2 HLA-DP as specific target for cellular immunotherapy in HLA-class II expressing B cell leukemia

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Abstract

Mismatching for HLA-DPB1 in unrelated donor hematopoietic stem cell transplantation (URD-SCT) has been associated with a decreased risk of disease relapse, indicating that HLA-DP may represent a target for graft versus leukemia (GVL) reactivity in HLA-class II expressing hematological malignancies. To investigate whether HLA-DP specific T-cells could mediate GVL-reactivity following HLA-DPB1 mismatched URD-SCT and donor lymphocyte infusion (DLI), we analyzed the immune response in a patient with leukemic lymphoplasmacytic lymphoma responding to DLI without GVHD. The emergence of leukemia reactive CD4+ T-cells during the clinical immune response was demonstrated by IFN-γ ELISPOT analysis. Following clonal isolation of these leukemia reactive CD4+ T-cells, blocking studies, panel studies and retroviral transduction experiments of both mismatched HLA-DPB1 alleles identified HLA-DPB1*02:01 and HLA-DPB1*03:01 as the targets of this immune response. The HLA-DP specific CD4+ T-cell clones were capable of recognizing and lysing several HLA-DP expressing myeloid and lymphoid hematological malignant cells. Since HLA-DP expression is mainly restricted to hematopoietic cells, HLA-DP may be used as a specific target for immunotherapy following T-cell depleted URD-SCT. Therefore, in patients with HLA-class II expressing hematological malignancies HLA-DPB1 mismatched SCT may be preferable over fully matched SCT allowing DLI to induce a GVL-effect.

Introduction

The graft-versus-leukemia (GVL) reaction that can be observed after allogeneic hematopoietic stem cell transplantation (SCT) is a beneficial immune response resulting in the elimination of residual leukemic cells in the patient. Donor derived T-cells play a major role in GVL-reactivity and graft versus host disease (GVHD), since depletion of T-cells from stem cell grafts resulted in a higher risk of leukemic relapse and a decrease of GVHD.[1;2] T-cells present in the graft as well as T-cells administered by donor lymphocyte infusion (DLI) after transplantation can induce GVL-reactivity and GVHD.[3;4] To minimize the risk for GVHD, patients are preferably transplanted with stem cells from an HLA identical sibling or an HLA-matched unrelated donor (URD).[5] Following HLA identical SCT, GVL-reactivity and GVHD are likely to be caused by donor derived T-cells recognizing minor histocompatibility antigens (MiHA). T-cells recognizing broadly expressed MiHAs may play a role in both GVL-reactivity and GVHD. In contrast, T-cells recognizing hematopoiesis restricted MiHAs may selectively mediate GVL-reactivity.[6-9]

Alloreactive T-cells recognizing HLA-mismatched alleles are present in high frequencies in peripheral blood.[10] An HLA-mismatched SCT may therefore result in a strong alloimmune response. Since HLA-class I molecules are ubiquitously expressed an HLA-class I mismatched SCT may cause severe GVHD. In contrast to ubiquitous expression of HLA-class I molecules, constitutive expression of HLA-class II molecules is mainly restricted to hematopoietic cells. A single HLA-class II locus mismatched SCT might therefore be anticipated to induce selective GVL-reactivity without GVHD. However, HLA-class II expression can be upregulated on various tissues after exposure to pro-inflammatory cytokines.[11;12] An anti-HLA-class II immune response following HLA-class II mismatched SCT may therefore also result in GVHD if HLA-class II molecules on non-hematopoietic tissues are upregulated as a consequence of the conditioning regimen or infections.[13] The results obtained from clinical transplantation studies concerning the impact of mismatching for different HLA-alleles have led to the current standard that patient and donor are usually preferably matched for the HLA-class II alleles HLA-DR and HLA-DQ in addition to the HLA-class I molecules.[14;15]

The role of HLA-DP as a transplantation antigen is less clear. Clinical reports on the impact of matching for HLA-DP on transplant outcome and GVHD often showed conflicting results.[16-20] A recent study, however, demonstrated that mismatching for HLA-DPB1 in T-cell depleted URD-SCT was associated with a significant decreased risk of disease relapse, whereas no effect on the incidence of severe GVHD was observed. It has been suggested that in T-cell depleted SCT HLA-DP may represent a relatively specific target for a GVL-reactivity in patients with HLA-class II expressing hematological malignancies.[21]

We hypothesize that administration of DLI several months after a T-cell depleted HLA-DPB1 mismatched SCT in the absence of an inflammatory environment, may cause a selective GVL-effect without risking severe GVHD. To investigate whether HLA-DP

specific T-cells could be demonstrated to be involved in GVL-reactivity following HLA-DPB1 mismatched URD-SCT, we analyzed the immune response in a patient with a refractory leukemic lymphoplasmacytic lymphoma responding to DLI following T-cell depleted SCT. We isolated the leukemia reactive T-cell clones during the clinical immune response and identified HLA-DP specific CD4+ T-cells as the likely mediators of persistent complete remission of the disease in the absence of GVHD.

