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Biology

Donor T Cells Administered Over HLA Class II Barriers Mediate Antitumor Immunity without Broad Off-Target Toxicity in a NOD/Scid Mouse Model of Acute Leukemia

Sanja Stevanović [1,*], Bart A. Nijmeijer [2], Marianke L.J. van Schie [1], Daniela C.F. Salvatori [3], Saskia Maas [3], Marieke Griffioen [1], J.H. Frederik Falkenburg [1]

[1] Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands
[2] Amsterdam Molecular Therapeutics, Amsterdam, The Netherlands
[3] Department of Anatomy and Embryology and Central Animal Facility, Leiden University Medical Center, Leiden, The Netherlands

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A B S T R A C T

Alloreactive (allo)-HLA–directed T cell responses after HLA-mismatched allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusion are typically considered detrimental responses mediating graft-versus-host disease (GVHD). Allo-HLA-reactive T cells with beneficial and selective graft-versus-leukemia (GVL) reactivity, however, can also be identified within an HLA-mismatched context. We investigated whether allo-HLA class II–directed T cells with beneficial GVL reactivity induced in NOD/scid mice engrafted with human chronic myelogenous leukemia in lymphoid blast crisis after treatment with donor lymphocyte infusion – mediated detrimental xenogeneic GVHD as a result of broad off-target cross-reactivity. The results demonstrate that beneficial GVL reactivity and xenogeneic GVHD are mediated by separate T cells. GVL reactivity was mediated by human T cells recognizing allo-HLA class II molecules, whereas xenoreactivity was exerted by human T cells recognizing H-2 molecules. Taken together, our data indicate a limited risk for detrimental off-target effects by allo-HLA class II–directed T cells and thereby provide a basis for the development of strategies for selecting allo-HLA–restricted T cells with selective GVL reactivity for adoptive transfer after HLA-mismatched allogeneic hematopoietic stem cell transplantation.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative treatment for patients with hematologic malignancies [1]. However, the beneficial graft-versus-leukemia (GVL) reaction after allo-HSCT is frequently accompanied by an undesired detrimental complication, graft-versus-host disease (GVHD). The incidence and severity of GVHD are reduced by depletion of donor T cells from the stem cell graft [2,3]; however, donor T cell depletion also increases the risk for relapse of the malignancy [2-4]. Although posttransplantation relapses can be prevented or treated with donor lymphocyte infusion (DLI), 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 allo-HSCT and DLI.

Because HLA-identical sibling donors are available for only 30% of allo-HSCT recipients, the majority of allo-HSCTs are performed with stem cell grafts from HLA-matched or HLA-mismatched unrelated donors (URDs) [7,8]. Treatment with HLA-mismatched allo-HSCT and DLI may result in profound immune responses owing to high frequencies of alloreactive 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 confined mainly to normal and malignant hematopoietic cells [11-15], and high HLA class II expression on nonhematopoietic cells is induced only under inflammatory conditions [16-18]. Thus, under noninflammatory circumstances, a disparity for HLA class II molecules is anticipated to induce a more selective GVL effect compared with HLA class I incompatibility. In support of this, we have previously shown 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 allo-HSCT are typically considered detrimental responses leading to graft rejection and GVHD, we and others have previously demonstrated that beneficial specificities exist within the allo-HLA–reactive T cell repertoire [20-24]. 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 beneficial reactivities [24]. Allo-HLA class I–directed $CD8^+$ T cells with detrimental reactivities recognized patient malignant cells as well as non-hematopoietic cells, whereas $CD8^+$ T cells with beneficial reactivities selectively recognized an overexpressed tumor-associated antigen on the malignant cells of the patient [24].

Recently, in 2 patients who converted to donor hematopoiesis with concomitant severe GVHD after HLA class

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* Correspondence and reprint requests: Sanja Stevanović, Department of Hematology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands.
E-mail address: s.stevanovic@lumc.nl (S. Stevanović).

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II–mismatched $CD4^+$ DLI, we demonstrated simultaneous induction of allo-HLA class II–directed $CD4^+$ T cells with both detrimental and beneficial reactivities [25]. Allo-HLA class II–directed $CD4^+$ T cells with detrimental reactivities recognized patient hematopoietic cells, as well as skin-derived fibroblasts cultured under inflammatory conditions to induce HLA class II expression, but $CD4^+$ T cells with beneficial reactivities selectively recognized patient hematopoietic cells [25]. These studies in individual patients showed that allo-HLA–reactive T cells with beneficial and detrimental reactivities are both retained within polyclonal allo-HLA–directed immune responses after HLA-mismatched allo-HSCT and DLI, providing a basis for further development of strategies for selecting allo-HLA–reactive T cells specifically recognizing (malignant) hematopoietic cells of the patient for adoptive transfer after HLA-mismatched allo-HSCT.

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 immune 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 the clinical application of allo-HLA–restricted T cells.

