective immune response in vivo
039	-	Gene optimization can generate cryptic epitopes that induce T cell responses
040	-	Immunotargeting human melanoma (MM) stem cells using combination of therapeutic vaccination and surgery
041	-	AdCD40L immunostimulatory gene therapy in combination with cyclophosphamide prolongs 6-months survival in a phase I/II trial for malignant melanoma

Abstract List (042 - 061)
Therapeutic Vaccination

No.:	*Short talk:*	*Title:*
042	-	Novel promising anti-cancer vaccine candidate based on HBcAg virus-like particles
043	-	Antitumor activity and immunogenicity of recombinant vaccinia virus expressing HPV 16 E7 protein SigE7LAMP is enhanced by high-level coexpression of IGFBP-3
044	-	Randomized phase II study of personalized peptide vaccination in patients with advanced bladder cancer progressing after chemotherapy
045	-	Robust induction of type-1 $CD8^+$ T-cell responses in WHO grade II low-grade glioma patients receiving peptide-based vaccines in combination with poly-ICLC
046	-	A phase I/II trial of TG01 and Gemcitabine as adjuvant therapy for treating patients with resected adenocarcinoma of the pancreas
047	*Yes*	Characterization of tumor-infiltrating lymphocytes following intratumoral administration of a chimeric adenovirus Ad5/3-D24-GMCSF (ONCOS-102) for refractory cancer patients with solid tumors
048	-	Immunostimulatory cancer therapy using dendritic cell-tumor cell hybrids
049	*Yes*	Innovative vaccination of melanoma patients with a plasmacytoid dendritic cell line - a phase I clinical trial
050	-	Combined chemoimmunotherapy of castrate-resistant prostate cancer with dendritic-cell based vaccine DCVAC/Pca
051	*Yes*	Common mutations in oncogenes/tumor suppressor genes as targets for long peptide anti-cancer vaccination
052	-	Development of a next generation dendritic cell-based immunotherapy for patients with castration-resistant prostate cancer
053	*Yes*	Induction of anti-tumor $CD8^+$ T cells and prominent infiltration of lymphocytes with a Th1 polarizing signature to pleural mesothelioma tumor following intratumoral injection of ONCOS-102
054	-	Evaluation of a combinatorial cancer immunotherapy approach
055	-	Phase II clinical trial of personalized peptide vaccination for previously treated advanced colorectal cancer
056	-	The immunogenicity of NY-ESO-1 peptides is primarily determined by the stability of their MHC-peptide complexes
057	*Yes*	Next-generation dendritic cell vaccination as postremission therapy in AML
058	-	Soluble MHC-dimer molecules activate antigen-specific T cells in vivo
059	-	Autologous tumor cells and SW742 allogeneic cell line have comparable stimulating effect on PBMCs of Gastrointestinal malignant patients In vitro
060	-	Idiotype vaccines produced with a non-cytopathic alphavirus vector induce potent antitumoral responses in a murine model of B-cell lymphoma
061	-	Combined immunotherapy against cancer: Limited efficacy of transcutaneous immunization and low-dose Cyclophosphamide

Abstract List (062 - 082)
Therapeutic Vaccination

No.:	*Short talk:*	*Title:*
062	-	Personalized pancreatic cancer vaccine
063	-	Closing the gap between bench and bedside: Manufacturing individual multi-peptide vaccines for phase I/II clinical trials in cancer immunotherapy
064	-	Integrating clinical and genomics data - challenges and opportunities
065	-	Towards an actively personalized cancer therapy: Patient specific multipeptide vaccination for primary liver cancers
066	-	A TLR agonist-based combination treatment enhances T cell recruitment into gastric tumors
067	-	Cancer vaccines with hTERT and Survivin mRNA transfected fast DCs - a simplified and effective cancer vaccine
068	-	Exploring dendritic cell (DC) vaccines targeting Wilms‘ tumour gene 1 (WT1) and survivin for uterine cancer
069	-	Protein transfer vectors: an efficient tool to induce antigen-specific cytotoxic T cell responses
070	-	Role of NK cells in antitumor immunization strategies
071	-	A mutated melanoma epitope identified by mutanome screening confers CD4 driven antitumoral immunity
072	-	Alphavirus-based immunization strategies targeting cervical cancer
073	-	Vaccine-induced cytokine-producing T cells synergize with cisplatin to cause massive infiltration of tumoricidal leukocytes
074	-	Re-vaccination after first vaccination for patients with high grade glioma: does it make sense?
075	-	Repeated intratumoral administration of ONCOS-102 leads to robust cellular and transcriptional immune activation at tumor site in an ovarian cancer patient
076	-	VicOryx - A therapeutic phase I/IIa vaccination trial with a $p16^{INK4a}$ peptide plus Montanide® ISA-51 VG in patients with human papillomavirus-associated cancer
077	-	Tumor-specific mutations as targets for individualized mRNA therapy in the murine 4T1 breast cancer model
078	-	A novel cell penetrating peptide-based vaccine for efficacious therapy of solid tumors
079	*Yes*	Synergistic effects of properly timed HPV16 synthetic long peptide vaccination during standard carboplatin-paclitaxel chemotherapy in animals and patients with metastatic cervical carcinoma
080	-	Vigorous T cell responses to neoantigen frameshift-derived peptides in Lynch syndrome patients treated with monocyte-derived dendritic cells
081	-	The src kinase Lyn impacts TLR4-triggering in human myeloid cells
082	*Yes*	Randomized phase II study of personalized peptide vaccination with cyclophosphamide pretreatment in refractory advanced biliary tractcancer patients

Abstract List (083 - 104)
New Targets & New Leads

No.:	*Short talk:*	*Title:*
083	-	Identification of unique HLA peptides presented on Glioblastoma cells and recovered from the plasma soluble HLA
084	-	Induction of tumor-specific cytotoxic $CD4^+$ T cells by 4-1BB agonist therapy
085	-	Development of a T cell activating bispecific antibody targeting carcinoma cells expressing the tight junction molecule CLDN18.2
086	-	Expression and turnover of proteins govern their sampling for HLA class I presentation
087	-	Treatment of melanoma with a serotype 5/3 chimeric oncolytic adenovirus coding for GM-CSF: results in vitro, in rodents and in humans
088	*Yes*	TRON Expression Atlas (TEA): A web - based platform for archival, retrieval, filtering and visualization of high throughput gene expression profiles
089	-	A novel synthetic Toll-like-receptor 7 agonist for tumor therapy
090	-	Rapid mimotope optimization for pharmacokinetic analysis of therapeutic antibody IMAB362
091	-	Fast and accurate identification of disease related variants using amplicon based targeted re-sequencing.
092	-	Effect and pharmacokinetics of ADC-1013, an agonistic CD40 antibody optimized for local immunotherapy of cancer, in hCD40tg mice
093	*Yes*	CetuGEX™, a novel anti-EGFR monoclonal antibody (mAb) with optimized glycosylation and antibody dependent cellular cytotoxicity - First in human experience
094	-	PankoMab-GEX™, a novel anti-TA-MUC1 monoclonal antibody (mAb) with optimized glycosylation - First in human experience
095	-	T-cell responses to oncogenic Merkel cell polyomavirus proteins distinguish patients with Merkel cell carcinoma from healthy donors
096	-	Treg-associated suppressor molecules are highly expressed on melanoma: New ways for immunotherapy
097	-	Treating tumors by targeting overexpressed Wilms´ Tumor protein 1
098	-	IMAB362, a novel first-in-class monoclonal antibody for treatment of pancreatic cancer
099	-	Understanding T cell responses against leukaemic fusion proteins
100	-	The HLA peptidome of small cell lung cancer cells
101	-	Identification of a new biomarker for ovarian cancer?
102	*Yes*	A new PD1-CD28 chimeric receptor overcomes PD-1-mediated immunosuppression in adoptive T cell therapy
103	-	HLA ligandome profiling identifies a novel category of frequently recognized CLL associated antigens
104	-	Refined targets for the peptide based immunotherapy of RCC

Abstract List (105 - 125)
New Targets & New Leads

No.:	*Short talk:*	*Title:*
105	-	Targeting leukemic stem cells in AML using the Bispecific CD33/CD3 BiTE® antibody AMG 330
106	-	Enabling variant detection in single cell transcriptomics using half cell sequencing
107	*Yes*	LTX-315 treatment induces complete and specific regression of disseminated tumors in a novel mesenchymal three tumor rat model
108	-	Identification of novel immune checkpoints as targets for cancer immunotherapy
109	-	Ratio of intratumoral macrophage phenotypes predicts local tumor outgrowth in malignant pleural mesothelioma patients
110	-	Novel oncolytic alphavirus vectors with increased tumor specificity
111	-	Novel T helper epitope peptides derived from Lck can induce cytotoxic T lymphocytes in HLA-A2^{+} cancer patients
112	-	High-throughput identification of immune-checkpoint molecules expressed by melanoma using patient-derived tumor-infiltrating lymphocytes
113	-	Preclinical rationale for the combination of IMAB362 with standard chemotherapy regimens for the treatment of gastro-esophageal cancer and pancreatic cancer
114	-	Diacylglyerol kinase: a reversible checkpoint blockade in CD8 T and NK cells from human carcinoma acting downstream of currently targeted checkpoint molecules
115	-	T cells from non-tolerized repertoires provide tools to discover the immunopeptidome for use in immunotherapy and vaccination
116	-	CXorf61 is a therapeutic vaccine target in triple negative breast cancer
117	-	HLA ligandome analysis identifies Histone Deacetylases as novel targets for T-cell mediated immunotherapy of ovarian carcinoma
118	-	A new method of testing T-cell reactivity to tumor antigens without prior knowledge of the T-cell epitopes
119	-	Selective stimulation of RIG-I with a novel synthetic 5`triphosphorylated RNA induces superior anti-tumor immunity in primary and transplanted mouse melanomas
120	-	Characterization of a genetic mouse model of lung cancer: A promise to identify non small cell lung cancer therapeutic targets and biomarkers
121	-	Identification of mutated neoantigens in the human melanoma model Ma-Mel-86 combining exome and transcriptome sequencing
122	-	Dual murine CT26 subclone model of heterogeneous cancer reveals dominance of interferon-sensitive clones in oncolytic alphavirus therapy
123	-	pMHCI-IgG redirect HCMV-specific CD8^{+} T-cells to eradicate cancer cells
124	-	Ex vivo therapy simulation of multivalent adenoviral immunotherapy in the human urinary bladder carcinoma microenvironment
125	*Yes*	A vaccine targeting mutant isocitrate dehydrogenase 1 induces anti-tumor immunity

Abstract List (126 - 135)
New Targets & New Leads

No.:	*Short talk:*	*Title:*
126	-	Developing a warehouse for off-the-shelf and personalized ovarian cancer peptide vaccination immunotherapy
127	-	Selective activation of ubiquitously expressed immune receptor RIG-I as novel target in solid tumors
128	-	From bedside to bench: Molecular benchmarking of an FcOptimized CD19 antibody used in treatment of relapsed and refractory pediatric B-lineage acute lymphoblastic leukemia
129	-	Identification of novel, naturally presented, immunogenic peptides of human cytomegalovirus
130	*Yes*	Efficient elimination of carcinoma cells expressing the tumor exclusive target CLDN6 with a bispecific antibody
131	-	Identification of novel tumor associated antigens in colorectal cancer
132	-	NLRP3 inflammasome-dependent contact hypersensitivity against pigmented cells by monobenzone-induced memory NK cells
133	-	Wet peptide HLA binding assay has added value in the selection of candidate $CD8^+$ T cell epitopes
134	-	The antigenic identity of human class I MHC phosphopeptides is critically dependent upon phosphorylation status
135	-	CD44v10, osteopontin and leukemia growth retardation by a CD44v10-specific antibody

Abstract List (136 - 156)
Tumor Biology & Interaction with the Immune System

No.:	*Short talk:*	*Title:*
136	-	New approaches of cancer vaccination and cancer immunotherapy based on the optimal stimulation of tumor-specific, MHC class II-restricted $CD4^+$ T helper cells
137	-	Treatment of pancreatic ductal adenocarcinoma with bifunctional siRNA targeting the immunosuppressive molecule galectin-1 and the cytosolic helicase RIG-I
138	*Yes*	Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL
139	-	Oxidative burst produced by the NOX2 complex does not play a role in methylcholanthrene induced sarcoma development
140	-	Establishment of a NY-BR-1 expressing breast cancer tumor model in HLA-transgenic mice
141	-	RIG-I stimulation induces the release of tumor-suppressing exosomes from melanoma cells
142	*Yes*	Activation of RIG-I-like helicases induce immunogenic cell death of pancreatic cancer cells leading to protective and therapeutic immunity in vivo
143	-	The abstract is withdrawn
144	-	Change in infiltration of immune cells during rejection after radioimmunotherapy in a syngeneic rat colon carcinoma
145	-	Study on the relation between heme oxygenase-1 and metallothionein expression and the tumour-associated local immune response in human cutaneous malignant melanoma
146	-	Attenuation of RIG-I-induced immune activation by hypoxia in murine melanoma cells
147	-	BRaf and MEK inhibitors differentially regulate cell fate and microenvironment in human hepatocellular carcinoma
148	-	Increased myeloid derived suppressor cells in glioblastoma patients
149	-	Modulation of the tumour microenvironment by the local delivery of an IL-12 expressing lentivirus to the lung promotes rejection of established tumours
150	-	A mouse tumor transplantation model mimicking the immune response to sporadic cancer
151	-	Local and systemic immune response in pancreatic cancer: multi-scale approach to tumor-immune interactions
152	*Yes*	MDA5-based immunotherapy can reprogram tumor myeloid-derived suppressor cells (MDSC) from a M2/G2 phenotype to a M1/G1 phenotype and reduce suppressive function
153	-	Enhanced immunogenic cell death and immune activation by synergism between therapeutic irradiation and RIG-I activation in murine melanoma cells
154	-	Tumor microenvironmental studies indicate controlled trafficking and preferential homing of immune cells towards the tumor
155	*Yes*	Myeloid-derived suppressor cells inhibit NK cell activity through prostaglandin-E2-regulated TGF-beta production
156	-	A new anti-ICOS monoclonal antibody prevents graft-vs-host disease but has no impact on a breast tumor growth in humanized mice

Abstract List (157 - 175)
Tumor Biology & Interaction with the Immune System

No.:	*Short talk:*	*Title:*
157	-	Immunogenic cell death specific danger associated molecular patterns induced by modulated electrohyperthermia in colorectal adenocarcinoma model
158	-	GMCSF armed oncolytic vaccinia virus induces anti-tumor immune response in immunocompetent Syrian hamster model
159	-	Characterizing tumor-specific memory stem like T cells in blood and bone marrow of breast cancer patients
160	-	Efficient lysis of malignant B lymphoid cells mediated by the T cell-recruiting triplebodies [19-3-19] and [33-3-19]
161	-	Ipilimumab and hypofractionated brain radiotherapy for brain metastases of malignant melanoma
162	-	Unrestricted T cell functionality in newly diagnosed and relapsed AML patients
163	-	Systematic analysis of changes in the antigen processing machinery in HPV transformed cells
164	-	Dysregulated glucose metabolism inversely correlates with $CD8^+$ T-cell infiltration in head and neck squamous cell carcinoma
165	-	Hormone induced tolerance of tumor associated dendritic cells
166	-	Standardized immunostaining and evaluation of HLA class I expression in tumor tissues -An impact on peptide-based cancer immunotherapy-
167	-	Role of immune effector cells in metastatic sites during the progression of breast cancer
168	-	Phenotypic characterization of the immune infiltrate in gynaecological tumours
169	-	Intraepithelial macrophage infiltration and high number of regulatory T cells promote whereas activated CD8 T cells prevent a progressive course of HPV-induced vulvar disease
170	-	Preclinical study on a new developed TLR7 agonist shows potent anti-tumor therapeutic effects
171	-	The relevance of differential expression of tolerogenic enzymes IDO-1, IDO-2 and TDO in commonly used mouse tumor models for testing novel therapeutics
172	-	HLA quantification in multiple myeloma reveals high and robust HLA class I and II expression on primary myeloma cells
173	-	Investigation of the spatial heterogeneity of specific immune cell phenotypes in the tumor microenvironment of follicular lymphoma
174	-	Interleukin-6 receptor and its ligand interleukin-6 are opposite markers for survival and infiltration with mature myeloid cells in ovarian cancer
175	*Yes*	Comparison of tumor infiltrating lymphocyte signatures across six cancer types and their association with disease prognostic factors

Abstract List (176 - 195)
Immunomonitoring

No.:	*Short talk:*	*Title:*
176	*Yes*	Survival of melanoma patients correlating with DTH skin reactions and CD8 T cell/Treg ratios in PBMC after vaccinations with CpG & peptides
177	-	TCR-Engineered Reference Samples (TERS) to control T-cell assay performance - towards an immuno-control kit
178	-	HLA typing from RNA-Seq sequence reads: a catalog of HLA type, HLA expression, and HLA presented somatic mutations in human cancer cell lines
179	-	Assessment of vaccine-induced immunity following intra lymph node administration of RIBOLOGICAL IMMUNOTHERAPY targeting NY-ESO-1 and tyrosinase in patients with advanced melanoma
180	-	Stability of peptides in long term -80°C storage
181	-	HLA-A2 multimer staining validation
182	-	Managing multi-center flow cytometry data for cancer immune monitoring
183	-	User vs. software-dependent variability of ELISPOT counts obtained from ten different laboratories
184	-	Skewed distribution of IL-7 receptor-α-expressing effector memory CD8(+) T Cells with distinct functional characteristics in oral squamous cell carcinoma
185	-	Validation of immunomonitoring of circulating myeloid-derived suppressor cells as a potential predictive tool for response to Ipilimumab treatment in melanoma patients
186	-	A xenograft model for studies of anti-leukemic effects of histamine dihydrochloride in acute myeloid leukemia
187	*Yes*	Resetting circulating CD8 T-cells to a tissue-like status permits sensitive detection of their responses to virus- and tumor-associated antigens
188	-	A bioinformatics platform for prediction and reporting of drug sensitivity from sample specific genomic data
189	-	MHC Multimer Proficiency Panel 2014: Consistent data obtained by the large majority of participants
190	*Yes*	Reversible NTAmer technology enables rapid and direct detection of monomeric TCR-pMHC kinetics at the cell surface and predicts tumor-specific T cell responsiveness
191	-	Expression of carbohydrate deficient bone sialoprotein compared to various tumor markers in metastatic cells and non-metastatic cells in vitro
192	-	Accurate detection of NKT cells using CD1d Dextramers
193	*Yes*	Presence of circulating Her-2-reactive CD8 T-cells is associated with lower frequency of MDSCs and better survival in elderly breast cancer patients.
194	-	Phenotyping of peripheral blood mononuclear cells of patients with advanced heavily pre-treated adenocarcinoma of the stomach and gastro-esophageal junction
195	*Yes*	High numbers of differentiated effector CD4 T cells are found in cancer patients and correlate with clinical response after neo-adjuvant therapy of breast cancer

Abstract List (196 - 212)
Immunomonitoring

No.:	*Short talk:*	*Title:*
196	-	Results of a multi-peptide vaccination trial for high risk renal cell carcinoma
197	-	CD14+CD11b+HLA-DR-/low MDSCs indirectly impact on the prognosis of late-stage melanoma patients by impairing the frequency and function of NY-ESO-1- and Melan-A-reactive peripheral T cells
198	-	The effect of glucocorticoids on the chromatin landscape
199	-	Immunomodulation induced by Stereotactic Ablative Radiotherapy (SABR) in oligometastatic breast cancer patients as a source of predictive biomarkers
200	-	ZO-1 as a potential prognostic marker in B cell lymphoma
201	-	Belatacept can inhibit allo-specific T cell responses in a donor-dependent manner but preserves virus-specific memory T cell-responses
202	-	The rapid antigen-reactive T Cell enrichment procedure enables direct ex vivo BKV-specific $CD4^+$ T cell characterization
203	-	Immune monitoring of absolute cell numbers and functional capacity of circulating γδ T cells and other immune cells from cancer patients
204	-	Egress of regulatory T cells from bone marrow of breast cancer patients
205	-	Dipeptides mediate peptide exchange on MHC class I molecules
206	-	Detection and functional assessment of regulatory T cells in blood of cancer patients
207	-	Immune status of patients with localized prostate carcinoma during primary radiotherapy
208	-	Early granulocyte effector function is not impaired by cyclosporin A
209	-	Identification of biomarkers for the response of patients with advanced gastroesophageal cancer to IMAB362 treatment
210	-	Thymic NK cells display a specific phenotype and functional profile
211	-	HTCC allows the characterization of differential $CD8^+$ T cell responses against 20+ antigens in a single patient sample
212	-	Investigation of humoral immune response towards persisting Epstein-Barr virus infections in multiple sclerosis and chronic fatigue syndrome using peptide microarrays

Abstract list (213 - 233)
Cellular Therapy

No.:	*Short talk:*	*Title:*
213	-	Redirection of T cells towards acute myeloid leukemia blasts via bispecific antibody-releasing human mesenchymal stem cells
214	-	Targeting melanoma and multiple myeloma using a high-affinity TCR specific for human MDM2 tumor-associated antigen
215	-	Optimisation of the isolation and expansion of tumour infiltrating lymphocytes from renal cell carcinoma
216	-	T-cell receptor transfer into human T-cells with ecotropic retroviral vectors
217	*Yes*	First clinical experience with a new generation of fast DCs transfected with mRNA from hTERT, survivin and autologous tumour
218	*Yes*	A photosensitizer delivered by bispecific antibody retargeted human T lymphocytes boosts cytotoxicity against carcinoma cells upon light irradiation
219	-	IL-12 enhances T-cell receptor-dependent and -independent tumor cell recognition by human effector T-cells
220	-	In vitro model for immunotherapy - Generation of a long-term growth factor-dependent DC culture
221	-	In depth analysis of in vitro isolated T cell receptors with specificity for HER2/neu-derived peptides
222	-	G-CSF directly affects $CD8^+$ cytotoxic antigen-specific T cells and impairs their functionality
223	*Yes*	One U-CAR - multiple targets: a new approach to provide multiple antigen specificities to genetically engineered T cells for cancer treatment
224	-	Generation of patient-individualized AML-reactive donor $CD8^+$ T cells under GMP-compliant conditions for adoptive transfer in leukemia patients after allogeneic hematopoietic stem cell transplantation
225	-	Analysis and applicability of different in vitro models of Glioblastoma multiforme
226	-	Characterization of a new T-cell subset specific for tumors with antigen processing defects
227	-	T-cell donor registry for third party adoptive immunotherapy in hematopoietic cell and solid organ transplantation
228	-	Allogeneic lymphocyte-licensed DCs expand TCR/CAR-engineered T cells, which are insensitive to oxidative stress and immunosuppressive factors
229	-	Generation of T-cell lines with broadened antigenic specificities to improve adoptive immunotherapy protocols for the treatment of nasopharyngeal carcinoma
230	-	GMP compliant expansion of CMV- and EBV-specific, donor derived, peptide-stimulated T-cells from G-CSF mobilized stem cell grafts
231	-	Genetically modified natural killer cells targeting EGFR-expressing malignancies
232	-	The mode of activation impacts on the differentiation status of gene modified T cells used in adoptive immunotherapy
233	*Yes*	New genetic adjuvants for improving T cell function in adoptive cell therapy of cancer

Abstract list (234 - 253)
Cellular Therapy

No.:	*Short talk:*	*Title:*
234	-	A novel form of dendritic cell (DC) for immunotherapy: CD137 ligand-generated DCs are more potent than conventional DCs in inducing cytotoxic T-cell responses
235	-	NY-ESO-1 108-116 and NY-ESO-1 120-128 are not epitopes for TCR gene therapy
236	-	TCRs from $CD4^+$ T cells are expressed and functional in both $CD4^+$ and $CD8^+$ T cells upon T-cell redirection
237	*Yes*	High-affinity CD20-specific TCRs suitable for adoptive immunotherapy can be readily isolated from the allo-repertoire using reverse immunology
238	-	$CD40L^+$ $CD8^+$ T cell-dependent antitumor immunity
239	*Yes*	Neo-antigen enriched TIL therapy mediates superior tumor eradication in a patient-derived xenograft model of human melanoma
240	-	Generation of T cell receptor transgenic antigen-specific HLA-A*0201-restricted cytotoxic T cells directed against Ewing Sarcoma associated target antigens
241	-	Isolation of a TCR with specificity for a naturally presented ITGA2B epitope for the treatment of therapy refractory myeloid leukemias by TCR-transgenic T cells
242	-	Leukoreduction system chambers as a novel and highly economic source of viable and functional monocyte-derived dendritic cells and lymphocytes
243	-	A new bispecific T cell recruiting antibody enhances anti-tumor activity of adoptive T cell transfer
244	-	Combination of adoptive T cell therapy with an EGFR x EpCAM-specific antibody for the treatment of a murine melanoma model
245	-	Pre-clinical models for the development of HER2-directed T-cell therapy
246	*Yes*	The influence of binding affinity and receptor density of chimeric antigen receptors on target-cell recognition after transfer by RNA electroporation
247	-	Towards clinical γδTCR gene therapy: a broadly applicable T cell product for cancer patients
248	-	Placenta-derived stem cells inhibit tumor progression on rat dimethylhydrazine-induced colon cancer model
249	-	T helper TCRs isolated from long term survivors after cancer vaccination for use in adoptive cell therapy
250	-	In vivo expansion of Epstein-Barr virus epitope-specific T lymphocytes following donor lymphocyte infusion for anti-CD20-refractory EBV-driven post-transplant lymphoproliferative disease
251	-	Human CD19 as a T-cell target antigen in the non tolerant host
252	-	Targeting Claudin-6 with CAR-engineered T cells for individualized immunotherapy of ovarian cancer
253	-	Invariant NKT cells generated by the TIL 1383I T cell receptor gene transfer with retroviral vectors allows efficient redirection of human antigen specificity

Abstract list (234 - 273)
Cellular Therapy

No.:	*Short talk:*	*Title:*
254	-	Establishment of GMP compliant process for the expansion of therapeutic doses of genetically modified NK-92 cells
255	-	Natural killer cells modified to express a targeted granzyme B fusion protein for enhanced antitumoral activity
256	-	Selective expansion of educated NK cells for cancer therapy
257	-	Genetically modified cytokine-induced killer (CIK) cells for targeted cancer therapy
258	-	T cells engineered to express two allo-restricted T cell receptors recognizing CD20/HLA-A*02:01 specifically and efficiently kill malignant B-lymphoid cells - a preclinical study
259	-	Dual specific cytotoxic potential and memory phenotype of IL-15-activated cytokine-induced killer cells targeting virus infection and leukemia in pediatrics
260	-	Human HLA-DR4-restricted high affinity T cell receptors against tumour-associated antigens
261	-	Characterization of freshly isolated and expanded tumor-infiltrating lymphocytes from pancreatic cancer patients
262	-	Targeting telomerase in B-cell chronic lymphocytic leukemia
263	-	HAdV-specific γ/δ and $CD8^+$ T cells generated by TCR transfection to treat adenovirus infection after allogeneic stem cell transplantation
264	-	Generation of transgenic antigen-specific, allogeneic HLA-A*0201-restricted cytotoxic T cells directed against Ewing Sarcoma specific target antigen STEAP1
265	-	Functional WT1-reactive T cells are present in the natural naïve and memory repertoire of healthy donors
266	-	Induction of $CD4^+$ stem cell-like memory T cells by inhibition of mTOR complex 1 and concomitant activity of mTOR complex 2
267	-	Natural human $BDCA1^+$ myeloid dendritic cells induce immunological and clinical anti-tumor responses in metastatic melanoma patients
268	-	Artificial antigen presenting cells, aAPC, a new approach for generating prostate cancer specific T cells
269	-	$CD47^+$ artificial APC demonstrate enhanced immune cell interaction
270	-	Recognition of fully processed Hepatitis C virus antigen and naturally occurring mutant HCV epitopes by HCV TCR gene modified T cells in the context of hepatocellular carcinoma
271	-	How is melanoma escaping us? An assessment of melanoma associated antigens before and after immunotherapy
272	-	The abstract is withdrawn
273	-	Lentivirus-induced dendritic cells accelerate de novo adaptive immune reconstitution against HCMV/pp65 in humanized mouse models of stem cell transplantation and are making progress in clinical development

Abstract list (274 - 285)
Cellular Therapy

No.:	*Short talk:*	*Title:*
274	-	Alteration of tumor microenvironment using immunomodulatory cytokines for combination treatment with adoptive T-cell therapy
275	-	Plasmacytoid dendritic cells - new killers for melanoma treatment
276	-	Rapid generation of clinical-grade antiviral T-cells: T-cell donor selection and manufacturing of T cells
277	-	Optimizing the efficiency of TCR transfer against malignant melanoma
278	-	Inhibition of CSF-1R supports T-cell mediated melanoma therapy
279	-	In vitro generation of mature, naive tumor antigen-specific $CD8^+$ T cells with a single T cell receptor from mobilized peripheral blood precursor cells
280	-	Generation of tumor specific T cells using DC-based vaccine for adoptive T cell therapy
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282	-	Membrane-attached cytokines expressed by mRNA electroporation: potent T cell adjuvants for adoptive cell therapy
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286	-	Molecular dynamics studies of the role of protein flexibility in immunological molecular recognition
287	*Yes*	Immune-cell poor Hgf-Cdk4 mouse melanomas benefit from antibody mediated PD-1 blockade after targeted activation of the type I IFN system
288	-	Transfer of a protective anti-tumor immunity from immunized mothers to the offspring: First evidence
289	-	Macrophage-dependent tumour immune rejection induced by local low dose irradiation
290	-	Response of monocytes and macrophages to temozolomide in mice
291	-	Modulation of heme oxygenase (HO)-1 enzyme activity by metalloporphyrins affects the antiviral T-cell response
292	-	Effect of local tumor irradiation on migration of antigen-specific CTLs and tumor growth
293	-	Reovirus activation of NK cells increases the efficacy of rituximab for the treatment of CLL
294	-	Impact of CTLA-4 antibodies Tremelimumab and Ipilimumab on the human immune system. Results of an ex vivo human melanoma model with the oncolytic parvovirus H-1 or cytotoxic drugs
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297	-	Engineering T-cell receptors to optimize anti-tumor immunity
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299	*Yes*	ImmTACs: Bi-specific TCR-anti-CD3 fusions for potent re-directed killing of cancer cells
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304	-	AFM13 - a bispecific anti CD30/CD16A TandAb® for the treatment of $CD30^+$ malignancies: Preclinical and first clinical results in patients with relapsed/refractory (R/R) Hodgkin Lymphoma (HL)
305	*Yes*	Microtubule-depolymerizing agents used in antibody-drug-conjugates induce anti-tumor immunity by stimulation of dendritic cells
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310	-	DNA vaccination in combination with an RNAi-based genetic adjuvant silencing STAT3 promotes migration of mature dermal dendritic cells and antitumor T cell responses against B16 melanoma
311	-	Protein Kinase B (PKB/Akt) limits the expression of Cbl-b via Glycogen Synthase Kinase-3 (GSK-3) and converts T cell tolerance to activation
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313	-	Allo-reactive $CD8^+$ T cell clones specific for the self-protein CD20 show superior avidity compared to autologous T cell clones of the same specificity
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315	-	Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8 T cells
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317	-	Stimulation of cytotoxic activity by killer dendritic cells and natural killer cells against HPV-positive tumor cells by a HPV vaccine
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319	-	Bacillus Calmette-Guérin (BCG) as an adjuvant immunotherapy for HPV-associated tumors
320	*Yes*	The human cationic amino acid transporter 1 (hCAT-1) is crucial for efficient T cell function
321	-	Engineering dendritic cells to secrete interferonα by mRNA transfection enhances their ability to promote tumor antigen-specific Tcell and antitumor NKcell effector functions

CIMT
Cancer Immunotherapy

Therapeutic Vaccination

Dendritic cell vaccination as adjuvant therapy after radical lymph node dissection prolongs overall survival in melanoma patients with regional lymph node metastasis

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Purpose: Currently, there is no effective adjuvant treatment in early stage melanoma at high-risk for recurrence and metastatic disease. We investigated the immunologic response and clinical outcome to adjuvant dendritic cell (DC) vaccination in melanoma patients with regional metastatic disease who underwent radical lymph node dissection (RLND).
Experimental design: In this retrospective study, 78 melanoma patients with regional lymph node metastasis who underwent RLND and received autologous DCs loaded with gp100 and tyrosinase were analyzed for tumor antigen-specific T cell responses in skin-test infiltrating lymphocyte cultures. Clinical outcome was compared with matched historical controls.
Results: Adjuvant DC vaccination in melanoma patients with regional lymph node metastases induced tumor-specific $CD8^+$ T cell responses in 72% of the patients. Overall survival was significantly improved compared to matched historical controls, 79 months versus 29 months. Five-year survival rate improved from 37% to 51%. The presence of tumor-specific $CD8^+$ T cells was highly correlated with a better overall survival.
Conclusion: Adjuvant DC vaccination in melanoma patients with regional lymph node metastases, who are at high risk for recurrence and metastatic disease after RLND, is extremely well tolerated without notable side effects and induced tumor-specific immune responses in the vast majority of patients, which were highly related to clinical outcome. Overall survival was improved significantly compared to matched historical controls.

IVAC MUTANOME - individualized vaccines for treatment of malignant melanoma - first feasibility assessment of on demand vaccine manufacturing

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Cancer is a primary cause of mortality in Europe. It arises from the accumulation of genomic alterations and epigenetic changes that constitute a hallmark of cancer. Owing to the molecular heterogeneity in cancer, only a minor fraction of patients profit from approved therapies. Available targeted therapies can only address alterations common to a particular type of cancer and induce transient effects due to the generation of resistant sub-clones. In contrast, the IVAC MUTANOME project aims to immunologically target multiple cancer mutations uniquely expressed in a given patient‘s tumor. The clinical translation of this approach is now being realized by collaboration of the Translational Research Center TRON and BioNTech RNA Pharmaceuticals.

A phase I study was approved and initiated in 2013 (NCT02035956). The objectives of the clinical trial are to study the feasibility, safety, tolerability and immunogenicity of the IVAC MUTANOME approach for malignant melanoma. Feasibility will be shown by the proven ability to provide the fully personalized IVAC MUANOME vaccine to patients within a time period of 155 days. Recruitment of a patient in the trial repetitively triggers the IVAC MUTANOME process covering (i) the receipt of tumor and blood sample specimens, (ii) the identification, prioritization and confirmation of mutations, (iii) testing of pre-existing immunity against private tumor mutations, (iv) the final selection of mutated sequences, (iv) design, production of a DNA lead structure, (v) GMP manufacturing and release of the patient-specific mRNA, (vi) shipment to the clinical trial site, and (vii) the administration of the IMP to patients.

The timelines for the IVAC mutanome process for the first 4 patients recruited to this first in man trial will be presented.

Developing an oncolytic virus "armed" to deliver antibodies to tumours

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Enadenotucirev (ColoAd1) is a chimeric Ad11p/Ad3 adenovirus, developed by bio-selection from a library of chimeric adenoviruses for the ability to replicate and exit rapidly from tumour cells. The virus is active against a broad range of cancer cell lines demonstrating a shorter time-to-lysis than either wild type Ad11p, Ad3 or Ad5. In normal cells, ColoAd1 is attenuated and shows little or no activity by either cytotoxicity or by quantitative PCR. *In vivo*, ColoAd1 shows efficacy in a range of subcutaneous and orthotopic metastatic tumour models following intra-tumoral, intravenous and intra-peritoneal injection (Kuhn et al, PLoS One, 3:e2409, 2008), and is currently being evaluated clinically for treatment of several different epithelial cancer types.

To develop ColoAd1 virus variants which are ‚armed' for delivery of therapeutic genes that should enhance ColoAd1 anti-tumour activity, we have developed a novel, efficient cloning system that enables rapid generation of modified vectors. To exemplify the utility of this approach, we chose to first encode anti-VEGF antibodies since targeting VEGF is clinically efficacious against several different types of cancer that are permissive to ColoAd1. We have successfully produced ColoAd1 variants encoding full-length (NG-135) and scFv (NG-76) forms of anti-human VEGF antibodies. These two variants exhibit similar virus activity profiles to ColoAd1 following infection of a variety of different colon cancer cell lines *in vitro*, in terms of virus replication, virus gene expression and oncolytic action, but in addition express and release the respective anti-VEGF antibody forms into the culture supernatant. *In vivo* tumour xenograft studies, using either HCT-116 or DLD human colon carcinoma cell lines implanted in CD1 nu/nu mice, have shown that following intra-tumoral injection the virus infection profile is similar to ColoAd1 (virus replication and Hexon gene expression). Anti-VEGF antibody expression by these tumours can be detected in the tissue as both mRNA and antibody protein which is able to bind its ligand VEGF in ELISA. Antibodies can be detected early (within 3 days of infection) and expression is sustained over several weeks. Furthermore, anti-VEGF antibody production levels are sufficient to allow detection in the blood. Studies to evaluate the impact of these anti-VEGF armed oncolytic viruses on tumour properties such as growth and their microenvironment are in progress.

Cancer vaccine development for hepatocellular carcinoma - HEPAVAC

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Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and represents the third and the fifth leading cause of cancer related death worldwide in men and women, respectively. Poor prognosis is due to the low efficacy of currently available treatments. In order to address such high a unmet medical need, the HEPAVAC Consortium aims to develop a highly innovative, novel cancer vaccine approach for HCC. The international project consortium consists of 9 European Partners from academia and the biotech industry with complementary and substantial expertise in developing immunotherapeutic strategies to treat cancer. The project is supported by the European Commission's 7th Framework Program (www.hepavac.eu).The main objective of HepaVac is to develop a novel cancer vaccine approach for HCC based on epitopes naturally processed and presented by HLA class I and II on primary liver cancer tissue. Combined in a multi-target, multi-peptide vaccination strategy, the aim is to elicit effective $CD4^+$ T helper and $CD8^+$ CTL responses able to target and kill hepatocellular carcinoma cells. The HEPAVAC strategy combines an off-the-shelf multi-peptide vaccine cocktail, suitable to treat HLA-A*02 and/or A*24 positive HCC patients, with an actively personalized approach (APVAC), targeting the individual immunopeptidome (HLA-ligandome) and mutanome of a specific patient and tumor. The latter step aims to take personalized medicine to a new level. Vaccines will be adjuvanted by the immune-stimulator RNAdjuvant® targeting toll-like receptor (TLR) 7. A randomized multi-center phase I/II clinical trial will be conducted to assess the HEPAVAC vaccine approach. Primary endpoints will include feasibility, safety and induction of specific cellular immune responses.The project has started in September 2013. Identification of specific HCC-associated target antigens is in progress by investigating the HLA I and II immunopeptidome of primary liver cancer. This is pursued using an integrated approach combining mass spectrometry, gene expression analysis and immunogenicity prediction through in vitro analysis of the human T-cell repertoire in HLA-matched healthy donors (XPRESIDENT® antigen discovery platform). To date, we have identified previously unknown HCC-associated target antigen candidates which are now entering a multi-step validation process. In parallel, the HEPAVAC Consortium is preparing its GMP facility at the University of Tuebingen, for customized manufacture of actively personalized treatment as well as to address logistical challenges of a personalized treatment approach. Preclinical studies assessing the formulation and combination of the immunological RNA-based adjuvant with peptide cocktails are underway.

Alphavirus-based vaccines encoding nonstructural proteins of Hepatitis C virus induce robust and protective T cell responses

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An absolute prerequisite for a therapeutic vaccine against hepatitis C virus (HCV) infection is the potency to induce HCV-specific vigorous and broad-spectrum T cell responses. Here, we generated three HCV vaccines based on a Semliki Forest virus vector (rSFV) expressing all- or a part of the conserved non-structural proteins (nsPs) of HCV. We demonstrated that an rSFV vector was able to encode a transgene as large as 6.1 kb without affecting its vaccine immunogenicity. Prime-boost immunizations of mice with rSFV expressing all nsPs induced strong and long-lasting NS3-specific $CD8^+$ T cell responses. The strength and functional heterogeneity of the T-cell response was similar to that induced with rSFV expressing only NS3/4A. Furthermore this leads to a significant growth delay and negative selection of HCV-expressing EL4 tumors in an *in vivo* mouse model. In general, as broad-spectrum T cell responses are only seen in patients with resolved HCV infection, this rSFV-based vector, which expresses all nsPs, inducing robust T cell activity has a potential for the treatment of HCV infections.

Development of a unique anti-AML immune therapy consisting of cord blood hematopoietic cell transplantation and cord blood stem cell-derived dendritic cell (CBDC) vaccination

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Background: Development of novel (immune) therapies is of utmost importance to improve survival in relapsed pediatric-AML (acute myeloid leukemia). We aim to develop a powerful and safe therapy consisting of two potentially synergistic components: Cord Blood (CB) hematopoietic (stem) cell transplantation (HCT) and vaccination with CB-derived Wilms Tumor 1 (WT1) mRNA-electroporated dendritic cells (DCs).

Materials and methods: After isolation, the $CD34^+$ CB stem cells were cultured using a two-step protocol. First, they were expanded using a combination of (growth) factors (Flt3L, SCF, IL-3 and IL-6). Next, the cells were differentiated towards DCs for one week using medium containing Flt3L, SCF, GM-CSF, IL-4 and human serum followed by a CYTOMIX (IL-1β, IL-6, TNF-α and PGE2)-induced maturation for the last 24 hours. Finally, the CBDC culture was electroporated with WT1-mRNA and both their phenotype (cell surface markers) and function (migration and antigen presentation) were assessed.

Results: Using the two-step protocol a total cell expansion of 300-500 fold was achieved. Based on surface marker expression, different DC subsets could be distinguished in our CBDC cultures. Since no differences in antigen presentation capacity between the DC subsets were detected, the whole CBDC culture was used in all phenotypic and functional assays. The maturation using CYTOMIX induced upregulation of costimulatory molecules (CD80, CD83 and CD86) and the chemokine receptor CCR7 on the CBDCs. These matured CBDCs showed enhanced CCR7-dependent migration towards CCL21 in a trans-well migration assay. The presentation of WT1 peptides by the CBDC culture, matured using CYTOMIX and electroporated with WT1 mRNA, was confirmed by the stimulation of a $WT1_{126-134}$ peptide-specific T cell clone . Furthermore, this same CBDC culture was able to induce expansion of the WT1-specific T cells from their autologous pool of naïve T cells in cord blood.

Conclusion: We have developed and tested an in vitro system for culturing large amounts of DCs from the $CD34^+$ CB stem cells. Both the phenotypic and functional data support the use of the whole CBDC culture as vaccine. We are currently translating our preclinical protocol to GMP production of a clinical grade vaccine.

Optimization of peptide-based vaccines using advanced technology

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Immune responses to Biologics are a major concern for the successful development of investigational products. PLATINE PHARMA SERVICES has developed a proprietary Immuno'Line™ technology to predict the immunogenicity in humans of proteins, protein-bound small chemicals or peptides, encompassed in therapeutic or prophylactic vaccines. This technology is based on quantitative and predictive T cell amplification assays using T cells harvested from naïve healthy donors displaying various allotypes, to address the immunogenicity prediction of peptide or protein candidates.

PLATINE notably worked with VAXEAL to select the optimal formulation of long synthetic peptides (LSP) encompassed in a therapeutic cancer vaccine developed by this company. In this study, Immuno'Line™ technology were used to determine the immunogenicity of the three LSP candidates either in their native form (S1, S2 and S3), after modification of their sequence (Mod-S1 and Mod-S2) or combined as a single long peptide containing the Mod-S1, Mod-S2 and native S3 peptides (LP). $CD4^+$ T cell lines from 12 naïve healthy donors, representative of the HLA II diversity encounter in the Caucasian population, were generated by restimulation with the different tested peptides. T cell reactivity was then tested against the naturally expressed native peptides, except for LP-stimulated T cell lines that were tested both against native and LP.

Results demonstrated that all 12 donors responded to at least one native peptide. The magnitude of the immune response was variable with mean frequencies of $CD4^+$ T cell precursors reactive to S1, S2 and S3, of 0.8, 0.5, and 0.7 per million of $CD4^+$ T cells respectively, corresponding to a moderate immunogenicity. Nevertheless, pooled together the native peptides are potentially able to induce T cell lines in all the tested donors associated with a high frequency of specific $CD4^+$ T cell precursors (1.9). This predicts a high immunogenicity of this pool of peptides in humans and this irrespective of individual's HLA class II type. The modified peptides were also capable of inducing peptide-specific immune response. However, the precursor frequency to Mod-S2 (0.1 per million) was drastically reduced compared to native S2 (0.5 per million). By contrast, Mod-S1 displayed equivalent to slightly higher immunogenicity compared to S1 with respectively 0.9 vs 0.8 $CD4^+$ T cell precursors per million. The long peptide appeared to be less immunogenic compared to the pool of corresponding individual peptides. This, combined with reactivity to neo-epitopes detected in 5 of 12 donors, suggested that the long peptide may not be the optimal candidate for vaccination.

In conclusion, based on Immuno'line™ analysis, the optimal vaccine-candidate proposed to Vaxeal was the pool of Mod-S1/ native S2 / native S3. Interestingly, Vaxeal observed equivalent results in subsequent *in vivo* preclinical studies.

A transcriptomic approach to generate better designer DC for clinical application

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Therapeutic vaccination aims to induce an immune response against the tumor in cancer-bearing patients. Dendritic cells (DC), representing "nature's own adjuvant", are frequently used in this context. DC for this purpose are usually generated from autologous monocytes and are matured and loaded with tumor-related antigen. Unfortunately, the clinical responses induced by the first generation of DC-vaccines fell behind the high expectations - nevertheless these trials showed immunogenicity and induction of specific T-cell responses. DC vaccines have since been optimized and improved, finally resulting in the generation of so called "designer DC" specifically equipped with features and molecules to increase their immunogenic efficiency. Several different approaches to achieve this are momentarily subjected to intensive research. We here present an approach to find candidate proteins suitable to improve the DC's capability to induce more effective and longer lasting immune responses in cancer immunotherapy. We and others observed that standard cytokine-cocktail-matured DC are unable to repetitively stimulate autologous naïve $CD8^+$ T cells in absence of additional activating stimuli. This deficiency could be overcome by different means, including antigen-specific $CD4^+$ help, CD40-activation on the DC, or activation of the NF-kappaB pathway within the DC, while all these signals had to be active during the DC-CTL encounter. To find effector molecules, which are induced by these different stimuli in a similar fashion, or which display an expression that correlates with observed differences in the induced CTL responses, we are now performing transcriptome analyses of the differentially treated DC. As a first prerequisite for these experiments, we showed that mRNA-electroporation *per se* does not affect the cytokine production pattern, surface marker expression, and transcriptome of cytokine-matured DC. Preliminary results of DC obtaining an additional activation stimulus already reveal that IL-12 is a relevant effector, which is not new, but reassuring that our approach is valid. We expect other candidates to emerge, which will help to further improve designer DC for clinical application and which will also contribute to a better understanding of the DC / T-cell interaction in CTL memory induction.

(authors StH, IP, KG, SP, SaH, and LB contributed equally)

Depleting the suppressors for the benefit of immunotherapy against cervical cancer

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Background: Cancer vaccines aim at inducing tumor-specific immune responses. However, in clinical studies so far these approaches have limited antitumor effect. Evidence is accumulating that MDSC (myeloid-derived suppressor cells) can suppress the antitumor immune response. Reversal of MDSC-mediated immune suppression by treatment with the tyrosine kinase inhibitor sunitinb can therefore possibly increase the efficacy of cancer vaccines.

Material and methods: We developed a method to assess MDSC depletion in a preclinical model of HPV-induced neoplasia. TC-1 (cells expressing HPV16-E7) tumor-bearing mice were injected with different dosages of sunitinib daily, for 9 days, with and without immunization with SFVeE6,7 (Semliki Forest virus encoding human papilloma virus E6,7 tumor antigens). Intra-tumoral, intra-splenic and circulating MDSC and CD8 T cell levels were assessed after treatment.

Results: Upon sunitinib treatment, the absolute numbers of intra-tumoral, intra-splenic and circulating MDSC decreased in a dose-dependent manner. Combined sunitinib and immunization therapy led to a marked decrease of intra-tumoral, intra-splenic and circulating MDSC levels as compared to non-treated control or immunization alone. The bi-therapy regimen markedly enhanced intra-tumoral, intra-splenic and circulating levels of CD8 T cells. The highest number of circulating CD8 T cells undergoing degranulation (CD107ab+) was observed after combined treatment. Most importantly, this combined sunitinib and immunization treatment regimen abrogated tumor growth.

Conclusions: In summary, we demonstrated that sunitinib alone or in combination with immunization can decrease intra-tumoral, intra-splenic and circulating MDSC levels. Also, combination of sunitinib treatment with immunization enhanced levels and degranulating activity of CD8 T cells, thus resulting in reversal of tumor growth. This study indicates that SFV-based immunotherapy combined with sunitinib treatment could improve treatment outcome.

Temozolomide promotes an increase in the proportion of regulatory T cells over effector T cells in patients with glioma

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Glioma is a dreadful disease with very poor prognosis and current treatments are not able to prolong life more than a few months. In an effort to propose new therapeutic options for glioma patients, antitumor vaccination is currently being tested in clinical trials. However, inducing an immune response in patients may be comprised by concomitant or previous temozolomide, an alkylating agent administered in most patients. Indeed, the impact of temozolomide on different immune cell subsets and, in particular, on T regulatory cells (Tregs), is controversial. Here, we assess this issue by analyzing in detail the phenotype and function of different subpopulations of immune cells in 25 patients with grade IV astrocytoma throughout their first line treatment (irradiation and temozolomide). 13 healthy individuals tested over the same period of time (i.e. 9 months) were included as controls. Our results show that temozolomide induces a severe and long-lasting decrease in T cells together with a concomitant increase of the T regulatory to T effector cell ratio (Treg/Teff ratio). However, patients were capable of mounting a T cell response to an influenza vaccine similar to that elicited in healthy individuals. Our results indicate that prophylactic vaccination is feasible in these patients despite an increase in the Treg/Teff ratio induced by temozolomide. A strict monitoring of Treg numbers during ongoing and future immunotherapy trials will be necessary to confirm whether successful therapeutic vaccination can be achieved in patients with different Treg levels.

Is the IMA950 multi-peptide vaccine applicable for grade II and III glioma patients?

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IMA950 is a vaccine encompassing multiple peptides which have been isolated from glioblastoma, and clinical trials are ongoing. In parallel to this clinical development for high grade glioma patients, it could be of high interest to investigate the value of IMA950 multi-peptide vaccine for patients with a less aggressive disease. Indeed, patients with grade II and III astrocytoma are younger, healthier and have longer survival then high-grade patients. However, their disease invariably relapses, with ultimate progression to glioblastoma in most cases. Here, we investigated expression at the mRNA level of the IMA950 glioma antigens in grade II and III astrocytoma and in other low grade brain tumors using the Nanostring nCounter technology. We observed a similar pattern of expression of the antigens in grade II and III astrocytoma as in glioblastoma, with 6 out of 9 antigens being expressed in more than 50% of samples (N= 20 for both grade II and III astrocytoma). The antigens were also expressed in a significant proportion of oligodendroglioma grade II and III and in ependymoma samples. Our results suggest that 6 among 9 peptides of IMA950 are appropriate candidates for clinical trials investigating the therapeutic potential of this vaccine composition for grade II/III astrocytoma patients

Towards a personalized immunotherapy by vaccination with exosome-loaded dendritic cells

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Introduction: Leukemia immunotherapy frequently does not meet expectation, one of the handicaps being tumor exosome (TEX)-promoted immunosuppression. We here asked, using the mouse leukemia WEHI3B, whether dendritic cell (DC) vaccination suffices to counterregulate TEX-induced immunosuppression and whether TEX could serve as tumor antigen for DC-loading.

Methods: DC were generated from bone marrow cells and were loaded with tumor lysate or TEX. TEX were isolated by standard protocols including sucrose density gradient purification from WEHI3B culture supernatant. WEHI3B-bearing mice were vaccinated with tumor lysate- or TEX-loaded DC or with TEX. Survival time and rate as well as immune response induction were monitored ex vivo. Lysate and TEX processing by DC were analyzed in vitro.

Results: DC-vaccination significantly prolonged the survival time of WEHI3B-bearing mice, TEX-loaded DC (DC-TEX) being superior to lysate-loaded DC (DC-lys), even an excess of TEX not interfering with immune response induction. The superior response to DC-TEX was accompanied by an increase in WEHI3B-specific $CD4^+$ T cells, evaluated by trogocytosis and proliferation. TEX had no negative impact on bone marrow cell-derived DC maturation in vitro and supported CD11c, MHCII and IL12 upregulation. Importantly, TEX were more efficiently taken up by DC than tumor lysate and uptaken TEX were recovered for a prolonged period and were, at least partly, recruited into the MHCII-loading compartment.

Conclusion: TEX did not drive DC into a suppressive phenotype and were a superior antigen due to highly efficient TEX-presentation that is supported by prolonged persistence in DC and preferential processing in the MHCII-loading compartment. As TEX can easily be recovered from patients´ sera, they provide an optimized, individual-specific antigen source for DC-loading

Integrated computational pipeline for epitope characterization for use in personalized genomic vaccination in solid tumors

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Background: Antigen-directed immunotherapy, such as anti-tumor vaccination, is a promising approach to treatment of solid tumors. Despite limited success in a subset of tumors with defined target antigens, therapeutic vaccine platforms have not yet achieved routine clinical efficacy. To expand the scope of antigen-directed immunotherapy the field will require computational approaches that accelerate antigen discovery. To this end, we combined sequencing data with a suit of bioinformatic tools to create an integrated antigen discovery platform, which may be used to identify targets for use in personalized immune-based therapeutics for patients with solid tumors.

Methods: We performed whole exome sequencing (WES) on DNA derived from a representative sample of primary tumor and matched normal tissue from a patient with pancreatic adenocarcinoma (PAAD). We identified all somatic non-synonymous mutations in the tumor-normal pair. We translate each tumor-specific variant in silico into a 21mer sequence corresponding to the variant residue plus symmetric flanking regions. Each variant peptide was then analyzed with a suite of algorithms which model peptide-MHC class I (pMHC) binding affinity. The results were filtered based upon absolute binding affinity (IC50) or relative binding rank to identify high affinity pMHC pairs. We then ranked candidate epitopes by predicted immunogenicity using a HLA and peptide sequence length invariant classifier. Our classifier translates amino acid strings to a reduced alphabet, transforms each string to n-gram frequencies, and then uses an ensemble of random forests to predict immunogenicity. Candidate peptides that ranked highly in both the pMHC binding and immunogenicity model were retained for further validation.

Results: WES of the tumor-normal pair identified 23 somatic non-synonymous mutations including a series of characteristic substitutions common in PAAD. We observed a G12D substitution in KRAS which is found in greater than 50% of all PAAD cases, and is known to confer constitutive signaling activity; as well as mutations in SMAD4, and TP53 both of which are observed in greater than 25% of all PAAD. The integrated ranking system identified few peptides of particular interest including: TEYKLVVVGADGV, GADGVGKSA derivatives of the observed KRAS substitution; as well as peptides WRWPDLHKNERK, and NERKHVKYCQYAF derivatives of variant SMAD4; and MCNSSCMGW from variant TP53—all of which are predicted to bind to non-competing MHC-I isoforms with good concordance in our immunogenicity scoring system.

Conclusion: We have developed an informatics-based approach that utilizes WES data to identify candidate peptides suitable for use in personalized vaccination. In future work, we will apply this analysis to additional solid tumors and to verify the quality of our predictions of using patient-derived autologous T cells exposed to synthetic peptides in vitro.

Vaccination of melanoma patient with native peptide promotes the early onset development of a dominant T cell repertoire

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Objective: Therapeutic vaccination against cancer aims at activating tumor-specific cytotoxic $CD8^+$ T cells while concomitantly inducing long-lasting memory responses. Previously we demonstrated that vaccination of advanced stage melanoma patients with low dose of native Melan-$A^{MART\text{-}1}{}_{26\text{-}35}$ peptide ("EAA") induces immune responses with superior memory and effector functions compared to the analog (A27L) peptide ("ELA"). However, major questions remain regarding the establishment of the increased polyfunctionality observed following native peptide vaccination. For instance, are there significant differences in clonotype selection between native and analog peptide-vaccinated patients? Can T cells display high levels of polyfunctionality already before vaccination? Or how early post-vaccination can one observe polyfunctionality?

Methods: We have studied three EAA- and four ELA-vaccinated patients from whom we obtained pre-vaccination, early (< 3 months or after ≤ 4 monthly vaccinations) and late (> 3 months or ≥ 4 monthly vaccinations) post-vaccine blood samples. From these PBMCs, individual Melan-$A^{MART\text{-}1}{}_{26\text{-}35}$-specific CD8 T cells were sorted by flow cytometry, followed by direct cell lysis and reverse transcription. The resulting cDNA was precipitated, globally amplified and subjected to semi-quantitative PCR for gene expression and TCR BV clonotype repertoire analyses.

Results: We observed that vaccination with the native/EAA peptide promoted the development of a dominant TCR clonotype repertoire earlier than with the analog/ELA peptide. Interestingly, there was an increased proportion of dominant clonotypes in the less-differentiated tumor-specific EM28+ T cell subset. Using single-cell gene expression analyses, we then characterized polyfunctionality by evaluating the co-expression of memory/homing-associated genes *(CD27, IL7R, EOMES, CXCR3 and CCR5)* and effector-related genes *(IFNG, KLRD1, PRF1 and GZMB)*. Prior to vaccination, T cells from both vaccination cohort displayed similar gene expression patterns, i.e. low effector and high memory gene expression. At early time point, we did not observed significant gene expression difference between EAA- and ELA-vaccinated patients. However, at late time points, EAA-vaccinated patients displayed augmented memory/effector gene co-expression.

Conclusions: These findings reveal the early onset of the establishment of a dominant T cell clonotype repertoire following vaccination with the native peptide when compared to patients vaccinated with the analog peptide. Conversely, the level of memory/effector T cell polyfunctionality appears to be established in time possibly through repeated boosting vaccination, likely through maturation of the tumor-specific T cell response.

Vaccination against the large T antigen as a model for immunotherapy of virally induced cancer

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One cancer that comprises a viral involvement is the Merkel cell carcinoma (MCC). MCC is a neuroendocrine skin tumor, which is relatively rare, but highly aggressive and its incidence rates have increased over the last decades. Aside from surgical excision no standard treatment exists for already established tumors. Recently the Merkel cell polyomavirus (MCV) was discovered to be associated with MCC (80 % of MCC tissues contain MCV, but only 8 % of healthy tissues). Under certain circumstances, this virus integrates into the host genome. Thereby, the production of most viral proteins is switched off, but a truncated form of one of its viral proteins, the large T antigen (LT) is still expressed. This protein contains conserved tumor suppressor-targeting motifs, e.g. the Rb binding domain, and serves as oncogenic driver. Moreover, LT is a foreign antigen (Ag) and similar in a large number of patients. Thus, LT seems to be an appropriate target for the immunotherapy of MCC.In contrast to overexpressed self-Ag, targeting a foreign Ag, such as truncated LT, does not require the breach of central tolerance and does not confer the risk of establishing autoimmunity against healthy tissue. Hence, the aim of this study is to develop an immunotherapeutic DC vaccination approach for the treatment of virally induced cancers by employing MCC with its LT as a model disease. These DC will be generated by the electroporation with truncated LT (truncLT)-RNA, but to improve the DC's immunogenicity, we will co-transfect constitutively active mutants of IkB kinases (caIKK) of the NF-kB signaling pathway. The introduction of caIKK - i.e. caIKKa and caIKKb - led to the up-regulation of maturation markers and co-stimulatory molecules and to an increased TNF, IL-6, IL-8, and IL-12p70 secretion. These caIKK-DC are highly immunogenic and potent stimulators for the generation of antigen-specific memory-like $CD8^+$ T cells. To further improve the DC's stimulatory capacity, we codon-optimized the caIKK constructs and compared the DC transfected with the optimized and standard constructs concerning surface expression of DC maturation markers, co-stimulatory molecules, and cytokine secretion in a time-dependent manner. We also intend to compare the changes induced by codon-optimized caIKKDC compared to standard caIKK-DC in their stimulatory potential. We will design the ongoing study either with the optimized or the standard constructs after further corroboration of the preliminary results. Thereby, we will assess whether the LT is a suitable target antigen for the DC vaccination therapy of MCC.

ASET study: Predictive parameters and immunological data of patients with locally recurrent or metastatic renal cell carcinoma (RCC) treated with the cell-based therapeutic cancer vaccine MGN1601

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Background: MGN1601, a cell-based therapeutic cancer vaccine, is a fixed combination of allogeneic tumor cells modified via MIDGE® vectors to express IL-7, GM-CSF, CD80, and CD154 plus the immunomodulator dSLIM®. In the ASET trial heavily pretreated patients with RCC were enrolled to assess safety, clinical efficacy, and immunological effects of MGN1601.

Methods: The treatment phase of the multicenter, single-arm phase 1/2 ASET study consisted of 8 intradermal vaccinations over 12 weeks. Following evaluation, patients with at least disease stabilization entered an extension phase (EP, 5 vaccinations until week 120). Overall, 19 patients were included and received at least one MGN1601 injection (ITT); the majority (N=17) had ≥3 lines of previous therapy.

Results: Ten out of 19 patients (53%) completed the treatment phase per-protocol (PP); 9 patients discontinued the study due to disease progression (PD) or rapid deterioration. Only 10 of 109 adverse events (9.2%) were drug-related and no drug-related serious adverse event was reported. Two patients achieved disease control (PR, SD) after 12 weeks and continued in the EP: One had PD during EP, the other remains in sustained partial remission after completion of the EP. Median OS was 24.8 weeks for ITT and 115.3 weeks for PP. Univariate analysis of pre-treatment characteristics revealed absolute lymphocyte counts, neutrophil to lymphocyte ratios, platelets, MSKCC score, and liver metastasis as putative predictive factors of longer overall survival. Upon stimulation with different recall antigens patients‘ PBMC showed a significant increase in IL-2 secretion in 7 of 10 analyzed patients in an LTT - including 2 patients with SD and PR. ELISpot assay using re-stimulation of patients‘ PBMC with MGN1601 cells or peptide mixes of tumor associated antigens resulted in at least two-fold increase of IFN-gamma secreting cells in 4 of 9 patients suggesting a cytotoxic anti-tumour response in the course of MGN1601 vaccination. Four of 5 patients showed an increase of single activation marker (CD169, CD86 or CD69) 48 hours after vaccination. All 5 patients developed vaccine cell binding antibodies. These immunological in-vitro analyses revealed improved cellular and humoral immune function during MGN1601 treatment.

Conclusions: MGN1601 administration was safe and showed promising OS in a population of heavily pretreated RCC patients who could receive therapy over 12 weeks (PP population). These results warrant further evaluation in a larger, controlled clinical trial. Baseline biomarkers and factors may allow identifying patients more likely to benefit from this innovative vaccination approach.

The great ability of antigen presenting cells, such as dendritic cells (DCs), to boost an immune response against an antigen is employed in new cancer immunotherapy strategies. One novel approach is therapeutic vaccination using long peptides (LPs) with overlapping sequences that span whole or parts of tumour-associated antigens (TAAs). LP vaccination has shown promising results when treating patients harbouring a HPV induced neoplasia; however, improvements are needed with respect to antigen uptake and optimal activation of DCs.

To improve LP vaccination we have linked LPs to a B cell epitope facilitating complex formation with specific antibodies. *In vivo* antigen-antibody complex formation has previously been shown to enhance antigen uptake and activate DCs which subsequently prime antigen-specific T cells[1]. We have therefore linked three identical linear peptides derived from tetanus toxin to a LP of interest. The linear tetanus derived peptide was chosen as we found that the majority of tested healthy individuals display IgG specific antibodies towards this epitope, as a result of tetanus vaccination, thereby facilitating *in vivo* conjugate-antibody complex formation. In a murine system conjugate-antibody complexes are superior, compared to conjugate alone, in activation of DCs that subsequently cross-present and activate LP-specific T cells (unpublished results).

We have recently evaluated our conjugate vaccine in a whole human blood loop system that uniquely contains intact complement system. Monocytes were found to internalise the conjugate in an antibody dependent manner but Fcγ

Patients treated with oncolytic adenovirus Ad5/3-hTERT-E2F-GMCSF induce immunological responses against the tumor

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Background: Oncolytic viruses that selectively replicate in tumor cells can be used to treat cancer. Accumulating data suggest that virus induced oncolysis can enhance anti-tumor immunity and break immune tolerance. To capitalize on the immunogenic nature of oncolysis, we generated a quadruple modified oncolytic adenovirus expressing granulocyte-macrophage colony-stimulating factor (GMCSF).

Results: Ad5/3E2FΔ24GMCSF (CGTG-602) was engineered to contain a tumor specific E2F1 promoter and 24bp deletion enabling replication only in tumor cells. The fiber features a knob from serotype 3 for enhanced gene delivery to tumor cells. The virus was first tested preclinically *in vitro* and *in vivo* and then 13 patients with solid tumors refractory to standard treatments were treated. Treatments were well tolerated and often tumor- and adenovirus-specific T-cell immune responses were seen. Overall, with regard to tumor marker or radiological responses, signs of antitumor efficacy were seen in 9/12 evaluable patients (75%). The radiological disease control rate with positron emission tomography was 83% while the response rate (including minor responses) was 50%. Tumor biopsies indicated accumulation of immunological cells, especially T-cells, to tumors after treatment. RNA expression analyses of tumors indicated immunological activation and metabolic changes secondary to virus replication. In blood samples responding patients showed changes in the amount/activity of circulating anti-cancer immunological cells and decrease in anti-tumor antigens were seen.

Conclusions: Ad5/3E2FΔ24GMCSF was found well tolerated in treatment of cancer patients and signs of immune activation and anti-tumor efficacy were seen. Clinical trials are needed to investigate its clinical utility further.

Development and characterization of TNF-alpha armed oncolytic adenovirus for treatment of solid tumors

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Tumor necrosis factor alpha (TNFa) has long been recognized to have anti-cancer characteristics, however, its systemic toxicity has limited its usability in clinics. Oncolytic viruses, selectively cancer-killing viruses, have shown great potency but still their efficacy needs to be improved to optimize clinical impact. Local production of TNFa from an oncolytic adenovirus would be a way to increase concentration of the molecule at tumors while limiting systemic exposure. The anti-tumor effects of TNFalpha include apoptotic cell death as well as activation of the host immune system against the tumor.

We generated a novel chimeric oncolytic adenovirus expressing human TNFa, Ad5/3-d24-hTNFa, whose efficacy and immunogenicity were tested *in vitro* and *in vivo*. TNFa has also been shown to sensitize cells to radiation and since the combination of adenoviruses and radiotherapy has shown synergistic antitumor effects in many studies, we also performed some studies to test the virus in combination with radiation.

We observed that hTNFa expressing virus had increased cancer eradicating potency both *in vitro* and *in vivo* compared to virus without the transgene. The potency of the virus to kill cancer cells was shown *in vitro* by MTS cell viability tests and in mouse models. Interestingly, we saw increase in immunogenic cell death markers (extracellular ATP, HMGB-1 and calreticulin exposure) in Ad5/3-d24-hTNFa treated cells. Moreover, the amount of tumor-specific T-cells was elevated in Ad5/3-d24-hTNFa treated B16-OVA tumors of C57BL/6 mice. We also observed that the sizes of tumor draining lymph nodes of Ad5/3-d24-hTNFa treated mice were notably larger than in control groups indicating immune activation. When tumor cells were treated *in vitro* with Ad5/3-d24-hTNFa and irradiated 24h later, the combination resulted in increased tumor cell death. *In vivo,* the combination of Ad5/3-d24-hTNFa with irradiation showed almost complete eradication of PC-3MM2 tumors in nude mice.

According to our knowledge this is the first report of an oncolytic adenovirus expressing human TNFa. Enhanced tumor eradication and anti-tumor immune responses mediated by Ad5/3-d24-hTNFa offer a new potential drug candidate for cancer therapy.

Photochemical Internalisation (PCI) - a new adjuvant technology for stimulating cytotoxic T-cell responses

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For generating a proper anti-tumour immunological response it is essential to stimulate cytotoxic T-cells (CTLs) to attack the tumour cells. Activation of CTLs is typically mediated through MHC Class I antigen presentation by antigen presenting cells (APCs), but unfortunately vaccines aimed at stimulating CTL responses are often ineffective because exogenously added vaccine antigens are primarily processed by the MHC class-II and not the MHC class-I pathway. The latter requires cytosolic delivery of antigen and this is often difficult to achieve with exogenously added antigens, making it important to develop new technologies for cytosolic delivery of such antigens. Photochemical Internalisation (PCI) is a technology for inducing cytosolic delivery of endocytosed molecules by illumination. In PCI a photosensitising molecule is used to make endocytic membranes light sensitive, with illumination inducing permeabilisation of the membranes. Fluorescence microscopy studies has shown that the photosensitiser $TPCS_{2a}$ (disulfonated tetraphenyl chlorin) co-localises with antigen in endocytic vesicles and that illumination releases the antigen into the cytosol. Thus, PCI has a clear potential for enhancing CTL responses by rerouting antigen presentation from MHC class II to MHC Class I, making access for the antigen to the MHC Class I presentation machinery in the cytosol of APCs. This principle has been investigated with *ex vivo* treatment of mouse bone-marrow-derived murine dendritic cells (DCs). After light activation, the DCs were cultured with antigen (ovalbumin)-specific CD8 T cells or used for immunisation of mice. PCI induced CD8 T-cell responses as measured by IFN-γ secretion *in vitro* and CD8 T-cell proliferation *in vivo*. The PCI adjuvant activity has also been demonstrated with *in vivo* vaccination where the antigen was mixed with $TPCS_{2a}$ and administered intradermally to mice, followed by illumination of the injection site. Also with this mode of vaccination PCI strongly increased the stimulation of CD8 T-cell responses as measured by antigen-specific proliferation and secretion of pro-inflammatory cytokines. In a murine model of melanoma, PCI-based vaccination prevented tumour growth as compared with vaccination without PCI. In conclusion, PCI has a completely new mechanism of action as an adjuvant technology, representing a new and potent tool for stimulation of cytotoxic CD8 T-cell responses, with a very interesting potential in cancer immunotherapy. This work demonstrated a first proof-of-principle for PCI-mediated immunisation in mouse models. PCI with the $TPCS_{2a}$ photosensitiser is currently in clinical development for enhancement of the effect of cancer cytotoxic drugs, and it has been shown that $TPCS_{2a}$ can be administered safely to patients in much higher doses than what is envisioned for the use in immunotherapy, paving the way for testing the technology in patients also in the area of cancer immunotherapy.

MultiPro: Development of multipeptide-based immunotherapy for prostate cancer patients independent of their HLA type

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Peptide-based immunotherapy has shown to induce tumor-specific T cells that are able to combat cancer. Especially cancer types with a high probability of recurrence or metastasis after a first line of treatment like prostate cancer are suited for immunotherapies. In a recent phase I/II clinical trial of therapeutic vaccination using 11 HLA-A*02 restricted synthetic peptides in 37 HLA-A*02 positive prostate carcinoma patients, a vaccine-induced immune response could be shown in 42% of the patients.

In contrast to most efforts made in this field, our group does not rely solely on peptides restricted to the most common HLA alleles (e.g. HLA-A*02), instead we are focusing on the identification of immunogenic prostate-specific peptides presented by diverse HLA alleles. Using a mass spectrometry-based approach our group identified about 60 novel prostate-associated peptides derived from differentiation antigens (PSA, PSMA, PAP) and other general tumor-associated antigens (HDAC1, HDAC2, WT1, CTNNB1) restricted to different HLA molecules. All identified peptides were eluted from either malignant or benign prostate tissue and validated by synthetic peptides to confirm the identified sequence. Immunogenicity testing of selected peptides was conducted by *in vitro* stimulation of healthy blood donor T cells with peptide-loaded artificial antigen presenting cells (aAPCs). Enriched CD8 positive T cells from peripheral blood mononuclear cells (PBMCs) are subsequently stimulated three times in weekly intervals with streptavidin-coated aAPCs, loaded with recombinant HLA-peptide complexes and costimulatory anti-CD28. Peptide-specific T cells were detected by tetramer staining and subsequent analysis via flow cytometry. Due to the high throughput character of testing the immunogenicity of more than 50 peptides, additional *in vitro* priming of candidate peptides was performed at collaborating institutes.

In preparation for a phase I/II multipeptide-based clinical trial (MultiPro) for patients with prostate cancer, the most promising peptides together with the immunogenic peptides from a preceding clinical study will be selected to develop a vaccine cocktail encompassing 20 to 30 peptides covering the most prevalent HLA alleles.

Oncolytic measles virus for tumor vaccination

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The concept of tumor vaccination is based on the induction of robust immune responses against tumor-associated antigens (TAA), which should be selectively expressed within the tumor. Whereas in the case of foreign antigens, an immune response can be induced quite easily, induction of immune responses against self-antigens needs potent antigen presentation to elicit an appropriate reaction. Tumor vaccines may be further improved by utilizing the properties of oncolytic viruses (OVs), which directly kill tumor cells and induce effective stimulation of the immune system. Immunogenic OVs additionally delivering a specific TAA may break immunological tolerance, induce a specifically redirected antiviral immune response, and thus lead to increased therapeutic efficacy. Attenuated measles virus (MV) derived from a vaccine strain is currently clinically tested as OV. Recombinant MVs of this type additionally reveal excellent vaccine characteristics, inducing potent and long lasting immune responses against endogenous and foreign antigens when the latter are additionally expressed by the recombinant virus. Therefore, we develop and aim to validate prototypic replicating MV for oncolysis and simultaneous directed *in vivo* vaccination.

For that purpose, we use a claudin family member, a recently well characterized tumor-confined TAA. Different antigen formats or epitopes were cloned into the MV genome and recombinant viruses were generated, which express those epitopes in different amounts. The expression gradient was achieved by cloning the extra gene into two different additional transcription units either behind the P (post P) or the H (post H) cassettes of Moraten vaccine strain-derived MV_{vac2} genome. Those engineered MVs are now being characterized and will be tested for their immunogenicity in MV-susceptible $IFNAR^{-/-}$-CD46Ge mice. In parallel, an immune competent, syngeneic tumor model has been established using transgenic C57/BL6-derived tumor cell lines expressing the target antigen as well as MV-receptors. The most promising recombinant MV inducing optimal humoral and cellular immune responses will be analyzed in this syngeneic model for therapeutic efficacy. Tumor growth and survival will be analyzed as well as induction of anti-tumoral immune responses and tumor infiltration of immune cells. In the case of long time survivors, those mice can be challenged with the appropriate tumor to test the long lasting memory effect of the therapy.

Vaccination with long NY-ESO79-108 peptide in combination with CpG leads to robust activation of CD4 and CD8 T cell responses in stage III and IV melanoma patients

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The cancer testis antigen NY-ESO-1 is one of the most frequently expressed antigen in patients with metastatic melanoma. Recombinant NY-ESO-1 protein and NY-ESO-1 peptides have been widely tested in immunotherapy trials in different types of human cancer. In this clinical trial (LUD-01-003), 19 patients received a mean of 8 (range 1-12) monthly s.c. vaccines, composed of the long synthetic NY-ESO$_{79\text{-}108}$ peptide, in combination with IFA and the immune adjuvant CpG (PF-3512676).

Immunomonitoring of NY-ESO-1 specific T cell responses in peripheral blood was performed at baseline and specific time-points during and post vaccination. To this aim, CD4 and CD8 T cells were stimulated *in vitro* for 13 days with overlapping 18-mers spanning the NY-ESO$_{79\text{-}108}$ region, followed by intracellular staining for IFNg and TNFa after re-challenge with either overlapping 13-mers for CD4 T cells, or short overlapping 9-mer for CD8 T cells. In 19/19 evaluable patients a very strong secretion of IFNg and TNFa was detected in in up to 40% of specific T cells, both CD4 and CD8, irrespective of patient‘s HLA. In contrast, no or minimal T cell responses were found at baseline. Immune responses were observed early after initiation of immunotherapy and were long-lasting. In nine patients, we could analyze the T cell specificities more in depth and found predominant HLA-DR and HLA-DP restricted responses, but only rare HLA-DQ restricted CD4 T cells. The most immunogenic region within the vaccine peptide was the NY-ESO-1$_{83\text{-}97}$ portion, which induced specific CD4 T cell responses (HLA-DR or -DP restricted) in all patients tested. Interestingly, besides the known MHC class II epitopes within the NY-ESO$_{79\text{-}108}$ sequence, we discovered a novel HLA-DR7-restriced, NY-ESO-1$_{87\text{-}99}$ immunogenic peptide. Using fluorescent HLA-DR7-multimers loaded with NY-ESO-1$_{87\text{-}99}$ we were able to detect reactive CD4 T cells in 5/5 HLA-DR7+ patients, after one round of *in vitro* stimulation. NY-ESO-1$_{87\text{-}99}$ Specific CD4 T cells were already present before immunotherapy, but were significantly expanded during peptide immunization. Even more remarkably, in preliminary experiments we also directly identified *ex-vivo* significant numbers of NY-ESO-1$_{87\text{-}99}$/DR7-specific CD4 T cells in the blood. These results show that vaccination with the long synthetic NY-ESO-1$_{79\text{-}108}$ peptide combined with the strong immune adjuvant CpG induced integrated, robust and functional CD4 and CD8 T cells responses in melanoma patients, supporting the further development of this immunotherapeutic approach.

Expanded γδT-APC - Vehicle for cancer immunotherapy

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Human Vγ9Vδ2 T cells are considered to play a vital role in protective immunity through cytokine secretion and cytokine activity. We have reported that human blood γδ T cells when activated with phosphoantigen, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), is of microbial origin and outperforms the structurally related phosphoantigen in eukaryotes, isopentenyl pyrophosphate (IPP), by < 10^3-folds are become a professional antigen presenting cells (γδ T-APCs) such as dentritic cells. γδ T cells show anti-tumor activity against a variety of allogeneic and autologous tumors *in vitro* and *in vivo*.

The present study is related to the *in vitro* expansion of Vγ9Vδ2 T cells and their functionality as an antigen presenting cell. This is a part of preclinical studies which is necessary to establish proof-of-principal before these γδ T-APCs can be used in a clinical setup. Autologous peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and melanoma patients, stimulated with zoledronate and expanded (purity >90%) for up to two weeks. Significantly higher expansion rates were observed in the presence of a combination of cytokines, IL-2 and IL-5 as compared to IL-2 alone. That substantially overcomes obstacles associated with γδ T cell expansion from cancer patients. Expanded γδ T cells can be generated in large numbers (>50×10^6/ml of blood) and express markers needed by professional APCs (MHC class-I/II, CD80, CD86, CD54, CD11a). They can uptake, process and present protein antigens (Flu M1 protein or purified protein derivates (PPD) from *mycobacterium tuberculosis*) to αβ-T cells and induced proliferation response *in vitro*. γδ T-APCs produce pro-inflammatory cytokines (IFNγ, TNFα and IL-6) and opposed immune-suppression. Therefore these expanded γδT-APCs can be used as a potential candidate for cancer vaccine.

MicOryx - a phase I/IIa clinical trial evaluating vaccination of MSI-H colorectal cancer patients with frameshift peptide antigens

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Background: Microsatellite instability (MSI-H) is caused by DNA mismatch repair deficiency and occurs in 15% of colorectal cancers. MSI-H colorectal cancers are characterized by a dense immune cell infiltration, which is compatible with a pronounced local anti-tumoral immune response. MSI-H cancers are characteristic of the inherited Lynch syndrome, but can also develop sporadically. In MSI-H cancers, insertion or deletion mutations at coding microsatellites may lead to shifts of the translational reading frame and to the generation of MMR deficiency-related frameshift peptides (FSPs). Expression of FSP antigens is restricted to MMR-deficient cells; therefore, FSP antigens are promising targets of vaccination approaches.

Study design: We have recently initiated a clinical phase I/IIa vaccination trial (Micoryx, ClinicalTrials.gov Identifier NCT01461148; Sponsor: Oryx GmbH&Co. KG) that evaluates vaccination with a combination of three FSP antigens (derived from frameshift variants of the coding microsatellite-containing genes AIM2, HT001, TAF1B) in the clinical setting. Inclusion criteria allow vaccination of patients with history of metastasized colorectal cancer (UICC stage III or IV) after the end of standard chemotherapy. Phase I of the trial evaluates safety and toxicity as the primary endpoint (6 patients); phase IIa addresses the induction of cellular and humoral immune responses (16 patients). T cell responses were monitored by interferon-gamma ELISpot, and antibody responses by ELISA from peripheral blood.

Results: Data from phase I demonstrate that no FSP antigen-associated severe adverse events have been observed after FSP vaccination. Significant induction of FSP-specific T cell immune responses was observed in 3 out of 4 evaluated patients. Antibody responses against FSP antigens were significantly induced in 9 out of 10 evaluated patients.

Conclusions: If vaccination with FSPs turns out to be well tolerated and leads to the induction of effector T cell immune responses, FSP vaccination may represent a promising novel approach for treatment of MSI-H colorectal cancer patients and for tumor prevention in Lynch syndrome mutation carriers.

Sequential intra-nodal immunotherapy with low-dose rituximab and dendritic cells in irradiated lymph nodes induces systemic anti-tumor immunity and regression of disseminated follicular lymphoma

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Purpose: Advanced stage follicular lymphoma (FL) is incurable by conventional therapies. Studies that have utilized the lymphoma immunoglobulin idiotype for immunization have so far not been a success. The present study explored the immunogenicity and clinical efficacy of a novel vaccination strategy involving repeated sequential administration of low-dose rituximab and dendritic cells (DC) in irradiated lymph nodes.

Patients and methods: Twenty patients with untreated or relapsed stage III/IV FL not in need of standard therapies were included. Work-up included CT, PET/CT and bone marrow specimens. Single cell suspensions from tumor were frozen for later immune studies and apheresis was performed for isolation of monocytes cultured to generate immature dendritic cells (DC). The first cohort (n=6) received radiotherapy to a solitary lymphoma node and intra-tumoral vaccination with immature autologous DC and GM-CSF in the same lesion. The treatment was performed twice with 6 weeks interval, targeting different sites. The second cohort (n=14) additionally received intra-nodal injections of 5mg rituximab prior to DC vaccination and now at three different sites, with two weeks intervals. Clinical outcomes were assessed by CT, PET/CT and bone marrow specimens. Systemic immune responses were assessed longitudinally by probing CD8 and CD4 T-cell reactivity against autologous tumor cells measured by proliferation ($CFSE^{low}$) and degranulation (CD107a/b).

Results: A total of 20 patients were enrolled in the study. In cohort 1 one patient showed a reduction in total tumor volume not qualifying for partial response (PR). This modest response was accompanied by an anti-tumor CD8 T cell response. Four patients had stable disease and two had progressive disease. None of these patients developed T cell responses.In cohort 2, five patients (36%) displayed objective clinical responses (2 CR, 3 PR), including one patient with primary cutaneous FL who showed regression of skin lesions. One CR patient is still in remission after 51 months, the other relapsed after 24 months. The best responses typically developed gradually over time and peaked at 8-12 months. All responding patients tested had strong systemic anti-tumor T cell responses. In the majority of cases there was an increase in the proliferation of $CD8^+$ as well as $CD4^+$ T cells post-treatment. However, the CD8 responses were generally stronger. The reduction in total tumor volume and prolonged time to next treatment correlated closely with CD8 T cell-mediated anti-tumor responses. Side-effects were limited to mild fever and flu-like symptoms grade I in some patients in conjunction with the rituximab injection. Auto-immune toxicities were not observed.

Conclusion: Sequential immunotherapy with irradiation, rituximab and DCs targeting single lymphoma nodes generated systemic T cell immunity accompanied by regression of disseminated FL.

Mining sequencing data to identify targets for cancer immunotherapy

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For the development of effective immunotherapies for cancer it is crucially important that the tumor-associated antigens and the T-cell epitopes encoded by these proteins are carefully chosen. Unique tumor antigens and tumor-specific somatic mutations provide a strong rationale for the design of patient-tailored therapies. Mutation-derived epitopes can be used for the development of effective epitope-defined adoptive T-cell transfer and multi-epitope-based vaccination strategies. Furthermore, mutated cell surface proteins can be targeted through antibody-based therapies. Cancer genomics allows high-throughput screening for tumor-specific mutations and epitope prediction algorithms can be utilized to assess the immunogenicity of epitopes containing these mutations. We developed a protocol for mining high-throughput exome and transcriptome data from patients to identify tumor-specific epitopes and mutated surface antigens suitable for cancer immunotherapy. Frequently mutated proteins or epitopes will then be generated and subsequently tested in T cell assays for recognition by extracted T cells from patient samples.

Here, the results from three patients are presented: DNA from different metastatic lesions and control samples from three patients was sequenced, analyzed for the presence of mutation-specific epitopes and T cell reactivity against a panel of mutation-specific epitopes was determined.

Self-adjuvanted RNActive® vaccine induces innate immune response at the site of injection that leads to a potent adaptive immunity in both mice and humans

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Two-component mRNA vaccine (RNActive®) combines high antigen expression with strong immune stimulation. We have previously shown that intradermal administration of RNActive® vaccines induced balanced, potent and long-lasting immune responses in both mice and humans. Here, we characterized early events upon injection to better understand the mechanism of action of RNActive® with a special focus on self-adjuvanticity potential of mRNA-based vaccines. We showed that shortly after vaccination, mRNA is present in both non-leukocytic and leukocytic cells and was also detected in dendritic cells (DCs) within the draining lymph nodes (dLNs) 24 h after intradermal administration. Full genome microarray analyses of the skin tissues showed that RNActive® vaccine transiently altered the gene expression profile at the injection site. Various chemokines whose pleiotropic functions include recruitment and activation of key cellular players of the innate immune system as well as multiple pro-inflammatory cytokines were up-regulated early upon RNActive® treatment. In corroboration with the gene expression data after injection of RNActive® we observed a strong production of chemokines including CXCL9, CXCL10, CCL3, CCL4, CCL5 as well as pro-inflamatory cytokines (IL-6, TNF-a) at the site of injection. The innate immune responses in the skin were followed by the increased cellularity as well as an enhanced activation of wide range of the immune cells in the dLNs including $CD8^+$ T-cells, B cells, γδ T-cells, NK and NKT cells. In summary, our data indicated that the self-adjuvanted RNActive® vaccine induces strong innate immune response at the site of administration followed by activation of the immune cells in the dLNs. These results provide a possible mechanistic explanation of a potent and balanced adaptive immunity induced by mRNA-based vaccine which is a novel vaccination platform for an efficient prophylactic and therapeutic immunotherapy.

