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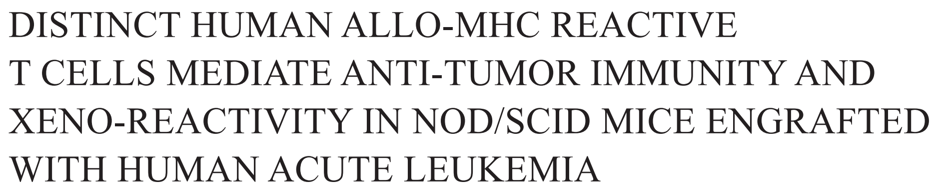
CHAPTER 3



DISTINCT HUMAN ALLO-MHC REACTIVE T CELLS MEDIATE ANTI-TUMOR IMMUNITY AND XENO-REACTIVITY IN NOD/SCID MICE ENGRAFTED WITH HUMAN ACUTE LEUKEMIA

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Submitted

ABSTRACT

Allo-HLA directed T cell responses after HLA-mismatched allogeneic stem cell transplantation (alloSCT) and donor lymphocyte infusion (DLI) are typically regarded as detrimental responses mediating Graft-versus-Host Disease (GvHD). However, allo-HLA reactive T cells with beneficial and selective Graft-versus-Leukemia (GvL) reactivity can also be identified within an HLA-mismatched context. We investigated whether beneficial allo-HLA-class II directed T cell response in NOD/scid mice engrafted with human chronic myeloid leukemia in lymphoid blast crisis induced detrimental xenogeneic GvHD as a result of off-target cross-reactivity or whether beneficial GvL reactivity and xenogeneic GvHD were mediated by separate T cells. The T cell response after HLA-class II mismatched DLI was shown to consist of allo-HLA-class II restricted leukemia-reactive T cells and allo-H-2-class I or allo-H-2-class II restricted xeno-reactive T cells. Our results show that T cells with beneficial and detrimental T cell reactivities can be clonally separated from an allo-MHC reactive T cell repertoire and indicate that *in vivo* allo-HLA directed T cells confer a limited risk for broad off-target allo-MHC cross-reactivity. Our data provide new insights into the nature of xenogeneic GvHD and illustrate that the NOD/scid mouse model for human acute leukemia permits analysis of *in vitro* generated leukemia-reactive allo-HLA directed T cells in a diversity of HLA-mismatched situations for their beneficial on-target efficacy and potential broad off-target toxicity.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (alloSCT) is a curative treatment for patients with hematological malignancies[1]. However, the beneficial Graft-versus-Leukemia (GvL) reaction after alloSCT is frequently accompanied by an undesired detrimental complication known as Graft-versus-Host-Disease (GvHD). The incidence and severity of GvHD is reduced by depletion of donor T cells from the stem cell graft[2;3]. Donor T cell depletion, however, also increases the risk for relapse of the malignancy[2-4]. Posttransplant relapses can be prevented or treated with donor lymphocyte infusion (DLI), but GvHD remains a significant cause of morbidity and mortality[5;6]. Therefore, therapies selectively stimulating GvL responses without GvHD are highly relevant to limit the toxicity and broaden the use of alloSCT and DLI.

Since HLA-identical sibling donors are available for only 30% of patients, the majority of alloSCT are performed with stem cell grafts from HLA-matched or HLA-mismatched unrelated donors (URD)[7;8]. Treatment of patients with HLA-mismatched alloSCT and DLI may result in profound immune responses due to high frequencies of allo-reactive donor T cells recognizing recipient disparate HLA molecules (allo-HLA)[9;10]. Due to constitutive HLA-class I expression on all nucleated cells, donor CD8+ T cells recognizing mismatched HLA-class I molecules are likely to mediate severe GvHD. In contrast to HLA-class I, constitutive expression of HLA-class II molecules is mainly confined to normal and malignant hematopoietic cells[11-15] and high HLA-class II expression on non-hematopoietic cells is only induced under inflammatory conditions[16-18]. Hence, under non-inflammatory circumstances, disparity for HLA-class II molecules is anticipated to induce a more selective GvL effect as compared to HLA-class I incompatibility. In support of this, we have previously demonstrated that clinically significant GvL effects can occur without GvHD after HLA-class II mismatched DLI administered in the absence of clinically evident inflammation[15;19], illustrating the potential therapeutic benefit of T cell based immunotherapy across HLA-class II barriers for selective induction of GvL immunity.

Although allo-HLA directed T cell immune responses after HLA-mismatched alloSCT are typically regarded as detrimental responses leading to graft rejection and GvHD, others and we have previously demonstrated that beneficial specificities exist within the allo-HLA reactive T cell repertoire[20-23]. By analyzing the specificity of T cells at the time of severe GvHD in a patient after HLA-class I mismatched DLI, we demonstrated induction of an allo-HLA-class I directed immune response consisting of T cells with detrimental as well as with beneficial reactivities[24]. Allo-reactive HLA-class I directed CD8+ T cells with detrimental reactivities recognized patient malignant cells as well as and non-hematopoietic cells, whereas CD8+ T cells with beneficial reactivities selectively recognized an over-expressed tumor-associated antigen on the malignant cells of the patient[24]. Recently, in two patients who converted to donor hematopoiesis with concomitant severe GvHD after HLA-class II mismatched CD4+ DLI, we demonstrated induction of allo-HLA-class II directed immune responses comprising of CD4+ T cells with detrimental reactivities recognizing

patient hematopoietic cells as well as skin-derived fibroblasts cultured under inflammatory conditions to induce HLA-class II expression, but also CD4+ T cells with beneficial reactivities selectively recognizing patient hematopoietic cells[25]. These studies in individual patients show that allo-HLA reactive T cells with beneficial and detrimental reactivities are both retained within polyclonal allo-HLA directed immune responses after HLA-mismatched alloSCT and DLI, supporting efforts for development of a model that permits assessment whether GvL and GvHD allo-HLA reactive T cell responses can be separated in a diversity of HLA-mismatched situations *in vivo*. This may provide a basis for further development of strategies for selection of allo-HLA reactive T cells specifically recognizing (malignant) hematopoietic cells of the patient for adoptive transfer after HLA-mismatched alloSCT.

The donor T cell repertoire has been shaped by selection events in the thymus on the basis of tolerance for antigens in HLA molecules that are shared between fully HLA-matched patients and donors, but not for antigens presented in allo-HLA molecules[10;26]. Therefore, in the HLA-mismatched setting, allo-HLA reactive T cell responses may contain T cells recognizing a variety of antigens presented in the context of allo-HLA molecules [27;28] as well as T cells recognizing multiple disparate HLA molecules[29]. This broad cross-reactive recognition by allo-HLA reactive T cells confers a risk for detrimental off-target reactivity in patients, and thus seriously hampers clinical application of allo-HLA restricted T cells.