Case report

A 55-year-old female patient with a leukemic lymphoplasmacytic lymphoma was referred to our center for URD-SCT. This chronic B cell leukemia was resistant to chemotherapy after receiving multiple courses of chlorambucil, cyclophosphamide, doxorubicin, vincristin and prednisone, and fludarabin. Following pre-treatment with rituximab, resulting in a partial response, the patient was transplanted with mobilized peripheral blood stem cells from a male HLA-matched unrelated donor. Patient and donor were fully matched for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 but differed for both HLA-DPB1 alleles. The HLA-DPB1 alleles were molecularly typed HLA-DPB1*02:01,03:01 for the patient and HLA-DPB1*04:02,05:01 for the donor. The non-myeloablative conditioning regimen consisted of fludarabin, anti-thymocyteglobulin and busulfan.[22] The graft was depleted of T-cells by in vitro incubation with 20 mg Alemtuzumab (Campath-1H) in the bag. No post-transplant GVHD prophylaxis was administered. Following transplantation rapid hematopoietic recovery occurred, and no GVHD developed. Chimerism analysis was performed on bone marrow samples by fluorescence in situ hybridization using X- and Y- chromosome specific probes as described previously.[8] The malignant cells in bone marrow (BM) and peripheral blood (PB) were identified as CD19+ and CD20 low B-cells by flowcytometry. One month after SCT persistent disease was observed with 7% malignant cells in BM. Chimerism studies showed 73% donor cells in BM. Five months after SCT the number of malignant cells gradually increased, and therefore a single dose of DLI containing $2.5*10^6$ T-cells / kg body weight was administered 7 months after SCT. After an initial further rise of the malignant cells to 58% in BM, the percentage leukemic cells started to decline from 6 weeks after DLI. Five month after DLI, complete remission and conversion to full donor chimerism was observed (Figure 1). During this clinical response transient minimal GVHD of the skin and mouth grade 1 developed, which was treated with topical corticosteroids. At present, 3 years later, the patient is in complete remission and good clinical condition without GVHD.

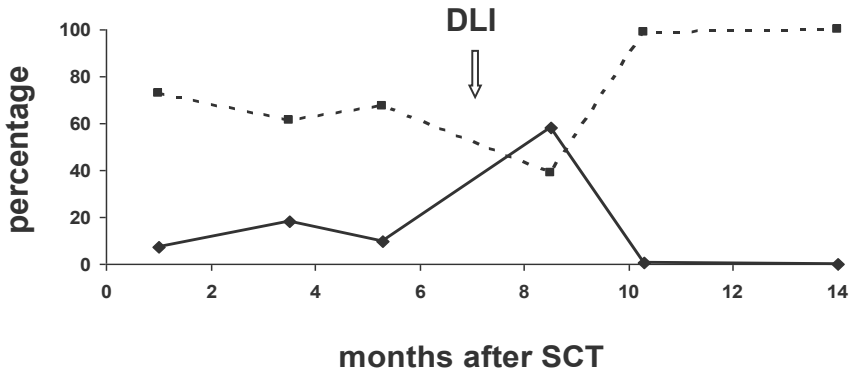


Figure 1. Clinical immune response to DLI following HLA-DPB1 mismatched URD-SCT. Percentage of malignant cells (—♦—) and percentage of donor chimerism (--■--) in BM after SCT. Arrow indicates DLI infusion. From 6 weeks after DLI the percentage malignant cells in BM started to decline. At the same time a rise in percentage donor chimerism was observed. Five months after DLI complete remission and conversion to full donor chimerism was observed.

Materials and methods

Cell collection and preparation

After informed consent, PB and BM samples were obtained from the patient, the unrelated stem cell donor as well as from other patients and healthy donors. Mononuclear cells (MNC) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable EBV-transformed B cell lines (EBV-LCL) were generated using standard procedures. Fibroblasts (FBs) were cultured from skin biopsies in DMEM with low glucose (BioWhittaker, Verviers, Belgium) and 10% fetal bovine serum (FBS, BioWhittaker). Renal cell carcinoma cell lines (RCC 02.20 and 91.04) and breast cancer cell lines (BCC MDA 231 and BT 549) were kindly provided by Dr. E. Verdegaal of the department of Clinical Oncology in the LUMC.

Flowcytometry

The monoclonal antibodies (moAb) anti-CD4 fluorescein isothiocyanate (FITC), anti-CD3 phycoerythrin (PE) and anti-CD19 allophycocyanin (APC) were obtained from Becton Dickinson (BD, San Jose, USA). Anti-CD8 FITC moAb was purchased from Caltag (Burlingame, USA). Anti-HLA-DP PE-labeled moAbs were obtained from Leinco Technologies (St. Louis, Missouri, USA), and anti-NGFR-PE was derived from Pharmingen (San Diego, USA). Flowcytometric analysis was performed on a BD flowcytometer.

Characterization of the anti-leukemic immune response by ELISPOT analysis

To determine the potential contribution of CD4+ T-cells and CD8+ T-cells to the clinical immune response following DLI, IFN-γ production of CD4+ and CD8+ T-cells in response to malignant cells from the patient was determined by ELISPOT analysis. PB-MNC obtained from the patient before DLI and at various time points during the clinical response were monocyte depleted using magnetic CD14 beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Next, CD4+ T-cells were isolated from the CD14 negative fraction using CD4 multisort isolation beads (Miltenyi Biotec GmBH). The CD4 positive fraction and the CD4 negative fraction, consisting of mainly CD8+ T-cells, were used in an IFN-γ ELISPOT (MabTech, Nacka Strand, Sweden) analysis according to the manufacturer's instructions. Briefly, 96-well nitrocellulose plates precoated with anti-IFN-γ antibodies (Mab-1 D1K) were seeded with $1x10^5$ T-cells and $0.5 x10^5$ leukemic cells in IMDM (BioWhittaker) supplemented with 10% human serum and 20 IU IL2/ml (Chiron, Amsterdam, The Netherlands), and incubated overnight at 37ºC. Cytokine secretion was detected by addition of a biotinylated anti-IFN-γ antibody (7-B6-1 biotin) for 2 hours at room temperature (RT). Following incubation with streptavidin (Extravidin-ALP E2636, Sigma, St Louis, USA) for 1 hour, substrate reagent (BCIP/NIBT-ALP-substrate, Sigma) was added to allow spot formation. Spots were counted using an automatic ELISPOT reader.

To analyze the specificity of the CD4+ T-cells responding to the leukemic cells, an ELISPOT assay was performed in the presence of anti-HLA-DP (B7.21) or anti-HLA-DQ (SPVL3) monoclonal antibodies (moAbs). Leukemic cells were pre-incubated with saturating concentrations of antibodies for 30 min at RT before seeding into the ELISPOT plates.