CpG conjugation to model tumour antigen ovalbumin leads to enhanced CD8$^+$ T-cell proliferation

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Activation of cytotoxic CD8$^+$ T-cells is crucial to developing an anti-tumor immune response. In order for T-cell activation to occur, tumor antigen needs to be cross-presented to activated dendritic cells. Dendritic cells cross-present tumor antigen on MHCI but need to be completely activated via danger signals to generate a potent CD8$^+$ T-cell response through co-stimulatory molecules. Linking a vaccine adjuvant, CpG oligodeoxynucleotide (ODN) chemically to a tumor antigen enables endocytosis of both entities into the same cell. The CpG-ODN - tumor antigen conjugate activates dendritic cells via the toll-like receptor 9 in the endosome and the tumor antigen is cross-presented on MHCI.

The model tumor antigen ovalbumin was first purified via gel filtration and only the monomer was used for conjugation. The vaccine adjuvant CpG ODN was conjugated with a stable bis-arylhydrazone bond to the model tumor antigen ovalbumin. A molar ratio of 2.5:1 of CpG ODN to ovalbumin was determined by UV-absorption of the bis- arylhydrazone bond. Purification of the conjugates was performed by gel filtration. Size-exclusion chromatography identified conjugates with a range modification. Murine bone marrow derived dendritic cells (BMDC) were cultured for six days and pulsed for 24h with either a conjugate or a mixture of antigen and adjuvant. Carboxyfluorescein succinimidyl ester stained CD8$^+$ T-cells were cocultured with stimulated murine BMDCs for 72h. BMDC activation and T-cell proliferation were analyzed using flow cytometry.

Effective activation of dendritic cells was shown by the upregulation of activation markers after stimulation with either the conjugate or the mixture of CpG ODN and ovalbumin. However the conjugate activated dendritic cells showed higher CD8$^+$ T-cell proliferation compared to dendritic cells activated with the mixture.

Conjugating the vaccine adjuvant CpG ODN to the model tumor antigen ovalbumin using a stable bis-arylhydrazone bond leads to a higher degree of CD8$^+$ T-cell proliferation compared to the CpG ODN antigen mixture.

Characterization of the IFNα response upon systemic administration of targeted mRNA vaccine delivery

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ORAL SHORT TALK

Antigen-encoding RNA has proven to be a potent tool for inducing T cell-mediated anti-tumoral immunity. We previously showed that complexing RNA with a certain liposomal carrier system resulted in RNA formulations (RNA-lipoplexes) suitable for intravenous administration. RNA-lipoplexes not only successfully transfected cDCs, pDCs and macrophages in the spleen, lymph nodes and bone marrow of mice, but also led to profound proliferation of tumor-specific $CD4^+$ and $CD8^+$ T cells, conferred complete tumor protection and mediated elimination of progressing CT26 and B16-OVA tumors in subcutaneous and metastasis models after intravenous administration. Intending to characterize the intrinsic immunostimulatory potential of these novel RNA-lipoplexes, we found that immunization induced both upregulation of activation markers on splenic cDCs, pDCs, macrophages, NK, T and B cells and a large variety of inflammatory cytokines, costimulatory molecules, chemokine ligands and receptors in different lymphoid compartments. This inflammatory milieu was found to be critically dependent on IFNα, and functional IFNAR-signaling during RNA lipoplex-induced T cell priming was essential for the generation of cytotoxic effector T cells and subsequent tumor rejection. The overwhelming majority of systemic IFNα was produced by splenic pDCs and macrophages in a sequential but overlapping manner, and whereas early IFNα secretion in mice devoid of IFNAR was only slightly affected in pDCs, macrophages completely depended on IFNAR signaling for IFNα synthesis. Surprisingly, macrophages were discovered to be the major IFNα contributors: Systemic IFNα was almost completely abrogated in mice depleted of macrophages, and consequently, activation of splenic immune cell populations was equally impaired. In contrast, upon depletion of pDCs in BDCA2-DTR mice, IFNα production by macrophages and activation of splenic immune cells remained unaffected.

Future studies will focus on the mechanistic characterization of RNA-lipoplex recognition in pDCs and macrophages while mounting the IFNα response and will determine whether there is a direct role for IFNα in T cell activation and differentiation.

Development of a high throughput protein production system for the efficient generation, screening and production of chimeric virus-like particles as cancer vaccines

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Virus-like particles (VLPs) consist of viral capsid proteins which self-assemble into supra-molecular, particulate structures. Chimeric VLPs can be used to display heterologous epitopes on their surface thereby transferring their potent intrinsic immunogenicity to the inserted epitope. Furthermore, they are promising candidates for therapeutic cancer vaccines due to their potential to induce auto-antibodies mediated cytocidal effector functions against target-positive cancer cells. Here, we use recombinant VLPs based on the Hepatitis B virus core antigen (HBcAg) as multivalent carrier platform for heterologous tumor antigen-derived epitopes. TU003 is a new target for cancer vaccination using VLPs. So far, the generation of functional chimeric VLPs has proven to be a time- and labor-intensive process with no guarantee of success. In order to generate a maximum number of functional VLPs targeting TU003 in a short period of time, a semi-automated system of chimeric VLP creation and validation (*high throughput protein production*, HTPP) was developed. Both, cloning and initial expression screening are conducted in 48- and 96-multiwell plates and are compatible with standardized thermal cyclers and liquid handling stations. In the further process, VLPs are characterized and purified using solubility analysis in high-throughput, size-exclusion chromatography (SEC), immunoblotting (Dot-Blot), NuPAGE and native agarose gel electrophoresis (NAGE). This high-throughput approach leads to significant acceleration of the vaccine development process allowing cloning and screening of >1000 VLP constructs in just a few weeks.

Targeting of antigen into XCR1$^+$ dendritic cells combined with a non-viral boost regime induces massive CD8$^+$ T cell cytotoxicity capable of eradicating established tumors

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ORAL SHORT TALK

Current immunization schemes do not induce potent CD8$^+$ T cell cytotoxicity unless combined with viral boost regimes, which are not easily applicable in the human. We have recently shown that the chemokine receptor XCR1 is selectively expressed on antigen cross-presenting dendritic cells (DC), the key players in the induction of CD8$^+$ T cell cytotoxicity. To target antigen into these DC, a mAb to murine XCR1 or alternatively XCL1, the natural chemokine ligand for XCR1, were fused to defined antigens using recombinant techniques. A single i.v. or s.c. injection of small amounts (1.0-2.5 µg) of either targeting reagent, applied together with an adjuvant, induced within 5 days a substantial expansion of antigen-specific CD8$^+$ T cells (3-5% of the CD8$^+$ T cell pool, 200,000 cells total, i.e. an approximately 1,000-fold expansion from the estimated 200 precursors in a naïve mouse). When this priming step was combined with a non-viral boost regime including complexed IL2, this resulted in a dramatic expansion of the antigen-specific cytotoxic CD8$^+$ T cells in the spleen on day 12 (70% of the CD8$^+$ T cell pool, 70% of all splenic cells, 200 x10^6 antigen-specific CD8$^+$ T cells total, i.e. 10^6-fold expansion of the original antigen-specific naïve T cells). To test the biological potency of this vaccination system, mice were inoculated with an aggressive tumor line s.c. When the tumor reached a substantial size (diameter of 5-7 mm, around day 6), mice were primed and boosted. Within hours of applying the boosting step, the tumor tissue showed signs of massive necrosis and was eliminated within few days. We believe that we have achieved a new dimension in the induction of CD8$^+$ T cell cytotoxicity in vivo with a system which could directly be transferred into the human field.

The GAPVAC consortium: Translating the concept of actively personalized cancer immunotherapy into the clinic

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Personalized medicine typically means biomarker-guided, selected use of a standard drug product. The Glioma Actively Personalized Vaccine Consortium (GAPVAC) takes personalization to the next level by engaging multiple tumor and immune biomarker profiles to guide the active tailoring and manufacture of a unique therapeutic cancer vaccine for an individual patient. The resulting highly personalized therapeutics are termed "actively personalized vaccines" (APVACs).

GAPVAC integrates multiple biomarker tools (e.g. genomics including next-generation sequencing (NGS), HLA peptidomics, transcriptomics and immunogenicity screening) and bioinformatics approaches to create two custom-made multi-peptide vaccines, manufactured for each patient participating in a phase I clinical trial.

A multi-disciplinary process for the APVAC drug development has been designed and its feasibility has been assessed in test runs prior to entering a clinical trial: Biomarker analyses have been performed on tumor samples from patients and data have been integrated for selection of two personalized drug products. The first set of peptides (APVAC1) is selected according to the individual immunopeptidome and immune system of a patient from a pre-defined warehouse consisting of shared tumor-associated peptides. APVAC2 peptides are selected based on three modules: (i) HLA-identified mutation-containing peptides that have been identified by NGS and in the tumor HLA ligandome, (ii) mutation-containing peptides with predicted MHC binding and (iii) non-mutated peptides not contained in the warehouse with strong over-presentation. The principles applied in the selection process will consider expression of selected gene candidates in the tumor (efficacy) and in healthy tissues (safety), predicted immuno-

genicity, and confirmed relevance for the sample specimen analysed.

As pre-requisite for entering into clinical development, we demonstrate that development of custom-made drug products - from target discovery to start of GMP manufacturing - is feasible within 7 and 14 weeks for APVAC 1 and APVAC 2, respectively. It is targeted to further decrease this turnover time with further development.

Based on principal discussions with the European Medicines Agency (EMA) Innovation Task Force (ITF) by the CIMT Regulatory Research group and a thorough discussion by the consortium of the GAPVAC clinical phase I study with the German national regulatory agency (PEI), a phase I study is now in preparation. The study aims to assess the feasibility, safety and biological efficacy of the APVAC concept for patients suffering from newly diagnosed glioblastoma. The trial will start mid 2014 in 6 European countries and the USA and will be accompanied by a comprehensive biomarker program to assess the mechanism-of-action and to support further clinical development.

The GAPVAC project is funded by the European Union FP7 and led by immatics biotechnologies GmbH (Tuebingen, Germany) and BioNTech AG (Mainz, Germany).

iVacALL: Personalized peptide-vaccination for pediatric acute lymphoblastic leukemia patients based on patient-individual tumor-specific variants determined by whole exome and transcriptome sequencing

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One prominent characteristic of cancer is the accumulation of somatic mutations. These often unique tumor-specific changes, e.g. single nucleotide variants (SNVs), can lead to altered protein sequences and thus to novel peptide ligands presented by MHC molecules of the transformed cell. Being truly tumor-specific, these mutated neoantigens represent an ideal T cell target.

Acute lymphoblastic leukaemia (ALL) is the most common pediatric malignancy. Although about 80% of the pediatric patients can successfully be treated by standard therapy, up to 25% develop a relapse with a dismal prognosis. Preventing a relapse after first-line chemotherapy or stem-cell transplantation is therefore mandatory. A vaccination against mutated leukemia-specific structures is promising, since neither tolerance mechanisms nor autoimmune reactions would impair the positive outcome of the therapy. Peptide vaccinations have been shown to be safe and effective in various settings.

Therefore we are establishing a patient-individual ALL-specific peptide-vaccination strategy combining next generation sequencing (NGS) of the whole exome and the transcriptome of the patients' tumor and normal tissue with *in silico* epitope prediction using SYFPEITHI, NetMHC and NetMHCpan for the patients' individual HLA type.

Whole exome sequencing was performed for 14 B-lineage ALL, one T-ALL blasts and autologous fibroblasts DNA. ALL-specific SNVs as well as insertions and deletions (InDels) were identified using a comparative bioinformatics pipeline. The expression of the mutations was determined by next generation RNA sequencing of the patients' ALL blasts. The determined variants were further validated by deep sequencing in 9/14 patients, with an average of 12 (± 8) validated mutations per patient. In the tumor cells of the T-ALL patient, 51 SNVs and 10 InDels were detected, 16 of 20 selected variants were validated. For all patients with validated variants, MHC class I and MHC class II epitopes were successfully predicted.

An individual peptide vaccine for one haploidentically transplanted patient with relapsed B-lineage ALL was compiled. Five peptides, MHC class I as well as MHC class II restricted, were selected out of epitope predictions for seven validated mutations. Four of the peptides were synthesized successfully. The patient has been vaccinated with the peptides in a prophylactic setting using GMCSF and Imiquimod as adjuvants. The vaccination has been well tolerated.

Two $CD4^+$ T cell clones from a healthy donor showed a specific recognition of a MHC class II peptide which was part of the vaccine cocktail. The cells did not respond to the wild type peptide, underlining the specificity of the approach.

Targeting c-MET and RIG-I with 5‘ppp-modified siRNA for immunotherapy of hepatocellular carcinoma (HCC)

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Background: HCC is the most common primary malignant type of liver cancer. Median survival in advanced tumors is less than one year. The tyrosine kinase c-MET, the receptor for hepatocellular growth factor (HGF), is frequently overexpressed in HCC and regulates tumor cell motility, proliferation and angiogenesis. Inhibition of this pathway, e.g. via siRNA-mediated gene silencing, is an interesting therapeutic option. Next to oncogenic pathways, immunosuppression plays an important role in tumor progression. Thus, novel therapeutic approaches should aim at breaking tumor-induced immune failure. Modification of siRNA as 5‘-triphosphat-RNA (ppp-RNA) allows combining gene silencing of oncogenic targets with activation of the cytosolic immune-receptor retinoic acid-inducible protein I (RIG-I). RIG-I recognizes viral ppp-RNA and activates innate and adaptive immunity via type I IFN and proinflammatory cytokines. In addition, it promotes the intrinsic pathway of apoptosis in tumor cells. This project focuses on the development of a bifunctional 5´ppp-siRNA targeting c-MET for HCC therapy.

Methods: Functional RIG-I expression was analyzed in human and the murine HCC cell line Hepa1-6 after stimulation with ppp-RNA. siRNAs were designed and evaluated for their silencing activity of c-MET. A bifunctional ppp-siRNA targeting c-MET (ppp-cMET) was generated via in vitro transcription using the T7 polymerase system and evaluated *in vitro* for its dual activities. The therapeutic *in vivo* efficacy was tested in a murine HCC model. For this purpose Hepa1-6 cells were orthotopically transplanted into the left liver lobe of C57BL/6 mice. Seven days after tumor induction RNAs complexed to *in vivo*-jetPEI™ were injected intravenously via the tail vein. 24 h later mice were sacrificed and tumor size was determined. Apoptosis was assessed by TUNNEL staining (IHC).

Results: Treatment of human and murine HCC cells with ppp-RNA upregulated RIG-I expression, indicative of intact RIG-I signaling. In addition, RIG-I activation induced phosphorylation of IRF3, production of IFN-b and CXCL10, as well as tumor cell apoptosis. Silencing of c-MET in tumor cells via siRNA significantly reduced cell viability. Both functions, c-MET silencing and RIG-I activation, were effectively combined in ppp-cMET, leading to profound tumor cell death. A single treatment of mice with orthotopic HCC with ppp-RNA, cMET siRNA and ppp-cMET significantly reduced tumor size in comparison to control RNA. A trend towards superior activity was seen in this short setting for ppp-cMET, leading to massive tumor cell apoptosis.

Conclusion: We could demonstrate that the RIG-I pathway is functional in HCC cells. Both, RIG-I activation and c-MET silencing lead to tumor cell apoptosis and can be combined in a single ppp-cMET molecule. *In vivo* experiments demonstrated high efficacy of RIG-I-based immunotherapy with most promising results for bifunctional ppp-siRNA.

Cripto-1 encoding DNA vaccine elicits tumor protective immune response in vivo

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DNA vaccines have a great potential in the field of tumor immunology. The *in vivo* expression of plasmid-encoded antigen generates innate and antigen-specific adaptive immune responses. Targeting broadly expressed tumor-associated antigens will increase the applicability of DNA vaccines against cancer. Cripto-1, a glycoprotein that plays a critical role during embryogenesis but is expressed in low amounts on normal differentiated tissues, has been shown to be overexpressed in more than 50% of human carcinomas. These include melanomas as well breast cancers. Cripto-1 is involved in multiple cellular processes such as cell-proliferation, migration, epithelial-mesenchymal-transition as well as tumor-related angiogenesis. DNA vaccines offer an attractive immunotherapeutic platform from which to study the feasibility of targeting Cripto-1 in mouse models. In this study, the ability of DNA vaccines encoding Cripto-1 to induce a tumor protective adaptive immune response was tested. We show protection against mouse Cripto-1 (mCripto-1) transduced D2F2 breast cancer model in Balb/c mice after immunization with human Cripto-1 (hCripto-1) or mCripto-1 encoding DNA vaccines. The vaccination with hCripto-1 as well as with mCripto-1 in Balb/c mice led to the generation of mCripto-1 specific antibodies. Immunization with hCripto-1 encoding DNA vaccine protected C57Bl/6 mice against endogenously mCripto-1 expressing melanoma B16F10. Tumor infiltrating T cells were found Balb/c mice vaccinated with mCripto-1 encoding DNA vaccine, while the same was not found for mice vaccinated with hCripto-1. This suggests that xenogenic hCripto-1 vaccine elicits a more humoral based immunity than syngenic mCripto-1 vaccine which generates cellular immunity as well. In summary, our data indicates that DNA vaccination with either hCripto-1 or mCripto-1 results in a protective immune response against Cripto-1 expressing tumors, which could lead to the development of therapeutic tumor vaccines to be tested in the clinic.

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Gene optimization can generate cryptic epitopes that induce T cell responses

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Codon optimization of nucleotide sequences is a widely used method to yield high levels of gene expression for gene therapy approaches or to increase the immunogenicity of nucleic acid-based vaccines. To overcome poor expression levels of human papillomavirus (HPV) E6 and E7, which play a causative role in cervical cancer, we codon-optimized both genes. E6 and E7 mRNA-pulsed dendritic cells were used to stimulate and screen for antigen-specific T cells. Candidate T cell receptor (TCR) genes were identified and cloned into a retroviral vector to characterize them with TCR-transduced peripheral blood lymphocytes (PBLs). We successfully isolated four TCRs with different V-alpha and V-beta chains from two unrelated donors, which recognized target cells harboring the codon-optimized E7 gene but not the wild type sequence. Epitope mapping surprisingly revealed a cryptic epitope at the 5‘ end of the codon-optimized E7 as target antigen for all TCRs. The -1nt alternative reading frame from the codon-optimized sequence translated for an artificial protein sequence, which is not encoded by the wild type E7 sequence due to the exchange of wobble nucleotides in the +0 reading frame for optimal codon usage. The resulting cryptic epitope is unrelated to any human or viral sequence, performs at excellent levels in epitope prediction servers regarding proteasome cleavage, TAP transport and binding affinity to the TCR restriction element HLA-B*27:05 and thus is an highly immunogenic antigen.

Mechanisms behind the phenomenon of protein translation initiated else but by the classical ATG start codon might be translational initiation noise or the active generation of defective ribosomal products (DriPs).

In conclusion, codon optimization of a nucleotide sequence can generate immunogenic cryptic epitopes from alternative open reading frames, which induce T cell responses that may lead to the rejection of engrafted cells after gene therapy or false positive immune responses after DNA vaccination. This novel finding is of great importance for the field of gene therapy and vaccination when designing nucleotide-based constructs to avoid unwanted immunogenic side effects.

Immunotargeting human melanoma (MM) stem cells using combination of therapeutic vaccination and surgery

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Introduction: Local tumor immunosuppression followed by systemic one is turning off the natural anti-cancer immune mechanisms and blocking effects of therapeutic vaccination. Targeting of immune check-points (a-PD1) may lead to tumor eradication providing the specific immune response exists. In contrast, CTLA4 eradicates only small faction of tumors, while extending survival of same patients. Combination of active immunostimulation with breaking tumor related immunosuppression is required. In adjuvant setting we have combined therapeutic vaccination with surgery of recurring tumors not responding to re-vaccination. Surgery was used to eradicate source of tumor related suppression.

Methods: Genetic whole cell MM vaccine (AGI-101H) comprises 2 cell lines modified with Hyper-IL-6 (H6) cDNA. H6 is composed of IL-6 and its soluble α receptor. H6 directly binds to IL-6 receptor β and activates JAK-STAT pathway. At the site of vaccination H6 delivers immune co-stimulatory signals. During vaccine manufacturing H6 auto-activates AGI-101 cells what alters their phenotype towards MM stem/initiating cells (CICs). 92% of AGI-101H cells show ALDH activity and lack of differentiation receptors. ALDH1A1 isoenzyme may account for direct induction of immune response that target CICs. Vaccine is administered 8 x every 2 weeks (induction) and then every month (maintenance). In progression re-induction is applied. If not successful, surgery is performed and maintenance continued until patient death. From selected HLA-A2 positive vaccinated MM patients PBMC were isolated and cryopreserved (before vaccine and after 6 or 11 days). Controls were untreated MM pts. and healthy donors. To enumerate ALDH1A1-specific $CD8^{t}$ T cells freshly isolated PBMC were stained with MHC Dextramer®. The effector functions of CTLs and T helper cryopreserved PBMC were analysed using granzyme-B, IFN-g and IL-2 ELISpot.

Results: Patients of two trials were evaluated (97 and 99, respectively). The primary endpoint was DFS, the secondary OS. Median followup was 10.5 and 6.2 years, respectively (as by 1.01.2011). An extension of DFS and OS was observed, as compared to non-treated controls. DFS probability at 5 years was respectively, 54.8% and 40.6% for stage IIIB, 25.0% and 24.0% for IIIC, and 8.5% and 17.7% for IV. OS probability at 5 years was respectively, 66.7% and 56.3% for IIIB, 43.8% and 39.8% for IIIC, and 26.1% and 41.2% for IV. Dextramer staining revealed increased number of ALDH1A1-specific $CD8^{+}$ T cells in vaccinated patients v. healthy controls. There was a significant increased of ALDH1A1-specific $CD8^{+}$ T cells after single vaccine dose. Preliminary data of ELISpot assays showed that vaccination with AGI-101H induces functionally active ALD1HA1-specific CTLs and T helper lymphocytes.

Conclusions: Combination of therapeutic vaccination and surgery seems to be effective strategy in advanced MM patients in adjuvant setting. The effectiveness may be related to targeting CICs.

AdCD40L immunostimulatory gene therapy in combination with cyclophosphamide prolongs 6-months survival in a phase I/II trial for malignant melanoma

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AdCD40L is an immunostimulatory gene therapy vector based on a replication deficient adenovirus serotype 5 that can deliver the human CD40 ligand (CD40L) gene into the tumor. CD40L is a potent stimulator of dendritic cells that in turn activate T cells, NK cells and M1 macrophages against the tumor. AdCD40L has shown promising effects in various tumor models, dog melanoma patients and a Phase I/II trial of bladder cancer. Herein, we present clinical results from 15 patients with disseminated malignant melanoma that were treated with local AdCD40L immunotherapy.

Fifteen patients were enrolled in a phase I/IIa trial. The trial was divided in Part A and Part B. In Part A, patients (n=6) were treated with ultrasound-guided intratumoral injection of AdCD40L (2.5x10e11 vp/mL) at four occasions, one week apart. In Part B, patients (n=9) were treated in the same manner. However, one day prior to the first and fourth AdCD40L injection patients received low dose intravenous cyclophosphamide (300mg/m2). At enrollment, the patients were analyzed for baseline tumor load using both MRI and PET. At week 6 and 9 post treatment, MRI and PET were repeated and the responses analyzed according to RECIST criteria. The patients were thereafter evaluated for 6-months survival. Biopsies and blood were taken before and at different time points post treatment for toxicity and immunological evaluation.

AdCD40L treatment was safe. The most common adverse event connected to AdCD40L was fever, transient increase of liver enzymes and pain at injection site. In Part A, occasional patients had reduced tumor activity according to PET but RECIST criteria revealed only one patient with stable disease (SD) whereas the remaining had progressive disease (PD). In Part B, six of the patients had reduced tumor activity but at week 9 only two of nine patients had SD and the remaining were in progression. However, at 6 months survival only one of six (17%) patients was still alive in Part A while six of nine (67%) were still alive in Part B. The log rank test showed that the survival curves were significantly different (p=0.0475). Hence, AdCD40L therapy reinforced by cyclophosphamide preconditioning did not significantly reduce tumor load according to RECIST criteria but prolonged the survival of melanoma patients in our Phase I/II trial.

AL and AM are shared first authors
THT and GU are shared last authors
THT is the sponsor

Novel promising anti-cancer vaccine candidate based on HBcAg virus-like particles

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Virus-like particles (VLPs) are formed by structural virus proteins which have the intrinsic property to self-assemble into multisubunit, highly repetitive protein structures. The strong humoral immunogenicity of the VLP is transmitted to inserted heterologous peptides including self-antigen-derived peptides. By combining the VLP carrier with tumor associated antigens, self tolerance can be overcome. This qualifies VLPs as an efficient antigen carrier system for anti-cancer vaccine development.

Recently, we developed a versatile antigen display platform based on modified hepatitis B virus core antigen (HBcAg) derived VLPs. We analyzed the effectiveness to elicit a strong cancer antigen specific humoral immune response in relevant animal models by chimeric HBcAg-VLPs displaying a surface epitope of the cancer associated tight junction protein claudin Claudin6.

Ultrapure chimeric VLPs were used for immunization studies in rabbits. We could demonstrate by immunofluorescence and FACS analysis that the induced auto-antibodies were capable to recognize Claudin6 in its native conformation on the surface of transfected HEK293 cells. This recognition is highly specific and no cross-reactivity to close claudin orthologs was detectable, which is a prerequisite for a successful vaccine development. Additionally, Claudin6 specific IgGs are effectively lysing CT26 tumor cells by Fc-mediated complement-dependent cytotoxicity (CDC).The potential of these chimeric VLPs to decelerate tumor growth was demonstrated in a CT26 tumor mouse model.

Antitumor activity and immunogenicity of recombinant vaccinia virus expressing HPV 16 E7 protein SigE7LAMP is enhanced by high-level coexpression of IGFBP-3

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The aim of this study was to investigate, whether the tumor- suppressive properties of Insulin-like growth factor-binding protein-3 (IGFBP-3) can be used to enhance the efficacy of therapeutic immunization against the HPV16 E7 oncogene associated with cervical cancer. We constructed recombinant vaccinia viruses (rVACV) co-expressing the immunogen SigE7Lamp and IGFBP-3. The expression of IGFBP-3 was controlled either by the early promoter H5 or by the synthetic early-late (E/L) promoter. Our results show that IGFBP-3 expression under the H5 promoter (P13-SigE7Lamp-H5-IGFBP-3) is significantly higher than under the E/L promoter (P13-SigE7Lamp-E/L-IGFBP-3) (***$p < 0.001$). The mice bearing TC-1 tumors treated with P13-SigE7Lamp-H5-IGFBP-3 had significantly smaller tumors than PBS treated mice by day 11 (*$p < 0.05$), whereas mice treated with P13-SigE7Lamp-E/L-IGFBP-3 or the control virus P13-SigE7Lamp-TK$^-$ had significantly smaller tumors by day 18 (***$p < 0.001$). Co-expression of IGFBP-3 increased T cell response against VACV antigen but not against HPV16 E7 antigen as determined by ELISPOT IFN-γ. *In vitro* analysis of virus multiplication has shown that P13-SigE7Lamp-H5-IGFBP-3 has significantly higher replication rate than P13-SigE7Lamp-TK$^-$ or P13-SigE7Lamp-E/L-IGFBP-3 (***$p < 0.001$) and that adding an IGFBP-3 neutralizing antibody significantly decreased yields of P13-SigE7Lamp-H5-IGFBP-3 (**$p < 0.01$).

Furthermore, high-level expression of IGFBP-3 was associated with a higher adsorption rate of P13-SigE7Lamp-H5-IGFBP-3 to CV-1 cells when compared with P13-SigE7Lamp-TK$^-$ (*$p = 0.0205$).

Structural analysis of the intracellular mature virion (IMV) of the IGFBP-3-expressing virus P13-SigE7Lamp-H5-IGFBP-3 has found two differences. Firstly, using western-blot we detected insertion of IGFBP-3 into virions of P13-SigE7Lamp-H5-IGFBP-3. Secondly, flow cytometry using microbeads covered with immobilized purified VACV virions, has shown that IMVs of the P13-SigE7Lamp-H5-IGFBP-3 have elevated phosphatidylserine (PS) exposure on the outer membrane that could result in increased uptake of IMVs by macropinocytosis.

Randomized phase II study of personalized peptide vaccination in patients with advanced bladder cancer progressing after chemotherapy

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Purpose: A personalized selection of the right peptides for each patient could be a novel approach for a cancer vaccine to boost anti-cancer immunity in the majority of patients along with the potential of survival benefits. The purpose of this study was to assess the efficacy and toxicity of personalized peptide vaccination (PPV) as second-line therapy in patients with advanced metastatic bladder cancer.

Methods: We conducted a multicenter, randomized phase II study to compare PPV plus best supportive care (BSC) with BSC alone in patients with advanced metastatic bladder cancer who failed or progressed after first-line platinum-containing regimens. PPV treatment was using maximum of four peptides chosen from 31 candidate peptides according to human leukocyte antigen (HLA) types and peptide-reactive immunoglobulin (IgG) titers, for 12 times of subcutaneously injections (8 injections, weekly; 4 injections, by-weekly). Primary outcome was progression free survival (PFS). Secondary outcomes were overall survival (OS), immune response and toxicity.

Results: From 2010 to 2013, 80 patients were randomly assigned; 38 patients were assigned to PPV plus BSC, and 42 patients were assigned to BSC alone. After median follow-up of 4.5 months, the median OS was 8.3 months on PPV plus BSC versus 4.2 months on BSC alone (hazard ratio [HR], 0.533; log-rank p = 0.0423). PFS was not significantly longer on PPV plus BSC (HR, 0.622; log-rank p = 0.0621). Both treatments were well tolerated, without serious adverse drug reactions. Peptide-specific IgG or cytotoxic T-lymphocyte responses were observed in 20 of 26 patients (77%), or 8 of 19 patients (42%) on PPV plus BSC, respectively.

Conclusions: PPV as second-line therapy in patients with advanced bladder cancer is active and well tolerated improving survival with immune responses. Further large scale, randomized trials are needed to confirm our preliminary results.

Robust induction of type-1 CD8$^+$ T-cell responses in WHO grade II low-grade glioma patients receiving peptide-based vaccines in combination with poly-ICLC

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Background: WHO grade II low-grade gliomas (LGGs) with high risk factors for recurrence are mostly lethal despite current treatments, and novel approaches are needed. We conducted a phase I study to evaluate the safety and immunogenicity of subcutaneous vaccinations with synthetic peptides for glioma-associated antigen (GAA) epitopes in human leukocyte antigen (HLA)-A2$^+$ adults with high-risk LGGs in the following three cohorts: 1) newly diagnosed patients without prior radiation therapy (RT); 2) newly diagnosed patients with prior RT, and 3) recurrent patients.

Methods: GAAs were interleukin-13 receptor (IL-13R)α2, EphA2, Wilms Tumor (WT)1, and Survivin, and synthetic peptides were emulsified in Montanide-ISA-51 and given every 3 weeks for 8 courses with intramuscular injections of Toll-like receptor 3 agonist Polyinosinic-Polycytidylic Acid Stabilized by Lysine and Carboxymethylcellulose (poly-ICLC), followed by q12-week booster vaccines. Primary endpoints were safety and CD8$^+$ T-cell responses against vaccine-targeted GAAs.

Results: Cohorts 1, 2, and 3 enrolled 12, 1, and 10 patients, respectively. No regimen-limiting toxicity has been encountered except for one case with Common Terminology Criteria for Adverse Events (CTCAE) Grade 3 fever (Cohort 1). Enzyme-linked Immuno-SPOT (ELISPOT) assays demonstrated robust and sustained interferon (IFN)-γ responses against at least 3 of the 4 GAA epitopes in 10 and 4 cases of Cohorts 1 and 3, respectively. Cohort 1 patients demonstrated significantly higher IFN-γ ELISPOT responses than Cohort 3 patients, suggesting newly diagnosed patients have superior vaccine-responsiveness to recurrent patients. IFN-γ ELISPOT response levels in this study is significantly higher than those observed in our previous phase I/II study in high-grade glioma patients (Okada et al. JCO 2011). Furthermore, IFN-γ ELISPOT response levels were significantly higher than those for IL-5, indicating effective type-1 skewing by the current regimen. Median progression-free survival (PFS) periods are 21 months (Cohort 1; since diagnosis; range 10-42) and 12 months (Cohort 3; since the 1st vaccine; range 3-26). The only patient with large astrocytoma in Cohort 2 has been progression-free for over 58 months since diagnosis. There was a positive trend between IFN-γ ELISPOT responses and progression-free survival (PFS) in Cohort 3 patients ($P = 0.08$ by The Cox proportional hazards model).

Conclusions: The current regimen is well tolerated and induces robust GAA-specific responses in WHO grade II LGG patients. These results suggest these patients may be suitable populations for vaccine therapy and warrant further evaluations of this approach.

A phase I/II trial of TG01 and Gemcitabine as adjuvant therapy for treating patients with resected adenocarcinoma of the pancreas

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Oncogenic mutations in the RAS genes are present in 80-90% of pancreatic cancers and are attractive targets for immunotherapy. It has previously been reported that RAS peptide vaccination of patients with pancreatic cancer induces specific cellular immune responses in approximately 50% of patients with inoperable/advanced disease; whereas 100% of patients that were treated following resection of their tumour showed induction of immune response *(Gjertsen MK et al., Int. J. Cancer: 92, 441-450 (2001), Weden S et al., Int. J. Cancer: 128, 1120-1128 (2011))*. In the previous studies, RAS peptide vaccination was given as monotherapy to the patients. In an ongoing phase I/II study the effect of RAS peptide vaccine TG01 given prior to and then with gemcitabine is being tested as adjuvant treatment of resected pancreatic cancer. By week 11, TG01 specific immune responses are detected in all of the patients with the combined treatment. Data from the phase I part of the study are presented.

Characterization of tumor-infiltrating lymphocytes following intratumoral administration of a chimeric adenovirus Ad5/3-D24-GMCSF (ONCOS-102) for refractory cancer patients with solid tumors

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ORAL SHORT TALK

Different viral vectors have been tested in clinical trials to generate vaccines to various human diseases. These studies consistently show that adenovirus can both prime and boost immune responses remarkably well. Oncos Therapeutics has developed a novel cancer immunotherapy platform based on engineered oncolytic adenoviruses armed with potent immune-stimulating transgenes. Transgene incorporation is intended to enhance a tumor-specific immune reaction. Presented here are results from immunohistochemical and gene expression analyses of tumor-infiltrating lymphocytes (TILs) in a phase I study testing chimeric oncolytic adenovirus ONCOS-102 in patients with refractory injectable solid tumors.

A total of 12 patients were treated with up to 9 intra-tumoral injections of ONCOS-102 at three different dose levels. Tumor biopsies were collected at baseline and 1 and 2 months after treatment initiation to assess the infiltration of immune cells and to facilitate microarray analysis of gene expression. PBMCs were isolated and the assessment of antigen specificity of $CD8^+$ T cells by IFN-gamma ELISPOT is on-going.

No dose limiting toxicity was identified by the independent data safety monitoring board. ONCOS-102 treatment triggered an innate immune response as measured by an immediate short-term increase in systemic pro-inflammatory cytokines. 4 out of 10 evaluable patients showed stable disease (SD) by RECIST evaluation at 3 months. Stabilization of tumor growth was detected both in injected and non-injected tumors. Systematic analysis of tumor biopsies demonstrated infiltration of $CD8^+$ T cells in 9 out 11 evaluable patients following treatment. Increase in other tumor infiltrating lymphocytes (TILs) was seen concomitantly. Increase in perforin and Granzyme B expression in microarray analysis was seen in biopsies showing the highest numbers of $CD8^+$ T cells suggesting that T cells had a cytotoxic phenotype.

Two patients showing the most striking post-treatment increase in TILs had also high expression levels for genes associated with activated Th1 cells (perforin, granzyme B, granulysin, IFN-gamma) and for genes associated with a Th1 type immunoprofile (IRF-1, X3CL1, CXCL9, CXCL10, CCL5, CCL2) in their post-treatment

biopsies. Both of these patients are still alive. Infiltration of immune cells at the tumor site appeared to be associated with prolonged overall survival (in an exploratory correlation analysis between tumor infiltrating lymphocytes [TILs] and overall survival, a positive association was seen). By the time of abstract submission, IFN-gamma ELISPOT analysis for PBMCs was completed for two patients, one of which showed a prominent post-treatment induction of tumor specific $CD8^+$ T cells.

Local ONCOS-102 treatment induced infiltration of Th1 cells into the tumor site. The apparent association between TILs following treatment and OS suggest an involvement of systemic immune activation in this refractory patient population.

Immunostimulatory cancer therapy using dendritic cell-tumor cell hybrids

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Dendritic cell-tumor cell hybrids (heterokaryons) are under investigation as a means for tumor immunotherapy since they should express MHC class I molecules from both cell types and, thus, present tumor antigens within an immunogenic context. The aim of this work was, therefore, to evaluate the immunostimulatory potential of the heterokaryons in order to understand and boost the efficiency of the vaccines based on these cells. Monocytes from peripheral blood of healthy donor were differentiated, in vitro, into mature dendritic cells (mDCs) in the presence of GM-CSF and IL-4 for seven days, and, in the last two days, also TNF-α. To generate the hybrids, mDCs were fused with cells from the breast cancer cell line MDA-MB-231 by applying an electric pulse. Flow citometry analysis showed that the hybrids expressed both the tumor antigen Her2 and the mDC marker CD11c (percentage of double-positive cells; Mix: 1.9 ± 0.3; Fusion: 16.4 ± 2.8; $p < 0.0001$; $n=14$) and high levels of the molecules CD40, CD83, CD80 and CD86. The hybrids also had higher expression of MHC class I molecules as measured by the median fluorescence intensity of HLA-ABC (Mix: 17.8 ± 3.4; Fusion: 33.2 ± 7.2; $p < 0.05$; $n=4$). The increased expression of MHC class I molecules in the hybrids was due to the co-expression of HLA-ABC from both cell types, as demonstrated by incubating each cell with an anti-HLA-ABC antibody conjugated with a different fluorochrome prior to fusion. The lymphostimulatory ability of the hybrid cells were evaluated after co-culture with autologous T lymphocytes. INF-γ was detected in the supernatant of the co-culture of T cells with the mix and the hybrids but not with the mDCs or tumor cells individually. T cell proliferation was detected in all groups. After re-stimulation with the hybrids, T cells expressed more CD45RO and less CD45RA, indicating the generation of memory T lymphocytes. Also, there was an increased expression of CD69 and a decreased expression of the lymph node homing receptor CD62L (L-selectin) and the negative regulatory protein PD-1 after the second stimulation with the hybrids. Hybrids also induced a lower percentage of $FoxP3^+$ regulatory T cells (13.1%), when compared to the untreated (18.0%) or mix-stimulated T cells (20.8%). Cytotoxic T cells capable of killing tumor cells were also observed after priming with the heterokaryons. Preliminary clinical data from vaccination of neuroblastoma patients with the hybrids showed clinical responses that ranged from complete clinical remission to none. So, these data indicate that dendritic cell-tumor cell hybrids are phenotypically competent to present antigens, express the costimulatory molecules and are able to prime T cells. Also, these hybrids can change the natural course of neuroblastoma disease in some patients.

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Innovative vaccination of melanoma patients with a plasmacytoid dendritic cell line - a phase I clinical trial

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ORAL SHORT TALK
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Clinical Trials

The clinical efficacy of treatments with immune checkpoints shows that natural in vivo cytotoxic T cells can be mobilized for the reduction of tumor mass in melanoma and raises a new interest for immunotherapy in cancer treatment. Numerous vaccine strategies, able to activate these cytotoxic cells, are under development. Regarding cell-based cancer vaccines, autologous myeloid dendritic cells loaded with different sources of tumor antigens have been used without leading to sufficient clinical efficacy.

We have developed and patented an innovative vaccine approach: GeniusVac, which is based on an HLA-A*02 plasmacytoid dendritic cell line (GEN cell line). In preclinical studies, its efficacy in melanoma was demonstrated in vivo in humanized mice and ex vivo with patients' PBMC (Aspord, PlosOne 2010, Aspord JID, 2012).

In June 2013, we initiated a phase I vaccination trial with GeniusVac-Mel4 (the irradiated GEN pDC line loaded with 4 melanoma HLA-A*02-restricted peptides) for 9 patients with metastatic melanoma (stage IIIC or stage IV) who do not respond to at least one line of systemic treatment. They will receive weekly sub-cutaneous injections of GeniusVac-Mel4, in 3 dose-escalating groups. The objectives are to evaluate (i) the tolerability and safety, and (ii) the immune response and clinical efficacy of multiple sub-cutaneous injections of GeniusVac-Mel4. The immune response will be analysed by measuring the percentage of Ag specific $CD8^+$ T lymphocytes, and their cytotoxicity and INFg secretion. The overall tumor response will be evaluated with the guidelines for evaluation criteria for solid tumors (RECIST 1.1) and using the immune-related response criteria (irRC).

The Drug product (DP) has been manufactured in the Grenoble cell therapy unit according to GMP guidelines. The DP is defined as the mix 4 Drug Substances, each one corresponding to GEN cells loaded with one melanoma antigenic peptide (MelA, Mage3, gp100 or Tyr), and irradiated. The DP is stored in freezing bags in liquid nitrogen until the prescription and then thawed, packaged in a syringe, and sent to the clinical center for injection. Identity, microbiological and viral security tests, and tumorigenicity tests are conformed to specifications.

Three patients have already been treated, receiving 3 injections of the 4 million dose. The treatment was safe, and no treatment-associated adverse events have been observed following vaccination. Immunomonitoring and clinical follow-up are ongoing.

If vaccination with GeniusVac-Mel4 turns out to be well tolerated, we will set-up a phase II clinical trial to fully evaluate the induction of specific T cell responses and improvement of overall survival. GeniusVac is a vaccine platform which represents a promising novel approach for treatment of melanoma, and, with few changes in associated peptides, for the treatment of many other cancers.

Combined chemoimmunotherapy of castrate-resistant prostate cancer with dendritic-cell based vaccine DCVAC/Pca

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Background: Appropriate combination of tumor mass reduction and neutralization of tumor-induced immunosuppression might potentiate the induction of anti-tumor immunity. We performed an open label, single arm Phase I/II clinical trial in patients with metastatic castrate resistant prostate cancer (mCRPC) eligible for docetaxel using autologous mature dendritic cells pulsed with killed LNCap prostate cancer cell line, DCVAC/PCa.

Methods: Eligible patients had progressive mCRPC despite androgen deprivation. None of the patients received abiraterone or enzalutamide. DCVAC/PCa treatment consisted of, on average ten doses of 1x107 dendritic cells injected s.c. Treatment comprised of initial 7d administration of metronomic cyclophosphamide, and subsequent 2 doses of DCVAC/PCa. Patients then started docetaxel (75 mg/m2) and prednisone (5 mg twice daily) treatment administered every 3-weeks and DCVAC/PCa was given every 6 weeks up to a maximum number of doses manufactured from one leukapheresis. The primary end point was safety, the secondary endpoint immune response. Overall survival (OS) was compared to the predicted OS according to Halabi and MSKCC nomograms.

Results: Data from twenty-five patients were evaluated. The mean age at the start of immunotherapy was 67 years, median PSA 109 ng/ml and Hb 11,9 g/dl. 48% patients had GS ≥8. No serious DCVAC/PCa-related adverse events have been reported. There were no clinical or laboratory signs of autoimmunity. Median OS was 19 months (95% CI: 14.69-23.31) while the predicted median OS was 12 months (95% CI: 11.19-12.81). We observed no significant changes of the peripheral blood Tregs and MDSCs during the course of the trial. Long-term administration of DCVAC/PCa led to the induction and maintenance of the stable levels of T cells specific against multiple tumor antigens including PSA, NY-ESO1, MAGE-A1 and MAGE-A3.

Conclusions: In patients with mCRPC, the alternate administration of DCVAC/PCa cancer immunotherapy and docetaxel results in the stabilization of the disease progression and longer than expected survival. Chemotherapy does not preclude the induction of tumor specific T cells.

Common mutations in oncogenes/tumor suppressor genes as targets for long peptide anti-cancer vaccination

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ORAL SHORT TALK

Promising targets for immunological therapeutic intervention in cancer therapy are tumor-associated antigens (TAAs). Particularly mutated genes are a source for true tumor-specific antigens (TSAs) because they are exclusively expressed in the tumor and are not shared with normal tissue. Moreover, TSAs reduce the risk of autoimmunity and increase the chance to overcome tolerance compared to non-mutated protein sequences. Taking this idea into consideration, we created a panel of peptides with sequences derived from the most frequently mutated variants of the tumor suppressor Trp53 and the oncoproteins Kras and Braf described for colorectal (CRC) and pancreatic carcinomas. More precisely, we designed long peptides (28-35 amino acids) representing wild-type (wt) or mutated sequences to facilitate a presentation of MHC I and II epitopes. In a first step we screened blood and bone marrow from 26 CRC patients for T cell responses against the chosen mutations by IFNγ ELISpot analysis. We found stronger responses against mutated peptides compared to wt peptides. Furthermore, correlation of the ELISpot results with the abundance of Trp53 and Kras mutations identified in the patients' primary tumors and metastases revealed that patients carrying mutations were more likely to be responsive against wt and mutated peptides than patients with no detectable mutation. Secondly, we vaccinated C57BL/6 mice as well as a HLA-Class I/II humanized mouse strain (β_2m-deficient, HLA-A2/D^b/hβ_2m [HHD chimera] and HLA-DR1 double transgenic) with wt or mutated peptides in a multi-peptide vaccination setting. T cell responses of immunized mice were monitored with flow cytometry by measuring cytokine secretion after antigen-specific *in vitro* restimulation. Thereby we observed simultaneous responses against the majority of the peptides in the vaccine suggesting the induction of a multi-epitope response. Interestingly, some of the mutated peptides induced a significantly higher secretion of cytokines than corresponding wt sequences in both $CD4^+$ as well as $CD8^+$ T cell subsets, indicating mutation-specificity. Next, we investigated the tumor-protective capacity of the vaccination approach. For this purpose, syngenic fibrosarcoma cell lines were generated in the HLA-Class I/II transgenic mice by carcinogen-induced tumorigenesis. In tumor challenge experiments we employed cell lines carrying intrinsic Kras/Trp53 mutations and cell lines which were engineered to express the most-immunogenic mutations found in our vaccination studies. Vaccination with mutated peptides resulted in retarded tumor outgrowth compared to vaccination with wt peptide counterparts for tumors with intrinsic mutations. Surprisingly, animals vaccinated with mutated peptides performed worse in case of highly immunogenic introduced mutations. Preliminary analysis points towards an involvement of T regulatory cells in the detrimental outcome after vaccination with highly immunogenic peptides.

Development of a next generation dendritic cell-based immunotherapy for patients with castration-resistant prostate cancer

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Prostate cancer is the second leading cause of cancer death in men. Anti-androgen therapy is the treatment of choice for patients diagnosed with metastatic disease. However, cancer growth progresses despite hormone ablation therapy in almost all patients after 2-3 years, resulting in castration-resistant prostate cancer (CRPC). CRPC patients have a poor prognosis and current treatment options can prolong overall survival for only a few months. Therefore, novel efficient therapeutic approaches are urgently needed.

Active immunotherapy using *ex vivo* generated dendritic cells (DCs) represents a promising treatment option - and the capacity of DC-based vaccines for activation of tumor-specific T cell responses has been demonstrated in numerous clinical trials. However, the clinical benefit is still not satisfactory. We have developed a new generation of DCs with improved immunogenicity and optimized for the use in cell-based immunotherapy of cancer. Currently this new generation of DCs is analyzed in a clinical trial phase I/IIa for the treatment of patients with acute myeloid leukemia using leukemia-associated antigens. In order to develop a DC vaccine formulation for the treatment of CRPC as a solid tumor indication, we performed an extensive preclinical evaluation of DCs expressing tumor antigens (TAs) associated with prostate cancer.

Monocyte-derived mature DCs were generated from healthy donors within three days by using a maturation cocktail containing a synthetic TLR7/8-agonist. DCs were loaded with mRNA encoding for different TAs and cryopreserved. After thawing DCs were analyzed regarding antigen expression, phenotype and function. Expression of TAs was high and not altered by cryopreservation. Additionally, DCs expressed high levels of CD83 and were negative for CD14, demonstrating a mature phenotype. Moreover, the expression of costimulatory molecules CD80, CD86 and CD40 was high compared to expression of inhibitory molecules CD274 and CD273. DCs secreted high levels of bioactive IL-12p70 and only low levels of IL-10, highlighting their capacity to polarize immune responses towards the Th1/Tc1 phenotype. Furthermore, TA-expressing DCs also had the capacity to induce antigen-specific T cell responses *de novo*.

These studies demonstrated the high potential of our vaccine cells for the activation of anti-tumor immune responses in prostate cancer. The three best candidate antigens will be selected for the final vaccine formulation.

(The last two authors share senior authorship)

Induction of anti-tumor CD8$^+$ T cells and prominent infiltration of lymphocytes with a Th1 polarizing signature to pleural mesothelioma tumor following intratumoral injection of ONCOS-102

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ORAL SHORT TALK
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Clinical Trials

Background: Adenoviruses are excellent immunotherapeutic agents with a unique ability to prime and boost immune responses. Recombinant adenoviruses cause immunogenic cancer cell death and subsequent release of tumor antigens for antigen presenting cells, resulting in the priming of potent tumor-specific immunity. This effect may be further enhanced by immune-stimulating transgenes expressed by the virus. We report a case of a 68-year-old male with asbestos-related malignant pleural mesothelioma with progressive disease in spite of 2 different chemotherapies and radiotherapy. 16 months after diagnosis the patient was treated in a Phase I study with a granulocyte-macrophage colonystimulating factor (GMCSF)-expressing oncolytic adenovirus, Ad5/3-D24-GMCSF (ONCOS-102), for the treatment of solid injectable tumors.

Methods: The patient was treated with 9 intratumoral injections of 3 x 10^{11} viral particles (VP) of ONCOS-102. For down-regulation of tumor supporting regulatory T cells low dose oral cyclophosphamide was included. Tumor biopsies were collected at baseline and 1 and 2 months after treatment initiation. Samples were analysed for tumor infiltrating lymphocytes (TILs) and gene expression with immunohistochemistry and microarray, respectively. Peripheral blood mononuclear cells (PBMCs) were analysed for tumor-specific CD8$^+$ T cell responses in IFNγ ELISPOT.

Results: Only Grade 1 and 2 adverse events according to common terminology for adverse events (CTCEA) were reported. Treatment-related innate immune response manifested by immediate short-term increase of pro-inflammatory cytokines was seen. A prominent post-treatment increase in the number of TILs was observed by immunohistochemical staining in biopsies after treatment compared to baseline. Expression levels of genes encoding cytotoxic factors (perforin, granzyme B, granulysin), Th1 associated factors (IFNγ, IRF1) and Th1 associated chemokines (CCL2, RANTES, CX3CL1, CXCL9 and CXCL10) were markedly elevated in post-treatment tumor biopsies. A prominent post-treatment induction of MAGE3-specific CD8$^+$ T-cells in PBMCs was seen in IFNγ ELISPOT. Further, a late metabolic response was observed in PET imaging 7.5 months after treatment initiation during the follow-up period after the end of trial, with a 47 % decrease in the metabolic activity as measured from the total tumor burden in comparison to the previous imaging at the 6 month time point. This patient is alive after 15 months from treatment initiation and 31 months from diagnosis

Conclusions: Local ONCOS-102 treatment shapes tumor microenvironment leading to increased tumor-specific immune reactivity as demonstrated by post-treatment infiltration of Th1 polarized lymphocytes and induction of MAGE3-specific CD8$^+$ T cells in PBMCs.

Evaluation of a combinatorial cancer immunotherapy approach

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In this work a combination therapy that acts upon the immune suppressive, the innate and specific arm of the immune system is proposed. This combination therapy, which consists out of intratumoral interleukin-12 (IL-12) gene therapy, human tyrosinase (hTyr) DNA vaccination and metronomic cyclophosphamide (CPX), was evaluated in a B16-F10 mice model. Following groups were compared: (1) no treatment, (2) control vector, (3) intratumoral IL-12 gene therapy, (4) intratumoral IL-12 gene therapy + metronomic CPX, (5) intratumoral IL-12 gene therapy + metronomic CPX + hTyr DNA vaccination. Next to clinical efficacy and safety, we characterized acute effects of IL-12 and anti-tumor immune response after a second tumor challenge. All treatment groups showed increased survival and higher cure rates than control groups. Survival of non-cured mice was increased when metronomic CPX was combined with IL-12 gene therapy. Furthermore, mice that received metronomic CPX had significantly lower percentages of regulatory T cells. Addition of the hTyr DNA vaccine increased cure rate and resulted in increased survival compared to other treatment groups. We also demonstrated that the manifest necrosis within days after IL-12 gene therapy is at least partly due to IL-12 mediated activation of NK cells. All cured mice were resistant to a second challenge. A humoral memory response against the tumor cells was observed in all groups that received IL-12 gene therapy, while a cellular memory response was only observed in the vaccinated mice. In conclusion, every component of this combination treatment contributed a unique immunologic trait with associated clinical benefits.

Phase II clinical trial of personalized peptide vaccination for previously treated advanced colorectal cancer

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Purpose: Colorectal cancer (CRC) is one of the major causes of cancer death in the world. Since the prognosis of advanced CRC (aCRC) still remains poor, the development of new therapeutic approaches, including cancer immunotherapy, would be highly desirable. We have developed a novel approach of cancer immunotherapy, named personalized peptide vaccination (PPV), in which vaccine peptides were selected from 31 cytotoxic T lymphocyte (CTL) antigen peptides, based on pre-existing host immunity. Recently conducted clinical trials of PPV for patients with various types of advanced cancers demonstrated the feasibility of this new approach. In the current study, we conducted a clinical study to examine the feasibility of PPV and to identify biomarkers that would be useful for prediction of overall survival (OS) in previously treated aCRC patients.

Patients and methods: A phase II clinical trial of PPV was conducted in aCRC patients, who had failed at least one regimen of standard chemo- and/or targeted therapies. For PPV treatment, a maximum of 4 peptides were selected from 31 candidate peptides based on the HLA class I types and antigen-specific humoral immune responses before vaccination, and subcutaneously administered (6 vaccinations, weekly; thereafter, bi-weekly). The primary and secondary endpoints were to analyze the clinical feasibility and safety of PPV and to identify biomarkers useful for prediction of OS after PPV in aCRC patients, respectively. Clinical findings and laboratory data before and after vaccination were statistically evaluated.

Results: Sixty previously treated aCRC patients underwent PPV without severe adverse events. Boosting of IgG responses specific to the vaccinated peptides was observed in 47% and 94% of the completed patients at the end of the first and second cycles of 6 vaccinations, respectively. Median OS time was 498 days with one and two year survival rates of 53% and 22%, respectively. Notably, among the enrolled patients, 32 patients, who were refractory to all of irinotecan, oxaliplatin, and fluoropyrimidines before enrollment, showed median OS time of 375 days with one-year survival rate of 51%. Multivariate Cox regression analysis of pre-vaccination factors showed that plasma IL-6, IP-10, and BAFF levels were significantly prognostic of OS [hazard ratio (HR) =1.503, P = 0.045; HR = 1.574, P = 0.025; HR = 0.510, P = 0.002; respectively]. In addition, genetic polymorphisms in the inflammatory factors were potentially prognostic.

Conclusions: The current study demonstrated that PPV induced substantial immune responses to vaccine antigens without severe adverse events and showed potential clinical benefits in previously treated aCRC patients, even in the refractory stage. Next step of clinical trials of PPV would be warranted for aCRC patients. Evaluation of the identified factors before vaccination might be useful for selecting the patients who would benefit from PPV.

The immunogenicity of NY-ESO-1 peptides is primarily determined by the stability of their MHC-peptide complexes

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The advent of personalized T cell cancer vaccines requires detailed knowledge of T cell epitopes of tumor-associated antigens (TAA). By proteomic analysis of MHC class I-bound peptides or *in silico* peptide predictions, hundreds of epitope candidates can be identified. Peptide binding affinity and MHC-peptide (pMHC) stability have been argued to determine CTL immunogenicity. To clarify which of these parameters is the more reliable predictor, we developed different high-throughput assays.

We performed 1) *in silico* prediction of HLA-A*0201 restricted 8 to 11-mer peptides of the NY-ESO-1 cancer testis antigen. Candidate peptides were subjected to 2) micro peptide-driven refolding of HLA-A*0201 heavy and light chain, 3) peptide rebinding to *in situ* peptide stripped MHC molecules 4) pMHC half-life measurements at 37 °C and 5) T2 cell stabilization assay.

Our results indicate partial reliability of *in silico* epitope prediction. Results obtained by micro peptide-driven refolding and pMHC renaturation partially diverge, with the latter assay being less sensitive. Surprisingly, little correlations were observed between peptide affinity for HLA-A*0201 protein and the stability of the complexes. Finally, the T2 stabilization assay was shown to be the less robust and sensitive assay. We classified candidate peptides into three different groups: a) good binder and good stability, b) good binder and poor stability and c) poor binder and poor stability. HLA-A*0201 transgenic mice were immunized with these peptides and preliminary results indicate that immunogenicity correlates primarily with the stability of pMHC complexes and not the binding affinity. In addition, our results uncover two new potential NY-ESO-I epitopes. Further optimization and miniaturization of our assays will enable scientists to determine new immunogenic epitopes and to guide the development of new therapeutic vaccines.

Next-generation dendritic cell vaccination as postremission therapy in AML

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ORAL SHORT TALK

Postremission therapy of patients with acute myeloid leukemia (AML) is critical for the elimination of minimal residual disease (MRD) and a prerequisite for achieving cure. Cellular immunotherapy is a highly effective treatment option as demonstrated by the low relapse rate after allogeneic stem cell transplantation (SCT). However, many patients are not eligible for this treatment. Therapeutic vaccination with autologous, antigen-loaded dendritic cells (DCs) is a promising strategy to induce cellular and humoral immune responses. Within recent years, we have developed a GMP-compliant protocol for the generation of *next-generation* DCs. A short, 3-day differentiation period is combined with a novel maturation cocktail that includes a TLR7/8 agonist. The resulting DCs are characterized by a positive costimulatory profile and a high production of bioactive IL-12p70. Both *in vitro* and *in vivo*, they have been shown to polarize $CD4^+$ T cells into Th1, to induce antigen-specific $CD8^+$ T cells and to activate NK cells.

We are currently conducting a proof-of-concept phase I/II clinical trial evaluating *next-generation* DCs as postremission therapy for AML patients with a non-favorable risk profile (NCT01734304). Standard exclusion criteria apply, and patients have to be ineligible for allogeneic SCT. DCs are generated from monocytes of the patients and then loaded with ivt-RNA encoding the leukemia-associated antigens WT1 and PRAME. Additionally, DCs transfected with RNA encoding CMV-pp65 are included as an adjuvant and surrogate antigen. Patients are vaccinated intradermally with one batch of $5x10^6$ DCs for each of the three antigens up to 10 times within 26 weeks. The primary endpoint of the trial is feasibility and safety. Secondary endpoints are immune responses and disease control, with particular focus on MRD conversion. Phase I will include 6 patients, and phase II another 14 patients. So far, two patients have been enrolled into the phase I of the trial. Pt. 1 was a 72 year-old man in CR with an adverse genetic risk profile (complex karyotype) and not eligible for allogeneic SCT. The differential blood count showed 11% monocytes of 7,6 G/l leukocytes (836 monocytes/µl). The leukapheresis yielded $3,4x10^9$ monocytes in total, and 14 vials of DCs per antigen were generated for clinical application. Pt. 2 was a 54 year-old man in CRi with an intermediate genetic risk group (cytogenetic abnormality not classified as favorable or adverse) and no HLA-matched donor. The differential blood count showed 7% monocytes of 5,9 G/l leukocytes (413 monocytes/µl). The leukapheresis yielded $2,2x10^9$ monocytes in total, and 6 vials of DCs per antigen were generated for clinical application. For both patients, the DCs fulfilled all quality criteria (cell count, viability, purity, sterility, phenotype). No adverse events have been observed so far except for slight erythema at the injection site. Up-to-date clinical and immunomonitoring data will be presented.

Soluble MHC-dimer molecules activate antigen-specific T cells in vivo

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Induction of a T cell mediated immune response is critical for the eradication of viral infections and tumors. Soluble peptide loaded major histocompatibility complex-Ig ($^{pep-}$MHC-Ig) bind their cognate ligands, T cell receptor (TCR), with high affinity and have been successfully used to visualize antigen-specific T cells. Furthermore, immobilized $^{pep-}$MHC-Ig can activate and expand antigen-specific T cells *in vitro*. Here we show data on the use of $^{pep-}$MHC-Ig as a potential immunization strategy in combination with or without anti-CD40 mAb pre-treatment to modulate antigen-specific T cell immune responses *in vivo*.

$^{SIY-}$K^{b}-Ig immunization of anti-CD40 mAb pre-treated C57BL/6 (B6) mice that previously received adoptively transferred 2C T cells, resulted in stronger T cell induction than in non-pretreated B6 mice. T cell responses after $^{SIY-}$K^{b}-Ig immunization in anti-CD40 mAb pre-treated B6 mice were antigen-specific, long lasting and of memory phenotype. Mechanistic studies using allogenic $^{QL9-}$L^{d}-Ig complexes to stimulate 2C T cell transferred into B6 mice demonstrated that the injected $^{pep-}$MHC-Ig molecules directly activate 2C T cells without antigen-uptake and reprocessing by antigen-presenting cells. Additionally we have demonstrated that $^{SIY-}$K^{b}-Ig immunization in combination with anti-CD40 mAb pre-treatment can induce antigen-specific T cells in naïve mice, which was confirmed by an in vivo killing assay utilizing SIY-pulsed target cells. Thus, soluble $^{pep-}$MHC-Ig molecules represent a powerful tool for in vivo T cell activation that, in contrast to peptide adjuvant formulations, directly stimulates antigen-specific T cells without the need for antigen reprocessing by antigen-presenting cells.

Autologous tumor cells and SW742 allogeneic cell line have comparable stimulating effect on PBMCs of Gastrointestinal malignant patients In vitro

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Background: Natural killer activity is believed to be important contributor of a patient's immune system to fight cancer. However, cancer patients have reportedly defective NK activity and the malignant target frequently has developed mechanisms to escape detection of NK cells. Our research is aimed at overcoming this NK cell deficiency.

Materials and methods: Malignant autologous epithelial cells of 10 colorectal carcinoma patients were separated by cell culture procedures. Peripheral blood mononuclear cells (PBMCs) were stimulated with their mitomycin treated autologous tumor cells or allogeneic SW742 colorectal carcinoma cell line. The expression of CD3, CD56, NKG2D and NKp44 were detected with flowcytometry and reverse transcription-PCR. NK activity of PBMCs against K562 target cell line was measured by MTT colorimetric assay.

Results: Stimulation with autologous tumor cells and allogeneic SW742 colorectal carcinoma cell line augmented $CD56^+$ and $CD56^+CD3^+$ cells and up-regulated NKG2D and NKp44 expression. NK activity of PBMCs after co-incubation with autologous tumor cells or SW742 was significantly raised.

Conclusions: Our results demonstrated that stimulation of PBMCs by SW742 can significantly improve NK activity as much as by autologous tumor cells which was confirmed by the higher expression of NKp44 and NKG2D. Since the separation of autologous tumor cells is difficult and time consuming the allogeneic tumor cell line could be a good replacement for large scale short term generation of activated NK cells. These data may help to improve cancer immunotherapy protocols.

Key words: Tumor immunotherapy, Natural Killer activity, Autologous Tumor cells, SW742, NKp44, NKG2D

Idiotype vaccines produced with a non-cytopathic alphavirus vector induce potent antitumoral responses in a murine model of B-cell lymphoma

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A promising therapy for patients with B-cell lymphoma is based on the use of idiotype antibodies as vaccines. This kind of vaccines are based on the use of immunoglobulins having a variable region with epitopes exclusively expressed by a lymphoma. Since these epitopes are different for each tumor, a specific idiotype vaccine has to be produced for each patient. Expression of immunoglobulins for human use often requires the use of mammalian cells because these cells are able to provide appropriate post-translational modifications that cannot be obtained with other systems. Production of proteins for clinical use can be greatly improved by the use of stable cell lines that can be scaled up to reach the desired level of production. We have used a noncytopathic Semliki Forest virus (ncSFV) RNA vector to generate stable cell lines expressing the murine follicular lymphoma-derived idiotype antibody A20. We generated ncSFV vectors harboring sequences of heavy (HC) and ligth (LC) chains of A20 using four different genetic constructs: 1) HC followed by LC using independent viral promoters, 2) LC followed by HC using independent viral promoters, 3) HC fused to LC using the foot and mouth disease virus 2A autoprotease sequence as a linker, and 4) LC and HC separated by an IRES sequence. All recombinant ncSFV vectors allowed rapid and efficient selection of stable BHK cell lines that expressed A20 in supernatants with a proper quaternary structure, as evidenced by non-reducing SDS-PAGE. Expression levels were similar in constructs 1-3, reaching up to 8 mg/L/24h, but they were about ten-fold lower when HC expression was driven by the IRES element (construct 4). A20 produced by SFV stable cell lines was highly glycosylated with a pattern similar to that of A20 produced by a hybridoma. A20 purified from the supernatant of ncSFV cell line harboring construct 1 was conjugated to KLH and used to immunize mice that received four monthly doses of 25 mg of idiotype. A secong group of mice received the same doses of A20 purified from the hybridoma. Both idiotypes were able to induce similar titers of anti-A20 antibodies. Interestingly, when mice were challenged with A20 lymphoma cells a stronger protection was observed in animals vaccinated with the ncSFV derived idiotype, resulting in much smaller tumors and longer survival in this group. These results indicate that the ncSFV vector could represent a quick and efficient system to produce patient-specific idiotypes with potential application as lymphoma vaccines.

Combined immunotherapy against cancer: Limited efficacy of transcutaneous immunization and low-dose Cyclophosphamide

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Transcutaneous immunization (TCI) is a novel vaccination strategy with a promising potential for combating tumors or persistent infectious diseases. Based on a T cell epitope and the Toll-like Receptor 7 agonist imiquimod, we have established a protocol that induces a potent cytotoxic T lymphocyte (CTL) response. However, TCI shows only limited effectiveness in terms of tumor protection, in part due to suppression by regulatory T cells (Treg). In another instance, cytotoxic drugs like cyclophosphamide (Cy) are currently the basis for the treatment of tumors including malignant lymphomas. Beyond this, low-dose Cy therapy can mediate inactivation of Treg. Hence, we conducted our present work to evaluate the efficacy and mechanisms of combined cytotoxic treatment with TCI.

Hence we performed TCI by applying imiquimod crème together with the CTL epitope SIINFEKL (d0/1) on the shaved back skin of C57BL/6 mice after i.p. injection of various doses Cy (25, 50, 100 mg/kg, d-1). Induction of CTL responses was assessed by the frequency of SIINFEKL specific $CD8^+$T cells and *in vivo* cytolysis of target cells (d6). Here, we observed no significant decrease in the induced CTL responses comparing TCI with or without Cy indicating that Cy does not impair TCI-induced CTL responses at the chosen dose levels.

To understand the mechanisms of Cy in this context, we first analyzed the frequency of Treg upon Cy/TCI and found a dose dependent decrease in Treg numbers compared to the untreated control. In contrast, TCI alone only led to a marginal increase in Treg numbers. Next we investigated its influence on DCs as crucial antigen-presenting cells in the draining lymph nodes and spleens upon vaccination. In Cy/TCI treated mice, we found an increased expression of CD80/CD86 compared to untreated or TCI alone with a shift to $CD4^-CD8^-$DCs, indicating stimulatory effects of Cy on DCs. However, despite an enhanced DC activation as well as Treg depletion, Cy/TCI did not result in an enhanced CTL response compared to TCI alone suggesting that Cy induces additional inhibitory factors after combined treatment. We hypothesized that this may be due to the induction of immunosuppressive immature myeloid cells that have been described after low-dose Cy treatment. Indeed, we detected increased numbers of monocytic as well as granulocytic immature myeloid cells in the spleens of Cy/TCI treated mice compared to TCI treated or controls.

Taken together, the combination of low-dose Cy with TCI leads to enhanced DC activation along with a reduction of Treg and does not diminish vaccination-induced immune responses. Therefore, such a combination appears feasible, but we suggest that Cy also induces inhibitory mechanisms that suppress vaccination induced immune responses and may impair the overall effectiveness of immunotherapeutic treatments. Our studies may contribute to the development of new therapeutic options against tumors by combining chemotherapy with immunomodulatory treatments.

Personalized pancreatic cancer vaccine

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Pancreatic cancer is a significant health issue due to the associated high mortality rate and a lack of improvement in survival rates over the last three decades. Therefore we have set out to develop a novel vaccine treatment platform utilizing heterologous prime:boost targeting either a PanC02 tumour associated antigen (TAA) expressed in our tumour model, or mutant peptides comprising the PanC02 mutanome.

To identify the PanC02 mutanome we utilized both whole exome and RNA sequencing technologies to identify mutated genes differentially expressed in the tumour cells compared to wild type C57Bl/6 DNA. We have identified 188 expressed mutated genes in PanC02; and through *in silico* analysis, 99 peptides were identified to have the potential to bind MHC class I. Confirmation of immunogenicity *in vivo* will be carried out using peptide vaccination and viral vectors, expressing multiple peptides, to determine which mutant peptides are truly immunogenic. In the first round of analyses mice were vaccinated with the 12 highest scoring peptides identified using multiple algorithms assessing ability to bind MHC class I. Using ELISpot and flow cytometry 5 of these peptides were confirmed to be immunogenic *in vivo*. We are currently building viruses to assess 24 additional peptides to determine their immunogenicity *in vivo*. Once all 36 peptides have been tested a viral mutanome vaccination platform will be generated using validated immunogenic peptides. These viral vectors will be used to vaccinate mice bearing orthotopic pancreatic tumours and will be compared to vaccination with more traditional tumour associated antigens to determine if a personalized approach to vaccination will be an improvement over vaccinating against a single tumour antigen. These proof-of-principle studies will help determine if a personalized approach to our prime:boost vaccination regimen is a viable option for a cancer in need of novel therapies.

Closing the gap between bench and bedside: Manufacturing individual multi-peptide vaccines for phase I/II clinical trials in cancer immunotherapy

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Current cancer research shows that more than ever new strategies for treatment of cancer patients are necessary; immunotherapy appears to be one very effective way and has witnessed a real breakthrough in the past year. Scientists at our Department have been investigating a huge amount of peptides for many years, which are highly related to tumors and their surroundings. Consequently a way has to be found to translate the research results into real patient treatment which includes the production of pharmaceuticals.

The *Wirkstoffpeptidlabor* at the University of Tübingen established both the production of GMP certified peptides as active pharmaceutical ingredients and a formulation process for vaccine peptide cocktails including different peptides. Multi-epitope peptide vaccines can be composed both specifically for distinct tumor entities and individually for each patient.

Drugs- also for clinical studies- are required to be produced in a highly regulated way. To produce high quality products our lab developed different strategies to supervise each single production step in a tight connection to quality controls. Reproducible product quality seen as a roof is carried by the pillars method validation, process validation, in process controls and qualified staff basing on a stable quality assurance system.

Integrating clinical and genomics data - challenges and opportunities

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Since the end of the Human Genome Project in 2003, rapid technological advances in DNA sequencing have resulted in a remarkable increase in sequencing throughput. The limitation of efficient knowledge discovery in genomics is no longer generation of data, but rather computational challenges around integration of genomics and clinical data. The increasing number of data types generated for patients in health care and clinical trials, together with the inadequate use of standards to support mapping across disparate data sources, vastly limits the utilization of data. Consequently, information integration has become one of the top priorities to be solved by the research community as it constitutes the basis from which further scientific discoveries can be made. In general, analyzing clinical data in combination with genomic data has the potential to reveal the genetic basis of cancer, infectious diseases and drug response, leading ultimately to improved health care and preventative measures for individual patients.

In the presentation we will discuss opportunities to accelerate the progress in biomedicine through the use of integrated patient data in medical research and challenges that have to be overcome before this can become a reality, fully realizing the promise of personalized medicine.

Towards an actively personalized cancer therapy: Patient specific multipeptide vaccination for primary liver cancers

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Over half a million people are diagnosed with hepatocellular carcinoma (HCC) every year. With alcohol abuse and hepatitis C being main risk factors, incidence rates are on the rise, positioning HCC among the leading causes of cancer related death worldwide. Cholangiocellular carcinomas (CCC) on the other hand are a rare tumor entity but associated with a very poor prognosis and often considered incurable. With chemotherapy and other adjuvant strategies showing only little benefit in both entities there is an urgent need for new and more effective therapeutic options. Personalized approaches, particularly immunotherapeutic ones, may represent promising alternatives with regard to effectiveness and safety profile. Here we demonstrate an approach to identify real tumor specific antigens with potential for future clinical application.

Our strategy focuses on naturally processed and presented HLA ligands eluted directly from individual patients´ tumor tissues. First we predict tumor specific peptides, which might arise from patient and tumor specific genetic alterations. To achieve this we perform next generation whole exome sequencing of malignant and benign tissues as reference. The gained knowledge is assembled in databases using bioinformatic pipelines, which enable the identification of tumor specific peptides by tandem mass spectrometry. Additionally we are interested in identifying tumor associated peptides. These are HLA ligands largely overrepresented on patient‘s tumor tissue and (virtually) absent on healthy tissues. Although originating from unmutated genetic regions, those peptides have shown the potential to prolong patient survival. Thus, we built a database of HLA ligands from diverse benign tissues. Crosschecking against this database assists to reject unsuitable peptides and increases the chance for selecting immunogenic ones. Analyzing samples from 18 patients (13 HCC/ 5 CCC), we were able to identify over 5000 different HLA ligands naturally presented on patients´ tumors and over 5000 on benign liver tissue respectively.

Using this strategy we are able to recommend individually tailored multi-peptide vaccines for each patient. Having a GMP peptide production facility available, we plan on applying this workflow for future clinical trials. We should be able to vaccinate patients with such a cocktail within three months after tumor resection, aiming at prevention of metastasis and/or relapse in an adjuvant setting. Once established, this approach should be applicable in many different malignancies.

A TLR agonist-based combination treatment enhances T cell recruitment into gastric tumors

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A major obstacle for the efficacy of T cell-based immunotherapy in gastric cancer is the lack of CD8 T cell infiltration in the tumor. In order to enhance T cell recruitment to the tumor site, we have developed a treatment protocol based on a combination of TLR agonists. To test the efficacy of the TLR-based combination therapy in gastric cancer, we have used transgenic CEA424-SV40 TAg mice that develop autochthonous tumors in the pyloric area of the stomach. In addition, the mice bear subcutaneous tumors that are induced by injection of a cell line derived from the original gastric tumor. Upon adoptive transfer of tumor-specific T cells and treatment with single TLR agonists, infiltration by CD8 T cells and tumor regression were achieved only in subcutaneous but not in autochthonous gastric tumors. To improve T cell recruitment, mice were treated with sequential combinations of TLR agonists that trigger different signaling pathways, leading to an enhanced production of Th1-type cytokines. The combinatorial treatment led to a high recruitment of CD8 T cells to the tumor site not only in subcutaneous tumors but also in autochtonous gastric tumors. In addition, this TLR-based therapy blocks tumor growth of mice with subcutaneously induced tumors. We are currently further investigating the mechanisms leading to the enhanced T cell recruitment. The results of this study may provide an important contribution to the development of complementary immunotherapeutic strategies to target gastric tumors.

Cancer vaccines with hTERT and Survivin mRNA transfected fast DCs - a simplified and effective cancer vaccine

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Prostate cancer (PC) is the most common cancer among men. Since 2005 more than 4000 new cases each year are diagnosed with PC in Norway and the incidence is increasing. In many cases prostate cancer is an indolent disease and patients often will die with the disease and not off it. If the patients are diagnosed with high Gleason Score they will develop relapse following primary therapy and when this occur there is no curative treatment available. We have previously reported that about 50% of hormone resistance patients mount specific immune responses following vaccination with Dendritic Cells (DCs) transfected with mRNA from autologous tumour. Immune responses were also related to overall survival. Initially DCs were produced over 7 days. Recently, we have reduced the production time down to 3 days (Fast DCs) and here we report about our clinical experiences with this new type of DCs. Five metastatic prostate cancer patients have been included in the study. Prior to the DC vaccination, 3 patients had bone metastasis while 2 were diagnosed with lymph node metastasis. The fast dendritic cells (DC) is produced by differentiation of autologous monocytes to mature DCs by adding GM-CSF (2500IU/mL) and IL-4 (1000IU/mL) for 48 hours, and 24 hours of maturation with GM-CSF (2500IU/mL), IL-4 (1000IU/mL), TNF-α (10ng/mL), IL-1β (10ng/mL) and PgE2 (1ug/mL) in CellGro DC medium. Mature Fast DCs were then transfected with hTERT- and Survivin-mRNA by electroporation. After over night incubation in medium without any cytokines added the vaccines were frozen and stored until use. Quality control of the DCs was performed. All mRNA transfected DC (mDCt) showed a mature phenotype with down-regulation of CD14 and up-regulation of CD80, CD83, CD86, CCR7, CD274, CD40 and HLA-DR compared to monocytes. All mDCt showed migration capacity towards CCL19. mDCt had no IL-12p70 secretion and except for one patient with high IL-10 secretion all showed low levels of IL-10. When immune responses were tested by T-cell proliferation, no CD4 T-cell responses could be detected. Two of the patient was HLA-A2 positive and dextramers was used to detect antigen specific CD8 positive T-cells in blood at several time points during the vaccination. Progression free survival (PFS) assessed by PSA measurement and MRI of the 5 patients were 7, 21, 24, 35 and 36 months. The patient vaccinated with DCs secreting high IL-10 has the shortest PFS. Two of the patients have been continuously vaccinated and one patient has been revaccinated following a treatment interval with chemotherapy and local radiotherapy of bone metastasis. Altogether, our fast DCs show objective clinical benefit in some of the patients. Only 2 patients were HLA-A2 positive and in both T-cell responses could be measured by dextramers during the vaccination. The reasons for the lack of CD4T-cell responses in our patients are not fully understood and are under investigation.

Exploring dendritic cell (DC) vaccines targeting Wilms' tumour gene 1 (WT1) and survivin for uterine cancer

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Background: Uterine cancer is the most frequent pelvic gynaecological cancer. For recurrent disease, therapeutic options are limited and short-lasting; hence, new and less toxic treatment modalities are needed. We identified Wilms' tumour gene 1 (WT1) and survivin as tumour-associated antigens expressed in this tumour. We previously applied *WT1*-specific dendritic cell (DC) immunotherapy in 6 uterine cancer patients and observed induction of WT1-specific immune responses and modest clinical responses. Here, we focused on the GMP manufacturing of a DC vaccine electroporated with mRNA encoding both *WT1* and *survivin*, linked to DC-LAMP for helper T cell induction, and co-electroporated with TriMix mRNA (CD40L, CD70 and caTLR4) for DC maturation.

Methods: Three healthy donors underwent leukapheresis and monocytes were isolated by elutriation. Monocytes were cultured in Cellgenix culture bags in the presence of GM-CSF and IL-4 until day 7. Immature DC were harvested, electroporated with mRNA encoding *WT1*-DC.LAMP and TriMix or *survivin*-DC.LAMP and TriMix, mixed and cultured for 3.5-4 h in the presence of rapamycin, IL-4 and GM-CSF before cryopreservation. After thawing, cells were cultured for either 1.5 h or 48 h. We analysed DC viability, phenotype, expression of WT1 and survivin, and cytokine secretion. From 1 HLA-A2 positive donor, DC were used for stimulation of autologous T cells in the presence of IL-2, IL-7, IL-15 and IL-21. Antigen specificity was checked using tetramer staining, CD137 upregulation, intracellular cytokine staining and CFSE proliferation.

Results: DC viability after thawing was more than 65% and purity (CD11c) was more than 75%, with B and T cells as main contaminants. While DC analysed 1.5 h after thawing still showed an immature phenotype, DC were fully mature after 48h, with upregulation of CD80, CD83, CD86 and CCR7. WT1 expression by DC was highest at 1.5 h after thawing and steadily decreased thereafter, while survivin only slightly decreased at 48h after thawing. DC secreted low levels of IL-12p70, intermediate levels of IL-1β and IL-18 and high levels of IL-6, IL-8, MCP-1 and MIP-1α. Upon stimulation of autologous T cells, we observed an increase of survivin-tetramer (survivin 95-104 peptide) positive T cells from almost undetectable (0.08% of $CD8^+$ T cells) to 0.56% of $CD8^+$ T cells after 1 stimulation (7-fold) and to 0.81% of $CD8^+$ T cells after 2 stimulations with DC (10-fold). No induction of WT1-specific T cells was noted. Analysis of CD137 upregulation, intracellular cytokine secretion and proliferation after restimulation of these T cells is ongoing.

Conclusion: We showed the feasibility of generating a monocyte-derived DC vaccine electroporated with mRNA encoding WT1-DC.LAMP and TriMix or survivin-DC.LAMP and TriMix, resulting in highly mature DC with the capacity to induce TAA-specific T cells. Since TAA expression is highest at 1.5 h after thawing, we postulate that this time point would be ideal for vaccination.

Protein transfer vectors: an efficient tool to induce antigen-specific cytotoxic T cell responses

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To induce and trigger innate and adaptive immune responses, antigen presenting cells (APCs) have to take up and process the antigen. Retroviral particles are capable of transferring not only the vectors´ genome, but also foreign cargo proteins into transduced cells when the cargo is genetically fused to structural proteins of the vector (Voelkel *et al.* 2010). Here, we demonstrate the advantage of lentiviral protein transfer vectors (PTV) for targeted antigen transfer directly into APCs and thereby induction of cytotoxic T cell responses.

Targeting of lentiviral PTVs is achieved analogous to gene transfer vectors by pseudotyping the vectors with truncated wild-type measles virus (MV) glycoproteins (GPs), which use human SLAM as main entry receptor. SLAM is expressed on stimulated lymphocytes and APCs, e.g. dendritic cells, and thus represents a potential target structure on APCs. As cargo proteins, the reporter protein eGFP or the model antigen Ova were analyzed.

Analysis of lentiviral eGFP-PTVs on receptor-transgenic CHO cells and B cell lines revealed that vectors pseudotyped with MV_{wt}-GPs mediated specific delivery only into SLAM-positive cells, in contrast to unspecific VSV-G pseudotyped vectors. Accordingly, specific protein transfer of Ova into SLAM-positive cells was evident. Moreover, *ex vivo* treatment of primary APCs, i.e. myeloid dendritic cells (mDCs), with Ova-PTVs resulted in stimulation of Ova-specific T lymphocytes upon co-cultivation with transduced mDCs, revealed by secretion of IL-2 and IFN-g. Thereby, $CD8^+$ T cells were efficiently activated by mDCs transduced with entry-competent PTVs, whereas $CD4^+$ T cells were most vigorously stimulated by mDCs incubated with bald vector particles. These data argue for cytosolic transfer of antigens by targeted PTVs into APCs followed by MHC-I presentation of peptides via the endogenous antigen presentation pathway, whereas solely particle-associated antigen is taken up via the endosomal route followed by MHC-II presentation via the exogenous pathway.

Concluding, SLAM-targeted PTVs have demonstrated specificity and efficacy of antigen transfer into primary APCs to trigger antigen-specific immune activation. As especially $CD8^+$ T cells were activated, SLAM-targeted PTVs may represent a suitable, and due to the absence of integration, safe vaccine especially for induction of cytotoxic immune responses. Since cytotoxic $CD8^+$ T lymphocytes are a mainstay of anti-tumoral immune responses, SLAM-targeted PTVs could be engineered for the transfer of specific tumor antigens provoking tailored anti-tumor immunity.

Role of NK cells in antitumor immunization strategies

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Classical tumor therapy is generally based on surgery combined with radio- and chemotherapy. However, recently additive immunotherapy has gained in impact. Particular vaccination strategies represent a promising approach strengthening the innate and adaptive immune response.

In order to identify immune stimulatory and editing mechanisms of different antitumor immunization strategies, we performed comparative analysis of whole tumor antigen vaccines induced by X-ray, hyperthermia, doxorubicin and high hydrostatic pressure. We characterized these different vaccination products both *in vitro* and by the use of *in vivo* tumor models.

Furthermore, we established a therapy induced *in situ*-immunization model closely resembling clinical treatment of established tumors with combination of hyperthermia and ionizing X-ray.

To describe effector-mechanisms and interactions between T-, B- and NK-cells, we used RAG1$^{-/-}$ as well as NK cell depleted mice in the different immunization models.

Collectively, our results demonstrated that the treatment regimen combining X-ray and hyperthermia induces the best immunogenic effects. Moreover, our *in vivo* results show the bivalent impact of NK cells on antitumor immunity. Despite their direct antitumor effects, NK cells mediate a negative influence on the generation of the favored antitumor immunity during the anti-tumor vaccination period.

Summarized, our preclinical studies indicate high importance for the development of immunotherapeutic protocols and the treatment of solid tumors.

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Cancer is driven by multiple genetic events followed by further clonal evolution, rendering disease elimination with single-targeted drugs a difficult task. The multiplicity of gene mutations derived from sub-clone heterogeneity may represent an ideal setting for multi-epitope tumor vaccination. Vaccines are particularly suited for the expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells and allow precise targeting of tumor-associated molecular alterations. As human cancers carry 100-300 non-synonymous mutations on average which are not subject to central immune tolerance, these mutations can be optimal candidates for vaccine development. We propose that cancer can be efficiently treated by T cells induced by poly-epitopic vaccines based on non-synonymous individual tumor-specific mutations. In order to test this hypothesis, we resorted to B16-F10 murine melanoma of which we identified more than 500 non-synonymous point mutations by whole exome sequencing (1). After selection of expressed genes and good potential MHC binders of the respective mutated epitopes, 50 mutations were chosen and validated by Sanger sequencing. Conductive to define the immunogenicity of the mutation-coding sequences, we designed 27-mer peptides (comprising all potential MHC class I and II epitopes of 8 to 14 amino acids in length carrying the mutation incorporating either the mutated or the wild-type amino acid to immunize C57BL/6 mice. Anti-tumor potency of all immunogenic epitopes was investigated in a transplantable B16-F10 melanoma model where mice immunized with mutation-encoding IVT-RNA revealed tumor control in the protective and the therapeutic setting for a substantial number of mutated epitopes. Surprisingly, we identified one MHC class II restricted epitope (Mut-30) that confers tumor control beyond the efficacy of known immunodominant tumor-associated antigens like Trp2 or gp100. To further examine the pharmacodynamics of this class II restricted mutated epitope, we identified the minimal epitope and characterized the functional profile in more detail. To investigate the relative role of $CD4^+$, $CD8^+$ and NK1.1 T cells in tumor control, we evaluated Mut-30 mediated control of tumor growth and survival in conjunction with administration of depleting Abs to deplete different T cell subpopulations. Mutation-specific IFN-γ and TNFα expressing $CD4^+$ T cells were shown to be increased in tumor after Mut-30 RNA vaccination. On-going studies aim to dissect the effector mechanisms induced by mutation-specific $CD4^+$ T in the B16 tumor microenvironment responsible for the observed strong anti-tumoral effect.