Development of a model that permits assessment of on-target efficacy as well as off-target toxicity of allo-HLA–directed T cells in diverse HLA-mismatched situations in vivo would be helpful in defining potential risks and benefits of the application of allo-HLA–restricted T cells. We previously used NOD/scid mice engrafted with human acute lymphoblastic leukemia or chronic myelogenous leukemia in lymphoid blast crisis to investigate the on-target efficacy of HLA class II–mismatched DLI [30,31]. Treatment of leukemia-engrafted mice induced strong GVL effects mediated by allo-HLA class II–restricted $CD4^+$ T cells. In those studies, however, the beneficial GVL effects after HLA class II–mismatched DLI were accompanied by the development of xenogeneic GVHD. Here, we investigated whether xenoreactivity in leukemia-engrafted NOD/scid mice treated with HLA class II–mismatched DLI occurred as a result of broad off-target cross-reactivity of allo-HLA class II–directed T cells. The results demonstrate that T cell responses induced in vivo after HLA class II–mismatched DLI consisted of leukemia-reactive T cells recognizing allo-HLA class II molecules and xenoreactive T cells restricted by H-2 class I or H-2 class II molecules, demonstrating that GVL reactivity and xenogeneic GVHD are mediated by separate T cells with distinct specificities. These data indicate the absence of broad off-target toxicity by allo-HLA class II–directed T cells and thus provide a basis for the development of strategies for selecting allo-HLA–restricted T cells with selective GVL reactivity for adoptive transfer after HLA-mismatched allo-HSCT.

MATERIALS AND METHODS

Patient and Donor Material

Bone marrow (BM) and peripheral blood (PB) samples were obtained from a patient, and PB samples were obtained from a healthy URD after approval by the Leiden University Medical Center's Institutional Review Board and provision of 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 myelogenous leukemia in lymphoid blast crisis. The 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 URD PB mononuclear cells (PBMCs) by negative selection using a Pan T Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity and composition of DLI were analyzed by flow cytometry after staining with FITC-labeled CD4 (BD Biosciences, San Jose, CA), 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 age 5 to 6 weeks were purchased from Charles River Laboratories (Saint-Germain-sur-l'Abresle, France). All animal experiments were conducted in accordance with institutional guidelines with permission from the Leiden University Medical Center's Animal Experiments Committee. Mice were engrafted with primary human leukemic cells from the patient and monitored as described previously [30]. In brief, after i.v. injection of leukemic cells (10×10^6), engraftment of leukemia was monitored weekly by flow cytometry analysis of PB samples after staining with FITC-labeled human CD19 (BD Biosciences) and PE-labeled mouse CD45 (Ly5, Caltag) monoclonal antibodies. On 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 i.p. injection. After DLI, PB samples were analyzed for the presence of leukemic cells and T cells by flow cytometry 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 killed during GVL response and xenogeneic GVHD at day 45 post-DLI (80 days after leukemic inoculation), 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 sorted by flow cytometry into single cells per well in U-bottomed 96-well plates containing irradiated (50 Gy) allogeneic PBMCs (0.05×10^6/well) as feeder cells in 100 μL of Isocove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) with 5% human serum, 5% FCS (BioWittaker), IL-2 (120 IU/mL; Chiron, Amsterdam, The Netherlands), and phytohemagglutinin (0.8 μg/mL; Murex Biotec, Dartford, UK). Proliferating T cell clones were selected and restimulated every 10 to 20 days.

Isolation of Stimulator Cells

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCLs) from patient and donor were generated using standard procedures and maintained in IMDM with 10% FCS. Donor EBV-LCLs were transduced with retroviral vectors encoding the mismatched HLA class II alleles of the patient, as described previously [30]. Dendritic cells (DCs) from NOD/scid and C57BL/6 mice were generated in vitro by culturing total BM from femora in RPMI-1640 medium (Invitrogen, Bleiswijk, The Netherlands) with 10% FCS and 20 ng/mL recombinant murine granulocyte macrophage colony-stimulating factor (Invitrogen) in 100-mm culture dishes. After 10 days, BM-derived DCs were phenotypically analyzed by staining with PE-labeled antibodies against mouse CD11c (clone HL3) and FITC-labeled antibodies against mouse $H\text{-}2K^d$ (clone SF1-1.1), $H\text{-}2D^b$ (clone 28-14-8), and $I\text{-}A^{g7}$ (clone OX-6) (all BD Biosciences). Single-cell suspensions of BM and spleen cells for functional studies were obtained from euthanized healthy mice on erythrocyte lysis. NOD/scid fibroblasts were established from skin specimens cultured in low-glucose DMEM (BioWhittaker) and 10% FCS with or without recombinant human (rh) IFN-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 $H\text{-}2K^d$, $H\text{-}2D^b$, and $I\text{-}A^{g7}$.

Functional Studies

T cell clones (5000 cells/well) were coincubated with stimulator cells (30,000 cells/well) in a final volume of 150 μL of IMDM with 5% human serum, 5% FCS, and 10 IU/mL IL-2 in 96-well U-bottomed plates. After overnight incubation at 37°C, release of IFN-γ in 50 μL of culture supernatants was measured by IFN-γ ELISA (Sanquin, Amsterdam, The Netherlands).

To determine H-2 restriction alleles of xenoreactive T cell clones, NOD/scid freshly isolated BM cells and in vitro cultured BM-derived DCs (30,000 cells/well) were preincubated 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 room temperature, followed by the addition of T cell clones (5,000 cells/well).

The cytolytic activity of xenoreactive human T cell clones and murine CD8$^+$ T cell clone B12i [32,33] against NOD/scid BM-derived DCs and NOD/scid fibroblasts was determined using a ^{51}Cr release cytotoxicity assay. Target cells were labeled with $Na_2^{51}CrO_4$ (PerkinElmer, Groningen, The Netherlands) for 1 hour at 37°C and then incubated (2000 cells) with T cell clones at a 10:1 effector:target ratio in 150 μL of culture medium. After 16 hours of incubation at 37°C, 25 μL of supernatant was collected and measured in a luminescence counter (TopCount NXT, PerkinElmer).

The cytokine release profiles of xenoreactive T cell clones (5000 cells/well) after overnight stimulation with BM-derived DCs (30,000 cells/well) and leukemia-reactive CD4+ T cell clones (5000 cells/well) on stimulation with primary human leukemic cells (30,000 cells/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).

