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HLA-DP specific responses in allogeneic stem cell transplantation

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HLA-DP specific responses in allogeneic stem cell transplantation

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1 General Introduction

General Introduction

Principles of allogeneic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a successful treatment for patients suffering from a variety of hematological malignancies.[1] Prior to SCT patients are treated with high dose chemotherapy combined with total body irradiation in order to eradicate malignant cells and to suppress the hematopoietic system of the patient to allow engraftment of donor hematopoietic cells. Initially the purpose of the SCT was to replace the lethally damaged patient hematopoietic cells by donor hematopoietic cells to reconstitute the hematopoietic system.[2] In this view autologous stem cells or stem cell from a syngeneic twin were supposed to be the best source of hematopoietic stem cells, since allo-SCT was associated with immunological complications such as graft versus host disease (GVHD) or graft rejection. However, clinical studies demonstrated that the occurrence of GVHD was associated with a decreased likelihood of disease relapse after transplantation, resulting in a better overall survival.[3] Donor derived T-cells appeared not only to be responsible for the detrimental GVHD but also for a reduced risk of disease relapse. Depletion of T-cells from the graft to prevent GVHD resulted in an increased risk of disease relapse illustrating that donor derived T-cells were capable of mediating a graft versus leukemia (GVL) effect.[4-6] Since transplantation between homozygous twins did not result in a GVL-effect it was concluded that the presence of T-cells in the graft was not sufficient to mediate GVL-reactivity but that alloreactive T-cells were essential for the antitumor effect.[7;8] Direct evidence that GVL-reactivity was mediated by donor derived T-cells was provided by the observation that donor lymphocyte infusion (DLI) could induce durable remissions in patients with persistent or relapsed leukemia. DLI as a treatment for recurrence of the malignant disease after allo-SCT has resulted in 20% to 90% complete remissions depending on the malignancy. In chronic myelogenous leukemia (CML) in chronic phase the best results were found. Although response rates in other hematological malignancies were much lower, evident clinical responses have been found in patients treated for relapsed acute myelogenous leukemia (AML), multiple myeloma (MM), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL) and acute lymphoblastic leukemia (ALL).[9-16]

The potential advantage of allo-SCT over autologous SCT appears to be the possibility to exploit the immune system to eradicate residual malignant cells after transplantation. Following allo-SCT normal hematopoietic and immune cells are of donor origin, whereas residual leukemic cells are of patient origin. In this setting where the hematopoietic system is of donor origin, donor derived T-cells can be infused to eliminate residual patient normal hematopoietic cells and leukemic cells without a risk for rejection. In other words, allo-SCT provides a platform for adoptive immunotherapy with DLI to eradicate residual leukemic

cells. In this approach conditioning regimens prior to transplantation may not be aimed at fully eradicating malignant cells but can be limited to doses sufficient to permit donor stem cell engraftment. Therefore toxicity of the conditioning regimen prior to SCT can be reduced.[17] However, the major complication following adoptive immunotherapy with DLI is GVHD. The main focus in hematopoietic SCT research is to develop strategies for immunotherapy of hematopoietic malignancies resulting in reduced GVHD while preserving GVL-reactivity.

GVL-reactivity and GVHD following HLA-matched allo-SCT

Since alloreactive T-cells recognizing HLA-mismatched alleles are present in high frequencies in peripheral blood, HLA-mismatched SCT may result in strong allo-immunity.[18] To reduce the risk of GVHD and allograft rejection, allo-SCT is preferably performed using an HLA-matched donor.[19;20] The optimal donor for an allo-SCT is a fully matched sibling of the patient. However, GVHD and GVL-reactivity coincide in the majority of patients following a fully HLA-matched allo-SCT with a T-cell repleted graft or after DLI. This illustrates that both GVL-reactivity and GVHD are triggered by genetic disparities other than HLA molecules. Single nucleotide polymorphisms present in the coding region of a gene may result in amino acid substitutions in a protein. In most cases this will not influence the biological activity of the protein, however processing of these polymorphic proteins may result in presentation of polymorphic peptides in the context of HLA-molecules. Differences between patient and donor in these polymorphic peptides presented on the cell-surface by HLA-class I or class II molecules are called minor histocompatibility antigens (MiHA) and may be recognized by CD8+ or CD4+ T-cells respectively. MiHA specific T-cell responses have been identified both in patients with selective beneficial GVL-reactivity as well as in patients suffering from GVHD.[10;17;21]

MiHAs in GVL-reactivity and GVHD

Several studies have demonstrated the presence of both CD4+ and CD8+ MiHA specific T-cells in patients suffering from GVHD following HLA-identical SCT. However, MiHA specific CD4+ and CD8+ T-cells have also been isolated from patients with selective GVL-reactivity. The appearance of both MiHA specific CD4+ T-cells and CD8+ T-cells in patients following allo-SCT and DLI who showed long term complete remissions of their malignancies illustrated their clinical relevance for GVL-reactivity. Detailed studies analyzing kinetics of MiHA specific immune responses showed that the appearance of MiHA specific T cells coincided with the disappearance of malignant cells from peripheral blood, further illustrating a GVL-effect of these T-cells.[22-27] Clonal isolation of these MiHA specific CD4+ and CD8+ T-cells from patients responding to DLI after HLA-matched allo-SCT demonstrated polyclonal immune responses directed against a significant number of distinct MiHAs.[22;23;27;28]

T-cell responses directed against MiHAs expressed on both hematopoietic cells and non-hematopoietic cells are likely to induce GVL-reactivity as well as GVHD. Since HLA-class I molecules are expressed on most nucleated cells, expression of HLA-class I restricted MiHAs on target cells is mainly determined by expression, translation and processing of genes encoding the MiHA in different target tissues. CD8+ T-cells recognizing broadly expressed MiHAs in the context of HLA-class I may mediate GVL-reactivity as well as cause damage in different organs resulting in profound GVHD. However, CD8+ T-cell responses directed to hematopoiesis restricted antigens may more selectively induce GVL-reactivity without GVHD.[24;29-31] Not only differential expression of MiHAs but also differential expression of HLA-molecules may contribute to hematopoiesis restricted allogeneic T-cell responses. In contrast to ubiquitous expression of HLA-class I molecules, expression of HLA-class II molecules is mainly restricted to hematopoietic cells. In patients with HLA-class II positive hematological malignancies, an HLA-class II restricted MiHA specific immune response may therefore more selectively induce GVL-reactivity. Several HLA-class II restricted MiHAs encoded by broadly expressed genes have been identified from immune responses in patients without severe GVHD.[28;32]

Identification of MiHAs

To gain more insight into the biology of MiHA specific T-cell responses in GVHD and GVL-reactivity, MiHAs have been molecularly characterized. Laborious techniques such as biochemical identification of peptides eluted from HLA-molecules and the use of cDNA libraries to express MiHAs in target cells were initially used to identify MiHA encoding gene transcripts. These strategies resulted in successful discovery of several MiHAs, however low efficiency of the techniques hampered identification of the majority of MiHAs recognized by T-cell clones.[25-28;33-41] To better understand the development of GVL-reactivity and GVHD, characterization of large numbers of MiHAs is essential. High-throughput identification and expression analysis of MiHAs in patients with various clinical responses after allo-SCT may provide insight into differences and allow manipulation of the balance between GVL-reactivity and GVHD. We developed an efficient strategy for MiHA identification using whole genome association scanning (WGAS). Multiple MiHA specific T-cell clones were isolated from clinical immune responses in patients who received an HLA-matched allograft followed by DLI. Using the WGAS-based strategy we identified multiple novel MiHAs.[27] This strategy may allow characterization of the biological relevance of immune responses directed against various MiHAs in different clinical immune responses. Analysis of different immune responses in patients after allo-SCT and DLI using the WGAS based strategy have demonstrated that immune responses with high amplitudo directed against broadly expressed antigens resulted in GVHD, whereas smaller immune responses directed against hematological restricted antigens resulted in more selective GVL-reactivity.[42;43]

Balance between GVHD and GVL-reactivity

Before HLA-matched allo-SCT, donor derived T-cells most likely have not been exposed to patient specific antigens and must be primed by professional antigen presenting cells (APC) in order to exert an efficient anti-tumor reactivity.[44-46] Residual patient derived dendritic cells, B-cells or macrophages which co-express the same antigens as patient leukemic cells can serve as APC for the induction of GVL-reactivity but also GVHD responses. Since APCs are hematopoietic cells, it may be expected that preferentially hematopoiesis specific antigens are presented, although also ubiquitously expressed antigens will be presented.[22;23] In the early post-transplant period, residual patient derived APCs may directly prime donor T-cells resulting in GVL-reactivity if the antigens are co-expressed by patient leukemic cells.[44;46] Leukemic cells themselves may also acquire an APC phenotype in vivo and function as APC to directly prime anti-leukemic T-cell responses.[47-49] However, in the context of a pro-inflammatory environment as a consequence of tissue damage caused by conditioning regimens or ongoing infections, APCs may not only express endogenous antigens, but can cross-present antigens from different cell types as well, resulting in the induction or amplification of anti-host responses which may induce GVHD.[50] Various conditions of the local microenvironment will determine whether T-cells are capable of recognizing different antigens and cause damage to target organs. This includes activation status of local environment, homing of T-cells and expression of adhesion molecules on target organs.[21;51] In contrast to the early post-transplant period, late after transplantation patient APCs will gradually be replaced by APCs of donor origin. In the absence of pro-inflammatory stimuli there might be a lower chance to develop a broad immune response with high amplitudo resulting in GVHD. In this period, leukemic cells may acquire an APC phenotype and directly trigger an immune response resulting in selective GVL-reactivity. However, if leukemic cells do not acquire an APC phenotype in this period, GVL-reactivity has to be induced by either limited number of residual patient APCs or by donor derived APCs cross-presenting patient derived antigens.[50] Although mice studies have demonstrated that this indirect antigen presentation does occur, the question remains how effective this is in humans.[10;17;50;52] The efficacy of DLI in this period may therefore either depend on the presence of residual patient APCs and their activation status or on the capacity of leukemic cells to acquire an APC phenotype.

Traditionally CD8+ T-cells are considered to be the primary effector cells in GVL-reactivity, whereas CD4+ T-cells are known to be required as helper cells for the induction and maintenance of CD8+ T-cell mediated immunity.[53] However, CD4+ T-cells with direct cytolytic activity against leukemic cells have also been isolated from patients with GVL-responses.[23;28;32;54;55] These data suggested that CD4+ T-cells may also serve as effector cells in GVL-reactivity. Since constitutive expression of HLA-class II molecules is predominantly restricted to hematopoietic cells, CD4+ T-cells may more selectively mediate GVL-reactivity. Indeed several clinical studies have shown that depletion of CD8+ T-cells from the graft or

DLI indeed resulted in a reduced incidence and severity of GVHD without compromising GVL-reactivity.[56;57] However, HLA-class II expression on non-hematopoietic cells can be upregulated after exposure to pro-inflammatory cytokines. An HLA-class II restricted immune response may therefore also result in GVHD if HLA-class II molecules on non-hematopoietic tissues are upregulated.

Unrelated donor stem cell transplantation

Allo-SCT is preferably performed using an HLA-matched donor to reduce the risk of GVHD and allograft rejection. The optimal donor for allo-SCT is a fully matched sibling of the patient. However, identical sibling donors are only available in about 30% of the patients.[20] In the absence of a family donor a good alternative is a matched unrelated donor (URD). In a recent large study it was shown that the highest survival in URD-transplantation was associated with high resolution DNA matching for HLA-A, -B, -C, and -DRB1. However, a perfectly matched donor is frequently not found. A single mismatch at one of these loci (7/8 match) was associated with lower survival, lower disease free survival, higher treatment related mortality and more acute GVHD (aGVHD) compared to 8/8 HLA matched pairs. This applied both high resolution (allele) and low resolution (antigen) mismatches. Remarkably, no differences for relapse and engraftment were found. Single mismatches at HLA-B or -C appeared to be better tolerated than mismatches for HLA-A or -DRB1. Mismatching for an additional allele was associated with even lower survival. A single HLA-DQB1 mismatch was not associated with adverse outcome. However, an HLA-DQB1 mismatch in a 7/8 matched pair was associated with a small but not statistically significant adverse effect on survival. Mismatching for HLA-DPB1 was not associated with a difference in survival.[19;20;58]

In URD-SCT preferably a fully matched donor for HLA-A, -B, -C, -DRB1 and HLA-DQB1 is used (10/10 match). However, in the absence of a full match, a donor matched at 9/10 alleles may be selected. A single mismatch at HLA-DQB1 may be preferred over another locus mismatch, since mismatching for a single HLA-DQB1 was not associated with adverse outcome, whereas a mismatch for HLA-A, -B, -C or -DRB1 resulted in lower overall survival.[19] Since mismatching for HLA-DPB1 did not result in a difference in transplant outcome in most studies, HLA-DPB1 is not taken into consideration in donor selection in most centers. Although the role of HLA-DP in GVL-reactivity and GVHD has been unclear for a long time, several studies have now demonstrated that HLA-DP does function as a classical transplantation antigen.[19;59;60]

The role of HLA-DPB1 in allo-SCT

HLA-DP is the sixth classic HLA-molecule. It consists of an alpha and a beta chain encoded by the HLA-DPA1 and HLA-DPB1 genes respectively. The genes are located centromeric to HLA-DR and HLA-DQ in the class II region of chromosome 6p21.3. HLA-DP shares structural similarities with the HLA-DR and HLA–DQ. For the HLA-DP alpha chain 34 alleles are known, whereas for the highly polymorphic beta chain 155 alleles are know to date.[62] Population studies have shown strong linkage disequilibrium between HLA-DPA1 and HLA-DPB1, but weak disequilibrium between HLA-DP and the other HLA-class II loci.[63;64] This may result from a recombination 'hotspot' in chromosome 6 between HLA-DPA1 and HLA-DQB1 genes. In sibling donors HLA-DPB1 mismatch has been reported up to 10.9%.[65] In HLA-A, -B, -C, -DRB1 and HLA-DQB1 matched URD hematopoietic SCT 70-89% HLA-DPB1 mismatch rates are reported.[60;66-70]

The relevance of matching for HLA-DPB1 in URD-SCT has been inconclusive for a long time. Several clinical studies failed to show a statistical significant difference in the incidence of severe GVHD or patient survival between HLA-DPB1 matched and mismatched patient-donor pairs.[71;72] As a result, HLA-DPB1 has not been taken into consideration in donor selection. Large clinical studies performed between 1999 and 2008 provided evidence for an immunogenic role of HLA-DPB1 in allo-SCT. In T-cell repleted allo-SCT mismatching for HLA-DPB1 was associated with an increased risk of severe GVHD and a decreased risk of disease relapse.[19;60;68;73;74] The overall effect on survival of patients who received an HLA-DPB1 matched or mismatched graft did not statistically differ, possibly due to a balanced effect of an increased risk of GVHD and reduced relapse rate. In contrast, in T-cell depleted allo-SCT mismatching for HLA-DPB1 was not associated with an increased risk of GVHD, whereas a significant decreased risk of disease relapse was still observed. This was particularly evident in patients transplanted for ALL, where HLA-DPB1 mismatched transplantation resulted in a significantly better overall survival.[59]

Further support that HLA-DPB1 mismatched transplantation can result in strong T-cell responses in vivo was provided by several studies demonstrating CD4+ T-cell responses directed against mismatched HLA-DPB1 molecules. HLA-DP specific CD4+ T-cells were isolated from skin biopsies in patients who developed GVHD following HLA-DPB1 mismatched SCT.[75;76] HLA-DP specific CD4+ T-cells were also isolated from an allograft rejection where patient and donor differed for a single HLA-DPB1 mismatch in the rejection direction.[77] It was speculated that HLA-DP specific CD4+ T-cells might also be able to induce a selective GVL-effect in the context of a T-cell depleted allo-SCT. To exploit this concept, HLA-DP expression on leukemic blasts was analysed. HLA-DP expression was found on the vast majority of leukemic cells analyzed. However considerable variability was found and expression was lower on AML than on B-ALL or B-CLL cells. Most leukemic

blasts were also susceptible to direct lysis by allogeneic HLA-DP specific T-cells.[61] However, thus far direct evidence that HLA-DP specific CD4+ T-cells were involved in GVL-reactivity had not been shown.