Castle JC, *et al.*: Exploiting the mutanome for tumor vaccination. *Cancer Res* 2012, 72:1081-1091

Vaccine-induced cytokine-producing T cells synergize with cisplatin to cause massive infiltration of tumoricidal leukocytes

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Treatment of patients with Human Papilloma Virus (HPV)-induced cancer with the clinically active HPV16 synthetic long peptide (SLP) vaccine does not result in clear clinical benefit, although in some patients clinical benefit was seen after subsequent treatment with chemotherapeutic agents, suggesting that the combination of both modalities can make a difference. In preclinical mouse models of HPV16-induced cervical cancer we studied whether SLP vaccination could be combined with chemotherapy to eradicate tumors. In most mice that received either peptide vaccination or chemotherapy treatment, only a temporary regression in tumor size was observed. Importantly, combined chemo-immunotherapy induced complete tumor eradication in nearly all mice. The chemotherapeutic agent cisplatin displayed the strongest synergy with SLP vaccination. This synergy was not due to increased sensitivity of cisplatin treated tumor cells to CTL-mediated killing, or to a stronger vaccine-induced circulating tumor-specific T cell response. Analysis of the intra-tumoral immune response revealed that combined treatment with cisplatin and SLP vaccination resulted in a strong increase in the density of intra-tumoral, TNFα and IFN-γ producing $CD8^+$ CTLs. Tumor cells incubated with Tumor Necrosis Factor alpha (TNF)α and Interferon gamma, together with cisplatin strongly enhance their chemokine expression, compatible with the abundant leukocyte infiltration in the tumor upon combined chemo-immunotherapy. Accordingly, when combined with systemic cisplatin treatment, SLP vaccine-induced CTLs appeared to migrate earlier from the tumor rim into the tumor beds. Moreover, analysis of the tumor cells *in vivo* showed that combined treatment not only caused a decrease in the proliferative capacity of tumor cells, but also a TNFα- induced enhancement of cisplatin-mediated tumor cell death. Cell death was accompanied by an increased expression of pro-apoptotic molecules. Together, our data show that combined peptide treatment with cisplatin leads to superior tumor eradication.

Re-vaccination after first vaccination for patients with high grade glioma: does it make sense?

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Multimodal strategies are developed to treat patients with high grade glioma (HGG). Active specific immunotherapy rapidly emerges as a new treatment modality. We provide immunotherapy for adults with primary diagnosis of GBM (HGG-2006) and for children/adults with relapsed HGG (HGG-IMMUNO-2003). In this retrospective analysis, we questioned whether second immunotherapy upon a new event was useful in patients who already got immunotherapy for their disease.

35 patients were treated with two vaccination treatments, 12 adults (26-69y) with primary diagnosis of GBM and 23 patients (7-55y) with relapsed HGG at time of first immunotherapy. At both times, leukapheresis was performed and DCs were loaded with lysate of the newly resected tumor tissue.

HGG-2006 patients treated with two immunotherapies had a median OS of 41.8m versus 14.8m in HGG-2006 patients (n=68) treated with one immunotherapy program. The age distribution of the former was younger than of the latter group. Similarly, HGG-IMMUNO-2003 patients treated with two immunotherapies had a median OS of 32m versus 11m in HGG-IMMUNO-2003 patients (n=163) with one immunotherapy program. The age of the former patient group was younger, and their HGG-IMMUNO-RPA risk profile was better. The time interval between the first and second leukapheresis was longer in the HGG-2006 than the HGG-IMMUNO-2003 patients. All second immunotherapy approaches were similar. There were less injections during second immunotherapy as compared to the first immunotherapy. The number of injections was similar to the numbers given to HGG-IMMUNO-2003 patients who got first vaccination at time of relapse. The OS calculated from the second leukapheresis in re-vaccinated patients was similar as the OS observed in HGG-IMMUNO-2003 patients treated for the first time at relapse. Second immunotherapy was feasible, and no extra vaccine-related toxicities were observed.

These retrospective results show that second immunotherapy is worth to be considered along the disease course of patients with HGG.

Repeated intratumoral administration of ONCOS-102 leads to robust cellular and transcriptional immune activation at tumor site in an ovarian cancer patient

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Advanced tumors have often metastasized at the time of diagnosis and are immunosuppressive, and are thus challenging to treat with standard therapies. We have completed a phase I trial with ONCOS-102, an oncolytic 5/3 capsid chimeric adenovirus coding for granulocyte macrophage colony-stimulating factor (GMCSF). We hypothesize that both oncolysis and local expression of GMCSF work together to initiate lymphocyte tumor infiltration, and optimally, to prime the anti-tumor immune response. To further enhance the effect, concomitant low-dose cyclophosphamide was included to down regulate tumor-promoting Treg-cells.

We report a 38-year-old patient with stage 3 metastatic micropapillary serous carcinoma of the ovary. She had previously been treated with surgery, followed by seven different chemotherapy regimens with varying responses of limited duration. The patient was treated intratumorally nine times with 3 x 10^{11} VP of ONCOS-102. There was evidence of GMCSF production remaining local, as there were signs of viral activity, but no elevation of GMCSF in the serum. Proinflammatory cytokines were temporarily elevated after each treatment. Only grade 1-2 adverse events were observed. The anti-adenoviral neutralizing antibodies increased throughout the 3-month observation period, which was not seen to reduce the effect of repeated local administration of the virus. Viral DNA was consistently detected in blood, suggesting an ongoing viral replication.

For the signals of activity, three tumors were followed, one of which was left untreated. T-cell infiltration was evaluated from a tumor biopsy, in which a dramatic increase in cells that are known to reduce cancer growth, namely, cytotoxic $CD8^+$ T-cells, $CD4^+$ helper T-cells, dendritic cells (DCs) and macrophages was seen at the peritumoral area 1 and 2 months after treatment initiation. Microarray data from the post-treatment biopsy further confirmed the immune activation at tumor site. High expression of favorable Th1-type immune mediators and tumor preventing M1-type macrophage markers were seen, as opposed to low expression of tumor promoting M2-macrophage markers. Disease stabilization was achieved concomitantly in both injected tumors, as evaluated by CT and PET at 3 months. At this time point, for the non-treated tumor PET showed ten percent reduction in activity, which led us to hypothesize that the treatment-primed immune cells may systemically lead to anti-tumor activity also in non-injected targets, which remains to be confirmed in an upcoming study. The patients´ performance status has remained good since the treatment (WHO1), and survival has now exceeded 8 months.

To summarize, favorable T-cell responses were observed in the tumor on both cellular and transcriptional level. Comparable radiological responses in injected and non-injected tumors further suggest the involvement of immune responses in disease control. This aspect will be thoroughly investigated in the upcoming phase 2 trial.

VicOryx - A therapeutic phase I/IIa vaccination trial with a p16^{INK4a} peptide plus Montanide® ISA-51 VG in patients with human papillomavirus-associated cancer

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Introduction: The tumor suppressor p16^{INK4a} is strongly overexpressed in HPV-associated cancers, including those of the oropharynx. While in normal tissues barely any p16^{INK4a} expression is detectable. Targeting this HPV type-independent antigen by vaccination could represent an interesting complementary therapeutic approach to E6/E7-based vaccination that are evaluated in clinical studies. We performed a phase I/IIa peptide vaccination trial to monitor toxicity and immunogenicity of p16^{INK4a} vaccination in patients with advanced HPV-associated cancers.

Study design: Patients with advanced p16^{INK4a}-overexpressing, HPV DNA-positive cancer (anogenital region, head and neck) were included after completion of standard treatment. The protocol comprised a total of 12 subcutaneous injections of a synthetic p16^{INK4} peptide mixed with Montanide® ISA-51 VG in weekly intervals. Objectives of the trial were clinical safety and changes of humoral and cellular immune responses against the p16^{INK4a} peptide. T cell responses were monitored by interferon-gamma ELISpot and antibodies by ELISA from peripheral blood (ClinicalTrials.gov Identifier: NCT01462838, Sponsor: Oryx GmbH und Co KG).

Results: Phase I is completed with 10 patients, phase IIa is ongoing with 14 patients recruited of 16 planned. No toxicity was observed during and after vaccination that was regarded as related to vaccination with the auto-antigen p16^{INK4a} in any of the patients. While pre-existing baseline T cell and antibody responses against the p16^{INK4a} peptide were rare, p16^{INK4a}-reactive T cells and antibodies were successfully induced in 8 of 16 patients analyzed to date. So far one head and neck cancer patient with lung metastases completed the entire study protocol with stable disease for now 18 months after the last vaccination. The remaining 9 patients of phase I had progressive disease.

Conclusions: This is the first study demonstrating that p16^{INK4a} peptide vaccination is safe and well tolerated and that immune responses against p16^{INK4a} can be induced by p16^{INK4a} peptide vaccination and are not accompanied by clinical autoimmune symptoms. Further trials will be designed to assess whether this approach may be an adjuvant therapeutic strategy for patients with HPV-associated cancers.

Tumor-specific mutations as targets for individualized mRNA therapy in the murine 4T1 breast cancer model

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Among woman, breast cancer is the most common malignant neoplasm with low five-year-survival rates in patients with metastasizing carcinomas.
Improved therapeutic strategies such as immunotherapeutic approaches are needed for breast cancer and therapeutic vaccination strategies hold promise for its treatment. As suitable vaccination targets, mutated antigens can be used for the induction of tumor reactive T-cells. High avidity T-cells that recognize neoantigens are unlikely to be deleted by central tolerance mechanisms or to cause autoimmune side effects because of the specific expression of these antigens in the tumor. Nevertheless, identification and immunocharacterization of such mutations as neoantigens for vaccine use is challenging. Our group investigates the identification of tumor-specific mutations via next generation sequencing (NGS) as targets for individualized mRNA based therapeutic cancer vaccination. In this study, we used the murine 4T1 mammary carcinoma, which closely resembles advanced breast cancer in human, as a preclinical tumor model to test the feasibility of a neoantigen-targeting personalized immunotherapy of human breast tumors.
Employing NGS technology for 4T1 tumor cells, we were able to identify 112 somatic point mutations, with 56 of those mutations in expressed genes. 38 Sanger sequencing validated mutations were then tested for the induction of an immune response. Accordingly, mice were repetitively immunized with i.v. injections of *in vitro* transcribed (IVT)-RNA encoding the mutated antigen sequence. Splenocytes of these mice were then tested for T-cell responses against the corresponding mutation by stimulation with IVT-RNA electroporated BMDC or peptides of the mutated antigen in an IFN-γ ELISpot assay. Of these 38 tested mutations, 17 were immunogenic and the majority of these T-cell responses were MHC-class-II restricted. Ongoing studies are investigating the anti-tumoral effects of vaccinations with IVT-RNA encoding the immunogenic mutations in lung metastatic and orthotopic 4T1 tumor models.

A novel cell penetrating peptide-based vaccine for efficacious therapy of solid tumors

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An ideal therapeutic vaccine stimulating T-cell mediated anti-tumor immunity should (i) stimulate multi-epitopic cytotoxic T lymphocyte-mediated immunity, (ii) induce helper T cells (T_h), and (iii) maintain durable effector and memory T cell responses. Moreover, for treatment of brain tumors it should promote T cell trafficking to the intracranial tumor site and maintain effector cell function sufficient to override inhibition mediated by immunosuppressive cells or molecules. We engineered a recombinant vaccine based on a fusion protein of a novel cell penetrating peptide (CPP) and a multi-antigenic protein. We first tested this chimeric protein *in vitro*: the protein cargo was efficiently delivered to antigen presenting cells and could be readily detected in the cytosol. This led to stimulation of monoclonal $CD4^+$ and $CD8^+$ T cells specific for 5 distinct epitopes, and restricted by different MHC class I and MHC class II alleles. Our vaccine promoted a polyclonal immune response *in vivo* with persistent $CD8^+$ effector T cells homing to the tumor site, as well as T_h1 polarized $CD4^+$ T cells. In tumor rejection studies, 3 vaccinations with only 10nM of CPP-based vaccine plus adjuvant were sufficient to induce regression of orthotopic GL261 glioma in the majority of treated mice. Furthermore, these mice remained tumor free for over 100 days.

Overall, the novel CPP-based chimeric protein vaccine that we have developed and tested for therapeutic efficacy in stringent pre-clinical models represents a major advance in vaccine technology. Its potency rivals that achieved by viral vectors, whilst retaining the simplicity and safety of peptide-based vaccines. These features will facilitate rapid clinical translation and application to gliomas, as well as for other malignancies with defined antigenic targets.

Synergistic effects of properly timed HPV16 synthetic long peptide vaccination during standard carboplatin-paclitaxel chemotherapy in animals and patients with metastatic cervical carcinoma

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ORAL SHORT TALK

We previously developed a synthetic peptide vaccine, covering human papillomavirus (HPV) type 16 E6 and E7 protein sequence by long overlapping peptides (HPV16-SLP), that was clinically active in patients with HPV16+ high-grade vulvar intraepithelial neoplasia. Complete lesion regression was related to a strong vaccine-prompted HPV16-specific effector T-cell response. However, in patients with HPV16-induced metastatic cervical cancer vaccine-induced T-cell responses were weaker and did not result in improved clinical outcome. A new study on the constitution of immune cells in blood of such patients revealed increased numbers of myeloid cells, low T-cell reactivity against common microbial recall antigens and a lower stimulatory capacity of antigen presenting cells, indicating an immunosuppressive status. When these patients were subsequently treated with standard carboplatin-paclitaxel chemotherapy a normalized immune profile similar to that found in healthy subjects was observed at a specific time point during this chemotherapy. In addition, experiments in HPV16+ tumor bearing mice showed that the tumor-induced abnormally high numbers of myeloid cells was normalized by this chemotherapy, both in blood and locally in the tumor. Moreover, combined HPV16-SLP vaccination and chemotherapy effectively led to a higher cure rate of mice with established HPV16+ tumors. Together, these data indicate that standard chemotherapy has an immune stimulatory effect by deletion of suppressive myeloid cells in the mouse tumor model and in patients with metastatic cervical cancer. Therefore, a clinical trial was started in which such patients were treated with standard chemotherapy in combination with HPV16-SLP vaccination. Comprehensive immune monitoring confirmed the beneficial effect of myeloid cell depletion associated with a robust induction of HPV16-specific T-cell responses that were sustained throughout several cycles of chemotherapy. The data of these studies will be reported. Currently, we have started a multicenter clinical trial in which HPV16-SLP vaccination is combined with carboplatin-paclitaxel and type I interferon to assess clinical and immunological outcome in a group of HPV16+ metastatic cervical cancer patients.

Vigorous T cell responses to neoantigen frameshift-derived peptides in Lynch syndrome patients treated with monocyte-derived dendritic cells

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Background: Individuals with Lynch syndrome have an up to 80% risk to develop colorectal cancer (CRC) due to a germline mutation in one of the DNA mismatch repair (MMR) genes. MMR-deficiency in tumor DNA causes shifts in the translational reading frame resulting in the production of peptides that are structurally altered as compared to the normal products. Frame-shift peptides (FSP) such as Caspase 5 and TGF-bRII are considered ‚foreign‘ by the immune system. MMR-deficient Lynch syndrome associated tumors, expressing these FSPs are characterized by a strong lymphocyte infiltration, and the degree of infiltration is correlated with a better prognosis. Cytotoxic T cells and helper T cells in blood of patients and healthy individuals with Lynch syndrome indicate that FSPs are immunogenic. Vaccination against FSPs may be promising for treatment of Lynch syndrome associated cancer. Dendritic cells (DC) are the professional antigen-presenting cells of the immune system and decisive in inducing immunity. This is the rationale for vaccination with monocyte-derived DC loaded with FSPs to stimulate T cells to combat Lynch syndrome associated CRC.

Patients and methods: After surgery for Lynch syndrome associated CRC, patients were vaccinated with DC loaded with CEA and FSP MHC class I binding peptides. Patients received up to three vaccination rounds, consisting of three weekly intradermal and intravenous DC injections. After each vaccination round, the presence of antigen-specific $CD8^+$ T cells was assessed in blood and challenged skin. Injection of minute amounts of the DC vaccine resulted in infiltration of immune cells into the skin. Specificity of these skin-infiltrating lymphocytes was assessed by flow cytometry with tetrameric MHC complexes binding to T cells that recognize the indicated peptides.

Results: In the first patient no specific T cells were found after the first round of three DC vaccinations. After the second and third round both FSP- and CEA-specific $CD8^+$ T cells were present. In the second patient $CD8^+$ T cells specific for Caspase 5 were already detectable after the first round. The functionality of these skin infiltrating T-cells was demonstrated in both patients by their capacity to produce high amounts of IFN-g upon stimulation with target cells loaded with CEA or one of the two FSPs. Antigen-specific $CD8^+$ T cells were not detected in peripheral blood lymphocytes by direct staining with tetrameric MHC complexes. Both patients reported flu-like symptoms during two days.

Conclusions: Cellular immunotherapy with DC vaccination against CEA and FSP-antigens is feasible and safe and immune responses towards Lynch syndrome tumor-specific peptides are induced. Our data emphasize the potency of DC-based immunotherapy to enhance the host‘s antitumor immunity and suggests that it may be considered for cancer prevention in healthy Lynch syndrome mutation carriers. The results warrant further investigation in a follow-up randomized trial.

The src kinase Lyn impacts TLR4-triggering in human myeloid cells

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Background: Sensing LPS and triggering via TLR4 is the initial step for myeloid cells to initiate an immunological response to pathogens. Src kinases regulate various key functions in cellular immune responses, depending on the functional status and the respective cell type. Among the 9 known src kinases, Lyn is known to be expressed in human dendritic cells. Dendritic cell-specific Lyn-deficient mice are hyperreactive to TLR-mediated triggering (Lamagna C et al. PNAS 2013) causing lupus-like disease. For human DCs, we recently identified a role for src kinases in the IL-12 production upon LPS-triggering (Wölfl M et al., Blood 2013). Now, we wanted to further analyse the mechanisms leading to increased IL-12 synthesis upon src-kinase blockade.

Methods: Monocytes-derived dendritic cells cultured in vitro with GM-CSF and IL-4, purified $CD14^+$ monocytes or Slan $(M\text{-}DC8)^+$ cells were stimulated with TLR-agonists and IFN-γ in the presence or absence of the src kinase inhibitor dasatinib. To assess signalling pathways associated with TLR-induced IL-12 production, the different signalling mediators downstream of TLRs were targeted with kinase inhibitors. Intracellular IL-12 production was subsequently measured by flow cytometry. In addition, the phosphorylation status of different protein kinases was analyzed by western blot.

Results: Dasatinib profoundly enhanced TLR4-mediated signalling in all myeloid subtypes tested. Increased IL-12 production correlated with an increase of NF-κB activation and a loss of Lyn and SHP-1-phosphorylation, which signals downstream of Lyn. Blockade of the Lyn pathway using inhibitory peptides led to results comparable to dasatinib. This data suggests a regulatory role of Lyn and SHP1 in the TLR4-mediated activation of human myeloid cells.

Randomized phase II study of personalized peptide vaccination with cyclophosphamide pretreatment in refractory advanced biliary tractcancer patients

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ORAL SHORT TALK
CIMT 2014
Clinical Trials

Purpose: We previously demonstrated the feasibility of personalized peptide vaccination (PPV), in which appropriate vaccine peptides are individually selected for each patient to boost anti-cancer immunity, in advanced biliary tract cancer (BTC) patients. This study was conducted to assess whether cyclophosphamide (CYP) pretreatment could improve the clinical efficacy of PPV in advanced BTC patients refractory to standard chemotherapies.

Methods: We conducted a randomized phase II study to examine the effect of CYP pretreatment (100 mg, daily for 7 days before each cycle of 6 vaccinations) on PPV for advanced BTC patients, who failed or progressed after standard chemotherapy regimens. For PPV treatment, a maximum of 4 peptides were selected from 31 candidate peptides based on the HLA class I types and antigen-specific humoral immune responses before vaccination, and subcutaneously administered (6 vaccinations, weekly; thereafter, by-weekly). The primary and secondary endpoints were to examine the immunological responses to vaccine antigens and overall survival (OS), respectively, after PPV with and without cyclophosphamide pretreatment (PPV plus CYP and PPV alone, respectively).

Results: Thirty-four BTC patients were randomly assigned with 16 patients for PPV plus CYP and 18 PPV alone groups, respectively. Severe adverse event was not observed in any patients with regard to either CPY pretreatment or PPV administration. There were no statistically significant differences between the two groups with regard to the frequency of either peptide-specific CTL responses or peptide-specific IgG responses. However, larger numbers of CTL spots per well were observed in PPV plus CYP group as compared to those in PPV alone group. OS in the PPV plus CYP group was significantly longer than that in the PPV alone group ($P = 0.03$ by Fleming-Harrington test). The median OS was 397 and 190 days in the PPV plus CYP and PPV alone groups, respectively.

Conclusions: Pretreatment with CYP did not enhance antigen-specific immune responses, but might improve the prognosis after PPV in refractory advanced BTC patients. Further larger scale, randomized trials would be needed to confirm this encouraging result.

CIMT
Cancer Immunotherapy

New Targets & New Leads

Identification of unique HLA peptides presented on Glioblastoma cells and recovered from the plasma soluble HLA

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The grim prognosis facing most Glioblastoma Multiforme (GBM) patients calls for the addition of the immunotherapy modality to the current treatments approaches. The HLA peptidomes (immunopeptidome) is a possible source for GBM immunotherapeutics. The GBM HLA peptidome can be recovered in large amounts from the tumor cells or from cultured cancer cells, but can also be recovered from the plasma soluble HLA molecules (sHLA), circulating in the cancer patients blood. Here we describe a large-scale analysis of the plasma sHLA peptidomes of GBM patients and membranal HLA peptidome of cultured human Glioma cells. We suggest that in addition to its potential usefulness to include lists of many thousands of identified HLA peptides, including many cancer vaccines candidates, the recovered sHLA peptidomes is a useful resource for early detection, improved prognosis and disease follow-up. The use of sHLA peptidome analysis for diagnosis and for selection of tumor antigens is based on the observation that some types of tumor cells tend to release large amounts of sHLA molecules to the circulation and these sHLA molecules carry with them their original loads of bound peptides. The sHLA molecules are immunoaffinity purified from the patients' blood in sufficient amounts for identification and quantification of a few hundreds to a few thousands of different peptides. The analysis is based on capillary chromatography and tandem mass spectrometry of the purified peptides, starting from a few milliliters of patients' blood.

In this (GAPVAC) study, we have analyzed the membranal HLA peptidomes of cultured GBM cells and of sHLA molecules recovered from patients' plasma. In total, we have identified more than 11,000 different HLA class I peptides derived from four cell lines: U-87, U-118, T98G, and LNT-229. These peptides were derived from more than 5,000 different proteins. In addition, we have identified more than 9400 different sHLA bound peptides, collected from the plasma of six healthy donors and twenty seven plasma samples of GBM patients. These sHLA peptides were derived from more than 4,500 different proteins. Some of the plasma samples we collected during different visits to the clinic of the same patients and thus allow us to follow the changes in the sHLA peptidome during the progression the disease and its treatment. The identified GBM HLA peptidomes includes peptides derived from putative tumor associated antigens, known to be expressed especially in cancer cells or in immune privileged tissues. Such peptides and their source proteins may have an advantage as putative cancer vaccine candidates or as biomarkers for early detection, improved prognosis and follow-up of the disease.

Induction of tumor-specific cytotoxic CD4$^+$ T cells by 4-1BB agonist therapy

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Over the last years the idea that effector CD4$^+$ T cells play a considerable role in direct protective anti-tumor responses has received growing attention. In our research we have used a murine retrovirus-induced tumor cell line of mouse origin, namely FBL-3 cells, as a model to study basic mechanisms of immunological control and escape during tumor formation. 4-1BB (CD137) is an activation-induced T cell cosimulatory molecule that upregulates survival genes, enhances cell division and induces cytokine and cytotoxins production by tumor-specific T cells. CD8$^+$ T cells are essential in FBL-3 tumor control. In our study we showed that the combination of *in vivo* CD8$^+$ T cell depletion and 4-1BB agonist therapy resulted in complete FBL-3 tumor regression. Following FBL-3 inoculation the population of activated CD4$^+$ effector T cells expands, but produces few cytokines or cytotoxic molecules. After 4-1BB agonist therapy an expansion of tumor-specific MHC II tetramer$^+$ CD4$^+$ effector T cells and enhanced production of the cytotoxic molecule granzyme B was observed. This therapy also resulted in improved cytokines production. 4-1BB agonist treatment was accompanied by the significant increased of *in vivo* killing activity of effector CD4$^+$ T cells in treated tumor-bearing mice compared to the non-treated group. These experiments suggest that tumor-specific cytotoxic CD4$^+$ T cell immune response can be improved by 4-1BB agonist therapy, leading to the enhanced resistance to oncovirus-associated tumors.

Development of a T cell activating bispecific antibody targeting carcinoma cells expressing the tight junction molecule CLDN18.2

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Multiple immune evasion mechanisms of cancer cells hinder conventional cancer immunotherapies to completely eradicate resisting malignant cells. Therefore, improved approaches are required to overcome the limitations of existing immunotherapies. Bispecific tandem single-chain antibodies (bsAb) are potent anti-cancer drugs enabling the patient's immune system to fight cancer cells. The potency of this approach has already been confirmed in clinical trials (Boder, Jiang 2011). Therefore we developed a bsAb targeting the tumor-associated antigen CLDN18.2. The efficacy of the bsAb was investigated in *in vitro* and *in vivo* experiments.

The bispecific antibody used in this study - termed 1BiMAB - is a tailor-made recombinant antibody, which consists of two fused single-chain variable fragments recognizing different antigens. 1BiMAB comprises a T cell-specific scFv targeting the CD3 epsilon chain of the T cell receptor complex, whereas the second specificity binds to the TAA CLDN18.2. It has been shown that CLDN18.2 is presented almost exclusively on the surface of 70 % of defined entities of cancer e.g. gastric, breast, ovarian (Sahin et al. 2008).

The goal of this bsAb approach is the specific recruitment of effector T cells to the tumor tissue. A physical linkage of T cells to TAA-expressing tumor cells is hereby mediated by the bsAb. Simultaneous binding of the bsAb to CLDN18.2 and CD3 induces then the clustering of CD3-receptor complexes on the surface of T cells resulting in their activation. Consequently, T cells up-regulate and release their cytotoxic enzymes thereby mediating lysis of the tumor cell. In our *in vitro* study we could show that 1BiMAB redirects the effector functions of the T cells towards the tumor cells. This could be demonstrated by strong induction of T cell activation and proliferation as well as up-regulation of pro-apoptotic proteins such as GrB only in the presence of CLDN18.2+ tumor cells and 1BiMAB. Thereby effective tumor cell lysis with EC_{50} values in nanomolar range could be reached. Furthermore, the therapeutic efficacy of 1BiMAB was investigated in a xenograft tumor mouse model. Therefore, immunodeficient mice engrafted with human PBMCs and carrying a subcutaneous tumor generated by CLDN18.2+ HEK293 cells were treated daily with 5 µg 1BiMAB for 22 days. Consequently, mice treated with 1BiMAB and engrafted T cells showed a significantly higher survival ($p < 0.0022$) than the control groups.

Taken together, our results demonstrate that simultaneous targeting of CLDN18.2 and CD3 induces potent T cell-mediated tumor cell elimination. 1BiMAB is able to activate T cells independent of signals, which are usually involved in the T cell recognition process such as antigen presentation, MHC restriction, and co-stimulatory effector molecules (Travers et al. 2008). Therefore, 1BiMAB will potentially enable to overcome several immune evasion mechanisms of tumor cells consequently supporting current solid cancer therapies.

Expression and turnover of proteins govern their sampling for HLA class I presentation

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T-cell responses against infected and cancer cells are initiated by recognition of HLA-I peptides (the peptidome) presented on the surface of nucleated cells. The repertoire of HLA-I peptides originates primarily from sampling the cytosolic degradation products of intracellular proteins. HLA-I peptides have been extensively studied in the last years because of they can potentially be used directly as immunotherapy based cancer vaccines. Even more advanced cell based therapeutic applications are being developed based on cancer specific HLA-I peptides. In this study, we used high resolution mass spectrometry and the MaxQuant bioinformatics environment to obtain a high accuracy and in-depth coverage of HLA peptidomes. We immuno-affinity purified the HLA-I peptidome from ten cancer and primary cell lines and identified an unprecedented number of peptides. This enabled us to shed new light on the mechanisms governing peptidome presentation and to determine which part of the proteome is sampled for presentation. We find that globally, protein abundance correlates strongly with the degree of HLA-I sampling. When computing HLA sampling density for proteins according to their expression levels, we discovered that proteins with a sampling density five-fold or higher had high turnover-rates pointing towards efficient degradation that enable the subsequent production of multiple epitopes. Our analysis validates known cancer epitopes and suggests many new ones. Better immunotherapeutic modules could possibly be developed based on wider and more accurate repertoires of HLA-I peptides, which in turn should increase the accessibility of these therapies for a larger cohort of patients.

Treatment of melanoma with a serotype 5/3 chimeric oncolytic adenovirus coding for GM-CSF: results in vitro, in rodents and in humans

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Metastatic melanoma is highly aggressive and refractory to irradiation and chemotherapy. Oncolytic immuno-virotherapy of malignant melanoma has gained attention in recent years, because melanoma is classically an “immunogenic tumor”, and because oncolytic virus replication is an immunogenic phenomenon.

We investigated the potency of the oncolytic adenovirus Ad5/3-D24-GMCSF (CGTG-102) on a panel of melanoma cell lines and animal models in combination with low-dose cyclophosphamide and summarized the melanoma-specific human data from the Advanced Therapy Access Program (ATAP).

The virus effectively eradicated human melanoma cells *in vitro* and subcutaneous SK-MEL-28 melanoma xenografts in nude mice, particularly when combined with low-dose cyclophosphamide. Furthermore, granulocyte-macrophage colony-stimulating factor (GM-CSF) expressed by the virus stimulated the differentiation of human monocytes into macrophages.

A total of nine patients with treatment-refractory melanoma received treatments in the ATAP. Four patients were evaluated for radiological benefit with Response Evaluation Criteria In Solid Tumors (RECIST) criteria: one patient showed a minor response, two showed stable disease, and one had progressive disease. Treatments appeared safe and well tolerated. Two patients survived through 559 and 1501 days after virus treatment.

Thus, Ad5/3-D24-GMCSF showed promising signs of efficacy in preclinical studies and possible antitumor activity in melanoma patients refractory to other forms of therapy. The data is in support of continued clinical development of oncolytic adenovirus for melanoma.

TRON Expression Atlas (TEA): A web - based platform for archival, retrieval, filtering and visualization of high throughput gene expression profiles

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ORAL SHORT TALK

Data handling of the steadily increasing amounts of genomic data has become an important issue in todays biomedical research. Vast amounts of high throughput gene expression profiling data exist, such as from next generation sequencing (NGS) and microfluidic qPCR platforms. However, most of the time this data is used to answer specific, experiment related questions. The results often end up in non - accessible excel files saved in seemingly random directories. Here, we introduce a comprehensive web-based platform, the TRON Expression Atlas (TEA), for the storage, visualization and filtering of high throughput gene expression profiling data. The TEA body atlas contains gene expression profiles of 28.000 genes in 2.000 human and over 70 mouse samples, including cancerous and non-cancerous tissues, cell lines and homogeneous cell types. The atlas database integrates both internally - generated and over 400 external datasets. All data are normalized and homogenized through the use of common internal processing pipelines (e.g. RNA-Seq pipeline). The web - based user interface includes search, visualization and filtering tools. Containing expression profiles from cancerous and normal adult tissues, TEA enables immunotherapy target discovery and personalized medicine biomarkers.

A novel synthetic Toll-like-receptor 7 agonist for tumor therapy

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Introduction: TLR7 is located in the endosome and is activated upon binding of viral ssRNA. This results in the release of proinflammatory cytokines such as IL-6, IL-12 and type 1 interferon and culminates in the activation of cells of the innate and adaptive immune system. This may be used for anti-tumoral immunotherapy. The clinical application of synthetic TLR7 agonists is currently limited to the topical treatment of skin tumors. New compounds are needed for safe and efficient systemic application.

Results and methods: All experiments were performed using the novel synthetic small molecule TLR7-agonist (SC1). We found SC1 to be specific for TLR7, as evidenced by a TLR7-HEK-reporter cell line and TLR7-/-splenocytes. SC1 induced a pro-inflammatory cytokine profile and lead to immune cell activation both *in vitro* and *in vivo*. SC1 induced IL-6,IL-12p70, MCP-1, MIP-1β, Interferon-γ and IP-10 in splenocytes and enhanced the expression of the early activation marker CD69 on T cells, B cells and natural killer cells (NK cells). Cytokine up-regulation and immune cell activation are TLR7-mediated as they were absent in TLR7-/- mice. *In-vivo,* treatment of mice with SC1 specifically killed β_2-microglobulin deficient target cells by NK cells as opposed to β_2-microglobulin competent target cells (92% specific lysis vs 0% specific lysis). Finally, treatment with SC1 of RMA-S tumor bearing mice (an NK cell sensitive lymphoma model) significantly reduced tumor growth as compared to untreated or vehicle-treated mice (mean tumor size at day 25: 148 mm^2 (vehicle); 139mm^2 (no treatment); 15mm^2 (SC1); n = 5) and resulted in a prolonged tumor-free survival (50 days after last treatment) in 5 out of 10 SC1-treated mice while the control groups did not survive to the last treatment time point.

Conclusion: The TLR7-agonist SC1 is a potent TLR7-specific immune activator and is effective in the treatment of an NK cell-sensitive murine tumor model. SC1 may be a promising candidate for use in anti-tumoral immunotherapy.

Rapid mimotope optimization for pharmacokinetic analysis of therapeutic antibody IMAB362

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Membrane proteins play a major role in a variety of life-threatening diseases (e.g. as selective tumor markers) and became the main focus in the development of therapeutic monoclonal antibodies. The process of drug development requires a set of tailor-made assays for the characterization of the antibodies and for monitoring their activity. Usually ELISA-based assays are being applied for this purpose using the corresponding antigen. This is challenging, because the targeted membrane proteins are often not available in a format which is compatible with a biochemical assay setup. This obstacle can be overcome by using anti-idiotypic antibodies which bind specifically to the antigen binding region of the therapeutic antibody. However, generating such anti-idiotypic antibodies is time-consuming and expensive. Therefore, peptidic mimotopes as easier-to-prepare structures are considered useful substitutes to mimic the full-length antigen, allowing tight specific binding of the therapeutic antibody. Peptides are easily accessible by chemical synthesis, show higher stability and are easier to handle compared to proteins. In general, peptide ELISA enables the analysis of proteins/protein interactions on the amino acid sequence level, e.g. for definition of protein interaction sites. Specifically binding peptides - or peptide-based mimotopes - for peptide ELISA can be discovered with several approaches which can be either knowledge based or based on random library approaches, like Phage Display. In this study optimal peptide mimotopes for the ELISA-based detection of the novel therapeutic antibody IMAB362 in biological samples were developed. IMAB362 is a highly tumor-specific monoclonal IgG1 antibody currently in clinical development among others for the treatment of advanced gastro-esophageal and stomach cancer. The antibody is directed against the cancer specific cell surface target Claudin 18 isoform 2, a 27.7 kDa gastric differentiation protein that spans the cell membrane with four transmembrane helices. IMAB362 binding peptides were selected from a peptide phage display library and optimized with the help of SAR analysis on peptide microarrays. The optimized peptides showed binding constants in the low nanomolar to picomolar range, an improvement by a factor of up to 30 compared to the initial hits. The best mimotope (apparent KD = 0.15 nM) was successfully used for the ELISA-based quantification of IMAB362 in samples from a mouse pharmacokinetic study. The described process allows the rapid discovery of mimotopes for targets which are difficult to produce or handle to be used in assays or for the purification of biological products.

Fast and accurate identification of disease related variants using amplicon based targeted re-sequencing.

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Background: Targeted exon enrichment followed by massive parallel sequencing is a method to selectively enrich genomic regions of interest and sequence them in a highly multiplexed manner. Off-the-shelf panels are available, as well as custom made panels.

Methods: To validate this method for the targeted sequencing of genes involved in breast cancer, we used a breast cancer panel consisting of 20 relevant genes. Genomic DNA from 3 different breast cancer cell lines (SK-BR-3, MCF7 and CAMA1) was isolated. PCR-based target enrichment was done in triplicates, generating nine libraries. The libraries were bar-coded and paired-end sequenced on the Illumina HiSeq2500 platform, pooled in 2 lanes of a rapid flow cell using 2X100 read length. Data was analyzed using the QIAGEN GeneRead online analysis tool (Bowtie2/GATK/variant calling based on 2000 reads). The same data were analyzed using an in-house pipeline for comparison (BWA/Samtools) Qiagen Ingenuity was used for fast identification of casual variants.

Results: After quality filtering, we obtained, on average, ~77M reads/sample. Approximately 94% of reads mapped to target regions with an average sequence coverage uniformity of 93% (% of bases covered at >=0.1 median). The number of detected variants was 98 in SK-BR-3 (86 SNPs/MMPs, 12 Indels), 96 in CAMA-1 (85 SNPs/MNPs, 11 Indels) and 92 for MCF-7 (87 SNPs/MMPs, 5 Indels). Examination of validated variants present in Cosmic showed 100% reproducibility between the triplicates. Also the reproducibility between the triplicates concerning all non-synonymous variants was 100%. We obtained 80% agreement with our in-house pipeline.

Conclusions: Validation of the 20-gene breast cancer panel for targeted sequencing showed very high reproducibility and specificity. The high flexibility in post-enrichment library preparation makes this panel suitable for high multiplexing, as we were able to use the panel in conjunction with the Illumina HiSeq2500. This method will be used to screen clinical samples for biomarker discovery and identifying targets for RNA-based vaccination using a customized gene panel.

Effect and pharmacokinetics of ADC-1013, an agonistic CD40 antibody optimized for local immunotherapy of cancer, in hCD40tg mice

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Local administration of immune activating antibodies may increase the efficacy and reduce the immune-related adverse events associated with systemic immunotherapy of cancer. Here we report the development of a fully human agonistic CD40 antibody (IgG1), ADC-1013, which has been optimized for local immunotherapy by increasing potency and tumor retention.

ADC-1013 activates CD40 receptors on antigen-presenting cells such as dendritic cells, resulting in up-regulation of co-stimulatory molecules and induction of IL-12. The anti-tumor effects related to the immune activating properties of ADC-1013 were studied in a human CD40 positive transgenic mouse (hCD40tg). This transgenic mouse strain has an intact immune system and fully functional dendritic cells that are activated upon ADC-1013 treatment. Furthermore, the dendritic cells obtained from this strain are able to induce antigen specific T cell activation *in vitro* upon stimulation with ADC-1013. Importantly, treatment with ADC-1013 in a syngeneic bladder cancer (MB49) model, which is hCD40 negative, demonstrated that ADC-1013 induce significant tumor protection and long term immunity independent of direct tumor targeting. In addition, the anti-tumor immunity was shown to be T-cell dependent.

Further, ADC-1013 is rapidly eliminated in hCD40tg mice. The levels of free ADC-1013 measured in blood were significantly lower than the isotype control, demonstrating target-dependent kinetics. This is in line with preclinical findings using a surrogate CD40 agonistic antibody and with clinical findings for CD40 targeted therapy. The results further indicates a non-linear kinetic profile of ADC-1013 in the hCD40tg mice.

To our knowledge, ADC-1013 represents the first immunomodulatory antibody optimized for local immunotherapy of cancer. It is currently in late preclinical development and will enter clinical trials in late 2014.

CetuGEX™, a novel anti-EGFR monoclonal antibody (mAb) with optimized glycosylation and antibody dependent cellular cytotoxicity - First in human experience

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ORAL SHORT TALK

Background: The epidermal growth factor receptor (EGFR) is a validated target in cancer. The full potential of this target is not yet clinically realized. CetuGEX is an IgG1 mAb against EGFR. A 10- to 250-fold enhancement of ADCC-mediated tumor cell killing is achieved by fully human and optimized glycosylation in all FcγRIIIa allotypes and KRAS status. Compared to cetuximab no immunogenic carbohydrate-chains are present.

Methods: Patients with advanced solid tumors, progressing after standard treatment, were enrolled into this multicenter, single agent dose escalation Phase I trial. PK, PD and immunological parameters were assessed. Safety, tolerability and also pharmacokinetics, immunogenicity and antitumor activity were assessed.

Results: 41 patients were treated on 8 q1w dose levels from 12 to 1,370 mg flat dose and one q2w (990 mg flat) schedule. Among these 25 pts were eligible receiving at least 8 weekly doses. The most frequently observed drug-related AE excluding IRR (only Grade 1 and 2) were nausea (20%), vomiting (20%), hypertension (20%), almost all low grade and acneiform dermatitis (25%). Infusion-related reactions (IRR), virtually only to the first infusion, were associated with cytokine secretion: IL-6, IL-8, TNFα, IFNγ and IP-10 as marker of macrophage activation. Premedication and optimization of infusion scheme reduced IRRs in severity and frequency from 76% to 57% mainly grade 1 and 2. In all tested patients circulating NK cells were reduced after CetuGEX treatment indicating a stimulation and migration of NK cells into the tissue. Favorable side effect profile was observed. Skin toxicity was rather low with 28% rash and 25% dermatitis acneiform (overlapping). All skin toxicities were grade 1 and 2 only. Activity was seen over all dose levels with 4 complete and strong partial responses each lasting at least 400 days, one NSCLC patientCR >850 d (ongoing), one mCRC , and 2 patients with esophageal and gastric cancer. Further 15 pts had stable disease lasting from 8 weeks to 414 d, with several minor responses. Clinical benefit rate was 46% (19/41) in all patients and 76% (19/25) in eligible patients with an average duration of >240 days (range: 71-850 d). All patients with clinical benefit were progressive prior treatment with CetuGEX. 78% (7/9) of colon cancer patients showed CB, with all patients either progressive upon EGFR treatments or KRAS mut. PK supports q1w and q2w dosing.

Conclusions: CetuGEX shows clear signs of activity and is well tolerated. Phase II is initiated in Patients with recurrent and/or metastatic SCCHN.

PankoMab-GEX™, a novel anti-TA-MUC1 monoclonal antibody (mAb) with optimized glycosylation - First in human experience

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Background: PankoMab-GEX is a humanized and glyco-optimized IgG1 mAb recognizing the novel carbohydrate-induced conformational TA-MUC1 epitope expressed in high incidences in a wide variety of cancers with high expression rates. The epitope comprises a tumor specific carbohydrate antigen (TF or Tn) together with the immunodominant peptide region of MUC1 and is virtually only expressed on tumor cells. PankoMab-GEX is tumor-specific and promotes potent tumor cell killing via ADCC, phagocytosis, apoptosis induction and proliferation inhibition.

Methods: Patients with TA-MUC1 positive (IHC reactivity score ≥3 on a 12 grade scale) advanced solid tumors, progressing after standard treatments, were enrolled into this phase I trial. Endpoints were safety, tolerability, pharmacokinetics (PK), immunogenicity and anti-tumor activity.

Results: 74 pts were included, 73 had at least one treatment: 52 (51) pts. were on 13 q3w dose levels from 1-2200 mg), 18 pts. on 5 q1w DL from 300-700 mg, 4 pts. received 1200 mg + 900 mg start dose one week prior in a q2w schedule. No MTD was reached. Infusion-related reactions (IRRs) mostly of °1/2 and during and after cycles 1 or 2 occurred in ~50 % of the pts, starting at the DL of 300 mg q1w. IRR consisted of dyspnea, rash, erythema and flushing, but no cytokine release, and no increases of factor C3a or eosinophilic cationic protein and hence no allergic reactions were observed. IRRs at the 1st infusion were decreased with premedication from 86% (6/7pts) to 53% (19/36pts) at DL ≥600 mg. PK was linear and dose-independent with a mean t1/2 of 184 h (q3w). In pts with at least 1 post-baseline CT (62) overall confirmed clinical benefit rate (CBR) was 32% (20/62) across all DL and schedules and 50% (17/34) at DL ≥600 mg including 1 CR in ovarian cancer with normalization of CA125 for 483 days at 1100 mg q3w, and 1 PR in NSCLC for 295 days at 600 mg q1w. Confirmed CBR in pts with OvCa was 45% (9/20) over all DL, and for DL ≥600 mg 60% (9/15). All 5 pts sensitive and 3 of 5 resistent to their last platinum based therapy experienced CB. Maximum CB duration was in a pseudomyxoma peritonei pt (-21% SLD ongoing for >760 d at 900 mg q3w).

Conclusions: PankoMab-GEX is safe, very well tolerated and demonstrated clinical activity as single agent in heavily pre-treated pts which were all progressive prior treatment. The q1w, q2w and q3w administration schedules were feasible and associated with clinical benefit. A Phase IIb study in advanced ovarian cancer is ongoing.

T-cell responses to oncogenic Merkel cell polyomavirus proteins distinguish patients with Merkel cell carcinoma from healthy donors

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Purpose: Merkel cell carcinoma (MCC) is a highly aggressive skin cancer with strong evidence of viral carcinogenesis. The association of MCC with the Merkel cell polyomavirus (MCPyV) may explain the explicit immunogenicity of MCC. Indeed, MCPyV-encoded proteins are likely targets for cytotoxic immune responses to MCC as they are both foreign to the host and necessary to maintain the oncogenic phenotype. However, to date only a single MCPyV-derived CD8 T-cell epitope has been described, thus impeding specific monitoring of T-cell responses to MCC and development of immunotherapeutic strategies targeting this oncogenic virus.

Method: To overcome this limitation, we scanned the MCPyV oncoprotein large T and small T antigens and the virus capsid protein VP1 for potential T-cell epitopes, and tested for MHC class I affinity. We confirmed the relevance of these epitopes using a high-throughput platform for T-cell enrichment and combinatorial encoding of MHC class I multimers.

Results: In peripheral blood from 38 patients with MCC and 30 healthy donors, we identified 53 MCPyV-directed CD8 T-cell responses against 35 different peptide sequences. Strikingly, T-cell responses against oncoproteins were exclusively present in patients with MCC, but not in healthy donors. We further demonstrate both the processing and presentation of the oncoprotein-derived epitopes, as well as the lytic activity of oncoprotein-specific T cells toward MHC-matched MCC cells. Demonstrating the presence of oncoprotein-specific T cells among tumor-infiltrating lymphocytes further substantiated the relevance of the identified epitopes.

Conclusion: These T-cell epitopes represent ideal targets for antigen-specific immune therapy of MCC, and enable tracking and characterization of MCPyV-specific immune responses.

Treg-associated suppressor molecules are highly expressed on melanoma: New ways for immunotherapy

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Metastatic melanoma is a highly immunogenic tumor and the most frequent form of skin cancer-related deaths worldwide. Therapeutic options are limited and poorly effective. One of the main reasons for tumor immune escape and therapy failure is the immunosuppressive tumor micro milieu. Herein, regulatory T cells (Treg) as well as inhibitory factors of the tumor itself avoiding anti-tumor immunity play a major role. A better understanding of inhibitory molecules and pathways regulating melanoma initiation and progression that could be used as therapeutic targets or biomarkers is needed. Several studies have reported expression of Treg specific markers in different tumor entities. Based on this, we investigated the expression of the Treg specific activation marker GARP (glycoprotein A repetitions predominant), which has been shown to be involved in the mediation of immunosuppressive function, in different human melanoma cell lines as well as in human melanocytes or primary melanoma in order to detect its possible functional relevance. We found that GARP is highly expressed on melanoma cells compared to normal melanocytes. We were able to figure out that GARP has suppressive capacity on different effector cells in the tumor milieu. Beside its tolerance inducing capacity in $CD4^+$ T cells, GARP suppressed the proliferation and reduced Granzyme B expression of $CD8^+$ T cell. Thus, molecules shared by both melanoma and Treg might be hopeful targets for immunotherapeutic strategies in cancer patients. Surprisingly, the addition of IFN-a a well-known drug used as therapeutic adjuvant in melanoma patients decreased the expression of GARP on melanoma cells.

Treating tumors by targeting overexpressed Wilms´ Tumor protein 1

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Wilms‘ Tumor protein 1 (WT1) is overexpressed in several kinds of adult leukemia and solid neoplasms, as well as in most primary renal malignancies in childhood, like Wilms‘ Tumor (WT). Although WT is a curable disease, current therapies frequently lead to harmful complications like skeletal abnormalities or cardiac dysfunction, what calls for an alternative treatment like immunotherapy by dendritic cell (DC) vaccination. WT1, a transcription factor involved in tumor cell proliferation, might thus be a suitable target for immunotherapy of various cancer types.

Our aim is to evaluate the immunogenicity of the wild-type antigen compared to a chimeric WT1 construct containing the MHC class II targeting signal DCLamp in various HLA-backgrounds, and to test their potential in a DC vaccine. Therefore, $CD8^+$ T cells were stimulated three times for 7 days with autologous, cocktail-matured DC, which had been transfected with either the wild-type WT1 or the modified WT1-DCLamp by RNA-electroporation. After 2 and 3 rounds of stimulation, the antigen-specific activity of the stimulated $CD8^+$ T cells was analyzed by Multi-Functional T-Cell assays (MFTC) and IFNγ-Elispots. Therefore, the $CD8^+$ T cells were electroporated with RNA encoding WT1 or WT1-DCLamp, thus acting as effectors and targets simultaneously in an overnight stimulation. Both assays revealed an antigen-specific increase in $CD8^+$ T cells producing TNFα and IFNγ, however, no difference could be observed regarding the immunogenic potential of WT1 and WT1-DCLamp.

As $CD4^+$ T cells also participate in cellular immune responses, the previous described experiments were repeated, this time using $CD4^+$ and $CD8^+$ T cells in a 1:1 ratio for the stimulations. Additionally, IL-15 was added. To analyze the antigen-specific activity of the induced T cells, chromium-release assays were performed. These assays indicated antigen-specific lysis of target cells loaded with the WT1 peptides WT1-126-134 or WT1-235-243 by T cells stimulated with WT1- or WT1-DCLamp-transfected DC. However, defining differences in the immunogenicity of WT1 and WT1-DCLamp requires further evaluation.

To advance the experiments, several WT1-peptides in two different HLA-backgrounds will be tested for their ability to elicit epitope-specific T-cell responses.

These will be analyzed not only for IFNγ production, but also for TNFα production. Additionally, dextramer-staining of WT1-stimulated T cells in different HLA-backgrounds will be performed, to finally allow sorting of WT1-specific T cells out of the stimulations.

WT1 seems to be a promising and versatile target for the immunotherapy of different types of cancer, albeit further experiments concerning the therapeutic application remain to be done.

The latter two authors contributed equally

IMAB362, a novel first-in-class monoclonal antibody for treatment of pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC), the most frequent subtype (>80%) of pancreatic cancer is characterized by a generally lethal progress within a short period of time following primary diagnosis. Despite high efforts, the treatment options are very limited and mainly of palliative nature. IMAB362 is a highly potent and tumor-cell selective therapeutic antibody which is currently in clinical development in gastro-esophageal cancer (in phase II and IIb trials). IMAB362 is directed against the tight junction molecule claudin 18.2 (CLDN18.2) whose expression is strictly restricted to short-lived gastric cells in the healthy human body. However, it is also expressed in several solid malignancies, such as cancers of the stomach, the esophagus, the lung, or the pancreas. More than 55% of patients with PDAC express CLDN18.2 and it can frequently be detected in metastases thereof. Therefore, we investigated whether IMAB362 might represent a potential treatment option for this patient population with a high unmet medical need by means of its *in vitro* and *in vivo* antitumoral activity.

IMAB362 exerts its antitumoral function by different modes-of-action including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) upon others. Therefore we assessed the capacity of IMAB362 to induce ADCC and CDC against CLDN18.2 expressing human pancreatic cancer cell lines *in vitro*. Depending on the expression level of CLDN18.2, pancreatic cancer target cells were killed via ADCC in the presence of IMAB362 and healthy donor peripheral blood mononuclear cells. IMAB362-induced cell killing ranged from 67-93% specific lysis with EC50 values ranging between 23-5,700 ng/mL. IMAB362's capacity to induce CDC was analyzed by incubation of CLDN18.2-positive pancreatic cancer cell lines with a serum pool from healthy donors as source for complement. We observed IMAB362 dose-dependent lysis rates with EC50 values ranging from 300-2,600 ng/mL for several cell lines.

In vivo administration of repeated doses of IMAB362 in mice bearing human pancreatic cancer xenografts significantly inhibited tumor growth and prolonged survival time compared to control. The antitumoral effect of IMAB362 strictly depended on the expression of its target CLDN18.2. IMAB362 did not only lead to growth retardation of subcutaneous human pancreatic cancer xenografts but it also reduced the metastatic load in murine lungs following intravenous injection of CLDN18.2-positive human pancreatic cancer cells.

In conclusion, single-agent IMAB362 has antitumoral activity in *in vitro* and *in vivo* models of human pancreatic cancer and metastasis. Therefore, the results from this study provide a strong rationale for clinical evaluation of this highly selective monoclonal antibody drug in patients with CLDN18.2-positive PDAC.

Understanding T cell responses against leukaemic fusion proteins

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Acute lymphoblastic leukaemia (ALL) is the most prevalent paediatric cancer (75% of all paediatric blood cancers). Despite a high initial response to chemotherapy (80-90%), a significant number of patients fail to respond to therapy and, importantly, relapse occurs in 20-25% of responding patients. Moreover, the intensity of the current chemotherapy treatments is associated with toxic side effects including growth impairment, heart and CNS disease and the development of secondary tumours. Consequently, there is a need for alternate therapeutic options with increased specificity and reduced toxicity. The TEL-AML1 fusion protein may be a suitable target for immunotherapy based approaches since it is the most frequent molecular lesion detected in 20-25% of the B cell precursor ALL (BCP-ALL or cALL) and, despite minor reported variations (9.6%), the available literature indicates that the fusion protein amino acid sequence is highly conserved. To test this, adenoviral vectors encoding the TEL-AML1 fusion breakpoint have been produced and are being tested for their ability to induce TEL-AML1 T cell responses in the C57 bl/6 mouse. Moreover, we are also generating a recombinant BCG vector encoding the TEL-AML1 fusion protein in order to test whether recombinant BCG offers significant advantages over an adenoviral vector. Importantly, in order to determine whether a TEL-AML1specific T cell can be generated to target antigen, various TEL-AML1 specific peptides are being used to stimulate cells from healthy donors. From the 23 donor samples being examined 7 responders have been identified, of which five are under the process of single cell cloning for further analysis and TCR isolation.

The HLA peptidome of small cell lung cancer cells

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Small cell lung carcinoma (SCLC) represents 13% of the newly diagnosed lung cancers, yet it is the more deadly of the different lung cancers. Treatment of SCLC remains challenging because of its rapid growth, early dissemination and development of drug resistance during the course of the disease. Thus, most SCLC patients face a grim prognosis, which calls for development of more specific and effective treatments, such as immunotherapy.

Our research focuses on the large-scale discovery of peptides presented by the HLA molecules on SCLC cells, while looking for peptides with potential to serve as targets for immunotherapy. Since the expression of class I HLA molecules on SCLC cells is significantly lower than on normal lung cells (due to the attempt of the SCLC cells to escape immune surveillance), we used two independent approaches to increase the level of expression of HLA molecules by SCLC cells in culture: First, treatment of the cells with interferon gamma (IFNγ), which increases the expression levels of the membranal HLA many folds. Second, we used recombinant expression of selected HLA alleles as induced the cells to express them as soluble HLA molecules (sHLA). The membranal HLA molecules were purified after solubilizing the IFNγ treated SCLC cells with mild detergents and the sHLA molecules were recovered from the growth medium of the transfected SCLC cells. Both approaches facilitate the recovery of large number of HLA peptides.

After immunoaffinity purification of the membranal or the soluble HLA molecules, the bound peptides were extracted and identified by capillary chromatography and tandem mass-spectrometry. In total, we were able to identify 13,544 different HLA peptides from three SCLC cells lines (NCI-H526, NCI-H69 and NCI-H82). We identified 12,932 membrane HLA class I peptides, 1,122 soluble HLA class I peptides. In addition, we identified 1,097 HLA class II peptides that were expressed by the NCI-H526 cells.

Tumor antigens potentially useful for vaccination were selected according to a number of criteria: 1) Antigens not expressed in normal tissues, such as tumor testis antigens; 2) Antigens that can elicit immune response. 3) Antigens encoded by genes known to be expressed in SCLC tumor cells. Using such databases and bioinformatics analyses we selected 72 such putative candidate HLA peptides for further testing as SCLC immunotherapeutics.

Identification of a new biomarker for ovarian cancer?

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Ovarian cancer affects approximately 6,500 women in the U.K. every year. It can occur at any age but is most common after menopause. Diagnosis at an early stage greatly improves the chances of effective treatment however diagnosis tends to be in the later stages of disease when patients present with pelvic or abdominal pain, urinary frequency or urgency, increased abdominal size. This diagnosis is confirmed by a pelvic examination, transvaginal ultrasonography and detection of carbohydrate antigen 125 (CA125) in the tumour tissue. However CA125 appears to have variable expression between patients and works better as part of a panel to improve specificity, sensitivity and differentiation of ovarian cancer from endometriosis. Human epididymis secretory protein 4 (HE4) is overexpressed in ovarian cancers however it has also been found in endometrial adenocarcinomas. HE4 provides optimal specificity when used to compare gynaecological malignancies with benign gynaecological disease but its sensitivity remains similar to CA125. However a panel of CA-125, HE4, CEA, and VCAM-1 was found to have 86% sensitivity for early-stage and 93% for late-stage ovarian cancer at 98% specificity. However there remains a need to identify a singularly good biomarker which could positively influence disease outcome by enabling early detection of ovarian cancer cells.

Using immunohistochemistry we have examined the expression of a panel of tumour antigens, in addition to CA125, in paraffin-embedded ovarian cancer microarrays containing 208 samples. Scoring was performed in a single blinded fashion and we identified a ovarian cancer protein which is expressed in more than 75% of ovarian cancer samples at every stage of disease (although early stage I and II samples predominate on the multiple tissue arrays). This frequency and the levels of expression of this protein exceeded the levels and frequency of expression of CA125 in sections from the same tissues.

Further study using an alternative antibody, specific for a single sub-variant of the ovarian cancer protein confirmed expression at all stages of ovarian cancer. Samples which were only differentially stained with haemotocylin were all negative however isotype control stained samples showed low level background staining (scoring mostly + and ++) which was expectable. Some background staining of both ovarian cancer protein and HE4 were observed in the normal tissue samples, again scoring + and ++, in a similar pattern to the isotype control. 51 of the samples were shown to express the ovarian cancer protein at high intensity (scoring mostly +++) predominantly in early stage disease compared to 19 samples for HE4. HE4 was found to be expressed at lower levels than ovarian cancer protein in the same tissues. We are now examining whether ovarian cancer protein is detectable in endometrial tissue and age/sex-matched healthy donors to see whether it would provide a good target for future immunotherapy treatments.

A new PD1-CD28 chimeric receptor overcomes PD-1-mediated immunosuppression in adoptive T cell therapy

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Introduction: Although tumor-specific, cytotoxic T cells are capable of killing tumor cells both *in vitro* and *in vivo*, treatment of immunocompetent hosts with adoptive T cell transfer does not lead to sufficient tumor regression without adjuvant therapy. This lack of efficacy has been proposed to be due to tumor-promoted T cell exhaustion and anergy. We and others have previously shown that programmed death receptor-1 (PD-1) upregulation is a hallmark of tumor-infiltrating, adoptively transferred T cells. PD-1 and its ligand (PD-L1) constitute a major immunosuppressive axis driven by tumor cells. Disruption of this axis may hit an Achilles´ heel of tumor immune escape.

Results and methods: In order to circumvent this direct immunosuppressive mechanism, a PD1-CD28 chimeric receptor was designed, cloned into the retroviral vector pMP71 and expressed in primary murine T cells specific for the model antigen ovalbumin (OT-1 cells). *In vitro,* ELISA measurement of supernatants revealed that PD-1-CD28 chimeric receptor-transduced primary T cells released 130-fold more interleukin-2 and 300-fold more interferon-γ than untransduced and control-transduced T cells when stimulated with anti-CD3e and PD-L1 ($p < 0.01$). In a protein-based array detecting various cytokines, PD-1-CD28 chimeric receptor-transduced T cells were found to secrete a CD28-like cytokine profile upon stimulation with anti-CD3 antibodies and recombinant PD-L1. Culture of transduced T cells in the presence of anti-CD3e antibodies and recombinant PD-L1 increased cell numbers 4-fold and significantly increased viability of cells compared to untransduced or control-transduced T cells using flow cytometric analysis. ($p < 0.01$). *In vivo,* treatment of mice carrying established (OVA and PD-L1 expressing) Panc02 subcutaneous tumors (mean tumor size at treatment onset 26 mm^3) with PD1-CD28-transduced OT-1 T cells delayed tumor growth and resulted in tumor rejection in 4 out of 8 mice (compared to 1 out of 8 mice treated with control-tranduced T cells). This demonstrates the functionality of the chimeric receptor in an immunocompetent mouse model.

Conclusion: Adoptive T cell therapy with PD-1-CD28 chimeric receptor-transduced T cells is a promising approach to overcome PD-1-PD-L1-mediated tumor-induced anergy and immunosuppression.

HLA ligandome profiling identifies a novel category of frequently recognized CLL associated antigens

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With the recent breakthrough developments in immune checkpoint inhibitors revealing the inherent potential of T cell based immunotherapy to effectively treat malignancies, a major remaining challenge is now to increase the specificity and frequency of anti-cancer immune responses. A rational and promising approach to achieve this goal is multi-peptide vaccination. For a long time, peptide-based immunotherapy mainly relied on reverse immunology and predictive approaches and most often fell short of its potential to achieve meaningful results *in vitro* as well as in the clinical setting. In contrast, a recent phase II vaccination study using confirmed, naturally presented HLA ligands observed specific, vaccine-induced immune responses, which were associated with improved clinical outcome (Walter, Weinschenk et al, Nat. Med. 2012).

Here, using the example of chronic lymphocytic leukemia (CLL), we further expanded on this strategy, defining a novel category of tumor associated T cell antigens strictly based on their exclusive and frequent representation in the HLA ligandome of CLL. Based on a cohort of 30 CLL patients and 30 healthy volunteers, we were able to comparatively and extensively map the HLA ligandome landscape of CLL, identifying more than 28,000 different HLA ligands. A set of 49 HLA ligand source proteins showed CLL-exclusive representation in >20% of CLL patients, thus falling into the novel category of ligandome-derived tumor associated antigens (LiTAAs). These LiTAAs were validated to be broadly and frequently represented across different stages and mutational subtypes of CLL and found to be robustly represented in HLA ligandomes of CLL patients undergoing standard chemo-/immunotherapy. Functional characterization of the corresponding HLA ligands (LiTAPs) by IFNγ-ELISPOT revealed peptide-specific immune recognition of 14/15 (93.3%) of evaluated LiTAPs exclusively in CLL patients. These immune responses were further verified to be strictly CLL- (LiTAP-) directed and mediated by functional, CLL patient derived $CD8^+$ T cells. Strikingly, for immunoreactive LiTAPs, a direct correlation between frequencies of representation in the CLL ligandomes and frequencies of immune recognition by CLL patient PBMC was observed. Furthermore, retrospective survival analysis suggests a possible benefit for patients showing immune reactions to more than one of the tested LiTAPs, although - not attaining statistical significance - these results have to be interpreted with caution, as only a relatively small number of patients, but also merely a fraction of identified LiTAPs have been evaluated.

Taken together these data clearly indicate tumor-dependent priming of LiTAP-specific T cells *in vivo* in CLL patients. This validates the newly defined category of LiTAAs to be physiologically relevant and demonstrates the effectiveness of our approach in target identification for future multi-peptide vaccination studies.

Refined targets for the peptide based immunotherapy of RCC

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The recent breakthrough developments in immune checkpoint inhibitors clearly demonstrated the inherent potential of T cell based immunotherapy to effectively treat malignancies. The major remaining challenge is now to increase the specificity and frequency of anti-cancer immune responses. With the field of peptide-based therapeutic vaccination consolidated by promising results in clinical trials (e.g. Immatics Biotechnologies IMA901 cocktail), an exciting and rational approach for achieving this goal presents itself. A pivotal factor for the coalescence of these two strategies is the identification of specific, confidently identified and relevant tumor associated T cell epitopes. As the HLA ligandome is the result of a complex antigen processing machinery, inferring information from upstream sources, such as the proteome and the transcriptome is virtually impossible. Direct scrutiny of the ligandome by modern mass spectrometry (LC-MS/MS) on the other hand is able to extensively map the HLA ligandome landscape.

Here, we implemented this approach for profiling the HLA ligandomes of 20 paired primary renal cell carcinoma (RCC) samples and devised a simplified target prioritization strategy, defining a novel category of ligandome derived tumor associated antigens (LiTAAs) strictly based on their frequent and exclusive representation in the HLA ligandomes of malignant tissue. From the bulk of more than 10,000 source protein identifications (>90% of the maximum achievable source proteome coverage as extrapolated from saturation analysis) we identified a panel of 291 HLA class I LiTAAs represented in ≥20% of RCC ligandomes. This finding is striking in context of the high degree of patient/tumor individuality we observed in patient-specific ligandome profiling by label-free quantification mass spectrometry (LFQ-MS), as well as in transcriptome analysis by RNAseq. Analysis of the HLA class II ligandomes enabled the identification of additional class II LiTAAs for synergistic vaccine design. Furthermore, naturally presented HLA class II ligands containing complete, embedded HLA class I LiTAA-derived peptides pointed out an exciting new option for optimized vaccines, targeting both, $CD4^+$ and $CD8^+$ T cells. Functional characterization of RCC-LiTAAs is ongoing.

Exhaustive analysis of the RCC-ligandome provided us with a refined set of frequently represented - and thus broadly applicable - targets for future off-the-shelf therapeutic vaccination of RCC.

Targeting leukemic stem cells in AML using the Bispecific CD33/CD3 BiTE® antibody AMG 330

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Antibody-based immunotherapy represents a promising strategy to target and eliminate chemoresistant leukemic cells in AML. In our previous work, we evaluated the cytotoxic effect of AMG 330 on AML cells. Using an AML long-term culture system, we could show efficient elimination of primary AML cells through AMG 330 activated and expanded autologous T-cells. As relapse rates in AML are high and are proposed to be caused by leukemic stem cells (LSCs), the ability of AMG 330 to redirect T-cells to LSCs was evaluated by assessing CD33 expression pattern within the LSC compartment and by limiting dilution transplantation (LDTA) assays in NOD SCID gamma null (NSG) mice. CD33 expression intensity on AML cells was assessed by flow cytometry (specific fluorescence intensity, SFI) and by surface antigen quantification (specific antigen binding sites, SABC). Median SFI and corresponding SABC was significantly higher on AML bulk cells compared to $CD34^+/CD38^-$ LSCs (AML bulk: SFI: 59.1, SABC: 47280; LSC SFI: 30.7, SABC: 24560, n=24, $p < 0.001$). Importantly, we could show significantly higher CD33 expression on LSCs compared to hematopoietic stem cells (HSC) (median SFI 8.1, 6480 SABC, n=7, $p = 0.047$). To further investigate how many CD33 molecules were needed for efficient AMG 330 mediated lysis, $CD33^{BRIGHT}$ cells (MV4-11: SFI 31) were mixed with $CD33^{DIM}$ cells (OCI-AML3: SFI 3) and co-incubated with healthy donor (HD) T-cells (E:T ratio 1:1). Lysis kinetics revealed a dependency on CD33 expression level (24h: OCI-AML3: 93%, MV4-11: 16%). However after 48 hours both cell lines were completely lysed suggesting that AMG 330 is active at low antigen density. As low CD33 expression levels might be sufficient for AMG 330 mediated lysis, we evaluated unwanted on-target toxicity in a colony-forming unit (CFU) assay. No significant difference in CFUs was detected between HD bone marrow samples pretreated with AMG 330 or control BiTE® ($p = 0.12$). LDTA assays were performed to test the potential of AMG 330 to effectively mediate lysis of LSCs. Patient-derived AML cells were lentivirally transduced with luciferase and co-cultured with HD T-cells and either AMG 330 or control BiTE® for 7 days. Residual $CD3^-$ cells were injected into NSG mice and monitored for AML outgrowth by *in-vivo* imaging and peripheral blood analysis. Control mice developed leukemia within 21 days post injection (3/3). In contrast, cells from AMG 330 treated cultures did not initiate leukemia in NSG mice (0/6; median follow up 119 days). We conclude from our data that AMG 330 has the potential to eliminate LSCs, while potentially sparing HSCs. Since our previous analysis of 621 AML patients demonstrated a highly variable CD33 expression pattern, we anticipate that AMG 330 mediated lysis will also differ between individual patients. Further investigations and clinical studies are needed to elucidate the impact of CD33 expression on response to AMG 330 mediated immunotherapy.

Enabling variant detection in single cell transcriptomics using half cell sequencing

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Next Generation Sequencing (NGS) is changing the way we understand cancer by providing deep insights into the tumor genome, development and progression. We can, for example, monitor expression of shared tumor antigens and identify immunogenic mutations for individualized therapeutic cancer vaccines (IVACs).

For applications ranging from tumor heterogeneity and evolution to circulating tumor cells (CTCs), we would like to profile gene expression and identify mutations in single cells. However, single cell genomics is complicated tremendously by the extremely small amounts of nucleic acids present in single cells and the high costs of sample profiling, particularly of exome profiling.

Here, we present a novel method to determine gene expression and identify mutations in single cells. The method is robust, reproducible, and costs less than existing methods. We isolate RNA from single cells; use a modified reverse transcription with 5‘ template switching; and use tagmentation-based library preparation methods to enable amplification with pico-gram amounts of input RNA, reduced hands-on time and lower costs.

Single cell sequencing is a challenging technique with a small signal to noise ratio. This leads to an uncertainty whether variants which have been detected are real or noise coming from reverse transcription, amplification and finally sequencing. A simple approach would be taking replicates into account. Biological replicates are difficult by the nature of transcription in single cells. Using biological replicates for mutation validation in CTCs assumes a model that all cells have the same mutations. This might be true for mutations which occurred early. Mutations gained later are missed in this process. However to circumvent this we decided to make technical replicates from single cells, the half-cell approach. By splitting up the first strand prior to amplification we keep technical errors stochastic distributed. Variations which are detected in both cells are unlikely to be false positives. This might be crucial for individualized medication based on CTC samples.

In our proof of concept experiments we were able to show that half cell sequencing (HCS) is capable of noise reduction and consistent mutation detection. Up to 10 % of validated mutations can be found in half cells from which up to 90 % are found in both halves and are confirmed by Bulk RNA-Seq. Variant detection from scratch without any reference reached up to 90 % accuracy by comparison to bulk RNA-Seq. Also non mutation events like RNA-editing could be detected and confirmed in half cells. This shows that HCS is a powerful tool for variant detection in single cells.

In summary, our method may enable to examine tumor heterogeneity, tumor evolution, and, using CTCs, identify patient specific immunotherapy targets in a minimally invasive manner.

LTX-315 treatment induces complete and specific regression of disseminated tumors in a novel mesenchymal three tumor rat model

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LTX-315 is a de novo designed peptide derived from a naturally occurring host defense peptide. LTX-315 has the potential to induce long-term specific protective immune responses by inducing the release of danger signals (e.g. HMGB1) and tumor associated antigens (TAAs).

In this study we have used a novel rat mesenchymal tumor model. The tumor cells maintain their mesenchymal somatic stem cell properties in vitro and show an aggressive growth pattern in vivo. Similar to what have been observed in several syngenic rodent tumor models a complete regression was obtained in this model following intratumoral (i.t.) treatment of subcutaneous tumors with LTX-315. The effect was T-cell-dependent since the intervention was inefficient in immune-deficient animals. Studies on treated tumor tissue confirmed infiltration of immune cells and a switch in the cytokine profile towards a Th1 response. Successfully cured animals were protected against re-challenge with the same tumor cell type, but not against other types of tumor cells. Moreover, tumor resistance could be adoptively transferred by spleen cells from LTX-315-treated animals to naïve animals. The resistance was abrogated by depletion of T-lymphocytes.

To clarify whether i.t. injections of LTX-315 in one tumor lesion could have an abscopal effect on metastatic disease, intraperitoneal tumors and two subcutaneous tumors were established in the animals. Thereafter, LTX-315 was injected into one of the subcutaneous lesions. As assessed by living imaging, all metastatic manifestations slowly regressed and all animals went into durable complete remission.

These studies indicate that LTX-315 has potential to locally activate the innate immune system by its membrane destabilizing effect on the tumor cells followed by a release of natural danger signals, providing a strong rationale for using LTX-315 in combination with other types of immune-modulatory therapies.

In summary, we show for the first time that LTX-315 mounts strong and specific immune responses that are able to control disseminated tumors in animals. The present findings further underline the potential use of LTX-315 as a novel treatment strategy to generate personalized tumor specific immune responses. LTX-315 is currently being tested in a Phase I dose escalation clinical study.

Identification of novel immune checkpoints as targets for cancer immunotherapy

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Members of the B7/CD28 family of immune checkpoints, such as CTLA4, PD1 and PDL-1, play critical roles in T cell regulation and have emerged as promising drug targets for cancer immunotherapy. We hypothesize that additional novel members of the B7/CD28 family play a role as negative immune regulators and thus may serve as targets for therapeutic mAbs. Utilizing Compugen's predictive discovery platform, we identified nine novel members of this family that may serve as immune checkpoints. The therapeutic relevance of three of these proteins, CGEN-15001T, CGEN-15022, and CGEN-15049, was confirmed following the validation of their immunomodulatory properties and their expression in various cancers. Two of these proteins, CGEN-15001T and CGEN-15022, are the basis of a license and collaboration agreement recently signed with Bayer as targets for cancer immunotherapy. Here we present results obtained for an additional novel immune checkpoint, CGEN-15049. Following its ectopic expression on cancer cell lines, CGEN-15049 inhibits the activity of NK cells and cytotoxic T cells (CTLs). The fusion protein, consisting of the extracellular domain of CGEN-15049 fused to an IgG Fc domain, displays robust inhibition of T cell activation and enhances iTregs differentiation. IHC studies indicate that CGEN-15049 is expressed in tumor cells of numerous types of cancers, as well as in tumor infiltrating immune cells. Based on its immunomodulatory activities on several types of immune cells which play key roles in cancer immune evasion, together with its expression pattern, CGEN-15049 may serve as mAb target for cancer immunotherapy.

Ratio of intratumoral macrophage phenotypes predicts local tumor outgrowth in malignant pleural mesothelioma patients

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Introduction: In patients with malignant pleural mesothelioma (MPM), local tumor outgrowth after invasive diagnostic procedures, e.g. biopsies and surgical incisions, is a relatively rare but painful complication. To prevent this tumor outgrowth, prophylactic radiotherapy to intervention sites (PIT) was introduced in an attempt to improve quality of life for these patients. However, studies regarding the efficacy of PIT have been hampered by the lack of proper biomarkers which enable the selection of patients prone to develop local tumor outgrowth. Tumor-associated macrophages (TAMs) are abundantly present in mesothelioma tumors and can be skewed towards an anti-tumor (M1) or pro-tumor (M2) phenotype. M2 TAMs are known to play an important role in wound healing, tissue remodeling and tumor invasion which suggests a potential role of this cell type in the development of tumor outgrowth. The aim of this study is to investigate whether the TAM phenotype in the tumor microenvironment can predict the development of local tumor outgrowth and therefore aid to the selection of patients who will benefit from prophylactic interventions.

Methods: 10 MPM patients who developed local tumor outgrowth were matched on age, sex, tumor histology, TNM stage, EORTC score, treatment and survival with 10 MPM patients who did not develop local tumor outgrowth after a local intervention. Immunohistochemistry was performed on diagnostic biopsies to determine the total TAM count (CD68) and the M2 TAM count (CD163). Two researchers and a pathologist independently assessed the TAM infiltration in tumor regions. Statistical analyses were performed using the Mann-Whitney U test.

Results: The total number of $CD68^+$ and $CD163^+$ cells was comparable between the tumor outgrowth and no tumor outgrowth group. However, the mean CD163/CD68 (M2/total TAM) ratio differed statistically significantly between the two groups: 0.90 ±0.09 in the tumor outgrowth group and 0.63 ±0.09 in the patients without tumor outgrowth ($p < 0.001$).

Conclusion: This study shows that patients who develop tumor outgrowth after a local intervention have a higher M2/total TAM ratio in their diagnostic tumor biopsies compared to patients who don't develop this outgrowth. In contrast with this ratio, the total (M2) TAM numbers did not differ between the two groups, indicating that the phenotype profile has more clinical value than the absolute cell numbers. Therefore, we propose that the intratumoral macrophage phenotype ratio is a new potential tool to predict which MPM patient will develop tumor outgrowth and could be used to select patients for a study regarding the efficacy of prophylactic irradiation.

Novel oncolytic alphavirus vectors with increased tumor specificity

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Avirulent Semliki forest virus (SFV) vector VA7 has been shown potency as oncolytic agent in immunocompromised cancer models, including glioma. However, in syngeneic glioma models displaying normal type-I interferon (IFN) response, the oncolytic efficacy was poor. Recently, neurovirulent alphaviruses have been shown to inhibit the induction of IFN response in cells. Here we studied if neurovirulent strains of SFV can be used to engineer potent but safe oncolytic agents. MicroRNA-mediated tissue de-targeting was used to inhibit unwanted SFV replication in healthy central nervous system tissue.

Inhibition of JAK/STAT-pathway in Vero(B) cells was studied by western blot. Oncolytic potency was tested *in vitro* by measuring cytopathic effect (CPE) in CT-2A mouse astrocytoma cell line. Target sequences against miR-122 (expressed in liver) or miR-124 (expressed in neurons) were inserted between SFV4 nsP3 and nsP4 genes. Tissue de-targeting *in vivo* was studied by infecting BALB/c mice with SFV4 carrying miR-122 or miR-124 target sequences (SFV4-miRT122 and SFV4-miRT124). Our results show that the neurovirulent SFV4 inhibits STAT1 phosphorylation thus disrupting type-I IFN mediated signaling. Inhibition is mediated by viral nsP3 and nsP4 genes *via* unknown mechanism. Correlating with this finding, SFV4 displays increased CPE in IFN-beta treated CT-2A cells *in vitro* as compared to interferon-sensitive SFV clones. Insertion of microRNA target sequences between nsP3 and nsP4 genes did not affect negatively on the STAT1 phosphorylation inhibitory capacity of SFV4. SFV4-miRT124 was detected to dominantly infect oligodendrocytes of the *corpus callosum* showing only limited replication in neurons. We conclude that neurovirulent SFV4 shows increased oncolytic potency *in vitro* and that safety of such virus can be increased by microRNA-mediated tissue de-targeting.

Novel T helper epitope peptides derived from Lck can induce cytotoxic T lymphocytes in HLA-A2$^+$ cancer patients

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Purpose: MHC class I-restricted epitopes have been mainly identified as antigen peptide candidates for cancer vaccines. However, tumor-specific helper T cell immunity has also been shown to be pivotal for the efficient eradication of solid tumors. For example, CD4$^+$ helper T cells are important for priming, differentiation, and expansion of tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs). In addition, they are also shown to be essential for the generation and maintenance of long-lasting CTL responses. In the current study, we attempted to identify peptide candidates, which contained both helper T cell epitopes and CTL epitopes, from Lck (p56^{Lck}).

Experimental Design: Based on the biding motif to the HLA-DR and -A2 alleles, 94 peptides were prepared from the amino acid sequence of Lck. The peptides were screened first for their ability to be recognized by immunoglobulin G (IgG) from cancer patients and subsequently for their potential to induce peptide-specific and cancer reactive CTLs from peripheral blood mononuclear cells (PBMCs) obtained from HLA-A2$^+$ cancer patients.

Results: The peptide candidates, which were recognized by IgGs of cancer patients, efficiently induced antigen-specific CTLs from PBMCs of HLA-A2$^+$ cancer patients. The antigen-specific CTLs lysed peptide-pulsed target cells as well as SW620 colon cancer cells in an HLA class I-dependent manner.

Conclusion: We identified novel T-helper peptide candidates derived from Lck, which can induce antigen-specific CTL responses in HLA-A2$^+$ cancer patients. The Lck protein (p56^{Lck}), the src family tyrosine kinase, which is essential for T-cell development and function, is known to also be aberrantly expressed in metastatic epithelial cancers. The identified novel T-helper peptides might be useful for development of peptide-based vaccines, especially in patients with cancer metastases.

High-throughput identification of immune-checkpoint molecules expressed by melanoma using patient-derived tumor-infiltrating lymphocytes

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Immunotherapeutic treatment of melanoma achieved major progress in recent years leading, for the first time, to improved survival. However, due to the fact that melanoma cells employ suppressive mechanisms in order to evade recognition and destruction by immune effector cells, the majority of patients still do not benefit from immunotherapy. These mechanisms are far more diverse than reflected by current clinically used immune modulatory drugs. In this study, we established a novel high throughput RNAi screening to identify new targets in melanoma using antigen-specific patient-derived tumor infiltrating lymphocytes (TIL) with primary HLA-matched melanoma cells. Using this approach, we screened a siRNA library containing more than 1200 G-protein coupled receptors (GPCR) and kinases as targets for immunotherapy.

Briefly, HLA-A2 and luciferase positive M579-A2-luc melanoma cells were reversely transfected with the GPCR and kinase library. Afterwards the target cells were co-cultured with MART1- and gp100-specific TILs to verify TIL-mediated tumor lysis. Local regression models (LOESS) were applied to generate a hit list of 48 candidates negatively regulating CTL cytotoxicity. Interestingly, 4 candidates of a related high-throughput breast cancer screen were among the top hits, suggesting reproducibility and robustness of the screening approach as well as a general role of those immune-checkpoint molecules in cancer.

In a first validation step, we abrogated candidate-mediated inhibition of TIL cytotoxicity by siRNA knock-down. We found that knock-down of several candidates in M579-A2-luc increased TIL-mediated killing and activity, measured by production of IFN-γ, IL-2 and TNFα. Furthermore, inhibition of the kinase activity of two candidates with small molecule inhibitors increased the tumor cell susceptibility to TIL-mediated killing in a concentration-dependent manner. Currently we are establishing a secondary high-throughput screening assaying multiple TC activation markers, including effector cytokines, to streamline the discovery process for large scale surface molecule libraries (consisting of around 3700 genes associated with the cell surface).

In summary, we established a novel antigen-specific screening approach for immune-checkpoint molecules expressed in melanoma and were able to identify a list of promising candidates for further investigation. This high-throughput screening offers a systematic platform to uncover the "immune-modulatome" of cancer and subsequently discover novel targets for immunotherapy.

Preclinical rationale for the combination of IMAB362 with standard chemotherapy regimens for the treatment of gastro-esophageal cancer and pancreatic cancer

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The tight-junction molecule claudin 18.2 (CLDN18.2) is a novel drug target for the treatment of patients with, e.g. gastric, esophageal, or pancreatic cancer. CLDN18.2 expression is strictly confined to short-lived gastric mucosa cells, but not present in any other healthy tissue. IMAB362 is the first-in-class monoclonal antibody against CLDN18.2 and is currently under clinical investigation in patients with gastroesophageal cancer (GEC). It bundles for independent, highly potent mechanisms of action: antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), cross-linking induced apoptosis, and inhibition of tumor-cell proliferation. IMAB362 exerts all 4 mechanisms of action *in vitro* in CLDN18.2-positive human gastric (GC) and pancreatic cancer (PC) cells and leads *in vivo* to tumor-growth retardation and a survival benefit in the respective xenograft models. Chemotherapeutics are the standard of care in solid cancers, such as GC or PC. Here we hypothesized that standard chemotherapy may augment IMAB362's antitumoral activity in a preclinical setting.

Therefore we studied the effect of IMAB362 in combination with standard chemotherapy *in vitro* and *in vivo* in models for GC (5-FU+O, EOF) as well as PC (gemcitabine, gemcitabine plus oxaliplatin). First we assessed the *in vitro* effect of cytostatic agents on IMAB362-mediated ADCC and CDC in GC and PC cell lines. Incubation with these agents enhanced the sensitivity of IMAB362-induced ADCC of both GC and PC cells with significantly reduced EC50 values. IMAB362-induced CDC was similarly affected by cytostatic agents in both models. Thus combination of IMAB362 with common chemotherapies enhanced its antitumoral activity *in vitro*.

For the analysis of the antitumoral activity of the combination of cytostatic agents with IMAB362 *in vivo*, mice were xenografted with either human GC or PC cells and treated with IMAB362 plus the respective chemotherapeutic agent. IMAB362 in combination with chemotherapy significantly inhibited tumor growth and led to a survival benefit in xenografted mouse models of both cancer types. IMAB362 plus the respective cytostatic agents were clearly superior to either treatment alone in both model systems *in vivo*.

In order to explain this effect we hypothesized that cytostatic agents might increase CLDN18.2 expression in cancer cells. Treatment with cytostatic agents increased CLDN18.2 gene expression and protein expression of human GC and PC cells. The increased target presence may at least in part facilitate the augmentation of IMAB362's antitumoral activity.

In conclusion, the addition of cytostatic agents augments IMAB362's antitumoral activity in preclinical models of GC and PC. This may in part be boosted by the increased target availability on the surface of cancer cells caused by cytostatic agents. Our data provide strong rationale for further clinical evaluation of IMAB362 combination therapies and studies are ongoing in patients with GEC.

Diacylglyerol kinase: a reversible checkpoint blockade in CD8 T and NK cells from human carcinoma acting downstream of currently targeted checkpoint molecules

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Recent experimental and clinical reports highlight the promise of using immune cell function to control tumor growth and even achieve rejection. Current activities focus on reversing immune checkpoints that are mediated by surface expressed molecules, i.e. PD-1, LAG-3, TIM-3 or CTLA-4. Therapeutics targeting these checkpoints show reassuring positive clinical results. Yet, a significant portion of patients does not respond and some tumor histologies appear to be completely unresponsive.

Our analysis of tumor-infiltrating lymphocytes (TILs) of human renal cell carcinoma revealed diacylglycerol kinase (DGK)-α as a key regulator of CD8 T lymphocyte function and NK cell function in the tumor milieu (1,2). DGK-α is a physiologic attenuator of immune cell activity and controls functional activities through moderating ERK and Akt signaling pathways. DGK-α was found highly expressed in CD8-TIL and NK-TIL compared to respective cell types from non-tumor kidney. Concomitantly, cytotoxic lymphocytes of TILs showed repressed ERK pathway activation as well as poor degranulation and cytokine production after stimulation. Inhibition of DGK-α as well as brief IL-2 culture restored cytotoxic lymphocyte function. Both interventions de-repressed the ERK pathway. DGK inhibition did not augment degranulation beyond the intrinsic response efficacy of the cytotoxic lymphocyte. This has important implication for a potential targeting of DGK in a clinical setting as this eases concerns about undesirable autoimmunity.

