Characterization and recognition of minor histocompatibility antigens

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General introduction

Stem cell transplantation

Cure from hematological malignancies depends on successful elimination of the malignant cells. Treatment regimens can consist of primary remission induction followed by myeoloablative consolidation therapy with high dose irradiation and or chemotherapy, which severely damages normal hematopoietic cells. To restore hematopoiesis, autologous stem cell transplantation (autoSCT) with hematopoietic stem cells (HSC) that were harvested and stored prior to consolidation therapy can be performed[1,2].

As alternative source, HSC can be harvested from a healthy donor. Allogeneic stem cell transplantation (alloSCT) requires matching of patient and donor major histocompatibility complex (MHC), also called human leukocyte antigens (HLA), to prevent host-*versus*-graft reactivity resulting in graft rejection, and graft-*versus*-host disease (GvHD), a life threatening complication caused by donor-derived alloreactive cells. Myeloablative consolidation therapy causes both tissue damage and lymphopenia thereby creating an inflammatory environment that supports activation and homeostatic proliferation of transplanted alloreactive cells, respectively. The cascade or 'cytokine storm' that follows is a major contributor to the development GvHD[3,4,5].

As compared to autoSCT, lower relapse rates are observed after alloSCT, which can be attributed to curative graft-*versus*-leukemia (GvL) reactions[6,7]. Patients who received alloSCT from an HLA-identical sibling also had lower relapse rates as compared to patients who were transplanted with a syngeneic donor (identical twin sibling)[8]. These observations indicate that GvL reactivity after alloSCT is the result of donor-to-patient alloreactivity that requires a certain degree of tissue incompatibility. In addition, many soluble and cell-bound ligands and receptors have been identified that play a role in activation or regulation of immune responses after alloSCT. The specific role for these factors in development of GvHD and how to manipulate these factors in order to selectively induce curative GvL reactions, however, is largely unknown[9].

Enhancing applicability and specificity of alloSCT

To treat patients with alloSCT who cannot tolerate the toxicity of myeloablative consolidation therapy, non-myeloablative (NMA) conditioning was developed.

NMA conditioning may however also lead to less efficient tumor reduction, thereby increasing the risk of relapse. Curative alloSCT following NMA conditioning is therefore likely to depend more on induction of GvL reactivity[10,11,12,13,14].

GvHD after alloSCT can be treated with immune suppression. Alternatively, alloSCT with a graft from which T cells are depleted results in reduced frequencies and severity of GvHD[15]. However, since GvL reactions are strongly correlated with occurrence of GvHD, these strategies also inhibit GvL reactions[16]. It was shown that postponed donor lymphocyte infusion (DLI) after T-cell depleted alloSCT could induce GvL reactivity with reduced incidence and severity of GvHD[17,18]. It is likely that at the moment of postponed DLI, consolidation therapy-induced tissue damage is resolved and donor HSC have restored normal hematopoiesis. Infused alloreactive donor T cells encounter fewer patient antigen presenting cells (APC) under less activating circumstances, thereby limiting development of GvHD[19,20,21]. Moreover, viral reactivations that occur frequently after alloSCT may be better controlled leading to a lower inflammatory environment[22,23,24]. Inflammatory conditions may also increase susceptibility of healthy non-hematopoietic tissues to T-cell damage because of increased expression of adhesion and costimulation molecules[25].

Although T-cell depleted alloSCT followed by delayed DLI illustrates that GvHD can be separated from GvL reactivity, GvHD remains a significant cause of morbidity and mortality in these patients.

AlloSCT and DLI for treatment of solid tumors

To exploit the T-cell mediated curative effect of alloreactivity, patients suffering from advanced stages of solid tumors have been treated with alloSCT[26,27,28,29]. Graft-*versus*-tumor (GvT) reactivity was induced by withdrawal of immune suppression shortly after alloSCT resulting in tumor regression in several patients albeit in most cases at the cost of severe GvHD. Similarly, in the setting of T-cell depleted alloSCT and DLI, tumor regressions in the presence of GvHD have been observed[18,30,31]. This demonstrates that in addition to undesired targeting of healthy tissues in GvHD, malignant non-hematopoietic cells can be eliminated by cellular immunotherapy.

