HLA-DP specific responses in allogeneic stem cell transplantation

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5 Patient HLA-DP specific CD4+ T-cells from HLA-DPB1 mismatched donor lymphocyte infusion can induce GVL-reactivity in the presence or absence of GVHD

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Abstract

Clinical studies have demonstrated that HLA-DPB1 mismatched stem cell transplantation (SCT) is associated with a decreased risk of disease relapse and an increased risk of graft versus host disease (GVHD) compared to HLA-DPB1 matched SCT. In T-cell depleted SCT, mismatching of HLA-DPB1 was not associated with an increased risk of severe GVHD, whereas a significant decreased risk of disease relapse was still observed. To investigate whether patient HLA-DP specific CD4+ T-cell responses were frequently induced after T-cell depleted HLA-DPB1 mismatched SCT and donor lymphocyte infusion (DLI), we developed a method to screen for the presence of HLA-DP specific CD4+ T-cells using CD137 as activation marker and analyzed 24 patient-donor combinations. Patients suffered from various B-cell malignancies, multiple myeloma and myeloid leukemias. In 13 out of 18 patients with a clinical response to DLI, patient HLA-DP specific CD4+ T-cells were detected after DLI, whereas these CD4+ T-cells were found in only 1 out of 6 patients without clinical responses to DLI. Eight patients developed significant GVHD. These data show that patient HLA-DP specific CD4+ T-cells frequently occur following HLA-DPB1 mismatched T-cell depleted SCT and DLI, and are associated with graft versus leukemia reactivity both in the presence and absence of GVHD.

Introduction

Following allogeneic hematopoietic stem cell transplantation (SCT), donor derived T-cells recognizing mismatched antigens on residual malignant cells can induce strong graft versus leukemia (GVL) reactions. Treatment of patients with relapsed leukemia, lymphoma or multiple myeloma with allogeneic hematopoietic SCT followed by donor lymphocyte infusion (DLI) can result in long lasting complete remissions.[1-6] Unfortunately, the beneficial GVL-effects of DLI are often accompanied by graft versus host disease (GVHD). To reduce the risk of GVHD, patient and donor are preferably matched for HLA-A, -B and -C, -DRB1 or also HLA-DQB1 (8/8 or 10/10 match).[7-9]

HLA-DPB1 is often not taken into consideration in donor selection since the overall mortality of patients who underwent HLA-DPB1 matched or mismatched SCT did not statistically differ. However, HLA-DPB1 matching status did have an impact on GVL-reactivity and GVHD.[8;10-13] In T-cell depleted SCT, mismatching for HLA-DPB1 has been associated with a significant decreased risk of disease relapse without an increased risk of severe GVHD.[11] The role of HLA-DP as transplantation antigen was confirmed by the isolation of polyclonal HLA-DP specific CD4+ T-cells from skin biopsies of patients with GVHD following HLA-DPB1 mismatched SCT.[14;15] We previously demonstrated a profound GVL-effect with only minimal skin GVHD caused by polyclonal HLA-DP specific CD4+ T-cells in a patient responding to HLA-DPB1 mismatched DLI for a refractory chronic B-cell leukemia, suggesting that HLA-DP specific CD4+ T-cells can be involved in GVHD, but also in selective GVL-reactivity.[5]

The beneficial effect of mismatching for HLA-DPB1 in T-cell depleted SCT on disease relapse has been reported to be more pronounced in acute lymphoblastic leukemia (ALL) compared to myeloid leukemias[16]. High expression of HLA-class-II molecules including HLA-DP is found on most B-ALL and B-chronic lymphocytic leukemia (CLL) cells, whereas myeloid leukemic cells show more variable expression of HLA-DP. However, HLA-DP expressing myeloid leukemic cells have been demonstrated to be recognized and lysed by HLA-DP specific CD4+ T-cells.[5;17] Both B-cell and myeloid hematological malignancies with sufficient HLA-DP expression may therefore be susceptible to an HLA-DP mediated GVL-effect.

The aim of this study was to investigate whether HLA-DP specific CD4+ T-cell responses frequently occur after HLA-DPB1 mismatched allo-SCT and DLI, and whether the development of patient HLA-DP specific CD4+ T-cell responses was associated with beneficial clinical responses (GVL-effect) or GVHD. We therefore analyzed HLA-DP specific immune responses and clinical responses in 24 patients after 10/10 matched, HLA-DPB1 mismatched T-cell depleted SCT who were treated with DLI.

To analyze the emergence of an allo-HLA-DP specific immune response after administration of DLI, we developed an assay to screen for allo-HLA-DP specific CD4+ T-cells. In this assay we used HLA-class-II negative HeLa-cells transduced with all molecules

relevant for HLA-class-II processing as stimulator cells and transduced patient or donor specific HLA-DP molecules into these cells to measure the emergence of patient HLA-DP specific CD4+ T-cells in peripheral blood (PB). Using these HLA-DP transduced HeLa-cells we introduced patient and donor HLA-DPB1 molecules as single variables to detect HLA-DP specific immune responses. Patient HLA-DP specific CD4+ T-cells were found in 72% of the patients in whom a clinical response was observed following DLI, but only in one out of six patients (17%) without a clinical response to DLI. Patient HLA-DP specific CD4+ T-cells were found in patients with GVHD and in patients with selective GVL-reactivity without GVHD. HLA-DP specific CD4+ T-cell responses were observed in patients suffering from B-cell malignancies, multiple myeloma and myeloid leukemias.

Materials and Methods

Cell collection and preparation

PB samples were obtained from patients and healthy stem cell donors after approval by the LUMC institutional review board and informed consent according to the Declaration of Helsinki. Samples were collected during standard follow-up following SCT and DLI (ie 6 weeks, 3 months and 6 months after DLI). In some individuals additional samples were available. Mononuclear cells (MNC) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable Epstein-Barr virus (EBV)-transformed B cell lines were generated using standard procedures. EBV-LCL and HeLa-cells were cultured in Iscove's modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, BioWhittaker).

Patient selection and characteristics

In total 24 patients who received a 10/10 matched, HLA-DPB1 mismatched T-cell depleted SCT followed by DLI between 2000 and 2008 were included in this study (Table 1). Patients suffered from multiple myeloma (n=8), B-cell malignancies (n=7) and myeloid leukemias (n=9). 15 patients received a single DLI and 9 patients received two or more DLIs. Indications for DLI included mixed chimerism, persistent or progressive disease and EBV-post-transplant-lymphoproliferative disorder. Leukocyte chimerism as determined by short tandem repeats was determined in bone marrow. In the absence of relapse or GVHD the level of mixed chimerism was stable in the first months after SCT. DLI was administered as intention to treat according to standard protocol. The first DLI was given between 4 months and 26 months after SCT. At 4 months 0.15*10e6 CD3+ T-cells were administered. At 6 months 1.5*10e6 CD3+ T-cells were given for mixed chimerism or persistent disease and 2.5*10e6 CD3+ T-cells for progressive disease. At 9 months 2.5*10e6 CD3+ T-cells were administered. Dose escalation to a maximum of 5*10e7 CD3+ T-cells was used in

patients receiving subsequent DLIs. The first DLI was freshly harvested and for subsequent DLIs cryopreserved material was used.