DGK inhibitors or activators of the ERK pathway emerge as promising therapeutic targets to improve antitumor immunotherapy in several ways. DGK inhibition restores suppressed function of both adaptive as well as innate cytotoxic lymphocytes in the tumor milieu. CD8 T cells and NK cells act synergistically in antitumor response with NK cells recognizing tumor cells that are resistant to T cell killing due to downregulation of MHC-class I molecules. Conjointly de-repressing the function of both cell types should lower the occurrence of immune escape. Moreover, DGK inhibition should act synergistically with current checkpoint therapeutics as it unblocks signaling pathways downstream of current checkpoint molecules. Further, DGK inhibition is reported to block key oncogenic pathways in tumor cells, pathways which are relevant for tumor cell survival (3). Therapeutic targeting of DGK, thus, may support the antitumor response not only by restoring cytotoxic lymphocyte activity but additionally by activating apoptotic pathways in tumor cells.

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Epitope discovery is a bottleneck for development of immunotherapy and vaccination in cancer and infectious disease. Here, we describe a new technology for the rapid and direct identification of multiple naturally processed and immunogenic cytotoxic T cell (CTL) epitopes, as well as of the T cells recognizing the epitopes. The high-throughput technology was based on the transfection of monocyte-derived dendritic cells (DCs) with mRNA encoding the full-length target protein and subsequent co-culture with T cells. CTLs reactive to processed and presented epitopes were directly

HLA ligandome analysis identifies Histone Deacetylases as novel targets for T-cell mediated immunotherapy of ovarian carcinoma

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Objective: Late diagnosis and resistance to chemotherapy are the main causes for the high mortality among women suffering from ovarian carcinoma (OvCa) emphasizing the urgent need for new treatment options. Meanwhile, several puplications provide evidence that OvCa is a highly immunogenic tumor entity making immunotherapies like peptide-based cancer vaccines promising approaches for the treatment of OvCa.

The main goal of this study was to perform HLA ligandome analysis of high-grade serous OvCas to identify tumor-associated peptides. In order to assess whether HLA ligandome reflects protein expression, candidates were validated by immunohistochemistry and correlated with immune cell infiltration and overall survival in a large OvCa cohort.

Material and methods: We isolated HLA class I molecules by affinity chromatography from 7 different OvCa samples and analyzed HLA ligands by liquid chromatography coupled mass-spectrometry. Candidate peptides were selected for immunogenicity analysis. *In vitro* priming of $CD8^+$ T cells from healthy blood donors was performed with artificial antigen-presenting cells (aAPCs). Presence of antigen-specific T cells was evaluated by tetramer staining and the functionality (cytokine production and cytotoxicity) was further assessed. Finally, the combined prognostic effect of tumor infiltrating lymphocytes (TILs) and the expression of candidate antigens was evaluated by immunohistochemistry in a tissue microarray of 136 cases of high-grade serous OvCas.

Results: HLA ligandome analysis identifies peptides derived from the established TAA HDAC1 to be highly overrepresented on tumor cells. In total, 4 different HDAC1 and HDAC2 derived peptides covering 3 different HLA restrictions were identified and tested for their immunogenic potential. *In vitro* priming confirmed 2 of these peptides to be immunogenic.

The most promising epitope is an HLA-A*02 restricted peptide which induced T-cell responses in 9 of 11 donors. To confirm this strong immunogenicity, *in vitro* primings were performed using peptide-loaded autologous DCs resulting in a positive IFNγ-response in 7 of 14 tested donors. T-cell multifunctionality has been shown by staining for TNFα, IL-2, CD107a and MIP-1β. In addition, HDAC1/2-specific T cells lysed HLA-A*02^+ tumor cells in cytotoxicity assays whereas HLA-A*02-negative cells were not killed. Immunhistochemistry revealed HDAC1 to be highly overexpressed in OvCa and showed a negative impact on prognosis (median 26 vs. 42 months, $p = 0{,}011$, log rank). Notably, high numbers of TILs can overcome this prognostic effect.

Conclusions: Based on our experimental data we plan to use the immunogenic HDAC1/2 derived peptides in an upcoming multipeptide Phase I/II vaccination trial in OvCa patients. Further we could show that the combined approach of proteomics and immunohistological evaluation is contributing to the identification and validation of reasonable antigens for the development of a multipeptide vaccine for OvCa.

A new method of testing T-cell reactivity to tumor antigens without prior knowledge of the T-cell epitopes

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The main goal of dendritic cell (DC) vaccination in melanoma is to induce a strong and tumor-specific T-cell response. Therefore, DC are manipulated to present tumor antigens to activate those T cells which are carrying a tumor-antigen-specific T-cell receptor. Classically, non-mutated tumor-associated antigens are used, but recently, also frequently occurring mutations of oncogenes, e.g. $BRAF^{V600E}$, $NRAS^{Q61K}$, have emerged as possible new targets of therapeutic tumor immunization. To detect and monitor the induced T cells without requirement for characterized T-cell epitopes and independent from HLA haplotypes we expressed these antigens intracellularly for natural processing. Testing several cell types identified the electroporation of autologous T cells themselves with the antigen-encoding mRNA as preferable. Taking advantage of their capability to present antigens on their own MHC molecules, the electroporated T cells were then capable to stimulate each other.

We verified the functionality of this read-out system with the melanoma antigen MelanA. Using tetramer staining, we observed an increase in the percentage of MelanA-specific $CD8^+$ T cells after repeated stimulation with MelanA-RNA electroporated DC. When these T cells were electroporated with RNA, they were able to express, process, and present the mRNA-encoded antigen and to stimulate each other, shown by cytokine-secretion-based assays. The numbers of cytokine-producing T cells after mRNA electroporation correlated with those observed after peptide pulsing, if intermediate peptide concentrations were used. These results indicate that T cells of high and intermediate affinity can be detected with our new screening method.

To examine the immunogenicity of the frequently occurring tumor driver mutations $BRAF^{V600E}$, $NRAS^{Q61K}$, we electroporated mature DC of healthy donors with mRNA containing the wild-type or the mutated antigen sequence in order to obtain the MHC presentation of the naturally processed antigen peptides. Afterwards, we stimulated autologous $CD8^+$ T cells up to four times with these DC to expand the tumor-specific T cells. To determine the percentages of tumor-antigen specific T cells, we electroporated them with mRNA of the mutated or non-mutated antigens, and used IFNg-ELISPOT assays and intracellular cytokine staining as read-outs.

Testing mutated antigens with this read-out system we could find a clear induction of immune responses. However, T cells of many donors expanded after stimulation with both, the mutated and the wild-type antigens. Cross-reactivity between the different antigen versions were observed, and is now systematically tested and compared in melanoma-bearing patients versus healthy persons.

The latter two authors contributed equally

Selective stimulation of RIG-I with a novel synthetic 5`triphosphorylated RNA induces superior anti-tumor immunity in primary and transplanted mouse melanomas

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Despite the introduction of new drugs like Vemurafenib and Ipilimumab, melanoma remains the most deadly form of skin cancer. As melanomas frequently become resistant or do not respond to current treatments, there is still a highly unmet medical need. Stimulation of the cytosolic RNA receptor retinoic acid inducible gene I (RIG-I) opens a new therapeutic strategy that could be well combined with modern immunotherapeutic regimens. RIG-I activation preferentially induces apoptosis in tumor cells and simultaneously activates the innate immune system via type I interferon (IFN) signaling. We previously developed an optimized fully synthetic 5´-triphosphate RNA (called ImOl100) ligand selective for RIG-I. We tested this immunostimulatory RNA in the genetic autochthonous Hgf-Cdk4 mouse melanoma model, which closely recapitulates human melanoma pathogenesis and therefore creates a more accurate prediction of therapeutic efficacy in patients than transplantable tumor models. Local delivery of ImOl100 into primary established HgfxCdk4^{R24C} mouse melanoma induced potent tumor regression in contrast to the TLR9 ligand CpG DNA. Histological analysis of the tumors revealed a strong infiltration of immune cells in ImOl100, but not in control RNA treated melanoma. ImOl100 induced a pronounced and durable type I IFN response after multiple local injections in IFNß-Luciferase reporter mice. Furthermore, ImOl100 synergized in the treatment of primary as well as transplanted melanoma with the inhibition of PD-1 signaling, opening the possibility of improved combinatorial treatment regimens. In summary, our RIG-I-selective ImOl100 shows strong anti-melanoma activity in a clinically relevant genetic mouse model synergizing with currently evolving treatment strategies.

Characterization of a genetic mouse model of lung cancer: A promise to identify non small cell lung cancer therapeutic targets and biomarkers

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Non-small cell lung cancer (NSCLC) accounts for 81% of all lung cancer cases and they are often fatal because 60% of the patients are diagnosed at an advanced stage. Besides the need for earlier diagnosis, there is a great need for additional effective therapies. We have characterized by non-invasive magnetic resonance imaging (MRI), histopathological and immunohistochemical analysis the lung cancer progression in a K-ras^{LA1}/p53^{R172HDg} mouse model which mimics features of human metastatic NSCLC. We have investigated the feasibility of this preclinical model as a biological reservoir for potential therapeutic targets and biomarkers.

Since the identification of oncoantigens expressed during tumor development could provide an unprecedented opportunity to address the immune system against these molecules, we performed a microarray analysis and RNA-seq profiling on total RNA extracted from lungs of 10,20 and 30 week-old wild type (wt) and K-ras^{LA1}/p53^{R172HDg} male and female mice to detect gene/exon-level differential expression and/or fusion genes associated to the increment of tumor mass.

Fusion events were not detected as well as genes with a differential expression at exon-level showed no significant correlation with the disease prognosis, while differential expression at gene-level allowed the identification of 1513 genes with a significant increase in expression associated to tumor mass increase. 74 genes, i.e. those secreted or expressed on the plasma membrane, were used for a meta-analysis of two transcriptomic datasets of human NSCLC sample. SPP1 (osteopontin) was the only molecule whose overexpression was statistically related to poor outcome in terms of both survival and metastasis formation. Two other molecules displaying over-expression associated with metastasis formation caused poor outcome, GM-CSF and ADORA3. GM-CSF is a secreted protein, and we confirmed its expression in the supernatant of a cell line derived from a K-ras^{LA1}/p53^{R172HDg} mouse tumor. ADORA3 is instead involved in the induction of p53-mediated apoptosis in lung cancer cell lines. Since p53 is inactivated in our model, ADORA3 does not negatively affect tumor growth, but remains expressed on tumor cells. Thus, it may be an interesting target for the development of antibody-targeted therapy on a subset of NSCLC which is p53 null and ADORA3 positive. Moreover, among those genes found differentially expressed during lung cancer progression in mice that fullfill the minimal requirement for an oncoantigen, ROS1 was identified as an interesting putative oncoantigen to be further investigated. Indeed, on the basis of the literature we consider ROS1 a potential candidate for the building of anti-tumor vaccines against NSCLC. In conclusion, our study provided a complete transcription overview of the K-ras^{LA1}/p53^{R172HDg} mouse NSCLC model. This approach allowed for the detection of ADORA3 and ROS1 as potential targets for new immunotherapeutic strategies to fight against NSCLC.

Identification of mutated neoantigens in the human melanoma model Ma-Mel-86 combining exome and transcriptome sequencing

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A series of antigens targeted by autologous TCRαβ$^+$ CD8$^+$ cytotoxic T-cell (CTL) clones have been identified via cDNA expression cloning in the human melanoma model Ma-Mel-86 (patient INTH) with four stable tumor cell lines derived from different lymph-node metastases. Most of them were melanocyte differentiation antigens, whereas HERPUD1^{G161S} (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) represented a mutated neoantigen targeted by HLA-B*15:01-restricted CTL. But a significant proportion of T cells did not recognize any of 40 known melanoma-associated antigens. To find out, whether they were directed against one or more individual neoantigens, we performed exome and transcriptome sequencing of the four melanoma cell lines and an autologous lymphoblastoid cell line to define expressed somatic mutations as a first step. All samples were sequenced as biological duplicates (exomes) or triplicates (transcriptomes) on the Illumina HiSeq 2000 platform. With these sequencing data 181 expressed somatic non-synonymous point mutations were identified, 63 of which were common to all four melanoma cell lines. We determined for all mutations the probabilities for the presentation of mutated 8-, 9- and 10-mer peptides by the patient's HLA class I molecules using NetMHC (http://www.cbs.dtu.dk/services/NetMHC/) and IEDB (http://tools.immuneepitope.org/mhci/). 174 peptides with a predicted binding affinity of ≤500 nM (NetMHC) and a percentile rank of ≤10 (IEDB) were synthesized and tested for their recognition by eight independent autologous mixed lymphocyte-tumor cell cultures (MLTCs) derived from four distinct blood samples and generated by stimulation with different autologous melanoma lines. Eleven of the predicted peptides were recognized by at least one of the MLTCs. They were encoded by four mutated genes: *HERPUD1*^{G161S} (see above), *INSIG1*^{S238F} (insulin-induced gene 1), *MMS22L*^{S437F} (methyl methanesulfonate-sensitivity protein 22-like), and *PRDM10*^{S1050F} (PR domain containing 10). All newly identified mutated peptide antigens were recognized in association with HLA-A*24:02 as demonstrated with MLTC-derived, clonal T cells. Final experiments will specify the optimal peptides and validate their intracellular processing. - The combination of both exome and transcriptome data allowed us to reliably determine expressed somatic mutations in Ma-Mel-86 cells. In independently generated autologous MLTCs, T-cell responses were observed against approximately 6% of the peptides carrying these mutations and predicted to bind to at least one of the patient´s HLA molecules according to the parameters indicated above. Retrospectively, the success rate could have been increased to 32% (8/25 peptides tested as positive) without missing any of the four immunogenic mutations using more strict selection criteria (NetMHC: binding affinity ≤200 nM, IEDB: percentile rank ≤1).

Dual murine CT26 subclone model of heterogeneous cancer reveals dominance of interferon-sensitive clones in oncolytic alphavirus therapy

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In our earlier studies, Semliki Forest virus vector VA7 completely eliminated type I interferon unresponsive human U87-luc glioma xenografts while interferon responsive mouse gliomas proved refractory. Here we describe in two clones of CT26 murine colon carcinoma, opposed patterns of type I interferon responsiveness and sensitivity to VA7. Both CT26WT and CT26LacZ clones secreted biologically active interferon *in vitro* upon virus infection but it protected only CT26WT cells. Focal virus infection in these cells was self-limiting but could be rescued using type I interferon pathway inhibitor Ruxolitinib or antibody against IFNβ. Whole transcriptome sequencing (RNA-Seq) and protein expression analysis revealed that CT26WT cells constitutively expressed 56 different genes associated with pattern recognition and type I interferon signaling pathways, spanning two reported anti-RNA virus gene signatures and 22 genes with reported anti-alphaviral activity. Whereas CT26WT tumors were strictly virus-resistant *in vivo*, infection of CT26LacZ tumors resulted in complete tumor eradication in immunocompetent and SCID mice. Despite successful eradication of CT26LacZ tumors from one flank CT26WT tumors continued growing in the other. The outcome was the same when CT26LacZ tumors contained only small fraction of CT26WT cells, demonstrating the dominance of IFN-responsiveness when heterogeneous tumors are targeted with interferon sensitive oncolytic viruses.

pMHCI-IgG redirect HCMV-specific CD8$^+$ T-cells to eradicate cancer cells

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Cancer and chronic viral infections challenge the human immune system and have generated multiple ways to evade an immune response. While cancer cells finally escape the immune system, many chronic viral infections are controlled lifelong by the adaptive immune response.

Antibody mediated targeting of recombinant pathogen-derived peptide MHCI-complexes (major histocompatibility complexes) can decorate tumor cells with antigenic peptide-MHCI-complexes thus mimicking viral infection and triggering elimination of these cells by CD8$^+$ cytotoxic T-cells. Therapeutic approaches were so far partly hampered by the infeasibility to produce those molecules as human IgG fusions and in reasonable quantities.

Employing extensive protein engineering, we generated novel formats of fully recombinant virus peptide-MHCI- fusions with full human IgG antibodies (pMHCI-IgG). These molecules can be expressed in standard mammalian expression systems with similar yield and purity as compared to normal antibodies. pMHCI-IgG molecules target tumor cells via the antibody-inherent specificity and thus deliver correctly folded viral peptide-HLA-A*0201 complexes to the tumor cell surface. We demonstrated that these molecules recruit and activate CD8$^+$ CMV (Cytomegalovirus) pp65 specific T-cells in a peptide-restricted manner exclusively upon target binding and polyvalent pMHCI delivery. We show that donor derived CMV pp65 specific CD8$^+$ T-cells within PBMCs (Peripheral Blood Mononuclear Cells) are numerically and functionally sufficient to eliminate tumor cells targeted with pMHCI-IgGs without any co- or prestimulation. As current anti-CD3 T-cell recruitment strategies (BITEs) harbor some important disadvantages like bulk T-cell activation and the subsequent massive release of cytokines, we investigated how pMHCI-IgGs compare to BITEs in two aspects. First, we show that the capacity of pMHCI-IgGs to recruit T-cells via pMHCI/TCR interaction is comparable to T-cell activation via high affinity anti-CD3 binders and that pMHCI-IgGs were similarly effective in inducing tumor cell killing under analogous preconditions. Secondly we show that even with donor derived PBMCs containing only small populations of peptide-MHC specific T-cells we are able to induce a potent eradication of tumor cells in vitro in a concentration- and time-dependent manner which was comparable to BITEs. Additionally, pMHCI-IgGs indeed seem to possess a much more favorable safety profile in comparison to BITEs. Consequently, pMHCI-IgGs constitute an attractive approach to induce highly functional but more specific and safer recruitment of T-cells to eradicate tumor cells. With the novel format of pMHCI-IgGs these molecules now advance in the focus of therapeutic application.

Ex vivo therapy simulation of multivalent adenoviral immunotherapy in the human urinary bladder carcinoma microenvironment

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The composition of the immunologic tumor microenvironment is crucial to disease progression and patient survival. Whereas marker profiles of favorable and non-favorable immunologic microenvironment compositions were described, therapeutic intervention remains a substantial challenge. Therapeutic targeting of the tumor microenvironment requires addressing different immune cell types and requires combinations of biological signals.

Urinary bladder carcinoma is an epithelial tumor qualified as immunotherapeutic target indication based on decades of therapeutic intravesical administration of the bovine mycobacterium Bacillus Calmette-Guérin (BCG). Repeated cycles of BCG administration induce unspecific inflammation and in turn can result in clinical response and prolonged survival of patients.

As therapeutic system addressing the tumor microenvironment and to induce concerted local induction of a cellular immune response we have developed an adenoviral vector system expressing the cytokines interleukin-2, interleukin-12 and the costimulatory protein 4-1BBL, termed Immunalon®. For the first time, molecular microenvironment reprogramming using this multivalent biological product was evaluated in an *ex vivo* human tumor tissue model of bladder carcinoma. Immunological changes are monitored over time by expression profiling using whole transcriptome analyses, by quantification of secreted therapeutic and response proteins and by histology to confirm cellular composition of stimulated samples.

Preliminary data in these resected tumor samples show that stimulation with Immunalon® leads to a broad activation of cellular immunological processes. This activation is dose-dependent and not observed by stimulation with control adenovirus without transgene expression. Tissue culture supernatants show elevation in Interleukin-12 and Interleukin-2 levels, as well as stimulation of a substantial Interferon-gamma response. Histologic evaluation indicates clear evidence for treatment-induced changes in infiltrating lymphocyte patterns.

Our data suggest primary urinary cancer tissues as valuable immunotherapy model. All features for treatment simulation by the adenoviral immunotherapeutic Immunalon® are provided, e.g. the dimension of tissue samples, their viability in culture over up to 14 days, and their non-dissociated microenvironment that retains the immunologic infiltrates serving as targets for local therapy approaches.

Based on the wide alterations in expression profiles detected upon Immunalon® exposure, further investigations on the cellular components of local immune activation are envisaged. Data on processes and effector cells that support the therapeutic potential of Immunalon® in the treatment of bladder carcinoma will be provided at the meeting.

A vaccine targeting mutant isocitrate dehydrogenase 1 induces anti-tumor immunity

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ORAL SHORT TALK

Point mutations in the *isocitrate dehydrogenase (IDH)* genes are an early, if not the earliest event during the development of gliomas and other tumors, such as acute myeloid leukemia (AML). Among grade II and grade III gliomas, more than 80 % carry an IDH mutation. In these entities, mutations almost exclusively affect the catalytically critical arginine residue of the cytoplasmic IDH1, leading to the amino acid exchange to histidine (R132H). This and other IDH mutations result in a neomorphic enzyme function and the production of the oncometabolite 2-hydroxyglutarate (2-HG) and thus genome-wide hypermethylation and malignant transformation.

The work at hand demonstrates the suitability of IDH1R132H as a target for immunotherapy in an MHC-humanized mouse model, A2.DR1. Peptides encompassing the mutated region bound MHC class II *in vitro* and induced a mutation-specific $CD4^+$ T helper (Th) response *in vivo*, whose antigen-specificity persisted in a specific T cell line and clone and which was accompanied by IDH1R132H-specific antibody production. To detect IDH1R132H-specific IgG in mouse and human serum, a peptide-coated ELISA was established. Several tested patients with IDH1R132H+ gliomas showed spontaneous IDH1R132H-specific antibody and $CD4^+$ Th1 cell responses. Preventive and therapeutic IDH1R132H peptide vaccination of A2.DR1 mice bearing syngeneic IDH1R132H+ sarcomas resulted in an effective mutation-specific antitumor immune response capable of controlling tumor growth in a $CD4^+$ T and B cell-dependent manner. Functionality of the vaccine was evidenced by loss of IDH1R132H expression in IDH1-transduced sarcomas and infiltration of IDH1R132H-specific $CD4^+$ T cells into the tumor bulk. Compared to therapeutic MHC II-mediated peptide vaccination against the well-established cancer testis antigen 1 (CTAG1B, NY-ESO-1), IDH1R132H is a relevant neoantigen of comparable efficacy.

In conclusion, IDH1R132H represents a potentially clinically meaningful tumor-specific neoantigen. Conceptually, patients with low-grade and anaplastic gliomas with a high prevalence of the IDH1R132H mutation represent a patient population, which may particularly benefit from a tumor vaccine, as there is currently no therapy preventing recurrence in this relatively young and immunologically competent patient population. Moreover, patient groups with other IDH1R132H-mutated tumors might potentially also benefit from such a vaccine.

Developing a warehouse for off-the-shelf and personalized ovarian cancer peptide vaccination immunotherapy

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Ovarian cancer remains the most lethal gynecological disease owing to late diagnosis and frequent resistance to platinum based chemotherapy. Despite recent improvements the overall prognosis of patients suffering from ovarian cancer remains poor and curative treatment is only possible by surgical resection at an early non-metastatic stage. The lack of treatment options and the high immunogenicity of ovarian cancer accompanied by frequent infiltration with lymphocytes have long demanded the use of an immunotherapeutic approach. Previous attempts involving peptide based vaccinations with WT1, p53 or NY-ESO however have failed to translate into clinical benefit. One major reason is that none of the tested antigens has ever been shown to be likewise presented by HLA molecules on ovarian cancer cells.

Here we report the design of a warehouse of naturally presented HLA peptides for off-the-shelf as well as personalized treatment of ovarian cancer patients. The constituents of this warehouse were identified by in depth HLA ligandome mapping of ovarian tumors and high throughput immunogenicity analysis of peptide vaccination candidates. We extracted HLA ligands from more than 30 different ovarian cancer samples and identified the presented peptides by state-of-the-art liquid chromatography coupled mass spectrometry yielding more than 35 000 unique peptides. In six cases we additionally took great effort to previously digest the cancer tissue into a single cell suspension in order to separate tumor cells, leukocytes and surrounding stroma cells by two consecutive MACS separations. Purified cell populations were again subjected to HLA ligandome analysis and label free quantification (LFQ) was used to determine the cellular origin of individual ligands and to verify the tumor association of peptides. All candidate antigens were additionally cross checked to be not presented on other tissues with an in house generated database of HLA ligands extracted from various benign samples of different origin (liver, kidney, ovary, PBMC…).

Analysis revealed CA-125 to be a key antigen for immunotherapy giving rise to more than 50 different HLA class I and several class II presented peptides that could be used synergistically in a vaccination cocktail. CA-125 presented peptides were found in more than 70% of tested patients making it a suitable antigen for an off-the-shelf cocktail. Notably, the presence of CA-125 presented peptides was not strictly dependent on elevated CA-125 serum levels. Additional peptides derived from further selected tumor associated antigens (IDO, Galectin-1, PTK2, ESR) can be used in a personalized approach directly addressing the antigenic profile of the patient‘s tumor.

In order to ensure that identified ligands can also be recognized by T cells and induce a functional T-cell response they were tested in an *ex vivo* high throughput immunogenicity analysis platform using healthy donor T cells and artificial antigen presenting cells.

Selective activation of ubiquitously expressed immune receptor RIG-I as novel target in solid tumors

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Retinoic acid inducible gene-I (RIG-I) is an ubiquitously expressed cytoplasmic receptor of the innate immune system. RIG-I detects viral 5'-triphsophorylated-RNA (3pRNA) within the cytosol of infected cells and triggers antiviral signaling pathways, including type I IFN induction in immune and non-immune cells. Tumor cells express RIG-I and respond to its activation by release of antiproliferative cytokines and pronounced apoptosis. Interestingly, non-malignant cells are considerably less susceptible to RIG-I induced apoptosis. Therefore, RIG-I ligands not only act on immune cells but directly on tumor cells and thus are different from classical immunostimulatory compounds acting only on immune cell subsets.

It was demonstrated that RIG-I activation leads to a strong anti-tumor effect in melanoma and glioblastoma by induction of direct apoptosis within the tumor cells and additionally by different immune mechanisms including type I IFN induction and NK cell activation. As RIG-I expression is not restricted to these tumor entities we generated a minimal synthetic 5'triphosphorylated RNA that is highly active and selective for RIG-I stimulation (ImOl100) and tested its antitumor activity in different tumor entities *in vivo* such as mammary cancer, fibrosarcoma, ovarial cancer and hepatocellular carcinoma. In an orthotopic breast cancer model and in a murine fibrosarcoma model intratumoral injections of ImOl100 led to effective tumor regression. In a syngenic murine ovarian cancer model ImOl100 treatment was administered intraperitoneally and induced complete elimination of the tumor cells judged by *in vivo* imaging of labeled tumor cells. Moreover, ImOl100 treatment offered protection against tumor rechallenge in this model. An intravenous application route was used in an orthotopic hepatocellular carcinoma (HCC) model. ImOl100 treated mice showed a distinct deceleration of HCC caused ascites and therefore a notably extention of survial.

In summary, RIG-I agonisation is effective in a broad range of different tumor entities and in different application routes *in vivo*. Therefore, activation of RIG-I is a promising anti-tumor mechanism with a broad potential for clinical application.

From bedside to bench: Molecular benchmarking of an FcOptimized CD19 antibody used in treatment of relapsed and refractory pediatric B-lineage acute lymphoblastic leukemia

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B-lineage acute lymphoblastic leukemia (ALL) is the most common childhood cancer. Although this disease can be curatively treated in 80% of patients by chemotherapy, prognosis for primary refractory or relapsed patients is very poor. Even after allogeneic stem cell transplantation (SCT), relapse rates are considerable and correlate significantly with persistent minimal residual disease (MRD) prior to or after SCT. Since a MRD constellation represents favorable effector-target ratios it is well suited for immunotherapy with therapeutic antibodies.

We developed and produced a third-generation CD19-specific monoclonal antibody (mAb) (4G7SDIE) in clinical-grade quality at a university-owned production unit. This high affinity Fcoptimized chimerized CD19-specific mAb mediates enhanced antibodydependent cellular cytotoxicity (ADCC) by NK cells through its improved capability to recruit FcγRIIIa bearing effector cells. In this study, 4G7SDIE was applied within the scope of a compassionate use program in pediatric patients with relapsed or refractory B-lineage ALL and characterized *in vitro* and *in vivo*.

As a first step, we confirmed expression of CD19 on leukemic blasts by quantitative flow cytometry. In initial cytotoxicity screenings, performed in an allogenic setting, we observed significantly increased lysis of B-lineage ALL blasts by PBMC after adding 4G7SDIE.

4G7SDIE was applied in 14 MRD-positive pediatric B-lineage ALL patients to reduce or eradicate MRD. In 9/14 patients, leukemic load was reduced by ≥ 1 log or below MRD-detection threshold during immunotherapy with 4G7SDIE. 2/9 responders received additional treatment. Cytotoxicity assays using B-lineage ALL patient PBMC on autologous blasts confirmed sustained functionality of patient NK cells over the course of 4G7SDIE treatment. *In vitro* cytotoxicity assays were performed using PBMC obtained at different time points of 4G7SDIE treatment from transplanted patients. Lysis of autologous leukemic blasts was found to be increased when adding 4G7SDIE or autologous patient serum taken after antibody treatment.

In a model with MCF7CD19transfectants, expressing various CD19 levels on the cell surface, a positive correlation between CD19 surface expression levels and lysis by PBMC of healthy donors coincubated with 4G7SDIE was shown. Strikingly, in 2/2 analyzed patients under 4G7SDIE therapy, a downmodulation of CD19 surface expression on the leukemic blasts was observed. *In vitro* antigenic shift assays on primary leukemic blasts showed considerable but very heterogeneous shift of CD19 surface expression. These observations hint at *in vivo* tumor escape mechanisms and furthermore indicate selective pressure exerted by immunotherapy with 4G7SDIE, underlining its therapeutic potential, but also delineating its limitations.

In conclusion, promising anti-leukemic effects have been observed *in vitro* and *in vivo* in this compassionate use program. We are currently setting up a phase I/IIa clinical trial.

Identification of novel, naturally presented, immunogenic peptides of human cytomegalovirus

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Human cytomegalovirus (HCMV) is a large ds DNA virus with a ~230 kb genome that belongs to the group of ß-herpesviruses. Whereas most healthy individuals control persistent HCMV infection well, HCMV can cause severe and sometimes lethal infections in immunocompromised persons.

Like most other viruses HCMV has evolved different strategies to escape the host immune response. One strategy to prevent recognition by immune cells of the host is to inhibit the presentation of peptides on MHC molecules. For example some of the viral unique short (US) genes encode proteins which interact at several stages with the generation of MHC ligands, peptide loading on MHC molecules or with the transfer of the peptide MHC complexes to the cell surface. As a result of this downregulation the isolation of naturally presented peptides of HCMV has not been very successful so far and to our knowledge all known HCMV derived T-cell epitopes are based on prediction. Surprisingly, despite the strong downregulation of HLA-peptide complexes there is an efficient control of the virus by healthy donors. Additionally, HCMV specific memory T cells could be detected in numerous studies indicating priming of peptide specific naïve T cells in any phase of HCMV infection.

In this study, primary human fibroblasts were infected with HCMV deletion mutants (ΔUS2-US11 or ΔUS2-US6+ΔUS11), HLA ligands isolated and characterized by liquid-chromatography-mass spectometry/mass spectometry (LC-MS/MS). In comparison to cells infected with the ΔUS2-US6 deletion mutant, the number of isolated peptides from the ΔUS2-US11 or ΔUS2-US6+ΔUS11 HCMV infected cells was significantly increased. We were able to identify 125 peptides from more than 80 viral proteins and tested their immunogenicity in healthy HCMV seropositive donors. IFNγ-ELISpot analysis after a 12-day stimulation with specific peptides and IL-2 revealed ~50% of the tested peptides to be immunogenic in at least one donor. The ability of these peptides to stimulate specific T cells to produce IFNγ and TNFα was determined by intracellular cytokine staining. Further, the ability of T-cells to bind these peptides specifically was analyzed by HLA tetramer staining.

Taken together, the artificial knockout of HCMV immunoevasins enabled the direct identification of HCMV-derived HLA ligands by LC-MS/MS. A substantial proportion of these peptides were found to be recognized by (memory) T cells of CMV-positive individuals, underscoring the physiological relevance of the identified HLA ligands.

The highly immunogenic HCMV peptides identified in this study could be used for specific generation of virus specific effector T cells for adoptive T-cell transfer after allogeneic stem cell transplantation because infection with or reactivation of this virus is frequently associated with life-threatening diseases in these patients.

Efficient elimination of carcinoma cells expressing the tumor exclusive target CLDN6 with a bispecific antibody

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Recombinant bispecific antibodies are potent anti-cancer drugs that redirect effector cells to specifically lyse tumor cells. The validity of this approach and its underlying bispecific molecular format has been shown in various proof-of-concept studies up to clinical phase II trials. Despite of impressive advances in the past, there is still a high unmet medical need for the treatment of solid cancers. Amongst others this is due to the only small number of addressable tumor specific markers which might enable the efficient and site-directed eradication of the malignant tissue. To overcome this problem, we selected CLDN6 as a target molecule for our studies. The expression of this antigen is highly tumor-specific, except for the expression in human placenta and during embryogenesis. CLDN6 positive tumor entities comprise ovarian, bladder, lung, gastric, pancreatic, breast, hepatic and other solid cancers.
We constructed a bispecific single chain molecule or short bi-$(scFv)_2$ recognizing simultaneously the T cell antigen CD3 and the tumor antigen CLDN6. The potency of the bi-$(scFv)_2$ was assessed by co-incubating CLDN6-expressing tumor cells with human T cells. Tumor cell lysis, T cell activation, proliferation and cytokine release were monitored by *in vitro* assays. Further, we tested the therapeutic efficacy of the CLDN6-specific bi-$(scFv)_2$ in a tumor mouse model. Therefore, the bi-$(scFv)_2$ was administered to immunodeficient mice engrafted with human PBMCs and carrying a subcutaneous tumor generated by human carcinoma cells that endogenously expressed CLDN6.
EC_{50} values in the pico- to low nanomolar range could be determined for the CLDN6-specific bi-$(scFv)_2$ -namely 6PHU3. Significant T cell activation started at a concentration of 0.1 ng/ml 6PHU3 and a total T cell activation of up to 75 % was achieved with 1000 ng/ml 6PHU3 after 48 hours of assay incubation. In the presence of target cells and 1 ng/ml 6PHU3, 80 % of T cells responded with proliferation when incubated for 96 hours. Moreover, T cell cytokine release mediated by 6PHU3 could be shown to be strictly target dependent. In a xenograft mouse model mice were treated daily or 3-times per week with 5 µg 6PHU3 by intraperitoneal injection. Tumors were eradicated or growth arrested by 6PHU3 treatment and survival of mice was significantly prolonged. Redirection of T cells to the tumor was shown to be selectively mediated by 6PHU3.
Our results show the *in vitro* and *in vivo* potency of the CLDN6-specific bi-$(scFv)_2$. Combination of the bispecific antibody concept with the selective targeting of this oncofetal tumor marker appears to be a promising strategy for the therapeutic treatment of solid tumors.

Identification of novel tumor associated antigens in colorectal cancer

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The establishment of distant metastasis is an important parameter affecting the prognosis of colorectal carcinoma (CRC) patients and it has been already shown that these arise from a small population of long term tumor initiating cells (LT-TIC). Furthermore, the T cell infiltrate in the primary tumor of CRC patients is also a major prognostic factor, suggesting the occurrence of tumor specific T cell responses and that the T cell mediated immune surveillance might play an important role in controlling tumor relapse, metastasis and response to treatment. Based on these observations we hypothesize that T cell responses against antigens expressed on LT-TIC might mediate more efficient immune surveillance.

To date little is known about the target antigens of the spontaneous T cell responses in CRC patients. For the purpose of identifying those antigens in a systematic and unbiased manner, we established a two-dimensional protein separation technique (PF2D). This technique allowed to fractionate lysates from primary and metastatic tumor tissue. The fractions, which were recognized by the patient´s T cell repertoire, as assessed by IFN-γ ELISpot assays, were selected and were further characterized with mass spectrometry (MS). The candidate proteins were validated by the use of synthetic peptides in ELISpot. As a result, we have identified 21 novel CRC-associated target antigens of spontaneous T cell responses. In a cohort of 20 patients, we have shown that the newly identified target antigens not only triggered responses more frequently than „canonical“ tumor antigens that are commonly used for immunotherapy (up to 50% of the tested patients), but the frequency of the T cells specific for the novel tumor associated antigens (TAA) was significantly higher. Furthermore, as shown by gene expression analysis some of the newly identified TAAs were selectively overexpressed on LT-TIC of CRC. We have demonstrated that they also represent target antigens of patients´ endogenous T cell responses, by means of MS of the immunogenic fractions subsequent to PF2D of three different LT-TIC enriched spheroid cultures. In addition, we have identified 30 amino acids long sequences of the these TAA, which elicit differential $CD8^+$ or $CD4^+$ T cell responses and we are currently working on identification of T cell epitopes and generation of T cell clones specific for these antigens. These would be further utilized to test the hypothesis that LT-TIC specific T cell clones have the capability to reduce the metastatic potential and tumorigenicity of TIC enriched spheroids in vitro and in vivo.

In conclusion we have identified novel set of antigens expressed in a therapeutically relevant subset of CRC cells, that can be highly immunogenic and might serve as better targets for immune monitoring and could be used for more efficient T cell based immunotherapy.

NLRP3 inflammasome-dependent contact hypersensitivity against pigmented cells by monobenzone-induced memory NK cells

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Monobenzone is a contact-sensitizer that induces a robust autoimmune response specifically against pigmented cells, including melanoma cells. We have previously characterized its mode of action in the exposed pigmented cell. However, it remains unclear how and which innate immune cells become activated to foster immune attack of monobenzone-exposed pigmented cells.

We now investigated monobenzone-induced immune activation using the established mouse model of contact hypersensitivity (CHS); mice were monobenzone-sensitized by abdominal skin exposure and a CHS immune response was elicited a week later by monobenzone-exposure of the right ear. Monobenzone CHS required the NLRP3 inflammasome and was dependent upon NK cells. Moreover, monobenzone CHS was fully intact in Rag-deficient mice, indicating T cell independence. Bone marrow chimera experiments showed NLRP3 to be pivotal outside the myeloid compartment. Further analysis of monobenzone-sensitized skin revealed induction of the cytokines CCL2, CXCL9, IL-1ß, IL-18 and the NKG2D-ligand MULT-1, suggestive of local NLRP3 inflammasome activation, lymphocyte recruitment and NK cell triggering. Additional analysis of treated ears revealed antigen-specific, NLRP3-dependent infiltration of $CD18^+$ $Ly49C\text{-}I^+$ $CD49b^+$ NK cells, indicating hepatic memory NK cell involvement. Adoptive transfer of hepatic NK cells from $CD45.2^+$ monobenzone-sensitized mice to $CD45.1^+$ naïve animals established $NKG2D^+$ hepatic memory NK cells to mediate monobenzone CHS. Screening of cutaneous lymph nodes upon monobenzone skin sensitization additionally showed recruitment of distinct activated dermal dendritic cells, interconnecting the memory NK cell response with ensuing T cell immunity.

Our results indicate that monobenzone induces pigment cell-specific immunity by specifically activating a NLRP3 inflammasome-dependent memory NK cell-mediated CHS response, without initial T cell involvement. These insights highlight a new immunological mechanism for activating antigen-specific immunity against pigmented cells, and allow for the further potentiation and development of monobenzone-based immunotherapy toward clinical application.

Wet peptide HLA binding assay has added value in the selection of candidate CD8$^+$ T cell epitopes

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Infected and cancerous cells can be recognized and killed by CD8$^+$ (cytotoxic) T cells. They are recognized via the foreign / altered proteins they produce, which are presented on the cell surface as peptides in context with HLA class I molecules. These peptides are therefore key candidates for the development of vaccines, immunotherapeutics and monitoring of disease-specific immune responses. The HLA-peptide binding is the most restrictive step in the peptide presentation pathway. Thus, the affinity of peptides to a given HLA is a strong indication of whether the peptide is a potential epitope or not. For prediction of peptide binding to HLA class I alleles several prediction methods have been developed. In a recent paper (Expert Rev Vaccines 11:43 (2012)) the NetMHCpan prediction tool was recommended. We show here that by adding a simple wet peptide HLA binding assay, candidate epitope selection can be improved while saving resources in further development. Using our UV-mediated peptide exchange technology, in silico-predicted binders and non-binders to different HLA alleles were tested for real binding. The peptide exchange efficiency was analyzed by ELISA. Data presented is on HLA-A*02:01 allele. Similar results were obtained for other HLA class I alleles like HLA-A*01:01, -A*11:01, -B*07:02 and -B*27:05. Comparison of peptide binding results (n = 309) obtained by peptide exchange assay and in silico analysis revealed that 20% of in silico-predicted HLA-A*02:01 binders do not bind/bind inadequately. Furthermore, 16% of in silico-predicted non-binders turned out to be binders. It was found that NetMHCpan cannot discriminate well between strong and weak binders. Most notably, ranking for best binders differed significantly. This has major consequences when candidate epitopes are selected for development based on in silico binding predictions: potential good candidates are missed while resources are unnecessarily spent on following up non-binders. As example, we analyzed eleven peptides screened for induction of a CD8$^+$ IFN-gamma response in patient cells (J. Hepatology 54:201 (2011)). Ranking of peptide binding using the peptide exchange assay showed a better correlation with immunogenicity than ranking based on prediction. In case the researchers would have selected 10 peptides based on in silico-predicted binding, they would have actually missed one of the most immunogenetic peptides. In conclusion: The peptide exchange assay improves the selection of CD8$^+$ T cell targets initially identified by in silico analysis and can further improve the success rate in epitope discovery important for vaccine development and monitoring of immunotherapy.

The antigenic identity of human class I MHC phosphopeptides is critically dependent upon phosphorylation status

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Dysregulated post-translational modification provides a source of altered self antigens that can stimulate immune responses in settings as diverse as autoimmunity, inflammation, and cancer. In recent years, phosphorylated peptides have emerged as a group of tumour-associated antigens presented by MHC molecules and recognised by T cells, and represent promising candidates for cancer immunotherapy. However, the impact of phosphorylation on the antigenic identity of phosphopeptide epitopes is unclear. Here we examined this by determining structures of HLA-A2-restricted phosphopeptides bearing canonical position 4-phosphorylations in the presence and absence of their phosphate moiety, and examining phosphopeptide recognition by the T cell receptor (TCR). Strikingly, two peptides exhibited major conformational changes upon phosphorylation, involving a similar molecular mechanism, which focussed changes on the central peptide region most critical for T cell recognition. In contrast, a third epitope displayed little conformational alteration upon phosphorylation. In addition, binding studies demonstrated TCR interaction with an MHC-bound phosphopeptide was both epitope-specific and absolutely dependent upon phosphorylation status. These results highlight the critical influence of phosphorylation on the antigenic identity of naturally processed class I MHC epitopes. In doing so they provide a molecular framework for understanding phosphopeptide-specific immune responses, and have implications for the development of phosphopeptide antigen-specific cancer immunotherapy approaches.

CD44v10, osteopontin and leukemia growth retardation by a CD44v10-specific antibody

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Background: A blockade of CD44 is considered a therapeutic option for the elimination of leukemia initiating cells. However, anti-panCD44 application is frequently burdened by severe side effects. Therefore we asked, whether side effects can be avoided by replacing anti-panCD44 with CD44 variant isoform (CD44v)-specific antibodies in CD44v-positive hematological malignancies, using the thymoma EL4 and CD44v10 transfected EL4 (EL4-v10) as model.

Methods: The therapeutic efficacy of anti-panCD44 and anti-CD44v10 was evaluated after subcutaneous application of EL4 / EL4-v10, supporting leukocyte recruitment by a local inflammatory reaction. Ex vivo and in vitro studies evaluated the impact of anti-panCD44 and anti-CD44v10 on EL4 and EL4-v10 as well as on draining lymph node cells and tumor-infiltrating leukocytes (TIL).

Results: CD44v10 cDNA transfection of EL4 had no major impact on thymoma marker and adhesion molecule expression, or in vivo growth rate. However, EL4-v10 had an advantage in adhesion to and migration towards osteopontin. Subcutaneous EL4/EL4-v10 growth was equally well inhibited by anti-panCD44 and anti-CD44v10, ex vivo analysis indicating NK cytotoxicity and ADCC as the dominating effector mechanisms. Yet, a local inflammatory reaction selectively strengthened the efficacy of anti-CD44v10. This was due to inflammation-induced chemokine, including osteopontin expression, the latter as a CD44v10 ligand being most abundantly recovered in EL4-v10 tumors. High level osteopontin in EL4-v10 tumors supported leukocyte recruitment and tumor-infiltrating T cell activation.

Conclusion: For hematological malignancies expressing CD44v, anti-panCD44 can be replaced by CD44v-specific antibodies without loss in efficacy. CD44v10, in particular, supports leukocyte recruitment and activation via osteopontin binding, where a local inflammation serves as initial trigger.

CIMT
Cancer Immunotherapy

Tumor Biology & Interaction with the Immune System

New approaches of cancer vaccination and cancer immunotherapy based on the optimal stimulation of tumor-specific, MHC class II-restricted CD4$^+$ T helper cells

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Recently we have shown that a number of murine tumors arising from mice of the Balb/c (MHC H-2d) strain, induced to express MHC class II molecule after transfection with CIITA (MHC class II transactivator) can be rejected effectively, generating tumor specific T helper cell (TH) triggering, immunological memory and protection even against parental tumor. The aims of this investigation were: a) - to assess whether the same results can be extended to tumors of different MHC background (H-2b); b) - to investigate, using dendritic cell knock-out C57Bl6 mice, whether CIITA transfected tumor cells can act as "surrogate APC" for their tumor antigens in vivo. To this end, LLC (Lewis lung carcinoma) cells were stably transfected with CIITA and selected for expression of MHC class II molecules. Parental tumor cells and CIITA-transfected cells (LLC-CIITA) were injected subcutaneously into C57/BL6 mice and tumor take and growth kinetics were assessed. Mice injected with LLC-CIITA cells fully rejected the tumor or remained tumor-free for longer time than mice injected with parental tumor cells. The growth kinetics and the size of CIITA-expressing tumors were significantly reduced compared to the parental tumor. Adoptive cell transfer of purified CD4$^+$ TH cells from mice injected with LLC-CIITA into naïve mice demonstrated that these cells were able to protect from LLC parental tumor growth. Taken together these results strongly suggest that, similarly to H-2d strain, also H-2b tumors can be rejected if expressing CIITA-mediated MHC class II molecules, confirming the general applicability of our tumor vaccination model. To achieve the second goal we performed in vivo experiments in a novel transgenic mouse model designated CD11.c DOG, in which it is possible to induce a conditional depletion of dendritic cells (DC) by diphtheria toxin (DT) treatment. These mice express the diphtheria toxin receptor (DTR) under the control of the promoter of the CD11c molecule, expressed preferentially in DC. Once injected with DT, dendritic cells are eliminated for the period of treatment up to 12 days. DT-treated CD11c.DOG mice where injected with LLC-CIITA tumor cells and tumor growth was followed during time. We found that LLC-CIITA cells can be recognized and rejected better than parental tumor even in CD11c-DOG mice. These results suggest that CIITA-tumor cells may act in vivo as surrogate APCs for their own tumor antigens and, most importantly,they can prime virgin CD4$^+$ TH cells to trigger a protective adaptive immune response against the tumor.

Treatment of pancreatic ductal adenocarcinoma with bifunctional siRNA targeting the immunosuppressive molecule galectin-1 and the cytosolic helicase RIG-I

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Background: Pancreatic ductal adenocarcinoma (PDAC) is characterized by an immunosuppressive microenvironment and sparse infiltration with lymphocytes. The novel bifunctional ppp-siRNA technology allows combining gene silencing of oncogenic or immunosuppressive targets with ppp-RNA-mediated immune activation via the cytosolic helicase RIG-I. An interesting immunosuppressive target is the β-galactoside-binding protein galectin-1 (Gal-1), which is secreted by tumor cells. Gal-1 suppresses T cells via induction of apoptosis of Th1 and Th17 T cells and inhibition of T cells migration into the tumor. Gal-1 also induces expansion of regulatory T cells and promotes angiogenesis. The aim of the present study was to assess the therapeutic efficacy of a novel bifunctional ppp-siRNA that combines gene silencing of Gal-1 with RIG-I-mediated immune activation in murine PDAC.

Methods: Murine PDAC cell lines were investigated for Gal-1 expression by IHC. We designed conventional and ppp-modified siRNAs targeting Gal-1 using a T7 polymerase (*in vitro* transcription) and evaluated these RNAs both *in vitro* and *in vivo* in the Panc02 mouse model of pancreatic cancer. *In vitro,* tumor cells were transfected with RNAs and Gal-1 expression, production of IFN-β and CXCL10, up-regulation of MHC class I molecules and cell viability were examined. *In vivo* the serum levels of Gal-1 were investigated in correlation to tumor growth. Lymphocyte activation, IFN-α and CXCL10 levels, and survival of mice in the orthotopic Panc02 model were examined after systemic treatment with unmodified and ppp-modified Gal-1 siRNA.

Results: The dual activities of ppp-Gal-1 were confirmed *in vitro*, leading to (i) reduced Gal-1 expression; and (ii) production of CXCL10 and IFN-b, MHC-I up-regulation and apoptosis of tumor cells via RIG-I activation. Systemic treatment of mice led to enhanced serum levels of IFN-α, CXCL10 and TNF-α. In addition, splenic $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and NKT cells up-regulated CD69 expression, indicative of systemic RIG-I activation. Treatment of mice with orthotopic pancreatic tumors with ppp-Gal-1 significantly prolonged survival, as compared to unmodified OH-Gal-1 or control RNA. In addition, 20% of the mice completely rejected their tumors leading to long-term tumor control.

Conclusion: 5'ppp-modified siRNA is a promising and versatile treatment strategy for PDAC. Silencing of oncogenic, metabolic (e.g. glutaminase) or immunosuppressive targets (e.g. TGF-b, Gal-1) can be combined with potent RIG-I mediated immune activation, leading to superior antitumor activity (Ellermeier et al., 2013; Meng et al., 2013).

Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL

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Even though melanoma is considered to be one of the most immunogenic solid tumors, handling its development remains a challenge. The basis for such escape from antitumor immune control has not yet been documented. Plasmacytoid dendritic cells (pDCs) are emerging as crucial but still enigmatic cells in cancer. In melanoma, the function of tumor-infiltrating pDCs remains poorly explored. We investigated the pathophysiologic role of pDCs in melanoma, both ex-vivo from a large cohort of melanoma patients and in-vivo in melanoma-bearing humanized mice. pDCs were found in high proportions in cutaneous melanoma and tumor-draining lymph nodes, yet associated with poor clinical outcome. We showed that pDCs migrating to the tumor microenvironment displayed particular features, subsequently promoting pro-inflammatory Th2 and regulatory immune profiles through OX40L and ICOSL expression. Elevated frequencies of IL5-, IL13- and IL10-producing T cells in melanoma patients correlated with high proportions of OX40L- and ICOSL-expressing pDCs. Strikingly TARC/CCL17, MDC/CCL22 and MMP2 found in melanoma microenvironment were associated with pDC accumulation, OX40L and ICOSL modulation or/and early relapse. Thus, melanoma actively exploits pDC plasticity to promote its progression. By identifying novel insights into the mechanism of hijacking of immunity by melanoma, our study exposes potential for new therapeutic opportunities.

Oxidative burst produced by the NOX2 complex does not play a role in methylcholanthrene induced sarcoma development

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Reactive Oxygen Species (ROS) produced by NOX2 complex are essential for proper immune cell function and immunomodulation. Classically, NOX2 in phagocytic cells produce super oxide to clear bacterial infection. Oxidative stress has been shown to modulate immune cell function, and is one of the key immune suppressive mechanisms, in MDSCs and Tregs, used to suppress T cell and NK cell effector functions. The tumor microenvironment is infiltrated by immune suppressive cells that are recruited from the periphery or polarized in the tumor microenvironment. In this study we aimed at examining the role of oxidative burst by immune suppressive cells in a methylcholanthrene induced sarcoma model. NOX2 requires p47^{phox} (NCF1) to organize the formation of the NOX2 complex on the cell membrane to produce super oxide. Homozygous mutant mice (NCF1*/*) have a functional loss of super oxide burst while heterozygous mice (NCF1*/+) retain this key immune cell function. These mice were injected with methylcholanthrene intramuscularly (25µg) to induce the development of sarcomas. We found that functionality of NOX2 did not lead to a difference in tumor incidence. Immunomonitoring by flow cytometry in tumor bearing mice showed that infiltrating immune cells experience an increase in oxidative state. Furthermore, infiltrating T cells showed an increase in effector-memory cell phenotype markers in both NCF1*/* and NCF1*/+ mice. Tumors established from both NCF1*/* and NCF1*/+ mice were tested for their *in vitro* proliferative capacity as well as their resistance to cisplatin treatment. These findings indicate that NOX2 and oxidative burst does not play a key role in tumor development or immune cell infiltration in the methylcholanthrene induced sarcoma model.

Establishment of a NY-BR-1 expressing breast cancer tumor model in HLA-transgenic mice

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Breast cancer is one of the leading causes of cancer related deaths in women worldwide. Current standard therapies show limited efficacy and are often accompanied by severe side effects. Immunotherapeutic approaches like adoptive T cell transfer have been proven successful in the treatment of metastatic melanoma, raising the question if this strategy can also be applied for the treatment of breast cancer. The differentiation antigen NY-BR-1 is overexpressed in 60% of breast carcinomas compared to healthy breast, thus representing an attractive target for T cell based immunotherapy approaches against breast cancer. The aim of this project is to establish a NY-BR-1-expressing, transplantable tumor model in HLA-transgenic mice that would allow to investigate the functional role of NY-BR-1-specific HLA-restricted $CD4^+$ T cells *in vivo* with respect to their capacity to sustain cytotoxic T lymphocyte (CTL)-mediated tumor attack and their capability to interact with tumor associated macrophages (TAMs) in an instructive way, thereby potentially promoting differentiation into type-1 macrophages (M1) in the tumor micro-environment. For this purpose, in a pilot study transfected clones of the murine lymphoma cell line EL4 stably expressing NY-BR-1 were established that gave rise to sub-cutaneous tumors with retained NY-BR-1 expression in HLA-DR4-transgenic mice. In addition, peptide immunization with a NY-BR-1-specific, $H2^b$-restricted CTL epitope identified in our previous studies led to the generation of a CTL line specifically recognizing EL-4/NY-BR-1-expressing transfectants *in vitro*. As several HLA-DR3- and HLA-DR4-restricted T cell lines specific for NY-BR-1-derived $CD4^+$ T cell epitopes recently identified by us are available, the results presented here outline the first NY-BR-1-expressing mouse tumor model, allowing the investigation of NY-BR-1-specific immune responses *in vivo*. Furthermore, the establishment of murine mammary gland cancer cell lines with stable expression of NY-BR-1 that give rise to orthotopic tumors is currently ongoing.

RIG-I stimulation induces the release of tumor-suppressing exosomes from melanoma cells

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Exosomes are vesicles 50-120 nm in size, derived from the endosomal compartment and have been shown to act as intercellular communication vehicles by transferring functional active molecules, including miRNA and mRNA. Exosomes released from tumor cells have been shown to promote tumor growth or to stimulate the immune system, e.g. activation of Natural Killer cells (NK cells). In this study we investigate the impact of the ubiquitously expressed innate immune receptor Retinoic acid-inducible gene I (RIG-I) on the release and function of melanoma cell derived exosomes. RIG-I is a cytosolic pattern recognition receptor of the innate immune system detecting viral nucleic acids by binding to 5'-triphosphorylated RNA (3pRNA) in the cytosol of infected cells. The activation of RIG-I by 3pRNA induces a potent anti-tumor immune response by cytokine induction (e.g. type I IFN) and innate immune cell activation (e.g. NK cells). Nevertheless, the mechanism mediating the intercellular communication between RIG-I activated tumor cells and the immune system remains elusive. Here we show that activation of RIG-I by 3pRNA in melanoma cells results in the release of RIG-I ligand containing tumor-exosomes with potent anti-tumor activity. Exosomes from RIG-I stimulated melanoma cells induced antiviral cytokines in recipient cells by transfer of RIG-I ligand. Moreover, 3pRNA induced tumor-exosomes caused direct apoptosis in melanoma cells. Additionally, 3pRNA induced melanoma derived exosomes showed an increased surface expression of the NK cell activating ligand BAG6 thereby triggering NK cell mediated cytotoxicity via NKp30. The treatment of subcutanouse murine melanomas with RIG-I ligand/ BAG6 carrying tumor-exosomes resulted in Th1 cytokine expression and led to an NK-dependent suppression of tumor growth *in vivo*.

Our data point out a novel mechanism to generate anti-tumor exosomes by the activation of the innate immune receptor RIG-I with interesting implications for tumor therapy and delivery of RNA-oligonucleotides.

Activation of RIG-I-like helicases induce immunogenic cell death of pancreatic cancer cells leading to protective and therapeutic immunity in vivo

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Background: RIG-I-like helicases (RLH) are immune receptors recognizing viral RNA in infected cells and induce a type I IFN driven immune response. Since immune mechanisms against viruses and tumors share many features, RLH ligands hold promise for tumor immunotherapy. We recently identified RLH as therapeutic targets in pancreatic cancer. RLH ligands counteracted immunosuppressive mechanisms and efficiently induced tumor cell apoptosis. Tumor cell death can occur immunologically silent or immunogenic, leading to tolerance or immune activation, respectively. Here, we investigated consequences of RLHinduced tumor cell death in regards to release of danger signals and cytokines, calreticulin exposure to the cell surface (eat-me signal), antigen uptake and activation of dendritic cells (DC) as well as antigen cross-presentation to CTL.

Material and methods: Murine pancreatic cancer cells (Panc02 and T110299 cells generated from Kras and p53 double-mutant mice) were treated with RLH ligands to induce apoptosis. Release of IFN-b, IL-6, CXCL10, HMGB1 and hsp70 were assessed by ELISA. Calreticulin translocation to the outer cell membrane, Fas (CD95) and MHC-I expression were measured by FACS analysis. Primary DC populations were isolated from spleens of WT and knock-out mice and cocultured with RLH-activated tumor cells. DC maturation marker expression, antigen uptake and antigen cross-presentation were assessed by FACS. Prophylactic and therapeutic vaccination experiments were performed in the syngeneic Panc02 tumor model.

Results: RLH ligands induced the release of IFN-b, IL-6, CXCL10, HMGB1 and Hsp70 from tumor cells. In addition, calreticulin translocated to the outer cell membrane. DC exposed to RLH-treated tumor cells upregulated CD80 and CD86 expression, which was mediated by tumor-derived type I IFN, whereas TLR, RAGE or inflammasome signaling was dispensable. $CD8a^+$ DC effectively engulfed apoptotic tumor material and cross-presented tumor-associated antigen to naïve $CD8^+$ T cells. In comparison, tumor cell death mediated by oxaliplatin, staurosporine or mechanical disruption failed to induce DC activation, antigen uptake or cross-presentation. Moreover, tumor cells treated with sublethal doses of RLH ligands upregulated MHC-I and Fas expression and were sensitized towards CTL- and Fas-mediated killing. Subcutaneous injection of a single dose of apoptotic, RLH treated tumor cells protected all mice from tumor challenge. Furthermore, intratumoral injection of RLH ligands mediated efficient tumor control and the emergence of tumor-specific CTL in peripheral blood.

Conclusions: RLH ligands induce a highly immunogenic form of tumor cell death linking innate and adaptive immunity. Tumor-derived type I IFN was identified as the main “danger signal” leading to DC activation. RLH-based immunotherapy holds promise for counteracting the potent immunosuppressive network found in pancreatic cancer.

The abstract is withdrawn

Change in infiltration of immune cells during rejection after radioimmunotherapy in a syngeneic rat colon carcinoma

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Introduction: In radioimmunotherapy, monoclonal antibodies (MAb) are conjugated with radionuclides e.g. ^{177}Lutetium (^{177}Lu). The antibodies direct the radionuclides to the tumor cells, where the radionuclide emit ionizing radiation damaging the tumor cells.

Others have shown that the therapeutic effects of external beam radiation can partly be derived to infiltration of immune cells. The abscopal effect (therapeutic response in untargeted tumors) is assumed to be, at least in part, immune mediated. The radiation dose and fractionation, which are able to induce immune response needs to be further evaluated.

The immune response during radioimmunotherapy is important to evaluate, since the therapeutic effects is a combination of continuous decay of the radionuclide and the effects of the antibody, which together is still mostly an unexplored area.

Aim: To evaluate the infiltrating immune cells during the rejection of established tumors after radioimmunotherapy with 400 MBq/kg ^{177}Lu-BR96 MAb (expecting to result in non-palpable tumors in about 90% of the animals within 2 weeks), compared to untreated tumors, in a syngeneic rat colon carcinoma.

Method: Of 30 animals with established tumors (at least 10x10 mm), 21 were treated with 400 MBq/kg ^{177}Lu-BR96 and 9 served as controls (untreated). In the treatment group, the therapy resulted in a complete response in 5 of 21 animals prior to sampling. Treated animals were sacrificed and tumors were excised on 1 day p.i. (3 animals with tumor), 2 days p.i. (3 animals with tumor), 3 days p.i. (3 animals with tumor), 4 days p.i. (2 tumors in 3 animals), 6 days p.i. (2 tumors in 3 animals), and 8 days p.i. (3 tumors in 6 animals). In the untreated group, tumors were excised on day 0 (when radioimmunotherapy was administered to the treatment group). The tumors were paraffin embedded. Consecutive sections were stained with anti-CD3 (Mature T cells, thymocytes), anti-CD8 (Thymocytes subset, Tc cells, NK cells, DC subset), anti-CD2 (Thymocytes, NK cells, T and B cells), anti-CD68 (Monocytes, macrophages, Neutrophils, Basophils, DC, myeloid progenitors), anti-CD163 (Monocytes, macrophages), and H&E. All histological sections are currently evaluated blindly by our clinical pathologist (OL).

Preliminary results: All evaluated antigens were present in untreated tumors. Infiltrations of immune cells were mainly located within the stromal compartment of the tumor and limited in tumor cell areas. Treated tumors displayed a decrease in the T cell associated antigens (CD3, CD8, and CD2). The few present cells were located within the stromal compartment. The staining of macrophage associated antigens (CD68 and CD163) was still mainly located within the stromal tissue. The fractions of stromal tissue and thereby the fraction of macrophage associated antigens increased during the treatment.

Study on the relation between heme oxygenase-1 and metallothionein expression and the tumour-associated local immune response in human cutaneous malignant melanoma

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Metastatic cutaneous malignant melanoma is promising target of cancer immunotherapy. Molecular biomarkers are required to identify a patient subset for which immunotherapy may be appropriate. Nevertheless, efficiency of an immunotherapy is supposed to be highly dependent on tumour microenvironment. In the present study, we aimed to compare heme oxygenase-1 (HO-1) protein expression of primary melanoma samples without metastases (n = 23) to samples with haematogenous metastases (n = 23) in a retrospective manner using tissue micro-array analysis of archived paraffin tissue blocks. Dendritic cell and macrophage markers (CD1a, CD68 and CD163) described previously in association with melanoma prognosis were also selected to investigate. In our previous study, using the same tissue micro-array, we observed the presence of metallothionein (MT) positive macrophages peritumourally, which has not yet been reported. Moreover, MT over-expression of the tumour cells was also observed and this correlated with the presence of tumour-infiltrating CD68 positive macrophages, a known predictive factor for melanoma progression, thereby suggesting a role for MT in the development of a defective host immune response. Peritumoural HO-1 expressing macrophages have been described in vertical growth melanomas. In the present study, we found HO-1 expression both in peritumoural macrophages and in melanoma cells, similarly to MT expression. It is proposed that MT and HO-1 might play a role in the communication between tumour cells and tumour microenvironment and can contribute to the switch from M1 macrophage activation towards M2-like phenotype, which is then able to promote metastasis by suppressing the anti-tumour immune response, enhancing the tissue remodelling and neoangiogenesis. In addition, our recent in vitro experiments showed a connection between zinc and redox signalling, which regulates expression of MT and HO-1 in HaCaT keratinocytes. In order to investigate whether characteristics of tumour microenvironment can be changed during immunotherapy, we analyzed immunohistochemically metastases of an ipilimumab responder patient before, during and after treatment with the anti-CTLA4 antibody.

Attenuation of RIG-I-induced immune activation by hypoxia in murine melanoma cells

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A common factor negatively influencing the efficacy of solid-tumor chemo- and irradiation therapy is hypoxia. Yet, the influence of hypoxia on tumor immunotherapy is largely unknown. In particular, novel regimens like the intratumoral triggering of cytosolic pattern-recognition receptors like retinoic acid-inducible gene I (RIG-I) could be likewise altered by hypoxia. We here investigated how hypoxia influences RIG-I function in murine melanoma cells.

A panel of murine melanoma cells was incubated at normoxia (pO_2=21%) or hypoxia (pO_2=2%) and stimulated with 5'-triphosphorylated RNA (3pRNA) to trigger RIG-I. Under hypoxia we observed attenuation of RIG-I-induced CXCL10 (IP10) secretion, a hallmark of RIG-I activation. Using B16.F10 melanoma cells as a model, we found weakened RIG-I efficacy under hypoxia to coincide with sharply reduced RIG-I protein-upregulation, downregulation of TOM70, intracellular ROS accumulation and cellular dedifferentiation. In contrast, RIG-I impairment was independent of IFNaR-expression, MAVS expression, mRNA decay rates or changed HSP90alpha chaperone function. Accordingly, co-cultures of 3pRNA-transfected B16.F10 cells with melanoma antigen-specific $CD8^+$ T cells or NK cells under hypoxia showed depressed T- and NK cell activation. Using vitamin C as ROS-scavenger or distinct NF-κB inhibitors to counteract cellular dedifferentiation, we successfully rescued 3pRNA-induced RIG-I expression under hypoxia.

Taken together, our results for the first time indicate the impairing effect of hypoxia on RIG-I function in melanoma cells and suggest an additional intervention preventing these effects. Our findings are of pivotal importance to the further development of cancer immunotherapies that rely on RIG-I-triggering within the tumor milieu.

BRaf and MEK inhibitors differentially regulate cell fate and microenvironment in human hepatocellular carcinoma

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Purpose: Small molecule inhibitors of the mitogen activated protein kinase (MAPK) pathway like sorafenib represent novel treatment options for advanced hepatocellular carcinoma (HCC). The aim of our study was to identify downstream targets as biomarker candidates that are directly linked to the oncogenic MAPK pathway in HCC and correlate with inhibition of this pathway by multi-kinase inhibitors.

Experimental Design: HCC cell lines and fresh tumor and tumor-free liver tissues from HCC patients were incubated with different BRaf or MEK inhibitors and analyzed for kinase phosphorylation, proliferation, induction of apoptosis and chemokine secretion.

Results: HCC cell lines responded differentially to these inhibitors in a dose-dependent manner even those targeting the same kinase. Sorafenib inhibited both MEK1 and ERK1/2 phosphorylation at high but increased signaling at low concentrations. Similarly, PLX4720 increased MEK/ERK signaling independently from mutations in BRaf or NRas. MEK inhibitors decreased ERK1/2 phosphorylation in a dose-dependent manner. These signaling characteristics correlated with inhibition of proliferation, induction of apoptosis and chemokine secretion. Fresh tissues derived from patients diagnosed with primary HCC responded to these inhibitors with changes in their microenvironment following the patterns observed in HCC cells.

Conclusions: Oncogenic signaling of the MAPK pathway influences HCC sensitivity to treatment with BRaf and MEK inhibitors regarding cell fate independently from mutations in BRaf and NRas. MAPK inhibitors have strong impact on chemokine secretion as consequence of interference with oncogenic signaling. Therefore, novel biomarker candidates associated with HCC microenvironment may be developed for prediction and monitoring of treatment response to small molecule inhibitors. Taken together, treatment of advanced HCC patients with sorafenib or other kinase inhibitors is associated with poor clinical response. Here, we demonstrate that low-dose sorafenib treatment of HCC cells increased instead of decreased signaling. Inhibition of the MAPK pathway was only achieved by high drug concentrations. This dose-dependent regulation of signaling correlated with proliferation, viability, apoptosis induction and secretion of chemokines and growth factors. These mechanisms are likely to be involved in the variable clinical response to kinase inhibitors. Therefore, we propose that chemokines, CXCL8 in particular, and growth factors represent biomarker candidates for sorafenib treatment of HCC patients because their plasma levels may correlate with HCC tissue response and allow an early discrimination between responders and non-responders. Therefore, we suggest that novel candidates for biomarkers of individual treatment responses can be further developed on the basis of our investigations.

Increased myeloid derived suppressor cells in glioblastoma patients

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Human glioblastoma (GBM) represents 15-20% of all intracranial tumors and approximately 50% of astrocytic tumors in adults. Despite multimodal treatment consisting of surgical resection, radiotherapy and chemotherapy, patients have a median survival of only 14,5 months. For long the brain has been regarded as an immune-privileged site based on the presence of the blood-brain-barrier, lack of conventional lymphatics and low immune cell trafficking. Recent studies now show that the brain rather is a specialized immune site where lymphocyte homing (adhesion/chemokine receptor expression) and immune response induction are tightly controlled and regulated in a unique fashion. These findings have paved the way for the development of immunotherapeutic intervention strategies. To exploit the potential of immune mediated tumor destruction, it is important to gain further insight in the immune-suppressive mechanisms active in glioma. Previously, we have reported on the presence and function of regulatory T cells in glioma. Recent studies show the increase of myeloid cells capable of suppressing T cell functioning, called myeloid derived suppressor cells (MDSCs).

Within the blood of high grade glioma patients, we could detect a significant increase of MDSCs defined by the expression of CD33 en the absence of MHC-II using facs relative to healthy controls ($P < 0.001$) and low grade glioma patients ($P < 0.05$). MDSCs can be divided in subpopulations based on the expression of $CD14^+$ (monocytic MDSCs) and $CD15^+$ (granulocytic MDSCs). We found that both populations were significantly increased within the blood of high grade glioma patients compared to healthy control or low grade glioma patients. The increase of these two populations showed a weak but significant correlation ($p = 0.012$, $R^2 0.304$).

Furthermore, cells with a similar phenotype can be found in paraffin embedded tissue sections using multiplex IHC. These cells are present around blood vessels, necrotic tissue and within nuclear dense areas, but are absent within healthy brain tissue. To quantify these cells within the tumor, we used ultrasonic sound aspirated tissue for facs analysis. We were able to detect up to 2% of granulocytic MDSC within this material of which the highest accumulation is found in high grade tumors. Altogether, our data indicates that cells with a MDSC phenotype are present within the periphery of glioma patients and within the tumor microenvironment.

Modulation of the tumour microenvironment by the local delivery of an IL-12 expressing lentivirus to the lung promotes rejection of established tumours

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Malignant tumours evade effective immune responses by the promotion of immunosuppression. Interleukin 12 (IL-12) is an immunomodulatory cytokine that may help overcome immune suppression by direct activation of innate and adaptive immune compartments. Initial phase -I and -II trials of systemic administered IL-12 in a mixed cohort of cancer patients encountered severe toxicity events including the deaths of a number of patients, suggesting that any treatment that includes the use of IL-12 would have to be delivered locally to the tumour.

The lung is a major site of metastasis for several types of solid tumour. With this considered, we hypothesized that locally delivered IL-12 may improve the anti-tumoural immune response without the toxicity associated with systemic delivery. To test this, we created an IL-12 encoding Lentivirus (IL-12Fc LV) for intranasal delivery in a murine model of metastaic B16 melanoma.

When administered via the intranasal route, mice treated with IL-12Fc LV exhibited a marked decrease in tumour metastases. Analysis of infiltrating effector subsets (Macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells) in the lung microenvironment confirmed that Macrophages are the recipients of the IL-12Fc LV and mass proliferation in all effector subsets was observed with the expression of inflammatory cytokines as well as Granzyme B and inducible nitric oxide synthase (iNOS). Survival of tumour bearing mice was significantly improved with IL-12Fc treatment. When IL-12Fc LV was administered to mice that had been depleted of $CD8^+$ T cells, NK cells and soluble IFNγ a loss of tumour rejection and a corresponding decrease in iNOS expression in Macrophages was observed.

We conclude that a locally delivered genetic vector encoding IL-12 activates immune responses and can effectively control metastatic melanoma in the lung. Future studies will aim to elucidate the exact mechanisms and effector subsets that mediate tumour rejection in IL-12Fc LV treated subjects with further development of a potent therapeutic tool for the treatment of lung cancer.

A mouse tumor transplantation model mimicking the immune response to sporadic cancer

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Immune surveillance was postulated first in the 1950s and proposes that early tumor lesions are controlled by the immune system. To test this hypothesis different tumor models have been developed. Autochthonous tumor models aim to mimic the formation of sporadic tumors in humans, but they are time consuming and the tumor antigens are often not known or not tumor-specific. Tumor transplantation models are time-saving and often comprise a tumor specific antigen, but they are artificial. The inoculation of cancer cells causes an acute inflammatory response and thereby induces an unintended recognition of tumor antigen by T cells. Therefore, a model would be desirable in which tumor antigen expression can be switched off during the first weeks after cancer cell inoculation and additionally cell growth is arrested. By later induction, antigen expression and tumor growth can be provoked under non-inflammatory conditions as it occurs similarly during autochthonous tumor development.

We established a transplantable tumor model by usage of a mouse gastric cancer cell line (TC200.09) that conditionally express SV40 large T antigen (Tag) and firefly luciferase (Fluc) as a fusion protein (TagLuc). In the presence of doxycycline (dox) TagLuc is expressed and acts as a cancer-driving antigen. In the absence of dox TagLuc expression declines to virtually undetectable levels and results in apoptotic cell death. However, a fraction of TC200.09 cells survive temporary oncogene inactivation and regain proliferative capacity *in vitro* after dox re-administration. This unique property enables us to prevent Tag expression during the inflammatory period within the first few weeks created by cancer cell inoculation. Our first results show that TC200.09 cells survive in immunodeficient mice in the absence of TagLuc as a cancer driving antigen. Furthermore, we were able to induce TagLuc expression in resting tumor cells by dox administration. In future experiments we will analyze the consequence of neo-antigen expression in nascent tumor cells in immunocompetent hosts. Will there be tolerance or immunity?

Local and systemic immune response in pancreatic cancer: multi-scale approach to tumor-immune interactions

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Pancreatic cancer is widely known as an aggressive entity. Patients are typically diagnosed in advanced stages and local rapid tumor growth and invasion occur early in this cancer. As tumor immunology evolves from bench to bedside in other cancers we aimed to develop an efficient work flow to elucidate patterns in tumor-immune interaction for pancreatic cancer with regard to the differential compartments of tumor epithelium and desmoplastic stroma as well as the serum of the corresponding patients.

Pancreatic cancer tissue and blood serum of therapy-naïve patients was acquired during surgery. Fresh frozen tissue was used for immunohistological staining, followed by computerized differentiated intratumoral and stromal quantitative cell count, as well as multiplex protein quantification. Upfront of the latter, tissue slides underwent laser microdissection to separate tumor epithelial and stromal cells. Serum protein levels were also analyzed by multiplex and compared to matched samples of healthy donors.

Density of immune cell infiltrates ranged from only 100 up to 3000 cells per mm^2. Cell distribution showed diverging patterns in tumor epithelial and stromal compartment. Diverging patterns could be identified for $CD4^+$ T-helper cells, cytotoxic $CD8^+$ T cells, tumor associated M2 macrophages ($CD163^+$) and B cells. Only few T-suppressor cells ($FoxP3^+$) were found, natural killer cells (NK) where even more scarce. Suppressive immune cells ($CD163^+$ & $FoxP3^+$) built a fraction of 23% (mean) of the infiltrate. Concentration of immunosuppressive cytokines exceeded stimulatory cytokines by 1-2 times the power of ten in local and systemic milieu. Cytokines of the tumor microenvironment appeared independent from and in part discordant to systemic cytokine levels.

In part contradictory and surprising, these novel findings have to be subsequently followed by further *in vivo* and *in vitro* studies focusing on single aspects to differentiate the intricate interactions between tumor-escape mechanisms and immune defense. Multi-scaled analyses of patient samples as depicted above provide solid data sets for acumination of tumor immunologic research.

MDA5-based immunotherapy can reprogram tumor myeloid-derived suppressor cells (MDSC) from a M2/G2 phenotype to a M1/G1 phenotype and reduce suppressive function

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Background: Pancreatic cancer is a highly aggressive disease characterized by poor therapy responses and dismal prognosis. The tumor entertains a potent immunosuppressive network with dense infiltration of myeloid cells, such as macrophages and MDSC. MDSC are versatile cells of monocytic (M-MDSC) and granulocytic (G-MDSC) origin exhibiting multiple immunosuppressive mechanisms that promote tumor growth. In the tumor environment, MDSC polarize in alternatively activated M2/G2-like cells, differing from the M1/G1 phenotype in function, receptor expression and cytokine production. The phenotype of M2/G2 MDSC is generally immunosuppressive and pro-tumorigenic. A new strategy for immunotherapy is reprogramming of MDSC to a M1/G1 phenotype. The cytosolic helicase melanoma differentiation-associated protein 5 (MDA5) is an immune receptor recognizing viral dsRNA that induces a type I IFN-driven immune response. Viral infection can be mimicked by transfecting the dsRNA analogue poly(I:C) into cells. Here we assessed the influence of MDA5-based immunotherapy on functional MDSC profiles in a murine pancreatic cancer model.

Methods: Subcutaneous Panc02 tumors were induced in C57BL/6 mice. On day 20 and 22 mice were treated with intravenous injection of 50 µg of control RNA or poly(I:C), each complexed with in vivo-jetPEI. The next day, mice were sacrificed and tumors and spleens were processed to single cell suspensions. For gene expression analysis by qRT-PCR cells were sorted in G-MDSC ($CD11b^+$, $Gr\text{-}1^{high}$) and M-MDSC ($CD11b^+$, $Gr\text{-}1^{int}$, $Ly6C^+$) fractions via FACS. For functional assays, MACS-sorted MDSC were cocultured with CFSE-labeled T-cells and T-cell proliferation was analyzed by flow cytometry. Therapeutic efficacy of MDA5-based immunotherapy was assessed in an orthotopic tumor model. Starting at day seven mice were treated twice weekly with RNAs for three weeks and survival was monitored.

Results: MDSC in tumors expressed an immunosuppressive phenotype with high levels of PD-L1, TGF-β, VEGF and arginase, as compared to splenic MDSC. Treatment with poly(I:C) significantly reduced expression levels of TGF-β, arginase, Adam17, galectin-9, as well as STAT3 and STAT5b, whereas NOX2 and NOS2 were significantly increased, indicative of a switch towards a M1/G1 phenotype. This correlated with a reduced suppressive function in T cell proliferation assays. Systemic treatment with the MDA5 ligand led to reduction of MDSC numbers in spleen and significantly prolonged survival in the orthotopic tumor model.

Conclusion: MDA5-based immunotherapy reduces the number and suppressive function of MDSC in murine pancreatic cancer. Reprograming MDSC towards a M1/G1 phenotype is an interesting and promising novel strategy for the treatment of pancreatic cancer.

Enhanced immunogenic cell death and immune activation by synergism between therapeutic irradiation and RIG-I activation in murine melanoma cells

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The efficacy of antitumor radiotherapy is often hampered by tumor radioresistance. Approaches to therapeutically enhance radiotherapy are limited. However, combinations between radiotherapy and immunotherapy are largely unexplored. Here, we demonstrate that activation of the retinoic acid inducible gene I (RIG-I) receptor within murine melanoma cells synergizes with simultaneous therapeutic irradiation. This approach can embody a new effective combinatorial immunoradiation regimen. Murine B16.F10 melanoma cells were irradiated with a therapeutic dose of 2 Gy under controlled conditions (Mevatron MD linear accelerator) and simultaneously RIG-I was activated by 5'-triphosphorylated RNA (3pRNA) transfection. In comparison to control conditions, melanoma cell apoptosis, HMGB-1 release, RIG-I protein expression, MHC class-I upregulation, IFNalpha and IP-10 cytokine secretion, and the ability of the B16 cells to activate melanocyte antigen-specific $CD8^+$ T cells were synergistically enhanced by simultaneous irradiation and RIG-I activation.

Activating RIG-I under simultaneous therapeutic irradiation showed synergism in both (immunogenic) cell death and RIG-I-induced immune activation. Our data warrant further exploration and development of such combinatorial immunoradiation regimens, to the benefit of future cancer immunotherapy.

Tumor microenvironmental studies indicate controlled trafficking and preferential homing of immune cells towards the tumor

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Introduction: Insights regarding the trafficking of immune cells within the tumor microenvironment are pivotal in order to optimize immunotherapeutic approaches. Malignant pleural mesothelioma (MPM) is a rare thoracic malignancy in which the immune infiltrate predominantly consists of tumor-associated macrophages (TAMs) of the M1 and M2 phenotype and T-cell subsets. Pleural effusion (PE) often accompanies MPM and is considered to be an easy-access opportunity to investigate the tumor microenvironment. The aim of the present study is to investigate the influx and interactions of TAM and T-cell subsets in PE and tumor tissue of MPM patients.

Methods: We measured TAMs, M2 TAMs, CD4 T-cells, CD8 T-cells, and regulatory T-cells in the PE of 30 MPM patients prior to treatment using flow cytometry. In addition, 10 associated cytokines were measured using a multiplex magnetic bead-based assay. An in vitro T-cell proliferation assay in the presence of PE and in co-culture with macrophages was performed in order to investigate their local interactions. In 5 MPM patients, the infiltration of TAMs and T-cells were compared between tumor biopsies and accompanying pleural effusions using immunohistochemistry.

Results: IL-6 and VEGF were the predominant cytokines present in pleural effusion. In general, TAMs were more prevalent in PE than T-cells, with clear patient-to-patient variation (mean % of alive cells ±SE: TAMs 46.8±6.1%, M2-TAMs 13.1±2.7%, CD3 T-cells 26.7±5.1, CD4 T-cells 15.4±3.4%, CD8 T-cells 8.5±1.6%, Tregs 6.7±1.3% of CD4 T-cells). The percentage of CD8 T-cells correlated negatively with the M2 TAMs in PE (Pearson $r=-0.63$, $p<0.001$, $n=30$). The proliferation assays showed that macrophages are able to suppress CD8 T-cell proliferation in the presence of PE. The distribution of M2 TAMs and CD8 T-cells in PE did not correlate with their presence in corresponding tumor biopsies.

Conclusion: TAMs and T-cell subsets are abundantly present and counter-regulated in PE of MPM patients. In addition, their presence in PE does not reflect their infiltration in the tumor. We propose that the immune cell fate in PE is determined by a complex interplay of local factors and therefore is not a direct reflection of the tumor. Furthermore, these data indicate that the trafficking of TAMs and T-cell subsets in the tumor microenvironment of MPM patients is controlled and warrants further research into the factors responsible for this preferential homing.

Myeloid-derived suppressor cells inhibit NK cell activity through prostaglandin-E2-regulated TGF-beta production

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ORAL SHORT TALK

Tumors suppress the host immune system by recruiting a variety of cellular immune modulators, such as regulatory T cells, tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs). MDSCs accumulate in high frequencies in peripheral blood of cancer patients and mediate potent inhibition of innate and adaptive anti-tumor immune responses. Emerging evidence has emphasized the importance of COX-2/PGE2 pathway in the induction of MDSCs.

In this study, we show that $CD14^{+}HLA\text{-}DR^{low/-}$ monocytic MDSCs isolated from blood of advanced stage melanoma patients significantly inhibited resting and activated NK cell cytotoxicity ($p < 0.05$) and ability to produce IFN-g ($p < 0.01$) via the production of TGF-β. In vitro, treatment of monocytes with PGE2 induced an MDSC-like phenotype with enhanced suppressive activity against autologous NK cells. Binding of PGE2 to EP2 and EP4 receptors on monocytes activated the p38MAPK/ERK pathway and resulting in elevated secretion of TGF-β. Moreover, neutralization of TGF-β produced by PGE2-treated monocytes significantly reversed the suppression of NK cell proliferation ($p < 0.05$) and cytotoxicity ($p < 0.01$). Furthermore, silencing COX-2 in murine 4T1 tumor cells reduced the accumulation of $CD11b^{+}Gr1^{+}$ MDSCs in the spleen ($p < 0.01$), and *in vivo* live imaging revealed an enhanced NK cytolytic capacity in mice bearing COX-2-silenced tumors.

Collectively, our results provide evidence in support of a direct involvement and molecular machinery of tumor-derived PGE2 in stimulating the production of TGF-β from MDSCs and its importance in suppressing NK cells.

A new anti-ICOS monoclonal antibody prevents graft-vs-host disease but has no impact on a breast tumor growth in humanized mice

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Pre-clinical assessment of new therapeutic antibodies for cancer immunotherapy is hampered by a lack of suitable animal models. Immunocompromised NOD.SCID.γc-/- (NSG) mice reconstituted with a human immune system, either through adoptive transfer of human PBMCs or human hematopoietic progenitors, hold the promise for such a model. Here, we investigated the antagonist activity of a new monoclonal antibody to the human ICOS molecule, a co-stimulatory molecule for T cells, on the xenogeneic graft-vs-host disease (GVHD) that develops after transfer of human PBMCs in NSG mice. We also evaluated the agonist activity of the mAb in a model of a breast cancer in mice humanized with cord blood-derived progenitors. In the xeno-GVHD setting, prevention of weight loss, tissue infiltration and improved survival was observed following a single treatment with a low dose of the mAb. This treatment was clearly associated with reduced T cell numbers in the blood and in peripheral lymphoid organs. Thus, the anti-ICOS mAb protected mice from lethal GVHD by affecting T cell numbers in the expansion phase of the disease. We next evaluated whether tumor rejection could be observed in humanized mice. In a first setting, an HLA-A2 breast cancer cell line (MDA-MB231) was implanted into cord blood-humanized NSG mice expressing a transgenic HLA-A2 molecule. A slight delay in tumor growth was observed in this setting, suggesting that the human immune system present in this mice only rejected a small fraction of tumor cells. Addition of the anti-ICOS mAb did not modify this low grade rejection despite reduced T cell numbers following treatment. In a second setting, humanized mice were immunized with a HLA-A2 restricted immunodominant peptide before tumor implantation but that did not ameliorate rejection either. However, no detectable IFNg production was observed by ELISPOT following in vitro T cell stimulation Thus, these negative results highlight the limitations of using humanized mice to study tumor rejection. We thus turned to a third model, where the tumor was implanted before the transfer of total human PBMCs to initiate a GVHD. In this setting, we observed a marked delay in tumor growth compared to empty mice. Thus, xeno-GVHD acted as an ´adjuvant´ for tumor rejection. This model constitute the basis for our current studies assessing the agonist activity of anti-ICOS mAb on tumor growth. Results of these studies will be reported at the meeting. Altogether, our results highlights some limitations of humanized mice for cancer immunotherapy studies. We thus propose a new model to observe tumor rejection by human T cells in vivo. Furthermore, using a pre-clinical model of GVHD, we show that anti-ICOS mAb represents a promising tool for management of this disease in human patients.