RESULTS

Isolation and Characterization of T Cells During GVL Response and Xenogeneic GVHD

For analysis of the specificity of the T cell responses during the GVL effect and xenogeneic GVHD, mice were engrafted with human leukemic cells and treated with DLI, consisting of 59% CD4$^+$ T cells and 35% CD8$^+$ T cells. 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, as described in 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 DCs by IFN-γ ELISA (Figure 1B). The murine DCs expressed H-2 class I and H-2 class II molecules (data not shown).

The reactivity of isolated CD4$^+$ T cell clones could be classified into groups based on the specific production of IFN-γ (>100 pg/mL) on stimulation with patient leukemic cells (112-306 pg/mL IFN-γ; n = 15) or NOD/scid DCs (131-363 pg/mL IFN-γ; n = 42). The majority of leukemia-reactive CD4$^+$ T cell clones were also shown to recognize patient-derived, but not donor-derived, EBV-LCLs, indicating recognition of alloantigens. In contrast to CD4$^+$ T cell clones, none of the isolated CD8$^+$ T cell clones recognized patient

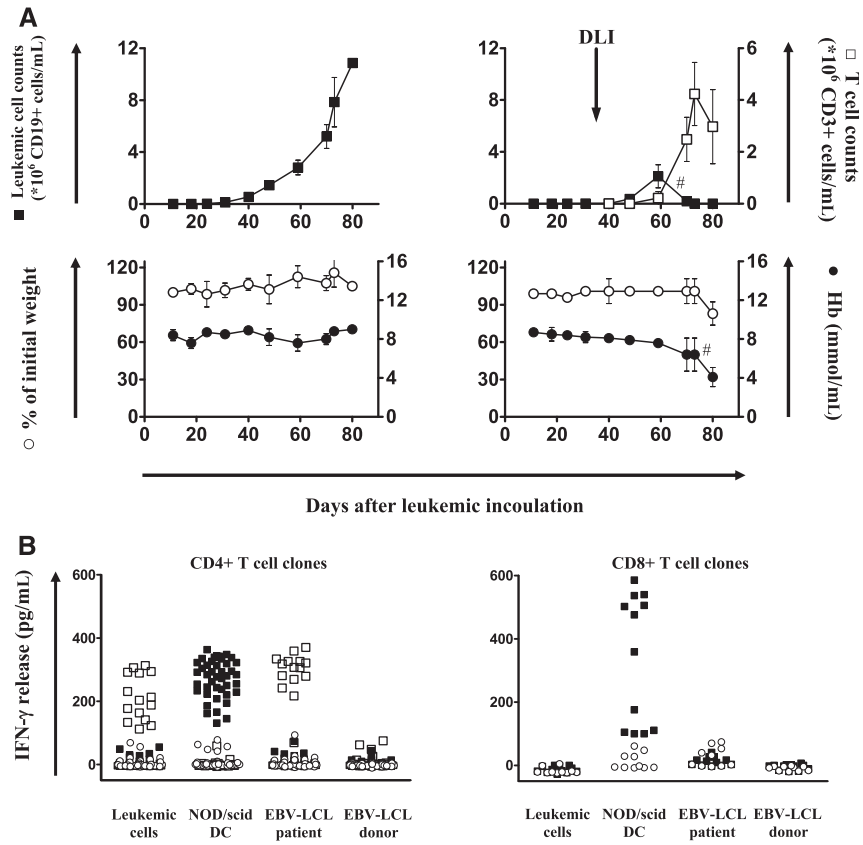

Figure 1. Isolation and characterization of T cell reactivity during GVL reactivity and xenogeneic GVHD after DLI. (A) NOD/scid mice were inoculated (day 0) with primary leukemic cells and then left untreated or given DLI at 35 days after leukemic inoculation (indicated by the arrow). (Upper) Absolute cell numbers of CD19$^+$ leukemic cells (–■–) and CD3$^+$ T cells (–□–) in peripheral blood of mice were assessed by flow cytometry. (Lower) Percentage of initial body weight (–○–) and hemoglobin (Hb) levels (mM) (–●–) in peripheral blood of mice were measured as well. Each symbol represents the mean values of untreated (n = 2) and treated mice (n = 2). Mice were killed during the GVL effect and xenogeneic GVHD at day 45 post-DLI (80 days after leukemic inoculation). # indicates the approximate starting time point of the GVL effect (Upper) and xenogeneic GVHD (Lower) in treated mice. (B) Human primary leukemic cells, NOD/scid BM-derived DCs, and patient- and donor-derived EBV-LCLs were tested for recognition by 91 CD4$^+$ and 22 CD8$^+$ T cell clones. The T cell clones were isolated from BM and spleens obtained from 2 mice at day 45 post-DLI (80 days after leukemic inoculation). Each symbol represents the mean release of IFN-γ (pg/mL) by a single T cell clone in 50 μL of culture supernatant in duplicate wells. □, T cell clones recognizing human leukemic cells but not NOD/scid BM-derived DCs; ■, T cell clones recognizing NOD/scid BM-derived DCs but not human leukemic cells; ○, nonreactive T cell clones.

leukemic cells or patient- and donor-derived EBV-LCLs, whereas a number of $CD8^+$ T cell clones recognized NOD/scid DCs (100-586 pg/mL; n = 12). The remaining $CD4^+$ (n = 34) and $CD8^+$ (n = 10) T cell clones were not reactive against any of the tested target cells. TCR-Vβ analysis demonstrated that the T cell clones expressed various different TCR-Vβ chains, illustrating that the leukemia-reactive and xenoreactive 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 DCs, demonstrating that GVL reactivity and xenogeneic GVHD in NOD/scid mice treated with DLI are mediated by distinct T cells. The data also show that the xenoreactive T cells were both $CD4^+$ and $CD8^+$, whereas all isolated leukemia-reactive T cells were $CD4^+$, probably because of selective mismatching for HLA class II alleles between 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-LCLs 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 found to be directed against mismatched HLA-DRB3*0101 (n = 4), -DQB1*0603 (n = 5), or -DPB1*0301 (n = 1) (Figure 2).

