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HLA-DP specific responses in allogeneic stem cell transplantation

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4 HLA-DPB1 mismatching results in the generation of a full repertoire of HLA-DPB1 specific CD4+ T-cell responses showing immunogenicity of all HLA-DPB1 alleles

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Abstract

Clinical studies have indicated that HLA-DP functions as a classical transplantation antigen in allogeneic stem cell transplantation. Mismatching for HLA-DPB1 was associated with an increased risk of graft versus host disease (GVHD) but also a decreased risk of disease relapse. However, specific HLA-DPB1 mismatches were associated with poor clinical outcome. It was suggested that this unfavorable effect was caused by a difference in immunogenicity between HLA-DPB1 alleles. To analyze whether immunogenicity of HLA-DPB1 mismatches could be predicted based on the presence or absence of specific amino acid sequences we developed a model to generate allo-HLA-DP responses in vitro. We tested in total 48 different stimulator/responder combinations by stimulating CD4+ T-cells from 5 HLA-DPB1 homozygous individuals with the same antigen presenting cells transduced with different allo-HLA-DP molecules. HLA-DPB1 molecules used for stimulation comprised 76-99% of HLA-DPB1 molecules present in different ethnic populations. We show that all HLA-DPB1 mismatches as defined by allele typing resulted in high frequency immune responses. Furthermore, we show that cross-recognition of different HLA-DPB1 molecules is a broadly observed phenomenon. We confirm previous described patterns in cross-recognition, and demonstrate that a high degree in similarity between HLA-DPB1 molecules is predictive for cross-recognition but not for immunogenicity.

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is an established treatment for a variety of hematological malignancies.[1] A significant part of the therapeutic effect of allo-SCT can be attributed to donor derived T-cells recognizing antigens on residual malignant cells thereby inducing a graft versus leukemia (GVL) effect.[2] When such antigens are not only present on hematopoietic cells but also on non-hematopoietic cells, donor derived T-cells can also mediate detrimental graft versus host disease (GVHD).[3,4] Since allo-reactive T-cells recognizing HLA-mismatched alleles are present in high frequencies in peripheral blood (PB)[5], HLA-mismatched SCT may result in strong allo-immunity. To reduce the risk of GVHD and allograft rejection, patient and donor are preferably matched for the HLA class I molecules HLA-A, -B, -C and the HLA class II molecules HLA-DRB1 and HLA-DQB1 (10/10 match).[6-8]

However, a perfectly matched donor is not always available. Several studies have demonstrated the importance of functional matching in order to select the best available donor. It has been suggested that mismatches at some loci may be better tolerated than others.[9-11] Minimal mismatches resulting in single amino acid substitutions have been associated with a significant increased risk of GVHD and transplant related mortality (TRM).[12,13] In contrast, highly diverged HLA class I mismatches with more than 5 amino acid substitutions in both the α-helix and β-sheet of an HLA class I molecule in combination with a negative cytotoxic T-lymphocyte precursor test have been shown to be better tolerated than less diverged mismatches.[14] These studies indicated that the number of amino acid substitutions between mismatched HLA-alleles was not predictable for the outcome of alloreactivity.

HLA-DPB1 is often not taken into consideration in donor selection. Recent large clinical studies showed that HLA-DP did function as a classical transplantation antigen since HLA-DPB1 mismatching was associated with both GVHD and GVL-reactivity.[6,15] The overall mortality of patients who received an HLA-DPB1 matched or mismatched SCT did not statistically differ, possibly due to a balanced effect of increased GVHD and reduced relapse rate. Remarkably, specific HLA-DPB1 mismatches have been associated with poor clinical outcome.[16-21] It was suggested that matching at an epitope level may be clinically more relevant in terms of transplant outcome than matching at allele level. An algorithm was developed in which permissive and non-permissive mismatches were defined.[19] HLA-DPB1 molecules were classified in 3 different immunogenicity groups according to their recognition by HLA-DPB1*09 specific CD4+ T-cell clones. Recently, the classification was modified introducing a fourth category in the algorithm.[21] Individuals were not supposed to elicit strong anti-HLA-DP responses to HLA-DPB1 molecules classified within the same immunogenicity group, based on the hypothesis that T-cells should not respond to foreign HLA-DPB1 molecules sharing specific amino acids with the 'self'-HLA-DPB1 allele. These mismatches were assigned to be permissive mismatches. In contrast, strong T-cell

responses were expected only to be generated against HLA-DPB1 molecules classified in higher immunogenic groups representing non-permissive mismatches.[19]

Recently, we demonstrated the in vivo occurrence of polyclonal HLA-DPB1 specific immune responses following both permissive and non-permissive HLA-DPB1 mismatched SCT and donor lymphocyte infusion (DLI).[22] In the present study we tested these CD4+ T-cells with different HLA-DPB1 specificity for cross-recognition of other HLA-DPB1 molecules and confirmed previously described patterns in cross-recognition.[19,20] In addition, cross-recognition of other HLA-DPB1 molecules was found which did not correspond to the previously proposed algorithm. To analyze whether immunogenicity of HLA-DPB1 mismatches could be predicted based on the presence or absence of specific amino acid sequences in the HLA-DPB1 molecule we developed a model to generate allo-HLA-DP responses vitro. Using this model we tested 48 different combinations. Responders were selected by homozygous expression of one of the four most common HLA-DPB1 alleles in northern European population. HLA-DPB1 molecules used for stimulation comprised 76-99% of the HLA-DPB1 alleles present in different ethnic populations.[15,23] We show that all HLA-DPB1 mismatches as defined by allele typing resulted in high frequency immune responses, also between individuals expressing HLA-DPB1 molecules that were frequently cross-recognized. Together, these data demonstrate that a high degree of similarity in amino acid sequence between HLA-DPB1 molecules is predictive for cross-recognition but not for immunogenicity and illustrate that T-cell recognition patterns may not predict allo-reactivity.