Immunogenic cell death specific danger associated molecular patterns induced by modulated electrohyperthermia in colorectal adenocarcinoma model

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Objective: Electric field and the concomitant heat (modulated electrohyperthermia, - mEHT) can synergistically provoke cell death in tumor tissue Due to elevated glycolysis (Warburg effect) a concomitant ion concentration elevation occur leading to elevated permittivity in cancer compared to non-malignant tissues. Here we studied the molecular mechanism of cell death and damage associated molecular patterns (DAMP) required for professional antigen presenting cells for inducing immunogenic cell death (ICD) upon mEHT treatment *in vivo* in colorectal cancer.

Methods: HT29 human colorectal carcinoma cells xenografted in BalbC (nu/nu) mice, were treated with a single shot mEHT for 30 minutes. 3 parallel samples were collected at 0, 1, 4, 8, 14, 24, 48, 72, 120, 168, 216h post-treatment. 4h post-treatment mRNA expression was compared to untreated samples. Human Apoptosis Arrays were also used on 8, 14 and 24h treated samples. Histomorphologic, immunohistochemical and TUNEL assay results were analyzed in tissue micro array (TMA) section using digital slides.

Results: Modulated electrohyperthermia treatment induced significant tumor cell death linked with DNA fragmentation (24-48h) using TUNEL assay, in line with the mitochondrial translocation of Bax (8-14h), cytochrome c release from mitochondria to the cytoplasm (8-14h) and concomitant nuclear translocation of apoptosis inducing factor AIF (14-24h). Activated caspase-3 was not detected in tumor cells. In mRNA assay at 4h, significant differential expression of 48 genes including heat shock proteins was seen upon mEHT treatment. Immunohistochemistry and apoptosis protein array confirmed elevated hsp70 expression (14-24h and 72-216h) and hsp90 expression (24-216h) in the morphologically intact peripheral parts of treated tumors. Furthermore, an early (4h) cytoplasmic to cell membrane exposure of carleticulin and later (48-216h) the release HMGB1 protein from cell nuclei was also revealed in the treated samples.

Conclusion: In our in vivo colorectal model mEHT caused a dominantly caspase independent programmed cell death involving AIF activation along with the spatiotemporal appearance of DAMP signals relevant to ICD.

GMCSF armed oncolytic vaccinia virus induces anti-tumor immune response in immunocompetent Syrian hamster model

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Engineered vaccinia viruses have shown promising results in the treatment of cancer. Tumor oncolysis is an immunogenic phenomenon and it has recently been proposed that the efficacy of oncolytic viruses is determined in part by activation of the immune system against tumor cells. This can be further enhanced by arming the viruses with immunostimulatory molecules such as granulocyte-macrophage colony-stimulating factor (GMCSF) which can mediate antitumor effects by e.g. recruiting and stimulating dendritic cells and natural killer cells. However, mechanistic and immunological aspects of this approach have not been studied much in model systems due to species incompatibility issues. Human GMCSF is active in Syrian hamsters and in addition, vaccinia virus has been shown to replicate in hamsters *in vitro* and *in vivo*. In this study, we describe the generation of vvdd-tdTomato-hGMCSF and preclinical testing in immunocompetent Syrian hamsters.

Both vvdd-tdTomato and vvdd-tdTomato-hGMCSF treatments induced remission of syngenic Hap-T1 hamster tumors and provided complete or partial protection when the animals were challenged with the same or a previously unencountered cell line respectively. Some tumors, especially in the vvdd-tdTomato-hGMCSF treatment group, showed pronounced heterophil infiltration in neoplastic tissue close to areas of necrosis. We also observed increased proliferation, migration and specific recognition and killing of syngenic tumor cells *ex vivo* by splenocytes derived from vvdd-tdTomato-hGMCSF treated hamsters.

Characterizing tumor-specific memory stem like T cells in blood and bone marrow of breast cancer patients

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Human memory T cells with stem cell-like properties have been identified recently in both humans and mice. The stem cell-like memory T cells (Tscm) have superior self-renewal, proliferation and mediate potent anti-tumor immunity upon adoptive transfer into a humanized mouse model. To date, their contribution to anti-tumor immune responses in cancer patients, their antigen specificities and physiological function has not been understood.

We analyzed the peripheral blood (PB) and bone marrow (BM) of breast cancer patients for the presence of Tscm cells by multicolor flow cytometry analyses with a panel of human T cell markers to distinguish between naïve (Tn), central memory (Tcm), effector memory (Tem) and Tscm ($CCR7^+$, $CD45RO^-$, $CD127^+$, $CD45RA^+$, $CD95^+$, $CXCR3^+$, $IL2R\beta^+$) populations. We detected Tscm cells in both $CD4^+$ and $CD8^+$ T cells in the PB and BM of breast cancer patients. Interestingly, the number of Tscm cells was significantly higher in breast cancer patients as compared to healthy donors for both $CD4^+$ (2-fold) and $CD8^+$ T cells (4-fold). We next assessed the expression of exhaustion markers (PD-1 & LAG3) and found that majority of Tscm cells (>95%) do not express PD-1 & LAG3 and comprised the least exhausted T cell compartment as compared to Tn, Tem and Tcm. Furthermore, to investigate the contribution of different subsets of memory T cells to tumor-specific T cells in breast cancer patients, we used breast cancer associated MHC class-I pentamers against human epidermal growth factor receptor 2 ($HER2_{348-356}$, $HER2_{435-443}$), Mucin-1 ($MUC1_{12-20}$) and Carcinoembryonic antigen ($CEA_{571-579}$). Cumulative analyses of tumor-specific pentamer$^+$ $CD8^+$ T cells in PB and BM showed that 20-25% of total tumor-specific T cells were of Tscm phenotype. We observed higher percentages of tumor-specific pentamer$^+CD8^+$ T cells among the Tscm cells in BM as compared to PB, however this difference was not significant. For functional characterization of tumor-specific Tscm cell we performed ex vivo re-stimulation assays using autologous dendritic cells loaded with several breast cancer-specific peptides with PB and BM of breast cancer patients and analyzed cytokine secretion. Cumulative analyses showed that 20-23% of tumor-specific IFN-γ^+ $CD8^+$ and 26-32% IL-2^+ $CD4^+$ had a Tscm phenotype. The tumor-reactive Tscm were polyfunctional (secreted both IFN-γ^+ and IL-2^+) and were less exhausted than other memory T cell subsets: Tcm and Tem.

In summary, we report the presence of Tscm cells in the PB and BM of breast cancer patients and a significant increase in their number as compared to healthy donors. Importantly, we could show that Tscm contribute to a population of functional and tumor reactive $CD8^+$ and $CD4^+$ T cells in PB and BM of breast cancer patients. Our further aim is to investigate the therapeutic potential of Tscm cells in adoptive T cell experiments with humanized mouse model of breast cancer.

Efficient lysis of malignant B lymphoid cells mediated by the T cell-recruiting triplebodies [19-3-19] and [33-3-19]

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Triplebodies [19-3-19] and [33-3-19] are antibody-derived fusion proteins, that carry three single-chain fragment variable (scFv) domains in tandem in a single polypeptide chain.
Beyond the clinically successful format of „bispecific T cell engagers“ such as Blinatumomab, triplebodies offer perceived advantages for future use as therapeutic agents, including a prolonged plasma half-life and the option to achieve a greater discrimination between malignant and healthy targets through their unique ability to bind two different antigens on the surface of the same target cell („dual-targeting“).
[19-3-19] was designed as a prototype for the recruitment of T cells as cytolytic effectors via triplebodies. Its two distal domains are specific for lymphoid antigen CD19, and the central domain for the T cell antigen CD3 epsilon. [19-3-19] binds CD19-bearing cells and primary T cells in an antigen-specific manner. Malignant cells from B-lymphoid cell lines and primary cells from patients with B-cell neoplasias were used as targets in cytotoxicity tests with pre-stimulated allogeneic T cells as effectors. The triplebody mediated up to 95 % specific lysis of CD19-positive tumour cells in a 3 hr reaction and had similar cytolytic potency as the clinically successful agent blinatumomab with EC_{50} doses in the low picomolar range. The agent was further capable of activating resting T cells from healthy unrelated donors and induced specific lysis of both autologous and allogeneic CD19-positive cells. In 15 hr cytotoxicity tests the agent led to the elimination of approximately 70% of CD19-positive target cells even with previously resting effector T cells used at an effector-to-target cell ratio of 1 : 10. The protein therefore was capable of mediating serial lysis of target cells by a single effector T cell.
The T cell recruiting triplebody [33-3-19] with specificity for myeloid lineage marker CD33 and B-cell antigen CD19 was designed to selectively bind biphenotypic leukemia cells. Results from initial binding and redirected lysis studies with this triplebody, which has the capacity for “dual-targeting”, are also shown.

Ipilimumab and hypofractionated brain radiotherapy for brain metastases of malignant melanoma

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Purpose: Immunomodulating effects of irradiation have been described in literature. Therefore we performed a retrospective analysis on patients with brain metastases from malignant melanoma who received Ipilimumab and whole brain radiotherapy (WBRT).

Methods: We analyzed patients with brain metastases from malignant melanoma who were treated with hypofractionated WBRT or stereotactic radiosurgery (STX) and received Ipilimumab in closely time relation. Patients treated between October 2010 and December 2012 were included. Follow-up was obtained until December 2013. The survival rate was estimated by Kaplan Meier plots. 25 patients with advanced melanoma and brain metastases who were treated with WBRT before 2010, and who had not received Ipilimumab, served as historic controls.

Results: A total of 16 patients were identified of whom 6 were treated with SRS, 2 with combination of SRS and WBRT and 8 with WBRT only. All patients received at least 2 doses of Ipilimumab. Three patients received Ipilimumab before and during radiotherapy. 13 patients received Ipilimumab after radiotherapy. The Median Time interval between radiotherapy and Ipilimumab was 1 month. Patients treated with radiotherapy and Ipilimumab had a censored median survival of **10,8 months**, compared with **3,6 months** for the patients who did not receive Ipilimumab (historic controls).

Conclusions: The combined treatment with Ipilimumab and radiotherapy is associated with a significantly improved overall surviving for patients with brain metastases from malignant melanoma. Further studies are needed to evaluate the relationship between the time interval and the sequence of Ipilimumab and radiotherapy. The magnitude of improved survival is suggesting a synergistic effect of hypofractionated radiotherapy with Ipilimumab treatment.

Unrestricted T cell functionality in newly diagnosed and relapsed AML patients

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The prognosis of acute myeloid leukemia (AML), particularly when associated with adverse chromosomal or molecular aberrations, is poor due to a high relapse rate after induction chemotherapy. Postremission therapy for elimination of minimal residual disease remains a major challenge. Immunotherapeutic strategies aim at the stimulation of AML-specific immunity, especially of $CD8^+$ T cells. However, the functionality of these cells in AML patients is not well described. T cell exhaustion has been suggested to contribute to immune evasion in various solid and hematological malignancies. Exhausted T cells are characterized by an increased expression of several inhibitory molecules, reduced proliferation and impaired cytokine secretion and cytotoxicity.

To characterize the T cell phenotype and function in AML, $CD8^+$ and $CD4^+$ T cells from patients at primary diagnosis, with refractory disease, at relapse and at relapse after allogeneic stem cell transplantation (alloSCT) (23, 4, 9 and 7 individuals, respectively) were analyzed by flow cytometry-based assays. Surface expression of CD244, CD160, PD-1, TIM-3 and LAG-3 was determined. T cell proliferation and production of the cytokines IFN-γ, TNF-α and IL-2 were measured in response to different stimuli. Results were compared to healthy controls (HC) (30 individuals), while untreated HIV-infected patients (10 individuals) served as positive controls for an exhausted T cell state.

In HIV-infected patients, we observed a pronounced upregulation of the inhibitory molecules CD244, CD160 and PD-1 on $CD4^+$ and $CD8^+$ T cells as well as globally impaired cytokine production. In contrast, T cells from AML patients at primary diagnosis showed an expression pattern of inhibitory surface molecules that was similar to T cells from age-matched HCs. Interestingly, AML patients with a relapse after alloSCT showed a 3- and 6-fold increased overall expression of PD-1 on $CD8^+$ ($p=0.0084$) and $CD4^+$ ($p< 0.0001$) T cells, respectively. This PD-1 expression pattern correlated to an increased proportion of memory T cells, which have an inherently higher expression of PD-1. Both, relapsed patients after conventional chemotherapy and relapsed patients after alloSCT, displayed a shift from the naive towards the memory T cell compartment. Functionally, no defect in T cell proliferation in any of the AML patient cohorts was detected. Of note, however, we observed a 2-fold decrease ($p=0.0068$) in IFN-γ production by $CD4^+$ T cells exclusively in patients at primary diagnosis. In summary, we show that T cells of newly diagnosed and relapsed AML patients are fully functional. Moreover, we demonstrate enhanced T cell differentiation in relapsed AML patients. We therefore hypothesize that bulk T cells in AML are in a status of activation, not exhaustion. Thus, immunotherapies that aim at eliciting tumor-specific immune responses, e.g. dendritic cell based vaccines, may be particularly suited for postremission therapy.

Systematic analysis of changes in the antigen processing machinery in HPV transformed cells

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Persistent infection with high-risk types of human papillomavirus (HPV) is a necessary prerequisite for malignant transformation. In order to establish persistent infection, HPV has developed multiple immune evasion strategies, among which modulation of expression of antigen processing machinery (APM) components directly interferes with T cell recognition of HPV-transformed cells.

The aim of this project was to systematically analyze the expression of all APM components in a comprehensive collection of HPV-positive cells. The cell lines are derived from cervical and head and neck squamous cell carcinomas, from primary tumors and metastases, and the collection includes long-standing and newly established cell lines. HPV-negative cells originating from primary or immortalized keratinocytes served as negative controls. Gene expression levels were determined by a quantitative real-time PCR screen, and protein levels by FACS and immunoblotting. To address the inducibility of interferon (IFN)-gamma-responsive APM components and the immunoproteasome, cells were analyzed with and without IFN-gamma exposure.

All HPV-negative keratinocytes showed a remarkably uniform expression of APM components. Interestingly, in HPV-positive cells the immunoproteasome subunits were consistently detectable even in the absence of IFN-gamma, and other components (e.g. endoplasmic reticulum aminopeptidase 1) were also found to be upregulated. All inducible APM components were induced upon IFN-gamma treatment in all cell lines, resulting in comparable APM component levels in HPV-positive and HPV-negative cell lines in this environment. Currently, we are examining the differentially expressed APM components on the protein level.

Taken together, this study will provide a systematic appraisal of HPV-mediated APM changes. In addition, the impact of IFN-gamma on antigen processing and thus epitope presentation in HPV-driven tumor cells will be assessed.

Dysregulated glucose metabolism inversely correlates with CD8$^+$ T-cell infiltration in head and neck squamous cell carcinoma

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Background: Recently, numerous reports have demonstrated that the number of CD8$^+$ T cells within a solid tumour predict for outcome. These findings have stimulated new interest in the development of CD8$^+$ T cell-based therapies that circumvent tumour-associated immunosuppressive mechanisms, and thereby release the brakes on pre-existing CD8$^+$ T-cell responses. The successful clinical application of such therapies will require delineation of the regulatory pathways that control CD8$^+$ T-cell activation and differentiation in the tumour environment.

Methods and Results: To identify sets of genes (modules) which correlated with low TIL abundance, we employed weighted correlation network analysis of two publicly available microarray data sets from primary head and neck squamous cell carcinoma (HNSCC) tissue. This approach identified an immune response module that was highly enriched for genes associated with T-cell activation (adj. $p = 9.7 \times 10^{-33}$). The expression of this module inversely correlated with a hypoxia-related meta-module ($r = -0.50$, $p = 2.3 \times 10^{-4}$), and with genes associated with aerobic glycolysis. Among the genes was the facilitated glucose transporter, member 1 (GLUT-1). We confirmed the inverse relationship between GLUT-1 expression and TIL density at the protein level, and modelled the impact of high GLUT-1 expression on glycolysis (measured using a Seahorse XF96 analyzer) and immune evasion.

Conclusion: We speculate that the prodigious appetite for glucose by HNSCC may limit the effectiveness of anti-tumour CD8$^+$ T cells, which are also addicted to glucose. Alternatively, lactate, the end product of glycolysis may impair CD8$^+$ T-cell expansion.

Hormone induced tolerance of tumor associated dendritic cells

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The immune system has the ability to recognize and destroy cancerous cells long before they grow, multiply and form tumors. However, once tumors develop they severely suppress anti-tumor immune responses. The underlying causes of immune suppression are complex and not completely understood. Recent studies indicate that tumor-associated dendritic cells (TADC) within the tumor microenvironment (TME) are key regulators of cytotoxic T-cell (CTL) function and may serve as a critical target for immunotherapy. The TADC-T cell interaction is critical to developing a robust anti-tumor response but can also result in T-cell tolerance to self-antigen.

Hormonal stimulation of tumor cells has been shown to promote tumor growth and hormone deprivation therapy is first line treatment of prostate and breast cancer. However, less is known regarding the impact of hormonal signaling on immune cell function in the TME. In this study we examined the effects of androgen receptor and estrogen receptor stimulation on bone marrow derived dendritic cells (BMDCs) and their effects on signaling pathways involved in DC induced tolerance.

A key mediator of tolerance in DCs is the transcription factor Forkhead Box O3 (FOXO3). FOXO3 is a transcriptional regulator of a variety of processes associated with cell cycle progression as well as regulation of nuclear receptors, including the androgen and estrogen receptors (AR and ER). Treatment of DCs with dihydrotestosterone (DHT) lead to an increased stability of the FOXO3 protein at all DHT levels tested. In addition we examined the effect of DHT on the transcription factor STAT3 which have previously been shown to exert tolerogenic effects in dendritic cells. Increasing exposure to DHT lead to an increase in the transcription factors STAT3 protein levels. Increased signaling in these either or both of these pathways due to DHT stimulation may promote tolerogenic functions in TADC. In contrast to AR stimulation, low level ER stimulation seems to increase the anti-tumor effect of TADC and lead to reduced expression of signaling molecules. However, high levels of estrogen were found to decrease the anti-tumor function of TADC as evidenced by the up-regulation of FOXO3 signaling. The insight gained from these studies will be valuable to targeting the treatment of cancer by immune therapy to individual needs.

Standardized immunostaining and evaluation of HLA class I expression in tumor tissues -An impact on peptide-based cancer immunotherapy-

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Down-regulation of HLA class I (HLA-I) expression is supposed to be one of major immune escape mechanisms of cancer and an important factor in the therapeutic resistance of cancer immunotherapy. However, lack of an anti-panHLA-I antibody suitable for immunostaining of formalin-fixed paraffin-embedded (FFPE) tissue sections has prevented the standardization and evaluation of HLA-I expression in cancer tissues. We developed a monoclonal antibody EMR8-5 that can react to HLA-A, B and C on FFPE tissue sections and conducted immunohistochemistry quality control exercises, including staining methods and interpretations for the standardization. Immunostaining of a variety of FFPE tumor tissues revealed that over 80% of cases have down-regulation or disappearance of HLA-I in breast cancer and prostate cancer. The loss of HLA-I expression became a significant prognostic marker in most epithelial cancers. Then, we evaluated HLA-I expression in cancer tissues of patients who underwent survivin2B peptide cancer vaccine. It was found that none of HLA-I-negative cases was responder in colon, lung and breast cancer cases in spite of increased HLA/peptide tetramer+ T-cells in their peripheral blood. Our study highlights the predictive significance of HLA-I expression and the importance of standardized immunostaining test using FFPE tissues in the development of cancer immunotherapy.

Role of immune effector cells in metastatic sites during the progression of breast cancer

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Breast cancer is a leading cause in cancer mortality of women worldwide. As in most cancers, the main cause of death is not the primary tumor but metastasis at distant sites in the body. Tumor/Immune interactions are critical in every step of tumorigenesis, from initiation to tumor progression and tissue invasion. Even though the molecular mechanisms that regulate the immune response in the primary tumor are slowly being characterized, little is known about those that operate in the metastatic sites. We used the 4T1 breast cancer model to dissect the role of immune effector cells in metastatic sites during the progression of mammary carcinoma. 4T1 cells were injected in the mammary fat pad of wildtype, Rag-/- and gamma(c)-/- mice. Primary tumor growth was similar between the different strains but metastatic burden was dramatically increased in lungs of gamma(c)-/- mice, pointing to an important role of innate lymphocytes in the regulation of lung metastasis. We phenotypically characterized Natural Killer (NK) cells isolated from lungs of 4T1 tumor bearing mice and compared them to the ones from the primary tumor. Interestingly, the expression of the activating receptors NKG2D and DNAM-1 in lung NK cells of tumor bearing mice increased during tumor progression, whereas the expression of these receptors in the primary tumor was clearly decreased. Moreover, the percentage of fully differentiated $CD11b^+CD27^-$ NK cells in tumor bearing lungs slightly decreased over time, but remained much higher than in primary tumors. Resection of the primary tumor resulted in increased levels of NKG2D and the $CD11b^+CD27^-$ subset in NK cells isolated from lungs. 4T1 mammary tumors induced the accumulation of $CD11b^+Ly6G^+$ and $CD11b^+F4/80^+$ myeloid cells in metastatic sites, which showed different status of activation, being $CD11b^+F4/80^+$ the most immunosuppressive population. Taken together, our data shows a differential state of activation for NK cells in metastatic sites during tumor progression. Whether this is translated into functional changes in these cells and how is this being regulated by the main myeloid subtypes that form the metastatic microenvironment constitutes the focus of our present research.

Phenotypic characterization of the immune infiltrate in gynaecological tumours

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Background: Tumours are infiltrated with various types of immune cells and depending on the composition of the immune infiltrate, these immune cells can elicit anti-tumour immunity or create immunosuppression. In gynaecological tumours (ovarian carcinoma, endometrial carcinoma and uterine sarcoma) it has been established that certain immune cell types are associated with improved (CD8 T cells) or worse (FoxP3 regulatory T cells) survival. However, the exact phenotype of the immune cells infiltrating these tumours, especially uterine tumours, is largely unexplored.

Materials and methods: Immune cells were isolated from fresh tumour biopsies using mechanical and enzymatical disruption. These infiltrating immune cells (TIL) were analysed using an extensive flow cytometric marker panel and compared to the phenotype of peripheral blood mononuclear cells (PBMC) from the same patients. In addition, immune cells from fresh tumour biopsies were also cultured in the presence of cytokines to assess antigen specificity.

Results and discussion: Regarding T cells, the CD4/CD8 ratio is slightly decreased in TIL versus PBMC and TIL contain higher percentages of regulatory T cells ($p = 0.0128$). $CD4^+$ T cells in TIL express significantly higher levels of the activation markers CD69, CD137 and HLA-DR and of the inhibitory markers ICOS, CTLA-4 and PD-1 compared to PBMC. $CD8^+$ T cells in TIL on the other hand express significantly increased levels of CD69, HLA-DR and PD-1 compared to PBMC. Both monocytic MDSC (Lin^- $HLA\text{-}DR^{lo}$ $CD11b^+$ $CD14^+$) and granulocytic MDSC (Lin^- $HLA\text{-}DR^{lo}$ $CD11b^+$ $CD14^-$) are detectable in TIL, with granulocytic MDSC being the most predominant subtype. No differences were observed in both MDSC subtypes in TIL versus PBMC. NK cells defined as $CD45^+$ $CD56^{lo/+}$ $CD16^+$ or $CD45^+$ $CD56^{lo/+}$ $CD16^-$ could be detected at comparable levels in TIL and PBMC. No differences in the expression of CD69, CD161 and HLA-DR on both types of NK cells could be found in TIL nor PBMC. These data indicate that gynaecological tumours contain T cells with a mostly activated and somewhat exhausted phenotype, along with monocytic and granulocytic MDSC and NK cells. We are currently analysing the memory differentiation stage of the T cells and investigating whether cultured TIL contain T cells recognizing tumour-specific antigens. These data will shed more light on the feasibility of tumour vaccination or adoptive T cell therapy in gynaecological malignancies.

Intraepithelial macrophage infiltration and high number of regulatory T cells promote whereas activated CD8 T cells prevent a progressive course of HPV-induced vulvar disease

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Usual vulvar intraepithelial neoplasia (uVIN) is caused by a failure of the immunesystem to clear a persistent HPV infection. While immunotherapeutic approaches to treat uVIN are achieving clinical success, many patients fail to show complete clinical responses. Data accumulate that local immune infiltrates play a determining role in the prognosis of disease and the outcome of immunotherapeutic approaches.

We conducted a comprehensive study of the local innate and adaptive immune response in uVIN by analyses of several types of myeloid cells, NK-cells and T-cells, as well as the expression of immune co-inhibitory molecules and their ligands in the microenvironment of 43 primary uVIN lesions, 20 recurrent uVIN lesions, 21 vulvarcarcinomas and 26 healthy controls by immunofluorescent confocal microscopy for CD14, CD33, CD163, CD3, CD8, FoxP3, PD1, TIM3, NKG2a and Galectins-1, -3 and -9, and immunohistochemistry for Tbet, IDO and NKp46.

The progressive course of vulvar neoplasia is characterised by an increase in epithelial and stromal mature M1 and M2 macrophages. While the M2 macrophages outnumber M1 macrophages in the stroma of uVIN and healthy controls, the M1 macrophages level up with M2 macrophages in cancer. A dense intraepithelial infiltration of mature $CD14^+$ macrophages (irrespective of M1 or M2 type) was found to be an independent prognostic factor for rapid recurrences ($p=0.004$) in uVIN. In particular, uVIN patients with an infiltration profile of innate immune cells resembling that of vulvar cancer, more rapidly displayed recurrences. Analysis of the T-cell response revealed that many of the T-cells expressed the co-inhibitory markers PD-1, TIM-3 and NKG2a. A dense infiltration by stromal $CD8^+TIM3^+$ T-cells as well as $CD3^+NKG2a^+$ T-cells associated with a prolonged recurrence free survival ($p=0.015$ and $p=0.001$, respectively), indicating that the expression of these markers is likely to reflect local activity of the infiltrating T cells. The expression of $TIM3^+$ was associated with galectin-9 expression, reflecting the local production of IFNg. HLA-E, the ligand for NKG2a was virtually absent in uVIN. Combinatorial analysis of the innate and adaptive immune cells showed that a dense intraepithelial infiltration by $CD14^+$ macrophages was associated with high numbers of intraepithelial Tregs and low numbers of stromal $CD8^+TIM3^+$ T-cells. Patients with low numbers of intraepithelial $CD14^+$ cells and high numbers of stromal $CD8^+TIM3^+$ cells showed the best recurrence free survival ($p=0.029$).

Our data clearly show a role for the local immune response. The expression of co-inhibitory markers is not necessarily an unfavourable sign as in the absence of the ligands T-cells may exert their function. Rather they indicate a truly active local microenvironment. An estimation of the number of intraepithelial $CD14^+$ cells and stromal $CD8^+TIM3^+$ T-cells may be of help in determining the prognosis of patients as well as the response of patients to immunotherapy.

Preclinical study on a new developed TLR7 agonist shows potent anti-tumor therapeutic effects

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Toll-like receptor (TLR) ligation activates both the innate and adaptive immune systems, and plays an important role in antiviral and anti-tumor immunity. Therefore, significant effort has been devoted to exploit the therapeutic potential of TLR agonists based on ssRNA, the natural ligand for TLR-7 and -8, and small molecule ligands.

So far, the only approved TLR agonists in clinical settings are imiquimod and resiquimod. Both compounds are applied topically since they display disadvantageous toxic effects after systemic application, while newer agents like 852A and VTX2337 just recently completed first clinical studies. In our study we used SC-1 a recently discovered small molecule with potent TLR7 agonist proprieties. *In vivo* pharmacodymamic and pharmacokinetic studies, using intravenous injection of SC-1, allowed us to find a non-toxic and tolerable dose in mice, which potently induces cytokine production (IFN-α TNF-α, IP-10, IL6,IL1β, IL10, IL12p40 and IFN-γ) detectable in plasma of treated mice.

Here we describe the first preclinical therapeutic study using SC-1 in mouse carcinoma models. CT26 tumor bearing mice were treated intravenously with SC-1 (3 mg/kg) every 5 days. All treated mice showed a strong delay in tumor growth, prolonged survival as well as a relevant anti-tumor response, which prevented tumor formation. Interestingly, we found that SC-1 therapy depends, at least in part, on expansion of $CD8^+$ T cells specific for tumor associated antigens (gp70). These gp70-tetramer$^+$ $CD8^+$ T cells, detectable in circulation as well as in spleen and in tumors, showed potent effector function as tested in IFN-γ ELISpot. Notably, SC-1 anti-tumor treatment is found beneficial also against tumor growth of the very immunosuppressive murine breast carcinoma, 4T1. Interestingly, adoptive cell transfer of $CD8^+$ T cells as well as $CD4^+$ T cells isolated from SC-1 treated tumor-free mice confers prophylactic tumor protection to recipient Balb/c mice challenged with CT26 tumor cells. In conclusion, this new TLR7 agonist SC-1, via repetitive systemic injections, is able to trigger the murine innate immune system to mount a potent and antigen specific, anti-tumoral adaptive immune response.

The relevance of differential expression of tolerogenic enzymes IDO-1, IDO-2 and TDO in commonly used mouse tumor models for testing novel therapeutics

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Objective: Inhibition of indoleamine-2,3-dioxygenase-1 (IDO-1), indoleamine-2,3-dioxygenase-2 (IDO-2), and/or tryptophan-2,3-dioxygenase (TDO) represent novel opportunities for reversing the immunosuppressed microenvironment found in patients with cancer. While these targets are clinically relevant to date there has not been a systematic assessment of the relative expression of these enzymes in both tumors and associated lymph nodes in preclinical tumor models. This report describes such a characterization in four of the most commonly used models.

Methods: The expression of IDO-1, IDO-2 and TDO was evaluated in the B16F10 melanoma and PAN02 pancreatic models in C57BL/6 mice, and 4T1 breast and CT26 colon models in Balb/c mice. PAN02 and CT26 cells were injected s.c. into the flanks of 8-week old female mice, while both the B16F10 and 4T1 tumors cells were administered by intradermal and mammary fat pad injection, respectively. In each model, tumors and draining lymph nodes were collected from separate cohorts of mice at an early and late time points correlating to mean tumor volumes of 100 and 1,000 mm^3, respectively. Expression of the 3 enzymes assessed using a variety of techniques, including PCR, Western blot and FACS based analysis.

Results: The results of these studies highlight distinct differences in the expression of these three enzyme targets, being dependent upon the choice of tumor cell, tumor vs. lymph node and the stage of growth. These differences are exemplified by the results obtained in the B16F10 and CT26 models. Prior to tumor inoculation both cells lines were negative for all three enzymes, however at both time points the CT26 tumors had significantly increased levels of IDO-1 and IDO-2, relative to the input cells. In contrast both of these enzymes remained undetectable in the B16F10 tumors.

Conclusion: It is important to ensure that the mouse models used for preclinical evaluation recapitulate the elements of the signaling pathway most relevant to the human condition. The results presented here will be of particular interest to those investigators targeting immunosuppression reversal as an approach to anti-cancer therapy.

HLA quantification in multiple myeloma reveals high and robust HLA class I and II expression on primary myeloma cells

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Multiple myeloma (MM) is a B-cell lymphoma, which is characterized by the expansion of monoclonal plasma cells. Despite of recent advances in the treatment of MM with proteasome inhibitors, immunomodulating drugs and autologous stem cell transplantation achieving long-term remissions in some patients the disease still remains incurable. This is due to the persistence of residual malignant plasma cells, i.e. minimal residual disease (MRD), which lead to high relapse rates.

The favorable immune effector-to-target cell ratios presented in this setting suggests that MRD in MM might be targeted effectively by T cell based immunotherapy. Essential prerequisite for effective T-cell recognition is the expression of HLA molecules on target cells. Therefore, we here investigated the absolute HLA class I and II surface expression on myeloma/plasma cells as well as on normal B cells, T cells and hematopoietic stem cells (HSC) of primary multiple myeloma samples (n=20), and bone marrow samples (n=15) of healthy volunteers (HV) respectively, using a quantitative flow cytometric assay. Furthermore, the impact of different therapeutic regimens on HLA class I and II expression of myeloma cells was examined in bone marrow samples of MM patients after 4 weeks of treatment. HLA expression levels were found to be heterogeneous on myeloma cells with total HLA class I molecule counts ranging from 120000-850000. Mean HLA class I expression on myeloma cells (415000 ± 55000) was shown to be significantly higher compared to the mean expression on autologous B cells (200000 ± 20000, $p=0.001$), T cells (165000 ± 15000, $p< 0.001$) and HSC (205000 ± 35000, $p=0.002$) in MM patients. In addition, HLA class I expression on primary myeloma cells was also found to be significantly higher than on normal plasma cells of HVs (290000 ± 25000, $p< 0.05$).

Mean HLA-DR expression on myeloma cells showed no significant difference compared to expression levels on autologous bone marrow cells as well as on normal plasma cells of HVs.

Different therapeutic regiments including monotherapy with the proteasome inhibitor carfilzomib (n=2) or immunomodulating drugs (pomalidomide (n=3) or lenalidomide (n=2)) did not induce significant loss or downregulation of mean HLA class I and II expression levels on primary myeloma cells after 4 weeks of treatment.

Furthermore longitudinal analysis of HLA ligandome composition in myeloma cell lines across different time points after incubation with carfilzomib as well as HLA ligandome analysis of primary myeloma cells using liquid chromatography coupled mass spectrometry (LC-MS/MS) are ongoing.

In conclusion these findings validate myeloma cells as a potential target for T cell based immunotherapy, which might take place after standard induction therapy to target MRD in MM patients.

Investigation of the spatial heterogeneity of specific immune cell phenotypes in the tumor microenvironment of follicular lymphoma

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Background: Tumor-infiltrating lymphocytes (TILs) are present in the tumor microenvironment of many cancers, with consequent tumor immunogenicity and association with survival. In particular, increased levels of regulatory T cells (Tregs) are associated with poorer prognosis in some cancers. However, given the complexity of TIL phenotype their visualization in situ is difficult, and impossible without multiplex immunophenotyping. An understanding of both the phenotype and spatial distribution of TILs *in situ* within the tumor microenvironment would be advantageous to understanding their role in tumour immunobiology, especially given growing interest in tumour immunotherapy. Here we present a multi-marker, computer-aided method for analysing the distributions of CD3/FOXP3 (Treg) and CD3/CD69 (Tact) T cells in follicular lymphoma sections using a multispectral imaging (MSI) and automated analysis approach. An hypothesized interaction distance (HID) analysis was used to determine whether the spatial patterns of Tregs and Tacts were prognostically significant.

Design: A single section of a tissue microarray containing triplex follicular lymphoma cores from 40 subjects [24 male, 16 female, age 35 to 75 years at diagnosis, median 55 years , 2- 171 months follow-up] was stained for CD3, FOXP3, CD69 and hematoxylin. Each core was imaged using MSI and the individual staining of each marker separated from each other using spectral unmixing. $CD3^+$ TILs were located using automated image analysis. The FOXP3 and CD69 status of each $CD3^+$ TIL was then determined and the spatial distributions of the CD3/FOXP3 and CD3/CD69 cells were used as input into the HID analysis.

Results: Multiplexed IHC staining, MSI and automated per-cell quantitative analysis was successful. Kaplan-Meier analysis demonstrated favourable outcome with higher numbers of $CD3^+$, $CD3^+$/$FOXP3^+$ and $CD3^+$/$CD69^+$ cells. HID analysis demonstrated the association of favourable outcome with a high entropy , representative of a diffuse spatial pattern, of $FOXP3^+$ and $CD69^+$ positive T cells.

Conclusion: In this study we report that higher Treg cell counts in a diffuse pattern was associated with favorable prognosis. This supports the importance of Tregs in the tumour microenvironment. It is pertinent to mention that contradictory findings are routinely reported from studies investigating the role of Tregs in solid and haematological malignancies. This is due to the complex interactions between pro-/anti- tumour immune factors present in the tumour microenvironment. The resultant effects are due to the summation of the activities of these factors. It is therefore even more relevant that a method such as exhibited here, capable of defining and measuring the effect on patient outcome of the spatial patterns of multiple cellular phenotypes in the tumor microenvironment is available.

Interleukin-6 receptor and its ligand interleukin-6 are opposite markers for survival and infiltration with mature myeloid cells in ovarian cancer

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Introduction: Increased levels of interleukin-6 (IL-6) in epithelial ovarian cancer (EOC) patients are correlated with a worse prognosis. IL-6 stimulates tumor-inflammation, -growth, and angiogenesis and is suggested to attract M2 macrophages and myeloid suppressor cells in EOC.

Materials and methods: We investigated the intricate interaction between the IL-6 signaling pathway and tumor-infiltrating myeloid cells to determine their prognostic and therapeutic impact in EOC. A tissue micro-array (TMA) containing 160 EOC samples was analyzed for the expression of IL-6, its receptor (IL-6R) and downstream signaling via pSTAT3 by immunohistochemistry. Triple color immunofluorescence confocal microscopy was used to identify myeloid cell populations (CD14, CD33, CD163). The relationship between these markers, tumor-infiltrating immune cells, clinicopathological characteristics and survival was investigated. Furthermore, a heatmap with all known immune markers was created by unsupervised clustering in order to define immune profiles in EOC.

Results: Tumors with a high expression of IL-6R displayed a low infiltration of mature myeloid cells ($p=0.017$) and were associated with longer disease-specific survival (DSS; $p=0.010$). High expression of IL-6R was an independent prognostic factor. In contrast, tumors with high IL-6 expression displayed a dense infiltration of mature myeloid cells ($p=0.012$) and were correlated with a shorter DSS ($p=0.034$). Clustering on all known immune parameters revealed roughly two types of tumor environments. A tumor-rejecting environment with high infiltration of cytotoxic $CD8^+$ T cells and fewer M2 macrophages and regulatory T cells (Tregs), and an immunosuppressive environment with high infiltration of Tregs and M2 macrophages, while having a low infiltration of $CD8^+$ T cells. In densely $CD8^+$ T-cell infiltrated tumors, the ratio between these lymphoid cells and CD163 myeloid cells was predictive for survival ($p=0.036$), indicating that the positive effect of $CD8^+$ T cells may be counteracted by suppressive $CD163^+$ cells, confirming the role as an immunosuppressive population.

Conclusion: Our data revealed two types of groups in EOC, the first containing patients with a high expression of IL-6R and low infiltration of mature myeloid cells, suggesting IL-6R might be useful as a prognostic marker. Treatment with a monoclonal antibody targeted against IL-6R might be used in these patients to prevent further tumor growth. The second group showed a high expression of IL-6 in tumor with a low expression of IL-6R, and was densely infiltrated with M2 macrophages. This group was associated with a worse clinical outcome. Here, treatment with anti-IL-6R might prevent differentiation of suppressive myeloid cells.

Comparison of tumor infiltrating lymphocyte signatures across six cancer types and their association with disease prognostic factors

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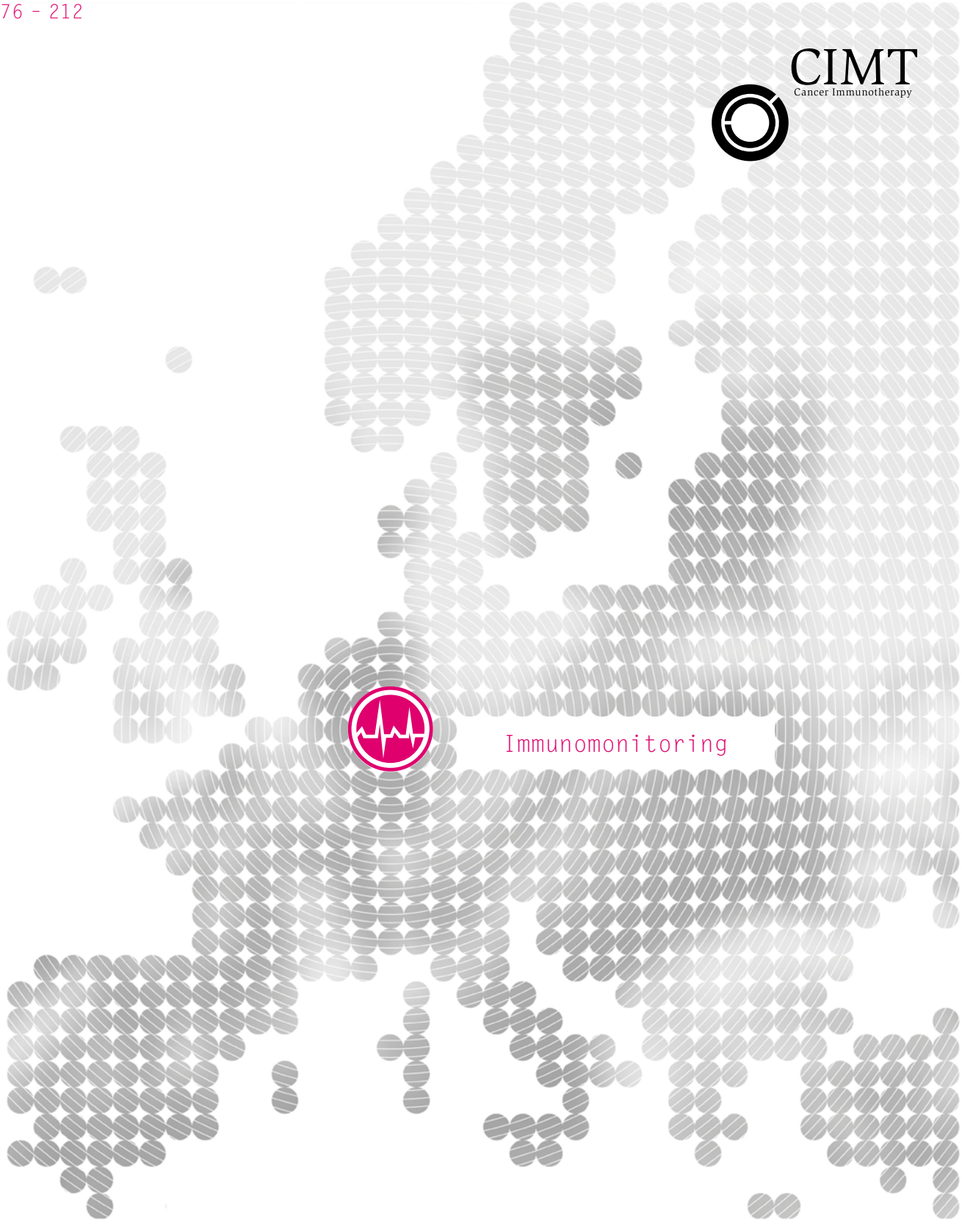
ORAL SHORT TALK

Recent clinical studies targeting the PD-L1/PD-1 pathway have shown the impact of restoring anti-tumor immunity in many cancer patients. While rapid and durable responses to blocking these pathways have been observed (Soria et al, ECC 2013, Topalian et al, NEJM, 2012; Hamid et al, NEJM, 2013), a proportion of patients do not respond to these therapies, thus prompting the assessment of additional factors that may further modulate or inhibit anti-tumor immunity. Studies evaluating the efficacy of PD-L1/PD-1 blockade in humans provide an opportunity to dissect the key factors that contribute to response or resistance to immune modulatory therapy.

We have applied highly sensitive immune gene expression assays (iCHIP) using the Fluidigm Biomark platform to interrogate the quality of the immune response across six cancer types including colorectal cancer (CRC; n=48), breast cancer (n=126), NSCLC (n=51), melanoma (n=35), renal cancer (RCC; n=48) and bladder cancer (n=42). The iCHIP platform consists of 96 genes that represent signatures associated with the interferon gamma (IFNγ) pathway, cytotoxic T cells, Th2 cells, T-regulatory cells, Th17 cells, myeloid cells, dendritic cells, NK cells, B cells and immune checkpoint markers. RNA was extracted from formalin-fixed paraffin embedded archival tissues that were derived from clinical collections or collected in the ongoing Phase I study of MPDL3280A, an engineered anti-PD-L1 antibody. Appropriate patient informed consents were obtained from the institutional review boards for the exploratory evaluation of biomarkers.

Immune gene expression analysis showed a unique pattern of immunosuppressive and immunoresponsive factors and cell types across indications. While indications, including triple-negative breast cancer (TNBC), NSCLC and bladder cancer represent the highest prevalence of INFγ signatures, CRC and hormone receptor-positive breast cancer constitute diseases with the lowest expression. In addition to a high IFNγ signature in TNBC, this subtype of breast cancer also consists of a high Treg signature when compared to melanoma, which represents the highest ratio of IFNγ:Treg gene expression. Th17 gene signatures are most prevalent in CRC compared to all other indications. Association of these gene signatures with disease stage, outcomes (where available) and other disease specific known prognostic factors including molecular subtypes and mutations in *KRAS, BRAF, PIK3CA* and *EGFR* will be presented.

CIMT
Cancer Immunotherapy



Immunomonitoring

Survival of melanoma patients correlating with DTH skin reactions and CD8 T cell/Treg ratios in PBMC after vaccinations with CpG & peptides

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ORAL SHORT TALK

CpG-based vaccination can efficiently induce high numbers of functionally competent tumor specific CD8 T cells, associated with favorable clinical outcome. Here we present data of a clinical trial that included 29 HLA-A2 positive patients with stage III-IV melanoma. They received up to 8 cycles of 4 monthly vaccines containing CpG, Montanide, and peptides derived from Melan-A/MART-1 and tyrosinase. All patients generated high numbers of Melan-A tetramer+ CD8 T cells, reaching 1.5 % (mean) of circulating CD8 T cells. These T cells secreted IFNγ in 24 patients. Although tyrosinase specific CD8 T cells were less frequent they doubled after vaccination in 8/13 evaluable patients. Statistical analysis showed that low Treg numbers at baseline predicted enhanced CD8 T cell responses. The function of tumor antigen-specific CD8 T cells was tested in vivo by **D**elayed **T**ype **H**ypersensitivity (DTH) reactions to i.d. injection of Melan-A peptide. Interestingly, these positively correlated not only with the numbers of tetramer+ and IFNγ+ CD8 T cells in blood, but also with the "ratio" of CD8 T cells to Treg cells. Remarkably, patient overall survival and disease free survival showed significant correlations with specific CD8 T cells, CD8/ Treg ratio, and DTH reactions. Together, the data show that CpG based vaccination induced long-lasting and favorable immune responses, supporting the further development of this immunotherapeutic approach.

TCR-Engineered Reference Samples (TERS) to control T-cell assay performance - towards an immuno-control kit

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The lack of standard control reagents for immunological T-cell assays prohibits comparability of results generated in one lab over time and across institutions. Therefore available TCR-engineered reference samples (TERS) that contain a defined number of antigen-specific T cells continuously leading to stable results are urgently needed. We established a simple and scalable technology to generate stable HLA-class I or II-restricted, tumor-associated antigen (TAA)-specific TERS batches that can be repetitively used to control the performance of commonly used T-cell assays over time. In the future, the users should manufacture TERS at large scale on their own. Therefore we developed TERS in a kit format consisting of TCR-RNA and a customized protocol for manufacturing.

As a first step in preparation for the immuno-control kit, we extensively tested six commonly used electroporation devices from four vendors (BioRad, Eppendorf, Harvard Apparatus and Lonza). We established the optimal electroporation conditions for the transfection of T cells with TCR-RNA leading to highest cell viability and TCR expression. Also, a large-scale RNA batch was manufactured and an extended RNA stability study was initiated testing RNA at accelerated and forced degradation for a period of 24 months. The data from this study will allow assigning appropriate shelf-life to TCR-RNA for the future immuno-control kit.

In preparation of a proficiency panel we organized a workshop with 4 participants who successfully produced HLA-class-I restricted TERS batches specific for NY-ESO-$1_{157\text{-}165}$ under guidance.

In November 2013, an exploratory proficiency panel (CIP_ID17_2013_RSKITMUL/E) was organized to test whether the TERS kit approach works if performed self-handed following a provided standard protocol. The kit for manufacturing the TERS at peripheral sites (n= 7 participants) consisted of two stability-tested RNAs: one TCR RNA specific for $NYESO1_{157\text{-}165}$ (SLLMWITQC) for the generation of the TAA-specific TERS and a reporter RNA coding for GFP which was used to optimize the electroporation settings of the available electroporation device prior to manufacturing the TERS. Additionally, a centrally manufactured TERS was provided. Both TERS were tested in parallel on two independent time points in MULTIMER staining experiments using a provided HLA-A2 NY-ESO-1 (SLLMWITQV) dextramer and a negative control dextramer (Immudex). We will show the preliminary results from the proficiency panel.

In summary, first results show that the immuno-control kit approach allows various users to produce their own TERS and to use it to control immune assay performance over time.

HLA typing from RNA-Seq sequence reads: a catalog of HLA type, HLA expression, and HLA presented somatic mutations in human cancer cell lines

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Cancer cell lines are a tremendous resource for cancer biology and therapy development. Given the advent of next-generation sequencing (NGS) technology, they are an ideal tool to examine the genetic origin of cancers; to identify potential novel tumor targets, such as tumor antigens for vaccine development; and to use in preclinical studies. For this purpose, we need highly characterized cell lines. Mutations, gene expression, and drug sensitivity have been determined for many cell lines. However, the HLA type and HLA expression of cell lines, characterizations necessary for the development of cancer vaccines, are largely incomplete and, when available, distributed in many publications.

Here, we used a previously described *in silico* method to determine the 4-digit HLA type and expression of publically available RNA-Seq samples from > 170 cancer and normal cell lines. We use standard NGS RNA-Seq short reads from "whole transcriptome" sequencing, map reads to known HLA types, and statically determine HLA type, heterozygosity, and expression. First, to our knowledge, we report previously unreported HLA class I and II genotypes and HLA expression of cancer cell lines. Second, these results provide a fundamental cell line "barcode" to track samples and prevent sample annotation swaps and contamination. Third, this provides insights into HLA downregulation and loss in cancer. Finally, these results are a fundamental resource for vaccine drug development. We show how to integrate the cancer cell-line specific HLA types and HLA expression determined here, with public available cell-line specific mutation information and existing MHC binding prediction algorithm to make a catalog of likely immunogenic mutations in each tumor cell line.

Assessment of vaccine-induced immunity following intra lymph node administration of RIBOLOGICAL IMMUNOTHERAPY targeting NY-ESO-1 and tyrosinase in patients with advanced melanoma

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There are about 160,000 new cases of melanoma worldwide each year. Despite advances in the treatment of early-stage melanoma, a significant fraction of patients will eventually develop metastatic melanoma, a subgroup with a poor prognosis. Immunotherapy may address the unmet medical need in patients with advanced melanoma.

A novel innovative RNA-based immunotherapy platform harboring proprietary chemical modifications and sequence elements that increase the bioavailability, immunogenicity and immunomodulatory properties of the investigational medicinal product was developed by BioNTech RNA Pharmaceuticals GmbH. Based on an extensive preclinical program and a favorable risk benefit analysis a first-in-human trial to test the safety, tolerability and pharmacodynamic activity of RIBOLOGICAL IMMUNOTHERAPY targeting the known tumor-associated antigens NY-ESO-1 and tyrosinase was initiated (NCT01684241).

So far 22 patients with stage III and IV malignant melanoma have been recruited to this phase I trial that included 4 dose escalation cohorts ranging from 50µg RNA up to 600µg RNA per injection. All 4 dose levels were well tolerated and no dose limiting toxicities were observed. Pharmacodynamic activity of RIBOLOGICAL IMMUNOTHERAPY was assessed by intradermal injections of RNA to induce DTH reactions, flow cytometric analysis of skin infiltrating lymphocytes, as well as IFN-gamma ELISPOT assays in peripheral blood mononuclear cells (PBMC).

We will present preliminary results from the immunological readouts made so far that are indicative of a high rate of vaccine-induced immunity. Immune responses against a microbial marker antigen and the cancer germline antigen NY-ESO-1 were more frequently observed than immune responses against the differentiation antigen tyrosinase.

Stability of peptides in long term -80°C storage

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Background and aims: Peptides of varying lengths are frequently used in cell based assays to monitor antigen specific responses. When these assays are used in clinical trials to monitoring patient responses to treatment it is of particular importance to know that all reagents in use are fit for purpose. We therefore underlook a study to assess the stability of peptides (9-meres) in long term storage at -80°C.

Method and Results: Six 9-mer peptides (Flu; GILGFVFTL, WT1-126; RMFPNAPYL, WT1-37; VLDFAPPGA, EBV; GLCTLVAML, HIV; ILKEPVHGV,CMV; NLVPMVATV) were purchased from PeptideSynthetics (UK) and dissolved in 100% DMSO before storage at -80°C. Following varying lengths of time an aliquot was sent back to PeptideSynthetics for assessment of peptide degradation by high-performance liquid chromatography (HPLC). The peptides were assessed at intervals for up to 320 weeks. The majority of peptides (4 of 6) were remarkably stable at -80°C and contained more than 90% intact peptide at the end of the study (range 150-202 weeks in storage). The EBV peptide had the fasted rate of degradation with less than 90% intact peptide already following 30 weeks in storage, falling to 68% intact peptide at the end of the study (320 weeks). The CMV peptide showed less than 90% intact peptide following 104 weeks at -80°C storage that further fell to 75% at the end of the study (157 weeks). To assess the impact of degradation on cell functionality, the degraded EBV and CMV peptides were compared to their newly synthesised counterpart (>95% intact) in ELISPOT assays. No significant difference in spot forming cells per million PBMCs were found between the degraded and newly synthesised EBV or CMV peptides, tested at 10-0.5 mg/mL, in any of the 10 HLA-A2^{+} donors tested.

Conclusions: Peptide integrity in long term storage at -80°C is generally well preserved. Although the kinetics and level of degradation can be peptide specific, partially degraded peptides are still fully functional in ELISPOT assays even at low concentrations (0.5 mg/mL). We therefore conclude that 9-mere peptides, dissolved in 100% DMSO and kept at -80°C for at least 3 years are suitable for use in endpoint ELISPOT assays to monitor responses in clinical trials.

HLA-A2 multimer staining validation

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Background and aims: Bioanalytical assay used to define success or failure in early phase clinical immunological trials (endpoint assays) require that the optimised method is suitable and reliable for its intended use. This process is referred to as assay validation and includes the confirmation of fundamental parameters such as accuracy, precision, selectivity, sensitivity and stability. Here we present the approach taken to validate two HLA-A2-restricted multimers, each containing a peptide from Wilms' tumor antigen (WT1-126: RMFPNAPYL, WT1-37: VLDFAPPGA), for use in flow cytometry endpoint assays.

Method and results: The WT1-37 multimer was validated using surplus samples from a patient with pre-existing WT1-37 immunity (WIN trial, GTAC173). The WT1-126 multimer was validated using a WT1-126 cell line (gift of Prof Greenberg, Milan, Italy). Prior to validation the background staining of both multimers on HLA-A2- donors was calculated as the 99th percentile (mean + 2.6 SD) and used as a cut off value for positive multimer staining (cut off 0.017% and 0.041% for WT1-37 and WT1-126, respectively). Multimer specificity was confirmed as the proportion of multimer+ cells was reduced by 57% and 21% for WT1-126 and WT1-37, respectively, following pre-incubation with the relevant but not a control irrelevant peptide (GLCTLVAML). The inter-assay variability was found to be very small with variances (CV) of 7.6% and 10.1% for the WT1-37 and WT1-126 multimer, respectively. The WT1-37 multimer was found to be very stable at 4°C with no loss of sensitivity detected following 17 weeks in storage. The WT1-126 multimer was less stable at 4°C with loss of sensitivity detected already following 2 weeks. The WT1-126 multimer is hence recommended to be used directly following thawing after -80°C storage. The sensitivity of the WT1-37 and WT1-126 multimer was also assessed in titration experiments. The lowest detection limit for the WT1-126 multimer was found to be equivalent to the calculated cut off value for non-specific binding. Numerically this was also true for the WT1-37 multimer, however, the presence of a WT1-37 multimer+ population was still visible even below this cut off value. This highlights the need for a more objective evaluation of multimer data also accounting for the visible appearance of multimer+ populations.

Conclusions: The ECMC group at Southampton has successfully validated two in-house made multimers specific for tumor-associated antigens (WT1-37 and WT1-126) that display good levels of specificity, sensitivity, inter-assay precision and stability. These multimers are therefore suitable for the identification of vaccine induced immunological response in clinical trials.

Managing multi-center flow cytometry data for cancer immune monitoring

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With the recent promising results of cancer vaccines and immunotherapy based on monoclonal antibodies to CTLA-4, PD-1 and PDL-1 in a range of metastatic cancers, immune monitoring has become increasingly relevant for cancer informatics. Immune monitoring requires accurate quantification of potentially rare cell subsets whose discrimination requires the measurement of multiple parameters on individual cells (e.g. antigen-specific T cells or other immune cell subsets like Tregs or MDSCs). The standard technology for single cell evaluation is flow cytometry, which can measure 10-20 parameters on a cell, with the next generation of flow and mass cytometers measuring up to 40 parameters. Because these parameters vary for each cell and 10^5−10^6 cells are analyzed per sample, the data complexity is comparable to that of proteomics or genomics. Many algorithms and pipelines have been developed for different aspects of flow cytometry analysis from normalization to clustering to phenotype correlations. However, these pipelines assume that the annotation required for data interpretation is correct and consistent, a necessary condition often not met in study data as annotation is typically manually entered without validation. We describe ReFlow, an informatics system organized around flow cytometric domain concepts that simplifies and validates data annotation for multi-center studies and the associated data transfer application programming interface for data sharing; examples of using Reflow as the frontend for automated analysis pipelines of proficiency panels organized by the Immunoguiding Program of the Association for Cancer Immunotherapy (CIP) will be given.

User vs. software-dependent variability of ELISPOT counts obtained from ten different laboratories

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IntroductIon: In each human donor's PBMC, there is a defined number of T cells specific for any given antigen. A major goal of immune monitoring with ELISPOT is to measure this number accurately and reproducibly between different laboratories. In ELISPOT assays, cytokine spots produced by antigen-specific T cells show a broad spectrum of sizes and densities over variable background. Therefore, even experienced investigators are likely to come up with different spot counts when subjectively judging the minimal spot size/density to be counted and the maximal spot size for the cut off between single cell-derived spots vs. those created by cell clusters. This study aims to find out whether statistics-based automated gating can harmonize spot counts obtained in different laboratories.

Methods: We studied PBMC plated in serial dilutions, with 24 replicates per dilution, to establish the distributional properties of HCMV pp65-induced IFN-γ ELISPOTs. We sent the physical ELISPOT plate and image files obtained from it to ten different laboratories for independent counting. The plate was machine counted by each laboratory relying on either Basic Count, which relies on subjective counting parameters set by the different investigators or SmartCount™, an automated counting method embedded in CTL's ImmunoSpot® Software that uses statistics-based autogating in conjunction with autothresholding.

Results: The IFN-γ spots were found to closely follow a Log Normal distribution. When spot counts were established by subjective judgment, Basic Count, the average coefficient of variation (CV) between the mean values for the independent laboratories was 26.7%. Counting with the SmartCount™ method produced counts with an average CV of 6.7% across the laboratories.

Conclusions: The Log Normal distributional properties of ELISPOTs permits one to automatically set the lower and upper gates for counting spots by means of statistics, achieving a target significance of 95%. Using SmartCount™, which relies on statistics-based autogating in conjunction with autothresholding, spot counts can be harmonized between different investigators and laboratories.

Skewed distribution of IL-7 receptor-α-expressing effector memory CD8(+) T Cells with distinct functional characteristics in oral squamous cell carcinoma

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CD8(+) T cells play important roles in anti-tumor immunity but distribution profile or functional characteristics of effector memory subsets during tumor progression are unclear. We found that, in oral squamous carcinoma patients, circulating CD8(+) T cell pools skewed toward effector memory subsets with the distribution frequency of CCR7(-)CD45RA(-)CD8(+) T cells and CCR7(-)CD45RA(+)CD8(+) T cells negatively correlated with each other. A significantly higher frequency of CD127(lo) CCR7(-)CD45RA(-)CD8(+) T cells or CCR7(-)CD45RA(+)CD8(+) T cells among total CD8(+) T cells was found in peripheral blood or tumor infiltrating lymphocytes, but not in regional lymph nodes. The CD127(hi) CCR7(-)CD45RA(-)CD8(+) T cells or CCR7(-)CD45RA(+)CD8(+) T cells maintained significantly higher IFN-γ, IL-2 productivity and ex vivo proliferative capacity, while the CD127(lo) CCR7(-)CD45RA(-)CD8(+) T cells or CCR7(-)CD45RA(+)CD8(+) T cells exhibited higher granzyme B productivity and susceptibility to activation induced cell death. A higher ratio of CCR7(-)CD45RA(+)CD8(+) T cells to CCR7(-)CD45RA(-)CD8(+) T cells was associated with advanced cancer staging and poor differentiation of tumor cells. Therefore, the CD127(lo) CCR7(-)CD45RA(-)CD8(+) T cells and CCR7(-)CD45RA(+)CD8(+) T cells are functionally similar CD8(+) T cell subsets which exhibit late differentiated effector phenotypes and the shift of peripheral CD8(+) effector memory balance toward CCR7(-)CD45RA(+)CD8(+) T cells is associated with OSCC progression.

Validation of immunomonitoring of circulating myeloid-derived suppressor cells as a potential predictive tool for response to Ipilimumab treatment in melanoma patients

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Background: Immune checkpoint inhibitors are a breakthrough new therapy for cancer and Ipilimumab, an anti-cytotoxic T lymphocyte-associated antigen-4 antibody, is becoming the leading treatment for the management of metastatic melanoma. The peculiar pattern of response to this antibody fostered the definition of new criteria for response assessment but, there is still an urgent need for reliable predictive biomarkers in order to identify responder patients. To this aim, immuno-monitoring of cancer patients revealed several possible candidate biomarkers that were predictive of better prognosis. Aim of this work was to monitor circulating levels of myeloid-derived suppressor cells (MDSCs) in metastatic melanoma patients receiving Ipilimumab treatment through a standardized flow cytometry approach.

Methods: We standardized flow cytometry analysis by monitoring cytometer and antibody performance by means of several quality controls based on fluorescent particles and reference cell-line staining. Taking advantage of this highly standardized method, we monitored the circulating levels of different subsets of MDSCs at baseline and at different time points after Ipilimumab treatment in 24 patients with metastatic melanoma.

Results: Melanoma patients had significantly higher levels of circulating $CD14^+/IL4R\alpha^+$ and $CD15^+/IL4R\alpha^+$ MDSCs and elevated frequencies of $CD14^+/HLA\text{-}DR^{low/-}$ MDSCs as compared to age- and gender-matched healthy donors. In addition, the percentages of MDSCs were significantly associated with serum levels increase in proteins involved in the induction and activation of these cells, such as VEGF, S100, and IL-6. Patients with higher MDSC frequencies presented also higher reactive C-protein levels and hence a greater cancer-related inflammatory state. We are currently monitoring MDSC levels in Ipilimumab-treated patients during the follow-up phase in order to identify a possible correlation between MDSC frequencies and clinical outcomes.

Conclusion: Our results indicate that MDSC levels were increased, when compared to healthy donors, in a cohort of metastatic melanoma patients and the levels of these cells correlated also with soluble factors known to sustain MDSC accumulation. In addition, we are investigating the possible predictive role of MDSC frequencies in melanoma patients during the follow-up phase of Ipilimumab treatment. In conclusion, our results indicate that standardization of sample acquisition and analysis by flow cytometry is a feasible method for assessing MDSC levels in novel immunotherapy approaches.

A xenograft model for studies of anti-leukemic effects of histamine dihydrochloride in acute myeloid leukemia

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Acute Myeloid Leukemia (AML) is characterized by expansion and accumulation of immature myeloid cells in the blood and bone marrow. Chemotherapy can temporarily hinder the disease, but relapses are common and the prospect of long-term survival for AML-patients is poor. Our research group has developed a relapse-preventing immunotherapy in AML - histamine dihydrochloride (HDC) in combination with IL-2 - which in 2008 was approved within the European Union.

The proposed mechanism of action for HDC is to prevent the production of oxygen radicals from myeloid cells, and thereby rescue anti-leukemic NK and T cells from radical-induced inactivation. AML is a genetically and morphologically heterogeneous disease and we recently discovered that leukemic cells from patients with monocyte-like leukemia produce immunosuppressive oxygen radicals, which could be inhibited by HDC, while leukemic cells from patients with non-monocytic leukemia did not produce oxygen radicals. In analogy with these results, post-hoc analysis of a clinical HDC/IL-2 trial showed that HDC/IL-2 was most efficacious in AML patients with monocytic leukemia.

The mechanism of action for HDC may, however, be pleiotropic. For example, histamine has also been proposed to exert pro-differentiating effects on the myeloid compartment. To further define the mechanism of action of HDC, and to verify its superior efficacy in monocytic AML, we are developing xenograft models where primary leukemic cells from AML patients are injected *i.v.* into sublethally irradiated immunodeficient NOD/SCID or NOD/SCID-IL2RG mice. This approach allows human AML cells to repopulate the murine immune system, which thereby enables *in vivo* analyses of human leukemic cells along with the assessment of anti-leukemic therapeutics.

In addition to *in vivo* injections, the patients AML cells have been cultured on methylcellulose in the presence or absence of HDC and CFU has been enumerated. For certain patients with monocytic AML, HDC has shown to clearly inhibit the CFU-forming capacity of the leukemic blasts.

In initial *in vivo* experiments using NOD/SCID mice we observed a transient engraftment of leukemic cells (i.e. 1-6% in the blood stream, four weeks after injection), whereafter the human cells were rejected. In later experiments, NOD/SCID-IL2RG (NOG) mice that have a lower capacity to reject grafts have been utilized, which has resulted in positive engraftments in more than two thirds of the animals.

In planned experiments, xenografted animals will be treated with *i.p.* injections of HDC and the leukemic cell burden will be followed using blood draws. In some animals we also plan to inject human NK cells prior to HDC treatment.

Resetting circulating CD8 T-cells to a tissue-like status permits sensitive detection of their responses to virus- and tumor-associated antigens

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ORAL SHORT TALK

99% of our body´s T-cells actively scan the surfaces of other cells in the tissues, a process which primes them for cognate antigen recognition. In contrast, less than 1% of body´s T-lymphocytes are in the circulation as suspended cells lacking such cell contacts. Accordingly, recent studies in mice and humans have shown that circulating CD4 T-cells are less sensitive to several T-cell activating mAb and to recall antigens as compared to tissue-resident cells. For practical and ethical reasons, however, immunological research and immunomonitoring in humans is almost exclusively performed using PBMC, i.e. functionally impaired T-cells. We have recently shown for CD4 T-cells that the impaired responsiveness of circulating T-cells could be restored by a short-term preculture step of PBMC at a high cell density which resets them to a tissue-like status (HD preculture, Römer P.S. et al. 2011).

We now addressed the question whether HD preculture of human PBMC would also allow more sensitive detection of CD8 T-cell responses to viruses and tumor-associated antigens, resulting in an improvement in immunomonitoring and the generation of CD8 T-cell lines and clones for cellular immunotherapy. By using interferon-gamma ELISPOT assays as a read out, we show that PBMC from leukemia patients who were treated by myeloablation and allogeneic HSCT respond with greatly increased sensitivity to Wilms tumor 1 (WT-1) derived peptides if the HD preculture method, also referred to as RESTORE protocol, is applied prior to antigen-specific stimulation. The simple but effective method increases the detection limit of anti-tumor CD8 T-cell responses that would otherwise go undetected and allows the generation of CD8 T-cell lines with an improved representation of clones responding to low antigen concentrations. Of note, HD preculture of PBMC does not change their cellular composition, nor does it increase background responses.

Besides tumor-specific responses, the sensitivity of T-cell responses to HCMV, EBV or Influenza is also enhanced by HD preculture of human PBMC. Direct comparisons of virus-specific CD8 memory T-cells from tissue-resident tonsillar cells, fresh and HD precultured PBMC of the same individual have shown that high anti-viral sensitivity is dependent on previous cellular interactions that occur in lymphoid tissues and during the HD preculture step, but not in the circulation, where T-cells lose integrin-mediated cell contact and “tonic” TCR signals and hence lower the activation threshold for a full response.

In conclusion, resetting circulating CD8 T-cells to a tissue-like status permits sensitive detection of their responses to virus- and tumor-associated antigens without prior in-vitro expansion of antigen-specific CD8 T-cell clones. Hence, HD preculture of PBMC better reflects in-vivo situations relative to conventional PBMC-based assay.

A bioinformatics platform for prediction and reporting of drug sensitivity from sample specific genomic data

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Genomic and bioinformatics platforms are providing copious amounts of molecular tumor data. To make the information actionable for clinicians, the impactful amount from these databases has to be extracted and presented clearly and concisely. Relevant information includes not only mutations and expression, but also therapeutic decision making, specifically connecting cancer mutations to targeted therapies. The goal of this project is to generate a patient report that predicts tumor drug sensitivities based on mutations and over-expressed genes. As a proof of concept, we built a platform that generates reports predicting drug sensitivities for cancer cell lines. Rules-sets, describing under which conditions these drugs are effective, were defined based on current publications in oncology. An algorithm to predict drug sensitivity based on these rules has been created and the predictions are presented in a well-structured web interface. The results show that a drug sensitivity prediction based on genomic cell line data is possible. Additionally, the framework is transferable to patient studies due to data resemblance.

MHC Multimer Proficiency Panel 2014: Consistent data obtained by the large majority of participants

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Starting last year, Immudex has taken over the MHC Multimer and Elispot Proficiency Panels, previously held by the CIC and CIMT.

Here, we report the results of the MHC Multimer Proficiency Panel. From the results obtained it is clear that the MHC Multimer assay is now a routine, reliable and accurate assay for the detection of antigen-specific T cells.

Each of the 50 worldwide participants received cell samples, together representing low, medium and high responses for two predefined MHC multimer specificities.

Participants were asked to determine the number of $CD8^+$, antigen-specific T cells corresponding to each of the two multimer specificities. Any MHC Multimer could be used; Dextramers were offered free of charge. 49 participants used MHC Dextramers, and one participant used Tetramers.

It was recommended to use additional antibody marker(s) for exclusion or inclusion of specific cell populations (e.g. anti-CD4 antibody, anti-CD3 antibody), and dead-cell dyes during data analysis.

After performing the MHC Multimer assay, the participants reported back their results, as "number of MHC multimer-specific, $CD8^+$ T cells". The participants performed well - even for the low responders the majority of participants got very consistent data.

The data set as a whole will now be analyzed, and each participating laboratory will receive a report detailing the individual laboratory's performance (in an anonymized format). The anonymized report will become publically available.

Thus, thanks to the harmonization efforts by the CIC and CIMT over many years, and possibly also because of the development of better reagents and protocols, the MHC Multimer assay now provides a simple and reliable means of accurate enumeration of antigen-specific T cells in blood.

Reversible NTAmer technology enables rapid and direct detection of monomeric TCR-pMHC kinetics at the cell surface and predicts tumor-specific T cell responsiveness

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ORAL SHORT TALK

Adoptive cell transfer has become a clinically successful therapy for cancer patients. However, large-scale screening methods for the rapid detection and isolation of T cells with high tumor killing potential are still needed to improve immune reactivity toward tumor-associated antigens. TCR affinity/avidity and binding kinetics for peptide-MHC (pMHC) represent major determinants of protective T cell immune responses. TCR-pMHC kinetics are typically assessed by surface plasmon resonance (SPR), but it requires the generation of soluble TCRs while underestimating the TCR-pMHC avidity effects associated with co-receptor binding and cell membrane complexity. We recently developed a two-color reversible pMHC multimer (NTAmer) approach allowing rapid flow cytometry-based visualization of monomeric TCR-pMHC dissociation kinetics (k_{off} rates) directly at the surface of living CD8 T cells. Due to the high stability and immediate reversibility of NTAmers, k_{off} rates can accurately be determined, providing the fine characterization of weak TCR-pMHC interaction kinetics, such as those found within the tumor-specific CD8 T cell repertoire.

We took advantage of a panel of A2-NY-ESO-$1_{157-164}$-specific CD8 T cells that express TCRs of incremental affinity for pMHC to validate the NTAmer technology, and found robust correlations between cell surface dissociation rates and soluble TCR-pMHC k_{off} rates measured by SPR. Using NTAmer variants containing mutations that abrogate CD8 binding to MHC, we next show that CD8 increases the dissociation half-lives ($t_{1/2}$) by a factor of 3 to 4, independently of TCR affinity. Using tumor-specific T cells expressing TCRs of very high affinities ($K_D \leq 0.1\mu M$), we were also able to precisely assess cell surface monomeric TCR-pMHC association rates (k_{on}). Our analysis reveals that CD8 attachment had only minimal impact on TCR-pMHC association kinetics, supporting the sequential model for T cell activation, in which the TCR initially binds to pMHC in a CD8-independent manner, followed by a CD8-dependent stabilization of the ternary complex. Importantly, we further demonstrate statistically significant correlations between NTAmer-based dissociation rates and the biological responses of T cells, such as calcium mobilization. Finally, we characterized a large set of NY-ESO-$1_{157-164}$-specific T cell clonotypes derived from a melanoma patient with naturally occurring anti-tumor CD8 T cell responses. The dissociation rates of multiple T cell clonotypes clustered within a narrow physiological range, yet strongly correlated with tumor cell recognition and killing, with TCR clonotypes of extended pMHC binding $t_{1/2}$ showing enhanced T cell responsiveness. In summary, the NTAmer technology enables efficient and direct interrogation of surface-based TCR-pMHC dissociation kinetics and opens novel perspectives for the rapid screening and isolation of functionally relevant CD8 T cells for adoptive cell transfer.

Expression of carbohydrate deficient bone sialoprotein compared to various tumor markers in metastatic cells and non-metastatic cells in vitro

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The expression of bone sialoprotein (BSP) has been demonstrated in breast cancer bone metastasis by many authors. Bäuerle et al. (2006) found that an anti-BSP antibody decreased proliferation, colony formation and migration of MDA-MB-231cells in vitro and reduced osteolysis besides inducing bone formation in a nude rat model. Later, it was revealed that tumor BSP is a carbodeficient BSP (CD-BSP) with threonine modified by O-glycans, leaving the C terminus of the protein free of glycans. Those results led to the question whether the formation of metastases is correlated to BSP or CD-BSP expression. To this aim, we investigated the expression of both glycoproteins and compared those to the expression of known binding partners of BSP such as matrix metalloproteinases 2 and 9, the integrin avß3, and the complement factor H which inhibits the complement-mediated cell-lysis.Osteonectin, osteopontin and the lectin Wisteria floribunda agglutinine (WFA) are other markers which are involved in the development of bone metastases.

We used the human breast cancer cell line MDA-MB-231 (metastatic), the human prostata cancer cell line PC-3 (metastatic), the human non-metastatic breast cancer cell line MCF-7, and the human immortalized cell line MCF-10 as control for the in-vitro-investigation.

In result, CD-BSP could be found in large metastatic cells. Small metastatic cells did not express CD-BSP, medium-sized cells showed various patterns of both CD-BSP and BSP expression. In large cells, the CD-BSP expression is always accompanied by BSP, mostly extracellularly distributed. In special cases, we found CD-BSP distribution in the cell nucleus as verified by 3D-confocal laser scanning microscopy (CLSM). The non-metastatic cells did not express CD-BSP. To gain further insight into the possible mechanism of action, we investigated the expression and cellular distribution of BSP and CD-BSP and compared those to WFA-binding sites. While hypo-glycosylated CD-BSP showed intracellular localization, the fully glycosylated BSP was located extracellularly. WFA- binding sites were found intra- and extracellularly, and WFA-staining was especially intensive in the metastatic cell line PC-3 and MDA-MB-231. Those findings support the hypothesis that altered protein glycosylation could affect the intercellular communication and may promote detachment of tumor cells leading to increased metastatic potential. CD-BSP might be a major player in this mechansm.

Accurate detection of NKT cells using CD1d Dextramers

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Natural killer T (NKT) cells are a subset of lymphocytes that can rapidly respond to the presence of tumor cells and participate in antitumor immune responses and surveillance. This has prompted interest in the development of innovative cancer therapies that are based on the manipulation of NKT cells. NKT cells act as potent activators of antitumor immunity when stimulated with the synthetic agonist α-galactosylceramide (α-GalCer) in experimental models. However, in some settings, NKT cells seem to act as suppressors and regulators of antitumor immunity. To date, the most well-characterized glycolipid ligand recognized by NKT cells is α-GalCer discovered initially in marine sponges (Agelas) from a screen of natural products with anticancer properties. α-GalCer has a strong affinity for CD1d molecules in both humans and mice. Recognition of CD1d-bound α-GalCer elicits a strong cytokine response by NKT cells. Due to this strong agonist activity, various chemical analogs of α-GalCer have been developed and used extensively to study the function of NKT cells. These approaches have also led to the use α-GalCer and its analogs as vaccine adjuvants, an approach that is aimed at boosting specific B and T cell responses to a vaccine candidate by concomitant activation of NKT cells in anti-tumor immunotherapy. We have developed a novel reagent, the CD1d Dextramer, that accurately and with high sensitivity directly detects NKT cells in blood samples using flow cytometry.

The CD1d Dextramer can be used to characterize phenotype and function of NKT cells, to better understand the effector functions, migratory patterns and survival properties of NKT cells in humans. We will demonstrate how various NKT cell subsets can be identified using this reagent. Increased understanding of the effector function of specific subsets of NKT cells in cancer will be essential to improve current strategies for NKT-cell-based immunotherapeutic approaches.

Presence of circulating Her-2-reactive CD8 T-cells is associated with lower frequency of MDSCs and better survival in elderly breast cancer patients.

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ORAL SHORT TALK

Breast cancer is one of the most common cancers among women. The risk of breast cancer has increased dramatically recently and larger numbers of elderly women are being diagnosed with the disease. Myeloid-derived suppressor cells (MDSCs) have been implicated in breast cancer prognosis. Their frequency has been associated with tumour burden and studies have shown a poor prognosis in metastatic breast cancer. MDSCs are known to impair the proliferation of T-cells and to promote T-cell apoptosis.The association between MDSC levels and immune responses of the host to tumour-associated antigens has not been investigated.

Here, we have studied peripheral T-cell responses to overlapping 15-mer peptides covering the entire Her-2 molecule, as well as the frequency of Tregs and MDSCs in blood of 78 breast cancer patients (aged 28-87years) at diagnosis prior to any treatment. After a 12-day in vitro expansion of memory cells and intracytoplasmic staining of TNF, IFN-γ, IL-2, IL-5, IL-10 and IL-17 in single cells by polychromatic flow cytometry, we found that CD4 T-cells reactive to Her-2 were detectable in 88% of the patients. In contrast, only 51% of patients had Her-2-reactive CD8 T-cells. The patients who lacked a CD8 response had significantly higher frequencies of Lineage(neg)CD14(pos)HLA-DR(neg) cells, defining a subset of MDSC ($p=0.002$). Importantly, the 4-year survival rate for patients who mounted a CD8 response and had lower frequencies of this particular subset of MDSC was 95% compared to only 75% of patients without Her-2-reactive CD8 T-cells and with higher frequency of MDSCs ($p=0.1$). This tendential survival advantage was more striking and highly significant in older patients (>65 years), where only 46% of those without a CD8 response to Her-2 and having high levels of MDSCs survived for 4 years. Strikingly, 100% of older patients who had a CD8 response to Her 2 and a lower frequency of MDSCs survived over this 4 year period ($p=0.016$). In marked contrast to the impact of MDSC, the frequency of Tregs did not correlate with patient CD8 responses or with survival.

These data emphasize the impact of MDSCs on survival of early-stage breast cancer patients, most likely by dampening favorable immune responses to tumor-associated antigens, and of over-riding importance for the survival of elderly patients.

Phenotyping of peripheral blood mononuclear cells of patients with advanced heavily pre-treated adenocarcinoma of the stomach and gastro-esophageal junction

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Immunotherapeutic approaches are emerging as promising new treatment options for patients with solid cancers. The host immune system in cancer patients is known to be compromised due to a number of reasons. The degree of immunosuppression in a patient varies at the time of diagnosis and depends on the particular type of cancer, stage, and prior anti-cancer therapies. For many tumor types, the immune alterations of the respective patient population are not further characterized even though the patient`s immunophenotype may be prognostic for the course of the disease or predictive with regard to benefit from specific types of treatments, including immunotherapy.

Therefore, we systematically monitored the immune status of 30 patients with heavily pretreated, advanced adenocarcinoma of the stomach and gastro-esophageal junction and 8 healthy donors as comparators by means of frequency, phenotype, and activation status of several immune-cell subsets. We have used quality-controlled, multi-colored flow-cytometry panels for the identification and characterization of the following immune cell subsets: B cells, T cells and regulatory T cells (Treg), natural killer (NK) cells, Vδ2 T cells, as well as several myeloid-derived suppressor cell (MDSCs) subsets.

We simultaneously performed a highly sensitive and robust monitoring of 24 different immune-cell subsets with state-of-art flow cytometry. We observed differences in frequency, phenotype, or activation status of major immune-cell subsets such as T cells, B cells, and NK cells, but also in rare subsets, that have so far not been studied extensively, such as Vδ2 T cells or MDSC subsets. The median frequency of B cells was decreased and NK-cell frequency was increased in patients compared to healthy donors. Regarding γδ T cells, the frequency of activated $CD25^{+}$ Vδ2 T cells was decreased in patients compared to healthy donors. On the other hand the frequency of activated Tregs ($CD4^{+}Foxp3^{hi}CD25^{hi}CD127^{lo}CD45RA^{-}$) was increased, whereas the frequency of resting Tregs ($CD4^{+}Foxp3^{lo}CD25^{hi}CD127^{lo}CD45RA^{+}$) was decreased in patients versus healthy donors. In addition, the frequency of several MDSC subsets was significantly increased in patients versus healthy donors.

In conclusion, our study provides comprehensive data on the immune status of patients with advanced adenocarcinoma of the stomach and gastro-esophageal junction compared to healthy donors. This knowledge may help to identify new prognostic markers and/or markers predictive of clinical responses of cancer patients to immunotherapies.

High numbers of differentiated effector CD4 T cells are found in cancer patients and correlate with clinical response after neo-adjuvant therapy of breast cancer

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ORAL SHORT TALK

The role of CD4$^+$ T-cells in anti tumor immune responses is poorly known, especially in humans. Herein, we characterized a population of differentiated effector CD4$^+$ T cells defined by low levels of the IL-2 and IL-7 receptors (CD25$^-$CD127$^-$) that expands in several types of cancers to represent 2-20% of total CD4$^+$ blood T lymphocytes. Contrary to the similar minor (0.2-2%) subset found in healthy donors, these cells bear effector markers such as CD244 and CD11b and low levels of CD27. However, these cells do not cycle (Ki67^{neg}) nor secrete IL-10 or IL-17 but display cytotoxic features. This subset encompasses oligoclonal expansions and its increase parallels the expansion of effector CD8$^+$ T cells that include tumor antigen-specific T cells. During neo-adjuvant chemotherapy in breast cancer patients, the increase in CD127$^-$CD25$^-$CD4$^+$ T cells correlated with tumor regression, suggesting that CD4$^+$ T cells may include tumor antigen specific cells, which could be generated by or participate in tumor regression during chemotherapy. Altogether, these data support the hypothesis that CD4$^+$ T cells are involved in anti-tumor response in humans. Moreover, the number and the characteristics of these effector CD4 T cells in the blood might be predictive bio-markers for prognosis and staging before chemotherapy.

Results of a multi-peptide vaccination trial for high risk renal cell carcinoma

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Limited treatment options are available for patients with advanced non metastatic renal cell carcinoma (RCC). Specific immunotherapy could be a promising approach, as depicted in this study. In this adjuvant phase I/II clinical trial, HLA-A*02 positive patients with advanced or metastatic RCC after complete resection were randomized to receive a multi-peptide cocktail either subcutaneously in Montanide ISA51 or intradermally together with GM-CSF. The vaccine was applied at 18 vaccination time points or until progression, and consists of four HLA-class II and ten HLA-A*02 binding peptides. These peptides represent T-cell epitopes or HLA-ligands derived from renal tumor-associated antigens. In addition, two viral-derived epitopes were included as recall and priming controls.

Progression-free survival was assessed by computed tomography every three months. Peripheral blood mononuclear cells were isolated at individual vaccination time points and frozen until the test. Vaccine-specific T-cell responses were measured *in vitro* by IFN-gamma Elispot, HLA-multimer staining and intracellular cytokine staining.

Results show that multi-peptide vaccination of patients with low tumor burden in stage III RCC is feasible and safe. Furthermore, immunomonitoring data indicate the activation of vaccine-specific $CD4^+$ and $CD8^+$ T cells. In more than 75% of analysed patients, $CD4^+$ T cells specific for 1-3 HLA-class II binding epitopes were detectable. These $CD4^+$ effector cells were shown to secrete several cytokines (IFN-gamma, TNF-alpha and IL-2). In all tested patients, we observed vaccine-induced specific $CD8^+$ T cells recognizing the virus-derived priming peptide. Tumor-associated peptides were also found to be immunogenic, albeit to a weaker extend. In 40% of the patients, $CD8^+$ T-cell responses were induced against up to three peptides.

At the last interim analysis, cancer-specific survival (CSS) in case of advanced RCC was 49.5±8.6 months, two patients progressed. In metastatic RCC after R0 resection, CSS was 49.2±6.3 months, and six patients progressed (time to progression: 10.5±2.9 months).

CD14+CD11b+HLA-DR-/low MDSCs indirectly impact on the prognosis of late-stage melanoma patients by impairing the frequency and function of NY-ESO-1- and Melan-A-reactive peripheral T cells

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The incidence of malignant melanoma is steadily increasing. While melanoma patients diagnosed at early stages of disease have a high survival probability, prognosis dramatically worsens with disease progression. The median survival of stage IV melanoma patients with unresectable distant metastasis is 9 months. Despite this, a small proportion of patients still does survive long-term, but the factors which are responsible for this survival advantage remain so far largely unknown. Here, we report the identification of peripheral immune signatures associating with longer survival and provide insight into the mechanistic basis predictive of long-term survival in melanoma patients.

This study investigated peripheral blood mononuclear cells (PBMCs) from 133 late-stage melanoma patients with distant metastasis and focused on the potential role of suppressive immune cell populations, i.e. myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) in tumor immunity. Percentages of circulating MDSCs of the CD14+CD11b+HLA-DR-/low phenotype inversely correlated with survival of advanced melanoma patients, while no association was found for CD4+CD25+FoxP3+ Tregs. We have previously reported that the presence of peripheral T cells capable of recognizing the tumor antigens NY-ESO-1 and/or Melan-A, *in vitro* is predictive of clinical outcome in late-stage melanoma. High *ex vivo* MDSC levels were found to be associated with the absence of melanoma antigen-specific T cells *in vitro*, implying a causal relationship. Whether the detection of functional antigen-specific T cells *in vitro* reflects the level of antigen-specific T cells *in-vivo* or is mainly driven by the amount of MDSCs during the *in-vitro* memory cell re-stimulation in our assay, remains to be determined.

Conclusions: MDSCs are likely to represent prognostic markers as well as therapeutic targets in advanced-stage melanoma patients. Inhibition of MDSCs should result in improved survival mediated by T cell responses to NY-ESO-1 and Melan-A or other target antigens released from suppression.