Characterization of H-2 restriction alleles in recognition of NOD/scid BM-derived DCs was performed with $CD4^+$ (n = 5; clones A-E) and $CD8^+$ (n = 5; clones F-J) xenoreactive T cell clones selected for expression of different TCR-Vβ chains to represent the diversity of the polyclonal xenoreactive T cell response (data not shown). Flow cytometry analysis demonstrated that NOD/scid DCs uniformly expressed H-2 class I and H-2 class II molecules (Figure 3A). Xenoreactive T cell clones were incubated with NOD/scid BM-derived DCs with and without blocking antibodies against H-2 class I ($H\text{-}2K^d$ and $H\text{-}2D^b$) and H-2 class II ($I\text{-}A^{g7}$) alleles, and recognition was measured by IFN-γ ELISA. Recognition of NOD/scid DCs by xenoreactive $CD4^+$ T cell clones could be blocked by monoclonal antibody against $H\text{-}2K^d$ for 1 T cell clone and by antibody against $I\text{-}A^{g7}$ for 4 T cell clones (Figure 3B). For xenoreactive $CD8^+$ T cell clones, recognition of NOD/scid DCs could be blocked by monoclonal antibodies against $H\text{-}2K^d$ (n = 3), $H\text{-}2D^b$ (n = 1), or $I\text{-}A^{g7}$ (n = 1) (Figure 3B).

To investigate whether the human CD8 and CD4 coreceptors as expressed by the xenoreactive T cell clones contributed to H-2–restricted recognition of murine DCs, xenoreactive T cell clones were also incubated with NOD/scid DCs with blocking antibodies against CD8 or CD4. Recognition of NOD/scid DCs by xenoreactive $CD8^+$ and $CD4^+$ T cell clones was inhibited to varying degrees on blockage of the coreceptors (Figure 3B). Some T cell clones displayed a modest decrease in recognition, whereas others demonstrated complete inhibition, indicating that human CD8 and CD4 can serve as coreceptors for H-2 class I– and H-2 class 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 molecules and H-2 class I or H-2 class II molecules, respectively. Furthermore, xenoreactive human T cells displayed H-2–restricted recognition of murine cells that depended on the contribution of CD4 and CD8 coreceptors to varying extents, illustrating genuine characteristics of T cell allorecognition.

Tissue Specificity and Reactivity of Xenoreactive T Cell Clones

To determine the tissue specificity of xenoreactive T cell clones, recognition of primary hematopoietic BM and spleen cells from NOD/scid mice by xenoreactive $CD4^+$ and $CD8^+$ T cell clones was measured by IFN-γ ELISA. Flow cytometry analysis showed that BM and spleen cells uniformly expressed H-2 class I molecules and contained low frequencies of H-2 class II+ cells (Figure 4A). H-2 class I– and H-2 class II–restricted xenoreactive T cell clones showed variable production of IFN-γ on incubation with primary hematopoietic cells (Figure 4B). This recognition was specific, as demonstrated by blocking with H-2–specific monoclonal antibodies (Figure 4C).

Along with hematopoietic cells, we tested the reactivity of xenoreactive T cell clones against nonhematopoietic primary NOD/scid skin-derived fibroblasts using IFN-γ ELISA. Because xenoreactive T cell clones produced the proinflammatory cytokine IFN-γ on stimulation with murine cells, we tested the reactivity of xenoreactive T cell clones against primary NOD/scid skin-derived fibroblasts cultured with and without

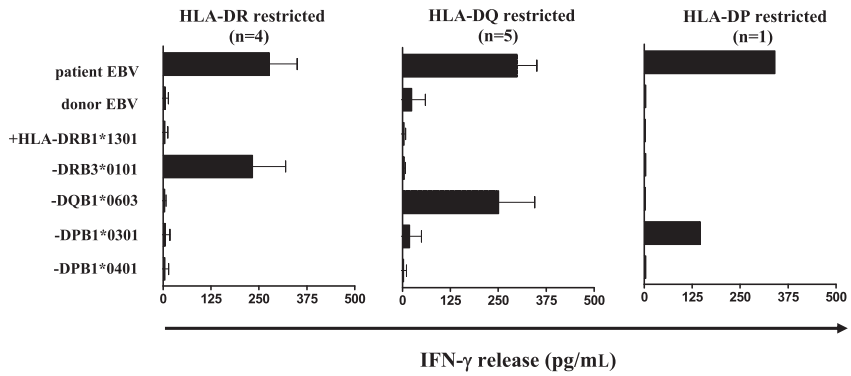

Figure 2. Leukemia-reactive $CD4^+$ T cell clones are restricted by HLA class II molecules. Leukemia-reactive $CD4^+$ T cell clones are restricted by different allo-HLA class II molecules. Recognition of patient and donor EBV-LCLs retrovirally transduced with HLA-DRB1*1301, -DRB3*0101, -DQB1*0603, -DPB1*0301, and -DPB1*0401 by representative leukemia-reactive $CD4^+$ T cell clones is shown as the release of IFN-γ (pg/mL) in 50 μL of culture supernatant. Of the 10 leukemia-reactive $CD4^+$ T cell clones tested, 4 clones were specific for HLA-DR, 5 clones were specific for HLA-DQ, and 1 clone was specific for HLA-DP.

