**Paramunity-inducing Factors (PINDs) in dendritic cell (DC) cultures lead to impaired antileukemic functionality of DC-stimulated T-cells**

***Christian Ansprenger1, Valentin Vogt1, Julia Schick1, Annika Hirn-Lopez1,Yvonne Vokac1, Ihor Harabacz2, Marion Braeu3, Tanja Kroell1, Axel Karenberg4, Hans-Jochem Kolb3, Helga Schmetzer1, 2***

*1 Dept for Hematopoetic Transplantations, MED3, University of Munich, Germany*

*2 VironPearl Biotech GmbH , Munich*

*3 Helmholtz Center Munich, CCG-HCT, Munich, Germany*

*4 Institute for the History of Medicine and Medical Ethics, University of Cologne, Germany*

short title: PIND-stimulated antileukemic T-cells

Correspondence:

Prof. Dr. Helga Schmetzer (PhD)

Med III, Department for Hematopoietic Transplantations, Klinikum Grosshadern

University Hospital of Munich - Grosshadern

Marchioninistr.15

81377 Munich

Germany

Phone: +49-89-4400-73137

Fax: +49-89-4400-76137

e-mail: Helga.Schmetzer@med.uni-muenchen.de

The project-idea is summarized and listed as inventional report of Hans-Jochem Kolb and Helga Schmetzer at the University Hospital of Munich; CONPIND is listed as Patents No DE3504940 A1 and US 6162600 A of Anton Mayr. This project was supported by RIEMSER pharmaceuticals and Viron Pearl Biotech GmbH.

***ABSTRACT***

***Introduction:*** Paramunity-inducing-Factors (PINDs) consist of attenuated/inactivated viruses of various poxvirus-genera, used in veterinary medicine as non-antigen-specific, non-immunising stimulators of the innate immune system against infectious and malignant diseases. Their danger-signaling-interactions were tested for their capacity to improve leukemic antigen-presentation on DC generated from AML-patients’ blasts (‘DCleu’) and DC-stimulation/activation of antileukemic T-cells.

***Methods:*** We analyzed, whether the addition of PINDs during DC cultures (15 healthy, 22 leukemic donors) and mixed lymphocyte culture (MLC, n=15) with autologous (n=6), allogeneic (n=2) or T-cells after stem cell transplantation (SCT; n=7) would alter the quality and quantity of DC, the composition of T-cell-subsets, and/or their antileukemic functionality (AF) as studied by FACS and functional Fluorolysis-cytotoxicity-assays.

***Results:* Effects on 1. DC-cultures:** PINDs in DC-cultures lead to increased proportions of mature DC and DCleu, but reduced proportions of viable and overall, as well as TLR4- and TLR9-expressing DC. **2. MLC:** PINDs increased early (CD8+ )T-cell activation (CD69+), but reduced proportions of effector-T-cells after MLC **3. AF:** Presence of PINDs in DC- and MLC-cultures reduced T-cells’ as well as innate cells’ antileukemic functionality. **4. Cytokine-release profile:** Supernatants from PIND-treated DC- and MLC-cultures resembled an inhibitory microenvironment, correlating with impaired blast lysis.

***Conclusions:*** Our data shows that addition of PINDs to DC-cultures and MLC result in a “blast-protective-capacity” leading to impaired AF, likely due to changes in the composition of T-/innate effector cells and the induction of an inhibitory microenvironment.

PINDs might be promising in treating infectious diseases, but cannot be recommended for the treatment of AML-patients due to their inhibitory influence on antileukemic functionality.

**Keywords:** AML, paramunity, dendritic cells, T-cells, immunotherapy, PIND, Zylexis

**Introduction**

Allogeneic hematopoietic cell transplantation (allo-SCT) represents the only potentially curative therapeutic strategy in acute myeloid leukemia (AML) [1], with donor T cells being the most important mediators of antileukemic reactions [2, 3], though relapses after SCT occur. Restoring remission in relapsed patients after allogeneic SCT can be augmented by a transfusion of donor T cells (DLI), thereby emphasizing the central role of T-cells. However, not all relapsed patients respond to a DLI-based therapy, moreover, graft versus host reactions can impair the effectiveness of SCT and immunotherapy of relapses [4]. Reasons for varying T-cell effects and methods to restore or improve the anti-leukemic capacity of effector-cells, such as T cells engineered to express chimeric antigen receptors (CARs) or engineered T cell receptors (TCRs) have provided promising results and are the subject of ongoing research [5].

DCs, amongst other tasks, act as key controllers of antigen-specific effector T cell responses, stimulating tumor cytotoxic T cells in particular. They thereby serve as an essential link between the innate and the adaptive immune systems. This makes DC-based tumor immunotherapies a highly interesting target for anti-tumor or anti-leukemic vaccination strategies, summarized under the term "DC vaccines". Molecular identification of tumor-specific antigens recognized by T lymphocytes has led to different strategies of their presentation through DC, e.g. antigen-pulsing [1, 6, 7]. A different approach, which is pursued by our group, employs DC of leukemic origin (DCleu), which can be generated by directly converting leukemic cells from AML patients in vitro (as described below), thereby co-presenting DC-typical antigens (e.g. CD40, CD86, CD80, CD1a, CD83), and thus regaining the stimulatory capacity of mature professional DC [6, 8, 9].

Our group has been able to further elicit DCleu-containing-DC induced T cell response patterns after mixed lymphocyte culture (MLC) of DC with T cells: T-cells, obtained from AML-patients (autologous) or donors (allogeneic), can be stimulated by those DCleu, resulting in very efficient cytotoxic effector cells with specific lytic activity against naïve blasts, although not in every case [10, 11], which prompted us to search for means to further improve the method.

Ithas been shown, that in addition to disease-specific effects, vaccines against infectious diseases can have nonspecific effects on the ability of the immune system to react to other targets [12]. The so called “Paramunity inducers” (PINDs) utilize this as their functional principal.

PINDs consist of highly attenuated (by 0.05 % β-propiolactone) and inactivated virus strains of various poxvirus-genera with closely linked protein complexes in the envelopes of the virus particles being responsible for their efficacy. Their development was based on observations of positive side-effects of smallpox vaccinations repeatedly described from the late 18th century onwards: healing of chronic skin rashes and reduced susceptibility to various infectious diseases, e.g. measles, scarlet fever and whooping cough. Even the prophylactic use of the vaccination, e.g. against syphilis, is described [13–15]. An early case report on long-term remission of chronic lymphatic leukaemia after smallpox revaccination dates back to the late 1970s [16].

The first *experimental* proof of non-specific effects from a specific trigger came from the observation of the so called “ring-zone-phenomenon”. This was based on the production of soluble antiviral substances in infected chicken embryos and cell cultures after being exposed to avi-pox-viruses, thereby protecting neighbouring cells from infections [17]. Based on these experiments Mayr et al. began to develop PINDs as non-immunising vaccines, with the capability of generating endogenic protective, non-antigen specific (“paramunising”) mechanisms. PINDs lead to an activation and regulation of the paraspecific, i.e. innate and unspecific immune system against noxious substances from the outside (e.g. bacteria) or the inside (tumours cells) [14, 15]. Ahne et al. [18] showed, that the PIND “CONPIND” initiated the production of major inflammatory mediators, most notably TNF-α, in whole blood and in human mononuclear cell cultures. The most notable evidence of their potential efficacy was shown with cats positive to feline leukaemia virus (FeLV), which recovered within two weeks after PIND-treatment [19]. Regardless of the promising results, there is a scarcity of non-veterinarian studies on PINDS and there are none in a malignant setting.

Interactions between pathogen-associated molecular patterns (PAMPs) on the surface of pathogens, and innate immune pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), control for both, innate and adaptive immunity, thereby offering a possible mechanism of action of PINDs. Cancer immunotherapy may utilize immune responses against PAMPs. For example TLRs are known to be able to activate a complex signaling cascade, leading to regulation of DC-activity, including phagocytosis, chemokine receptor expression, migration from peripheral tissue to draining lymph nodes, enhanced antigen presentation by antigen presenting cells (APCs), eventually resulting in increased production of cytokines, chemokines, adhesion molecules and antimicrobial peptides [20–24]. TLR4 and TLR9 (which we analyzed in this study) are receptors involved in immune reactions against bacterial and/or viral ‘danger signals’ [25, 26]**.**

The aim of our study was to determine, whether the addition of PINDs to DC/DCleu cultures could 1) optimize their antigen-presenting potential, 2) improve the composition and function of DC-stimulated T-cells in MLC (in the presence of additionally added PINDs) and 3) improve the function of cells of the innate immune system.

Therefore we added PINDs to DC-cultures of AML blasts to allow PIND-phagocytosis/processing in maturating DC differentiating to ‘leukemia-derived DC’, as well as to MLC-cultures to additionally stimulate T-cells. Our hypothesis was that immunogenity, enhanced this way, would lead to an improved activation and subsequently superior antileukemic functionality of T-cells. Therefore, we compared the composition, quantity and quality of defined DC- and T-cell-subtypes before and after respective cultures, and correlated the data with the antileukemic functionality of stimulated T-cells in the presence and absence of PINDs. In addition we performed some experiments using mononuclear cells, containing T- as well as NK cells, as effector cells.