Isolation of leukemia reactive T-cell clones

To allow further characterization of leukemia reactive T-cells, T-cells producing IFN-γ in response to stimulation with the leukemic cells were clonally isolated. PB-MNC or BM-MNC were depleted of monocytes using CD14 beads and overnight incubated with equal numbers of irradiated (30Gy) BM derived leukemic cells harvested from the patient before transplantation. Cell suspensions were incubated at a final concentration of $2x10^6$ cells/ml in IMDM containing 10% human serum and 10 IU IL2/ml. After overnight stimulation, activated IFN-γ secreting T-cells were stained using the IFN-γ capture assay (Miltenyi Biotec GmbH) according to manufacturer's instructions, and isolated by cell sorting as described previously.[23] Briefly, cells were incubated with IFN-γ catch reagent (anti-CD45/anti-IFN-γ-antibody) and cultured for 45 min at 37°C under continuous rotation. After incubation with IFN-γ-detection antibody (PE labeled) for 10 minutes, cells were stained with FITC-conjugated anti-CD4 or FITC-conjugated anti-CD8 moAbs. Cell suspensions were counterstained with propidium iodide (PI, Sigma, St Louis, USA) immediately prior to cell sorting to exclude dead cells. Viable (PI-negative), CD4 positive or CD8 positive, IFN-γ secreting lymphocytes were sorted single cell per well or 50 cells per well into U-bottom

microtiter plates (Greiner Bio-One, the Netherlands) containing 100 µl of feeder mixture consisting of culture medium, IL2 (120 IU/ml), phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK), and 50 Gy-irradiated allogeneic third-party PB-MNC ($0.5x10^5$/ml). Proliferating T-cell clones were selected, and further expanded using non-specific stimulation and third party feeder cells.

Characterization of T-cell clones

To analyze the specificity of the expanded T-cell clones, IFN-γ production in response to patient derived leukemic cells, donor EBV-LCL, a panel of unrelated EBV-LCL and various hematological malignant cells was tested. To determine IFN-γ production, 5 000 T-cells were cocultured with 30 000 stimulator cells in a final volume of 150 µl IMDM culture medium supplemented with 20 IU IL2/ml. After overnight incubation, supernatants were harvested, and IFN-γ production was measured by ELISA (CLB, Amsterdam, The Netherlands). Recognition of non-hematopoietic cells was analyzed using FBs, RCC and BCC as stimulator cells. Following cell culture for 6 days in the presence or absence of 200 U/ml IFN-γ (Immukine, Boehringer Ingelheim bv, Alkmaar, The Netherlands), cells were thoroughly washed and 10 000 stimulator cells were cocultured with 5 000 T-cells in a final volume of 150 µl IMDM culture medium supplemented with 20 IU IL2/ml. IFN-γ production was measured by ELISA after overnight incubation.

To determine the HLA-restriction molecules essential for recognition, blocking studies were performed using anti-HLA-class I (W6/32), anti-HLA-class II (PdV5.2), anti-HLA-DR (B8.11-2), anti-HLA-DP (B7.21) and anti-HLA-DQ (SPVL3) antibodies. Target cells were pre-incubated with saturating concentrations of moAbs for 30 minutes at RT before addition of T-cells. After overnight incubation supernatants were harvested, and IFN-γ production by the T-cell clones was determined by ELISA.

To determine the cytotoxic capacity of the isolated T-cell clones against leukemic cells, a CFSE (carboxyfluorescein diacetate succinimidyl ester; molecular Probes Europe, Leiden, The Netherlands) based cytotoxicity assay was performed as described before.[24] Briefly, 25 000 CFSE labeled leukemic cells from the patient or 15 000 CFSE labeled PB-MNC from unrelated patients were incubated with 75 000 unlabeled T-cells for 24 hours. Cell suspensions were collected and stained with APC labeled antibodies specific for B cells (CD19) or myeloid cells (CD33 / CD34) to detect leukemic cells. To exclude dead cells from analysis 0.2 µg/ml PI was added immediately prior to measurement. Survival of CFSE labeled, marker positive target cells was analyzed on a flowcytometer. To allow quantitative analysis 10 000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA) were added to each sample and 2 000 microbeads were acquired. The percentage of lysis was calculated as follows: % lysis = 100 - ([absolute no. viable $CFSE^+$ target cells ^{exp}]/[absolute no. viable $CFSE^+$ target cells ^{control}]) x 100). T-cell clones showing more than 20% lysis of the leukemic cells were determined to be cytotoxic T-cell clones.

Retroviral transduction of donor EBV-LCL with cDNA encoding patient HLA-DPB1

To confirm specific recognition of HLA-DPB1*02:01 and HLA-DPB1*03:01 by the isolated T-cell clones, donor EBV-LCL were retrovirally transduced with patient derived HLA-DPB1 alleles. Total RNA from patient PB-MNC was extracted using Trizol (Invitrogen, Carlsbad, CA) and transcribed into cDNA by reverse transcriptase using random oligo dT primers (Pharmacia, Uppsala, Sweden). HLA-DPB1 fragments were obtained by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and cloned into LZRS retroviral vectors containing the marker gene truncated nerve growth factor receptor (ΔNGF-R). The identity of the PCR products was verified by sequence reactions. φ-NX-A packaging cells were transfected with these retroviral vectors using a calcium phosphate transfection method (Life Technologies, Gaithersburg, USA). Retroviral supernatants containing the HLA-DPB1*02:01 or HLA-DPB1*03:01 gene were harvested and used for transduction of donor-derived EBV-LCL using recombinant human fibronectin fragments CH-296 (Bio Whittaker) as described before.[25] HLA-DPB1 transduced EBV-LCL were stained with PE-conjugated anti-ΔNGF-R moAbs and purified using anti-PE isolation beads (Miltenyi Biotec GmbH).