Materials and methods

Cell collection and preparation

Peripheral blood (PB) samples were obtained from healthy donors after approval by the LUMC institutional review board and informed consent according to the Declaration of Helsinki. Mononuclear cells (MNC) were isolated by Ficoll-Isopaque separation and cryopreserved. Stable EBV-transformed B cell lines (EBV-LCL) 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% bovine fetal serum (FBS, BioWhittaker).

Flowcytometry

The monoclonal antibodies (moAb) anti-CD4 fluorescein isothiocyanate (FITC), anti-CD14 phycoerythrin (PE), anti-Nerve Growth Factor Receptor (NGFR)-PE, anti-CD3 Peridinin Chlorophyll Protein (PerCP), anti-CD19 allophycocyanin (APC) and anti-interferon-γ (IFN-γ)-APC were obtained from Becton Dickinson (BD, San Jose, CA, USA). Anti-CD56-APC and anti-

CD154-PE were obtained from Beckman Coulter (Fullerton, CA, USA). Anti-CD8-APC moAbs ware purchased from Caltag Laboratories (Burlingame, CA, USA) and anti-HLA-DP-PE moAbs were obtained from Leinco Technologies (St. Louis, Missouri, USA). Flowcytometric analysis was performed on a BD flowcytometer using CellquestPro software, and cell sorting was performed using a BD FACSAria cell-sorting system.

Transduction of HeLa cells with different retroviral constructs

HLA class II negative HeLa cells were retrovirally transduced with 12 different combinations of HLA-DPB1 and HLA-DPA1 molecules as described in table 1. In order to allow appropriate co-stimulation and processing of the HLA-DP molecules HeLa cells were first transduced with CD80, Invariant chain (Ii) and HLA-DM. HLA-DMA, HLA-DMB and CD80-Ii were cloned into separate pLZRS retroviral vectors. HLA-DMA was combined with the marker gene ΔNGF-R and HLA-DMB with enhanced green fluorescence protein (eGFP). HLA-DPA1 and HLA-DPB1 molecules were cloned into separate MP71-IRES retroviral vectors containing the marker genes eGFP or ΔNGF-R respectively. The identity of all constructs was verified by sequencing. Retroviral supernatants were generated using packaging Φ-NX-A cells as previously described[24,25] and used for transduction of HeLa cells or EBV-LCL using recombinant human fibronectin fragments CH-296 (BioWhittaker, Verviers, Belgium)[25,26]

HLA-DM and CD80-Ii transduced HeLa (HeLa-II) cells were purified by flowcytometric cell sorting based on expression of both marker genes and CD80 staining. Selected cells were subsequently transduced with HLA-DPA1 and HLA-DPB1 constructs. HLA-DP transduced HeLa-II cells were selected based on positive staining for anti-HLA-DP-PE. EBV-LCL expressing the specifically transduced HLA-DPA1 and HLA-DPB1 molecules were isolated by selecting for eGFP and ΔNGF-R double positive cells.

Characterization of CD4+ T-cell clones

To analyze cross-recognition of different HLA-DPB1 molecules by our previously isolated HLA-DPB1 specific CD4+ T-cell clones[22], IFN-γ production in response to HeLa-II cell lines transduced with different HLA-DPB1 molecules and HLA-DP negative HeLa-II cells was determined. To determine IFN-γ production, 5 000 T-cells were co-cultured with $0.03*10^6$ stimulator cells in culture medium consisting of IMDM supplemented with 5% human serum and 5% FBS supplemented with 10 IU Interleukine-2 per ml (IL-2, Chiron, Amsterdam, The Netherlands). After overnight incubation, supernatants were harvested, and IFN-γ production was measured by enzyme-linked immunosorbent assay (ELISA; Centraal Laboratorium voor Bloedtransfusiedienst, CLB, Amsterdam, The Netherlands).

To identify HLA-DPB1 specific CD4+ T-cell clones with the same specificity but derived from different clonal origin, T-cell receptor (TCR)-Vβ-chain analysis was performed by flowcytometric analysis. CD4+ T-cell clones were stained with specific antibodies for

different TCR-Vβ chains using a TCR-Vβ repertoire kit (Beckman Coulter) and analyzed by flowcytometry.

Generation of HLA-DPB1 specific CD4+ T-cell lines

To study immunogenicity of HLA-DPB1 in different stimulator/responder combinations we developed a model to generated allo-HLA-DP responses in vitro. Twelve different HLA-DP transduced HeLa-II cell lines were used to stimulate purified CD4+ T-cells derived from different HLA-DPB1 homozygous responders. Responder cells were typed HLA-DPB1*02:01, *03:01, *04:01 and *04:02. CD4+ T-cells were first purified from PB-MNC using magnetic untouched CD4+ T-cell isolation beads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Next, negative fractions were stained with anti-CD4-FITC, anti-CD14-PE and anti-CD8-APC, anti-CD56-APC and anti-CD19-APC. CD4+, CD14-/CD8-/CD56-/CD19- cells were selected using flow-cytometric cell sorting. $1*10^6$ purified CD4+ T-cells were stimulated with $0.1*10^6$ irradiated (30Gy) HLA-DP transduced HeLa-II cells in culture medium supplemented with 50 IU IL-2/ml. At day 14, CD4+ T-cell lines were analyzed for specific recognition of various cell lines.

Characterization of CD4+ T-cell lines

To analyze specificity of the CD4+ T-cell lines stimulated with different HLA-DPB1 molecules, IFN-γ production in response to HeLa-II cells transduced with 12 different HLA-DPB1 alleles (including the autologous control) and HLA-DP negative HeLa-II cells was determined. To further confirm specificity for allo-HLA-DPB1, IFN-γ production in response to autologous responder EBV-LCL transduced with the relevant HLA-DPB1 molecules used for stimulation was determined. IFN-γ production was determined in harvested supernatants or by intracellular cytokine staining.

To determine IFN-γ release in supernatants 25 000 , 10 000 or 2 000 CD4+ T-cells were co-cultured with $0.05*10^6$ stimulator cells in a final volume of 150μl culture medium. Supernatants were harvested following overnight incubation, and IFN-γ production was measured by ELISA.