The effect of glucocorticoids on the chromatin landscape

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Emotional distress is a common response to a diagnosis of cancer. This distress activates the hypothalamic pituitary adrenal axis resulting in excessive glucocorticoid production. High levels of circulating glucocorticoids are well known to disrupt immune function. We have modeled the impact of glucocorticoids on the immune system *in vitro* by treatment of the NK cell line, NK92, with dexamethasone. Such treatment reduces natural killer cell activity of the cell line by reduction of perforin and granzyme B levels as well as the reduction of both the constitutive and stimulated production of IFN gamma, IL-6 and TNF alpha. These effects were a consequence of epigenetic modifications at the regulatory regions of each of these genes. The purpose of this investigation was to extend these observations to an analysis of the effect of this glucocorticoid on the chromatin landscape by assessment of the intensity and localization of nuclear epigenetic marks and architectural proteins. Immunofluorecent detection increased for the transcriptionally repressive epigenetic marks H3K9me3 and H3K27me3 (as judged by global mean fluorescent intensity, MFI) as well as nuclear localization, with significant accumulation at the nuclear periphery. In contrast, the transcriptionally active epigenetic mark H4K8ac was unaffected by glucocorticoid and exhibited diffuse nuclear staining. The effect of glucocorticoid on three nuclear architectural proteins; CTCF, Mediator, and Cohesion was also assessed. Glucocorticoid increased global MFI and signal aggregation of Med1 (mediator) and SMC-1 (cohesion) with no effect on the CTCF. Med1 localized to the nuclear periphery with SMC-1 localized to the nuclear center. No change in the localization of CTCF was observed. These results demonstrate the impact of glucocorticoids on the chromatin landscape and suggest the effects of glucocorticoids on immune function to be in part mediated by not only changes in the detection of epigenetic marks and architectural proteins but also their sub-nuclear localization. Detection of such effects in the peripheral blood of cancer patients may not only provide a means by which to dissect the effect of emotional distress upon the immune system but may serve to identify those cancer patients at immunologic risk.

Immunomodulation induced by Stereotactic Ablative Radiotherapy (SABR) in oligometastatic breast cancer patients as a source of predictive biomarkers

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Question: In oligometastatic Breast Cancer (BC) patients, the use of Stereotactic Ablative Radiotherapy (SABR) favours the local control of the treated lesions minimizing normal tissue damage. SABR may contribute to break local tolerance and release tumor-associated antigens (TAAs), improving host anti-tumor immune responses. A careful immunomonitoring of BC patients undergoing SABR may allow the identification of predictive/prognostic biomarkers, particularly for patients concomitantly treated with drugs acting through immune-mediated mechanisms, as Trastuzumab.

Methods: This phase II study enrols oligometastatic BC patients with ≤ 6 metastatic lesions diagnosed by FDG-PET/CT, controlled loco-regional disease, and no documented brain metastases. SABR consists of 30 Gy in 3 consecutive fractions. Immune profiling included the analysis of serum cytokine levels (IL-1β, IL-6, IL-8, IL-10, TNF-α) by ELISA, the characterization of circulating immune populations (T and B lymphocytes, NK cells, myeloid-derived suppressor cells, regulatory T cells), and the analysis of polyfunctional T-cell responses against known BC TAAs (survivin, mammaglobin-A, Her2) by flow cytometry. The efficacy of patients' NK cells in mediating Trastuzumab-dependent ADCC was evaluated by an in vitro assay. Analyses were carried out before SABR, 24h, 1 and 4 months after SABR.

Results: Ten patients resulted evaluable; 6 had luminal A, 2 luminal B, 1 Her2+ luminal B, 1 Her2+ non luminal BC. Three patients presented 1 single metastasis. The site of metastasis was bone only in 8 cases, bone and axillary lymph node in 1 case, and lung in 1 case. Six patients received concomitant hormonal therapy, 1 patient Trastuzumab, 1 Trastuzumab+steroids, and 1 chemotherapy only. With a median follow-up of 6 months, tumor control of the treated lesions was documented in all patients.

At diagnosis, patients disclosed higher levels of IL-6 ($p < 0.01$) and lower amounts of IL-8 ($p < 0.01$) in comparison to healthy women ($n = 5$). One month after SABR, IL-6 levels were still significantly higher ($p < 0.01$), while IL-8 levels were similar to those of controls. SABR treatment seemed to induce a decrease of circulating B cells ($p < 0.05$), and an increase ($p = 0.01$) in CD56bright NK cells at a follow-up of 1 month. In patients treated with Trastuzumab ($n = 2$), in vitro ADCC efficacy increased 24h after treatment. In comparison to diagnosis levels, a higher number of HLA-A*02 and B*35-restricted survivin-specific T cells was noticed in 3/4 patients 1 month after SABR. Two/5 patients disclosed the appearance of polyfunctional T cells against HLA-A*02- and A*24-restricted Her2-derived epitopes 1 month and 4 months after SABR.

Conclusions: These preliminary data suggest that SABR may improve host anti-tumor immune responses, possibly contributing to the induction of clinical responses to treatment, both locally and systemically. The possible predictive value of immune biomarkers will be assessed after completion of the study.

ZO-1 as a potential prognostic marker in B cell lymphoma

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We have shown previously that lymphocytes can form homocellular gap junctions, but consequences of these contacts still stays unclear. One of the intracellular proteins associated with GJs is ZO-1, which is responsible for cell cycle regulation. There were no published data on use of ZO-1 expression for the prognosis of lymphoma/leukemia. The aim of this study was to determine ZO-1 expression in B CLL cells compared to healthy donor B cells.

Methods: 113 periferal blood samples from B CLL patients and 50 blood samples from healthy donors were examined. Lymphocytes were isolated on percoll gradient following PBS washing. Following diagnostic flow cytometry panel was used: CD3/CD5/CD10/CD13/CD19/CD20/CD22/CD23/CD25/CD33/CD34/CD38/CD79/CD103/CD138/cytoKappa/CytoLambda/ZAP70/FMC-7/Cx43 (Beckman Coulter, R&D). We used Beckman Coulter FC500 for flow cytometry. B CLL was confirmed by prevalence of cells with the following phenotype: CD19+/CD20+dim/CD22+dim/CD23+/CD5+/CD10-. For the further analysis we isloated CD19 expressing cells, using MagCellect Human B Cell Isolation Kit (R&D Systems). Cells were incubated in 24 well plates, 5% CO_2, 37°C, in RPMI1640 medium. 1-octanol and carbenoxolone were used as GJ inhibitors. Apoptosis was evaluated using Annexin V-FITC Apoptosis Detection Kit (Sigma). Cells were lysed in a concentration of 5×10^7 using 150mM NaCl, 2mM MgCl2, 0.5mM CaCl2, 1% Triton X, 0.1% SDS, 50mM TRIS pH 7.4, 1mM EDTA and CompleteTM protease inhibitor cocktail (Roche Diagnostics) on ice during 20 minutes, than centrifuged for 1 min at 15000 rpm. ZO-1 expression was assesed by western blotting assay using primary antibodies: Anti-ZO1 tight junction protein antibody [mAbcam 61357] (abcam). Cx43 expression was assayed using Human Connexin 43/GJA1 Allophycocyanin MAb (Clone 578618), Mouse IgG2A (R&D). Detection was performed using Immun-Blot Goat Anti-Mouse IgG (H + L)-AP Assay Kit (Bio Rad). Membrane was scanned and image was analysed in ImageJ software.

Qantitative data are shown as Mean ± SD, data compared using Student T test. Qualitative data shown as absolute frequencies, compared using Pearson Chi square test. A p-value of < 0.05 is considered statistically significant. Data were analysed using SPSS 12.0 software.

Results:

1. ZO-1 and Cx43 expressed at significantly lower levels in B CLL than in healthy B cells.
2. ZO-1 expression levels negatively correlated with expression of CD38 and Zap70 as assessed by flow cytometry. ZO-1 expression was reduced only in 5 CD38-/Zap70- patient samples (which constituted 54% of all patient samples), but in 13 C D38+/Zap70+ (28%) samples. At the same time, 80% normal lymphocytes expressed ZO-1.
3. B cell apoptosis was reduced when incubated with anti-Cx43 antibodies. Apoptosis was less reduced in lymphoma cells, and correlated with ZO-1 levels. On addition of 1-octanol or carbenoxolone, apoptosis was reduced even more. Our data suggest that ZO-1 expression may negatively correlate with lymphoma prognosis.

Belatacept can inhibit allo-specific T cell responses in a donor-dependent manner but preserves virus-specific memory T cell-responses

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Purpose: Belatacept (Bela) blocks T cell activation via inhibition of the interaction between CD28 and B7.1/2. As it acts specifically, side effects in comparison to calcineurin inhibitors (CNI) are minimal but it is known that patients under Bela medication have increased risk of early rejections after kidney transplantation (KTx). Reactivation with Ebstein-Barr virus (EBV) in kidney recipients represents a huge problem. EBV-vaccination would be a solution, but there is no vaccine available. However, EBV-derived virus like particles (VLPs) could be a feasible strategy. Little is known about the effect of Bela on virus-specific T cell activation and if it is possible to induce a virus specific immune reaction with EBV-VLPs.

Methods: IFNg-ELISpots with PBMCs of healthy donors (n=5) or KTx patients (n=5) in the presence of Bela, CTLA-4-Ig or CNI were performed. PBMCs were stimulated with a pool of CMV, EBV, Flu peptides (CEF), an allogeneic B cell line, EBV-specific VLPs or PHA as positive control. In parallel, supernatants were collected and cytokines were measured via multiplex analysis. Binding of Bela to B7.1/2$^+$ cells was compared to CTLA-4-Ig by flow cytometry.

Results: Bela was not able to reduce the CEF-peptide-specific IFNg production in contrast to CNI. Allo-specific IFNg responses were reduced by Bela and CTLA-4-Ig in some donors (healthy controls and KTx patients). Interestingly, the allo-specific IL-2 production was diminished significantly by Bela and CTLA-4-Ig while IL-10 secretion was unaffected. Binding of Bela and CTLA-4-Ig to B7.1/2$^+$ cells showed that Bela binds stronger to B7.1/2 on Monocytes and B cells. EBV-specific VLPs induced weak IFNg production in healthy donors whereas they failed in most KTx patients to induce a VLP-specific immune response.

Conclusions: In contrast to CNI, the memory CD8$^+$ T cell response to viruses was not impaired by costimulation blockade (Bela, CTLA-4-Ig). However, the allo-specific immune response was partially blocked by costimulation blockers which primarily affected the IL-2 production while all other cytokines remained unaffected. This difference may explain the increased number of rejections early after Tx. EBV-specific VLPs may represent one possibility to vaccinate immunosuppressed patients. Our studies indicate that sensitivity towards Bela varies among healthy donors and Tx recipients which implies that predisposition of the immune response may determine the susceptibility to Bela treatment.

The rapid antigen-reactive T Cell enrichment procedure enables direct ex vivo BKV-specific CD4$^+$ T cell characterization

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BK virus (BKV) is a ubiquitous polyomavirus, which persists in a latent and asymptomatic state in healthy individuals after primary infection. In immunocompromised patients BKV reactivation may occur, which then can cause, e.g. haemorrhagic cystitis after allogenic stem cell transplantation or loss of the allograft after renal transplantation. Therefore, the characterization of BKV specific T cells for better understanding the BKV immune response is necessary to develop strategies like the adoptive transfer of virus specific T cells for the prevention or treatment of infections in immunocompromised patients.

BKV-specific T cells are rare in healthy donors and conventional flow cytometric detection methods are limited in sensitivity to characterize these cells. Recently a protocol for sensitive and broad functional analysis of rare antigen-specific CD4$^+$ T cell repertoires directly *ex vivo* was developed. This antigen-reactive T cell enrichment (ARTE) approach utilizes after short-term antigen stimulation the magnetic selection of activated CD154$^+$ CD4$^+$ T cells to subsequently uncover its phenotypical and functional profile by flow cytometry. In our work we focused on the analysis of BKV-specific T cells using a streamlined ARTE protocol with regard to time period and hand-on time.

Up to 2.5x10^7 PBMC of healthy donors were stimulated with BKV peptide pools, covering the complete sequence of the large T antigen (LT) and virion protein1 (VP1). Both proteins are immunodominant targets for T cell immunity. After six hours cells were fluorescently labeled for detection of various cell surface markers like CD4, CD45RA, and CCR7, and in parallel magnetically labeled for CD154, before cell fixation. All steps were performed directly in the cell culture plate without cell harvesting and washing to simply the procedure and to reduce hand-on time. Afterwards the cell surface labeled and fixed cells are harvested and directly applied onto a column in a magnetic field to enrich BKV antigen activated CD154$^+$ T cells. The retained cells are permeabilized, intracellular stained for cytokines and washed directly on the column to avoid centrifugation steps. Finally, CD154$^+$ T cells are eluted from the column for subsequent flow cytometric analysis. The ARTE approach allows us to process larger cell numbers compared to flow cytometric detection only and therefore facilitate the detection and enumeration of TNF-a, IFN-g, IL-2, and IL-10-producing memory T cells within the entire BKV-reactive CD4$^+$ T cell pool.

In summary, we developed a rapid and convenient protocol to detect and characterize in blood very rare antigen-specific CD4$^+$ T cell subpopulations combining magnetic cell enrichment and flow cytometric analysis.

Immune monitoring of absolute cell numbers and functional capacity of circulating γδ T cells and other immune cells from cancer patients

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γδ T lymphocytes are of interest in the context of T cell-based immunotherapeutic strategies due to their capacity to kill various tumor cells HLA-independently and to acquire antigen-presenting features. The success of immunotherapies, where the cytotoxic activity of circulating γδ T cells is activated by aminobisphosphonates, requires a profound knowledge about cell numbers and functional capacity of patients´ γδ T lymphocytes. To this end, we established an immune monitoring system that allows the determination of absolute cell numbers of not only conventional αβ T- and B lymphocytes and Natural Killer (NK) cells, but also of total γδ T lymphocytes and γδ T cell-subpopulations in small volumes (50µl) of whole blood. A possible influence of radio- or chemotherapy on the cell number of circulating γδ T cells or other cells of the immune system can be easily determined. Additionally, we analyzed the functional cytotoxic activity of circulating γδ T cells as well as of αβ T cells and NK cells from patients within a low number of peripheral blood mononuclear cells (PBMC). To demonstrate the efficacy of the established immune monitoring system, we analyzed the blood of patients with pancreatic ductal adenocarcinoma (PDAC). PDAC is a very aggressive malignancy characterized by a desmoplastic stromal microenvironment where conventional treatment approaches including surgery, chemotherapy and radiation are often not effective. Our results revealed no changes in the distribution of different γδ T cell subpopulations in untreated patients with advanced PDAC compared to age-matched healthy donors. To overcome the immunosuppression by PDAC stromal cells such as myofibroblasts on cytotoxic cells, we designed novel bispecific antibodies with specificity for CD3 on T cells, for CD16 on NK cells and γδ T cells and for Vγ9 on γδ T cells, and for human epidermal growth factor receptor Her2/neu expressed on PDAC cells. Besides their capacity to selectively target T cells or NK cells to tumor antigens and to enhance the cytotoxic activity of these immune cells, the bispecific antibodies were used to examine the functional capacity of cytotoxic cells within PBMC by Real-Time Cell Analyzer. Taken together, our results revealed that the determination of absolute cell numbers of different cell populations as well as the analysis of the cytotoxic capacity of circulating T cells or NK cells from patients against tumor cells of interest can give a better assessment of a personalized tumor treatment.

Egress of regulatory T cells from bone marrow of breast cancer patients

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Background: FoxP3+ CD4$^+$ Regulatory T cell (Treg) infiltration in tumor correlates to reduced survival. We observed reduced percentage of Treg in bone marrow of breast cancer patients that correlates to an increase in peripheral blood. We hypothesize that Treg mobilize out of bone marrow and infiltrate the tumor via blood route. On Contrary, CD4 T effector cells (Teff) are not efficiently mobilized from bone marrow.

Rationale: Sphingosine-1-Phosphate receptor 1 (S1P1) is well studied for its role in T cell egress guided by a gradient of its ligand Sphingosine-1-phosphate (S1P). CCR7 has been reported to play a role in cell retention in lymph nodes. CXCR4 - CXCL12 interaction is also reported in homing and retention of cells in bone marrow. We studied surface expression levels of S1P1, CXCR4 and CCR7 on Teff and Treg and analyzed the ligands for these receptors in bone marrow and blood plasma of breast cancer patients in comparison to healthy individuals.

Results: Plasma S1P levels revealed the existence of an S1P gradient with low to high concentrations of S1P in bone marrow to blood respectively. While expression levels of CCR7 ligands remained unaltered, CXCL12 expression levels in bone marrow plasma of patients was drastically reduced in comparison to healthy individuals but not to patient blood plasma. S1P1 transcript and protein expression levels between Teff and Treg was not altered. Interestingly, surface S1P1 expression was detected in around 5% of Treg but not Teff. CCR7 surface expression was high on Teff whereas CXCR4 surface expression levels between Teff and Treg were similar. Using MHC II tetramers detecting mammaglobin (Mam) specific T cells we find that the percentage of Mam specific Treg was low in bone marrow and high in blood. The percentage of Mam specific Teff was high in bone marrow. Higher surface S1P1 expression was detected on Mam specific Treg compared to Teff. Peripheral blood Teff and Treg had no/very low S1P1 surface expression. Influence of T cell activation on receptor expression was investigated by co-culturing primary antigen presenting cells (APC) with sorted Teff and Treg along with polyclonal stimulation. High surface S1P1 expression was detected 3 days after activation both in Teff and Treg while surface CXCR4 expression was reduced. Interestingly, surface CCR7 expression on Teff was not reduced upon activation.

Conclusion: Higher S1P1 and lower CCR7 expression on the surface of bone marrow Treg and presence of reduced CXCL12 levels in bone marrow could lead to preferential exit of Treg from bone marrow of breast cancer patients. Retention of Teff in bone marrow could be attributed to higher CCR7 and lower S1P1 expression. Exit signals coupled with loss of retention signals thus may guide cell egress from bone marrow.

Dipeptides mediate peptide exchange on MHC class I molecules

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MHC (major histocompatibility complex) class I molecules are membrane proteins that play a central role in mammalian antiviral immune response by presenting the viral proteome to the cell surface for the recognition by cytotoxic T lymphocytes. In our previous work[1], we have shown that peptides as small as two amino acids (dipeptides) can efficiently fold the class I allotypes HLAA*02:01 and H-2K^b *in vitro*. Such dipeptides also accumulate and stabilize the peptide receptive A2 and K^b molecules on the cell surface. Now, we have looked at the exchange of class I bound peptide for an exogenous peptide of interest in the presence of dipeptide. We found that dipeptides accelerate the dissociation of high-affinity peptides both on the recombinant and on the cell surface A2 and K^b molecules in an allotype-specific manner. We also demonstrate that cell surface peptide exchange, catalyzed by dipeptides, can be used to activate peptide specific T lymphocytes. We successfully employed this peptide exchange technique to produce epitope-specific MHC tetramers from MHC monomers that were originally folded with a different peptide. This technique allows us to create different tetramers of a desired specificity from a single starting monomer in one day. We could show that these tetramers are equally efficient in the detection of virus specific T cells from human blood samples.

Reference:

1. Dipeptides promote folding and peptide binding of MHC class I molecules. Saini et al. (Proc Natl Acad Sci U S A. 2013 Sep 17;110(38):15383-8.)

Detection and functional assessment of regulatory T cells in blood of cancer patients

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It is well established that accumulation of regulatory T cells (Tregs) in cancer patients is associated with tumor progression, poor prognosis and the suppression of anti-tumor immune effector functions, and Treg-mediated immunosuppression is therefore considered a major obstacle for successful cancer immunotherapy. Given their profound effect on the outcome of immunotherapy trials, Treg frequency and function are being studied extensively. Unfortunately, no consensus has been reached on a) the (minimal number of) markers required to define the frequency of human Tregs and b) which assay is the best to measure Treg suppressor function.

Here we propose essential marker sets for flow cytometric enumeration of Tregs and report on the side-by-side comparison of two flow cytometric approaches that measure Treg suppressor function.

Based on the outcome at the workshop organized by the CIMT immunoguiding program (CIP) workgroup on the detection and functional testing of (antigen-specific) Tregs and subsequent discussions with leading experts in the field, we propose the following context-dependent (i.e. in peripheral blood / tumor / lymph node (LN)) marker set for flow cytometric Treg analysis: CD3, CD4, CD25, CD127 (IL7-R), FoxP3, CD45RA and helios. Yet, as some of these markers are also expressed on activated conventional effector T cells (Tconv), measurement of Treg suppressive function is crucial for the discrimination of Tregs from activated Tconv. Classically, in suppressor cell assays the inhibition of responder cell proliferation or cytokine production is measured in a 3-7 days long period. However these assays require large numbers of Tregs and are technically challenging. Recently, an assay measuring Treg-mediated prevention of CD69 and CD154 up-regulation on $CD4^+$tT cells has been described as an alternative and shorter (7-20 hours) method. So far, correlation between these two methods to determine suppressive function has not been formally shown. To study the suitability of the latter assay, the ability of Tregs to inhibit CD25, CD69, CD154 and CD137 up-regulation on $CD4^+$ and $CD8^+$ T cells was determined, and compared to the capacity of Tregs to inhibit proliferation of the effector T cells (as assessed by cell trace violet dye dilution). We show that indeed Tregs, which were isolated through CD4 and CD25 magnetic beads, are able to inhibit aCD3/CD28-bead-induced up-regulation of CD25, CD69 (on CD4 and CD8) and CD154 (on CD4), but not CD137 (on CD8) T cell activation markers on responder T cells. Moreover, this inhibition of activation marker up-regulation significantly correlates with suppression of their proliferation, indicating that measuring inhibition of T-cell activation is well-suited for analyzing Treg suppressive function. In conclusion, the T-cell activation marker assay is a simple, fast and robust assay to measure Treg suppressive activity and should be considered as an alternative short-time assay for functional Treg testing.

Immune status of patients with localized prostate carcinoma during primary radiotherapy

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The long-term goal of our study is to establish a T-cell based immune therapy for patients with localized prostate carcinoma (PCa) in combination with dose escalated radiation therapy (RT). To this aim, we monitored the cellular immune status of PCa patients treated with RT.

Twenty-one HLA*A2-positive patients were included in the study. All patients received external beam RT (78Gy in 39 fractions) to the prostate and the seminal vesicles. In a subset of patients with high risk PCa, the pelvic lymphatics were treated to 50.4Gy in 28 fractions. Most patients received concomitant hormone ablative treatment. Peripheral blood mononuclear cells (PBMC) were obtained at four timepoints: prior to the first RT fraction, after four weeks of treatment, on the day of the last day of treatment and three months after completion of treatment. PBMC were frozen until the day of analysis, which was conducted by multicolor flow cytometry.

Comparing the median percentages of various cell subsets at the beginning vs. at the last day of the RT, we found some changes in the immune status (19 patients). While the T- and B-cell populations within viable lymphocytes decreased (T: 63.3 vs. 58.6%; B: 4.1% vs. 1.9%), the percentages of natural killer -NK- cells and regulatory T-cells -Treg- increased (NK: 31.1% vs. 38.8%; $CD4^+CD25^+Foxp3^+$-Treg: 4.2% vs. 6.3%). An increase in the proliferation rate was observed for NK-, B-, T-, and Treg-cells (NK: 5.9% vs. 12.6%; B: 3.7% vs. 11.2%; T: 2.5% vs. 5.8%; Treg: 11.2% vs. 14.7% -fraction of $Ki67^+$ cells-). The proportion of the CD4- and CD8-positive T-cell subsets did not change significantly.

From five patients, we also performed complete blood counts with differentials at each timepoint. The absolute lymphocyte counts decreased from 1323.0/µl before RT to 552.1/µl at the last day of RT.

The data obtained so far show a significant change of several immune cell subsets during RT in PCa patients. The potential effect of the treatment on the cell viability, cell-differentiation and function of viral-specific $CD8^+$ T-lymphocytes is under investigation and will be presented.

Early granulocyte effector function is not impaired by cyclosporin A

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Severe infections with *Aspergillus fumigatus* play a relevant role in immunodeficient patients e.g. after allogeneic hematopoietic stem cell transplantation. In these patients prolonged neutropenia is a major risk factor for fungal infections indicating an essential role of innate immune system in antifungal defense. Data from previous studies suggest that NFAT is a key player in signaling pathways of innate immunity e.g. in polymorphonuclear neutrophils (PMN) and that it can be modulated by commonly used agents like cyclosporin A (CsA). The aim of our work is to elucidate the relevance of NFAT-dependent activation signals in PMN, their potential modulation by calcineurin/NFAT inhibitors and their significance in immune responses against *Aspergillus fumigatus*.

We firstly performed *in vitro* experiments using human (healthy donors) PMN after purification and from whole blood regarding their effector functions in absence or presence of CsA in titrated doses appropriate to therapeutic levels. Phagocytosis and activation-induced shedding of CD62L was measured by flow cytometry using polychromatic microspheres and appropriate surface markers (CD11b-PB, CD62L-APC, CD66b-FITC). Generation of reactive oxygen species was analyzed by dichlorofluorescein assay (DCF) and activation-induced synthesis of IL-8 by enzyme-linked immunosorbent assay (ELISA) and intracellular flow cytometry. Secondly, we applied a murine *Aspergillus fumigatus* pneumonia model to study the antifungal innate immune response *in vivo* with or without intraperitoneal administration of CsA. Here, PMN recruitment to the lungs, pulmonary clearance of *Aspergillus fumigatus* and survival of infected mice was examined. PMN-depleted mice using an murine Gr1-antibody served as controls.

Administration of CsA had no significant influence on human granulocyte expression of activation markers and shedding of CD62L. Moreover, additional functional testing indicated also no substantial influence on generation of reactive oxygen species (5245 RFU ± 354 vs. 5763 ± 520 (control) after stimulation with LPS, mean ± SEM) compared to controls. Likewise the activation-induced synthesis of IL-8 was not reduced in presence of CsA *in vitro* (519pg/ml ± 81 vs. 463 ± 131 (control) after stimulation with LPS). In contrast, phagocytosis was rather stimulated in presence of CsA (83.5% ± 1.7 vs. 71.0 ± 1.5 (control) after stimulation with LPS). Regarding survival of *Aspergillus fumigatus* infected mice, successful granulocyte-dependent clearance of fungal infection was not impaired in relevant degree through CsA medication.

Early granulocyte-based innate immune function is not inhibited by CsA, suggesting that NFAT may play a more crucial role in granulocyte differentiation under steady state conditions. Therefore, we are planning *in vivo* experiments with long-term CsA-pretreated mice and also *ex vivo* analysis of patient blood samples under continuous CsA medication.

Identification of biomarkers for the response of patients with advanced gastroesophageal cancer to IMAB362 treatment

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Background: IMAB362 is a novel monoclonal antibody specifically binding to the tight junction protein CLDN18.2 which is almost exclusively present on tumor cells. It is currently undergoing Phase II clinical testing in patients with advanced gastroesophageal cancer. In order to identify patients who would benefit the most from treatment with IMAB362, we examined parameters that could serve as potential biomarkers.

Materials and methods: Patients included in these ancillary analyses were enrolled in an international, multi-center, Phase IIa clinical trial evaluating the safety and efficacy of repeated intravenous infusions of IMAB362 at 300 mg/m^2 and 600 mg/m^2. We examined the CLDN18.2 positivity, several immune cell phenotypes, the capacity of patient-derived blood components to exert antibody-mediated modes of action (antibody-dependent cellular cytotoxicity [ADCC] and complement-dependent cytotoxicity [CDC]) in vitro, and the presence of immune-related genetic single-nucleotide polymorphisms (SNPs).

Results: CLDN18.2 is expressed on primary tumors as well as on metastasis thereof. More patients with adenocarcinoma of the stomach and the gastroesophageal junction express CLDN18.2 compared to patients with adenocarcinomas of the esophagus. Regarding immune effector cells administration of a single-dose of IMAB362 did not alter frequency, phenotype, or activation status of evaluated immune cell populations. ADCC and CDC with patient material did not differ pre- and post-treatment with IMAB362. Finally SNPs showed a variant allele pattern which allows testing for frequency shifts of SNP alleles between patient subpopulations and ideally the identification of a putative responder population.

Conclusions: Analysis of the correlation of the evaluated parameters with clinical outcome data is currently ongoing. This will help to identify biomarkers predictive for a response to IMAB362 in patients.

Thymic NK cells display a specific phenotype and functional profile

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Until recently, studies on human NK cells mainly focused on cells isolated from peripheral blood. Little is known about the development and functional role of human NK cell subpopulations in secondary lymphoid organs. In this study, we report on a detailed phenotypic and functional characterization of human NK cell subsets isolated from thymic tissues.

NK cells can be classified according to the expression of CD56 and CD16 into immunoregulatory, cytokine-producing $CD56^{high}CD16^{dim}$ and cytotoxic $CD56^{dim}CD16^{high}$ NK cells. It is widely accepted that NK cells derive from bone marrow and that the final development takes place in secondary lymphoid tissues. Recently, it has been reported that thymic $CD34^{+}$ cells can differentiate into NK cells under treatment with IL-2 *in vitro*. However, it is still unsolved if NK differentiation also occurs in secondary lymphoid tissues such as the thymic organ.

The main objective of our study was the profound analysis of the phenotypical and functional profile of NK cell subsets from thymic tissues. Therefore, we performed detailed immune monitoring and characterization of NK cells isolated from more than 200 thymus preparations obtained from differently aged children undergoing heart surgery.

Our flow cytometry analysis revealed a different subset composition of child thymic NK cells compared to peripheral blood NK cells of both children and adults. Thymic NK cells have not only a lower CD56-expression but also a different ratio of $CD16^{high}/CD16^{dim}$ NK cells. Furthermore, thymic NK cells display a specific phenotype with low expression of KIRs, CD117, CD57, CD62L, and CX3CR1, and upregulation of NKG2A, NKG2D, CD27, and CD127 on at least one NK cell subset.

Importantly, functional assays of sorted thymic NK cell subsets demonstrated the capability to kill tumor cells in a perforin-dependent manner and to produce a large range of cytokines. However, a prolonged stimulation period or a specific trigger was required to gain cytotoxic and cytokine-producing functions.

In summary, this study provides deep insight on the phenotype and function of human NK cells in thymic tissue being of great value for further understanding NK cell development and education.

HTCC allows the characterization of differential CD8$^+$ T cell responses against 20+ antigens in a single patient sample

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Introduction: HLA tetramer combinatorial coding (HTCC) technology is an epitope discovery and immunomonitoring platform in which directly ex vivo 20+ different antigen (Ag)-specific CD8$^+$ T cell populations can be detected simultaneously within a single sample. We here combine HTCC with additional markers to simultaneously characterize the individual Ag-specific T cells. Immunomonitoring of Ag-specific T cells becomes more important, e.g. for determining treatment effect in early trials. Phenotypic and functional characterization gives extra insight in T cell responses induced by natural infection or vaccines.

Material and methods: In the HTCC approach T cells are stained with tetramers and visualized by flow cytometry. For each peptide-HLA combination 2 tetramers are created, each conjugated with a different fluorochrome using a unique combination of colors (i.e. each peptide-HLA combination has a unique ‚2-color code'; 8 different fluorochromes yield 28 unique dual coding combinations).

We have standardized and validated the technology for implementation in clinical trials. Peripheral blood mononuclear cells (PBMCs), derived from buffy coats of healthy subjects, were used for reproducibility studies. HLA-A2-negative PBMCs containing an HLA-B*07:02 EBV (RPPIFIRRL)-specific T cell response were spiked with a decreasing number of HLA-A2-restricted influenza A (GILGFVFTL)-specific T cells and analyzed by combinatorial coding flow cytometry using a LSRII in order to test sensitivity.

Results: The detection of Ag-specific CD8$^+$ T cells by dual coding (n = 26) was found to be as efficient as detection with conventionally fluorescently-labeled HLA multimers (n = 8). Importantly, dual coding resulted in reduced background staining, thereby making it a more sensitive method than the classical assay. Intra and inter assay variations were low and demonstrate that HTCC is a robust technology. We were able to detect influenza A-specific T cells even at a frequency of 0.004%.

In patient samples, 20-plex HTCC was combined with 4 phenotypic markers defining naive, memory and effector Ag-specific CD8$^+$ T cells. We observed that T cell populations directed against different epitopes of a single virus had different characteristics. Therefore this HTCC gives immediate insight in the differential development of Ag-specific T cell responses.

Conclusion: Our data show that the HTCC technology is robust, more sensitive than classical tetramer staining and can be combined with phenotypic analysis. It is in particular suitable for the characterization of the breadth of T cell responses induced by natural infection or vaccines, or for the monitoring of therapeutic interventions using limited patient material.

Investigation of humoral immune response towards persisting Epstein-Barr virus infections in multiple sclerosis and chronic fatigue syndrome using peptide microarrays

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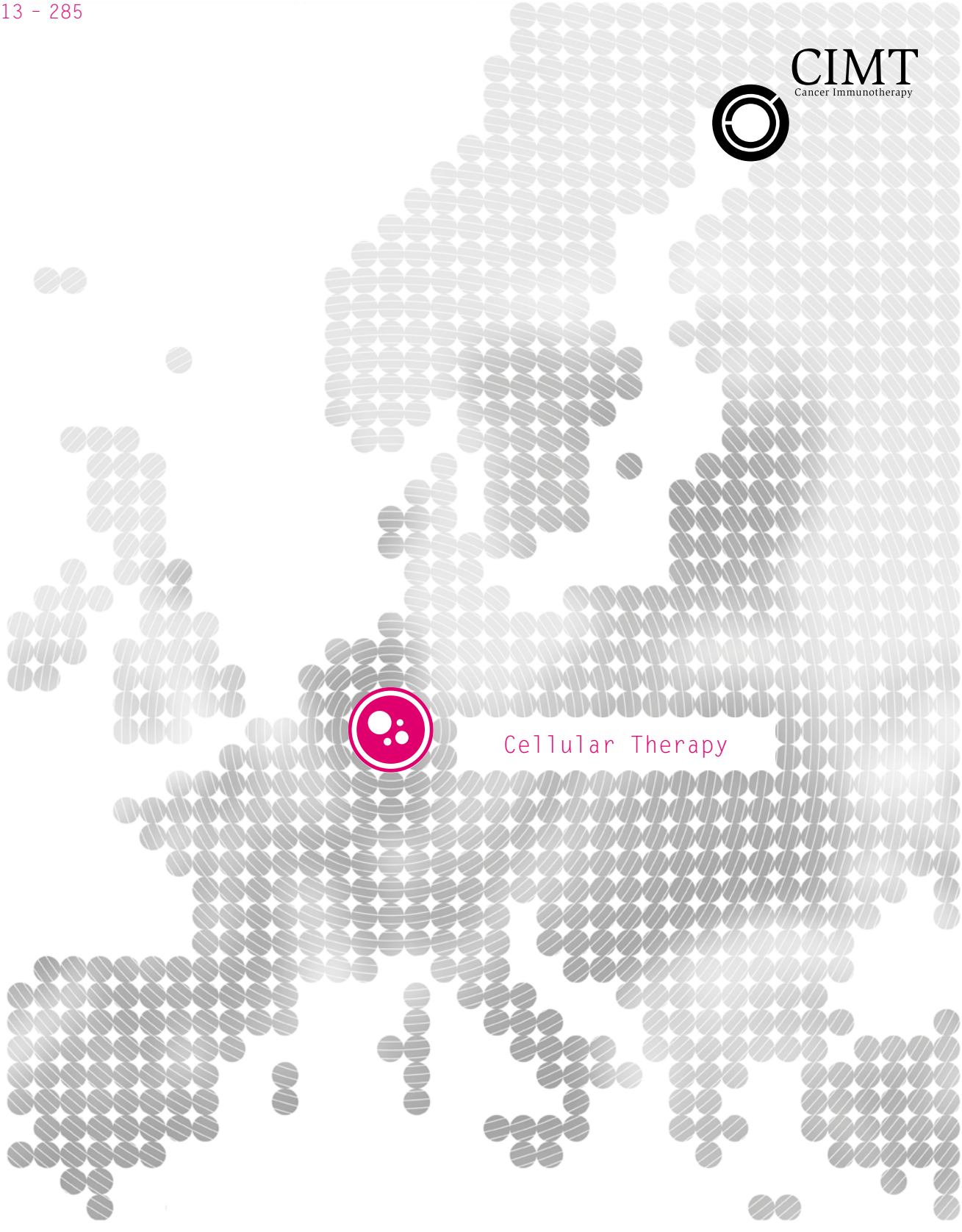
Most humans carry a considerable number of persisting viral infections, frequently in a latent state. Chronic infections may intermittently reactivate especially under immune suppression and play an important role as trigger or cofactor for autoimmune diseases and cancer. Correlation of such infections with diseases is difficult. An analysis of antibody repertoire in chronic infection may provide information about patterns of virus reactivation.

Following primary infection the herpes virus 4 (EBV) results in persistent mostly asymptomatic latent infection. About 95% of the adult population are EBV seropositive. EBV infections are known as cofactors for various diseases such as lymphomas, multiple sclerosis and chronic fatigue syndrome. The detailed mapping of humoral immune response in human serum samples allows a high resolution analysis of the antibody repertoire against EBV antigens.

We developed a peptide microarray platform with peptide libraries of up to 6900 members. Here we present data using a library of peptide scans through major EBV antigens from incubations with serum samples of healthy human donors, patients diagnosed with chronic fatigue syndrome or multiple sclerosis.

Even though there are shared epitopes between the specimens and cohorts, different epitope patterns are observed for a number of antigens and antigen areas. The patterns of antibody responses could be of diagnostic value for multiple sclerosis and chronic fatigue syndrome.

CIMT
Cancer Immunotherapy



Cellular Therapy

Redirection of T cells towards acute myeloid leukemia blasts via bispecific antibody-releasing human mesenchymal stem cells

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In recent decades, bispecific antibodies (bsAb) have emerged as promising tools for antigen-specific cancer immunotherapy. In general, bsAb molecules are composed of two different antigen binding moieties that bind simultaneously two different cell types. Most bsAb are designed to redirect cytotoxic immune cells to cancer cells, which results in the specific lysis of the latter. The potential of bsAb to mediate tumor cell eradication has already been successfully substantiated in various *in vitro* and animal studies as well as in pilot clinical trials.

Recently, a novel humanized bsAb for the redirection of T cells was developed in our group, that targets the CD3-complex on T cells and the surface molecule CD33, which is widely overexpressed on acute myeloid leukemia (AML) blasts. Our *in vitro and in vivo* data have already demonstrated that this bsAb facilitates an efficient and specific eradication of AML blasts, with low risk of side effects. However, one downside of bsAbs is their short half-life *in vivo* which leads to the continuous administration of such agents to achieve the desired effect, resulting in high costs treatment.

To address this problem we engineered an immortalized human bone-derived mesenchymal stem cells (hMSCs) cell line, known as single-cell-picked clone 1 (SCP-1), in order to develop a cellular vehicle for the constant delivery of the humanized CD33-CD3 bsAb to the tumor microenvironment. *In vitro* assays have shown that the produced bsAb by the engineered hMSCs is effective in redirecting T cells toward $CD33^+$ AML target cells, leading to an high specific T cell-mediated tumor cell killing. In addition to the parental CD33-CD3-secreting hMSC line a sub-line was further generated, which expresses on the surface the T cell costimulatory ligand 4-1BBL (CD137L), a member of the tumor necrosis factor receptor (TNFR) family, which provides an additional stimulation of T cells, resulting in a higher T cell expansion, an enhanced release of pro-inflammatory cytokines and a more efficient anti-tumor effect, as it has been recently demonstrated by our group. Indeed, our preliminary *in vitro* data have shown that the application of additional stimulation of T cells through the 4-1BB and 4-1BBL-hMSCs (CD137/CD137L) interaction leaded to a significant improvement of the anti-tumor activity and prolong the immune response.

In sum, our data show that continuous *in situ* delivery of bsAb CD33-CD3 by genetically modified hMSCs facilitates efficient activation of T cells for specific and efficient killing of AML blasts over prolonged period of time. Therefore, the idea presented here could be a promising alternative to continuous exogenous administration of short-lived antibodies for AML immunotherapy. *In vivo* antibody production would result in effective and persistent levels of these therapeutic agents.

Targeting melanoma and multiple myeloma using a high-affinity TCR specific for human MDM2 tumor-associated antigen

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The human homologous of the murine double-minute 2 protein (MDM2) tumor-associated antigen (TAA) is overexpressed in a variety of human tumors, including soft tissue sarcoma, multiple myeloma (MM) and melanoma. MDM2 protein overexpression was particularly observed in invasive and metastatic melanoma and was associated with enhanced survival of MM cells. Adoptive transfer of TAA-specific T cell receptor (TCR) T cells is an attractive approach to treat cancer patients. We have already described that using HLA-A*0201 (A2.1) transgenic mice can circumvent self tolerance to human TAAs. We have generated an HLA.A2-restricted CD8-dependent MDM2 (81-88)-specific TCR from a high-avidity murine CTL clone derived from CD8 x A2K^b transgenic mice to redirect human T cells. We focused on a new approach in adoptive T cell immunotherapy where cancers overexpressing MDM2 can be targeted by human T cells transduced with the MDM2 (81-88)-specific TCR.

Human T cells were retrovirally transduced with the wild-type (wt) MDM2-TCR. We analyzed the TCR expression level by flow cytometry and measured the affinity to bind HLA.A2 molecules loaded with the MDM2 (81-88)-peptide using labeled tetramers. We screened melanoma and MM cell lines for MDM2 protein expression via Western Blot and analyzed the HLA.A2 expression by flow cytometry. Cell lines which were highly HLA.A2 positive and also expressing MDM2 were tested for recognition by the wt MDM2 (81-88)-specific TCR in a cytolytic assay. Our data demonstrated that these cell lines can be lysed by human T cells transduced with wt MDM2-TCR compared to melanoma or MM cell lines which do not express HLA.A2 or exhibit weak MDM2 levels.

We also modified and optimized the wt MDM2-TCR-construct to increase the expression level of introduced TCR. To increase the expression level of transduced TCR we codon-optimized the sequence and cloned it in a bicistronic retroviral vector containing the self-cleaving 2A virus-derived peptide. The risk of TCR heterodimers formation with unknown specificity and generation of potentially autoreactive T cells was reduced via addition of an inter-chain disulfide bond between TCR α and β constant domains.

These results demonstrate that melanoma and MM can be targeted by MDM2-specific TCRs. In addition we are currently engineering an optimized single chain MDM2-TCR to prevent any residual formation of mixed heterodimer TCRs and to improve safety for its use in adoptive T cell therapy. This can be a promising approach for targeting cancer cells overexpressing MDM2.

Optimisation of the isolation and expansion of tumour infiltrating lymphocytes from renal cell carcinoma

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Background: Encouraging results have been achieved after tumour infiltrating lymphocytes (TILs) transfer in patients with metastatic melanoma with objective responses ranging from 50 to 72% including 20% complete remissions. Unfortunately, attempts to use TIL therapy with other solid tumours failed to obtain comparable clinical responses. We focused on renal cell carcinoma (RCC) and investigated a modified protocol to isolate and expand TILs in order to overcome the issues previously encountered when applying the melanoma protocol.

Methods: Tumour samples were enzymatically disaggregated overnight or with the GentleMACS™ dissociator and activating beads were added to the culture with high-dose IL-2. Remaining digest was frozen and used as autologous tumour target to test TILs' *in vitro* functionality via IFN-γ secretion in ELISA assays at the end of the expansion period.

Results: We managed to successfully expand TILs in 90% of RCC samples processed. 33% of TILs expanded after overnight digestion and 55% of TILs grown after GentleMACS™ dissociation showed reactivity to the autologous tumour as measured by IFNγ production in ELISA assays. The difference between the two methods was likely to be due to downregulated expression of surface markers on the tumour cells (in particular of class I) rather than lack of activity of the expanded TILs, as TILs expanded after overnight digest did not show any reduction in reactivity when co-cultured with tumour cells derived from samples processed with the GentleMACS™ dissociator. Surface staining of each tumour digest using flow cytometry confirmed this hypothesis.

Conclusions: A robust method to isolate and expand TILs has been established and we aim to incorporate it into future clinical work.

T-cell receptor transfer into human T-cells with ecotropic retroviral vectors

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The transduction of human T-cells with recombinant T-cell receptors (TCRs) requires amphotropic pseudotyped retroviral vetors (RV). These are considered safe in principle, however, TCR-coding transfer vectors and packaging vectors could theoretically recombine to produce replication competent vectors (RCV). Therefore TCR transduced T-cell preparations intended for adoptive transfer must be proven free of RCV. To eliminate the need for RCV testing, we transduced human T-cells with retroviral vectors (RV) which were pseudotyped with the ecotropic envelope of murine leukemia virus. Ecotropic RV by themselves transduce exclusively rodent cells and thus potentially arising RCV would be non-infectious for human cells. To enable TCR transduction into human T-cells, we created susceptibility for ecotropic RV by transfecting mCAT-1, the receptor for murine retroviruses, before proceeding to transduction. Here we show that electroporation of mRNA encoding mCAT-1 enabled ecotropic transduction of human T-cells with rates ranging between 60% and 80%. mCAT-1-dependent ecotropic transduction rates correlated to the mRNA dose used for electroporation. At optimal doses, ecotropic transduction was more efficient and robust than amphotropic transduction performed in parallel. Moreover, after electroporation with mCAT-1 mRNA T-cells lost susceptibility for ecotropic retroviral vectors within a week, which corresponded to a functional mCAT-1 half-life of about 3h. We could further show that ecotropic transduction is suitable for reprogramming of T-cell reactivity. Upon re-stimulation, $CD8^+$ T-cells transduced with a TCR targeting the CMV-pp65 model antigen secreted IFNg and TNFa in CMV-pp65-dependent manner.
We conclude that ecotropic RV represent a versatile, safe and potent tool to prepare T-cells for adoptive transfer.

First clinical experience with a new generation of fast DCs transfected with mRNA from hTERT, survivin and autologous tumour

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ORAL SHORT TALK
CIMT 2014
Clinical Trials

We have previously shown in preclinical models that a new generation of fast DCs, using a maturation cocktail containing IL-1β, TNFα, IFNγ, PGE2 and the TLR7/8 ligand R848, is more efficient than the standard 7 day DCs. These DCs show a high up-regulation of HLA-DR and co-stimulatory molecules like CD80, CD83, CD86 and CD40 combined with a down regulation of CD14. They have a good migratory capacity towards CCL19 and are especially characterized by IL-12p70 production when stimulated with CD40L transfected mouse fibroblasts, whilst IL-10 production is low.

Here we investigated if the new generation DCs maintain their properties when produced by GMP standards and if they are able to mount specific immune responses in patients.

Monocytes were enriched using the Elutra cell separator and cultivated either fresh or after cryopreservation. Monocytes were cultured in Teflon bags in the presence of IL-4 and GM-CSF and the maturation cocktail was added on day 2 or 3. After 24 hours DCs were harvested and electroporated with mRNA. After 2-4 hours recovery, cells were frozen with either 2.5E+6 or 5E+6 transfected DCs per vial. Productions from one lung cancer, one prostate cancer and 4 glioblastoma patients showed the same characteristics with only some slight variations in the amounts of IL-12p70 released after co-culture with CD40L transfected mouse fibroblasts.

The first patient treated with the new generation DCs transfected with hTERT and survivin mRNA suffered from stage IV lung cancer with brain metastases. Following diagnosis in June 2011 she was treated with chemotherapy and radiotherapy. Since December 2011 she has been vaccinated and has obtained a status of stable disease. DC vaccination was interrupted in 2/2013 when an attempt was made to re-open an occluded bronchus with radiotherapy. During irradiation the patient developed an inflammation of the pleura, which was treated with high dose cortisone. During cortisone therapy the patient developed 2 new brain metastases, which were treated with Dexamethasone and local radiotherapy using Cyberknife. DC vaccination was continued in 6/2013 and health conditions gradually improved bringing the patient again into a status of stable disease.

The second patient receiving DC vaccination had a hormone resistant prostate cancer in a very advanced stage and dropped out immediately after start of treatment.

Treatment of four glioblastoma patients with hTERT and survivin transfected DCs plus either with autologous tumour mRNA or hCMVpp65 mRNA DCs has started. All patients show strong local DTH responses and flu-like symptoms after vaccination at an earlier time point, but it is too early to evaluate clinical outcome.

Altogether our results show that the new generation DCs can successfully be used clinically in different kinds of cancer. Whether the strong DTH reactions and flu like symptoms observed following the DC vaccinations turn into strong specific T-cell responses is under investigation.

A photosensitizer delivered by bispecific antibody retargeted human T lymphocytes boosts cytotoxicity against carcinoma cells upon light irradiation

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ORAL SHORT TALK

Photodynamic therapy (PDT) is an emerging treatment modality for cancer. In PDT a light-sensitive photosensitizer (PS) is administered and after a latency period the malignant lesions are selectively irradiated with light of a specific wavelength, thereby exciting the photosensitizing agent. In the presence of oxygen, activated PS-molecules generate singlet oxygen and reactive oxygen species, which provoke lethal oxidative damage in aberrant cells. However, tumor selectivity after systemic application of PS is limited and healthy tissues also accumulate PS. The general distribution of the photosensitizing agent leads to adverse effects, like eye and skin photosensitivity. In order to circumvent these obstacles, tumor-targeting PDT strategies are under investigation.

Here, we report on the feasibility of a cell-based drug targeting concept, using bispecific antibody (bsAb, EpCAM×CD3) redirected human T lymphocytes as selective transport vehicles for the model photosensitizer 5,10,15,20-tetrakis(3-hydroxyphenyl)-porphyrin (mTHPP) in vitro. This photosensitizing agent is the parent porphyrin of mTHPC, which is the active pharmaceutical ingredient of Foscan. In the context of adoptive cell transfer (ACT), the concept aims at enhancing the selectivity and efficacy of PDT, while simultaneously decreasing adverse effects. Most notably, the approach intends to combine the phototoxicity of PS-molecules delivered by redirected $CD4^+$ and $CD8^+$ T cells (drug effect) with the cytotoxicity of retargeted $CD8^+$ T cells (T cell effect) in a synergistic manner.

We have demonstrated that ex vivo activated human polyclonal T lymphocytes take up water-soluble complexes composed of hydrophobic mTHPP and poly(styrene sulfonate) sodium salt (PSS) after short-term incubation. In absence of light and when drug loading occurred at a tolerable concentration of PSS/mTHPP-complexes, viability and cytotoxic function of carrier cells were not impaired. When "drug-enhanced" T cells were co-cultivated with EpCAM expressing human carcinoma cells, mTHPP was transferred to target cells. Interestingly, in the presence of bsAb, which cross-links effector and target cells thereby inducing the cytolytic activity of cytoxic T lymphocytes, significantly more PS was transferred. Therefore, the bsAb does not only provide tumor cell specificity but also indirectly enhances drug specificity. Consequently, redirected drug-loaded T cells were more effective in killing A549 lung carcinoma and SKOV-3 ovarian carcinoma cells than retargeted unloaded T cells upon irradiation of co-cultures. Notably, the additive approach using redirected unloaded T cells in combination with separately applied PSS/mTHPP equal to the amount of complex carried by T cells was less efficient, as well. Thus, the combined cytotoxicity of transferred photosensitizer molecules and T lymphocytes exhibited synergistic antitumor effects. Our findings support the feasibility of the envisioned T cell-based targeted PDT approach.

IL-12 enhances T-cell receptor-dependent and -independent tumor cell recognition by human effector T-cells

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Adoptive T-cell Transfer is a promising therapeutic approach for a variety of malignancies. However, in vitro expansion of T-cells specific for tumor-associated antigens is hindered by the preferential expansion of T-cells with low functional avidity, defined by their lacking capacity to lyse tumor cells presenting the cognate antigen endogenously.

However, T-cell function is also context-dependent, as an inflammatory or suppressive microenvironment affects the T-cell response. We therefore asked how inflammatory cytokines affect functionality of in vitro-primed, human T-cells specific for tumor-associated antigens.

Highly purified naïve $CD8^+$ T-cells were primed in optimized conditions using IL-12-producing, peptide loaded DCs. At the peak of antigen-specific proliferation, 8 days after the initial TCR trigger, various inflammatory cytokines were added alone or in certain combinations. 48h later antigen-specific function was evaluated using intracellular cytokine staining and cytotoxicity assays as readout. Only IL-12 pretreatment resulted in increased antigen-specific cytokine production and tumor cell killing in these short-term cultured, policlonal T-cell lines, whereas the other inflammatory cytokines had no antigen-specific effect.

We next wanted to evaluate T-cell function on a clonal level and analyzed individual T-cell clones generated against the six-transmembrane epithelial antigen of the prostate 1 (STEAP-1) as well as clones against preferentially antigen expressed in melanoma (PRAME). In contrast to the short-term cultures, which display an early effector memory phenotype ($CD62L^+$; $CCR7^+/^-$, $CD27^+$, $CD28^{+-}$), T-cell clones obtained after 3 rounds of restimulation and an approximate culture time of 6 weeks, are terminally differentiated ($CD62L^-$; $CCR7^-$, $CD27^-$, $CD28^-$)). Similar to early effector memory T-cells, IL-12 increased antigen-specific cytotoxicity in T-cell clones in a dose-dependent manner. Surprisingly - and in contrast to early effector T-cells - TCR- and HLA-independent cytotoxicity was also clearly increased. Careful analysis of this observation, revealed a critical role of signaling through the NK-cell activating receptor DNAM-1, but not NKG2D. Increased TCR-independent lysis could be blocked by DNAM-1-inhibiting antibodies and correlated with an increase of DNAM-1 expression in cultured T-cell clones.

In conclusion, we show a dual effect of IL-12 on TCR-dependent and TCR-independent activation of human T-cells. Less differentiated T-cells respond to IL-12 with higher functional TCR avidity, whereas DNAM1-mediated, antigen-independent effects dominate the T-cell response after IL-12 preincubation in terminally differentiated T-cells.

IL-12 may be suitable for fine-tuning T-cell activity prior to adoptive transfer, however caution is required when interpreting effects of fully differentiated T-cells in an inflammatory environment.

In vitro model for immunotherapy - Generation of a long-term growth factor-dependent DC culture

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) of the immune system. Originating from hematopoietic precursors in the bone marrow, DCs migrate to the periphery as immature cells. They become mature in presence of microbial stimuli and proinflammatory cytokines or by interacting with CD40L-expressing T cells during an infectious or inflammatory event. The mature DC is characterized by its expression of high levels of MHC II costimulatory molecules.
They play an important role in orchestrating the cell-mediated immune response. Thus, DCs are of great interest for manipulation of immunity in context of cancer therapy. To study the effects of new therapeutical approaches on DCs *in vitro* systems are needed. We compared different protocols for generating mouse bone marrow-derived DCs (BMDCs) and established a long-term growth factor-dependent DC culture. Cultured with GM-CSF these cells display an immature phenotype. In the presence of additional IL-4 the BMDCs exhibit an intermediate maturation stage. The cells are stimulable by i.e. LPS, CpG, and TNF-alpha to full maturation. Since the capacity to induce antitumor immune responses correlates with degree of DC maturation, these BMDCs are a good basis for *in vitro* experiments testing new DC-based immunotherapies.

In depth analysis of in vitro isolated T cell receptors with specificity for HER2/neu-derived peptides

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The Human Epidermal Growth Factor Receptor 2 (HER2) represents one of the most extensively studied antigens in the field of targeted tumor therapies and is found to be overexpressed in many different cancers. Engaging immunotherapeutic approaches, treatment options for advanced stage malignancies with HER2 overexpression have improved dramatically. However, it is questionable if HER2 actually serves as a favourable target antigen for T cell receptor (TCR) responses as independent studies of several research groups revealed that HER2 expression modifies adaptive immune responses, especially cytotoxic T cell reactivity.

In order to elucidate the actual functionality of HER2-specific T cells, we stimulated healthy naïve T cells in the autologous or single HLA-mismatched setting by using different conditions. After Multimer specific enrichment, clonal expansion and functional testing, we were able to isolate seven different TCR recognizing two previously described HER2 epitopes (aa369-377, aa689-697).In these studies, we confirmed large similarities between CDR3 regions of the alpha chains of TCR with specificity for HER2 aa369-377. During subsequent characterization, despite defined peptide reactivity, the TCR displayed recognition patterns against diverse malignant cell lines derived from various primary tumors which did not always correspond to endogenous HER2 expression of the respective cell line. Moreover, recognition and lytic capacity of our TCR against various target cells stably transduced with the complete HER2 construct or the extra- or intracellular domain did not show a clear correlation to HER2 expression. In addition, we investigated a possible cross-recognition of peptides derived from other members of the HER family (HER1, HER3 and HER4) by titrating peptide reactivity for each analogous epitope. Indeed, some of our TCR specifically cross-reacted against selected peptides, although not all recognized target cells expressed the correspondent HER protein.

As our technical approach resulted in highly specific T cell populations when targeting other antigens, especially differentiation antigens, our profound studies of HER2-specific T cell stimulation and isolated TCR with specificity for HER2-derived peptides further confirm that HER2 may be a problematic tumor associated antigen for T cells. Cross-reactivity of TCR for peptides derived from other HER2 family members was partially present and cannot be certainly ruled out for alternative proteins which would imply that selected peptides may not be optimal. However, our data would be also explainable by published data about a negative impact of HER2 expression on peptide processing and presentation potentially being responsible for the lack of correlation between mRNA expression and MHC peptide recognition previously communicated also by others. Future analyses will aim to improve understanding of the molecular mechanism of HER2 dependent antigen specific target reactivity.

G-CSF directly affects CD8$^+$ cytotoxic antigen-specific T cells and impairs their functionality

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Cellular therapies have become a powerful tool to complement impaired immune reconstitution in patients after hematopoietic stem cell transplantation (HSCT). HSCT patients are at risk of experiencing recurrent reactivation of persisting viruses like Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) or lytic viruses like Adenovirus (ADV). If available, seropositive stem cell donors may serve as a source for antiviral T cells. However, we and others showed that granulocyte-colony stimulating factor (G-CSF)-mobilization has negative effects on T-cell function in an antigen-dependent and independent way. *In vivo* and *in vitro* G-CSF treated antiviral T cells were found to be impaired in their cytotoxicity, the secretion of effector molecules and degranulation capacity. So far, the mechanisms of G-CSF treatment affecting CD8$^+$ T cells are not known. An indirect effect via antigen presenting cells (APCs) or CD4$^+$ T cells is assumed.

As the G-CSF receptor (G-CSFR) was found to be expressed on CD8$^+$ T cells, we aimed to elucidate the mechanism of G-CSF treatment on antigen-specific T cells. Isolated CD8$^+$ T cells were stimulated *in vitro* in the presence of bead-based viral peptide-loaded artificial APCs (aAPCs). This setup was chosen to exclude any influence from G-CSF-treated non-T-cell populations. After stimulation the proliferation capacity, cytotoxicicity in response to peptide-loaded target cells and the secretion of effector molecules was determined. Additionally, mRNA expression profile and telomere length analysis were performed in CD8$^+$ T cells from G-CSF-mobilized donors.

Our results showed for the first time, that G-CSF treatment directly affects antiviral CD8$^+$ T cells resulting in a decreased T-cell functionality. Interestingly, the proliferation capacity and the viability of the antigen-specific T cells are not hampered. The secretion of IFN-γ in response to peptide-loaded target cells was reduced by 33% after *in vitro* treatment. *In vivo* analysis of mRNA expression levels showed changes in surface marker expression like CD57, CD95L or CD137 and proteins involved in signaling pathways associated with G-CSF. The length of CD8$^+$ T-cell telomers of *in vivo* G-CSF treated donors was unaffected.

In further studies we investigate the influence of G-CSF treatment on miRNA-21, - 139, -150 and -155 in antiviral T-cells and the expression of the G-CSFR isoforms I and IV and observed differences in miRNA expression and functionality with respect to the different isoforms expressed. Freshly isolated T cells and the cytotoxic T-cell line TALL-104 were transduced with lentiviral vectors encoding either isoform I or IV and their impact on cytotoxicity was evaluated.

Taken together, our results demonstrate that G-CSF directly affects CD8$^+$ antigen-specific T cells. Our study shows that during G-CSF mobilization, the functional activity of antiviral memory T cells is impaired, indicating that even stem cell donors may not be the best source of T cells.

One U-CAR - multiple targets: a new approach to provide multiple antigen specificities to genetically engineered T cells for cancer treatment

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ORAL SHORT TALK

The adoptive transfer of autologous, patient-derived T cells engineered with chimeric antigen receptors (CARs) is currently considered as a highly promising therapeutic option for treatment of otherwise incurable malignant diseases. CARs combine the cellular and humoral arm of the immune response by assembling a single-chain fragment variable (scFv) as binding moiety which provides the antigen-specificity and an activating immune receptor. It has been demonstrated both *in vitro* and *in vivo*, that CAR engrafted effector T cells mediate long-lasting anti-tumor responses. Moreover, in recent clinical trials CAR modified T cells efficiently eradicate leukemia of B cell origin in late-stage cancer patients.

Despite these promising results, there are still some critical issues remaining to be solved before the CAR technology can be widely applied for cancer treatment. One major limitation of the CAR approach is that the fixed binding moiety of CARs allows the recognition of only a single predefined antigen by each CAR construct. However, a monotherapeutic approach targeting only a single antigen implies the risk for development of tumor escape variants, which have lost the target antigen during treatment as reported in recent clinical trials (Grupp et al. 2013). To overcome limitations of the conventional CAR approach a flexible modular targeting system for CAR engrafted T cells was developed. First, T cells are engineered to express a universal CAR (U-CAR), which has specificity for a short peptide motif of 10 amino acids derived from a human nuclear protein. The second components of the modular system are individualized target module (TM), which provides tumor antigen specificity for U-CAR armed T cells. Here we provide first prove of concept for this new approach. Antigen-specific redirection of T cells armed with the universal CAR in the presence of different targeting modules against various antigens (PSCA, PSMA, CD33, CD123, CD19) was effective even at femtomolar concentrations of the targeting module. The modular approach of the U-CAR system also lowers the risk for life-threatening "on target, off site" side effects which can develop in case that CAR engrafted T cells recognize their target antigen on healthy tissue as reported from two clinical studies (Lamers et al. 2006, Morgan et al. 2010). Half-life time of small molecules like TMs in humans is very short, most likely restricted to a few hours, so in case of adverse reactions against healthy tissue or occurrence of excessive tumor lysis syndromes halting of TM infusion will dampen ongoing adverse immune reactions. Taken together, the modular nature of U-CAR technology will allow retargeting of autologous, patient-derived T cells to several antigens under controlled pharmacological conditions and has the potential to become a highly effective treatment option for late stage cancer patients with reduced risks for side effects.

Generation of patient-individualized AML-reactive donor CD8$^+$ T cells under GMP-compliant conditions for adoptive transfer in leukemia patients after allogeneic hematopoietic stem cell transplantation

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Effective treatment of patients undergoing allogeneic hematopoietic stem cell transplantation (AHSCT) for chemo-refractory acute myeloid leukemia (AML) depends on the graft-versus-leukemia (GvL) effect mainly mediated by donor T cells. Unfortunately, this effect is frequently hampered by alloreactivity of donor lymphocytes resulting in graft-versus-host disease (GvHD) and insufficient GvL-reactivity to prevent relapse. Thus, protocols that include additional cellular therapy to improve antileukemia responses are warranted. In the present study we therefore sought to establish a GMP-compliant manufacturing protocol for producing highly AML-reactive, patient-individualized donor CD8$^+$ T lymphocytes to confer sustained antileukemic immunity while reducing the risk of GvHD in leukemia patients post AHSCT. Based on a reliable protocol published previously (Albrecht et al., 2011, Cancer Immunol. Immunother.) naïve CD45RA$^+$CD8$^+$ T cells from healthy donors were immuno-magnetically isolated by the MACSTM technology and stimulated with fully HLA class I-matched, primary AML blasts under optimized cytokine conditions comprising Interleukin (IL)-7/-12/-15 and -21 in allogeneic mixed lymphocyte/leukemia cultures (MLLCs) for 28 - 42 days (d). IL-12 was replaced by IL-2 after 14d of culture. In comparative studies MLLCs were then substituted with research-grade reagents or available GMP-grade components (IL-2, -7, -15, -21, TexMACS® medium, human serum) to define optimal GMP-culture conditions. Moreover, a newly developed 96/24 well micro structured cell culture system was tested in first studies to allow the setup of GMP-compliant MLLCs. So far, five different HLA class I-identical patient-donor MLLCs using naive CD8$^+$ T cells either isolated by the non-GMP-grade Naïve CD8 T cell isolation Kit or a GMP-guided CD45RO depletion/CD8 isolation procedure were performed and compared for purity of CD45RA$^+$CD8$^+$ cells, growth/expansion rates and numbers of individual AML-reactive T cell populations obtained from these MLLCs following culture in titrated standard research-grade versus (vs) GMP-grade components. In addition, we examined AML-reactivity of these individual populations by IFN-g ELISpot analyses. While purity was slightly higher (~100% vs ~90%) using the Naïve CD8 T cell isolation Kit, the frequency and AML-reactivity of T cells obtained upon culture in the presence of research-grade vs GMP-grade cytokines was overall comparable. Finally, generation of leukemia-reactive T cells required the presence of human serum despite the use of T cell compatible GMP-medium. In summary, AML-reactive T cells can be generated in vitro under GMP-grade conditions with comparable frequency and reactivity when compared to antileukemic T cells cultured at standard research-grade level. Further studies to evaluate the biological activity of these GMP-compliant T cells in a patient-tailored preclinical AML xenograft model and to validate the upscaling procedure are in progress.

Analysis and applicability of different in vitro models of Glioblastoma multiforme

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Glioblastoma multiforme (GBM) is the most common and lethal brain tumor in adults with a very dismal prognosis. Numerous efforts are made to investigate the cellular properties of GBM cells and to develop more effective therapies. In the era of personalized therapy a variety of new drugs and immune approaches with specific molecular targets are in the focus of research.

For the development of novel treatment strategies optimal in vitro models of GBMs are essential, generated from individual patients and analysed at early passages to avoid culture artefacts. Currently, a variety of cell culture methods with different properties are available. However, differences between the classical monolayer cell culture, spheroid culture and cultures in cancer stem cell selective medium (= serum-free, supplemented with growth factors) are not defined in detail. Thus, the aim of our study is to analyse differently cultured GBM cells established from the same primary tumor with a focus on their molecular biology and response to numerous drugs. First results show that some individual GBM cell lines grow as adherent monolayers in cancer stem cell selective medium, whereas GBM cell lines derived from other primary tumors only grow as multicellular spheroids under the same culture conditions. In consistency with the cancer stem cell hypothesis individual GBM cancer stem cell lines show an increased tumorigenicity in vitro compared to their respective serum culture counterparts as demonstrated by anchorage independent growth assays.

Proposed cancer stem cell markers like CD133 and Nestin appear to be not exclusively expressed in cancer stem cell cultures of our individual GBM models, but also in their respective standard culture counterparts. However, cancer stem cell cultures established from different primary GBMs show an elevated expression of FoxP3 compared to tumor cells cultured under standard conditions. These observations point towards a specific cell signalling and marker expression architecture in the cancer stem cell subpopulation that differs from the standard serum containing culture. The identification of cancer stem cell specific antigens has a great potential in specifically targeting this subpopulation of tumor cells with targeted drugs or immune approaches to prevent regrowth of the tumor.

Characterization of a new T-cell subset specific for tumors with antigen processing defects

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Tumors frequently evade immune recognition through shutdown of the antigen processing machinery, resulting in failure to present tumor antigens to cytotoxic T cells. The bottleneck in the processing pathway is the peptide transporter TAP, which translocates peptides into the ER for loading MHC class I molecules. We previously revealed that TAP-deficiency induces an unique alternative peptide repertoire and some of these peptides are immunogenic neo-antigens. We named these peptides TEIPP: 'T-cell epitopes associated with impaired peptide processing' (Van Hall, Nat Med, 2006). The prototypic TEIPP antigen is derived from the housekeeping protein Trh4, which is ubiquitously expressed, but selectively emerges in MHC-I on TAP-deficient cells.

We investigated the generation and behavior of TEIPP-specific T cells using a TCR-transgenic mouse, as these peptides are hidden ‚self antigens'. We observed an efficient thymic selection of TEIPP-specific $CD8^+$ T-cells and a strong activation by encountering cognate antigen *in vitro*, indicating that this novel anti-tumor T-cell subset is not hampered by central tolerance. Importantly, vaccination with synthetic peptide or irradiated tumor cells with forced overexpression of the Trh4 antigen resulted in massive expansion and effector functions of *in vivo* transferred T-cells. TAP-deficient tumors, however, failed to elicit T cell responses, unless co-stimulatory molecules were co-expressed or Trh4 was over-expressed, pointing at a weak capacity to cross-present this novel type of tumor antigens via host APC. Collectively, these findings implied that TEIPP-T cells are still naïve and unspoiled in tumor-bearing mice, but are ready to be exploited by vaccination. We are currently optimizing the protocols for effective eradication of TAP-deficient tumors that are otherwise resistant to conventional T cell specificities. Our study reveals unique characteristics of TEIPP T-cells as a new subset of anti-tumor lymphocytes and opens up a salvage treatment for tumors that display antigen processing defects.

T-cell donor registry for third party adoptive immunotherapy in hematopoietic cell and solid organ transplantation

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Infection and reactivation of human cytomegalovirus (CMV), Epstein-Barr virus (EBV) and adenovirus (ADV) are common complications of hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT). In recent years increasing numbers of viral pathogens expanding from herpes-simplex virus (HSV), polyoma virus BK, herpes virus (HHV)-6, respiratory syncytial virus, parainfluenza and influenza virus have been shown to cause substantial morbidity and mortality in the immunocompromised host. Adoptive immunotherapy with virus-specific T cells can effectively reconstitute antiviral immunity with limited acute toxicity or risk of GvHD. Third-party partially HLA-matched antiviral T cells from virus-seropositive individuals would offer an alternative option in patients receiving an allogeneic cord blood (CB) transplant or a transplant from a seronegative donor and since donor blood is generally not available for solid organ recipients. Furthermore, stem cell donors seem to be unsuitable T-cell donors because of an impaired functionality of antiviral memory T cells during and after mobilization with granulocyte colony-stimulating factor (G-CSF).

Frequency assessments of virus-specific memory T cells in more than 450 HLA-typed healthy donors as well as in HSCT/SOT donors using a high throughput T-cell assay were performed over a period of 4 years at Hannover Medical School. To identify the most efficient antigens for adoptive immunotherapy, we assessed the frequencies of CMV, EBV, ADV, HHV6 and BK-specific memory T cells in healthy donors in response to HLA-restricted peptides and overlapping peptide pools.

For each virus, we identified at least >60% potential CTL donors with highly significant differences in frequencies of T cells against viral antigens of different species as well as against different viral epitopes from one species. Among the antigens tested frequencies for CMVpp65, EBV-BZLF1, and HHV6-U90 peptide pools were highest. Overall frequencies of antiviral T cells detected by T-cell receptor staining were lower than those of the corresponding peptide pools. Especially in the case of ADV a donor response to a certain peptide may not be determined without prior short-term in vitro stimulation. In addition, confirmatory testing for CMV serology using western blot technique revealed approximately 15% false-positive results, possibly influencing future analysis and selection of potential SCT and T-cell donors.

We established a registry of potential T-cell donors ("T cells of interest" registry, TOI registry) which provides detailed information's about donors' HLA type (class I and II high resolution), virus serology, virus-specific T-cell frequencies, best T-cell detection method, and results of functional and alloreactivity assays. TOI registry will facilitate rapid availability of T cells for adoptive immunotherapy of virus-associated diseases in transplant recipients without an adequate T-cell donor.

Allogeneic lymphocyte-licensed DCs expand TCR/CAR-engineered T cells, which are insensitive to oxidative stress and immunosuppressive factors

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Adoptive transfer of genetically engineered, autologous T cells is a treatment strategy that has accomplished good clinical responses in cancer patients. Peripheral lymphocytes are isolated, stimulated, equipped with a new TCR or CAR receptor and expanded *ex vivo* before being reinfused back to the patient. Once transferred, the T cells should be able to circulate, expand, recognize and kill tumor cells for as long as tumor cells remain in the host. There are many aspects to consider for optimization of T cell therapies. One such aspect is how to best culture T cells *ex vivo* in order to retain their full potency upon transfer.

The two most commonly used protocols for expanding T cells in association with adoptive T cell therapy of cancer is to use beads with immobilized anti-CD3 and anti-CD28 antibodies together with IL-2 or to use the rapid expansion protocol (REP), which utilizes soluble anti-CD3 antibody (OKT-3), IL-2 and irradiated allogeneic feeder cells. However, bead- or REP-expanded T cells are sensitive to the harsh tumor microenvironment and often short-lived after reinfusion.

We have developed a new T cell expansion protocol based on preactivated, allo-sensitized allogeneic lymphocytes (ASALs) as helper cells to license allogeneic mature dendritic cells (DCs). The ASALs and DCs are prepared in advanced and can then be used together with OKT-3 and IL-2 to expand T cells upon request. We found that the ASAL/DC combination yields an enriched Th-1 polarizing cytokine environment (IFN-γ, IL-12). Furthermore, the ASAL-licensed DCs express co-stimulatory (CD80, CD86) and survival (CD70, CD40) molecules for T cell activation/stimulation as well as CD64 (FcgRI), which anchors the OKT-3 antibody and induce optimal proliferation/differentiation signals to $CD8^+$ T cell. This is reflected by the dual benefit of anti-CD3-TCR crosslinking and the costimulation provided by cell-cell interaction between T cells and $Fc\gamma RI^+$ DCs.

When genetically engineered T cells were expanded by this co-culture system, they showed better survival and cytotoxic efficacy under oxidative stress and when exposed to immunosuppressive cytokines, as well as superior proliferative response during tumor cell killing. Our results suggest a new method to expand T cells with improved quality for adoptive T cell therapy of cancer.

Generation of T-cell lines with broadened antigenic specificities to improve adoptive immunotherapy protocols for the treatment of nasopharyngeal carcinoma

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Background: Although adoptive infusion of EBV-specific T cell lines constitutes a promising strategy for nasopharyngeal carcinoma (NPC) therapy, however, the clinical benefit of current protocols is still poor. One major limitation is constituted by the restricted number of EBV antigens that can be targeted and their poor immunogenicity. The oncogenic EBV protein BARF1 is expressed in the majority of NPC cases and may constitute an optimal target. We have previously demonstrated that NPC patients have strong spontaneous CD4 and CD8 T cell responses against BARF1 protein and derived epitopes. Moreover, BARF1-specific cytotoxic T lymphocytes (CTLs) can be easily generated from healthy donors. Nevertheless, T cell lines obtained with current protocols for adoptive immunotherapy of NPC include only negligible numbers of BARF1-specific CTLs, pointing to the need to implement these T-cell cultures in BARF1 specificities.

Aim: To develop optimized, GMP-upgradable protocols for adoptive immunotherapy of NPC based on the generation of T-cell lines enriched in BARF1-specific effectors.

Methods: Lymphoblastoid cell lines (LCLs) were treated with different EBV lytic cycle inducers at doses able to induce abortive or limited EBV replication while preserving cell viability. Expression of BARF1, LMP1, ZEBRA and EA mRNA was quantified by qRT-PCR. EBV-specific donor- and patients-derived CTLs were generated with LCLs treated with the different drugs from PBMCs. Standard cytotocxicity assays were used to assess the specificity of CTLs. Content in Granzyme B granules was assessed by multispectral imaging flow cytometry.

Results: T reatment with low doses of doxorubicin (DX) proved to be the most suitable and simple protocol to enhance BARF1 expression (3.3 fold increase), without down-regulating other viral antigens that are targeted by EBV-specific CTLs. By contrast, TPA/Na-butyrate (TPA+NaB) or cisplatin (CSP) were less effective in up-regulating BARF-1 and induced higher levels of cell apoptosis. CTLs induced with DX-treated LCLs (DX-CTLs) showed high levels of specific cytotoxicity against NPC cells endogenously expressing BARF1 (c666.1-A2, >90% of specific lysis) or T2A2 cells loaded with BARF1 or LMP1 HLA-A2 peptides (30% specific lysis). CTLs generated with LCLs either untreated or exposed to TPA+NaB of CSP induced only low levels of BARF1 or LMP1 specific cytotoxicity. Notably, the extent of specific lysis induced by DX-CTLs was higher against the BARF-1 peptides. In addition, qRT-PCR and Western blot showed up-regulation of HLA class I and immunogenic cell death markers activation in DX-LCLs. DX-CTLs have also a higher content in Granzyme B granules. The identified protocol were also verified using patients-derived LCLs.

Conclusions: These findings provide the rationale for a rapid up-grading at the GMP level of the use of DX-treated LCLs for the generation of CTL lines enriched in BARF1 specificities for adoptive immunotherapy of resistant or relapsing NPC.

GMP compliant expansion of CMV- and EBV-specific, donor derived, peptide-stimulated T-cells from G-CSF mobilized stem cell grafts

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Reactivation of CMV and EBV after allogeneic stem cell transplantation (aSCT) impacts negatively on outcomes. Following aSCT approximately 20-30% of all patients reactivate EBV and 40-50% CMV, respectively. Specific antiviral therapy is only available for CMV. With the exception of Ganciclovir all drugs are being used outside their approved indication. Similarly, the use of Rituximab as B-cell depleting antibody seems to be effective in case of EBV reactivation, but bears strong side effects including long term B-cell depletion making frequent transfusion of immunoglobulins a must. Furthermore, all therapies are cost-intensive. We have developed a manufacturing protocol according to GMP standards that allows the generation and expansion of T-cells with specificity for CMV and EBV out of the G-CSF mobilized stem cell graft from EBV and CMV seropositive donors. G-CSF mobilized peripheral blood mononuclear cells (PBMC) were purified using Ficoll gradient centrifugation and stored in liquid nitrogen. After thawing, up to $1x10^9$ PMBC were stimulated with 21 peptides derived from CMV and 29 peptides derived from EBV. All peptides exhibit a defined HLA restriction covering 80% of the central European population with at least one HLA class I haplotype. After peptide stimulation, cells were incubated in closed system culture bags for 9 days at 37°C, 5% CO_2 in GMP certified media supplemented with 50IU/ml IL-2.

Reactivity of PBMC for both viruses prior to peptide stimulation ranged from 0.1% to 0.7%, as tested by ELISpot; on average 15% of all cells showed reactivity against EBV and CMV after peptide restimulation, resulting in a more than 20-fold expansion of reactive T-cells. Flow cytometric analyses by pentamer staining confirmed ELISpot results showing virus-specificities up to 60% as well as an activated phenotype. Activation marker is up-regulated upon 9 days of culture with CMV/EBV-peptides.

This protocol exhibits several advantages: (i) The stem cell graft can be used as a source for PBMC, no second apheresis is required. (ii) PBMC as raw material can be stored stably until generation of T-cells is desired. (iii) The manufacturing process makes use of standard clean room equipment. (iv) The protocol can be easily adapted to local requirements and preferences. (v) Costs for production would be competitively low compared to current prices for antiviral therapies and associated complications.

Genetically modified natural killer cells targeting EGFR-expressing malignancies

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Natural killer (NK) cells are the body´s first line of defense against viral infections and malignant cells. In addition to primary NK cells, continuously expanding cytotoxic cell lines such as NK-92 hold promise for cancer immunotherapy. The therapeutic utility of such cells can be further enhanced by genetic modification with chimeric antigen receptors (CARs), which recognize antigens differentially expressed on the surface of tumor cells. CARs typically consist of an extracellular antigen-specific single-chain antibody fragment (scFv) fused via a flexible hinge and transmembrane region to an intracellular signaling moiety such as CD3 zeta chain or a composite CD28-CD3 zeta fusion molecule. Here we generated second generation CAR constructs specific for the epidermal growth factor receptor (EGFR) and its variant EGFRvIII. Overexpression and malignant activation of EGFR has been described for many tumors of epithelial origin. Likewise, enhanced EGFR levels and expression of the constitutively active EGFR mutant form EGFRvIII are a particular feature of glioblastoma. We developed a panel of humanized and codon-optimized CARs that employ scFv antibody fragments either recognizing epitopes restricted to wildtype EGFR or ERGFRvIII, or an epitope common to both target receptors. For signaling these CARs carry a composite CD28-CD3 zeta domain. Upon lentiviral transduction of NK-92 cells, single cell clones were derived and characterized for CAR expression. Functional analysis *in vitro* revealed high and specific cytotoxicity of CAR-expressing NK-92 cells towards established and otherwise NK-resistant glioblastoma cells carrying EGFR or EGFRvIII. Furthermore, anti-tumoral activity of retargeted NK-92 cells was demonstrated in a subcutaneous glioblastoma xenograft model in NOD/SCID common gamma chain knockout (NSG) mice. Ongoing work now aims at the evaluation of retargeted NK-92 cells in orthotopic tumor models based on established and primary glioblastoma cells.

The mode of activation impacts on the differentiation status of gene modified T cells used in adoptive immunotherapy

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Introduction: Generating bulk T cell populations with defined anti-tumour specificity requires genetic manipulation, which requires the combination of viral vectors with strong mitogenic stimulation to ensure high surface expression of the receptor. However, this simultaneously drives T cell differentiation. Current thinking suggests T cells of an earlier differentiation state are more capable of long-term persistence, potentially driving improved anti-tumour response in the solid tumour setting. We therefore characterised the degree of differentiation promoted by several activation agents, in both mixed and early differentiated human T cells populations.

Methods: Mixed T cells and CCR7$^+$ cells from healthy donors were activated with either αCD3/αCD28 coated plates; αCD3/αCD28 coated microbeads or αCD3/αCD28 coated artificial antigen presenting cells. T cells were transduced with a retrovirus containing the MART-1 specific DMF5 TCR before phenotypic and functional characterisation after 14 days in IL-2 culture.

Results: Both mixed and CCR7$^+$ T cell populations activated with αCD3/αCD28 coated microbeads demonstrated significantly faster growth kinetics and although all T cells exhibited a degree of differentiation, mixed and CCR7$^+$ cells activated with αCD3/αCD28 coated microbeads had significantly more central memory T cells with enhanced co-stimulatory marker expression. Bead activated cells also produced the lowest IFNγ levels upon co-culture with HLA-matched MART-1$^+$ melanoma cell lines. Interestingly, no difference in functionality was noted in late differentiated CCR7$^-$ populations, irrespective of the activation stimulus used.

Conclusion: Activation with αCD3/αCD28 coated microbeads maintains gene modified T cells at a less differentiated stage which may be beneficial for studies of long-term adoptive T cell transfer

New genetic adjuvants for improving T cell function in adoptive cell therapy of cancer

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ORAL SHORT TALK

Recent results obtained in clinical trials of cancer immunotherapy evaluating the adoptive transfer of anti-tumor T cells show great promise. One approach in this field exploits tumor-infiltrating lymphocytes (TILs) isolated from fresh tumor biopsies, which are capable of recognizing tumor antigens through their endogenous TCR. Another approach which gains momentum utilizes autologous T cells genetically engineered to recognize tumor through either MHC-bound peptides by exogenous TCR genes or surface antigens with genes encoding chimeric antigen receptors (CARs). While reports of high clinical response rate are encouraging, new strategies are needed for improving T cell survival and tumoricidal activity and reducing the severe toxicity often associated with the clinical protocols. To enhance the survivability and functional properties of anti-tumor T cells and minimize toxicity we created three classes of genetic adjuvants designed to operate autonomously, independently of immunomodulatory agents. One class encodes membrane-attached derivatives of IL-2, single-chain IL-12 and IL-15. The other produces constitutively-active (ca) toll-like receptors devoid of their ligand-binding domain. The third encodes a novel homo-oligomerizing configuration of tumor necrosis factor receptors, inducing ligand-free activation. Aside from caCD40 presented here, evaluation of ca4-1BB and caOX40 is underway. For gene delivery into human TILs or polyclonal T cells we have been using electroporation of in-vitro-transcribed mRNA.

In the complete absence of exogenous cytokines, mRNA-encoded membrane IL-2, IL-12 and IL-15 supported the proliferation of human CD8 and CD4 T cells for at least 6 days post-transfection, comparably to exogenous IL-2. In CD8 T cells adjuvants elevated IFN-γ, OX40, CD25 and CD69. In anti-melanoma TILs they induced a panel of cytokines and chemokines, including high level IFN-γ, displaying a marked synergistic effect. Even when this initial effect waned, a considerably higher fraction of transfected, compared to control-transfected TILs, responded robustly to autologous, but not mismatched, melanoma. Transfected short-term cultured ´young´ TILs revealed exceptional up-regulation of 4-1BB, OX40, CD25 and CD28 and an increase in IFN-γ production, but no change in the inhibitory receptors PD-1 and CTLA-4.

Our findings so far suggest that this new panel of genetic adjuvants can substantially improve the functional properties of anti-tumor T cells, offering a new tool in cancer immunotherapy through adoptive T cell transfer.

A novel form of dendritic cell (DC) for immunotherapy: CD137 ligand-generated DCs are more potent than conventional DCs in inducing cytotoxic T-cell responses

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Dendritic cells (DCs) are professional antigen-presenting cells that are essential in regulating and orchestrating T-cell-based immunity. This property has prompted intense research in the use of DC-based immunotherapy. Nevertheless, DC vaccination is still in its infancy and improvements in terms of DC preparation, antigen-loading and increasing their immunostimulatory capacity are required. Here, we describe a potent and novel type of DC generated by CD137-ligand reverse signalling into peripheral monocytes.

The ligand for CD137 (CD137L) is expressed on peripheral human monocytes and delivers a potent activating signal via reverse signalling. Treatment of monocytes with a recombinant CD137 protein that induces reverse signalling through CD137L reduces typical macrophage characteristics such as phagocytosis and CD14 expression. However, distinct DC characteristics including endocytosis, costimulatory molecule expression and the ability to stimulate proliferation of allogeneic naïve T-cells are induced. Allogeneic $CD8^+$ T cells activated by CD137L-generated DCs (CD137L-DCs) are able to induce the demise of target cells more potently than T cells activated by conventional DCs. Additionally, CD137L-DCs can be further matured which leads to an increase in DC marker expression such as CD83, CD86 and HLA-DR. This in turn enables a stronger activation of allogeneic T cells.

Using gene expression arrays and protein expression analysis, we deduced that CD137L-DCs express high levels of ALCAM, a ligand for CD6 on T cells and which their long-term interaction has been reported to contribute to T-cell proliferation. Indeed, we find that the blockade of ALCAM-CD6 engagement using antagonist antibodies partially abrogates T-cell proliferation, suggesting that ALCAM expression is important for CD137L-DC's function. Next, we also tested the ability of CD137L-DCs to initiate antigen-specific T-cell activation in an autologous setting. Using cytomegalovirus (CMV) pp65 peptides, we show that mature CD137L-DCs are able to activate pp65-specific T cells more potently than conventional DCs as evidenced by an enhanced IFN-gamma production by both $CD4^+$ and $CD8^+$ pp65-specific T cells. More importantly, these T cells are able to induce a stronger antigen-specific killing of HLA-A2 matched target cells as compared to T cells activated by conventional DCs. These *in vitro* data show that CD137L-DCs are potent antigen-presenting cells and should be evaluated for human anti-tumour immunotherapy.