According to our hypothesis based on physiological functions of DC, PINDs administered to AML patients could be phagocytized by patients` spontaneously arising DC/ leukemia-derived DC *in vivo*, thereby leading to ‘improved leukemic antigen presentation’ and induction of antileukemic T-cell-immunity in vivo. Moreover PINDs – based on observations of Horber and Mayr [14, 19] – could directly induce cells of the innate immune system. These mechanisms could contribute to stabilization of disease or remissions in AML patients.

**Materials and methods**

**1. Patients**

Heparinized blood samples were taken by aspiration of peripheral blood (PB) or bone marrow (BM), after obtaining informed consent. Samples were collected from healthy test persons and from AML-patients. Clinical characteristics of samples which were used in cytotoxicity fluorolysis assays are given in Table 1.

**2. Diagnostics**

Diagnostic reports were provided by accredited laboratories of the patients’ treating institution. Diagnosis of AML was based on the French-American-British (FAB) classification [27] and flowcytometry to define a blast phenotype [28, 29].

**3. Sample Collection**

Mononuclear cells (MNCs) were separated from whole blood samples by density gradient centrifugation (Ficoll-Hypaque, Biochrom, Berlin, Germany), then washed and suspended in phosphate-buffered saline (PBS) without Ca2+ and Mg2+ (Biochrom, Berlin, Germany). After quantification MNC were frozen with standardized procedures and stored in liquid nitrogen until use.

T-cells were obtained through positive selection by CD3+antibodies (Milteney Biotech, Bergisch-Gladbach, Germany 1 x 106 cells/well) from MNC from AML patients or from healthy stem cell donors, as described [10, 11].

**4. DC Generation**

MNCs were pipetted in 12-well tissue culture plates in 1ml Xvivo (Bio Whittaker Europe, Verviers, Belgium) FCS-free medium: Dendritic cells (DCs) were generated (in a preliminary experiment) in parallel with 4 different DC generating methods: MCM-Mimic (‘MCM’) [30], Ca-Ionophor (‘Ca’) [31], and Picibanil (‘Pici’) [32], Intron (‘Int’) [33]. Subsequently, the method resulting in highest DC counts was chosen for quantitative generation of DC as described by us [6, 8, 9] in the main experiments. These were performed in parallel settings with or without added PINDs, as given in the experimental workflow (figure 1).

**“MCM”**: DC were generated from 2-2.5 x106 MNC/ml in ‘MCM-Mimic’ medium containing 800 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF; Sandoz, Holzkirchen, Germany), 500 U/ml Interleukin 4 (IL-4; Cell Concepts, Umkirch, Germany) and 40 ng/ml FLT3-Ligand (FLT; PromoCell, Heidelberg, Germany) for 10-14 days, adding the same cytokines after 4-5 days again. On day 7 - 8 half medium exchange was performed, and 150ng/ml IL-6 (Cell Concepts, Umkirch, Germany), 5 ng/ml IL-1β (Cell Concepts, Umkirch, Germany), 1µg/ml Prostaglandin E2 (PGE2, Pfizer, Vienna, Austria) and 200U/ml Tumor necrosis factor (TNF) α (Cell Concepts, Umkirch, Germany) were added. After 10-14 days, cells were harvested for subsequent experiments [6, 8, 30]**.**

**“Ca”**: DC were generated from 7x105 MNC/ml in ‘Ca-Ionophore’ medium adding 375ng/ml A23187 (Sigma-Aldrich, Thum, Germany) and 250 U/ml IL-4. After 3-4 days, cells were harvested for subsequent experiments [6, 31].

**“Pici”**: DCs were generated with ‘Picibanil’, a lysis product of *Streptococcus pyogenes* which has nonspecific immunomodulatory effects, from 2 - 2.5 x106 MNC/ml in the presence of 500 U/ml GM-CSF and 250 U/ml IL-4. After 7 to 8 days in culture, 10 µl/ml OK-432 (Chugai Pharmaceuticals, Kamakura City, Japan) and 1µg/ml PGE2 were added. Cells were harvested after 9 - 11 days in culture [8, 32].

**“Int”**: DC were generated from 2-2.5x106 MNC/ml in ‘Intron’-medium containing 800 U/ml GM-CSF, and 5µl/ml Interferon alpha (Int; PromoKine, Heidelberg, Germany) for 10-14 days, adding the same cytokines after 4-5 days for a second time. On day 7 - 8 half medium exchange was performed, and 800 U/ml GM-CSF, 5µl/ml Int and 200 U/ml TNFα were added. After 10-14 days, cells were harvested for subsequent experiments [33, 34].

**“PINDs”**: In order to potentially further improve their T-cell stimulating capacity, we cultured DC using the methods described above, in parallel adding PINDS consisting of Parapox-ovis, Parapox-avis or a combination of both virus stems (see Table 2), to the respective medium. Zylexis is commercially available from Pfizer AH, Inc. (formerly Baypamun from Bayer, Inc.). HP and 3P were prepared and provided by I. Harabacz, HP-3P is their 1:1 combination. Conpind is also a combination of both virus stems, prepared and provided by B. and A. Mayr.

Lyophilized products were resolved in aqua ad injectionem as recommended, resulting in PIND-concentrations of 25 mg/ml (46 IFN-Units). Dilutions of 1:5, 1:10 and 1:20 were used, resulting in end-concentrations of 9.2, 4.6 or 2.3 IFN-Units in cultures. Dilutions of 1:5 and 1:10 of our settings were chosen according to personal recommendation of Prof. A. Mayr and used for previous experiments. Finally we used the 1:20 dilution because of an increased accumulation of dead cells in the 1:5 and (to a lesser extent) in the 1:10 concentrations (see “Results - 1.2 Usage of PINDs in a concentration of 1:20” for further information).

All experiments were performed according to the strategy given in chapter 11 (experimental setup).

**5. Flow cytometry**

Flow cytometric analyses with a panel of mouse monoclonal antibodies (moABs) directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), tandem CY7-PE-conjugation (PC7), or allophycocyanin (APC) was performed to evaluate and quantify amounts and phenotypes of leukemic cells, DCs, B-cells, T-cells and NK-cells in the PB/BM samples as described [10]. Antibodies were purchased from Immunotech/Beckman Coulter (a), Becton Dickinson(b), Caltag (c), Serotech (d) and Invitrogen (e). The following conjugated moABs were used: **FITC:** CD40c, CD80ab, CD83a, CD86c, CD3a, CD25a, CD39d, CD45ROa and CD122d; **PE:** TLR9a, CD4a, CD8b, CD56a,CD73b, CD80a, CD83a, CD86bc, CD127a, CD152a and CD206a; **PC7**: CD1aa, CD3a, CD4a, CD25a, CD40c, CD56a, CD83bc, CD86c, CCR7b, TLR4aand 7AADa; **APC:** CD1aa, CD3a, CD8b, CD25e, CD33a, CD34a c, CD40c, CD45ROe, CD69b, CD71b, CD117a and CD206a.

Cells were suspended in PBS with 20% FCS (Biochrome) and incubated with the moABs according to manufacturer’s instructions. For intracellular staining, we used FIX & PERM reagents (CALTAG Laboratories, manufactured by An Der Grub Bio Research GmbH, Austria) according to manufacturer’s instructions for fixation and permeabilization, in order to facilitate antibody access to intracellular structures, leaving the morphological scatter characteristics of the cells intact. Proportions of positive events in the defined gate compared with the isotype controls were calculated using CellQuest Software (BD), as described [6, 8, 10].

**6. Quantification and characterization of DC by Flow Cytometry**

DCs were generated, harvested, counted and quantified by flow cytometry as described above. Before culture mononuclear cell samples were characterized to identify the blast marker with the highest expression, as well as DC-markers with low/no expression in MNC. After culture these blast and DC markers were combined to identify the DC-method which yielded the highest DC-count (characterized by expression of DC-markers, that were not expressed on uncultured cells) and DCleu (characterized by co-expression of DC- and blast markers). DCs were characterized by the proportionally highest expressed DC-marker (DCopt). For further analysis and quantification of DCs, and especially leukemia-derived DC, a refined gating strategy was applied [9]. This strategy takes into consideration different scatter profiles of blasts and DC and enables a sensitive detection and quantification of blasts, which were not converted to DCleu, of DC without proof of leukemic derivation and of DC with leukemic derivation (DCleu). In addition viable (DCopt not co-expressing 7AAD; DCvia) or mature (DCopt co-expressing CD83; DCmat) DC in DC-fractions were identified (Table 3 a).