Flowcytometry

The monoclonal antibodies (moAb) anti-CD3 fluorescein isothiocyanate (FITC), anti-Nerve Growth Factor Receptor (NGFR)-phycoerythrin (PE), anti-CD4 Peridinin Chlorophyll Protein (PerCP), anti-CD137 allophycocyanin (APC) and anti-interferon-γ (IFN-γ)-APC were obtained from Becton Dickinson (BD, San Jose , CA, USA). Anti-CD154-PE was obtained from Beckman Coulter (Fullerton, CA, USA). Anti-HLA-DP-PE moAbs were purchased from Leinco Technologies (St. Louis, MO, USA). Flowcytometric analysis was performed on a BD flowcytometer. Cell sorting was performed using a BD FACSAria cell-sorting system.

Transduction with different HLA-DP constructs

HeLa-cells and EBV-LCL were transduced with different HLA-DPA1 and HLA-DPB1 molecules as described previously.[18] Briefly, HeLa-cells were transduced with CD80, HLA-DM and Invariant chain (HeLa-II cells) to allow appropriate costimulation and processing of HLA-DP molecules. Purified HeLa-II cells were subsequently transduced with different combinations of HLA-DPA1 and HLA-DPB1 molecules and selected based on positive staining with anti-HLA-DP-PE antibodies. HLA-DP transduced EBV-LCL were selected based on marker gene expression.

Characterization of HLA-DP specific CD4+ T-cells in PBMNC

To validate the use of HLA-DP transduced HeLa-II cells as stimulator cells to detect HLA-DP specific CD4+ T-cells in PB, we used PBMNC from a patient in whom we previously demonstrated a profound HLA-DPB1*03:01 specific immune response. To determine the optimal method to detect HLA-DP specific CD4+ T-cells, percentages of activated CD4+ T-cells using different activation markers after various incubation periods were compared. Cryopreserved PBMNC were thawed, and CD4+ T-cells were positively selected using magnetic CD4-beads (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. 0.1x10e6 purified CD4+ T-cells were stimulated with 0.03x10e6 control HeLa-II cells or cells transduced with patient HLA-DPB1*03:01 or donor HLA-DPB1*04:02 for 6 to 44 hrs in 150µl culture medium supplemented with 10% human serum and 10 IU/ml IL-2 (Chiron, Amsterdam, the Netherlands).

For intracellular IFN-γ and CD154 staining, cells were incubated in the presence of 10µg/ml Brefeldin-A (Sigma-Aldrich, St Louis, MO, USA), harvested after 6, 10 or 18 hrs of incubation, surface stained with CD4-FITC and CD3-PerCP-labeled MoAbs, fixed with 1% paraformaldehyde and permeabilised using 0.1% Saponin (Sigma-Aldrich). Next, cells were stained intracellularly with CD154-PE and IFN-γ-APC-labeled MoAbs and analyzed by flowcytometry.

Table 1. Patient characteristics

Patient #	Disease	DLI (months after SCT)	Indication DLI	HLA-DP specific CD4+ T-cells (%)	GVL/ GVM
1	Multiple myeloma	9	Persistent disease	0.30	Yes
2	Multiple myeloma	9	Mixed chimerism	0.29	Yes
3	Multiple myeloma	2	EBV-PTLD	0.17	Yes
4	Multiple myeloma	9	Persistent disease	0.43	Yes
5	MCL	4	Mixed chimerism	0.16 / 0.25	Yes
6	CLL	10	Progressive disease	4.50	N.I [1]
7	Multiple myeloma	9 36	Progressive disease Progressive disease	0.84 / 0.19 No	No No
8	AML	8	Mixed chimerism	0.15	No
9	CML	10	Molecular relapse	0.31	Yes
10	AML	4	Mixed chimersm	0.21	Yes
11	WM	7	Persistent disease	3.08 / 0.92	Yes
12	CML	26	Molecular relapse	0.17	Yes
13	AML	10 28 36	Mixed chimerism Mixed chimerism Mixed chimerism	0.19 No 0.15	No No Yes
14	Multiple myeloma	4 11 18 23 29	Persistent disease Persistent disease Progressive disease Progressive disease Progressive disease	No No - - No	No No No No Yes

aGVHD (weeks after DLI)	cGVHD (months after DLI)	Treatment for GVHD	Treatment prior to DLI	Remarks
Grade III (2)	No	Systemic	Thalidomide	Deceased 2 months after DLI due to GVHD
Grade I (9)	Extensive [4]	Systemic		
Grade IV (5)	Extensive [4]	Systemic	R-CHOP for PTLD	Measured during prednisone treatment for PTLD
Grade I (13)	Extensive [4]	Systemic		
Grade II (13)	No	Systemic		
Grade IV (5)	No	Systemic	R-CHOP and Radiotherapy	Deceased 2 months after DLI due to pulmonary distress possibly GVHD/ BOOP
Grade II (6) No	Extensive [4] No	Systemic	Radiotherapy	
Grade I (12)	Extensive [4]	Systemic		
No	No			
No	No			
Grade I (11)	No	Local		
No	No			
Grade I (9) No No	No No No	Local		
No No No No Grade I (9)	No	Local	Bortezomib Thalidomide Lenalidomide Cyclophosphamide + prednisone + Lenalidomide	IFN-α [3]

Table 1. Continued

Patient #	Disease	DLI (months after SCT)	Indication DLI	HLA-DP specific CD4+ T-cells (%)	GVL/ GVM
15	NHL	15	Mixed chimerism	No	No
		19	Mixed chimerism	No	Yes
16	CML	15	Molecular relapse	No	Yes
17	CML	15	Cytogenetic relapse	No	No
		20	Cytogenetic relapse	No	Yes
		61	Cytogenetic relapse	No	No
		75	Cytogenetic relapse	No	Late [2]
18	CML	20	Molecular relapse	No	Yes
		43	Molecular relapse	No	No
		49	Molecular relapse	No	No
	Lymphocyte blast crisis	53	Progressive disease	No	No
19	NHL	10	Mixed chimerism	No	No
		16	Mixed chimerism	No	No
20	B-ALL	6	Mixed chimerism	No	No
21	Multiple myeloma	6	Persistent disease	No	No
		14	Persistent disease	No	No
22	Multiple myeloma	6	Mixed chimerism	No	No
23	MDS/AML	7	Progressive disease	No	No
		10	Progressive disease	No	No
24	NHL	8	Progressive disease	0.36	No

GVM indicates graft versus myeloma; WM Waldenstrom macroglobulinemia; MCL Mantle cell lymphoma; CLL, Chronic lymphocytic leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; EBV-PTLD, Ebstein-Barr virus-induced posttransplantation lymphoproliferative disease; R-CHOP, Rituximab-Cyclophosphamide Doxorubicin Vincristine Prednisolone; BOOP, Bronchiolitis obliterans with organizing pneumonia; and AIHA, Auto Immune Hemolytic Anemia.