Permissive and non-permissive mismatches

Since different studies resulted in conflicting outcomes concerning the role of HLA-DPB1 in allo-SCT, it was suggested that there might be a difference in immunogenicity between different HLA-DPB1 mismatches. In primary mixed lymphocyte reaction (MLR) a variety in response values to different HLA-DPB1 mismatches was found. This observation resulted in the hypothesis that not all HLA-DPB1 incompatibilities would elicit measurable T-cell responses in MLR. In vitro studies showed that specific amino acid sequence differences between donor and recipient were associated with high T-cell responses.[78-80] From these studies it was suggested that patient and donor needed to be matched only for specific regions in the hypervariable regions of the HLA-DPB1 allele to prevent GVHD. In 2008 a study was performed in which it was indeed shown that incompatibilities at distinct positions in the hypervariable regions of HLA-DPB1 were risk factors for developing acute GVHD. A specific amino acid mismatch at position 69 of the HLA-DPB1 molecules was associated with a higher rate of treatment related mortality.[73] In two large studies around 5 000 patients who underwent an allo-SCT through the Japan Marrow Donor Program were analyzed for specific mismatches associated with an increased risk of GVHD or a decreased risk of disease relapse. Two specific HLA-DPB1 mismatch combinations were found to be statistically significantly correlated with severe aGVHD and 6 different specific HLA-DPB1 mismatch combinations were associated with a decreased risk of disease relapse and a better overall survival.[81;82] It was suggested that these observations should be taken into consideration in donor selection.

The suggestion that matching at an epitope level might be clinically more relevant in terms of transplant outcome than matching at allele level was translated into a clinical algorithm by Zino et al.[83] In this algorithm permissive and non-permissive mismatches were defined. HLA-DPB1 molecules were classified in different immunogenicity groups based on T-cell recognition patterns by HLA-DPB1*0901 specific CD4+ T-cell clones. Individuals were not supposed to elicit strong anti-HLA-DP responses to HLA-DPB1 molecules classified within the same immunogenicity group. This was 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 called permissive mismatches. In contrast, strong T-cell responses were expected to be generated in response to HLA-DPB1 molecules classified in higher immunogenic groups, representing non-permissive mismatches. Retrospective analysis of a patient cohort showed a significantly higher probability of 2-year survival in permissive compared to non-permissive mismatches. However, no significant

effect on aGVHD or disease relapse was observed in 10/10 matched patients.[84;85] These different observations have not been confirmed in other studies.[73;85]

Mismatched HLA-DPB1 as a specific target for GVL-reactivity

As discussed before, immune responses directed against hematopoiesis restricted antigens can be expected to result in selective GVL-reactivity, whereas immune responses directed against broadly expressed antigens may result in both GVL-reactivity and GVHD. We demonstrated in patients with selective GVL-reactivity low frequencies of MiHA specific T-cells, whereas in patients with both GVL-reactivity and GVHD higher frequencies of MiHA specific T-cells directed against multiple MiHAs were found.[42;43] Since constitutive expression of HLA-class II molecules may be expected to be predominantly restricted to hematopoietic cells, an immune response directed against a single HLA-class II restricted antigen can be hypothesized to induce selective GVL-reactivity. In case of an immune response directed against an HLA-class II restricted MiHAs, a relatively restricted immune response inducing selective GVL-reactivity may be expected to occur. However, alloreactive T-cells recognizing mismatched HLA-molecules are present in high frequencies in peripheral blood.[18] A single HLA-class-II locus mismatched SCT may therefore result in a broad allo-immune response, thereby increasing the risk for GVHD. Mismatching for HLA-DPB1 has indeed been shown to induce strong HLA-DPB1 specific CD4+ T-cell responses in vivo.[75-77] In T-cell repleted SCT mismatching for HLA-DPB1 was associated with both a decreased risk of disease relapse and an increased risk in GVHD.[19;60] In contrast, in T-cell depleted SCT, mismatching for HLA-DPB1 was associated with a decreased risk of disease relapse without an increased risk in GVHD.[59] These data suggest that in HLA-DPB1 mismatched allo-SCT the transplant regimen used may be important for the balance of GVL-reactivity and GVHD.

HLA-class II expression can be upregulated on non-hematopoietic tissues by pro-inflammatory cytokines released as a consequence of tissue damage caused by the conditioning regimen, ongoing infections, or other immune responses. In T-cell repleted SCT, infusion of T-cells derived from an HLA-DPB1 mismatched donor in an inflammatory environment may therefore result in a strong immune response resulting in GVHD. In contrast, HLA-DPB1 mismatched allo-SCT followed by donor derived T-cells at a later time point may more selectively induce GVL-reactivity when tissue damage is largely restored and HLA-class II expression may be anticipated to be restricted to hematopoietic cells. In HLA-DPB1 mismatched allo-SCT local circumstances in the host at the time of T-cell infusion may therefore determine whether GVL-reactivity develops in the presence or absence of GVHD.

Aim of this study

The overall survival between patients transplanted with an HLA-DPB1 matched or mismatched SCT did not statistically differ in most studies.[19;60] However, it has been shown that HLA-DP can function as a classical transplantation antigen and can induce strong allo-immune responses in vivo.[75;76;86;87] In T-cell repleted SCT mismatching for HLA-DPB1 is associated with an increased risk of GVHD. In contrast, in T-cell depleted SCT no association with an increased risk of GVHD was found, whereas a decreased risk of disease relapse was still observed, suggesting that in T-cell depleted SCT mismatching for HLA-DPB1 may induce a selective GVL-effect.[59] Furthermore, it has been suggested that specific HLA-DPB1 mismatches were associated with an increased risk of GVHD or treatment related mortality.[73;81;83;84] Together these data suggest that different outcomes can be expected following HLA-DPB1 mismatched allo-SCT depending on the transplant regimen used and possibly also influenced by specific circumstances present in the host at the time of transplantation.

The aim of this thesis is to investigate whether mismatching for HLA-DPB1 can result in selective GVL-reactivity without GVHD. Furthermore, we determined whether all HLA-DPB1 mismatches can be expected to result in an allo-immune response in vivo. Finally, we investigated whether following HLA-DPB1 mismatched SCT HLA-DP specific immune responses are often induced and whether the occurrence of HLA-DP specific immune responses is associated with GVL-reactivity, GVHD or both.

To analyze whether HLA-DP specific CD4+ T could induce a specific GVL-effect without GVHD, we analyzed in ***chapter 2*** the immune response in a patient transplanted with a 10/10 matched, HLA-DPB1 mismatched SCT followed by DLI for relapsed chronic B-cell leukemia. In this patient a profound GVL-effect with minimal skin GVHD resulting in a persistent complete remission of the disease was observed. We analyzed the kinetics of the immune response and clonally isolated leukemia reactive T-cell clones from the peak of the immune response. The specificity of the immune response was unraveled. Furthermore, differential recognition patterns of HLA-DP specific CD4+ T-cells for various hematological malignant cells and non-hematopoietic cells were tested.

Various studies have suggested that some HLA-DPB1 mismatches would result in stronger allo-reactivity than others. It has been suggested that matching at an epitope level would be clinically more relevant in terms of transplant outcome than matching at allele level.[73;81;83;84] An algorithm defining permissive and non-permissive HLA-DPB1 mismatches had been tested in a retrospective analysis of a patient cohort. Higher overall survival in patients transplanted with a permissive mismatch compared to non-permissive mismatches was found, however no significant effect on GVHD or disease relapse was observed.[84] In other retrospective analyses other specific HLA-DPB1 mismatches were suggested to be associated with GVL-reactivity, GVHD or transplant related mortality.[73;81;82]

These different observations from various research groups were not consistent and have not been confirmed in other studies.[85]

In ***chapter 3*** we analyzed whether permissive HLA-DPB1 mismatches could result in HLA-DPB1 specific immune response in vivo. Two patients responding to DLI after HLA-A, -B, -C, -DRB1, -DQB1 matched, HLA-DPB1 mismatched SCT were analyzed for the presence of patient HLA-DPB1 specific CD4+ T-cells. The patients received a permissive or non-permissive HLA-DPB1 mismatched SCT followed by DLI. $CD4^+$ T-cells were isolated from peripheral blood obtained during the clinical immune response to DLI, and tested for specific recognition of stimulator cells transduced with patient and not donor HLA-DP molecules.

To analyze whether different HLA-DPB1 mismatches would result in differential frequencies of immune responses, we analyzed in ***chapter 4*** 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 HLA-DPB1 responses in vitro and tested in total 48 different stimulator/responder combinations by stimulating CD4+ T-cells from 5 HLA-DPB1 homozygous individuals with the same APC transduced with different allo-HLA-DP molecules. Furthermore, we analyzed whether CD4+ T-cell clones with different HLA-DPB1 specificity showed similar cross-recognition patterns as previously demonstrated and we analyzed whether we could identify additional patterns in cross-reactivity of HLA-DPB1 molecules by HLA-DPB1 specific CD4+ T-cells.

To analyze whether HLA-DP specific immune responses are frequently induced after T-cell depleted HLA-DPB1 mismatched SCT and DLI we developed in ***chapter 5*** a method to screen patients for the presence of HLA-DP specific CD4+ T-cells. We analyzed 24 patient-donor combinations. Patients analyzed suffered from various B-cell malignancies, multiple myeloma and myeloid leukemias. Furthermore, we analyzed whether the presence of HLA-DP specific CD4+ T-cells was associated with clinical outcome in terms of GVHD or GVL-reactivity, and whether HLA-DP specific T-cell responses could be found after permissive as well as non-permissive HLA-DPB1 mismatched DLI.

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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.

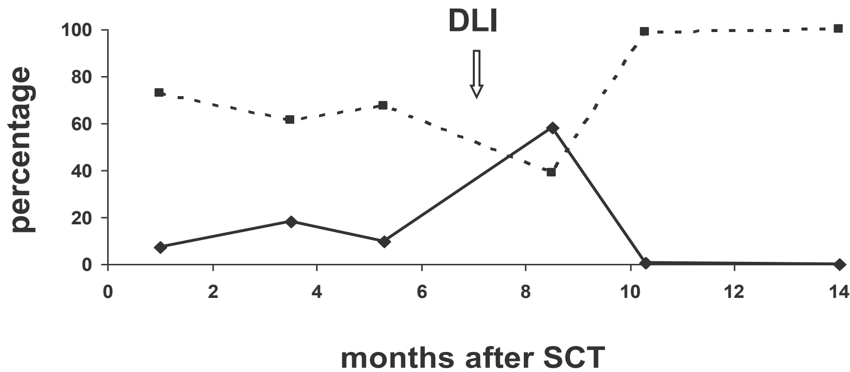


Figure 1. Clinical immune response to DLI following HLA-DPB1 mismatched URD-SCT. Percentage of malignant cells (—♦—) and percentage of donor chimerism (--■--) in BM after SCT. Arrow indicates DLI infusion. From 6 weeks after DLI the percentage malignant cells in BM started to decline. At the same time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

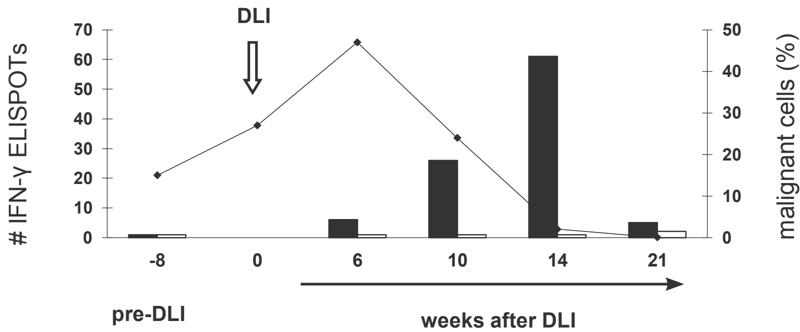


Figure 2. **Determination of an anti-leukemic CD4+ T-cell response starting 6 weeks after DLI.** Number of IFN-γ ELISPOTs produced by CD4+ T-cells (■) and CD8+ T-cells (□) before and after DLI in response to stimulation by leukemic cells from the patient. Leukemic cells as a percentage of PB-MNC is shown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

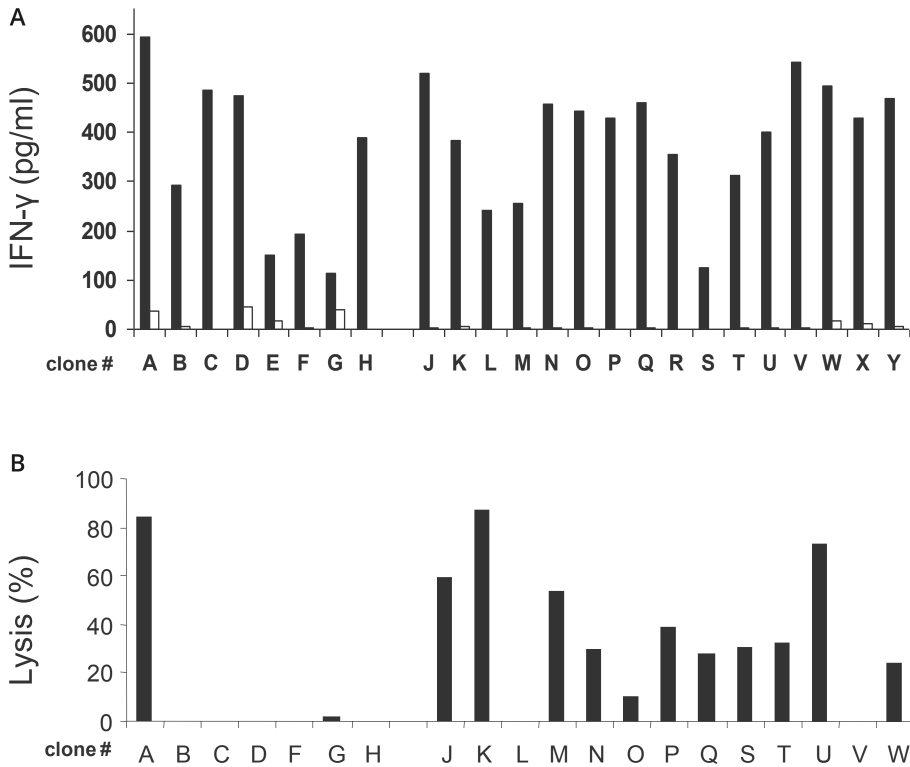


Figure 3. Reactivity of isolated CD4+ T-cell clones against patient malignant cells. Clones A - H represent CD4+ T-cell clones isolated from peripheral blood obtained 10 weeks after DLI. Clones J - Y were isolated from bone marrow obtained 14 weeks post DLI. (A) patient leukemic cells (■) and donor derived 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.

