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**HLA CLASS II RESTRICTED MINOR HISTOCOMPATIBILITY
ANTIGENS**

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IDENTIFICATION, PROCESSING AND BIOLOGY

ANITA KREMER

HLA class II restricted minor histocompatibility antigens - identification, processing and biology

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**HLA CLASS II RESTRICTED MINOR HISTOCOMPATIBILITY
ANTIGENS**

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IDENTIFICATION, PROCESSING AND BIOLOGY

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INTRODUCTION

INTRODUCTION

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (aSCT) is a potentially curative treatment for benign and malignant hematopoietic diseases¹. Originally being meant as pure replacement of patient hematopoiesis to allow administration of lethal dosages of radio- and chemotherapy, it now represents the dawn of cellular immunotherapy.

In classical myeloablative transplantation, patient hematopoiesis is completely eradicated by radio-/chemotherapy to eliminate residual malignant cells and to allow engraftment of donor cells. Subsequently, donor hematopoietic stem cells are infused intravenously to repopulate the hematopoietic system and to rescue the patient from the otherwise lethal treatment. Unfortunately, the benefit of the therapy is severely limited by substantial toxicity of the conditioning regimen and even more so by the occurrence of a side-effect called graft-versus-host disease (GvHD), the major cause of morbidity and mortality after allogeneic stem cell transplantation^{1,2}. This wasting syndrome was originally described by Bellingham and Brent in the last 1950's as "secondary disease" in mice that survived direct irradiation toxicity ("primary disease") and were successfully engrafted with allogeneic bone marrow³. The authors concluded that this "secondary disease" was due to an immune response of the donor cells against tissue of the host. This notion was further strengthened by the observation that administration of immunosuppressive drugs could ameliorate GvHD⁴. Clinically, acute GvHD mainly affects skin, gut and liver and is subdivided in four grades of severity⁵. Affection of the skin is usually the first clinical manifestation and presents as pruritic maculo-papular rash. In severe cases this can culminate in a bullous exanthema with desquamation of the skin. Gut GvHD presents mainly with voluminous watery diarrhea, nausea and vomiting. GvHD of the liver presents as cholestatic hyperbilirubinemia and is difficult to differentiate from other causes of liver dysfunction after aSCT, such as veno-occlusive disease, drug toxicity and viral infections. Despite immunosuppressive treatment grade III and IV GvHD have poor prognosis with 5-year survival of 25% and 5%, respectively.

The identification of human leukocyte antigens (HLA) revealed that mismatching for these highly polymorphic antigens that are encoded in the major histocompatibility complex (MHC) is a dominant factor in the occurrence of GvHD, and that the degree of HLA-mismatching correlated with the incidence of GvHD. The development of high resolution HLA typing, but also improved antibiotic therapy, infectious prophylaxis, modern immunosuppressive drugs and sophisticated supportive care highly enhanced the success rate of aSCT, but GvHD remains a major complication limiting the outcome. It was, however, also observed that the occurrence of GvHD inversely correlated with the incidence of leukemic relapses^{6,7}. This and higher risk of relapse in syngeneic and autologous compared to allogeneic transplantation indicated the existence of a graft-versus leukemia (GvL) effect which is based on genetic differences between patient and donor⁸. It became evident that this effect was mediated by donor T-cells when depletion of T-lymphocytes from the graft was introduced into clinical practice to prevent GvHD. Although depletion of T-lymphocytes from the graft dramatically reduced the incidence of GvHD, the beneficial effect was negated by higher incidence of leukemic relapses, resulting in no improvement in overall survival.

The concept of an immune response of donor-derived T-cells against leukemic cells also laid the basis for the development of reduced intensity conditioning^{9,10}. The lethal conditioning treatment before infusion of the stem cell graft mainly aimed at destruction of residual malignant cells and the preparation of an immunocompromised host obviating graft rejection. Reduced intensity conditioning (RIC) regimens still cause sufficient immunosuppression to allow engraftment and to induce a state of host-versus-graft tolerance to allow donor derived T-cells to recognize and eradicate residual malignant cells. While RIC regimens lead to a substantial reduction in transplantation related toxicity thereby enabling application in older patients with more co-morbidities, GvHD still remains a major problem.

T-cells

T-lymphocytes play a central role in adaptive immunity against different pathogens. The mature T-cell compartment consists of CD4⁺ and CD8⁺ single positive cells mainly expressing an α/β -T-cell receptor to recognize antigens presented on HLA class II and class I,

respectively. T-cell progenitors arising from the bone marrow do not display any of these lineage commitments yet. In the thymus these T-cell progenitors develop to mature T-cells and selection for useful T-cell receptors (TCR) takes place ¹¹. In the earliest stage of development thymocytes do not express CD4, CD8 or a TCR yet and are referred to as double-negative. First, thymocytes rearrange their TCR β -chain by recombination of V (variable), D (diversity) and J (joining) gene regions. The variability of this process enables an antigen diversity of about 10^{16} for mature α/β TCRs ¹². After rearrangement of their TCR $CD4^+/CD8^+$ double-positive thymocytes are subjected to thymic selection. To ensure interaction with self-HLA, thymocytes displaying a TCR with no or low affinity to self-HLA do not receive any survival signal and will undergo apoptosis or “death by neglect”, a process called positive selection. Those TCR recognizing self-HLA with sufficient affinity will further undergo negative selection. Hereby, T-cells with too high affinity to self peptide-HLA complexes will be eliminated to sustain a state of self-tolerance. During these selection processes, recognition of HLA class I and class II molecules, respectively, leads to transition to CD8 and CD4 single-positive T-cells ¹³. These naive T-cells then leave the thymus and migrate to the periphery to await antigen encounter.

Upon activation by antigen (signal 1) combined with co-stimulatory molecules (signal 2) on antigen-presenting cells, naive T-cells rapidly start to proliferate forming a population of clonal effector cells. After successful elimination of the antigen, T-cells will enter a contraction phase, during which about 90-95% of effector cells die. A residual population of antigen-specific T-cells, however, will remain as long-lived memory cells to allow a faster response after re-exposure to the same antigen ^{14,15}.

CD4⁺ T-cells

CD4⁺ T-cells - also referred to as T-helper cells - orchestrate the adaptive immune response to pathogens and play a central role in the induction of various autoimmune and allergic diseases. Accordingly, T-lymphocytes expressing the CD4 co-receptor and recognizing peptides presented in HLA class II molecules do not form a homogenous population. Upon encounter of antigen by the TCR, naive CD4 T-cells can differentiate in at least four different

subsets: Th1, Th2, Th17 and iTreg¹⁶⁻¹⁸. Depending on the cytokines produced by the innate immune system, naive CD4⁺ T-cells activate different transcription factors, which in their turn control their cytokine profile and effector function.

Beside the cytokine milieu at the time of antigen encounter, other factors such as the nature of the antigen, its affinity to the TCR and available costimulants are also important for CD4 T-cell polarization. Furthermore, the presence of different combinations of cytokines during activation can also lead to Th cells with mixed phenotypes.

Thus, T-helper cells have multiple roles *in vivo* and a strong impact on cellular immunity in the field of tumor immunology.

Donor lymphocyte infusion

The positive influence of donor derived T-lymphocytes on eradication of malignant cells led to development of donor lymphocyte infusion (DLI) as anti-tumor therapy. In the late 1980's the first patients were treated with DLI for relapsed leukemia after a SCT, and the proof that donor lymphocytes without additional treatment can induce long lasting complete remissions laid the cornerstone for T-cell based immunotherapy^{19,20}. It has been shown that DLI can induce complete remissions in 20-80% of patients, with the best results obtained for chronic myeloid leukemia (CML) in chronic phase, but also patients with relapsed multiple myeloma (MM) and acute myeloid (AML) or acute lymphoid leukemia (ALL) can show profound clinical responses after administration of DLI^{19,21-24}. However, beside the beneficial GvL effect, infusion of donor lymphocytes is often accompanied by detrimental GvHD^{25,26}. Several factors such as cell dose, timing and microenvironment significantly affect clinical outcome after DLI^{25,27}. The inflammatory environment created around transplantation due to tissue damage caused by the conditioning regimen and (viral) infections during the early post-transplant period leads to up-regulation of HLA molecules and increased presentation of cellular debris²⁸. This can lead to activation of infused T-cells, which in their turn release cytokines and further increase tissue damage and immune activation. Tissue damage can be (partially) avoided by T-cell depleted aSCT and subsequent application of donor T-cells late

after transplantation when the inflammatory environment has subsided. The delayed administration of donor T-cells after T-cell depleted aSCT indeed reduces GvHD while preserving GvL effect. However, T-cell depleted aSCT also delays immune reconstitution after transplantation resulting in increased rates of infections and early relapses. Separation of GvL effect and GvHD therefore remains a major objective in allogeneic SCT, which stimulated researchers to investigate in more detail the specificities of alloreactive T-cells.

In HLA-mismatched transplantations, the majority of alloreactive T-cells are directed against non-polymorphic, and to lesser extent also polymorphic peptides presented in the mismatched HLA molecules. However, also in HLA-matched aSCT, about 40% of patients develop acute GvHD requiring systemic treatment. This is due to genetic disparities between donor and recipient that lie outside the HLA loci and encode for polymorphic peptides presented on shared HLA molecules that are able to elicit allogeneic T-cell responses. These polymorphic peptides are referred to as minor histocompatibility antigens (MiHA) ²⁹. The molecular characterization of these MiHA may enable selective application of T-cells recognizing antigens with restricted tissue distribution and therefore allows specific targeting of malignant hematopoietic cells without inducing GvHD. The replacement of patient hematopoiesis with donor cells after stem cell transplantation thereby spares healthy donor-derived hematopoiesis from T-cells recognition.

Minor histocompatibility antigens

The first minor histocompatibility antigen (MiHA) has been identified in 1995 by elution of HLA bound peptides. This hematopoiesis-specific antigen - designated HA-2 - was derived from the plasma membrane associated class I myosin MYO1G and is presented in HLA-A*0201 ³⁰. Shortly thereafter, the second hematopoiesis specific MiHA HA-1 was molecularly characterized ³¹. The identification of MiHA that are exclusively expressed in hematopoietic cells and the observation that T-cells against these MiHA can inhibit engraftment of leukemia in NOD/scid mice led to the notion that selective induction of GvL effect by these T-cells might be possible ³². Since that time an increasing number of MiHA was identified by different methods at an exponential rate. While from 1995 to 2008 about 20

MiHA have been characterized by peptide elution from HLA molecules^{30,31,33}, screening of plasmid DNA libraries³⁴⁻³⁷ and genetic linkage analysis^{38,39}, the recent development of whole genome association scanning⁴⁰⁻⁴² doubled the number of HLA class I restricted MiHA in the past few years. However, the number of MiHA with therapeutic value based on restricted expression in hematopoietic cells is still limited. A range of factors determine the applicability of a MiHA specific T-cell in patient care. (1) The peptide epitope has to be presented on a common HLA molecule to allow treatment of considerable numbers of patients. (2) The allele frequency of the immunogenic variant should range between 30 to 70% to enable matching of a negative donor with a positive patient. (3) The MiHA has to be sufficiently expressed in malignant cells to allow GvL effect to take place. And (4), to restrict T-cell reactivity to residual patient hematopoiesis and malignant cells, the MiHA should not be presented on non-hematopoietic tissues.

The last requirement might be circumvented by exploiting HLA class II restricted MiHA. In contrast to HLA class I molecules, HLA class II consisting of HLA-DR, -DQ and -DP are mainly expressed on cells of the hematopoietic system. MiHA specific CD4⁺ T-cells are therefore expected to mediate less GvHD especially if administered late after aSCT when inflammation induced up-regulation of HLA class II on non-hematopoietic cells has subsided. The identification of HLA class II restricted MiHA, however, proved to be difficult and was hampered by technical limitations. Techniques applied for identification of HLA class I restricted MiHA could not successfully be transferred to HLA class II restricted MiHA, which significantly delayed their characterization. Finally in 2008, the first HLA class II restricted MiHA was identified as a polymorphism in phosphatidylinositol-4-kinase type 2 beta (LB-PI4K2B-1S) presented on HLA-DQB1*0603⁴³. LB-PI4K2B-1S was identified by screening a recombinant bacteria cDNA library⁴⁴. Thereby, mRNA of the patient was isolated and cloned into a bacterial expression vector under control of an inducible promoter. These plasmids were transfected into bacteria, and pools of about 50-100 different bacteria were loaded on EBV-immortalized B-cells (EBV-LCL) of the donor. The exogenous antigens could be processed and presented on donor EBV-LCL in endogenous HLA class II molecules that are shared with the recipient. T-cell clones with specific recognition of patient, but not donor, EBV-LCL were co-incubated with bacteria loaded donor EBV-LCL, and IFN- γ production was measured after overnight co-incubation.

Shortly after identification of LB-PI4K2B-1S, another autosomal HLA-DQ-restricted MiHA encoded by the B cell-specific CD19 gene was discovered using a combination of genetic linkage analysis and subsequent fine mapping with single nucleotide polymorphism (SNP) markers ⁴⁵. In addition to LB-PI4K2B-1S and CD19L, however, more HLA-class II-restricted MiHA need to be discovered to further elucidate their role in GVL and GVHD, and to explore their use ⁴⁶as targets for CD4⁺ T cells to treat hematological malignancies.

Molecular basis of MiHA

Different mechanisms have been shown to lead to formation of HLA class I restricted MiHA. Most MiHA are encoded by “missense” single nucleotide polymorphisms in coding gene regions that directly lead to an amino acid change in the protein. In addition, MiHA can be created by SNPs in coding gene regions that are synonymous in the normal reading frame, but missense in an alternative reading frame. Proteins can be translated in an alternative reading frame as a result of a frame-shift due to the presence of a polymorphic nucleotide insertion or deletion, such as for MiHA LRH-1 ³⁸ or due to the presence of a non-polymorphic alternative start codon as shown for LB-ECGF-1H ³⁴ and LB-ADIR-1F ³³. Even SNPs in non-coding gene regions can create MiHA as a result of alternative mRNA splicing, as reported for PANE1 ⁴⁶ and ACC-6 ³⁷. Finally, MiHA can be encoded by genes that are present in the patient, but homozygously deleted in the donor as shown for the MiHA encoded by UGT2B17 ⁴⁷.

Besides a direct difference in interaction of the presented peptide with the TCR, also posttranslational differences between patient and donor variant can determine the immunogenicity of a polymorphism. It has been described that differential processing precludes surface presentation of the donor variant of MiHA HA-3 ⁴⁸, HA-8 ⁴⁹ and possibly HA-2 ⁵⁰. These donor variants fail to activate MiHA-specific T-cells when endogenously expressed, but induced strong T-cell activation after exogenous peptide loading, demonstrating that insufficient processing of the donor variant into the HLA class I pathway is the cause for lack of T-cell recognition. Finally, it has been shown for HA-1 that impaired

binding affinity of the donor variant to the HLA-A*02:01 restriction molecule leads to an increased dissociation rate and insufficient surface presentation ³¹.

The two known HLA class II restricted MiHA are directly encoded by missense SNPs and exogenously loading of the donor variant did not activate T-cells, suggesting differential interaction of patient and donor type peptides with the TCR. However, no in depth studies have so far been performed.

Antigen processing - Classical pathway

For eradication of malignant cells by MiHA specific T-cells, surface presentation of endogenous antigens on tumor cells is required. Traditionally, it was believed that endogenous, cytosolic proteins are exclusively presented on HLA class I molecules to cytotoxic CD8⁺ T-cells ^{51,52}. Cytosolic proteins are thereby degraded by the proteasome and peptides are transported via the transporter associated with antigen processing TAP into the endoplasmatic reticulum (ER). Here, the HLA class I α -chain assembles with β 2-microglobulin, and the α/β complex is loaded with antigenic peptides. This process is supported by various proteins such as tapasin, calnexin and other chaperones including calreticulin and Erp57. The peptide loaded HLA complex is then transported via the secretory pathway to the cell surface.

In contrast, HLA class II molecules are regarded to present exogenous antigens to CD4⁺ T-cells. Extracellular proteins enter the cell via different mechanisms including scavenger-receptors, clatherin-dependent endocytosis or pinocytosis, and finally all end-up in the endosome. Subsequently, the endosome matures resulting in an increasingly acidic pH and finally fuses with the lysosome. Proteases like cathepsin B, D, S and L, but also other enzymes such as asparagine endopeptidase (AEP) thereby degrade the extracellular proteins in a pH-dependent manner. In the ER, the HLA class II α - and β -chain are synthesized and already form a heterodimer. As this “empty” HLA molecule is unstable, it is supported by a chaperone known as the invariant chain (Ii). The Ii fulfills two biological functions ^{53,54}. First, its C-terminal part also referred to as CLIP (class II associated invariant chain peptide)

occupies the peptide binding groove formed by the HLA class II α - and β -chain thereby preventing premature peptide loading in the ER. Secondly, its N-terminal cytoplasmic tail contains a signaling motif that targets HLA class II molecules through the trans-Golgi network to the endocytic pathway. In late endosomes, also called MHC class II containing compartments (MIIC), HLA class II complexes encounter antigenic peptides. The Ii is proteolytically cleaved until only the remnant CLIP remains in the peptide binding groove⁵⁵. The non-polymorphic, non-classical class II molecule HLA-DM then catalyzes the release of CLIP, thereby enabling binding of antigenic peptides that form a stable HLA-peptide complex⁵⁶. The loaded HLA-peptide complex is subsequently transported to the cell surface, while HLA-DM remains in the endocytic compartment.

Some cell types, such as B-cells, mature dendritic cells and thymic epithelial cells, express the non-classical HLA molecule HLA-DO⁵⁷⁻⁵⁹. The biological role of this molecule is not fully understood, but it has been shown to closely bind to HLA-DM and to modulate its function. The activity of HLA-DM can be monitored by measuring surface expression of CLIP, the remnant of the Ii, as presented in HLA-DR.

While the above-described classical pathway has been studied in detail for presentation of exogenous antigens, the role of these molecules in HLA class II presentation of endogenous antigens is less clear.

Antigen processing - Alternative pathways

In contrast to the traditional paradigm, it is known by now that the dichotomy between HLA class I and class II is not as strict. Dendritic cells can present exogenous antigens in HLA class I by a process called cross-presentation⁵¹ and, vice versa, endogenous antigens can enter the HLA class II pathway^{60,61}. The first indications came from peptide elutions from HLA-DR molecules, revealing a substantial number of self peptides being presented in HLA class II. While a majority of those peptides were derived from membrane proteins, which enter the endocytic pathway via their natural turn-over, peptides derived from cytosolic proteins were also found^{62,63}.

Several pathways have been shown to be implicated in HLA class II mediated presentation of intracellular antigens, among which autophagy pathways are the most established. Autophagy is a constitutive process, which can be additionally induced by cellular stress such as starvation. At least three distinct subtypes of autophagy can be distinguished: (macro)autophagy, chaperone-mediated autophagy (CMA) and microautophagy. (Macro)autophagy and CMA have both been implicated in HLA class II presentation of cytosolic proteins. During (macro)autophagy cytosolic proteins and organelles are sequestered into *de novo* forming double-membrane structures known as autophagosomes⁶⁴. The outer membrane of these autophagosomes eventually fuses with the lysosome forming an autolysosome in which the autophagic body (inner membrane and content) is degraded by lysosomal enzymes. So far, a number of proteins including self (complement c5)⁶⁵, viral (EBNA1)⁶⁶ and tumor antigens (MUC1)⁶⁷ have been shown to enter the HLA class II processing pathway via (macro)autophagy.

During CMA a specific homologous pentapeptide motif within the target protein promotes binding to a molecular chaperone complex of several heat-shock proteins including the heat shock cognate 70kDA protein (hsc70). This complex can bind to the lysosomal membrane protein LAMP2A and chaperone-mediated substrate unfolding likely enables translocation across the lysosomal membrane⁶⁸. Involvement of CMA in HLA class II antigen processing was shown for the cytoplasmic antigen glutamic acid decarboxylase (GAD) and a mutant form of human Igκ L chain⁶⁹.

During microautophagy, small cytosol-containing vesicles bud directly into the lysosomal lumen. Until very recently, studies of this pathway have been limited to yeast and cell-free systems^{70,71}. Now, Sahu et al⁷² described a microautophagy-like pathway in mammalian cells which leads to uptake of soluble cytosolic proteins into intraluminal vesicles (ILV) in the late endosome. Proteins thereby bind to chaperone hsc70 via the same pentameric motif as described for CMA. In contrast to CMA, however, microautophagy does not rely on lysosomal membrane protein LAMP2A, but rather requires electrostatic binding of hsc70 to acidic phospholipids in the endosomal-limiting membrane and endosomal sorting complex required for transport (ESCRT) I and III for formation of ILV. These ILV can be released as exosomes upon fusion of the late endosome with the plasma membrane or degraded upon

fusion with the lysosome. So far, a role for microautophagy in HLA class II antigen processing and presentation has not been demonstrated. Involvement of the classical MHC class I processing machinery for MHC class II restricted epitopes has been described by Tewari et al ⁷³. Both the proteasome and TAP were shown to be required for generation and transport of two MHC class II restricted epitopes derived from two distinct transmembrane glycoproteins from influenza virus. So far, this is the only study showing involvement of TAP in surface presentation of MHC class II restricted antigens.

Finally, it was proposed by Taylor et al. ⁷⁴ that intercellular transfer of antigens resulting in endocytosis and processing within the acceptor cell leads to HLA class II restricted antigen presentation. The authors showed that HLA class II restricted epitopes from two EBV nuclear antigens (EBNA2, EBNA3C) entered the endosomal/lysosomal pathway via intercellular transfer. The nature of this transport, however, was not further elucidated, and the existence of a second pathway providing direct access to the HLA class II processing pathway after intracellular protein synthesis cannot be definitely excluded.

In conclusion, despite the accepted importance of CD4⁺ T-cells in cellular immunotherapy as helper and effector cells and the requirement for surface presentation of endogenous (tumor) antigens to benefit from their effector potential, little is known about processing and presentation of endogenous antigens into HLA class II. More insight into these pathways and mechanisms might provide a platform to improve CD4⁺ T-cell based immunotherapy.

AIM OF THE STUDY

The GvL effect mediated after allogeneic SCT by MiHA specific donor-derived T-cells recognizing residual malignant cells of the patient is a very potent form of cellular immunotherapy. However, the benefit of this treatment is limited by induction of detrimental GvHD, the major cause of morbidity and mortality after aSCT. The exploitation of CD4⁺ T-cells recognizing MiHA presented on HLA class II molecules might enable selective induction of GvL effect without GvHD. Therefore, more HLA class II restricted antigens need to be identified to gain more insight in the biology and potency of these antigens as therapeutic targets. The aim of this study was to discover novel HLA class II restricted MiHA and to analyze intracellular processing and presentation of these endogenous antigens into the HLA class II pathway.

Very recently the first two autosomal HLA class II restricted MiHA have been characterized. LB-PI4K2B-1S has been found by screening a recombinant bacteria cDNA library and CD19L could be identified by a combination of genetic linkage analysis with subsequent fine mapping based on marker SNPs. In **chapter 2**, the aim was to identify novel HLA-class II restricted MiHA. For this purpose activated CD4⁺ T-cell clones were isolated from peripheral blood of a patient with relapsed CML who responded to DLI after HLA-matched aSCT. MiHA specificity of these T-cell clones was determined by selective recognition of patient, but not donor, EBV-LCL and characterization of the MiHA was performed by screening a recombinant bacteria cDNA library. The identified target antigens were further characterized in terms of precise HLA restriction, minimal epitope, population frequency and tissue distribution.

The HLA class II processing pathway is in our understanding optimally designed for presentation of exogenous antigens. Despite recent evidence of alternative pathways shuttling cytosolic and even nuclear proteins into the endocytic compartment, little is known about the influence of molecules with known functions in HLA class II processing on surface presentation of endogenous ligands. In **chapter 3**, we analyzed the influence of the Ii, HLA-DM and HLA-DO on processing and presentation of six endogenous HLA class II ligands in a human cell line model.

The results as obtained in chapter 3 imply a possible separation of GvL effect and GvHD based on differential expression of HLA-DO in professional versus non-professional antigen presenting cells. In **chapter 4**, we determined expression levels of the molecules involved in HLA class II processing Ii, HLA-DM and HLA-DO in primary leukemic cells and T-cell recognition experiments were performed to confirm their influence on surface presentation of endogenous antigens in HLA class II.

CD4⁺ T-cells play a central role in orchestrating adaptive immune responses and as “helper cells” they have the ability to support other immune effector cells. In **chapter 5**, we analyzed the helper potential of a MiHA specific CD4⁺ T-cell clone and described development of a coordinated B- and T-cell response against intracellular antigen PTK2B.

For certain HLA class II restricted antigens it has been described that release of antigen and subsequent uptake by surrounding cells can lead to presentation of the epitope. For male antigen DBY it has even been shown that this kind of indirect antigen presentation can lead to eradication of MHC class II negative tumors in mice. In **chapter 6**, we analyzed whether our novel HLA class II restricted MiHA could be indirectly presented, and we studied in detail the mechanism of antigen transfer between viable cells.

In **chapter 7** the results obtained in these studies are summarized and discussed in the context of CD4 T-cell based immunotherapy.

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2

Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity

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ABSTRACT

Potent graft-versus-leukemia (GVL) effects can be mediated by donor-derived T cells recognizing minor histocompatibility antigens (mHags) in patients treated with donor lymphocyte infusion (DLI) for relapsed hematologic malignancies after HLA-matched allogeneic stem cell transplantation (alloSCT). Donor-derived T cells, however, may not only induce GVL, but also mediate detrimental graft-versus-host disease (GVHD). Because HLA-class II is under noninflammatory conditions predominantly expressed on hematopoietic cells, CD4⁺ T cells administered late after alloSCT may selectively confer GVL without GVHD. Although a broad range of different HLA-class I-restricted mHags have been identified, the first 2 autosomal HLA-class II-restricted mHags have only recently been characterized. By screening a recombinant bacteria cDNA expression library, we identified 4 new HLA-class II-restricted mHags recognized by CD4⁺ T cells induced in a patient with relapsed chronic myeloid leukemia who achieved long-term complete remission and experienced only mild GVHD of the skin after DLI. All CD4⁺ T cells were capable of recognizing the mHags presented by HLA-DR surface molecules on primary hematopoietic cells, but not on skin-derived (cytokine-treated) fibroblasts. The selective recognition of hematopoietic cells as well as the balanced population frequencies and common HLA-DR restriction elements make the novel mHags possible targets for development of immunotherapeutic strategies.

INTRODUCTION

Relapsed hematologic malignancies after HLA-matched allogeneic stem cell transplantation (alloSCT) can be efficiently treated with donor lymphocyte infusion (DLI).^{1–3} This beneficial graft-versus-leukemia (GVL) immunoreactivity is mediated by donor T cells directed against minor histocompatibility antigens (mHags). These mHags are polymorphic peptides that differ between patient and donor, and are able to elicit CD8⁺ or CD4⁺ donor T-cell responses in the context of self-HLA.⁴ A variety of HLA-class I–restricted mHags have been identified,^{5–17} and the appearance of CD8⁺ T cells specific for several of these mHags was closely followed by complete remissions of the malignancies,^{12,14,16,18} indicating the clinical relevance of these T cells.

Because HLA-class I molecules are expressed on all nucleated cells in malignant and nonmalignant tissues of the patient, CD8⁺ T cells recognizing mHags in the context of HLA-class I molecules may not only mediate GVL, but also induce detrimental graft-versus-host disease (GVHD). GVHD is still the main cause of transplant-related morbidity and mortality,¹⁹ and therefore separating GVL and GVHD is a major objective of immunotherapy after alloSCT. One approach is the use of mHags that are encoded by hematopoiesis-restricted genes such as HA-1⁶ and HA-2,⁵ to specifically stimulate cytotoxicity of donor-originated CD8⁺ T cells against the malignant hematopoietic cells of the patient.

It is well established that also CD4⁺ T cells play an important role in antitumor immunity, both as helper cells indispensable for the induction and maintenance of CD8⁺ T cells^{20–22} and as effector cells with direct cytolytic activity against HLA-class II⁺ tumor cells.^{23–26} Because under noninflammatory or low-inflammatory conditions HLA-class II molecules are predominantly expressed on hematopoietic cells, stimulating CD4⁺ T-cell immunity late after alloSCT may be another promising strategy to selectively target residual leukemia without inducing severe GVHD.^{27–29}

Although several HLA-class I–restricted mHags have been identified by various methods,^{5–8,10,11,13–17,30,31} the discovery of HLA-class II–restricted mHags proved to be more difficult and was hampered by technical limitations. We developed a method by which recombinant

bacteria cDNA expression libraries were used to identify antigens as targets for CD4⁺ T cells.³² The feasibility of this method was demonstrated by the recent identification of the first autosomal HLA-class II–restricted mHag LB-PI4K2B-1S.³³ Using a combination of genetic linkage analysis and subsequent fine mapping with single nucleotide polymorphism (SNP) markers, another autosomal HLA-DQ–restricted mHag encoded by the B cell–specific *CD19* gene was recently identified.³⁴ However, identification of more HLA-class II–restricted mHags is required to further elucidate their role in GVL and GVHD, and to explore their use as targets for CD4⁺ T cells to treat hematologic malignancies.

Here, we present the molecular identification of 4 new HLA-DR–restricted autosomal mHags recognized by CD4⁺ T cells induced in a patient who was successfully treated with DLI for relapsed chronic myeloid leukemia (CML). Our data show that CD4⁺ T cells directed against these mHags recognized primary hematopoietic cells, but not skin-derived (cytokine-treated) fibroblasts. These *in vitro* findings and the clinical observation that a long-lasting complete remission was induced with only limited GVHD of the skin suggest an important role of the novel HLA-DR–restricted mHags in antitumor reactivity.

METHODS

Isolation of hematopoietic cells

Peripheral blood and bone marrow (BM) samples were obtained from patients with hematologic malignancies and healthy persons after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque separation and cryopreserved. Activated CD4⁺ T cells were isolated from BM of the patient 5 weeks after DLI. BM cells were stained with fluorescein isothiocyanate (FITC)–labeled anti-CD4 (BD Biosciences) and phycoerythrin (PE)–labeled anti-HLA-DR (BD Biosciences) and single cell sorted by flow cytometry. For isolation of patient-derived hematopoietic cells, peripheral blood mononuclear cells (PBMCs) obtained from the patient before alloSCT were stained with FITC-labeled anti-CD14, PE-labeled anti-CD3, and allophycocyanin-

labeled anti-CD19 (BD Biosciences), and monocytes (CD14⁺), T cells (CD3⁺) and B cells (CD19⁺) were isolated by flow cytometry. CD34⁺ CML progenitor cells were isolated from BM cells obtained from the patient during the relapse before DLI by flow cytometric sorting after staining with allophycocyanin-labeled anti-CD34 (BD Biosciences). CD34⁺ progenitor cells from other patients with CML were isolated by magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). CD33⁺/CD14⁻ acute myeloid leukemia (AML) cells from 3 patients were isolated by flow cytometric sorting after staining with CD14-FITC and CD33-allophycocyanin (BD Biosciences).

Cell culture

Epstein-Barr virus transformed B-cell lines (EBV-LCLs) were cultured in Iscove modified Dulbecco medium (IMDM; Lonza BioWhittaker) with 10% fetal calf serum (FCS; Cambrex), 1% penicillin/streptomycin (Lonza BioWhittaker), and 1.5% L-glutamine (Lonza BioWhittaker). T-cell clones were cultured in IMDM with 5% human serum, 5% FCS, and 100 IU/mL interleukin-2 (IL-2; Chiron), and restimulated every 10 to 20 days with irradiated allogeneic PBMCs and 0.8 µg/mL phytohemagglutinin (Oxoid) as previously described.^{14,16} Immature dendritic cells (DCs) were generated by culturing isolated monocytes in medium with 100 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Novartis) and 500 IU/mL IL-4 (Schering-Plough) for 7 days. During the final 2 days, 100 ng/mL GM-CSF, 10 ng/mL tumor necrosis factor α (TNF-α; Cellgenix), 10 ng/mL IL-1β (Cellgenix), 10 ng/mL IL-6 (Cellgenix), 1 µg/mL prostaglandin E₂ (Sigma-Aldrich), and 500 IU/mL interferon-γ (IFN-γ; Boehringer-Ingelheim) were added for maturation. Isolated CD34⁺ CML progenitor cells were modified to leukemic antigen-presenting cells (APCs) as described previously.³⁵ Fibroblasts were cultured in Dulbecco modified Eagle medium (Lonza, BioWhittaker) with 8% FCS, 1% penicillin/streptomycin, and 1.5% glutamine in the absence or presence of IFN-γ (100 IU/mL) for 5 days.

ELISA

Stimulator cells (3×10^4 cells/well) were coincubated with CD4⁺ T cells (5×10^3 cells/well) overnight at 37°C in U-bottom 96-well plates. Peptide pulsing was performed by

incubating donor EBV-LCLs (10^6 cells/mL) for 2 hours with synthetic peptides (1 μ g/mL) in IMDM containing 2% FCS. Peptide-pulsed donor EBV-LCLs were washed twice and subsequently used as stimulator cells. Cytokine release was measured in 50- μ L supernatants by IFN- γ enzyme-linked immunosorbent assay (ELISA; Sanguin) or multi-Th1/Th2/Th17 cytokine ELISA (SABioscience) according to the manufacturer's instructions.

⁵¹Cr-release cytotoxicity assay

Target cells were labeled for 1 hour at 37°C with 100 μ Ci (3.7 MBq) $\text{Na}_2^{51}\text{CrO}_4$ (Amersham). After washing, target cells (10^3 cells/well) were incubated with CD4^+ T cells at effector-target ratios of 10:1 for 10 hours. ^{51}Cr was analyzed in 25- μ L supernatants. The percentage of specific lysis was calculated as follows: [experimental release (cpm) – spontaneous release (cpm)]/[maximal release (cpm) – spontaneous release (cpm)] $\times$ 100%.

Construction of recombinant bacteria cDNA expression library

A recombinant bacterial cDNA expression library was constructed as described previously.³³ In brief, RNA of the patient was isolated with Trizol (Invitrogen) and purified (RNeasy kit; QIAGEN). cDNA was synthesized with oligo-dT and random primers, respectively. Both primers contained an *Asc*I restriction site. After cDNA synthesis, *Bam*HI-*Eco*RI (Stratagene) adapters were ligated, followed by digestion with *Asc*I and size fragmentation by column chromatography. cDNA fragments were ligated into *Bam*HI and *Asc*I restriction enzyme sites of vector pKE-1,³⁶ which contains an isopropyl β -D-thiogalactoside (IPTG)-inducible *tac* promoter and resistance genes for ampicillin and kanamycin. Vectors were electroporated into *Escherichia coli* BL21 (DE3) and recombinant bacteria were selected for ampicillin resistance.

Screening of recombinant bacteria cDNA expression library

Pools of 50 to 100 recombinant bacteria were screened for T-cell recognition, as described previously.³³ Bacteria were grown to 600-nm optical density of 0.5 with 50 μ g/mL ampicillin (Sigma-Aldrich) and protein expression was induced by 1 mM IPTG (Promega). Subsequently, bacteria were opsonized by adding human serum with 17% (vol/vol)

complement (Sigma-Aldrich) and incubated for 1 hour. EBV-LCLs of the fully HLA-matched donor (3×10^4 cells/well) were pulsed with complement-opsonized bacteria in IMDM with 10% FCS and 30 $\mu\text{g/mL}$ gentamycin (Sigma-Aldrich) overnight at 37°C. Mixes of up to 3 CD4⁺ T-cell clones (3×10^3 cells/well per clone) were coincubated with bacteria-pulsed EBV-LCLs for 20 hours. Supernatants were harvested for IFN- γ ELISA. In a second round, single bacterial clones derived from a positive pool were screened for T-cell recognition. Positive bacterial clones were grown overnight and plasmids were isolated by the plasmid mini prep kit (QIAGEN). Inserted cDNA fragments were sequenced using vector-specific primers.

Isolation and retroviral transduction of HLA-DR alleles

Total RNA of patient-derived EBV-LCLs was obtained using Trizol (Invitrogen) and transcribed into cDNA by reverse transcriptase (Invitrogen) using oligo-dT primers (Roche Diagnostics). HLA-DR genes were amplified with HLA-DRB1- and HLA-DRB3-specific primers. Polymerase chain reaction (PCR) products were cloned into retroviral vector MP71, which contains the marker nerve growth factor receptor ($\Delta\text{NGF-R}$).³⁷ PCR products were verified by sequencing. Wild-type $\phi\text{nx A}$ packaging cells were transfected with these vectors as previously described,³⁷ with the exception that the Fugene HD transfection kit (Roche Diagnostics) was used. Viral supernatants were used for transduction of EBV-LCLs on plates coated with recombinant human fibronectin CH 296 (Takara Shuzo) as previously described.³⁷ All EBV-LCLs showed retroviral transduction efficiencies of 3% to 7% based on staining with PE-conjugated anti- $\Delta\text{NGF-R}$ (BD Biosciences).

SNP genotyping assays

Genomic DNA was isolated from 1400 patient and donor samples (PBMCs or BM cells) by the Gentra Systems PureGene genomic isolation kit (Biocompare). The SNPs rs751019 (PTK2B), rs2236225 (MTHFD1), and rs12692566 (LY75)³⁸ were analyzed using SNP genotyping assay (Applied Biosystems) containing forward and reverse primer for amplification and 2 TaqMan MGB probes labeled with VIC and FAM dyes to detect the different alleles. SNP rs2236410 (MR1) was analyzed using allele-specific primers labeled

with VIC and FAM dyes, respectively (KBioScience). Genotyping was performed according to the manufacturer's instructions.

Quantitative RT-PCR

Quantitative real-time reverse-transcriptase (RT)-PCR was performed as described previously.¹⁴ Using Primer Express (Applied Biosystems), the following primers were designed: MR1: 5'-AAGGTGGAAGTGAAGCGCCT-3', 5'-GTGGTGCTTCCATCCTCCAG-3', and probe 5'-(VIC)-CACTACAATCACTCAGGGTCTCACACTTACCAGA-(TAMRA)-3'; PTK2B: 5'-GGAAAGATGGTGAGAAGCGG-3', 5'-AGCTCTCTGAGAGGTGGGACC-3', and probe 5'-(VIC)-TGCCCCAGATCCCCATGCTAAACC-(TAMRA)-3'; LY75: 5'-TGCCAGCAATCACAGCTTTC-3', 5'-TATTGGCCACTCGCTAATTCTTATC-3', and probe 5'-(VIC)-CTAAAAGCCATCAAAAACAAAATAGCAAATATATCTGGTG-(TAMRA)-3'; MTHFD1: 5'-GGCCTGATGGGAAATACGTG-3', 5'-GCCCCGATTGTAGTTGTGCTTTT-3', and probe 5'-(VIC)-TGACTGGAATAACTCCAACACCCCTGGG-(TAMRA)-3'. PCR was performed in 25 μ L reaction mixture containing 2.5 μ L Taqman buffer, 300 nM of each primer, 180 nM probe (LY75: 200 nM), 200 μ M deoxynucleotide triphosphate, 3 mM MgCl₂ (MTHFD1: 4 mM, PTK2B: 1.5 mM), 0.625 U AmpliTaq Gold polymerase, and 10 and 100 ng cDNA sample. Amplification was started with 10 minutes at 95°C, followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 60°C. Expression of the genes was normalized as ratio with the housekeeping gene porphobilinogen deaminase (*PBGD*).

RESULTS

Isolation and characterization of mHag-specific CD4⁺ T-cell clones

We analyzed the specificity of the CD4⁺ T-cell response induced in a patient with relapsed CML after HLA-matched alloSCT who responded to DLI with complete donor chimerism and remission of the leukemia, and developed only mild GVHD of the skin. CD4⁺ T cells

were single cell sorted from bone marrow mononuclear cells obtained 5 weeks after DLI based on expression of the activation marker HLA-DR (Figure 1A). A total number of 25 CD4⁺ T-cell clones were shown to recognize mHags based on differential recognition of patient and donor EBV-LCLs in an overnight IFN- γ ELISA. By pulsing donor EBV-LCLs with a peptide comprising previously identified mHag LB-PI4K2B-1S, 9 of the 25 T-cell clones were shown to be specific for HLA-DQB1*0603–restricted LB-PI4K2B-1S (data not shown). Of the 16 remaining T-cell clones, 8 clones (55, 62, 78, 79, 80, 87, 92, and 100) produced high levels of IFN- γ (> 250 pg/mL) upon incubation with patient EBV-LCLs (Figure 1B), and were selected for further characterization.