[1] N.I. indicates Not Interpretable; patient died due to multi organ failure before follow-up analysis was performed

[2] Late indicates response observed > 6 months after DLI

[3] IFN-α indicates IFN-α given at time of DLI to enhance immune response

[4] acute GVHD followed by chronic GVHD

aGVHD (weeks after DLI)	cGVHD (months after DLI)	Treatment for GVHD	Treatment prior to DLI	Remarks
No	No		Rituximab 4x + prednisone	For AIHA and thrombocytopenia
No	No			
No	Limited (7)	Local		
No	No			
Grade I (9)	No	No		IFN-α [3]
No	No			IFN-α [3]
No	No			
No	No			
No	No			
No	No			
No	Limited (6)	No	Polychemotherapy + Imatinib	IFN-α [3]
No	No			
No	No			
No	Limited (6)	Local		
No	No			
No	No			
No	No			
No	No			IFN-α [3]
No	No			
No	Limited (3)	Local	Rituximab	Deceased 4 months after DLI due to progressive disease

To determine the expression of surface CD154 and CD137, cells were harvested after 16, 22 or 44 hrs of incubation, washed, surface stained with CD4-FITC, CD3-PerCP, CD154-PE and CD137-APC-labeled MoAbs and analyzed.

To confirm specificity of CD137 expressing CD4+ T-cells, 0.75x10e6 purified CD4+ T-cells were stimulated with 0.15x10e6 HLA-DPB1*03:01 transduced HeLa-II cells and after 44 hrs of incubation, CD137 expressing CD4+ T-cells were sorted single cell per well into U-bottom microtiter plates (Greiner Bio-One, the Netherlands). Proliferating CD4+ T-cell clones were expanded using nonspecific stimulation and third-party feeder cells. IFN-γ production in response to HLA-DPB1*03:01 or HLA-DPB1*04:02 transduced HeLa-II cells, donor EBV-LCL or HLA-DPB1*03:01 expressing EBV-LCL was tested. To determine IFN-γ production, 5 000 CD4+ T-cells were cocultured with 30 000 stimulator cells in 150µl medium. After overnight incubation, supernatants were harvested and IFN-γ production was measured by enzyme-linked immunosorbent assay (ELISA; CLB, Amsterdam, the Netherlands).

Analysis of HLA-DP specific CD4+ T-cells after allo-SCT and DLI in 24 patients

To quantify the presence of HLA-DP specific CD4+ T-cells in PBMNC, CD4+ T-cells derived from donors and from patients after SCT before and after DLI were analyzed for the expression of CD137 in response to stimulation with patient or donor HLA-DPB1 molecules. Samples obtained 6 weeks to 6 months after DLI were analyzed according to availability and moment of clinical response. CD4+ T-cells were positively selected from PBMNC using magnetic CD4-beads (Miltenyi Biotec) according to the manufacturer's instructions. 0.15x10e6 CD4+ T-cells were stimulated with 0.03x10e6 HeLa-II cells, HeLa-II cells transduced with donor or shared HLA-DPB1 molecules or HeLa-II cells transduced with patient specific HLA-DPB1 molecules in 150µl IMDM supplemented with 10% human serum and 10 IU/ml IL-2. After 44 hrs of incubation, CD137 expression on CD3+/CD4+ T-cells was analyzed using flowcytometry.

Clinical responses following HLA-DPB1 mismatched T-cell depleted allo-SCT and DLI

Clinical responses to DLI were defined as beneficial clinical responses or GVHD. Beneficial clinical responses were defined as a sustained increase in donor chimerism to ≥ 99% donor cells, or a decrease of malignant cells or disease marker resulting in either complete disappearance or a reduction of more than 90%. Clinically important GVHD was considered present in case of acute GVHD (aGVHD) grade 2 or more or extensive chronic GVHD (cGVHD). aGVHD was graded according to Glucksberg criteria and cGVHD according to Shulman criteria. Lesions of the mouth matching criteria for cGVHD in the absence of other signs of cGVHD were classified as limited cGVHD. Clinical data were analyzed by a hematologist who was not informed of the presence or absence of HLA-DP specific CD4+ T-cells.

Statistical analysis

To determine whether there was a significant association between the presence of patient HLA-DP specific CD4+ T-cells and clinical responses two tailed Fisher's exact test was used. To determine whether there was a statistically significant difference in magnitude between T-cell responses directed against permissive compared tot non-permissive mismatches, Student's T-test after logarithmic transformation was used.