**7. Mixed Lymphocyte Culture (MLC)**

Previously selected CD3+ ***T-cells*** from MNC from AML patients or from healthy stem cell donors were co-cultured and stimulated with irradiated (20Gy) AML-blast containing MNC in the presence/absence of Zylexis (5x104 ‘MNC’; T(MNC), T(MNC+Z)) and in parallel with irradiated DCleu-containing DC (5x104 ‘DC’; T(DC)), irradiated DCleu-containing DCz and additional Zylexis (5x104 ‘DC’; T(DCz +Z)) in 1 ml MLC-Medium (RPMI 1640 medium (Biochrom) containing 15% human serum (PAA) and 50U/ml IL-2 (Proleukin R5, Chiron)). In these comparative settings the influences of Zylexis should be optimized: Zylexis was not only added to DC cultures (giving DC the chance to phagocyte, process and present Zylexis-antigens), but also to MLC in order to benefit from DC-mediated as well as direct influences of Zylexis on T-cells. Various experimental settings are given in table 3 b. Total DC-counts in the MLC were adjusted to 5x104 DC per 1x106 T-cells. Cells were restimulated with 5x104 ‘DC’ or 5x104 ‘MNC’ and supplementation with IL-2, as described [8] with a half-medium-exchange on day 4. On day 7 cells were harvested and the cytotoxicity assay was carried out. Some experiments were performed using ***MNC*** fractions (containing T- as well as NK or other cells of the cellular immune system) as effector cells. For better comparability with results obtained with T-cells as effector cells cell counts in the MLC using MNC as effector cells were adjusted to T-cell counts in the effector cell suspension using only T-cells; the remaining experimental settings were performed in analogy to MLC using T-cells with/without addition of Zylexis and stimulation with DCleu containing DC (MNC(DC), MNC(DCz+Z) or blast-containing MNC (MNC(MNC), MNC(MNC+Z); Table 3b).

Antigen expressions on (allogeneic or autologous) CD3+ T-cells were evaluated by FACS-analyses comparing the co-expression of CD28, CD154, CD4, CD8, CD45RA, CD45RO, CD25, CD71 before and after blast derived ‘MNC’- or ‘DC’-contact. This let us evaluate proportions of proliferating, costimulatory, naïve, memory, central memory or CD4+/CD8+ or migratory T-cells before and after ‘MNC’ or ‘DC’ co-culture [10, 35, 36]. Subtypes of T cells are given in Table 3 c.

**8.** **Cytotoxicity Fluorolysis Assay**

The lytic activity of effector T-cells or MNC (containing T- and NK-cells) was measured by a Fluorolysis-Assay by counting viable blast target cells, labeled with specific fluorochrome antibodies, before and after effector cell contact as described [10].

Stimulated effector-cells (T-cells or MNC) from healthy HLA-matched donors (=allogeneic) or from AML and MDS patients (=autologous) as well as non-stimulated T-cells (=uncultured, Tu) as a control (see Table 3 c) were co-cultured with thawn blasts as target cells, which were stained for 15 min with two FITC- and/ or PE conjugated ‘blast’ specific antibodies before culture. The effector- to target-cell ratio was adjusted to 1:1 and the cells were co-cultured for 24 h. To evaluate amounts of viable (7AAD-) target cells and to quantify the cell-loss after 24 h of incubation time, cells were harvested, washed in PBS and re-suspended in a FACS flow solution containing 7AAD (BD, Biosciences Pharmingen) and a defined number of fluorosphere calibration-beads (Becton Dickinson, Heidelberg, Germany). Viable cells were gated in a SSC/7AAD-gate. Afterwards, viable, 7AAD-negative cells co-expressing a specific blast marker (combinations) were quantified by taking into account defined counts of calibration beads as described. Cells were analyzed in a FACS Calibur Flow Cytometer using CELL Quest software (Becton Dickinson, Heidelberg, Germany). The percentage of lysis was defined as the difference between proportions of viable blasts before and after the effector cell contact [8].

**9. Cytometric bead assay (CBA) for cytokine and chemokine analysis**

During DC-generation (n=11) and MLC (n=5) 500μl-supernatants of each sample were taken and stored at -80ºC until analysis. The TH1/TH2-CBA-kit II with antibodies for IL-4, IL-6, IL-10, TNF-α and IFN-γ was used for analysis. As a control cytokine levels in cell culture media (RPMI+20% human serum, Biochrom, Berlin, Germany) without cells added were measured. The standard serial dilutions of positive and negative controls of the respective cytokines were included. 50μl mixed human TH1/TH2 Cytokine capture beads and 50μl PE Detection Reagent were incubated with 50μl of each test sample in assay tubes (12x75mm, BD Falcon) for 3 h at room temperature. After a washing step with 1ml Wash Buffer the assay tubes were centrifuged at 200g for five min, the bead pellets resuspended in 300μl Wash Buffer and analyzed by flow cytometry. Each sample was tested once, as recommended. Cytokines or chemokines measured in the samples were quantified using the standard curve [37].

**10. Statistical methods**

Mean and standard deviation, median and range, two-tailed t-tests and analyses of variance (ANOVA) were performed with a personal computer using Excel 2010 (Microsoft) and Graph Pad PRISM (GraphPad Software, Inc.). In detail we used the ANOVA-test when comparing parameters from *more than two* sets of data, e. g. values of a DC-parameter obtained in a given experiment without PINDs (w/o) and with several different PINDs (e.g. Zylexis, Conpind, etc.). The best result among any of the four DC-generating-methods was individually chosen for w/o and each PIND and selected for comparison.

We used the t-test when comparing parameters from *two* sets of data, e.g. w/o and a single PIND (Zylexis) or differently pooled averages of DC-parameters from different PINDs (Ø). In both cases we either used the value obtained with the best DC-generating-method in the presence of the PIND, or selected the value in analogy to the best method obtained w/o.

Differences were considered as significant, if the p-value was < 0.05.

**11. Experimental setup**

Influences of various PINDs on peripheral blood-cells (from healthy or AML-donors) was to be studied in various experiments: 1) DC were generated in the presence versus absence of PINDs. 2) Compositions of DC- (or MNC) stimulated T-cells after MLC (again in the presence vs absence of PINDs) were evaluated. 3. T-cells’ antileukemic effects after MLC (cytotoxicity assay).

Preliminary experiments were performed to compare 1) efficiency of freshly prepared (‘new’) vs up to ten day old PIND-solutions (‘old’), as well as 2) the efficiency and toxicity of different PIND-dilutions (1:5, 1:10, 1:20). Additionally we performed 3) multiple comparative analyses of different combinations of PINDs and DC-generating methods.

Experimental workflow (figure 1): Since cell quantity for all experiments was limited, we had to determine the ‘best method’ for every given sample. 1) In a first step a small number of DC were generated in parallel with 4 different DC generating methods, but without the addition of Zylexis. 2) Subsequently, the method resulting in highest DC counts was chosen for quantitative generation of DC (in the presence vs absence of Zylexis, analysis of cell composition by flow cytometry and cytokine profiles by CBA). 3) Subsequently a MLC was performed by co-culture of effector cells (T-cells or MNC) with stimulatory cells (DC and MNC), with addition of Zylexis to MLCs with stimulatory cells previously exposed to Zylexis (resulting in various effector cell settings as detailed in table 3 b). Cellular compositions (in comparison to unstimulated Tcells) as well as cytokine profiles were evaluated by Flowcytometry and CBA-assays. 6) Antileukemic functionality of various settings was assessed through Cytotoxicity Fluorolysis Assay.

**Results**

**1. Preliminary testing**

**1.1 *Dissolved ten-day-old Zylexis shows similar activity compared to Zylexis prepared directly before use***

In a clinical setting, PINDs, such as Zylexis, are used immediately after preparation from lyophilisates (“new”). In an experimental setting only small quantities are used. Therefore we compared results from PINDs being prepared ten days in advance and stored at 8°C until use (“old”) to its corresponding “new” preparation.

In this trial we used Zylexis as a representative of all PINDs, and analyzed quantity (DCopt), maturation (DCmat), and viability (DCvia) of DC generated from healthy donors through the Ca-, MCM-, Pici-, and Int-methods with “old” or “new” Zylexis in concentrations of 1:10 and 1:20. Statistical analysis showed no significant differences between the use of “old” or “new” Zylexis in 1:10 and 1:20 settings in either DC-generating-method (data not shown).

We decided to use Zylexis, and the other PINDs, up to ten days after preparation in our future experiments.

**1.2 *Usage of PINDs in a concentration of 1:20***

Treatment of animals with PINDs is performed by injecting one vial (containing 25 mg/ml) into one animal, independent of weight, species or body mass index. Following personal communication with Mayr et al. (no publications on this topic are available) we diluted the out of the box Zylexis-preparation (intended for injection in animals) of 25 mg/ml (46 IFN-Units) 1:5, 1:10 and 1:20. This resulted in end-concentrations of 9.2, 4.6 or 2.3 IFN-Units in cultures. We observed numerous dead cells in light microscopy during our pre-preliminary testing when using 1:5 and to a lesser extent 1:10 dilutions. Due to the extraordinary accumulation of dead cells in the 1:5 dilutions, we refrained from further testing of 1:5 dilutions and further compared 1:10 and 1:20 concentrations. Analysis of proportions of DC subtypes generated from 9 healthy donors under the influence of PINDs (Zylexis, Conpind, HP, 3P, HP-3P) in the aforementioned concentrations showed less dead cells and in addition (non-significantly) higher levels of DCopt, DCmat and DCvia in the 1:20 dilution (data not shown). Therefore we conducted our further experiments using 1:20 dilutions.