Targeting of alloantigens by donor-derived T cells on solid tumors requires primary T-cell activation by patient-derived professional antigen presenting cells (APC)[19]. GvT reactivity without GvHD can only be obtained if the targeted alloantigens are selectively expressed on tumor cells but not on healthy tissue cells. Many solid tumors originate from epithelial cells and it can therefore be expected that they share many antigens, which may explain the strong association between GvT and GvHD. Selective induction of alloreactivity to treat transplanted patients with solid tumors requires identification of alloantigens that are expressed by both professional APC and solid tumor cells, but not by normal tissue cells. Thus far, such alloantigens have not been identified.

Responding cells: The T-cell repertoire

The T-cell repertoire develops in the thymus, where committed lymphoid progenitor cells mature into T cells that express a functional T-cell receptor (TCR). TCR are heterodimers consisting of an α-chain (*TRA*) and β-chain (*TRB*), or a γ-chain and δ-chain. Whereas T cells that express γδ-TCR are primarily involved in first-line defense, αβ-T cells can specifically recognize peptides presented by HLA[32].

Genomic TCR gene segments require somatic recombination in order to become a functional TCR gene. The diversity of TCR is generated by recombination of variable, diversity, joining and constant gene segments, random insertion and deletion of nucleotides at the newly formed junctions between the gene segments as well as pairing of different α and β TCR chains[33]. *TRB* gene segments are rearranged until a productive β-chain is obtained which can dimerize with a pre α-chain to form a pre T-cell receptor. Since each single *TRB* allele can rearrange twice at the two TRB loci, four attempts per cell can be made to produce a functional TCR. Theoretically, 80% of thymocytes succeed in rearranging a functional β-chain. In a next step, CD4 and CD8 co-receptors are expressed and T cells acquire the capacity to produce progeny, leading to expansion of a T-cell clone in which all cells express identical β-chains. At that time, simultaneous rearrangement of the 2 *TRA* loci commences. If *TRA* rearrangement fails to generate a functional α-chain, developing T cells die by apoptosis.

Rearrangement is a random process resulting in a repertoire of TCR that may recognize ‘self’ and ‘non-self’ antigens. The ‘negative selection model’ proposes

that only T cells expressing TCR that lack binding to self-peptides will pass thymic selection thereby preventing autoimmunity[34]. Mature naive CD4 and CD8 T cells are then released from the thymus. Due to this negative selection in the thymus, DLI is devoid of T cells recognizing antigens that are shared between patient and donor. In contrast, T cells expressing high-affinity TCR recognizing patient-specific antigens that are non-self to the donor can be present within the donor T-cell repertoire.

In addition to thymic selection, peripheral tolerance provides mechanisms to prevent T-cell responses against self-antigens[35]. These mechanisms include suppression by regulatory T cells and induction of anergy or apoptosis following inappropriate TCR stimulation. It can be speculated, however, that peripheral tolerance to self-antigens may be broken if strong T-cell responses against non-self antigens occur. If true, strong alloreactive T-cell responses may stimulate T-cell responses against self-antigens, which may explain the frequent development of autoimmune diseases following alloSCT[36,37].

Minor histocompatibility antigens (MiHA)

In HLA-matched alloSCT, alloreactive T cells that mediate GvL reactivity and GvHD recognize minor histocompatibility antigens (MiHA), which are polymorphic peptides presented by self human leukocyte antigens (HLA)[38,39]. Peptides are generated in the cytosol by proteasomes, which are large enzymatic complexes. Proteasomes can hydrolyze the peptide bond between amino acid residues of proteins resulting in peptides of variable length that are further degraded by amino peptidases. 'Transporters associated with antigen processing' (*TAP1* and *TAP2*) can translocate peptides of 8-12 amino acids from the cytosol to the endoplasmic reticulum (ER). In the ER, these peptides may bind in the groove of HLA-class I molecules and peptide-HLA complexes are subsequently transported via the Golgi complex to the cell membrane where they are presented to the TCR of CD8 T cells. Peptides from extracellular proteins are taken up, degraded via endosomes, and assembled in HLA-class II molecules for presentation to CD4 T cells[40].

The groove of HLA class I molecules can bind peptides non-covalently by the presence of specific anchor amino acid residues at both the N- and C-terminus of the peptide. *HLA-A*, *HLA-B* and *HLA-C* encode three human class I HLA proteins with similar structure. Within these genes, allelic variations encode

structural changes that reside predominantly in the peptide-binding groove and thereby influence binding and presentation of peptide subsets[41,42]. In self-HLA, peptides degraded from endogenous proteins are considered as 'self'. Following viral infection, peptides derived from viral proteins can be recognized as 'non-self' resulting in a T-cell mediated anti-viral response. Similarly, in the setting of HLA-matched alloSCT, donor T cells can recognize polymorphic, patient-specific peptides as 'non-self' antigens and elicit an alloreactive response. Non-self peptides can be encoded by genomic patient-to-donor variations that lead to patient-specific amino acid polymorphisms. The absence of these patient-specific polymorphisms in the donor allows the presence of alloreactive donor-to-patient high-affinity TCR within the donor T-cell repertoire.