In this study, we investigated the potential risk for off-target toxicity in an allo-HLA directed T cell response in a preclinical NOD/scid mouse model for human acute lymphoblastic leukemia and chronic myeloid leukemia in lymphoid blast crisis. Previously, we demonstrated that GvL effects in leukemia-engrafted NOD/scid mice treated with human T cells can develop in the absence or presence of xeno-reactivity[25;30;31]. Here, we investigated whether xeno-reactivity in leukemia-engrafted NOD/scid mice treated with HLA-class II mismatched DLI occurred as a result of off-target cross-reactivity of allo-HLA-class II directed T cells or whether GvL reactivity and xeno-reactivity were mediated by separate T cells. Our data show that T cell responses induced *in vivo* after HLA-class II mismatched DLI consisted of allo-HLA-class II restricted leukemia-reactive T cells and H-2-class I or allo-H-2-class II restricted xeno-reactive T cells, demonstrating that GvL reactivity and xenogeneic GvHD are mediated by different T cells with distinct specificities. These data show a limited risk for detrimental off-target effects by allo-HLA-class II directed T cells, and therefore provide a basis for development of strategies for selection of allo-HLA restricted leukemia-reactive T cells with selective GvL reactivity for adoptive transfer after HLA-mismatched alloSCT.

MATERIALS AND METHODS

Patient and donor material

Bone marrow (BM) and peripheral blood (PB) samples were obtained from a patient, and PB samples from a healthy unrelated donor (URD) after approval by the Leiden University Medical Center Review Board and informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by centrifugation over a Ficoll-Hypaque gradient and cryopreserved. Leukemic cells were obtained from a patient with chronic myeloid leukemia in lymphoid blast crisis. Patient and donor were fully matched for HLA-A, -B and -C alleles, but mismatched (underlined) for HLA-DR (patient: DRB1*1301, DRB1*1302, DRB3*0101, DRB3*0301; donor: DRB1*0701, DRB1*1302, DRB3*0301, DRB4*0101), HLA-DQ (patient: DQB1*0603, DQB1*0604; donor: DQB1*0202, DQB1*0604) and HLA-DP (patient: DPB1*0301, DPB1*0401; donor: DPB1*0201, DPB1*0501) alleles.

For DLI, CD3+ T cells were isolated from donor PB mononuclear cells (PBMC) from the URD by negative selection using the pan T isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity and composition of DLI were analyzed by flowcytometry after staining with FITC-labeled CD4 (Becton Dickinson (BD) Biosciences, San Jose, USA), PE-labeled CD8 (Caltag, Invitrogen, Oslo, Norway) and PECy5-labeled CD3 (BD Biosciences) monoclonal antibodies.

In vivo NOD/scid mouse experiments

Female NOD/scid mice aged 5 to 6 weeks were purchased from Charles River (Saint-Germain-sur-l'Abresle, France). All animal experiments were conducted according to Institutional Guidelines with permission from the Leiden University Medical Center Animal Experiments Committee. Mice were engrafted with primary human leukemic cells from the patient and monitored as described previously[25]. Briefly, upon intravenous injection of leukemic cells ($10*10^6$), engraftment of leukemia was monitored weekly by flowcytometric analysis of PB samples after staining with FITC-labeled human CD19 (BD Biosciences) and PE-labeled mouse CD45 (Ly5, Caltag) monoclonal antibodies. Upon detection of leukemic cells in PB, mice were treated with DLI consisting of CD4+ ($5*10^6$ cells) and CD8+ ($3*10^6$ cells) T cells by intraperitoneal injection. After DLI, PB samples were analyzed for the presence of leukemic cells and T cells by flowcytometry after staining with FITC-labeled human CD19 and CD4, PE-labeled human CD8 and PECy5-labeled human CD3 monoclonal antibodies. During the experiments, mice were monitored for the appearance of xenogeneic GvHD. Symptoms including weight loss, anemia, hunched posture, ruffled fur and reduced mobility were used to diagnose xenogeneic GvHD.

Isolation of T cell clones

Mice were sacrificed during GvL response and xenogeneic GvHD, and T cell cloning was performed from BM and spleen samples by single cell sorting. In brief, organ suspensions were stained with FITC-labeled human CD4 antibody and PE-labeled human CD8 antibody, and CD4+ and CD8+ cells were separately sorted single cell per well using flowcytometry into U-bottom 96-wells plates containing irradiated (50 Gy) allogeneic PBMC ($0.05*10^6$/well) as feeder cells in 100 ml Isocove's modified Dulbecco's medium (IMDM, BioWhittaker, Verviers, Belgium) with 5 % human serum, 5 % fetal calf serum (FCS, BioWittaker), IL2 (120 IU/ml) (Chiron, Amsterdam, The Netherlands) and phytohemagglutinin (0.8 mg/ml) (Murex Biotec Limited, Dartford, UK). Proliferating T cell clones were selected and restimulated every 10-20 days.

Isolation of stimulator cells

Epstein-Barr Virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL) from patient and donor were generated using standard procedures and maintained in IMDM with 10 % FCS. Donor EBV-LCL were transduced with retroviral vectors encoding the mismatched HLA-class II alleles of the patient as previously described[25]. Dendritic cells (DC) from NOD/scid and C57BL/6 mice were generated *in vitro* by culturing total BM from femora in RPMI-1640 (Invitrogen, Bleiswijk, The Netherlands) with 10% FCS and 20 ng/ml rmGM-CSF (Invitrogen) in 100mm culture dishes. After 10 days, BM-derived DC were phenotypically analyzed by staining with PE-labeled antibodies against mouse CD11c (clone HL3) and FITC-labeled antibodies against mouse H-2K^d (clone SF1-1.1), H-2D^b (clone 28-14-8) and I-A^{g7} (clone OX-6) (all BD Biosciences). Single cell suspensions of BM and spleen cells for functional studies were obtained from euthanized healthy mice upon erythrocyte lysis. NOD/scid fibroblasts were established from skin specimens cultured in low glucose Dulbecco modified Eagle medium (BioWhittaker) and 10% FCS with or without rhIFN-γ (500 IU/ml) for 5 days. Phenotypical analysis of BM and spleen cells, and skin-derived fibroblasts was performed after staining with FITC-labeled monoclonal antibodies against mouse H-2K^d, H-2D^b and I-A^{g7}.

ELISA

T cell clones (5,000 cells per well) were co-incubated with stimulator cells (30,000 cells per well) in a final volume of 150 ml IMDM with 5% human serum, 5% FCS and 10 IU/ml IL2 in 96-wells U-bottom plates. After overnight incubation at 37°C, release of IFN-γ in 50 ml culture supernatants was measured by IFN-γ ELISA (Sanquin, Amsterdam, The Netherlands).