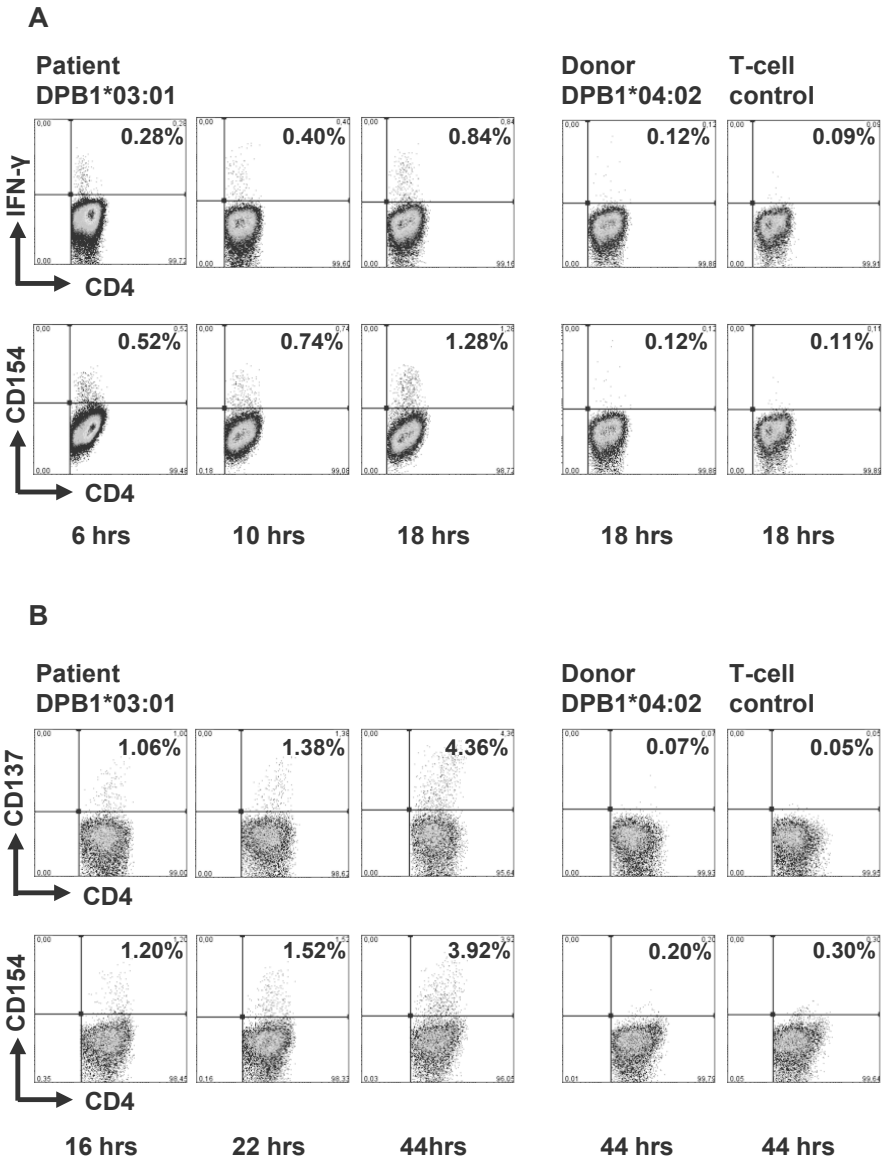
Results

Detection and isolation of HLA-DP specific T-cells

To allow quantitative analysis of patient specific allo-HLA-DP responses in patients treated with HLA-DPB1 mismatched T-cell depleted allo-SCT and DLI, we developed a method to screen for patient HLA-DP specific CD4+ T-cells in PB using HLA-class-II negative HeLa-cells transduced with all molecules relevant for HLA-class-II processing and different HLA-DP molecules as stimulator cells. Transduction of the same stimulator cells with patient or donor specific HLA-DP molecules as single variables allowed detailed analysis of patient HLA-DP specific CD4+ T-cell responses.

To validate the use of HLA-DP transduced HeLa-II cells as stimulator cells to detect HLA-DP specific CD4+ T-cells in PB, we used PBMNC from a patient in whom we previously demonstrated a profound HLA-DPB1*03:01 specific CD4+ T-cell response. This patient was transplanted with a 10/10 matched, HLA-DPB1 mismatched SCT followed by DLI for relapsed chronic B-cell leukemia.[5] Purified CD4+ T-cells isolated from PBMNC from the peak of the immune response 14 weeks after DLI were stimulated with HeLa-II cells transduced with patient HLA-DPB1*03:01 or donor HLA-DPB1*04:02. We compared the percentages of activated CD4+ T-cells using different markers after various incubation periods. Specific activation of CD4+ T-cells was found after stimulation with HeLa-II cells transduced with patient HLA-DPB1*03:01, but not with donor HLA-DPB1*04:02, indicating that HLA-DP transduced HeLa-II cells can be used as stimulator cells to detect allo-HLA-DP specific CD4+ T-cells (Figure 1). Upon stimulation with patient HLA-DPB1*03:01, maximum intracellular IFN-γ and CD154 staining was found after overnight incubation showing 0.84% and 1.28% activated CD4+ T-cells, respectively. Stimulation with donor specific HLA-DPB1*04:02 did not result in activated CD4+ T-cells as compared to background staining (0.12% for both IFN-γ and CD154 expression) (Figure 1A). After stimulation with patient HLA-DPB1*03:01, maximum surface CD137 or CD154 expression was found after 44 hrs of incubation, resulting in 4.36% and 3.92% activated CD4+ T-cells respectively. Stimulation with donor HLA-DPB1*04:02 resulted again in only background CD137 (0.07%) and CD154 (0.20%) expression (Figure 1B).

Analysis of CD137 expression after different incubation times was repeated in independent experiments with similar results (Figure 1C). Based on these results and favorable kinetics of CD137 expression on CD4+ T-cells shown by us and others[19-21], CD137 was selected as activation marker to perform further experiments.



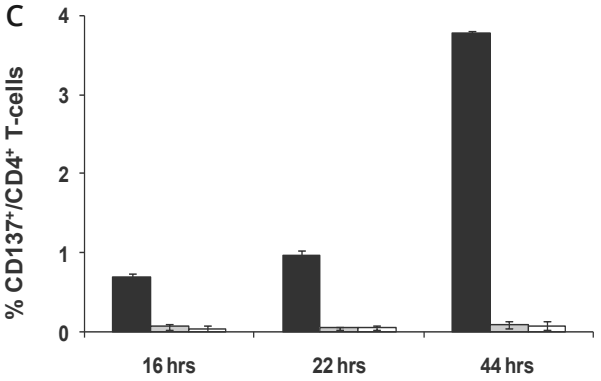


Figure 1. Kinetics of different activation markers to detect patient HLA-DP specific CD4+ T-cells directly ex vivo. PBMNC from a patient (HLA-DPB1*02:01,03:01) transplanted for a chronic B cell leukemia with an HLA-DPB1 mismatched donor (HLA-DPB1*04:02,05:01) were analyzed for the presence of HLA-DPB1*03:01 specific CD4+ T-cells. Purified CD4+ T-cells obtained during the clinical response to DLI were stimulated with HeLa-II cells transduced with donor or patient specific HLA-DPB1 molecules. (A) After 6 - 18 hrs of incubation, intacellular IFN-γ (upper panel) or CD154 (lower panel) staining was determined by flowcytometry. (B) After 16 – 44 hrs of incubation, surface CD137 (upper panel) and CD154 (lower panel) expression was determined by flowcytometry. (C) Percentages of CD137 expressing CD4+ T-cells after incubation with either patient HLA-DPB1*03:01 (■), donor HLA-DPB1*04:02 (■) or medium alone (□) was determined at different time points. Mean results ± SD of 2-4 individual experiments are shown

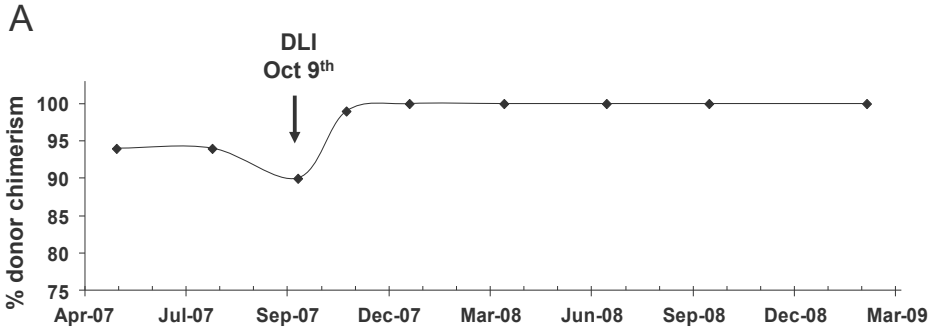
To confirm the specificity of CD137 expressing CD4+ T-cells for patient HLA-DPB1*03:01, we clonally isolated CD137+/CD4+ T-cells by flowcytometry, and tested growing T-cell clones for specific recognition of HLA-DPB1*03:01. A total number of 46 CD4+ T-cell clones were expanded, and 41 (89%) of these CD4+ T-cell clones showed specific recognition of both HeLa-II cells transduced with HLA-DPB1*03:01 as well as EBV-LCL with endogenous HLA-DPB1*03:01 expression, whereas HeLa-II cells transduced with donor HLA-DPB1*04:02 and EBV-LCL with endogenous HLA-DPB1*04:02 were not recognized. These results confirmed specificity of CD137 expressing CD4+ T-cells for the allo-HLA-DPB1*03:01 molecule, and confirmed that this method can be used to measure HLA-DP specific CD4+ T-cells directly ex vivo.