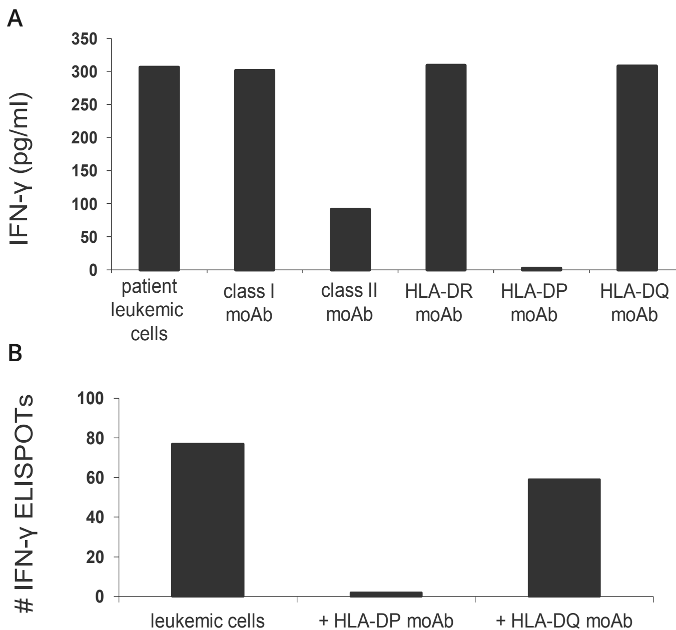
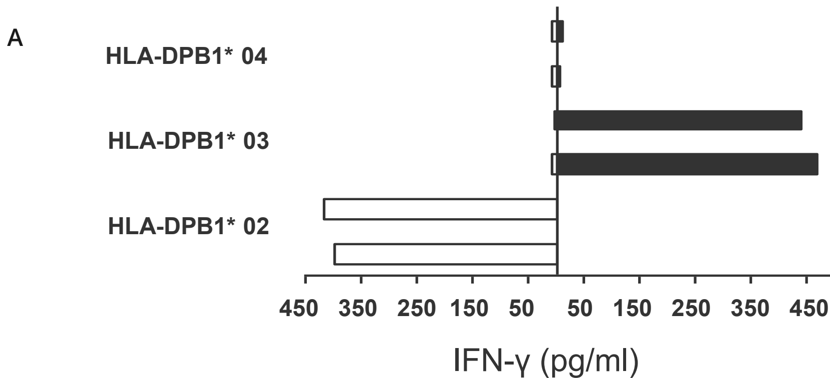


Figure 4. HLA-DP restricted recognition of patient leukemic cells by CD4+ T-cell clones. (A) Representative example of HLA-DP restricted recognition of patient leukemic cells by the isolated CD4+ T-cell clones. IFN-γ release (pg/ml) of clone M in response to patient malignant cells in the absence or 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).



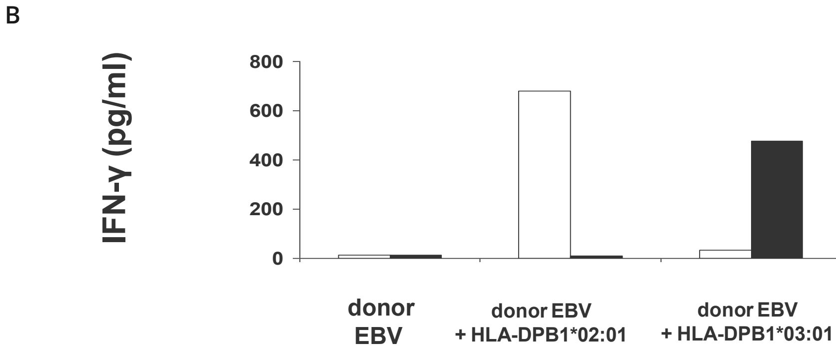


Figure 5. Differential recognition of HLA-DPB1*02:01 and HLA-DPB1*03:01 by the HLA-DP restricted T-cell clones. (A) Recognition of a panel of EBV-LCL expressing HLA-DPB1*02, HLA-DPB1*03 or HLA-DPB1*04 by two representative T-cell clones. Two representative examples of each target are shown. White bars 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.

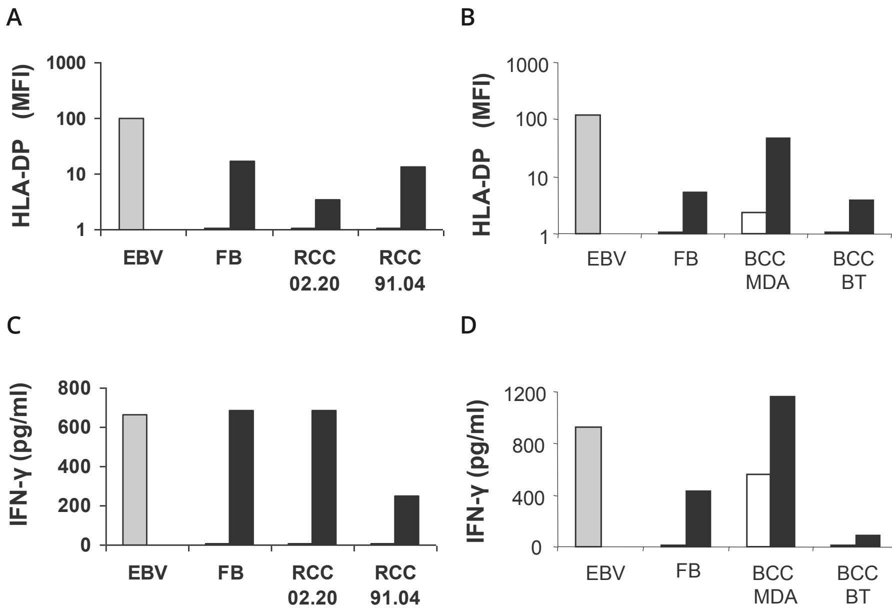


Figure 6. Expression of HLA-DP on non-hematopoietic cells of various origins and recognition by HLA-DP specific T-cells. Mean fluorescence intensity (MFI) of HLA-DP-PE staining on (A) HLA-DPB1*03 typed EBV-LCL, fibroblasts (FBs) and renal cell carcinoma (RCC) cells and (B) HLA-DPB1*02 typed EBV-LCL, 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.

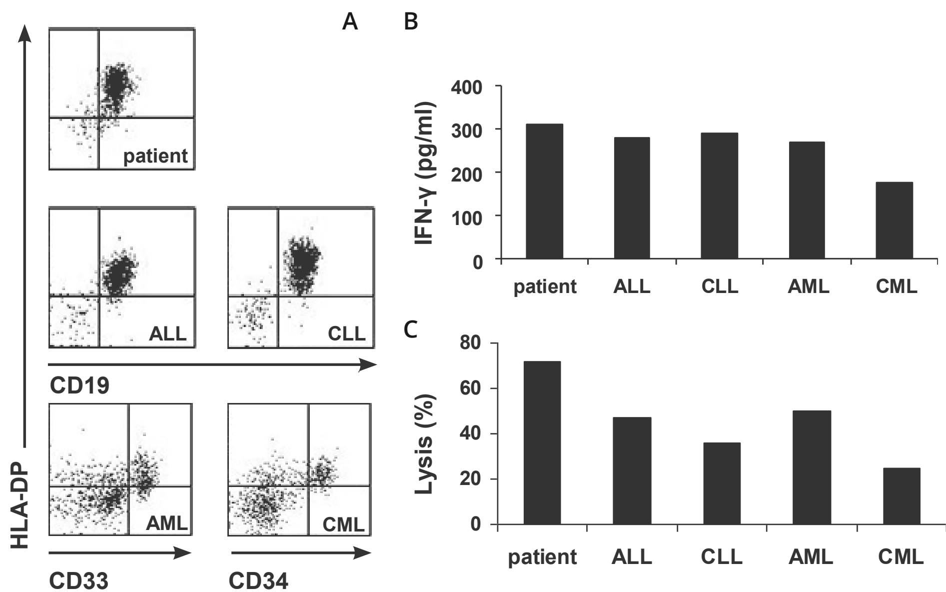


Figure 7. HLA-DP expression on various lymphoid and myeloid malignant cells and their recognition by an HLA-DPB1*03:01 restricted T-cell clone. PB-MNC from the patient analyzed in this study and from HLA-DP*0301 positive ALL, CLL, AML and CML patients were used in this experiment. (A) Expression of HLA-DP 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.

Acknowledgments

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3 Both permissive and non-permissive HLA-DPB1 mismatches can induce polyclonal HLA-DPB1 specific immune responses in vivo and in vitro

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Letter to the editor

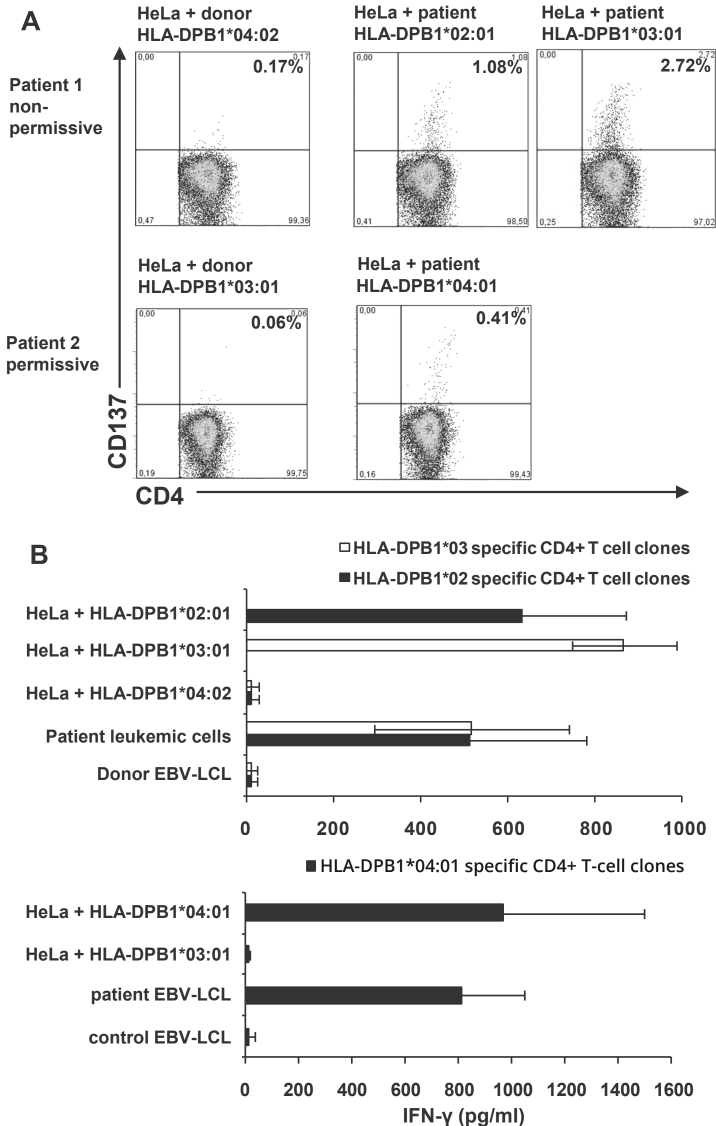
Clinical studies have indicated that human leucocyte antigen (HLA)-DPB1 functions as a classical transplantation antigen in allogeneic stem cell transplantation (SCT). Mismatching for HLA-DPB1 was associated with an increased risk of graft versus host disease (GVHD) but also a decreased risk of disease relapse.[1;2] However, some studies showed that specific HLA-DPB1 mismatches were associated with poor clinical outcome.[3] It was suggested that this unfavorable effect was caused by differences in immunogenicity between HLA-DPB1 alleles. An algorithm defining permissive and non-permissive HLA-DPB1 mismatches was developed based on cross-reactive T-cell reactivity patterns. It was suggested that permissive mismatches would not result in T-cell responses, whereas strong T-cell responses were expected to be generated against non-permissive mismatches.[3;4] Clinical analysis of different patient cohorts indeed showed a significant higher risk of mortality in the non-permissive mismatched group compared to the permissive mismatched group. However, the risk of GVHD and relapse did not significantly differ between these groups, implying that the effect on overall survival may not have directly resulted from differences in alloreactivity.[3;5;6]

To analyze whether permissive HLA-DPB1 specific immune responses occur in vivo, we analyzed immune responses in two patients responding to donor lymphocyte infusion (DLI) following HLA-A,-B,-C,-DRB1,-DQB1 matched, HLA-DPB1 mismatched SCT. The patients received a permissive or non-permissive HLA-DPB1 mismatched SCT, respectively. CD4+ T-cells were isolated from peripheral blood obtained during the clinical immune response to DLI, and stimulated with HLA-class II negative HeLa cells transduced with the specific HLA-DP molecules derived from patient or donor. In both patients, HLA-DPB1 specific CD4+ T-cells were demonstrated by CD137 upregulation in response to stimulation with HeLa cells transduced with patient and not donor HLA-DP molecules (Figure 1A). CD137 expressing CD4+ T-cells were clonally isolated and specific recognition of patient HLA-DPB1 molecules was confirmed for 58-78% of the T-cell clones (Figure 1B). T-cell receptor-Vβ analysis showed a polyclonal origin of these HLA-DPB1 specific CD4+ T-cell responses.

