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CHAPTER 2

HLA-CLASS II DISPARITY IS NECESSARY FOR EFFECTIVE T CELL MEDIATED GRAFT-VERSUS-LEUKEMIA EFFECTS IN NOD/SCID MICE ENGRAFTED WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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LETTER TO THE EDITOR

Relapses of hematological malignancies after HLA-matched allogeneic stem cell transplantation (alloSCT) are a major cause of treatment failure\(^1\). Although posttransplant relapses of chronic myeloid leukemia (CML) in chronic phase can be effectively treated with donor lymphocyte infusion (DLI), curative responses in patients with B-lineage acute lymphoblastic leukemia (ALL) and CML in lymphoid blast crisis (CML-BC) are limited\(^1\). The efficacy of DLI after HLA-matched alloSCT is mediated by donor T cells targeting polymorphic antigens presented on patient-derived malignant and non-malignant cells, but not on donor-derived cells, in the context of shared HLA molecules\(^2\). These T cells are known to reside in the naïve T cell repertoire, and require priming by professional antigen presenting cells (APC) for functional activation\(^3\)\(^-\)\(^6\). Early after alloSCT, residual patient-derived professional APC may trigger Graft-versus-Leukemia (GvL) responses against antigens shared between APC and patient malignant cells. Late after alloSCT, however, when professional APC are of donor origin, the ability of malignant cells to elicit effective GvL responses after HLA-matched DLI may depend on their capacity to serve as professional APC. ALL and CML-BC cells lack a professional APC phenotype, and their in vitro modification into leukemic-APC is probably necessary for efficient generation of leukemia-reactive T cells from HLA-matched donors\(^7\)\(^-\)\(^9\).

In this study, we investigated whether primary GvL responses can be efficiently elicited against established human ALL and CML-BC in NOD/scid mice after treatment with HLA-matched DLI. NOD/scid mice were engrafted with leukemic cells from different patients and treated with DLI from HLA-matched (12/12 alleles) unrelated donors (URD) (Supplementary Table 1). Leukemic cells from both patients expressed HLA-class I and HLA-class II molecules (data not shown). Progressive leukemic development was observed in control mice that did not receive treatment with DLI (Figure 1A). In mice treated with HLA-matched DLI, CD4+ and CD8+ T cells appeared in PB from 19 days after DLI. Despite significant T cell expansion in vivo, leukemic cells were delayed in growth compared with non-treated mice (patient 1: P=0.1; patient 2: P<0.01), but not eliminated (Figure 1A). During in vivo T cell expansion, despite absence of profound GvL reactivity, the majority of treated mice developed xeno-reactive symptoms\(^10\), prohibiting long-term follow-up (>60 days) and mice were therefore sacrificed. To analyze the specificity of the T cells, CD4+ and CD8+ T cells were clonally isolated from treated mice. Expansion was obtained for 6 CD4+ and 56 CD8+ T cell clones from patient 1 and 12 CD4+ and 75 CD8+ T cell clones from patient 2. All T cell clones were analyzed for reactivity against patient leukemic cells, NOD/scid bone marrow-derived DC (murine DC) and donor-derived EBV-LCL in IFN-γ ELISA (Figure 1B). None of the isolated CD4+ and CD8+ T cell clones recognized leukemic cells or donor-derived EBV-LCL, whereas a number of CD4+ (patient 1: n=4; patient 2: n=5) and CD8+ T cell clones (patient 1: n=31; patient 2: n=40) were xeno-reactive, based on specific recognition (>100 pg/ml IFN-γ) of murine DC.
Figure 1: In vivo induction of GvL reactivity after HLA-matched DLI.