To determine H-2 restriction alleles of xeno-reactive T cell clones, NOD/scid BM-derived DC (30,000 cells per well) were pre-incubated with monoclonal antibodies against H-2K^d (clone SF1-1.1; BD Biosciences), H-2D^b (clone 28-14-8; BD Biosciences) or anti-IA^{g7} (OX-6; Hybridoma Facility, Utrecht, The Netherlands) for 30 minutes at RT before addition of T cell clones (5,000 cells per well).

Cytokine release profile of xeno-reactive T cell clones (5,000 cells per well) upon overnight stimulation with BM-derived DC (30,000 cells per well), and leukemia-reactive CD4+ T cell clones (5,000 cells per well) upon stimulation with primary human leukemic cells (30,000 cells per well) was determined by multi-Th1/Th2/Th17 cytokine ELISA (Qiagen, Venlo, The Netherlands).

IFN-γ and TNF-α levels in plasma of treated and leukemia-engrafted untreated mice were determined by IFN-γ ELISA and TNF-α ELISA (Sanquin).

Histopathologic analyses

Histopathologic analyses were performed on spleen, iliac lymph nodes, intestine, lungs, kidneys and skin of mice upon sacrifice. Tissues were fixed in 4% buffered formalin, embedded in paraffin, sectioned at 5 mm thickness and stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry, sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the slides in 0.01M sodium citrate buffer (pH 6.0, 98°C) and blocking of endogenous peroxidase was performed by fixing the slides in PBS/0.3% hydroxyperoxide. Subsequently, sections were blocked with 3% bovine serum albumin in PBS/0.05% Tween-20 before overnight incubation with unconjugated rabbit anti-human CD20 (clone SP32, Abcam, Cambridge, United Kingdom) or rabbit anti-human CD3 (clone SP7, Abcam) diluted in PBS/0.05% Tween-20 to detect human leukemic cells and T cells, respectively. Control sections were incubated with PBS/0.05% Tween-20 alone. All sections were subsequently incubated with biotinylated goat anti-rabbit secondary antibody (DAKO, Glosstrup, Denmark) in the presence of 1.5% normal goat serum and processed for streptavidin immunoperoxidase staining using 3,3-diaminobenzidine substrate followed by counterstaining with hematoxylin.

RESULTS

Isolation and characterization of T cells during GvL response and xenogeneic GvHD

To analyze the specificity of the T cell responses during GvL effect and xenogeneic GvHD, mice were engrafted with human leukemic cells and treated with DLI, which consisted of 59% CD4+ and 35% CD8+ T cells. The patient leukemic cells and donor lymphocytes were completely matched for all HLA-class I alleles and mismatched for HLA-DRB1, -DRB3, -DQB1 and -DPB1 alleles (Materials and Methods). The expansion of human CD4+ and CD8+ T cells correlated with the occurrence of GvL effects and xenogeneic GvHD symptoms (Figure 1A). During GvL reactivity and xenogeneic GvHD, human CD3+ T cells were clonally isolated from BM and spleens of treated mice and expanded. A total of 91 CD4+ and 22 CD8+ T cell clones were obtained. All T cell clones were analyzed for reactivity against patient leukemic cells and NOD/scid BM-derived DC using IFN-γ ELISA (Figure 1B). The murine DC expressed H-2-class I and H-2-class II molecules

(data not shown). The reactivity of isolated CD4+ T cell clones could be divided into groups based on their specific production of IFN-γ (>100 pg/ml) upon stimulation with patient leukemic cells (112-306 pg/ml IFN-γ; n=15) or NOD/scid DC (131-363 pg/ml IFN-γ; n=42). The majority of leukemia-reactive CD4+ T cell clones were also shown to recognize patient-, but not donor-derived, EBV-LCL, indicating recognition of allo-antigens. In contrast to CD4+ T cell clones, none of the isolated CD8+ T cell clones recognized patient leukemic cells, or patient- and donor-derived EBV-LCL, whereas a number of CD8+ T cell clones recognized NOD/scid DC (100-586 pg/ml; n=12). TCR Vb analysis demonstrated that the T cell clones expressed various different TCR Vb chains, illustrating that the leukemia-reactive and xeno-reactive T cell responses were polyclonal (data not shown).

In conclusion, these data show induction of high frequencies of human T cells with distinct specificities for human leukemic cells and murine DC, demonstrating that GvL reactivity and xenogeneic GvHD in NOD/scid mice treated with DLI are mediated by distinct T cells. Furthermore, the data show that the xeno-reactive T cells were both CD4+ and CD8+, whereas all isolated leukemia-reactive T cells were CD4+, probably due to selective mismatching for HLA-class II alleles between the donor and recipient.

Identification of MHC restriction elements of isolated T cell clones

To identify the HLA-class II restriction molecules involved in recognition of patient leukemic cells, leukemia-reactive CD4+ T cell clones (n=10) were screened for recognition of donor EBV-LCL retrovirally transduced with patient mismatched HLA-DR (B1*1301 or B3*0101), HLA-DQ (B1*0603) and HLA-DP (B1*0301 or B1*0401) alleles using IFN-γ ELISA. CD4+ T cell clones were shown to be directed against mismatched HLA-DRB3*0101 (n=4), -DQB1*0603 (n=5) or -DPB1*0301 (n=1) (Figure 2A).

Characterization of H-2 restriction alleles in recognition of NOD/scid BM-derived DC was performed with CD4+ (n=5, clones A to E) and CD8+ (n=5, clones F to J) xeno-reactive T cell clones selected for expression of different TCR-Vb chains to represent the diversity of the polyclonal xeno-reactive T cell response (data not shown). Xeno-reactive T cell clones were incubated with NOD/scid BM-derived DC with and without blocking antibodies against H-2 class I (H-2K^d and H-2D^b) and H-2 class II (I-A^{g7}) alleles, and recognition was measured by IFN-γ ELISA. Recognition of NOD/scid DC by xeno-reactive CD4+ T cell clones could be blocked by monoclonal antibody against H-2K^d for one T cell clone, and antibody against I-A^{g7} for four T cell clones (Figure 2B, *Upper*). For xeno-reactive CD8+ T cell clones, recognition of NOD/scid DC