To demonstrate the generation of HLA-DPB1 specific immune responses directed against permissive and non-permissive HLA-DPB1 mismatches in different individuals, we developed a model to generate allo-HLA-DP responses in vitro. Purified CD4+ T-cells of 4 different responders were stimulated with HeLa cells transduced with permissive (n=2) or non-permissive (n=2) HLA-DPB1 molecules. 14 days after stimulation, CD4+ T-cells were restimulated with HeLa cells transduced with HLA-DP molecules used for stimulation and HeLa cells transduced with responder HLA-DP molecules. Both stimulation with permissive and non-permissive mismatched HLA-DPB1 molecules resulted in the generation of HLA-DPB1 specific CD4+ T-cells as measured by specific IFN-γ production in response to the HLA-DPB1 molecules used for stimulation (Figure 1C).

In conclusion, we demonstrated that in vivo both permissive and non-permissive

HLA-DPB1 mismatches resulted in strong polyclonal immune responses. Furthermore, we demonstrated in vitro for 4 additional individuals that permissive and non-permissive HLA-DPB1 responses were equally effectively generated. These data show immunogenicity of permissive mismatched HLA-DPB1 alleles. We suggest that the difference in overall survival between patients transplanted with permissive and non-permissive HLA-DPB1 mismatched donors observed in clinical studies[3;4;6] may not result from direct differences in alloreactivity.



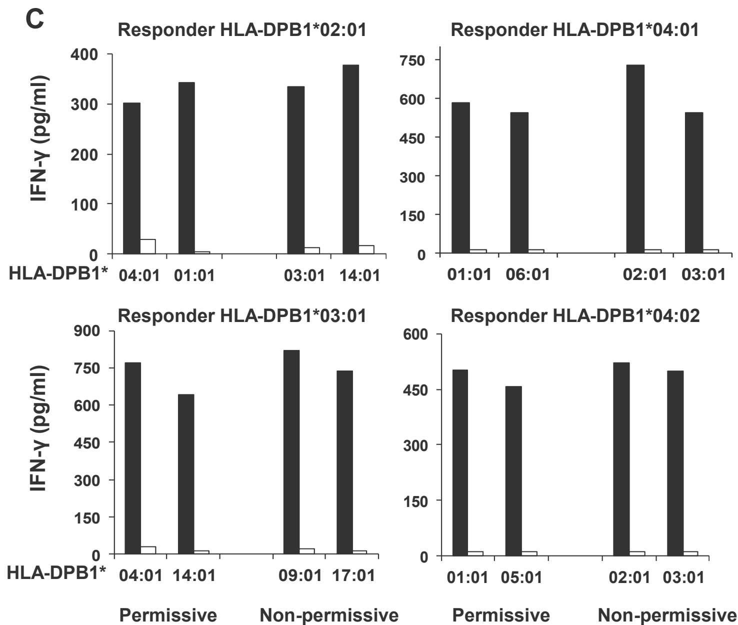


Figure 1. Permissive and non-permissive HLA-DPB1 mismatches induced HLA-DPB1 specific immune responses in vivo and in vitro
Two patients transplanted with an HLA-A,-B,-C,-DRB1,-DQB1 matched, HLA-DPB1 mismatched SCT were analyzed for the presence of HLA-DPB1 specific CD4+ T-cells following DLI. Patient 1 (HLA-DPB1*02:01,03:01) was transplanted for a chronic B cell leukemia with a non-permissive HLA-DPB1 mismatched donor (HLA-DPB1*04:02,05:01). Patient 2 (HLA-DPB1*04:01) was transplanted for Multiple Myeloma with a permissive HLA-DPB1 mismatched donor (HLA-DPB1*03:01,04:02) in the GvH direction. (A) Purified CD4+ T-cells obtained during the clinical immune response to DLI were stimulated with HLA-class II negative HeLa cells transduced with either donor or patient HLA-DP molecules. After 48 hours, CD137 expression on CD4+ T-cells was determined using flowcytometry. Percentages of CD137 positive CD4+ T-cells in response to stimulation with different HLA-DPB1 molecules are shown. (B) CD137 expressing CD4+ T-cells shown in figure 1A were clonally isolated and tested for recognition of different target cells. IFN-γ production (pg/ml) was determined in 50 μl supernatant. Mean results ± SD of a selection of 11 HLA-DPB1*02:01 specific CD4+ T-cell clones, 15 HLA-DPB1*03:01 specific CD4+ T-cell clones and 15 HLA-DPB1*04:01 specific CD4+ T-cell clones are shown. (C) Purified CD4+ T-cells derived from 4 different healthy individuals were stimulated with HeLa cells transduced with permissive mismatched HLA-DPB1 molecules (n=2) or non-permissive mismatched HLA-DPB1 molecules (n=2). At day 14, 25,000 CD4+ T-cells from each cell line were restimulated with 50,000 HLA-DP transduced HeLa cells used for stimulation (■) or HeLa cells transduced with control donor HLA-DP molecules (□). IFN-γ release (pg/ml) measured in 50μl supernatant upon restimulation is shown.

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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 ($0.05x10^6$/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.

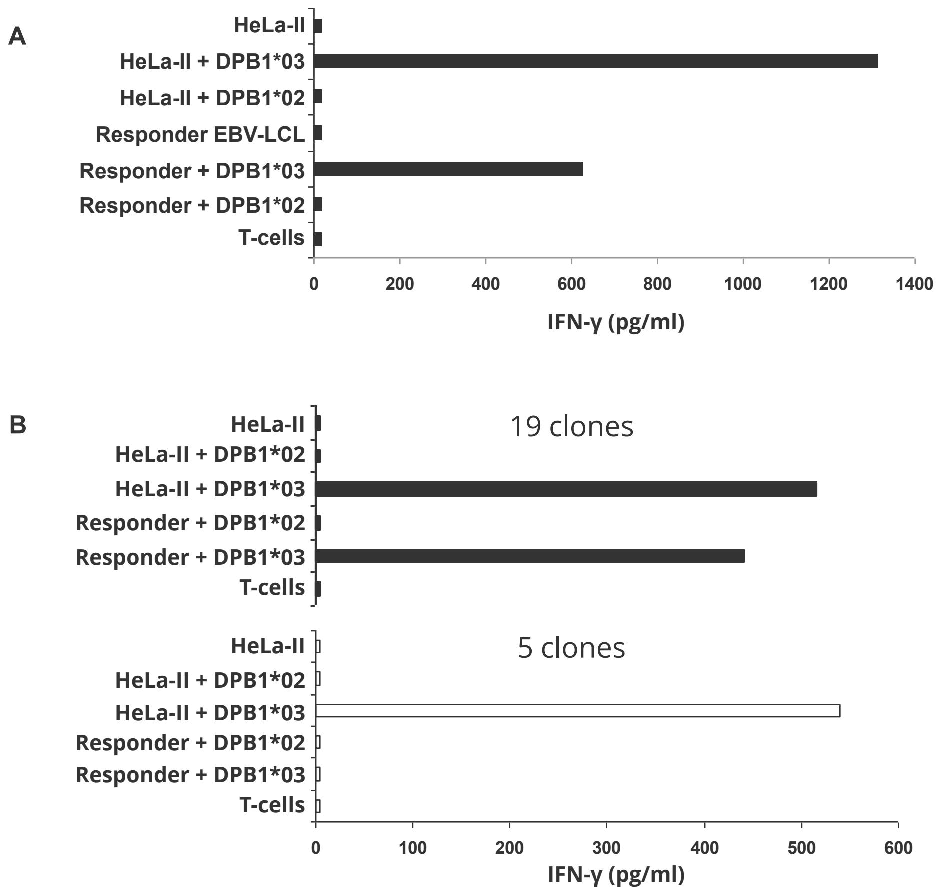

Figure 2. In vitro generation of an HLA-DPB1*03 specific CD4+ T-cell line. (A) CD4+ T-cells purified from an HLA-DPB1*04:02,05:01 typed responder were stimulated with HeLa-II cells transduced with HLA-DPB1*03:01. At day 14, CD4+ T-cells were tested for recognition of untransduced HeLa-II cells or responder 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

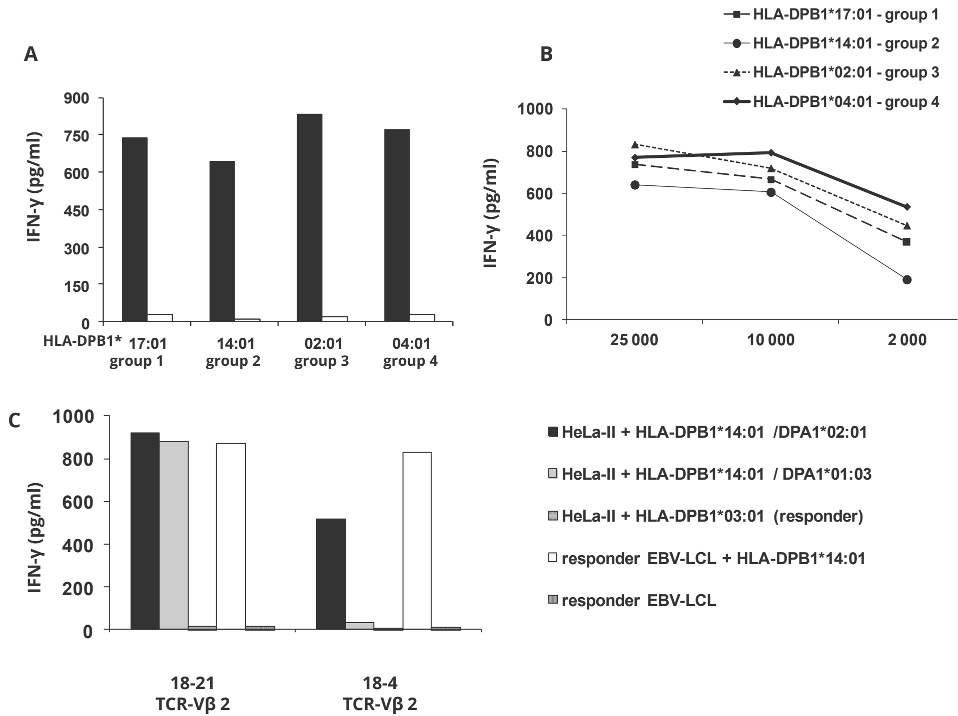

Figure 3. Comparable HLA-DPB1 specific immune response generated against HLA-DPB1 molecules classified in 4 different groups. (A) Purified CD4+ T-cells derived from a healthy HLA-DPB1*03:01 typed individual were stimulated with HeLa-II cells transduced with HLA-DPB1*17:01, *14:01, *02:01 or *04:01. At 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.

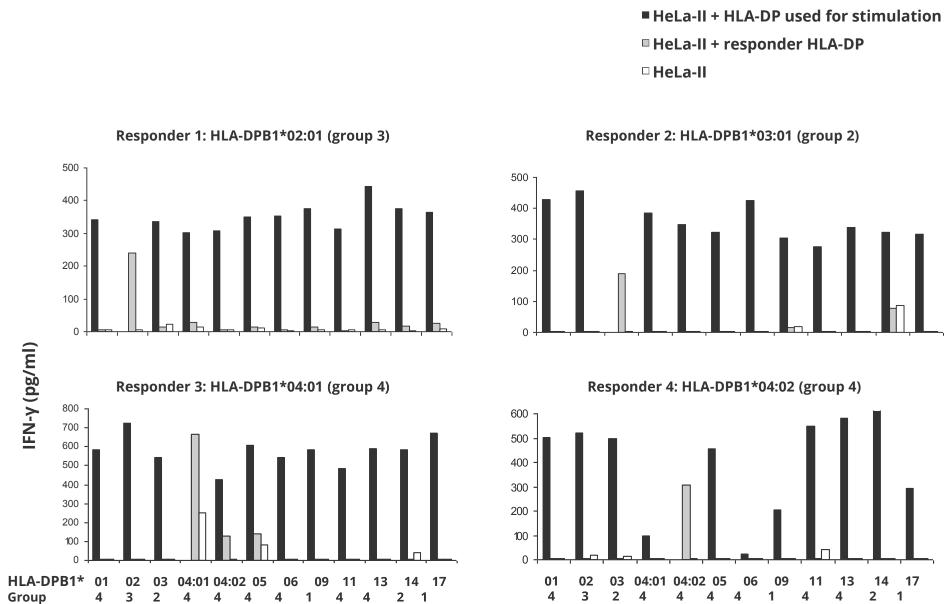

Figure 4. HLA-DPB1 specific immune responses can be generated from all HLA-DPB1 mismatch combinations. Purified CD4+ T-cells derived from 4 different responders were stimulated with HeLa-II cells transduced with 12 different HLA-DPB1 molecules. At day 14, 25 000 CD4+ T-cells from each cell line were 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.

Acknowledgments

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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	Progressive disease	0.84 / 0.19	No
		36	Progressive disease	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	Mixed chimerism	0.19	No
		28	Mixed chimerism	No	No
		36	Mixed chimerism	0.15	Yes
14	Multiple myeloma	4	Persistent disease	No	No
		11	Persistent disease	No	No
		18	Progressive disease	-	No
		23	Progressive disease	-	No
		29	Progressive disease	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)	Extensive [4]	Systemic	Radiotherapy	
No	No			
Grade I (12)	Extensive [4]	Systemic		
No	No			
No	No			
Grade I (11)	No	Local		
No	No			
Grade I (9)	No	Local		
No	No			
No	No			
No				
No			Bortezomib	
No			Thalidomide	
No			Lenalidomide	
Grade I (9)	No	Local	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.