(A) NOD/scid mice were inoculated (day 0) with primary leukemic cells (10*10^6 cells) from patients 1 (ALL) and 2 (CML-BC), and were left untreated or received DLI 33 and 29 days after leukemic inoculation, respectively (indicated by the arrow). The infused T cell population in DLI-treated mice consisted of 58% CD4+ (5*10^6 cells) and 35% CD8+ (3.6*10^6) T cells for URD1 and 73% CD4+ (5*10^6 cells) and 24% CD8+ (1.8*10^6 cells) T cells for URD2. Absolute cell numbers of CD19+ leukemic cells (●) in non-treated and treated (■) mice, and CD3+ T cells (%) in treated mice in PB were assessed by flowcytometry. Each symbol represents the mean values of non-treated (n=2) and DLI treated mice (n=4). * Indicates statistically significant difference using an unpaired t test. Treatment of leukemia-engrafted mice with HLA-matched DLI resulted in delayed leukemia progression, but no effective GvL reactivity. (B) Reactivity of CD4+ (○) and CD8+ (■) T cell clones isolated at sacrifice from spleen of mice engrafted with leukemic cells from patients 1 and 2 were tested for recognition of human primary leukemic cells, NOD/scid bone marrow-derived DC and donor-derived EBV-LCL in IFN-γ ELISA. T cell clones producing >100 pg/ml IFN-γ upon incubation with stimulator cells were considered reactive. Each dot represents the mean release of IFN-γ (pg/ml) by a single T cell clone in 10 ml culture supernatants. None of the isolated T cell clones from leukemia-engrafted mice after treatment with HLA-matched DLI were leukemia-reactive. Only xeno-reactive CD8+ and CD4+ T cell clones were found. Materials and methods are described in Supplementary Information.

The poor immunogenicity of ALL and CML-BC may be overcome by treatment with HLA-mismatched alloSCT, since donor T cells targeting antigens presented in mismatched HLA molecules can also be derived from the memory T cell repertoire. Memory, as compared to naïve, T cells have less APC requirements and a lower threshold for activation. GvL responses in vivo against poorly immunogenic malignancies may therefore be more efficiently induced across HLA barriers. HLA disparity, however, is associated with an increased risk of Graft-versus-Host Disease (GvHD), and patients are therefore preferably transplanted with donors that are identical for HLA-A, -B, -C and -DRB1.
mismatched for HLA-DQB1 and/or HLA-DPB1\textsuperscript{13}. In these patients, HLA-class II restricted T cell responses as induced after HLA-class II mismatched DLI may result in profound GvL reactivity with controllable Graft-versus-Host Disease, since HLA-class II molecules are preferentially expressed on (non)-malignant hematopoietic cells and not on non-hematopoietic tissues under non-inflammatory conditions\textsuperscript{14}. Here, we indeed demonstrate and confirm our previous finding that HLA-class II mismatched DLI induces strong anti-tumor immunity in human ALL engrafted NOD/scid mice\textsuperscript{15}. NOD/scid mice engrafted with leukemic cells from patient 2 were treated with DLI from HLA-class II mismatched URD3 (\textit{Supplementary Table 1}). CD4+ and CD8+ T cells appeared in PB from 23 days after DLI, and \textit{in vivo} T cell expansion coincided with disappearance of leukemic cells (P<0.001) (Figure 2A).

Similar to mice treated with HLA-matched DLI, mice developed xeno-reactive symptoms and were sacrificed. Clonal isolation and characterization of 64 CD4+ and 30 CD8+ T cell clones from treated mice showed that 32 CD4+ T cell clones were leukemia-reactive and recognized patient-derived EBV-LCL (data not shown) based on specific recognition (>100 pg/ml IFN-γ) of these target cells, but not donor-derived EBV-LCL or murine DC (Figure 2B). None of the isolated CD8+ T cell clones were leukemia-reactive or reactive against patient-derived EBV-LCL, but a number of CD4+ T cell clones (n=4) and CD8+ T cell clones (n=24) were xeno-reactive. Twenty leukemia-reactive CD4+ T cell clones were tested for HLA-restriction against donor-derived EBV-LCL transduced to express patient mismatched HLA-class II alleles (Figure 2C). CD4+ T cell clones reactive against donor-derived EBV-LCL transduced to express HLA-DRB3*0101 (n=3), -DQB1*0603 (n=15), -DPB1*0301 (n=1) or -DPB1*0401 (n=1) were found, illustrating induction of a polyclonal CD4+ T cell mediated anti-tumor response over all patient mismatched HLA-class II loci.

In conclusion, our NOD/scid mouse model shows impairment of HLA-matched DLI to mediate GvL reactivity against established ALL and CML-BC, whereas the \textit{in vivo} immunogenicity of the leukemic cells was sufficiently high to generate effective T cell responses across HLA-class II barriers. The failure of HLA-matched DLI to induce anti-tumor immunity in NOD/scid mice suggests that patients with relapsed ALL and CML-BC after alloSCT, when professional APC are of donor origin, may be unlikely to respond to HLA-matched DLI. Our data emphasize the relevance of HLA-class II disparity for efficient induction of GvL immunity against poorly immunogenic ALL and CML-BC, and support the use of HLA-class II mismatched unrelated alloSCT and DLI as treatment modality to improve clinical outcome for patients with HLA-class II positive high-risk ALL and CML-BC.
Figure 2: *In vivo* induction of GvL reactivity after HLA-class II mismatched DLI.