**1.3 *None of the four* *methods is favored or disadvantaged by the addition of PINDs to DC-generation***

We determined if certain combinations of PINDs and DC-generating methods, would generally result in superior/inferior DC-counts.

We compared DC- subtypes (as listed in table 3a) from 14 healthy and 9 leukemic donors after culture with MCM, Ca, Pici and Int. Each method was employed five times with the individual addition of either Zylexis, Conpind, HP, 3P or HP-3P. No significant differences in proportions of DC-subtypes could be found for any specific combination (ANOVA, data not shown).

Therefore we continued to use all four DC-generating methods in combination with all PINDs in our further experiments.

**2. Comparison of Quality and Quantity of DC cultured with and without PINDs**

**2.1 The presence of *PINDs in DC-cultures with healthy or leukemic MNC decreases DC’s viability and increases their maturation***

Our first step was to determine the effects of PINDs on the proportions of DC-subtypes (by flowcytometry) obtained from the 4 standard DC-generating-methods in general. Therefore we compared results obtained with the different DC-methods w/o, with Zylexis or with a pooled average from all PINDs (øPINDs; ). For the latter each sample’s best results from any of the four methods were used.

In healthy donors (n=15) a decrease in DCopt and significantly in DCvia (p=0.02) could be observed in the presence vs. absence of PINDs in DC-cultures. Proportions of DCmat increased on a non-significant scale (figure 2a).

The addition of PINDs to DC generation from leukemic donors (n=12) resulted in decreased DCopt, DCvia and DCleu/bla, while DCleu/DC or DCleu/MNC remained similar. Again DCmat increased significantly (p= 0.01, t-test; see figure 2b).

Additional refined analyses comparing DC-parameters through different modes of selection underlined the overall observation of decreased viability and increased maturation in DC generated in the presence of PINDs (data not shown).

In conclusion the presence of any PIND decreased viability and increased maturation of DC from healthy and leukemic samples compared to DC generating methods w/o the addition of PINDS.

**2.2 *Zylexis benefits Maturation of DC***

Due to limited cells available we focused further testing on Zylexis, as the only currently commercially available PIND.

We analyzed the influence of Zylexis on DC-cultures from healthy (n=15) and leukemic samples (n=12) in analogy to section 2.1. In both cases we observed a (significant) decrease of DCvia (healthy samples: p=0.004, t-test) and DCopt as well as a significant increase in DCmat (healthy samples: p=0.04, leukemic samples: p=0.02, t-test) in the presence of Zylexis (figures 2 a and b). Comparisons between Zylexis and the other PINDs did not show significantly different influences on DC-proportions (ANOVA, data not shown).

**2.3 The presence of Zylexis in DC cultures decreases TLR4- and TLR9-expression on DC/DCleu**

TLR4 and TLR9 are known to facilitate “danger signaling” in DC-mediated T-cell activation. Therefore we studied their expression under the influence of PINDs.

DC were generated in parallel with all four DC-generation methods. Similar average proportions (no statistical differences, ANOVA) of TLR9+ and TLR4+ DC were found in all settings in the *absence* of Zylexis, (data not shown).

In parallel experiments the *presence* of Zylexis in all DC-culture-media (significantly) decreased expression levels of TLR9 and TLR 4 on blasts and DCleu, while their expression on DC was not affected (figure 3 a and b; t-test).

**3. Effects of Zylexis on compositions and functions of T-cells in Mixed Lymphocyte Cultures (MLC)**

We compared different surface marker profiles on T-cells (autologous and HLA-matched T-cells after SCT from AML-patients, n=8) cultured with DC generated in the absence (T(DC)) or in the presence of Zylexis, which was additionally added to the culture-medium of the latter (T(DCz+Z)), as described in the materials and methods section, chapter 7.

**3.1 *Zylexis induces early T-cell Activation, but impairs their differentiation to effector T-cells***

We analyzed influences of Zylexis on T-cell activation and proliferation in MLR. No significant differences could be found in these T-cell-subtype proportions (t-test), although CD69+ T-cells as well as CD25+CD8+ T-cells increased under the influence of DCz+Z(arrows, figure 4 a).

We analyzed the influence of Zylexis on proportions of the following T-cell-subtypes: naïve-, central-memory-, effector-memory-, effector-T-cells. We observed a lower decrease of Tnaive as well as a lower increase of Teff under the influence of Zylexis in MLC (figure 4 b), whereas proportions of Tcm, Tem and Treg were similar in both groups (data not shown).

In conclusion this means that the presence of Zylexis in MLC increases early CD8+ T-cell proliferation, but impairs differentiation to effector T-cells.

**4. Effects of Zylexis on T-cells’ antileukemic functionality**

Prior results from our group have shown, that DC/DCleu stimulated T-cells (T(DC)) are the most efficient antileukemic effector cells (although not effective in every given case). We studied, whether the presence of Zylexis in MLC would increase antileukemic activity compared to cultures w/o Zylexis or untreated T-cells (Tu).

**4.1 *T-cells stimulated with DCz or MNC in the presence of Zylexis lose their antileukemic functionality (figure 4)***

We performed parallel investigations of **DC/DCleu** stimulated T-cell-cultures *without* [MLC (T(DC)] or *with* Zylexis added to DC cultures and [MLC (T(DCz+Z)] as well as T-cells stimulated with blast-containing MNC *without* [MLC T(MNC)] and *with* Zylexis added to MLC [MLC (T(MNC+Z)], and evaluated T-cells’ antileukemic activity after 24 h of incubation with blast target cells.

Our results showed, that the presence of Zylexis in the culture decreased the antileukemic reactivity of T-cells (figure 5 a, arrows): In the Zylexis-groups only 1 of 15 T(DCz+Z)-samples (6.7%) showed lytic activity, whereas there was none in the T(MNC+Z)-group. At the same time lysis was highest with T(DC) (53.3%, 8 of 15 cases), while unstimulated T-cells (Tu) or blast-stimulated T-cells T(MNC) displayed blast-lysis in 2 of 8 (25.0%) and 2 of 14 (14.3%) cases after 24 h-contact with blast-target-cells (p=0.01; ANOVA). Especially direct comparison between matching effector cells - T(DC) and T(DCz+Z), as well as T(MNC) and T(MNC+Z) - statistically pronounced the decreased antileukemic functionality, especially under the influence of DC (p=0.004; p=0.165; t-test). In cases in which lysis occurred, similar decreases of blast-proportions were observed. T(DC) showed an average of -41% lysed blast-cells and the one case in which Lysis occurred in T(DCz+Z) showed -44% lysed blasts. In cases without Lysis, the amount of blast proliferation did not show significant differences among Tu, T(DC), T(DCz+Z), T(MNC) and T(MNC+Z) after 24 h (ANOVA).

To obtain more detailed information on T-cells’ compared to MNCs’ antileukemic effector cell reactivity and efficacy, we used T-cells or MNC (containing T-cells as well as cells of the innate immune system; six cases) as *effectors* (stimulation with MNC and DC, in the presence/absence of Zylexis): T-cells led to lysis in 4 of 24 cases, while antileukemic activity could only be observed in 1 of 24 cases using MNC as effector cells. Use of the latter also resulted in a significantly higher rate of proliferating blasts in cases without lysis (p= 0.01, t-test, data not shown). Proportions of cases with antileukemic activity from differently stimulated effector cells (MNC or T-cells) are given in figure 5 b. No antileukemic reactivity in the presence of Zylexis could be demonstrated, regardless of the effector cells (T-cells and MNC). A significant increase of lysis from DC-stimulated T-cells compared to all other effector/stimulator cell combinations regardless of the presence of Zylexis could be shown (p=0.04, ANOVA). This increase was also significant when directly comparing DC-stimulated T-cells’ antileukemic activity in the absence compared to the presence of Zylexis (p=0.05, t-test). Again, in cases without lysis, the amount of blast proliferation ( if lysis did not occur) did not show significant differences in the groups compared (data not shown).

So although comparable effector-cell counts were added to MLC cultures in both settings, antileukemic reactivity of those using MNC, which additionally contained other immune reactive cells, showed decreased antileukemic function compared to “pure” T-cells. Addition of Zylexis to MNC-settings did not result in lysis in any case.

We conclude that the presence of Zylexis lead to a significant loss or inhibition of antileukemic T- as well as antileukemic innate- (MNC)cell functionality, potentially benefitting blast-proliferation. No special benefit on the “innate immunity” could be observed under the influence of Zylexis.