All 8 T-cell clones showed specific lysis of patient, but not donor, EBV-LCLs in a 10-hour ⁵¹chromium release assay (Figure 1C), and produced IFN- γ , IL-13, IL-5, and IL-2 (Figure 1D), but not IL-4, IL-6, IL-10, IL-12, IL-17A, TNF- α , granulocyte CSF, or transforming growth factor β 1 as determined by multicytokine ELISA.

To determine the mHag specificity of the 8 T-cell clones, a panel of 22 EBV-LCLs sharing one or more HLA-class II alleles with the patient was tested for recognition. Similar EBV-LCL recognition patterns were observed for T-cell clones 55, 78, and 92 as well as T-cell clones 79 and 100 (data not shown), indicating that the 8 T-cell clones recognized 5 different HLA-class II–restricted mHags. Further characterization and mHag identification were performed for 1 representative clone of each specificity, that is, 62, 78, 80, 87, and 100.

The capacity of the mHag-specific CD4⁺ T cells to recognize nonmalignant and malignant hematopoietic cells was determined by IFN- γ ELISA. B cells, T cells, and monocytes were purified from PBMCs of the patient before alloSCT, and tested for recognition. In addition, immature and mature dendritic cells (DCs) were generated from isolated monocytes *in vitro*, and malignant CD34⁺ CML progenitor cells were purified from BM obtained from the patient during the relapse after alloSCT before treatment with DLI.

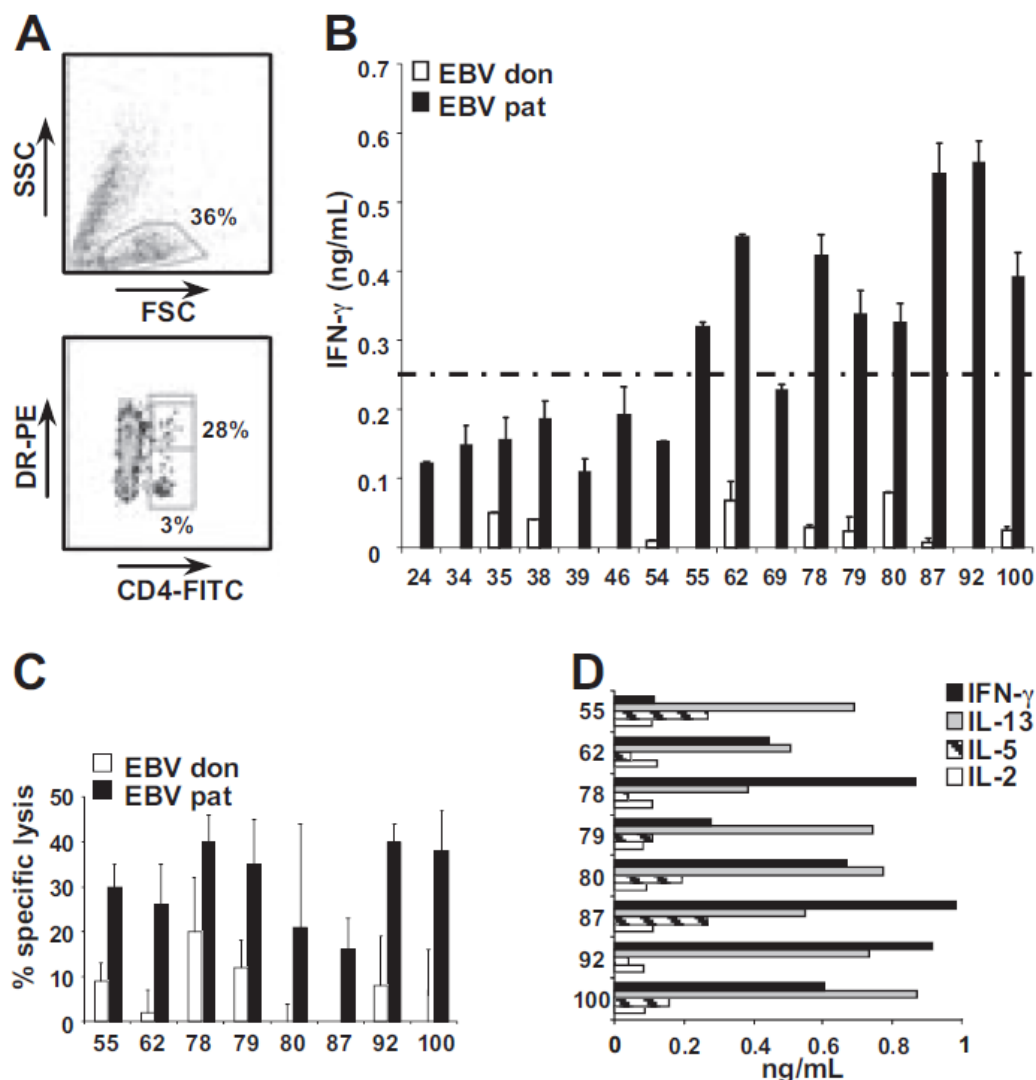


Figure 1: Isolation and characterization of mHag-specific CD4⁺ T-cell clones.

(A) Activated (HLA-DR⁺) CD4⁺ T cells were single cell sorted from bone marrow cells obtained from the patient 5 weeks after DLI by flow cytometry. Indicated are the percentages of bone marrow cells expressing CD4 (3%) and CD4⁺ cells expressing HLA-DR (28%). A total number of 106 CD4⁺ T-cell clones were growing out of 1100 single sorted cells. (B) Sixteen CD4⁺ T-cell clones were directed against unknown mHags based on differential recognition of patient (pat) and donor (don) Epstein-Barr virus transformed B-cell lines (EBV-LCLs). Mean release of IFN- γ (ng/mL) in 50- μ L supernatants of duplicate wells is shown. (C) All CD4⁺ T-cell clones recognizing unknown mHags and releasing > 250 pg/mL IFN- γ upon incubation with patient EBV-LCLs were tested for specific lysis of patient and donor EBV-LCLs in a 10-hour 51Cr release assay. Mean specific lysis of triplicate wells at effector-target ratios of 10:1 is shown. (D) CD4⁺ T-cell clones were tested for cytokine release by multicytokine ELISA. Release of IL-2, IL-5, IL-13, and IFN- γ in 50- μ L single supernatants is shown (ng/mL). CD4⁺ T-cell clones did not produce IL-4, IL-6, IL-10, IL-12, IL-17A, TNF- α , granulocyte CSF, and transforming growth factor β 1 (data not shown).

Figure 2A shows that all T-cell clones strongly recognized mature DCs, expressing high levels of HLA-class II and costimulatory molecules, whereas monocytes and immature DCs were recognized at variable levels. Clones 62, 78, 80, and 87 weakly recognized monocytes and immature DCs, whereas these cells were strongly recognized by clone 100. All T-cell clones recognized high HLA-class II-expressing B cells, although to a different extent, whereas T cells, lacking expression of HLA-class II, were not recognized (Figure 2B). Purified CD34⁺ CML progenitor cells from the patient were weakly recognized by clones 62, 78, and 80, whereas strong recognition was observed for clones 87 and 100 (Figure 2B). In addition to purified CD34⁺ CML progenitor cells of the patient, CD34⁺ cells were isolated from other patients with CML and differentiated to immature and mature leukemic APCs. The data in Figure 2C show that, upon induction of an APC phenotype *in vitro*, CML cells were recognized by all T-cell clones.

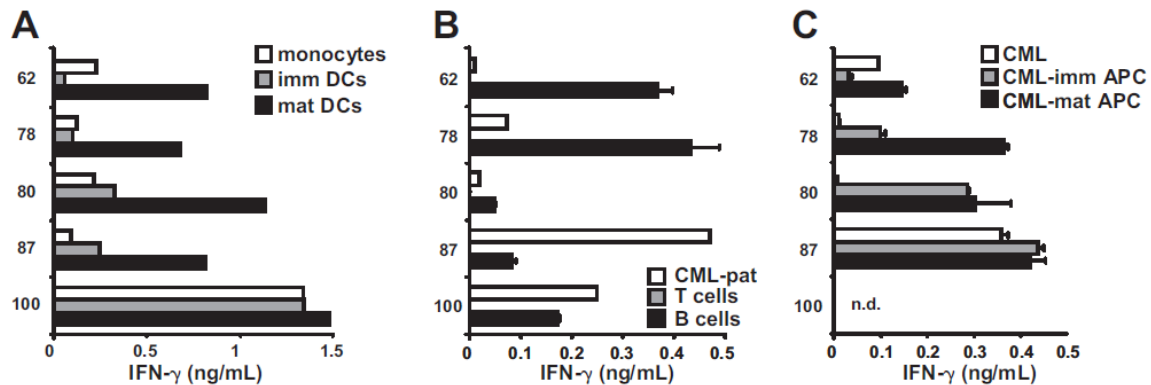


Figure 2. Recognition of nonmalignant hematopoietic cells and chronic myeloid leukemia.

Monocytes (CD14⁺), B cells (CD19⁺), and T cells (CD3⁺) were isolated from PBMCs from the patient obtained before alloSCT by flow cytometry. Isolated monocytes were cultured *in vitro* to immature and mature DCs. CD34⁺ chronic myeloid leukemia (CML) progenitor cells were isolated from bone marrow cells obtained from the patient after alloSCT during the relapse before treatment with DLI. In addition, CD34⁺ cells were isolated from other patients with CML and cultured *in vitro* to professional APCs. CD4⁺ T-cell clones were tested against (A) patient-derived monocytes, immature and mature DCs; (B) patient-derived T cells, B cells, and CD34⁺ CML progenitor cells; and (C) CD34⁺ CML progenitor cells from other patients with and without *in vitro* induced APC phenotype. Mean release of IFN-γ (ng/mL) in 50-μL supernatants of single (monocytes, immature DCs, mature DCs, patient-derived CD34⁺ CML progenitor cells) or duplicate wells is shown. nd indicates not determined

In conclusion, these data suggest that the mHag-specific CD4⁺ T cells have been induced in this patient by malignant CML cells with mature APC phenotype and that the CD4⁺ T cells

then played various roles as effector cells in the direct elimination of the malignant CD34⁺ CML progenitor cells according to their capability to recognize these cells. Induction by nonmalignant B lymphocytes of the patient is not likely, because before DLI more than 99% of the B cells were of donor origin, whereas 23% of bone marrow cells were bcr-abl–positive leukemic cells (data not shown).

Identification of 4 HLA-class II–restricted mHags as targets for CD4⁺ T cells

To identify the mHags recognized by CD4⁺ T-cell clones 62, 78, 80, 87, and 100, recombinant bacterial cDNA expression libraries were constructed from EBV-LCLs of the patient, and screened for T-cell recognition as previously described.³³ Two different cDNA libraries were constructed, each containing 4×10^6 random-primed or oligo-dT–primed cDNAs cloned into vector pKE-1 under control of an isopropyl β -D-thiogalactoside (IPTG)–inducible promoter.³⁶ The different cDNAs were size-separated by column chromatography and divided into different fractions. For each fraction, a total number of 960 different pools of approximately 50 different bacteria were screened for T-cell recognition by IFN- γ ELISA. By screening 2 different fractions of the random-primed cDNA library and one fraction of the oligo-dT–primed cDNA, we identified 4 of the 5 HLA-class II–restricted mHags, designated LB-MR1-1R, LB-PTK2B-1T, LB-LY75-1K, and LB-MTHFD1-1Q.

LB-MR1-1R

In a first screening of the fraction containing the largest random-primed cDNA fragments, 1 bacterial pool stimulated IFN- γ release by T-cell clone 62. Subcloning of this pool revealed 2 positive single cDNAs, each comprising a 1556-bp sequence identical to “major histocompatibility complex, class I–related” (*MR1*) gene (GenBank accession no. NM_001531).³⁹ The *MR1* gene contained 5 known missense SNPs, and sequencing of cDNA of the patient and donor EBV-LCLs revealed that the patient was G/A heterozygous at position 121 bp, whereas the donor was A/A homozygous. This A-to-G transition creates a His-to-Arg substitution at amino acid position 39 (H→R39) of the MR1 protein (SNP database ID no. rs2236410).

To identify the epitope recognized by clone 62, 3 overlapping 19-mer peptides all comprising the R at position 39 were synthesized and tested for T-cell recognition (Figure 3A). Two of these peptides stimulated IFN- γ release by clone 62. A 15-mer peptide comprising amino acids shared by the 2 19-mer peptides also stimulated IFN- γ release, whereas a 15-mer peptide containing the donor variant H at position 39 was not recognized (Figure 3A). Serial truncations of the 15-mer peptide at the N and C termini led to the identification of a minimal 12-mer YFRLGVSDPIRG peptide (amino acids 29-40), designated LB-MR1-1R (Figure 3A).

LB-PTK2B-1T

Screening the same fraction of the random-primed cDNA library revealed 2 other bacterial pools that stimulated IFN- γ release by clone 78. Subcloning of these 2 pools revealed 2 cDNAs containing 813- to 2558-bp and 2318- to 3414-bp regions of the gene encoding “protein tyrosine kinase 2 beta, variant 4” (*PTK2B*; GenBank accession no. NM_173175). The 241-bp region that was shared by the 2 cDNAs contained only 1 known missense SNP at position 2542 bp. Sequencing of the donor and the patient confirmed disparity for this SNP, with the patient being C/A heterozygous and the donor A/A homozygous. This A-to-C transition creates a Lys-to-Thr substitution at amino acid position 796 (K→T 796) of the PTK2B protein (SNP database ID no. rs751019).

Three overlapping 19-mer peptides comprising the T at position 796 and subsequently a series of truncated peptides were synthesized and tested for T-cell recognition. The data show that a core region of 9 amino acids (VYMNDTSPL) at positions 791 to 799 is crucial, but not sufficient, for T-cell recognition. T-cell recognition was shown to require addition of at least one additional residue at either N- or C-terminus of the core region. A 15-mer peptide containing the donor variant K at position 796 was not recognized, confirming the identification of LB-PTK2B-1T as the mHag recognized by clone 78 (Figure 3B).

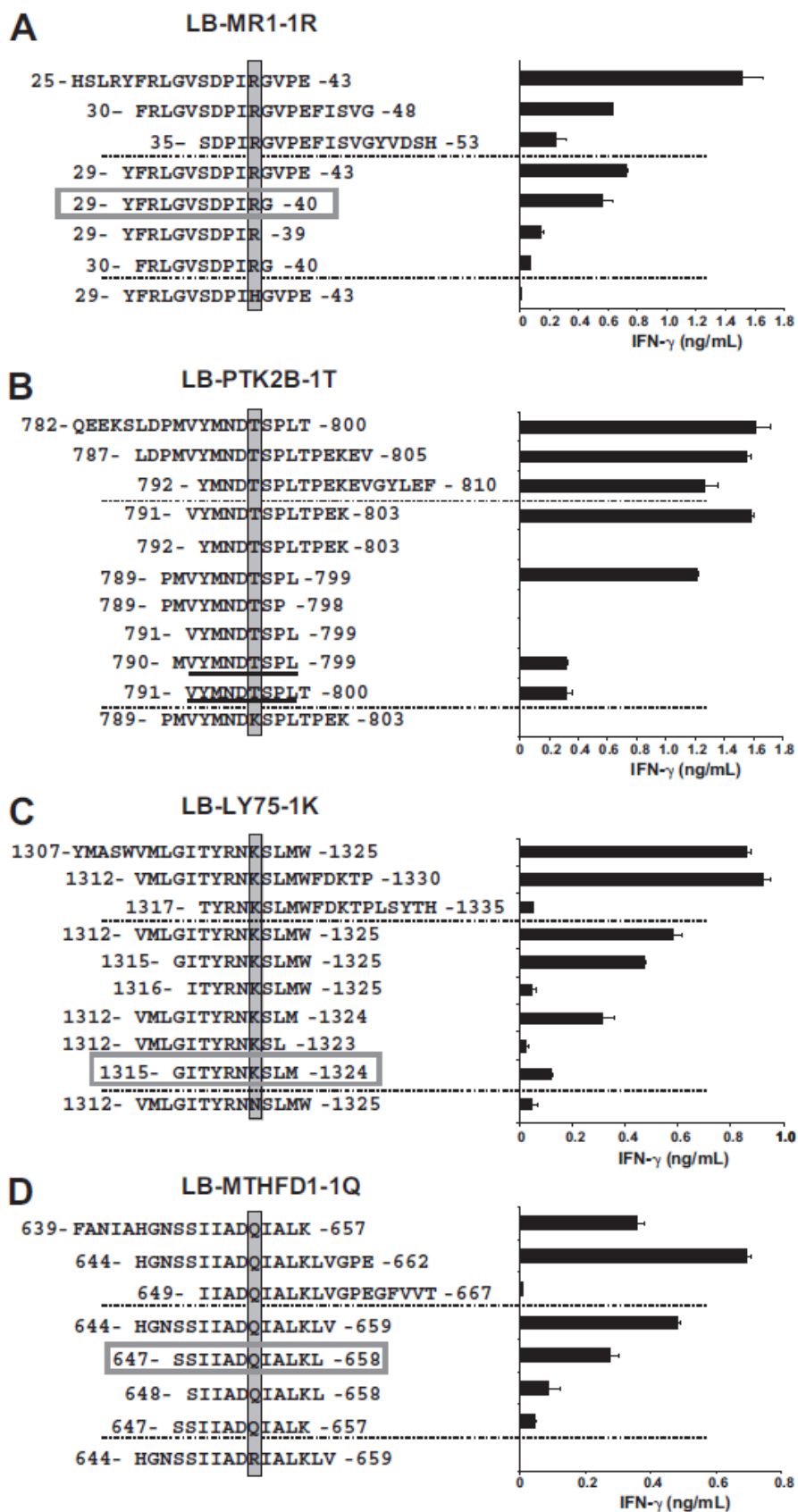


Figure 3. Identification of the minimal epitopes of the HLA-class II–restricted mHags.

Three partially overlapping 19-mer peptides comprising a region of 14 amino acids upstream and downstream of the identified polymorphisms (*top*) as well as series of peptides containing N- and C-terminal truncations (*middle*) and a peptide comprising the polymorphism of the donor (*bottom*) were synthesized, pulsed onto donor EBV-LCLs, and tested for recognition by the mHag-specific CD4⁺ T-cell clones in IFN- γ ELISA. Indicated are the variant residue (shaded), the minimal epitope (boxed), and the core region of the minimal epitope (underlined). (A) T-cell clone 62 was tested against peptides comprising LB-MR1-1R. The minimal epitope is the 12-mer YFRLGVSDPIRG. (B) T-cell clone 78 was tested against peptides comprising LB-PTK2B-1T. The core region is the 9-mer VYMNDTSPL. (C) T-cell clone 80 was tested against peptides comprising LB-LY75-1K. The minimal epitope is the 10-mer GITYR NKSLM. (D) T-cell clone 87 was tested against peptides comprising LB-MTHFD1-1Q. The minimal epitope is the 12-mer SSIIADQIALKL. Mean release of IFN- γ (ng/mL) in 50- μ L supernatants of duplicate wells is shown.

LB-LY75-1K

In another screening containing shorter fragments of the random cDNA library, 3 bacterial pools stimulated IFN- γ release by clone 80. Subcloning of these 3 pools revealed 3 cDNAs containing 2176- to 5739-bp, 2281- to 5739-bp, and 3558- to 4673-bp regions of the gene encoding “lymphocyte antigen 75” (LY75i GenBank accession no. NM_002349). The 1116-bp region shared by the 3 cDNAs contained 5 known missense SNPs. The only observed SNP disparity between patient and donor was at position 4032 bp, with the patient being heterozygous G/T and the donor homozygous T/T. This T-to-G transition creates an Asn-to-Lys substitution at amino acid position 1321 (N→K1321) of the LY75 protein (SNP database ID no. rs12692566).

Following a similar strategy as described for LB-MR1-1R and LB-PTK2B-1T, a minimal epitope of 10 amino acids (GITYR NKSLM) of LB-LY75-1K was shown to be recognized by clone 80 (Figure 3C). A 15-mer peptide containing the donor variant N at position 1321 did not stimulate the release of IFN- γ .

LB-MTHFD1-1Q

Finally, the fraction with the largest fragments of the oligo-dT–primed cDNA library was screened for T-cell recognition. Three bacterial pools stimulated IFN- γ release by clone 87. Subcloning of these 3 pools revealed 2 cDNAs containing 3- to 3116-bp and 1166-to 3116-bp regions of the gene encoding “methylene tetrahydrofolate dehydrogenase (NADP⁺ dependent) 1” (MTHFD1; GenBank accession no. NM_005956). In the 1951-bp region

shared by the 2 cDNAs, 7 missense SNPs were known. Sequencing of the patient and the donor revealed one disparity at position 2011 bp, with the patient being heterozygous G/A and the donor homozygous G/G. This G-to-A transition creates an Arg-to-Gln substitution at amino acid position 653 (R→Q653) of the MTHFD1 protein (SNP database ID no. rs2236225).

Synthesis of 3 overlapping 19-mer peptides and a subsequent series of truncated peptides led to the identification of a 12-mer peptide (SSIIADQIALKL) as the minimal epitope of LB-MTHFD1-1Q. A peptide comprising the donor variant R at position 653 did not stimulate IFN- γ release by T-cell clone 87 (Figure 3D).

Frequencies of HLA-class II–restricted mHag-specific T-cell clones

To determine the frequencies of CD4⁺ T cells specific for the new mHags, all T-cell clones isolated based on expression of HLA-DR were tested for recognition of donor EBV-LCLs pulsed with the LB-MR1-1R, LB-PTK2B-1T, LB-LY75-1K, and LB-MTHFD1-1Q peptides, respectively. In addition to clone 62, LB-MR1-1R was recognized by T-cell clone 69. Clones 62 and 69 expressed the same T-cell receptor (TCR) beta chain (BV) and complementarity determining region 3 (CDR3) (data not shown). LB-PTK2B-1T was recognized by clones 78, 55, and 92. These 3 clones expressed different TCR BV chains with CDR3 regions variable in length and amino acid composition, demonstrating a polyclonal CD4⁺ T-cell response against LB-PTK2B-1T (data not shown). LB-LY75-1K was recognized by clones 34 and 80. These 2 clones also expressed different TCR BV chains. LB-MTHFD1-1Q was recognized only by T-cell clone 87.

Population frequencies of HLA-class II–restricted mHags

To determine the population frequencies of the new HLA-class II–restricted mHags, SNP genotyping assays for rs2236410 (LB-MR1-1R), rs751019 (LB-PTK2B-1T), rs2236225 (LB-MTHFD1-1Q), and rs12692566 (LB-LY75-1K) were performed for 1400 patient and donor samples available at our laboratory. The analysis showed balanced population frequencies of 25% (LB-MR1-1R), 33% (LB-LY75-1K), 68% (LB-MTHFD1-1Q), and 70% (LB-PTK2B-1T; Table 1). These population frequencies slightly differ from the white

population frequencies in the dbSNP database,³⁸ which were 33%, 40%, 50%, and 60%, respectively.

Table 1. Population frequencies of HLA-DR–restricted mHags

	Immunogenic		Nonimmunogenic
	+/+	+/-	-/-
LB-MR1-1R	2	23	75
LB-PTK2B-1T	21	49	30
LB-LY75-1K	3	30	67
LB-MTHFD1-1Q	20	48	32

Genotyping was performed for SNP rs2236410 (LB-MR1-1R), rs751019 (LB-PTK2B-1T), rs12692566 (LB-LY75-1K), and rs2236225 (LB-MTHFD1-1Q) in 1400 patient and donor samples. Frequencies (%) of homozygous-positive (+/+), heterozygous (+/-), and homozygous-negative (-/-) samples are shown.

Identification of the HLA-class II restriction elements

Based on the SNP genotyping results, a panel of mHag⁺ EBV-LCLs sharing one or more HLA-class II alleles with the patient was composed and tested for T-cell recognition. The data strongly suggested HLA-DRB3*0202, -DRB3*0101, -DRB1*1301, and -DRB1*0301 as restriction elements for LB-MR1-1R, LB-PTK2B-1T, LB-LY75-1K, and LB-MTHFD1-1Q, respectively (data not shown). Restriction by these HLA-class II alleles was confirmed by retroviral transduction of mHag⁺ EBV-LCLs lacking the appropriate HLA molecule with patient-derived cDNAs encoding the respective HLA-class II molecules (Figure 4).

Tissue-specific recognition of HLA-class II–restricted mHags

CD33⁺/CD14⁻ cells from mHag⁺ patients with AML expressing the appropriate HLA-DR molecules were purified by flow cytometry and tested for T-cell recognition. Figure 5 shows recognition of primary AML cells by all mHag-specific CD4⁺ T-cell clones. In addition, fibroblasts derived from a skin biopsy of the patient were cultured with and without IFN- γ and tested for recognition. Flow cytometric analysis demonstrated significant up-regulation of HLA-DR surface molecules after treatment with IFN- γ (Figure 6A). Similar results were obtained for skin-derived keratinocytes. None of the CD4⁺ T-cell

clones, however, was capable of recognizing (IFN- γ -treated) fibroblasts (Figure 6B). MHag⁺ keratinocytes expressing the appropriate HLA-DR molecule were available only for clones 62 and 78. Both clones failed to recognize (IFN- γ -treated) keratinocytes (data not shown), demonstrating that the novel mHags are efficiently presented by HLA-DR surface molecules on various hematopoietic cells, including primary AML cells, but not on non-hematopoietic fibroblasts and keratinocytes.

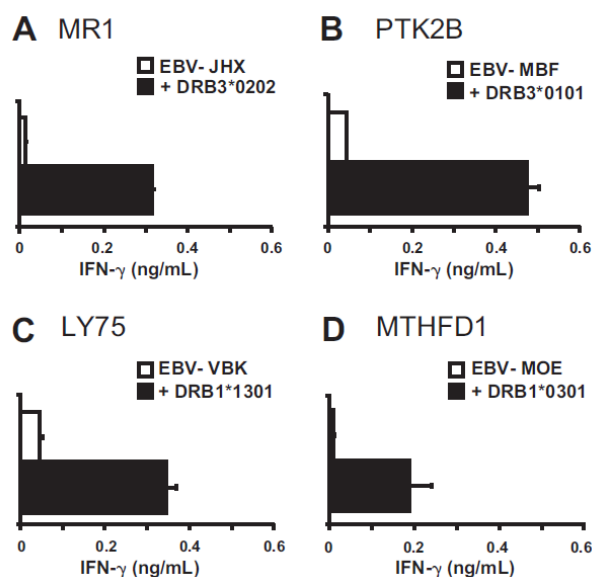


Figure 4. Identification of the HLA-DR restriction elements.

All 4 HLA-DR alleles of the patient (HLA-DRB1*0301, -DRB1*1301, -DRB3*0101, and -DRB3*0202) were isolated and retrovirally transduced into mHag⁺ EBV-LCLs lacking the appropriate HLA-DR molecules. CD4⁺ T-cell clones were tested against nontransduced and HLA-DR-transduced EBV-LCLs in IFN- γ ELISA. (A) T-cell clone 62 recognized LB-MR1-1R in the context of HLA-DRB3*0202, (B) T-cell clone 78 recognized LB-PTK2B-1T in the context of HLA-DRB3*0101, (C) T-cell clone 80 recognized LB-LY75-1K in the context of HLA-DRB1*1301, and (D) T-cell clone 87 recognized LB-MTHFD1-1Q in the context of HLA-DRB1*0301. Mean release of IFN- γ (ng/mL) in 50- μ L supernatants of duplicate wells is shown.

Tissue-specific expression of the mHag-encoding genes

To investigate the tissue distribution of the mHag-encoding genes, non-hematopoietic cells of different origins were cultured (fibroblasts, keratinocytes, biliary epithelial cells, proximal tubular epithelial cells, and gut epithelial cells) or isolated (hepatocytes), and gene expression was analyzed and compared with hematopoietic cells (PBMCs and EBV-LCLs) by quantitative real-time RT-PCR. The data showed overexpression of the *LY75* (> 10-fold) and *PTK2B* (> 4-fold) genes in hematopoietic versus non-hematopoietic cells (Table 2). Expression of the *MR1* gene was comparable between hematopoietic cells and fibroblasts,

but showed lower expression levels in other non-hematopoietic cells. The *MTHFD1* gene was expressed at comparable levels in all analyzed cell types.

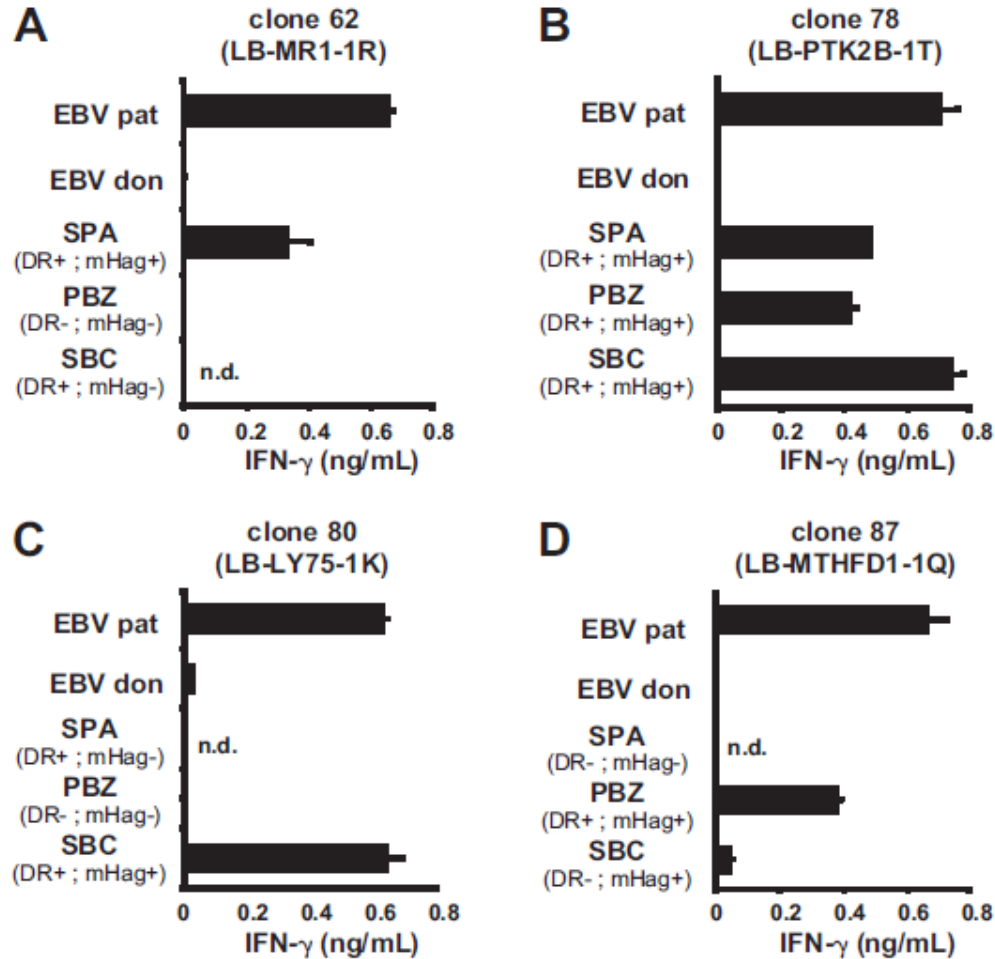


Figure 5. Recognition of other leukemic cells. Acute myeloid leukemia (AML) cells (CD33+/CD14-) from 3 patients with known HLA and mHag typing were isolated from PBMCs (PBZ, SBC) or bone marrow mononuclear cells (SPA) by flow cytometry. The isolated AML cells were tested for recognition by T-cell clone 62 (A), clone 78 (B), clone 80 (C), and clone 87 (D). EBV-LCLs of donor and patient served as negative and positive control, respectively. The SNP genotyping data (mHag+ or mHag-) and expression of the respective HLA-DR restriction molecule (DR+ or DR-) are indicated for each of the 4 mHags. All 4 T-cell clones recognized mHag+ AML cells expressing the appropriate HLA-DR restriction molecules. Mean release of IFN- γ (ng/mL) in 50- μ L supernatants of duplicate wells is shown. nd indicates not determined.

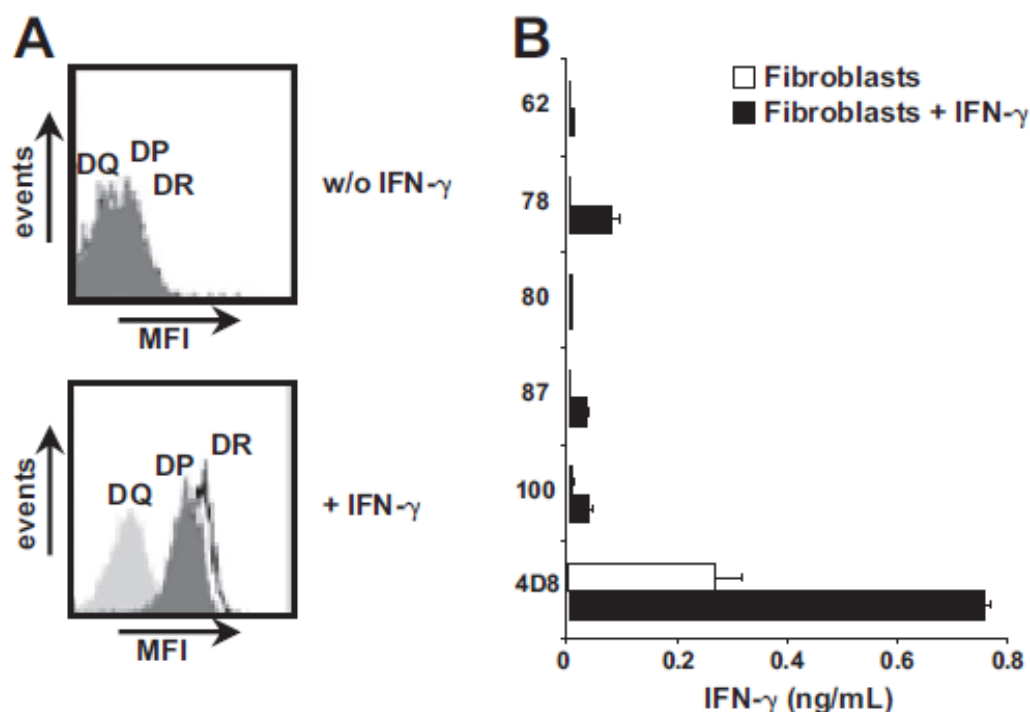


Figure 6. Fibroblast recognition by HLA-class II-restricted T cells. Primary fibroblasts of the patient were cultured with and without addition of 100 IU/mL IFN- γ . (A) Up-regulation of HLA-DR, -DQ, and -DP was measured by flow cytometry after 5 days of IFN- γ treatment. (B) T-cell recognition of pretreated and untreated fibroblasts was tested in an IFN- γ ELISA. A CD8⁺ T-cell clone (4D8) recognizing an unknown mHag in HLA-B8 served as positive control. Mean release of IFN- γ (ng/mL) in 50- μ L supernatants of duplicate wells is shown.

Table 2. mRNA expression of the mHag-encoding genes in hematopoietic and non-hematopoietic cells

Cell type	MR1, %	PTK2B, %	LY75, %	MTHFD1, %
EBV-LCLs, n = 2	100	100	100	100
PBMCs, n = 2	71	99	34	67
Fibroblasts,* n = 2	58	23	0	64
Keratinocytes,* n = 2	15	9	2	64
Biliary epithelium,† n = 1	5	3	0	73
Gut epithelium,* n = 2	33	7	3	38
Proximal tubular epithelium,‡ n = 2	5	26	0	81
Hepatocytes,§ n = 1	3	2	0	49

Expression of the mHag-encoding genes is corrected for expression of the PBGD housekeeping gene and shown relative to the level of gene expression in EBV-LCLs (100%). All measurements have been performed in duplicate with 100 and 10 ng cDNA.

EBV-LCLs indicates Epstein-Barr virus transformed B-cell lines ; PBMCs, peripheral blood mononuclear cells; and n, number of different samples per cell type.

* Fibroblasts, keratinocytes, and gut epithelial cells were isolated from biopsies.

† Biliary epithelial cells were purchased from ScienCell.

‡ Proximal tubular epithelial cells were kindly provided by Dr C. van Kooten, Department of Nephrology, Leiden University Medical Center (LUMC).

§ Hepatocytes were kindly provided by Dr A. E. Kremer, Liver Center, Academic Medical Center, University of Amsterdam.

DISCUSSION

In patients with relapsed hematopoietic malignancies after alloSCT, infusions with donor lymphocytes have proven to mediate potent GVL effect resulting in complete remissions.¹⁻³

In this study, we demonstrated that CD4⁺ T cells directed against 6 different HLA-class II-restricted mHags, including previously identified HLA-DQ-restricted mHag LB-PI4K2B-1S,³³ were induced in a patient with relapsed CML after alloSCT who showed long-term complete remission after treatment with DLI.

A broad range of HLA-class I-restricted mHags has been identified using a variety of techniques, including screening of plasmid cDNA libraries,^{7,10,14,15,17} elution of HLA-bound

peptides,^{5,6,8,11,13,16} genetic linkage analysis,^{9,12} and recently whole genome association scans.^{30,31} Most of these techniques did not allow or were difficult to adapt for identification of HLA-class II–restricted mHags. By screening a recombinant bacteria cDNA library,³² we could now identify 4 unknown CD4⁺ T-cell specificities. This technique is not only extremely efficient for identification of mHags, but may also have broad value for identification of nonpolymorphic antigens in antitumor or autoimmunity. Thus far, most nonpolymorphic HLA-class II–restricted epitopes have been characterized by screening antigens that were known targets for CD8⁺ T cells or antibodies.⁴⁰ Although this approach led to the identification of useful targets for T-cell therapy, HLA-class II epitopes from unknown and perhaps clinically more relevant antigens might have been missed.

It is well established that CD4⁺ T cells are required for efficient induction of *in vivo* immunity.^{21,22} In this study, all CD4⁺ T-cell clones showed not only recognition, but also specific lysis of patient-derived EBV-LCLs, suggesting a role in antitumor immunity as effector cells. Isolated CML progenitor cells, however, were strongly recognized only by T-cell clones 87 and 100, whereas all mHag-specific T-cell clones showed strong recognition of CML cells upon induction of an APC phenotype. Therefore, we hypothesize that all mHag-specific CD4⁺ T cells were key players as helper cells in the induction of antitumor CD8⁺ T cells. CD4⁺ T cells specific for LB-MTHFD1-1Q, represented by clone 87, may have played a more dual role as helper cells as well as effector cells directly eliminating CD34⁺ CML progenitor cells. In contrast to the variable recognition of isolated CML progenitor cells, all CD4⁺ T-cell clones strongly recognized AML cells, indicating that all 4 mHags can serve as direct targets for CD4⁺ effector T cells when presented by the appropriate HLA-DR restriction molecules at the surface of AML cells.

It is unclear why isolated CML progenitor cells, despite significant HLA-DR expression, were not or were hardly recognized by CD4⁺ T cells specific for LB-MR1-1R, LB-PTK2B-1T, and LB-LY75-1K, whereas CML-APCs were strongly recognized. Possible explanations are overexpression of the mHag-encoding genes or increased surface expression of HLA-DR or costimulatory molecules in CML cells upon induction of an APC phenotype. The latter possibility seems unlikely, because no difference in expression of

HLA-DR and various costimulatory (CD40, CD80, CD83, CD86) molecules was observed between CD34⁺ CML and AML cells (data not shown). A third, and perhaps more likely, explanation is a difference in processing and presentation of endogenous antigens into the HLA-class II pathway in CML-APCs compared with CML progenitor cells. This is supported by recent findings suggesting that maturation of APCs could enhance HLA-class II-restricted antigen presentation of endogenous proteins.⁴¹

In several patients treated with alloSCT, high frequencies of mHag-specific CD4⁺ T cells have been shown to precede and closely correlate with the onset of clinical GVHD.^{23,42–45} Although constitutive HLA-class II expression is restricted to hematopoietic cells, expression can be induced on non-hematopoietic cells by proinflammatory cytokines. CD4⁺ T cells recognizing mHags in HLA-class II may therefore contribute to development of GVHD when high levels of proinflammatory cytokines are released as a consequence of conditioning regimens or high pathogenic loads early after transplantation.⁴⁶

Under noninflammatory conditions, however, mHag-specific CD4⁺ T cells may exert a selective GVL effect without GVHD. This is supported by one of our recent studies demonstrating high numbers of HLA-DP-specific alloreactive CD4⁺ T cells in a patient who responded to DLI without GVHD late after HLA-DP-mismatched alloSCT,²⁹ as well as by clinical studies showing that CD8⁺ T-cell depletion reduces the incidence of GVHD associated with DLI without adversely affecting conversion to donor hematopoiesis.^{27,28} We here isolated mHag-specific CD4⁺ T cells from a patient who responded to DLI with only mild GVHD more than 1 year after alloSCT. Our *in vitro* data demonstrated no recognition of fibroblasts and keratinocytes by the mHag-specific CD4⁺ T cells, even after up-regulation of HLA-DR by IFN- γ . For LY75 and PTK2B, the lack of recognition can be explained by low gene expression levels in non-hematopoietic cells. For MR1 and MTHFD1, however, gene expression is similar between hematopoietic and non-hematopoietic cells. Our data therefore demonstrate that gene expression is not predictive for T-cell recognition of non-hematopoietic cells, and do not support a role for these mHag-specific CD4⁺ T cells in development of GVHD. The lack of recognition of non-hematopoietic cells by the mHag-specific CD4⁺ T cells may be explained by altered protein

translation or degradation or inefficient processing and presentation of the novel mHags into the HLA-class II pathway, as postulated for isolated CML progenitor cells.

