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# Meeting report

# An update on graft-versus-host and graft-versus-leukemia reactions: a summary of the sixth International Symposium held in Schloss Ellmau, Germany, January 22–24, 2004

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**Summary:**

**The Sixth International Symposium on Graft-versus-Host and Graft-versus Leukemia Reactions was held in Schloss Ellmau (near Garmisch-Partenkirchen, Germany) between January 21 and 24, 2004. A total of 110 invited participants (scientists and clinicians working in the area of allogeneic stem cell transplantation) discussed current topics. Major topics of the 2004 meeting were: clinical results of donor lymphocyte infusions, basic biology, immunogenetics, function and clinical relevance of natural killer cells, haplo-identical stem cell transplantation, immune monitoring and immune modulation. Further highlights were: adoptive immunotherapy, vaccination and antibody-mediated strategies. As can be seen in the summaries of the individual presentations, important advances have occurred in our understanding of GVH and GVL reactions. Each session was followed by an animated discussion, which resulted in new ideas, insights and projects both for basic research and clinical transplantation. This year's symposium ('From Marrow Transplantation to Cell Therapy') was jointly organized by the Ludwigs-Maximilians-University of Munich (Sonderforschungsbereich 455), GSF (National Research Center for Environment and Health) and the EBMT Immunobiology Working Party. The organizers and authors of the conference proceedings would like to extend their gratitude to all participants for sharing their ideas, slides and manuscripts and making this event possible.**

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## Clinical results of donor lymphocyte infusions (DLI)

Cesare Guglielmi (Rome, Italy) presented the EBMT data of DLI for relapsed chronic myelogenous leukemia (CML). In a retrospective study, 298 patients with CML who had DLI before January 1999 were analyzed.[1] The major aim of the study was to determine how the initial dose of DLI affected the ultimate outcome. Three groups of patients were compared (median dose 0.1 *vs* 1 *vs* $3.5 \times 10^8$/kg). Overall, graft-versus-host disease (GVHD) was observed in 46% of patients, myelosuppression in 19% and any response in 74%. The first group had the best survival, showing a significant difference from the other groups up to 9 months after DLI. The data shown by Guglielmi demonstrate that patients with relapsed CML who start DLI with a dose higher than $0.2 \times 10^8$ mononuclear cells (MNC)/kg are exposed to significant morbidity and mortality. The incidence of major side effects can be markedly reduced by starting with a cell dose inferior or equal to $0.2 \times 10^8$ MNC/kg. He also presented a current protocol of the EBMT Chronic Leukemia Working Party for CML relapse after transplant in which escalating doses of DLI are compared to 12 months of imatinib, followed by the same escalating doses of DLI.

*Christoph Schmid* (Munich, Germany) reviewed the results of adoptive immunotherapy in acute myelogenous leukemia (AML). In contrast to CML, adoptive immunotherapy using donor cells (DLI) has been less effective in the treatment of AML relapse post allo-transplant. In a retrospective analysis by the EBMT, data on 120 patients were analyzed in order to define risk factors for response and outcome.[2] The overall complete remission rate was 42%, and the duration of remission post transplant, the use of chemotherapy prior to or with DLI, and the development of acute GVHD of $\geqslant$grade II had a significant influence on response. Responding patients showed a median disease-free survival of 304 days, and a 1-year survival of 55%. Interestingly, the use of chemotherapy by itself was not associated with better survival. Experiments were performed to improve antigen presentation *in vitro* by treating leukemic blasts with distinct cytokine cocktails containing GM-CSF and interleukin-4. With this technique, leukemia-derived dendritic cells showing

immunophenotypic and functional properties of professional antigen-presenting cells (APCs) could be generated.[3] This approach was translated into a clinical trial using mobilized donor blood cells and systemic application of GM-CSF for treatment of AML relapse post transplant in 24 patients. The response rate was 67%, and long-term remissions could be induced.[4] More recently, DLI were also used prophylactically after allo-transplants for high-risk AML. In all, 12 patients have been treated so far in Munich using an escalating dose regimen. Toxicity was very low, and the patients showed a 2 year DFS of 82% (manuscript in preparation). In conclusion, DLI may also be effective in AML, but candidates probably will have to be selected according to defined risk factors. Improvement may also come from immune-modulatory strategies using cytokines or growth factors, and from the use of DLI in a prophylactic or minimal residual disease situation.[5]

*Bart Nijmeijer* (Leiden, The Netherlands) discussed experimental studies on DLI for acute lymphoblastic leukemia (ALL) using a mouse model in which immune-deficient NOD/SCID mice were engrafted with primary human ALL blasts. The mouse model had been established earlier and was demonstrated to permit the monitoring of leukemic progression *in vivo*[6] as well as monitoring of the antileukemic efficacy of human effector cells.[7] After leukemic engraftment, human donor lymphocytes were infused and the blood of the animals was continuously monitored for leukemic cells and effector cells. Clinically, ALL and lymphoid blast crisis in general respond poorly to DLI after allogeneic stem cell transplantation (SCT). The underlying reasons are likely to be multifactorial and may include disease progression, GVHD and lack of co-stimulatory molecules. *In vitro*, Nijmeijer and his colleagues could generate conventional MHC-restricted T-cell responses against ALL cells in HLA-disparate settings, demonstrating that, in a situation of high antigenic disparity, the establishment of conventional T-cell responses against ALL cells is feasible. Also, *in vivo* in leukemic animals T-cell responses against HLA-identical and non-HLA-identical lymphoid blast cells were observed after DLI. However, these responses displayed a distinctly limited expansion potential, and T-cell proliferation stopped abruptly after a 40-day expansion period irrespective of the achievement of remissions. When tested *in vitro*, T cells recovered from treated animals were demonstrated to exert leukemia-specific cytotoxicity, but this cytotoxicity did not appear to be HLA-restricted. The nature of this limited and unconventional T-cell response against ALL cells as well as the reasons for imminent regression of the responses despite continuous presence of leukemic cells are under further evaluation. Since the establishment of conventional T-cell responses against ALL cells appears to be dependent on the antigenicity of the tumor cells, *in vivo* application of *in vitro* manipulated ALL blasts that express co-stimulatory molecules may lead to more durable and efficient responses. This strategy may improve the efficacy of cellular immunotherapy in ALL and will be evaluated in the animal model.

*Karl Peggs* (London, UK) showed preliminary data from the United Kingdom Collaborative Group, indicating an allogeneic graft-versus-tumor effect in Hodgkin's lymphoma (HL). In the past, allogeneic transplantation for relapsed or refractory HL had a high transplant-related mortality and resulted in few cures according to the IBMTR data. The UK group performed reduced intensity conditioning transplants in 49 patients using alemtuzumab, fludarabine and melphalan as conditioning.[8] DLI were given for progression (16 cases) or mixed chimerism (three cases) following the transplant. According to Peggs and his collaborators, reduced-intensity transplants have a low toxicity especially if a related fully matched donor is used and potentially lead to lasting responses. Responses to DLI provide evidence for a GVT effect in HL. Further exploration of reduced-intensity transplants in HL appears warranted.

*Jürgen Finke* (Freiburg, Germany) reviewed the results and perspectives of DLI in multiple myeloma.[9–11] So far only a limited number of patients have been treated with DLI. An overall response of up to 50% (including complete responses in 22–40% of the patients) was reported. The main toxicity was GVHD. High numbers of transfused T cells (up to $3 \times 10^8$/kg) as well as effective disease control prior to allo-transplantation and DLI were associated with better response. Repeated DLI and dose escalation seemed to improve the results. Protocols using prophylactic DLI for prevention of relapse post transplant were effective but had a slow response, reminiscent of the pattern seen in CML. Prophylactic DLI led to a significant survival advantage in a study using DLI after T-cell-depleted primary transplantation. A complete or partial response had a 30–40% relapse rate. In some patients, a relapse at extramedullary sites was observed. So far, the mechanisms of the graft-versus-myeloma effect are not well understood. Identification of target antigens as idiotypes, tumor-associated antigens, MAGE or other neo-antigens might enhance the efficacy of the procedure. In the clinical setting, reducing the tumor burden prior to allo-transplantation using high-dose chemotherapy and autologous stem cell rescue ('auto-allo tandem protocol') appears promising.

*Anthony D Ho* (Heidelberg, Germany) analyzed the relationship between GVHD and allogeneic antitumor effects in multiple myeloma. He started by reviewing the data in CML, where graft-versus-tumor effects may occur in the absence of serious GVHD. This is exemplified by DLI which effect graft-versus-tumor (graft-versus-hematopoiesis) reactions and may induce stable remissions in relapsed CML. The introduction of reduced intensity transplants for multiple myeloma gives a chance to separate graft-versus-tumor and graft-versus-hematopoiesis effects. In Heidelberg, a total of 55 patients had an allogeneic transplant. In most patients, the indication for allotransplant was relapse after one or more autotransplants. The median duration of disease at the time of allotransplant was 3 years from the time of diagnosis. According to Ho's data, severe GVHD is not always associated with antitumor effects.[12] Outcome was independent of acute GVHD, interval between diagnosis and transplant, or donor type. Approximately 60% of patients with less extensive pretreatment ($\leqslant 8$ cycles) had derived long-term benefit (ie with progression-free survival) from allogeneic transplantation. Patients with progressing myeloma after allogeneic transplantation did not benefit from

graft-versus-hematopoiesis reactions. According to Ho, an early allogeneic transplantation in patients with high-risk myeloma and alternative strategies including vaccination against the so-called shared tumor antigens would be needed to increase the chance of cure for multiple myeloma.