**4.2 Cases without lysis presented with higher Treg and Teff counts in MLC-samples of T(DC) and T(DCZ+Z) on the day of harvest (n=8)**

We correlated T-cells’ composition with their antileukemic functionality in the presence or absence of Zylexis. We observed similarly increased proportions of CD4+ and CD8+ Treg in cases *without* lysis after stimulation with DC in the presence or absence of Zylexis (figure 6 a and b). However, in cases *with* antileukemic activity, DC-stimulated T-cells were able to mediate antileukemic activity *despite* upregulated CD8+ Treg counts, but not in the presence of Zylexis (figure 6 a). Cases *with* antileukemic activity also showed upregulated Teff in the presence or absence of Zylexis (figure 6 c).

This data points to a different influence on the T-cell composition through Zylexis in DC-cultures, than DC alone.

**4.3. Effects of Zylexis on Cytokine-Profiles in DC- or MLC-culture supernatants**

In previous studies we have shown, that high cytokine-concentrations (especially IL-6, IFN-γ) correlated with antileukemic T-cell-activity [37]. We analyzed whether the addition of Zylexis would alter cytokine release profiles in **DC**-culture supernatants. We measured the levels of IL-2, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-γ and MCP1 in *supernatants after DC-generation* from different DC-generating methods (Ca; MCM; Pici; Int), with and without Zylexis (n=11). Pooling the results of the DC-generating methods we were able to show (significantly) lower levels of IFN-γ, TNF-α and IL-2 in supernatants containing Zylexis (figure 7 a). Levels of the inhibitory IL-10 were (non- significantly) higher under the influence of Zylexis (3.0±7.9 vs. 3.6±14.2, p=0.83).

In analogy we analyzed cytokine-release-profiles (IL-2;IL-4; IL-6; IL-8; IL-10; IL-12p70; IL-17A; TNF-α; IFN-γ; MCP1) in supernatants taken from **MLC**-media containing different effector cells (n=5). The presence of DC vs. blast containing MNC increased the release of IL-4 after MLC, especially under the influence of Zylexis (p=0.001; ANOVA; figure 7 b). Levels of the remaining cytokines did not differ in the groups compared (data not shown).

We conclude, that the presence of Zylexis influences the cytokine-release-profiles of cells in DC- as well as DC-stimulated MLC-cultures by favoring a more inhibitory cytokine-environment.**Discussion**

**1. How to improve antileukemic T-cell-activity – with a special focus on DC**

A better prognosis has been reported for combinations of chemotherapy and immunotherapy than for chemotherapy alone to treat leukemia [38, 39]. However, allo-HCT remains the only curative treatment option for many haematological malignancies, although long-term follow-up data beyond 5 years remains scarce. Its curative potential is mainly attributed to the graft-versus-leukemia effect, which in turn is primarily driven by donor derived immune-effector-cells, mainly donor-T-cells primed against leukemic targets. Their concerted infusion after allo-SCT in order to treat relapsed leukemia has become known as DLI [4, 40, 41]. Dendritic Cells (DC) are known to be the main T-cell stimulators due to their professional antigen presenting functionality [42]. DC-based immunotherapy involves their activation and subsequent presentation of tumor-antigens to effector-lymphocytes, initiating an immune response against cells expressing the equivalent antigens [7].

Due to DC’s unique antigen presenting capacity immunosuppressive features of the leukemic blasts can be circumvented [43].

Different methods of loading antigens in or onto DC have been used in order to optimize antitumor responses. One strategy involves pulsing DC with leukemic cells or fusion of blasts with DC. Those DC are then able to present their tumor antigens to (donor) lymphocytes and thereby convert them into cytotoxic T cells [44]. Although several concepts exist, the identification and low immunogenicity of tumor-specific peptides remain a problem [7].

A strategy reserved to myeloid malignancies is to convert leukemic blasts directly to leukemia-derived dendritic cells (DCleu), thereby creating APCs presenting the whole leukemic antigen repertoire and possessing the stimulatory capacity of mature professional DC - thereby bypassing the highly complex procedures mentioned above. We could show that T-cells, obtained from AML-patients (autologous) or donors (allogeneic), can be stimulated by these DCleu, resulting in very efficient cytotoxic effector cells with specific lytic activity against naïve blasts, although not in every case [10, 11].

Observations of tumor remissions occurring in a context of infectious diseases have been known for centuries. Many physicians followed the credo of the ancient Greek philosopher Parmenides (ca. 500 B.C.): “Give me the power to induce fever and I will cure all diseases” [45]. According to a medieval legend, a priest called Peregrine Laziosi was immediately cured of his cancerous leg ulcer after a serious local infection occurred; because of this “miracle healing” he became the patron saint of all cancer victims [46]. Several surgeons of the 18th and early 19th century induced “targeted” infections by cutting wounds directly at the sites of tumors, especially in breast cancer. Jean Cruveilhier, the first professor of pathological anatomy in Paris, recommended that inflammation may be induced “by incisions or irritating applications” [47]. Around 1900, the New York surgeon William Coley refined this empirical approach by systematically using a bacterial vaccine (“Coley’s vaccine”) which is said to have induced regression of metastatic cancer in a considerable number of his patients [48]. With the emergence of aseptic surgery, antibiotics, antipyretics and modern therapeutic options for cancer, Coley’s vaccine fell into oblivion.

In recent years research on therapeutic strategies involving the nonspecific, innate immune activation has become more important. For example clinical data shows a correlation between improved outcome and tumor-infiltration of both, innate natural-killer- and adaptive T-cells. Recently a role of iNKT (invariant natural killer cells), CIK (cytokine induced killer cells) or NK (natural killer) -cells in the mediation of DC-stimulated antileukemic responses was described [49].

One of the few cancer immunotherapies in widespread clinical use, the intravesical administration of Bacillus Calmette–Guerin for superficial bladder cancer, is innate and nonspecific in its action, utilizing antimicrobial immunity for antitumor effects [50, 51]. Efforts to turn on the immune system against cancers with inactivated tumor vaccines or intra-tumor injections of bacterial products to induce local inflammation and recruit an antitumor immune response have led to anecdotal successes [52].

A biological employing a nonspecific activation of the innate immune system as its mode of action is Ipilimumab. It is an antibody, which blocks CTLA-4, a receptor on T-cells transmitting inhibitory signals during antigen-presentation through DC, and thereby leads to activation of the immune system [53]. Another example of an immune response modifying drug is Imiquimod, a synthetic imidazoquinoline that activates TLR7, currently used for the treatment of actinic keratosis, superficial basal cell carcinoma and condylomata acuminate [54]. The drug does not exhibit direct antiviral or antiproliferative activity when tested in a number of cell culture systems. Cells activated by Imiquimod secrete cytokines (primarily INF-α, IL-6, and TNF-α), thereby stimulating several other aspects of the innate immune response, as well as cellular immunity (e.g. Langerhans-cells, natural killer cells or macrophages) [55].

**2.** **PINDs have a negative impact on antileukemic T-cell activity**

PINDs are known as (pox-derived) substances “optimizing” immune responses not only in infectious, but also in malignant diseases of animals due to stimulating/co-stimulating capacities [12, 15]. Similarly, PINDs are known to act as non-immunising vaccines, with the capability to activate and regulate the paraspecific, i.e. innate, immune system, as shown in veterinarian fields. Their mechanism of action has not been analyzed in depth [15].

The aim of this study was to determine, whether the addition of PINDs to DC-generating media or to MLC-cultures (going along with PIND-phagocytosis/processing during DC- differentiation) would lead to an increased antileukemic stimulatory capacity of DC/DCleu, in MLC with T-cells.

In the past our group was able to demonstrate DCleu containing DC could be generated ex vivo in every given case with at least one of the DC-generating methods presented above, and that the composition and quality of DC is predictive for a successful ex vivo antileukemic response, especially with respect to proportions of mature and leukemia-derived DC [10].

An astonishing finding was, that the presence of PINDs in DC cultures benefitted maturation of DC, but decreased DCs’ viability as well as TLR4- and 9-expression on blasts *and* DC DCleu during culture. This was true for all kinds of PINDs tested, whether they were derived from “ovis” or “avi” virus strains, or the combination of both. TLRs have been linked to virus detection and the induction of immune responses. As PINDs consist of highly attenuated and inactivated virus strains, we expected an increase in TLR-expression, although it is known, that inactivated Parapoxvirus ovis mediates its immunostimulatory properties by TLR-dependent as well as TLR-independent pathways [56]. We conclude that PINDs either act through different TLRs/ PRRs than tested, or exclusively on TLR-independent pathways [57].

We found, that the presence of Zylexis induced early (CD8+) T-cell activation, while differentiation to effector-T-cells was impaired. Moreover, no special activation of cells of the innate immune system (using the total MNC-fraction as effector cells) under the influence of Zylexis could be observed.