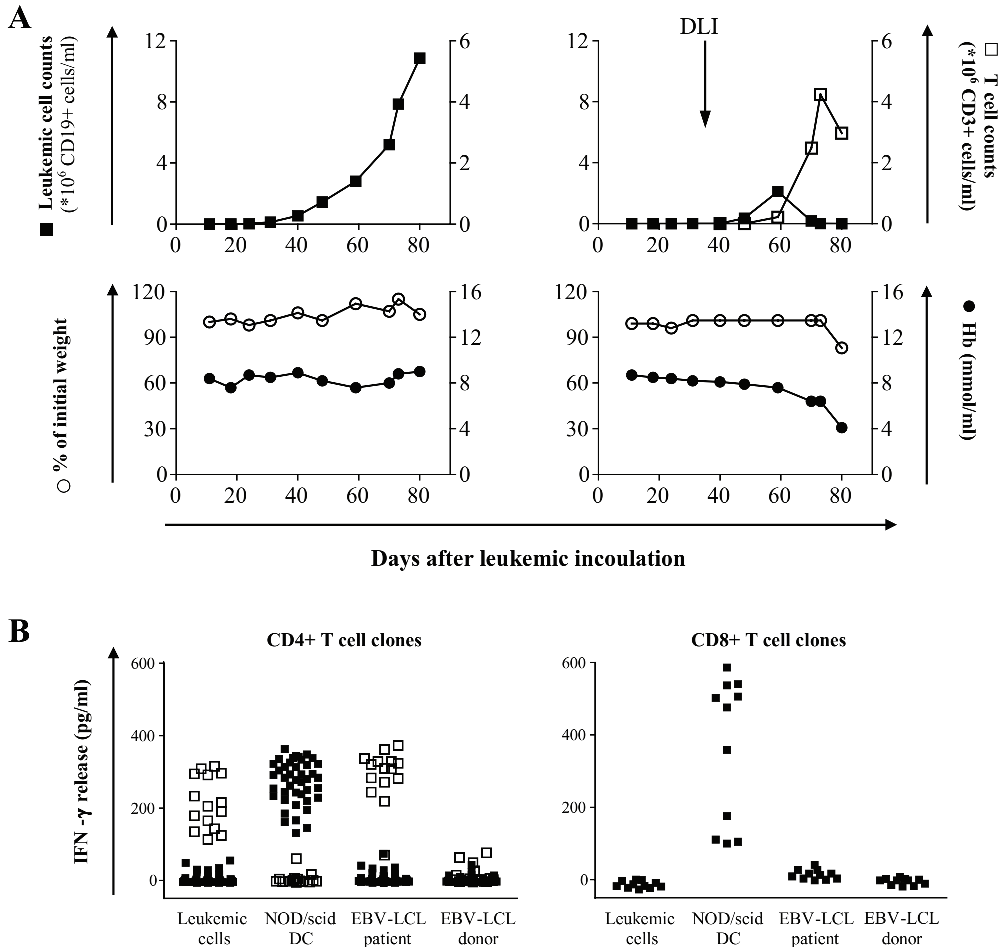

Figure 1: Isolation and characterization of T cell reactivity during GvL reactivity and xenogeneic GvHD after DLI.

(A) NOD/scid mice were inoculated (day 0) with primary leukemic cells, and were left untreated or received DLI 35 days after leukemic inoculation (indicated by the arrow). Absolute cell numbers of CD19+ leukemic cells (--■--) and CD3+ T cells (--□--) in peripheral blood of mice were assessed by flowcytometry (*Upper*). In addition, percentage of initial body weight (--○--) and hemoglobin (Hb) levels (mM) (--●--) in peripheral blood of mice (*Lower*) were measured. Each symbol represents the mean values of untreated (n=2) and treated mice (n=2). **(B)** Human primary leukemic cells, NOD/scid BM-derived DC, and patient and donor derived EBV-LCL were tested for recognition by 91 CD4+ and 22 CD8+ T cell clones. T cell clones were isolated from BM and spleen obtained from two mice at 45 days (80 days after leukemic inoculation) after DLI. Each dot represents the mean release of IFN-γ (pg/ml) by a single T cell clone in 50 ml culture supernatants of duplicate wells. T cell clones recognizing human leukemic cells, but not NOD/scid BM-derived DC, are indicated by open squares (□), whereas T cell clones recognizing NOD/scid BM-derived DC, but not human leukemic cells are represented are represented by closed squares (■). Non-reactive T cell clones (34 CD4+ and 10 CD8+ T cell clones) are not displayed.

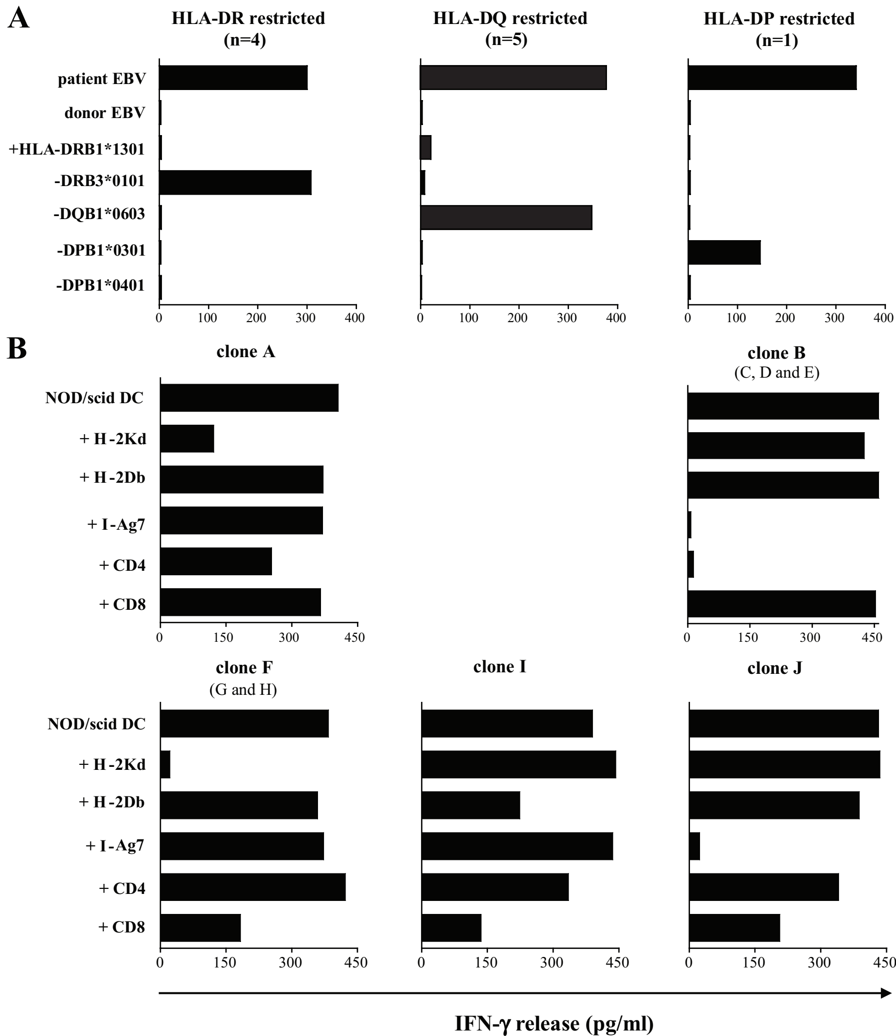

Figure 2: Leukemia- and xeno-reactive T cell clones recognize allo-antigens in different MHC molecules.