NY-ESO-1 108-116 and NY-ESO-1 120-128 are not epitopes for TCR gene therapy

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T cell receptor (TCR) gene therapy, the grafting of a new antigen specificity onto patients' T cells by gene transfer, is a promising approach for cancer treatment. For TCR gene therapy, the cancer-testis antigen NY-ESO-1 is an interesting target, since it appears to be expressed only in testis, placenta trophoblasts, presumably the thymus, and also cancer cells. So far, only NY-ESO-1 157-165 with suboptimal binding to HLA-A2*0201 (IC_{50} = 1262 nM), was described. Here, I tested two other potential epitopes with better prediction bindings, NY-ESO-1 108-116 (IC_{50} = 63 nM) and NY-ESO-1 120-128 (IC_{50} = 326 nM), to determine their immunogenicity in transgenic AB*ab*DII mice carrying the human TCR alpha-beta gene loci and the human MHC I *HLA-A2*0201* gene. These transgenic mice are non-tolerant to NY-ESO-1 and avoid the problem of deletion of high avidity T cells due to self-tolerance in humans. My results demonstrate that NY-ESO-1 108-116 elicits a specific $CD8^+$ T cell response. However, the peptide was not endogenously processed, since specific $CD8^+$ T cells did not respond when co-cultured with NY-ESO-1 endogenously expressing cells. Furthermore, I found no evidence that NY-ESO-1 120-128 is immunogenic. Taken together, the results demonstrate that NY-ESO-1 108-116 is immunogenic but not endogenously processed, whereas NY-ESO-1 120-128 is not immunogenic. Both results suggest that these peptides are not suitable epitopes, although they are predicted as stronger binders to HLA-A2*0201 in comparison with NY-ESO-1 157-165.

TCRs from CD4$^+$ T cells are expressed and functional in both CD4$^+$ and CD8$^+$ T cells upon T-cell redirection

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Adoptive transfer of genetically engineered T cells offers great opportunities in cancer immunotherapy. Most studies have focused on the transfer of HLA class I-restricted T-cell receptors (TCRs), however, several preclinical and clinical trials have shown the importance of CD4 T-cell help during this type of therapy.

We have identified and cloned HLA class II-restricted CD4$^+$ T cells isolated from patients vaccinated with the long peptide GV1001 derived from telomerase (hTERT 611-626). In some patients with stage III lung cancer and stage IV melanoma we observed an outstanding clinical response to vaccination with >10-year survival. Furthermore, strong T-cell responses against hTERT peptides other than the vaccination peptide were detected, suggesting epitope spreading. After studying several such patients, T-cell responses against certain peptides associated with clinical benefit were identified and formed the basis for a second generation peptide vaccine currently tested in the clinic. CD4$^+$ T-cell clones recognizing novel hTERT peptides were isolated. These HLA-DR and -DQ restricted T-cell clones recognized target cells loaded with a 30-mer peptide (hTERT 660-689) at concentrations as low as 0.06 µM and one of the CD4$^+$ Tc clones was also reactive against a melanoma cell line with the corresponding HLA allele.

The TCRs from these CD4$^+$ T-cell clones were identified and separately cloned into an mRNA expression vector. Transient TCR expression represents a safer alternative compared to stable gene transfer for the first clinical evaluation of novel TCRs, but requires multiple T-cell infusions to compensate for the short-lasting transgene expression.

Electroporation of mRNA encoding the TCRs into polyclonal, *in vitro* expanded human T cells showed that both CD8$^+$ and CD4$^+$ T cells expressing the TCR were functional following co-incubation with peptide-loaded targets. Redirected T cells produced TNF-α, IFN-γ and some IL-2 upon target recognition and became CD107a positive.

T-cell redirection using such HLA class II-restricted TCRs may be useful both in haematopoietic malignancies and in melanoma where tumour cells often express HLA class II. In addition, combining the redirection of T cells with both HLA class I- and class II-restricted TCR may provide a more powerful therapeutic effect in adoptive T cell therapy.

High-affinity CD20-specific TCRs suitable for adoptive immunotherapy can be readily isolated from the allo-repertoire using reverse immunology

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ORAL SHORT TALK

Studies using Tcell receptor (TCR) or chimeric antigen receptor (CAR) transduced T cells have shown the effectiveness of adoptive immunotherapy to treat different malignancies. The efficacy and safety of such interventions greatly depends on good target selection to prevent off-tumor toxicity. Tumor-associated self-antigens which are either overexpressed in malignant tissue or demonstrate tissue-restricted expression present attractive targets. However, the broad application of TCR-based adoptive immunotherapy directed against such targets is hampered by a lack of an effective immune response against self-antigens. Through self-tolerance, T cells carrying high-affinity TCRs reactive to self-antigens are deleted during negative thymic selection. Exploiting the immunogenicity of allogeneic human leukocyte antigen (alloHLA) molecules to generate an effective immune response against self-antigens can be an attractive strategy to overcome self-tolerance. Here, we describe a protocol to efficiently isolate highavidity alloHLA-restricted T cells targeting the B-cell compartment.

From a B-cell peptide elution library 15 peptides derived from genes exhibiting Bcell restricted expression were identified and peptide-MHC multimers (pMHC) of HLAA*0201 were generated. Via MACSorting and FACSorting a plethora of pMHCbinding Tcell clones from HLA-A*0201-negative individuals were isolated. Generated Tcell clones were selected based on peptide-specificity and avidity for further characterization.

We successfully isolated two distinct T-cell clones carrying high-affinity TCRs specific for a CD20 peptide presented in HLAA*0201. CD20 dependent recognition could be demonstrated by genetically engineering CD20-negative K562-A2 cells to express CD20. The isolated Tcell clones efficiently recognized CD20expressing HLAA*0201 primary chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and mantle cell lymphoma (MCL), while recognition of CD20-negative hematopoietic and non-hematopoietic cell-subsets was absent. In addition, the CD20 specific T-cell clones were able to more efficiently recognize ALL cell-lines than CD20 specific antibodies. We demonstrated that on target cells with only very low CD20 surface expression, the CD20 specific T cell clones could still efficiently recognize endogenously processed CD20 derived peptides in the context of HLA-A*0201.

In summary, we developed a platform for the rapid identification of high-affinity TCRs of therapeutic relevance targeted to self-antigens by combining gene expression data with valuable information on peptide processing from peptide elution studies and exploiting the immunogenicity of allogeneic HLA. Using this platform we successfully isolated CD20-specific TCRs which could broaden the application of immunotherapies targeted to CD20 in cases were CD20-cell surface expression is low. Based on its general principle the developed platform could easily be adapted to target other malignancies.

$CD40L^+$ $CD8^+$ T cell-dependent antitumor immunity

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Adoptive T cell therapies are a promising approach to treat cancer. However, which subset and functional quality of $CD8^+$ T cells is more suitable for achieving effective and durable responses in the strong immunosuppressive context of cancer is still a matter of debate and investigation. We detected recently that in average 25% of human memory $CD8^+$ T cells express CD40L, a strong immunostimulatory molecule of activated $CD4^+$ T helper cells that is even able to overcome cancer-induced immunosuppression. Therefore, we analyzed the role of this distinct $CD8^+$ T cell population in antitumor responses.

First, we assessed the expression of CD40L on $CD8^+$ T cells in a tumor model where B6 mice were challenged with SV40 TAg-expressing cancer cells. Here $CD40L^+CD8^+$ T cells represented 50% of the protective tumor-specific $CD8^+$ T cell response. To analyze the impact of CD40L expression on $CD8^+$ T cells *in vivo* we challenged $Rag1^{-/-}$ mice with cancer cells and adoptively transferred wt or $CD40L^{-/-}CD8^+$ T cells. Application of wt $CD8^+$ T cells prevented the formation of solid tumors, whereas transfer of $CD40L^{-/-}CD8^+$ T cells alone or together with wt $CD4^+$ T cells resulted in a non-controlled tumor progression. The necessity of CD40L on $CD8^+$ T cells for tumor rejection was further demonstrated by injecting cancer cells in mice that lack CD40L expression only on mature $CD8^+$ T cells. This newly generated E8Icre x CD40Lflox mouse strain was significantly more susceptible to tumor formation than wt mice. Since the injection of the cancer cells in complete $CD40L^{-/-}$ but not in $CD40^{-/-}$ mice resulted in tumor formation, we assume that CD40L-mediated rejection does not depend on interaction with $CD40^+$ host cells but more on CD40 expression by the cancer cells.

Taken together, our data reveal that the presence of tumor-specific $CD40L^+CD8^+$ T cells may represent a crucial element in control and rejection of tumors. Therefore the capability of $CD8^+$ T cells to express CD40L should be considered and implemented in future adoptive T-cell therapies against cancer.

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Neo-antigen enriched TIL therapy mediates superior tumor eradication in a patient-derived xenograft model of human melanoma

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ORAL SHORT TALK

The mutational load in melanoma is high relative to that in most other human malignancies, resulting in the possible expression of large numbers of patient-specific mutated antigens. This may, in part, explain the immunogenicity of this disease and the high rate of responsiveness to immunotherapeutic strategies such as tumor-infiltrating lymphocyte (TIL) therapy. Indeed, recent data have shown that cytotoxic T-cell reactivity targeting neo antigens may be common in human melanoma TILs. Importantly though, the clinical relevance of neo-antigen specific T cell populations remains uncertain.

To directly address the tumoricidal potential of defined neo-antigen specific T cell populations, we first identified two neo-antigen specific T-cell populations within a bulk melanoma TIL culture by the combination of exome sequencing and MHC multimer-based T cell screens. Subsequently, we generated TIL products that are highly enriched for these neo-antigen reactivities and we compared the anti-tumor activity of these neo-antigen enriched TIL with that of ‚standard' bulk TIL in NSG mice bearing the autologous tumor. We observed outgrowth of the tumors in mice treated with standard TIL. In contrast, tumors in mice treated with enriched TILs were controlled long-term. Additional experiments showed that this superior activity of neo-antigen enriched TIL was caused by the increased numbers of T cells with a high anti-tumor activity, rather than the depletion of cell populations with inhibitory activity. Furthermore, once tumors eventually recurred these were still recognized *in vitro* by cells from the initially infused TIL culture.

Together, these preclinical data demonstrate that neo-antigen reactive T cells within bulk TIL cultures form a critical component of anti-tumor reactivity and provide a further basis to target mutated antigens with cancer immunotherapy.

Generation of T cell receptor transgenic antigen-specific HLA-A*0201-restricted cytotoxic T cells directed against Ewing Sarcoma associated target antigens

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Introduction: Advanced Ewing Sarcomas (AES) are associated with poor prognosis. Despite multimodal therapeutic approaches including surgery, irradiation and chemotherapy, overall survival does not exceed 15 per cent. Allogeneic stem cell transplantation (allo-SCT) has been applied as an experimental option for patients with AES with mixed results. We aim to generate T cell receptor (TCR)/CD20 co-transduced allorestricted T-cells directed against ES specific or selective antigens for adoptive transfer.

Methods and Results: ES associated antigens PAPPA, ADRB3, and PRSS35 were elected as target antigens. Suitable peptide candidates with high HLA-A*0201 affinity were selected and tested for their MHC binding. Antigen specific, allogenic HLA-A*0201 restricted cytotoxic T cells were generated by priming HLA-A*0201- T cells with HLA-A*0201+ dendritic cells (DCs). Positive cells were isolated via FACS sorting. After single cell dilution antigen specific T cells were identified and characterized via ELISpot assays for interferon gamma and granzyme B. For further characterization T2 peptide binding assays with decreasing peptide concentrations were used. LCL from different donors were used to test for cross reactivity. For the most promising clones the alpha and beta chain of the corresponding TCR have been identified via PCR and cloned into a retroviral vector system. Sequences underwent codon optimization and minimal murinisation of the constant region. After retroviral transduction, donor T-cells expressed the transgenic TCR, stained highly positive in FACS analyses and showed peptide specific recognition in ELISpot assays using T2 cells. Transduction of T cells with codon optimized CD20 already resulted in $CD20^+CD8^+$ double positive T cells, rendering these cells susceptible for Rituximab (RTX) in case of unexpected cross-reactivity *in vivo*.

Conclusion: We have generated specific cytotoxic T cells directed against tumor-associated antigens in ES. *In vitro* assays verified their target specificity, avidity, and lack of cross-reactivity. After identification of the TCR and viral transduction, donor T-cells still show specificity towards their peptide-MHC complex. Further a transduction of CD20 for a suicide switch generates $CD8^+CD20^+$ double positive T-cells for a complement depletion of these cells using RTX in case of an adverse event. These cells constitute a tool to eradicate minimal residual disease after allo-SCT in the future.

Isolation of a TCR with specificity for a naturally presented ITGA2B epitope for the treatment of therapy refractory myeloid leukemias by TCR-transgenic T cells

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Acute myeloid leukemias are highly aggressive diseases with often unfavorable prognosis. Allogeneic hematopoetic stem cell transplantation and subsequent donor lymphocyte infusions are effective, still treatment failure is frequent. Redirection of T cells by chimeric antigen receptors or T cell receptors (TCR) are currently considered as novel therapeutic strategy in order to selectively target leukemia cells. In order to identify suitable target structures for TCR we recently used the immunopeptidomic approach and identified novel HLA ligands that are derived from genes with restricted expression to the hematopoetic system and presented on different HLA molecules. We furthermore established a workflow that allows isolation of peptide specific T cells after stimulation of naïve T cells in the single HLA-mismatched setting with the aim to generate a biobank of leukemia-reactive TCR. Using that workflow we were previously able to isolate a leukemia reactive TCR with specificity for an HLA-*B*07:02*-restricted MPO epitope by using HLA multimer based cell sorting (Klar *et al.* Leukemia *in revision*). We furthermore identified an HLA-*B*15:01*-restricted epitope ($ITGA2B_1$) of Integrin alpha IIb (ITGA2B), which is expressed in megakaryocytes and platelets and shows high expression in different myeloid neoplasms. As the generation of $ITGA2B_1$-HLA multimers was not successful, we aimed to isolate T cells with specificity for this peptide by IFN-γ capture assay.

Therefore, naïve T cells of an HLA-*B*15:01* negative healthy donor were co-incubated with peptide-pulsed single HLA-mismatched dendritic cells and restimulated on day 10. On day 20, cells were co-incubated with peptide-pulsed C1R-B15 cells and IFN-γ producing cells were isolated with the *IFN-γ Cell Enrichment Kit* (Miltenyi). After cloning by limiting dilution we were able to isolate a clone and its TCR (b5-7D) that shows peptide specific lysis of T2-B15 cells as well as IFN-γ production after co-incubation with $ITGA2B_1$ pulsed T2-B15, C1R-B15 and K562-B15 cells. We furthermore could observe a high functional avidity of TCRb5-7D-transgenic T cells with an EC_{50} value of $1.5x10^{-9}$M. Most importantly, reactivity against the HLA-*B*15:01*-transduced myeloid leukemia cell lines HL-60, NB4, KG1a and K562 has been observed but not against the lymphoid cell lines C1R and Molt4. Further experiments to analyze recognition of healthy cell populations and primary leukemia cells are currently ongoing.

Taken together, we herein present a leukemia-reactive TCR with specificity for a previously identified HLA-*B*15:01* ligand of the megakaryocytic antigen ITGA2B that was isolated in a single HLA-mismatched setting by IFN-γ capture assay. This is a further proof - beneath the previously described MPO-specific TCR2.5D6 - that the combination of immunopeptidomics and isolation of TCR in the single HLA-mismatched setting is a powerful method to develop effective tools for the treatment of therapy-resistant leukemias.

Leukoreduction system chambers as a novel and highly economic source of viable and functional monocyte-derived dendritic cells and lymphocytes

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The demand for human monocyte-derived dendritic cells (moDCs), as well as for primary human B and T lymphocytes for immunological research purposes has increased in recent years. Classically, these monocytes are isolated from blood, leukapheresis products or buffy coats of healthy donors by plastic adherence of peripheral blood mononuclear cells (PBMCs), followed by stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, while lymphocytes are usually isolated from the non-adherent fraction (NAF) by magnetic cell sorting. However, donor-blood is a limited resource and not every blood bank offers leukapheresis products or buffy coats for laboratory use. Additionally, a leukapheresis is very expensive and also the generation / isolation of cells is time- and cost-intensive. To overcome some of these obstacles, we evaluated if low-cost leukoreduction system chambers (LRSCs), which arise after routine donor plateletpheresis procedures, and are usually discarded, would be an alternative and appropriate source of PBMCs to generate moDCs and to isolate lymphocytes. By analyzing the number and phenotype of immature and mature dendritic cells (DCs), as well as of B and T lymphocytes derived from LRSCs, we found all cells to be of high quantity and quality. Further investigations on DCs comprising transwell migration assays, allogeneic mixed lymphocyte reactions (MLR), cytokine secretion assays, and cytotoxic T cell induction assays revealed high migratory, as well as stimulatory capacity of these cells. In addition, DCs and T cells were efficiently electroporated with mRNA and showed characteristic cytokine production after co-culture, demonstrating LRSCs as an efficient, valid, and economic source for generation of moDCs and lymphocytes for research purposes.

A new bispecific T cell recruiting antibody enhances anti-tumor activity of adoptive T cell transfer

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Introduction: A limiting step for adoptively transferred tumor-specific T cells is their recruitment from the blood circulation to the proximity of tumor cells and subsequent engagement in direct tumor cell contact. We hypothesized that a bispecific antibody recruiting T cells to a target antigen on tumor cells could enhance T-cell-tumor interaction and thus increase the efficacy of adoptively transferred T cells.

Methods: A new bispecific murine IgG2a antibody (BsAb) was generated that recognizes EpCAM as a tumor antigen and truncated EGFR (D-EGFR) as an inert surface marker protein on transduced T cells. T cells from transgenic mice for TCR specific for the SV40 large T antigen (TCR-1) were retrovirally transduced with D-EGFR. S.c. tumors were induced in C57Bl/6 mice by injecting mGC8 cells derived from a syngeneic large T antigen expressing EpCAM-positive gastric tumor.

Results: *In vitro,* the BsAb increased (4-fold) binding of transduced T cells to EpCAM positive tumor cells. In the presence of the BsAb, tumor-directed T cells efficiently lysed EpCAM-positive cells (83 % at a 10:1 effector to target ratio). *In vivo*, the antibody reached EpCAM+ tumor cells as evidenced by immunofluorescence. mGC8 tumor-bearing mice were treated twice with a combination of the BsAb and transduced TCR-I T cells. Tumor growth was significantly reduced for over 30 days (n=12) compared with control groups (transduced T-cells or BsAb alone) and survival was prolonged by > 30 days ($p < 0.001$). Therapeutic success was accompanied by an increase in T cell infiltration in the combination group, while no T cell recruitment towards other EpCAM positive organs was observed.

Conclusions: Co-administration of a BsAb bridging adoptively transferred tumor-specific T cells via an inert surface molecule to a tumor-associated surface antigen enhances the efficacy of therapeutic T cell transfer without off-target recruitment in healthy tissues.

Combination of adoptive T cell therapy with an EGFR x EpCAM-specific antibody for the treatment of a murine melanoma model

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Background: Adoptive T cell therapy represents a powerful approach for the treatment of cancer. However, its efficacy is limited by a lack of T cell infiltration within the tumor tissue. We have previously shown, that transducing antigen-specific T cells with a marker antigen (EGFR) not expressed by normal T cells and combining T cells with an EGFR x EpCAM bispecific antibody can mediate potent anti-tumor effects in a murine gastric cancer model. To test the applicability of the approach to different T cell antigens and tumor models, we hypothesized that a similar synergy could be seen in the B16-OVA-melanoma model using ovalbumin-specific T cells (OT-1).

Methods and Results: We generated a B16-OVA-mEpCAM cell line through retroviral transduction, as a model with a T cell targetable antigen (ovalbumin). We expanded $CD8^+$ T cells from OT-1 transgenic mice *in vitro* to generate a population consisting mainly of effector memory T cells ($CD44^+$ $CD62L^-$). C57Bl/6 mice were subcutaneously injected with B16-OVA-EpCAM melanoma cells and were treated with OT-1 T cells or the combination of the bispecific antibody with T cells (n=6 per group). Tumor growth was delayed in the combination group (mean tumor size on day 15, 77 mm^2 vs 137 mm^2). When we repeated the experiment comparing the two groups above with a group treated only with the bispecific antibody, we found surprisingly, that the treatment success relied entirely on the bispecific antibody alone, while the T cells did not contribute to efficacy (mean tumor size on day 18, 8 mm^2 in antibody-treated vs 106 mm^2 in untreated mice). We hypothesized that antibody dependent cellular cytotoxicity may be the mode of action of the bispecific antibody. We thus sublethally irradiated (5 Gy) mice 24 h after subcutaneous injection of B16-OVA-EpCAM tumor cells. We included groups with no radiation, with T cell-treatment alone, with bispecific antibody treatment alone, with the combination or with neither (n=6 per group). In mice without further treatment sublethal irradiation lead to slight benefit (mean tumor size on day 22, 40 mm^2 in irradiated vs 152 mm^2 in non-irradiated mice) In contrast, irradiation completely abrogated the effect of the bispecific antibody treatment (mean tumor size on day 29, 146 mm^2 in irradiated vs 117 mm^2) in non-irradiated mice, both antibody-treated).

Conclusions: Combination of antigen-specific OT-1 T cells and a bispecific antibody does not synergize in the B16-OVA-EpCAM model. The bispecific antibody was, however, effective in this model, most likely due to ADCC. Other strategies need to be investigated to enhance ACT in this model.

Pre-clinical models for the development of HER2-directed T-cell therapy

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The adoptive transfer of autologous, tumor-reactive T cells to treat cancer patients is a promising tool in immunotherapy. One of our clinical goals is the adoptive transfer of autologous T cells transduced with a human epidermal growth factor receptor 2 (HER2)-directed TCR in patients with advanced breast cancer. We successfully established cytotoxic T-cell clones and $CD8^+$ T cells transduced with a TCR specific for the tumor and self-antigen HER2 and tested their lytic capability in vitro.

Since HER2 is a self antigen that is not only expressed by malignant cells but also in normal tissue, e.g. the heart, we tested the recognition of normal $HER2^+$-cell lines by HER2-directed T cells in vitro. For this purpose HER2-directed $CD8^+$ T cells were used to analyze the recognition and lysis of HLA-$A2^+$ cardiomyocyte cell lines in IFNg-ELISA and chromium-release-assay. Compared to HER2-overexpressing breast cancer cell lines recognition of the cardiomyocytes by HER2-directed T cells was much lower. From these data we hypothesize that there might be a therapeutic window, which may allow the treatment with HER2-directed T cells without having any unwanted side effects, like cardiac dysfunction.

To further analyse this, we are establishing a mouse model using HLA-A2-transgenic NOD/SCID-HHDII mice. We injected a human breast cancer cell line s.c. into NOD/SCID-HHDII mice and evaluated the responsiveness of tumor cells towards adoptive T cell transfer. Our data show a significant tumor growth inhibition after adoptive T cell transfer and IL-15 treatment. Furthermore, in the NOD/SCID-HHDII mice we are able to detect potential autoimmune reactions. So far no signs auf autoimmunity could be detected after adoptive T-cell therapy. These pre-clinical studies, as well as the in vitro tests, are important for the evaluation of possible unwanted side effects after adoptive transfer of TCR-transduced T cells specific for self-antigens like HER2.

The influence of binding affinity and receptor density of chimeric antigen receptors on target-cell recognition after transfer by RNA electroporation

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ORAL SHORT TALK

During the last decade chimeric antigen receptors (CARs) have become a potent and promising tool in the immunotherapy of cancer. Although CAR-transduced T cells showed strong anti-tumor responses in cancer patients, severe side-effects occurred in several patients. These side effects were due to an on-target, off-tissue reaction of the transduced T cells, as most CARs target self-antigens, which are overexpressed in tumor tissues, but can also be found on healthy tissue.

It is, therefore, desirable to enable the T cells to discriminate between high and low antigen levels on tumor cells and other tissues, respectively. We thus wanted to explore whether this can be achieved via different CAR affinity and CAR density.

To investigate these issues, it was crucial to adjust the surface density of the different CAR molecules to desired levels, to allow for a direct comparison of CARs with a different affinity to the target antigen. In contrast to retroviral transduction, where CAR expression levels are difficult to manipulate, the electroporation of CAR-encoding mRNA allows to directly influence CAR-expression density by adjusting the amount of transfected mRNA.

In this study we efficiently electroporated T cells from healthy donors with mRNA encoding different CAR-molecules, which target the same antigen with different affinities. We show that the amount of RNA used during electroporation directly impacts CAR-expression levels and that the receptor density on the T-cell surface can be adjusted to identical levels for different receptors. This enabled us to investigate the influence of CAR affinity and CAR density on the recognition of cells with high or low antigen levels. We explored the cytokine secretion profiles and cytolytic capacity of the CAR-transfected T cells in regard to CAR density and binding affinity after stimulation with tumor cell lines with different levels of the target antigen.

Taken together we provide a tool to investigate the function of CAR molecules targeting the same antigen with respect to antigen affinity and receptor density. The method allows for the selection of CARs for a potential clinical application by lowering the risk of unwanted T-cell activation upon recognition of low-level antigen expression on healthy tissue.

(C.K. and B.S. conributed equally; J.D. and N.S. share senior authorship)

Towards clinical γδTCR gene therapy: a broadly applicable T cell product for cancer patients

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γδT cells are innate immune cells with strong anti-tumor activity and provide anti-tumor receptors that are interesting tools in immune therapy against cancer. The introduction of γδTCR genes into αβT-cells allows the adoptive transfer of T cells that are not limited by HLA-restriction, in any patient with any cancer (Marcu-Malina et al., Blood 2011). Combinatorial γδTCR-exchange (CTE) has been applied to design a γ9δ2 TCR with strong and broad anti-tumor reactivity, referred to as TCR γ9-G115/δ2-cl5 (Gründer et al, Blood 2012).

Aim of the study is to design a clinical grade procedure which guarantees an efficient expression of the introduced γδTCR in αβT-cells, an adequate enrichment of engineered cells, as well as functional efficacy.

Firstly, γ and δ chains were introduced into the retroviral vector pMP71, separated by a 2A peptide sequence. Gene-engineered human αβT cells expressed the γδTCR and recognized tumors of different hematological origin as well as solid tumors. Secondly the orientation of the γ and δ chains and the particular 2A peptide sequence influenced TCR expression as well as anti-tumor function as previously reported for αβTCR genes. Thirdly, introduction of the optimal γδTCR transgene cassette into αβT cells was followed by the depletion of non-transduced αβTCR positive cells using a clinical grade anti-αβTCR antibody. This depletion procedure resulted in a highly pure, but untouched, population of γδTCR-engineered T cells with increased γδTCR expression and improved anti-tumor function both *in vitro* but also *in vivo* in a humanized mouse model. Importantly, introduction of a γδTCR into αβT cells followed by clinical-grade depletion of non-transduced T-cells abolished residual alloreactivity.

All together, we developed a clinical grade GMP vector which allowed a deliberate choice for the most potent clinical γδTCR receptor cassette. In addition, clinical grade depletion of non-transduced and transduced T-cells expressing high levels of endogenous αβTCRs results in a potent γδTCR-engineered T cell product suitable for an autologous but also allogeneic clinical scenario.

Placenta-derived stem cells inhibit tumor progression on rat dimethylhydrazine-induced colon cancer model

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Background: As is generally known systemically administered mesenchymal stem cells could migrate to sites of malignant tumor and potentially be an effective delivery vehicle for therapeutics. Genetic modification of stem cells to overexpress antitumor genes has provided prospects for clinical use as anticancer therapy. However the data about the effects of stem cells themselves on tumor formation and progression are controversial.

Purpose: To determine the effect of intravenous allogenic and xenogenic transplantation of placenta-derived mesenchymal stem cells (PDSC) at 1,2-dimethylhydrazine (DMH) induced colon cancer in rats.

Materials and methods: rat PDSC were obtained from placentas of Albino rats by spontaneous migration from the tissue onto the culture plate and cultivated at DMEM high glucose medium with 10% FBS for 4 passages. Real time PCR, FISH, flow cytometry and immunocytochemistry methods were used for obtained cells description and immunophenotyping. Human PDSC were obtained from human placentas after normal labors and cultivated at the same conditions. Male Albino rats were injected of DMH (20 mg/kg weekly) subcutaneously for 20 weeks and treated with rat or human PDSC intravenously ($0.5*10^6$ and $2.0*10^6$ cells per animal) at 22nd week (after adenocarcinomas formation equal $T_{1-2}N_{0-1}M_0$ stage of human colorectal cancer). Animals were euthanized at 27th week, tumor number and size in dissected colon were measured, tumors and normal colonic mucosa slides stained with hematoxylin-eosin-orange were examined (light microscopy), morphometry analysis was carried out.

Results: The trophoblast derivation and the "stemness" of PDSC were confirmed. Rat PDSC didn't alter the bowel mucosa of healthy rats when applied at any dose, as opposed to human PDSC, which caused inflammation in rectum mucosa. Allogenic transplantation of PDSC to DMH-induced animals at dose of $2.0*10^6$ cells decreased the tumor number twice, the tumor size by 20% and the total dysplasia/cancers area tripled. PDSC allogenic transplantation at dose of $0.5*10^6$ cells since PDSC xenogenic transplantation at any dose had no effect on tumor progression. Rat PDSC protective effect against carcinogen-induced alterations of bowel mucosa with no tumors was observed: inflammation decrease, microvascular violations diminution, mucosa morphological parameters renewal caused by rat PDSC applied at high dose have been occured. On the contrast human PDSC escalated the DMH-induced inflammation in both colon and rectum mucosa.

Conclusions: Allogenic transplantation of PDSC at high dose inhibits tumor progression and protects the normal bowel mucosa under rat DMH-induced colon cancer model.

Thus: 1) PDSC have a prospect for clinical use as anticancer therapy when applied in adequate dose; 2) *in vivo* investigations of human stem cells on animal models require corrections.

T helper TCRs isolated from long term survivors after cancer vaccination for use in adoptive cell therapy

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Adoptive cell therapy with retargeted T cells has produced remarkable clinical responses in recent trials, but also serious toxicity. For most cancer forms, there is a lack of safe targets for therapy with cytotoxic T cells. We hypothesize that therapy with redirected T helper cells may transform the inflammatory milieu and induce epitope spreading, while circumventing key toxicity concerns. Human telomerase reverse transcriptase (hTERT) is overexpressed in most human cancers. We have conducted a series of hTERT cancer vaccine trials. From long term survivors after vaccination, we isolated >100 $CD4^+$ T helper cell clones recognizing hTERT epitopes. These T cell clones were characterized with regard to HLA restriction, affinity, fine specificity, proliferative capacity, cytokine profile and recognition of naturally processed epitopes. Based on their characteristics, two DP4-restricted T cell clones (A1 and A2) were selected for molecular T cell receptor (TCR) cloning. DP4 is among the most prevalent HLA molecules in Caucasians.

After TCR sequence identification, TCR A1 and A2 were cloned into the retroviral vector MP71, together with the GMP-applicable suicide/marker/sorter gene RQR8. The RQR8 gene comprises an epitope from CD34, facilitating identification and purification of transduced cells, and two minimal epitopes for rituximab, allowing for effective elimination of transduced cells if needed. We investigated the expression and functionality of TCR A1 and A2, after retroviral transduction. The results demonstrated that both TCRs were expressed well and that that the transduced T cells acquired the desired hTERT specificity. Interestingly, the T helper TCRs were expressed and functional both in $CD4^+$ and $CD8^+$ T cells. Both cell populations specifically produced IFNγ and CD107a upon hTERT peptide stimulation. Next, we cloned TCR A1 and A2 into the mRNA expression vector pCIpA102, for transient redirection of T cells with biodegradable TCRs. This strategy allows for safer clinical testing and dose escalation, based on repeated injections of redirected T cells that express the TCR for 4-7 days. We transfected T cells with TCR mRNA by use of electroporation. The results showed that both TCR A1 and A2 were highly expressed in T cells, and that the transfected T cells specifically recognized APCs pulsed with the relevant hTERT peptide.

The findings demonstrate that the cloned TCRs confer recipient T cells with the desired hTERT-specificity and functionality *in vitro*. Preclinical experiments may provide limited information on the efficacy or toxicity of these TCRs, as T helper cell therapy depends on mobilizing the host immune system. We therefore plan to use mRNA-based biodegradable TCRs for a phase I trial evaluating these receptors.

In vivo expansion of Epstein-Barr virus epitope-specific T lymphocytes following donor lymphocyte infusion for anti-CD20-refractory EBV-driven post-transplant lymphoproliferative disease

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Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disease (PTLD) remains an important complication of T lymphocyte deplete allogeneic haematopoietic stem cell transplantation (allo-HSCT). In this setting, almost all cases of PTLD arise from EBV-transformed B lymphocytes, which expand as a result of iatrogenic disruption of normal immune surveillance, in particular virus-specific T lymphocyte responses. Although the introduction of Rituximab (a B lymphocyte-specific anti-CD20 monoclonal antibody) has dramatically improved survival from PTLD, up to 30% of patients remain refractory to Rituximab and have a very high risk of mortality. Unselected donor lymphocyte infusion (DLI) has been established as successful salvage therapy, and in vivo expansions of total lymphocytes during the early weeks after adoptive transfer have been shown to correlate with clinical response. Nevertheless, the specificity of expanded T lymphocytes and their functional characteristics have, to date, remained unknown.

Here we report two cases of Rituximab-refractory PTLD complicating allo-HSCT, successfully treated with DLI. In both cases, PTLD arose 60-100 days following transplant, associated with elevated circulating EBV and radiological evidence of disease. Both patients received standard therapy with 4 weekly infusions of Rituximab without sustained response. As rescue therapy, both subsequently received DLI, comprising 1x10^6 unselected $CD3^+$ cells/kg derived from their stem cell donors. This led to complete resolution of EBV loads to below the detection limit of 500 copies/ml blood, as well as complete clinico-radiological responses. We prospectively followed the in vivo kinetics of EBV epitope-specific T lymphocytes present in the DLI following administration. For the first time, we have shown that functional EBV-specific $CD8^+$ and $CD4^+$ T lymphocytes against a range of viral epitopes expand in vivo following DLI. These expansions coincide with both the increase in absolute lymphocyte numbers and the decrease in EBV load in the blood. Interestingly, hierarchies of immunodominance in the in vivo expanded EBV-specific T lymphocytes match those seen in the unselected donor lymphocytes prior to infusion. Importantly, expression of the viral protein targets of these expanded T lymphocytes was detected in PTLD tumour cells of patient biopsies. Although unfortunately Patient B died several months after DLI from non-PTLD related mortality, Patient A remains disease free after 2 years, and maintains viral load and EBV-specific memory T lymphocyte responses in the ranges seen in healthy EBV carriers. This study demonstrates the clinical importance of adoptive therapy for the treatment of EBV-associated disease, and provides invaluable insights into the in vivo dynamics of transferred virus-specific T lymphocytes.

Human CD19 as a T-cell target antigen in the non tolerant host

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Adoptive T-cell therapy represents an increasingly important option for the treatment of hematologic malignancies. Due to the lack of specific antigens, so far only tumor associated antigens (TAA) can be targeted. However, TAAs derive from self-antigens and therefore antigen specific T-cells are eliminated in the thymus resulting in tolerance. To overcome central tolerance we utilized CD19 deficient animals or CD19 deficient animals on a HLA*A02:01 (HHDII) transgenic background. Within this CD19 non tolerant host we then identified human CD19 derived peptides giving rise to specific T-cell responses *in vitro* and *in vivo*.

CD19 deficient or wild type animals were lethally challenged with murine CD19 deficient autologous B-cell lymphoma cells transduced to express human CD19. As control recipients humanized CD19 animals were used. In CD19 deficient animals only antigen negative lymphomas grew out and 26% of animals rejected lymphomas long term (>100 days). In contrast, due to 66% homology between human and murine CD19, none of the wild type animals rejected lymphomas and antigen loss was found only in 50%. Humanized CD19 animals neither rejected lymphomas nor antigen loss was found confirming tolerance towards human CD19. T cells isolated from surviving CD19 deficient mice displayed a CD19 specific response when restimulated *in vitro*. Transfer of CD19 specific T cells into CD19 deficient hosts resulted in full protection from lethal lymphoma challenge and 100% of the animals survived.

We next crossed CD19 deficient animals onto the HHDII background to obtain HLA*A02:01 restricted murine T cells specific for CD19. Using repetitive peptide pool immunization we identified single peptide specific HLA*A02:01 restricted $CD8^+$ T cells. When restimulated *in vitro* T cells show a strong secretion of IFNγ and expansion. Flow cytometric T-cell receptor analysis revealed a Vβ5.1 restriction. Repetitive stimulation *in vitro* resulted in expansion of $CD8^+$ cells binding peptide loaded MHC multimers.

Collectively our results demonstrate that human CD19 serves as a T-cell antigen in the context of murine MHCI and in the context of human HLA*A02:01. By utilizing the xenogenic approach central tolerance can be circumvented and human CD19 specific T cells can be generated and expanded. Cloning of the T-cell receptor will allow to test this approach in preclinical model.

Targeting Claudin-6 with CAR-engineered T cells for individualized immunotherapy of ovarian cancer

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The potential of T cells to eradicate tumors provoked the development of novel T cell based immunotherapeutic strategies. Chimeric antigen receptors (CARs) represent a promising new approach for the adoptive immunotherapy of cancer and CAR-modified T cells mediated impressive antitumor reactivity in early clinical trials. CARs are recombinant receptors that combine HLA independent scFv-mediated antigen-binding with T cell signaling.

We designed a second generation CAR with specificity for Claudin-6 (CLDN6), coupled with CD28 and CD3ζ signaling domains.

CLDN6 is an onco-fetal gene that belongs to the family of tight junction proteins and is expressed in human stem cells and during early stage of epidermal morphogenesis. As it is absent in adult healthy tissues, but overexpressed in different cancers, CLDN6 represents an ideal target antigen for immunotherapy based on CAR-engineered T cells.

For functional validation we used mRNA transfer for rapid expression of different CAR mutants in T lymphocytes and evaluated CLDN6-CAR-mediated effector functions by IFNγ-ELSPOT, luciferase-based cytotoxicity and CFSE-based proliferation assays.

CAR-expressing T cells exhibited specific cytokine secretion and proliferation in response to CLDN6 expressing target cells. Furthermore, the CAR-engineered T cells efficiently killed CLDN6-expressing tumor cells *in vitro*.

Based on these experiments we selected the best performing receptor candidate and evaluated the viral vector most suitable for clinical application. We compared three different retroviral vectors regarding transduction rate, strength of CAR surface expression and antigen-specific induction of cytokine secretion, proliferation and cytotoxicity in short-term and long-term assays.

Finally, we selected one self-inactivating (SIN) retroviral vectors with a favorable biosafety profile for stable gene transfer of our CLDN6-CAR providing the final lead structure for GMP production, preclinical testing and finally clinical validation for the therapy of ovarian cancer.

Invariant NKT cells generated by the TIL 1383I T cell receptor gene transfer with retroviral vectors allows efficient redirection of human antigen specificity

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Human invariant NKT cells (iNKT cells) are a novel lymphocyte population characterized by an invariant T-cell receptor Vα24 chain paired with Vβ11. Human iNKT cells activated by a specific glycolipid antigen, α-Galactosylceramide (α-GalCer) in a CD1d-dependent manner have been shown to produce large amounts of cytokines such as IFN-Y and also have potent killing activity against various tumor cell lines. One major hurdle to studying the anti-tumor activity of human iNKT cells is they don't recognize antigens presented by classical MHC molecules and their tumor antigen specificity is unknown. Therefore, we expressed the MHC class I restricted TIL 1383I TCR into human iNKT cells to better understand their biology and anti-tumor activity. The 1383I TCR is a high affinity TCR that recognizes the melanoma antigen tyrosinase presented by HLA-A2. In this study, we report the production and function of the TIL 1383I TCR transduced human iNKT cells. Human iNKT cells were activated and expanded from normal PBMCs by stimulating them with α-GalCer pulsed dendritic cells (DCs) in the presence of IL-2, IL-7 and IL-15. The cultured cells were transduced using retroviral vectors encoding the TIL 1383I TCR and were isolated using CD34 magnetic beads. The TIL 1383I TCR transduced human iNKT expression was measured by flow cytometry (FACS) and their anti-tumor activity by intracellular cytokine staining. The TIL 1383I TCR transduced human iNKT cells specifically produced IFN-γ in response to tyrosinase peptide loaded T2 cells and HLA-A2^{+}/tyrosinase^{+} human melanomas. These results indicate that these engineered iNKT cells can lead to better understanding of their biology and can help generate new effector populations for the treatment of cancer patients.

Establishment of GMP compliant process for the expansion of therapeutic doses of genetically modified NK-92 cells

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Introduction: The continuously growing NK-92 cell line derived from a non-Hodgkin's lymphoma with $CD56^+CD3^-CD16^-$ phenotype is highly cytotoxic against broad spectrum of tumor targets and has completed phase I clinical trials in humans. To further improve the anticancer activity, NK-92 cells from an FDA-licensed master cell stock were transduced under GMP-compliant conditions with an ErbB2-specific humanized CAR construct by lentiviral gene transfer.The aim was to generate and characterize a GMP compliant Master Cell Stock of genetically modified NK-92 cells and to get a deeper insight into target-effector cell interactions to better understand the alternative activation pathway triggered by CAR in ErbB2-targeted NK-92 cells.

Methods: Cells were expanded in X-Vivo 10 media supplemented with 5% heat inactivated human fresh frozen plasma and 500U/ml of IL-2 in VueLife bags to a density of 5x10e5 cells/ml and frozen in human serum albumin supplemented with 7,5% DMSO according to the established cryopreservation protocol. The identity ($CD56^+$, $CD16^-$) and CAR expression stability were monitored using two - step staining with ErbB2/IgG fusion protein and anti-human IgG F(ab)2-APC. The functionality was analyzed using.

FACS-based cytotoxicity assay and methylcellulose assay where effector cells were coincubated with either ErbB2 positive or negative cancer targets.

Results: After expansion (490 fold) MCS comprising 200 ampules of 2,5x10e7 genetically modified NK-92 cells was established and qualified to meet predefined specifications with regard to post - thaw recovery (>50%), stability of CAR expression (>99%) and specific cytotoxicity (>80%). MCS is analyzed in accordance with the principles of good manufacturing practice (GMP) for qualification and release.

The ongoing testing has already confirmed the identity and biological safety of MCS.

Conclusion: Efficient, tumour-specific retargeting of NK-92 cytotoxicity can be achieved. The MCS of NK-92 cells expressing a humanized ErbB2-specific CAR may serve as a platform for future clinical trials in novel ErbB2-targeted cell-based anticancer therapies.

Natural killer cells modified to express a targeted granzyme B fusion protein for enhanced antitumoral activity

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Natural killer cells play a crucial role in the defense against viral infection and elimination of neoplastic cells. Natural cytotoxicity of NK cells can be triggered rapidly upon appropriate stimulation, and is regulated by a complex balance of signals from germline-encoded activating and inhibitory cell surface receptors. In contrast to B and T lymphocytes, NK cells are intrinsically programmed to detect common modifications in cellular metabolism or gene expression that are concurrent with an oncogenic process. Hence, different strategies are being developed to employ NK cells for cancer immunotherapy. These include activation of autologous NK cells, adoptive transfer of allogeneic NK cells, and pharmacological and genetic modulation of NK-cell activity.

As a novel approach, we investigated expression of a tumor-specific granzyme B (GrB) fusion protein in human NK cells as a means to augment their antitumoral activity. The pro-apoptotic serine protease GrB plays a crucial role in NK-cell mediated cytotoxicity. In cytotoxic lymphocytes, GrB is stored together with other granzymes and perforin in the dense core of lytic granules. Following target cell recognition and activation, lytic granules within the effector cells polarize towards the immunological synapse, fuse with the plasma membrane and release their contents into the synaptic cleft between the effector and target cell. Upon release, GrB enters target cells in cooperation with perforin, and activates apoptotic cell death by various mechanisms. NK cells possess all pathways required for processing, packaging and triggered release of endogenous wildtype GrB, which may also be employed by an ectopically expressed retargeted GrB derivative.

For selective targeting to tumor cells, we fused the epidermal growth factor receptor (EGFR) peptide ligand transforming growth factor α (TGFα) to human pre-pro-GrB. Established human NK cells transduced with a lentiviral vector encoding the GrB-TGFα molecule expressed this fusion protein in amounts comparable to endogenous wildtype GrB. Following activation of genetically modified NK cells by cognate target cells, correctly processed and enzymatically active GrB-TGFα protein was released together with endogenous granzymes and perforin. Thereby, cooperation of the tumor-specific fusion protein with endogenous factors augmented natural cytotoxicity of the modified effectors against NK-sensitive targets. Furthermore, GrB-TGFα protein enriched from supernatant of artificially activated NK cells displayed EGFR-specific binding and specifically killed EGFR-expressing tumor cells in the presence of an endosomolytic activity.

Ongoing work focuses on co-expressing targeted GrB variants with tumor-specific chimeric antigen receptors (CARs) in NK cells. While CARs can ensure selective targeting and efficient NK-cell activation at the tumor site, the released GrB fusion protein may potentiate the antitumoral response by providing a dual lethal hit to the tumor cell.

Selective expansion of educated NK cells for cancer therapy

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New insights into the fine specificity and molecular regulation of NK cell functionality hold promise for the design of more efficient strategies to activate, expand, and deliver NK cells for cancer immunotherapy. We recently reported that education by self-specific inhibitory KIRs is required for the proliferation of NK cells in response to stimulation with cellular targets expressing HLA-E. Based on this finding, we have developed a protocol for selective expansion of educated, NKG2C$^+$ NK cells. We generated NK cell cultures with distinct specificities (2DL1 or 2DL3) and tested these at day 14 against a panel of primary leukemic blasts from 24 children with acute lymphoblastic leukemia (ALL) in a flow-cytometry-based killing assay. The malignant cells were identified based on the minimal residual disease phenotype determined by the NOPHO panel. The expanded NK cells displayed strictly specific recognition patterns based on the HLA genotype of the leukemic blasts (HLA-C1/C2) and significantly more potent cytotoxicity (on average 80% killing at the E:T ratio 5:1) than short-term activated NK cells or the NK92 cell line. The possibility to selectively expand educated NK cells represent a promising first step towards exploiting the adaptive-like behaviour of NK cells in cancer therapy.

Genetically modified cytokine-induced killer (CIK) cells for targeted cancer therapy

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Pre-emptive immunotherapy based on minimal residual disease (MRD) status with donor lymphocyte infusions (DLI) using cytokine-induced killer (CIK) cells may be beneficial to prevent relapse without causing graft-versus-host-disease (GvHD). CIK cells are a heterogeneous effector cell population including T cells ($CD3^+CD56^-$), natural killer (NK) cells ($CD3^-CD56^+$) and natural killer T (NKT) cells ($CD3^+CD56^+$). They are generated by *ex vivo* expansion of peripheral blood mononuclear cells (PBMC) in the presence of interferon (IFN)-γ, anti-CD3 antibody, IL-2 and IL-15, and exhibit non-MHC-restricted cytotoxic activity.

While CIK cells have shown potent *in vivo* activity against various cancer types such as lymphomas or colorectal cancer, their cytotoxicity against B-ALL, characterized by the expression of CD19, has been limited. Hence, retargeting of CIK cells using chimeric antigen receptors (CARs) to facilitate selective target cell recognition and enhance specific cytotoxicity represents a promising approach. CARs comprise an extracellular scFv antibody fragment as an antigen-binding domain, linked via a flexible hinge region and a transmembrane domain to an intracellular signaling moiety such as CD3 zeta chain (first generation CAR), or zeta chain fused to a co-stimulatory protein domain such as CD28 (second generation CAR).

We established an optimized protocol for transduction of CIK cells with CD19-specific lentiviral CAR constructs, and characterized cells for expression of an EGFP marker gene and CAR surface expression. Effects of exposure to lentiviral vector particles on the development of CIK cell subpopulations were monitored over four weeks of continuous culture. In *in vitro* cytotoxicity assays we could demonstrate potent and selective cytotoxicity of retargeted CIK cells towards established cancer cell lines expressing CD19 and primary pre-B-ALL blasts.

To increase safety of CAR-expressing CIK cells and allow control of potential adverse effects in a clinical setting, we are investigating inducible caspase-9 (iCasp9) as a suicide gene. iCasp9 is a modified human caspase-9 fused to an FKBP12 protein domain, which allows conditional dimerization and activation by binding of the small molecular weight compound AP20187. So far we could demonstrate expression of iCasp9 upon lentiviral transduction of CIK cells, and selective elimination of transduced cells upon treatment with AP20187. Ongoing work now aims at co-expression of CARs together with iCasp9, and characterization of retargeted CIK cells in suitable *in vivo* models in NOD/SCID common γ chain knockout (NSG) mice.

T cells engineered to express two allo-restricted T cell receptors recognizing CD20/HLA-A*02:01 specifically and efficiently kill malignant B-lymphoid cells - a preclinical study

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Patient T cells genetically modified to express cancer-targeted T-cell receptors have recently shown great efficacy in the induction of clinical responses in malignant melanoma and synovial sarcoma. The challenge is currently lack of high-affinity TCRs that recognize cancer-restricted antigens for the majority of tumor types.

Although shared self-proteins represent attractive cancer targets, the autologous T-cell repertoire is normally depleted of T cells with TCRs recognizing peptides from such antigens with high affinity, during negative thymic selection. We have previously generated high-avidity T cells that recognize a peptide from CD20 (SLFLGILSV) in the context of allogeneic HLA-A*02:01. The T cells were shown to efficiently and specifically kill patient-derived leukemia and lymphoma cells (*Abrahamsen et al, Leukemia 2010 and Int J Cancer 2012, Kumari et al, PNAS 2013*). A number of T cell clones were functionally tested and two were selected for cloning and expression of the TCRs and subsequent pre-clinical evaluation. The TCRs A94 and A23 were cloned into the retroviral vector pMP71 as 2A-bicistronic constructs encoding WT sequence or variants modified with codon optimization, murinization of the constant part and addition of an extra cystein bond in the constant part, as described by others. The genetic optimization led to a doubling in A23-transduced peripheral blood T cells staining positively for the HLA-A2/CD20p multimer, whereas a manifold higher expression of the A94 was observed. The A23^{opt} and A94^{opt} also mediated correspondingly stronger degranulation (>90%) than the WT TCRs in response to target cells induced to express a single-chain trimer containing the CD20p, or to peptide-loaded HLA-A2^{pos}SupT1 cells. The re-directed T cells showed no cross-reactivity to a wide range of HLA-A2^{pos} CD20^{neg} cell lines of different histologies, including colon carcinoma, keratinocytes, HEK, liver carcinoma, lung adenocarcinoma and mesenchymal stem cells. Responses were rescued when target cell lines were loaded with the CD20p. The peptide affinity for the two TCRs was measured in response to target cells loaded with titered amounts of peptide. The A94^{opt} had an EC50 between 100-10pM and was approx. 10-fold more sensitive than A23^{opt}. In comparison, the MART-1-specific TCR DMF5, which has proven efficient in a clinical trial utilizing TCR re-directed T cells, showed almost 100-fold lower sensitivity for the cognate WT MART-1 peptide.

Finally, the re-directed T cells responded to 5 different HLA-A2^{pos} lymphoma cell lines, and to chronic lymphocytic leukemia (CLL) cells from 4 HLA-A2^{pos} patients, all endogenously expressing CD20. In contrast, no responses were seen to two HLA-A2^{neg}CD20^{pos} lymphoma cell lines and CLL donors. In conclusion, the results show that T cells re-directed with A2/CD20p specific TCRs efficiently and specifically kill malignant B cells, paving the way for clinical testing of these TCRs.

Dual specific cytotoxic potential and memory phenotype of IL-15-activated cytokine-induced killer cells targeting virus infection and leukemia in pediatrics

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Introduction: Viral reactivation or de novo infection with human pathogenic Adenovirus (AdV) and Cytomegalovirus (CMV) are frequent complications after allogeneic stem cell transplantation. In addition relapse of the underlying disease is the other major cause of treatment failure. Screening for minimal residual disease (MRD) allows identification of impending relapse and pre-emptive immunotherapy such as the application of donor lymphocyte infusions (DLI) or the transfer of cytokine induced killer (CIK) cells may in principle prevent relapse in a group of high risk pediatric patients.

Methods: CIK cells are generated from peripheral blood mononuclear cells (PBMC) of CMV- seropositive healthy donors according to the standard protocol. Besides the standard stimulations, IL-15 is supplemented and viral CMV-antigen in the form of a peptide pool is presented in the medium to increase the frequency of existing virus-specific cytotoxic T-lymphocytes (CTL) identified by flow cytometry stained with specific MHC-Multimers. The cell-mediated cytotoxicity is evaluated *in vitro* targeting leukemia cell line, antigen-loaded cell line and primary viral infected cells.

Results: Besides the main population, containing $CD3^+CD56^-$cells, T-NK cells ($CD3^+CD56^+$) an essential subpopulation is expanding which consist mainly of a terminal differentiated activated $CD8^+$ TEMRA ($CD45RA^+CD62L^-$) or TEM ($CD45RO^+CD62L^-$) phenotype, respectively. The incubation with viral antigen leads to an up to 11.0 fold donor-dependent increase regarding CMV-specific $CD8^+$ cells but results also in responder (12 donors) and non-responder (6 donors) . The simultaneous peptide stimulation during the culture period has no negative influence on the anti-tumor effect directed to the M4 AML subtype cell line THP-1 *in vitro*. In subsequent *in vitro* experiments the enhanced cytotoxic capacity of antigen-stimulated CIK cells targeting viral antigen-loaded T2 cell line is shown. In follow-up *in vitro* experiments donor-mismatched (matched in only one HLA antigen) fibroblasts are infected with CMV and first results may indicate to an increased lysis of the infected fibroblasts by $CIK_{antigen\ pos}$ cells compared to $CIK_{antigen\ neg}$ cells. Uninfected fibroblasts representing healthy recipient tissue and therefore function as a control for Graft-versus-Host disease (GvHD) are not killed at all. Cytokines production like IL-6, IL-10, IP10, TNFα, IL12/23 and Granzyme B during the expansion period of the CIK cell cultures were monitored using Cytometric Bead Array.

Discussion: The generation of dual specific cytotoxic CIK cells may be an improved immunotherapy after stem cell transplantation inducing cytotoxicity against leukemia cells and might help to clear specifically virus reactivation. Beyond that the minimal alloreactive potential and therefore the low risk of inducing GvHD disease, of the CIK cells is already shown in murine and human *in vivo* settings.

Human HLA-DR4-restricted high affinity T cell receptors against tumour-associated antigens

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The adoptive transfer of tumour-reactive T cells for cancer treatment has proven effective in various murine cancer models. However, isolation and ex vivo expansion of functional T cells from patients' tumours have been shown to be difficult and succeeded only in a minority of cases. One underlying reason is that tolerance mechanisms either during T cell development or induced by the tumour lead to absence of high avidity T cells in the host. Furthermore, the importance of CD4 T cells in anti-tumour responses not only by giving essential help to CD8 T cells but also by independent tumour destruction has been appreciated. Therefore, in this project we aim to generate MHC class II-restricted high affinity T cell receptors (TCRs) that can be used to render autologous T cells tumour-reactive for adoptive T cell therapy of cancer. To generate MHC class II-restricted TCRs we immunize TCR gene loci/HLA-DR4-IE transgenic (ABabDR4) mice with human tumour antigens. The advantage of this model is that T cells in ABabDR4 mice express a diverse human TCR repertoire but were not subject to negative selection for human tumour antigens during T cell development. Thus, immunization of ABabDR4 mice with tumour-associated antigens might result in the generation of high affinity TCRs that cannot be found in humans due to negative selection during T cell development. Moreover, these TCRs should not cause problems by immunogenicity, because they are fully human. So far, we demonstrated by immunization with a pan DR binding peptide that ABabDR4 mice are capable of mounting specific CD4 T cell responses. We will immunize the ABabDR4 mice with tumour-associated antigens and test isolated TCRs for their tumour-destroying potential in a mouse model of adoptive T cell therapy of cancer.

Characterization of freshly isolated and expanded tumor-infiltrating lymphocytes from pancreatic cancer patients

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In contrast to the general belief that pancreatic ductal adenocarcinoma (PDA) is a poorly immunogenic tumor, we find evidence for an adaptive immune response in this aggressive cancer type.
Immune infiltration can readily be detected by immunohistochemistry and flow cytometry in tumor biopsies from PDA patients. The majority of tumors contain prominent T cell infiltrates, which are frequently dominated by $CD4^+$ T cells.
These PDA tumor-infiltrating lymphocytes (TIL) can be isolated and expanded *ex vivo* with similar efficiency as in melanoma and can be tumor-reactive. While comparison of the T cell receptor repertoire between PDA TIL and PBMC suggests selective expansion of T cell subsets in the tumor, such prominent T cell clones can be lost during culture.
Our long-term aim is the development of T cell-based therapies for recurrent PDA. Based on our data this is a promising approach, though cultures of PDA TIL for therapeutic application will benefit from selection/enrichment of $CD8^+$ tumor-reactive T cells.

Targeting telomerase in B-cell chronic lymphocytic leukemia

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Telomerase reverse transcriptase (TERT), the enzyme primarily implicated in the elongation of telomeres in mammalian cells, is also considered as an important universal tumor antigen, since it is overexpressed in more than 85% of tumor cells irrespective of origin and histological type. When TERT is reactivated in tumor cells, the enzyme is processed and its antigenic peptides are presented within class I MHC molecules, promoting tumor recognition by specific cytotoxic T lymphocytes (CTL) in both preclinical and clinical settings. Recently, our group demonstrated that the adoptive cell transfer (ACT) of mouse (m)TERT-specific CTLs was able to control the progression of several transplantable tumor models but, at the same time, the repeated TERT-specific CTL administration induced a temporary autoimmune depletion of B cells. The higher susceptibility of activated/transformed B cells to mTERT-specific CTL lysis was also successively confirmed *in vitro*. These results represent a promising background for the treatment of haematologic malignancies, such as B cell leukemia (B-CLL).

Therefore, we explored the feasibility of telomerase as a promising immunotherapeutic target in a transgenic mouse model, the IgH.TEμ mouse. In this model, the sporadic expression of SV40 large T antigen in mature B cells generates a murine B-CLL displaying common characteristics with human B-CLL. In order to normalize the tumor system, we created homogeneous B-CLL-like cases by expanding malignant bone marrow cells in immune-deficient mice. This new model is characterized by the expansion of $CD19^+$ cells expressing high levels of active telomerase, which are recognized by mTERT-specific CTLs both *in vitro* and *in vivo*.

Starting from these preliminary mouse data, we translated our working hypothesis to a human setting, to test the ability of human (h)TERT-specific $CD8^+$ CTL to selectively recognize transformed B cells as a proof of concept of future clinic translation. Full-length rearranged TCR α/β genes were isolated from a hTERT-specific CTL clone and inserted in a retroviral vector system, allowing a simultaneous and balanced cell surface expression of both TCR subunits. Human $CD3^+$ T cells transduced with hTERT-specific TCR were able to recognize *in vitro* immortalized B cell malignancies expressing hTERT. These findings suggest that naturally processed hTERT/HLA-$A2^+$ complexes presented on the surface of immortalized B-malignancies are sufficiently immunogenic to be recognized by hTERT-specific CTL.

HAdV-specific γ/δ and CD8$^+$ T cells generated by TCR transfection to treat adenovirus infection after allogeneic stem cell transplantation

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Human adenovirus infection is life threatening after allogeneic haematopoietic stem cell transplantation (HSCT). Immunotherapy with donor-derived adenovirus-specific T cells is promising; however, 20% of all donors lack adenovirus-specific T cells. To overcome this issue, we transfected CD8$^+$ T cells with mRNA encoding an HLA-A*01-restricted, adenovirus-specific T-cell receptor (TCR). Since allo-reactive endogenous TCR of donor T lymphocytes would induce graft-versus-host disease (GvHD) in a mismatched patient, we transferred the TCR into γ/δ T cells, which are not allo-reactive.

TCR-transfected γ/δ T cells secreted low quantities of cytokines after antigen-specific stimulation, which were increased dramatically after co-transfection of CD8α-encoding mRNA. In direct comparison with TCR-transfected α/β T cells, TCR-CD8α-co-transfected γ/δ T cells produced more tumor necrosis factor (TNF), and lysed peptide-loaded target cells as efficiently. Most importantly, TCR-transfected α/β T cells and γ/δ T cells, co-transfected with the TCR and CD8α, efficiently lysed adenovirus-infected target cells. Here we show for the first time that not only CD8$^+$ T cells, but also γ/δ T cells can be equipped with an adenovirus specificity by TCR-RNA electroporation. Thus, our strategy offers a new means for the immunotherapy of adenovirus infection after allogeneic HSCT.

Background: Ewing Sarcoma (ES) is the second most common bone malignancy in children and young adolescents with a high potential of dissemination into lung and bones. Patients with localized disease receiving current treatment, have an approximate long-term survival of >65%. If the tumor disseminates into the lung or bone the rate decreases to 30% or 10%, respectively, compelling the search for new therapeutic treatment modalities.

Immunotherapy of cancer is an attractive treatment option, however, the development of a successful immunotherapy against autochthonous tumors is hampered by an ineffective T cell repertoire against tumor antigens and the inability of the patient‘s immune system to overcome tolerance-inducing mechanisms. Here, the specific recognition and lytic potential of transgenic allo-restricted $CD8^+$ T cells directed against the ES-associated antigen Six-Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) was examined.

Methods: Following repetitive $STEAP1^{130}$ peptide-driven stimulations with HLA-A*0201^+ dendritic cells, allo-restricted HLA-A*0201^- $CD8^+$ T cells were stained with HLA-A*0201/peptide multimers, sorted and expanded by limiting dilution. After functional analysis of suitable T cell clones via IFN-gamma and Granzyme B ELISpot, flow cytometry and xCelligence assay, TCR alpha and beta chains were identified by specific RT-PCR, cloned into retroviral vectors, codon optimized, transfected into HLA-A*0201^- primary T cell populations and tested again for specificity and their lytic capacity.

Results: Initially generated as well as derived transgenic T cells specifically recognized $STEAP1^{130}$ pulsed or transfected cells in the context of HLA-A*0201 as determined by specific IFN-gamma release, lysed cells and inhibited growth of HLA-A*0201^+ ES lines more effectively than HLA-A*0201^- ES lines. Transgenic T cells, furthermore, could be stained by HLA-A*0201/peptide multimers and sorted via magnetic beads for rapid expansion of TCR transgene bearing populations.

Conclusion: These results identify T cell clones capable of recognizing and inhibiting growth of STEAP1 expressing HLA-A*0201^+ ES *in vitro* in a highly restricted manner. Its receptor could be cloned and transgenic T cells will be used for subsequent analysis in a preclinical study.

Induction of CD4$^+$ stem cell-like memory T cells by inhibition of mTOR complex 1 and concomitant activity of mTOR complex 2

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Introduction: Robust immune responses are thought to depend on less differentiated memory T cells which are long-lived, capable to self-renew and able to give rise to further differentiated progeny. Recently, a cell type with such properties has been described and termed stem cell-like memory T (TSCM) cell. Of note, *in vitro* activation of Wnt signalling during T cell priming has been suggested to trigger the formation of CD8$^+$ TSCM cells. However, by contrast, CD4$^+$ TSCM cells are currently only known to exist, but the signalling pathways leading to their induction and their functional characteristics remain elusive.

Aims: We are exploring pharmacological strategies aiming at the induction of CD4$^+$ TSCM cells and are characterizing CD4$^+$ TSCM cells in terms of function.

Results: Here, we describe the inhibition of mTOR complex (mTORC) 1 with concomitant activity of mTORC2 as underlying mechanism to arrest a fraction of naive CD4$^+$ T cells in a stem cell-like state during T cell priming. This arrest is accompanied by a metabolic switch from glycolysis to oxidative phosphorylation. Further evidence is brought to this finding by the observation that T cell specific Raptor knockout mice, which lack mTORC1 activity in T cells, show increased numbers of TSCM cells. Of importance, *in vitro* induced CD4$^+$ TSCM cells display features distinctive of stem cells as low mitochondrial membrane potential, limited glucose uptake rate and a superior engraftment and long-term persistence after adoptive transfer in highly immunocompromised mice.

Significance: Our data reveal a novel immunostimulatory effect of mTORC1 inhibition by drugs like Rapamycin which are at present exclusively in clinical use due to their immunosuppressive function. The induction of CD4$^+$ TSCM cells might enrich the range of T cell-based immunotherapeutic approaches which are currently mainly based on CD8$^+$ T cells.

Natural human BDCA1$^+$ myeloid dendritic cells induce immunological and clinical anti-tumor responses in metastatic melanoma patients

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Dendritic cell (DC)-based anti-cancer immunotherapy exploits the capacity of DCs to initiate immune responses. Tumor-antigen-loaded DCs are injected into cancer patients to stimulate T cells and initiate tumor eradication. In experimental clinical trials this approach demonstrated to be feasible and safe and to results in effective tumor-specific immune responses and some favorable clinical outcomes. Up to now, DC-vaccines were mostly based on ex vivo-cultured monocyte-derived DCs. However, our recent clinical study exploiting blood-derived plasmacytoid DCs (pDC) showed that naturally circulating DCs are a promising alternative.

Here, we report on a clinical study exploiting another naturally occurring DC subset: BDCA1$^+$ myeloid DCs (myDCs). Fifteen patients with metastatic melanoma received intranodal injections of BDCA1$^+$ myDCs activated and loaded with tumor antigen-associated peptides ex vivo. Despite the limited number of myDCs administered, almost all patients mounted anti-vaccine T cell responses. Several patients also mounted robust tumor-specific CD8$^+$ T cell responses, which coincided with improved clinical outcome. In two patients evident tumor regression was observed and one patient is in ongoing complete remission for 16+ months.

Our results indicate that therapeutic vaccination against cancer with naturally occurring blood DCs is feasible with minimal toxicity and that in patients with metastatic melanoma, it induces favorable immune responses, which correlate with clinical outcome.

Artificial antigen presenting cells, aAPC, a new approach for generating prostate cancer specific T cells

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There are several well-defined prostate-specific target antigens for immunotherapy of prostate cancer. Two such molecules are the prostate-specific membrane antigen (PSMA) and the tyrosine kinase receptor EphA2, which are expressed on normal prostatic epithelial cells and over expressed in most prostate cancers. Studies have mapped the HLA-A2-restricted target antigens from PSMA and EphA2 that can be recognized by antigen-specific cytotoxic T lymphocytes (CTL). These studies are the basis of a variety of different experimental immunotherapies, currently under evaluation, for treatment of prostate cancer. Still there are several hurdles complicating the development of effective prostate cancer specific therapies. First, prostate specific protein targets are often natural self-antigens thus the immune system may be tolerant to these; and second it has been reported that dendritic cells (DC) in prostate cancer patients are often impaired due to pre-treatment and the underlying disease. Therefore, new approaches that stimulate potent immune response outside the body independent of autologous DC are especially interesting.

Previously, we have shown that HLA-A2-Ig based aAPC can be used to replace autologous DC in protocols for the generation of antigen-specific CTL. Therefore, we performed aAPC stimulation for multiple donors to generate prostate cancer specific T cells directed at antigens from PSMA and EphA2. The generated T cells were highly specific and showed good in vitro activity as demonstrated in intracellular cytokine and killing assays. Experiments to generate PMSA and EphA2-specific T cells from PBMC of prostate cancer patients are ongoing. Together the performed experiments demonstrate that aAPC can be used to generate functional active prostate cancer specific CTL *in vitro* for potential use in adoptive immunotherapy

CD47$^+$ artificial APC demonstrate enhanced immune cell interaction

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Over the past decade we and others have been focused on the development of an artificial Antigen-Presenting Cells (aAPC) for generation and expansion of antigen-specific T cells. To date aAPC have successfully been used to stimulate antigen-specific T cell responses in vitro as well as in vivo. To further improve the in vivo efficiency of our bead based aAPC technology we investigated the addition of CD47 to the surface of the aAPC to enhance their *in vivo* half live. CD47 is a species-specific marker of self, as it delivers a negative signal to SIRP-a expressed on resident macrophages subsequently inhibiting the clearance of intact hematopoietic cells. Furthermore, CD47 has been shown to interact with SIRP-b2 expressed on T cells resulting in modulation of cell signalling pathways. We therefore hypothesised that adding CD47 onto our aAPC, will minimize macrophage mediated phagocytosis without negative interference on antigen-specific T cells generation.

To test this we coated aAPC with varying amounts of rhCD47-Ig and co-cultured them in vitro for up to 3h with human macrophages. Re-isolated aAPC from those cultures displayed increasing amounts of remaining surface proteins that could be positively correlated to the amount of rhCD47-Ig. In summary, our data revealed a CD47 concentration dependent inhibition of aAPC phagocytosis. Furthermore we tested the ability of CD47$^+$ aAPC to generate antigen-specific T cells directed at Mart-1 and FluM1 from multiple donors. Functionality of those T cells was confirmed in a standard ^{51}Cr-release assay and by intra cellular cytokine staining. Finally, co-culture experiments of macrophages, T cells and CD47$^+$ aAPC demonstrated an enhanced stimulatory capacity of CD47$^+$ aAPC over normal aAPC.

Thus these data support our hypothesis that addition of CD47 on aAPC has the potential to further improve aAPC efficiency in future *in vivo* applications.

Recognition of fully processed Hepatitis C virus antigen and naturally occurring mutant HCV epitopes by HCV TCR gene modified T cells in the context of hepatocellular carcinoma

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Hepatitis C virus (HCV) infects approximately 3% of the world's population, often causing associated diseases including cirrhosis of the liver and hepatocellular carcinoma (HCC). Subsequent chronic infections are thought to be attributed to the rapidly mutating HCV genome leading to immune escape variants. With limited effective therapies and slow vaccine development, alternative approaches for treatment and prevention of infection and its associated diseases is imperative. An immune-based approach for targeted therapy utilizes adoptive T cell transfer to genetically engineer an individual's T cells to become reactive to an antigen of choice. Our lab has firmly demonstrated its ability to use retroviral vectors encoding T cell receptor (TCR) genes to redirect the specificity of normal peripheral blood lymphocyte (PBL)-derived T cells to recognize tumor and viral antigens. We have previously identified and cloned two novel TCRs from HLA-A2-restricted, HCV NS3:1406-1415- or HCV NS3:1073-1081-reactive T cell clones. Here, we demonstrate PBL-derived T cells transduced with a recombinant retroviral vector encoding for the respective TCR can recognize peptide-loaded targets and HCV$^+$ HCC cells with cytokine production in a CD8-independent manner, a property consistent with other high affinity TCRs . We have measured the affinity of NS3:1406-1415 peptide/MHC to its receptor. Additionally, T cells engineered to express HCV NS3:1406-1405- or NS3:1073-1081-reactive TCRs can recognize peptide-loaded targets and tumor cells expressing a variety of mutant HCV antigens *in vitro*, indicating that these TCRs have broad crossreactivity against naturally occurring HCV mutant epitopes. These transduced T cells can recognize tumors expressing an epitope-specific minigene as well as the full NS3 protein, indicating these TCRs transfer the ability to recognize fully processed antigen. Initial studies also show that adoptive transfer of PBL-derived human T cells engineered to express an HCV NS3:1406-1415-reactive TCR can mediate regression of human HCC tumors in a mouse xenograft model. Based on these observations, we hypothesize that the ability of a TCR to have broad reactivity is not unique to such isolated clones and that in patients with resolved HCV infection there may exist a variety of TCRs with the capability of recognizing many mutant HCV epitopes. We believe the approach to redirect T cells to recognize mutagenic HCV antigens may not only have benefits in further understanding TCR biology, but that despite the instability in the HCV genome, TCR gene modified T cells may be a promising clinical therapy for treating HCV infection and associated HCC.

How is melanoma escaping us? An assessment of melanoma associated antigens before and after immunotherapy

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Introduction: Tumor escape mechanisms, including tumor manipulation of melanocyte-associated antigens (MAA), have been implicated as a factor in metastatic melanoma (MM) and in immunotherapy non-responders. In clinical practice we often see this as loss of expression of melanoma associated antigens by immunohistochemical staining methods. Here we aimed to quantify this phenomenon and determine if there is a difference in MAA expression in patients who had received immunotherapy compared to those who have not.

Methods: Immunohistochemical stains for Mart-1, HMB-45, S100, tyrosinase, and MITF were performed on formalin-fixed, paraffin-embedded tissue sections from 11 patients treated with immunotherapy and 13 untreated. Of the MM specimens, there were 9 regional lymph nodes (LN) and 15 visceral metastases. All tumors were reviewed and staining was assessed semiquantitatively by a board-certified dermatopathologist as positive (expression by 1-100% of tumor cells) or negative (expression by < 1% of tumor cells). Data was assessed for (1) differences between overall and individual MAA expression between patients with and without immunotherapy, (2) differences between overall and individual MAA expression between LN versus visceral metastases, and (3) assessment of relative loss of individual MAA expression as a percentage of total immunohistochemical stains performed for each antigen. Results were compared using a two-tailed Fisher´s t-test.

Results: There was no statistically significant difference between overall or individual MAA expression for patients with or without immunotherapy or LN versus distant metastases. Although no statistical significance was reached between loss of expression of individual antigens, Melan-A, HMB-45 and tyrosinase demonstrated the largest relative loss of MAA expression (25-26%), when compared with MITF (10%) and S100 (4.3%).

Conclusion: MAA expression pre- and post-therapy has clinical importance, as it can impact the design of immunotherapeutic protocols. This study demonstrated that MAA expression remained stable despite immunotherapy. Additionally, the MAA expression profile of our patient population for LN versus distant metastases and relative percentage of MAA loss was consistent with published literature. More extensive studies with larger numbers of patients pre- and post-therapy are important to improve immunotherapeutic efficacy and patient outcomes in the future.

The abstract is withdrawn

Lentivirus-induced dendritic cells accelerate de novo adaptive immune reconstitution against HCMV/pp65 in humanized mouse models of stem cell transplantation and are making progress in clinical development

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Hematopoietic cell transplantation (HCT) with allogeneic hematopoietic stem cells represents the only curative treatment for high-risk hematologic malignancies, but human cytomegalovirus (HCMV) illness harmfully impacts survival. Novel approaches to accelerate immune reconstitution and *de novo* adaptive antiviral effects are warranted particularly for patients receiving HCT from HCMV negative grafts, as adoptive donor T cells cannot be expanded *in vitro*. Therefore, we are developing a novel approach for donor-derived adoptive dendritic cell transfer. We produced an integrase-defective tricistronic lentiviral vector co-expressing GM-CSF, IFN-a and full length pp65 HCMV antigen to transduce monocytes overnight (MOI of 5). The outgrown long-lived Self-differentiated Myeloid-derived Lentivirus-induced dendritic cells internally loaded with pp65 (SmyleDC/pp65) showed low number of integrated tricistronic vector copies (day 30 of culture: 0.1 copies/cell). The clonal contribution of the genetically modified monocytes was monitored with a high-throughput integration site (IS) analysis. The majority of ISs were below 5% and outside genes. *In vitro* autologous T cell stimulation assays confirmed the activation and expansion of $CD4^+$ and $CD8^+$ T cells and pp65-specific CTL. NodRag$^{-/-}$IL2gc$^{-/-}$ mice receiving HCT from G-CSF mobilized blood or from cord blood and adoptively transferred with donor-derived SmyleDC/pp65 showed faster reconstitution of mature effector memory human $CD8^+$ T cells producing anti-pp65 reactive T cell responses. Mature human B cells were demonstrated in reconstituted lymph nodes, human immunoglobulins (IgG1, IgG2, IgG3, IgG4 and IgM) were evident in plasma and IgG reactivity against pp65 was detectable, demonstrating human adaptive Ig class switch *in vivo*. Notably, thymus analyses (absolute $CD4^+/CD8^+$ double positive cell numbers) demonstrated that adoptive transfer of SmyleDC/pp65 promoted *de novo* T cell development *in vivo*. The tricistronic ID-LV-G2app65 vector was produced under GMP-like conditions. The quality control analyses entailed purity, sterility and viral titer (titer after purification 5.7×10^7 infective particles/ml). Three independent pilot GMP-like batches of SmyleDC/pp65 were produced following a 24-28h transduction protocol under standard operating procedures with closed bag systems and cryopreserved. SmyleDC/pp65 thawed and cultured for 7 days without exogenous cytokines showed 38-60% recovery (relative to thaw). Preliminary Q/C analyses of SmyleDC/pp65 showed the expected identity and potency immunophenotypic markers ($CD45^+$, $CD14^-$, $CD11c^{hi}$, $HLA\text{-}DR^+$, $CD86^+$, $CD80^+$). Intracellular staining for detection of the pp65 antigen showed 20-31% positive cells. Requirements for a manufacturing authorization will be clarified in cooperation with the competent authorities for a multicentric phase I clinical trial.

Alteration of tumor microenvironment using immunomodulatory cytokines for combination treatment with adoptive T-cell therapy

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Background: Novel immunologic therapies are constantly developed for cancer treatment. Adoptive cell therapies (ACT) are a recent potent approach for treating cancer and adoptive transfer of autologous tumor infiltrating lymphocytes or genetically redirected peripheral blood mononuclear cells have successfully been used to treat patients with advanced solid tumors as well as patients with hematologic malignancies. During the first two decades of adoptive cell therapy, safety of cell transfer *per se* has generally been good, but significant toxicities and even mortality has been associated with the concomitant treatments used to enhance the therapy, including preconditioning chemotherapy and radiation, and the administration of systemic IL-2 after transfer. Other possible obstacles of ACT include the lack of trafficking of transferred cells to tumors, inability to kill tumor cells due to anergy or halted lymphocyte proliferation resulting in a rapid decline in cell numbers.

Hypothesis: To further enhance the existing treatment regimens, we aimed to study whether concomitant local treatment with immunomodulatory cytokines would increase the efficacy of adoptive T-cell transfer. We hypothesize that cytokine injections in combination with ACT may cause a danger signal that recruits T cells and other immune cells to tumors and thereby can favourably alter the tumor microenvironment by reducing immunosuppression and cellular anergy.

Methods: Immunocompetent B16.OVA bearing C57BL/6 female mice were adoptively transferred with $2*10^6$ $CD8a^+$ enriched OT-I lymphocytes on day 0 and treated with intratumoral injections of recombinant murine cytokines (diluted in saline) on 5 consecutive days per treatment round. A set of mice was sacrificed after each treatment round (#1 and #2), tumors were harvested and both total lymphocytes as well as tumor (OVA) specific OT-I cells were analyzed by flow cytometry.

Results: To our surprise, we observed that cytokine injections induced accumulation of $CD8^+$ OVA^- T cells at the tumor site over time, while the count of $CD8^+$ OVA^+ T cells in different treatment groups remained lower than in the saline treated or untreated control groups. Moreover, the total amount of $CD3^+$ T lymphocytes and $CD19^+$ B lymphocytes varied between treatment groups indicating alternative mechanisms of action for each cytokine studied.

Conclusions: Results based on our animal model suggest that intratumoral cytokine injections can result in redistribution of immune cell subsets and thus improve adoptive cell therapy by affecting the cellular composition of tumor microenvironment.

Plasmacytoid dendritic cells - new killers for melanoma treatment

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Malignant melanoma is one of the most treatment-refractory skin malignancies with increasing incidence, frequent metastasis, and poor prognosis. On the other hand, melanoma is a very immunogenic type of cancer with repeatedly reported spontaneous regressions. The success of immunotherapies using tumor antigen-pulsed monocyte-derived dendritic cells for advanced stages of melanoma is significantly correlated with the induction of tumor-specific cytotoxic T lymphocytes. However, these therapies do not sufficiently induce cytotoxic adaptive immune responses, and therefore, the success is limited so far. Our aim is to improve these tumor-vaccination approaches using virus-activated plasmacytoid dendritic cells (PDC). PDC and their most prominent cytokine IFN-alpha are already used in immunotherapies against cancer, because they can link innate and adaptive immunity. Our hypothesis is that upon viral stimulation with herpes simplex virus type 1 (HSV-1), PDC are able to induce immune responses against melanoma cells. We activated PDC using the infectious, but replication-deficient HSV-1 *d*106S mutant, kindly provided by collaboration partners. The cells or the cell-free supernatants (s/n) were cocultivated with five melanoma cell lines and six patients' primary isolates. PDC s/n marginally reduced the growth of some melanoma cell lines. In contrast, cocultures with virus-activated PDC had a significant cytotoxic effect on nearly all melanoma cells. We excluded NK cell contamination by titration of these cells in melanoma cell cocultures. Interestingly, HSV-1-activated PDC exerted similar cytotoxicity to melanoma cells as NK cells. More importantly, the vaccine construct HSV-1 *d*106S showed similar cytotoxic effects compared to wildtype virus in cocultures, although *d*106S is replication-incompetent. Even with UV-inactivated *d*106S virus, PDC could inhibit melanoma cell growth. To define the underlying mechanism, we first defined the cytotoxic potential of PDC and then used neutralizing antibodies against the most prominent candidates in coculture experiments: the IFN-a receptor (IFNaR Ab), TNF-alpha, TRAIL, and inhibitors against granzyme B and prostaglandins. The effects of soluble components were mainly due to IFN-a, whereas effects of activated PDC could not be neutralized by the IFNaR Ab. Taken together, our results suggest that virus-stimulated PDC inhibit melanoma cell growth via soluble factors and cell-contact dependent mechanisms.

Rapid generation of clinical-grade antiviral T-cells: T-cell donor selection and manufacturing of T cells

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Efficient early or pre-emptive treatment intervention by adoptive antiviral T-cell transfer in high risk patients requires (i) manufacturing without long-term *ex vivo* stimulation maintaining antiviral $CD4^+$ and $CD8^+$ cells and (ii) undelayed recruitment of a preferably pre-registered T-cell donor.

Over 4 years, frequencies of CMV-, EBV-, ADV- and HHV6-specific memory T cells in blood and stem cell donors were analyzed to establish a T-cell donor registry ("T cells of interest" registry, TOI registry). Potential donors are first identified by IFN-g EliSpot followed by detailed phenotyping and functional analysis. Specific eligibility was defined if >0.03% $CD3^+IFN\text{-}g^+$ T cells respond to the antigen in cytokine secretion assay (CSA). Additionally, enrichment efficiency of TOIs should result in a purity of >60% $CD3^+IFN\text{-}g^+$ cells. To obtain a manufacturing license according to the German Medicines Act (AMG), enrichment of clinical grade antiviral T cells from three donors was performed on the example of CMV under GMP conditions using the CliniMACS cytokine capture system (CCS) and CMVpp65 peptide pool.

Quality control of the final products starting with 0.05-1.7% IFN-g secreting $CD3^+$ T cells resulted in 19%-81% $CD3^+IFN\text{-}g^+$ T cells. A total of 0.3-1.8x10^6 $CD3^+CD56^-CD45^+$ and 0.54-14.2x10^5 $CD3^+IFN\text{-}g^+$ T cells (purity 19.2%-81.2%) were enriched. Among $CD3^+$ T cells we found 12-68% $CD8^+IFN\text{-}g^+$ and 5-53% $CD4^+IFN\text{-}g^+$ cells. Despite this low purity of IFN-g secreting T cells the number of contaminating $IFN\text{-}g^-$ T cells was acceptable (2.3-6.7x10^5). In all preparations contaminating B cells (4-5%), granulocytes (5-27%), monocytes (16-30%), and NK cells (12-21%) were found.

In a first trial we generated clinical-grade EBV-specific T-cell using the CliniMACS CCS and the EBV-derived EBNA-1 peptide pool. As expected, the number of enriched EBV-specific $IFN\text{-}g^+$ T cells (recovery 13.7%) was much lower to the number of enriched CMV-specific $IFN\text{-}g^+$ T cells (recovery 67.9%). In the final T-cell product we found sufficient number of total $CD3^+$ T cells (3.15x10^5) and $IFN\text{-}g^+$ T cells (1.6x10^4) with monocytes (1.66x10^5) the most common impurity.

The manufacturing of clinical grade antiviral T cells by large-scale CliniMACS CCS was successfully validated and tested using antigens derived from CMV and EBV, respectively. The donor pre-testing results indicate, that a starting frequency of ≥0.03% is sufficient for successful purification of TOIs. High proportions of $CD4^+$ T cells negatively influence purity and therefore the efficacy of the cellular preparation.

Optimizing the efficiency of TCR transfer against malignant melanoma

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Background: Immunotherapy by adoptive T cell transfer is a known approach to reject tumors in cancer patients. To induce tumor regression T cells can be equipped in vitro by a T cell receptor (TCR) engineered to detect tumor antigens. The efficiency of TCR gene transfer can be impeded by mispairing of the introduced TCRα- or β chains with the endogenously expressed TCR chains. To reduce mispairing and to increase expression and functionality of transferred TCR we optimized a human TCR specific for the melanoma antigen gp100.

Methods: We constructed a single chain (sc) TCR by covalently linking the variable domain of the TCRα- chain to the TCRβ-chain and co-expressed the constant domain of the TCRα-chain (Cα) to achieve stable scTCR expression. Since the human scTCR/Cα was not expressed at the cell surface due to instability, we substituted the constant TCR domains by mouse TCR domains (chim scTCR/Cα). As mouse-derived proteins could be immunogenic in patients, we minimally murinized the constant domains by replacing only selected amino acids in the human constant domains of the scTCR/Cα by their corresponding mouse amino acids (mm scTCR/Cα). To increase stability, additional disulfide bonds between the scTCR and the Cα were introduced. For further improvement, we linked the gene of the scTCR with the Cα by a 2A-element and expressed it on one retroviral vector.

Results: By retroviral transduction of human T cells with the scTCR/Cα, we detected a high expression at the cell surface for the chim scTCR/Cα and the mm scTCR/Cα. T cells transduced with the different TCR constructs were able to lyse melanoma cells and cells generated from melanoma metastases in a chromium release assay. T cell degranulation induced by incubation with gp100 peptide loaded cells was equal in T cells transduced with the chim scTCR/Cα and the mm scTCR/Cα. This assay also showed a degranulation of CD4 T cells, showing that the TCR reacts CD8-independent. Affinity of both constructs as determined by K_D measurement with different tetramer concentrations was equal. Furthermore we are running an in vivo experiment to prove rejection of melanomas by the scTCR/Cα constructs in a NSG mouse model. Therefore, melanoma cells were transduced with a luciferase gene to detect tumor growth and metastasis by in vivo imaging. A control of melanoma growth was especially seen in mice with a high frequency of transferred T cells in the blood. During the experiment, we plan also to scan for a panel of cytokines and chemokines to reveal potential immune escape mechanisms.