For intracellular IFN-γ staining $0.05*10^6$ CD4+ T-cells were restimulated with $0.05*10^6$ stimulator cells in culture medium supplemented with 10μg/ml Brefeldin-A (Sigma-Aldrich, St Louis, MO, USA). After 4 hours incubation, CD4+ T-cells were surface stained with CD4-FITC and CD3-PerCP-labeled MoAbs. Cells were washed, fixed and permeabilised using 0.1% Saponin (Sigma-Aldrich) for 20 min at 4°C. Next, cells were stained intracellularly with CD154-PE and IFN-γ-APC-labeled MoAbs, and analyzed by flowcytometry.

Generation of HLA-DPB1 specific CD4+ T-cell clones

To analyze the diversity of HLA-DPB1*14:01 specific CD4+ T-cell responses, HLA-DPB1*14 specific CD4+ T-cells were clonally isolated from a T-cell line generated as described above. At day 14 restimulation was performed, and after 4 hours of incubation, activated IFN-γ producing CD4+ T-cells were stained using the IFN-γ capture assay (Miltenyi Biotec GmbH) according to the manufacturer's instructions. PE labeled IFN-γ producing CD4+ T-cells were stained with anti-CD4-APC conjugated moAbs and counterstained with propidium iodide (PI, Sigma) immediately prior to cell sorting to exclude dead cells. Viable (PI-negative), CD4 positive IFN-γ secreting lymphocytes were sorted single cell per well into U-bottom microtiter plates (Greiner Bio-One, the Netherlands) containing 100μl feeder mixture consisting of culture medium supplemented with IL2 (120 IU/ml), phytohemagglutinin (PHA, 0.8μg/ml, Murex Biotec Limited, Dartford, UK), and 50 Gy-irradiated allogeneic third-party PB-MNC (0.05×10^6/ml). Proliferating T-cell clones were selected and further expanded using non-specific stimulation and third party feeder cells.

Results

HLA-DP transduced HeLa-II cells as antigen presenting cells

To study recognition of HLA-DP molecules we used HLA class II negative HeLa cells transduced with different HLA-DP molecules as antigen presenting cells (APC). By using the same APC in all experiments, the variability between different stimulations was limited to the expression of different HLA-DP molecules. Since HeLa cells do not endogenously express HLA class II molecules we first transduced CD80, Ii and HLA-DM into the HeLa cells (HeLa-II) in order to allow appropriate co-stimulation and processing of transduced HLA-DP molecules. Next, HeLa-II cells were transduced with different combinations of HLA-DPA1 and HLA-DPB1 molecules. HLA-DPB1 molecules were co-transduced with HLA-DPA1 molecules found in positive linkage disequilibrium in different populations.[23] (table 1)

Table 1. HLA-DP constructs used for transduction

Contruct [1] HLA-DPB1*	HLA-DPB1*	HLA-DPA1*	Frequency [2] (%)	Classification [3] (Group)
01:01	01:01	02:01	3,6	4
02:01	02:01	01:03	10,9	3
03:01	03:01	01:03	12,6	2
04:01	04:01	01:03	40,9	4
04:02	04:02	01:03	13	4
05:01	05:01	02:02	3,2	4
06:01	06:01	01:03	2,4	4
09:01	09:01	02:01	1,2	1
11:01	11:01	02:01	2,4	4
13:01	13:01	02:01	1,2	4
14:01	14:01	02:01	1,6	2
17:01	17:01	02:01	2,4	1

[1] Construct names for combination of HLA-DPB1 and HLA-DPA1 molecules used for transduction into HeLa-II cells or EBV-LCL.
[2] Frequencies of HLA-DPB1 molecule in northern European po pulation according to Begovich et al.[23]
[3] HLA-DPB1 molecules were previously classified in 4 immunogenicity groups: highly immunogenic (group 1), intermediate immunogenic (group 2 and 3) or low immunogenic (group 4)[21]

Cross-recognition of HLA-DPB1 molecules by different HLA-DPB1 specific CD4+ T-cell clones

To analyze whether CD4+ T-cell clones with different HLA-DPB1 specificities showed similar cross-recognition patterns as previous demonstrated[19,20] we tested HLA-DPB1*02:01, HLA-DPB1*03:01 or HLA-DPB1*04:01 specific CD4+ T-cell clones expressing different TCR-Vβ for recognition of 12 different HLA-DPB1 molecules. HLA-DPB1 specific CD4+ T-cells have previously been isolated from 2 patients during clinical immune responses following HLA-DPB1 mismatched SCT and DLI.[22] In figure 1 cross-recognition patterns of the different

	Clone	TCR-Vβ	Group 1		Group 2		Group 3	Group 4						
			09:01	17:01	03:01	14:01	02:01	01:01	04:01	04:02	05:01	06:01	11:01	13:01
A. HLA-DPB1*02:01 Specific CD4+ T cell clones	24-a	20	-	337	-	-	726	-	-	-	-	-	-	-
	24-b	7.1	-	-	-	-	764	-	-	-	-	-	-	-
	24-c	12	-	-	-	-	632	-	-	-	-	-	-	-
	24-d	9	-	346	-	-	834	-	-	-	-	-	-	-
	24-e	?	-	-	-	-	733	-	-	-	-	-	-	-
B. HLA-DPB1*03:01 Specific CD4+ T cell clones	25-a	13.1	-	-	930	446	-	-	-	-	-	-	-	-
	25-b	12	-	-	871	-	-	-	-	-	-	-	-	-
	25-c	17	-	-	869	-	-	163	-	-	-	-	437	-
	25-d	7.1	-	-	950	840	-	-	-	-	-	914	919	-
	25-e	9	-	-	867	500	-	-	-	-	-	-	-	-
	25-f	?	-	-	857	610	-	-	-	-	-	-	-	-
C. HLA-DPB1*04:01 Specific CD4+ T cell clones	28-a	12	-	-	-	-	-	-	437	-	-	-	-	-
	28-b	17	-	-	-	-	-	181	651	-	-	-	-	-
	28-c	13.2	-	-	-	-	-	-	484	-	-	-	-	-
	28-d	21.3	-	-	-	-	-	868	679	-	-	-	-	-
	28-e	?	-	-	-	-	-	-	915	633	-	-	-	749