(A) Leukemia-reactive CD4+ T cell clones are restricted by different allo-HLA-class II molecules. Recognition of patient EBV-LCL and donor EBV-LCL retrovirally transduced with HLA-DRB1*1301, -DRB3*0101, -DQB1*0603, -DPB1*0301 and -DPB1*0401 by representative leukemia-reactive CD4+ T cell clones are shown as the release of IFN-γ (pg/ml) in 50 ml culture supernatants. Of the ten leukemia-reactive CD4+ T cell clones tested, four were specific for HLA-DR, five for HLA-DQ and one for HLA-DP. **(B)** Xeno-reactive CD4+ and CD8+ T cell clones are restricted by different H-2-class I and II molecules, and dependent on co-receptors for recognition. Recognition of NOD-scid BM-derived DC by representative xeno-reactive CD4+ (*Upper*) and CD8+ (*Lower*) T cell clones in the presence or absence of blocking antibodies is shown as measured by IFN-γ ELISA. T cell clones indicated between brackets displayed similar recognition patterns as the representative T cell clone. Blocking was performed using monoclonal antibodies against H-2 class I (H-$2K^d$ and H-$2D^b$), class II (I-A^{g7}), human CD8 (FK18) and human CD4 (RIV6). Of the five xeno-reactive CD4+ T cell clones tested (*Upper*), one T cell clone was H-$2K^d$ restricted (A) and four T cell clones were I-A^{g7} restricted (B, C, D and E). Of the five xeno-reactive CD8+ T cell clones tested (*Lower*), three T cell clones were H-$2K^d$ restricted (F, G and H), one T cell clone was H-$2D^b$ restricted (I) and one T cell clone was I-A^{g7} restricted (J). All T cell clones showed reduced IFN-γ production upon blocking of the co-receptor. The release of IFN-γ (pg/ml) in 50 **m**l culture supernatants is shown.

could be blocked by monoclonal antibodies against H-2K^{d} (n=3), H-2D^{b} (n=1) or I-A^{g7} (n=1) (Figure 2B, *Lower*). To investigate whether the human CD8 and CD4 co-receptors as expressed by the xeno-reactive T cell clones contributed to H-2 restricted recognition of murine DC, xeno-reactive T cell clones were also incubated with NOD/scid DC with blocking antibodies against CD8 or CD4. Recognition of NOD/scid DC by xeno-reactive CD8+ and CD4+ T cell clones was inhibited to different extents upon blocking of the co-receptors (Figure 2B, *Lower*). Some T cell clones displayed a modest decrease in recognition, whereas complete inhibition was observed for other T cell clones, demonstrating that human CD8 and CD4 can serve as co-receptors for H-2-class I and II restricted recognition.

In conclusion, these data illustrate that T cells induced during GvL reactivity and xenogeneic GvHD in NOD/scid mice after HLA-class II mismatched DLI were restricted by various disparate allo-HLA-class II, and H-2-class I or H-2-class II molecules, respectively. Furthermore, xeno-reactive human T cells displayed H-2 restricted recognition of murine cells that depended on the contribution of CD4 and CD8 co-receptors to different extents, illustrating genuine characteristics of T cell allo-recognition.

Tissue specificity and reactivity of xeno-reactive T cell clones

To determine the tissue specificity of xeno-reactive T cell clones, primary hematopoietic BM and spleen cells were isolated from NOD/scid mice and compared with *in vitro* cultured NOD/scid BM-derived DC for H-2-class I and H-2-class II expression and recognition by xeno-reactive CD4+ and CD8+ T cell clones as measured by IFN-γ ELISA (Figure 3). Flowcytometric analysis demonstrated that NOD/scid DC, BM and spleen cells uniformly expressed H-2-class I molecules (Figure 3A). NOD/scid DC were also uniformly positive for H-2-class II, whereas primary BM and spleen samples contained low numbers of H-2-class II+ cells (Figure 3A). All H-2-class I and H-2-class II restricted xeno-reactive T cell clones showed specific recognition (>30 pg/ml IFN-γ) of NOD/scid DC, as illustrated by the lack of recognition of C57BL/6 BM-derived DC, which only share the H-2D^{b} allele with NOD/scid mice (Figure 3B). The only exception is T cell clone I that recognized C57BL/6 DC, which is in line with the observed inhibition of recognition of NOD/scid DC by blocking antibodies against H-2D^{b} (Figure 2B). H-2-class I and H-2-class II restricted xeno-reactive T cell clones showed variable but specific recognition of primary NOD/scid BM and spleen cells, as the recognition could be blocked with H-2 specific monoclonal antibodies (data not shown).