Applying Zylexis to the DC- and MLC-culture-medium resulted in a loss of antileukemic functionality, almost producing a “blasts protective capacity”. This finding was further supported by the detection of an “inhibitory soluble microenvironment” (low Il-2, TNF-α, IFN-γ, high IL-4) in DC- or MLC-culture supernatants in the presence of Zylexis. Such an inhibitory microenvironment through soluble factors was described before by Vignali et al. [58]. Although alternatively an induction of a (antileukemic) B-cell-/ antibody- response can be discussed [59] in the presence of PINDs in DC/MLC-settings, we suggest a minor role of IL-4 (in the presence of low IL-2, TNF-α, IFN-γ and higher IL-10 and Treg-subtypes). An interesting finding was, that DC stimulated T-cells were able to lyse AML-blasts despite high CD8+Treg, however not in the presence of Zylexis – pointing to an influence of Zylexis on the composition of (regulatory) T-cells. Further analyses are necessary to characterize these (regulatory) T-cells in more detail: CD39+, CD73+ or other Treg-subtypes might mediate the inhibitory effects described.

MUTZ-3 is a myeloid leukemia cell line, that can acquire a DC-like phenotype and function as potent antigen presenting cells (MUTZ-3-derived DC = MuDC). Kim et al. [60] were able to demonstrate, that MuDC did not show an enhanced level of CCR7 or cytokine production after lipopolysaccharid (LPS) stimulation, and LPS-stimulated MuDC ultimately failed to stimulate primary NK cells. On the other hand our group could show recently, that cells of the innate immune system (NK-, CIK- and iNKT-cells) are characterized by different cellular profiles in patients with AML, ALL or CLL, and play a role in the mediation of antileukemic reactions after DC-stimulation [49]. This suggests, that in analogy the leukemia derived DC used in our experiments might as well show an impaired reaction from stimulation through other PAMPs (e.g. PINDs). The underlying mechanism to this remains unknown.

In the past we were able to show that an inhibitory microenvironment as established by blasts can impair an antileukemic T-cell response, but can be overcome by the conversion of leukemic blasts to DCleu [61]. Recent findings of our group support these cellular effects on a soluble level: we showed that ‘DC’-/’MNC’-stimulation of T-cells resulted in increased cytokine-levels in culture-medium compared to serum. High cytokine-concentrations (especially IL-6, IFN-γ) in MLC –supernatants after T-cells’ ‘DC’-/’MNC’-stimulation correlated with lytic T-cell-activity [37]. In the context of this study we could (at least in part) confirm the following observations: in the presence of Zylexis lower concentrations of IL-2, TNF-α and IFN-γ were found in MLC supernatants. This might add to the blast-protective effect described above, as these cytokines have been described as important mediators of the immune response [62]. This is in contrast to the findings of Ahne et al., who described an increase in inflammatory mediators, most notably TNF-α, when studying effects of the PIND Conpind [18]. These cytokines decreased in our experiments, which might be due to other factors influencing PIND-dependent immunomodulation.

‘Viral therapies’ using smallpox-, herpes zoster-, hepatitis-, influenca-, varicella-, measles-, rabies- or other viruses used in the treatment of cancer were shown to induce (transient) tumor regressions [63]. Moreover, our group could show, that a ‘danger signaling’ via PGE-2, Picibanil or PGE-1 gives rise to improved DC-generation, T-cell composition and soluble factor release after MLC, as well as an improvement of the antileukemic reactivity of DC-stimulated T-cells in MNC or even whole blood culture settings, containing the total cellular and soluble microenvironment of the individual leukemic patient [64].

Various escape mechanisms (inhibitory cytokines, regulatory cells), or unknown ‘blast protective mechanisms’ can be overcome by carefully selected immunomodulatory strategies: oncolytic viruses were shown to limit the efficacy of an virus based immunotherapeutic approach [65]. Combinations of viral-based therapeutic strategies as listed above with low-dose chemotherapies or IL-2, improved strategies concerning application (timing, route of administration and dosage) in combination with thorough quality controls in manufacture of uniform virus products and monitoring of unexpected or adverse events (e.g. elevated antibodies against the viral products, inhibitory cytokine profiles in serum, clinical events) could contribute to improve the viral products and their clinical use in a context of antitumor- or immune-therapy [62, 63].

Our findings support the central role of T-cells as the most important mediators of antileukemic reactions by showing their superior antileukemic functionality as effector cells before and/or after SCT. However, these T-cell effects can be variable due to several ‘influences’ in the microenvironment [2, 66]. Additional assays to quantify leukemia-specific cells (e.g. by Tetramer-, CSA-, ELISPOT, intracellular cytokine or degranulation assays) could further contribute to understand the whole functional repertoire of PINDs.

**Conclusions**

In summary our data points to Zylexis’ induction of early T-cell activation and reduced effector cell function – resulting in a downregulated antileukemic T-cell functionality in general, that might be caused by altered DC-characteristics (e.g. decreased TLR-expression in combination with increased maturation, decreased viability) and/or changed microenvironment - protecting blasts from attacks by immune-reactive cells. This means that although the use of PINDs might be promising in curing patients with infectious diseases, in patients with myeloid leukemias the “blast protective capacity” induced by PINDs might lead to unspecific and/or inhibitory influences in the cellular/innate immunity. The need for a thorough analysis on PINDs’ whole range of effects in different settings (e.g: in/ex vivo environment, infectious/malignant) arises.

Therefore PIND-supported antileukemic trials cannot be recommended for the treatment of AML-patients.

**Table and Figure Legends**

***Table 1:*** *Characteristics of patients and samples used for PIND experiments and functional assays.*

***Table 2:*** *Parapox-Virus Stem(s) in different PINDS used for experiments.*

***Table 3:*** *Abbreviations of* ***a.*** *DC-subtypes,* ***b.*** *different MLC-settings by type of effector cells* ***c.*** *T-cell-subtypes as evaluated by flow cytometry with the respective surface-marker profiles after staining with fluorochrome-labelled antibodies.*

***Figure 1:*** *Experimental workflow to study the influence of Zylexis on DC-generation as well as cells in MLC and cytotoxicity:* ***a.*** *Pretesting with few cells and identification of the ‘best’ ( ) DC-generating method for individual samples (without PINDs). Here MCM was chosen (exemplarily) as ‘best method’* ***b.*** *Quantitative DC-generation with the previously chosen ‘best DC-generating method’ with and without the addition of Zylexis in parallel(evaluated by flowcytometry). Asservation of culture-supernatans for CBA.* ***c.*** *MLC of effector cells (T-cells or MNC) with stimulator cells (DC or MNC), with and without the (repeated) addition of Zylexis (resulting in the effector cells as detailed in table 3 b, evaluated by flowcytometry). Asservation of culture-supernatans for CBA.* ***d.*** *Functional cytotoxicity assay of various MLC-cell compositions to detect antileukemic capacity. The Various analytical methods and their timing are given below.*

***Figure 2:***Comparison of proportions of DC-subtypes (in % of cells) obtained with DC-methods w/o, with Zylexis or with average of pooled result*s from all PINDs (øPINDs; ): Each sample’s best results from any of the four methods in (****a.****) healthy (n=15) and (****b.****) leukemic samples (n=12) were used. Statistically significant values from t-tests between w/o and Zylexis as well as w/o and øPINDs are given (t-test). No significant differences between DC-values under the influence of Zylexis and øPINDs could be found.* ***a.*** *Upregulated proportions of early proliferating CD3+ and activated CD8+ T-cells found in the presence of Zylexis after MLC* ***b.*** *Downregulated production of Teff (and lower decrease in Tnaive) in the presence of Zylexis*

***Figure 3:*** *Expression of TLR on cells of leukemic origin (blasts and DCleu) (significantly) decreased after culture in different DC-generating media in the presence of Zylexis (t-test). Proportions of all cells (“TLR-9/4+”), DC, blasts and DCleu expressing TLR9 (****a.****) and TLR4 (****b.****) in cultures w/o or with Zylexis are given. Results obtained after culture with 4 different DC-generating methods showed similar average proportions of TLR-expression and are pooled.*

***Figure 4:***C*hanges of different T-cell subtypes after MLC with DC in the presence or absence of Zylexis, compared to day 0 (n= 8) in percentage points****a.*** *Upregulated proportions of early proliferating CD3+ and activated CD8+ T-cells found in the presence of Zylexis after MLC* ***b.*** *Downregulated production of Teff (and lower decrease in Tnaive) in the presence of Zylexis*

***Figure 5:*** *The presence of Zylexis significantly decreased the effector cells’ antileukemic activity.* ***a.*** *Proportions of cases with antileukemic activity from untreated T-cells (Tu), and T-cells stimulated with MNC or DC - without or with Zylexis (arrows).* ***b.*** *Proportions of cases with antileukemic activity from 6 parallelly tested cases with T-cells or MNC as effector cells, either stimulated with blast-containing MNC or DCleu containing DC and in the absence or presence of Zylexis.*

***Figure 6:*** *Gain or loss in percentage points of selected T-cell proportions in MLC-samples (n=8) of T(DC) and T(DCz+Z) on day of harvest (compared to d0) in correlation with achieved Lysis/non-Lysis. DC-stimulated T-cells are able to mediate antileukemic activity and compensate function of upregulated CD8+Tregs (****a.****), but not in the presence of Zylexis, whereas CD4+Tregs (****b.****) and Teff (****c.****) are not affected.*

***Figure 7:*** *The presence of Zylexis decreased release of IL-2, TNF-α and IFN-γ in DC-culture supernatans (****a.****) and increased release of IL-4 in MLC-supernatans after MLC of Tcells with DC-stimulated T-cells in the presence of Zylexis (****b.****). Cytokine levels evaluated by CBA in parallelly analysed supernatans are given.* ***a.*** *Cytokine levels* (IL-2, *TNF-α and IFN-γ) in DC-culture supernatans in the presence or absence of Zylexis (n=11).* ***b.*** *IL-4 levels in MLC-culture supernatans after T-cells’ stimulation with blast-containing MNC or DCleu-containing DC in presence or absence of Zylexis (n=5).*

**Funding and declaration of interests**

This project was supported by RIEMSER pharmaceuticals.