Figure 1. Differential cross-recognition of HLA-DPB1 molecules by CD4+ T-cell clones with the same HLA-DPB1 specificity. Different HLA-DPB1*02:01 (n=5), *03:01 (n=6) or *04:01 (n=5) specific CD4+ T-cell clones, identified by different TCR-Vβ expression, were tested for recognition of 12 different HLA-DP transduced HeLa-II cell lines. Each row represents recognition of the 12 different HLA-DPB1 molecules by one CD4+ T-cell clone. Overnight IFN-γ production measured in 50µl supernatant is shown (pg/ml). "- " indicates less than 100 pg/ml IFN-γ measured. (A) HLA-DPB1*02:01 specific CD4+ T-cell clones were isolated during the clinical immune response to DLI from an HLA-DPB1*04:02, 05:01 typed donor. (B) HLA-DPB1*03:01 specific CD4+ T-cell clones were isolated during the clinical immune response to DLI from an HLA-DPB1*04:02, 05:01 typed donor. (C) HLA-DPB1*04:01 specific CD4+ T-cell clones were isolated during the clinical immune response to DLI from an HLA-DPB1*03:01,04:02 typed donor.

CD4+ T-cell clones is shown. Individual CD4+ T-cell clones directed against the same HLA-DPB1 allele exhibited different patterns in cross-recognition demonstrating that the CD4+ T-cell clones were specific for different epitopes. Interestingly, in accordance to previous reports[19,27,28], most HLA-DPB1*03 specific CD4+ T-cell clones showed cross-recognition of HLA-DPB1*14:01, illustrating the similarity of these two HLA-DPB1 molecules. In addition, HLA-DPB1*02:01 and HLA-DPB1*03:01 specific CD4+ T-cell clones showed cross-recognition of other HLA-DPB1 molecules which did not correspond to the previously proposed algorithm.[19,21]

Generation of HLA-DPB1 specific CD4+ T-cell responses in vitro

To analyze whether frequently observed cross-reactivity between specific HLA-DPB1 molecules influenced mutual immunogenicity, we developed a model to generate allo-HLA-DP responses in vitro. In this model we stimulated purified CD4+ T-cells form healthy individuals with HeLa-II cells transduced with different HLA-DPB1 molecules and analyzed their specificity at day 14 after stimulation.

First, we analyzed the capacity of HLA-DP transduced HeLa-II cells to induce HLA-DPB1 specific CD4+ T-cell responses in vitro. We purified CD4+ T-cells from a cryopreserved fraction of DLI stored for one of the patients described above[22,29], and stimulated these donor derived CD4+ T-cells with HLA-DPB1*03 transduced HeLa-II cells. 14 days after stimulation CD4+ T-cells were tested for specific recognition of the cell line used for stimulation by intracellular IFN-γ measurement as well as IFN-γ ELISA. Specific IFN-γ production was observed by 8-12% of CD4+ T-cells in response to restimulation with HLA-DPB1*03 transduced HeLa-II cells and not in response to HeLa-II cells transduced with control HLA-DPB1 molecules or HLA-DP negative HeLa-II cells, illustrating a high frequency of HLA-DPB1 specific CD4+ T-cells (data not shown). Similarly, in IFN-γ ELISA specific recognition of HLA-DPB1*03 transduced HeLa-II cells and not HeLa-II cells transduced with control HLA-DPB1 molecules or HLA-DP negative HeLa-II cells was shown. Specificity for allo-HLA-DPB1*03 of the CD4+ T-cell line was further confirmed by specific recognition of HLA-DPB1*03 transduced autologous responder EBV-LCL (Figure 2A).

To confirm specific recognition of HLA-DPB1*03 transduced HeLa-II cells and HLA-DPB1*03 transduced responder EBV-LCL by a single CD4+ T-cell, HLA-DPB1*03 specific CD4+ T-cells were clonally isolated from this CD4+ T-cell line using the IFN-γ capture assay. In total 24 CD4+ T-cell clones using at least 8 different TCR-Vβ chains were expanded and tested for specific recognition of HLA-DPB1*03 (Figure 2B). The data showed that 19 CD4+ T-cell clones specifically recognized both HLA-DPB1*03 transduced HeLa-II cells and HLA-DPB1*03 transduced autologous responder EBV-LCL. Five CD4+ T-cell clones only recognized HLA-DPB1*03 transduced HeLa-II cells probably representing recognition of polymorphic peptides or monomorphic cell type specific peptides presented in the transduced HLA-DP molecules. These results showed that HeLa-II cells transduced with HLA-DP molecules can be used as stimulator cells for in vitro induction of polyclonal HLA-DPB1 specific CD4+ T-cell responses.