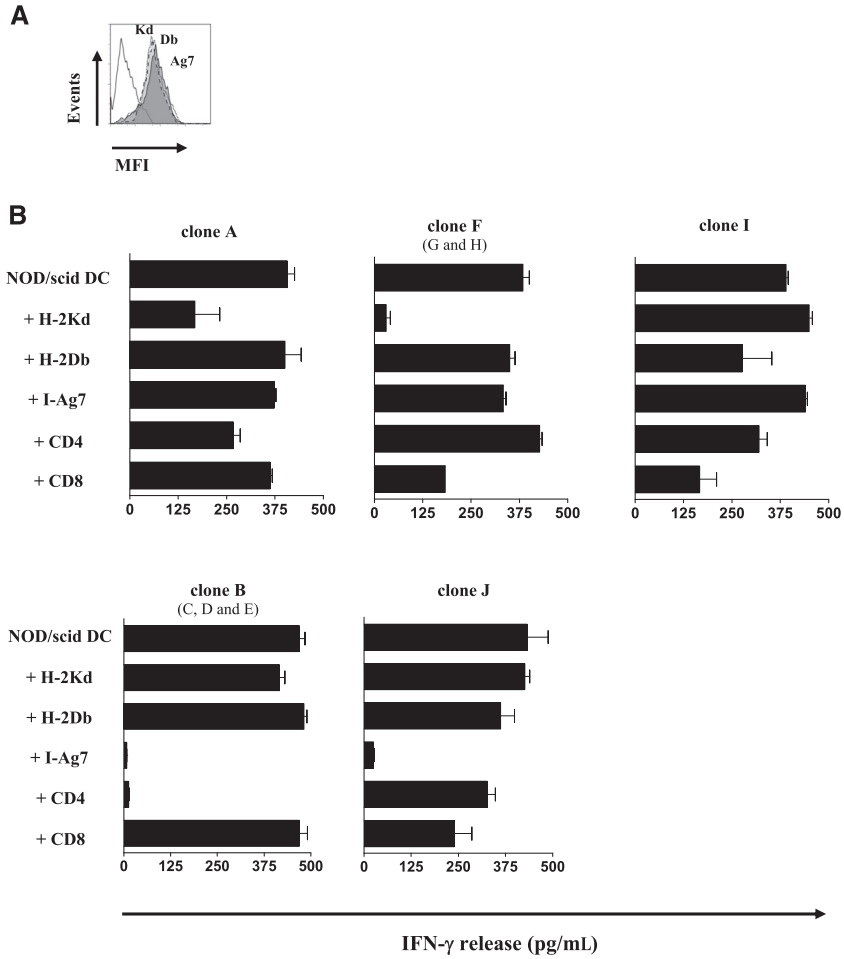

Figure 3. Xenoreactive T cell clones are restricted by H-2 molecules. (A) H-2 class I (H-2K^d and H-2D^b) and H-2 class II (I-A^{g7}) expression on NOD/scid in vitro–generated BM-derived DCs was measured by flow cytometry. (B) Xenoreactive CD4$^+$ and CD8$^+$ T cell clones are restricted by different H-2 class I and H-2 class II molecules and are dependent on coreceptors for recognition. Recognition of NOD-scid BM-derived DCs by representative xenoreactive H-2 class I–restricted (Upper) and H-2 class II–restricted (Lower) CD4$^+$ and CD8$^+$ T cell clones in the presence or absence of blocking antibodies was measured by IFN-γ ELISA. The T cell clones in 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), H-2 class II (I-A^{g7}), human CD8 (FK18), and human CD4 (RIV6). Of the 5 xenoreactive CD4$^+$ T cell clones tested, 1 clone was H-2K^d –restricted (A), and 4 clones were I-A^{g7}–restricted (B-E). Of the 5 xenoreactive CD8$^+$ T cell clones tested, 3 clones were H-2K^d–restricted (F-H), 1 clone was H-2D^b–restricted (I), and 1 clone was I-A^{g7}–restricted (J). All T cell clones showed reduced IFN-γ production on blocking of the coreceptor. The release of IFN-γ (pg/mL) in 50 μL of culture supernatant is shown.

rhIFN-γ. Flow cytometry analysis showed that surface expression of H-2 class I molecules was up regulated after treatment with rhIFN-γ, whereas surface expression of H-2 class II remained absent [34-36] (Figure 5A). In line with H-2 surface expression, xenoreactive H-2 class I–restricted T cell clones recognized nontreated as well as cytokine-treated skin fibroblasts, whereas H-2 class II–restricted T cell clones showed no reactivity (Figure 5B).

Cytolytic Capacity and Cytokine Profile of Xenoreactive T Cell Clones

To determine whether xenoreactive human T cell clones were capable of lysing murine cells, ^{51}Cr-release cytotoxicity assays were performed with all xenoreactive T cell clones for which H-2 class I and H-2 class II restriction alleles were identified. Murine CD8$^+$ T cell clone B12i, which has been shown to exert cytolytic activity against murine H-2b and H-2d haplotype–expressing target cells, was included as a positive control [32,33]. In contrast to murine CD8$^+$ T cell clone B12i, none of the xenoreactive T cell clones exhibited specific lysis against NOD/scid BM-derived DCs or primary skin-derived fibroblasts in ^{51}Cr-release cytotoxicity assays (Figure 6A).