Conclusions: Our results show equal expression and functionality of mm and chim scTCR/Cα. These optimized gp100-specific TCRs represent promising candidates for adoptive T cell transfer by providing effective antitumor responses.

Inhibition of CSF-1R supports T-cell mediated melanoma therapy

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Tumor associated macrophages (TAM) can promote angiogenesis, invasiveness and immunosuppression. The cytokine CSF-1 (or M-CSF) is an important factor of TAM recruitment and differentiation and several pharmacological agents targeting the CSF-1 receptor (CSF-1R) have been developed to regulate TAM in solid cancers. We show that the kinase inhibitor PLX3397 strongly dampened the systemic and local accumulation of macrophages driven by B16F10 melanomas, without affecting Gr-1$^+$ myeloid derived suppressor cells. Removal of intratumoral macrophages remarkably coincided with retained tissue macrophages in healthy organs and a modest, but statistically significant, delay in melanoma outgrowth. Importantly, CSF-1R inhibition strongly enhanced tumor control by immunotherapy using tumor-specific CD8 T cells. Elevated IFNγ production by T cells was observed in mice treated with the combination of PLX3397 and immunotherapy. These results support the combined use of CSF-1R inhibition with CD8 T cell immunotherapy, especially for macrophage-stimulating tumors.

In vitro generation of mature, naive tumor antigen-specific CD8$^+$ T cells with a single T cell receptor from mobilized peripheral blood precursor cells

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Peripheral blood mononuclear cells transduced with a tumor antigen-specific T cell receptor (TCR) are becoming an effective treatment modality against malignancies. However, major, in some cases, lethal toxicities including colitis, cardiac and neurologic toxicities were reported. It was shown in animal studies that cross-pairing of the TCR α and β chains of the endogenous TCR with the α and β chains of the transduced TCR chains may cause 1.limited expression of the tumor antigen-specific T cell receptor with loss of avidity for the tumor cells, and 2. may generate de novo auto-reactive TCR causing life threatening auto-immunity. We here studied a strategy for the efficient generation of naive CD8$^+$ T cells with a single TCR. Transduced adult mobilized peripheral blood progenitor cells were differentiated to TCR-expressing double positive cells using OP9-Delta-like 1 stromal cells. Addition of the tumor-specific peptide induced double positive precursors to cross-present the peptide, leading, in the absence of co-stimulation, to cell cycle arrest and differentiation into mature CD8$^+$ T cells. Comprehensive phenotypic, molecular and functional analysis demonstrated the generation of naive and resting CD8$^+$ T cells through a process similar to thymic positive selection. These mature T cells contain germline TCRA and TCRB loci and express high levels of the specific multimer-reactive TCR. Upon activation, specific cytokine production as well as efficient killing of tumor cells are induced.

Comparative expression analysis of the in vitro derived T cells and peripheral blood CD8 T cells put in evidence their very similar characteristics. The major difference was observed in the expression of some costimulatory/inhibitory molecules: CD28 expression was absent whereas on the other hand inhibitory molecule PD-L1 and CTLA4 were not or to a much lower degree expressed on in vitro generated T cells. This was confirmed at the protein level. To remediate the absence of costimulatory molecule CD28, precursor cells were transduced to express chimeric TCR in which the cytoplasmic tail is replaced with the CD3ζ and CD28 signalling intracellular part. The results obtained with these TCR chimeric constructs will be reported at the conference.

Using this strategy, large numbers of high-avidity tumor-specific naive T cells can be generated from readily available hematopoietic progenitor cells without TCR chain cross-pairing.

Generation of tumor specific T cells using DC-based vaccine for adoptive T cell therapy

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During the last decades, the adoptive T cell therapy (ACT) is gradually finding its stable place among the other contemporary immunotherapeutic strategies and expands the spectrum of available treatment options in cancer therapy. We have developed a DC-based vaccine currently being tested in multiple clinical trials. LNCap loaded mature DC-based vaccine has proved the ability to induce antigen-specific T cells in vivo. Due to low frequencies of such induced tumor-specific T cells we try to find approaches that might increase their numbers. Our ongoing work focuses on the establishment of GMP-compliant ACT large scale *ex vivo* protocol, using autologous tumor-specific T-lymphocytes induced by the DC-based vaccine. In our experiments on healthy donors we included antigen-specific pre-stimulation step by the DC-based vaccine prior to non-specific stimulation (using Dynabeads anti-CD3/CD28) in order to increase absolute number of specific T cells in the final product. *In vitro* stimulation of autologous T cells with DC-based vaccine induced on average 3 % tumor-specific $CD8^+$ and 19. 3 % tumor-specific $CD4^+$ T cells. Our current protocol setting enables the generation of T cell product that is of early/intermediate phenotype associated with long term *in vivo* persistence, survival and effector functions. Following *ex vivo* stimulation and expansion, T cells show low expression of CCR7 and CD57, and high expression of CD27 and CD28. To date, our results demonstrate that antigen-specific T cells can be expanded up to 1, 42 x10/9 using Dynabeads antiCD3/antiCD28 and WAVE bioreactor culture system. In addition, final T cell product was analyzed for its proliferative potential (analysis with KI-67) and the frequency of regulatory T cells. Based on these preliminary results, we transfer the protocol on patient-derived T cells, starting with the expansion step and testing several GMP culture conditions. Further characterization and functional analysis of expanded T cells are under way.

Keywords: Cancer immunotherapy, adoptive T cell therapy, tumor specific T cell expansion

Co-stimulation with CD40(L)Ligand enhances the immunostimulation of human dendritic cells (hDC) and induces apoptosis towards hepatocellular carcinoma cells

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Background and aims: Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide with poor prognosis. Dendritic cells (DCs) are professional antigen-presenting cells able to prime antigen-specific T-cells. CD40/CD40 ligand (CD40L) interaction between DCs and T-cells is a strong co-stimulation necessary to initiate and activate an effective immune response. The aim of this study was to analyze the impact of CD40L on DC in order to improve a DC-based immune response towards HCC.

Methods: Human monocyte-derived DCs were isolated by ficoll densitiy gradient centrifugation and cultured with IL-4 and GM-CSF. DCs were adenovirally transduced on day 6 with AdhCD40L encoding human CD40L or AdMock as a control. On day 8, DCs were analyzed for specific maturation markers by flow cytometry and for cytokine expression by ELISA. The supernatant of AdhCD40L-transduced DCs was analyzed in an apoptosis assay towards different HCC cell lines.

Results: After transduction of DCs with AdhCD40L an efficient CD40L-expression could be detected on the surface of the cells and in the supernatant. AdhCD40L-transduced DCs expressed significantly higher maturation markers such as CD83 and CD86 compared to AdMock-transduced DCs. CD40L significantly increased the expression of potent DC-typical Th1-cytokines, such as IL-12 without increasing immunosuppressive cytokines, such as IL-10. Furthermore, CD40L could even activate the expression of untypical DC-cytokines, such as IFNy on hDC. HCC-cells cultured with the supernatant of CD40L-expressing DC showed an increase in apoptosis rates: in more than 80% of Huh7, up to 60% in HepG2 and to about 30% in CCL-13 cells the sub-G1-fraction was increased compared to the controls.

Conclusions: Transduction of hDCs with AdhCD40L leads to an increased immune stimulation of DC and a switch to Th1 cytokines secretion. Moreover, CD40L was able to induce apoptosis in HCC cells. Thus, these findings emphasize the potential of CD40L to improve DC-based immunotherapies of HCC.

Membrane-attached cytokines expressed by mRNA electroporation: potent T cell adjuvants for adoptive cell therapy

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Th1 immunostimulatory cytokines, in particular IL-2, are commonly applied in different protocols for adoptive cell therapy (ACT) of cancer for enhancing function and longevity of the transferred T cells. Yet, the systemic administration of high-dose IL-2 is often accompanied by adverse physiological effects and the same is true for IL-12. Moreover, IL-2 consumption by tumor-residing Tregs augments suppression and severely reduces its availability for the effector T cells. Alternative application modes for IL-2 and other key Th1 cytokines such as IL-12 and IL-15 may reduce toxicity and improve the clinical efficacy of ACT.

We chose to tackle these problems by expressing IL-2, IL-12 and IL-15 as integral membrane constituents in tumor-reactive T cells via the electroporation of in-vitro-transcribed mRNA as a safe and efficient delivery method which allows the co-expression of multiple genes. Membrane expression is expected to avoid systemic dissemination and associated toxicity, render cytokines constantly available for the effector cells and minimize competition by counteracting cells. The use of mRNA prevents prolonged growth signaling and obviates the risk of cellular transformation.

We assembled mRNA templates encoding membrane (mem) human IL-2, IL-12 (single-chain) and IL-15 and their 3 mouse counterparts. We first confirmed proper surface expression by flow cytometry. Ex-vivo, all 3 membrane cytokines supported the growth of mRNA-transfected human CD8 and CD4 T cells for at least 6 days post-transfection in the absence of IL-2, comparably to high-dose soluble IL-2. Similarly, memIL-2 and memIL-12 and, to a lesser extent, memIL-15, were comparable to their respective soluble cytokines in supporting the ex-vivo growth of splenic mouse CD8 T cells. In a series of experiments we co-expressed each of the 3 membrane cytokines with constitutively-active derivatives of TLR4 and CD40 in peripheral blood-derived human T cells and anti-melanoma tumor-infiltrating lymphocytes (TILs). Collectively, the mere expression of different combinations of these genes elevated the production of IFN-γ and additional cytokines and chemokines and enhanced the expression of 4-1BB, OX40, CD25, CD28 and CD69 but not of CTLA4 and PD1, in some cases exhibiting synergistic effects. Furthermore, memIL-2 enhanced the ability of mRNA-transfected TILs to specifically respond to autologous melanoma even 3 days post-transfection. Of note, in all CD25 flow cytometry analyses, each transfection which included memIL-2 mRNA resulted in complete loss of signal, a phenomenon which likely reflects a cis mode of action, manifested in blocking the CD25 epitope of the detecting mAb by the bound memIL-2.

These results offer memIL-2 as a straightforward and utterly safe solution to systemic IL-2 toxicity and point to the 3 membrane cytokines (and additional potential candidates) as potent T cell adjuvants, alone or in conjunction with other genetic or non-genetic agents.

The Xuri W5 cell expansion system can produce clinically relevant cells

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Lymphocytes, expanded for clinical use, often consist of a small selected starting population, which requires multiple rounds of replication to achieve therapeutic doses. By using perfusion culture with the Xuri W5 Cell Expansion System, high cell density cultures which are sufficient for therapeutic doses, can be generated. The Cellbag™ bioreactors, used together with the Xuri W5 system, are functionally closed, single-use bioreactors that are delivered pre-sterilized and suitable for cGMP production. Perfusion is automatically maintained by the Xuri W5 system, which removes metabolites through an internal filter while supplying the culture with nutrients, thus keeping the culture in a steady state.

The handling of only one culture using the Xuri W5 system, compared to having to manipulate multiple T-flasks, simplifies the sampling process. Cells produced for use in clinical trials often need to meet specific release criteria, which can include showing that the product contains low levels of endotoxin and is sterile. Since the cells grown in the Cellbag bioreactor are contained in one homogenous mixture, only one sample is required to get a representative cell count or to analyze release criteria. Such samples can be withdrawn from the Cellbag through the sample clave port, by connecting a Luer syringe or other device, without the need of opening the system or transferring it to a laminar air flow hood, thus keeping the system functionally closed.

Efficient strategy to overcome immunosuppression of the stromal composition on γδ T cell activity in pancreatic disease

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Chronic pancreatitis (CP) as well as pancreatic ductal adenocarcinoma (PDAC), which is an extremely aggressive gastrointestinal malignancy, are characterized by a dense stromal microenvironment. CP is regarded as high risk factor for the development of PDAC. This study aimed to investigate whether human γδ T lymphocytes, which have the ability to kill PDAC cells, infiltrate to the same extent in the inflammatory stroma in CPs compared to the desmoplastic stroma in PDACs. We observed that human $CD3^+$ $CD8^+$ γδ T cells were localized at increased frequency throughout the entire stromal compartment in CP tissues. In addition, γδ T cells accumulated in the lymphoid follicles of CP patients suggesting a recruitment of γδ T cells to the site of inflammation. In contrast, the frequency of γδ T cells in the stromal and ductal epithelial/tumoral compartment in patients with PDAC was lower. However, γδ T cells in PDAC tissues were distributed more intensively in the ductal epithelium and the stroma close to the ductal epithelium, which demonstrates mobilization and infiltration to the tumor site. To overcome immunosuppression of the stromal composition on γδ T cell-activity and to improve their cytotoxicity, a novel bispecific antibody with specificity for Vγ9 on γδ T cells and for human epidermal growth factor receptor Her2/neu expressed on PDAC cells was designed. $[(Her2)_2xV\gamma9]$ selectively enhanced the cytotoxicity of γδ T cells from PDAC patients *in vitro*, and thereby the release of perforin and granzyme B. Moreover, the adoptive transfer of a low numbers of γδ T cells together with the $[(Her2)_2xV\gamma9]$ antibody reduced growth of pancreatic tumors grafted into SCID-Beige immunocompromised mice. Taken together, our results demonstrate that $[(Her2)_2xV\gamma9]$ selectively targets γδ T cells to tumor antigens, which indicates the tractable use of endogenous γδ T cells for immunotherapy. A further advantage for a γδ T cell-based immunotherapy might be given by their low frequency in the peripheral blood as well as in the tissue of PDAC patients avoiding a cytokine storm as it could be observed when the majority of the αβ T cells instead of γδ T cells is activated.

ErbB2/HER2-specific natural killer cells for adoptive immunotherapy of glioblastoma

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Significant progress has been made over the last decade towards realizing the potential of natural killer (NK) cells for cancer immunotherapy. NK cells can respond rapidly to transformed and stressed cells, and have the intrinsic potential to extravasate and reach their targets in almost all body tissues. In addition to donor-derived primary NK cells, also continuously expanding cytotoxic cell lines such as NK-92 are being considered for adoptive cancer immunotherapy. High cytotoxicity of NK-92 has previously been shown against malignant cells of hematologic origin in preclinical studies, and general safety of infusion of NK-92 cells has been established in phase I clinical trials. To enhance their therapeutic utility, here we genetically modified NK-92 cells to express a chimeric antigen receptor (CAR), consisting of an ErbB2-specific scFv antibody fragment fused via a linker to a composite CD28-CD3 zeta signaling domain.

GMP-compliant protocols for vector production, lentiviral transduction and expansion of a genetically modified NK-92 single cell clone (NK-92/5.28.z) were established. Functional analysis of NK-92/5.28.z cells revealed high and stable CAR expression, selective cytotoxicity against ErbB2-expressing but otherwise NK-resistant tumor cells of different origins in vitro, as well as homing to ErbB2-expressing tumors in vivo. Ongoing work now focuses on the development of these cells for adoptive immunotherapy of ErbB2-positive glioblastoma. Glioblastoma (GBM) is the most common and aggressive intracranial malignant tumor in humans. Standard therapy for GBM includes maximal safe surgical resection, radiotherapy and chemotherapy with temozolomide. However, recurrence of GBM is very frequent, and the median survival of glioblastoma patients is only 12 to 15 months. We evaluated the activity of NK-92/5.28.z cells against a panel of glioblastoma cell lines and primary glioblastoma cultures in in vitro cytotoxicity assays and demonstrated selective cell killing that was dependent on the level of ErbB2 expression by the target cells and the time of their exposure to the effector cells. Antigen specificity and selective cytotoxicity of NK-92/5.28.z cells were retained in vivo, resulting in antitumoral activity against subcutaneous and orthotopic glioblastoma xenografts in NSG mice. Our results suggest adoptive transfer of ErbB2-specific NK-92/5.28.z cells as a promising new immunotherapy approach for ErbB2-positive glioblastoma.

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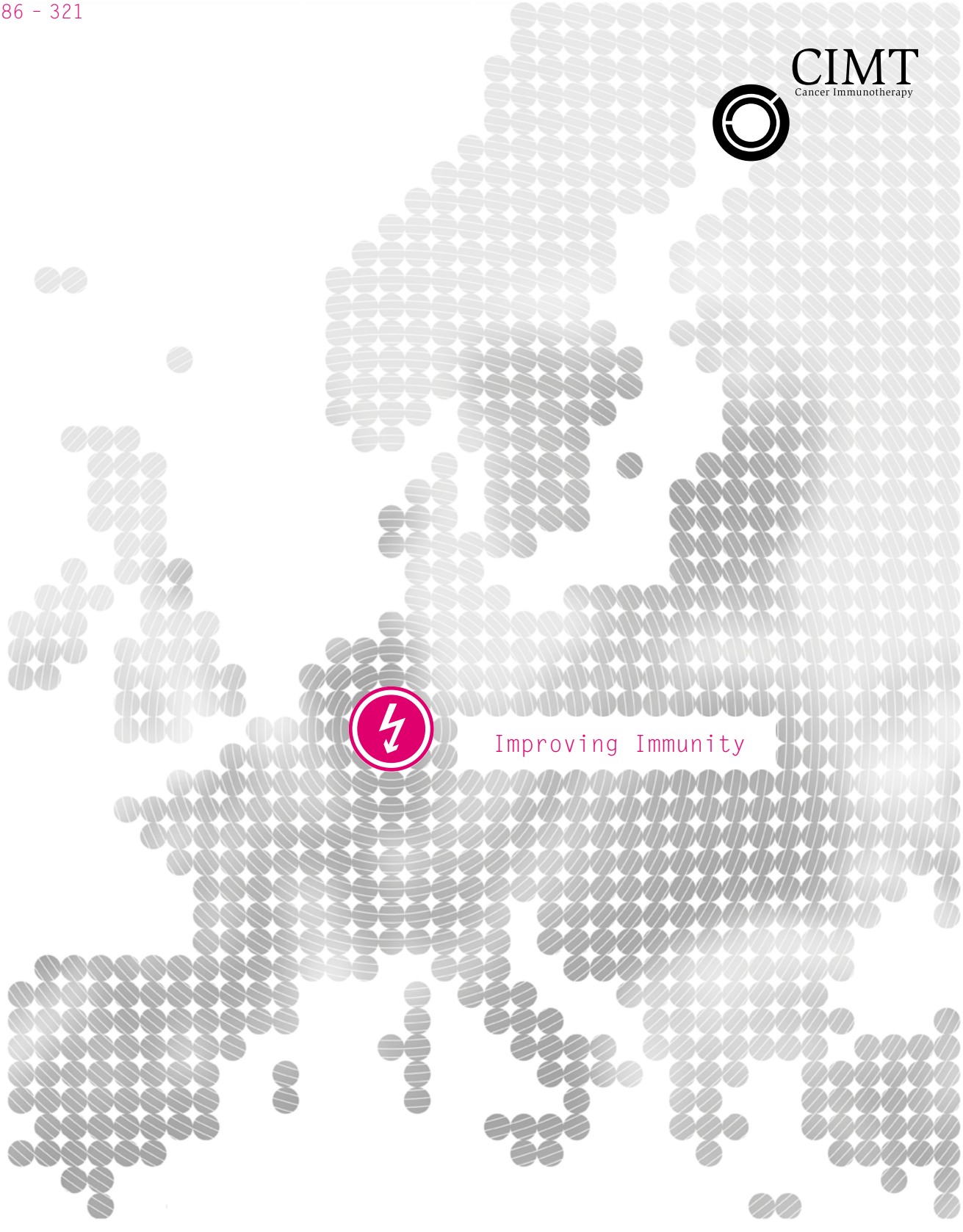
Cancer Immunotherapy



Improving Immunity

Molecular dynamics studies of the role of protein flexibility in immunological molecular recognition

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Activation of the cellular immune response involves the recognition of an antigenic peptide presented by either the class I or class II major histocompatibility complex (MHC) by T-cell receptors (TCRs). Because of the large population of potential antigenic peptides in comparison to the number of available TCRs, TCRs must be cross-reactive, yet must also maintain a degree of specificity to avoid indiscriminate T cell activation. A commonly implicated factor for TCR specificity and cross reactivity, as well as the current focus for this study, involves the flexibility of TCR complementarity-determining region (CDR) loops. This study focuses on the DMF5 TCR, which is specific for epitopes of the MART-1 protein, upregulated in the majority of melanomas. Beyond its potential utility in cancer therapy, this particular TCR is of interest because of the apparent rigidity of its CDR loops, which contrasts with what traditionally has been expected of TCR binding loops. Molecular Dynamics simulations of both the unligated and ligated DMF5 TCR were performed in order to assess its flexibility and to gain insight into the roles of mobility in DMF5 binding.

Immune-cell poor Hgf-Cdk4 mouse melanomas benefit from antibody mediated PD-1 blockade after targeted activation of the type I IFN system

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ORAL SHORT TALK

Infiltration of human primary melanomas with cytotoxic immune cells correlates with the spontaneous activation of the type I interferon (IFN) system and a favorable prognosis. Therapeutic antibody mediated blockade of immune inhibitory receptors in patients with pre-existing lymphocytic infiltrates prolongs survival. However, new complementary strategies are needed to efficiently activate innate and adaptive anti-tumor immunity in immune cell-poor human melanomas. In this study we experimentally show that primary cutaneous melanomas in Hgf-Cdk4(R24C) mice, which imitate a sub-group of human immune cell-poor melanomas with a low type I IFN response signature, escape type I IFN-induced immune surveillance as well as immunoediting. Peritumoral injections of the immunostimulatory RNA polyinosinic:polycytidylic acid (polyI:C) initiated a cytotoxic inflammatory response in the tumor microenvironment and significantly impaired tumor growth of primary and transplanted Hgf-Cdk4 melanomas. This critically required the coordinated activation of the type I IFN system by dendritic, myeloid, NK and T cells. However, targeted activation of the type I IFN system led to an up regulation of PD-L1 on melanoma cells and increased numbers of $PD1^{+}CD8^{+}$ circulating T cells. Importantly, antibody-mediated blockade of the IFN-induced immune-inhibitory interaction between PD-L1 and PD-1 receptors significantly prolonged survival of melanoma bearing mice. These results highlight important interconnections between the type I IFN system and immune-inhibitory receptor signaling in melanoma pathogenesis which serve as targets for combination immunotherapies.

Transfer of a protective anti-tumor immunity from immunized mothers to the offspring: First evidence

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Recent studies involving maternal immunization against neonatal tetanus and influenza virus have shown that this approach was very effective in providing newborns with protection from these pathogens. Here we studied the effect of an anti-tumor DNA vaccine on the progression of mammary carcinogenesis in cancer prone rat Her-2(neu) transgenic female (BALB-neuT) mice born from mothers immunized against the extracellular and transmembrane domain of neu. High levels of specific anti-neu IgGs were detected in both milk from vaccinated mothers and in sera of their offspring. The progression of spontaneous mammary carcinogenesis was hampered in BALB-neuT female offspring born and fed by vaccinated mothers as compared to controls. Immunized mothers KO for the μIg chain failed to transfer protection to their offspring. Likewise the protection was nil when the newborns were KO for FcγRI/III. Furthermore we observed the expansion of low avidity $CD8^+$ T cells bearing a TCR rearrangement reacting with neu dominant peptide (p63-71) in offspring from vaccinated mothers and a T-cell-mediated cytotoxic response against p63-71 was found. This phenomenon could be due to the uptake of immune complexes of IgGs together with part of the neu protein found in breast milk. Taken together our results show for the first time that maternal immunization against an oncoantigen could offer effective protection to the offspring genetically predestined to develop mammary carcinomas, indicating the potential application of this approach in neonatal cancer diseases.

Macrophage-dependent tumour immune rejection induced by local low dose irradiation

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Inefficient T cell immigration is a major factor of poor prognosis in many cancers and a critical limitation of immunotherapy. Tumours induce an immunosuppressive environment, preventing the entry of anti-tumour immune effector cells. It has been shown that this barrier can be overcome by activating the tumour microenvironment, in particular by modifying the tumour vasculature through high dose total body irradiation. However, irradiation at such doses cannot be applied in a clinical setting. In RIP1-Tag5 (RT5) mice, a model of spontaneous insulinoma, we could show that local low dose irradiation (LDI) was sufficient to activate the tumour microenvironment. In combination with adoptively transferred tumour specific T cells, local LDI led to a normalization of aberrant vasculature, increased T cell infiltration, tumour rejection and prolonged survival. Furthermore, we show that T cell infiltration is mediated by iNOS+ macrophages that are recruited into the tumour upon irradiation. Moreover, adoptive transfer of low dose irradiated macrophages into unirradiated RT5 mice resulted in effects corresponding to the combination treatment demonstrating that macrophages are indispensable for tumour immune rejection. To determine the effects of combination treatment on signalling pathways we plan to perform an RNA sequencing of tumour infiltrating macrophages. For this purpose we are currently sorting the macrophages by FACS from tumours of respectively treated mice. We finally assessed the effects of local LDI in advanced human pancreatic cancers in the frame of a controlled clinical study. Similar to the murine RT5 tumour model, local low dose irradiation of human pancreatic cancer resulted in accumulation of iNOS+ intratumoural macrophages and a strong accumulation of intraepithelial T cell infiltrates. Our findings reveal an indispensable role for iNOS+ macrophages in the joint regulation of T cell recruitment and angiogenesis in tumours and open new options for therapeutic exploitation.

Response of monocytes and macrophages to temozolomide in mice

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Immunodeficiency is a severe and therapy limiting side effect of anticancer therapy resulting in acute hematotoxicity if DNA damaging agents are applied. In previous studies we analysed human monocytes and compared them with macrophages, which were derived from them, as to DNA repair and sensitivity to the anticancer drug temozolomide (TMZ). We observed that monocytes were more sensitive than macrophages to the killing effect of TMZ. We also showed that the expression of the base excision repair (BER) proteins XRCC1, LigIII3, PARP-1 and DNA-PK is lacking in monocytes, but increased during their differentiation into macrophages [1, 2]. Here, we translated this study to mouse where we investigated monocytes *in vivo*. We compared mouse monocytes isolated from bone marrow (BM) of C57BL/6 mice with T lymphocytes collected from spleen by Western blot analysis. As shown previously for human cells, mouse monocytes lack the expression of XRCC1, LigIII, PARP-1 and DNA-PK on protein level. To verify this, we investigated the mRNA expression in monocytes, macrophages and T lymphocytes by qPCR. We isolated these populations stained with CD115 and GR-1 (monocytes), F4/80 (macrophages) and CD3 (T lymphocytes) by FACS. As expected, we observed less *XRCC1* expression in monocytes compared to macrophages and T lymphocytes. Furthermore, we assessed the sensitivity of mouse monocytes compared to macrophages after TMZ treatment. Following TMZ injection in C57BL/6 mice, apoptosis of monocytes, isolated from BM, and macrophages, which were collected from the peritoneum, was measured by annexin V staining and flow cytometry. The data revealed that monocytes were more sensitive than macrophages to TMZ. Additionally, peripheral blood was analyzed after TMZ treatment of C57BL/6 mice and a depletion of different cell populations, including monocytes was observed. In summary, we showed that murine monocytes are more sensitive than macrophages to TMZ, which is likely the result of downregulation of base excision and DNA double-strand repair.

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Modulation of heme oxygenase (HO)-1 enzyme activity by metalloporphyrins affects the antiviral T-cell response

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Adoptive transfer of virus-specific T cells can prevent reactivation of latent viruses such as CMV and EBV and have been demonstrated to lead to a sustainable reconstitution of antiviral immunity. *In vitro* stimulation and expansion of virus-specific T cells is required for clinical applications.
The multifunctional role of heme oxygenease (HO)-1 as immune modulator makes this an attractive target for the modification of T-cell responses. In the present study we asked whether modulation of HO-1, which is the inducible isoform of enzymatic heme degradation, might have effects on *in vitro* T-cell activation. We investigated whether (1) induction of HO-1 by cobalt-protoporphyrin (CoPP) or inhibition by tin-mesoporphyrin (SnMP) can affect expansion and function of virus-specific T cells, (2) application influences other cell populations (DCs, Tregs, NK cells) mediating effects on proliferating T cells and (3) HO-1-modulated antiviral T cells would be suitable for the purpose of adoptive immunotherapy.
Inhibition of HO-1 via SnMP in CMVpp65-peptide-pulsed PBMCs resulted in an increased antiviral T-cell activation and in the generation of a higher proportion of effector memory T cells (CD45RA-CD62L-). A more effective phenotype was determined by an increased capability to secrete IFN-g and granzyme B. These results showed that despite proliferation HO-1 inhibition also improve the functionality of antiviral T cells. In addition, depletion of Tregs and SnMP exposure lead to a 15-fold increase in the number of antiviral T cells.
As this approach opens the stage for GMP-conform improvements the clinical applicability of SnMP was proven by isolating antiviral T cells using the cytokine secretion assay. SnMP-treatment resulted in a higher cell amount and purity compared to untreated samples without negative impact on quality and effector function (CD107a, IFN-γ, TNF-α levels are stable).
The results underline the important role of HO-1 in the modulation of the adoptive immune response. Inhibition of HO-1 resulted in a much more effective generation of functionally active T cells applicable for adoptive T-cell therapy.

Effect of local tumor irradiation on migration of antigen-specific CTLs and tumor growth

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Current treatment of cervical cancer is based on aggressive surgery and/or chemoradiotherapy. Non-invasive therapies, like immunotherapy, might enhance treatment efficacy. Recruitment of cytotoxic T lymphocytes (CTLs) into tumors is a critical component of effective cancer immunotherapies. Local irradiation therapy can induce chemokines that orchestrate this recruitment.

Here we use local low-dose tumor irradiation, alone or with a therapeutic immunization based on Semliki Forest virus (SFV) against human papillomavirus (HPV)-related cancer.

We demonstrate that immunization with SFVeE6,7 or SFVeOVA, replicon particles expressing either HPV16 E6/E7 or ovalbumin, results in an antigen-specific migration of $CD8^+$ T cells into HPV- and OVA-specific tumors. Irradiation alone results in a 2-fold increase of anti-tumoral $CD8^+$ T cells. Irradiation, combined with immunization, leads to a 10-fold increase of intra-tumoral $CD8^+$ T cells and to a 20-fold increase of antigen-specific $CD8^+$ T cells. Most importantly, irradiation potentiates the SFV-induced decrease in tumor size.

Reovirus activation of NK cells increases the efficacy of rituximab for the treatment of CLL

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Background: Chronic Lymphocytic Leukaemia (CLL) is a heterogeneous disease associated with the accumulation of $CD19^+/CD5^+$ malignant B lymphocytes in the blood, bone marrow and secondary lymphoid organs. CD20 is expressed on both normal and malignant B cells but is absent from B cell precursors, mature plasma cells and non-lymphoid normal tissues, and has been targeted for antibody-based immunotherapy. Rituximab is a chimeric anti-CD20 monoclonal antibody which can induce its cytotoxic effects via antibody-dependent cellular cytotoxicity/phagocytosis (ADCC/ADCP) utilising Fc Receptor engagement on monocytes, macrophages or NK cells. It is currently used in combination with chemotherapy and has yielded excellent response rates in approximately 50% of CLL patients. Despite this, CLL remains incurable due to Minimal Residual Disease (MRD) and subsequent relapse.

Reovirus exerts its anti-cancer activity either by direct oncolysis or activation of innate and/or adaptive anti-tumour immunity. Reovirus treatment of PBMCs increases NK-mediated lysis of tumour cell targets, *in vitro*. Importantly, NK cell activation has also been observed in colorectal cancer patients after systemic delivery of reovirus, prior to surgical resection. This study investigates the direct cytotoxic effects of reovirus against CLL and examines whether reovirus could be used to increase the efficacy of rituximab-based immunotherapy.

Results: These data show that CLL cells are relatively resistant to direct reovirus oncolysis, at earlier time points, but demonstrate increase sensitivity at 7 days. CLL cell lines are susceptible to NK cell-mediated lysis and reovirus activation, in the context of PBMCs, increases lysis of CLL cell targets. Importantly, NK cells from CLL patients are activated by reovirus, to a similar extent as healthy donors, and patient NK cells are activated in autologous tumour systems. Finally, this study demonstrates that reovirus activation of PBMC increases the efficacy of rituximab-based immune-therapy.

Conclusion: These results suggest that oncolytic viruses could be used to increase the efficacy of antibody-based immunotherapy and provide a novel treatment approach for the treatment of CLL.

Impact of CTLA-4 antibodies Tremelimumab and Ipilimumab on the human immune system. Results of an ex vivo human melanoma model with the oncolytic parvovirus H-1 or cytotoxic drugs

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Tumor-directed and immune-stimulating therapies are of special interest in cancer treatment. We analysed the impact of the oncolytic parvovirus H-1 and cytotoxic drugs like temozolomide, fotemustine, dacarbacine and combination of paclitaxel and carboplatin to trigger melanoma cell death and its immunogenicity to induce human dendritic cell (DC). Maturation.. Adding of anti-CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) antibodies (AB) Tremelimumab (Treme) and Ipilimumab (Ipi) may additionally strengthen DC maturation.

Human Sk29Mel melanoma cells expressed CTLA-4 on cell surface. In immature DCs (iDC) coculture with H-1PV infected or cytotoxic drugs pretreated Sk29Mel tumor cell lysates (TCL) induced maturation of iDCs shown by increased expression of CD80, CD83 and CD86. Treme and Ipi did not negatively affect this DC maturation. Using ELISA, coculture experiments with H-1PV infected TCLs additionally increased cytokine production (IL-6, IFNγ, IL-12 and TNFα). The addition of Ipi or Treme did not further strengthened DC maturation. Furthermore, we analysed these DC TCL cocultures with regulatory T cells (Tregs) to overcome the negative Tregs effects on DC maturation by anti-CTLA-4 AB. Here, both Treme or Ipi significantly increased IL-6 and decreased TGF-ß.

H-1PV or cytotoxic drug induced cell killing strengthened immunogenicity of melanoma tumor cell lysates. Tremelimumab and Ipilimumab additionally blocked Tregs favouring a pro-inflammatory milieu and reinforced T-cell activation. This combination of active enhancement of tumor immunogenicity and concomitant blockade of the CTLA-4 silencing process by tumor cells and Tregs is therapeutically promising.

Promiscuous HPV16-derived T-helper epitopes for improving HPV targeted immunotherapy

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High-risk types of human papillomavirus (HPV) are associated with several malignant diseases, including cervical carcinoma, other anogenital tumors and oropharyngeal carcinomas. Fortunately, most HPV infections are successfully eradicated by the immune system. Accumulating data show that both cytotoxic T cell and T helper cell responses play a pivotal role in the control and clearance of HPV infection. To date, therapeutic HPV vaccines have mostly been developed to elicit tumor-specific CTL responses. The exclusive targeting of HLA class I-restricted HPV epitopes may lead to suboptimal and short-lasting $CD8^+$ T cell responses as. Furthermore, these responses might be insufficient as HLA class I surface expression can be reduced as a result of HPV-mediated antigen processing machinery changes. In contrast, HLA class II molecules are expressed in high-grade cervical lesions and cervical cancer. Therefore, we believe that for design of immunotherapeutic approaches against HPV-mediated lesions, it is beneficial to include $CD4^+$ T helper cell epitopes derived from HPV target antigens. In this study, we combined *in silico* HLA class II epitope prediction methods with *ex vivo* immunological evaluation to identify promiscuous HPV16 E2-, E5-, E6-, and E7-derived $CD4^+$ T cell epitopes. Candidate selected HPV16-derived epitopes were found to able to be presented by up to nine out of eleven tested HLA-DR molecules. Furthermore, they were capable to induce frequent and robust HPV16 peptide-specific T cell responses in healthy donors and CIN/ cervical cancer patients, as monitored by IFN-γ ELISpot and cytokine secretion assays. The observed HPV16 peptide-specific immunity was of the Th1 phenotype, and not associated with $CD4^+$ regulatory T cells. These responses to the identified $CD4^+$ T-helper epitopes most likely represent a memory response that correlated with viral clearance in healthy donors. We conclude that the identified T helper epitopes are valuable candidates for the development of a comprehensive therapeutic HPV vaccine.

Oncolytic virotherapy as emerging immunotherapy - Influence of GM-CSF encoding vaccinia virus JX-594 on cell death and human immune system in the human ex vivo melanoma model

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Introduction: Preclinical data are needed to understand the interaction of oncolytic viruses with tumor cells. Of special interest is the influence on tumor-associated immune cells and the role of immunogenic cell death.The oncolytic virus pexastimogene devacirepvec (Pexa-Vec, JX-594) is a vaccinia virus derived from the Wyeth vaccine strain. JX-594 is genetically modified to express GM-CSF. GM-CSF is associated with activation of dendritic cells. Another modification is the deletion of the thymidine kinase (TK) gene to guarantee a selective replication in cells with high levels of TK, which applies to cancer cells. In this case we want to demonstrate the type of cell death induced by JX-594 in melanoma cells but also specify its effects on dendritic cell (DC) maturation by tumor cell lysates (TCL) and cytokine secretion. Therefore we used our human *ex vivo* melanoma model with cytotoxic T cells (CTL) and corresponding melanoma cells from the same patient. The data will be presented in contrast to the non-modified wildtype parvovirus H-1 (H-1PV).

Methods: Melanoma cells SK29Mel or its HLA-A2 loss clone SK29Mel 1.22 were infected with several MOI´s of viruses and incubated alone or in combination with cytostatics for different time periods. Monocytes were isolated via adherence, and differentiation into iDCs was achieved by stimulation with IL-4 and GM-CSF. Mature DCs (mDCs) were generated by stimulation with a cytokine cocktail. DCs were co-cultivated with pre-treated tumor cells and activation and maturation marker CD80, CD86 and CD83 were measured via FACS. Cytokine levels were compared by ELISA.

Results: Infection with JX-594 presented a MOI-dependent reduction of cell viability (MOI 0.0001 - 1). Combination with cytostatic drugs could even strengthen these effects. Especially 5-FU and Docetaxel were potent synergistically agents and increased reduction of cell viability.JX-594 induced TCL further increased DC maturation with higher expression of CD80 and CD83. In combination with 5-FU CD86 expression was also enhanced. JX-594 also induced a slight increase of cytokine levels of TNF-α and IL-6.Immunogenic cell death markers will be presented at the meeting to further define the type of cell death which is induced by JX-594 and H-1PV.

Conclusions: The oncolytic vaccinia virus JX-594 presented strong reduction of cell viability for even lower MOIs.Increased expression of activation and maturation markers could be measured. Combination with specific cytostatic agents could even strengthen these effects.Besides H-1PV, JX-594 is a potent candidate for the immunotherapeutic approach of oncolytic virotherapy.

Engineering T-cell receptors to optimize anti-tumor immunity

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Recognition of malignantly transformed or virally infected cells by T-cells is mediated through the T-cell receptor (TCR). Malignant melanoma is one such malignancy that is immunosensitive. One of the melanoma antigens presented by the MHC is the MART-$1_{27\text{-}35}$ (AAGIGILTV) nonameric peptide, which is recognized by the TCRs DMF4 and DMF5. Clinical trials involving adoptive cell therapy (ACT) of melanoma patients showed cancer regression of 13% and 30% for clonally expanded T-cells genetically engineered to express DMF4 and DMF5. Our work involves using structure-guided computational design to enhance the affinity of DMF5 towards the MART-$1_{27\text{-}35}$ peptide, with the eventual goal of assessing the impact of enhanced affinity on anti-tumor immunity in mouse models of melanoma. Thus far, we have generated five higher affinity mutants of DMF5; αD26Y, αD26W, βL98W, and two double-mutants αD26Y/βL98W and αD26W/βL98W, and are optimizing a retroviral expression system to generate gene-modified human T cells.

ImmTACs: Bi-specific TCR-anti-CD3 fusions for potent re-directed killing of cancer cells

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ORAL SHORT TALK

The human immune system has the potential to clear tumours but fails to do so in many patients, as thymic selection removes high affinity T cell responses to self-antigens. This problem is compounded by the immunosuppressive tumour microenvironment, involving a complex array of mechanisms. To address the issues of low T cell affinity, tumour tolerance and low expression of tumour specific target antigens, we have engineered bi-specific ImmTAC reagents comprising soluble, pico-Molar affinity T cell Receptors (TCRs) fused to an anti-CD3 specific scFv. The TCRs target peptide epitopes derived from cancer-associated antigens presented on the cell surface by Class I MHC, and the anti-CD3 moiety re-directs a potent anti-tumour T cell response. Target epitopes are selected to meet several criteria including Mass Spectrometry validated tumour presentation, highly restricted expression in normal tissues, and frequent expression in common tumour types. T cell lines are derived via in house cloning. Subsequently, TCR chains are identified by RACE and affinity matured before generation of ImmTAC proteins and cellular testing against both antigen positive and negative cell lines and normal tissues.

We have demonstrated that ImmTACs are very sensitive, recognising as few as 10 epitopes per cell; this sensitivity far exceeds that of tumour-specific T cell clones directly isolated from cancer patients. ImmTACs demonstrate potent (pico-Molar EC_{50}) activity *in vitro* and *in vivo*, re-directing T cell killing of cancer cells with very low levels of cell surface MHC whilst sparing antigen negative cells from normal tissues. Our lead candidate, IMC-gp100, an ImmTAC specific for the HLA-A2 presented gp100 (280-288) epitope, is undergoing Phase I/II clinical testing in melanoma patients; it is well tolerated and induces T cell mobilisation and tumour shrinkage. Several other well validated candidates are currently progressing through the company pipeline.

Tumor bearing humanized mice: Identification of a novel feature of anti-EGFR antibodies in cancer therapy

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ORAL SHORT TALK

Numerous humanized monoclonal antibodies (mAb) have been generated for cancer therapy and constitute a promising approach for treatment of patients with metastatic tumors. However, limitations in the predictive value of available animal models restricted the in vivo analyses of these antibody-mediated properties so far. Humanized mice hold promise to overcome these limitations, being able to concentrate on role of the human immune system in cancer defense. As a proof of concept we established a humanized mouse model to evaluate mAb in an epidermiod cancer setting using EGFR-expressing A431 tumor cells. In this context, Cetuximab has been designed to inhibit EGFR signaling and has been postulated as a potent mediator of antibody dependent cytotoxicity (ADCC). However, induction of ADCC in vivo is controversially discussed and could not be addressed in conventional animal models so far. Here, we established a human tumor-bearing mouse model in which human immune cells can engraft and mediate anti-tumor responses. Using immunodeficient NOD/Scid mice transgenic for human MHC class I molecule HLA-A2 and adoptively transferred human HLA-A2$^+$ PBMC after engraftment of human epidermoid cell carcinoma A431 lead to a solid coexistence without evidence of rejection. Addition of high dose anti-EGFR mAb in absence of an immune system induced a strong tumor regression. However, tumor regression by low doses of anti-EGFR mAb treatment was immune cell-dependent and surprisingly not mediated by NK cells but tumor infiltrating CD8$^+$ T effector cells. This CD8$^+$ T cell-mediated ADCC was restricted to IgG1 anti-EGFR mAb. CD8$^+$ T cell-mediated ADCC was also depending on binding to CD16 and could be inhibited after blockade. Furthermore, enhanced glycosylation of the Fc portion of anti-EGFR mAb and presence of IL-15 markedly improved ADCC activity of CD8$^+$ T cells. Taken together, our results provide strong evidence of a novel mechanism of CD8$^+$ T cell-mediated cytotoxicity induced by anti-EGFR mAb with an IgG1 isotype and demonstrate that novel humanized mouse models allow the preclinical testing of therapeutic mAb and the characterization of their functional crosstalk with human immune cells in vivo.

Human IL-2 Mutein with higher antitumor efficacy than wild Type IL-2

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IL-2 has been used for the treatment of melanoma and renal cell carcinoma, but this therapy has limited efficacy and severe toxicity. Currently, it is assumed that part of the limited efficacy is due to the IL-2-driven preferential expansion of regulatory T cells, which dampen the antitumor immunity. In this study, we characterize a human IL-2 mutant with higher antitumor efficacy and lower toxicity than wild type human IL-2 (wtIL-2). The mutant differs from wtIL-2 by four mutations at the interface with the a subunit of IL-2R. The IL-2 mutant induces in vitro proliferation of $CD8^+$CD44hi and NK1.1 cells as efficiently as does wtIL-2, but it shows a reduced capacity to induce proliferation of $CD4^+Foxp3^+$ regulatory T cells. The IL-2 mutant shows a higher antimetastatic effect than does wtIL-2 in several transplantable tumor models: the experimental metastasis model of MB16F0 melanoma and the experimental and spontaneous metastasis models for the mouse pulmonary carcinoma 3LL-D1222. Relevantly, the IL-2 mutant also exhibits lower lung and liver toxicity than does wtIL-2 when used at high doses in mice. In silico simulations, using a calibrated mathematical model, predict that the properties of IL-2 mutein are a consequence of the reduction, of at least two orders of magnitude, in its affinity for the a subunit of IL-2R (CD25). The human IL-2 mutant described in the present work could be a good candidate for improving cancer therapy based on IL-2

Oncolytic immunotherapy with an adenovirus coding for trastuzumab for the treatment of HER2-positive cancer

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Purpose: Introduction of trastuzumab, a monoclonal antibody to human epidermal growth factor receptor 2 (HER2), has significantly improved the survival of HER2-positive breast and gastric cancer patients. Nonetheless, therapeutic antibody production is expensive and systemic administration of trastuzumab carries the risk of cardiomyopathy while local tumor concentrations may remain low. Oncolytic viruses can be armed with therapeutic transgenes and have potential to kill infected cancer cells *per se*. Therefore, we hypothesized that by arming an oncolytic adenovirus with trastuzumab, we could achieve local production and antibody activity combined with oncolysis at the tumor site.

Experimental design: Antibodies are normally produced by B-cells and it is uncertain if functional antibody can be produced in cancer cells. Here, we constructed an oncolytic adenovirus with dual anticancer activity: First, Ad5/3-D24-tras is a chimeric serotype 5 oncolytic adenovirus entailing selective replication and oncolysis in cancer cells. Second, virus encodes human trastuzumab antibody heavy and light-chain genes, linked with an internal ribosomal entry site, thus coupling viral protein expression with local sustained production of trastuzumab. We assessed the trastuzumab production, mechanism of action and efficacy of Ad5/3-D24-tras *in vitro* and *in vivo*.

Results: Ad5/3-D24-tras showed potent breast, gastric and esophageal cancer cell killing *in vitro*. Importantly, cancer cells were able to produce full-length assembled antibody, which was released from infected cells to supernatant as detected by ELISA. Trastuzumab heavy and light chain expression was confirmed by Western blot *in vitro*. In HER2-positive gastric cancer, Ad5/3-D24-tras treatment induced antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by peripheral blood mononuclear immune cells. Moreover, Ad5/3-D24-tras treatment significantly inhibited tumor-growth over controls in a HER2-positive gastric cancer mouse model; improved efficacy was coupled with higher local concentrations of trastuzumab in Ad5/3-D24-tras treated tumors compared to oncolytic parent virus or commercial trastuzumab ($p < 0.05$, both). As indication of immune activation in xenograft model, Ad5/3-D24-tras treatment showed dendritic cell and natural killer cell induction in local lymph nodes.

Conclusions: Ad5/3-D24-tras showed promising *in vivo* efficacy together with functional antibody production and immune cell induction in HER2-positive gastric cancer. Local trastuzumab activity combined with oncolytic potency of Ad5/3-D24-tras is an attractive anticancer approach for HER2-positive cancer and might also potentiate the other immunotherapeutic effects of oncolytic viruses.

Characterization of a novel human IL-15 superagonist to promote engraftment and long-term persistence of human virus- and alloantigen-specific CD8$^+$ T cells in humanized NOD/Scid IL2Rcg-null mice

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Adoptive transfer of donor-derived cytolytic T cells (CTL) directed to leukemia has evolved as a promising strategy to improve antileukemic responses in immunocompromised patients following allogeneic hematopoietic stem cell transplantation. However, durable clinical responses are often hampered by detrimental graft-versus-host disease and limited capability of transferred effector T cells to establish sustained antileukemia immunity. Among various strategies such as the transfer of less differentiated, central memory T cells, Interleukin (IL)-15 has emerged as a promising cytokine to improve adoptive CTL therapy. Upon high affinity binding to the IL-15Rα-chain (Rα) and in trans-presentation to the common IL-2Rβγ-chains, it facilitates the in vitro generation of leukemia-reactive CTL from naïve CD8$^+$ CD45RA$^+$ precursors, supports engraftment of adoptively transferred CD8$^+$ T cells in lymphopenic hosts and promotes homeostasis and formation of T cell memory.

We therefore generated a novel human IL-15 superagonist (IL-15N72D-IL-15RαSu/Fc referred to as ILR-Fc) by first substituting asparagine (N) to aspartic acid (D) at position 72 previously reported to increase the biological activity of IL-15. The modified IL-15 sequence was then linked to the 65 amino-acid spanning IL-15-binding Sushi-domain of IL-15Rα. To improve the bioavailibility of the IL-15 complex we finally fused the truncated IL-15Rα to the Fc-domain of human IgG1 via a second linker. Production of soluble ILR-Fc using the Expi293™ Expression System (Life Technology) and protein-A affinity based purification resulted in ≥ 1mg recombinant protein/25 ml culture. Bioactivity analyses of ILRN72D-Fc as compared to IL-15 (N72D) produced in E coli revealed that ILR-Fc was 10-20 fold more active on supporting proliferation of IL-15 dependent Mo-7e cells than E.coli derived IL-15. Moreover, ILR-Fc supported the generation and expansion of human HLA-A2 reactive CD8$^+$ CTL in vitro at an efficacy superior to commercially available IL-15.

To further test the pharmakokinetic prolife of ILR-Fc in vivo we adoptively transferred EBV- and HLA-A2-specific CD8$^+$ CTL into NSG and HLA-A2 transgenic NSG (NSG-A2) mice and observed marked expansion and prolonged persistence in mice receiving weekly i.p. injections of e.g. 25 ug/ml ILR-Fc/mouse. To achieve comparable effects with E.coli-derived IL-15, mice had to be i.p. injected every 2nd. day with 10 ug/ml/mouse, whereas no sustained CD8 survival was detectable in the absence of IL-15. This extended bioactivity was partially attributed to an increased half-life of ILR-Fc in the serum as determined by IL-15 ELISA. Moreover, ILR-Fc promoted immune-responses of transferred CD8$^+$ CTL in EBV$^+$ B cell-engrafted NSG and NSG-A2 mice, respectively.

In summary, ILR-Fc has potent and prolonged bioactivity to facilitate CD8$^+$ T cell persistence and activity in lymphopenic hosts and might thus serve as a valuable tool to improve selective CD8$^+$ T cell-mediated immunotherapy.

AFM13 - a bispecific anti CD30/CD16A TandAb® for the treatment of CD30$^+$ malignancies: Preclinical and first clinical results in patients with relapsed/refractory (R/R) Hodgkin Lymphoma (HL)

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Introduction: AFM13 is a novel, tetravalent bispecific antibody (Ab) belonging to Affimed's RECRUIT TandAb platform. AFM13 recruits NK-cells to kill CD30$^+$ tumor cells. HL is characterized by CD30$^+$ Reed-Sternberg cells. Salvage treatment of HL is of high medical need despite the establishment of brentuximab vedotin, an anti CD30 antibody-drug conjugate.

Method: Binding and cytotoxic activity of AFM13 were assessed and compared to other CD30 targeting Abs including those with Fc-enhanced activity. Based on these preclinical data, a phase I study in heavily pretreated patients (pts.) with R/R HL was initiated. Pts. received stepwise escalated doses of i.v. AFM13 (0.01 to 7.0 mg/kg) weekly or 4.5 mg/kg twice weekly over 4 weeks. Primary objectives were safety and tolerability, secondary objectives were pharmacokinetics, pharmacodynamics (PD), and efficacy measured using Cheson criteria (2007).

Results: *In vitro* AFM13 demonstrated stronger affinity to CD16A compared to other Abs. NK cell mediated cytotoxicity (EC_{50}) was independent of CD16A allotypes and required the presence of CD30$^+$ cells. In the clinical phase I study, 24 patients received AFM13 weekly, 4 patients were treated twice weekly. AFM13 was well tolerated with mainly mild to moderate adverse events (AEs). The maximum tolerated dose was not reached. AFM13 was detectable in plasma up to 168 h (highest dose) with a half-life of 15-22 hours. AFM13 treatment resulted in NK cell activation and significant decrease of sCD30. The clinical effect was more pronounced in cohorts receiving ≥1.5 mg/kg with an overall response rate of 23% (3/13) and a disease control rate of 77%. AFM13 treatment was effective in patients refractory to brentuximab vedotin.

Conclusion: AFM13 is a highly effective NK-cell recruiter. It was well tolerated and showed anti-tumor activity in a clinical phase I study. PK data indicate that the dose regimen investigated was sub-optimal. A phase II study with an optimized dose regimen is in preparation.

Microtubule-depolymerizing agents used in antibody-drug-conjugates induce anti-tumor immunity by stimulation of dendritic cells

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ORAL SHORT TALK

Antibody drug conjugates (ADCs) are emerging as powerful treatment strategies with outstanding target specificity and high therapeutic activity in cancer patients. Brentuximab vedotin represents a first-in-class ADC directed against CD30-positive malignancies. We hypothesized that its sustained clinical responses could be related to the stimulation of an anti-cancer immune response. We here demonstrate that the dolastatin family of microtubule inhibitors, from which the cytotoxic component of brentuximab vedotin is derived, comprises potent inducers of phenotypic and functional DC maturation. In addition to the direct cytotoxic effect on tumor cells, dolastatins efficiently promoted antigen uptake and migration of tumor-resident DCs to tumor-draining lymph nodes. Exposure of murine and human DCs to dolastatins significantly increased their capacity to prime T cells. Underlining the requirement of an intact host immune system for the full therapeutic benefit of dolastatins, the anti-tumor effect was far less pronounced in immune-compromised mice. When combining dolastatins with tumor-antigen-specific vaccination or blockade of the PD-1/PD-L1 and CTLA-4 co-inhibitory pathways, we observed substantial therapeutic synergies. Ultimately, antibody-MMAE-conjugates induce DC homing and activate cellular anti-tumor immune responses in patients. Our data reveal a novel mechanism of action for dolastatins and provide a strong rationale for clinical treatment regimens combining dolastatin-based therapies, such as brentuximab vedotin, with immune-based therapies.

Combining antibody-directed presentation of IL-15 and 4-1BBL in a trifunctional fusion protein for cancer immunotherapy

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Taking influence on the cytokine-receptor network that modulates the immune response holds great potential for cancer immunotherapy. Although encouraging results have been obtained by focusing on individual members of the common γ-chain (γc) receptor and tumor necrosis (TNF) receptor family so far, combination strategies might be required to further improve the effectiveness of the antitumor response. Here, we propose the combination of IL-15 and 4-1BBL in a single, tumor-directed molecule. Therefore, a trifunctional antibody-fusion protein was generated, composed of a tumor-specific recombinant antibody, IL-15 linked to a fragment of the IL-15Rα chain (RD) and the extracellular domain of 4-1BBL. In soluble and targeted form, the trifunctional antibody-fusion protein RD_IL-15_scFv_4-1BBL was shown to stimulate activated T cell proliferation and induce T cell cytotoxicity to a similar degree as the bifunctional scFv_RD_IL-15 fusion protein. On the other hand, in targeted form, the trifunctional fusion protein was much more effective in inducing T cell proliferation and IFN-γ release of unstimulated PBMC. Here, the additional signal enhancement could be attributed to the costimulatory activity of 4-1BBL, indicating a clear benefit for the simultaneous presentation of IL-15 and 4-1BBL in one molecule. Furthermore, the trifunctional antibody-fusion protein was more effective than the corresponding bifunctional fusion proteins in reducing metastases in a tumor mouse model in vivo. Hence, the targeted combination of IL-15 and 4-BBL in form of a trifunctional antibody-fusion protein is a promising new approach for cancer immunotherapy.

Development of recombinant bispecific antibodies for selective stimulation of the CD95 death receptor on activated B cells and lymphoma cells

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Multimeric monoclonal antibodies directed to the death receptor (CD95/APO-1/Fas) are capable of inducing apoptosis in CD95 expressing and - sensitive cells. However, clinical application of such agonistic antibodies, e.g. to achieve killing of CD95 expressing human cells is precarious, due to expression of CD95 on normal tissue, such as liver.
In 2001 we reported that CD95 antibodies, chemically hybridized to a second antibody directed against a different target antigen on the same cell, such as CD20, induce apoptosis of the cells if the expression of the target antigen exceeds a certain threshold level.
Based on these results we constructed a recombinant CD20 x CD95 antibody. We demonstrate that this reagent effectively and specifically kills lymphoma cells *in vitro* and *in vivo*.
In addition it induces the lysis of activated B cells and the suppression of antibody production *in vitro*.
Both suppression of lymphoma cells and killing of normal activated B cells by the recombinant CD20 x CD95 antibody is more pronounced than that achieved with monospecific CD20 antibodies.

Betulin - a plant-derived cytostatic drug - enhances antitumor immune response

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Conventional cytostatic cancer treatments are rarely curative. New therapeutic strategies are more promising by targeting the tumor microenvironment, inhibiting angiogenesis and antagonizing the immunosuppressive activity of established tumors. Along these lines, plants provide a broad spectrum of potential drugs for cancer therapy, such as betulin, a pentacyclic triterpene of the birch tree representing the reduced congener of betulinic acid. The anticancer activity of betulinic acid has been linked to its ability to directly trigger mitochondrial membrane permeabilization, a central event in the apoptotic process. In contrast to the potent cytostatic action towards different tumor cell lines, non-neoplastic cells and normal tissue remain relatively resistant.

This study aimed to investigate the antitumor activity on melanoma cells and the immune modulating effect on dendritic cells and T cells of phytocompounds, especially betulin. By means of MTT and CSFE proliferation assay as well as nuclear DAPI staining we could confirm that betulin decreased the proliferation rate of both the highly metastatic B16F10 and the low metastatic B164A5 melanoma cell line. In addition, annexin V/7-AAD staining showed that betulin induced a higher rate of apoptosis in melanoma cells as compared to primary bone marrow derived dendritic cells (BMDCs) of C57BL/6 mice. Furthermore, as evaluated by co-incubation assays and ELISA we found that betulin significantly stimulated the TLR-4-dependent IL-12p70 production of murine BMDCs. We could further show that this increased secretion of IL-12p70 protein was due to an increased IL-12p35 mRNA expression, while IL-12p40 mRNA level remained unchanged. Interestingly, these results are in contrast to data of genistein, another plant metabolite with antitumor properties, which caused a down regulation of IL-12p70 production of BMDCs. Subsequent *ex vivo* experiments utilizing OT I spleen cells revealed that the betulin-enhanced activation of dendritic cells loaded with ovalbumin resulted in an increased, antigen-specific T cell stimulation as demonstrated by an induction of IL-2 and IFN-γ. A next step would be to load dendritic cells with specific melanoma antigens to receive a directed immune response against the tumor tissue.

In summary, cytostatic agents like betulin that simultaneously exhibit immune stimulatory activity hold great promise as a novel approach for an integrated cancer therapy.

Enhanced humoral responses following antigen delivery to different receptors on murine dendritic cells

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Background: The superior capacity of dendritic cells (DCs) to regulate adaptive immune responses offers the potential for using DCs in various clinical settings. Monoclonal antibodies directed at DC surface receptors can be employed for targeted delivery of disease-specific antigens to DCs. For this purpose, DCs express a unique pattern of cell surface receptors that can be employed as target structures for such targeted delivery of antigens. Different surface receptors on DCs will, to a varying extent, lead to internalization, processing and presentation of antigenic peptides to T cells. Depending on the intracellular routing of the antigen, antigenic peptides are presented on MHC class I and/ or class II facilitating induction of different T cell responses. While some DC surface molecules, e.g. CD205 and Clec9A, have already been shown to constitute useful targets for antigen-delivery, it is, however, likely that other structures could turn out to be even better vaccine targets.

Methods: Mice were immunized subcutaneously with 5 µg of monoclonal rat antibody specific for each of the ten target receptors. As non-targeted controls, mice were immunized with a non-targeting isotype-matched monoclonal antibody. Mice were immunized twice and serum samples were collected at two weeks intervals until day 84. The levels of mouse anti-rat IgG antibodies were determined by quantitative ELISA.

Results: The results presented here demonstrated that antigen-delivery to DCs even in the absence of adjuvants may mediate strong humoral responses. Interestingly, considerable difference in both the strength and IgG subclass composition were observed between the ten target receptors. The target receptors that elicited the strongest antibody responses were Clec7A and CD11c. Targeting of Ag to CD36, CD205, Clec6A and PDC-TREM did also induce potent humoral responses although not as strong as the responses obtained with CD11c and Clec7A. Targets like CD209, Clec9A and Siglec H did not lead to potent induction of antibody responses as they only showed responses comparable to the ones elicited by the non-targeted control. The levels of the IgG subclasses were for the majority of targets dominated by an IgG1 response. Clec7A were the only target receptor that lead to robust amounts of IgG2a indicating the presence of a manifest Th1 component following antigen-delivery to this receptor on DCs.

DNA vaccination in combination with an RNAi-based genetic adjuvant silencing STAT3 promotes migration of mature dermal dendritic cells and antitumor T cell responses against B16 melanoma

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DNA vaccines represent an attractive alternative for cancer immunotherapy. However, plasmids used for DNA vaccination can induce overexpression and activation of STAT3, a transcription factor that inhibits the maturation and function of dendritic cells and the subsequent induction of antitumor T cell immunity. Therefore, developing an RNAi-based adjuvant targeting STAT3 represents an attractive strategy to overcome this potential limitation and enhance the potency of DNA vaccines encoding the tumor antigens. Plasmids encoding a short hairpin RNA (shRNA) against STAT3 (shSTAT3) and the melanoma antigen tyrosinase-related protein 2 (TRP2) were generated. STAT3 knock-down was corroborated *in vitro* by western blot and RT-qPCR and *in vivo* by RT-qPCR of mouse skin intradermally electroporated with plasmid DNA. STAT3 expression was up-regulated in transfected cells and electroporated tissue when using empty plasmid or control shRNA. In contrast, shSTAT3 was able to decrease STAT3 expression. We also evaluated the migration and maturation status of skin-derived dendritic cells in draining lymph nodes and skin explants by flow cytometry. Intradermal DNA electroporation induced a rapid migration of highly mature dermal dendritic cells, which was further promoted by co-administration of shSTAT3, as compared to control shRNA or empty plasmid. Antigen-specific $CD8^+$ T cell responses were evaluated after two vaccinations by *in vitro* peptide stimulation followed by intracellular cytokine staining for flow cytometry. Mice immunized with pTRP2 mounted a TRP2-specific $CD8^+$ T cell response that was further increased in shSTAT3 co-electroporated mice. Vaccinated mice were then intravenously challenged with B16F10 melanoma cells and lung melanoma foci were counted after three weeks. Animals treated with pTRP2 and shSTAT3 showed enhanced protection against melanoma as noted by decreased lung metastasis formation compared to control groups. In conclusion, we showed that silencing STAT3 during intradermal DNA electroporation promotes migration of mature dermal dendritic cells and antitumor CTL responses in a mouse melanoma model.

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Protein Kinase B (PKB/Akt) limits the expression of Cbl-b via Glycogen Synthase Kinase-3 (GSK-3) and converts T cell tolerance to activation

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Several animal models have shown that increased activity in the PI3K/PKB signaling cascade alters T cell homeostasis and contributes to the development of autoimmune disease. To understand the mechanisms that initiate disease, we examined whether PKB could alter the outcome of T cell tolerance induction *in vivo* and promote T cell function and autoimmunity. Peptide antigen administration generally leads to the induction of T cell tolerance *in vivo* that can be measured as the deletion of T cells and/or the induction of anergy. Here, we have found that the persistant presence of active PKB signaling subverts the induction of tolerance to peptide antigen and instead results in autoimmunity. Furthermore, we show that PKB promotes autoimmunity by limiting the expression of Cbl-b via a novel post-translational, GSK3-dependent mechanism. Phosphorylation of Cbl-b by GSK-3 on residues Ser^{476} and Ser^{480} was found to be required for the stable expression of the Cbl-b protein. We found that reduced expression of Cbl-b protein was sufficient to convert tolerance to activation. These results define a new molecular pathway that regulates the induction of tolerance versus activation *in vivo*. Furthermore, they identify GSK-3 as a key regulator of Cbl-b expression and a target for modulation in adoptive T cell therapy for tumors.

Antigen-specific T cell Redirectors (ATR): a nanoparticle based approach for antigen-specific redirection of T cells to tumors

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Immunotherapy is the modulation of a patient's immune system to treat illness. Unfortunately many T cell based attempts have failed due to the fact that existing tumor-specific T cells are mostly anergic or tolerized and ex vivo generated T cells are often already of exhausted phenotype. Therefore, investigators have developed alternative approaches including bispecific antibody technology to redirect fully functional non-tumor specific T cells to the tumor. This has been primarily accomplished through targeting CD3 which is expressed on all T cells to engage and redirect them towards a molecule that is expressed on the tumor cells. Here we present a novel nanoparticle based approach to selectively target cytotoxic T cells (CTL) and redirect them to kill tumors, termed ATR (Antigen-specific T cell Redirectors).

ATR were generated by coupling either MHC-Ig dimer or clonotypic anti-TCR antibody 1B2 to target the effector T cell population and an anti-CD19 to re-direct those to $CD19^+$ tumor target cells onto 50-100nm nanoparticles. Flow cytometry and microscope based data confirm that the described ATR phenotype efficiently and stably stain tumor and T cells in a dose dependent manner and ATR mediate antigen-specific conjugate formation of effector T cells and tumor target cells. We further developed two clinically relevant protocols to test and optimize our ATR in vitro. First a pre-treatment approach in which the effector T cells are pre-incubated with ATR mimicking an adoptive transfer approach and second a co-culture protocol that mimics an active immunotherapy approach of direct ATR injection. Antigen-specific ATR mediated re-direction of T cells to tumor target cells was demonstrated in ^{51}Cr-release killing assays at low E:T ratios. Variation of ATR target-cell : effector-cell targeting molecule ratio could further increase efficacy. Finally, intra tumoral ATR injection induced T cell re-direction and reduced tumor growth in a s.c. Raji/SCIDbeige treatment model.

In summary this data demonstrates that ATR target and redirect antigen-specific CTL to tumor cells that would otherwise not be recognized and mediates their lysis. ATR can be used to develop new innovative immunotherapeutic approaches for all cancers that can be targeted with antibodies or antibody-like molecules. Furthermore, ATR could also be used in conjunction with virus-specific immunization to specifically increase the targeted CTL population. Ultimately, we expect ATR and their potential for clinical applications to increase our understanding of tumor immunotherapy through T cell redirection.

Allo-reactive CD8$^+$ T cell clones specific for the self-protein CD20 show superior avidity compared to autologous T cell clones of the same specificity

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As most tumor antigens represent self-proteins, high-avidity T cells are largely deleted from the autologous repertoire. Allogeneic T cells from human leukocyte antigen (HLA)-mismatched donors provide repertoires wherein such cells have not been systematically deleted. We have previously reported the successful generation of allo-reactive cytotoxic T cells (CTLs) from HLA-A*02:01^{neg} donors, specific for a peptide (SLFLGILSV) from the B cell antigen CD20 in complex with HLA-A*02:01 (A2/CD20p). These CTLs efficiently killed HLA-A*02:01^{pos}/CD20^{pos} target cells, including primary cancer cells from patients with chronic lymphocytic leukemia, follicular lymphoma or acute lymphoblastic leukemia, while a range of other HLA-A*02:01^{pos}/CD20^{neg} target cells were spared, indicating a high degree of specificity (Abrahamsen et al., Leukemia 2010; Abrahamsen et al., Int J Cancer 2011; Kumari et al., PNAS 2013). Another study demonstrated that A2/CD20p-specific CTLs could be generated from HLA-A*02:01^{pos} donors, but these CTLs killed HLA-A*02:01^{pos}/CD20^{pos} targets only when high effector-to-target ratios were used, suggesting low avidity. The aim of the present study was to further investigate and directly compare the avidity of autologous and allo-reactive A2/CD20p-specific T cells. Peripheral blood mononuclear cells from HLA-A*02:01^{pos} or HLA-A*02:01^{neg} healthy donors were co-cultured with antigen-loaded autologous monocyte-derived dendritic cells (moDCs). HLA-A*02:01^{neg} moDCs were transfected with HLA-A*02:01 mRNA prior to antigen loading. A2/CD20p-specific CTL clones were established from the co-cultures following flow cytometric sorting of single CD8$^+$ T cells staining positively with fluorescent multimers of monomeric HLA-A*02:01/CD20p. A2/CD20p multimer positive CTL clones were tested for degranulation in response to peptide-loaded HLA-A*02:01-transduced SupT1 cells or target cells naturally expressing HLA-A*02:01 and CD20, as measured by the percentage of CD8$^+$ cells that were CD107a/b$^+$ by flow cytometry. When target cells were pulsed with high peptide concentrations of 10 nM-1 µM, the responses of autologous and allo-reactive CTL clones were comparable. However, at a concentration of 100 pM CD20p, the median degranulation response in the allo-reactive CTL clones was > 7-fold higher than in the auto-reactive CTL clones. Whereas the median degranulation response was only 4.64% (range 1.75%-34.0%, n=9) among the auto-reactive CTL clones at 100 pM, the median response for allo-reactive CTL clones was 42.2% (range 12.7%-72.6%, n=20) even at the lowest peptide concentration of 1 pM. Thus, allo-reactive T cell clones specific for a self-peptide in complex with HLA-A*02:01 seem to be of higher avidity as compared to autologous clones specific for the same peptide-HLA complex, as indicated by manifold higher responses at low peptide concentrations and responses to 100-fold lower peptide concentrations.

Combinatorial strategy to improve immune response to cancer vaccine for hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and represents the third and the fifth leading cause of cancer-related death worldwide in men and women, respectively. Hepatitis B (HBV) and hepatitis C (HCV) virus chronic infections account for more than 80% of primary HCC. Prognosis of HCC is generally poor because of the low effectiveness of available treatments and the overall 5-year survival rate is approximately 5-6%.

Immunotherapeutic interventions, including cancer vaccines, may represent a novel and effective strategy. However, only few immunotherapy trials for HCC have been conducted so far with contrasting results, suggesting that improvements in several aspects of the immunotherapy approaches need to be implemented.

In the present study a novel combinatorial strategy, based on chemotherapy plus vaccine, is evaluated in a mouse model. The chemotherapy is a multidrug cocktail including taxanes and alkylating agents, which is administered in a metronomic-like fashion. The vaccine is a multi-peptide cocktail including HCV epitopes, derived from NS3 and core viral proteins, as well as universal tumor antigen hTERT epitopes. The combinatorial strategy designed and evaluated in the present study induces a significant enhancement in eliciting specific T cell responses, when compared to vaccine alone. The full evaluation of the observed enhanced cellular immune response is currently ongoing and will be presented.

Such results are highly promising and may pave way to relevant improvement of immunotherapeutic strategies for HCC and beyond.

Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8 T cells

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Cross-presentation of cell-associated antigen is important in the priming of CD8 T-cell responses to proteins that are not expressed by antigen-presenting cells (APCs). *In vivo*, dendritic cells are the main cross-presenting APC, and much is known regarding their ability to capture and process cell-associated antigen. In contrast, little is known about the way death effector pathways influence the efficiency of cross-priming. We hypothesized that the molecular mechanism of cell death influences the immunologic instruction given to the APC and we focused our attention on two of the major classes of programmed cell death (PCD): type I PCD (or classical apoptosis) and type II PCD (referred to as autophagic cell death or caspase-independent cell death).

In our system we used wild-type mouse embryonic fibroblasts (MEFs) for classical apoptosis, and for caspase-independent cell death, with increased features of autophagy, we used *Bax/Bak*$^{-/-}$ double knockout (DKO) fibroblasts. Both were virally infected with influenza as a source of cell-associated antigen. We found that immunization with cells undergoing autophagy before cell death, were superior in facilitating the cross-priming of antigen-specific CD8 T cells. Silencing of a*tg5* expression rendered the DKO fibroblast less immunogenic, illustrating the need of the atg5 cascade for efficient cross-priming . Type II PCD resulted also in a more efficient cross-presentation of viral antigen and the availability of MHC I/peptide complexes was increased as monitored by proliferation of antigen specific transgenic CD8 T-cells. Interestingly we found no supporting evidence for enhanced immunogenic cell death, as caspase-independent cell death did not expose calreticulin on the cell surface undergoing Typ II PCD but autophagy in the antigen donor cell triggered the production of type I IFNs in phagocytic DCs.

We conclude that autophagy is an interesting form of “immunogenic death” with the enhanced priming efficiency being a result of persistent MHC I cross-presentation and the induction of type I interferons. We suggest to expand the research on non-viral antigens and suspect that targeting the autophagy cascade may provide a therapeutic strategy for achieving robust cross-priming of viral and tumor-specific CD8^{+} T cells.

Natural killer cells are potently activated by interleukin-15 dendritic cells

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Introduction: Given their prime role in the generation of antitumor immunity, dendritic cells (DCs) have attracted great interest for anticancer vaccination. After 2 decades of clinical trials, it is clear that further optimization efforts are still needed to fully unlock the therapeutic activity of these cells. Recently, evidence has emerged that natural killer (NK) cells may hold the key to effective DC-based immunotherapy in cancer patients. This underscores the need to design novel DC vaccine formulations endowed with capacity to stimulate the innate arm of the immune system.

Method: Human monocyte-derived DCs were generated according to our rapid DC culture protocol involving interleukin (IL)-15 for DC differentiation and a Toll-like receptor agonist for DC maturation (Anguille *et al.* J Transl Med 2009). These DCs, hereafter termed IL-15 DCs, were compared with the "gold-standard" IL-4 DCs used in clinical vaccination trials today, in terms of their capacity *(1)* to induce phenotypic NK cell activation, *(2)* to stimulate NK cell cytokine secretion (IFNγ), and *(3)* to harness the antitumor cytotoxic effector function of NK cells.

Results: NK cells are highly activated following stimulation with autologous IL-15 DCs, as evidenced phenotypically by the upregulation of activation markers such as CD56, CD69 and HLA-DR. Importantly, expression of NKG2D and natural cytotoxicity receptors (NCRs) NKp30 and NKp46 on NK cells is enhanced after IL-15 DC exposure, whereas IL-4 DCs appear to have an adverse effect on expression of NKG2D and NCRs. With regard to the cytokine secretion function of NK cells, no raise in NK cell-derived IFNγ is observed after DC stimulation, nor after stimulation with DCs in combination with tumor target cells. The cytotoxic capacity of NK cells, however, is radically increased by IL-15 DCs but not by IL-4 DCs. This effect is not only observed against the classical NK-tumor target cell line K562, but also against the otherwise NK-resistant Daudi tumor cell line. IL-15 DC-activated NK cells exert no "off-target" toxicity towards DCs themselves or towards autologous peripheral blood lymphocytes. Transwell experiments show that the enhancement of NK cell-mediated tumor cell killing by IL-15 DCs is predominantly contact-dependent. Neutralization of membrane-bound IL-15 indicates that IL-15 is the prime mediator in this cell-to-cell interaction.

Conclusion: Here we show that IL-15 DCs, but not conventional IL-4 DCs, induce phenotypic NK cell activation and harness the cytotoxic antitumor effector function of NK cells in a contact- and IL-15-dependent fashion. Given the importance of NK cells in the effectiveness of DC-based immunotherapy, the results of this study provide further support for the implementation of IL-15 DCs in DC cancer vaccine protocols.

Stimulation of cytotoxic activity by killer dendritic cells and natural killer cells against HPV-positive tumor cells by a HPV vaccine

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Cervarix™ is approved as a preventive vaccine against infection with the human papillomavirus (HPV) strains 16 and 18, which are causally related to the development of cervical cancer. We are the first to investigate *in vitro* the effects of this HPV vaccine on interleukin (IL)-15 dendritic cells (DC) as proxy of a naturally occurring subset of blood DC, and natural killer (NK) cells, two innate immune cell types that play an important role in antitumor immunity. Our results show that exposure of IL-15 DC to the HPV vaccine results in increased expression of phenotypic maturation markers, pro-inflammatory cytokine production and cytotoxic activity against HPV-positive tumor cells. These effects are mediated by the vaccine adjuvant, partly through Toll-like receptor 4 activation. Next, we demonstrate that vaccine-exposed IL-15 DC in turn induce phenotypic activation of NK cells, resulting in a synergistic cytotoxic action against HPV-infected tumor cells. Our study thus identifies a novel mode of action of the HPV vaccine in boosting innate immunity, including killing of HPV-infected cells by DC and NK cells.

Antitumor efficacy of anti-CTLA4 antibody is dependant on gut microbiota.

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The cytotoxic T lymphocyte antigen-4 (CTLA-4)-blocking antibody ipilimumab is an FDA and EMEA-approved treatment for metastatic melanoma, which induces immune-mediated long-term control in 15% cases. However, ipilimumab also results in substantial immune related side effects including grade III-IV colitis. To uncouple efficacy and toxicity we analyzed the mode of action of anti-CTLA4 therapy in preclinical mouse models. Recently, the gut microbiota was implicated in response to anti-cancer therapies (Viaud et al 2013, Iida et al. 2013), but little is known about the microbiota in anti-tumor responses or toxicity development post anti-CTLA4 therapy. Here, we show that disruption of the microbial balance by a broad spectrum antibiotic cocktail impairs the antitumor response following anti-CTLA4 immunotherapy. The anti-CTLA4 efficacy relies on Gram negative bacteria, since the use of specific antibiotics that target Gram negative bacteria (Colistin) resulted in reduced tumor control, whereas the antitumor response is maintained or ameliorated in vancomycin-treated mice (antibiotic against Gram positive bacteria). Under a broad spectrum antibiotic regimen, the intratumoral infiltrate of leukocytes is reduced, $CD8^+$ tumor infiltrating T cells respond poorly to therapy, with decreased interferon gamma (IFN-γ)-production and no upregulation of ICOS, while $CD4^+Lag3^+$ cells fail to lose Foxp3, yet a normal response to mouse ipilimumab is observed. Supporting a role for gut microbiota in the antitumor effects of CTLA4 blockade, a loss of efficacy of the antibodies was observed in germ free mice paralleling a reduced accumulation of TH1 TILs in tumor beds.

Loss of epithelial barrier integrity induced by anti-CTLA4 after 1 or 3 Ab injections is observed with reduced mucosal thickness, disorganization of the crypts at the colon level, as well as increased Goblet and Paneth cell numbers in the small intestine. Finally, we observed dramatic dysregulation of the lamina propria T cells with anti-CTLA4 which elicited a raise in IFN-γ producing $CD4^+$ T cells and a higher proportion of RORγt-expressing $CD4^+$ T cells and γδ T cells while Treg remained stable. Thus, anti-CTLA4 modifies the balance of immune cells in the gut toward a pro-inflammatory pattern. These modifications may facilitate Gram negative bacterial translocation to secondary lymphoid organs. Although no overt colitis was observed with CTLA4 blockade, the combination of anti-CTLA4 and anti-IL-10 antibodies aggravated the epithelial damage, promoted pTH17 in the spleen culminating in synergistic antitumor effects. Data on the biofilm pyrosequencing will be available to delineate which Gram negative bacteria plays a probiotic role in these synergistic effects.

Our data confirm the importance of the gut microbiota in the clinical efficacy and potentially the toxicity of CTLA4 blockade and highlight the complex interplay between the gut mucosa, systemic and antitumor immunity.

Bacillus Calmette-Guérin (BCG) as an adjuvant immunotherapy for HPV-associated tumors

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Human papillomavirus (HPV) infection has been recognized as a major public health problem. High-risk HPV infection accounts for about 5 percent of all cancers worldwide, including virtually all cervical, and the majority of oropharyngeal localizations. Recurrent respiratory papillomatosis (RRP) is the most common benign neoplasm of the larynx caused by the low-risk HPV-6 and HPV-11. The disease is characterized by recurrent proliferation of squamous papillomas within the respiratory tract resulting in severe airway obstruction. Despite its benign nature, in 3-5% of patients, respiratory papillomas may undergo malignant transformation to squamous cell carcinoma with quite poor prognosis. Currently, the main therapeutic approach in RRP is surgical removal of the laryngeal papillomas. The adjuvant therapy as IFN-a, some antiviral drugs (Ribavirin, Cidofovir) and inhibitors of cyclooxygenase-3 or EGFR do not prevent efficiently the recurrence of the papillomas.

Bacillus Calmette-Guérin (BCG) is a potent stimulator of Th1 response and has been successfully applied for treatment of superficial bladder tumors and malignant melanoma. However, data about the effects of combined surgical/ BCG immunotherapy in RRP patients are scarce.

The present study investigates the effects of BCG on antiviral immune response in RRP patients subjected to combined CO_2 microsurgery /BCG therapy.

Materials and methods: RRP patients (n = 17) subjected to combined CO_2 laser microsurgery / BCG-immunotherapy were studied before (0), 6, 12 and 20 months after the start of immunomodulation. The percentage of plasmocytoid dendritic cells (pDCs), mature dendritic cells, effector Tc1, Th1, Th17 and regulatory T-cells, as well as the *in vitro* stimulated secretion of Th1/Th2 cytokines were determined by flow cytometry in comparison to healthy controls.

Results: Significantly decreased levels of plasmocytoid and mature DCs were established in untreated RRP patients (average 4.8% and 32% respectively), that approached healthy control levels after 12 months of BCG application (8.5 vs. 8, and 55 vs. 60, $p > 0.5$ for both). In addition, 12 months of BCG immunotherapy significantly decreased the share of proinflammatory Th17 (0.4 vs. 0.69, $p < 0.05$) and increased the level of circulating Treg (4.6 vs. 7.1, $p < 0.05$). Finally, the initially decreased IFNg/IL-4, and IFNg/IL-10 ratios were restored after 20 months of BCG application.

These results indicate that RRP is characterized by ineffective antiviral response due to disturbed Th1/Th2/Th17 cytokine background and imbalanced differentiation of proinflamatory, effector and Treg cells.

Conclusion: BCG immunotherapy shows promising results in treatment of laryngeal papillomas as a result of the enhanced antiviral T-cell response. Further studies are warranted to confirm the potential role of BCG as adjunctive therapy approach in other HPV-associated tumors.

The human cationic amino acid transporter 1 (hCAT-1) is crucial for efficient T cell function

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ORAL SHORT TALK

The semi-essential amino acid L-arginine is crucial for the activation and the efficient function of primary human T lymphocytes. The depletion of L-arginine, as found in tumor tissues and sites of acute and chronic inflammation, is followed by both, the profound decline of T cell proliferation and down-regulation of their IFN-γ secretion. This is at least partially responsible for a deficiency of functional T cells in the tumor micromilieu. We were therefore wondering which transporters are responsible for L-arginine transport into human T cells and if L-arginine transporters play a functional role in human T lymphocyte activation.

The analysis of the expression of L-arginine transporters in resting primary human T lymphocytes revealed a notable expression of the system y^+L amino acid transporter y^+LAT2 mRNA, but only a weak expression of hCAT-1 mRNA. In contrast, upon stimulation with anti-CD3/anti-CD28 coupled beads, hCAT-1 mRNA expression was significantly induced and surpassed the expression level of y^+LAT2. Maximal hCAT-1 mRNA expression was already observed after 6 hours of stimulation. On protein level, hCAT-1 expression increased up to 48 hours of stimulation, our latest point of analysis. This was also true under L-arginine starvation, a condition in which translation of most proteins is decreased or shut down. Arginine transport was then measured by quantification of ^{3}[H]arginine influx in resting and stimulated T cells. In unstimulated T lymphocytes uptake of ^{3}[H]arginine was barely detectable, whereas in activated cells L-arginine uptake was strongly induced. Additionally, the time courses of the rise in L-arginine uptake and hCAT-1 protein correlated well.

The irreversible hCAT-1 inhibitor N-ethylmaleimide considerably inhibited L-arginine transport in stimulated T lymphocytes, demonstrating that the L-arginine transport was indeed hCAT-mediated. To elucidate the functional role of hCAT-1 in stimulated T cells, hCAT-1 expression was suppressed by siRNA. This resulted in a strong reduction of hCAT-1 mRNA and protein as well as of activation-induced L-arginine uptake. T cell proliferation in these cells was markedly attenuated. IFN-γ release remained unaffected by siRNA-mediated down-regulation of hCAT-1.

In summary, our data indicate that the hCAT-1 transporter is predominantly responsible for L-arginine influx into primary activated human T lymphocytes and that hCAT-1 plays a crucial role for their proliferation upon TCR- and costimulation-mediated activation.

Enhancement of hCAT-1-mediated L-arginine transport in tumor-infiltrating T cells in combination with arginase inhibition in the tumor microenvironment might become part of novel immune-enhancing antitumoral treatment strategies.

Engineering dendritic cells to secrete interferonα by mRNA transfection enhances their ability to promote tumor antigen-specific Tcell and antitumor NKcell effector functions

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Background: Dendritic-cell (DC) vaccination is widely being investigated as a promising new treatment in different malignancies. However, objective and durable clinical response rates remain low. Type-I interferon (IFN) signaling on DCs was recently reported to be essential in mice for tumor rejection by the adaptive and innate immune system. Hence, targeted delivery of IFN-α by DCs to immune cells could boost the generation of anti-tumor immunity following DC cancer vaccination while avoiding the side effects frequently associated with systemic administration. In the present work, we hypothesized that engineering human monocyte-derived DCs to secrete IFN-α enhances their ability to promote adaptive and innate immunity.

Methods: Mature human monocyte-derived DCs were generated according to our two-step, clinical-grade culture protocol with GM-CSF and IL-4 for 5 days, followed by the addition of TNF-α and PGE_2 for an additional 2 days (Van Tendeloo *et al.*, Proc Natl Acad Sci USA, 2010). After harvesting, DCs were mock-electroporated or electroporated with *WT1* mRNA, *IFN-α* mRNA, or both *WT1* and *IFN-α* mRNA. DCs were assessed *in vitro* for IFN-α secretion, viability, phenotype, and their capacity for stimulating T and NK cells.

Results: *IFN-α* mRNA electroporation of DCs resulted in high levels of IFNα secretion, independently of coelectroporation with *WT1* mRNA, and increased viability and phenotypic maturation. While *IFNα* mRNA electroporation reduced the induction of allogeneic $CD4^+$ T-cell proliferation by DCs in a mixed lymphocyte reaction, it significantly enhanced IFN-γ secretion by a WT1specific $CD8^+$ T-cell clone in response to *WT1* mRNA-electroporated DCs. In addition, *IFN-α* mRNA-electroporated DCs were capable of expanding autologous WT1-specific $CD8^+$ T cells. Finally, *IFN-α* mRNA-electroporated DCs also promoted the survival, phenotypic activation, and cytotoxicity and IFNγ secretion against K562 and Daudi tumor cells of autologous NK cells in an IFN-α-dependent manner.

Conclusions: Collectively, we show that *IFN-α* mRNA-electroporated DCs are potent stimulators of both adaptive and innate anti-tumor immunity *in vitro* through the secretion of IFN-α. Our work thus offers a new and immediately applicable strategy which could improve the therapeutic efficacy of DC cancer vaccination, and paves the way for clinical trial evaluation.

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