Non-self peptides can be encoded by patient-specific single nucleotide polymorphisms (SNPs). SNPs may encode patient-specific amino acid substitutions that result in structurally different peptides for which high-affinity TCR exist in the donor T-cell repertoire. Missense SNPs can encode an amino acid substitution in the normal protein sequence that is present in the antigenic peptide[43,44,45,46,47,48,49]. SNPs that do not cause an amino acid substitution in the normal protein sequence (synonymous SNPs) can, as a result of an upstream alternative start codon which is out-of-frame, encode an amino acid substitution in an alternatively translated polypeptide[50]. It has been hypothesized that MiHA derived from such 'defective ribosome products' make up a considerable part of the MiHA repertoire [51,52].

Furthermore, SNPs that substitute a non-anchor amino acid by an anchor amino acid can lead to binding of the peptide to HLA and presentation to donor T cells[43,44]. Differential peptide expression can also be the result of SNPs that are not located in exons but in introns. During mRNA maturation, introns are removed from the pre-mRNA by spliceosomes requiring donor-, branch- and acceptor-splice motifs contained within the intron. Intron SNPs may introduce or disturb such motifs resulting in transcripts with differences in exon composition or length, leading to disparate protein sequences between patient and donor[53,31]. One observation was made in which a SNP-encoded amino acid polymorphism introduced proteasomal splicing and annealing of two noncontiguous peptide segments to form a MiHA[54].

Not only single nucleotide polymorphisms, but also single nucleotide insertions or deletions can encode patient-to-donor protein disparities by shifting the

translation reading frame[55]. In addition, complete proteins can be absent in the donor if the encoding genes are absent due to homozygous gene deletions[56,57]. Although it appears that MiHA encoded by missense SNPs are the most common, this observation may be biased by the fact that these MiHA are identified easier as compared to MiHA that are encoded by other SNPs and insertions/deletions.

MiHA identification strategies

The first MiHA were identified by mass spectrometric analysis of the antigenic peptide[46,58,44,43,59,45]. This requires laborious isolation of peptide-HLA complexes from billions of MiHA^{pos} cells followed by HPLC purification and fractionation. A different approach aims at identification of the MiHA-encoding transcript in plasmid libraries that contain cDNA generated from mRNA of MiHA^{pos} cells. Subcloning of a positive cDNA pool may identify MiHA^{pos} single plasmids that can be sequenced to detect the MiHA-encoding SNP. As compared to biochemical purifications, cDNA library methods do not require laborious handling of large cell numbers, but still, large numbers of plasmids need to be produced and tested. Nevertheless, several HLA class I restricted MiHA were discovered by this method[60,50,61,62,54]. In addition, by adapting the cDNA library method, HLA class II restricted MiHA targeted by CD4 T-cell clones were identified[48,63].

Whole genome association scanning (WGAs) for MiHA identification utilizes a panel of cells of which SNP genotype data are available. All panel cells are tested for recognition by the T-cell clone, MiHA^{pos} and MiHA^{neg} cells are separated, and the SNP-genotype data are searched for SNPs with similar distribution between MiHA^{pos} and MiHA^{neg} cells. If low-resolution SNP-genotype data are used, WGAs can identify the region or gene containing the MiHA-encoding SNP[47,64,65,55,49,66,67]. By using high-resolution SNP-genotype data, WGAs can directly identify the MiHA-encoding SNP, provided that the MiHA encoding SNP is present in the data[68,69,49,70]. If the MiHA-encoding SNP is not included in the SNP genotype data, SNPs that are in linkage disequilibrium with the MiHA-encoding SNP may be identified. Subsequent Sanger sequencing of gene transcripts derived from both patient and donor can reveal the patient-to-donor disparate MiHA-encoding SNPs that are absent in the SNP genotype data.