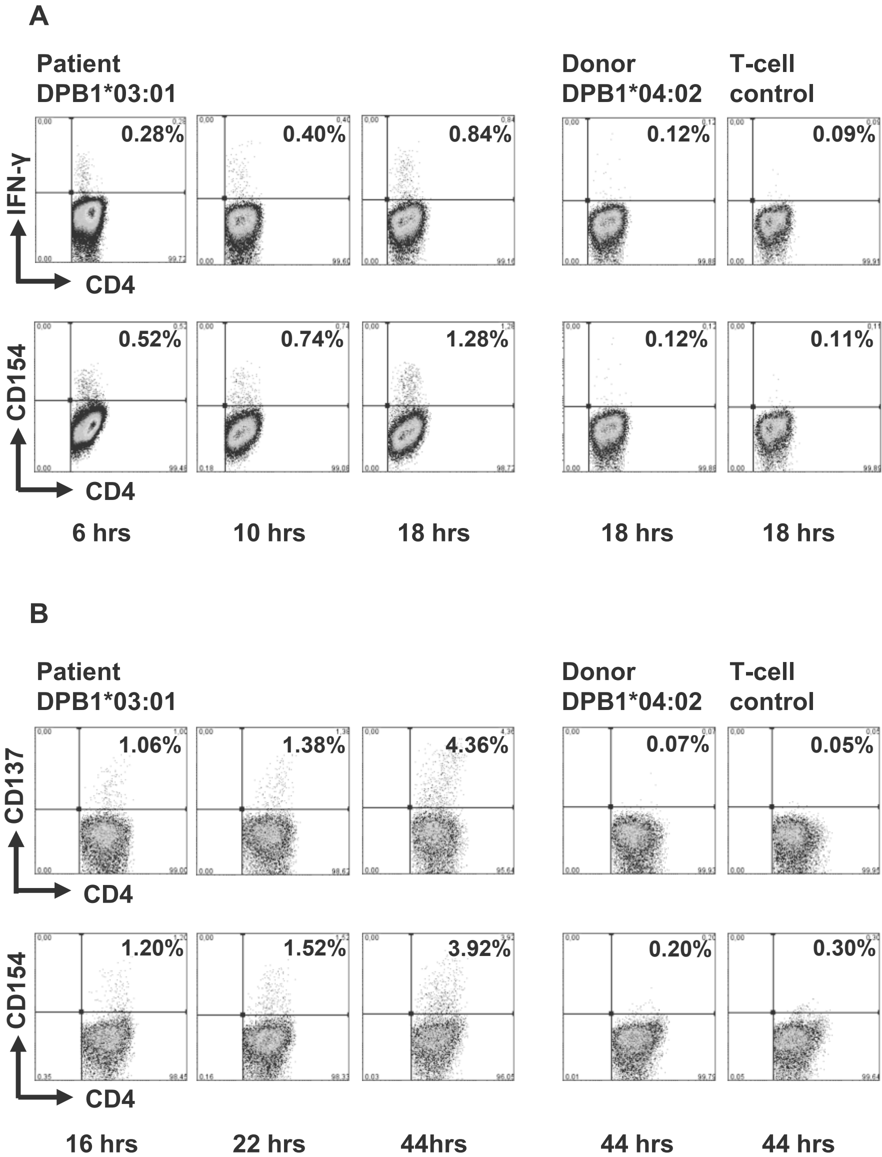
Results

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.



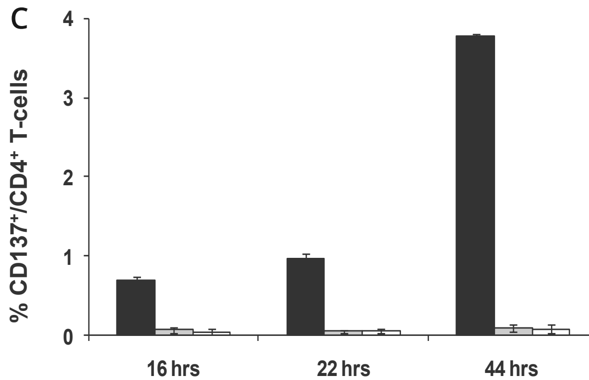


Figure 1. Kinetics of different activation markers to detect patient HLA-DP specific CD4+ T-cells directly ex vivo. PBMNC from a patient (HLA-DPB1*02:01,03:01) transplanted for a chronic B cell leukemia with an HLA-DPB1 mismatched donor (HLA-DPB1*04:02,05:01) were analyzed for the presence of HLA-DPB1*03:01 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

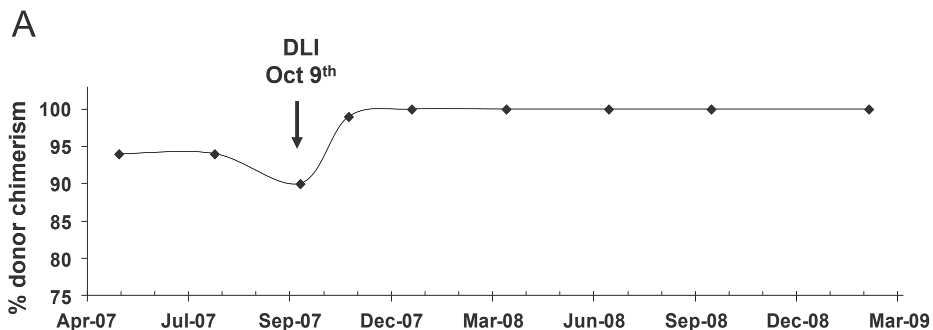
To confirm the specificity of CD137 expressing CD4+ T-cells for patient HLA-DPB1*03:01, we clonally isolated CD137+/CD4+ T-cells by flowcytometry, and tested growing T-cell clones for specific recognition of HLA-DPB1*03:01. A total number of 46 CD4+ T-cell clones were expanded, and 41 (89%) of these 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.



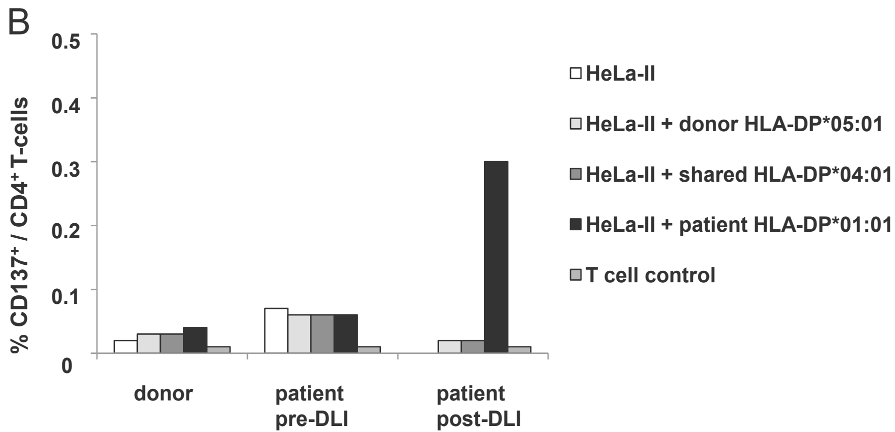


Figure 2. Emergence of patient HLA-DP specific CD4+ T-cells following HLA-DPB1 mismatched allo-SCT and DLI. (A) Percentage of donor chimerism in bone marrow after SCT. Arrow indicated DLI. Conversion to 100% donor chimerism was observed 3 months after DLI. (B) CD4+ T-cells purified from donor PBMNC, 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.

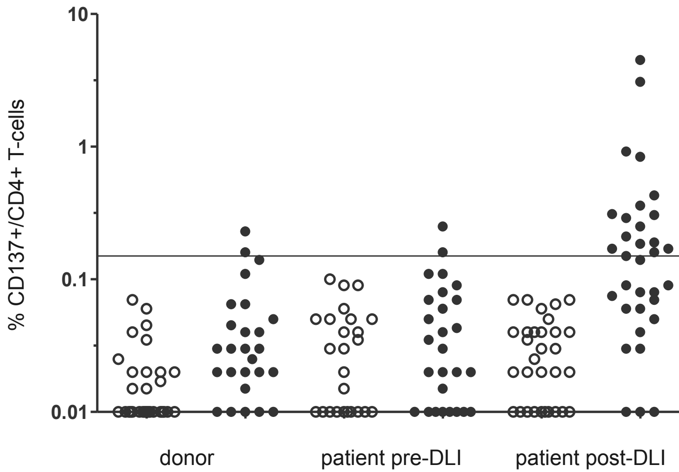


Figure 3. HLA-DP specific CD137+/CD4+ T-cells observed after HLA-DPB1 mismatched allo-SCT and DLI in 24 individuals. A total number of 24 patient-donor combinations were analyzed for the presence of patient HLA-DP specific CD4+ T-cells. In total, percentages of CD137+/CD4+ T-cells in response to 39 donor 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.

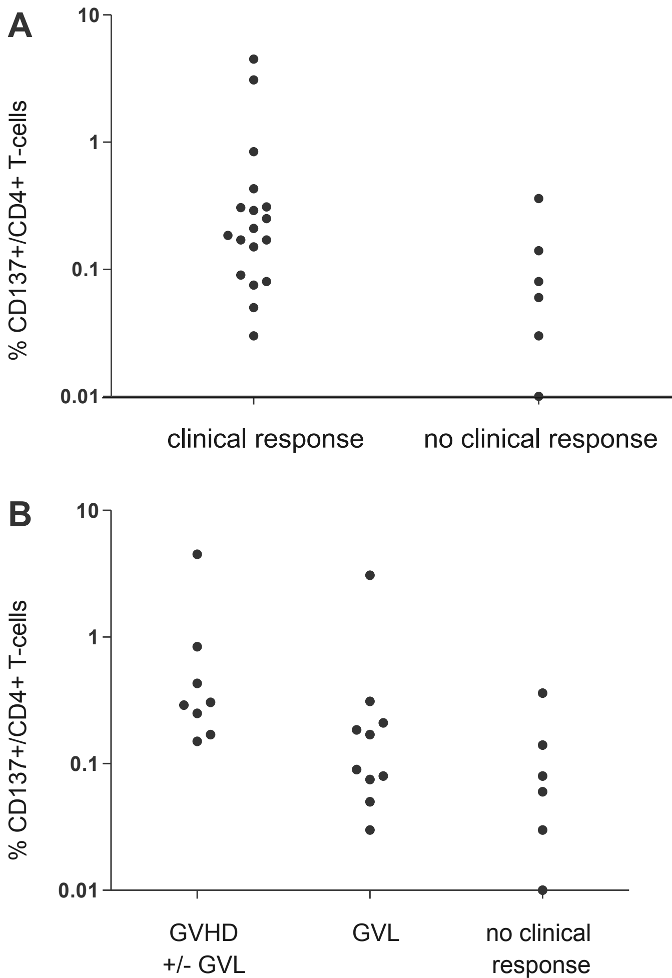


Figure 4. HLA-DP specific CD137+/CD4+ T-cells in patients with and without clinical responses to DLI. Percentage of CD137+/CD4+ T-cells in response to stimulation with HeLa-II cells transduced with patient HLA-DPB1 molecules obtained after allo-SCT and DLI are shown. Results were corrected for background 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).

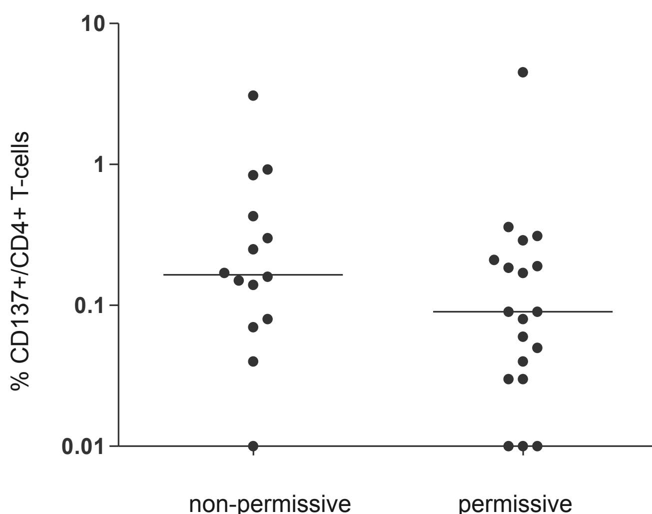


Figure 5. HLA-DP specific CD137+/CD4+ T-cells in response to permissive and non-permissive mismatches Percentage of CD137+/CD4+ T-cells in response to stimulation with HeLa-II cells transduced with patient HLA-DPB1 molecules obtained after allo-SCT and DLI are shown. Results were corrected for background 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	% HLA-DP specific CD4+ T-cells		Clinical response
#	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	CD4+ T-cells	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

6 Summary and General Discussion

Summary

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.[1-4] 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).[5-7]

HLA-DPB1 is not taken into consideration in donor selection, since mismatching for HLA-DPB1 is not associated with decreased survival. HLA-DPB1 mismatched allo-SCT is however associated with an increased risk of graft versus host disease and a decreased risk of disease relapse compared to HLA-DPB1 matched SCT.[6;8] In T-cell depleted allo-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, suggesting a selective GVL-effect.[9] In this thesis we investigated whether HLA-DP could be used as a relatively specific target for anti-leukemia reactivity following HLA-DPB1 mismatched T-cell depleted allo-SCT.

To investigate whether HLA-DP specific T-cells could mediate GVL-reactivity following HLA-DPB1 mismatched URD-SCT and DLI we analyzed in ***chapter 2*** the immune response in a patient with a refractory chronic B cell leukemia responding to DLI after HLA-DPB1 mismatched SCT. Patient and donor were fully matched for HLA-A, -B, -C, -DRB1 and –DQB1. The patient was typed HLA-DPB1*02:01,03:01 and the donor HLA-DPB1*04:02,05:01. We observed a profound GVL-effect with minimal skin GVHD resulting in a persistent complete remission of the disease. By ELISPOT analysis we identified the antileukemic CD4+ T-cell response starting 6 weeks after DLI. Following clonal isolation of these leukemia reactive CD4+ T-cells, blocking studies, panel studies and retroviral transduction experiments of the mismatched HLA-DPB1 alleles, identified both mismatched HLA-DPB1*02:01 and HLA-DPB1*03:01 as the targets of this immune response. The GVL-effect was caused by a polyclonal immune response comprising both T-helper and cytotoxic CD4+ T-cells. Since the emergence and kinetics of the leukemia-reactive CD4+ T-cells corresponded to the disappearance of the malignant cells, these HLA-DP specific CD4+ T-cells were likely to have mediated the anti-leukemic effect. Furthermore, the isolated HLA-DP specific CD4+ T-cells were capable of recognizing and lysing several HLA-DP-expressing myeloid and lymphoid hematological malignant cells. Recognition of non-hematopoietic cells was analyzed by testing for recognition of fibroblasts, renal cell carcinoma cell lines or breast cell carcinoma cell lines. Expression of HLA-DP was absent on most of these resting non-hematopoietic cells. Incubation with IFN-γ resulted in upregulated expression of HLA-DP and specific HLA-DP restricted recognition. These results illustrated that HLA-DP may

represent a relatively specific target for GVL-reactivity and that HLA-DPB1 mismatched allo-SCT may be preferable over a fully matched SCT for HLA-class II expressing hematological malignancies, making use of HLA-DP as a target for immunotherapy.