Parts of the results presented in this manuscript were worked out in the course of the MD thesis of Christian Ansprenger, Dr Julia Schick, Dr Valentin Vogt, Annika Hirn-Lopez and Yvonne Vokac at the University of Munich and the Helmholtz- Centre Munich.

The project-idea is summarized and listed as intentional report of Prof. Hans-Joachim Kolb and Prof. Helga Schmetzer at the University Hospital of Munich; CONPIND is listed as Patents No DE3504940 A1 and US 6162600 A of Anton Mayr.

**Acknowledgements**

The authors thank nurses and physicians on the wards for their support and diagnostic laboratories for the contribution of diagnostic reports of the patients. Moreover, we thank all of our colleagues for helpful discussion. We would like to thank Prof Barbara and Prof Anton Mayr as well as Dr Ihor Harabacz for providing us with Conpind, HP and 3P as well as guidance and discussion regarding PINDs and their employment.

**Obituary**

On the death of Prof. Dr. Barbara Mayr (died 2015) and Prof. Prof Anton Mayr (died 2014)

Prof Mayr was appointed to the Chair of Microbiology and Animal Diseases at the LMU in Munich in 1963. His later wife Prof Barbara Mayr became a professor at the same institute in 1972. In scientific terms, the Mayr couple worked mainly in the field of immunization in infectious diseases, especially of viral diseases in animals and the development of live vaccines. The MVA virus (Modified Vaccinia Ankara Virus) developed by Prof Mayr finally created the basis for the development of safe pox vaccines.

Prof B. and A. Mayr provided all their knowledge about ' paramunity inducers' (PINDs) made from highly attenuated poxvirus products, as well as the substances themselves for the project described here, as well as discussed the first results together with us. Unfortunately, the two could not experience the completion of our project. We, the authors of the manuscript thank them for all the enthusiasm, all the advice and accompaniments in the project. The expertise of the two we have to owe the execution of this very special project.

My special thanks go to Prof B. Mayr for many encouraging and personal words throughout the project phase.

Helga Schmetzer (in the name of all authors)

References

1 Rosenblatt J, Stone RM, Uhl L, Neuberg D, Joyce R, Levine JD, Arnason J, McMasters M, Luptakova K, Jain S, Zwicker JI, Hamdan A, Boussiotis V, Steensma DP, DeAngelo DJ, Galinsky I, Dutt PS, Logan E, Bryant MP, Stroopinsky D, Werner L, Palmer K, Coll M, Washington A, Cole L, Kufe D, Avigan D: Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. Sci Transl Med 2016;8:368ra171.

2 Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, Ljungman P, Ferrant A, Verdonck L, Niederwieser D, van Rhee F, Mittermueller J, Witte T de, Holler E, Ansari H: Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995;86:2041–2050.

3 Stamouli M, Gkirkas K, Tsirigotis P: Strategies for improving the efficacy of donor lymphocyte infusion following stem cell transplantation. Immunotherapy 2016;8:57–68.

4 Schmid C, Labopin M, Nagler A, Niederwieser D, Castagna L, Tabrizi R, Stadler M, Kuball J, Cornelissen J, Vorlicek J, Socie G, Falda M, Vindelov L, Ljungman P, Jackson G, Kroger N, Rank A, Polge E, Rocha V, Mohty M: Treatment, risk factors, and outcome of adults with relapsed AML after reduced intensity conditioning for allogeneic stem cell transplantation. Blood 2012;119:1599–1606.

5 Fesnak AD, June CH, Levine BL: Engineered T cells: the promise and challenges of cancer immunotherapy. Nat Rev Cancer 2016;16:566–581.

6 Dreyssig J, Kremser A, Liepert A, Grabrucker C, Freudenreich M, Schmid C, Kroell T, Scholl N, Tischer J, Kufner S, Salih H, Kolb HJ, Schmetzer HM: Various 'dendritic cell antigens' are already expressed on uncultured blasts in acute myeloid leukemia and myelodysplastic syndromes. Immunotherapy 2011;3:1113–1124.

7 Kitawaki T: DC-based immunotherapy for hematological malignancies. Int J Hematol 2014;99:117–122.

8 Kremser A, Dressig J, Grabrucker C, Liepert A, Kroell T, Scholl N, Schmid C, Tischer J, Kufner S, Salih H, Kolb HJ, Schmetzer H: Dendritic cells (DCs) can be successfully generated from leukemic blasts in individual patients with AML or MDS: an evaluation of different methods. J Immunother 2010;33:185–199.

9 Schmetzer HM, Kremser A, Loibl J, Kroell T, Kolb HJ: Quantification of ex vivo generated dendritic cells (DC) and leukemia-derived DC contributes to estimate the quality of DC, to detect optimal DC-generating methods or to optimize DC-mediated T-cell-activation-procedures ex vivo or in vivo. Leukemia 2007;21:1338–1341.

10 Grabrucker C, Liepert A, Dreyig J, Kremser A, Kroell T, Freudenreich M, Schmid C, Schweiger C, Tischer J, Kolb HJ, Schmetzer H: The quality and quantity of leukemia-derived dendritic cells from patients with acute myeloid leukemia and myelodysplastic syndrome are a predictive factor for the lytic potential of dendritic cells-primed leukemia-specific T cells. J Immunother 2010;33:523–537.

11 Liepert A, Grabrucker C, Kremser A, Dreyssig J, Ansprenger C, Freudenreich M, Kroell T, Reibke R, Tischer J, Schweiger C, Schmid C, Kolb HJ, Schmetzer H: Quality of T-cells after stimulation with leukemia-derived dendritic cells (DC) from patients with acute myeloid leukemia (AML) or myeloid dysplastic syndrome (MDS) is predictive for their leukemia cytotoxic potential. Cell Immunol 2010;265:23–30.

12 Benn CS, Netea MG, Selin LK, Aaby P: A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends Immunol 2013;34:431–439.

13 Flanagan KL, van Crevel R, Curtis N, Shann F, Levy O: Heterologous ("nonspecific") and sex-differential effects of vaccines: epidemiology, clinical trials, and emerging immunologic mechanisms. Clin Infect Dis 2013;57:283–289.

14 Mayr A: Development of a non-immunising, paraspecific vaccine from attenuated pox viruses: a new type of vaccine. New Microbiol 2003;26:7–12.

15 Mayr A: Taking advantage of the positive side-effects of smallpox vaccination. J Vet Med B Infect Dis Vet Public Health 2004;51:199–201.

16 Hansen RM, Libnoch JA: Remission of chronic lymphocytic leukemia after smallpox vaccination. Arch Intern Med 1978;138:1137–1138.

17 Mayr A, Wittmann G: Observations on local spread of pox viruses in tissue. Science 1957;125:1034–1035.

18 Ahne W, Mayr A: Poxvirus preparation CONPIND initiates production of the major inflammatory mediators IL-1 alpha and TNF-alpha in human whole blood and in blood mononuclear cell cultures. Comp Immunol Microbiol Infect Dis 1997;20:139–145.

19 Horber D, Mayr B: [Paramunization of FeLV-positive cats with PIND-AVI]. Tierarztl Prax 1991;19:311–314.

20 Iwasaki A, Medzhitov R: Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–995.

21 Kawai T, Akira S: Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 2011;34:637–650.

22 Maruyama K, Selmani Z, Ishii H, Yamaguchi K: Innate immunity and cancer therapy. Int Immunopharmacol 2011;11:350–357.

23 Oda K, Kitano H: A comprehensive map of the toll-like receptor signaling network. Mol Syst Biol 2006;2:2006 0015.

24 Penack O, Henke E, Suh D, King CG, Smith OM, Na IK, Holland AM, Ghosh A, Lu SX, Jenq RR, Liu C, Murphy GF, Lu TT, May C, Scheinberg DA, Gao DC, Mittal V, Heller G, Benezra R, van den Brink, M. R.: Inhibition of neovascularization to simultaneously ameliorate graft-vs-host disease and decrease tumor growth. J Natl Cancer Inst 2010;102:894–908.

25 Barton GM, Kagan JC, Medzhitov R: Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol 2006;7:49–56.

26 Zhang L, Qin H, Guan X, Zhang K, Liu Z: The TLR9 gene polymorphisms and the risk of cancer: evidence from a meta-analysis. PLoS One 2013;8:e71785.