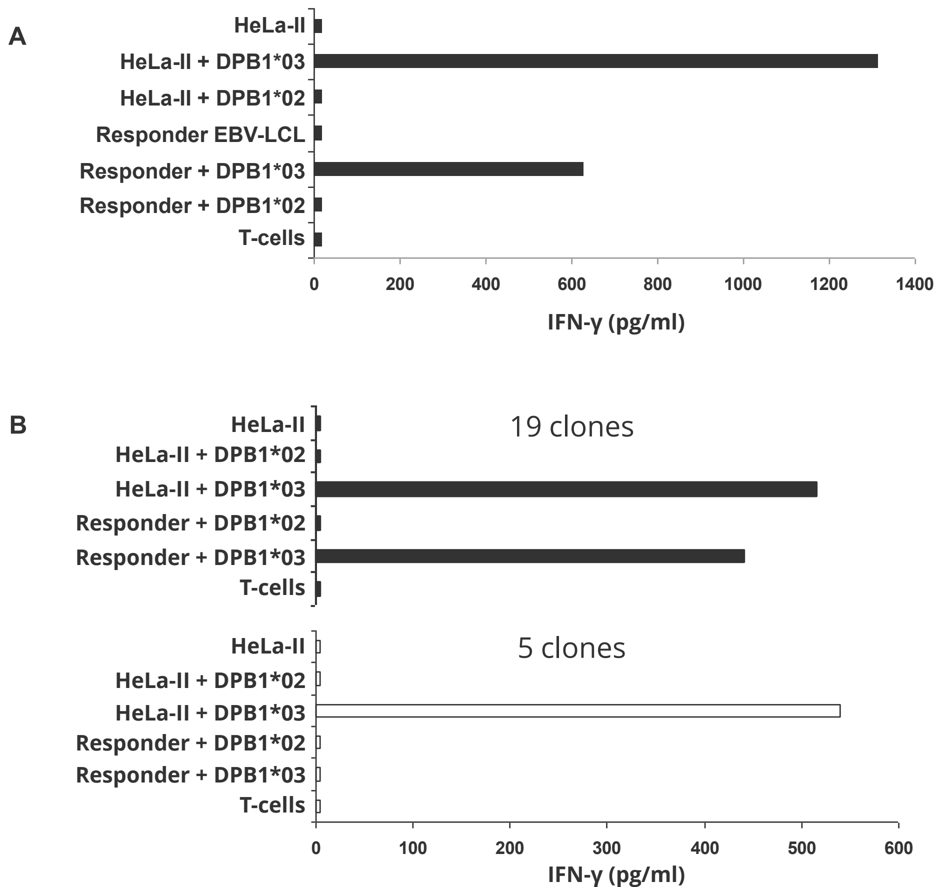

Figure 2. In vitro generation of an HLA-DPB1*03 specific CD4+ T-cell line. (A) CD4+ T-cells purified from an HLA-DPB1*04:02,05:01 typed responder were stimulated with HeLa-II cells transduced with HLA-DPB1*03:01. At day 14, CD4+ T-cells were tested for recognition of untransduced HeLa-II cells or responder EBV-LCL and HeLa-II cells or responder EBV-LCL transduced with HLA-DPB1*03 or random control HLA-DPB1*02. IFN-γ release (pg/ml) in 50μl supernatant is shown after overnight incubation with different stimulator cells. (B) IFN-γ production from 24 HLA-DPB1*03 specific CD4+ T-cell clones in response to different stimulators was determined in 50μl supernatant. Recognition pattern shown in black bars was observed for 19 CD4+ T-cell clones whereas the recognition pattern shown by the white bars was observed for 5 CD4+ T-cell clones.

Strong and diverse HLA-DPB1 specific immune responses were generated between individuals expressing frequently cross-recognized HLA-DPB1 alleles.

To analyze whether frequently observed cross-reactivity between HLA-DPB1*03:01 and HLA-DPB1*14:01 resulted in low mutual immunogenicity we generated an HLA-DPB1*14:01 specific immune response from an HLA-DPB1*03:01 typed responder using our model. We generated HLA-DPB1*17:01, HLA-DPB1*02:01 and HLA-DPB1*04:01 specific immune response from the same individual as a comparison. HLA-DPB1 molecules were selected based on their classification in different immunogenicity groups.[21] Purified CD4+ T-cells were stimulated with HeLa-II cells transduced with the one of these 4 different HLA-DPB1 molecules. 14 days after stimulation CD4+ T-cells were tested for specific recognition of the relevant HLA-DPB1 molecules used for stimulation. The generation of these four HLA-DPB1 specific immune responses was demonstrated by specific IFN-γ production in response to restimulation with the HLA-DPB1 molecule used for stimulation and not in response to responder HLA-DPB1 molecules (Figure 3A). Specificity for allo-HLA-DPB1 of the CD4+ T-cell lines was confirmed by specific recognition of autologous responder EBV-LCL transduced with the relevant HLA-DPB1 molecules used for stimulation (data not shown).

To analyze whether there was a difference in frequencies of HLA-DPB1 specific CD4+ T-cells generated against the different HLA-DPB1 molecules, the number of responder cells used for restimulation were titrated in IFN-γ ELISA (Figure 3B). All 4 HLA-DPB1 specific CD4+ T-cell lines showed significant IFN-γ release using 2 000 responder cells demonstrating a high frequency of HLA-DPB1 specific CD4+ T-cells in all these cell lines.

To determine the diversity of this HLA-DPB1*14:01 specific immune response, CD4+ T-cells were clonally isolated. Twelve HLA-DPB1*14 specific CD4+ T-cell clones using 4 different TCR-Vβ chains were tested for cross-reactive recognition of other HLA-DPB1 molecules. Only one HLA-DPB1*14:01 specific CD4+ T-cell clone showed cross-recognition of HLA-DPB1*09:01, whereas 11 CD4+ T-cell clones showed no cross-recognition of other HLA-DPB1 molecules (data not shown).

Next, we analyzed whether the HLA-DPA1 molecule contributed to the specificity of this allo-HLA-DP response. We analyzed whether recognition of allo-HLA-DPB1*14 depended on expression of the HLA-DPA1*02:01 molecule used for stimulation. Seven CD4+ T-cell clones were tested for recognition of HeLa-II cells tranduced with HLA-DPB1*14:01/DPA1*02:01 and HeLa-II cells transduced with HLA-DPB1*14:01/HLA-DPA1*01:03. Similar membrane expression of the different HLA-DP constructs was confirmed by flowcytometry (data not shown). For 4 CD4+ T-cell clones recognition depended on the presence of HLA-DPA1*02:01 used for stimulation since no IFN-γ production was observed in response to HeLa-II cells transduced with HLA-DPB1*14:01/DPA1*01:03 (Figure 3c). For 3 HLA-DPB1*14 specific CD4+ T-cell clones recognition in response to stimulation with HeLa-II