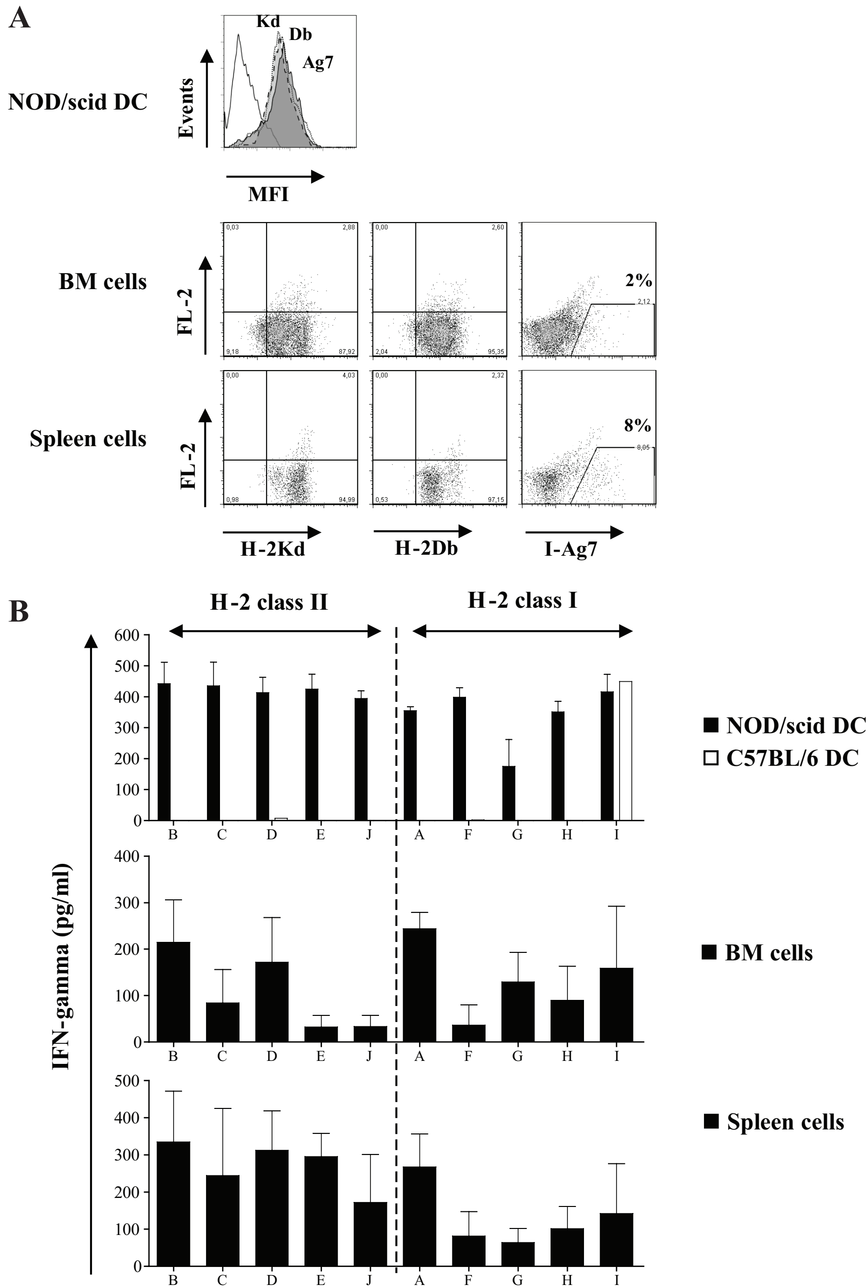

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Figure 3: Xeno-reactive human T cell clones recognize primary NOD/scid derived hematopoietic cells.

(A) H-2-class I (H-2K^d and H-2D^b) en class II (I-A^{g7}) expression on NOD/scid *in vitro* generated BM-derived DC and primary BM and spleen cells was measured by flowcytometry. **(B)** *In vitro* generated DC (*Upper*) from NOD/scid (black bars) and C57BL/6 (white bars) mice and NOD/scid BM (black bars, *Middle*) and spleen cells (black bars, *Lower*) were tested for recognition by xeno-reactive H-2-class II restricted CD4+ (B, C, D and E) and CD8+ (J) T cell clones and xeno-reactive H-2-class I restricted CD4+ (A) and CD8+ (F, G, H and I) T cell clones in IFN-γ ELISA. The mean release of IFN-γ (pg/ml) in 50 ml culture supernatants of 4 independent experiments is shown.

In addition to hematopoietic cells, reactivity of xeno-reactive T cell clones against non-hematopoietic primary NOD/scid skin-derived fibroblasts was tested using IFN-γ ELISA. As xeno-reactive T cell clones produced the pro-inflammatory cytokine IFN-γ upon stimulation with murine cells, we tested the reactivity of xeno-reactive T cell clones against primary NOD/scid skin-derived fibroblasts cultured without and with rhIFN-γ. Flowcytometric analysis demonstrated that surface expression of H-2-class I molecules was upregulated after treatment with rhIFN-γ while surface expression of H-2-class II remained absent[32-34] (Figure 4A). In line with H-2 surface expression, xeno-reactive H-2-class I restricted T cell clones recognized non-treated as well as cytokine-treated skin fibroblasts, while H-2-class II restricted T cell clones showed no reactivity (Figure 4B).

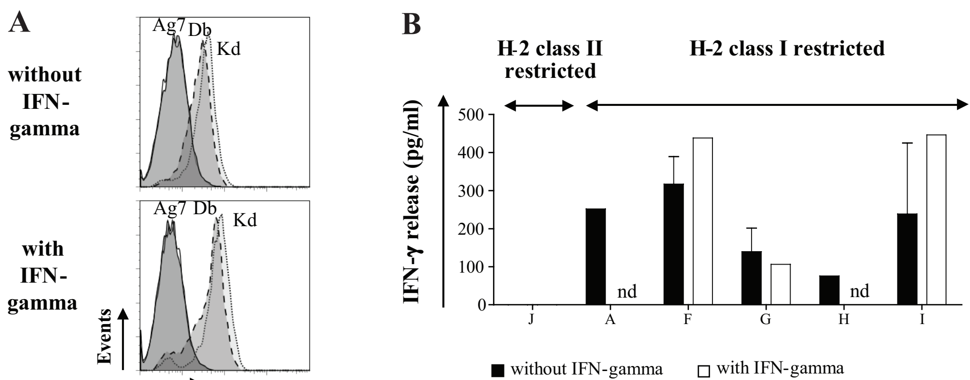

Figure 4: Xeno-reactive H-2-class I restricted T cells recognize primary non-hematopoietic skin fibroblasts.

(A) Primary NOD/scid skin-derived fibroblasts were cultured with or without human IFN-γ. After 5 days of cytokine treatment, H-2-class I and II expression was measured by flowcytometry using antibodies against H-2K^{d} (dotted), H-2D^{b} (dashed) and I-A^{g7} (solid). Indicated are the mean fluorescence intensities (MFI) of non-stained and stained cells. **(B)** Non-treated and cytokine treated primary skin-derived fibroblasts were tested for recognition by xeno-reactive T cell clones in IFN-γ ELISA. Indicated are the H-2-class II restricted CD8+ T cell clone J, the H-2-class I restricted CD4+ T cell clone A, and H-2-class I restricted CD8+ T cell clones F, G, H and I. The mean release of IFN-γ (pg/ml) in 50 ml culture supernatants of single or duplicate wells is shown. nd indicates not determined.

To determine whether the xeno-reactive T cell clones were capable of lysing murine cells, ^{51}Cr-release cytotoxicity assay was performed with all H-2-class I and H-2-class II xeno-reactive T cell clones. None of the xeno-reactive T cell clones displayed specific lysis against NOD/scid BM-derived DC or primary skin-derived fibroblasts in the ^{51}Cr-release cytotoxicity assay (data not shown).

As xeno-reactive human T cells produced high levels of IFN-γ, but did not show cytolytic capacity against murine cells, we analyzed and compared the cytokine release profiles of xeno-reactive CD4+ and CD8+ T cell clones and leukemia-reactive CD4+ T cell clones in more detail by multicytokine ELISA. Xeno-reactive T cells and leukemia-reactive CD4+ T cell clones displayed a similar cytokine profile, and produced predominantly IFN-γ, TNF-α and IL-13 (Figure 5A). As IFN-γ and TNF-α have previously been suggested to be involved in the development of xenogeneic GvHD, we measured these cytokines in plasma of mice after treatment with DLI. As a control, cytokine levels in plasma of non-treated mice were measured. Levels of TNF-α were undetectable in plasma of treated and non-treated mice (data not shown). In contrast, high levels of IFN-γ were detected in treated mice at the time of human T cell proliferation and development of GvL and xenogeneic GvHD responses (Figure 5B), whereas levels of IFN-γ were undetectable in plasma of non-treated mice.