Because xenoreactive human T cells produced high levels of IFN-γ but showed no cytolytic capacity against murine cells, the cytokine release profiles of xenoreactive CD4$^+$ and CD8$^+$ T cell clones and leukemia-reactive CD4$^+$ T cell clones were analyzed and compared in more detail by multi-cytokine ELISA. Xenoreactive T cells and leukemia-reactive CD4$^+$ T cell clones displayed similar cytokine profiles and produced predominantly IFN-γ, TNF-α, and IL-13 (Figure 6B). Given that IFN-γ and TNF-α have been implicated in the development of xenogeneic GVHD, these cytokines were measured in plasma of mice after treatment with DLI. Cytokine levels in plasma of nontreated mice served as a control. Levels of TNF-α were undetectable in plasma of both treated and nontreated 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 6C), whereas IFN-γ was undetectable in the plasma of nontreated mice.

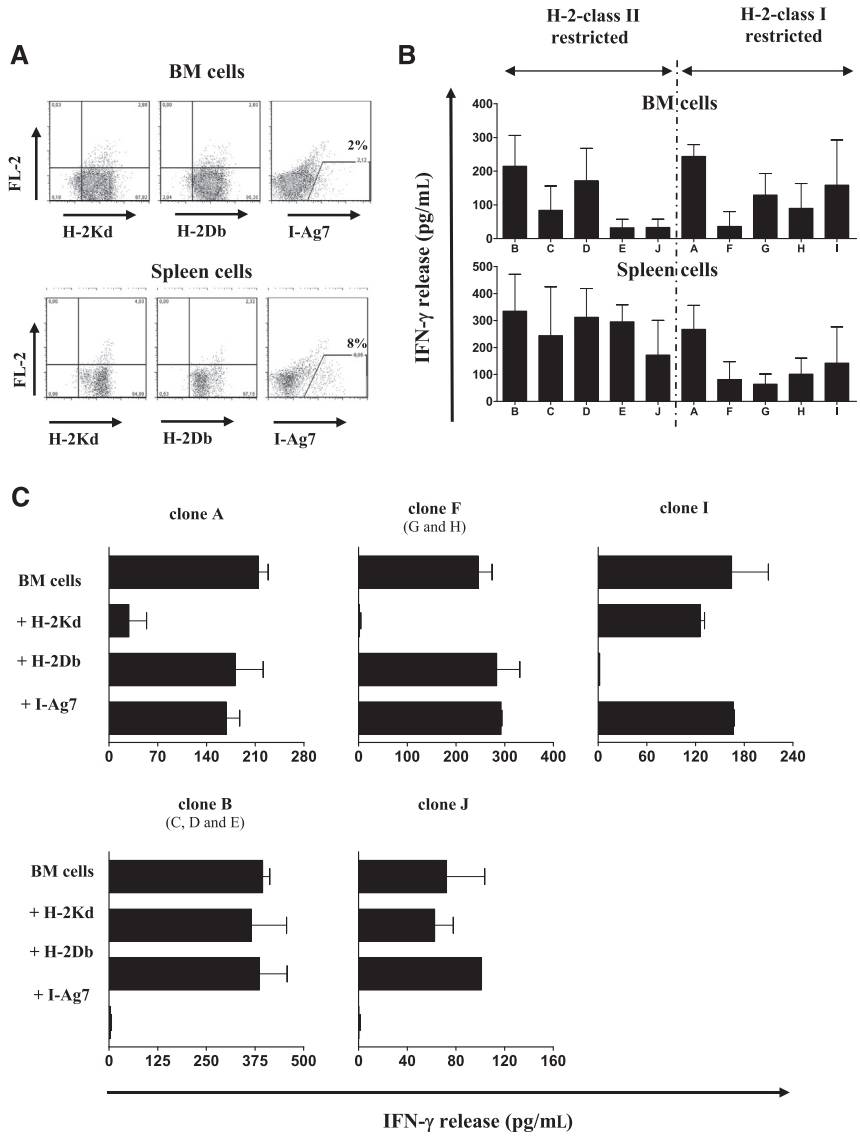

Figure 4. Xenoreactive human T cell clones recognize primary NOD/scid-derived hematopoietic cells. (A) H-2 class I (H-2K^d and H-2D^b) and H-2 class II (I-A^{g7}) expression on primary BM and spleen cells was measured by flow cytometry. (B) NOD/scid BM (black bars, Upper) and spleen cells (black bars, Lower) were tested for recognition by xenoreactive H-2 class II–restricted CD4$^+$ (B-E) and CD8$^+$ (J) T cell clones and xenoreactive H-2 class I–restricted CD4$^+$ (A) and CD8$^+$ (F-I) T cell clones by IFN-γ ELISA. The mean release of IFN-γ(pg/mL) in 50 μL of culture supernatant in 4 independent experiments is shown. (C) Recognition of primary NOD/scid BM cells by representative xenoreactive H-2 class I–restricted (Upper) and H-2 class II–restricted (Lower) CD4$^+$ and CD8$^+$ T cell clones in the presence or absence of MHC-blocking antibodies as measured by IFN-γ ELISA. The T cell clones in 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) or H-2 class II (I-A^{g7}). The release of IFN-γ (pg/mL) in 50 μL of culture supernatants is shown.

In conclusion, our data show that xenoreactive CD4$^+$ and CD8$^+$ T cell clones produced high levels of IFN-γ but failed to mediate cytolysis. Furthermore, high levels of IFN-γ were detected in the 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.