## Physiology of natural killer (NK) cells and their involvement in graft-versus-host and graft-versus-leukemia responses

*Andrea Velardi* (Perugia, Italy) gave an update on the impact of NK cell alloreactivity on mismatched hematopoietic transplantation.[13] As background, the effector function of NK cells is negatively regulated by receptors for MHC class I. Some receptors (killer-immunogobulin-like receptors (KIRs)) are specific for epitopes (KIR ligands) shared by certain HLA-C and HLA-B alleles. NK cells make up a repertoire which has a simplified view of class I polymorphisms. As far as the selection of donors is concerned, to date, HLA-C group 1 and 2 inhibitory NK receptor genes (KIR2DL1 and KIR2DL2/3) have been detected in almost all individuals tested (114/116). Concurring with this evidence, HLA typing predicted donor-versus-recipient NK alloreactivity in all 95 donors who were HLA-C group mismatched with their recipients. Therefore, for donor selection, HLA typing predicts NK cell alloreactivity in HLA-C group mismatches. Differently, although most individuals possessed the HLA-Bw4 inhibitory NK receptor gene (KIR3DL1), only two-thirds had the corresponding NK clones. Indeed, alloreactive NK clones were found in 19/29 HLA-Bw4 group mismatched donors. In HLA-Bw4 mismatches, Velardi and his colleagues recommend direct functional assessment of the donor alloreactive NK repertoire. Updated transplantation outcomes were presented for all 85 advanced stage AML patients (80% $\geqslant$second CR) transplanted from haploidentical donors (follow-up: 7–120 months). Transplantation from NK alloreactive donors enhanced engraftment (rejection rate 9% in the absence of NK alloreactivity, *vs* 2% in its presence), and helped to protect from GVHD (10 *vs* 3%). But, even more impressive was the antileukemic effect of NK alloreactivity. The probability of relapse was 79% for the 44 patients transplanted from non-NK alloreactive donors *vs* 17% ($P<0.005$) for the 36 patients transplanted from NK alloreactive donors. Lack of an NK alloreactive donor was the strongest independent risk factor predicting relapse (transplantation from non-NK alloreactive *vs* NK alloreactive donor: hazard ratio = 4.24, CI = 1.34–13.45, $P=0.014$), when compared with disease status at transplant (relapse *vs* remission: hazard ratio = 2.76, CI = 1.06–7.21, $P=0.038$). The probability of event-free survival was 52% for patients with NK alloreactive donors *vs* 7% for those without ($P<0.005$). NK alloreactivity improved survival also in unrelated BMT (87 *vs* 39%, $P<0.0007$) using a large BM graft and ATG.[14] Thus, NK cell alloreactivity, as observed in haploidentical transplants and in certain unrelated transplant protocols, appears to provide a survival advantage over matched transplants. In a mouse model using human BCR/ABL-positive cells injected into SCID mice, the co-administration of alloreactive NK cells led to the clearance of human leukemic cells (unpublished data).

*Lorenzo Moretta* (Genova, Italy) reviewed the molecular basis and recent advances of NK cell function.[15–19] Classically, NK cells exert cytolytic activity against some tumor cells, virally infected cells, IgG-coated target cells, possibly bacteria, and participate in cytokine production. Activating signals are received from MHC class I negative tumor cells, cells infected by certain viruses, IgG immune complexes and interleukin-2. Human NK cells have a complement of inhibitory receptors including KIR on their cell surface (p140, p70, p58.1/2, LIR-1/ILT-2 and CD94/NKG2A). These molecules interact with various HLA molecules and types on the surface of normal and malignant cells. In most instances, NK cells express several activating receptors and target cells several ligands involved in natural cytotoxicity, if the activating signal is present and no inhibitory signal is received (eg due to lack of a certain HLA molecule, cytotoxicity is triggered) (see Figure 1). The activating receptors on NK cells and some of their ligands have been characterized: NKp46, NKp30, NKp44, 2B4, DNAM-1 and NKp80. More recently, two ligands for DNAM-1 were identified: CD155 (PVR) and CD112 (Nectin-2). There are two checkpoints in NK cell activation: (1) surface expression of ligands for triggering receptors. These ligands are expressed *de novo* by potentially harmful cells (cytokine-activated cells, proliferating cells, cells 'stressed' by temperature, viral infection or malignant transformation); (2) interaction between molecules and MHC class 1, their specific receptors. The latter sense the possible loss of MHC-class I molecules (eg tumor cells or virus-infected cells). Dendritic cells are at the interface between adaptive and innate immunity. Dendritic cells can as well stimulate the proliferation and expansion of NK cells, when incubated with NK cells; however, they are also lysed by NK cells. The NK subset responsible for

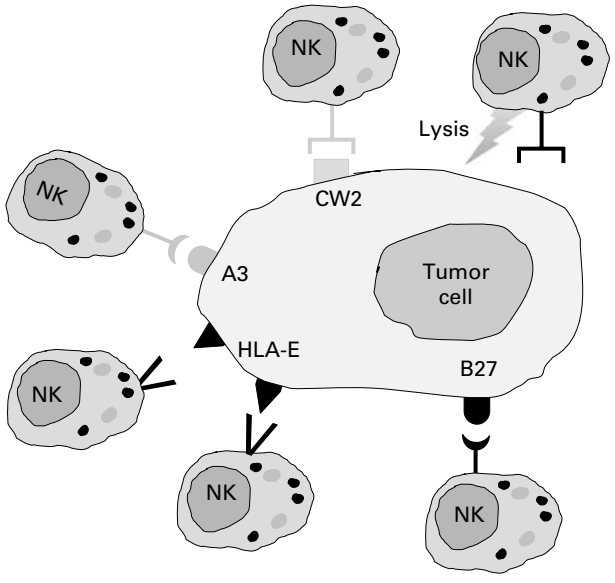


**Figure 1** Concept of NK cell-mediated lysis of tumor cells due to lack of inhibitory signals.

the killing of dendritic cells was identified: it expresses CD94/NKG2A, but lacks KIR. The expression of HLA-E (the ligand of CD94/NKG2A) is downregulated in immature dendritic cells. There is heterogeneity among CD94/NKG2A+, KIR-NK cells in the killing of immature dendritic cells (inverse correlation between CD94/NKG2A surface density on NK cells and magnitude of their cytolytic activity). Finally, the downregulation of HLA-B and HLA-C is not sufficient to allow cytotoxicity in NK cells expressing KIR3DL1 or KIR2DL. Taken together, the balance of triggering and inhibitory molecules regulates the function of NK cells and may be critical in graft-versus-host and graft-versus-leukemia reactions.

*Christine S. Falk* (Munich, Germany) discussed the differential regulation of activating and inhibitory receptors on human NK cells and NK-like T cells. Although NK-like T cells display classical T-cell phenotypes, they are functionally related to NK cells. Like molecular densitometers, both effector cells are regulated by a delicate balance between activation and MHC class I-mediated inhibition. Therefore, the recognition of leukemic cells by NK and NK-like T cells *in vitro* depends on the one hand on individual MHC class I levels and on the other hand on the expression of activating ligands by the malignant cells. In preliminary analyses, primary human leukemia cells were screened for sensitivity to non-MHC restricted T cells and NK cells and cytotoxicity was correlated to the expression and density of HLA-A, B, C and E molecules. Despite their relatively high HLA-A, -B and -C expression, B cells of chronic lymphocytic leukemia of different patients were efficiently lysed by NK cells as well as NK-like T cells, indicating that in these constellations, activating signals dominated over MHC-mediated inhibition. Leukemic cells were characterized by substantial variation with respect to expression of NK receptor ligands that may be responsible for the induction of cytotoxicity against leukemic cells. According to Falk's data, the expression of NKG2D and other receptors by NK and NK-like T cells may account for this cytotoxic activation and may permit discrimination between normal and leukemic and cells on nonhematopoietic origin, respectively. The gain of knowledge regarding NK and T-cell regulation in recent years may provide the basis for innovative immunotherapy approaches for the treatment of leukemic diseases.