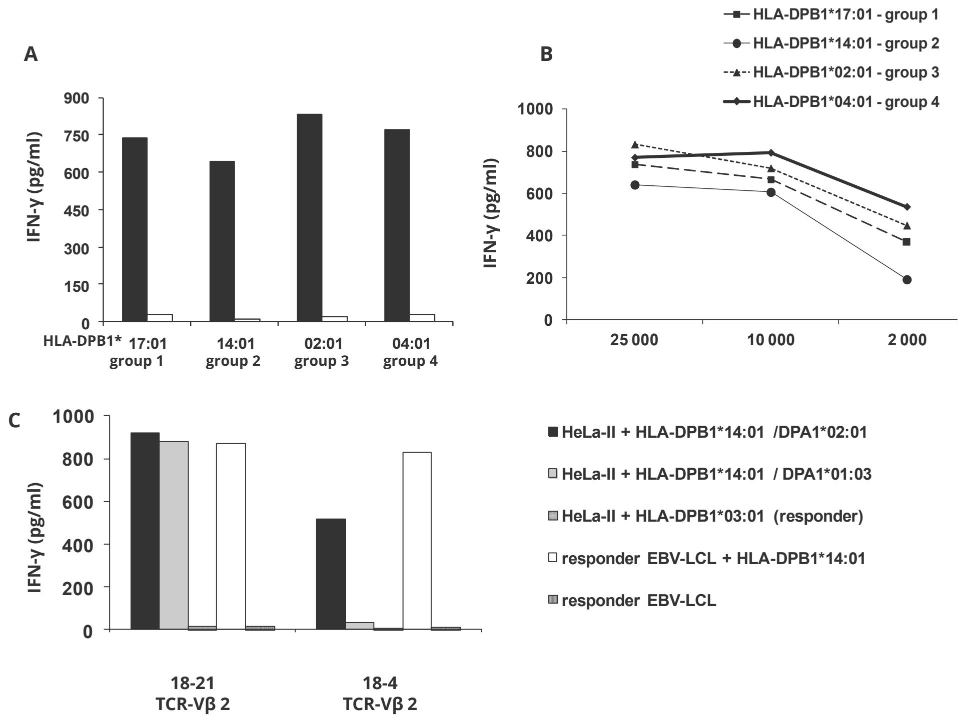

Figure 3. Comparable HLA-DPB1 specific immune response generated against HLA-DPB1 molecules classified in 4 different groups. (A) Purified CD4+ T-cells derived from a healthy HLA-DPB1*03:01 typed individual were stimulated with HeLa-II cells transduced with HLA-DPB1*17:01, *14:01, *02:01 or *04:01. At day 14, 25 000 CD4+ T-cells from each cell line were restimulated with 50 000 HLA-DPB1 transduced HeLa-II cells used for stimulation (■) or HeLa-II cells transduced with control responder HLA-DPB1*03:01 molecules (□). IFN-γ release (pg/ml) measured in 50µl supernatant upon restimulation is shown. (B) IFN-γ production of the 4 different HLA-DPB1 specific CD4+ T-cell lines in response to HLA-DPB1 transduced HeLa-II cells used for stimulation is shown using different numbers of responder cells. IFN-γ release was determined in 50µl supernatant following overnight incubation using 25 000, 10 000 or 2 000 responder cells. Immune responses generated against different HLA-DPB1 molecules are depicted using different symbols and lines. (C) Recognition of two representative HLA-DPB1*14:01 specific CD4+ T-cell clones in response to HeLa-II cells transduced with HLA-DPB1*14:01 combined with different HLA-DPA1 molecules. Specificity for HLA-DPB1*14 of the CD4+ T-cell clones is shown by recognition of responder EBV-LCL transduced with HLA-DPB1*14. IFN-γ production in 50µl supernatant was determined by ELISA.

cells transduced with a different HLA-DPα chain was preserved. CD4+ T-cell clones with the same TCR-Vβ as identified by antibody staining showed different recognition patterns further demonstrating the diversity of this immune response.

These data demonstrate that minor differences between HLA-DPB1 molecules expressed by stimulator and responder cells resulted in a high-frequency polyclonal CD4+ T-cell response.

HLA-DPB1 specific immune responses were generated from all HLA-DPB1 mismatch combinations

To determine immunogenicity of HLA-DPB1 molecules in multiple different stimulator/ responder combinations, CD4+ T-cells purified from 4 healthy individuals were stimulated with HeLa-II cells transduced with 11 different allo-HLA-DPB1 molecules. Responders were selected based on homozygous expression of HLA-DPB1*02:01, HLA-DPB1*03:01, HLA-DPB1*04:01 or HLA-DPB1*04:02. In total 44 different stimulator/responder combinations were analyzed for the generation of allo-HLA-DPB1 specific immune responses. At day 14 after stimulation, CD4+ T-cells were tested for specific recognition of the relevant HLA-DPB1 molecules used for stimulation. HLA-DPB1 specific immune response were generated in 42 out of 44 stimulations as demonstrated by specific IFN-γ production in response to restimulation with the HLA-DPB1 transduced HeLa-II cell lines used for stimulation and not in response to HeLa-II cells transduced with responder HLA-DPB1 molecules or HLA-DP negative HeLa-II cells (Figure 4). Some CD4+ T-cell lines showed minor IFN-γ release in response to HeLa-II cells transduced with responder HLA-DPB1 molecules and HLA-DP negative HeLa-II cells which may be explained by non-HLA-DP restricted recognition of HeLa-II cells.

As shown in figure 4, CD4+ T-cells were also stimulated with HeLa-II cells transduced with the autologous responder HLA-DPB1 molecule. IFN-γ production observed in response to HeLa-II cells transduced with the autologous HLA-DPB1 molecule illustrated that CD4+ T-cell responses could also be generated against polymorphic minor histocompatibility antigens[30, 31] or cell-type specific peptides presented in 'self'- HLA-DP.

These data demonstrate that HLA-DPB1 specific CD4+ T-cell responses can result from all HLA-DPB1 mismatch combinations.