T-cell receptor (TCR) – Vβ chain analysis

To determine whether T-cell clones with the same specificity were derived from the same clonal origin, TCR-Vβ chain analysis was performed. First, CD4+ T-cell clones were stained with specific antibodies against different TCR-Vβ chains using a TCR-Vβ repertoire kit (Beckman Coulter, Fullerton, USA) and analyzed by flowcytometry. T-cell clones expressing the same TCR-Vβ were further analyzed by PCR using TCR-Vβ specific upstream primers in combination with a downstream TCR-BC primer. TCR-Vβ PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and sequenced using the dye terminator cycle sequencing kit (ABI-PRISM; PerkinElmer, Foster City, CA), according to the manufacturer's instructions to obtain a complete identification of the TCR-Vβ genes.

Results

Identification of leukemia reactive CD4+ T-cells during the clinical response to DLI

To determine which T-cells were involved in the anti-tumor response following DLI in the patient, IFN-γ production by CD4+ T-cells and CD8+ T-cells in response to stimulation with patient leukemic cells was determined using ELISPOT. PB samples obtained before DLI and at various intervals following DLI were analyzed. Specific production of IFN-γ by CD4+ T-cells but not CD8+ T-cells was observed during the clinical immune response to DLI from six weeks after DLI (Figure 2). The correlation between the kinetics of the anti-leukemic CD4+ T-cell response and the disappearance of leukemic cells resulting in persistent complete remission of the disease suggests that these CD4+ T-cells mediated the anti-leukemic effect.

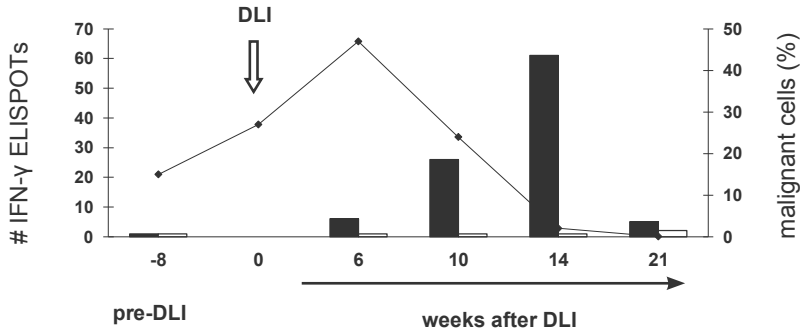


Figure 2. **Determination of an anti-leukemic CD4+ T-cell response starting 6 weeks after DLI.** Number of IFN-γ ELISPOTs produced by CD4+ T-cells (■) and CD8+ T-cells (□) before and after DLI in response to stimulation by leukemic cells from the patient. Leukemic cells as a percentage of PB-MNC is shown on the secondary axis (—♦—).

Isolation and characterization of leukemia reactive T-cells

To further characterize the anti-leukemic T-cell response, leukemia reactive T-cell clones were isolated from PB-MNC obtained 10 weeks post-DLI and from BM-MNC obtained 14 weeks post-DLI. After overnight stimulation with patient leukemic cells, activated IFN-γ producing T-cells were isolated using cell sorting. The percentage of IFN-γ producing T-cells 10 weeks and 14 weeks post-DLI was 0.2% and 0.7% respectively. Isolation and expansion of IFN-γ producing T-cells resulted in 24 CD4+ T-cell clones, 19 CD8+ T-cell clones and 6 natural killer (NK) cell clones for further analysis.

All clones were tested for recognition of patient malignant cells and donor derived EBV-LCL as measured by IFN-γ production. All CD4+ T-cell clones showed specific production of IFN-γ (114-592 pg/ml) in response to patient malignant cells, but not to donor derived cells (Figure 3a). The CD8+ T-cell clones and NK-cell clones did not produce IFN-γ in response to patient leukemic cells, indicating that these clones were isolated probably due to spontaneous ex vivo production of IFN-γ or non-specific staining (data not shown).

To determine whether these CD4+ T-cell clones were capable of lysing the leukemic cells, a CFSE based cytotoxicity assay was performed. Of the 20 CD4+ T-cell clones tested, 11 CD4+ T-cell clones (55%) showed specific lysis (20-84%) of the malignant CD19+ B cells (Figure 3b). Due to contamination, four CD4+ T-cell clones were lost before analysis. The isolated CD8+ T-cell clones and NK-cell clones did not show lysis of patient leukemic cells (data not shown). These results illustrate that leukemia reactive cytokine producing and cytotoxic CD4+ T-cell clones were isolated from the immune response following DLI.