Some studies showed, in mostly T-cell repleted transplant regimens, that specific HLA-DPB1 mismatches were associated with poor clinical outcome.[10-14] It was suggested that this unfavorable effect was caused by a difference in immunogenicity between various HLA-DPB1 alleles. An algorithm defining permissive and non-permissive HLA-DPB1 mismatches was developed based on cross-reactive T-cell recognition patterns. It was suggested that permissive mismatches would not result in T-cell responses, whereas strong T-cell responses were expected to be generated against non-permissive mismatches.[10;13]

To analyze whether permissive HLA-DPB1 specific immune responses occur in vivo, we analyzed in ***chapter 3*** immune responses in 2 patients responding to donor lymphocyte infusion (DLI) after HLA-A, -B, -C, -DRB1, -DQB1 matched, HLA-DPB1 mismatched SCT. One of the patients received a permissive HLA-DPB1 mismatched SCT, the other patient a non-permissive HLA-DPB1 mismatched SCT. CD4+ T-cells were isolated from peripheral blood obtained during the clinical immune response to DLI, and stimulated with HLA-class II negative HeLa cells transduced with the specific HLA-DP molecules derived from patient or donor. In both patients, HLA-DP specific CD4+ T-cells were demonstrated by CD137 up-regulation in response to stimulation with HeLa cells transduced with patient and not donor HLA-DP molecules. Clonal isolation of these CD4+ T-cells confirmed specific recognition of the mismatched patient HLA-DPB1 molecules. Furthermore, we demonstrated in vitro for 4 additional individuals that permissive and non-permissive HLA-DPB1 responses were equally effective generated, illustrating immunogenicity of both permissive and non-permissive mismatches.

In ***chapter 4*** we further analyzed 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 and 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 showed that all HLA-DPB1 mismatches as defined by allele typing resulted in high frequency immune responses. Furthermore, we demonstrated that cross-recognition of different HLA-DPB1 molecules by HLA-DP specific CD4+ T-cell clones was a common observation. Individual CD4+ T-cell clones directed against the same HLA-DPB1 molecule exhibited different patterns in cross-recognition demonstrating that these CD4+ T-cell clones were specific for different epitopes. We confirmed previously described patterns in cross-recognition but also demonstrated cross-recognition patterns which did not correspond to the proposed algorithm. Overall, the data illustrated that frequently observed cross-recognition patterns

between specific HLA-DPB1 molecules did not preclude allo-HLA-DP responses between individuals expressing these HLA-DPB1 molecules. Together the 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.

To investigate whether patient HLA-DP specific immune responses were frequently induced following T-cell depleted HLA-DPB1 mismatched allo-SCT and DLI, we developed in ***chapter 5*** a method to screen for the presence of HLA-DP specific CD4+ T-cells. 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 peripheral blood in a simple and effective manner. We demonstrated that patient HLA-DP specific CD4+ T-cell responses were frequently found following T-cell depleted HLA-DPB1 mismatched SCT and DLI (14 of 24 patients: 58%). The presence of HLA-DP specific CD4+ T-cells seemed to correlate 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. HLA-DP specific CD4+ T-cells were most dominantly found in patients who developed GVHD following HLA-DPB1 mismatched T-cell depleted SCT and DLI. However, patient HLA-DP specific CD4+ T-cells were also found in a significant number of patients who developed beneficial clinical responses after DLI without GVHD. 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.

In summary, HLA-DPB1 mismatched allo-SCT followed by DLI late after transplantation can result in selective GVL-reactivity. Furthermore, all HLA-DPB1 mismatches as defined by allele typing are immunogenic and resulted in vitro in high frequency immune responses. In a cohort of 24 patients transplanted for various hematological malignancies with a 10 out 10 matched HLA-DPB1 mismatched SCT followed by DLI, HLA-DP specific immune responses were frequently found. The presence of HLA-DP specific CD4+ T-cells correlated with clinical immune responses including both selective GVL-reactivity and GVHD. It is likely that in each individual local environmental circumstances possibly in combination with the induction of other immune responses may finally determine the balance between GVHD and GVL-reactivity.

General Discussion

In this thesis we demonstrated that HLA-DPB1 mismatched SCT followed by DLI late after transplantation can result in selective GVL-reactivity. Furthermore, we showed that all HLA-DPB1 mismatches are immunogenic and that HLA-DPB1 mismatched SCT followed by DLI frequently resulted in an HLA-DP specific immune response. The presence of patient HLA-DP specific CD4+ T-cells correlated with clinical immune responses. However, in the analyzed patient cohort not only selective GVL-reactivity was observed but a significant number of patients also developed GVHD.

Of the 24 patients analyzed, 8 patients developed clinical significant GVHD. This included both patients who received DLI early after SCT and patients who received DLI later after SCT. GVHD was observed in patients receiving DLI for various indications including both persistent- and progressive disease and mixed chimerism. Furthermore, patients who developed GVHD suffered from various diseases, including multiple myeloma, B-cell malignancies and myeloid leukemia. Although this patient cohort was rather diverse en small, no clear correlation between clinical circumstances and development of GVHD was found. The results indicated that it is not easily predictable in which patients GVHD can be expected to develop.

Balance between GVL-reactivity and GVHD in HLA-class II restricted immune responses

In a fully HLA-matched setting, it has been shown that in patients with selective GVL-reactivity relatively limited immune responses directed against a few MiHAs were found. In contrast, in patients with GVHD, higher frequencies of multiple different MiHA specific T-cells have been found.[15;16] These data suggest that the development of a broader immune response may be correlated with the development of GVHD. In an HLA-matched setting, MiHA specific T-cells recognize non-self polymorphic peptides in the context of self-HLA molecules. During thymic development, T-cells are positively selected for recognition of self-HLA-molecules, followed by deletion of T-cells specific for a self-peptide in a self-HLA-molecule. This selection process results in a T-cell repertoire recognizing only non-self-peptides in self-HLA.[17] In allo-HLA-reactivity, T-cells recognize a peptide in the context of non-self HLA molecules. Allo-HLA-reactivity is therefore due to cross-reactivity of T-cells educated in the thymus to recognize a non-self peptide in the context of self-HLA. Any T-cell may by chance be cross-reactive with a peptide presented in a non-self HLA-molecule. Since there is no thymic selection for non-self HLA-molecules, no selection for cross-recognition of allo-HLA molecules occurs. The frequencies of T-cells directed against allo-HLA molecules can therefore be expected to be much higher than the frequencies of allo-reactive T-cells in a HLA-matched setting. Frequencies of allo-reactive T-cells in an HLA-

mismatched setting have been demonstrated to be a 1000 fold higher than the frequencies of allo-reactive T-cells in HLA-identical setting.[18;19] Following HLA-mismatched allo-SCT and DLI a broad immune response is therefore likely to develop, possibly increasing the risk for GVHD. Furthermore, in adult donors the T-cell compartment consists of both naïve and memory T-cells. Since any T-cell can exhibit reactivity against a peptide in the context of non-self HLA, allo-HLA-reactive T-cells may reside both in the naïve and in the memory repertoire.[18] An allo-HLA-DP specific CD4+ T-cell in the naïve T-cell repertoire requires professional antigen presenting cells (APC) for priming and has a relatively high threshold of activation.[20] In contrast, memory T-cells have a low threshold of activation and do not require professional APCs for priming of the immune response.[21] If by chance, T-cells specific for a peptide/allo-HLA-DP-molecule are present in the memory compartment of the donor, a vigorous immune response may be expected to be generated following HLA-DPB1 mismatched allo-SCT, whereas an allo-HLA-DP specific CD4+ T-cell from the naïve repertoire may result in a more limited immune responses.

T-cell responses directed against ubiquitously expressed antigens may be involved in both GVL-reactivity and GVHD, whereas T-cell responses directed against antigens specifically expressed on hematopoietic cells, including malignant cells may induce GVL-reactivity without severe GVHD. Immune responses directed against HLA-class II restricted MiHAs may also result in selective GVL-reactivity since expression of HLA-class II molecules is relatively restricted to hematopoietic cells. Indeed, T-cell responses directed against several HLA-class II restricted MiHAs encoded by broadly expressed genes have been identified in patients with GVL-reactivity accompanied by only mild GVHD.[22;23] This is further supported by the observation from clinical studies showing that CD8+ T-cell depletion reduces the incidence of GVHD associated with DLI without adversely affecting conversion to donor hematopoiesis.[24;25] However, HLA-class II expression can be induced on non-hematopoietic cells by pro-inflammatory cytokines.[26;27] HLA-class II restricted CD4+ T-cell responses may therefore contribute to development of GVHD when high levels of pro-inflammatory cytokines are released as a consequence of conditioning regimens or high pathogenic loads early after transplantation. High frequencies of MiHA-specific CD4+ T-cells have indeed been shown in several studies to precede and closely correlate with the onset of clinical GVHD.[28;29] Furthermore, allo-HLA-DP specific CD4+ T-cells have been isolated from skin biopsies of patients suffering from GVHD.[30-32] These data suggest that when a broad HLA-class II specific immune response is generated local circumstances at the time of DLI may influence the balance between GVL-reactivity and GVHD.

HLA-class II expression on non-hematopoietic tissues

We hypothesized that mismatched HLA-class II molecules may be used as a relatively specific target for cellular immunotherapy since most leukemic cells are expected to express HLA-class II molecules. In chapter 2 we demonstrated the expression of HLA-DP

on various leukemic cells. B-ALL and CLL cells showed high expression of HLA-DP whereas more variable expression of HLA-DP was found on myeloid leukemic cells. Furthermore, we demonstrated that various leukemic cells were recognized and lysed by HLA-DP specific CD4+ T-cell clones. Under normal conditions HLA-class II expression on non-hematopoietic tissues is anticipated to be restricted to antigen presenting cells. However, upon exposure to pro-inflammatory cytokines HLA-class II expression on non-hematopoietic cells may be upregulated[26;27] and thereby become a target for GVHD. We demonstrated that HLA-DP expression was not found on resting non-hematopoietic cells including fibroblasts and renal- or breast-cell carcinoma cell-lines, and showed that co-incubation with IFN-γ can result in upregulation of HLA-DP and specific recognition by HLA-DP specific CD4+ T-cell clones.

HLA-class II expression on non-hematopoietic tissues following conditioning regimens or in the presence of immune responses against pathogens has been described to result from a local release of cytokines which induce upregulation of co-stimulatory molecules and HLA-class II molecules on APC. These APC then induce activation and proliferation of T-cells, resulting in a further release of pro-inflammatory cytokines and more upregulation of HLA-class II molecules. In the end this cascade may result in cell damage or tissue destruction.[33;34]

To further support the hypothesis that mismatched HLA-class II molecules might be used as a relatively specific target for cellular immunotherapy in hematological malignancies we analyzed the expression of HLA-class II molecules on non-hematopoietic tissues at various moments during the course of SCT. Skin biopsies were collected after conditioning regimen at the time of SCT and during GVHD and stained for HLA-class II expression. In normal skin biopsies, HLA-class II expression was only found on dendritic cells in the epidermis. In the dermis clumps of HLA-class II expressing cells, possibly close to vessel structures were found. No upregulation of HLA-class II compared to normal skin biopsies was found following conditioning regimens at time of SCT. However, three months after SCT upregulation of HLA-class II molecules was found. In patients with GVHD also increased expression of HLA-class II molecules was demonstrated in the dermis. We did not find increased expression of HLA-class II molecules on keratinocytes in the epidermis. The results may indicate that not the conditioning regimen itself but possibly repair mechanisms or lymphopenia-induced homeostatic proliferation of donor T-cells induced upregulation of HLA-class II molecules on non-hematopoietic tissues found prior to the development of GVHD. Possibly the inflammatory status in the patient at time of DLI infusion greatly influences the outcome of GVHD. (unpublished data)

CD4+ DLI resulting in selective GVL-reactivity or GVHD

In allo-SCT, T-cell depletion of the graft has shown to reduce the risk of severe GVHD. However, in these protocols post-transplantation anti-viral and anti-tumor immunity is

also significantly impaired. To accelerate immune reconstitution for viral antigens and prevent relapse of malignancies, after T-cell depleted allo-SCT, prophylactic DLI can be administered. However, early intervention is associated with a risk of GVHD. CD8+ T-cells are known to be potent effector cells in both anti-tumor and anti-viral immunity, but they also play an essential role in GVHD. Although CD4+ T-cells are generally regarded as helper cells in induction and maintenance of CD8+ T-cell immunity, direct cytolytic activity in anti-tumor and viral responses has been shown. Administration of CD4+ DLI may reduce the risk of severe GVHD while preserving anti-tumor and antiviral activity.[24;25]

At this moment in our center a phase II open label single center randomized clinical study is ongoing in which immunologic effects of prophylactic infusion of purified donor CD4+ T-cells early after T-cell depleted allo-SCT for various hematological malignancies are evaluated. In patients receiving DLI from a matched related donor, no increase in GVHD was observed. Three patients received prophylactic CD4+ DLI from a 10/10 matched unrelated donor. Two of these patients suffered from AML and received a graft from a fully HLA-A, -B, -C, -DRB1 and -DQB1 matched, HLA-DPB1 mismatched donor. Three months after SCT, both patients received CD4+ DLI for mixed chimerism. This resulted in an increase in donor chimerism, which coincided with initially limited acute GVHD of the skin requiring only topical corticosteroid treatment. Subsequently, three to 8 weeks later, both patients developed grade 3/4 acute GVHD of the colon that was successfully treated with systemic immune suppression. At 15 months after alloSCT, both patients were still in CR. The clinical course and specificity of the T-cell immune response was analyzed in detail in these patients. Both patients experienced an episode of cytomegalovirus (CMV) reactivation early after alloSCT and were shown to contain significant numbers of patient-derived CMV specific T-cells with an activated phenotype as reflected by expression of HLA-class II at the time of prophylactic CD4+ DLI. A profound polyclonal CD4+ T-cell immune responses directed against mismatched HLA-DPB1 alleles in both patients was found. Allo-reactive HLA-DP specific CD4+ T-cells were shown to recognize HLA-class II expressing patient hematopoietic cells as well as skin-derived fibroblasts of the patients cultured with pro-inflammatory cytokines. In addition, colonic biopsies of both patients at the time of GVHD showed predominant infiltration with CD4+ T-cells and colonic epithelial cells displayed expression of HLA-class II. CMV specific T-cells may have contributed to the upregulation of HLA-class II expression on patient hematopoietic as well as non-hematopoietic cells. As a consequence, HLA-class II expressing residual patient-derived T-cells and non-hematopoietic tissues became targets for allo-reactive HLA-DP specific CD4+ T-cells. HLA-class II expression may have been particularly upregulated on non-hematopoietic cells in CMV-infected tissues due to a strong local inflammatory response mediated by CMV specific T-cells and activated inflammatory cells. The subsequent recognition of HLA-class II expressing non-hematopoietic cells by HLA-DP specific CD4+ T-cells may have resulted in local exacerbation of GVHD. These data support the hypothesis that active viral infection at

the time of HLA-DPB1 mismatched T-cell infusion may trigger HLA-DP specific CD4+ T-cells to mediate both a beneficial GVL-reactivity and detrimental GVHD.[35]