All methods for MiHA discovery as described above are based on read-out systems that use MiHA specific T-cell clones as tools to identify MiHA^{pos} peptide fractions or cDNA pools, or to separate MiHA^{pos} from MiHA^{neg} test cells. Consequently, each of these approaches for MiHA discovery depends on the generation of stable T-cell clones from samples of patients who responded to alloSCT (and DLI).

Detection of MiHA-specific T cells

T cells that are involved in alloreactive immune responses can be detected and isolated by secretion of cytokines[71,72,73] and expression of cell surface markers[74,75,76]. Isolation and *in vitro* clonal expansion allows subsequent MiHA identification and detailed analysis of the tissue specificity of isolated T-cell clones. After identification of the MiHA peptide, HLA-peptide monomers can be produced and used to generate multimers coupled to a fluorescent label to validate T-cell binding to newly discovered MiHA[77]. Flowcytometric analyses can be performed to analyze sequentially taken blood samples from patients with GvL reactivity, which demonstrated vigorous expansion followed by retraction of MiHA-specific cells.[78,50]. Detection levels of approximately 0.01% can be reached, and lower frequencies may be visualized after peptide stimulation to obtain expansion of MiHA-specific T cells[79].

Since some HLA class I monomers seem to be difficult to produce stably, applicability of multimers to detect alloreactive T cells is limited by the ability to produce monomers *in vitro*. As a consequence, research has been focused on common HLA class I alleles such as HLA-A*02 and B*07, while neglecting less frequent HLA alleles. Therefore, our knowledge on frequencies and composition of MiHA specific T-cell responses in peripheral blood may be biased and not completely cover *in vivo* immune responses after alloSCT and DLI.

Tissue distribution of MiHA

After activation and expansion of infused MiHA specific donor T cells, it is likely that induction of GvL reactivity without or with GvHD depends on the tissue distribution of the MiHA that are targeted by the alloimmune response. Several MiHA specific T-cell clones that specifically recognized hematopoietic cells have been isolated indicating that MiHA can be differentially expressed [44,43,60,55,50,49,44,43,60,69,55,50,49]. Whether a MiHA is selectively expressed by malignant hematopoietic cells or by all hematopoietic cells of the patient is not

relevant. After alloSCT, patient hematopoiesis is replaced by donor HSC thereby allowing eradication of both the malignant and normal hematopoietic cells of the patient. Targeting of patient hematopoiesis restricted MiHA that are also expressed by the malignant cells can therefore induce GvL reactivity without GvHD. In contrast, targeting of MiHA that are presented by non-hematopoietic tissue cells is likely to result in GvHD[46,80,45,62,81].

To test whether MiHA are broadly expressed or have a hematopoiesis-restricted expression pattern, *in vitro* T-cell recognition of non-hematopoietic cells can be performed. Skin-derived fibroblasts of the patient are relatively easy to obtain and are suitable targets for first screening of MiHA distribution. They however represent only a fraction of tissues that can be targeted during GvHD. To generate a comprehensive test cell panel, non-hematopoietic tissue cells and cell lines from third party individuals can be used. This however requires that test cells are positive for both the HLA-restriction molecule and the MiHA-encoding SNPs, which may be difficult to achieve for MiHA with low frequencies and MiHA presented by rare HLA-alleles.

Testing of MiHA recognition requires that several different T-cell clones with different TCR that recognize the same MiHA are used. Different T-cell clones with similar recognition of hematopoietic test cells may display different strength of recognition of non-hematopoietic cells due to sub-optimal adhesion and costimulation of non-hematopoietic cells[25]. Under these conditions, lower TCR affinity and decreased effector function of the T-cell clone may lead to under-estimation of MiHA expression by non-hematopoietic cells.

In addition to measurement of recognition by MiHA-specific T-cell clones, expression levels of MiHA-encoding genes can be quantified to predict absence or presence of MiHA on cells from various tissue types. Gene-specific primers and probes are used in both real-time PCR and array-based platforms to detect and quantify mRNA of MiHA-encoding genes. Whereas absence of gene expression is expected to correlate with lack of T-cell recognition, it remains difficult to predict how gene expression is related to the amount of protein that is produced and the actual epitope density that is displayed on the cell surface. Estimation of MiHA tissue distribution should therefore be based on both functional analyses using MiHA-specific T-cell clones and investigation of MiHA-encoding gene expression profiles.