27 Hayhoe FG: Classification of acute leukaemias. Blood Rev 1988;2:186–193.

28 Campana D, Behm FG: Immunophenotyping of leukemia. J Immunol Methods 2000;243:59–75.

29 Graf M, Reif S, Hecht K, Pelka-Fleischer R, Pfister K, Schmetzer H: High expression of urokinase plasminogen activator receptor (UPA-R) in acute myeloid leukemia (AML) is associated with worse prognosis. Am J Hematol 2005;79:26–35.

30 Lee AW, Truong T, Bickham K, Fonteneau JF, Larsson M, Da Silva I, Somersan S, Thomas EK, Bhardwaj N: A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. Vaccine 2002;20 Suppl 4:A8-A22.

31 Houtenbos I, Westers TM, Stam AG, Gruijl TD de, Scheper RJ, Ossenkoppele GJ, van de Loosdrecht, A. A.: Serum-free generation of antigen presenting cells from acute myeloid leukaemic blasts for active specific immunisation. Cancer Immunol Immunother 2003;52:455–462.

32 Sato M, Takayama T, Tanaka H, Konishi J, Suzuki T, Kaiga T, Tahara H: Generation of mature dendritic cells fully capable of T helper type 1 polarization using OK-432 combined with prostaglandin E(2). Cancer Sci 2003;94:1091–1098.

33 Chen X, Regn S, Raffegerst S, Kolb HJ, Roskrow M: Interferon alpha in combination with GM-CSF induces the differentiation of leukaemic antigen-presenting cells that have the capacity to stimulate a specific anti-leukaemic cytotoxic T-cell response from patients with chronic myeloid leukaemia. Br J Haematol 2000;111:596–607.

34 Hirn-Lopez A, Deen D, Vokac Y, Kroell T, Kraemer D, Schuster F, Borkhardt A, Schmid C, Kolb HJ, Tischer J, Schmetzer HM: Role of IFN alpha in DC-cocktails fort he generation of (leukemia-derived) dendritic cells from AML-blasts, the induction of antileukemic functionality of DC-stimulated T-cells and in correlation with clinical response to immunotherapy. Preparation 2017.

35 Schick J, Vogt V, Zerwes M, Kroell T, Kraemer D, Kohne CH, Hausmann A, Buhmann R, Tischer J, Schmetzer H: Antileukemic T-cell responses can be predicted by the composition of specific regulatory T-cell subpopulations. J Immunother 2013;36:223–237.

36 Vogt V, Schick J, Ansprenger C, Braeu M, Kroell T, Kraemer D, Kohne CH, Hausmann A, Buhmann R, Tischer J, Schmetzer H: Profiles of activation, differentiation-markers, or beta-integrins on T cells contribute to predict T cells' antileukemic responses after stimulation with leukemia-derived dendritic cells. J Immunother 2014;37:331–347.

37 Fischbacher D, Merle M, Liepert A, Grabrucker C, Kroell T, Kremser A, Dreyssig J, Freudenreich M, Schuster F, Borkhardt A, Kraemer D, Koehne C-H, Kolb H-J, Schmid C, Schmetzer HM: Cytokine Release Patterns in Mixed Lymphocyte Culture (MLC) of T-Cells with Dendritic Cells (DC) Generated from AML Blasts Contribute to Predict anti-Leukaemic T-Cell Reactions and Patients' Response to Immunotherapy. Cell Commun Adhes 2015;22:49–65.

38 Arpinati M, Curti A: Immunotherapy in acute myeloid leukemia. Immunotherapy 2014;6:95–106.

39 Lake RA, Robinson BW: Immunotherapy and chemotherapy--a practical partnership. Nat Rev Cancer 2005;5:397–405.

40 Kolb HJ, Simoes B, Schmid C: Cellular immunotherapy after allogeneic stem cell transplantation in hematologic malignancies. Curr Opin Oncol 2004;16:167–173.

41 Orti G, Barba P, Fox L, Salamero O, Bosch F, Valcarcel D: Donor lymphocyte infusions in AML and MDS: Enhancing the graft-versus-leukemia effect. Exp Hematol DOI: 10.1016/j.exphem.2016.12.004.

42 Brossart P: Dendritic cells in vaccination therapies of malignant diseases. Transfus Apher Sci 2002;27:183–186.

43 van de Loosdrecht, A. A., van den Ancker W, Houtenbos I, Ossenkoppele GJ, Westers TM: Dendritic cell-based immunotherapy in myeloid leukaemia: translating fundamental mechanisms into clinical applications. Handb Exp Pharmacol 2009:319–348.

44 Blair A, Goulden NJ, Libri NA, Oakhill A, Pamphilon DH: Immunotherapeutic strategies in acute lymphoblastic leukaemia relapsing after stem cell transplantation. Blood Rev 2005;19:289–300.

45 Hobohm U: Fever and cancer in perspective. Cancer Immunol Immunother 2001;50:391–396.

46 Pötscher A: Peregrin Laziosi. Salzburg, Verlag St. Peter, 2001.

47 Hoption Cann SA, van Netten JP, van Netten C, Glover DW: Spontaneous regression: a hidden treasure buried in time. Med Hypotheses 2002;58:115–119.

48 Hoption Cann SA, van Netten JP, van Netten C: Dr William Coley and tumour regression: a place in history or in the future. Postgrad Med J 2003;79:672–680.

49 Boeck C, Amberger D, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, Schuster F, Salih H, Babor F, Borkhardt A, Schmetzer HM: Significance of frequencies, compositions and/or antileukemic activity of (DC-stimulated) invariant NKT-, NK- and CIK-cells on the outcome of patients with AML, ALL and CLL. Press in J Immunother 2017.

50 Alexandroff AB, Jackson AM, O'Donnell MA, James K: BCG immunotherapy of bladder cancer: 20 years on. Lancet 1999;353:1689–1694.

51 Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, Martos JA, Moreno M: The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer 1997;79:2320–2328.

52 Ribas A: Tumor immunotherapy directed at PD-1. N Engl J Med 2012;366:2517–2519.

53 Ribas A: Combination Therapies Building on the Efficacy of CTLA4 and BRAF Inhibitors for Metastatic Melanoma. Am Soc Clin Oncol Educ Book 2012:675–678.

54 Vacchelli E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, Galluzzi L: Trial Watch: Toll-like receptor agonists for cancer therapy. Oncoimmunology 2013;2:e25238.

55 Miller RL, Gerster JF, Owens ML, Slade HB, Tomai MA: Imiquimod applied topically: a novel immune response modifier and new class of drug. Int J Immunopharmacol 1999;21:1–14.

56 Siegemund S, Hartl A, Buttlar H von, Dautel F, Raue R, Freudenberg MA, Fejer G, Buttner M, Kohler G, Kirschning CJ, Sparwasser T, Alber G: Conventional bone marrow-derived dendritic cells contribute to toll-like receptor-independent production of alpha/beta interferon in response to inactivated parapoxvirus ovis. J Virol 2009;83:9411–9422.

57 Schaefer L: Complexity of Danger: The Diverse Nature of Damage-associated Molecular Patterns\*. J Biol Chem 2014;289:35237–35245.

58 Vignali DA, Collison LW, Workman CJ: How regulatory T cells work. Nat Rev Immunol 2008;8:523–532.

59 Xia F, Deng C, Jiang Y, Qu Y, Deng J, Cai Z, Ding Y, Guo Z, Wang J: IL4 (interleukin 4) induces autophagy in B cells leading to exacerbated asthma. Autophagy 2018:1–35.

60 Kim KD, Choi SC, Noh YW, Kim JW, Paik SG, Yang Y, Kim, K., 2nd, Lim JS: Impaired responses of leukemic dendritic cells derived from a human myeloid cell line to LPS stimulation. Exp Mol Med 2006;38:72–84.

61 Schmetzer HM: Antileukemic T-cell-mediated immune reactions: limitations and perspectives for future therapies. Immunotherapy 2011;3:809–811.

62 Mogensen TH: Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009;22:240-73, Table of Contents.

63 Nemunaitis J: Live viruses in cancer treatment. Oncology (Williston Park) 2002;16:1483-92; discussion 1495-7.

64 Amberger D, Vokac Y, Hirn-Lopez A, Deen D, Kroell T, Schmid C, Kolb HJ, Tischer J, Schmetzer HM: Released soluble factors in serum or supernatants of leukemia-derived dendritic cell or mixed lymphocyte cultures are predictive for T-cells’ antileukemic functionality or clinical response to immunotherapy. J Immunother 2017.

65 Altomonte J, Ebert O: Replicating viral vectors for cancer therapy: strategies to synergize with host immune responses. Microb Biotechnol 2012;5:251–259.

66 Dickinson AM, Norden J, Li S, Hromadnikova I, Schmid C, Schmetzer H, Jochem-Kolb H: Graft-versus-Leukemia Effect Following Hematopoietic Stem Cell Transplantation for Leukemia. Frontiers in Immunology 2017;8:496.