DISCUSSION

Using a NOD/scid mouse model for human acute leukemia, we investigated the potential risk for broad off-target toxicity by allo-HLA-reactive T cells induced after HLA class II–mismatched DLI. Our results show that the in vivo T cell response induced after HLA class II–mismatched DLI consisted of human T cells exerting separate reactivity toward human leukemic cells and murine cells. GVL reactivity was mediated by human T cells recognizing allo-HLA class II molecules, whereas xenoreactivity was exerted by other human T cells recognizing H-2 molecules. Thus, our data show that allo-HLA class II–directed T cells induce beneficial GVL effects without mediating broad off-target toxicity.

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]. Considering that allo-HLA molecules are not encountered during thymic development, and thus are not subjected to negative selection, allo-HLA–reactive T cells may potentially exert cross-reactive recognition of multiple allo-HLA–antigen complexes [27-29]. In this

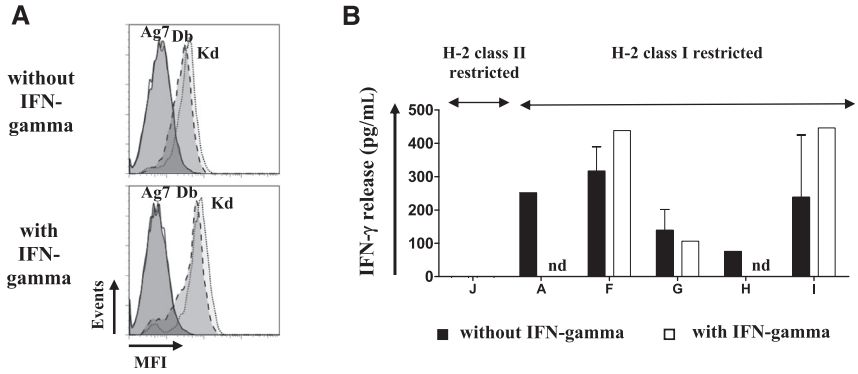

Figure 5. Xenoreactive H-2 class I–restricted T cells recognize primary nonhematopoietic skin fibroblasts. (A) Primary NOD/scid skin-derived fibroblasts were cultured with or without human IFN-γ. After 5 days of cytokine treatment, H-2 class I and H-2 class II expression was measured by flow cytometry using antibodies against H-2K^{d} (• • • •), H-2D^{b} (- - - -), and I-A^{g7} (—). The mean fluorescence intensity (MFI) of stained and nonstained cells is shown. (B) Nontreated and cytokine-treated primary skin-derived fibroblasts were tested for recognition by xenoreactive T cell clones in IFN-γ ELISA. Shown 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 μL of culture supernatant of single or duplicate wells is shown. nd, not determined.

study, we demonstrated that the allo-HLA class II–restricted CD4^{+} T cells isolated during an in vivo allo-HLA class II–directed immune response did not exert broad allo-MHC cross-reactivity. Restricted off-target toxicity toward specific allo-HLA complexes by cross-reactive T cells with a defined specificity, as previously demonstrated for virus-specific T cells [37], cannot be ruled out in our model, however. For virus-specific T cells, this off-target toxicity was shown to be restricted to specific mismatched HLA alleles for which no thymic selection had occurred. For allo-HLA class II–directed CD4^{+} T cells, restricted off-target toxicity may be particularly relevant for patients who have undergone transplantation over multiple HLA class I and class II barriers, as often occurs in haploidentical or cord blood allo-HSCT. Off-target toxicity toward ubiquitous allo-HLA class I molecules may potentially cause detrimental GVHD in these allo-HSCT settings. Furthermore, in addition to restricted off-target toxicity, on-target reactivity by allo-HLA–reactive CD4^{+} T cells might lead to detrimental GVHD owing to up-regulation of HLA class II expression on nonhematopoietic tissues during inflammatory circumstances. These inflammatory circumstances are likely to depend on complex (combinations of) in vivo factors and thus cannot be accurately investigated or predicted in our NOD/scid mouse model.

We recently investigated the GVL effects of HLA-matched (12/12 allele match) and HLA class II–mismatched DLI in leukemia-engrafted NOD/scid mice [31] and demonstrated impaired ability of HLA-matched DLI to mediate effective GVL effects, in contrast to the profound and efficient GVL response seen after HLA class II–mismatched DLI. These data strongly emphasize the relevance of HLA class II–mismatched DLI as a treatment modality to combat relapsed high-risk HLA class II–positive acute leukemia. In this study, we show that allo-HLA class II–directed T cells lack broad off-target reactivity, further supporting the use of HLA class II–mismatched allo-HSCT and DLI to treat aggressive leukemia. Our NOD/scid model provides a platform for investigating GVL effects of specific minor and major HLA disparities, as well as allo-HLA–restricted T cells with selective GVL reactivity for adoptive transfer after HLA class II–mismatched allo-HSCT.

Several in vivo models for xenogeneic GVHD developed in immunodeficient mice through the administration of human PBMCs or purified T cells have been proposed as models for human GVHD as well [38-42]. In these models, however, whether xeno-reactivity as mediated by human T cells sufficiently resembles HLA-restricted alloreactivity in humans remains unknown. In this study, we found that xenoreactive human CD4^{+} and CD8^{+} T cells were restricted by H-2 class I and H-2 class 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 xenoreactive CD4^{+} and CD8^{+} T cell clones was shown to depend to a varying extent on co-receptor interaction with H-2 molecules. These findings demonstrate the genuine characteristics of the alloreactive T cell repertoire, similar to that observed for allo-HLA–restricted T cell reactivity in humans. The xenoreactive 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 might be explained by a species barrier in accessory molecules required for adequate T cell activation and lysis of target cells. Considering that we and others have demonstrated that CD8 and CD4 coreceptors contributed to the avidity of the interaction between human T cell and murine cells [43-46], a species barrier between other accessory molecules is the more likely explanation of the lack of lytic effector function of xenoreactive T cells. This idea is supported by studies showing that the introduction of human CD54 and/or CD58 molecules into murine cells is required for human T cell–mediated cytotoxicity [47,48] and is in line with our observations [49] that CD54 expression on human cells is also essential for high-avidity interaction with alloreactive T cells. Thus, our in vitro findings suggest that the effector phase of xenogeneic GVHD in vivo is likely mediated by the release of soluble factors by xenoreactive T cells rather than by direct cytotoxicity. Previous mouse studies with murine T cells have shown that inflammatory soluble factors play an essential role in GVHD by mediating bone marrow suppression [50] and inducing characteristic cutaneous and intestinal lesions [51], as illustrated by the absence of these detrimental effects in the presence of neutralizing antibodies against IFN-γ and TNF-α [50,51]. In addition, rapid-onset lethal anemia, a characteristic feature of xenogeneic GVHD, can result from sustained systemic exposure to IFN-γ, as was