CD4+ T-cells as helper cells in GVL-reactivity

CD4+ T-cells are known as helper cells for the induction and maintenance of CD8+ T-cell immune responses. Naïve CD8+ T-cells require priming by activated APCs to proliferate and differentiate in effector cells. APCs can be activated by inflammatory signals derived from pathogens. Alternatively, in the absence of inflammatory signals, CD4+ T-cells can activate APCs by CD40-CD40Ligand interaction and cytokine production.[36-38] In patients successfully treated with DLI for relapsed hematological malignancies after allo-SCT, both MiHA specific CD4+ T-cells and CD8+ T-cells have been isolated suggesting that the MiHA specific CD4+ T-cells may have provided help for the induction of CD8+ T-cell responses.[22;23;39] Animal models have shown that in the absence of tissue damage or infection, CD8+ T-cell mediated GVL-reactivity following DLI administration required priming of CD4+ T helper cells by host APCs.[40]

It has been suggested that efficacy of DLI to induce an anti-tumor response may depend on the capacity of leukemic cells to become APC in vivo. Recently it was shown that cross-talk between CD4+ T-cells and leukemic cells in vivo can change leukemic cells into an APC phenotype. In this study, leukemic cells in mice treated with DLI acquired an APC phenotype in vivo, whereas leukemic cells from non-treated mice remained unchanged. In vitro experiments confirmed that co-culture of primary leukemic cells with leukemia-reactive CD4+ T-cells induced an APC-phenotype on leukemic cells, whereas this was not observed from non-specific CD4+ T-cells. In this study allo-reactive CD4+ T-cells fulfilled a dual role in the anti-tumor response. First, as effector cells by directly eliminating leukemic cells, and secondly, as helper cells by producing cytokines with induced an APC-phenotype on leukemic cells. This cross-talk between leukemic cells and leukemia specific CD4+ T-cells may have had a significant role in the overall magnitude of the antitumor response.[41]

CD4+ T-cells as effector cells

Although CD4+ T-cells have mostly been studied in their role as helper cells for CD8+ T-cell immunity, in several studies CD4+ T-cells with direct cytolytic activity have been isolated from patients with GVL-responses after allo-SCT.[22;23;42;43] These CD4+ T-cells have been suggested to be involved in mediating direct anti-tumor responses as effector cells. This is supported by both murine and human studies showing that CD4+ DLI mediated GVL-reactivity.[25;44] Direct evidence that CD4+ T-cells can mediate an anti-tumor response in a mouse model was provided by Stevanovic et al. Anti-tumor effect of CD4+ T-cells was investigated by administering highly purified CD4+ DLI, obtained after positive isolation of CD4+CD8- T-cells by flowcytometry. The CD4+ DLI did not contain contaminating CD8+ T-cells and in vivo no expansion of CD8+ T-cells was observed. Since the emergence

and kinetics of activated CD4+ T-cells corresponded to the disappearance of leukemic cells, conclusive evidence was provided that CD4+ T-cells are capable of mediating GVL-reactivity.[41] In chapter 2, we demonstrated using ex vivo ELISOT analysis the presence of only CD4+ and not CD8+ leukemia reactive T-cells in both peripheral blood and bone marrow indicating that CD4+ T-cells can elicit a profound anti-leukemia response in the absence of leukemia-reactive CD8+ T-cells. In these studies both CD4+ T-cells producing high levels of IFN-γ without cytolytic activity and CD4+ T-cells which were capable of direct lysis of leukemic cells were found. The results demonstrated that CD4+ T helper cells and CD4+ cytotoxic CD4+ T-cells are sufficient for direct effector function in GVL-reactivity in patients.

In conclusion

In chapter 5, it was shown that following HLA-DPB1 mismatched allo-SCT and DLI a variety of responses can occur. In a few patients no response was observed, whereas in other patients GVL-reactivity in the presence or absence of GVHD was observed. From these data it could not be easily predicted in which patient population a selective GVL-effect may be expected. We demonstrated in chapter 3 that allo-HLA-DP specific immune responses can be generated from all HLA-DPB1 mismatch combinations. Therefore, we hypothesize that not only the T-cell repertoire of the donor, but possibly more importantly local circumstances in the host determines the balance between GVL-reactivity and GVHD. Expression of HLA-class II in different target tissues influenced by inflammatory conditions may determine the magnitude and diversity of immune responses. To further support this hypothesis research analyzing local circumstances in the host like expression of HLA-class II molecules, adhesion and co-stimulatory molecules and their relation to GVHD is essential.

As long as it is not possible to predict which patients are especially at risk to develop GVHD following HLA-DPB1 mismatched SCT and DLI, it may be preferable to use HLA-DPB1 matched SCT over HLA-DPB1 mismatched SCT in low-grade disease, in order to reduce the risk for GVHD as much as possible. However, in high risk patients, in whom a strong anti-tumor response is required, an HLA-DPB1 mismatched donor may be favorable over an HLA-DPB1 matched donor to provoke a GVL-effect while accepting a risk for concurrent GVHD.

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7 Nederlandse Samenvatting

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Allogene stamceltransplantatie (SCT) is een succesvolle behandeling voor patiënten met allerlei kwaadaardige hematologische ziekten. Voorafgaand aan de SCT krijgen patiënten hoge dosis chemotherapie en bestraling om kwaadaardige cellen te vernietigen en om het hematopoietische- en dus afweersysteem van de patiënt te onderdrukken om zo plaats te maken voor donor stamcellen. Oorspronkelijk was het doel van de SCT het vervangen van het lethaal beschadigde hematopoietische systeem van de patiënt. Autologe stamcellen of stamcellen van een genetische identieke tweeling werden verondersteld de beste bron voor hematologische stamcellen te zijn. Transplantatie met stamcellen van een allogene donor bleek namelijk gepaard te gaan met immunologische complicaties zoals afstoting of *graft versus host ziekte* (GVHD). Bij GVHD worden gezonde weefsels van de patient, zoals huid, darmen en lever, aangevallen door het afweersysteem van de donor. Deze afstotingsreactie kan mild zijn, maar ook een levensbedreigende complicatie vormen. Klinische studies toonden echter aan dat het optreden van GVHD ook gepaard ging met een verminderde kans op recidief van de ziekte wat resulteerde in een betere uiteindelijke overleving. Afweercellen (T cellen) van de donor, die ook in het stamceltransplantaat aanwezig zijn, bleken niet alleen verantwoordelijk voor het optreden van GVHD maar zijn ook in staat een zogenaamd *graft versus leukemie* (GVL) effect te bewerkstelligen. Verwijdering van T cellen uit het transplantaat verminderde de kans op GVHD, maar vergrootte de kans op een recidief van de leukemie. Daarnaast bleek een donor lymfocyten infusie (DLI) bij een recidief van de ziekte na SCT, een langdurige remissie van verschillen hematologische maligniteiten te kunnen induceren. Het grote voordeel van een SCT met een allogene donor boven eigen stamcellen is dus dat het immuunsysteem van de donor gebruikt kan worden om resterende kwaadaardige cellen van de patiënt na de transplantatie op te ruimen. Echter het grote probleem van immunotherapie door middel van DLI is GVHD. Een belangrijk doel in SCT onderzoek is dan ook om strategieën voor immunotherapie te ontwikkelen waarbij er minder GVHD optreedt maar met behoud van GVL-reactiviteit.

GVL-reactiviteit en GVHD bij HLA-identieke SCT

Om het risico op afstoting en GVHD te beperken, wordt voor een SCT bij voorkeur gebruik gemaakt van een HLA-identieke donor. HLA-moleculen zijn eiwitten op het celoppervlak van cellen, die antigenen aan T cellen presenteren. HLA-klasse I moleculen (HLA-A, -B en -C) zitten op vrijwel alle kernhoudende cellen. HLA-klasse II moleculen (HLA-DR, -DQ en -DP) komen voornamelijk op hematopoietische cellen tot expressie. Allo-reactieve T cellen die vreemde HLA-moleculen herkennen, zijn aanwezig in hoge frequenties in perifeer bloed. Een HLA-niet-identieke SCT kan daarom leiden tot sterke allo-reactiviteit met daarbij een hoog risico op GVHD. Echter ook na een HLA-identieke SCT of DLI treedt vaak GVHD op. Dit wordt veroorzaakt door genetische verschillen tussen patiënt en donor anders dan HLA-moleculen, zogenaamde minor histocompatibility antigenen (MiHA). Dit zijn stukjes

eiwitten, verschillend tussen patient en donor, die in HLA-moleculen gepresenteerd worden. Donor T cellen kunnen MiHA van de patiënt als lichaamsvreemd herkennen en een immuunrespons hiertegen ontwikkelen. Verschillende studies hebben aangetoond dat MiHA-specifieke T cellen betrokken zijn bij GVHD en GVL-reactiviteit. Kinetiek studies hebben laten zien dat het opkomen van deze T cellen gepaard gaat met het verdwijnen van kwaadaardige cellen uit het bloed wat het GVL-effect illustreert. In patiënten die gerespondeerd hebben op DLI na HLA-identieke SCT zijn polyklonale immuunresponsen gevonden gericht tegen verschillende MiHAs.

T cel responsen gericht tegen MiHA die zowel op hematopoietische cellen als op niet-hematopoietische cellen tot expressie komen zullen waarschijnlijk leiden tot zowel GVL-reactiviteit als GVHD. Een immunologische respons van CD8+ T cellen tegen een breed tot expressie komend MiHA gepresenteerd in een HLA-klasse I molecuul leidt tot zowel GVL-reactiviteit als ook herkenning en dus schade in verschillende organen, resulterend in ernstige GVHD. Een CD8+ T cel respons specifiek voor een hematopoiese gerestricteerd antigeen leidt tot selectieve GVL-reactiviteit. In tegenstelling tot de HLA-klasse I moleculen komen HLA-klasse II moleculen vooral tot expressie op hematopoietische cellen. Bij patienten met een HLA-klasse II positieve hematologische maligniteit zou dus een immuunrespons tegen een HLA-klasse II gerestricteerd MiHA kunnen leiden tot een selectief GVL-effect. Inderdaad zijn deze CD4+ T cel responsen gevonden in patiënten bij wie geen GVHD plaats vond. Uitgebreide analyse van immuunresponsen in patiënten met en sterk anti-leukemie effect met en zonder GVHD, heeft laten zien dat immuunresponsen met een hoge amplitude gericht tegen breed tot expressie komende antigenen leidt tot zowel GVL-reactiviteit als GVHD, waar beperkte immuunresponsen gericht tegen hematopoiese gerestricteerde antigenen vaker resulteerden in selectieve GVL-reactiviteit.

Traditioneel worden CD8+ T cellen gezien als primaire effector cellen in GVL-reactiviteit, en CD4+ T cellen als helper cellen voor het induceren en in stand houden van CD8+ T cel immuunresponsen. In patiënten met selectieve GVL-responsen zijn echter ook CD4+ T cellen gevonden met een direct cytotoxische activiteit tegen leukemie cellen. Dit suggereert dat CD4+ T cellen ook als effector cellen kunnen fungeren in GVL-reactiviteit. Omdat de expressie van HLA-klasse II moleculen dus met name gerestricteerd is tot hematopoietische cellen zou een CD4+ T cel immuunrespons kunnen leiden tot selectieve GVL-reactiviteit. Klinische studies hebben inderdaad laten zien dat het verwijderen van CD8+ T cellen van het SCT of van DLI kan leiden tot minder GVHD met behoud van GVL-reactiviteit. Echter, expressie van HLA-klasse II moleculen op niet-hematopoietische cellen kan worden opgereguleerd bij expositie aan pro-inflammatoire cytokines. Een HLA-klasse II gerestricteerde immuunrespons kan dan alsnog resulteren in GVHD.

SCT van een onverwante donor

Een allogene SCT wordt bij voorkeur uitgevoerd met een HLA-identieke donor om het risico van GVHD en afstoting te beperken. De optimale donor is een HLA-identieke broer of zus van de patiënt. Deze is echter niet altijd beschikbaar. Als er geen geschikte familie donor is, is een HLA-identieke onverwante donor een goed alternatief. Bij voorkeur wordt een volledig identieke donor voor HLA-A,-B,-C, -DRB1 en -DQB1 gebruikt. Grote studies hebben laten zien dat een mismatch voor HLA-A, -B, -C en –DRB1 geassocieerd is met een slechtere uitkomst. Een mismatch voor alleen HLA-DQB1 resulteerde niet in een slechtere overleving, echter in combinatie met een andere mismatch werd een nog slechtere uitkomst gezien. Een mismatch voor HLA-DPB1 resulteerde niet in een slechtere overleving en daarom wordt er bij de donor selectie niet gekeken naar HLA-DPB1.

De rol van HLA-DPB1 in allogene SCT

HLA-DP is het zesde klassieke HLA-molecuul. Vanwege een recombinatie 'hotspot' tussen de HLA-DPB1 genen en de overige HLA-genen, wordt HLA-DP niet gelinkt aan de andere HLA-genen overgeërfd. Dit resulteert een mismatch kans voor HLA-DPB1 tot 10% bij een broer of zus en een mismatch kans tot 90% bij onverwante donoren. Lange tijd is de rol van HLA-DPB1 in onverwante SCT niet duidelijk geweest. Studies toonden wisselende resultaten in data betreffende een risico op GVHD en overleving. HLA-DPB1 werd daarom niet meegenomen in donor selectie. Grote klinische studies uitgevoerd tussen 1999 en 2008 hebben echter laten zien dat HLA-DP zich gedraagt als een klassiek transplantatie antigen. Bij een SCT met T cellen werd bij een HLA-DPB1 niet-identieke donor meer GVHD en een lagere recidief kans gezien in vergelijking met een HLA-DPB1 identieke donor. Dit resulteerde in een gelijke totale overleving. In T cel gedepleteerde SCT werd geen verhoogd risico op GVHD gezien, maar wel een lagere kans op recidief van de ziekte.

Verder bewijs van een immunologische rol voor HLA-DP werd geleverd door verschillende studies waarbij na allogene HLA-DPB1 niet-identieke SCT, CD4+ T cellen gericht tegen het niet-identieke HLA-DP molecuul werden gevonden. HLA-DP specifieke CD4+ T cellen werden geïsoleerd uit huidbiopten van patiënten die GVHD ontwikkelden en uit een patiënt die het SCT afstootte. Er werd gesuggereerd dat HLA-DP specifieke T cellen wellicht selectieve GVL-reactiviteit konden induceren bij T cel gedepleteerde SCT. Echter direct bewijs hiervoor werd niet geleverd.