Patient HLA-DP specific CD4+ T-cells are frequently detected following T-cell depleted HLA-DPB1 mismatched SCT and DLI

To analyze the presence of HLA-DP-specific CD4+ T-cell responses after HLA-DPB1 mismatched allo-SCT and DLI, we analyzed immune responses in 24 patients. All patients

received a T-cell depleted HLA-A, -B, -C, -DRB1, -DQB1 matched, HLA-DPB1 mismatched allo-SCT. Patients received DLI for various indications, including mixed chimerism, persistent or progressive disease (Table 1). CD4+ T-cells were obtained from donors, and from patients after SCT, before and after DLI. For each patient-donor combination, purified CD4+ T-cells were stimulated with control HeLa-II cells, and with HeLa-II cells transduced with donor specific, shared or patient specific HLA-DPB1 molecules. After 44 hrs of incubation CD137 expression on CD4+ T-cells was determined.

Figure 2 shows a representative example of the analysis of HLA-DP specific CD4+ T-cells in a patient with a clinical response to DLI. This patient with multiple myeloma was typed HLA-DPB1*01:01,04:01 and the donor was typed HLA-DPB1*04:01,05:01. The patient received a single dose of 2.5x10e6 CD3+ T-cells for mixed chimerism 9 months after SCT resulting in conversion to 100% donor chimerism 3 month later (Figure 2A). Stimulation of donor derived CD4+ T-cells or CD4+ T-cells derived from the patient after SCT before DLI with HeLa-II cells transduced with donor HLA-DPB1*05:01, shared HLA-DPB1*04:01 or patient specific HLA-DPB1*01:01 molecules did not result in significant CD137 expression (0.01%-0.07%). CD4+ T-cells obtained 6 weeks after DLI, however, showed 0.3% CD137+/CD4+ T-cells upon stimulation with patient HLA-DPB1*01:01, whereas no increase in CD137+/CD4+ T-cells was found in response to shared or donor specific HLA-DPB1 molecules (Figure 2B). These results illustrate the emergence of HLA-DPB1*01:01 specific CD4+ T-cells in this patient 6 weeks after DLI.



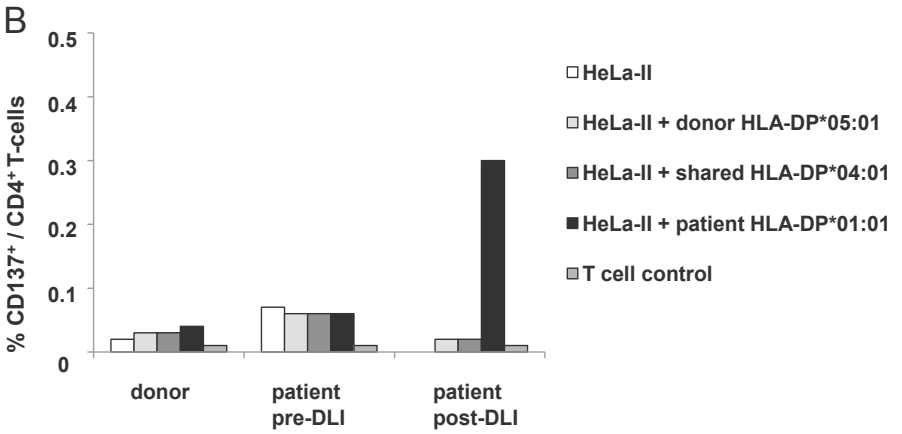


Figure 2. Emergence of patient HLA-DP specific CD4+ T-cells following HLA-DPB1 mismatched allo-SCT and DLI. (A) Percentage of donor chimerism in bone marrow after SCT. Arrow indicated DLI. Conversion to 100% donor chimerism was observed 3 months after DLI. (B) CD4+ T-cells purified from donor PBMNC, patient PBMNC obtained after SCT before DLI, and 6 weeks after DLI were stimulated with HeLa-II cells, HeLa-II cells transduced with donor HLA-DPB1*05:01 or shared HLA-DPB1*04:01, HeLa-II cells transduced with patient HLA-DPB1*01:01 or left unstimulated. Percentages of CD137 expressing CD4+ T-cells are shown after 44 hrs of incubation.

Using this method, we screened the 24 patients for the presence of patient HLA-DP specific CD4+ T-cell responses. HLA-DP specific immune responses were defined as detection of >0.15% CD137+/CD4+ T-cells after stimulation with HeLa-II cells transduced with patient specific HLA-DPB1 molecules but not in response to donor or shared HLA-DPB1 molecules. The cutoff of 0.15% was defined as a positive response based on background CD137 expression in response to HeLa-II cells or HeLa-II cells transduced with donor HLA-DPB1 molecules of less than 0.10% in all cases. Of the 24 patients, 9 individuals exhibited 2 patient specific HLA-DPB1 alleles different from the donor and 15 patients had only one patient specific HLA-DPB1 allele. In figure 3, percentages of CD137+/CD4+ T-cells for these 24 patients in response to stimulation with the 33 patient specific HLA-DPB1 molecules and 39 donor specific or shared HLA-DPB1 molecules are shown.