(A) NOD/scid mice were inoculated (day 0) with primary leukemic cells (10^6 cells) from patient 2 (CML-BC), and were left untreated or received DLI 36 days after leukemic inoculation (indicated by the arrow). The infused T cell population in DLI treated mice consisted of 62% CD4+ (5*10^6 cells) and 32% CD8+ (3*10^6 cells) T cells. Absolute cell numbers of CD19+ leukemic cells (--●--) in non-treated and treated (--■--) mice, and CD3+ T cells (--○--) in PB were assessed by flowcytometry. Each symbol represents the mean values of non-treated (n=2) and DLI treated mice (n=2). ** Indicates statistically significant difference using an unpaired t test. Treatment of leukemia-engrafted mice with HLA-class II mismatched DLI resulted in profound and effective GvL reactivity. (B) Reactivity of CD4+ (○) and CD8+ (■) T cell clones isolated from spleens of mice at sacrifice were tested for recognition of human primary leukemic cells, NOD/scid bone marrow-derived DC and donor-derived EBV-LCL in IFN-γ ELISA. T cell clones producing >100 pg/ml IFN-γ upon incubation with stimulator cells were considered reactive. Each dot represents the mean release of IFN-γ (pg/ml) by a single T cell clone in 10 ml culture supernatants. Leukemia-reactive CD4+ and xenoreactive CD8+ and CD4+ T cell clones were isolated from leukemia-engrafted mice after treatment with HLA-mismatched DLI. (C) Recognition of patient-derived EBV-LCL, non-transduced and retrovirally transduced donor-derived EBV-LCL with HLA-DRB3*0101, -DQB1*0603, -DPB1*0301 and -DPB1*0401 by representative leukemia-reactive CD4+ T cell clones is shown as release of IFN-γ (pg/ml) in 10 ml culture supernatants. Out of 20 CD4+ T cell clones tested, 3 were specific for HLA-DRB3*0101, 15 for HLA-DQB1*0603, one for HLA-DPB1*0301 and one for HLA-DPB1*0401. Isolated leukemia-reactive CD4+ T cell clones were restricted by the mismatched HLA-class II alleles of the patient. Materials and methods are described in *Supplementary Information*. 
SUPPLEMENTARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS

Patient and donor material
Peripheral blood (PB) samples were obtained from patients and healthy unrelated donors (URD) after approval by the Leiden University Medical Center Review Board and informed consent according to the Declaration of Helsinki. Leukemic cells were obtained from patients with B-lineage ALL (patient 1) and CML-BC (patient 2) (HLA-typing available in Supplementary Table I).

In vivo NOD/scid mouse experiments
All animal experiments were conducted according to Institutional Guidelines with permission from the Leiden University Medical Center Animal Experiments Committee. NOD/scid mice (Charles River, Saint-Germain-sur-l’Abresle, France) were engrafted with primary human leukemic cells, treated with DLI and monitored, as described previously15. For DLI, CD3+ T cells were isolated from PB mononuclear cells from URD using the pan T isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Significance between DLI-treated and non-treated mice was determined using an unpaired t test.

Generation of T cell clones and target/stimulator cells
T cell cloning was performed from spleen samples of mice by limiting dilution as previously described15. Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCL) from patients and donors were generated, and donor EBV-LCL were transduced with retroviral vectors encoding mismatched HLA-class II alleles as previously described15. Dendritic cells (DC) from NOD/scid mice were generated in vitro by culturing bone marrow in RPMI-1640 (Invitrogen, Breda, The Netherlands) with 10% fetal calf serum (BioWhittaker, Verviers, Belgium) and 20 ng/ml rmGM-CSF (Invitrogen). T cell clones (2,000 cells/well) were co-incubated with stimulator cells (10,000 cells/well), and IFN-γ production was measured as described previously1.
## SUPPLEMENTARY TABLE

### Supplementary Table 1: HLA types of patients and donors.

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HLA-typing was performed by standard serology methods and sequence specific polymerase reactions.