In summary, alloSCT and DLI can be performed to treat patients who suffer from malignant diseases. It has been shown that recognition of MiHA by donor-derived alloreactive T cells is important for both curative GvL reactivity and detrimental GvHD. MiHA discovery implies both elucidation of the antigenic structure of MiHA, which can be achieved by different technical approaches, and investigation of expression patterns of MiHA-encoding genes by malignant and normal cell types. Identification of MiHA that are specifically expressed by malignant cells allows development of future adoptive immunotherapies that can induce GvL reactivity without GvHD.

Aim of the study

In alloSCT, depletion of donor T cells from the stem cell graft leads to lower prevalence and severity of GvHD, illustrating that T cells play a major role in GvHD. However, this coincides with an increase in relapse rates, which requires additional treatment with DLI. The aim of the study as described in this thesis is to analyze the role of MiHA-specific CD8 T cells in patients with GvL reactivity without or with GvHD after alloSCT and DLI. Immune responses were studied at the clonal T-cell level and various tools for MiHA discovery have been developed and validated. Recognition strength and specificity of T-cell clones for different types of MiHA were investigated for their potential role in GvL reactivity and GvHD.

In ***chapter 2***, we explored biochemical purification and mass spectrometry for characterization of MiHA peptides. From a patient who was cured from multiple myeloma after T-cell depleted alloSCT and DLI, multiple T-cell clones were isolated, including T-cells targeting HA-1 and LB-ECGF-1H. A T-cell clone that was isolated at a high frequency recognized an unknown MiHA presented by HLA-A*02. It was therefore likely that this T-cell clone played a dominant role in the graft-*versus*-myeloma effect and we investigated the capacity of this T-cell clone to recognize myeloma cells and both hematopoietic and non-hematopoietic normal cells. Characterization of the MiHA recognized by this T-cell clone involved culturing of large numbers of patient EBV-LCL followed by biochemical purification, fractionation and mass spectrometric analysis.

To increase the throughput of MiHA identification, in ***chapter 3*** we applied whole genome association scanning (WGAs) as method for MiHA discovery. This method requires a panel of EBV-LCL test cells from which detailed SNP genotype data have to be generated by micro-array techniques. By testing of recognition of individual EBV-LCL in the panel and combining this with the SNP genotype data, MiHA-encoding SNP can be identified. We aimed at analyzing multiple T-cell clones restricted to HLA-A*02 or B*07 that were isolated from 2 patients who obtained long-term remissions following alloSCT and DLI. Therefore, we composed a panel of 80 EBV-LCL that endogenously expressed both HLA-A*02 and HLA-B*07. Characterization of the potential antigenic peptides was performed by prediction of HLA-binding, and by testing of recognition at low concentrations. Generation of tetramers of identified MiHA was performed to investigate *in vivo* kinetics of MiHA-specific T cells.

In ***chapter 4***, we wanted to improve applicability of WGAs by batch-wise retroviral transduction of non-endogenously expressed HLA-molecules into EBV-LCL of the WGAs panel. We analyzed three HLA B*40:01 restricted T-cell clones that were isolated from a patient with CML who achieved long term clinical remission following alloSCT and DLI. In addition, one HLA B*40:01 restricted MiHA was analyzed using cDNA cloning techniques. To estimate the role of the individual T-cell clones in GvL reactivity and GvHD, we selected a comprehensive panel of both normal and malignant hematopoietic cells and tested recognition by the T-cell clones.

In addition to patients suffering from hematological malignancies, T-cell depleted alloSCT and postponed DLI was applied to treat patients with solid tumors. Remissions were observed, but often coincided with severe GvHD. In ***chapter 5*** we report application of alloSCT and DLI to treat a patient who suffered from metastatic renal cell carcinoma (RCC). Treatment resulted in stable disease at the cost of severe long term GvHD. T-cell clones were isolated at the onset of GvHD after DLI and analyzed for their potential role in the observed graft-versus-tumor reactivity and GvHD. We further investigated the likelihood that effective GvT reactivity in the absence of GvHD can occur by using micro-array gene expression data.

In ***chapter 6*** we analyzed GvL reactivity that was observed after alloSCT and DLI in 6 patients without and 5 patients with GvHD. We focused on MiHA-specific CD8 T cells and determined the magnitude of the alloreactive CD8 T-cell response. Tissue specificity of the isolated T-cell clones was analyzed by testing the capacity of individual T-cell clones to respond to hematopoietic and non-hematopoietic stimulator cells. As non-hematopoietic stimulator cells, fibroblasts were cultured from patient derived skin biopsies in the absence or presence of IFN-γ to mimic inflammation. Finally, WGAs was performed to identify the repertoire of targeted MiHA thereby enabling investigation of the tissue distribution of the MiHA-encoding genes by microarray gene expression analysis.