*Hermann Wagner* (Munich, Germany) discussed the potential involvement of Toll-like receptors in graft-versus-host and graft-versus-leukemia reactions. Members of the Toll-like receptor (TLR) 9 subfamily are evolutionary related and sense viral and bacterial infections. While TLR9 directly recognizes as ligand viral and bacterial CpG-DNA motifs, murine TLR7 and human (h) TLR7 and hTLR8 sense viral single-stranded RNA motifs. Upon ligand-driven activation, members of the TLR9 subfamily bridge innate and adaptive immunity by activating T helper cell independently immature antigen-presenting dendritic cells (APCs) into professional APCs. Cellular activation is strictly dependent on the adaptor molecule MyD88, as opposed to activation mediated by the TLR2–4 subfamilies, in which the adaptor molecules TIRAP, TRIF and TRAM come into play.[20]

## Haploidentical SCT, immune reconstitution and immune modulation

*Megan Sykes* (Boston, USA) used lymphohematopoietic GVH reactions to separate GVHD and GVL reactions. In mice, nonmyeloablative conditioning including *in vivo* T-cell depletion of the recipient, followed by bone marrow transplantation (BMT) that was T-depleted *in vivo*, produced a state of mixed chimerism without inducing GVHD. Delayed DLI given at day +35 resulted in conversion to complete chimerism and showed marked GVL activity. This concept was translated into clinical protocols using anti-T-cell antibody as well as thymic irradiation for recipient and donor marrow T-cell depletion, and, most recently, using *ex vivo* donor CD34 selection from peripheral blood stem cells (PBSC). Preliminary clinical data confirmed the feasibility in patients with advanced and chemo-refractory lymphoid malignancies. Delayed DLI led to complete chimerism. Mixed chimerism can indeed induce stronger GVL effects than full chimerism. In further studies, Sykes and her co-workers studied the activation and expansion patterns of donor lymphocytes. GVH-reactive CD4 and CD8 cells underwent expansion, developed effector function and converted to the memory phenotype in mixed chimeric DLI recipients without causing GVHD, whereas similar expansion was associated with GVHD in freshly conditioned mice (R Chakraverty *et al*, unpublished data). Administration of DLI to established full donor chimeras was ineffective at mediating GVL because DLI-T cells are not activated (R Chakraverty *et al*, unpublished data), presumably due to a lack of host professional APC. Sykes also showed data on six patients who underwent combined kidney and nonmyeloablative bone marrow transplants. Paradoxically, although some of the patients lost donor chimerism secondary to host T-cell recovery, they all achieved renal allograft tolerance.

*Hans-Jochem Kolb* (Munich, Germany) reviewed the Munich experience with haploidentical SCT. Several concepts are in use to reach tolerance of one haplotype-mismatched transplants. In the megadose concept, high doses of CD34 selected cells are given. In the T-cell energy concept, cytotoxic T lymphocyte (CTL)A4-Ig blocks B7-CD28 interactions and produces energy. In the mixed chimerism concept, donor and host hematopoieses coexist and prevent GVHD without interfering with the GVL reactions. In the alloreactive NK-cell concept, KIR discordant NK cells suppress host-versus-graft reactions and GVHD without losing the GVL reactivity. The Munich approach to haploidentical transplantation is shown in Figure 2. At day 0, haploidentical bone marrow is given. On day +6, CD6-depleted PBSCs are transfused. In addition, an *in vitro* T-cell depletion is performed with anti-T-cell globulin (ATG). The CD6-depletion is based on dog experiments in which CD6-negative marrow cells suppress the generation of cytotoxic cells against autologous and allogeneic target cells. A total of 54 patients were transplanted (36 males, 18 females, median age 44 years, most with advanced hematologic malignancies, including 29 cases of AML and 14 cases of acute lymphoblastic leukemia). The 1-year survival was close to

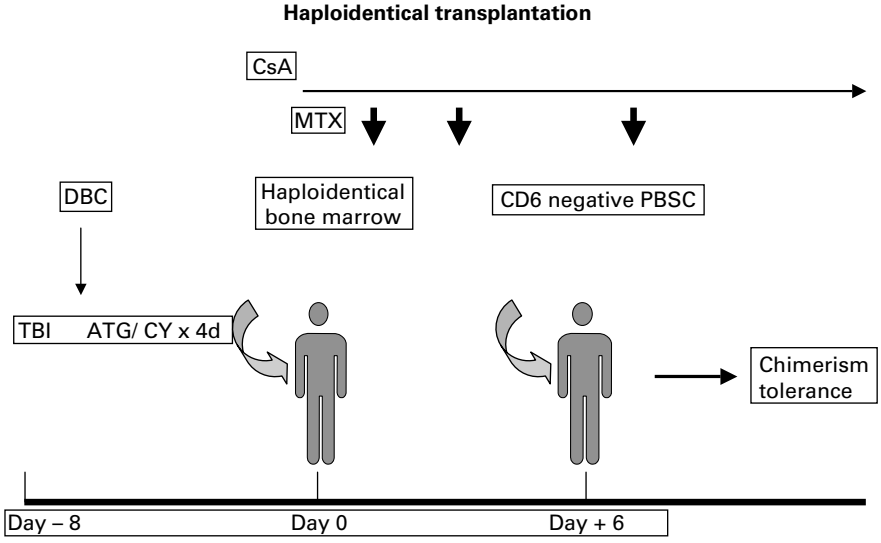


**Figure 2** Munich concept for haploidentical SCT using bone marrow and CD6 depleted peripheral blood stem cells.

30% in a Kaplan–Meier analysis. At 5 years, 18% survived. Interestingly, patients transplanted in first remission had a 5-year survival of approximately 60%. Recently, Kolb's group inaugurated a reduced intensity conditioning (using 4 Gy instead of 12 Gy total body irradiation) for haploidentical transplants. It is concluded from the Munich data that HLA-haploidentical SCT is feasible without complete T-cell depletion and nonmyeloablative conditioning. Rejection and GVHD can be controlled, but viral and fungal infections remain problematic. As a conclusion, Kolb recommended that HLA-haploidentical transplants should be considered earlier in patients who do not have a fully matched donor. Future studies will consider reducing postgrafting immunosuppression and reinforcing the suppressor activity of CD6− CD+ cells.

Using a rodent BMT model, *Markus Mapara* (Berlin, Germany) presented data on the role of host APC in the GVL reaction. Extending earlier studies, Mapara could show that, using a fully MHC-mismatched strain combination, DLI-mediated GVL effects are dependent on direct allorecognition and are therefore significantly stronger in mixed chimeras than in full chimeras. Using a nonmyeloablative conditioning regimen, DLI-mediated GVL reactions were transient. A maximal GVL effect correlated with an initial brisk expansion of alloreactive cells after administration of DLI and was lost over time concomitantly with the rapid contraction of the alloreactive T-cell population. Investigating the role of the conditioning regimen for the development of GVH, Mapara showed that the expression of chemokines in GVHD target organs (e.g. colon) is closely related to the intensity of the conditioning and is also influenced by recipient strain. Thus, in contrast to B6 mice BALB/*c* recipients show an increased and prolonged chemokine expression following a nonmyeloablative preparative regimen. Overall, chemokine expression showed a strong pattern of redundancy. Therefore, tissue infiltration by GVHD-inducing effector cells will probably not be prevented by blocking any single chemokine alone.

*Philippe Martiat* (Brussels, Belgium) discussed new approaches to haploidentical SCT. Haploidentical SCT is feasible, but leads to profound immune deficiencies and NK KIR mismatch does not seem to add to the GVL effect of allo-SCT in ALL and AML with HvG NK reactivity. DLI may improve the immune competence, but has a significant risk of GVHD. In the protocol developed in Brussels, patients were conditioned with melphalan, fludarabine, ATG and total body irradiation. Stem cells were selected for CD34 positivity and depleted for T and B cells. To decrease the risk of rejection, cyclosporine is given pretransplant. The protocol for which 27 patients are currently evaluable (18 very high risk) is conducted in three phases. In phase I, G-CSF was given post transplant and in a dose-finding study donor lymphocytes were infused ($3 \times 10^4$ CD3/kg at day 28, continued at increasing dose monthly). In phase II, GM-CSF was given post transplant and a fixed dose of donor lymphocytes was administered on day 28. Preliminary results show that in phase I a high incidence of relapse was observed, whereas in phase II more GVHD-related complications were observed. Now, in phase III, which he plans to apply to a larger group of patients, an individualized strategy (depending on the type of disease and KIR-mismatch) is followed and CMV- and aspergillus-specific donor cells are infused post-transplant. In addition, the effects of NK cell alloreactivity will be evaluated.

*Yair Reisner* (Rehovot, Israel) discussed the concept of megadose SCTs across major genetic barriers.[21,22] Donor-type human CD34 stem cells induce potent specific reduction of host antidonor CTL precursors in mixed lymphocyte cultures. The attributes of these CD34-positive veto cells are: they act in the first 24 h of culture and they mediate a reduction of CD3+IL-2+ and CD3+ g-interferon (IFN)+ effector cells. Contact with T cells and recognition of HLA class I molecules on the CD34+ cells is essential. Finally, the regulatory activity is sensitive to low-dose radiation. Tentative mechanisms of tolerance induction are: energy, deviation towards Th2 dominance,

active suppression and deletion. A transgenic mouse model was generated in which 2c transgenic mice bear large number of CD8 T cells expressing T-cell receptor (TCR) against H-2Ld. Veto cells of H-2Ld background, but not of other H-2 origins, are indeed able to delete 2c transgenic CD8 T cells. In more recent experiments, a synergism between rapamycin and host-nonreactive veto CTLs was found. Reisner concluded that the induction of tolerance in radiation chimeras involves a balance between host antidonor CTL precursors, donor dendritic cells and veto cells.