Permissive en non-permissive mismatches

Vanwege conflicterende resultaten in verschillende studies over de rol van HLA-DP in allo-SCT, is gesuggereerd dat er wellicht een verschil in immunogeniciteit tussen verschillende HLA-DPB1 mismatches zou zijn. Verschillen in aminozuren in specifieke delen van het HLA-DP molecuul zouden gunstig of ongunstig zijn. Verschillende onderzoeksgroepen vonden

andere specifieke mismatches die geassocieerd zouden zijn met meer GVHD of een slechtere overleving. Zino en medewerkers ontwikkelden een algoritme waarbij HLA-DPB1 mismatches werden ingedeeld in 3 en later 4 verschillende groepen. Dit was gebaseerd op het herkenningspatroon van enkele T cel klonen. Een mismatch tussen HLA-DPB1 moleculen met grote onderlinge gelijkenis werd 'permissive' genoemd en een mismatch tussen HLA-DPB1 moleculen uit verschillende groepen werd 'non-permissive' genoemd. In een cohort studie bleek een 'non-permissive' mismatch geassocieerd met een slechtere overleving.

Diverse bevindingen van de verschillende onderzoeksgroepen konden echter niet bevestigd worden in andere studies.

Mismatched HLA-DP als een target voor GVL-reactiviteit

Een immuunrespons gericht tegen een hematopoiese gerestricteerd antigen wordt verwacht te resulteren in selectieve GVL-reactiviteit, terwijl een immuunrespons tegen een breed tot expressie komend antigen kan resulteren in zowel GVL-reactiviteit als GVHD. Tegelijkertijd blijken beperkte immuunresponsen met lage frequenties MiHA-specifieke T cellen GVL-reactiviteit te induceren, waar bredere immuunresponsen gericht tegen meerdere antigenen zowel GVL als GVHD induceren. Omdat allo-reactieve T cellen in hoge frequenties aanwezig zijn in perifeer bloed, zou een immuunrespons gericht tegen een niet-identiek HLA-DPB1 molecuul kunnen leiden tot een sterke immuunrespons en daarmee naast GVL-reactiviteit ook GVHD kunnen induceren. Bij HLA-DPB1 niet-identieke SCT met T cellen wordt zowel een hoger risico op GVHD gezien als een lager risico op recidief van de ziekte. Bij T cel gedepleteerde SCT wordt wel een lager risico op recidief gezien, maar niet een verhoogd risico op GVHD. Deze data wijzen erop, dat bij HLA-DPB1 niet-identieke SCT het gebruikte transplantatie regiem in combinatie met individuele omstandigheden in de patiënt, de balans tussen GVHD en GVL-reactiviteit zouden kunnen bepalen.

Dit proefschrift

HLA-DPB1 wordt niet meegenomen in donor selectie voor een hematologische SCT. Dit komt omdat een HLA-DPB1 niet-identieke SCT niet geassocieerd is met lagere overlevingskansen vergeleken met een HLA-DPB1 identieke SCT. Mismatchen voor HLA-DPB1 is echter wel geassocieerd met een verhoogd risico op GVHD en een lagere kans op recidief van de ziekte. Bij een T cel gedepleteerde HLA-DPB1 niet-identieke SCT werd geen verhoogd risico op GVHD gevonden, maar wel een significant lager risico op recidief van de ziekte. In dit proefschrift is bestudeerd of HLA-DP gebruikt kan worden als een relatief specifiek target voor anti-leukemie reactiviteit na een T cel gedepleteerde HLA-DPB1 niet-identieke SCT. Verder is er gekeken of er bij alle HLA-DPB1 mismatch combinaties verwacht kan worden dat er een immuunrespons optreedt. Daarnaast is bestudeerd of

een HLA-DP specifieke immuunrespons ook daadwerkelijk vaak optreedt na HLA-DPB1 niet-identieke SCT en DLI, en of het optreden van een HLA-DP specifieke immuunrespons dan ook geassocieerd is met een anti-leukemie effect of GVHD.

Om te kijken of HLA-DP specifieke T cellen een anti-leukemie reactie kunnen bewerkstelligen na HLA-DPB1 niet-identieke SCT en DLI hebben we in ***hoofdstuk 2*** de immuunrespons in een patiënt met een chronische B cel leukemie die goed reageerde op DLI na HLA-DPB1 niet-identieke SCT bestudeerd. Patiënt en donor waren volledig identiek voor HLA-A, -B, -C, -DRB1 en –DQB1. De patiënt was getypeerd HLA-DPB1*0201;0301 en de donor HLA-DPB1*0402;0501. Er vond een sterke anti-leukemie respons plaats met slechts beperkte GVHD van de huid wat resulteerde in een complete remissie van de ziekte. Met behulp van ELISPOT analyse werd een CD4+ T cel respons geïdentificeerd die 6 weken na DLI begon. Na isolatie en opgroeien van de leukemie reactieve CD4+ T cellen tot T cel klonen, kon met behulp van experimenten met blokkerende antilichamen, panel experimenten waarbij herkenning van cellijnen met verschillende HLA-DPB1 moleculen getest werd, en retrovirale transductie van beide gemismatchte HLA-DPB1 moleculen, HLA-DPB1*02 en HLA-DPB1*03 geïdentificeerd worden als het doelwit van deze immuunrespons. De anti-leukemie respons bleek veroorzaakt door een polyklonale T cel respons die zowel uit helper als uit cytotoxische CD4+ T cellen bestond. Omdat het ontstaan en de kinetiek van de anti-leukemie specifieke CD4+ T cellen overeen kwam met het verdwijnen van de kwaadaardige cellen uit het bloed, lijkt het waarschijnlijk dat deze HLA-DP specifieke CD4+ T cellen de antitumor respons veroorzaakt hebben. De geïsoleerde HLA-DP specifieke CD4+ T cellen waren ook in staat om myeloide en lymfatische maligne hematologische cellen van andere patiënten te herkennen en te lyseren. Herkenning van niet-hematologische cellen werd bekeken door herkenning van fibroblasten, niercelcarcinoom cellijnen en borstkanker cellijnen te bestuderen. HLA-DP kwam niet tot expressie op de meeste van deze cellijnen in rust en deze cellen werden dus ook niet herkend door de CD4+ T cellen. Echter, behandeling met IFN-γ om een pro-inflammatoire situatie na te bootsen, resulteerde in opregulatie van HLA-DP en specifieke HLA-DP gerestricteerde herkenning van deze niet hematopoietische cellen. De resultaten illustreren dat HLA-DP mogelijk een relatief specifiek doelwit kan zijn voor anti-leukemie reactiviteit en dat een HLA-DPB1 gemismatchde SCT de voorkeur zou kunnen krijgen over een HLA-DPB1 identieke SCT bij HLA-klasse II positieve hematologische maligniteiten om op deze manier gebruik te kunnen maken van HLA-DP als een target voor immunotherapie. Het toedienen van donor T cellen na een T cel gedepleteerde HLA-DPB1 niet-identieke SCT zou op die manier kunnen resulteren in een sterk anti-leukemie effect zonder een groot risico op ernstige GVHD.

Een aantal studies heeft laten zien dat specifieke HLA-DPB1 mismatches waren geassocieerd met een slechtere overleving. Er is gesuggereerd dat dit ongunstige effect werd veroorzaakt door een verschil in immunogeniciteit tussen verschillende HLA-

DP allelen. Op basis hiervan werd een algoritme ontwikkeld waarin permissive en non-permissive HLA-DPB1 mismatches werden gedefinieerd. Dit algoritme was gebaseerd op kruisherkenningspatronen van HLA-DP specifieke CD4+ T cellen. Er werd gesuggereerd dat bij permissive mismatches geen T cel responsen geïnduceerd zou worden door hoge mate van overeenkomst tussen het HLA-DPB1 molecuul van patient en donor. Bij non-permissive mismatches zou er juist wel een sterke T cel respons plaatsvinden en daarmee een grotere kans op het ontstaan van GVHD.

In ***hoofdstuk 3*** hebben we gekeken of 'permissive' HLA-DPB1 specifieke immuunresponsen in vivo voorkomen. We hebben de immuunrespons in 2 patiënten geanalyseerd die reageerden op DLI bij een HLA-A, -B, -C, -DRB1, -DQB1 identieke, HLA-DPB1 niet-identieke SCT. De ene patiënt had een 'permissive' HLA-DPB1 niet-identieke SCT gekregen en de andere een 'non-permissive' HLA-DPB1 niet-identieke SCT. CD4+ T cellen werden uit het bloed geïsoleerd op het moment van de klinische respons op DLI en getest op specifieke herkenning van patiënt HLA-DP moleculen. In beide patiënten werd specifieke herkenning van patiënt en niet donor HLA-DP moleculen gevonden. Verder laten we voor vier andere individuen zien dat zowel 'permissive' als 'non-permissive' HLA-DPB1 specifieke immuunresponsen even effectief gegenereerd kunnen worden. Dit illustreert immunogeniciteit van zowel 'non-permissive' als 'permissive' mismatches.

In ***hoofdstuk 4*** gaan we hier verder op in. We hebben bestudeerd of immunogeniciteit van HLA-DPB1 mismatches voorspeld kan worden gebaseerd op de aan- of afwezigheid van specifieke aminozuur sequenties. We hebben een model ontwikkeld waarin we in vitro allo-HLA-DP immuunresponsen konden genereren. In totaal hebben we 48 verschillende stimulator/responder combinaties getest door CD4+ T cellen van 5 HLA-DPB1 homozygote individuen te stimuleren met dezelfde antigen presenterende cellen waarin verschillende HLA-DP moleculen getransduceerd waren. We laten zien dat vrijwel alle combinaties resulteerden in hoge frequente immuunresponsen. Verder laten we in dit hoofdstuk zien dat kruisherkenning van verschillende HLA-DPB1 moleculen door HLA-DP specifieke T cellen een veel voorkomende observatie is. Individuele CD4+ T cel klonen met dezelfde specificiteit lieten verschillende herkenningspatronen in kruisreactiviteit zien. Dit illustreert dat ze specifiek voor een ander epitoop zijn. We bevestigen eerder beschreven patronen in kruisherkenning, maar laten ook patronen zien die niet overeenkomen met het voorgestelde algoritme. Deze data samen illustreren dat een hoge mate van overeenkomst tussen HLA-DPB1 allelen voorspellend is in kruis-reactieve herkenning, maar niet voor immunogeniciteit. Ook laten we zien dat T cel herkenningspatronen allo-reactiviteit niet kunnen voorspellen.

Om te kijken of HLA-DP specifieke immuunresponsen vaak voorkomen na T cel gedepleteerde HLA-DPB1 niet-identieke SCT en DLI hebben we in ***hoofdstuk 5*** een methode ontwikkeld om bloed van patiënten te screenen op de aanwezigheid van HLA-DP specifieke CD4+ T cellen. Door HeLa cellen getransduceerd met verschillende HLA-

DP moleculen te gebruiken als stimulator cellen, konden we het opkomen van patiënt HLA-DP specifieke T cel responsen op een eenvoudige en effectieve manier meten. We laten zien dat patiënt HLA-DP specifieke immuunresponsen vaak gevonden worden na T cel gedepleteerde HLA-DPB1 niet-identieke SCT en DLI. (14 van de 24 patiënten: 58%). De aanwezigheid van HLA-DP specifieke CD4+T cellen leek te correleren met klinische respons op DLI omdat HLA-DP specifieke CD4+ T cellen werden gevonden in 13 (72%) van de 18 patiënten met een klinische respons op DLI en slechts in 1 (17%) van de 6 patiënten waarbij geen klinische respons werd gezien. HLA-DP specifieke T cellen werden vooral gevonden in patiënten die ernstige GVHD ontwikkelden, maar ook in een significant aantal patiënten dat alleen een GVL-reactie na DLI ontwikkelde zonder GVHD. HLA-DP specifieke CD4+ T cellen werden gevonden in patiënten met verschillende hematologische maligniteiten, waaronder de ziekte van Kahler, B-cel maligniteiten en myeloide leukemieën.

De resultaten van het onderzoek beschreven in dit proefschrift laten zien dat HLA-DPB1 niet-identieke SCT gevolgd door DLI laat na de transplantatie kan resulteren in een selectief GVL-effect. We hebben laten zien dat alle HLA-DPB1 mismatches immunogeen zijn en kunnen resulteren in sterke immuunresponsen. In een cohort van 24 patiënten die een SCT gevolgd door DLI ontvingen voor allerlei hematologische ziekten van een HLA-DPB1 niet-identieke donor, werden zeer frequent HLA-DP specifieke immuunresponsen gevonden. Bovendien correspondeerde de aanwezigheid van HLA-DP specifieke T cellen met het optreden van een klinische respons, waarbij er zowel selectieve GVL-reacties werden gezien als GVHD. Waarschijnlijk wordt de uiteindelijke balans tussen GVHD en GVL-reactiviteit na HLA-DPB1 niet-identieke T cel gedepleteerde SCT en DLI in ieder individu bepaald door het ontwikkelen van HLA-DP specifieke immuunresponsen, de lokale omstandigheden in de patiënt in combinatie met de aan- of afwezigheid van andere immuunresponsen.

Nawoord

Curriculum Vitae

List of Publications

Nawoord

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Curriculum Vitae

Caroline Elisabeth Rutten is geboren op 22 maart 1979 te Leiden. In 1997 behaalde zij haar gymnasium β diploma aan het Jeanne d'Arc College te Maastricht. In hetzelfde jaar begon zij met de studie Biomedische Wetenschappen en in 1999 met de studie Geneeskunde aan de Universiteit van Leiden. Haar afstudeeronderzoek voor Biomedische Wetenschappen deed ze aan de Universiteit van Stellenbosch in Kaapstad, Zuid-Afrika, waarna ze in 2005 haar doctoraal behaalde. In datzelfde jaar behaalde ze haar arts-examen (cum laude). In oktober 2005 startte ze met het in dit proefschrift beschreven promotieonderzoek bij het laboratorium voor Experimentele Hematologie van het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van prof. dr. J.H.F. Falkenburg. In januari 2010 begon ze aan de opleiding tot internist in het LUMC (opleider prof. dr. J.A. Romijn). In 2011 zette ze deze opleiding voort in het Bronovo Ziekenhuis in Den Haag (opleider dr. J.W. van 't Wout). Sinds januari 2012 is ze weer werkzaam in het LUMC (opleider prof. dr. J.T. van Dissel).

List of Publications

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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