All 4 novel mHags have potential relevance as targets in antitumor immunity after HLA-matched alloSCT. LB-LY75-1K and LB-PTK2B-1T are attractive based on overexpression of their genes in hematopoietic versus non-hematopoietic cells, whereas LB-MTHFD1-1Q may be useful as direct target for CD4⁺ T cells against CD34⁺ CML progenitor cells. Of special interest may be the *PTK2B* gene, also known as *Pyk2/RAFTK/FAK2/CAKβ*. The nonreceptor tyrosine kinase has been reported to play a role in dysregulation of various cellular processes in tumor cells, and may be interesting for immunotherapy as potential tumor-associated antigen.^{47–51} This is supported by the finding that antibodies against PTK2B have been observed in 3 of 19 patients with CML who responded to CD4⁺ DLI.⁵² Because PTK2B is an intracellular protein, antibodies against PTK2B are not expected to contribute directly to the elimination of tumor cells *in vivo*. The presence of antibodies, however, may indicate induction of a coordinated B- and CD4⁺ T-cell response against PTK2B, emphasizing the potential relevance of LB-PTK2B-1T as target in antitumor immunity after HLA-matched alloSCT.

By screening a recombinant bacteria cDNA expression library, we identified 4 novel HLA-DR–restricted mHags that contributed to GVL reactivity in a patient with complete remission of relapsed CML after DLI. Identification of HLA-class II–restricted mHags is needed to elucidate the role of CD4⁺ T cells in GVL and GVHD, and is crucial for development of new T-cell–based immunotherapies. The newly identified mHags provide a variety of possible applications. All HLA-DR–restricted mHags have balanced population frequencies and common HLA restrictions, and therefore allow selection of HLA-matched and mHag-mismatched patient-donor pairs for allogeneic stem cell transplantation. The HLA-class II–restricted mHags also open possibilities to specifically induce antileukemic CD4⁺ T cells, either *in vitro* for adoptive transfer or *in vivo* by vaccination. Finally, the identified mHags may be of relevance as helper epitopes to induce alloreactive or autoreactive CD8⁺ T cells against (HLA-class II[−]) solid tumors. However, all mHags have been identified as targets for CD4⁺ T cells isolated from 1 patient, and it therefore remains

to be shown whether these mHags are broadly immunogenic in other mHag-disparate patient-donor pairs.

In conclusion, our detailed analysis of the *in vivo* induced CD4⁺ T-cell response after DLI provides insight into the role of CD4⁺ T cells as helper and/or effector cells in therapeutic alloreactivity. The findings establish a basis for fundamental understanding of these phenomena, which is required for optimal use of HLA-class II–restricted mHags in the development of new T cell–based immunotherapies.

ACKNOWLEDGMENTS

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3

Endogenous HLA class II epitopes that are immunogenic *in vivo* show distinct behavior toward HLA-DM and its natural inhibitor HLA-DO

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ABSTRACT

CD4⁺ T cells play a central role in adaptive immunity. The acknowledgment of their cytolytic effector function and the finding that endogenous antigens can enter the HLA class II processing pathway make CD4⁺ T cells promising tools for immunotherapy. Expression of HLA class II and endogenous antigen, however, does not always correlate with T-cell recognition. We therefore investigated processing and presentation of endogenous HLA class II epitopes that induced CD4⁺ T cells during *in vivo* immune responses. We demonstrate that the peptide editor HLA-DM allowed antigen presentation of some (DM-resistant antigens) but abolished surface expression of other natural HLA class II epitopes (DM-sensitive antigens). DM sensitivity was shown to be epitope specific, mediated via interaction between HLA-DM and the HLA-DR restriction molecule, and reversible by HLA-DO. Because of the restricted expression of HLA-DO, presentation of DM-sensitive antigens was limited to professional antigen-presenting cells, whereas DM-resistant epitopes were expressed on all HLA class II-expressing cells. In conclusion, our data provide novel insights into the presentation of endogenous HLA class II epitopes and identify intracellular antigen processing and presentation as a critical factor for CD4⁺ T-cell recognition. This opens perspectives to exploit selective processing capacities as a new approach for targeted immunotherapy.

INTRODUCTION

CD4⁺ T cells are important in antitumor immunity as helper cells for the induction and maintenance of cytotoxic CD8⁺ T cells.^{1–3} Moreover, evidence is emerging that CD4⁺ T cells alone can provide efficient antitumor reactivity.^{4–7} In a NOD/SCID mouse model for human acute lymphoblastic leukemia, infusion of human CD4⁺ T cells led to eradication of the malignant cells.⁸ Clinical trials in allogeneic stem cell transplantation (alloSCT) found that depletion of CD8⁺ T cells from donor lymphocyte infusion preserved the ability of the T cells to induce conversion to donor chimerism,^{9,10} highlighting the direct effector function of CD4⁺ T cells.

Antigen presentation to T cells by HLA surface molecules depends on protein degradation mediated by proteasomes and lysosomal proteases as the 2 major cellular breakdown mechanisms. Traditionally, it has been assumed that HLA class II molecules present exogenous antigens which are degraded by proteases within the endosomal/lysosomal system, whereas endogenous cytosolic antigens are degraded by the proteasome and presented by HLA class I molecules. In the past decade, however, evidence emerged that HLA class I molecules can also present exogenous antigens via a process called cross-presentation,¹¹ and that intracellular antigens can enter the HLA class II pathway.^{12,13}

In antitumor immunity, professional APCs such as dendritic cells (DCs) take up and present antigens derived from tumor cells. The encounter of these exogenous antigens presented on DCs may be sufficient for the induction of CD4⁺ T cells and their function as helper cells. However, to serve as specific targets for cytolytic CD4⁺ T cells, tumor cells need to present endogenous peptides in HLA class II molecules as expressed at their cell surface. We and others, however, observed that some HLA class II–positive target cells are not recognized by the respective CD4⁺ T-cell clones,^{14,15} despite appropriate HLA class II and antigen expression. Already in 1996, Harris et al provided evidence for cell type–specific differences in presentation of endogenous antigens in HLA class II.¹⁶ By eluting peptides from HLA-DR, professional APCs were shown to present an extended repertoire of self-peptides^{17,18} compared with nonprofessional APCs. The investigators also found that significant amounts of class II–associated invariant chain peptide (CLIP), the remnant of

the invariant chain (Ii), were presented at the cell surface of professional APCs, whereas CLIP was absent on nonprofessional APCs.¹⁶ Surface expression of CLIP on professional APCs may be attributed to a molecule called HLA-DO. Although its precise biologic role is still unclear, HLA-DO has been shown to increase CLIP surface presentation by inhibition of HLA-DM in a pH-dependent manner,^{19,20} and its expression has been reported to be confined to professional APCs, such as B cells, DCs, and thymic epithelial cells.²¹

Here, we investigated whether endogenous antigens identified as natural targets for CD4⁺ T cells are differentially processed and presented on HLA class II–positive cells. We previously identified 6 antigens derived from endogenous proteins as targets for CD4⁺ T cells during *in vivo* immune responses after alloSCT. These antigens comprise 5 HLA class II–restricted autosomal minor histocompatibility antigens (MiHAs)^{14,22} and 1 male-specific MiHA.²³ We analyzed the contribution of the Ii, HLA-DM, and HLA-DO to the processing and presentation of these antigens via HLA class II, and we have shown that natural HLA class II epitopes can be divided into 2 groups. For 1 group of antigens, presentation is preserved in the presence of HLA-DM (DM-resistant), whereas for another set of antigens presentation is abolished by expression of HLA-DM (DM-sensitive). Presentation of DM-sensitive antigens in HLA class II molecules can be restored by coexpression of HLA-DO, suggesting that *in vivo* presentation of DM-sensitive antigens is restricted to professional APCs. In conclusion, our study suggests the existence of a category of endogenous HLA class II epitopes *in vivo* that behave like CLIP and whose presentation depends on a delicate balance between HLA-DM and DO. This insight opens possibilities for the use of these antigens for selective targeting of specific types of HLA class II–positive cells in (antitumor) immune responses.

METHODS

Hematopoietic cell isolation

PBMC and bone marrow samples were obtained from healthy persons after approval by the Leiden University Medical Center Institutional Review Board and informed consent

according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque separation and cryopreserved. CD14⁺ monocytes were isolated from PBMCs by magnetic beads according to the manufacturer's instructions (Miltenyi Biotec).

Cell culture

HeLa, MJS, Raji, COS, and EBV-transformed B-cell lines (EBV-LCLs) were cultured in IMDM (Lonza BioWhittaker) with 10% FCS (Cambrex), 1% penicillin/streptomycin (Lonza), and 1.5% L-glutamine (Lonza). T-cell clones were cultured in IMDM with 5% human serum, 5% FCS, and 100 IU/mL IL-2 (Chiron) and were restimulated every 10-20 days with irradiated allogeneic PBMCs and 0.8 µg/mL PHA (Oxoid).²²

Immature DCs were generated by culturing monocytes in medium with 100 ng/mL GM-CSF (Novartis) and 500 IU/mL IL-4 (Schering-Plough) for 7 days. During the final 2 days 100 ng/mL GM-CSF, 10 ng/mL TNF-α (Cellgenix), 10 ng/mL IL-1β (Cellgenix), 10 ng/mL IL-6 (Cellgenix), 1 µg/mL prostaglandin E₂ (Sigma-Aldrich), and 500 IU/mL IFN-γ (Boehringer-Ingelheim) were added for maturation.

Fibroblasts were cultured in DMEM with low glucose (Lonza), 8% FCS, 1% penicillin/streptomycin, and 1.5% glutamine with or without IFN-γ (100 IU/mL) for 4 days.

T-cell library

CD4⁺/CD56⁻ T cells were isolated by flow cytometry (Aria; BD Biosciences) and seeded at 2000 cells/well and expanded with 500 IU/mL IL-2, 0.8 µg/mL PHA, and 50 000 irradiated allogeneic feeders per well.²⁴

After 2-3 weeks of expansion CD4⁺ T-cell bulks were washed twice and plated at 2×10^5 cells/well for each target. As target cells HeLa-Ii and HeLa-Ii/DM cotransduced with the relevant HLA class II molecules were used.

Flow cytometry

Flow cytometric analyses were performed on a FACSCalibur (BD Biosciences) and cell sorting on a FACSaria (BD Biosciences) with the use of PE-labeled monoclonal antibodies

against HLA-DR (L243; BD Biosciences), CLIP (Cer.CLIP; Santa Cruz Biotechnology), NGFR (C40-1457; BD PharMingen), CD80 (L307.4; BD Biosciences), HLA-DQ (1a3; Meridian Life Science), CD20 (L27; BD Biosciences), and CD4 (RPA-T4; BD PharMingen). FITC-labeled anti-CD14 (M5E2) and anti-CD34, (581) APC-labeled anti-CD19 (HIB19) and anti-CD56 (B159), and peridinin chlorophyll protein complex-labeled anti-CD3 (SP34-2) were purchased from BD PharMingen.

Retroviral constructs and transduction

Ii, HLA-DM α , and HLA-DM β were cloned into pLZRS vectors. For Ii, CD80 was used as marker gene, for HLA-DM α truncated Δ NGFR, and for HLA-DM β enhanced green fluorescence protein. As control pLZRS with only CD80 was cloned, and the Ii was recloned in MP71 with CD20 as marker gene. HLA-DQB1*0603, DQB1*0501, DQA*0103, and DQA*0101 were cloned into pLZRS vectors with enhanced green fluorescence protein (α chains) and truncated Δ NGFR (β chains). HLA-DRB1*0301, DRB1*1301, DRB3*0101, DRB3*0202, and DRA*0102 were cloned in MP71 vectors with the use of truncated Δ NGFR as marker gene.

Full-length PTK2B, MR1, PI4K2B, DBY, and MTHFD1 were cloned into MP71 with CD20 as marker gene. HLA-DO α was cloned into MP71 with bicistronic HLA-DQA*0103 and HLA-DO β with bicistronic HLA-DQB1*0603. Chimeric constructs and mutated DR α chains were cloned by 2-step PCR. For chimera A the 39-bp sequence of the PTK2B epitope was inserted at positions 220-262 bp in the PI4K2B sequence (NM_018323), whereas the 42-bp sequence of the PI4K2B epitope was inserted at positions 2371-2410 bp in the PTK2B sequence (NM_173175) for chimera B. Mutated DR α chains were cloned by inserting single-point mutations at position 193 (G/A) and 227 (T/C), respectively.²⁵ Chimeric constructs were cloned into MP71 with CD20, mutated DRA in MP71 vectors with Δ NGFR. All constructs were verified by sequencing. Retroviral supernatant fluid was obtained by transfecting wild-type Φ nx-A packaging cells as previously described,²⁶ with the exception that the Fugene HD transfection kit (Roche) was used. Viral supernatant fluids were used for transduction on plates coated with recombinant human fibronectin CH 296 (Takara Shuzo).²⁶

Antigen presentation assays

Stimulator cells (3×10^4 cells/well) were coincubated with CD4⁺ T-cell clones (5×10^3 cells/well) overnight at 37°C in 96-well plates. For recombinant protein loading *Escherichia coli* cells with a pKE-1 vector encoding the respective antigen under an isopropyl thiogalactoside-inducible promoter were grown to OD₆₀₀ of 0.5 with 50 µg/mL ampicillin (Sigma-Aldrich), and protein expression was induced by 1mM isopropyl thiogalactoside for 4 hours (Promega). Subsequently, bacteria were opsonized for 1 hour by adding human serum with 17% (v/v) complement (Sigma-Aldrich). Target cells (3×10^4 cells/well) were pulsed with complement-opsonized bacteria in culture medium with 30 µg/mL gentamycin (Sigma-Aldrich) overnight at 37°C. T-cell clones were added, and cytokine release was measured after overnight incubation in 25-µL supernatant fluids by IFN-γ ELISA (Sanguin).

Microarray gene expression analysis

Total RNA was isolated with small and micro scale RNAqueous isolation kits (Ambion) and was amplified with the TotalPrep RNA amplification kit (Ambion). After preparation with the use of the whole-genome gene expression direct hybridization assay (Illumina), cRNA samples were dispensed onto Human HT-12 Version 3 Expression BeadChips (Illumina). Hybridization was performed in the Illumina hybridization oven for 17 hours at 58°C. Microarray gene expression data were analyzed with R 2.15 (R Project). Normalization was done in the lumi package,²⁷ using the variance stabilizing transformation and quantile normalization. All microarray data are available at the Gene Expression Omnibus under accession no. GSE38798.

RESULTS

HLA-DM has divergent effects on the presentation of natural HLA class II epitopes.

To study processing and presentation of endogenous HLA class II epitopes, we selected 6 polymorphic antigens identified as targets for CD4⁺ T cells during *in vivo*-induced immune

responses after HLA-matched alloSCT. Because of single amino acid differences between patient and donor, endogenous peptides of the patient presented in shared HLA molecules are recognized by donor T cells. Five of these polymorphic peptides were derived from autosomal proteins (LY75, MR1, PTK2B, PI4K2B, and MTHFD1) and were identified as targets for MiHA-specific CD4⁺ T cells.^{14,22} One male-specific MiHA was derived from the DBY protein.²³ The 6 proteins included 2 transmembrane proteins (LY75 and MR1), 2 cytosolic kinases (PTK2B and PI4K2B), 1 cytosolic enzyme (MTHFD1), and 1 nuclear/cytosolic RNA helicase (DBY). The antigens were restricted by 4 different HLA-DR and 2 different HLA-DQ molecules: DRB1*0301/A*0102 (MTHFD1), DRB1*1301/A*0102 (LY75), DRB3*0101/A*0102 (PTK2B), DRB3*0202/A*0102 (MR1), DQB1*0603/A*0103 (PI4K2B), and DQB1*0501/A*0101 (DBY). To investigate the role of molecules with known functions in processing and presentation of HLA class II epitopes, we retrovirally transduced the HLA class II–negative cervix carcinoma HeLa cell line with the genes encoding the Ii, HLA-DM α/β , or both. Cell lines were sorted by flow cytometry on the basis of marker gene expression for Ii (CD80) and HLA-DM α/β (GFP/NGFR). Subsequently, all HeLa variants were transduced with the appropriate HLA class II and isolated on the basis of HLA-DR or -DQ surface expression. Expression of the Ii and HLA-DM in the transduced cell lines was confirmed by Western blot analysis (data not shown), and their functionality was confirmed by monitoring cell surface expression of CLIP, the remnant of the Ii occupying the peptide binding groove. Whereas surface expression of the introduced DR or DQ restriction alleles was comparable between the different HeLa variants (Figure 1A; data not shown), staining of CLIP in DR was markedly increased on HeLa cells stably expressing the Ii and completely removed by coexpression of HLA-DM (Figure 1A).

The 4 HeLa variants cotransduced with the relevant HLA class II restriction molecules were pulsed with recombinant proteins to induce antigen expression. Protein uptake, processing, and subsequent surface presentation of the peptide were quantified by IFN- γ release of the specific CD4⁺ T-cell clones. Presentation of all antigens was increased by expression of the Ii.

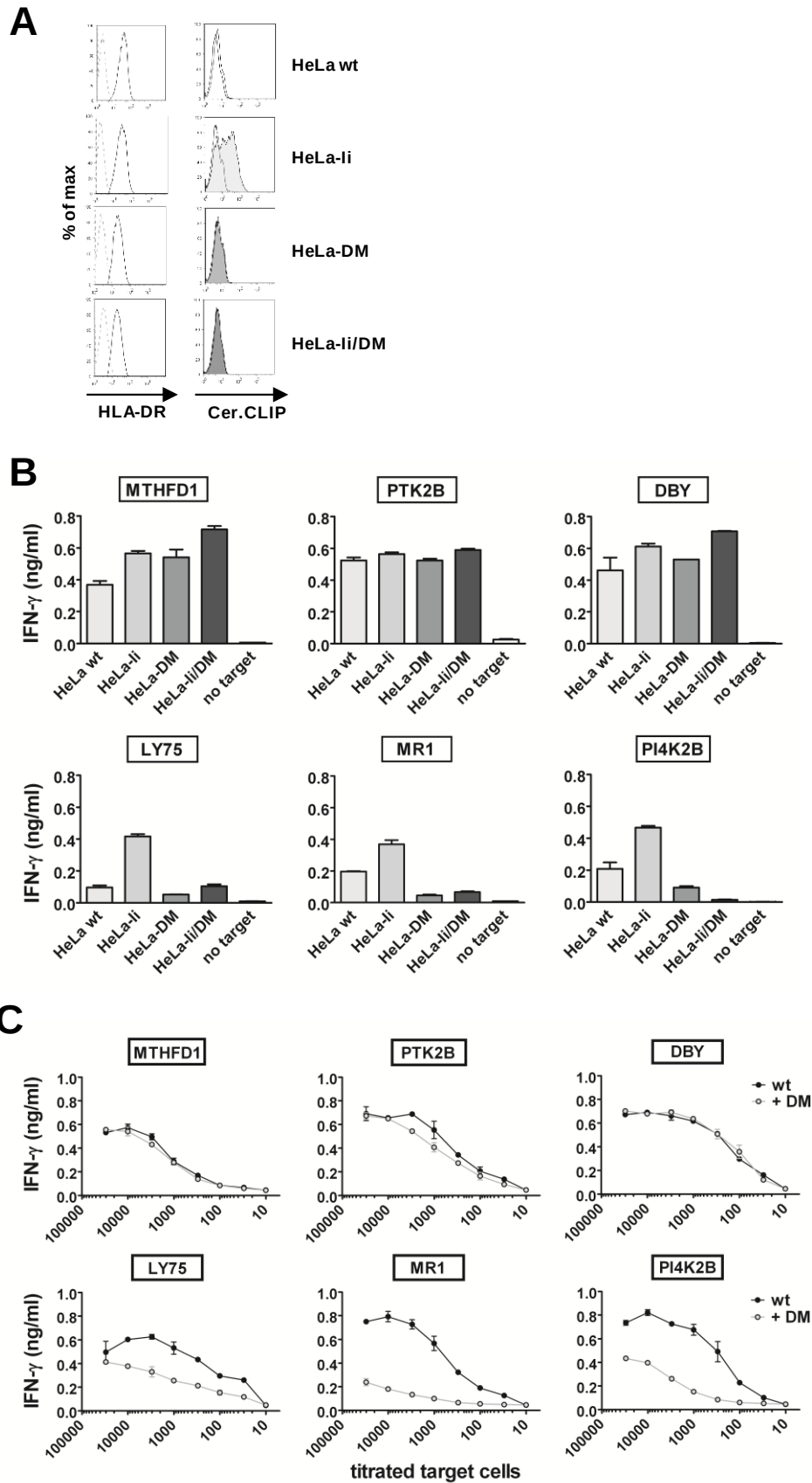


Figure 1. Natural HLA class II epitopes can be divided in HLA-DM-sensitive and -resistant antigens.

The cervix carcinoma HeLa cell line was retrovirally transduced with the invariant chain (Ii), HLA-DM, or a combination of both. (A) Indicated is the HLA-DR (left) and class II-associated invariant chain peptide (CLIP; right) surface expression as measured by flow cytometry. (B) All HeLa variants were cotransduced with the appropriate HLA class II restriction molecules, and specific release of IFN- γ by the CD4⁺ T-cell clones after exogenous loading with recombinant proteins was measured in ELISA. Antigens in the upper row display a DM-resistant phenotype as defined by retained antigen presentation on expression of HLA-DM, whereas DM-sensitive antigens with impaired presentation in the presence of HLA-DM (DM-sensitive) are depicted in the lower part. Mean $\pm$ SD release of IFN- γ in duplicate wells is shown. (C) HLA-DM was retrovirally transduced into EBV-transformed B-cell lines (EBV-LCL) from patient MRJ, and tested for T-cell recognition in IFN- γ ELISA. CD4⁺ T-cell clones specific for PI4K2B, MR1, PTK2B, LY75, and MTHFD1 were isolated from patient MRJ and selected on the basis of recognition of patient-derived EBV-LCL. HLA-DM was also introduced into the HLA-DQB1*0502-positive Raji cell line derived from a male patient with Burkitt lymphoma to measure T-cell recognition of endogenous DBY antigen. Solid squares and open circles indicate T-cell recognition of the endogenous antigens as expressed in the wild-type and HLA-DM transduced EBV-LCL, respectively. Numbers of titrated target cells are depicted on the x-axis. Mean $\pm$ SD release of IFN- γ in duplicate wells is shown.

Coexpression of HLA-DM did not affect or slightly increased presentation of 3 antigens (MTHFD1, PTK2B, and DBY), whereas recognition of the other 3 antigens (LY75, MR1, and PI4K2B) was abolished in the presence of HLA-DM (Figure 1B). To exclude that the observed increase in recognition of the HeLa-Ii variant was because of expression of the marker gene CD80, we recloned the Ii in a construct with CD20 and yielded the same results (data not shown).

To confirm the divergent effect of HLA-DM on presentation of endogenously expressed antigens, we retrovirally transduced HLA-DM into patient-derived EBV-LCL. As described earlier these EBV-LCL expressed all HLA class II epitopes from LY75, MR1, PTK2B, PI4K2B, and MTHFD1 and were strongly recognized by the specific T-cell clones. In addition, to study presentation of the male antigen DBY, HLA-DM was transduced into the Raji cell line, which is derived from a male patient with Burkitt lymphoma and expresses HLA-DQB1*05 and DBY. Similar as observed for the HeLa variants pulsed with recombinant proteins, T-cell recognition of the endogenously expressed antigens could be again divided in 2 groups. Recognition of MTHFD1, PTK2B, and DBY was not affected by overexpression of HLA-DM, whereas presentation of LY75, MR1, and PI4K2B was markedly decreased in the presence of transduced HLA-DM (Figure 1C). Finally, we confirmed the effect of HLA-DM in another cell line. Antigen-

negative COS cells were transduced with Ii alone or Ii in combination with HLA-DM and subsequently with the HLA-DR restriction allele for PTK2B or the HLA-DQ restriction allele for PI4K2B. Next, the genes encoding PTK2B or PI4K2B were introduced, and T-cell recognition was measured by IFN- γ ELISA. The data confirmed that HLA-DM did not affect T-cell recognition of DM-resistant antigen PTK2B, whereas recognition of DM-sensitive antigen PI4K2B was significantly inhibited (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

In conclusion, these data indicated the existence of 2 different groups of natural HLA class II epitopes. Presentation of 1 group of antigens is not or hardly influenced by HLA-DM (DM-resistant), whereas the other group of natural HLA class II epitopes behaves like CLIP and their presentation is removed by HLA-DM (DM-sensitive). This difference in behavior toward HLA-DM cannot be explained by the binding affinity of the peptides for their HLA class II restriction molecules or affinity of the T-cell receptors as expressed on the different T-cell clones, because no consistent difference in these variables between DM-resistant and DM-sensitive antigens was observed (supplemental Figure 2). Moreover, because the effect of HLA-DM on T-cell recognition of HLA class II-restricted antigens was measured after retroviral transfer into the same target cells, variables such as density of HLA class II surface molecules and avidity of the T-cell clones as influenced by adhesion and costimulatory molecules can be excluded as relevant factors.

HLA-DM sensitivity was epitope specific

To analyze whether the sensitivity or resistance toward HLA-DM was a characteristic of the protein or the epitope, we made wild-type and epitope exchange constructs of PTK2B and PI4K2B (Figure 2A). The T-cell epitope of DM-resistant antigen PTK2B (a 13-aa epitope in HLA-DRB3*0101) was exchanged with the T-cell epitope of DM-sensitive antigen PI4K2B (a 14-aa epitope in HLA-DQB1*0603) and vice versa. The constructs were transduced in HeLa cells expressing HLA-DRB3*0101/A*0102 and DQB1*0603/A*0103, respectively. T-cell recognition assays found that exchange of the T-cell epitopes still allowed presentation in the context of their original HLA class II restriction molecule. Moreover, the exchanged T-cell epitopes showed the same behavior toward HLA-DM as in

their natural configuration (Figure 2B). Thus, DM sensitivity is an intrinsic property of a T-cell epitope that does not depend on the context of the epitope within the protein.

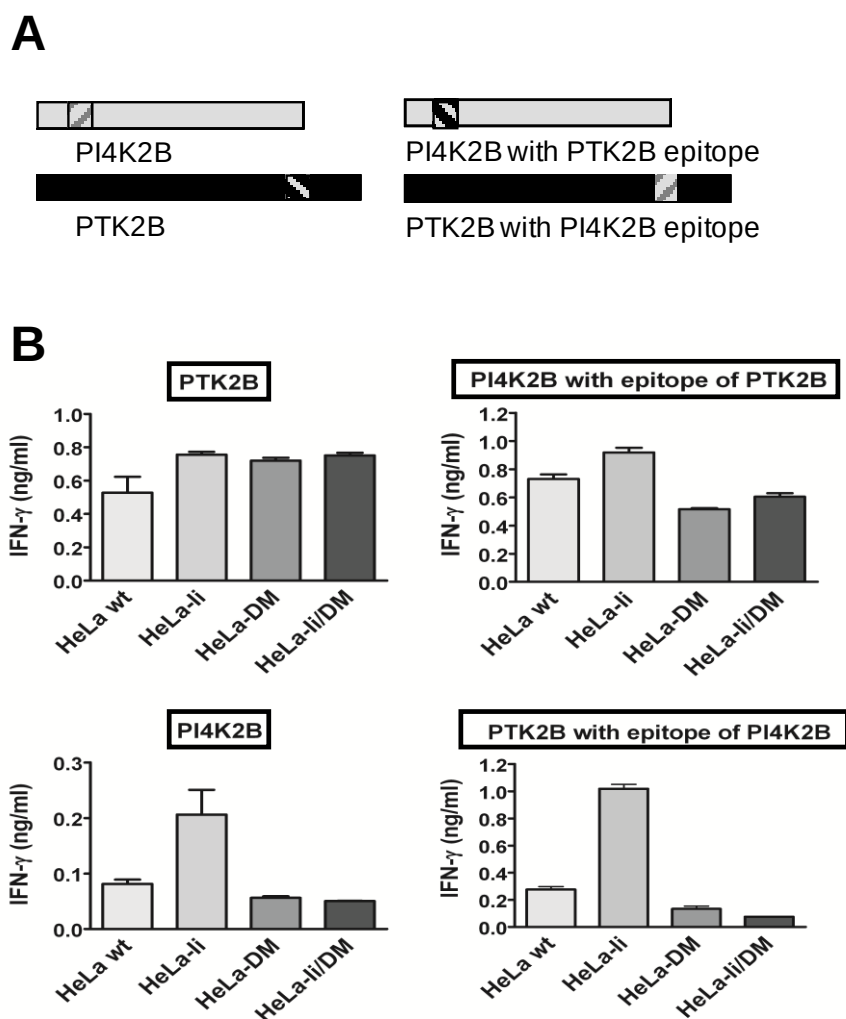


Figure 2. DM sensitivity is determined by the T-cell epitope.

(A) Schematic overview of wild-type proteins PI4K2B and PTK2B (left) and proteins with exchanged T-cell epitopes (right). T-cell epitopes are indicated by the hatched areas. (B) T-cell recognition in IFN- γ ELISA is shown for the 2 wild-type PI4K2B and PTK2B antigens (left) and proteins with exchanged T-cell epitopes (right) after retroviral transduction into HeLa variants cotransduced with DRB3*0101/DRA*0102 (top) and DQB1*0603/DQA*0103 (bottom), respectively. Mean $\pm$ SD release of IFN- γ of duplicate wells on coinubation with the specific CD4⁺ T-cell clones is shown.

HLA-DM precluded presentation of DM-sensitive epitopes via interaction with the HLA-DR α chain

Removal of CLIP by HLA-DM has been described to be mediated via interaction between HLA-DM and HLA-DR, and certain amino acid residues in the HLA-DR α and β chain have been described as indispensable for HLA-DM to elicit its function.²⁵ We investigated whether the same interaction between HLA-DR and HLA-DM was required for removal of the natural DM-sensitive HLA class II epitopes. Because all HLA-DR-restricted antigens use the same α chain (DRA*0102), we mutated a known interaction residue within this molecule (E40K). This mutation hampered the formation of SDS-stable DR dimers (data not shown), indicating loss of interaction with HLA-DM.²⁵ For T-cell recognition, HeLa variants transduced with wild-type or mutated DR α were pulsed with recombinant proteins to induce antigen expression. No effect on presentation of DM-resistant antigens MTHFD1 and PTK2B was observed. However, the mutation rescued presentation of the DM-sensitive antigens LY75 and MR1 in the presence of HLA-DM (Figure 3).

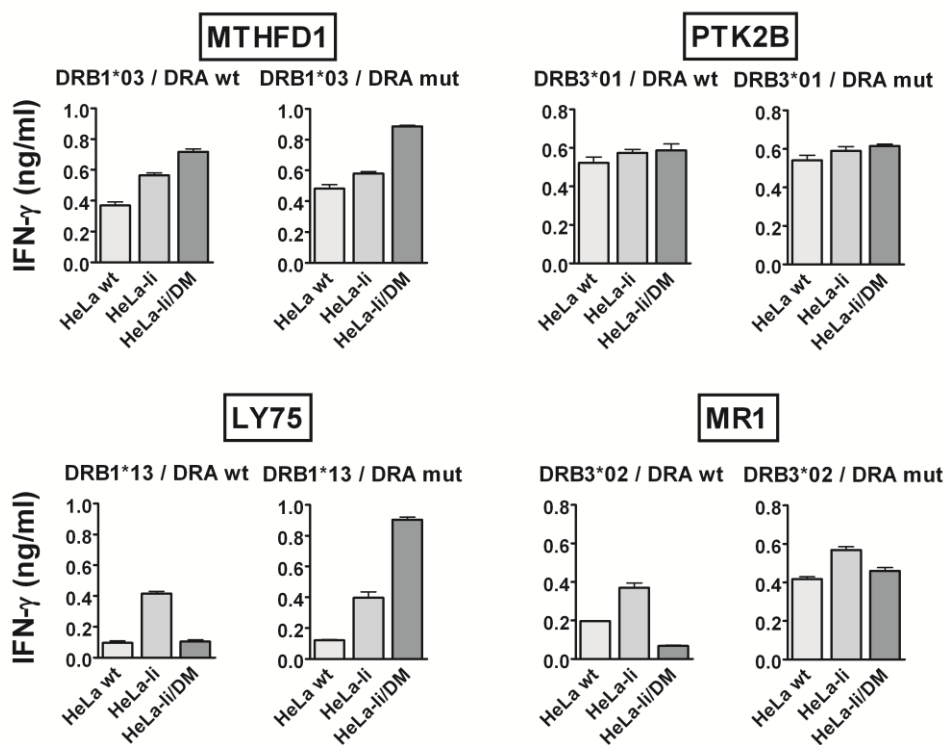


Figure 3. HLA-DM sensitivity is mediated via interaction with HLA-DR α .

The effect of point mutation E40K in DR α (DRA mut) versus wild-type DR α (DRA wt) was tested on the presentation of DM-resistant antigens MTHFD1 and PTK2B (top) as well as DM-sensitive antigens LY75 and MR1 (bottom). HeLa variants were transduced with the genes for DRA wt or DRA mut in combination with the appropriate DRB chains and tested for T-cell recognition after exogenous loading of the indicated recombinant proteins. Mean $\pm$ SD release of IFN- γ after coincubation with the specific T-cell clones is depicted.

Mutation of another interaction residue within DR α (F51S) gave similar results (data not shown). Peptide loading showed that the mutation neither interfered with recognition of the HLA-peptide complex by the T-cell receptor nor prohibited binding of the peptide to the HLA molecule (data not shown). In conclusion, our data indicated that DM sensitivity of natural T-cell epitopes is mediated via interaction between HLA-DM and the HLA class II restriction molecule in a manner similar as described for CLIP.

HLA-DO restored presentation of DM-sensitive antigens

HLA-DO has been described as a natural inhibitor of HLA-DM, and its expression has been mainly confined to B cells, mature DCs, and thymic epithelial cells. We therefore investigated whether expression of HLA-DO could restore presentation of DM-sensitive antigens in HLA-DM-positive cells. We transduced the HLA-DO α/β chains into the HLA-DO-negative and HLA class II-positive melanoma MJS cell line, which endogenously expresses Ii and HLA-DM. The functionality of the HLA-DO construct was confirmed by occurrence of CLIP surface expression (Figure 4A). MJS cells endogenously express HLA-DRB3*0101, and we retrovirally introduced HLADQB1*0603/A*0103 either alone or in combination with HLA-DO. MJS cells with and without HLA-DO were subsequently loaded with recombinant PTK2B and PI4K2B proteins or retrovirally transduced with the genes encoding these proteins and were compared for presentation of the HLA class II epitopes. HLA-DO increased presentation of the DM-sensitive antigen PI4K2B, whereas it did not alter presentation of DM-resistant antigen PTK2B (Figure 4B-C).

These data indicated that there are 2 distinct subsets of HLA class II-restricted antigens: one that behaves like CLIP and whose presentation is determined by a delicate balance between Ii, HLA-DM, and HLA-DO expression and another set of HLA class II epitopes whose presentation is not or less dependent on this balance.

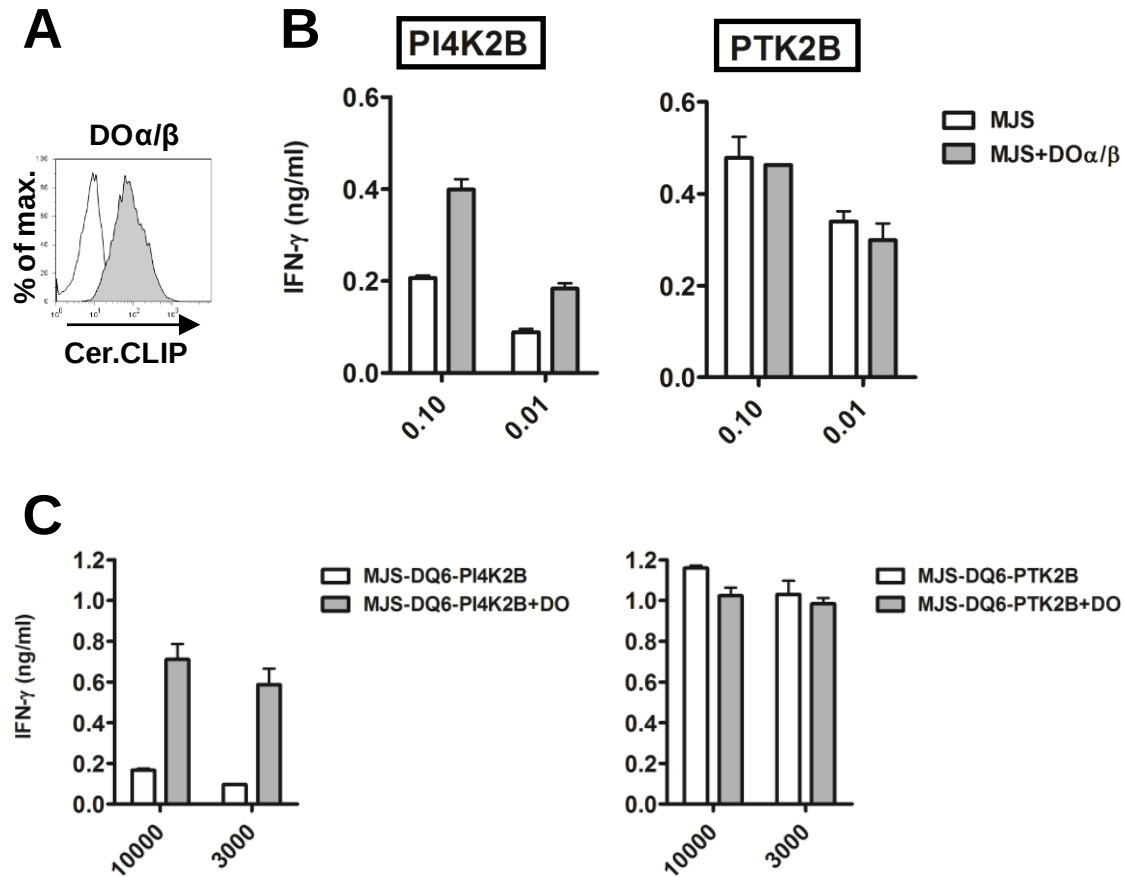


Figure 4. HLA-DM sensitivity is reversible by HLA-DO.

(A) Histograms are shown for class II-associated invariant chain peptide (CLIP) surface expression on MJS wt (open) and MJS cells transduced with HLA-DO α/β (filled) as measured by flow cytometry. (B) The influence of HLA-DO on presentation of the DM-sensitive antigen PI4K2B and DM-resistant antigen PTK2B was analyzed by testing T-cell recognition of MJS wt (open bars) and MJS transduced with HLA-DO α/β (closed bars). MJS melanoma cells endogenously express the invariant chain and HLA-DM but lack expression of HLA-DO. The HLA-DRB3*0101 restriction allele for PTK2B is endogenously expressed by MJS cells and the HLA-DQB1*0603/DQA1*0103 restriction allele for the HLA class II epitope from PI4K2B was introduced retrovirally. To induce antigen expression, MJS cells were loaded with recombinant proteins in 2 different concentrations. Mean $\pm$ SD release of IFN- γ in duplicate wells is shown. (C) MJS-DQ6 (open bars) and MJS-DQ6-DO (closed bars) cells were transduced with retroviral vectors encoding PI4K2B and PTK2B to test the influence of HLA-DO on endogenously expressed antigens. T-cell recognition was tested against titrated numbers of target cells (10 000 and 3000), and mean $\pm$ SD release of IFN- γ in duplicate wells is shown.