*Antoine Toubert* (Paris, France) discussed immune reconstitution after cord blood transplantation (CBT). As of now, more than 1500 umbilical CBTs have now been performed from related and unrelated donors for treatment of many high-risk hematologic disorders. Advantages of cord blood (CB) as an alternative source of hematopoietic stem cells are: relative ease of procurement, absence of risk to donors, reduced risk of transmitting infection, large donor pool and faster allocation process. Patients receiving HLA-identical sibling CBT have slower engraftment (neutrophil and platelet recovery), but also a lower risk of acute and chronic GVHD, especially in children. Some properties are specific to CB lymphocytes: they are functionally and phenotypically naive compared with adult blood lymphocytes, a unique cytokine profile and a fully constituted polyclonal TCR repertoire. Immaturity and naivety of CB T cells could explain the lower GVHD risk, but also raises some concern about the quality of immune reconstitution in CB-transplanted patients. Evaluation of immune reconstitution after HSC transplantation has recently improved through the development of direct methods for analysis of T-cell diversity (Immunoscope or spectratyping), *ex vivo* thymic function (TCR rearrangement excision circles or TRECs) and viral-specific immune responses (HLA class I tetramers). Regeneration of the T-cell population proceeds normally in two different pathways. The thymic-independent pathway includes transfer of graft-derived mature donor T cells followed by antigen-driven expansion, especially in case of non-T-cell depleted graft. This provides the first wave of T-cell reconstitution after transplantation, but this pathway is nearly absent in CBT due to the lack of memory T cells. The thymic-dependent pathway involves selection of graft-derived precursor cells, accounts for the more durable reconstitution of the T-cell compartment and generates a more diverse TCR repertoire. Differences in the naive/memory T-cell populations of CB and bone marrow (BM) explain the lack of T-cell expansions in CBT and probably account for less GVHD in this situation. Analysis of T-cell immune reconstitution after CBT compared to identical sibling BMT precisely matched for age and GVHD grade indicates that, despite the much lower number of CD34+ cells infused, recovery of T lymphocyte diversity towards normal was comparable, or even better, in CBT recipients.[23] Peculiar properties of lymphoid progenitors in CB could favor a stronger thymic rebound and prompt a more durable long-term reconstitution of the TCR diversity. These data are fully consistent with the favorable clinical outcome of CBT once engraftment and hematological recovery have been achieved. However, in the short term after transplant (first 3 months), specific immune responses against herpes viruses (EBV, CMV) measured by the frequency of antigen-specific CD8 T cells may be delayed and less efficient in CBT compared to BMT.[24] There is a high risk of EBV reactivation and development of post-transplant lymphoproliferative disorders in such a naïve immune environment. Therefore, these patients need a close monitoring of EBV reactivation and of the EBV-specific T-cell immune responses in order to initiate early specific therapies such as rituximab (anti-CD20 monoclonal antibody). These patients would also benefit eventually of viral-specific adoptive cellular therapies.

*James Ferrara* (Ann Arbor, USA) showed data that a histone deacetylase inhibitor reduces acute GVHD, yet preserves the GVL effect. Chromatin remodeling by acetylation or deacetylation of histones plays an essential role in the regulation of gene expression. Histone deacetylases decrease the acetylation of histone lysine tails and thereby condense chromatin structure and repress gene transcription. Inhibitors of histone deacetylases result in hyperacetylation and modify gene expression either positively or negatively depending on the context in a cell-specific manner. Since the complications of bone marrow transplantation depend on a complex network of inflammatory cytokines, Ferrara and co-workers hypothesized that the inhibition of histone deacetylases might influence the cytokine release and the complications during allogeneic transplantation. Suberoylanilide hydroxamic acid (SAHA) is a reversible inhibitor of histone deacetylases and has antitumor activity at micromolar concentrations. At nanomolar concentrations, SAHA has been found to reduce the secretion of cytokines in experimental endotoxemia. In two mouse models of acute GVHD (B6> B6D2F1 and BALBc> B6), SAHA was administered intraperitoneally on days 3–7 after BMT. Indeed, when SAHA was injected at 35 mg/kg, serum levels of pro-inflammatory cytokines were decreased, the intestinal histopathology was less affected, and the clinical severity and mortality from acute GVHD were decreased compared with vehicle-treated animals. In contrast, SAHA had no effect on donor T-cell proliferative and cytotoxic responses to host antigens *in vivo* or *in vitro*. Importantly, when mice received lethal doses of tumor cells at the time of BMT, administration of SAHA did not impair GVL activity and resulted in significantly improved leukemia-free survival using two different tumor and donor/recipient combinations. Further studies are underway to determine the target genes of SAHA. Taken together, these findings reveal a critical role for histone deacetylase inhibition in the pro-inflammatory events contributing to GVHD and suggest that SAHA and similar compounds may provide a strategy to reduce GVHD while preserving cytotoxic T-cell responses to host antigens and maintaining beneficial GVL effects.[25]

## Immune monitoring, vaccination and proteomics

*Catherine Wölfel* (Mainz, Germany) presented a novel approach to the identification of T-cell-defined minor histocompatibility (mHag) or leukemia-associated antigens using cDNA expression cloning with nonclonal allo-mixed

lymphocyte culture (allo-MLC) responders. A cDNA library was constructed from pretransplantation peripheral blood mononuclear cells (PBMC) of an HLA-A2.1-positive leukemia patient. cDNA pools were transfected together with HLA-A*02011 into COS-7 cells. The transfectants were tested in IFN-g ELISPOT assays for recognition by MLC responder lymphocytes derived from an HLA-identical sibling donor after repeated stimulation with irradiated patient pre-transplantation PBMC. cDNA pools leading to recognition by MLC responders were cloned. Antigen-coding cDNA clones were sequenced and compared to the respective sequences within the expressed genome of the donor, because allelic differences are regarded as candidate genes encoding for mHag or leukemia-associated antigens. Using this approach, Wölfel and her co-workers identified a one-base pair patient–donor mismatch within the TRIM22 gene. Homologous HL-A2.1-binding peptides containing the polymorphic residues of both alleles were synthesized and then loaded on an HLA-A2.1-positive cell line. Loading with nona- and decamer peptides derived from the patient's allele led to lysis by MLLC responders in chromium release assays, while the corresponding donor peptides did not. T cells against the TRIM22 mismatch were still detectable at low frequencies in the patient's *ex vivo* PBMC 5 years after successful allo-transplantation with HLA/peptide tetramers. Allo-TRIM22-specific T cells could be enriched and expanded from these post-transplantation lymphocytes by stimulation both with patient-derived pre-transplantation PBMC and with allo-TRIM22 peptide-loaded donor PBMC. Further patient/donor pairs with TRIM22 mismatches will be investigated to evaluate their role for GVHD and GVL effects. Using this strategy might allow the identification of mHag and leukemia-associated antigens as a basis for active and passive immunotherapy.

*Ulrich Keilholz* (Berlin, Germany) discussed the status of vaccination studies in AML.[26,27] The rationale for vaccine-based strategies is to induce leukemia-specific T-cell responses. The advantages are that peptides are easy to produce, that the vaccine needs no *in vivo* processing, and that CD8+ T cells can be activated directly via MHC-class-I restricted peptides. As a target for immunotherapy, the transcription factor WT1 was chosen. WT1 is overexpressed in AML and essential for the proliferation of AML blasts. It has been shown in a murine model that the HLA-A2-binding peptide 126–134 induces a protective response. A phase II study was initiated vaccinating patients with AML or MDS with a WT1-HLA-A2-binding peptide plus GM-CSF plus KLH. A T-cell response (monitored by tetramer staining and cytokine flow cytometry) and clinically relevant anti-leukemic activity were observed. Keilholz plans to extend these studies to a multicenter setting and to target other epitopes or antigens like proteinase 3.

*Andreas Moosmann* (Munich, Germany) discussed the use of Epstein–Barr virus (EBV) vectors for the activation of virus-specific T-cell responses.[28] He used mini-EBV vectors to generate immortalized B-cell lines (LCL). Mini-EBV vectors are an alternative to stimulate anti-viral CTL. In contrast to complete EBV, this vector contains a mini-genome within an intact EBV coat, thereby supporting latent infection and immortalization, whereas the lytic cycle is no longer supported. The vector is produced by an EBV packaging cell line that substitutes for the missing functions, and is able to effectively infect B cells and establish 'mini-LCL'. Without releasing wild-type EBV, the mini-LCL leads to similar T-cell expansion and EBV-specific T-cell function as compared to conventional LCL. Further, the mini-EBV vector may be used to produce T cells with other specificities. As a model antigen, pp65 from human CMV was introduced into the mini-EBV genome, and mini-LCL-expressing pp65 were generated. Using these stimulators, both functional EBV- and CMV-specific T cells (CD8 positive in their majority) could be generated easily from healthy seropositive donors.