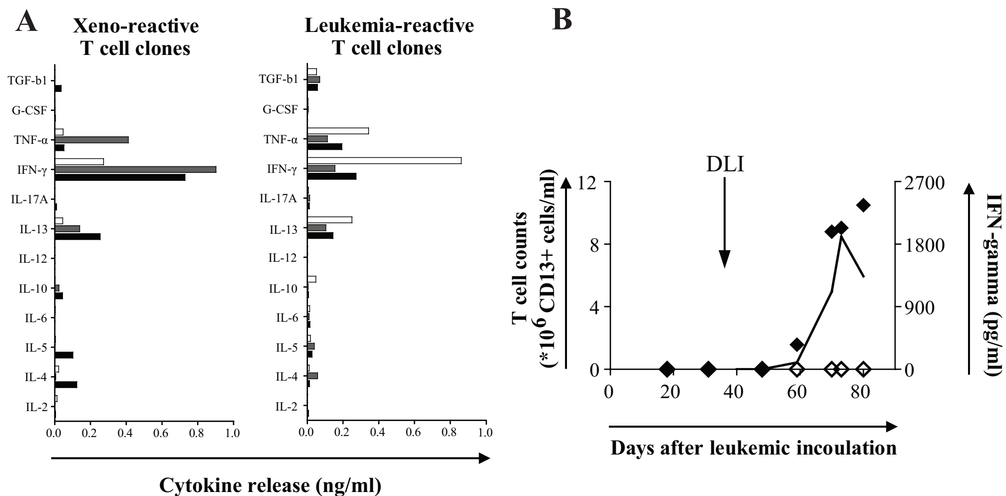

Figure 5: Measurement of human cytokines.

(A) Cytokine release of 3 xeno-reactive T cell clones (B, F and J) upon stimulation with NOD/scid BM-derived DC and 3 leukemia-reactive CD4+ T cell clones upon stimulation with primary human leukemic cells was measured by multi-cytokine ELISA. The different T cell clones are represented by open, grey and filled bars. Release of cytokines in 50 µl supernatant is shown (ng/ml). **(B)** Levels of IFN-γ and TNF-α in plasma of treated (n=2) and untreated leukemia-engrafted (n=2) mice were determined in ELISA. Levels of TNF-α were undetectable in treated and untreated mice (data not shown). Average IFN-γ levels (pg/ml) in 10 ml plasma of treated mice (♦) and untreated leukemia-engrafted mice (◇) is shown. Solid line shows expansion of T cells in treated mice after DLI.

In conclusion, these data show that xeno-reactive CD4+ and CD8+ T cell clones displayed broad H-2 specific recognition of NOD/scid derived hematopoietic and non-hematopoietic cells, and produced high levels of IFN-γ, but failed to mediate cytolysis. Furthermore, high levels of

IFN-γ were detected in plasma of mice at the time of xenogeneic GvHD, suggesting that T cell mediated IFN-γ release *in vivo* plays a major causative role in the development of xenogeneic GvHD.

Infiltration of human T cells in murine organs during GvL and xenogeneic GvHD after DLI

Since our experiments demonstrated that GvL and xenogenic GvHD were mediated by separate T cells, we investigated whether different organs were targeted during these clinical responses. We performed immunohistochemical stainings of the spleen, iliac lymph nodes, lungs, kidneys and skin of two mice during GvL and xenogeneic GvHD after DLI. Organs from two untreated leukemia-engrafted mice and a healthy mouse were analyzed as controls. In untreated leukemia-engrafted mice, infiltration of human leukemic cells was observed in all tissues, with exception of the skin (Figure 6). In mice treated with DLI, co-localization of human leukemic cells and human T cells was detected in all tissues, but the skin. In the skin of treated mice, infiltration of human T cells was observed in the absence of human leukemic cells, and histopathological analysis on hematoxylin-eosin stained sections of the skin demonstrated concentrated areas of hyperkeratosis and moderate acanthosis, which was not observed in untreated leukemia-engrafted and healthy mice. Histopathological analysis on spleen, lymph nodes, lungs and kidneys sections of treated mice showed (interstitial) fibrosis and (peri-)vasculitis, suggestive of local inflammation. These histological parameters were absent in untreated leukemia-engrafted and healthy mice.

In conclusion, the data showed co-localization of human leukemic cells and human T cells in murine hematopoietic and lymphoid tissues, and detection of human T cells infiltrating the skin in the absence of human leukemic cells, further supporting that GvL and xenogeneic GvH responses in leukemic NOD/scid mice after treatment with DLI are exerted by separate human T cells.

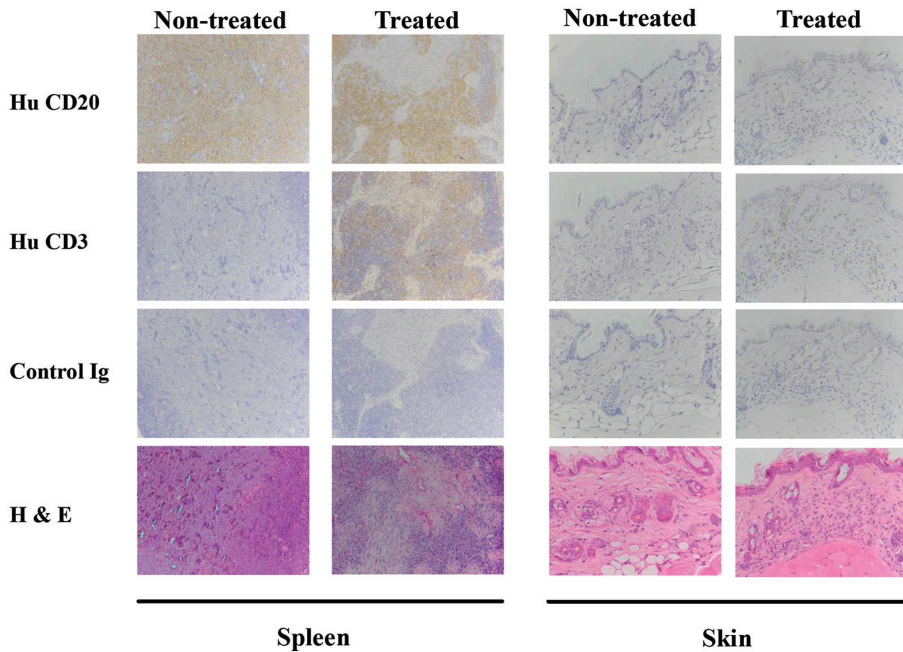

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Figure 6: Infiltration of human leukemic cells (CD20+) and human T cells (CD3+) in the organs of NOD/scid mice during GvL and xenogeneic GvHD after DLI.