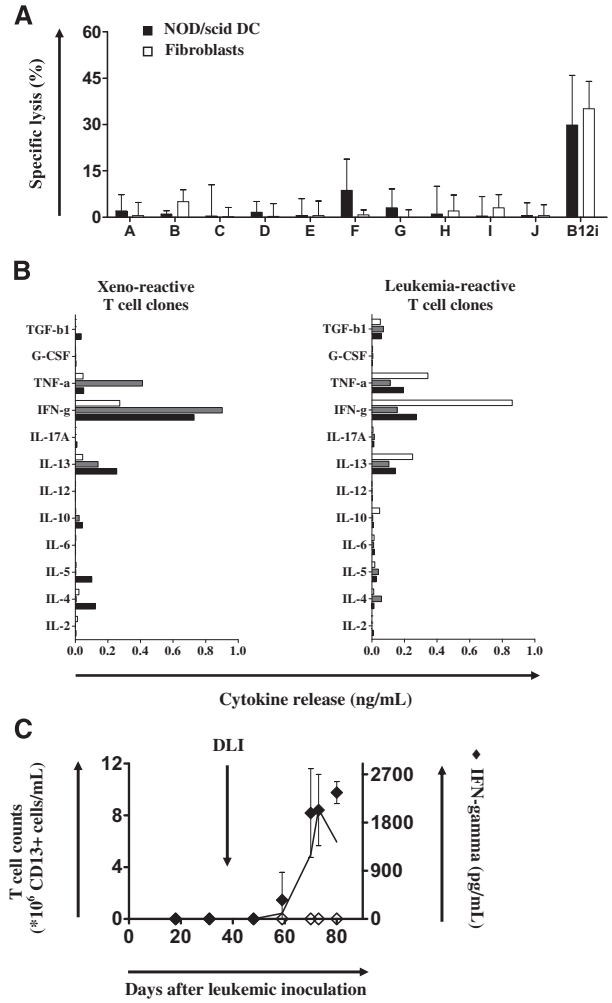

Figure 6. Cytolytic capacity and cytokine profile of xenoreactive T cell clones. (A) The cytolytic capacity of xenoreactive H-2 class I– and H-II class II–restricted $CD4^+$ and $CD8^+$ T cell clones was measured against NOD-scid BM-derived DC (black bars) and skin-derived fibroblasts (white bars) in a 16-hour ^{51}Cr-release cytotoxicity assay (effector:target ratio of 10:1). A murine $CD8^+$ T cell clone (B12i) served as positive control. (B) Cytokine release in 3 xenoreactive T cell clones (B, F, and J) after stimulation with NOD/scid BM-derived DCs and in 3 leukemia-reactive $CD4^+$ T cell clones on stimulation with primary human leukemic cells was measured by multicytokine ELISA. The different T cell clones are represented by clear, gray, and black bars. The release of cytokines (ng/mL) in 50 μL of supernatant is shown. (C) Levels of IFN-γ and TNF-α in plasma of treated (n = 2) and untreated (n = 2) leukemia-engrafted mice were determined by ELISA. Levels of TNF-α were undetectable in treated and untreated mice (data not shown). Average ± SD IFN-γ levels (pg/mL) in 10 μL of plasma from treated mice (◆) and untreated (◆) leukemia-engrafted mice. The solid line indicated the rapid expansion of T cells in treated mice after DLI.

recently shown in mice during infection and IFN-γ infusion [52]. In the present study, we found pathological levels of IFN-γ in mice treated with DLI at the time of anemia, further supporting the idea that a cytokine storm, rather than direct cytolysis, is induced by xenoreactive human T cells, and that this cytokine storm is the main cause of xenogeneic GVHD in immunodeficient mice after DLI.

It has been suggested that non–MHC-restricted effector natural killer T cells play a role in antitumor immunity [53] and xenogeneic GVHD [54] in immunodeficient mice. For all $CD4^+$ and $CD8^+$ T cell clones analyzed in the present study, however, recognition of human and murine target cells could be blocked by anti-MHC–specific monoclonal antibodies, and MHC restriction molecules could be identified. Thus, our data indicate a dominant role for conventional MHC-restricted T cells in GVL effects and xenogeneic GVHD induced in leukemia-engrafted NOD/scid mice after treatment with human DLI.

In conclusion, in this study, we have shown that allo-HLA class II–directed T cells induced in vivo in leukemia-engrafted NOD/scid mice after HLA class II–mismatched DLI displayed on-target GVL reactivity without broad off-target toxicity. Our results provide a basis for the development of strategies for selecting allo-HLA–restricted T cells with selective GVL reactivity for adoptive transfer after HLA-mismatched allo-HSCT.

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