*Eva Weissinger* (Hannover, Germany) applied proteomics to allogeneic SCT. She and her group collected sera and urine from 34 patients who underwent an allogeneic transplantation (15 from related and 19 from matched unrelated donors) and investigated these samples by capillary electrophoresis and mass spectroscopy. As controls, healthy volunteers and five patients who underwent an autologous transplant were studied. A total of 700–2500 polypeptides were identified and analysed in three-dimensional dot plots.[29] In all, 11 patients developing GVHD between days +14 and +60 showed significant differences with completely new polypeptides appearing and significant changes in concentration of the proteins excreted. Comparison of the plots of all patients with GVHD yielded about 25 polypeptides only present in these patients, while they were never seen in any of the other patient groups. Polypeptides 'significant' for GVHD could be detected at least 6 days prior to clinical parameters like skin rash or increase of liver enzymes. The so-called 'GVHD pattern' was compared to patterns seen in patients with no complications, sepsis and healthy individuals. Statistical evaluation showed that the GVHD pattern allowed discrimination from healthy individuals, patients without complications and sepsis from GVHD with high significance.[30] According to Weissinger's data, proteomics may serve as a useful tool for the identification and early diagnosis of complications associated with allogeneic SCT.

## Non-HLA genetics, GVH and GVL reactions

*Anne Dickinson* (Newcastle, UK) gave a broad overview about non-HLA immunogenetics and allogeneic SCT.[31] A number of genes with polymorphisms have been described in recent years, which have an impact on transplant outcome (cytokine genes, cytokine receptor genes and other non-HLA encoded genes). Biologically, polymorphisms within the 5′ or 3′ regulatory sequences of genes alter the structure of the transcription factor-binding sites within gene promoters and thereby regulate cytokine production. Polymorphisms of TNFα, TNFRII, IL-1,IL-Ra, IL-6, IL-10, vitamin D receptor and estrogen receptor were found to have an impact on graft-versus-host disease and survival. More recently, polymorphisms of mannose-binding lectin, FcγRIIa and myeloperoxidase were found to have an impact on infectious complications. A polymorphism of IL-10 was recently identified, which is associated with a

decreased risk of acute GVHD and death in remission.[32] Dickinson presented data on 230 patients from four European centers combining classic risk factors with six cytokine polymorphisms and a functional test (skin explant assay) in different donor–recipient pairs and performed a multivariate analysis. As far as acute GVHD grades II–IV are concerned, reactivity in the skin explant assay, donor IL-1Ra and patient IL-6 polymorphisms were highly predictive. As for chronic GVHD, a polymorphism of patient IL-6 (exon 174 GG) was highly predictive. Taken together, the study of genetic polymorphisms improves the prediction of complications in matched allogeneic sibling transplantation and allows the potential establishment of a clinical-immunogenetic risk index. Based on this index or score, the risk in specific patients may be targeted. Future work will combine cytokine polymorphisms with pharmacogenomics and will extend the present studies to different ethnic groups and unrelated SCT.

*Markus Uhrberg* (Düsseldorf, Germany) reviewed the immunogenetics and the regulation of NK cell receptors. At the basis of NK cell biology is the 'missing self' concept: NK cells have inhibitory receptors on their surface, which if not triggered lead to lysis of target cells. There are three types of the so-called KIRs: (a) inhibitory KIR (2DL1,2,3/ 2DL5/ 3DL1/ 3DL2), (b) stimulatory KIR (2DS1,2,3,4,5 and 3DS1) and (c) KIR2DL4 which has both an activating structure and a cytoplasmic inhibitory motif. The major inhibitory ligands are:

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| for KIR2DL1, | group 2 HLA-C (S77/N80); |
| for KIR2DL/2/3, | group 1 HLA-C (N77/K80); |
| for KIR3DL1, | HLA-B Bw4 epitope (As77–83). |

KIR genes are polymorphic and clonally distributed. The inheritance of KIR haplotypes has been defined by family segregation analysis. The KIR repertoire results from combinatorial diversity and clonal selection. The repertoire of NK cells is oligoclonal, which correlates with the activity of NK cells in the early phase of the immune response. Uhrberg gave examples how the KIR repertoire of the donor reconstitutes following haplo-identical SCT both for matched and mismatched KIR epitopes. Recent studies concerning the regulation of KIR expression revealed that the expression of KIR genes is epigenetically regulated. Indeed, the methylation status of KIR CpG islands correlates with the expression of KIR in freshly isolated NK cells. Upon treatment of NK cells with inhibitors of DNA methyltransferases, formerly silent KIR genes are readily reactivated and expressed on the cell surface. Uhrberg proposed the hypothesis that KIR promoters become demethylated during NK cell differentiation. In summary, the detailed analysis of NK cell receptors on the immunogenetic as well as expression level will be necessary to define future strategies, which will exploit the antileukemic potential of NK cells in SCT.

*Ernst Holler* (Regensburg, Germany) discussed NOD2/ CARD15 mutations as potential new players in the inflammatory cascade after allogeneic transplantation. NOD2/CARD15 proteins have been described as intracytoplasmatic counterparts of TLR. They act as sensors for bacterial muramyl dipeptide in cells of monocytic lineage as well as in specialized gastrointestinal epithelial cells and mediate activation of NF-$\kappa$B and subsequent cytokine and defensin production. Recently, a significant association of mutated single-nucleotide polymorphisms (SNPs) 8,12 and 13 of the NOD2/CARD15 gene resulting in a diminished antibacterial defense with an increased risk of Crohn's disease has been reported. Due to the similarities of inflammatory changes in Crohn's disease and GVHD, Holler and his collaborators speculated that NOD2/ CARD15 SNPs might play a role in the pathogenesis of GVHD. DNA samples in two cohorts of 169 recipient/ donor pairs from Regensburg and 101 recipient/donor pairs from Newcastle and Vienna were typed for the presence of these mutations using a sensitive taqman PCR. Distribution of mutated (30%) and wild-type SNPs in SCT recipients and donors was not different from normal controls reported in the literature. In both cohorts, the presence of NOD2/CARD15 mutations was a significant risk factor for severe GVHD and transplant-related mortality. This held true both for matched related and unrelated transplants with the highest risk when donor and recipients had the mutations. Holler's observations suggest a relevant role of NOD2/CARD15 mutations in complications following allogeneic SCT: a deficient antibacterial response in both, epithelial cells of the recipient GI tract and in donor monocytes/macrophages, might result in increased bacterial translocation and subsequently altered or enhanced activation of donor cells. These data might help to explain the observations on the role of the GI microflora in induction of GVHD. Further studies are ongoing, but it appears that typing of donors and recipients for NOD2/CARD15 mutations might contribute to pretransplant risk assessment or even donor selection in the future.

*Stefan Stevanoviç* (Tübingen, Germany) discussed how new minor histocompatibility antigens as mediators of graft-versus-host reactions and graft-versus-tumor reactions can be discovered. GVHD is mainly caused by allorestricted T-cell recognition of foreign MHC, although minor histocompatibility (minor H) antigens may also play a role. In contrast, GVL effects are mediated exclusively by T-cell recognition of minor H antigens. Any of the thousands of different proteins expressed within a cell may function as a minor H antigen, provided a short peptide that is polymorphic between donor and recipient in SCT is presented on the recipient's HLA molecule(s). At present, only few human minor H antigens have been characterized, as listed in the database SYFPEITHI (www.syfpeithi.de), including well-known HA-1 and HA-2. Many polymorphisms in human proteins are known – some of them described to play a role in GvH or GvL – but T-cell epitopes and HLA restrictions of these remain uncharacterized. He proposed two strategies for the identification of novel minor H antigens by bioinformatic screening. The first involves the examination of all sequence variations in human proteins for the probability of presentation by HLA molecules, which is carried out using T-cell epitope prediction. The second is to examine all HLA-presented peptides known so far (either characterized in the Stevanoviç lab or listed in SYFPEITHI) for polymorphic counterparts in, for example, Genbank by sequence comparison algorithms such as tfasta or tblastn.