T cells recognizing DM-sensitive HLA class II epitopes represented a significant part of the natural repertoire

Our data showed that 3 of 6 identified HLA class II epitopes are DM sensitive. We next investigated the overall contribution of DM-sensitive epitopes to natural T-cell responses. For this purpose, we generated and screened CD4⁺ T-cell libraries according to the method described by Geiger et al.²⁴ Briefly, CD4⁺ T cells were purified and seeded at 2 000 T cells per well. CD4⁺ T cells were nonspecifically expanded in the presence of irradiated allogeneic PBMCs, PHA, and IL-2. After 3 weeks of *in vitro* expansion, all T-cell pools were tested against HeLa variants expressing the Ii with and without cotransduced HLA-DM in IFN- γ ELISA. CD4⁺ T-cell libraries from 3 different donors were screened against HeLa variants transduced with the HLA class II molecules as expressed by the donors. T-cell pools with no or low (< 200pg/mL IFN- γ) reactivity were scored as “nonreactive,” whereas the remaining pools were separated into DM-resistant or -sensitive phenotypes depending on differential recognition of HeLa-Ii and HeLa-Ii/DM. HLA class II epitopes were considered DM sensitive when recognition in the context of HLA-DM was decreased by more than 50%. The data showed that separation of HLA class II epitopes into DM-resistant and DM-sensitive epitopes was not restricted to the 6 antigens analyzed in more detail but represented a widely occurring phenomenon observed for HLA class II epitopes in natural T-cell responses. Our data furthermore showed that the majority of epitopes recognized in the context of HLA-DQ display a DM-sensitive phenotype (Figure 5A), whereas for HLA-DR alleles a tendency toward more DM-resistant epitopes was observed (Figure 5B).

HLA-DQ and HLA-DO β are predominantly expressed in B cells and mature DCs

Our data found that presentation of DM-sensitive epitopes depended on balanced expression of Ii, HLA-DM, and HLA-DO. Because most of the natural HLA class II epitopes presented by HLA-DQ are DM sensitive, we analyzed the surface expression patterns of HLA-DR and -DQ in more detail by flow cytometry. In addition, expression of the genes encoding Ii, HLA-DM α/β , and HLA-DO α/β was evaluated in hematopoietic cells and fibroblasts. To investigate the effect of IFN- γ on the HLA class II machinery in non-hematopoietic cells, fibroblasts were analyzed with and without pretreatment with IFN- γ .

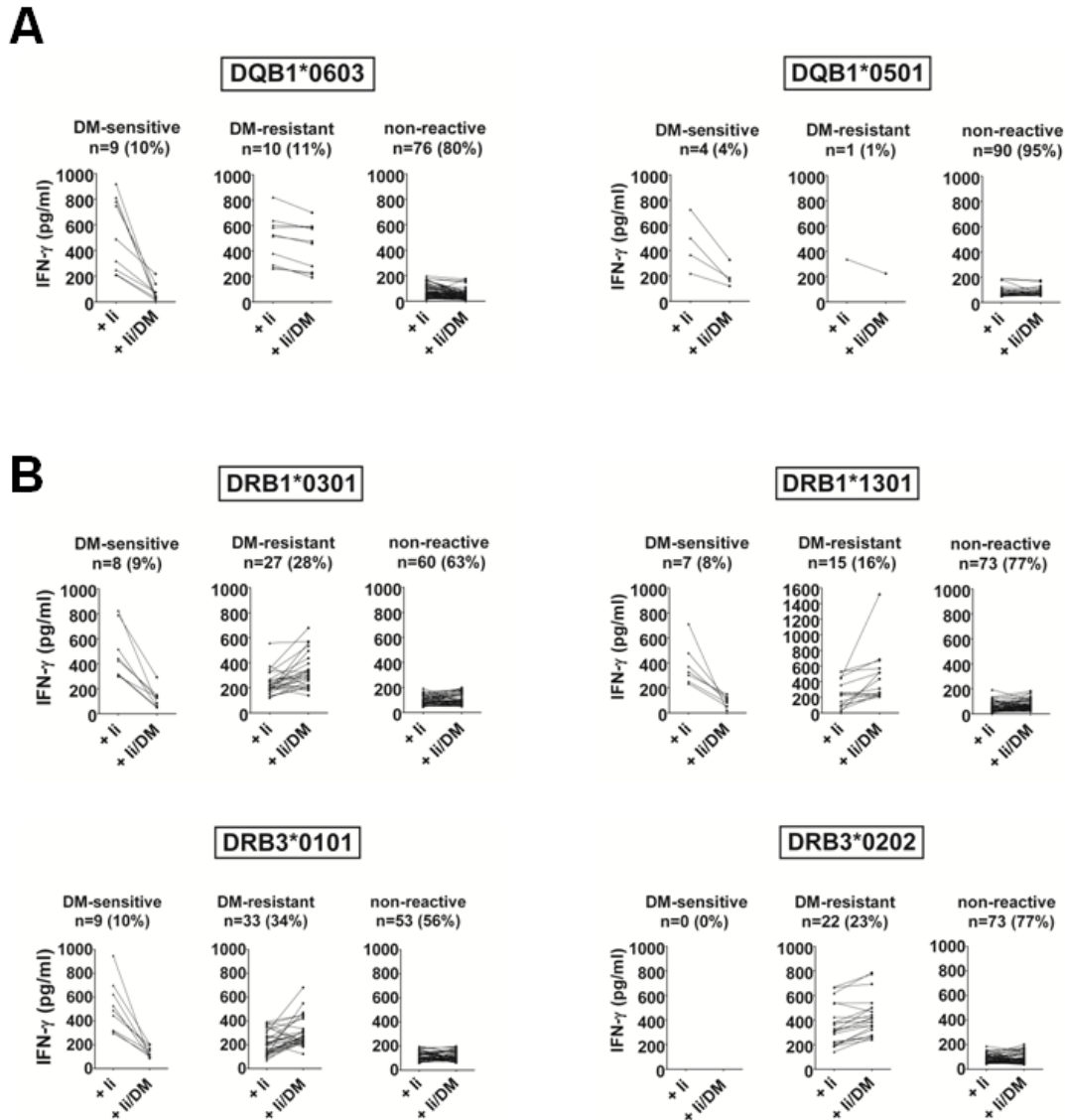


Figure 5. DM-resistant and DM-sensitive antigens as recognized by the natural CD4⁺ T-cell repertoire.

Purified CD4⁺ T cells were seeded in one 96-well plate, each well containing 2000 different T cells. CD4⁺ T cells were nonspecifically expanded and tested for recognition of HeLa-invariant chain (Ii) and HeLa-Ii/DM transduced with different HLA class II molecules. T-cell pools with no or low (< 200 pg/mL IFN- γ) recognition of both HeLa variants are depicted as “nonreactive.” The remaining pools were separated into DM-sensitive and DM-resistant phenotype, based on the difference in recognition between HeLa-Ii and HeLa-Ii/DM. Pools with > 50% decrease in recognition of HeLa-Ii/DM versus HeLa-Ii were assigned DM-sensitive, whereas all other pools were counted as DM-resistant. CD4⁺ T-cell libraries were generated from 3 different donors, and CD4⁺ T-cell pools were tested against HeLa-Ii and HeLa-Ii/DM variants transduced with HLA-DQB1*0603/A*0103 (2 donors), DQB1*0501/A*0101 (1 donor), DRB1*0301 (1 donor), DRB1*1301 (2 donors), DRB3*0101 (1 donor), and DRB3*0202 (1 donor). Representative results are shown for 2 HLA-DQ alleles (A) and 4 HLA-DR molecules (B). The total number and percentages of DM-resistant and DM-sensitive pools for each HLA class II allele are indicated.

HLA-DR was strongly expressed on B cells, monocytes, CD34⁺ cells, immature and mature DCs, and IFN- γ -treated fibroblasts, whereas no HLA-DR expression was observed on T cells and nontreated fibroblasts. In contrast, HLA-DQ expression was high on B cells and mature DCs but significantly lower than HLA-DR on monocytes, immature DCs, CD34⁺ cells, and IFN- γ -treated fibroblasts (Figure 6A). This indicated that HLA-DQ was preferentially expressed on professional APCs, including B cells and mature DCs. Expression analysis of the genes encoding Ii, HLA-DM α/β , and HLA-DO α/β by microarray gene expression profiling revealed lack or only weak expression in HLA class II-negative T cells and nontreated fibroblasts. In contrast, all HLA-DR-positive cells, including monocytes, B cells, immature and mature DCs, CD34⁺ cells, and IFN- γ -treated fibroblasts, showed gene expression for Ii, HLA-DM α/β , and HLA-DO α , albeit at different levels. However, different patterns of gene expression were observed for HLA-DO β . Whereas HLA-DO β was strongly expressed in B cells and mature DCs, no significant expression could be measured in the other cell types (Figure 6B). The data therefore showed that HLA-DQ and HLA-DO β are coexpressed on professional APCs, including B cells and mature DCs, suggesting that these cells may represent the subset of HLA class II-positive cells capable of inducing CD4⁺ T cells against DM-sensitive antigens.

DM-sensitive antigens are efficiently presented on mature DCs

Because HLA-DM α/β gene expression was diminished and HLA-DO α/β gene expression was strongly induced on DC maturation, we hypothesized that mature DCs may be particularly relevant as primary cell type for *in vivo* presentation of DM-sensitive antigens, whereas DM-resistant antigens may also be efficiently presented on immature DCs. To investigate this, we tested T-cell recognition of immature and mature DCs generated from monocytes of patient MRJ cells that expressed all 5 antigens and their respective HLA class II restriction alleles. The data confirmed that presentation of DM-sensitive antigens benefited more from maturation of DCs compared with DM-resistant antigens (Figure 7A)

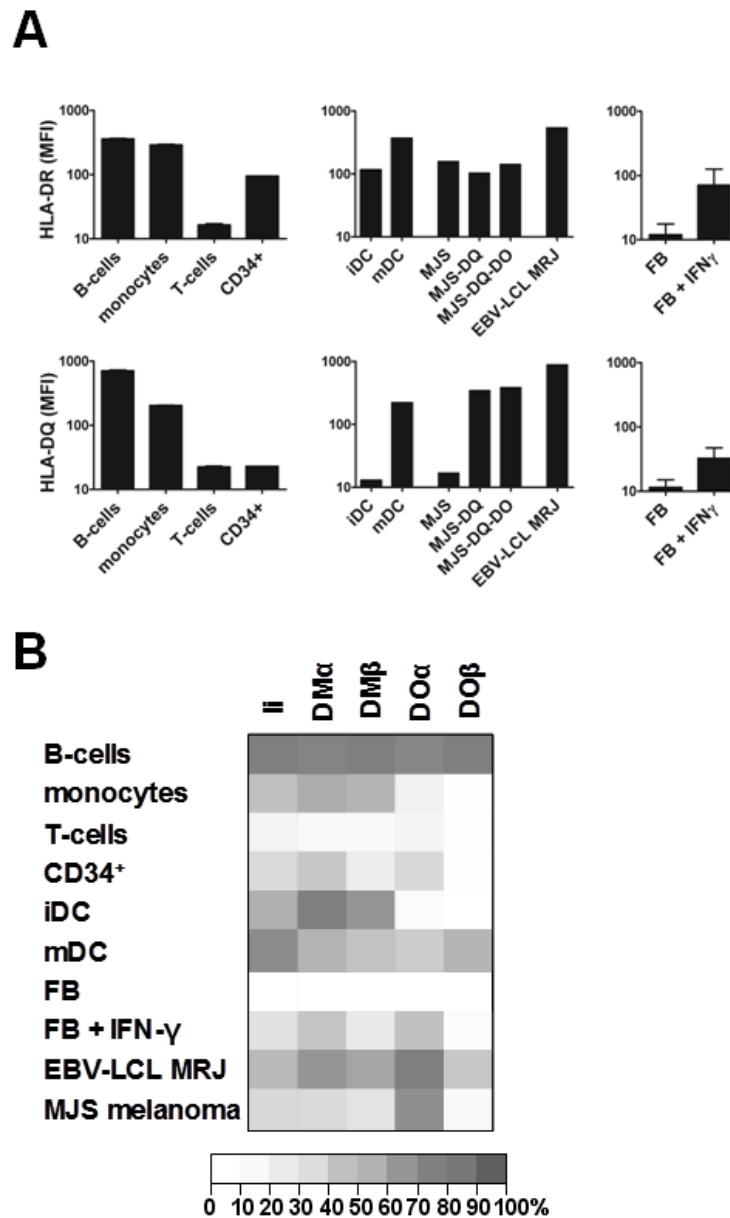


Figure 6. Coordinated expression of HLA-DQ and HLA-DO β in professional APCs.

(A) The mean fluorescence intensity (MFI) of flow cytometric staining for HLA-DR (left) and HLA-DQ (right) are shown for primary B cells (CD19⁺), monocytes (CD14⁺), T cells (CD3⁺), hematopoietic stem cells (CD34⁺), immature and mature dendritic cells (DCs), MJS melanoma cells with and without transduced HLA-DQ and HLA-DO, EBV-transformed B-cell lines (EBV-LCL) from patient MRJ, as well as fibroblasts with and without IFN- γ treatment. For primary cells means and SDs of duplicate stainings are shown. (B) mRNA expression of invariant chain (Ii), DM α and DM β , and DO α and DO β was measured by microarray gene expression analysis in primary B cells, monocytes, T cells, hematopoietic stem cells (CD34⁺), immature and mature DCs, MJS melanoma cells, EBV-LCL from patient MRJ, as well as fibroblasts with and without IFN- γ treatment. Relative gene expression is depicted for the mean of 3 independent samples for each cell type.

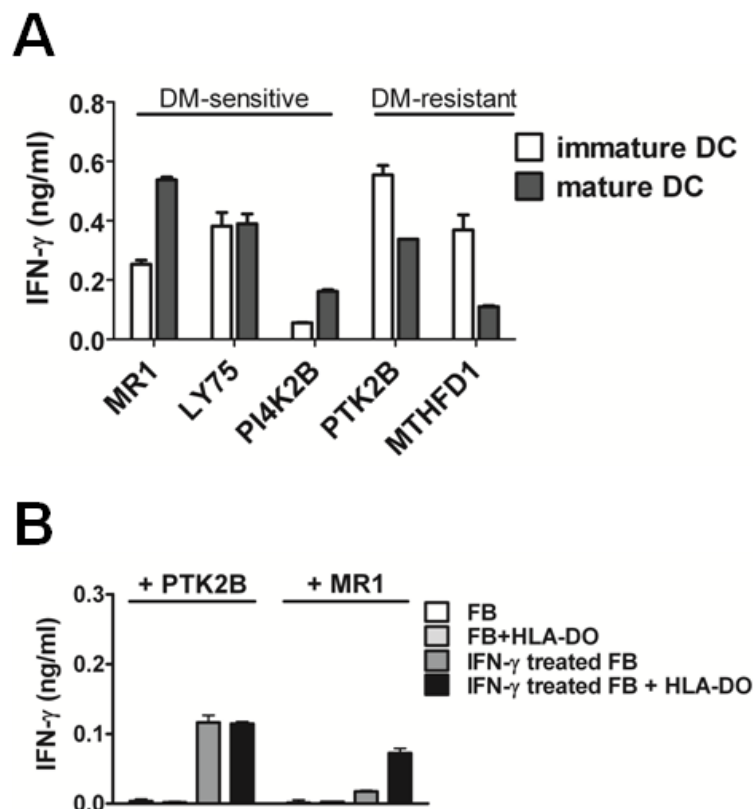


Figure 7. Presentation of DM-sensitive antigens on primary hematopoietic and IFN- γ -treated nonhematopoietic cell types is regulated by HLA-DO. (A) Monocyte-derived immature (open bars) and mature (closed bars) dendritic cells (DCs) were generated from patient MRJ expressing all 5 antigens as well as their respective HLA class II restriction alleles, and recognition by the specific T-cell clones was tested by IFN- γ ELISA. Indicated is the T-cell recognition of mature versus immature DCs. (B) Primary fibroblasts expressing HLA-DRB3*0101 and DRB3*0202 with and without transduced HLA-DO were pulsed with recombinant MR1 (DM-sensitive) and PTK2B (DM-resistant) proteins, and recognition by the specific T-cell clones was tested without cytokine pretreatment or after 3-4 days of treatment with IFN- γ . Mean $\pm$ SD release of IFN- γ in duplicate wells is shown. The fibroblasts used for T-cell recognition were included in the microarray analysis as depicted in Figure 6B.

DM-sensitive antigens are inefficiently presented on cytokine-treated non-hematopoietic cells because of lack of HLA-DO

Furthermore, in contrast to Ii, HLA-DM α/β , and HLA-DO α , we could confirm earlier reports that expression of HLA-DO β is hardly induced in non-hematopoietic cells on IFN- γ treatment. This suggested that, even under inflammatory conditions, DM-sensitive antigens are not or inefficiently presented on non-hematopoietic cells. We here tested processing and presentation of a DM-sensitive (MR1) and DM-resistant (PTK2B) antigen in skin-derived

fibroblasts endogenously expressing the relevant HLA-DR restriction alleles. Fibroblasts with and without IFN- γ treatment were pulsed with recombinant proteins to induce antigen expression and were tested for T-cell recognition. In line with the absence of HLA-DR expression, untreated fibroblasts were not recognized by the respective T-cell clones (Figure 7B). After up-regulation of HLA class II by IFN- γ treatment, the DM-resistant antigen PTK2B was clearly recognized, whereas the DM-sensitive antigen MR1 hardly induced T-cell recognition. Introduction of HLA-DO enhanced presentation of DM-sensitive antigen MR1, whereas it did not affect presentation of DM-resistant antigen PTK2B. In conclusion, the data confirm that DM-sensitive antigens are inefficiently presented on cytokine-treated non-hematopoietic cells because of lack of HLA-DO.

DISCUSSION

CD4⁺ T cells play a key role in adaptive immunity. They promote induction and maintenance of CD8⁺ T cells and induce humoral immunity.^{2,3} In addition, they can function as potent cytolytic effector cells.⁴⁻⁷ According to this central role in the immune system, presentation of HLA class II epitopes to CD4⁺ T cells is tightly regulated, a task attributed to the peptide editing function of the nonclassic HLA class II molecule HLA-DM.²⁸⁻³⁰ Here, we show that endogenous HLA class II epitopes that are immunogenic *in vivo* consist of 2 groups. One group of antigens is presented on all HLA class II-positive cells (DM-resistant), whereas the other group displays a CLIP-like behavior and their presentation depends on a delicate balance between Ii, HLA-DM, and HLA-DO (DM-sensitive).

It is well established that HLA-DM promotes the removal of CLIP, and this is believed to be necessary for efficient presentation of antigenic peptides.^{29,30} Surface expression of CLIP has therefore been regarded as a marker for defective antigen presentation. Here, we demonstrate that 3 of 6 natural HLA class II epitopes that were recognized by CD4⁺ T cells in *in vivo* immune responses display a similar behavior toward HLA-DM as CLIP. Moreover, in our cell line models, surface expression of CLIP, either because of absence of HLA-DM or co-expression of HLA-DO, correlated with presentation of a broad peptide

repertoire. The existence of DM-sensitive antigens as a relevant category of HLA class II epitopes has been further confirmed by our T-cell library experiments as well as an earlier mouse study,³¹ showing that at least a quarter of undefined self-antigens in natural T-cell responses are recognized in a DM-sensitive manner. These data are, however, in striking contrast with the general assumption that HLA-DM is necessary to promote presentation of bona fide HLA class II epitopes. A possible explanation for this contradiction might be the origin of the presented epitope. Most studies have focused on exogenous antigens, whereas we here analyzed presentation of endogenous proteins. It has been shown by biochemical studies^{31–34} that HLA-DM promotes the formation of long-lived HLA-peptide complexes.³⁵ This should ensure that exogenous antigens, which are taken up and processed by DCs in the periphery, are presented sufficiently long to allow migration of the DCs to the draining lymph node and to prime naive T cells.³⁶ The requirement for long-lived HLA-peptide complexes may, however, be less stringent for endogenous antigens because of continuous assembly and export of intracellular HLA-peptide complexes to the cell surface.³⁷ This may explain why T-cell responses against endogenous antigens are not skewed toward DM-resistant epitopes but also allow recognition of DM-sensitive antigens which are exclusively intracellularly processed (supplemental Figure 3). HLA-DM is supposed to mediate peptide exchange on interaction with HLA class II molecules, and the global conformation of a HLA-peptide complex enables or disrupts interaction with HLA-DM. Mutagenesis studies found that the lateral surface of HLA-DM and HLA-DR are important for the DM-DR interaction.^{25,38} In line with this, mutation of residues E40 and F51 in the DR α chain completely alleviated the inhibitory effect of HLA-DM on presentation of our DM-sensitive epitopes. These residues were previously found to be necessary for HLA-DM-mediated removal of CLIP,²⁵ further supporting the CLIP-like behavior of our DM-sensitive epitopes. Mutations in the DR α chain, however, did not significantly alter presentation of DM-resistant antigens. Thus, although others have postulated that the function of HLA-DM is to keep the HLA restriction molecule in an “open, peptide-receptive” conformation,³⁹ our data support an “open, peptide-exchange” configuration in which HLA-DM stimulates peptide release until a peptide binds that induces a conformational change in the HLA restriction molecule that disrupts interaction with HLA-DM.

We demonstrated that presentation of DM-sensitive epitopes could be restored by HLA-DO. This nonclassic class II molecule is selectively expressed in B cells, DCs, and thymic epithelial cells,^{19,40} and, although it is known to alter the presented peptide repertoire by interacting with HLA-DM, its biologic function is unclear. Mice lacking H2-O (murine HLA-DO) show impaired ability to generate humoral immune responses with slow and reduced antibody responses, but, when growing older, these mice spontaneously develop high titers of autoantibodies. This contradictory phenotype indicates a dual role for HLA-DO. Because we here show that a broad repertoire of endogenous peptides is presented in the presence of HLA-DO, it can be speculated that expression of HLA-DO in professional APCs may be relevant for inducing a broad T-helper response.^{41,42} If true, slow and reduced antibody responses in mice lacking H2-O may reflect impaired T-cell help against exogenous antigens. HLA-DO expression in thymic epithelial cells, however, might be necessary for central tolerance by inducing apoptosis or anergy of T cells against a maximum repertoire of self-peptides. In T-cell library experiments, we noticed a dominance of DM-sensitive antigens in HLA-DQ. Because HLA-DQ and HLA-DO are coordinately expressed in professional APCs, it can be assumed that DM-sensitive antigens in HLA-DQ may be particularly relevant in the onset of immune responses to induce a broad CD4⁺ T-helper response without stimulating reactivity against HLA class II-positive cells lacking HLA-DO.

The restricted expression profile of HLA-DO and its influence on the presented peptide repertoire implicates that there are 2 types of HLA class II-positive cells, presenting overlapping but distinct sets of peptides. This is also supported by peptide elutions from B cells and melanoma cell lines with and without transfected HLA-DO, indicating that a fraction of peptides is specific for HLA-DO-expressing cells (10%-26%).^{20,43,44} In line with this, we could show that lack of HLA-DO in cell lines and primary fibroblasts precludes efficient presentation of DM-sensitive antigens. HLA-DO expression might therefore explain earlier observations that certain cell types are not recognized by CD4⁺ T-cell clones despite substantial expression of HLA class II and the antigen. We noticed that the T-cell clones for DM-sensitive antigens MR1 and LY75 did not recognize patient-derived CD34⁺ chronic myelogenous leukemia progenitor cells,¹⁴ which is in line with low HLA-DO expression in these cells. Others showed that 2 CD4⁺ T-cell clones against MELOE-1 failed

to recognize melanoma cells.¹⁵ Because the melanoma cells expressed sufficient antigen and HLA class II, it is tempting to speculate that the antigens are DM-sensitive epitopes that are inefficiently presented because of lack of HLA-DO.

To exploit adaptive immunity against cancer, the existence of 2 types of HLA class II-expressing cells presenting different peptide profiles based on expression of HLA-DO opens an interesting perspective. In alloSCT donor-derived T cells recognize and eradicate patient-derived malignant cells, thereby mediating beneficial GVL effect.⁴⁵ These T cells, however, may also react against healthy non-hematopoietic tissues of the patient, thereby inducing GVHD, the main cause of morbidity and mortality.⁴⁶ Many efforts have been directed toward separation of the GVL effect and GVHD, and clinical data indicate that these 2 phenomena can indeed arise independently.⁶ One option to shift the balance toward GVL effect is to exploit CD4⁺ T cells as HLA class II expression is mainly restricted to cells of the hematopoietic system. The main drawback of this approach is that non-hematopoietic cells up-regulate HLA class II under inflammatory conditions which often occur in the setting of alloSCT because of the conditioning regimen or (viral) infections,⁴⁶ and this may trigger CD4⁺ T cells to cause GVHD.

We show that DM-sensitive antigens are not efficiently presented on cytokine-treated non-hematopoietic cells because of lack of up-regulation of HLA-DO β by IFN- γ .⁴⁷ On the basis of these data, T cells recognizing DM-sensitive antigens are not expected to mediate GVHD. Intriguingly, our preliminary experiments found broad expression of HLA-DO in primary leukemic cells of different origins. T-cell therapy targeting DM-sensitive antigens may therefore allow selective eradication of DO-expressing malignant cells with reduced risk of GVHD. Because CLIP-expressing leukemias have been reported to exhibit a particularly bad prognosis,⁴⁸ specific targeting of DM-sensitive antigens with CLIP-like behavior may have additional therapeutic value.

In conclusion, our detailed analysis of processing and presentation of natural HLA class II epitopes provides novel insights into the relative roles of HLA-DM and -DO in presentation of endogenous antigens. The findings underline that, in addition to HLA class II and antigen expression, antigen processing should be considered as a main factor for optimal use and development of CD4⁺ T cell-based immunotherapy. Furthermore, these data might

lay the basis for a new approach of targeted therapy that is not based on selective antigen expression but on selective processing capacities of the target cells.

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SUPPLEMENTARY INFORMATION

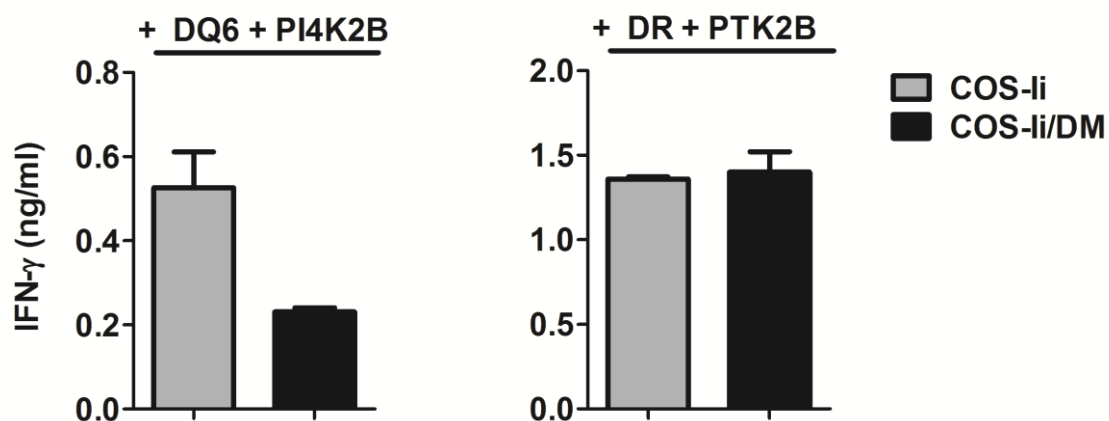


Figure S1: The effect of HLA-DM on T-cell recognition of the PI4K2B and PTK2B antigens after retroviral transfer into COS cells.

T-cell recognition was compared between COS cells transduced with Ii and HLA-DM and COS cells transduced with Ii alone after retroviral introduction of the PTK2B and PI4K2B genes and their respective HLA-class-II restriction alleles. Indicated is the mean release of IFN- γ in duplicate wells.

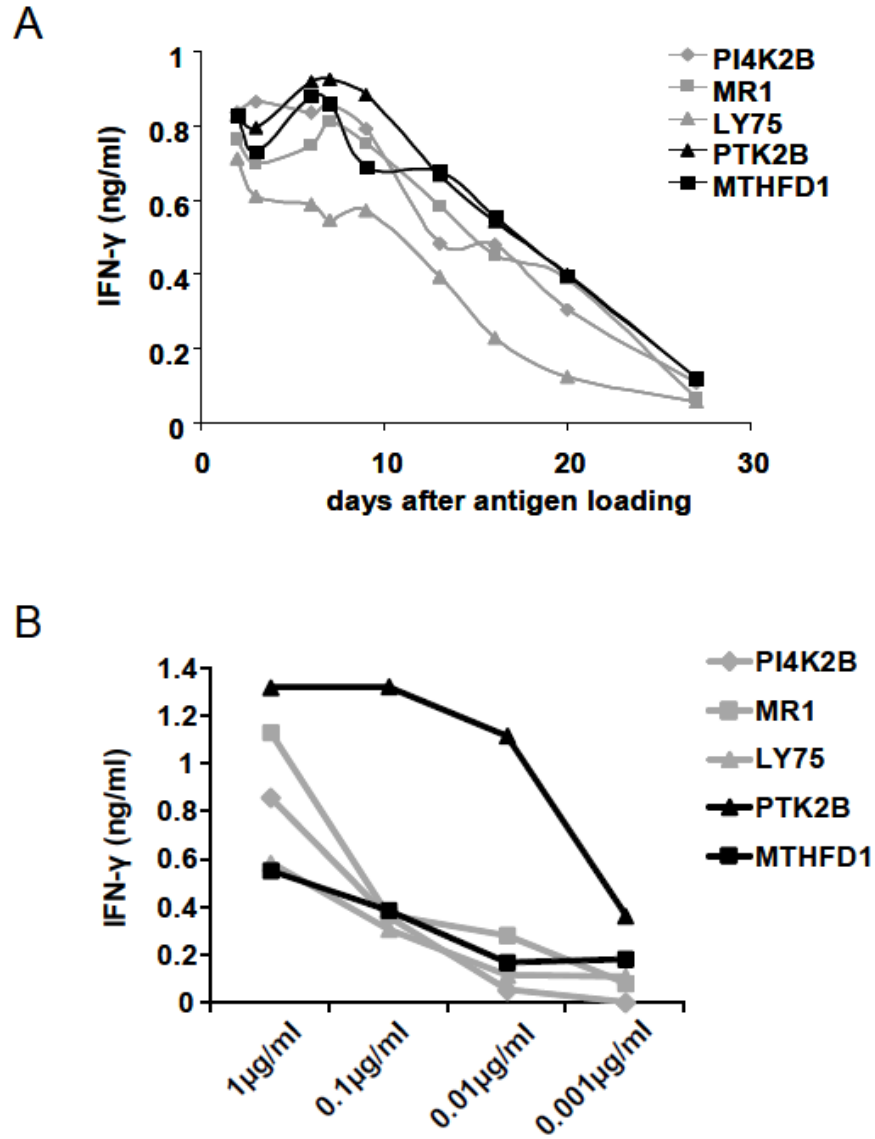


Figure S2: Persistence of HLA-peptide complexes and affinity of TCRs for DM-resistant and DM-sensitive antigens.

(A) Recombinant proteins were loaded on antigen negative EBV-LCL IZA expressing all relevant HLA-class-II restriction alleles. After 4 hrs of incubation at 37°C, protein pulsed EBVLCL were washed twice, and T-cells were added on different days after pulsing to measure the persistence of naturally processed peptides in their presenting HLA molecules. DM-resistant and DM-sensitive antigens are depicted in black and grey, respectively.

(B) Antigen negative EBV-LCL IZA were loaded with titrated amounts of synthetic peptides for 2 hours at 37°C, washed twice and tested for T-cell recognition by IFN- γ ELISA after overnight incubation. DM-resistant and DM-sensitive antigens are depicted in black and grey, respectively.

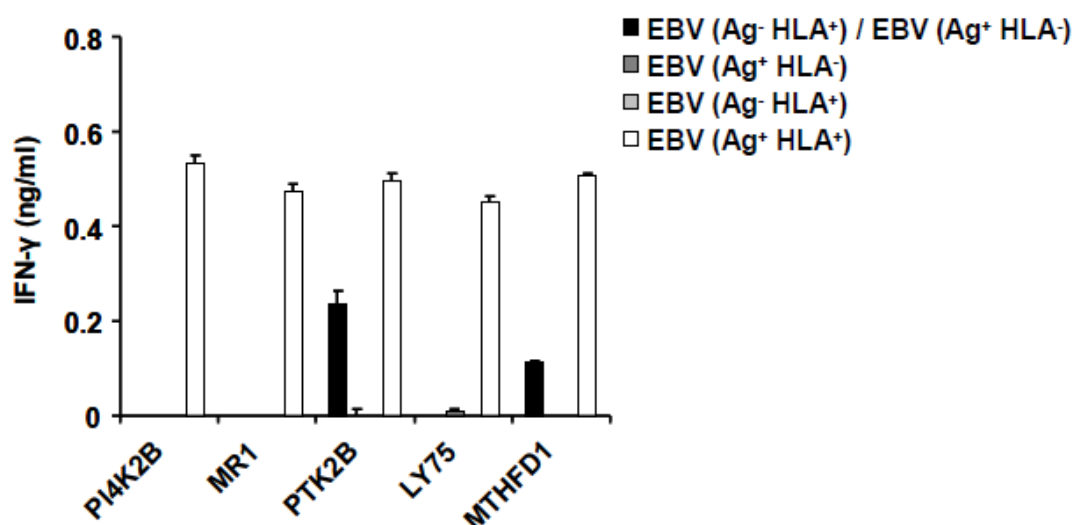


Figure S3: DM-sensitive antigens are exclusively intracellularly processed.

To determine whether endogenously expressed DM-resistant and DM-sensitive antigens can follow an extracellular pathway of antigen secretion and re-uptake, antigen negative EBVLCL expressing all relevant HLA-class-II restriction alleles (IZA) were co-cultured together with antigen positive EBV-LCL lacking expression of the relevant HLA-class-II molecule(s). After overnight co-culturing, T-cell clones were added and IFN- γ release was measured by ELISA. Mean release of IFN- γ in duplicate wells is shown.

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4

HLA-DO regulates surface presentation of HLA-class-II restricted antigens on B-cell malignancies

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ABSTRACT

Hematological malignancies often express surface HLA-class-II, making them attractive targets for CD4⁺ T-cell therapy. We previously demonstrated that HLA-class-II ligands can be divided into DM-resistant and DM-sensitive antigens. In contrast to DM-resistant antigens, presentation of DM-sensitive antigens is suppressed by HLA-DM, but can be rescued by HLA-DO. We also showed that HLA-DO expression remains low in non-hematopoietic cells under inflammatory conditions, suggesting that DM-sensitive antigens may be ideal T-cell targets with a low risk for GvHD. Here, we demonstrated that B-cell malignancies often express HLA-DO and that levels are in particular high in chronic lymphocytic leukemia. Moreover, we showed that surface presentation of DM-sensitive antigens is regulated by HLA-DO, and that DM-sensitive antigens are relevant T-cell targets in B-cell malignancies and especially chronic lymphocytic leukemia. These data open the perspective to target HLA-class-II ligands with specific processing and presentation behavior for CD4⁺ T-cell therapy of hematological malignancies.

INTRODUCTION

Allogeneic stem cell transplantation (aSCT) in the treatment of hematological malignancies is the most successful form of cellular immunotherapy.¹ The beneficial graft-versus-leukemia (GvL) effect is mediated by donor derived T-cells recognizing residual leukemic cells of the patient.² These T-cells, however, may also react against non-hematopoietic tissues of the patient, thereby inducing graft-versus-host disease (GvHD), the major cause of morbidity and mortality after aSCT.^{3,4} A possible approach to shift the balance towards GvL is to exploit CD4⁺ T-cells, since HLA-class-II is under normal conditions mainly expressed on cells of hematopoietic origin.^{5,6} However, HLA-class-II can be upregulated on non-hematopoietic tissues under inflammatory conditions.

Recently, we identified a number of HLA-class-II restricted minor histocompatibility antigens^{7,8} and analyzed their processing and presentation behavior. The data showed that HLA-class-II ligands can be divided into HLA-DM-resistant and -sensitive antigens.⁹ DM-resistant antigens are presented on all HLA-class-II^{pos} cells, whereas presentation of DM-sensitive antigens is decreased by HLA-DM and restored by co-expression of its natural inhibitor HLA-DO. Interestingly, in contrast to HLA-DM, HLA-DO is hardly up-regulated by pro-inflammatory cytokines.^{9,10} We therefore hypothesized that DM-sensitive antigens are inefficiently presented on non-hematopoietic cells under inflammatory conditions. This makes DM-sensitive antigens attractive targets for CD4⁺ T-cell therapies with a low risk for GvHD. A prerequisite for selective GvL is, however, that DM-sensitive antigens are presented on leukemic cells.

We here investigated expression of HLA-DM and -DO in primary leukemic cells, and demonstrated that HLA-DO is often expressed in B-cell malignancies, in particular in chronic lymphocytic leukemia (CLL). We further confirmed the functional relevance of HLA-DO as regulator in surface presentation of DM-sensitive antigens on leukemic cells. Our data show that CD4⁺ T-cells directed against DM-sensitive antigens are promising tools in aSCT, and emphasize the relevance of HLA-DO expression in cell types of different origins for optimal design of CD4⁺ T-cell based immunotherapy.

MATERIALS AND METHODS

Cell samples and culture

Peripheral blood and bone marrow samples were obtained from patients and healthy individuals after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque separation. T-cells, fibroblasts (FB), dendritic cells (DC) and cell line LB-ALL-SK were cultured as previously described.^{9,11}

Microarray gene expression analysis

CD14⁺ monocytes, CD19⁺ B-cells, CD3⁺ T-cells and CD34⁺ hematopoietic stem cells (HSC) were isolated from (G-CSF mobilized) peripheral blood from healthy donors by flow-cytometry. Acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) were isolated from patient samples by flow cytometry based on expression of CD33, CD34, CD19, CD19/CD5, and CD38 expression, respectively. FB were cultured from skin biopsies in the absence or presence of IFN- γ (100 IU/ml) for 4 days. Isolation of total RNA and microarray gene expression analysis was performed as described recently.⁹

Quantitative RT-PCR

Expression of HLA-DOA and HLA-DOB mRNA was measured by quantitative real-time RT-PCR using the Brilliant III Ultra Fast SYBR Green QPCR Mastermix (Agilent) and the following primers: DOA: 5'- TTTGCCCCGCTTTGACCCGCA -3' and 5'- TCACCCGTGGAGGCACGTTG -3', DOB: 5'- AGGAGCAGACAGGCCGTGGA -3' and 5'- CCTCTGGTTGCACTTTTCTCCCCA -3'. Gene expression for DOA and DOB was normalized as ratio with 18S RNA expression. Amplifications were started with 10 min at 95°C, followed by 50 cycles of 30 sec for denaturing at 95°C, 30 sec of annealing at 60°C, and 30 sec extension at again 60°C. After amplification, specificity of the PCR products was confirmed by melting curve analysis.

Western blot analysis

Cells were lysed on ice in 1% triton-X lysis buffer containing protease inhibitors (Roche Diagnostics, Almere, The Netherlands). The sub-nuclear fraction was obtained by centrifugation at 10,000g for 30 min. SDS-Page was run on precast NuPage[®] Novex 10% Bis-Tris Mini gels (Life Technologies, Bleiswijk, The Netherlands) for 35 min. at 30V and blotted on PVDF membranes. HLA-DOB expression was detected by mouse monoclonal antibody s-69739 (Santa Cruz Biotechnology, Dallas, Texas 75220) and biotinylated goat anti-mouse IgG (Life Technologies).

Antigen presentation assays

Stimulator cells (3×10^4 cells/well) and CD4⁺ T-cells (5×10^3 cells/well) were overnight co-incubated at 37°C in 96-well plates. IFN- γ release was measured by ELISA (Sanquin, Amsterdam, The Netherlands). As stimulator cells, Raji and LB-ALL-SK cell lines and primary CLL and ALL cells were used. Raji and LB-ALL-SK were retrovirally transduced with HLA-DQA1*01:03/DQB1*06:03 and HLA-DOA/DOB as recently described.⁹ In addition, Raji and LB-ALL-SK expressing HLA-DQA1*01:03/DQB1*06:03 were transduced with lentiviral pLKO.1-puro containing short hairpin RNA for HLA-DOA (TRCN0000057268) or scrambled shRNA (SHC002) from the MISSION library (Sigma-Aldrich, Zwijndrecht, The Netherlands).

Flow cytometry based cytotoxicity

Target cells (1×10^4 cells/well) and CD4⁺ T-cells (3×10^4 cells/well) were overnight co-incubated at 37°C in 96-well plates. LB-PI4K2B-1S peptide was added at 1 μ g/ml. Co-cultures were stained with PE-labeled anti-CD3, and analysed by flow cytometry after addition of a fixed number of flow-count beads and propidium iodide (PI) to allow quantitative measurement of viable (PI negative) cells. As target cells, FB transduced with HLA-DQA1*01:03/DQB1*06:03 were used after pre-treatment with IFN- γ (100 IU/ml) for 4 days.