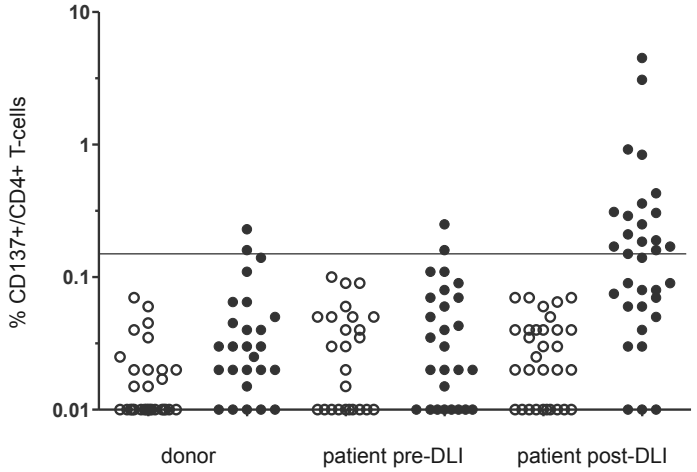


Figure 3. HLA-DP specific CD137+/CD4+ T-cells observed after HLA-DPB1 mismatched allo-SCT and DLI in 24 individuals. A total number of 24 patient-donor combinations were analyzed for the presence of patient HLA-DP specific CD4+ T-cells. In total, percentages of CD137+/CD4+ T-cells in response to 39 donor or shared HLA-DPB1 molecules and 33 patient specific HLA-DPB1 molecules are shown. For each patient-donor combination, CD4+ T-cells purified from donor PBMNC and patient PBMNC obtained after SCT before DLI and after DLI were stimulated with HeLa-II cells transduced with donor or shared HLA-DPB1 molecules (○) or HeLa-II cells transduced with patient specific HLA-DPB1 molecules (●). Percentages of CD137+/CD4+ T-cells are shown after 44 hrs of co-incubation with HeLa-II cells. Results were corrected for background CD137 expression on unstimulated CD4+ T-cells. For each patient-donor combination, representative results obtained after only one DLI are shown. HLA-DP specific immune responses were defined as detection of >0.15% CD137+/CD4+ T-cells after stimulation with HeLa-II cells transduced with patient specific HLA-DPB1 molecules based on <0.10% CD137+/CD4+ T-cells in response to donor specific or shared HLA-DPB1 molecules. Line represents the threshold of 0.15% for positive results. Results post-DLI represent the first DLI except for patients 14, 15 and 17. In these patients results to the first DLI with a clinical response are shown.

In 14 individuals, patient HLA-DP specific CD4+ T-cells were detected after DLI. Specific CD137+/CD4+ T-cells varying between 0.15% and 4.5% were measured after stimulation with HeLa-II cells transduced with patient HLA-DPB1 molecules but not in response to donor HLA-DPB1, including shared HLA-DPB1 molecules (0 – 0.07%). Patient HLA-DP specific CD4+ T-cells were determined in 9 of the 15 patients with a single HLA-DPB1 mismatch, and in 5 of the 9 individuals with two HLA-DPB1 mismatches. Of the latter 5 patients, 3 patients showed CD4+ T-cell responses to both mismatched HLA-DPB1 alleles.

In 12 of the 14 patients with detectable HLA-DP specific CD4+ T-cells, patient HLA-DP specific immune responses were likely to be induced by DLI, since CD137+/CD4+ T-cells were not found before DLI (0% - 0.09%). In the remaining 2 patients (patient 8 and 9), low percentages of HLA-DP specific CD4+ T-cells were also measured after SCT before DLI (0.16 and 0.25%).

In summary, in 14 out of 24 (58%) patient-donor combinations analyzed patient HLA-DP specific CD4+/CD137+ T-cells were found, indicating that HLA-DP specific CD4+ T-cell responses frequently occurred after HLA-DPB1 mismatched allo-SCT and DLI.

Clinical responses following HLA-DPB1 mismatched T-cell depleted allo-SCT and DLI

We evaluated whether the presence of patient HLA-DP specific CD4+ T-cells following HLA-DPB1 mismatched T-cell depleted allo-SCT and DLI was related to clinical outcome. Clinical responses to DLI were defined as beneficial clinical responses or GVHD. In 18 of 24 patients (75%), clinical responses to at least one of the DLIs were observed. In 5 patients (Table 1: patients 1-5) both a beneficial clinical response and GVHD were observed. 3 patients (patients 6-8) developed GVHD without a beneficial clinical response. In 10 patients (patients 9-18) selective beneficial responses without GVHD were observed. The 6 remaining patients (patients 19-24) did not show any clinical response to DLI.

In figure 4A, percentages of patient HLA-DP specific CD137+/CD4+ T-cells are shown for patients with and without clinical responses after DLI. Patient HLA-DP specific CD4+ T-cells were observed in 13 out of 18 patients (72%) with clinical responses after DLI, and in only one of 6 patients (17%) without clinical responses following DLI. The latter patient (patient 24) suffered from lymphoma and died due to progressive disease 4 months after DLI. These results illustrate that the presence of patient HLA-DP specific CD4+ T-cells was associated with development of clinical responses after DLI (p=0.05).

In figure 4B, percentages of patient HLA-DP specific CD137+/CD4+ T-cells are shown for patients with GVHD after DLI and for patients who developed selective beneficial clinical responses. CD137+/CD4+ T-cells were detected in all patients with GVHD (patients 1-8), and in 5 out of 10 patients with selective beneficial clinical responses (patients 9-13). These data illustrate an association between the presence of patient HLA-DP specific CD4+ T-cells and development of clinical significant GVHD (p=0.036). However, patient HLA-DP specific CD4+ T-cells were also found in patients with selective GVL-responses.

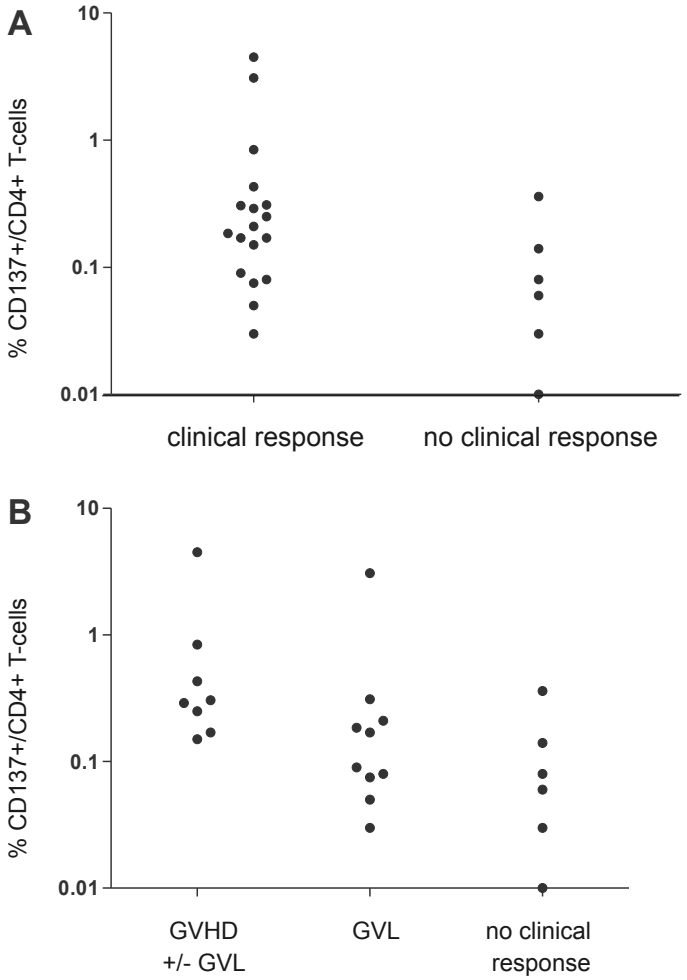


Figure 4. HLA-DP specific CD137+/CD4+ T-cells in patients with and without clinical responses to DLI. Percentage of CD137+/CD4+ T-cells in response to stimulation with HeLa-II cells transduced with patient HLA-DPB1 molecules obtained after allo-SCT and DLI are shown. Results were corrected for background CD137 expression on unstimulated CD4+ T-cells. For 9 patients who were screened for CD4+ T-cells specific for 2 different patient specific HLA-DPB1 molecules, results for only one HLA-DPB1 molecule with the highest percentage of CD137+/CD4+ T-cells are depicted. (A) Patient HLA-DP specific CD4+ T-cells were found in 13 out of 18 patients with clinical responses to DLI, and in one out of six patients without clinical response to DLI. (B) CD137+/CD4+ T-cells were found in all 8 patients with GvHD (in presence or absence of GVL), in 5 out of 10 patients who developed beneficial clinical responses without GVHD (selective GVL-reactivity). CD137+/CD4+ T-cells were detected in only one patient without clinical response to DLI.