*Eliane Gluckman* (Paris, France) discussed the association of pharmacogenetic polymorphisms with the toxicities and GVHD following HLA-identical sibling BMT. As background, both the donor and recipient inflammatory and immune defense gene polymorphisms are associated with neutrophil recovery, infections, acute and chronic GVHD after HLA-identical BMT.[33] She hypothesized that a polymorphism of genes that interfere with metabolism of drugs used in conditioning regimens (such as *P*450 cytochrome family genes) and GVHD prophylaxis (such as methylene tetrahydrofolate reductase (MTHFR) or Vitamin-D receptor genes) may also be associated with toxicities and outcomes after HLA-identical BMT. A total of 107 patients with acute and chronic leukemias, who had a HLA-identical sibling transplant between 1989 and 1999 at Hôpital St Louis, were analyzed. The median age was 35 years, the median follow-up 77 months. The genotype of *P*450 cytochrome family members, MTHFR, vitamin D receptors, glutathione-*S*-transferases and other enzymes was studied both in the recipients and donors. The data show that cytochrome *P*450 recipient CY2PB6 gene polymorphism, involved in the metabolism of cyclophophamide, is associated with mucositis, hemorrhagic cystitis and VOD after BMT. The incidence and severity of acute GVHD is associated with VDR and MTHFR gene polymorphisms. However, only VDR gene polymorphisms (TaqI) are also associated with transplant-related mortality and overall survival. According to Gluckman's data, polymorphisms of genes that interfere with drugs used in conditioning regimens are important factors associated with outcomes after HLA-identical BMT in patients with leukemia. Genetic risk assessment together with other clinical factors should be analyzed prospectively in order to individualize conditioning regimens, GVHD prophylaxis, antibiotic prophylaxis and to help the choice of donors in the allogeneic SCT setting.

### *TCRs, T-cell responses and selective T-cell depletion*

*Frederik Falkenburg* (Leiden, The Netherlands) discussed new *in vitro* studies transferring minor H antigen-specific TCR into CMV-specific T cells to treat leukemia. The minor histocompatibility antigens HA-1 and HA-2 are exclusively expressed in hematopoietic cells. HA-1- and HA-2-specific CTLs recognize normal and malignant hematopoietic cells, but not nonhematopoietic cells. In a patient who was treated with HA-1 and HA-2 mismatched DLIs, CTLs specific for these minor histocompatibility antigens could be identified. The emergence of the CTLs was associated with an antileukemic response. In order to expand and generate large numbers of HA-1- and HA-2-specific CTLs, the TCR genes derived from antigen-specific T cells were introduced into primary T cells. An approach to maintain the long-term specificity of expanded and transfected T-cell clones will be to take advantage of the immune response to common latent herpes viruses (EBV and CMV). Thereby, based on the restricted endogenous TCR repertoire, the formation of new complexes with unknown specificity due to pairing of endogenous TCR with the retrovirally induced TCR should be minimized. Falkenburg concluded from his data that a redirection of the immune response of peripheral T cells with specificity for minor histocompatibility antigens is feasible. Indeed, the HA-2-TCR-transferred T cells from HLA-A2-negative individuals did not exert alloreactivity against HLA-A2, yet preserved their CMV specificity. Further work will show if the latent presence of CMV antigens will maintain the long-term functional properties *in vivo*.

*Hans Stauss* (London, UK) showed data about the possible use and limitations of allogeneic TCRs for the immunotherapy of leukemias. In the past, his group in collaboration with the group of M Theobald (Mainz) had demonstrated that high-avidity CTLs against HLA-A2-presented peptides of human MDM2 can be isolated from A2 transgenic mice or from A2-negative human donors. Transfer of murine TCRs into human cells generated CTL that can effectively kill human HLA-A2-positive cancer cells expressing the MDM2 transcription factor. A similar approach was used in mice, and high-avidity CTLs specific for H2-$K^b$-presented MDM2 peptides were isolated from BALB/*c* mice. These CTLs were adoptively transferred into tumor-bearing B6xBALB/*c* F1 and B6-Rag−/− mice to assess their ability to inhibit tumor growth, and their risk of causing GVHD. The histological analysis showed no signs of GVHD, while the CTL delayed tumor growth in both groups of mice. However, protection was limited since most mice eventually developed tumors. Despite being present in tumor-bearing mice, MDM2-specific CTL had lost their effector function (induction of unresponsiveness). The same allo-restricted strategy was also used to isolated high-avidity CTL of HLA-A2-negative donors against a Wilms tumor antigens 1 (WT1)-derived peptide epitope presented by A2. The WT1 protein is overexpressed in most leukemias and in a large number of solid human cancers. Detailed analysis showed that the allo-restricted CTLs were able to kill CD34+ cells isolated from leukemia patients, but not normal CD34+ cells that express low levels of WT1. Stauss *et al* isolated the TCR genes of allo-restricted CTL and used retroviral vectors to transfer these genes into T lymphocytes of HLA-A2-positive individuals. The engineered CTLs displayed specificity for the WT1 peptide and were able to lyse leukemia cells expressing the WT1 protein. Therefore, a WT1 TCR transfer could be used to equip patient CTL with selective killing specificity for autologous leukemia cells.

*Matthias Theobald* (Mainz, Germany) discussed experimental studies aimed at reprogramming T helper lymphocytes by modulating class I major histocompatibility-specific TCR affinity. A condition for immunotherapy of leukemia and lymphoma is the expression and presentation of tumor-associated antigens. There is increasing evidence that the immune system and particularly CTLs respond efficiently to malignant target cells. Accumulation and subsequent overexpression of human MDM2 and altered p53 protein is associated with high-level presentation of MDM2 and wild-type (wt) p53-derived peptides by major histocompatibility complex (MHC) class I molecules on a wide range of malignant cells. A major barrier to the design of broad-spectrum MDM2 and p53-specific immunotherapeutics for leukemia and cancer, however, has been the observation that low-level expression of MDM2 and wt p53 peptides by nontransformed tissues and cells results in

self-tolerance of T lymphocytes with high avidity for self-class I MHC/self-peptide complexes. HLA-A2.1 (A2.1) transgenic (Tg) mice models provide a conceptual basis that exploits species differences between human and murine protein sequences in order to circumvent self-tolerance and obtain A2.1-restricted CTLs and T helper (Th) cells specific for epitopes derived from MDM2 and p53 self proteins. High-affinity MDM2 and p53-specific TCRs have been gained from A2.1-restricted Tg CTLs and delivered into human T lymphocytes in order to transfer antigen (Ag) specificity, affinity, and class I MHC restriction.

*Chiara Bonini* (Milan, Italy) discussed a new protocol administering donor lymphocytes transduced with a suicide gene in the setting of haploidentical SCT. Bonini's group had previously shown that the infusion of donor lymphocytes transduced by a retroviral vector to express the herpes simplex virus thymidine kinase (TK) is an efficient tool for controlling GVHD while preserving anti-tumor activity. A multicenter clinical protocol was designed to give donor lymphocytes transduced with TK to enhance immune reconstitution and prevent relapse after haploidentical SCT. In all, 11 patients with high-risk hematologic malignancies received escalating doses of TK-DLI starting at 42 days after SCT: four patients received $1 \times 10^6$/kg and seven patients received $1 \times 10^7$/kg CD3+/TK+ cells. Circulating transduced cells were documented in 9/10 evaluable patients (median day of engraftment +21). While only 1/4 patients achieved immune reconstitution after infusion of the lower dose, five out of seven patients who received the higher dose of TK+ cells reached 50 CD3+/$\mu$l by day 50, increasing steadily thereafter. In those patients who achieved immune reconstitution after TK-DLI, no additional antiviral treatment was required. Analysis on *ex vivo* lymphocytes showed that engrafted T cells were fully functional, being able to proliferate and produce high levels of IFN-$\gamma$ after stimulation with mitogens and viral antigens. A full repertoire of naïve, memory and effector cells was obtained. Five out of six patients who achieved immune reconstitution remained in CR (median follow-up 6 months, ranging between 3 and 37 months). In one out of five patients who achieved immune reconstitution with the higher dose of TK-DLI, a visceral acute GVHD was observed. The administration of multiple doses of ganciclovir (10 mg/kg/day), in the absence of immunosuppressive drugs, quickly resulted in the complete resolution of all clinical and biochemical signs of GVHD. In conclusion, the higher dose of TK-DLI is recommended to promote immune reconstitution after haploidentical SCT, while providing an effective and selective treatment for GVHD.

*John Barrett* (Bethesda, USA) studied the immune repertoire and type of T lymphocytes participating in GVL and GVH reactions. First, low frequencies of peripheral blood T cells specific for the nonallelic PR1and WT-1 peptides were quantitated using a highly sensitive PCR-based method detecting antigen-induced INF-$\gamma$ production. This assay allows the identification of as few as 1/100 000 antigen-specific T cells. These studies showed that low frequencies of memory/effector CD8+ T cells recognizing nonallelic antigens occur in normal donors.[34] They have high avidity for self antigens and are probably identical to the clonally expanded populations seen posttransplant. Some donor cells with GVL reactivity are central memory cells recognizing nonallelic antigens. To identify other peptides that elicit T-cell responses, a WT-1 peptide library was made. This has revealed that patients and donors recognize multiple WT-1 epitopes through both MHC class I and II HLA antigens. These studies underpin the possibility of separating harmful alloresponses from useful nonallelic GVL reactive T cells prior to transplant. In a clinical study, a selective depletion of CD25+ alloreactive cells was performed by challenging the donor with nonleukemic cells of the patient and then depleting CD25+ cells with an immunotoxin. These patients had significantly less GVHD with some preservation of a GVL effect. These results indicate that some of the GVL response is mediated by memory CD8 and CD4 T cells recognizing nonallelic leukemia-restricted antigens.