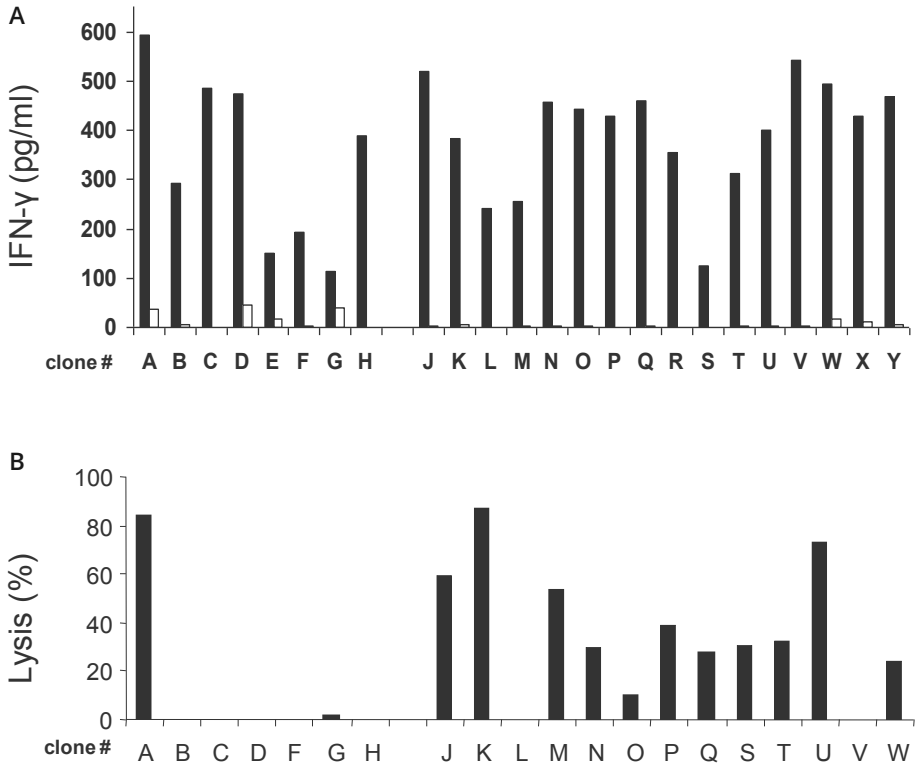


Figure 3. Reactivity of isolated CD4+ T-cell clones against patient malignant cells. Clones A - H represent CD4+ T-cell clones isolated from peripheral blood obtained 10 weeks after DLI. Clones J - Y were isolated from bone marrow obtained 14 weeks post DLI. (A) patient leukemic cells (■) and donor derived EBV-LCL (□) were tested for recognition by all isolated CD4+ T clones. Release of IFN-γ (pg/ml) in 50 µl supernatant is shown. (B) Percentage lysis of patient malignant cells by the isolated CD4+ T-cell clones in a 24h CFSE based cytotoxicity assay. The experiment was performed in duplicate, mean percentage lysis of leukemic cells is shown. Clones E, R, X, Y could not be tested in the cytotoxicity assay due to contamination.

Identification of HLA-DPB1*02:01 and HLA-DPB1*03:01 as targets for leukemia reactive CD4+ T-cells

To determine the HLA restriction molecules essential for recognition by the isolated CD4+ T-cell clones, blocking studies were performed. Addition of HLA-class II moAbs reduced IFN-γ production by all isolated CD4+ T-cell clones. Pre-incubation of the leukemic cells with specific moAbs for the HLA-class II molecules resulted in abrogation of IFN-γ production by all CD4+ T-cell clones upon blocking with HLA-DP, but not with HLA-DR or HLA-DQ specific

moAbs, illustrating HLA-DP restricted recognition. The mean percentage of inhibition of IFN-γ production for all T-cell clones upon addition of HLA-class II moAbs was 53 +/- 28%. The mean percentage of inhibition of IFN-γ production upon addition of an HLA-DP specific moAb was 87 +/- 19%. A representative example of the inhibition of IFN-γ production upon addition of blocking moAbs is shown in figure 4a.

To analyze whether the circulating CD4+ T-cells that responded ex vivo to the leukemic cells by production of IFN-γ as demonstrated in the ELISPOT analysis were all HLA-DP specific, the ELISPOT analysis was performed in the presence of blocking antibodies. Addition of HLA-DP specific moAbs to the leukemic cells completely abrogated production of IFN-γ ELISPOTs by the CD4+ T-cells isolated at the maximal anti-leukemic response 14 weeks post-DLI (Figure 4b). These results indicate that the profound anti-tumor response observed following DLI was mediated by HLA-DP specific CD4+ T-cells.

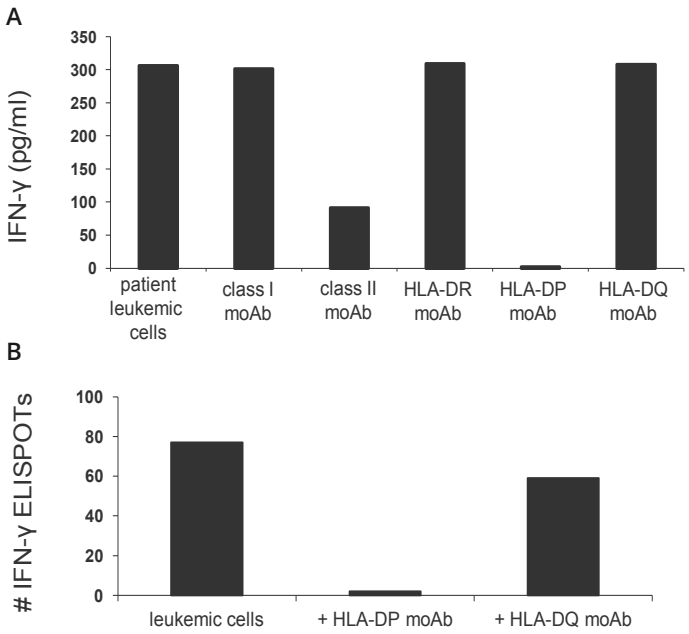
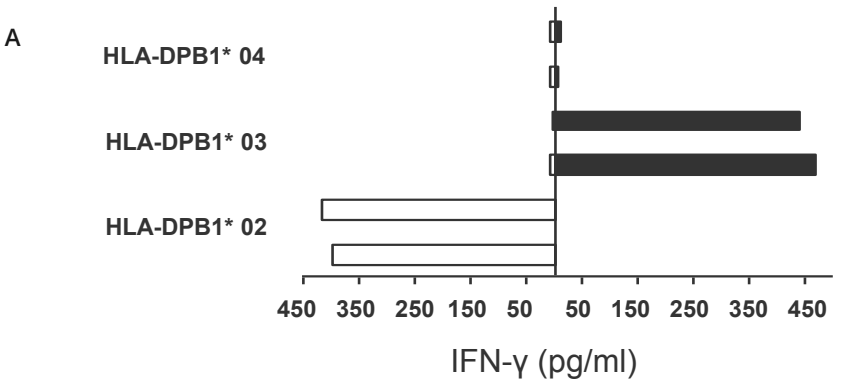


Figure 4. HLA-DP restricted recognition of patient leukemic cells by CD4+ T-cell clones. (A) Representative example of HLA-DP restricted recognition of patient leukemic cells by the isolated CD4+ T-cell clones. IFN-γ release (pg/ml) of clone M in response to patient malignant cells in the absence or presence of various blocking antibodies is shown. (B) Production of IFN-γ ELISPOTs by CD4+ T-cells obtained 14 weeks after DLI in response to stimulation with malignant cells from the patient, in the presence or absence of various blocking antibodies.