RESULTS

We recently proposed that DM-sensitive antigens may be relevant targets for T-cells with a selective GvL effect and low risk for GvHD. To investigate whether DM-sensitive antigens are relevant targets for GvL, primary leukemic cells of different origins were collected and compared for expression of HLA-DM (DMA and DMB) and DO (DOA and DOB) with non-malignant hematopoietic cells by microarray gene expression analysis (Figure 1A). The data showed that primary leukemic cells (AML, CML, ALL and CLL) expressed high levels of HLA-DMA and DMB, except for two AML-M3 which were also low in HLA-class-II (data not shown). HLA-DMA and DMB expression was low in all HLA-class-II^{neg} samples, which included MM, T-cells and FB cultured in the absence of IFN- γ . Similar as HLA-DMA and DMB, HLA-DOA expression was low in HLA-class-II^{neg} samples, but significant expression was observed in HLA-class-II^{pos} AML, CML, ALL and CLL, except for 7 AML, which included the two AML-M3 with low HLA-class-II. In contrast to HLA-DMA, DMB and DOA, expression of HLA-DOB was not or hardly induced in FB by IFN- γ , and we previously demonstrated that DM-sensitive antigens are inefficiently presented on FB in an inflammatory environment due to low levels of induced HLA-DOB.⁹ Remarkably, a strong difference in HLA-DOB expression was observed between myeloid and lymphatic malignancies, and especially CLL. Except for mature DC, all samples from myeloid origin showed low expression of HLA-DOB, whereas strong expression was observed in CLL and healthy B-cells. Variable HLA-DOB expression was observed in ALL samples. HLA-DOB expression was high in the majority of MM, but these cells were HLA-class-II^{neg} (data not shown). The strong difference in HLA-DOB expression between CLL and myeloid malignancies could be confirmed by quantitative RT-PCR (data not shown) and Western blot analysis (Figure 1B). Strong HLA-DOB protein expression was observed in all CLL, whereas expression could not be detected in AML (Figure 1B). ALL cells showed variable HLA-DOB expression also on protein levels. The slight up-regulation of HLA-DOB in FB cultured under inflammatory conditions, which was observed on mRNA level, did not translate into detectable protein expression on Western blot (Figure 1B). In conclusion, our data show that HLA-DO in its full heterodimer is expressed at high levels in primary CLL and a subgroup of ALL.

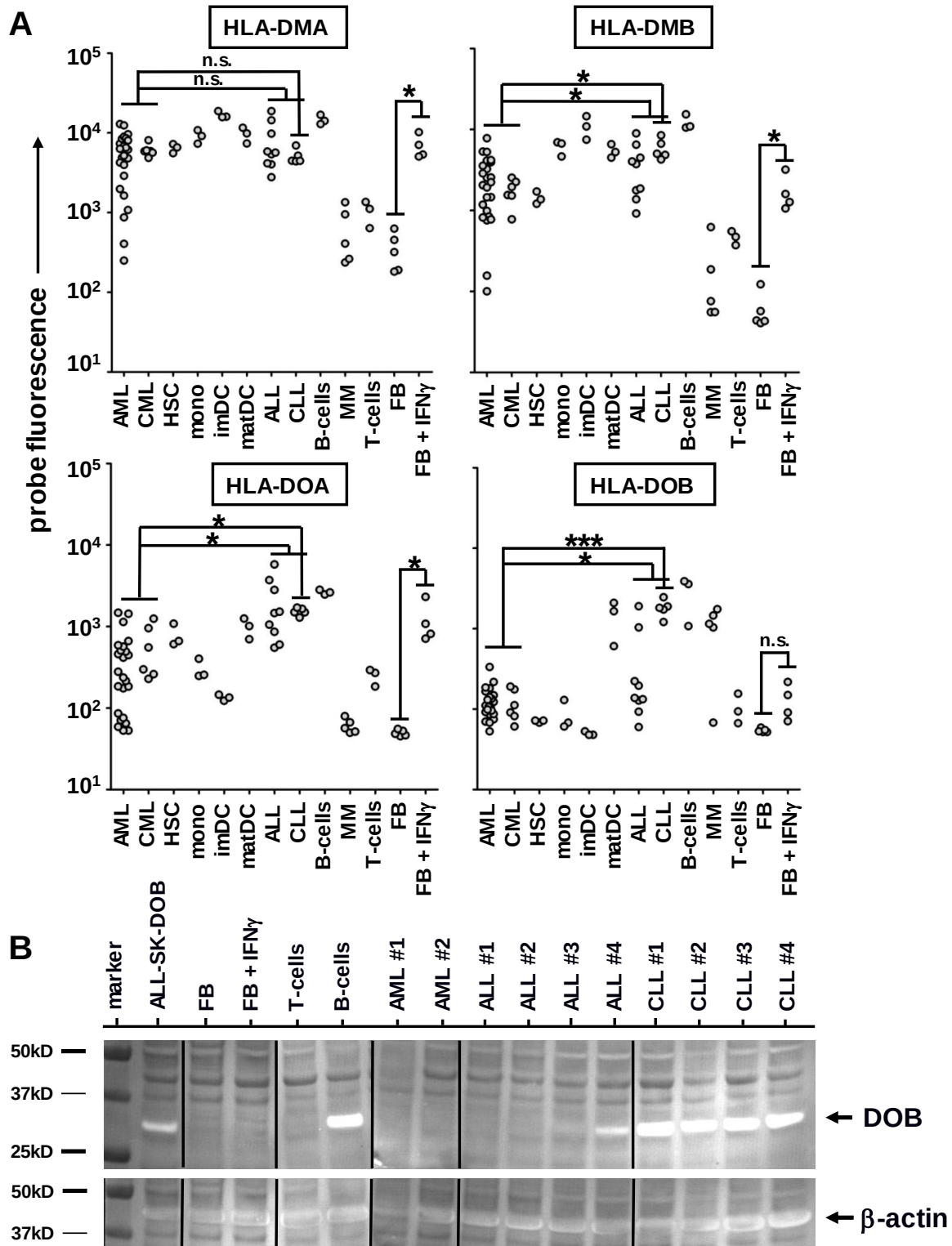


Figure 1. HLA-DM and DO expression in primary leukemic cells.

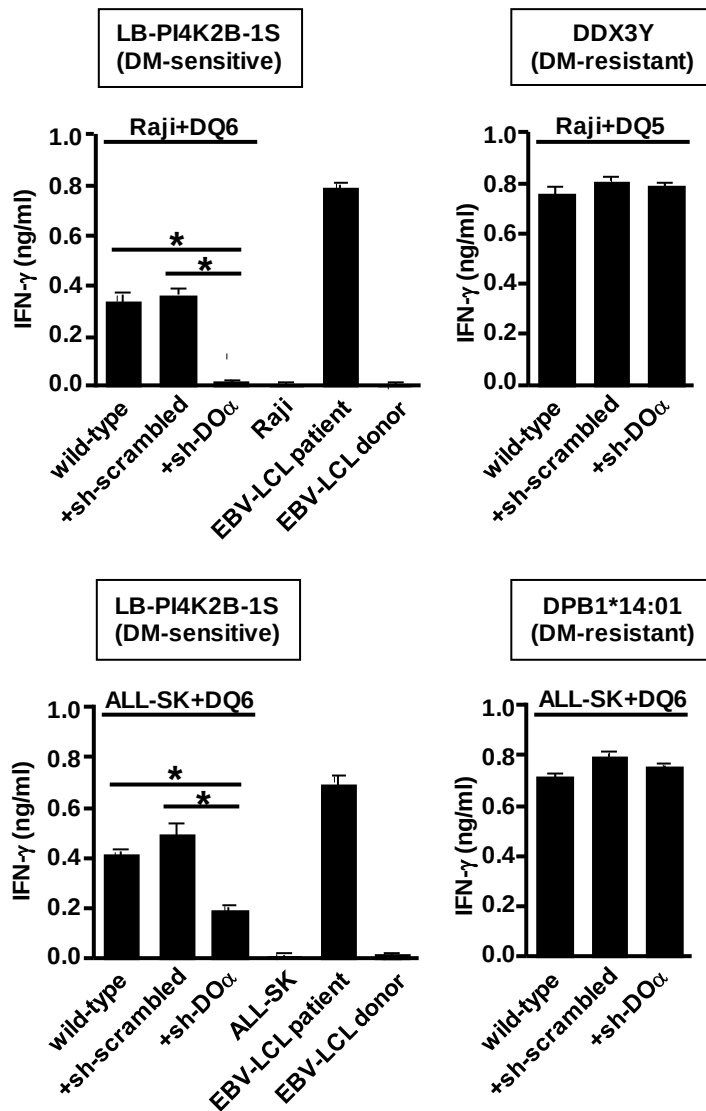
(A) The genes encoding the alpha and beta chains of HLA-DM (DMA and DMB) and HLA-DO (DOA and DOB) were analyzed by microarray gene expression analysis in acute myeloid leukemia (AML; n=18), chronic myeloid leukemia (CML; n=6), acute lymphoblastic leukemia (ALL; n=9), chronic lymphocytic leukemia (CLL; n=5) and multiple myeloma (MM; n=5) as well as in non-malignant hematopoietic stem cells (HSC; n=3), B-cells (n=3), T-cells (n=3), monocytes (n=3), monocyte-derived immature and mature dendritic cells (imDC and matDC; n=3), and non-hematopoietic fibroblasts (FB) cultured in the presence (n=4) or absence (n=5) of IFN- γ . Normalized probe fluorescence intensities are depicted for individual samples. Statistical analysis was performed using student's t-tests (n.s.; not significant; * p<0.01; ** p<10⁻¹⁰; *** p<10⁻²⁰).

(B) HLA-DOB protein expression was measured by Western blot analysis in cell line LB-ALL-SK after retroviral transfer of the HLA-DOB gene, primary FB cultured in the absence or presence of IFN- γ , T-cells, B-cells, AML, ALL and CLL. Expression of β -actin is shown as loading control.

To investigate whether HLA-DO was functionally active in malignant B-cells and regulated surface presentation of DM-sensitive antigens, we first targeted HLA-DO in B-cell lymphoma cell line Raji and ALL cell line LB-ALL-SK by lentiviral introduction of HLA-DOA specific shRNA. Raji and LB-ALL-SK showed similar expression of HLA-DOA by microarray gene expression analysis, but expression of HLA-DOB was significantly different and HLA-DOB protein could only be detected in the Raji cell line (data not shown). Raji and LB-ALL-SK both endogenously expressed DM-sensitive antigen LB-PI4K2B-1S and the HLA-DQB1*06:03 restriction allele was retrovirally introduced. Quantitative RT-PCR confirmed a decrease in HLA-DOA mRNA expression upon introduction of specific, but not scrambled, shRNA (Figure S1A). T-cell experiments demonstrated that upon silencing of HLA-DOA, presentation of DM-sensitive antigen LB-PI4K2B-1S was significantly diminished, whereas presentation of DM-resistant control antigens were not affected (Figure 2A).

Next, we enforced HLA-DO expression in LB-ALL-SK by retroviral introduction of the HLA-DOA and DOB genes. Overexpression of HLA-DOA and DOB was confirmed by quantitative RT-PCR (Figure S1B) and Western blot analysis (Figure 1B). In T-cell experiments, presentation of LB-PI4K2B-1S was enhanced after HLA-DOB gene transfer, whereas presentation of a DM-resistant control antigen was not altered (Figure 2B).

A



B

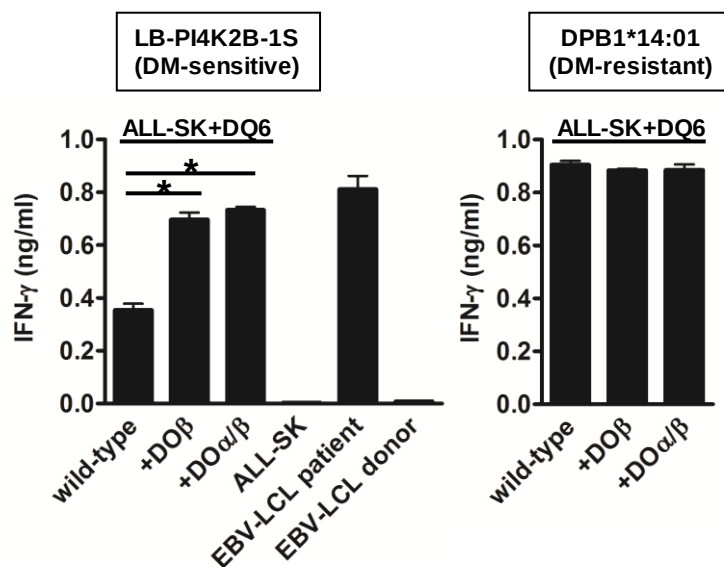


Figure 2. HLA-DO regulates surface presentation of DM-sensitive antigens on B-cell malignancies.

(A) Silencing of HLA-DO by HLA-DOA specific shRNA reduces presentation of DM-sensitive antigen LB-PI4K2B-1S. B-cell lymphoma cell line Raji (upper) and ALL cell line LB-ALL-SK (lower) both endogenously express DM-sensitive antigen LB-PI4K2B-1S and the HLA-class-II restriction allele was expressed after retroviral transfer of the HLA-DQA1*01:03 and DQB1*06:03 genes. In a next step, Raji and LB-ALL-SK were transduced with lentiviral vectors containing silencing shRNA for HLA-DOA (TRCN0000057268) or scrambled control shRNA (SHC002) from the MISSION library. The T-cell clone for LB-PI4K2B-1S is an allo-reactive CD4⁺ T-cell clone isolated from a patient with CML after HLA-matched alloSCT. For specificity of the T-cell clone, EBV-LCL from patient and donor were included as positive and negative controls, respectively. For the Raji cell line, a CD4⁺ T-cell clone recognizing male-specific DM-resistant antigen DDX3Y was included as control, and the HLA-class-II restriction allele was expressed after retroviral transfer of the HLA-DQA1*01:01 and DQB1*05:01 genes. For LB-ALL-SK, an allo-reactive CD4⁺ T-cell clone recognizing an unknown DM-resistant antigen in the endogenous HLA-DPB1*14:01 molecule was included. Mean release of IFN- γ in duplicate wells of one representative experiment is shown, but similar results were obtained in two other experiments. Significant differences in student's t-tests are indicated (* p<0.05).

(B) Enforced expression of HLA-DO by retroviral transfer of the HLA-DOB gene enhances presentation of DM-sensitive antigen LB-PI4K2B-1S. Cell line LB-ALL-SK expressing LB-PI4K2B-1S and its HLA-DQ restriction allele was retrovirally transduced with HLA-DOB alone or together with HLA-DOA. T-cell recognition of DM-sensitive antigen LB-PI4K2B-1S and the unknown DM-resistant antigen in HLA-DPB1*14:01 was measured by IFN- γ ELISA. Mean release of IFN- γ in duplicate wells of one representative experiment is shown, but similar results were obtained in two other experiments. Significant differences in student's t-tests are indicated (* p<0.05).

Finally, we investigated whether high expression of HLA-DOB in primary CLL as compared to ALL also translated into strong T-cell recognition of DM-sensitive antigens. Primary CLL and ALL samples were selected for expression of DM-sensitive antigen LB-PI4K2B-1S and an unknown DM-resistant antigen recognized by an allo-reactive HLA-DQB1*06:03-specific T-cell or for expression of DM-sensitive antigen LB-LY75-1K and DM-resistant antigen LB-PTK2B-1T and their relevant HLA-DR restriction alleles. The data showed that all leukemic samples were recognized by the T-cells, but that presentation of DM-sensitive antigens was superior on CLL as compared to ALL, whereas presentation of DM-resistant antigens was comparable (Figure 3A). Increased expression of HLA-DOA and DOB in these CLL as compared to ALL samples was confirmed by quantitative RT-PCR (Figure S1C). In conclusion, our data illustrate that HLA-DO regulates surface presentation of DM-sensitive antigens on B-cell malignancies, and that DM-sensitive antigens are relevant T-cell targets in particular for primary CLL which often express high HLA-DO. Since HLA-DO is not or hardly induced in non-hematopoietic cells by inflammatory cytokines^{9,10}, DM-sensitive antigens have been suggested to be T-cell targets

with a low risk for GvHD. To test the capacity of non-hematopoietic cells to present DM-sensitive antigens under inflammatory conditions, we retrovirally introduced HLA-DQB1*06:03 into skin derived FB endogenously expressing LB-PI4K2B-1S and tested T-cell mediated lysis of these FB after pre-treatment with IFN- γ and overnight co-incubation at an E:T ratio of 3:1. No or only low specific lysis of FB endogenously expressing LB-PI4K2B-1S was measured, whereas FB exogenously loaded with LB-PI4K2B-1S peptide showed strong specific lysis (Figure 3B). Our data illustrate that DM-sensitive antigens are inefficiently presented on non-hematopoietic cells under inflammatory conditions, and therefore support the therapeutic value of DM-sensitive antigens as T-cell targets with a low risk for GvHD.

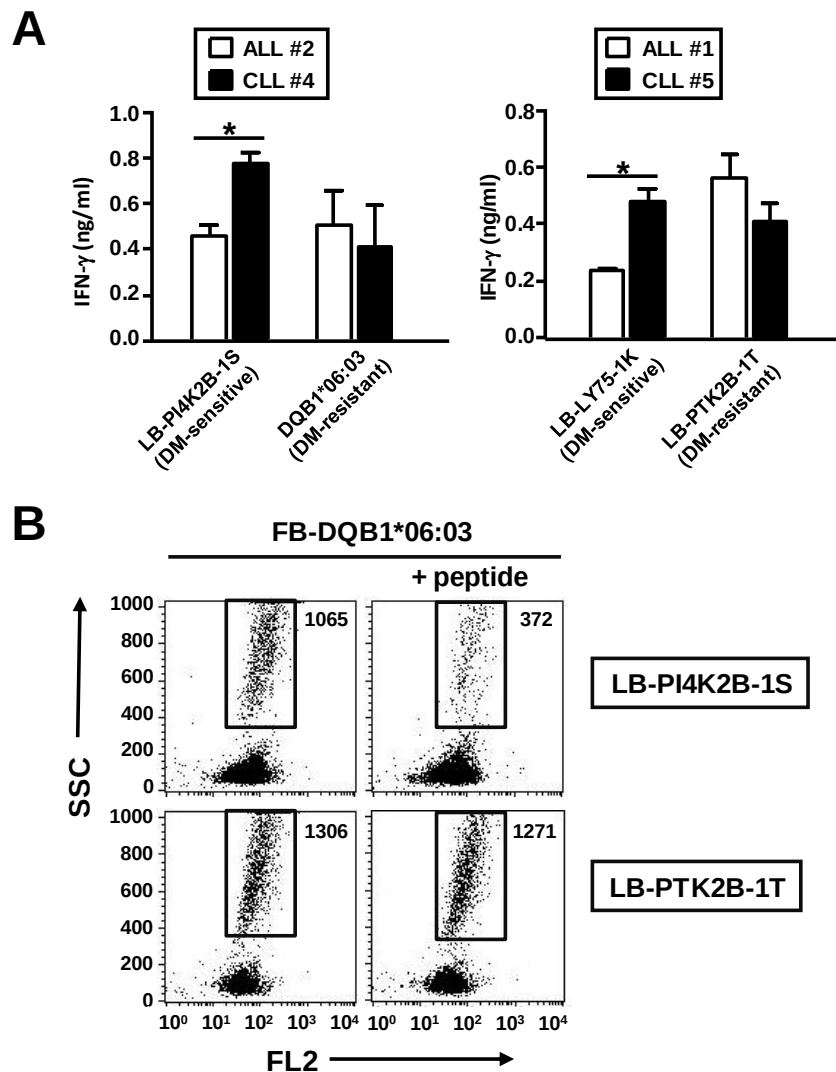


Figure 3. DM-sensitive antigens are efficiently presented on primary CLL, but not on skin derived FB under inflammatory conditions.

(A) DM-sensitive antigens are efficiently presented on high HLA-DO expressing CLL cells. Primary ALL and CLL were selected for expression of LB-PI4K2B-1S and HLA-DQB1*06:03 (left) or expression of LB-LY75-1K and LB-PTK2B-1T and their respective HLA-DRB3*01:01 and DRB1*13:01 restriction alleles (right). Primary ALL and CLL cells were sorted by flow cytometry and T-cell recognition of DM-sensitive antigens LB-PI4K2B-1S and LB-LY75-1K and DM-resistant antigen LB-PTK2B-1T and an unknown DM-resistant antigen recognized by an allo-reactive HLA-DQB1*06:03-specific T-cell clone was measured by IFN- γ ELISA. Mean release of IFN- γ of duplicate wells of two independent experiments are depicted.

(B) LB-PI4K2B-1S positive FB transduced with HLA-DQB1*06:03 were tested for lysis by LB-PI4K2B-1S specific T-cells after pre-treatment with IFN- γ . T-cells and FB were co-incubated overnight at an E:T ratio of 3:1, and numbers of viable (PI negative) FB were measured relative to a fixed number of flow-count beads in a quantitative flow-cytometry based cytotoxicity assay. T-cells for LB-PTK2B-1T were included as negative controls, since the FB did not express the HLA-DR restriction allele for these T-cells, and HLA-DQB1*06:03 transduced FB were exogenously loaded with LB-PI4K2B-1S peptide as positive control to confirm the lytic capacity of LB-PI4K2B-1S specific T-cells. Indicated is the side scatter and fluorescence as measured in FL2 for co-cultures of T-cells and FB. Fluorescence in FL2 for gated FB has been measured as a result of auto-fluorescence, whereas fluorescence in FL2 for remaining T-cells indicates positive staining with PE-labeled anti-CD3 antibody. Numbers of viable (PI negative) FB after overnight co-incubation with the indicated T-cells are shown. One representative of two experiments is shown.

DISCUSSION

We recently demonstrated that DM-sensitive antigens are inefficiently presented on non-hematopoietic cells under inflammatory conditions, suggesting that DM-sensitive antigens may be attractive targets to induce GvL with a reduced risk for GvHD. For GvL effect, however, DM-sensitive antigens should be presented on the surface of leukemic cells. We here show that HLA-DO is widely expressed in leukemic cells and that expression is sufficient for T-cell recognition of DM-sensitive antigens. Moreover, we demonstrate that HLA-DO expression is particularly high in primary CLL, and that these levels are functional as demonstrated by superior recognition of DM-sensitive antigens on CLL as compared to ALL.

HLA-DM has been described as chaperone that greatly accelerates peptide exchange in MHCII¹² and various studies have shown that HLA-DM induces release of peptides binding to MHCII with low affinity, resulting in a selection process that favors presentation

of high affinity peptides.¹²⁻¹⁴ DM-resistant T-cell epitopes are therefore expected to bind with higher affinity to HLA-class-II than DM-sensitive T-cell epitopes. Our T-cell epitopes, however, are presented by different HLA-class-II alleles, each with its own binding characteristics and kinetics of HLA-DM catalysed peptide editing¹⁴, and an accurate comparison of HLA-class-II binding affinity and half-lives between our DM-sensitive and DM-resistant T-cell epitopes is therefore not possible.

Our data show that HLA-DO regulates presentation of DM-sensitive antigens by HLA-class-II surface molecules on leukemic cells. Recently, Guce et al.¹⁵ elucidated the crystal structure of the DO-DM complex, and demonstrated that DO and DM bind in a side-by-side arrangement similar to that proposed for MHC class II and DM. HLA-DO thus acts as a substrate mimic that tightly binds to DM, thereby preventing access to MHC class II. Guce et al.¹⁵ also demonstrated that HLA-DO inhibits DM-mediated peptide release in a dose-dependent manner. Since DM is expressed at higher levels than DO, free DM and DO-DM complexes are both present, and the major mechanism for shifting their balance is via transcription of the DOB subunit¹⁶. This is also represented by our data showing constitutive expression of HLA-DMA, DMB and DOA in all HLA-class-II positive cells, whereas DOB expression is different between lymphoid and myeloid cells, showing high expression in healthy B-cells and CLL cells and variable expression in ALL. We further demonstrated that high HLA-DO expression indeed translates to T-cell recognition of DM-sensitive antigens, since (low-affinity) DM-sensitive peptides are efficiently presented on lymphatic leukemias and especially on CLL. Moreover, HLA-DOB is not expressed at detectable levels in skin-derived FB under inflammatory conditions, and T-cells for DM-sensitive antigens also failed to lyse cytokine pre-treated FB.

In conclusion, our data demonstrate and confirm the unique tissue distribution of HLA-DO and its function to inhibit DM-mediated peptide release in leukemic cells. These features of HLA-DO may be exploited for development of novel therapies in which CD4⁺ T-cells for DM-sensitive antigens are used to induce GvL reactivity after aSCT with a low risk for GvHD.

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SUPPLEMENTARY INFORMATION

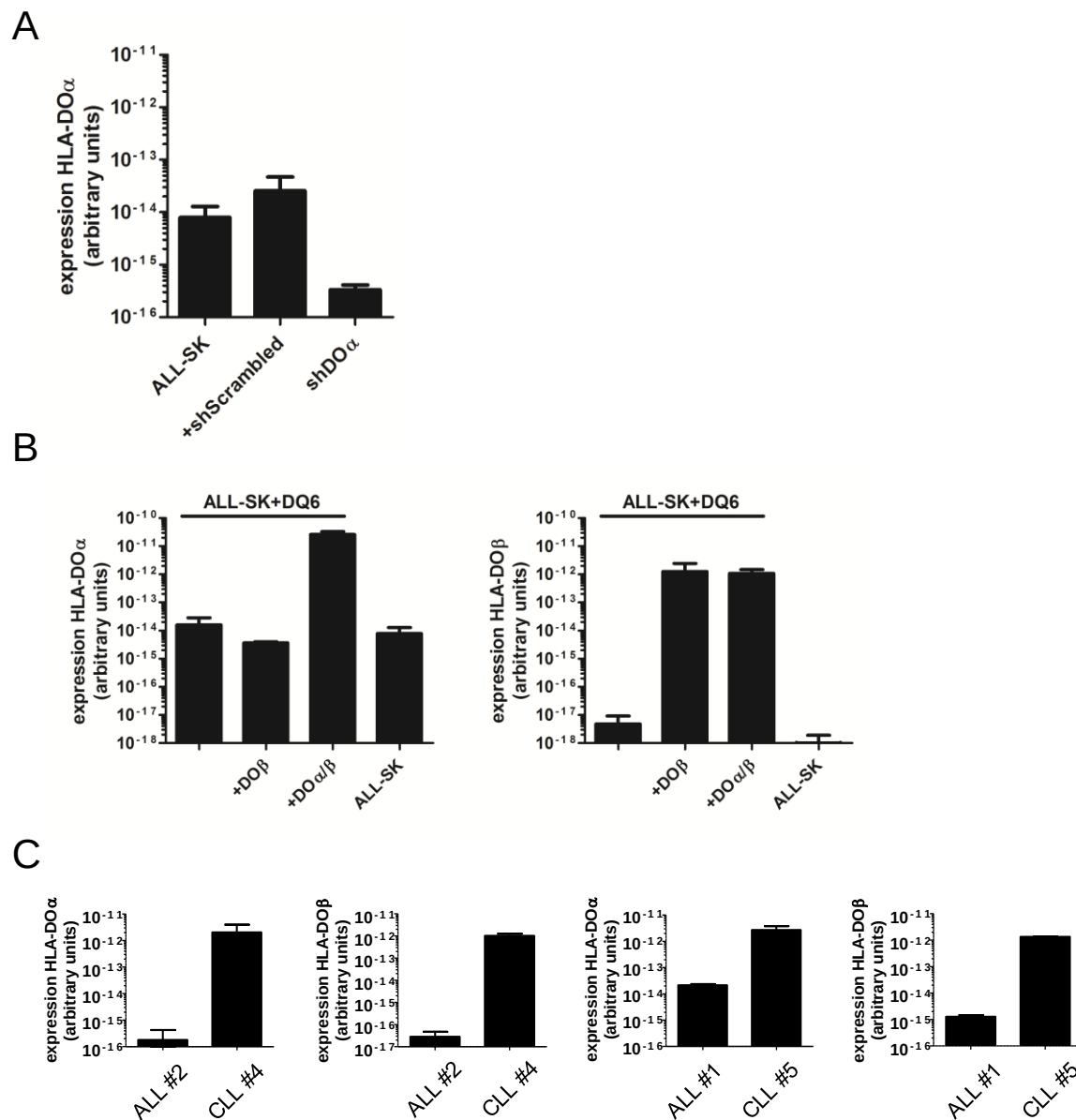


Figure S1. HLA-DOA and -DOB mRNA expression by quantitative RT-PCR.

(A) HLA-DOA mRNA expression corrected for expression of 18S RNA is shown for cell line LB-ALL-SK after transduction with scrambled and DOA specific short hairpin RNA.

(B) Relative mRNA expression of HLA-DOA (left) and -DOB (right) corrected for 18S RNA expression is shown for cell line LB-ALL-SK after retroviral transfer of HLA-DOB alone or together with HLA-DOA.

(C) HLA-DOA and -DOB mRNA expression corrected for 18S RNA for primary ALL and CLL.

In all graphs means and standard deviation of duplicate measurements of one representative experiment are shown.

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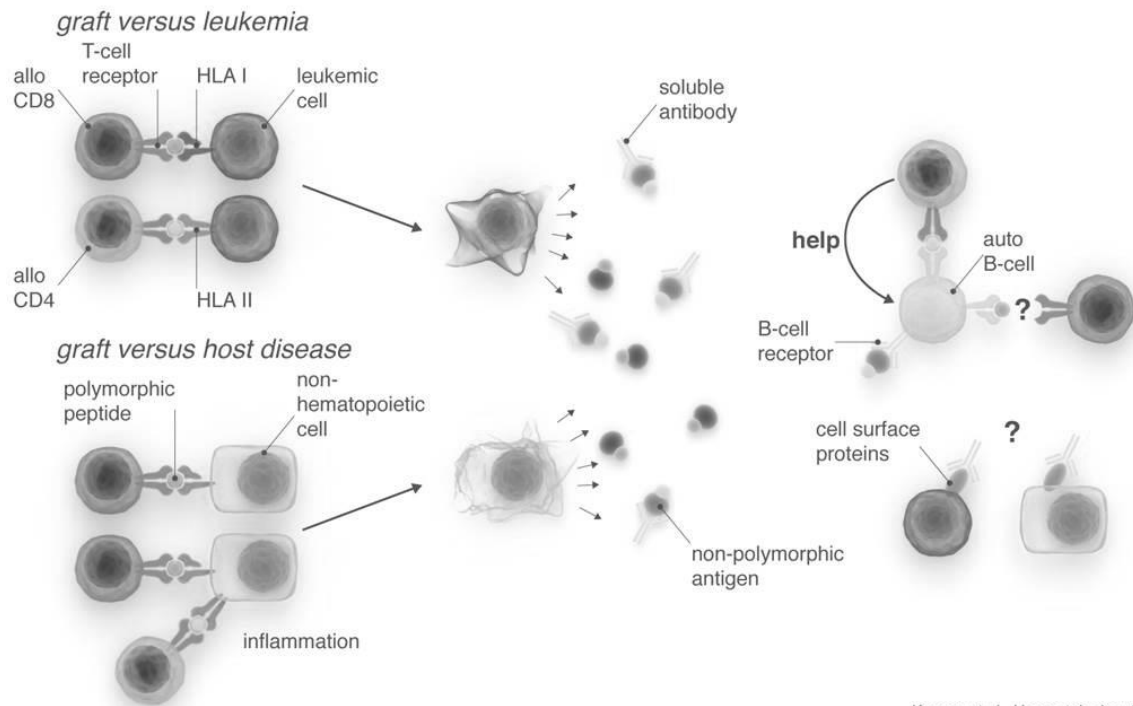
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Development of a coordinated allo T-cell and auto B-cell response against autosomal PTK2B after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

It is well known that allo-reactive T-cells play a crucial role in graft-versus-leukemia and graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Allo-reactive CD4⁺ T-cells can mediate direct cytotoxicity, but may also stimulate production of IgG antibodies as helper cells. Immune complexes may subsequently be processed and presented by professional antigen presenting cells and stimulate induction of specific CD8⁺ T-cells. As such, proteins targeted in coordinated T- and B-cell responses may represent a class of immunodominant antigens in clinical responses after alloSCT. We previously identified LB-PTK2B-1T as HLA class II restricted polymorphic antigen in a patient treated with donor lymphocyte infusion for relapsed chronic myeloid leukemia after HLA-matched alloSCT. Since PTK2B has also been described as antibody target, we here investigated whether a coordinated T- and B-cell response against PTK2B was induced. Patient serum before and after alloSCT and DLI was screened for antibodies, and we indeed observed development of a humoral immune response against PTK2B. Antibodies against PTK2B were only found after DLI and, in contrast to the CD4⁺ T-cells, recognized a monomorphic region of the protein. To our knowledge this is the first description of a coordinated allo-reactive CD4⁺ T-cell and auto-reactive antibody response against an autosomal antigen.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (alloSCT) is often the only therapeutic option for patients with hematological malignancies. During this treatment patient hematopoiesis is eradicated by high-dose radio-/chemotherapy and the patient is rescued by infusion of hematopoietic stem cells of an (HLA-matched) donor.¹ While in the beginnings of alloSCT infusion of donor hematopoiesis was solely meant to rescue the patient from the otherwise lethal radio-/chemotherapy, it is known by now that its beneficial effect is actually mediated by an immune response of donor cells against residual malignant cells of the patient, the so-called graft-versus-leukemia (GvL) effect.² The same immune reaction, however, can also be directed against healthy non-hematopoietic tissues of the patient causing detrimental graft-versus-host disease (GvHD).³

It is well established that beneficial GvL effect as well as undesired GvHD are mediated by donor derived allo-reactive T-cells. While CD8⁺ T-cells confer GvL effect and GvHD via their direct cytolytic activity, the role of CD4⁺ T-cells might be more diverse. Although they can exert direct cytolytic activity, CD4⁺ T-cells are generally believed to play a central role as helper cells in immune responses.^{4,5} CD4⁺ T-cells provide “help” by inducing maturation of dendritic cells and stimulation of CD8⁺ T-cells⁶, and they are also known to deliver essential signals for B-cells to produce IgG antibodies.⁷ Although the capacity of CD4⁺ T-cells to stimulate production of antibodies is well-known, and induction of auto- and allo-reactive antibodies after HLA-matched alloSCT has been demonstrated by various studies⁸⁻¹⁵, the link between CD4⁺ T- and B-cell immunity after alloSCT has only been demonstrated for DDX3Y, a male specific antigen encoded on the Y-chromosome.^{16,17}

We previously identified 5 HLA class II restricted polymorphic antigens as targets for allo-reactive CD4⁺ T-cells in a patient with relapsed chronic myeloid leukemia who responded to donor lymphocyte infusion (DLI) after HLA-matched alloSCT.^{18,19} One of these antigens has been shown to be derived from human PTK2B, which is a member of the focal adhesion kinase family of proteins. PTK2B, also known as PYK2 or RAFTK, has previously been described as target for antibodies in 3 of 19 patients treated with DLI for relapsed CML after HLA-matched alloSCT.¹⁰ It has, however, not been addressed whether

these antibodies were allo- or auto-reactive and whether T-cells specific for PTK2B were induced in these patients.

We here investigated whether allo-reactive CD4⁺ T-cells against PTK2B exerted helper function *in vitro* and whether a coordinated B-cell response was induced *in vivo*. Allo-reactive CD4⁺ T-cells against LB-PTK2B-1T were shown to induce DC maturation and B-cell activation *in vitro*. Moreover, we showed *in vivo* induction of a humoral immune response against PTK2B after DLI. This antibody response was mapped to a small non-polymorphic region of the protein. In conclusion, this is the first report demonstrating induction of a coordinated allo T-cell and auto B-cell response against an autosomal antigen after alloSCT.

METHODS

Antigen presentation assays

EBV-LCL were loaded with 1 µg/mL of synthetic peptides in IMDM with 2% FCS and incubated for 2 hours at 37°C. Cells were washed twice and plated at 30.000 cells/well in a 96-wells plate. Effector cells were added at 5000 T-cells/well. After overnight co-incubation IFN-γ production was measured by ELISA (Sanquin, Burton upon Trent, UK) following instructions of the manufacturer.

Helper function assays

To induce maturation of dendritic cells (DC), CD4⁺ T-cell clones and immature DC were seeded in a 1:1 ratio in a 24-wells plate. After 4 days of co-incubation, cells were harvested, washed and stained with antibodies for FACS analysis. For induction of B-cell activation, CD4⁺ T-cell clones and isolated B-cells were seeded in a 1:1 ratio and after 2 days of co-incubation, cells were harvested, washed and stained for FACS analysis.

Western blot analysis

Whole cell lysates of transfected HEK293T cells were obtained from OriGene (Rockville, USA). HeLa cells were retrovirally transduced with MP71 vector containing PTK2B or PI4K2B constructs. 20µg of protein was loaded on each lane. SDS-Page was run on precast NuPage[®] Novex 10% Bis-Tris Mini gels (Invitrogen) for 35 minutes at 30V under reducing conditions. Gels were blotted on PVDF membranes using XCell SureLock[®] Mini-Cell blotting system (Invitrogen) according to manufacturer's instructions. Blots were blocked for 1 hour at room temperature in phosphate-buffered saline with 0,05% Tween-20 and 5% BSA, and subsequently incubated with diluted (1:40) serum samples overnight at 4°C. Subsequently, the membrane was incubated with biotinylated anti-human IgG and streptavidin-QDots₆₂₅ (Invitrogen) for one hour each and visualized under UV illumination.

Suspension bead array

The suspension bead array was performed as previously described.²¹ Purified proteins (20 µg) were coupled to carboxylated beads (Bio-Rad Laboratories B.V.) according to manufacturers' instructions. Diluted serum samples (1:100 and 1:300) were pre-absorbed with 0.5% (w/v) polyvinylalcohol and 0.8% (w/v) polyvinylpyrrolidone (Sigma) (PVX) and 1% bovine serum albumin (BSA) for 1 hour prior to incubation with the protein-coupled bead mix at room temperature on a shaker for 1 hour. In specific blocking experiments, serum samples were pre-absorbed with purified recombinant proteins at 0.5 or 2.0 µg/mL in PVX and 1% BSA. Beads were washed and incubated for another hour with PE-labeled anti-human IgG, and fluorescence was measured on a Bioplex-100 (BioRad).

RT-PCR

Clonotypic RT-PCR was designed with a forward primer specific for the variable T-cell receptor β-chain and a reverse primer specific for the CDR3 region of clone 78. Expansion of the PCR product was followed by measuring fluorescence of the DNA-intercalating fluorophore EvaGreen (Biotium, San Francisco, CA), and specificity of the PCR product was confirmed by melting curve analysis.

RESULTS AND DISCUSSION

LB-PTK2B-1T specific CD4⁺ T-cells can provide specific help

LB-PTK2B-1T specific CD4⁺ T-cell clone 78 has been isolated 5 weeks after DLI based on expression of activation marker HLA-DR on bone marrow cells from a patient with relapsed CML after HLA-matched alloSCT.¹⁹ The specificity of this T-cell clone was demonstrated by IFN- γ production upon stimulation with patient, but not donor, EBV-LCL as well as donor EBV-LCL loaded with LB-PTK2B-1T peptide (Figure 1A). To investigate *in vivo* dynamics of the T-cell response, we developed a clonotypic PCR for the CDR3 region of clone 78 and detected a specific PCR product in patient PBMC 9 weeks after DLI (Ct = 36), but not in patient samples collected before alloSCT or prior to DLI and in a donor sample, whereas the PBGD housekeeping gene was similarly expressed in all samples (Ct = 25-27) (data not shown).

These data demonstrate that LB-PTK2B-1T specific CD4⁺ T-cells specifically expanded *in vivo* after treatment with DLI. To address the helper potential of PTK2B-specific CD4⁺ T-cells, we analyzed whether clone 78 was capable of inducing maturation of dendritic cells and activation of B-cells in an antigen specific manner. We isolated CD14⁺ monocytes from PBMC endogenously expressing LB-PTK2B-1T and the HLA-DRB3*01:01 restriction allele, and subsequently differentiated these monocytes into immature dendritic cells in the presence of GM-CSF and IL-4. After 4 days of co-culture with PTK2B-1T specific CD4⁺ T-cell clone 78, the dendritic cells acquired a mature phenotype as measured by up-regulation of HLA-DQ, costimulatory molecule CD86 and adhesion molecule CD54 (Figure 1B). Maturation of dendritic cells could not be measured after co-culture with a control T-cell clone. In addition, we isolated CD19⁺ cells and measured specific B-cell activation by up-regulation of activation marker CD86 after 2 days of co-culture with T cell clone 78 (Figure 1C). In conclusion, the experiments show that PTK2B specific CD4⁺ T-cell clone 78 is a *bona fide* T helper cell as illustrated by its ability to induce DC maturation and B-cell activation *in vitro*.