*Jean Claude Gluckman* (Paris, France) described a novel CB cell population of fetal dendritic, T and NK cell progenitors.[35] CD34+CD45RA++CD7++ progenitor cells were sorted and expanded according to previously published methods. Both by limiting dilution assays and clonal analysis, these progenitors display a higher NK than B-lymphocyte potential. This population does not overlap with the CD34+CD45++Lin−CD10+ progenitor cell population found in the CB and postnatal bone marrow and also giving rise to cells of the lymphoid lineages and dendritic cells. These populations are shown to correspond to lineage-polarized lymphoid progenitors, toward the T/NK (CD34+CD45RA++CD7++) or the B lineage (CD34+CD45++Lin−CD10+). Gene expression profiling showed that CD34+CD45RA++CD7++ progenitors express T, NK, granulo-monocytic or B lineage-associated genes, whereas the CD34+CD45++Lin−CD10+ progenitors essentially display a pro-B profile. Both populations can be demonstrated in the fetal bone marrow. The CD34+CD45RA++CD7++ cells were also found to accumulate in the fetal thymus and may be fetal thymus-colonizing cells. Conversely to the B-lymphocyte progenitors, which persist throughout life, the CD34+CD45RA++CD7++ progenitors become undetectable in the postnatal bone marrow. Gluckman concluded that the presence of CD34+CD45RA++CD7++ progenitors in umbilical CB could account for some of the differences in the outcome of CBT *vs* BMT or blood SCT.

*Günther Eissner* (Regensburg, Germany) showed data that support the concept that endothelial cell damage is the primary event of complications following allogeneic SCT. He described three steps of the transplant-related endothelial cell damage: (1) primary endothelial damage is caused by the pretransplant conditioning with release of host-derived inflammatory cytokines, (2) in the aplastic phase post transplant, the damage is enhanced by lipopolysaccharide and other factors, (3) in the progression phase, acute (and chronic) GVHD is caused by activated donor T cells and monocytes. Transmembrane tumor necrosis factor modulates the antiendothelial cell cytotoxicity of allogeneic effector cells. Eissner suggested studies to monitor endothelial cell damage and activation during allogeneic transplantation: screen patient sera for cytotoxicity against endothelial cells, monitor circulating endothelial cells as a

marker for endothelial cell damage or activation and use of new techniques to cultivate and immortalize patient specific endothelial cells.

*Christoph Huber* (Mainz, Germany) discussed the topic of separating GVH reactivity from favorable immune responses.[36] Initial work compared the autologous T-cell response with the allogeneic tumor-reactive T-cell repertoire. Using the model of renal cell cancer, tumor-specific responses of CD8+ CTLs and CD4+CD8+NK−T cells were analyzed in a HLA-A1-matched healthy donor.

## Antibody-mediated responses

*Michael Schleuning* (Wiesbaden, Germany) reviewed recent data on the use of ATG in matched unrelated stem cell transplants. The rationale for using ATG is that it provides an *in vivo* host T-cell depletion and thus facilitates engraftment. However, since free ATG can still be detected at the time of engraftment, it also is believed to reduce the rate of acute GVHD. There are different preparations of ATG with different kinetics and antigen specificity available. For example, rabbit ATG raised against a lymphoblastic T-cell line blocks the interaction between donor T cells and dendritic cells more efficiently than ATG raised against thymocytes. It activates CTLA4, thus interfering with the CD28-B7 signaling; in addition, it also directly inactivates CD28 and B7.1 and B7.2 on APCs. Several studies indicate that ATG is effective in matched unrelated transplants in patients with CML by reducing the rate of acute and chronic GVHD and thus improving the outcome. If a sufficient dose of ATG is used, ATG raised against a T-cell line seems to be superior to other preparations, possibly due to its shorter biological activity half-life and a more pronounced inhibition of costimulatory molecules. However, depending on the half-life of its biological activity and the kinetics of the underlying disease, the beneficial effects of ATGs sometimes may only be achieved at the costs of an impaired graft-versus-leukemia effect. Therefore in acute leukemia, the role of ATG during conditioning for MUD transplants remains to be defined. The use of routine postgrafting G-CSF, which might promote a TH1/TH2 shift, may actually have deleterious effects in acute leukemia patients conditioned with ATG.

*Roland Mertelsmann* (Freiburg, Germany) reviewed the expanding treatment options for patients with malignant lymphoma and presented data on the clinical application of a new anti-idiotype vaccine. A production strategy based on anchored RT-PCR cloning of the variable segments of the idiotype genes from lymphoma biopsies was developed for transcripts of $\mu$, $\gamma$ and $\alpha$ isotypes, and for $\kappa$ and $\lambda$ light chains. Identified clonal $V_H$ and $V_L$ segments were inserted into an inducible dicistronic expression vector. Recombinant idiotype Fab fragment were expressed in *Escherichia coli* and purified from the bacterial periplasma by affinity chromatography. Vaccine production was successful in 89% of attempted cases. In all, 18 B-non-HL patients (seven follicular lymphoma, three CLL, three multiple myeloma, two immunocytoma, two mantle cell lymphoma, one diffuse large-cell lymphoma) who had relapsed after previous anthracycline- or fludarabine-based chemotherapy received repeated intradermal vaccinations with 0.5–1.65 mg Fab fragment mixed with a lipid-based adjuvant in increasing intervals of 2–4 weeks concomitant with GM-CSF. No major side effects occurred. Only 7/18 patients had normal CD4+ T-cell counts prior to vaccination. As assessed by ELISA for IgM, IgG, and opposite light chain antibodies, 5/17 evaluable patients developed anti-Fab antibodies. In 6/16 patients, trace amounts of autoantibodies were detectable after immunization. In 6/15 evaluable patients, anti-Fab T-cell responses were induced by the vaccinations as detected by ELISPOT quantitation of IFN-$\gamma$-secreting cells upon *in vitro* stimulation with Fab-presenting autologous dendritic cells. Complete regression of pre-vaccination lymphadenopathy occurred in two patients (one follicular lymphoma, one diffuse large-cell lymphoma). In total, 10/17 evaluable patients and 10/14 nonsecreting B-NHL have progression-free survival of at least 4 months after the start of immunizations. One patient with blastoid mantle cell lymphoma progressed after 10 months, and another patient with pleomorphic immunocytoma after 6 months. There was no evidence for any influence on the natural course of disease progression in four patients with monoclonal gammopathy. These results indicate the feasibility, tolerability and potent immunogenicity of the tested idiotype vaccine formulation in highly immunosuppressed NHL patients and encourage further clinical trials.[37]

*Gundram Jung* (Tübingen, Germany) discussed the selective stimulation of the CD95 death receptor pathway with bispecific antibodies.[38] Monoclonal antibodies like rituximab and herceptin have established their activity, but the full promise of serotherapy for cancer has not been realized. As the next step, the effector function of antibodies needs to be improved. Theoretically, an optimal way of 'arming' a 'naked' antibody would be to replace its natural effector part, the Fc portion, with an effector moiety which is more efficient but retains its selectivity, that is the dependence upon binding to the target antigen. One elegant way to achieve this is to construct bispecific antibodies directed to a tumor-associated antigen (TAA) and a 'biomimetic' antibody which triggers a cell surface receptor with antitumor activity only if the TAA antibody has bound. This principle has been used in the past to redirect immune effector cells towards tumor cells via 'immune-activating receptors' like CD2, CD3, CD28, CD16 or CD64. Recently, Jung has used this principle to selectively trigger the CD95 death receptor on tumor cells. An important question to be answered in animal experiments is whether selective CD95 stimulation *in vivo* induces CD95-mediated 'pleiotropic' effects like inflammation and antiangiogenesis in addition to apoptosis. If this is so, an attractive new type of reagent would be available for experimental cancer therapy.

*Ralph Mocikat* (Munich, Germany) generated triomas for the immunotherapy of human B-cell lymphomas.[39] Triomas are lymphoma cells that are modified by fusion to hybridomas to express an immunoglobulin directed against surface receptors of APCs. In a mouse model, triomas generate long-lasting immunity against A20 lymphomas and even eradicate established tumors. Immunity is T-cell-mediated and does not depend on the lymphoma idiotype.

The immune response is polyclonal, includes the lymphoma idiotype and potentially other tumor-associated antigens. The generation of human triomas was attempted in 15 cases of B-cell neoplasias (11 cases of CLL, two cases of follicular lymphomas and one case each of plasmacytoma and centroblastic lymphoma), and was successful in 11 cases. The triomas expressed markers of human B lineage, human HLA alleles, and have human chromosomes detected by FISH analysis. In a preclinical evaluation, human triomas were able to induce tumor-specific responses of autologous patient T cells.