To further identify the specificity of the HLA-DP restricted CD4+ T-cell clones, recognition of a panel of EBV-LCL was analyzed. Since patient and donor differed for both HLA-DPB1 alleles, the T-cell clones were likely to recognize allo-HLA-DPB1 of the patient. To analyze whether there was differential recognition of the patient derived HLA-DPB1*02:01 and HLA-DPB1*03:01 alleles, a panel of EBV-LCL expressing either HLA-DPB1*02:01 or HLA-DPB1*03:01 was tested for recognition. Of the 20 CD4+ T-cell clones tested, 17 CD4+ T-cell clones recognized all EBV-LCL expressing HLA-DPB1*03:01 (n=7) but not EBV-LCL expressing HLA-DPB1*02:01 (n=5). Three CD4+ T-cell clones recognized all EBV-LCL expressing HLA-DPB1*02:01 and not HLA-DPB1*03:01. EBV-LCL expressing the donor specific HLA-DPB1*04 allele (n=3) were not recognized by the T-cell clones. Representative examples of the recognition pattern of an HLA-DPB1*02:01 specific T-cell clone and an HLA-DPB1*03:01 specific T-cell clone are shown in figure 5a.

To confirm specific recognition of HLA-DPB1*02:01 and HLA-DPB1*03:01, donor EBV-LCL were retrovirally transduced with the patient derived HLA-DPB1*02:01 or HLA-DPB1*03:01 genes. Transduction with HLA-DPB1*02:01 only induced recognition by the HLA-DPB1*02:01 specific T-cell clones, and not by HLA-DPB1*03:01 specific clones. Transduction with HLA-DPB1*03:01 resulted only in recognition by the HLA-DPB1*03:01 specific clones. These experiments confirm specific recognition of HLA-DPB1*02:01 and HLA-DPB1*03:01 (Figure 5b).



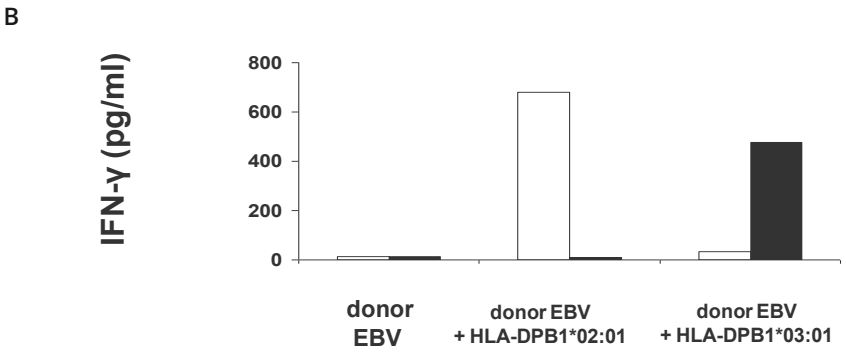


Figure 5. Differential recognition of HLA-DPB1*02:01 and HLA-DPB1*03:01 by the HLA-DP restricted T-cell clones. (A) Recognition of a panel of EBV-LCL expressing HLA-DPB1*02, HLA-DPB1*03 or HLA-DPB1*04 by two representative T-cell clones. Two representative examples of each target are shown. White bars represent the recognition pattern of a T-cell clone (clone B) recognizing only EBV-LCL expressing HLA-DPB1*02:01. Black bars represent the recognition pattern of a T-cell clone (clone A) recognizing only EBV-LCL expressing HLA-DPB1*03:01. In total 3 HLA-DPB1*02:01 restricted T-cell clones and 17 HLA-DPB1*03:01 restricted T-cell clones were identified. (B) Recognition of donor EBV-LCL upon retroviral transduction of patient HLA-DPB1*02:01 and HLA-DPB1*03:01 by HLA-DPB1*02:01(□) or HLA-DPB1*03:01 (■) specific T-cell clones.

To analyze whether the recognition of HLA-DP by the T-cell clones was solely determined by the expression of HLA-DP or whether the T-cell clones recognized the allo-HLA-DP molecules in combination with a peptide only expressed by hematopoietic cells, we tested several HLA-DPB1*02 or HLA-DPB1*03 typed non-hematopoietic cell lines for recognition. Since constitutive expression of HLA-class II molecules was expected to be absent on non-hematopoietic cells two FBs, two RCC cell lines and two BCC cell lines were cultured for 6 days in the absence or presence of 200 U/ml IFN-γ to induce HLA-class II expression. As shown in figure 6a and 6b expression of HLA-DP was absent on resting FBs, both RCC cell lines and on BCC BT 549. BCC MDA 231 showed expression of HLA-DP without incubation with IFN-γ. Incubation with IFN-γ resulted in upregulated expression of HLA-DP for all cell lines analyzed. All non-hematopoietic cell populations were recognized by the specific T-cells clones if sufficient HLA-DP was expressed. (figure 6c and 6d) Specific recognition of HLA-DP was confirmed by blocking studies (data not shown). Since the T-cell clones recognized cells derived from various tissues, specific recognition by the T-cell clones was determined by the expression of allo-HLA-DP and not by the recognition of a hematopoiesis specific peptide presented in the allo-HLA-DP molecule.