In ***chapter 7***, we summarize the analyses presented in chapters 2 to 6 of T-cell mediated immune responses in various patients who responded to the infusion of donor lymphocytes to treat recurring disease or incomplete donor chimerism. We discuss strategies for selection of alloreactive T cells and subsequent MiHA discovery, including ‘reversal’ of MiHA discovery. The influence of MiHA tissue

distribution and environmental factors is discussed as well as possibilities and limitations to elicit GvL reactivity without GvHD in the setting of alloSCT to treat recurring hematological malignancies and solid tumors.

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Chapter 2

Multiple myeloma–reactive T cells recognize an activation-induced minor histocompatibility antigen encoded by the ATP-dependent interferon-responsive *(ADIR)* gene

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Abstract

Minor histocompatibility antigens (MiHA) play an important role in both graft-*versus*-tumor effects and graft-*versus*-host disease (GvHD) after allogeneic stem cell transplantation. We applied biochemical techniques and mass spectrometry to identify the peptide recognized by a dominant tumor-reactive donor T-cell reactivity isolated from a patient with relapsed multiple myeloma who underwent transplantation and entered complete remission after donor lymphocyte infusion. A frequently occurring single nucleotide polymorphism in the human ATP-dependent interferon-responsive *(ADIR)* gene was found to encode the epitope we designated LB-ADIR-1F. Although gene expression could be found in cells from hematopoietic as well as non-hematopoietic tissues, the patient suffered from only mild acute GvHD despite high percentages of circulating LB-ADIR-1F–specific T cells. Differential recognition of non-hematopoietic cell types and resting hematopoietic cells as compared with activated B cells, T cells, and tumor cells was demonstrated, illustrating variable LB-ADIR-1F expression depending on the cellular activation state. In conclusion, the novel MiHA LB-ADIR-1F may be a suitable target for cellular immunotherapy when applied under controlled circumstances.

Introduction

Allogeneic stem cell transplantation (SCT) is a curative treatment in patients with hematologic cancers. In addition to the antitumor effects of chemotherapy, antibody treatment and/or irradiation administered as the conditioning regimen prior to transplantation, an allogeneic graft-*versus*-tumor (GvT) immune-reactivity significantly contributes to the curative potential of this therapy.[1–5] This GvT reactivity following HLA-matched SCT has been demonstrated to be mediated primarily by T cells from the donor. Allo-reactive T cells from donor origin may not only mediate the beneficial GvT effect but are also responsible for the development of graft-*versus*-host disease (GvHD), which is the major detrimental complication after allogeneic SCT.[6,7] T-cell depletion of the stem cell graft removes both GvHD and GvT effect.[8–10] The antitumor reactivity can be reintroduced in case of persistent or relapsed hematologic malignancies after transplantation by donor lymphocyte infusion (DLI).[11–15] Whereas profound antitumor effects are frequently associated with GvHD in patients responding to DLI, postponed administration of DLI has been associated with a decreased risk of severe GvHD, and clinical observations indicate that more subtle antitumor reactivities can also be observed in the absence of GvHD.[16–18]

The main targets of both GvHD and GvT reactivity after HLA-matched allogeneic SCT are minor histocompatibility antigens (MiHA).[19–21] MiHA are peptides differentially expressed by donor and recipient that can be recognized in the context of self-HLA molecules. MiHA may arise from differential processing of peptides due to polymorphisms in the gene encoding the protein or by direct polymorphisms in the peptide sequence that is presented in the HLA molecules.[22,23]

The clinical manifestation of immune responses against MiHA is likely to be determined by the specific tissue expression of the proteins encoding these antigens. Whereas MiHA constitutively expressed in many tissues are likely to be targets for combined alloreactive GvHD and GvT responses, T-cell responses directed against antigens that are restricted to the hematopoietic cell lineages including the malignant cells of hematopoietic origin are likely to mediate a GvT reactivity without severe GvHD.[21,24–31] However, antigens with variable expression in tissues may also be targets for relatively specific GvT responses because these proteins may not be expressed or recognized on normal tissues under steady state conditions.[19,32,33] By specific stimuli like

inflammatory reactions they may be induced in target tissues of GvHD, leading to a detrimental local immune response.[7,34,35] Characterization of MiHA from patients responding to cellular immunotherapeutic interventions following allogeneic SCT in the presence or absence of GvHD will lead to a better understanding of the pathogenesis of GvHD and GvT and may lead to the development of specific antitumor T-cell therapy.