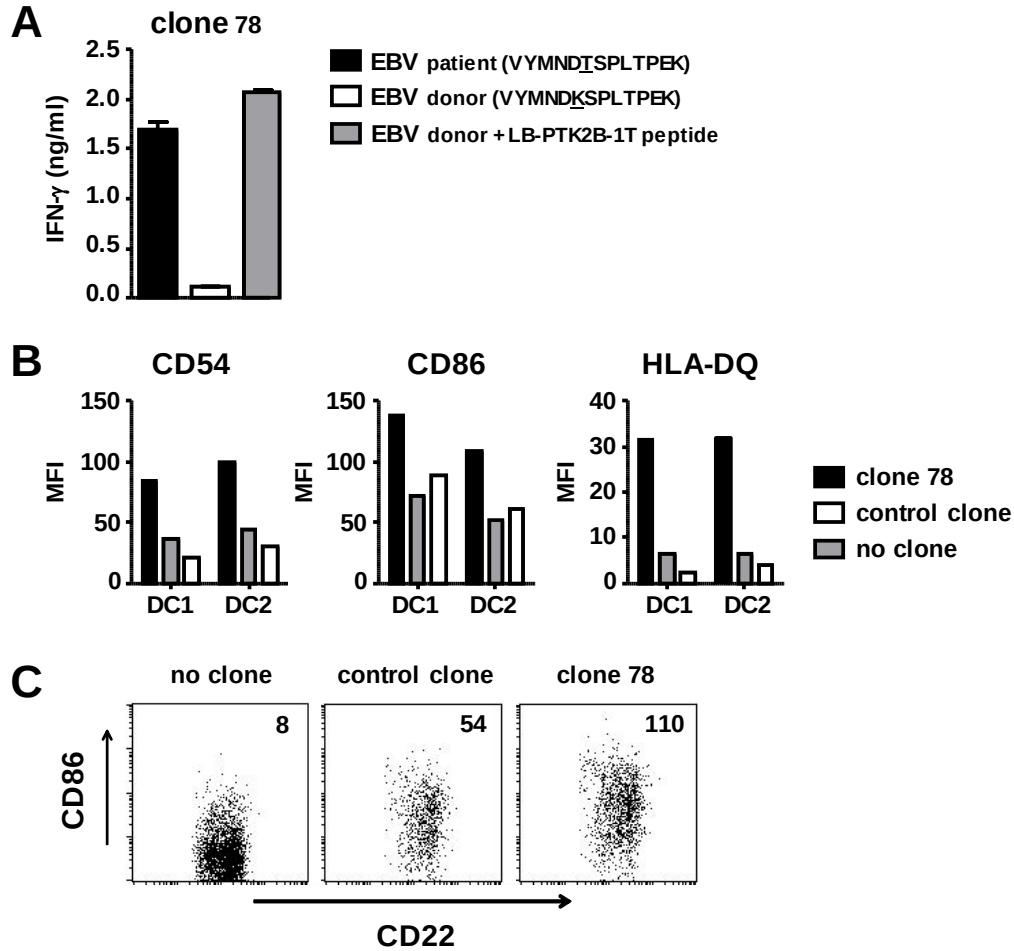


Figure 1: LB-PTK2B-1T specific CD4⁺ T-cells can provide specific help.

(A) LB-PTK2B-1T specific T-cell clone 78 has been isolated by flowcytometry based on expression of activation marker HLA-DR and CD4 from a bone marrow sample obtained five weeks after DLI from a patient with relapsed CML after HLA-matched alloSCT. The T-cell clone strongly recognized patient, but not donor, EBV-LCL as well as donor EBV-LCL loaded with LB-PTK2B-1T peptide in IFN- γ ELISA. Mean and standard deviation of 50- μ l supernatants in duplicate wells is shown.

(B) Immature DC of two LB-PTK2B-1T positive donors expressing HLA-DRB3*01:01 were co-cultured in the absence (open bars) or presence of CD4⁺ T-cell clone 78 (filled bar) or a CD4⁺ T-cell control clone (grey bar) specific for another previously identified HLA-DR restricted MiHA which is not expressed by the DC. Maturation of DC was assessed by measuring surface expression of CD54, CD86 and HLA-DQ after co-culture for 4 days.

(C) LB-PTK2B-1T positive B-cells expressing HLA-DRB3*01:01 were isolated and analyzed by flowcytometry after 2 days of culture in the absence (left) or presence of CD4⁺ T-cell clone 78 (right) or a CD4⁺ T-cell control clone (middle). Activation of B-cells was measured by surface expression of CD86 and mean fluorescence intensities for gated B-cells are depicted.

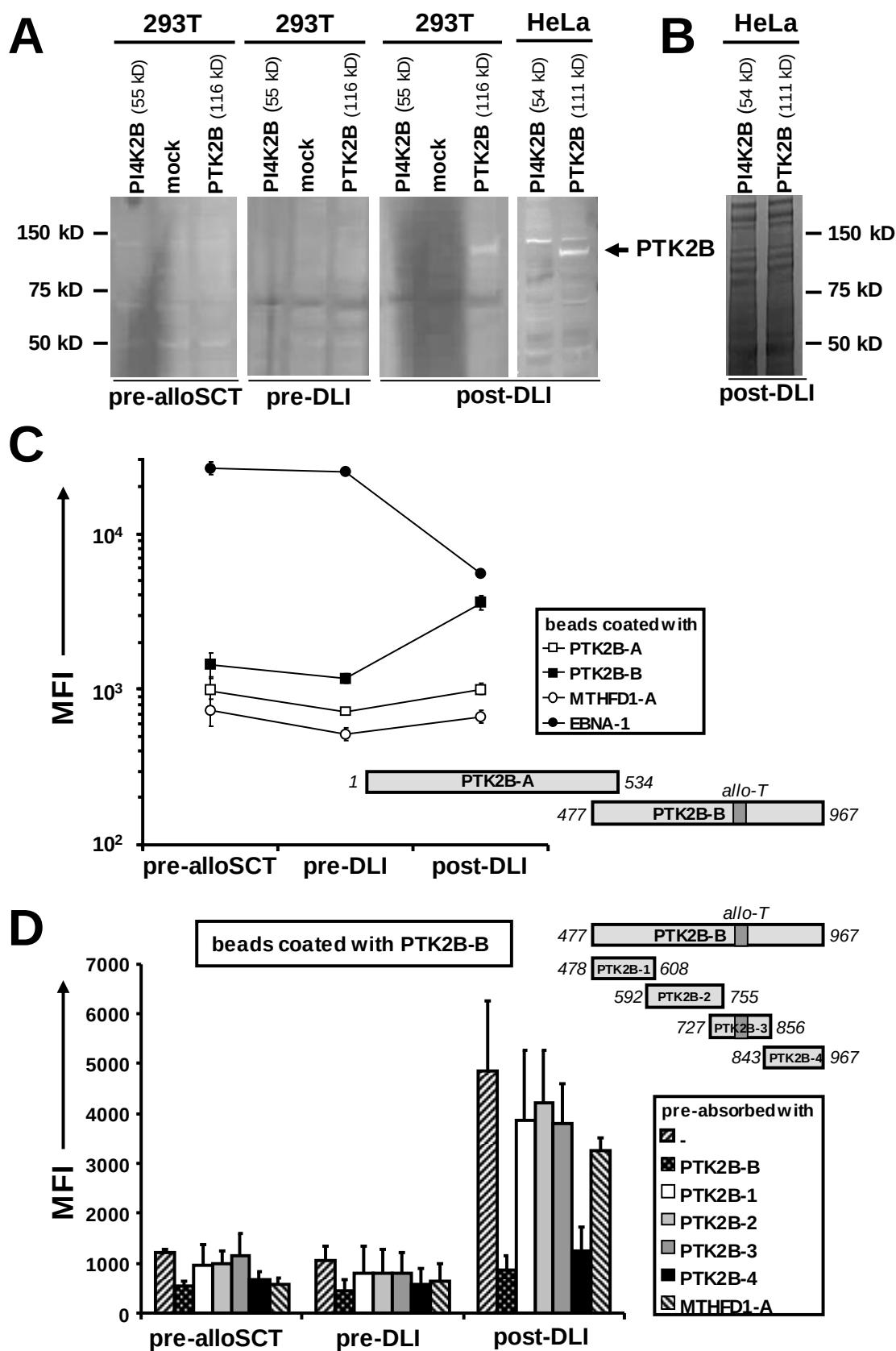


Figure 2: Detection of induced PTK2B specific auto-antibodies after donor lymphocyte infusion

(A) Western blots containing whole cell lysates of HEK293T cells transfected with PI4K2B, PTK2B or empty vector (mock) were incubated with diluted (1:40) serum samples from patient MRJ obtained before aSCT (left), after aSCT / before DLI (middle) and after DLI (right). The binding of serum antibodies was visualized by a secondary antibody against human IgG. HeLa cells retrovirally transduced with PI4K2B or PTK2B vector were incubated with diluted serum from patient MRJ obtained after DLI to confirm specific binding of serum antibodies to PTK2B.

(B) As a control for protein loading, SDS-PAGE with HeLa cells transduced with PI4K2B or PTK2B were stained with Coomassie Brilliant Blue.

(C) Multiplexed analysis of antibody recognition sites was conducted with recombinant proteins PTK2B-A (aa 1-534) and -B (aa 477-967) comprising the N- and C-terminal part of PTK2B, respectively, and EBV-derived antigen EBNA1 as positive control and the N-terminal part of MTHFD1 (MTHFD1-A) as negative control. Protein fragments were isolated, purified and coupled to color-coded suspension beads. Subsequently, serum samples (1:300) were incubated with protein-coated beads and binding of specific antibodies was measured by staining with PE-labeled anti-human IgG. Mean fluorescence intensities of single measurements of two independent experiments are shown. Indicated is the allo-T-cell epitope at aa 791-800 in PTK2B-B.

(D) To further map for the antibody epitope of PTK2B, inhibition assays were performed by adding soluble recombinant protein fragments as competitors. Serum samples (1:100 and 1:300) were pre-absorbed with recombinant PTK2B fragments 1 (aa 478-608), 2 (aa 592-755), 3 (aa 727-856), 4 (aa 843-967) or PTK2B-B (aa 477-967) prior to incubation with protein-coated beads. MTHFD1-A was included as negative control. Mean fluorescence intensities of single measurements with suspension beads coated with PTK2B-B of two independent experiments are shown. Inhibition of mean fluorescence intensity after pre-absorption with PTK2B-B and fragment 4 indicates specific binding of antibodies. Pre-absorption with these fragments did not inhibit antibody binding to EBNA-1 coated beads (data not shown).

Induction of PTK2B specific auto-antibodies after donor lymphocyte infusion

To address the *in vivo* helper function of PTK2B-1T specific CD4⁺ T-cells in more detail, we analyzed whether a coordinated T- and B-cell response against this protein was induced after DLI. Patient serum obtained before alloSCT, between T-cell depleted alloSCT and DLI, and after DLI were screened for anti-PTK2B IgG antibodies by Western Blot analysis (Figure 2A/B) and multiplexed suspension bead arrays (Figure 2C/D), and antibody reactivity against PTK2B could be measured by both methods. Whereas antibody reactivity against CMV or EBV antigens was detectable in all serum samples, reactivity against PTK2B specifically evolved after DLI, and was not found before alloSCT or prior to DLI. Antibody reactivity against PTK2B after DLI could not be explained by an overall increase in antibodies after immune recovery, as witnessed by decreased binding of antibodies to EBV-derived antigen EBNA1 and lack of reactivity against other cellular proteins on

Western blot. To further determine the immunogenic region of PTK2B, we produced partially overlapping recombinant protein fragments and mapping for recognition sites showed that antibody reactivity was directed against the C-terminal part of PTK2B (PTK2B-B; Figure 2C). Further fragmentation of the C-terminus of PTK2B revealed that the antibody epitope was located in a non-polymorphic region between amino acids (aa) 843 - 967 (PTK2B-4; Figure 2D), whereas the polymorphic T-cell epitope is located between aa 791 - 800. By sequencing PTK2B cDNA of patient and donor, we confirmed that no differences were present within this region (data not shown), illustrating that the humoral immune response after DLI consisted of auto-antibodies against PTK2B.

CONCLUDING REMARKS

In this report, we demonstrate induction of a coordinated allo CD4⁺ T- and auto B-cell response against autosomal antigen PTK2B after alloSCT. In contrast to the coordinated B- and T-cell response against DDX3Y in which an allo-reactive antibody specific for the protein variant encoded on the Y-chromosome has been shown to co-develop with an auto-reactive CD4⁺ T-cell recognizing both protein variants encoded on the Y- and X-chromosomes^{16,17}, we here demonstrate induction of an allo-reactive CD4⁺ T-cell specific for a polymorphic epitope combined with an antibody directed against a monomorphic region of the same protein. It has been shown that approximately 20% of the mature peripheral B-cell repertoire is self-reactive^{22,23}, but that the majority of B-cells are anergic to prevent production of auto-antibodies.^{24,25} Self-tolerance can be broken by molecular mimicry, in which antibodies generated in response to an infectious agent are cross-reactive with self proteins. In these cases, antibody responses are usually transient as the involved T-cells are directed against foreign pathogen derived antigens and not against self-proteins. In the situation of DLI for leukemic relapse it is conceivable that release of cytosolic contents due to tissue damage mediated by activated allogeneic T-cells may have triggered a break in B-cell tolerance and production of auto-antibodies against intracellular self-antigens.¹⁰ Although antibodies against intracellular antigens are not expected to mediate direct cytotoxicity, immune complexes may be formed, which can be efficiently processed and

presented by professional antigen presenting cells, thereby stimulating induction or amplification of specific T-cell responses.²⁶⁻²⁸ As such, proteins targeted in coordinated T- and B-cell responses may represent a class of immunodominant antigens in clinical responses after alloSCT. However, it should be noted that, although simultaneous development of T- and B-cell responses against the same protein is suggestive for cognate T-cell help, non-cognate T-cell responses and direct stimulation by TLR-ligands cannot be ruled out and should also be considered as stimulators for auto-reactive B-cells.

In conclusion, our data demonstrate development of a coordinated CD4⁺ T- and B-cell response against autosomal antigen PTK2B. Based on this patient case, it is tempting to speculate that allo-reactive CD4⁺ T-cells as administered by DLI may break B-cell tolerance and stimulate development of auto-antibodies after alloSCT.

ACKNOWLEDGMENTS

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SUPPLEMENTARY INFORMATION - METHODS

Hematopoietic cell isolation

Peripheral blood and bone marrow samples were obtained from patients and healthy individuals after approval by the Leiden University Medical Center Institutional Review Board and informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque separation and cryopreserved.

Cell culture

Epstein-Barr virus transformed B-cell lines were cultured in IMDM (Lonza BioWhittaker, Basel, Switzerland) with 10% FCS, 1% penicillin/streptomycin (Lonza) and 1.5% L-glutamine (Lonza). T-cell clones were cultured in IMDM with 5% human serum, 5% FCS and 100 IU/mL interleukin-2 (IL-2) (Chiron, Ringskiddy, Ireland), and restimulated every 10-20 days with irradiated allogeneic peripheral blood mononuclear cells (PBMC) and 0.8 µg/mL phytohemagglutinin (PHA) (Oxoid, Cambridge, UK) as previously described.¹⁹ Immature dendritic cells (DC) were generated by culturing isolated monocytes in medium with 100ng/mL GM-CSF (Novartis) and 500 IU/mL IL-4 (Schering-Plough, Kenilworth, NJ) for 5 days.

Antigen presentation assays

EBV-LCL were loaded with 1 µg/mL of synthetic peptides in IMDM with 2% FCS and incubated for 2 hours at 37°C. Cells were washed twice and plated at 30.000 cells/well in a 96-wells plate. Effector cells were added at 5000 T-cells/well. After overnight co-incubation IFN-γ production was measured by ELISA (Sanquin, Burton upon Trent, UK) following instructions of the manufacturer.

Helper function assays

To induce maturation of dendritic cells (DC), CD4⁺ T-cell clones and immature DC were seeded in a 1:1 ratio in a 24-wells plate. After 4 days of co-incubation, cells were harvested, washed and stained with antibodies for FACS analysis. For induction of B-cell activation,

CD4⁺ T-cell clones and isolated B-cells were seeded in a 1:1 ratio and after 2 days of coinubation, cells were harvested, washed and stained for FACS analysis.

Antibodies

Flowcytometric analysis was performed on a FACS Calibur (BD Biosciences, Breda, The Netherlands) using PE-labeled monoclonal antibodies against HLA-DQ (1a3; Bio-Connect B.V., Huissen, The Netherlands), CD86 (BD Biosciences), APC-labeled CD22 (BD Biosciences), and FITC-labeled antibodies against CD54 (MorphoSys AbD GmbH, Düsseldorf, Germany). B-cell isolation was performed by magnetic cell sorting with anti-CD19 bead (Miltenyi). Biotinylated goat anti-human IgG (Sanbio B.V., Uden, The Netherlands) and PE-labeled goat anti-human IgG (Bio-Connect B.V.) were used as secondary antibodies in Western blot analysis and suspension bead assay, respectively.

Western blot analysis

Whole cell lysates of transfected HEK293T cells were obtained from OriGene (Rockville, USA). HeLa cells were retrovirally transduced with MP71 vector containing PTK2B or PI4K2B constructs. 20µg of protein was loaded on each lane. SDS-Page was run on precast NuPage® Novex 10% Bis-Tris Mini gels (Invitrogen) for 35 minutes at 30V under reducing conditions. Gels were blotted on PVDF membranes using XCell SureLock® Mini-Cell blotting system (Invitrogen) according to manufacturer's instructions. Blots were blocked for 1 hour at room temperature in phosphate-buffered saline with 0,05% Tween-20 and 5% BSA, and subsequently incubated with diluted (1:40) serum samples overnight at 4°C. Subsequently, the membrane was incubated with biotinylated anti-human IgG and streptavidin-QDots 625 (Invitrogen) for one hour each and visualized under UV illumination.

Production of recombinant proteins

Gene sequences encoding full length and partial PTK2B proteins were cloned into a Gateway vector with a N-terminal His-tag (pDEST17; Invitrogen) according to instructions of the manufacturer. The identity of the constructs was verified by DNA sequencing. Recombinant proteins were obtained from bacterial cultures after addition of 0.4 mM IPTG

for 4 hrs by isolation of inclusion bodies as described by Palmer et al.²⁰ with minor modifications.

Suspension bead array

The suspension bead array was performed as previously described by Schwenk et al.²¹ Purified proteins (20 µg) were coupled to carboxylated beads (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) according to manufacturers' instructions. Diluted serum samples (1:100 and 1:300) were pre-absorbed with 0.5% (w/v) polyvinylalcohol and 0.8% (w/v) polyvinylpyrrolidone (Sigma) (PVX) and 1% bovine serum albumin (BSA) for 1 hour prior to incubation with the protein-coupled bead mix at room temperature on a shaker for 1 hour. In specific blocking experiments, serum samples were pre-absorbed with purified recombinant proteins at 0.5 or 2.0 µg/mL in PVX and 1% BSA. Beads were washed and incubated for another hour with PE-labeled anti-human IgG, and fluorescence was measured on a Bioplex-100 (BioRad).

RT-PCR

Clonotypic RT-PCR was designed with a forward primer 5'-CCAGTGCCCATCCTGAAGAC-3' specific for the variable T-cell receptor β -chain and a reverse primer 5'-GCGTATCTGTGCTAGGATTGA-3' specific for the CDR3 region of clone 78. RT-PCR for the PBGD housekeeping gene was performed with forward primer 5'-GGCAATGCGGCTGCAA-3' and reverse primer 5'-GGGTACCCACGCGAATCAC-3'. Expansion of the PCR product was followed by measuring fluorescence of DNA-intercalating fluorophore EvaGreen (Biotium, San Francisco, CA) in 10 µl reactions containing 10 ng cDNA, 0.4 U FastStart-Taq polymerase (Roche Applied Science, Almere, The Netherlands), 200 nM dNTPs, 2 mM MgCl₂ and 200 nM (TCR) or 300 nM (PBGD) primers. RT-PCR conditions were as follows: 10 min at 95°C, followed by 50 cycles of 15 sec for denaturing at 95°C, 30 sec of annealing at 65°C, and 20 sec extension at 72°C, and final elongation for 5 min at 72°C. After amplification, specificity of the PCR products was confirmed by melting curve analysis.

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6

Intercellular transfer of natural HLA class II ligands is mediated by full length proteins that are sequestered and secreted into microvesicles

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ABSTRACT

CD4⁺ T-cells play a central role as helper cells in adaptive immunity. Presentation of exogenous antigens in MHC class II by professional antigen presenting cells (APC) is a crucial step in induction of specific CD4⁺ T-cells in adaptive immune responses. For efficient induction of immunity against intracellular threats such as viruses or malignant transformations, antigens from HLA class II-negative infected or transformed cells need to be transferred to surrounding APC to allow efficient priming of naïve CD4⁺ T-cells. Here we investigate the mechanism of indirect antigen presentation of six natural HLA class II epitopes. We demonstrate that 3 of the 6 natural HLA class II epitopes could be transferred between viable cells, and that intercellular transfer was mediated by full-length proteins that were secreted within microvesicles. Transfer of these microvesicles, which have been identified as exosomes, led to T-cell recognition of previously antigen-negative acceptor cells. Furthermore, we demonstrated that antigen transfer was not dependent on the T-cell epitope, but that sequestration and secretion into microvesicles was largely determined by protein characteristics such as cellular abundance and specific binding sequences. Our data show that certain natural HLA class II epitopes can be indirectly presented via intercellular transfer of full-length proteins within secreted microvesicles. This mechanism of antigen transfer from HLA class II-negative cells to surrounding APC may play a crucial role in induction of immunity against intracellular viruses or transformed cells.

INTRODUCTION

CD4⁺ T-cells play a central role as helper cells in adaptive immunity¹. MHC class II positive macrophages, B-cells and dendritic cells are well equipped to take up, process and present exogenous antigens in their MHC class II molecules, and priming of naïve CD4⁺ T-cells by these professional antigen presenting cells (APC) is a crucial step in efficient induction of immune responses². Upon recognition of epitopes in MHC class II molecules, antigen-specific CD4⁺ T-cells become activated and secrete cytokines, and as a result provide help for induction and maintenance of CD8⁺ cytotoxic T-lymphocytes^{1,3}, stimulation of B-cells to produce antibodies⁴, maturation of dendritic cells¹ and induction of delayed type hypersensitivity reactions by activation of macrophages^{4,5}. As such, CD4⁺ T helper cells are crucial in eliciting potent immunity against exogenous pathogens.

For efficient induction of immunity against intracellular threats such as viruses or malignant transformations, intracellular antigens from HLA class II-negative infected / transformed cells need to be transferred to surrounding APC in order to prime naïve CD4⁺ T-cells. This concept of antigen transfer is well known from solid organ transplantation where presentation of donor antigens on recipient APC has been linked to chronic rejection^{6,7}. Also in a mouse model of minor histocompatibility antigen mismatched bone marrow transplantation, donor APC presenting recipient antigens have been shown to stimulate CD4⁺ T-cells and mediate Graft-versus-Host disease⁸. A similar mechanism in which CD4⁺ T helper cells are primed by APC presenting differentiation antigens taken up from highly specialized HLA class II negative tissues may explain the predisposition of human individuals with specific HLA class II alleles to develop autoimmune diseases⁹. Finally, in tumor immunology, indirect presentation of tumor-associated antigens by professional APC¹⁰⁻¹² has been shown to induce beneficial immune responses in which MHC class II negative tumors can be rejected in a CD4⁺ T-cell dependent fashion.

The exact mechanism(s) of indirect antigen presentation in HLA class II after intercellular transfer *in vivo* is unknown. While cellular debris and antigen release from dying cells has often been proposed as primary source of indirectly presented cytosolic antigens, lack of indirect presentation for certain antigens rather suggests a more selective mechanism^{13,14}.

Moreover, as T-cell help is critical in the induction phase of an immune response, it seems unlikely that priming of CD4⁺ T-cells would be solely dependent on death of infected / transformed cells.

We here show that a subset of cytosolic proteins can be transferred between viable cells leading to indirect antigen presentation in HLA class II molecules of the acceptor cells. This intercellular transfer of natural HLA class II restricted antigens is mediated by secreted microvesicles that have been identified as exosomes, and leads to CD4⁺ T-cell recognition of previously antigen-negative acceptor cells. Furthermore, we demonstrated that intercellular antigen transfer was not dependent on the T-cell epitope, but rather on protein-specific characteristics. We showed that sequestration and secretion of full length cytosolic proteins closely correlates with cellular abundance, but that selective processes as illustrated by involvement of an hsc70 chaperone binding site, also contribute to the final amount of protein that is transferred to acceptor cells.

MATERIALS AND METHODS

Cell culture

Cervix carcinoma HeLa, melanoma MJS, and lymphoma Raji cell lines and Epstein-Barr virus transformed B-cell lines (EBV-LCL) from patient MRJ and her HLA-matched donor IZA as well as EBV-LCL from other individuals were cultured in IMDM (Lonza BioWhittaker, Basel, Switzerland) with 10% fetal calf serum (FCS) (Cambrex, Verviers, Belgium), 1% penicillin/streptomycin (Lonza) and 1.5% L-glutamine (Lonza). CD4⁺ T-cell clones were cultured in IMDM with 5% human serum, 5% FCS and 100 IU/mL interleukin-2 (IL-2) (Chiron, Ringskiddy, Ireland), and restimulated every 10-20 days with irradiated allogeneic peripheral blood mononuclear cells (PBMC) and 0.8 µg/mL phytohemagglutinin (PHA) (Oxoid, Cambridge, UK) as previously described¹⁶. For isolation of exosomes, HeLa cells were cultured in serum-free IMDM with 1% penicillin/streptomycin, 1.5% L-glutamine, 5 µg/mL insulin (Sigma-Aldrich B.V., Zwijndrecht, The Netherlands), 5 µg/mL transferrin (Invitrogen, Breda, The Netherlands),

5 ng/mL EGF (PeproTech, Rocky Hill, NJ), 100 nM hydrocortisone (Sigma-Aldrich B.V.) and 20 ng/ml FGF2 (Biaffin GmbH & Co KG, Kassel Niederzwehren, Germany) as previously described (Hutchings SE, PNA, 1978).

Antibodies

Flowcytometric analysis was performed on a FACS Calibur (BD Biosciences, Breda, The Netherlands) and cell sorting on a FACS Aria (BD) using PE-labeled monoclonal antibodies against HLA-A2 (BB7.2; BD Pharmingen, San Diego, CA), HLA-DR (L243; BD), HLA-DQ (1a3; Meridian Life Science, Saco, ME), NGFR (C40-1457; BD Pharmingen) or CD20 (L27; BD). For Western blotting, mouse antibodies against hsc70 (sc-7298; Santa Cruz Biotechnology, Santa Cruz, CA), gp96 (ab63469; Abcam, Cambridge, UK), 6xHis (ab18184; Abcam), and rabbit antibodies against CD9 (ab65230; Abcam), PTK2B (ab24798; Abcam), and PI4K2B (ab37812; Abcam) were used. As secondary antibodies, biotinylated goat anti-mouse or goat anti-rabbit antibodies (Invitrogen) were used and visualized by Streptavidin-QDots 625 (Invitrogen) under UV illumination.

Retroviral constructs and transduction

HLA-DQB1*0603, DQB1*0501, DQA*0103 and DQA*0101 were cloned into separate pLZRS vectors with truncated nerve growth factor receptor (Δ NGFR) as marker gene for the DQB chains and enhanced green fluorescence protein (EGFP) for the DQA chains. HLA-DRB1*0301, DRB1*1301, DRB3*0101, DRB3*0202 and DRA*0102 were cloned into MP71 vectors using truncated Δ NGFR as marker gene. HLA class II negative HeLa cells transduced with retroviral vectors encoding HLA class II molecules were sorted based on cell surface expression of HLA-DR or DQ. EBV-LCL transduced with HLA-DRB or DQB chains were sorted based on expression of the marker gene Δ NGFR. The genes encoding full length PTK2B, MR1, PI4K2B and MTHFD1 were cloned into MP71 vectors with human CD20 as marker gene. Full length DBY was cloned into a pLZRS vector containing EGFP as marker gene. Chimeric and mutated constructs were cloned by two-step PCR. Chimera A contained full-length PI4K2B (NM_018323) with the 42 bp sequence encoding its T-cell epitope at positions 220-262 bp replaced by the 39 bp sequence

encoding the PTK2B epitope. Chimera B contained full-length PTK2B (NM_173175) with the 39 bp sequence encoding its T-cell epitope at positions 2371-2410 bp replaced by the 42 bp sequence encoding the PI4K2B epitope. Fusion chimera 1 contains full-length PI4K2B fused to the last 621 bp of the PTK2B gene. Fusion chimera 3 contains the first 270 bp of the PI4K2B gene fused to full-length PTK2B. Chimeric constructs A, B, 1 and 3 were cloned into MP71 vector with CD20 as marker gene. For construct 5 6xHis-tags was fused to the N-terminus of PTK2B and the C-terminus of PI4K2B and both sequences were linked with a T2A sequence (His-PTK2B-T2A-PI4K2B-His). The whole construct was cloned into a MP71 vector using eGFP as marker gene. Construct 11 encoded full-length PTK2B (1-2901 bp) and construct 14 (2317-2901 bp) contained truncated PTK2B cloned into MP71 vectors with EGFP as marker gene. A natural methionine served as start codon for construct 14. Constructs 22 and 23 contained two amino acids in the most C-terminal putative hsc70 binding motif (2548-2562 bp) mutated to alanines (LELKN to LELAA). Construct 22 encoded a mutated version of full-length PTK2B, and construct 23 a mutated version of truncated PTK2B as encoded by construct 14. All constructs were verified by DNA sequencing. Retroviral supernatants were obtained by transfecting wild-type Φ nx-A packaging cells as previously described³³, with the exception that the Fugene HD transfection kit (Roche) was used. Viral supernatants were used for transduction of cells in culture plates coated with recombinant human fibronectin CH 296 (Takara Shuzo, Otsu, Shiga, Japan) as previously described³³.

Antigen presentation assays

Stimulator cells (10.000 or 30.000 cells/well) were co-incubated with CD4⁺ T-cells (5000 cells/well) overnight at 37°C in 96-well plates. In intercellular transfer experiments, donor and acceptor cells were plated at 1:1 ratios in 96-wells plates overnight before adding the T-cell clones. As acceptor cells, we used HeLa cells transduced with a retroviral vector encoding the invariant chain and human CD80 as marker gene. This HeLa-Ii variant has been demonstrated to efficiently process and present all six natural HLA class II ligands³⁴. For loading with recombinant proteins, *E.coli* with a pKE-1 vector encoding the respective antigen under an IPTG-inducible promoter were grown to OD₆₀₀ of 0.5 with 50 µg/mL ampicillin (Sigma-Aldrich B.V.) and protein expression was induced by 1 mM IPTG for 4

hrs (Promega, Madison, WI). Subsequently, bacteria were opsonized by adding human serum with 17% (vol/vol) complement (Sigma-Aldrich B.V.) for 1 hr. Target cells were pulsed with complement-opsonized bacteria in IMDM with 10% FCS and 30 µg/mL gentamycin (Sigma-Aldrich B.V.) overnight at 37°C before T-cell clones were added. Culture supernatants were depleted of cells by centrifugation with 300g for 10min and separated through 5, 30 (Vivaproducts, Littleton, MA) or 100 (Millipore, Amsterdam, The Netherlands) kDa filters. Concentrated flowthrough fractions (50 µl) or isolated exosomal fractions (50 µl) were loaded on target cells in 96-wells plates overnight before T-cell clones were added. Cytokine release was measured in 50-µl supernatants by IFN-γ ELISA (Sanquin, Burton upon Trent, UK) according to manufacturer's instructions.

Western blot analysis

Cells were harvested and washed once with phosphate-buffered saline (PBS). Cell pellets were resuspended in 1% triton-X (Sigma-Aldrich B.V.) lysis buffer containing protease inhibitors (Roche, Woerden, The Netherlands) for 20 min on ice. Whole cell lysates were obtained after centrifugation at 10.000g for 30 min. Protein concentration was assessed by the Bradford protein assay (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). SDS-Page was run with 10-25 µg protein in each lane on precast NuPage® Novex 10% Bis-Tris Mini gels (Invitrogen) for 35 min at 30V under denaturing conditions. Gels were blotted on PVDF membranes using XCell SureLock® Mini-Cell blotting system (Invitrogen) according to manufacturer's instructions. Blots were blocked overnight with 5% BSA in PBS with 0.05% Tween 20 and subsequently incubated with primary antibody, biotinylated secondary antibody and streptavidin-QDots₆₂₅. Each incubation step was performed for 1 hr at RT. Antibody binding was visualized under UV illumination.

Isolation of exosomes

HeLa cells were grown in serum-free medium for three 3 days. Culture supernatant was depleted of cells by centrifugation at 300g for 10min. Supernatants were subsequently depleted of cellular debris by centrifugation at 3000g for 20 min, followed by centrifugation at 10.000g for 30min. Finally, supernatants were centrifuged at 100.000g for 15 hrs, pellets were resuspended in a large volume of PBS and centrifuged again at

100.000g for 15 hrs in a Beckman-Coulter SW28 rotor. Exosome pellets were resuspended in 150-200 µl of culture medium and immediately used in T-cell recognition experiments. Exosome pellets were resuspended in 50 µl of 1% triton-X lysis buffer for Western blot analysis, and in PBS/4% glutaraldehyde (Brunschwig Chemie, Amsterdam, The Netherlands) for electron microscopy.

RESULTS

We analyzed the occurrence of indirect antigen presentation for six natural HLA class II epitopes. All six polymorphic antigens were targets for T-cells in immune responses after allogeneic hematopoietic stem cell transplantation, and included two membrane proteins (LY75 and MR1), two cytosolic kinases (PTK2B and PI4K2B), one cytosolic enzyme (MTHFD1) and one nuclear / cytosolic RNA helicase (DBY). The antigens were restricted by four different HLA-DR and two different HLA-DQ molecules: DRB1*0301/A*0102 (MTHFD1), DRB1*1301/A*0102 (LY75), DRB3*0101/A*0102 (PTK2B), DRB3*0202/A*0102 (MR1), DQB1*0603/A*0103 (PI4K2B) and DQB1*0501/A*0101 (DBY). Five of the proteins (LY75, MR1, PTK2B, PI4K2B and MTHFD1) were identified as targets for autosomal minor histocompatibility antigen (MiHA) specific CD4⁺ T-cells in a female patient (MRJ) after alloSCT with her HLA-matched sister (IZA)^{15,16}, whereas the remaining protein (DBY) is encoded on the Y-chromosome, and recognized by CD4⁺ T-cells in a male patient after alloSCT with his HLA-identical sister¹⁷.

A subset of natural HLA class II restricted antigens are intercellularly transferred.

To investigate the occurrence of indirect antigen presentation, we co-cultured antigen positive EBV-LCL cells lacking the respective HLA class II molecules (donor cells) with antigen-negative HeLa cells transduced with the relevant HLA class II restriction alleles (acceptor cells). Both donor cells and acceptor cells were not recognized by the respective T-cell clones when analyzed separately. Upon co-culturing, however, recognition was observed for three antigens (MTHFD1, PTK2B and DBY). HeLa acceptor cells were pulsed with recombinant proteins as positive controls for their capacity to process antigen

from an extracellular source (Figure 1A). Further analysis revealed that direct cell-cell contact was not required, since the three cytosolic proteins were efficiently transferred between cells via culture supernatants (Figure 1B). Antigen transfer as measured by T-cell recognition did not occur when flow-throughs of culture supernatants from 10, 30 or 100 kDa filters were loaded (Figure 1B and data not shown), indicating that antigens were secreted as proteins or particles with sizes >100 kDa.

Co-culture experiments using EBV-LCL as donor cells and HeLa as acceptor cells have some drawbacks. First, different EBV-LCL are required as donor cells for different antigens, and these EBV-LCL may differ in their ability to induce non-specific release of IFN- γ by T-cells. Moreover, the antigens as encoded by endogenous genes in EBV-LCL may show differences in expression levels. Furthermore, HeLa acceptor cells normally lack HLA class II expression, and intrinsic processing and presentation mechanisms in these cells may therefore be inappropriate and show some defects. Finally, HeLa acceptor cells endogenously express the PI4K2B epitope, thereby complicating data interpretation for this antigen. We therefore developed a reversed system for two selected antigens, one antigen (PTK2B) for which we saw transfer between cells, and one antigen (PI4K2B) for which we did not observe transfer. We cloned and transduced the genes for PTK2B and PI4K2B into HLA class II negative HeLa cells, which were subsequently used as donor cells together with antigen-negative EBV-LCL IZA, which endogenously express all relevant HLA class II restriction alleles, as acceptor cells. Using this reversed system, we again demonstrated and confirmed that PTK2B was efficiently transferred between cells. Despite induced over-expression of PI4K2B in HeLa cells, no transfer was observed for this antigen (Figure 1C).

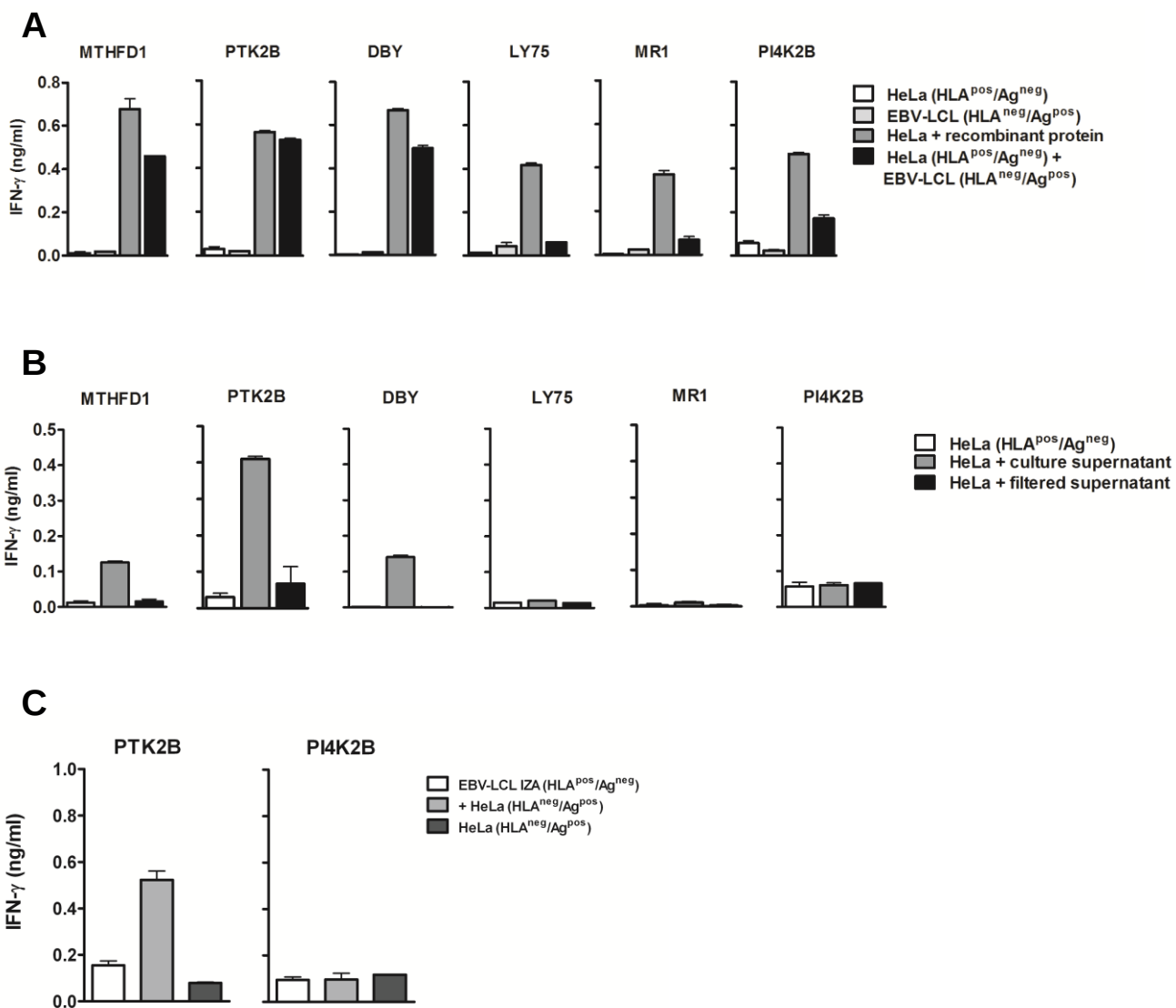


Figure 1: A subset of natural HLA class II restricted antigens are intercellularly transferred.