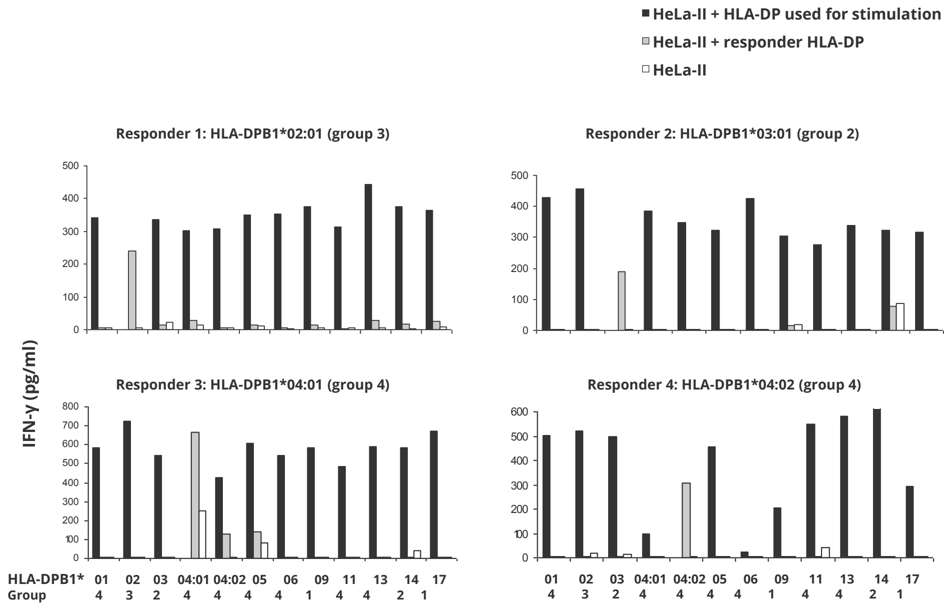

Figure 4. HLA-DPB1 specific immune responses can be generated from all HLA-DPB1 mismatch combinations. Purified CD4+ T-cells derived from 4 different responders were stimulated with HeLa-II cells transduced with 12 different HLA-DPB1 molecules. At day 14, 25 000 CD4+ T-cells from each cell line were restimulated with 50 000 HLA-DPB1 transduced HeLa-II cells used for stimulation (■), HeLa-II cells transduced with autologous responder HLA-DPB1 (■) or HeLa-II cells without HLA-DP expression (□). IFN-γ release (pg/ml) measured in 50μl supernatant upon restimulation is shown.

Cross-recognition of HLA-DPB1 molecules by HLA-DPB1 specific CD4+ T-cells

To investigate whether we could identify additional patterns in cross-reactivity of HLA-DPB1 molecules by HLA-DPB1 specific CD4+ T-cells, all HLA-DPB1 specific CD4+ T-cell lines shown in figure 4 were analyzed for cross-recognition of 12 different HLA-DPB1 molecules. Complete data sets of experiments from two representative responders are shown in figure 5. In figure 5A, cross-recognition of different HLA-DPB1 molecules by 12 HLA-DPB1 specific CD4+ T-cell lines generated from an HLA-DPB1*03:01 typed responder (group 2)[21] is shown, and in figure 5B data from an HLA-DPB1*04:01 typed responder (group 4)[21] are shown.

A broad diversity in cross-reactivity between different HLA-DPB1 specific CD4+ T-cell lines was observed (Figure 5). CD4+ T-cell lines with the same HLA-DPB1 specificity generated from different responders showed cross-recognition of different HLA-DPB1 molecules. This phenomenon is likely to be caused by a different TCR-repertoire present in each responder.

A. Responder HLA-DPB1*03:01 (group 2)

HLA-DPB1*		Group 1		Group 2		Group 3	Group 4						
		09:01	17:01	03:01	14:01	02:01	01:01	04:01	04:02	05:01	06:01	11:01	13:01
Group 1	09:01	++	++										+
	17:01	+	++				++				+		+
Group 2	03:01			+									
	14:01		+		++								
Group 3	02:01		++			++		+	++	+			
Group 4	01:01						++						
	04:01							++	+				
	04:02					+		+	++				
	05:01									++			
	06:01		+						+		++		
	11:01											++	
	13:01												++

B. Responder HLA-DPB1*04:01 (group 4)

HLA-DPB1*		Group 1		Group 2		Group 3	Group 4						
		09:01	17:01	03:01	14:01	02:01	01:01	04:01	04:02	05:01	06:01	11:01	13:01
Group 1	09:01	++	++			+							+
	17:01	++	++								++		+
Group 2	03:01			++	+						+		
	14:01	+		+	++		+						+
Group 3	02:01		+			++					++		
Group 4	01:01			++			++						
	04:01							++	++				
	04:02							+	++				
	05:01	++	++		++		++		+	++			+
	06:01	+	+								++		
	11:01											++	
	13:01	+					+						++

Figure 5. Cross-recognition of 12 different HLA-DPB1 molecules by HLA-DPB1 specific CD4+ T-cells. 12 CD4+ T-cell lines specific for 12 different HLA-DPB1 alleles were tested for recognition of HeLa-II cells transduced with 12 different HLA-DPB1 molecules. Each row in one figure represents recognition of the 12 different HLA-DPB1 molecules by one CD4+ T-cell line. Gray boxes represent recognition of the specific HLA-DPB1 molecule used for stimulation for each CD4+ T-cell line. '++' indicates more than 100pg/ml IFN-γ production in 50μl supernatant by 10 000 CD4+ T-cells upon restimulation with a specific HLA-DPB1 transduced HeLa-II cell line. '+' indicates more than 100pg/ml IFN-γ production in 50μl supernatant by 25 000 CD4+ T-cells upon restimulation with a specific HLA-DPB1 transduced HeLa-II cell line. (A) Results of 12 different HLA-DPB1 specific CD4+ T-cell lines generated from an HLA-DPB1*03:01 typed responder. (B) Results of 12 different HLA-DPB1 specific CD4+ T-cell lines generated from an HLA-DPB1*04:01 typed responder.