Recently, we studied in detail several patients treated for relapsed hematologic malignancies after allogeneic SCT with DLI. During the clinical GvT response, we isolated tumor-reactive T cells based on their ability to produce IFN-γ in response to specific activation by bone marrow containing the malignant cells.[28,36] From one of the patients who were treated with DLI for relapsed multiple myeloma (MM) after transplantation with DLI and IFN-α we isolated a dominant cytotoxic T lymphocyte (CTL) clone capable of recognizing the malignant MM cells from the patient. At the time of the clinical response the patient suffered from mild acute GvHD of skin and liver (grade II), which resolved after discontinuation of IFN-α and short-term treatment with prednisone and cyclosporine. The patient entered a complete remission and is now, 6 years later in persistent complete remission without GvHD.

In the present study we identified the MiHA recognized by this CTL clone to be encoded by the ATP-dependent interferon-responsive *(ADIR)* gene.[37] This gene was found to be highly expressed in the MM cells, in other hematopoietic tumors, as well as non-hematopoietic tumor cell lines. Recognition of normal nonmalignant cells appears to be minor under steady state conditions, but activation of the target cell populations resulted in enhanced recognition by the CTL clone. We hypothesize that T-cell responses against MiHA encoded by the *ADIR* gene may lead to a strong GvT reactivity, potentially coincident with GvHD depending on the activation state of the target tissues.

Materials and methods

CTL generation and culture

The HLA-A2–restricted MiHA-specific CTL clone RDR2 was previously isolated using the IFN-γ secretion assay from a peripheral blood (PB) sample of a patient at the time of clinical response to DLI as treatment for relapsed MM after SCT.[36] CTL clone RDR2 and the allo–HLA-A2 control clone MBM13 were expanded by stimulation with irradiated (50 Gy) allogeneic mononuclear cells (MNCs) and patient-derived Epstein-Barr virus lymphoblastoid cell line (EBV-LCL) in IMDM (Cambrex, Verviers, Belgium) supplemented with penicillin-streptomycin (Cambrex), 3 mM L-glutamine (Cambrex), 5% fetal bovine serum (FBS) (Cambrex), 5% pooled human serum, 100 U/mL IL-2 (Chiron, Amsterdam, The Netherlands), and 0.8 μg/mL PHA (Remel, Dartford, United Kingdom).

Target cell populations

After informed consent was obtained in accordance with the Declaration of Helsinki, with approval from Leiden University Medical Center's Institutional Review Board, BM and PB samples were collected from patients and donors, and MNCs were isolated by Ficoll-Isopaque separation. Recognition of target cells was measured in cytotoxicity assays, and stimulation of responder cells was measured using INF-γ secretion. Lineage-specific hematopoietic cell populations were isolated from PBMCs by magnetic-activated cell sorting using magnetic microbeads coupled to CD4, CD8, CD14, and CD19 monoclonal antibody (mAb) (Mylteni Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. PHA blasts were generated from PBMCs by stimulation with 0.8 μg/mL PHA and subsequent culturing in IMDM supplemented with 100 IU/mL IL-2 and 10% FBS. EBV-LCLs were cultured in IMDM supplemented with 10% FBS. The HLA-A2–positive lymphoblastoid-processing defective cell line T2[38] was cultured in IMDM supplemented with 10% FBS. Hela/A2 was generated by retroviral transduction of HLA-A*02:01 in Hela Tk^- cells and cultured in IMDM supplemented with 10% FBS. Mesenchymal stem cells (MSCs) were generated from bone marrow cells by culturing adherent cells in low-glucose DMEM (Invitrogen, Paisley, Scotland) supplemented with 10% FBS.[39] Commercially obtained biliary epithelial cells (BECs) (Sciencell, San Diego, CA) were grown in DMEM F12 (Cambrex) supplemented with 5% FBS, 2×10^{-9} M T3 hormone, 8.2×10^{-7} M

hydrocortisone, 2 ng/mL epidermal growth factor, and 5 µg/mL insulin and were retrovirally transduced with HLA-A*02:01. IFN-α modulation of stimulator cells was performed by addition of 1000 IU/mL IFN-α-2a (Roche, Woerden, The Netherlands). Adherent solid tumor cell lines from thyroid carcinoma (TT), breast adenocarcinoma (MCF7), and cervical carcinoma (CaSki) from the American Type Culture Collection (ATCC, Manassas, VA) and a melanoma cell line (Brown) were cultured in RPMI supplemented with 10% FBS.