Permissive and non-permissive HLA-DPB1 mismatches

To determine whether patient HLA-DP specific CD4+ T-cells were preferentially found in non-permissive mismatch combinations compared to permissive mismatch combinations[19;22], we analyzed the percentages of CD137+/CD4+ T-cells found after SCT and DLI in these two different groups. In 9 patients two mismatch HLA-DPB1 alleles were present in GVH direction. Each mismatched HLA-DPB1 allele was analyzed separately. (Supplemental Table S1)

In total, 19 permissive HLA-DPB1 mismatches and 14 non-permissive HLA-DPB1 mismatches were present. Figure 5 shows the percentages of CD137+/CD4+ T-cells for each category. Patient HLA-DP specific CD4+ T-cells were found in both permissive and non-permissive mismatched combinations, showing a slightly higher median percentage in the non-permissive group (p=0.22).

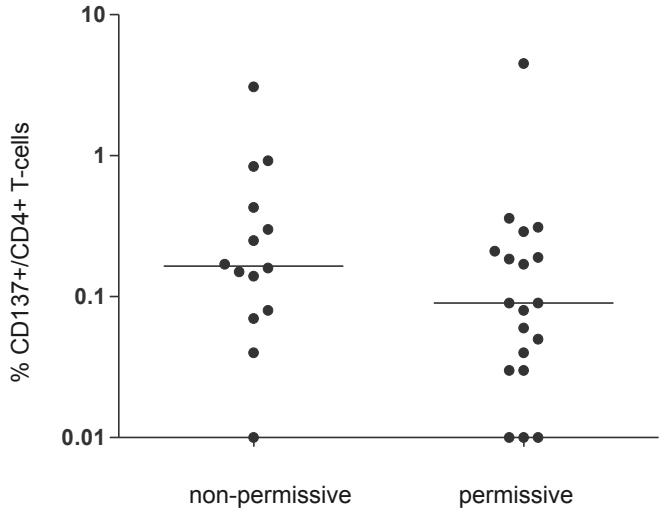


Figure 5. HLA-DP specific CD137+/CD4+ T-cells in response to permissive and non-permissive mismatches Percentage of CD137+/CD4+ T-cells in response to stimulation with HeLa-II cells transduced with patient HLA-DPB1 molecules obtained after allo-SCT and DLI are shown. Results were corrected for background CD137 expression on unstimulated CD4+ T-cells. CD137+/CD4+ T-cell responses to each HLA-DPB1 allele are depicted separately. The modified algorithm classifying HLA-DPB1 alleles in 4 categories was used.[19] Results of non-permissive and permissive HLA-DPB1 mismatch combinations are shown. Horizontal line represents median values.

Discussion

In this study, we developed an assay to screen patients for HLA-DP specific immune responses following HLA-DPB1 mismatched T-cell depleted SCT and DLI. Using HeLa-cells transduced with various genes encoding relevant molecules for appropriate HLA-class-II processing in conjunction with different HLA-DP molecules as stimulator cells, we were able to measure the emergence of patient HLA-DP specific CD4+ T-cells in PB in a simple and effective manner. Patient HLA-DP specific CD4+ T-cell responses were frequently found following HLA-DPB1 mismatched DLI (58%). The presence of HLA-DP specific CD4+ T-cells correlated with clinical responses to DLI, since patient HLA-DP specific CD4+ T-cells were measured in 13 (72%) out of 18 patients with clinical responses to DLI, and in only one (17%) out of 6 patients without clinical responses (p=0.05). HLA-DP specific CD4+ T-cells were most dominantly found in patients who developed GVHD, but patient HLA-DP specific CD4+ T-cells were also found in a significant number of patients who developed beneficial clinical responses without GVHD after DLI. HLA-DP specific CD4+ T-cell responses were induced in patients suffering from a variety of hematological malignancies, including multiple myeloma, B-cell malignancies and myeloid leukemias.

Since alloreactive T-cells recognizing mismatched HLA-alleles are present in relatively high frequencies in PB[23], HLA-mismatched SCT is likely to frequently induce alloimmune responses. Our study indeed demonstrates that mismatching for HLA-DPB1 frequently resulted in the generation of patient HLA-DP specific immune responses. In the majority of patients, HLA-DP specific CD4+ T-cells were not found in patient PBMNC obtained prior to DLI, indicating de novo induction of HLA-DP specific T-cell responses by DLI. Moreover, the high frequencies of patient HLA-DP specific T-cells after DLI reflected in vivo development and expansion of an anti-HLA-DP response in the patients, since in only two cases low frequencies of patient HLA-DP specific CD4+ T-cells could directly be detected in donor PBMNC.

In this study, GVHD was associated with the presence of patient HLA-DP specific CD4+ T-cells. In the majority of patients, GVHD coincided with a beneficial clinical response. Patient HLA-DP specific CD4+ T-cells were, however, also found in patients with selective GVL-reactivity without GVHD, illustrating that HLA-DP specific CD4+ T-cells may induce GVL-responses in the presence or absence of GVHD.

HLA-DPB1 mismatched SCT may induce selective GVL-reactivity without GVHD since constitutive expression of HLA-class-II molecules is mainly restricted to hematopoietic cells. However, HLA-class-II expression on various tissues can be upregulated after exposure to pro-inflammatory cytokines. An HLA-DP specific immune response may result in GVHD if HLA-class-II molecules on non-hematopoietic tissues are upregulated as a consequence of conditioning regimens, infections or ongoing immune responses. In some patients with GVHD in this study, a pro-inflammatory environment may have

been induced by chemotherapy or radiotherapy administered prior to DLI. Alternatively, a profound HLA-DP specific GVL-response may induce pro-inflammatory cytokines, resulting in upregulation of HLA-class-II molecules on non-hematopoietic cells, thereby inducing GVHD. Furthermore, both GVHD and GVL-reactivity may have been mediated by the simultaneous development of immune responses directed against other molecules than HLA-DP. An inflammatory environment induced by minor histocompatibility antigen (MiHA) specific CD4+ or CD8+ T-cell responses[4;6;24-27] may have provoked or enhanced HLA-DP specific immune responses. Since HLA-DP specific CD4+ T-cells were found both in the presence and absence of GVHD, it is likely that the development of GVHD is not solely determined by the induction of an HLA-DP specific immune response, but also by other immune responses and factors influencing HLA-class-II expression on non-hematopoietic cells.