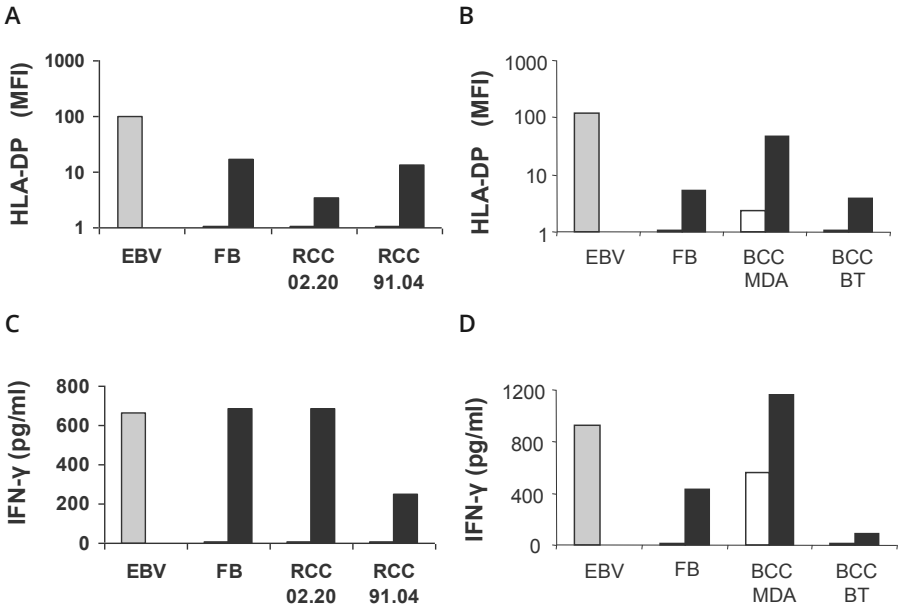


Figure 6. Expression of HLA-DP on non-hematopoietic cells of various origins and recognition by HLA-DP specific T-cells. Mean fluorescence intensity (MFI) of HLA-DP-PE staining on (A) HLA-DPB1*03 typed EBV-LCL, fibroblasts (FBs) and renal cell carcinoma (RCC) cells and (B) HLA-DPB1*02 typed EBV-LCL, FBs and breast cancer (BCC) cells. HLA-DP expression on FBs, RCC and BCC cells is shown after 6 days culturing in the absence (□) or presence (■) of 200 U/ml IFN-γ. (C) IFN-γ release (pg/ml) of an HLA-DPB1*03 specific T-cell clone (clone J) in response to HLA-DPB1*03 stimulator cells cultured in the absence (□) or presence (■) of IFN-γ. (D) IFN-γ release (pg/ml) of an HLA-DPB1*02 specific T-cell clone (clone M) in response to HLA-DPB1*02 stimulator cells cultured in the absence (□) or presence (■) of IFN-γ.

T-cell clones with the same specificity are derived from different clonal origin

To analyze whether T-cell clones with the same specificity were derived from the same clonal origin, TCR-Vβ chain usage was determined. Different TCR-Vβ expression was found for all 3 T-cell clones specific for HLA-DPB1*02:01. Seven different TCR-Vβ chains were identified for the HLA-DPB1*03:01 specific T-cell clones, illustrating a polyclonal origin of this immune response. T-cell clones using the same TCR-Vβ, as determined by PCR and sequence reactions, were isolated at different time points following DLI, illustrating the persistence of this anti-HLA-DP immune response (data not shown).

HLA-DP as a specific target for immunotherapy

To investigate whether HLA-DP could more broadly be used as a target for cellular immunotherapy, the expression of HLA-DP on various leukemic cells was analyzed. B-ALL (n=4) and CLL (n=5) showed high expression of HLA-DP, whereas the expression of HLA-DP

was variable in myeloid leukemias, including AML (n=3) and CML (n=4). Two T-ALL samples analyzed did not express HLA-DP (data not shown).

To analyze whether various hematological malignant cells that expressed HLA-DP (Figure 7a) could be recognized by the isolated HLA-DP specific T-cell clones, HLA-DPB1*03:01 expressing ALL, CLL, AML and CML samples were tested for recognition and lysis by an HLA-DPB1*03:01 specific cytotoxic T-cell clone in an IFN-γ ELISA and cytotoxicity assay respectively. All samples tested were recognized comparable to the patient leukemic cells as measured by IFN-γ production (Figure 7b), and specific lysis (30-60%) of the malignant cell populations was observed (Figure 7c). HLA-DP restricted recognition and lysis was confirmed in blocking experiments (data not shown). These results indicate that various hematological malignancies with sufficient expression of HLA-DP may be susceptible to an HLA-DP mediated GVL-effect.