Immunohistochemical (IHC) and histopathological analysis was performed on tissue sections from four leukemia-engrafted NOD/scid mice during GvL and xenogeneic GvHD after DLI (treated), two leukemia-engrafted NOD/scid (non-treated) and a healthy NOD/scid mouse (healthy, not shown). IHC staining (DAB-brown) with human CD20, human CD3, control Ig and histology (hematoxylin-eosin (H&E)) of tissue sections of the spleen **(A)** and the skin **(B)** from representative mice are shown. Magnification, x 20 (spleen), x 40 (skin).

DISCUSSION

Using a NOD/scid mouse model for human acute lymphoblastic leukemia, we investigated the potential risk for off-target toxicity by allo-HLA reactive T cells induced after HLA-class II mismatched DLI. Our results show that T cell response induced after HLA-class II mismatched DLI consisted of human T cells exerting separate reactivity towards human leukemic cells or murine cells. Beneficial GvL reactivity was mediated by human T cells recognizing allo-HLA-class II molecules, whereas detrimental xeno-reactivity was exerted by other human T cells recognizing H-2 molecules. Furthermore, we performed histological analysis of various organs during GvL effect and xenogeneic GvHD, and demonstrated that the skin of DLI-treated mice was infiltrated with human T cells in the absence of leukemic cells, further supporting the finding that

GvL reactivity and xenogeneic GvHD in leukemia-engrafted NOD/scid mice treated with human DLI are separate clinical entities.

Allogeneic T cell responses across HLA barriers are exerted by T cells that have been educated to recognize self-HLA/antigen complexes, but are cross-reactive to allo-HLA/antigen complexes[10;26]. As allo-HLA molecules are not encountered during thymic development, and therefore not subjected to negative selection, allo-HLA reactive T cells may potentially exert cross-reactive recognition of multiple allo-HLA/antigen complexes[27-29]. The presence of such cross-reactivities in the allo-HLA T cell repertoire may have detrimental effects in the patients. In this study, we demonstrated that allo-reactive T cells isolated during an *in vivo* allo-HLA-class II directed immune response do not exert broad allo-MHC cross-reactivity. Leukemia-reactive T cells were shown to be restricted by allo-HLA-class II molecules, whereas xeno-reactive T cells recognized allo-H2 molecules, demonstrating that beneficial and detrimental T cell reactivities derived from an allo-reactive T cell repertoire can be separated at a clonal level. Based on these results, we postulate that steering an allo-HLA immune response towards a defined target antigen may allow selection for allo-HLA reactive T cells with high on-target avidity, limiting the possibility for broad cross-reactive off-target recognition. This is supported by previous studies in leukemia-engrafted NOD/scid mice showing that treatment with leukemia-specific T cell lines or single T cell clones resulted in GvL effect but did not cause xenogeneic GvHD[30;31]. Our NOD/scid mouse model therefore provides a platform for testing *in vitro* generated leukemia-reactive T cells derived from the allo-HLA T cell repertoire for use in adoptive cellular therapy.

Several *in vivo* models for xenogeneic GvHD have been developed in immunodeficient mice by administration of human PBMC or purified T cells and were proposed as a model for human GvHD[35-39]. In these models, however, it remained unknown whether xeno-reactivity as mediated by human T cells sufficiently resembled HLA-restricted allo-reactivity in humans. In this study, we demonstrated that xeno-reactive human CD4+ and CD8+ T cells were restricted by H-2-class I or II molecules. H-2-class I restricted CD4+ and H-2-class II restricted CD8+ T cell clones were also isolated, and H-2 restricted recognition of murine target cells by xeno-reactive CD4+ and CD8+ T cell clones was shown to depend to variable extent on co-receptor interaction with H-2 molecules. These findings demonstrate the genuine characteristics of the allo-reactive T cell repertoire, similarly as observed for allo-HLA restricted T cell reactivity in humans. The xeno-reactive human CD8+ and CD4+ T cells produced significant levels of IFN-γ, but failed to exert direct cytolytic activity against murine target cells. This lack of cytolytic activity may be explained by a species barrier in accessory molecules required for adequate T cell activation and lysis of target cells. Since we and others demonstrated that co-receptors CD8 and CD4 contributed to the avidity of the interaction between human T cell and murine cells[40-43], a species barrier between other accessory molecules is more likely to explain the lack of lytic effector function of xeno-reactive T cells. This is supported by studies, showing that introduction of human CD54 and/or CD58 molecules into murine cells is required for human T cell mediated cytotoxicity[44;45]. Our *in*

vitro findings therefore suggest that the effector phase of xenogeneic GvHD *in vivo* is likely to be mediated by release of soluble factors by xeno-reactive T cells rather than by direct cytotoxicity. Murine studies with murine T cells have demonstrated that inflammatory soluble factors play an essential role in GvHD by mediating bone marrow suppression[46] and inducing characteristic cutaneous and intestinal lesions[47], as illustrated by the absence of these detrimental effects in the presence of neutralizing antibodies against IFN-γ and TNF-α[46;47]. In addition, rapid-onset lethal anemia, which is a characteristic feature of xenogeneic GvHD, can result from sustained systemic exposure to IFN-γ, as was recently shown in mice during infection or IFN-γ infusion[48]. In this study, we demonstrated pathological levels of IFN-γ in mice treated with DLI at the time of anemia, further supporting the assumption that a cytokine storm, rather than direct cytolysis, is induced by xeno-reactive human T cells and that this cytokine storm is the main cause for development of xenogeneic GvHD in immunodeficient mice after DLI.

In conclusion, in this study, we have demonstrated that beneficial leukemia-reactive T cells and detrimental xeno-reactive T cells can be separated at a clonal level within an *in vivo* induced HLA-class II directed T cell response after HLA-class II mismatched DLI. Allo-HLA-class II directed T cells displayed beneficial on-target reactivity without off-target allo-MHC cross-reactivity. Based on these results, we postulate that the preferential expression of HLA-class II molecules on hematopoietic cells may allow specific targeting of HLA-class II + hematopoietic malignancies with *in vitro* generated allo-HLA-class II directed T cells exhibiting high on-target avidity, limiting the risk for cross-reactive off-target allo-reactivity. These findings are particularly relevant for recipients of alloSCT from donors mismatched for multiple HLA-class and HLA-class II alleles, as is prevalent in haplo-identical and cord blood alloSCT. Cross-reactive recognition of ubiquitously expressed allo-HLA-class I molecules by adoptively transferred allo-HLA-class II directed T cells may potentially cause detrimental GvHD in patients. Our NOD/scid mouse model for acute leukemia represents a suitable *in vivo* model for analysis of *in vitro* generated and selected leukemia-reactive allo-HLA-class II directed T cells in a diversity of HLA-mismatched situations for their beneficial on-target efficacy and potential to exhibit broad cross-reactive off-target toxicity.