Antigen presenting cells (APC) are required for efficient induction of anti-tumor immunity in vivo. In this study, HLA-DP specific CD4+ T-cells were found in patients with relapsed or persistent disease as well as in patients who received DLI for mixed chimerism. In patients with relapsed or persistent disease, malignant cells with sufficient HLA-DP expression may have served as APC to induce HLA-DP specific immune responses. Alternatively, crosstalk between CD4+ T-cells and leukemic cells may have induced an APC phenotype on leukemic cells, thereby amplifying HLA-class-II specific immune responses.[28] In other patients, residual patient derived hematopoietic cells may have served as APC to induce HLA-DP specific CD4+ T-cell responses. Previous in vitro studies have demonstrated that HLA-DP specific CD4+ T-cells were capable of recognizing and lysing multiple myeloma cells, B-cell malignancies and myeloid leukemic cells with sufficient HLA-DP expression.[5;17;29;30] Based on these in vitro experiments, HLA-DP specific CD4+ T-cells are expected to induce anti-tumor reactivity against a variety of hematological malignancies in-vivo. Indeed, in our study beneficial clinical responses after HLA-DPB1 mismatched alloSCT and DLI were found in patients suffering from multiple myeloma, various B-cell malignancies, and myeloid leukemia.

In this study, 6 patients did not respond to DLI. Two patients had progressive disease at the time of DLI. In one of these patients HLA-DP specific CD4+ T-cells were found, but this patient died too early to benefit from the treatment. The other patient suffered from AML and immunotolerizing effects may have hampered the induction of an immune response. In 3 patients, DLI was administered at 99% donor chimerism. It is likely that in these patients numbers of patient derived HLA-class-II expressing APC were insufficient to induce an HLA-DP specific immune response. Accordingly, levels of patient chimerism did not change in time, reflecting a stable clinical situation in these patients. In the last patient mixed chimerism did not convert to 100% donor chimerism within 6 months after DLI.

In 5 patients with a selective beneficial response to DLI, no HLA-DP specific CD4+ T-cells were found in samples available for analysis. Two patients (patients 14-15) received other treatments in the period that DLI was given. One patient received several drugs for multiple myeloma in combination with multiple DLIs, finally resulting in a sustained complete remission. The other patient received Rituximab and prednisone for repeated episodes of autoimmune hemolytic anemia and thrombocytopenia. In both patients the specific contribution of each drug or treatment to the clinical response could not be identified and the other treatments are likely to have hampered the analysis of HLA-DP specific CD4+ T-cells.

The other 3 individuals (patients 16-18) were treated with DLI for minimal residual disease as illustrated by molecular or cytogenetic but no hematological relapse. By clonal isolation of activated CD4+ T-cells we could demonstrate low numbers of patient HLA-DP specific CD4+ T-cells in all 3 patients. (4%-20% of expanded CD4+ T-cell clones, data not shown). In these patients overall percentages of patient HLA-DP specific CD4+ T-cells were below the threshold of sensitivity of our screening method (0.10%). Since we could not unequivocally demonstrate that these HLA-DP specific CD4+ T-cells contributed to clinical responses we did not score them as positive results. However, even considering these responses as negative, statistical analysis revealed a significant correlation between the presence of HLA-DP specific CD4+ T-cells and development of clinical responses. Alternatively, we may have missed a significant response if PBMC were not collected at the optimal moment during immune responses. For MiHA specific CD8+ T-cells, we previously demonstrated that peak responses may not last more than 2 weeks.[4;6;31;32] Kinetics of CD4+ T-cells are more difficult to study due to lack of HLA-class-II tetramers, but several studies showed long term persistence of alloreactive CD4+ T-cells.[5;27;33] Alternatively, in some patients there may have been immune responses directed towards other mismatched antigens, including MiHAs. In two of these patients, we indeed successfully isolated low frequencies of MiHA specific CD8+ T-cell clones after DLI (data not shown).

In conclusion, in this study we show that HLA-DP specific CD4+ T-cells were frequently present in patients treated with T-cell depleted HLA-DPB1 mismatched allo-SCT and DLI for various hematological malignancies. HLA-DP specific CD4+ T-cells were found in patients with beneficial clinical responses both in the presence and absence of GVHD. It is likely that local environmental circumstances and induction of other immune responses may determine the balance between GVHD and GVL-reactivity in each individual. To investigate more specifically the effect of HLA-class-II mediated immune responses on GVL-reactivity and GVHD, a randomized clinical study is presently ongoing in our center to analyze the effect of purified CD4+ DLI early after transplantation on donor-chimerism, disease relapse and GVHD.

Acknowledgments

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Supplementary Table S1. Permissive and Non-Permissive HLA-DPB1 mismatches in GVH direction

Patient #	Donor HLA-DPB1*		Patient HLA-DPB1*		Permissive / Non-Permissive		DP-specific CD4+ T-cells	% HLA-DP specific CD4+ T-cells		Clinical response
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2		Allele 1	Allele 2	
1	04:01	04:02	03:01	04:01	NP	shared	Yes	0.30		Yes
2	04:01	05:01	01:01	04:01	P	shared	Yes	0.29		Yes
3	03:01	04:02	04:01		P		Yes	0.17		Yes
4	04:01		03:01		NP		Yes	0.43		Yes
5	01:01	13:01	02:01	10:01	NP	NP	Yes	0.16	0.25	Yes
6	01:01	03:01	02:01	03:01	P	shared	Yes	4.5		Yes
7	02:01		04:02	10:01	P	NP	Yes	0.19	0.84	Yes
8	04:01		03:01	04:01	NP	shared	Yes	0.15		Yes
9	04:02		04:02	06:01	shared	P	Yes		0.31	Yes
10	02:01	04:01	03:01	13:01	NP	P	Yes	0.07	0.21	Yes
11	04:02	05:01	02:01	03:01	NP	NP	Yes	0.92	3.08	Yes
12	01:01	02:01	03:01	04:01	NP	P	Yes	0.17	0.09	Yes
13	02:01	04:01	04:01	05:01	shared	P	Yes		0.19	Yes
14	04:01		04:01	01:01	shared	P	No		0.08	Yes
15	04:01		04:01	04:02	shared	P	No		0.09	Yes
16	02:01		03:01	04:01	NP	P	No	0.08	0.01	Yes
17	03:01	04:02	02:01		P		No	0.03		Yes

18	03:01	04:01	02:01	03:01	P	shared	No	0.05		Yes
19	04:02	11:01	02:01	04:01	NP	P	No	0	0	No
20	02:01	13:01	03:01	04:01	NP	P	No	0.08	0.06	No
21	02:01		01:01	04:02	P	P	No	0.04	0.06	No
22	02:01	04:01	03:01	04:01	NP	shared	No	0.14		No
23	02:01	03:01	03:01	04:01	shared	P	No		0.03	No
24	04:01		04:01	04:02	shared	P	Yes		0.36	No

NP = non-permissive mismatch
P = permissive mismatch