In total, 33 out of 42 HLA-DPB1 specific CD4+ T-cell lines showed cross-recognition of at least one other HLA-DPB1 molecule and a broad variety in cross-recognition of the different HLA-DPB1 specific CD4+ T-cell lines was observed. However, consistent patterns in cross-recognition reported in previous studies were observed. All HLA-DPB1*09 specific CD4+ T-cell lines (n=4) recognized HLA-DPB1*17 and vice versa. Furthermore, all HLA-DPB1*03 specific CD4+ T-cell lines (n=3), generated from responders who did not express HLA-DPB1*03, showed cross-recognition with HLA-DPB1*14 and vice versa. Finally, HLA-DPB1*04:01 and HLA-DPB1*04:02 specific CD4+ T-cell lines generated from HLA-DPB1*02:01 or HLA-DPB1*03:01 typed responders (n=2) showed cross-reactivity with each other. These cross-recognized HLA-DPB1 molecules were previously classified in different groups.[19,21] Furthermore, we did not find additional patterns in cross-recognition. These data showed that the previously reported classification of HLA-DPB1 molecules in different immunogenicity groups was predictive for cross-recognition by HLA-DPB1 specific CD4+ T-cells.

Discussion

In this study we showed that all HLA-DPB1 mismatch combinations as defined by allele typing resulted in high-frequency polyclonal immune responses. As reported previously, cross-recognition of different HLA-DPB1 molecules by HLA-DPB1 specific CD4+ T-cell clones was a common observation. However, we demonstrated that frequently observed cross-reactivity between specific HLA-DPB1 molecules did not preclude the capacity to generate HLA-DPB1 specific immune responses between individuals expressing these HLA-DP molecules. These data illustrate that an algorithm defining permissive and non-permissive mismatches for HLA-DPB1 alloreactivity can not be developed merely based on T-cell recognition patterns.

We demonstrated that a single stimulation with a cell population expressing HLA-DP molecules in combination with relevant molecules involved in the HLA class II processing pathway resulted in high-frequency of HLA-DPB1 specific CD4+ T-cells in more than 95% of the different stimulator/responder combinations analyzed. Responder cells expressed one of the four most common HLA-DPB1 molecules present in northern-European population[23] and 97% of SCT donors in our center expressed at least one of these HLA-DPB1 molecules. HLA-DPB1 molecules used for stimulation comprised 76-99% of HLA-DPB1 molecules present in different ethnic populations.[23] We demonstrated that all HLA-DPB1 mismatches as defined by allele typing were immunogenic.

Our data confirmed previously described patterns in cross-recognition of HLA-DPB1 molecules[19,27,32], illustrating that the proposed classification of HLA-DPB1 molecules in different immunogenicity groups[19, 21] was predictive for cross-recognition. The high degree

in similarity of amino acid sequences in the hypervariable region of HLA-DPB1 molecules classified within group 1 (HLA-DPB1*09 and HLA-DPB1*17) and group 2 (HLA-DPB1*03 and HLA-DPB1*14) is likely to explain the frequently observed cross-reactivity between HLA-DPB1 molecules classified within these groups. In addition, we demonstrated a broad diversity of cross-recognition patterns by several other HLA-DPB1 specific CD4+ T-cell lines. We demonstrated that allo-HLA-DPB1 specific immune responses comprised a variety of T-cell clones apparently generated against different immunogenic epitopes. Cross-recognition patterns of these T-cell clones could often not simply be explained by the presence or absence of specific shared amino acid sequences in the hypervariable region of HLA-DPB1 (data not shown), illustrating the complexity of the three-dimensional structure of an HLA-DP-peptide complex for T-cell recognition.

In this study we demonstrated a redundancy of epitopes against which allo-reactive T-cells can be generated. Not only differences in the HLA-DPB1 molecule but also the HLA-DPA1 molecule contributed to the specificity of allo-HLA-DP responses. Furthermore, previous reports have shown that substitutions of single amino acids in the HLA-DPB1 molecule influenced T-cell recognition either by direct contact with the TCR or indirectly by changing the conformation of peptides presented in the groove.[33] We demonstrated by generating immune responses against HeLa-II cells transduced with 'self'-HLA-DPB1 molecules that polymorphic peptides presented in 'self'-HLA-DP were also capable of stimulating allo-responses. Although the magnitude of these immune responses was lower than allo-HLA-DP responses, it is known from HLA-matched donor recipient pairs that allo-immune responses directed against mHags presented in HLA class I or HLA class II are capable of inducing clinically significant allo-responses.[34-37] In conclusion, although individuals expressing highly similar HLA-DPB1 molecules may clonally delete T-cells specific for shared epitopes, our data demonstrate that sufficient differences between the HLA-DPB1 molecules remain to mount strong allo-HLA-DPB1 specific immune responses.

Although we demonstrated immunogenicity of all HLA-DPB1 mismatches, clinical studies showed a significant decrease in overall survival and higher risk for transplant related mortality for specific HLA-DPB1 mismatches.[18,21,38] However, the studies also showed that these specific mismatches did not enhance GVHD or GVL-reactivity. Since both the risk of disease relapse and the risk of aGVHD did not significantly differ, the observed effect on overall survival may have resulted from mechanisms other than merely differences in alloreactivity between the HLA-DPB1 molecules. Specific HLA-DPB1 molecules have been associated with an increased susceptibility to particular diseases.[39-41] The observed adverse effect on overall survival of some HLA-DPB1 mismatches may therefore be the result of indirect (immunologic) factors. For example the presence of a single nucleotide polymorphisms within the innate immunity receptor NOD2/CARD15 between recipient and donor has been associated with a significant reduction in overall survival.[42-44] Although the exact mechanisms by which these data can be explained are

not yet resolved, the observed effects are likely to be caused by indirect influences on the immune system as well.

In conclusion, we show that all HLA-DPB1 mismatches as defined by allele typing resulted in high frequency CD4+ T-cell responses. Furthermore, we show that cross-recognition of HLA-DPB1 molecules is a broadly observed phenomenon and confirm previously described patterns in cross-reactivity. We demonstrate that frequently observed cross-reactivity between specific HLA-DPB1 molecules did not preclude allo-HLA-DPB1 responses between individuals expressing these HLA-DPB1 molecules. Together these data demonstrate that a high degree in similarity between HLA-DPB1 alleles is predictive for cross-reactivity but not for immunogenicity and illustrate that T-cell recognition patterns may not predict allo-reactivity.

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