(A) HeLa cells retrovirally transduced with the relevant HLA class II restriction alleles were co-cultured with antigen-positive EBV-LCL lacking the appropriate HLA class II molecules. As positive controls, HeLa cells retrovirally transduced with the relevant HLA class II restriction alleles were exogenously loaded with the six selected antigens as recombinant proteins. Antigen processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN- γ ELISA. Mean values and standard deviation of duplicate wells is shown.

(B) Culture supernatants from antigen positive EBV-LCL lacking the appropriate HLA class II molecules were centrifuged with 300g to remove viable cells and cell debris, and loaded on antigen negative HeLa variants expressing the relevant HLA class II restriction alleles. In addition, culture supernatants were passed through different filters to remove all proteins and particles with sizes >10, 30 or 100 kDa, and flowthroughs were loaded on antigen negative HeLa variants expressing the relevant HLA class II restriction alleles. Antigen uptake and subsequent processing and presentation into HLA class II was tested by measuring T-cell recognition in IFN- γ ELISA. A representative experiment with culture supernatants and flowthroughs of 30 kDa filters is shown, but results were similar for 10 and 100 kDa filters. Mean values and standard deviation of duplicate wells is shown.

(C) Antigen-negative EBV-LCL IZA expressing all relevant HLA class II molecules and antigen-transduced, HLA class II negative HeLa cells were tested for T-cell recognition in IFN- γ ELISA either separately or after co-culture. Mean values and standard deviation of duplicate well is shown.

Intercellular transfer is mediated by secreted microvesicles.

As antigen transfer was consistently more pronounced after co-culture with viable cells as compared to culture supernatants, we investigated whether the cytosolic proteins were actively secreted in microvesicles. We isolated secreted microvesicles from culture supernatants from transduced HeLa cells by differential high speed ultracentrifugation. The nature of the isolated fraction was confirmed as exosomes, which are released intraluminal vesicles of the late endosome, by enrichment of tetraspanin CD9, absence of ER resident gp96 (glycoprotein 96) and presence of chaperone hsc70 on Western blot (Figure 2A) as well as their size and cup-shaped morphology in electron microscopy (Figure 2B). By pulsing antigen-negative EBV-LCL IZA with the microvesicular pellet or the supernatant after high-speed centrifugation, we could demonstrate T-cell recognition of the microvesicular fraction, whereas the supernatant after centrifugation with 100.000g was depleted of antigen (Figure 2C). Additionally, we investigated the presence of PTK2B and PI4K2B in the exosomal fraction on Western blot. In line with T-cell recognition data, we found full length PTK2B in the exosomal fraction, whereas PI4K2B was absent (Figure 2A). Notably, although over-expression of PTK2B and PI4K2B in HeLa cells was induced by the same type of retroviral vector, we observed a strong difference in protein expression

in cellular lysates on Western blot. In conclusion, our data demonstrated that the intracellular antigen PTK2B could be processed and presented into HLA class II by intercellular transfer, and that indirect presentation of this cytosolic protein is mediated by secretion of microvesicles.

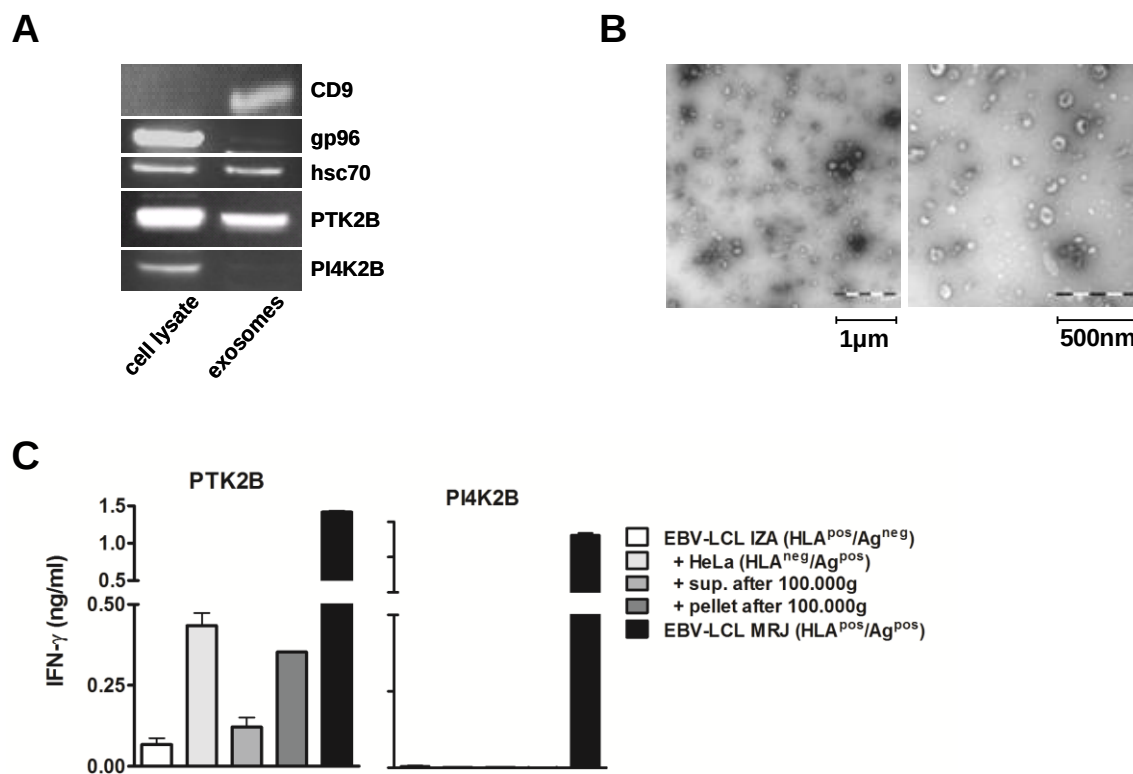


Figure 2: Intercellular transfer is mediated by secreted microvesicles.

(A) Whole cell lysates as well as lysates from exosomal pellets from culture supernatants from HeLa cells retrovirally transduced with PTK2B or PI4K2B were analyzed for the presence of tetraspanin CD9, ER resident gp96, and constitutive heat shock protein hsc70 as well as for the presence of PTK2B and PI4K2B on Western blot.

(B) Pellets obtained after differential high-speed ultracentrifugation at 100.000g were analyzed by electron microscopy. The presence of cup-shaped vesicles with sizes of 30-100nm confirmed isolation of exosomes.

(C) HeLa cells expressing PTK2B (left) or PI4K2B (right) after retroviral transduction, but lacking expression of the relevant HLA class II restriction alleles, were used as donor cells in co-culture experiments with antigen negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles as acceptor cells. In addition, exosomes were isolated from culture supernatants from transduced HeLa cells. The exosomal pellets as well as the depleted supernatants were loaded onto EBV-LCL IZA. Antigen uptake, and subsequent processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN-γ ELISA. Mean values and standard deviation of duplicate wells is shown.

Intercellular transfer of natural HLA class II ligands is independent of the T-cell epitope

Although all six antigens were efficiently processed and presented if administered exogenously as recombinant proteins (Figure 1A), intercellular transfer in co-incubation experiments was only observed for three antigens. To investigate whether intercellular transfer was mediated by the T-cell epitope or whether other protein sequences were relevant in this process, we made retroviral constructs containing the T-cell epitopes exchanged between PTK2B and PI4K2B (Figure 3A), and transduced HeLa cells with these chimeric transgenes (exchange chimera A and B). Culture supernatants from transduced HeLa cells were subsequently loaded on antigen-negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles. We again found transfer of wild-type PTK2B, but not PI4K2B. For the chimeric proteins, we now observed transfer of the PI4K2B T-cell epitope when expressed in the context of the PTK2B protein (Chimera B), whereas transfer of the PTK2B T-cell epitope in the context of the PI4K2B protein (Chimera A) was markedly diminished as compared to wild-type PTK2B (Figure 3B). These data demonstrated that intercellular transfer is not dependent on the T-cell epitope, but rather relies on surrounding protein sequences.

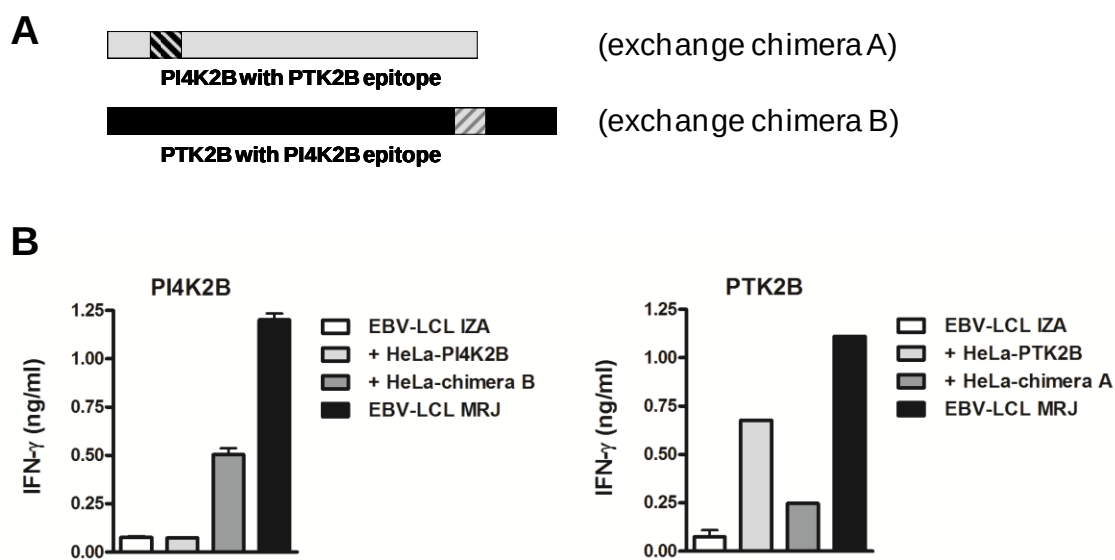


Figure 3: Intercellular transfer of natural HLA class II ligands is independent of the T-cell epitope

(A) Schematic picture of retroviral constructs encoding chimeric PI4K2B and PTK2B proteins with exchanged T-cell epitopes. Exchange chimera A consists of full length PI4K2B with the T-cell epitope of PTK2B, whereas chimera B consists of full length PTK2B with the T-cell epitope of PI4K2B. Protein sequences for PI4K2B and PTK2B are shown in grey and black, respectively, and T-cell epitopes are indicated by hatched areas.

(B) Antigen negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles were loaded with concentrated culture supernatants from HeLa cells transduced with wild-type PTK2B or PI4K2B or epitope-exchange chimeras A and B. Antigen uptake and processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN- γ ELISA. Antigen positive EBV-LCL from patient MRJ served as positive control. Mean values and standard deviations of duplicate wells are shown.

Intercellular transfer via secreted microvesicles is dependent on protein characteristics

To further investigate whether a specific region within the PTK2B or PI4K2B protein is required to mediate or abrogate intercellular transfer, fusion constructs 1 and 3 were generated. Chimera 1 consisted of full-length PI4K2B fused to the C-terminal 207aa of PTK2B, whereas chimera 3 contained the N-terminal 90aa of PI4K2B fused to full-length PTK2B (Figure 4A). Both fusion proteins contained the PI4K2B and PTK2B T-cell epitopes as well as antibody epitopes at their N- and C-terminal regions, respectively. Similar as described for chimera A and B, HeLa cells were transduced with retroviral vectors encoding chimera 1 and 3, and culture supernatants from these cells were loaded on antigen-negative EBV-LCL IZA. For both fusion proteins, we observed transfer of the PI4K2B as well as PTK2B T-cell epitopes, but transfer of both epitopes was significantly lower for chimera 1 as compared to chimera 3 (Figure 4B). Intercellular transfer of chimeric proteins 1 and 3 as measured by T-cell recognition correlated with the amount of protein in the exosomal fractions as detected on Western blot using PTK2B and PI4K2B specific antibodies. All proteins that were efficiently transferred were also abundantly measured in exosomal fractions (PTK2B, chimera B and chimera 3), whereas low recognition of chimera 1 was represented by a weak band on Western blot and no band was visible for proteins that were not transferred (PI4K2B and chimera A) (Figure 4C). Notably, the amount of protein in exosomal fractions correlated closely with the amount of protein as detected in cellular lysates.

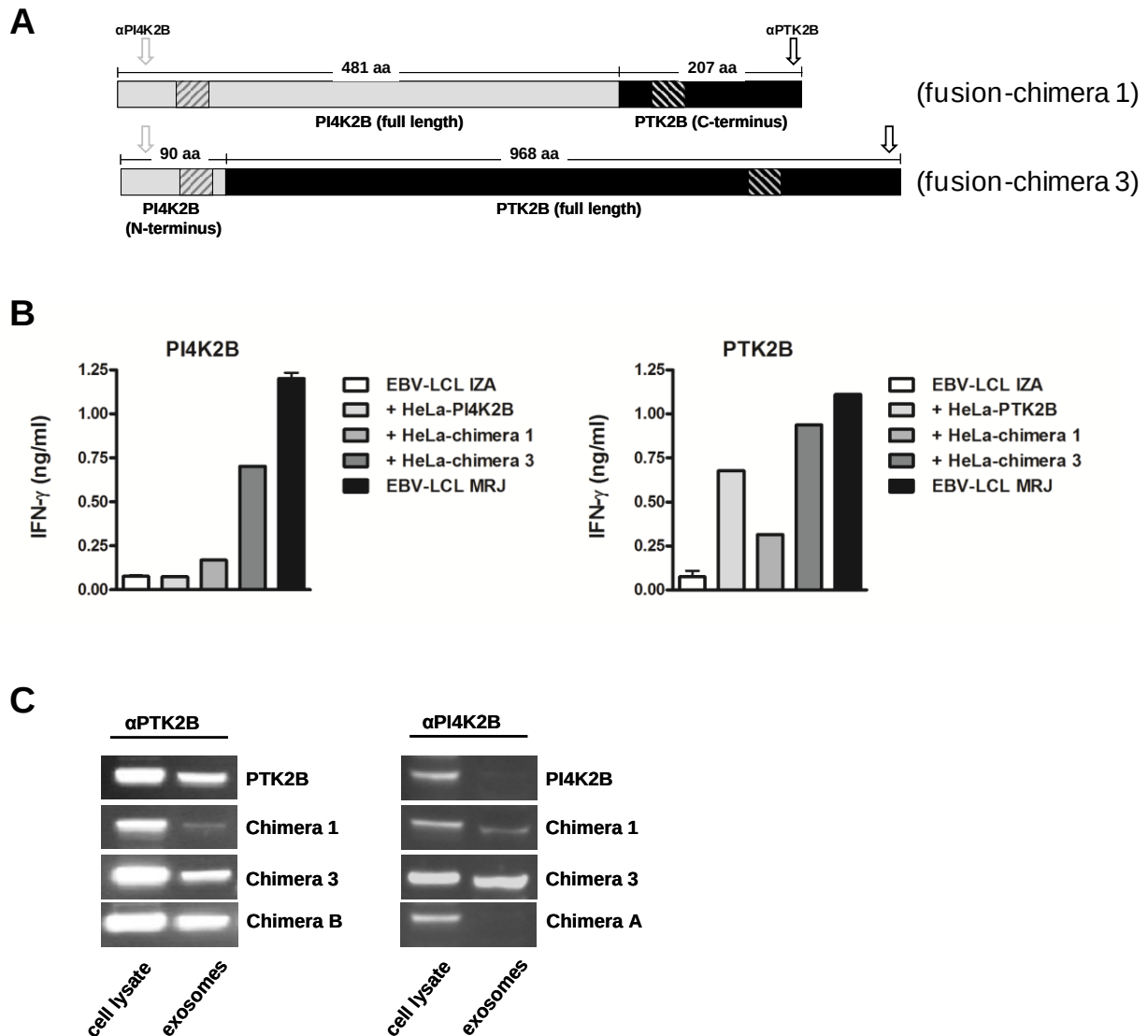


Figure 4: Intercellular transfer via secreted microvesicles is dependent on protein characteristics

(A) Schematic picture of PI4K2B - PTK2B fusion proteins. Fusion-chimera 1 consists of full length PI4K2B fused to the C-terminal 207aa of PTK2B, whereas fusion-chimera 3 consists of the N-terminal 90aa of PI4K2B fused to full length PTK2B. Protein sequences for PI4K2B and PTK2B are shown in grey and black, respectively, and T-cell epitopes are indicated by hatched areas. Arrows indicate the binding sites of antibodies used for detection of PI4K2B and PTK2B on Western blot.

(B) Antigen negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles were loaded with concentrated culture supernatants from HeLa cells transduced with wild-type PTK2B or PI4K2B or fusion-chimeras 1 and 3. Antigen uptake and processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN- γ ELISA. Mean values and standard deviations of duplicate wells are shown.

(C) Whole cell lysates as well as lysates from exosomal pellets from culture supernatants from HeLa cells retrovirally transduced with the genes for wild-type PTK2B or PI4K2B, chimeric protein A or B, or fusion protein 1 or 3 were analyzed on Western blot using antibodies against PTK2B (left) and PI4K2B (right).

In conclusion, our data confirmed that intercellular transfer of cellular proteins by secretion of microvesicles is not mediated by the T-cell epitope, but rather dependent on protein-specific characteristics. By making chimeric proteins, however, a specific region required to mediate or abrogate intercellular transfer could not be identified, but dramatic variations in cytosolic expression of wild-type and chimeric proteins were observed, which correlated closely with the amount of protein that is sequestered into microvesicles.

Intercellular transfer is associated with cellular abundance of the protein.

Since our data demonstrated that protein sequestration into exosomes, and as a consequence intercellular transfer of natural HLA class II ligands is correlated with cytosolic abundance of the protein as measured on Western blot (Figure 4C), we investigated whether the difference in intensity between PTK2B and PI4K2B could be explained by the use of different antibodies for Western blotting or by differences in gene transcription. We therefore generated a retroviral construct encoding PTK2B and PI4K2B both fused to His-tags to allow detection of both proteins by the same anti-His antibody (Figure 5A). Moreover, this retroviral construct encoding PTK2B and PI4K2B linked by a T2A sequence allows equimolar translation of both proteins by a single transcript, thereby excluding differences in gene transcription and protein translation efficiencies between the two transgenes. The retroviral construct was transduced into antigen negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles, and translation of the PI4K2B and PTK2B epitopes was confirmed by measuring recognition by the respective T-cell clones (data not shown). The retroviral construct was subsequently introduced into HeLa cells, which were used as donor cells in co-culture experiments with antigen negative EBV-LCL IZA as acceptor cells. Co-culture experiments again showed transfer of PTK2B, but no transfer of PI4K2B (Figure 5B). Western blot analysis of transduced HeLa cells with an α -His antibody revealed strong expression of PTK2B, whereas only a weak band was visible for PI4K2B (Figure 5C). These experiments showed that despite the use of the same anti-His antibody and equimolar translation of both proteins from a single retroviral transcript, PTK2B was more abundantly expressed than PI4K2B in cellular lysates on Western blot, indicating that the difference in intercellular transfer between PTK2B and PI4K2B is largely determined by a difference in protein stability.

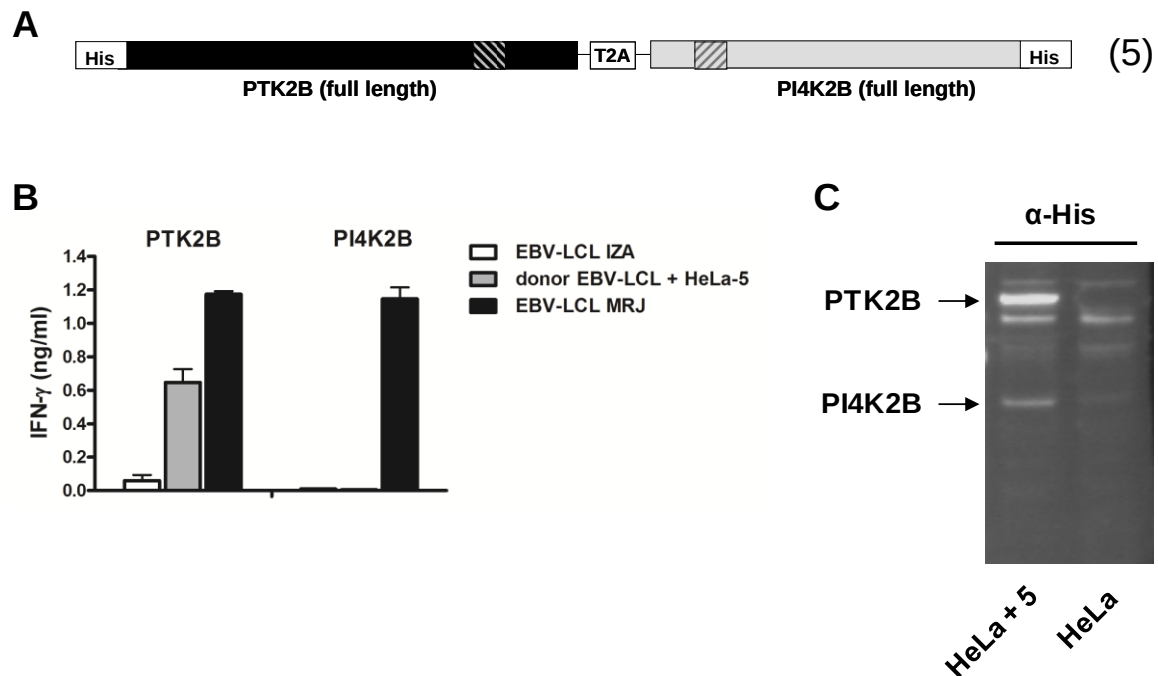


Figure 5: Intercellular transfer correlates with cellular abundance of the protein.

(A) Schematic picture of a retroviral construct encoding full length PTK2B and PI4K2B both fused to His-tags and linked by a T2A sequence (construct 5).

(B) Retroviral construct 5 encoding full length PTK2B and PI4K2B linked by T2A was introduced into HeLa cells lacking the relevant HLA class II restriction alleles. These cells were co-cultured with antigen negative EBV-LCL IZA expressing the relevant HLA class II restriction alleles. Antigen transfer and subsequent processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN- γ ELISA. Mean values and standard deviations of duplicate wells are shown. The data show selective transfer of PTK2B, but not PI4K2B.

(C) Expression of PTK2B and PI4K2B in HeLa cells retrovirally transduced with construct 5 encoding full length PTK2B and PI4K2B linked by T2A was analyzed on Western blot using an α -His antibody. Despite equimolar translation of PTK2B and PI4K2B by a single retroviral transcript, abundant expression of PTK2B and weak expression of PI4K2B is shown, demonstrating that selective transfer of PTK2B via exosomes correlates with increased protein stability.

Selective processes also contribute to intercellular transfer of HLA class II ligands

Recently, a microautophagy-like process has been described to deliver soluble cytosolic proteins into the vesicles of late endosomes / multivesicular bodies¹⁸. Upon fusion of the late endosome with the plasma membrane these intra-endosomal vesicles are released as exosomes. Entry into this pathway was described to be mediated by hsc70-mediated engulfment of KFERQ-containing proteins¹⁸. Although we found that protein sequestration into intraluminal vesicles and subsequent intercellular transfer of natural HLA class II

ligands closely correlated with cellular abundance, evidence was also obtained for involvement of more selective processes. For example, cellular expression of wild-type PI4K2B and chimera 1 was comparable on Western blot (Figure 4C), whereas chimera 1 was more pronounced than wild-type PI4K2B in the exosomal fraction, pointing to a role for the C-terminal 207 AA of PTK2B in selective sequestration into exosomes. Searching the protein sequence of PTK2B revealed the presence of a putative hsc70 binding motif in the C-terminal part of the protein downstream of the T-cell epitope. To investigate the contribution of this putative hsc70 binding motif to intercellular transfer, we mutated two amino acids in this motif to alanine residues (LELKN mutated to LELAA) in order to impair binding to chaperone hsc70 as described by Sahu et al.¹⁸. This mutation was introduced into constructs encoding full-length PTK2B (constructs 11 (wt) and 22 (mut)) and into constructs encoding a truncated version of PTK2B (constructs 14 (wt) and 23 (mut)) containing the T-cell epitope as well as the C-terminal putative hsc70 binding site. The retroviral constructs encoding wild-type and mutated PTK2B were transduced into wild-type HeLa cells and sorted for comparable levels of GFP marker gene expression (Figure 6A). Western blotting of cellular lysates confirmed similar levels of expression of wild type and mutated PTK2B in their full length as well as truncated protein configuration (Figure 6B). Despite similar levels of expression, intercellular transfer of both full length as well as truncated PTK2B bearing the mutation was markedly decreased as compared to the wild-type proteins (Figure 6C, D). In conclusion, our data demonstrate that intercellular transfer of natural HLA class II ligands is mediated by secretion of microvesicles and that protein sequestration into these intraluminal vesicles within antigen positive donor cells closely correlates with cellular abundance. Moreover, in addition to cellular abundance, selective processes also contribute to the total amount of protein that is sequestered into microvesicles as illustrated by involvement of the hsc70 binding motif for PTK2B. Cellular abundance in combination with selective processes within antigen positive donor cells therefore determine the final levels of natural HLA class II ligands that are expressed on the surface of acceptor cells.

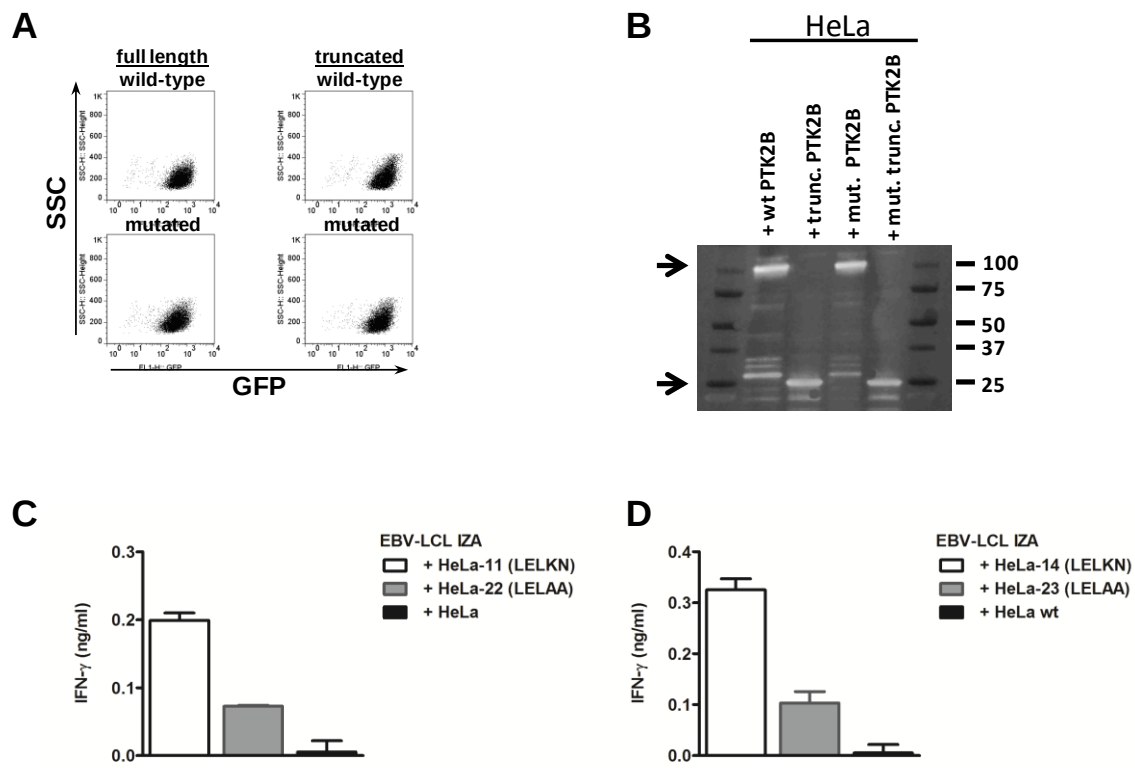


Figure 6: Selective processes also contribute to intercellular transfer of natural HLA class II ligands.

(A) Retroviral constructs encoding wild type (constructs 11/14) and mutated (constructs 22/23) full length (constructs 11/22) and truncated (constructs 14/23) PTK2B were introduced into HeLa cells lacking the relevant HLA-DRB3*0101 restriction allele. Mutated constructs contained two altered amino acids in the most C-terminal putative hsc70 binding site (LELKN mutated to LELAA). Truncated constructs started at position 773 aa and contained the T-cell epitope from position 791 to 800 aa. HeLa cells transduced with the different PTK2B variants were sorted by flowcytometry based on comparable expression of the EGFP transgene.

(B) Western blot analysis of whole cell lysates of HeLa cells transduced with wild-type and mutated full-length or truncated PTK2B constructs. Due to the C-terminal binding site of the anti-PTK2B antibody all constructs could be visualized by the same antibody.

(C) HeLa cells transduced with retroviral constructs encoding wild type (LELKN, construct 11) and mutated (LELAA, construct 22) full length PTK2B were co-cultured with antigen negative EBV-LCL IZA expressing the relevant HLA-DRB3*0101 restriction allele. Antigen transfer and subsequent processing and presentation into HLA class II was assessed by measuring T-cell recognition in IFN- γ ELISA. One representative of three independent experiments is shown.

(D) Intercellular transfer was tested as described above for constructs encoding wild-type (LELKN, construct 14) and mutated (LELAA, construct 23) truncated PTK2B.

DISCUSSION

Priming of naïve CD4⁺ T-cells by professional APC is a crucial step in the initiation of a potent immune response. The HLA class II processing pathway is perfectly equipped to efficiently present exogenous threats to CD4⁺ T-cell surveillance². However, when it comes to intracellular dangers such as viruses or malignant transformation in HLA class II negative cells, efficient CD4⁺ T-cell priming requires release of antigen by cellular death or active transport of intracellular antigens to professional APC. We here demonstrate that certain cytosolic proteins can be actively transferred between viable cells and that transfer is mediated via secreted microvesicles. Sequestration into microvesicles has been shown to depend on certain protein characteristics, which include cellular abundance and, in case of low abundance, on a putative binding site to heat shock cognate hsc70.

Most viral infections and malignant transformations do not occur in professional APC and transport of foreign or mutated cytosolic proteins from infected/transformed cells to professional APC is therefore beneficial or even essential for early and efficient induction of cellular immune responses. For CMV and influenza, it has been shown that viral proteins are transported from infected cells to professional APC via exosomes, and that this leads to activation of CD4⁺ T-cells^{19,20}. It is by now also well established that most tumor cell lines release exosomes²¹, and isolation of these vesicles from several body fluids, such as ascites, pleural effusion, urine and serum, emphasizes their existence *in vivo*^{22,23}. The downstream effects of these tumor-derived vesicles, however, are rather controversial²⁴. While several studies show immunosuppressive properties, there is also considerable evidence for immunosupportive effects, what may be explained by antigen transfer and subsequent presentation to T-cells with either regulatory or effector function.

For MHC class I restricted tumor antigens it has been reported by Wolfers et al²⁵ that immunization of mice with tumor derived exosomes was more potent in tumor rejection than vaccination with tumor lysates or apoptotic bodies. Furthermore, they demonstrated that differentiation antigen MART-1/Melan-A is sequestered as full length protein into exosomes and that this antigen can be cross presented in APC leading to activation of CD8⁺ T-cells. In another mouse study in which different tumor cell vaccines were compared for

their immunogenicity, targeting OVA to exosomes was shown to be superior in inducing CD8⁺ T-cell mediated tumor rejection as compared to membrane bound or soluble OVA²⁶. Increased vaccine potency upon antigen targeting to exosomes has also been demonstrated by various other studies^{27,28}.

We here analyzed intercellular transfer of six natural HLA class II ligands, and demonstrated that three antigens could be transferred via exosomes. Several studies have shown that exosomes harbor a distinct set of proteins^{18,25}. Exosomes are known to be derived from intraluminal vesicles of the late endosome where they originate from inward budding of the endosomal membrane thereby allowing invagination of cytoplasmic proteins²⁴. Sahu et al.¹⁸ described a microautophagy-like process in which cytoplasmic proteins bind to hsc70 which are then directed via the ESCRT complex to inward budding parts of the endosomal membrane. This mechanism has been described for proteins with a KFERQ-like motif, and may (partly) explain the distinct protein composition of exosomes. Our data also point to a role of the C-terminal KFERQ-like motif in intercellular transfer of PTK2B, as illustrated by hampered intercellular transfer of PTK2B upon mutation of this motif and induced transfer of a protein which cannot be transferred in its natural configuration (PI4K2B) upon fusion to this motif. Interestingly, Sahu et al.¹⁸ also demonstrated that high cellular expression of proteins without KFERQ-like motif can overrule this selective process leading to sequestration of abundant proteins into exosomes via bulk microautophagy. This is supported by our observation that intercellular transfer closely correlates with cellular abundance of the protein. The significance of bulk and selective microautophagy for induction of immune responses *in vivo*, however, is still unknown and needs to be unraveled.

From a biological point of view it is reasonable to argue that abundantly expressed proteins are transported to professional APC as dividing intracellular pathogens mostly reach high cytosolic copy numbers, and optimal priming of immune cells against these pathogenic infections requires antigen presentation on professional APC. The *in vivo* role of the KFERQ-like motif, however, is unclear. As described above, the KFERQ-like motif is involved in a microautophagy-like process, but also plays a central role in chaperone-mediated autophagy where cytosolic proteins bind to hsc70 to facilitate transport via

LAMP2A across the lysosomal membrane²⁹. Studies have shown that approximately one third of cytosolic proteins harbor a KFERQ-like motif, and in the early nineties when this motif has first been described, it was shown both *in vitro* and *in vivo* that upon starvation cytosolic proteins with a KFERQ-like motif are preferentially degraded indicating their uptake into lysosomes³⁰. The benefit of the KFERQ-like motif and the checkpoint of multi-vesicular bodies directing them to lysosomal degradation or exosomal release, however, are unresolved and remain to be elucidated.

We here demonstrated indirect presentation of three natural HLA class II ligands, including antigen DDX3Y which is encoded on the Y-chromosome. Some years ago, it has already been shown that this protein can lead to CD4⁺ T-cell mediated tumor rejection independent of MHC class II expression of the tumor¹². Furthermore, recipient derived DDX3Y presented on donor APC has been reported to induce CD4⁺ T-cell mediated graft-versus-host disease in mice³¹. CD4⁺ T-cell mediated rejection of an MHC class II negative tumor requires several steps. First, antigens should be released and transferred from MHC class II negative tumor cells to MHC class II positive APC. We here show that DDX3Y can be intercellularly transferred and that this transfer is mediated by secreted microvesicles. Sequestration of DDX3Y into exosomes has also been demonstrated by others, showing that mice injected with exosomes isolated from male cells were capable of mounting a DDX3Y-specific CD4⁺ T-cell response³². In a next step, activated CD4⁺ T-cells need to recruit other effector cells in order to mediate rejection of the MHC class II negative tumor¹⁰⁻¹². In mouse models macrophages and NK-cells have been suggested to play a role, but also other immune cells might be of importance. Further studies are required to elucidate whether one special type of immune cell plays a crucial role in rejection of MHC class II negative tumors, or whether it is mediated by an orchestrated immune response with the CD4⁺ T-cell as central regulator.

In conclusion, we here showed that a subset of natural HLA class II ligands can be intercellularly transferred via secreted microvesicles, and that sequestration into microvesicles and subsequent transfer is dependent on protein characteristics, including cellular abundance and presence of specific hsc70 binding sites. Indirect antigen presentation has been shown to mediate beneficial but also detrimental effects, and it is

therefore relevant to take intercellular transfer into consideration when selecting a tumor antigen for cellular immunotherapy or vaccination.

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7

SUMMARY AND GENERAL DISCUSSION

SUMMARY

Allogeneic stem cell transplantation is a potentially curative treatment for many hematological malignancies. The beneficial Graft-versus-Leukemia (GvL) effect is thereby mediated by donor-derived T-cells recognizing the malignant cells of the patient as foreign. However, the same T-cells might also target healthy non-hematopoietic cells of the patient such as skin, liver and gut causing a detrimental and sometimes lethal side effect, referred to as Graft-versus-Host disease (GvHD). Although, removal of T-lymphocytes from the stem cell graft significantly reduces severity and frequency of GvHD, it also results in a higher frequency of infections and leukemic relapses after allogeneic stem cell transplantation. Early administration of unmanipulated donor lymphocyte infusion (DLI) again lowers the risk or even treats leukemic relapses but it is also often associated with induction of GvHD.

In HLA-matched stem cell transplantation donor-derived T-cells recognize polymorphic peptides presented in self-HLA, so called minor histocompatibility antigens (MiHA). As these peptides are derived from intracellular proteins one strategy to induce GvL effect without GvHD is to selectively transfer T-cells directed against hematopoiesis restricted antigens. This however requires identification of a multitude of MiHA to select for antigens with a suitable expression profile. Another strategy is to exploit CD4⁺ T-cells, as HLA class II molecules are normally predominantly expressed on the hematopoietic system. However, under inflammatory conditions HLA class II might be up-regulated on non-hematopoietic tissues. Furthermore, until recently the identification of HLA class II restricted MiHAs was hampered by technical limitations. In this thesis we sought to identify and characterize HLA class II restricted MiHA and to analyze their intracellular processing to obtain insights on their role in GvL effect and GvHD.

In **chapter 2** we identified four new HLA class II restricted MiHA that induced CD4⁺ T-cell responses in a patient who underwent DLI for relapsed chronic myeloid leukemia after HLA-identical allogeneic stem cell transplantation. We isolated activated CD4⁺ T-cell clones from patient bone marrow obtained 5 weeks after DLI. By screening a recombinant bacterial cDNA library we identified LB-PTK2B-1T, LB-MR1-1R, LB-LY75-1K and LB-

MTHFD1-1Q as targets of MiHA specific CD4⁺ T-cell clones. We further showed that these HLA class II restricted MiHA were presented by HLA-DRB3*0101, DRB3*0202, DRB1*1301 and DRB1*0301, respectively. All four MiHA had a balanced population frequency between 25 and 70% and two of the identified antigens, PTK2B and LY75, showed preferential expression in hematopoietic cells. All MiHA specific CD4⁺ T-cell clones were able to recognize primary AML and ALL cells, however, only the T-cell clone specific for LB-MTHFD1-1Q was capable of recognizing primary CML progenitor cells of the patient, while all of them were able to recognize activated CML cells.

We observed that some cell types were not recognized by the MiHA specific CD4⁺ T-cell clones despite significant expression of the MiHA encoding gene and the HLA restriction molecule. Therefore, we hypothesized that cell-type specific processing of these antigens into the HLA class II pathway might play a role in determining their presentation. To analyze this in more detail we developed a cell line model based on the HLA class II negative cell line HeLa and investigated the influence of known molecules within the HLA class II processing route on the presentation of our newly identified MiHA. In **chapter 3** we describe that HLA class II restricted antigens can be divided in two distinct groups based on their behaviour towards HLA-DM. One group of antigens is strongly presented in the presence of HLA-DM (DM-resistant), whereas presentation of the other group of antigens is diminished or completely abrogated by HLA-DM and restored upon co-expression of HLA-DO (DM-sensitive). We could demonstrate in different cell line models that LB-MR1-1R, LB-LY75-1K and previously identified LB-PI4K2B-1Q⁴³ were DM-sensitive, while LB-PTK2B-1T, LB-MTHFD1-1Q and DBY displayed a DM-resistant phenotype. We further showed that DM-sensitivity is dependent on the T-cell epitope and was mediated via interaction between HLA-DM and the HLA-DR restriction molecule. Lack of interferon- γ mediated up-regulation of the HLA-DO β -chain precluded efficient presentation of DM-sensitive antigens on non-hematopoietic cells even under pro-inflammatory conditions. This suggests that CD4⁺ T-cells directed against DM-sensitive antigens might bear a lower risk of inducing GvHD and might therefore be attractive candidates for immunotherapy. In **chapter 4** we analyzed whether DM-sensitive antigens would be good targets for mediating GvL effect. We showed that HLA-DO is widely expressed in primary leukemic cells and that in particular CLL and some ALL cells express

high levels of HLA-DO α - and β -chain comparable with expression levels observed in healthy B-cells and mature dendritic cells. We further demonstrated by silencing of HLA-DO in ALL and Burkitt lymphoma cell lines that endogenous levels are sufficient and necessary to present DM-sensitive antigens. In line with their higher HLA-DO expression levels primary CLL cells were superior to ALL cells in presenting DM-sensitive antigens.