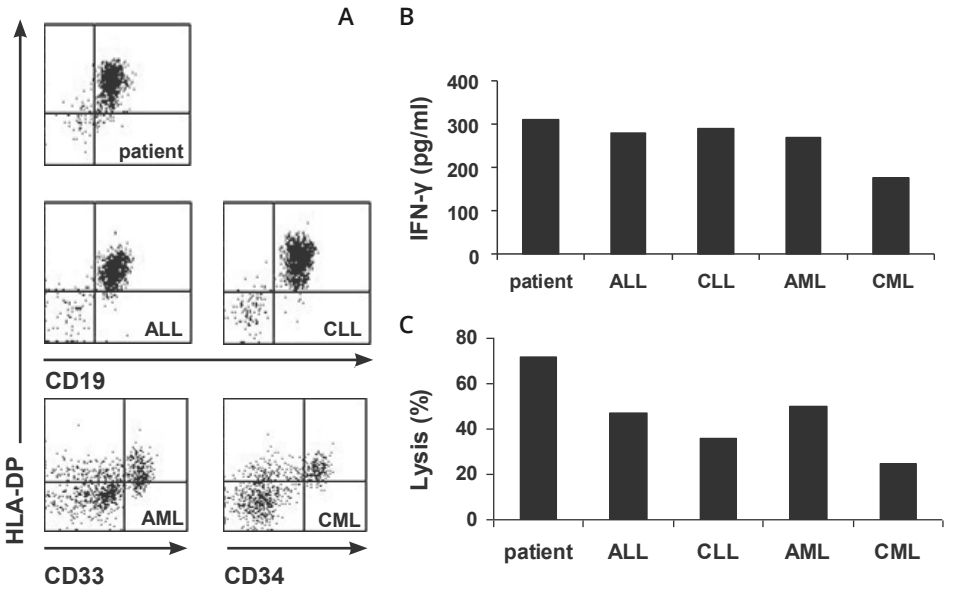


Figure 7. HLA-DP expression on various lymphoid and myeloid malignant cells and their recognition by an HLA-DPB1*03:01 restricted T-cell clone. PB-MNC from the patient analyzed in this study and from HLA-DP*0301 positive ALL, CLL, AML and CML patients were used in this experiment. (A) Expression of HLA-DP on malignant B-cells (CD19) or myeloid cells (CD33 / CD34). (B) Recognition of total PB-MNC by an HLA-DPB1*03:01 specific T-cell clone (clone A) as measured by IFN-γ production. (C) For the B cell malignancies lysis of the CD19 positive leukemic cells by an HLA-DPB1*03:01 specific cytotoxic CD4+ T-cell clone (clone A) in a 24h CFSE based cytotoxicity assay is shown. For the myeloid malignancies lysis of the CD33 or CD34 malignant cells is shown. The experiment was performed in triplicate wells. Mean percentage of lysis is shown.

Discussion

In this study we analyzed the immune response in a patient with a refractory chronic B cell leukemia responding to DLI after an HLA-DPB1 mismatched SCT. We observed a profound GVL-effect with minimal skin GVHD resulting in a persistent complete remission of the disease. Our results indicate that the GVL-effect was caused by a polyclonal immune response comprising both T helper and cytotoxic CD4+ T-cells directed against both mismatched HLA-DPB1 alleles. By ELISPOT analysis we identified the anti-leukemic response starting at 6 weeks after DLI. Since the emergence and kinetics of the leukemia-reactive CD4+ T-cells corresponded to the disappearance of the malignant cells the HLA-DP specific CD4+ T-cells were likely to mediate the anti-leukemic effect.

Since we demonstrated using ex vivo ELISPOT analysis the presence of only CD4+ and not CD8+ leukemia reactive T-cells both in peripheral blood and bone marrow, our study indicates that HLA-DP specific CD4+ T-cells can elicit a profound anti-leukemic response in the absence of leukemia reactive CD8+ T-cells. Although CD4+ T-cells have mostly been studied in their role as helper cells for development of CD8+ T-cell immunity[26;27], CD4+ T-cells with direct cytolytic activity have been isolated from patients with GVL responses after SCT, and have been suggested to play a direct role in anti-tumor immunity as effector cells.[28-31] Some of the HLA-DP specific CD4+ T-cells that were isolated in this study were capable of direct lysis of leukemic cells indicating that CD4+ T helper cells and CD4+ cytotoxic T-cells are sufficient for a direct effector function in GVL-reactivity.

The expression of HLA-DP on normal peripheral blood cells is found most profoundly on B-cells.[32] Similarly, high expression of HLA-DP is found on most B-ALL and CLL cells whereas myeloid leukemic cells show more variable expression of HLA-DP.[33] Especially B cell malignancies may therefore be susceptible to an HLA-DP mediated GVL-effect. Indeed, the beneficial effect of mismatching for HLA-DPB1 in T-cell depleted URD-SCT on the risk of disease relapse has been reported to be present most pronounced in ALL as compared to AML and CML.[21] In our study however, HLA-DP expressing myeloid leukemias were also demonstrated to be recognized and lysed by HLA-DP specific CD4+ T-cells, and therefore both B-cell malignancies and myeloid malignancies with sufficient HLA-DP expression may be susceptible to an HLA-DP mediated GVL-effect. As illustrated by the tumor cell lines, occasionally non-hematological tumor cells may also constitutively express HLA-DP and be therefore a target for HLA-DP specific immunotherapy.

The conflicting results of clinical reports on the impact of mismatching for HLA-DPB1 on GVHD may be explained by different transplantation regimens. HLA-DP reactive T-cells administered at the time of SCT may result in both GVHD and GVL-reactivity as a consequence of upregulated HLA-DP expression on non-hematopoietic cells caused by pro-inflammatory cytokines released as a consequence of tissue damage induced by the conditioning regimen or infections.[11;12] T-cell depleted SCT followed by the infusion of donor T-cells at a later time point, however, may more selectively induce a GVL response

when tissue damage is largely restored, and the expression of HLA-DP is anticipated to be restricted to hematopoietic cells. In this study we showed that resting FBs and most other non-hematopoietic cell lines did not express HLA-DP and were therefore not recognized by the T-cell clones. However, long-term incubation of non-hematopoietic cells with high amounts of IFN-γ as a model to mimic an inflammatory environment, induced substantial upregulation of HLA-DP and thereby recognition by the T-cell clones.

It has been suggested that not all HLA-DPB1 differences as defined by allele typing will be able to induce a T-cell response. Several groups have attempted to identify permissive and non-permissive HLA-DPB1 mismatch combinations to prevent GVHD based on the hypothesis that specific amino acid substitutions in the hypervariable region of the HLA-DPB1 allele are essential for T-cell reactivity.[34-37] Mixed lymphocyte reactions (MLR) showed that in 30% of HLA-DP mismatched pairs a negative MLR was observed and substantial differences in MLR reactivity between different HLA-DPB1 mismatched combinations were found. Strikingly, HLA-DPB1*03 and HLA-DPB1*02, which were the targets of the immune response in our patient, were found to be the most potent stimulators in a MLR.[34] These studies suggest that only certain combinations of HLA-DP mismatches may be able to induce a potent T-cell reactivity, and further explain controversial observations described in different studies on the outcome of HLA-DPB1 mismatched SCT.

In summary, our results illustrate that HLA-DP may represent a relatively specific target for GVL-reactivity. HLA-DPB1 mismatched SCT may be preferable over a fully matched SCT in HLA-class II expressing hematological malignancies, making use of HLA-DP as a target for immunotherapy. After T-cell depleted URD-SCT, the administration of donor T-cells may induce an allo-HLA-DP specific immune response resulting in a potent GVL-effect without the risk of severe GVHD.

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