*Belinda Pinto-Simoes* (Ribeirão Preto, Brazil) presented a new CD20 × CD3 bispecific trifunctional antibody (Bi20), which activates T cells and directs T cells against CD20 positive lymphoid malignancies. *In vitro* experiments have shown a significant T-cell activation and an increased killing of B-cell lines and fresh CLL cells when Bi20 was added to a co-culture of normal PBMC and the respective target cells. Furthermore, T cells that had been activated in the presence of Bi20 were able to lyse fresh target cells even without further addition of Bi20. The proposed mechanisms of action are direct tumor cell killing by the simultaneously bound T cell, the induction of inflammatory cytokine release by macrophages activated via FcR binding, and the presentation of tumor cell antigens to naïve T cells following phagocytosis by the activated macrophages. In a clinical phase I trial, four heavily pretreated patients with advanced lymphoid malignancies received between eight and 25 infusions of Bi20, using a dose-escalating regimen that started at dose as low as 10 $\mu$g. Besides moderate fever and chills, transient granulocytopenia Grade III and IV was the most serious side effect in one case. Clinically, disease stabilization (two cases) and disappearance of a p53-deleted population (one case) were observed. Based on these results, more detailed analysis of the exact mechanism of action and the modalities for the clinical use of Bi20 seem justified and are currently under way.

*Helga Bernhard* (Munich, Germany) used a HLA/peptide-guided strategy to isolate and expand antigen-specific CTL clones. T cells were stimulated by autologous dendritic cells loaded with a given tumor antigen. Using tetramer-based flow cytometry, reactive T cells could be sorted and selectively expanded. In functional tests, the expanded CTL demonstrated their ability of Ag-specific lysis. Since survival *in vitro* was shortened by the presence of nonreversible multimers (tetramers), a reversible multimer, called streptamer, was introduced and led to significant improvement of clonal proliferation without interfering with functional properties. Bernhard also presented preliminary data on the clinical use of CTL that were generated using this strategy. In a case of refractory metastatic, Her2neu overexpressing breast cancer, specific CTLs against the $Her2_{369–377}$ peptide could be generated from several clones and were transfused using an escalating dose regimen. The transfused cells could be detected in the peripheral blood and the bone marrow up to 4 and 24 h after transfusion, respectively. Using *in vivo* imaging, the systemic distribution of the transfused CTL could be followed and enrichment in liver and spleen was shown. After the adoptive transfer of HER2-specific T cells, micrometastatic carcinoma cells were no longer detectable in the bone marrow. Finally, candidate antigens for CTL generation in myeloma patients were investigated: the cancer/testis antigen Ny-Eso-1 and MAGE-C1 being promising targets. Ny-Eso-1 was found to be upregulated by the demethylating agent 5-aza-2′-deoxycytidine. Clinical targets for transfer of specific CTL may be the situation of minimal residual disease after autologous or allogeneic SCT.

## Adoptive immunotherapy of solid tumors

*Dietger Niederwieser* (Leipzig, Germany) reviewed the European experience of allogeneic SCT for solid tumors. Between 1999 and 2001, the number of transplants has increased several fold, mainly due to patients with renal cell cancer and to a lesser extent with breast cancer. In all, 75 patients who had an allogeneic transplant for metastatic breast cancer were analyzed (39 myeloablative, 36 non-ablative; Ueno *et al*, unpublished). Some of the patients had a clear response to immunological manipulation like DLI. A multivariate analysis showed that relapse was inversely correlated (especially in the nonmyeloablative cohort) with the presence of acute GVHD. The Solid Tumor Working Party of the EBMT recently analyzed 61 patients with renal cell cancer transplanted at different centers between 2000 and 2003. Three patients rejected their graft and 19 patients developed acute GVHD $\geqslant$grade 2. At a median follow-up of 200 days, 24 patients were alive and 37 had died, 27 of tumor progression, 10 of transplant-related causes. Tumor responses were observed in 17 patients (nine partial remissions and eight minor responses). These responses were usually concurrent with GVHD and/or DLI. The duration of responses was 2–22+ months. The survival following allogeneic transplants depended on certain risk factors (median survival for poor prognosis patients 5 months, *vs* 25 months for good prognosis patients). Further research will focus on defining the immunogenic peptides on tumor cells as targets for the allogeneic response. EBMT has initiated a phase III study for patients with metastatic renal cell cancer having progressed after at least one prior immunotherapy (allogeneic transplant in patients with a HLA-matched sibling *vs* no transplant in patients without a donor).

*Andreas Mackensen* (Regensburg, Germany) discussed adoptive immunotherapy using autologous and allogeneic tumor-specific T cells. Melan-A specific T cells were generated *in vitro*, expanded and transferred to patients with malignant melanoma.[40] The cells were shown to react with the tumor antigen by tetramer staining and had the phenotype of memory/effector cells (CCR7-/$CD45RA^{low}$). Combined *ex vivo* analysis of Melan-A-tetramers and IFN-$\gamma$ secretion demonstrated survival of functional active Melan-A-specific CTL in the peripheral blood for at least 2 weeks. Clinically, most patients developed fever and chills after the transfer and 2/12 patients reached a partial remission, one had a minor response. Six of 12 patients developed eosinophilia and two patients lost melan-A expression in lymph node metastasis. The homing of adoptively transferred CTL was monitored using

$^{111}$In-labeling. Data were shown about the generation of artificial APCs since it was found that patients with melanoma have functional defects in their APCs.[41] Future studies will use tumor-specific CTL in the setting of nonmyeloablative conditioning and explore other antigens and epitopes as targets for immunotherapy.

*Thomas Blankenstein* (Berlin, Germany) showed data from experimental animals implicating IFN-$\gamma$, IFN-$\gamma$ receptor expression on stromal cells and angiogenesis in the immunological rejection of tumors. IFN-$\gamma$ produced by either CD4+ or CD8+ T cells acts on nonhematopoietic tumor cells and, either directly or indirectly, induces angiostasis.[42–44]

## Conclusion

*Alejandro Madrigal* (London, UK) summarized the meeting. He mentioned that an amazing progress has been made in the last 10 years since the first workshop on GVH and GVL reactions in Munich, 1994. The focus of the treatment of leukemia has shifted from pure chemotherapy and palliation to immunotherapy, immunologic manipulation and cure. The pace of progress is not always predictable, but it is safe to predict further advances in the next 10 years. The meeting spanned a wide array of topics, from T-cell immunology to CB stem cells, gave new insights into the biology of NK cells, discussed non-HLA genetics as determinants both for GVH and GVL reactions and mentioned cytokines, proteomics and antibody-mediated approaches. Clinically, since the description by Kolb, the use of DLI has expanded to multiple myeloma, Hodgkin's disease, renal cell cancer and other diseases; yet, it still faces the problems of dosage, time of intervention, interaction with the genetic background and with other treatment modalities. GVHD has not been eradicated but may be modulated or prevented by immune manipulation. New treatments like histone deacetylase inhibitors are under development, which may play a role in preserving GVL while modulating GVH reactions. The expansion and adoptive transfer of T cells specific for infectious agents and leukemia-associated antigens may shift the balance of alloimmune responses from GVH to graft-versus-tumor reactions. Beyond major and minor HLA antigens, multiple genes are involved in specific immune responses. Examples for these are cytokine genes and the genes metabolizing drugs (pharmacogenes). Ultimately, a specific treatment may be developed for each patient eradicating leukemia, yet preserving its anti-infectious immune responses. New questions will be asked: is it possible to measure the immune response to particular antigens, is it clinically relevant? Can T-cell responses be made more efficient? The proof of concept has already been made using T cells equipped with suicide genes. Triggering suicide genes, specific or nonspecific immune responses, can be stopped using pharmacologic manipulation. To implement these therapies, GMP facilities will be needed in all major centers. At 10 years from now, specific T cells, specific NK cells, tumor-specific vaccines and specific antibodies or B cells will be available for specific patients or specific clinical situations. Major progress was made unraveling the function and genetics both of activating and inhibitory receptors on NK cells. The 'disparity' or 'alloreactivity' of NK receptors was shown to be of major importance in haplo-identical transplantation. The interplay of NK cells and GVH and GVL responses in other types of SCT is under further investigation. The question was raised whether is it useful and clinically relevant to select matched unrelated stem cell donors according to their NK receptors. NK cells reach beyond their classical targets and may therefore be used in adoptive immunotherapy. More work is underway to elucidate the killing mechanisms of and the resistance mechanisms to NK cells. The isolation of new minor HLA antigens and determination of their protein structure is a further task. The specificity of B-cell responses needs to be explored in more detail. Bispecific and even trispecific antibodies are under development. Further progress depends on the close collaboration of basic and clinical scientists. According to Madrigal, the GVH/GVL meetings have been a spectacular success, establishing a network for collaboration and exchange of ideas between basic and clinical scientists. This multicenter international collaboration will guarantee future advances.

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