After having so far focused on the direct effector function of MiHA specific CD4⁺ T-cells, in **chapter 5** we analyzed their potential as helper cells. Despite the known function of CD4⁺ T-cells as helper cells and their ability to promote production of IgG antibodies, coordinated CD4⁺ T- and B-cell responses after alloSCT have not extensively been characterized, and have only been described for an antigen encoded on the Y-chromosome in male patients transplanted with female donors. One of the here identified MiHA containing proteins, PTK2B, has also been described as antibody target in earlier studies. We therefore investigated whether a coordinated T- and B-cell response against PTK2B was induced. We indeed observed development of a humoral immune response against PTK2B after DLI. In contrast to the T-cell epitope, the antibody target was located in a C-terminal monomorphic region of the protein, suggesting a coordinated allo-reactive CD4⁺ T-cell and auto-reactive B-cell response against an autosomal antigen.

These data again underline the central role of CD4⁺ T-cells as helper cells in adaptive immunity. Presentation of exogenous antigens in MHC class II by professional antigen presenting cells is believed to be the first step in induction of adaptive immune responses. For efficient induction of immunity against intracellular threats such as viruses or malignant transformations, intracellular antigens from infected or transformed cells need to be transferred to surrounding APC to allow efficient priming of naïve CD4⁺ T-cells. In **chapter 6** we investigate the mechanism of indirect antigen presentation of six natural HLA class II epitopes. We demonstrate that 3 of the 6 HLA class II epitopes could be transferred between cells, and that intercellular transfer was mediated by full-length proteins. Transfer of these antigens led to T-cell recognition of previously antigen-negative acceptor cells. Furthermore, we demonstrated for PTK2B that intercellular antigen transfer was not dependent on the T-cell epitope, but that release was largely determined by protein characteristics such as cellular abundance and specific binding sequences. This mechanism

of antigen transfer from HLA class II-negative cells to surrounding APC may play a crucial role in induction of immunity against intracellular viruses or transformed cells.

GENERAL DISCUSSION

Characterization of HLA class II restricted MiHA

In chapter 2 we reported the identification of four new HLA class II restricted MiHA. By testing T-cell recognition of serial truncations of the identified epitopes we sought to determine the minimal amino acid sequences necessary for T-cell activation. In contrast to often clear-cut results for HLA class I restricted epitopes^{33,34,42,75}, definition of a minimal epitope for HLA class II restricted antigens is more difficult. For three of the four novel HLA class II restricted MiHA, a minimal peptide could be identified that still gave T-cell activation. However, the level of T-cell activation after stimulation with this minimal peptide was much weaker and required higher concentrations than upon loading with 15- or 19-mer peptides, suggesting that the minimal peptide may not be the epitope that is naturally presented at the cell surface and recognized by the T-cell. For LB-PTK2B-1T, serial peptide truncations revealed a core region of 9 amino acids that was necessary but not sufficient for T-cell recognition similar as described for the CD19L specific CD4⁺ T-cell clone by Spaapen et al⁴⁵. For T-cell recognition of a minimal peptide, an additional amino acid was needed either at the N- or C-terminus of the core region. It has been described for HLA class II restricted epitopes that the classical 9-mer core region lies in the center of a longer peptide stretch and that the peptide-HLA complex is stabilized by N- and C-terminal residues⁷⁶. We did not investigate whether limited T-cell activation by our minimal epitopes is due to ineffective binding of the peptide to the HLA molecule or whether N- and C-terminal residues are directly contacted by the T-cell receptor. Interestingly, DM-sensitivity as described in chapter 3 might be due to lower affinity between the peptide and the HLA restriction molecule making this interaction vulnerable to the interference of HLA-DM^{77,78}. It might therefore be interesting to investigate whether DM-resistant epitopes can become DM-sensitive by cleaving or exchanging specific N- or C-terminal residues. Evidence supporting this hypothesis is given by the recognition profile of the 12-mer LB-PTK2B-1T epitope when expressed in the context of the PI4K2B protein in HeLa cells transduced with the invariant chain +/- HLA-DM. In these experiments, LB-PTK2B-1T surrounded by non-cognate amino acids acquires a less stringent DM-resistant phenotype as compared to LB-PTK2B-1T in its natural protein context. To demonstrate

that HLA-peptide stability is indeed the underlying factor that determines DM-sensitivity, it can be investigated whether the DM-sensitive phenotype becomes more pronounced when a 10-mer, instead of a 12-mer, LB-PTK2B-1T epitope is introduced into PI4K2B and expressed in HeLa cells transduced with the invariant chain +/- HLA-DM.

To determine the actual length of the naturally presented MiHA epitopes, peptides need to be eluted from HLA class II surface molecules. These experiments are, however, more complicated than for HLA class I and may show that not a single peptide, but different length variants are generated within the lysosome and presented on the cell surface.

MiHA specific CD4⁺ T-cells in anti-tumor immunity

In chapter 2 we describe the isolation of 25 MiHA specific CD4⁺ T-cell clones from bone marrow of a patient with relapsed CML. The disease relapse was treated with DLI and the bone marrow sample was taken 5 weeks after intervention. After treatment with DLI, the patient experienced a complete and on-going remission and developed only mild GvHD of the skin. The specificity of 19 T-cell clones could be determined. Of these T-cell clones, 9 clones recognized LB-PI4K2B-1S in HLA-DQB1*0603⁴³, 3 clones LB-PTK2B-1T in HLA-DRB3*0101, 2 clones LB-MR1-1R in HLA-DRB3*0202, 2 clones LB-LY75-1K in HLA-DRB1*1301 and one clone LB-MTHFD1-1Q in HLA-DRB1*0301. The specificity of the 2 remaining T-cell clones has been identified by whole genome association scanning⁴² as an epitope from the PIP4K2A protein presented by HLA-DRB1*0301 (data not shown in this thesis). In total 6 HLA class II restricted MiHA have been identified as targets for CD4⁺ T-cells in this patient after DLI. While all CD4⁺ T-cell clones recognized activated CML cells, only two MiHA (LB-MTHFD1-1Q and LB-PIP4K2A-1S) were efficiently recognized on CD34⁺ CML progenitor cells of the patient, indicating that CD4⁺ T-cells for LB-MTHFD1-1Q and LB-PIP4K2A-1S are relevant effector cells in eradication of the disease. In addition, CD4⁺ T-cells for these two MiHA may have contributed to induction of CML-APC via CD40-CD40L interaction and cytokine production⁷⁹⁻⁸¹. Subsequently, these CML-APC may have induced CD4⁺ T-cells for other HLA class II restricted MiHA, which in their turn may have played a critical role as helper cells in augmenting the anti-

tumor response by activation of CD8⁺ T-cells and B-cells. Induction of MiHA specific T-cells by APC of patient origin different from CML-APC is unlikely as the patient achieved more than 99% donor chimerism in non-malignant hematopoietic cells before DLI.

The precise contribution of MiHA specific CD4⁺ T-cells to the overall anti-tumor response is difficult to determine as the patient received unmodified DLI and also developed a CD8⁺ T-cell response against at least 7 different HLA class I restricted MiHA, including HA-1 and HA-2 in HLA-A*0201, 4 identified MiHA in HLA-B*4001 and one MiHA in HLA-B*0801^{75,82}. It has, however, been shown previously that administration of CD8 depleted DLI has no negative impact on conversion to full donor chimerism^{83,84} and that application of highly purified CD4⁺ DLI in a mouse model of human ALL can lead to rejection of leukemia⁷⁹. Furthermore, CD4⁺ DLI was shown to induce expansion of MiHA specific CD8⁺ T-cells in the patient⁸⁵, underlining the importance of CD4⁺ T-cells not only as effector cells but also as helper cells in anti-tumor immunity.

MiHA specific CD4⁺ T-cells as helper cells

In chapter 5 we further focused on the helper potential of the CD4⁺ T-cell clone directed against LB-PTK2B-1T. We demonstrated that *in vitro* this T-cell clone was able to induce maturation of dendritic cells and activation of B-cells in an antigen-dependent manner. Furthermore, we could show that this T-cell clone expanded in the patient after DLI, and that T-cell expansion was paralleled by development of a PTK2B-specific antibody response. It has been shown earlier that antibody responses emerge after DLI^{86,87}, but development of a coordinated T- and B-cell response after alloSCT against antigens derived from the same protein has only been shown for male antigen DBY⁸⁸. For this male antigen, the T-cell was shown to be auto-reactive recognizing both DBX and DBY, whereas the antibody was specific for the Y-chromosome variant. On the contrary, the here described coordinated immune responses against PTK2B includes a patient-specific allo-reactive CD4⁺ T-cell, while the antibody is auto-reactive recognizing a monomorphic part of the protein that is identical between patient and donor. The antibody response was detected in patient serum obtained two years after DLI, indicating a profound and sustained immune

response against this intracellular protein. Development of the antibody response after DLI was not associated with clinical GVHD or autoimmune reactions in this patient probably due to the intracellular location of PTK2B. It is, however, intriguing that allogeneic CD4⁺ T-cell responses might induce a break in B-cell tolerance and thereby cause induction of antibodies, which upon binding to cell-surface antigens may contribute to development of clinical responses (GvL, GvH, or autoimmunity) after allSCT. Although antibodies against intracellular proteins such as PTK2B are not expected to mediate direct cytotoxicity, the formation of immune complexes with released antigen from dying cells may have stimulated antigen presentation by professional APC, and as such, antibodies against intracellular proteins may stimulate patient-specific T-cells and augment the anti-tumor immune response.

DM sensitivity is a common phenomenon for endogenous HLA class II antigens

In chapter 3 we described the processing of 5 HLA class II restricted MiHA and one HLA class II restricted male antigen. We demonstrated that these antigens can be separated in two distinct groups based on their behavior towards HLA-DM. While DM-resistant antigens are efficiently presented in the context of HLA-DM, presentation of DM-sensitive antigens is abolished by expression of HLA-DM. HLA-DM has been described as chaperone that greatly accelerates peptide exchange in MHCII⁷⁷ and various studies have shown that HLA-DM induces release of peptides binding to MHCII with low affinity, resulting in a selection process that favours presentation of high affinity peptides^{77,78,89}. The here described DM-resistant T-cell epitopes are therefore expected to bind with higher affinity to HLA class II than the DM-sensitive T-cell epitopes. Nevertheless, we observed the DM-sensitive phenotype for 3 of 6 of our identified HLA class II restricted MiHA, and screening of T-cell libraries from healthy donors also revealed a high frequency of circulating T-cells for DM-sensitive antigens. This seems contradictory to the notion that HLA-DM serves to select for stable HLA-peptide complexes to allow optimal priming of CD4⁺ T-cells. While this might be true for exogenous antigens which are available in limited amounts and need to be stably presented until the peripheral APC has migrated to the lymph node to present the antigen to CD4⁺ T-cells, different rules might apply for

endogenous antigens. Our findings clearly demonstrate that significant numbers of circulating T-cells are specific for DM-sensitive antigens, illustrating that these HLA class II antigens are immunogenic and efficiently presented by (specific subsets of) professional APC *in vivo*. We demonstrated that the behavior of HLA class II antigens towards HLA-DM is independent of the delivery pathway, i.e. whether the antigens were endogenously expressed from transduced genes or exogenously delivered as recombinant proteins. However, in the context of continuous antigen supply from endogenous sources and constant assembly with the presenting HLA molecule, the need for long-lived HLA-peptide complexes is possibly outpaced allowing sufficient presentation of DM-sensitive antigens.

The analyzed T-cell epitopes are presented by different HLA class II alleles, each with its own binding characteristics and kinetics of HLA-DM catalysed peptide editing⁷⁸, and an accurate analysis and comparison of HLA class II binding affinity and half-lives between the here described DM-sensitive and DM-resistant T-cell epitopes is therefore difficult. However, we investigated the persistence of HLA-peptide complexes by loading EBV-LCL with recombinant proteins and measured T-cell recognition at different time points after protein loading and washing. Surprisingly, all HLA-peptide complexes were shown to be extremely stable, as demonstrated by detectable T-cell recognition up to 4 weeks after protein loading. In this experiment, we failed to show any clear difference in HLA-peptide stability between DM-resistant and DM-sensitive antigens. However, it would be interesting to analyze whether the presented peptide pool differs dependent on the source of the antigen and whether exogenous antigen delivery screws the repertoire towards DM-resistant antigens.

Another factor that may influence the behavior of the epitope towards HLA-DM may be the presenting HLA class II restriction molecule. The interaction between HLA-DR and HLA-DM has been located to the lateral surface of HLA-DR and certain amino acids have been shown to be crucial for this interaction^{90,91}. Doebele et al.⁹⁰ demonstrated the relevance of these amino acids for HLA-DRB1*0301, and we could confirm that the two described residues in the DR alpha chain are also essential for the DM-sensitive behavior of LB-LY75-1K and LB-MR1-1R that are presented in DRB1*13:01 and DRB3*02:02, respectively. Our third DM-sensitive antigen LB-PI4K2B-1S, however, is presented in

HLA-DQB1*06:03. The alpha chain of HLA-DQ is far more polymorphic than for HLA-DR and, although the two residues at position 40 and 51 are conserved in HLA-DQA1*01:03, mutating these amino acids had no influence on presentation of DM-sensitive antigen LB-PI4K2B-1S (data not shown in this thesis), indicating that the interaction between HLA-DQ and HLA-DM follows different rules. Interestingly, T-cell library experiments demonstrated that the majority of HLA-DR restricted T-cells recognize DM-resistant antigens, whereas the proportion of T-cells for DM-sensitive antigens was clearly higher for HLA-DQ.

The role of HLA-DO

In chapter 3 and 4 we demonstrated in cell line models and primary leukemic cells that presentation of DM-sensitive antigens is dependent on expression of HLA-DO. This non-classical HLA class II molecule is expressed in mature dendritic cells, B-cells and thymic epithelial cells and has been identified as inhibitor of HLA-DM⁵⁷⁻⁵⁹. Recently, Guce et al.⁹² elucidated the crystal structure of the DO-DM complex, and demonstrated that DO and DM bind in a side-by-side arrangement similar to that proposed for MHC class II and DM. HLA-DO thus acts as a substrate mimic that tightly binds to DM, thereby preventing access to MHC class II. The authors also demonstrated that HLA-DO inhibits DM-mediated peptide release in a dose-dependent manner. Since DM is expressed at higher levels than DO, free DM and DO-DM complexes are both present, and the major mechanism for shifting their balance is via transcription of the DOB subunit⁹³. Interestingly, it has been shown by us and others that the β -subunit of HLA-DO is not or hardly up-regulated by inflammatory cytokines⁹⁴. We hypothesized that this unique characteristic might be exploited for selective cellular therapy against DM-sensitive antigens to reduce the risk or severity of GvHD. In order to establish a GvL response, however, DM-sensitive antigens need to be efficiently presented on leukemic cells. We could show that primary CLL cells highly express HLA-DO both on mRNA and protein levels and that CLL cells are very well capable of presenting DM-sensitive antigens. Most ALL and AML cells were also able to activate T-cell clones for DM-sensitive antigens, but the activation levels were often considerably lower and correlated with HLA-DO expression (data not shown in this thesis).

Notably, AML cells and cytokine pre-treated fibroblasts show comparable levels of HLA-DOA and –DOB expression in microarray gene expression analysis as depicted in chapter 4. Nevertheless, most AML cells are recognized by specific T-cell clones whereas cytokine pre-treated fibroblasts are only recognized after additional transduction with HLA-DOB. These experiments indicate that HLA-DOA and DOB expression are not the only factors that determine the threshold for T-cell recognition, but that other factors such as surface expression of adhesion and co-stimulatory molecules also play a role. In contrast to IFN- γ pre-treated fibroblasts, AML cells express a variety of co-stimulatory and adhesion molecules, including CD11c, CD54, CD83 and CD86 (data not shown in this thesis), and T-cell reactivity against DM-sensitive antigens on AML cells may therefore be stimulated at relatively low HLA-DO expression levels. A minimal level of HLA-DO expression is, however, necessary for presentation of DM-sensitive antigens as demonstrated by decreased recognition after silencing of HLA-DOA in ALL cell line LB-ALL-SK (chapter 4) and complete lack of recognition of AML cells co-expressing CD33 and CD14 (data not shown in this thesis). Microarray gene expression analysis demonstrated a difference in HLA-DOA expression between CD33⁺ CD14⁺ (HLA-DOA positive) and CD33⁺ CD14⁻ (HLA-DOA negative) AML cells isolated from the same patient, and these HLA-DOA expression levels correlated with a difference in T-cell recognition of DM-sensitive antigens, whereas DM-resistant antigens were similarly presented on both AML subsets (data not shown in this thesis). The data presented in chapter 3 and 4 show how intracellular processing mechanisms might be used in the future to develop selective cellular therapy.

Intercellular transfer of HLA class II restricted antigens

In chapter 6 we demonstrate that some of the identified HLA class II restricted epitopes can be processed and presented after intercellular transfer of the antigen. Remarkably, intercellular transfer of HLA class II ligands has only been observed for DM-resistant antigens. Although this observation seems to support the hypothesis that exogenous antigen delivery may screw the HLA-peptide repertoire towards DM-resistant antigens, we also demonstrated intercellular transfer of a DM-sensitive antigen when introduced at the

position of a DM-resistant antigen in another protein. This experiment demonstrates that intercellular transfer is not dependent on the behavior of the epitope towards HLA-DM, but rather on protein sequences located outside the T-cell epitope. Our data provide evidence that protein stability is a relevant factor that determines whether HLA class II ligands can be intercellularly transferred, but other factors and mechanisms are also likely to play a role.

Intercellular transfer of HLA class II ligands is probably not of high clinical relevance for HLA class II expressing malignancies, such as highly immunogenic chronic phase CML. However, one could imagine that cellular therapy of leukemia with lower immunogenicity such as ALL or blast crisis CML might benefit from T-cell induction by professional APC after intercellular transfer of antigens. Apart from hematological malignancies, indirect antigen presentation may be of strong clinical relevance for solid tumors of which the majority are HLA class II negative. It has already been shown in mouse studies, that CD4⁺ T-cells can lead to tumor rejection of MHC class II negative tumors via indirect presentation of the antigen ^{95,96}. While the precise effector mechanism is not clear, it has been shown that activation of CD4⁺ T-cells by tumor-derived antigens on surrounding APC is crucial ⁹⁷. Various studies have shown that chemokines and cytokines as secreted by activated CD4⁺ T-cells during initiation of the immune response may attract and activate macrophages, which subsequently play a relevant role in the effector phase of the anti-tumor response. In contrast to direct targeting of tumor cells by CD8⁺ T-cells, indirect presentation of antigens to CD4⁺ T-cells bears the attractive benefit that tumor cells cannot escape via downregulation of MHC molecules. The question remains whether transferred antigen is released from dying cells or whether an active mechanism is involved. Although experiments in chapter 6 of this thesis as well as recent unpublished data point towards a selective secretion mechanism, confounding factors such as protein expression, stability, peptide-MHC interaction and TCR affinity complicate data interpretation.

Concluding remarks

To exploit CD4⁺ T-cells as delayed DLI after T-cell depleted allogeneic stem cell transplantation is a promising strategy. It combines the effector and helper function of CD4⁺ T-cells to prevent leukemic relapses and infections with the benefit of selectively targeting hematopoietic cells due to the restricted expression of HLA class II. Additional modification of CD4⁺ T-cells by selecting T-cells recognizing DM-sensitive antigens appears a promising strategy to further reduce the risk for GvHD. Depletion of donor T-cells that are reactive with cytokine pre-treated fibroblasts of the patient may be a possible strategy to enrich for CD4⁺ T-cells recognizing DM-sensitive antigens. It needs to be shown, however, whether administration of T-cells for DM-sensitive antigens leads to induction of effective GvL responses, and thus whether isolation of these T-cells is truly advantageous and pays out the additional efforts.

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8

SYNOPSIS IN DUTCH - NEDERLANDSE SAMENVATTING

ACKNOWLEDGEMENTS

CURRICULUM VITAE

LIST OF PUBLICATIONS

NEDERLANDSE SAMENVATTING

Allogene stamceltransplantatie

Allogene stamceltransplantatie is een succesvolle behandeling voor patiënten met kwaadaardige aandoeningen van het bloedvormend systeem. Voorafgaande aan de transplantatie wordt de patiënt behandeld met chemotherapie vaak gecombineerd met bestraling om het eigen bloedvormend systeem en de eigen afweer te vernietigen. Oorspronkelijk was de transplantatie bedoeld om residuele leukemie cellen met een lethale dosis chemotherapie te vernietigen en aansluitend het bloedvormend systeem met stamcellen van een gezonde donor te herstellen. Het anti-leukemie effect was echter veel sterker en langduriger dan verwacht, wat verklaard kan worden door een afweerreactie van donor cellen tegen de leukemie cellen van de patiënt. Helaas kan hetzelfde effect ook tegen gezonde weefsels van de patiënt optreden. Afstotingsreacties tegen organen zoals huid, darm en lever kunnen mild zijn, maar ook een levensbedreigende complicatie vormen bekend als graft-versus-host disease (GvHD).

Met de ontdekking van de humane leukocyt antigenen (HLA) werd duidelijk dat één of meerdere verschillen tussen patiënt en donor voor deze hoog polymorfe antigenen een belangrijke factor is in de ontwikkeling van GvHD. Door de implementatie van hoge resolutie HLA typering maar ook door verbeterde antibiotica, profylaxe tegen infectie en moderne immuun suppressie is de uitkomst van allogene stamceltransplantatie aanzienlijk verbeterd, maar GvHD blijft een groot probleem en is nog steeds de belangrijkste oorzaak van mortaliteit na allogene stamceltransplantatie. Door T-lymfocyten uit het transplantaat te verwijderen neemt het risico op GvHD af, maar het effect op overleving blijft beperkt vanwege een grotere kans op recidief van de leukemie.

Donor lymfocyten infusie

Het inzicht dat T-lymfocyten van de donor een anti-leukemie effect kunnen bewerkstelligen gaf aanleiding tot het ontwikkelen van donor lymfocyten infusie (DLI) als behandeling

voor recidief van leukemie. Met DLI kan in 20-80% van de patiënten een remissie worden bereikt, met de beste resultaten voor chronisch myeloïde leukemie (CML) in chronische fase, maar ook in patiënten met multipel myeloom of acute myeloïde leukemie (AML) worden remissies bereikt. Naast het gewenste anti-leukemie effect, kan echter ook GvHD optreden na DLI.

Na niet-HLA-identieke allogene stamceltransplantatie zijn donor afkomstige T-lymfocyten voornamelijk gericht tegen het lichaamsvreemde HLA molecuul dat tot expressie komt op cellen van de patiënt. Maar ook na HLA-identieke transplantatie ontwikkelt ongeveer 40% van de patiënten GvHD. Dit wordt veroorzaakt door genetische verschillen tussen patiënt en donor die buiten het HLA complex liggen. De genetische verschillen coderen voor polymorfe peptiden, die gepresenteerd worden door HLA moleculen op cellen van de patiënt en die herkend worden door donor T-lymfocyten als lichaamsvreemd. Deze polymorfe peptiden worden minor histocompatibiliteits antigenen genoemd.

Minor histocompatibiliteits antigenen

Het eerste minor histocompatibiliteits antigen, bekend als HA-2, is in 1995 ontdekt en afkomstig van een hematopoiese specifiek eiwit. Sindsdien is met verschillende methodes een toenemend aantal minor histocompatibiliteits antigenen die gepresenteerd worden door HLA klasse I moleculen geïdentificeerd. Minor histocompatibiliteits antigenen die tot expressie komen op cellen van het bloedvormend systeem, maar niet op niet-hematopoietische weefsels, zoals HA-1 en HA-2, zijn belangrijk voor het ontwikkelen van therapie om hematologische maligniteiten na allogene stamceltransplantatie te behandelen. T-lymfocyten die gericht zijn tegen hematopoiese specifieke minor histocompatibiliteits antigenen zullen namelijk de kwaadaardige cellen van de patiënt aanvallen, maar niet de normale bloedcellen omdat deze na allogene stamceltransplantatie van donor oorsprong zijn. Omdat hematopoiese specifieke minor histocompatibiliteits antigenen niet tot expressie worden gebracht op niet-hematopoietische weefsels, zullen T-lymfocyten die gericht zijn tegen deze antigenen niet of nauwelijks bijwerkingen veroorzaken. Het aantal minor histocompatibiliteits antigenen met therapeutische waarde is echter beperkt. De

therapeutische waarde wordt bepaald door een aantal factoren: (1) Het peptide moet worden gepresenteerd in een HLA molecuul dat frequent voorkomt in de humane populatie, (2) de frequentie van de immunogene variant van het peptide moet 30-70% zijn om met een redelijke kans een negatieve donor te kunnen vinden voor een positieve patiënt, (3) het antigen moet tot expressie komen op de kwaadaardige cellen van de patiënt om het gewenste anti-leukemie effect te kunnen veroorzaken en (4) het antigen mag niet tot expressie komen op niet-hematopoietische weefsels om afstotingsreacties tegen gezonde organen van de patiënt te voorkomen of te minimaliseren.

Het optreden van afstotingsreacties tegen gezonde niet-hematopoietische organen zou kunnen worden voorkomen of verminderd door donor $CD4^+$ T-lymfocyten toe te dienen die gericht zijn tegen minor histocompatibiliteits antigenen die gepresenteerd worden door HLA klasse II moleculen. In tegenstelling tot HLA klasse I moleculen komen de HLA klasse II moleculen HLA-DR, -DQ en -DP voornamelijk tot expressie op cellen van het hematopoietische systeem. Om die reden kunnen donor $CD4^+$ T-lymfocyten mogelijk een selectief anti-leukemie effect induceren zonder of met een geringe kans op ernstige bijwerkingen. Om dit nader te bestuderen was het noodzakelijk de antigenen die gepresenteerd worden door HLA klasse II moleculen te identificeren. De technieken die beschikbaar waren om HLA klasse I bindende minor histocompatibiliteits antigenen te ontdekken waren echter niet bruikbaar en vereisten aanpassingen. In 2008, meer dan 10 jaar na de ontdekking van het eerste HLA klasse I bindende minor histocompatibiliteits antigen, is LB-PI4K2B-1S geïdentificeerd; het eerste minor histocompatibiliteits antigen in HLA klasse II (DQB1*06:03).

Antigen processing

De oorspronkelijke gedachte was dat peptiden van endogene eiwitten in HLA klasse I moleculen worden gepresenteerd, terwijl HLA klasse II voor presentatie van peptiden van exogene eiwitten verantwoordelijk is. Inmiddels is bekend dat peptiden van zowel endogene als exogene eiwitten kunnen worden gepresenteerd in HLA klasse I en II. HLA klasse II processing begint met de synthese van HLA klasse II moleculen in het

endoplasmatisch reticulum (ER). Hier worden lege HLA klasse II moleculen gestabiliseerd door een chaperone bekend als de invariante (Ii) keten. Naast dit stabiliserend effect heeft de Ii keten twee biologische functies. Het C-terminale gedeelte van de Ii keten, dat CLIP wordt genoemd, ligt in de peptide bindingsgroef van het HLA klasse II molecuul en voorkomt te vroege binding van peptiden in het ER. Het N-terminale gedeelte van de Ii keten ligt in het cytoplasma en heeft een signaal motief waardoor het HLA-Ii complex via het trans-Golgi netwerk naar de endocytische route wordt gebracht. In het late endosoom wordt de Ii keten afgebroken door proteolytische enzymen zodat alleen het resterende CLIP peptide in het HLA klasse II molecuul gebonden blijft. Het niet-polymorfe HLA molecuul HLA-DM katalyseert vervolgens het proces waarbij CLIP wordt uitgewisseld voor andere antigene peptiden, die vervolgens in HLA klasse II moleculen op het celoppervlak worden gepresenteerd.

Sommige cellen zoals B-cellen, dendritische cellen en enkele thymus epitheel cellen brengen naast HLA-DM nog een ander niet-polymorf HLA klasse II molecuul tot expressie; HLA-DO. De precieze biologische functie van HLA-DO is niet duidelijk, maar diverse studies laten zien dat HLA-DO kan binden aan HLA-DM en zo diens functie kan beïnvloeden.

Dit proefschrift

Bij HLA-identieke allogene stamceltransplantatie herkennen donor afkomstige T-lymfocyten polymorfe peptiden die worden gepresenteerd door HLA moleculen op cellen van de patiënt, maar niet op cellen van de donor. Een strategie om het gewenste anti-leukemie effect te induceren zonder ernstige bijwerkingen is het toedienen van donor CD8⁺ T-lymfocyten tegen minor histocompatibiliteits antigenen met hematopoiese specifieke expressie. Om deze strategie breed toe te kunnen passen moet echter een groot aantal antigenen in verschillende HLA klasse I moleculen met een geschikt expressie profiel zijn geïdentificeerd. Een andere strategie is het toedienen van CD4⁺ T-lymfocyten die minor histocompatibiliteits antigenen herkennen die door HLA klasse II moleculen worden gepresenteerd. In tegenstelling tot HLA klasse I moleculen die tot expressie komen op

zowel hematopoietische als niet-hematopoietische cellen, worden HLA klasse II moleculen met name tot expressie gebracht op cellen van het bloedvormend systeem. Op basis hiervan kunnen donor CD4⁺ T-lymfocyten mogelijk een selectief anti-leukemie effect induceren.

In dit proefschrift hebben wij minor histocompatibiliteits antigenen geïdentificeerd die door HLA klasse II moleculen worden gepresenteerd en hun intracellulaire processing en presentatie bestudeerd om inzicht te krijgen in de rol van donor CD4⁺ T-lymfocyten in het induceren van het gewenste anti-leukemie effect en ongewenste bijwerkingen en hun toepasbaarheid voor therapie.

In **hoofdstuk 2** hebben wij 4 nieuwe minor histocompatibiliteits antigenen die gepresenteerd worden door HLA klasse II moleculen geïdentificeerd. De antigenen worden herkend door donor CD4⁺ T-lymfocyten die zijn opgewekt in een patiënt die met succes is behandeld met DLI voor een CML recidief na HLA-identieke allogene stamceltransplantatie. Geactiveerde (HLA-DR⁺) CD4⁺ T-lymfocyten zijn geïsoleerd uit beenmerg van de patiënt 5 weken na DLI. Met behulp van een bacteriële cDNA expressie bank hebben wij aangetoond dat de geactiveerde CD4⁺ T-lymfocyten gericht waren tegen LB-PTK2B-1T, LB-MR1-1R, LB-LY75-1K en LB-MTHFD1-1Q. Deze polymorfe peptiden worden in verschillende HLA-DR allelen gepresenteerd, namelijk HLA-DRB3*01:01, DRB3*02:02, DRB1*13:01 en DRB1*03:01. De HLA-DR bindende antigenen hebben allemaal een gebalanceerde frequentie in de populatie van 25-70%, en de genen die coderen voor LB-PTK2B-1T en LB-LY75-1K komen hoger tot expressie in hematopoietische cellen. De specifieke T-lymfocyten herkenden allemaal primaire AML en ALL cellen, maar primaire CML voorloper cellen werden alleen herkend door T-lymfocyten tegen LB-MTHFD1-1Q. CML voorloper cellen die in vitro waren gemodificeerd tot professionele antigen presenterende cellen (APC) werden door alle T-lymfocyten herkend.

Bij het testen van herkenning van verschillende celtypes door CD4⁺ T-lymfocyten was het opvallend dat sommige celtypes niet werden herkend ondanks detecteerbare expressie van het antigen coderende gen en expressie van het relevante HLA klasse II restrictie molecuul. Om die reden veronderstelden wij dat celtype specifieke mechanismes een rol zouden kunnen spelen in processing en presentatie van antigenen in HLA klasse II. Om dit

nauwkeurig te bestuderen hebben wij een cellijn model ontwikkeld gebaseerd op HLA klasse II negatieve HeLa cellen. Met dit model hebben wij de bijdrage van bekende moleculen op processing en presentatie van onze nieuw geïdentificeerde minor histocompatibiliteits antigenen in HLA klasse II onderzocht.

In **hoofdstuk 3** laten wij zien dat antigenen die gepresenteerd worden door HLA klasse II moleculen kunnen worden verdeeld in twee groepen op basis van hun reactie op HLA-DM. Een groep antigenen wordt goed gepresenteerd in aanwezigheid van HLA-DM (DM-resistent), terwijl presentatie van de andere groep antigenen door HLA-DM wordt verminderd of afgeschakeld (DM-sensitief). Presentatie van deze laatste groep DM-sensitieve antigenen kan worden hersteld door co-expressie van HLA-DO. In verschillende cellijnen hebben wij aangetoond dat LB-MR1-1R, LB-LY75-1K en het al eerder geïdentificeerde LB-PI4K2B-1Q tot de groep DM-sensitieve antigenen behoren, terwijl LB-PTK2B-1T, LB-MTHFD1-1Q en DBY DM-resistente antigenen zijn. Verder hebben wij laten zien dat DM-sensitiviteit afhankelijk is van het T-cel epitoom en dat voor een DM-sensitief fenotype een interactie nodig is tussen HLA-DM en het HLA-DR restrictie molecuul. Onze data hebben ook aangetoond dat in niet-hematopoietische cellen de expressie van de β -keten van HLA-DO niet kan worden opgereguleerd door interferon- γ met als gevolg dat DM-sensitieve antigenen niet goed kunnen worden gepresenteerd op niet-hematopoietische cellen die HLA klasse II tot expressie brengen in een ontstekingsomgeving. Dit suggereert dat donor CD4⁺ T-lymfocyten die gericht zijn tegen DM-sensitieve antigenen een laag risico hebben op GvHD en daarom geschikt zouden kunnen zijn voor immuuntherapie.

In **hoofdstuk 4** hebben wij nader onderzocht of T-lymfocyten die gericht zijn tegen DM-sensitieve antigenen in staat zijn leukemie cellen te herkennen en dus mogelijk een anti-leukemie effect kunnen geven in vivo. HLA-DO komt in veel primaire leukemie cellen tot expressie, en met name CLL en sommige ALL cellen brengen de α - en β -keten van HLA-DO hoog tot expressie vergelijkbaar met de expressie in gezonde B-cellen of rijpe dendritische cellen. Door de expressie van de α -keten van HLA-DO te remmen in ALL en Burkitt lymfoom cellijnen hebben wij aangetoond en bevestigd dat endogeen HLA-DO de presentatie van DM-sensitieve antigenen op het celoppervlak reguleert. Bovendien hebben

wij laten zien dat DM-sensitieve antigenen efficiënter worden gepresenteerd op primaire CLL cellen dan op ALL cellen, hetgeen overeenkomt met de hoge expressie van HLA-DO in CLL ten opzichte van ALL.

Naast de effector functie van $CD4^+$ T-lymfocyten, die uitgebreid is bestudeerd in bovenstaande hoofdstukken, hebben wij in **hoofdstuk 5** meer hun vaardigheden als helper cellen onderzocht. Ondanks de bekende helper functie van $CD4^+$ T-lymfocyten bij productie van IgG antilichamen, zijn gecoördineerde $CD4^+$ T- en B-cel responsen na allogene stamceltransplantatie niet in detail bestudeerd, en alleen beschreven voor mannelijke antigenen die worden gecodeerd op het Y-chromosoom in mannelijke patiënten na transplantatie met vrouwelijke donoren. Eén van de HLA klasse II bindende minor histocompatibiliteits antigenen die we hebben geïdentificeerd is afkomstig van PTK2B, dat door anderen is beschreven als eiwit dat wordt herkend door antilichamen na allogene stamceltransplantatie. Dit heeft ons ertoe gebracht te onderzoeken of in onze patiënt een gecoördineerde T- en B-cel respons was opgewekt tegen PTK2B. Inderdaad hebben wij een humorale immuunrespons tegen dit eiwit kunnen meten na DLI. In tegenstelling tot de $CD4^+$ T-lymfocyten, herkennen de antilichamen een niet-polymorfe structuur in het C-terminale gedeelte van het eiwit, waarmee is aangetoond dat een gecoördineerde allo-reactieve $CD4^+$ T-cel en auto-reactieve B-cel respons tegen het autosomale PTK2B eiwit is opgewekt in een patiënt die met succes is behandeld met DLI voor een CML recidief na HLA-identieke allogene stamceltransplantatie.

De data in hoofdstuk 5 onderstrepen de centrale rol van $CD4^+$ T-lymfocyten als helper cellen in adaptieve immuniteit. In het algemeen wordt aangenomen dat opname en presentatie van exogene eiwitten in MHC klasse II van professionele APC een belangrijke eerste stap is in de inductie van een adaptieve immuunrespons. Voor efficiënte inductie van afweer tegen intracellulaire gevaren zoals virussen of kwaadaardige transformaties is het dus noodzakelijk dat intracellulaire eiwitten van geïnfecteerde of getransformeerde cellen worden opgenomen door omliggende APC om zo een efficiënte primaire $CD4^+$ T-cel respons te induceren. In **hoofdstuk 6** hebben wij het mechanisme van indirecte presentatie bestudeerd voor 6 HLA klasse II bindende minor histocompatibiliteits antigenen. Van de 6 HLA klasse II gepresenteerde epitopen bleken 3 antigenen van de ene naar de andere cel te

kunnen worden overgebracht. Intercellulair antigeen transfer werd gemedieerd door het hele eiwit en resulteerde in T-cel herkenning van cellen die het antigeen oorspronkelijk niet tot expressie brachten. Verder konden wij voor PTK2B laten zien dat intercellulair transfer niet afhankelijk was van het T-cel epitoom maar van andere eiwit eigenschappen zoals de totale hoeveelheid beschikbaar eiwit in de cel en specifieke bindingssequenties. Het mechanisme van antigeen transfer van HLA klasse II negatieve cellen naar omliggende APC speelt mogelijk een belangrijke rol in het opwekken van afweer tegen intracellulaire bedreigingen zoals virussen en getransformeerde cellen.

De resultaten van het onderzoek zoals beschreven in dit proefschrift laten zien dat het toedienen van donor $CD4^+$ T-lymfocyten als DLI na T-cel gedepleteerde allogene stamceltransplantatie een veelbelovend strategie is. $CD4^+$ T-lymfocyten kunnen als effector en helper cellen een recidief van leukemie en infecties voorkomen en hebben mogelijk een geringe kans op ernstige bijwerkingen vanwege het feit dat HLA klasse II moleculen selectief tot expressie komen op het oppervlak van hematopoietische cellen. Echter, niet-hematopoietische weefsels kunnen onder ontstekingsomstandigheden HLA klasse II positief worden, en klinische studies zullen dus moeten uitwijzen of donor $CD4^+$ T-lymfocyten inderdaad bruikbaar zijn om een gunstig effect op de balans tussen het gewenste anti-leukemie effect en ongewenste bijwerkingen te bewerkstelligen. De resultaten in dit proefschrift suggereren bovendien dat het risico op ernstige bijwerkingen verder kan worden verminderd door donor $CD4^+$ T-lymfocyten te selecteren die DM-sensitieve antigenen herkennen. $CD4^+$ T-lymfocyten gericht tegen DM-sensitieve antigenen kunnen mogelijk worden verrijkt door donor T-lymfocyten te verwijderen die reactief zijn tegen niet-hematopoietische cellen van de patiënt, zoals fibroblasten gekweekt uit een huidbiopt na voorbehandeling met inflammatoire cytokines om expressie van HLA klasse II te induceren. Het is echter onbekend of $CD4^+$ T-lymfocyten gericht tegen DM-sensitieve antigenen in staat zijn een effectieve anti-leukemie respons te induceren, en meer studie is dus nodig om te onderzoeken of het verrijken van $CD4^+$ T-lymfocyten tegen DM-sensitieve antigenen een goede strategie is om de balans tussen het gewenste anti-leukemie effect en ongewenste bijwerkingen na allogene stamceltransplantatie te verbeteren.

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CURRICULUM VITAE

Anita Natalie Kremer werd geboren als Anita Stumpf op 28 augustus 1980 te München in Duitsland. In 1999 behaalde ze haar gymnasium diploma aan het Theresien-Gymnasium te München en begon in hetzelfde jaar met de studie Geneeskunde aan de Ludwig-Maximilians-Universiteit van München. Tijdens haar co-schappen ging ze voor interne geneeskunde naar de aan Harvard geaffilieerde Massachusetts General Hospital en de Brigham and Women Hospital in Boston in de USA. Haar keuze co-schap liep ze op de afdeling Neurologie aan het Kantonspital te Aarau in Zwitserland. In 2006 behaalde ze haar artsexamen en begon ze met haar opleiding interne geneeskunde in het universitair ziekenhuis München-Großhadern. In 2007 onderbrak ze haar opleiding en begon ze aan het in dit proefschrift beschreven promotieonderzoek onder begeleiding van Dr. Marieke Griffioen en Prof.dr. Fred Falkenburg op het laboratorium voor Experimentele Hematologie van de afdeling Hematologie in het Leids Universitair Medisch Centrum. In 2011 trouwde ze met Andreas Kremer. In 2012 ging ze terug naar Duitsland om haar opleiding tot internist voort te zetten aan het universitair ziekenhuis te Erlangen.

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