Cytotoxicity assays

CTL-induced specific cytotoxicity of defined cell types in a heterogeneous target cell population was determined in CFSE-based cellular cytotoxicity assays as described before.[40] Briefly, target cells were labeled with 2.5 µM CFSE (Molecular Probes, Leiden, The Netherlands) and incubated with target cells at a 1:1 ratio. After 4 and 24 hours, specific cell populations were counterstained with PE- or APC-labeled CD138, CD3, CD4, CD8, CD14, or CD19 mAb (Becton Dickinson, Erembodegem-Aalst, Belgium), and propidium iodide was added to exclude dead cells. To allow quantification of the surviving cell numbers in each sample, 10^4 Flow-count Fluorospheres (Coulter, Miami, FL) were added immediately before flow cytometric analysis. Cytotoxicity of CTL clones in standard ^{51}Cr release assays was performed as described previously.[41] HPLC-purified natural peptides or diluted synthetic peptides were tested for reactivity by loading ^{51}Cr-labeled T2 cells for 1 hour at 37°C and 5% CO_2 prior to addition of CTLs.

IFN-γ secretion assays

Quantification of CTL stimulation was performed by IFN-γ secretion assays. CTLs were cocultivated with various freshly isolated PBMC cell populations, EBV-LCLs, PHA blasts, transfected Hela/A2 cells, MSCs, or BECs. Stimulator cells and CTLs were diluted in IMDM supplemented with 10% FBS and cultured in 96-well microtiterplates for 24 hours at various responder-stimulator ratios. In each well 5×10^3 CTLs were stimulated with 2×10^4 PBMCs or Hela/A2, 5×10^3 BECs, or 1×10^3 MSCs. Supernatant was harvested, and IFN-γ was measured by standard enzyme-linked immunosorbent assay (ELISA) (Sanquin, Amsterdam, The Netherlands).

Peptide isolation, purification, and characterization

Purification of peptides from 8×10^{10} EBV-LCLs was performed as described before.[24,42] Briefly, frozen cell pellets were lysed and affinity purified. Peptides

were separated from proteins by size-exclusion centrifugation, and peptide concentrates were injected on a Smart System (Amersham Biosciences, Freiburg, Germany) and subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on a 10 cm × 2.1 mm C2/C18 3 µm particle column at 0.2 mL/min. A gradient from 20% to 50% organic phase containing 0.1% TFA was run while 0.1 mL fractions were collected in siliconized vials and stored at −80°C. Isopropanol or acetonitrile was used as organic phase. Reactive fractions were injected on a 15 cm × 75 um C18-Pepmap nano column (LC Packings/Dionex, Breda, The Netherlands), which was directly coupled to a QTOF1 mass spectrometer (Micromass Waters, Manchester, United Kingdom). The flow was delivered by a conventional JASCO binary gradient system (JASCO, Maarssen, The Netherlands), which was reduced to approximately 300 nL/min by an in-house–constructed flow splitter. Injections were performed with a FAMOS (LC Packings/Dionex). On-line HPLC tandem mass spectrometry analysis on the HCT^{plus} (Bruker Daltonics, Bremen, Germany) was performed in a similar fashion. In this case the flow was delivered by an Ultimate (LC Packings/Dionex).

Sequence analysis of the ADIR gene

Trizol reagent (Invitrogen) was added to patient and donor cell pellets, RNA was isolated and purified, and 4 µg was reverse transcribed into cDNA for 1 hour at 37°C using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions. Polymerase chain reactions (PCRs) of nucleotides 1 to 327 of the *ADIR* gene were performed in 50 µL GeneAmpII PCR buffer containing 1.5 mM $MgCl_2$, 250 µM dNTPs, 800 nM forward primer (nucleotides 1 to 18), 800 nM reverse primer (nucleotides 309 to 327), 2% DMSO, and 1.5 units of AmpliTaq DNA polymerase. Amplification on an Applied Biosystems GeneAmp PCR system 2400 was achieved in 35 cycles of 15 seconds at 95°C and 30 seconds at 58°C. Sequence reactions were performed on 1 µL of purified PCR product using the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA) and 1 µM reverse primer in 25 cycles of 10 seconds at 96°C, 5 seconds at 58°C, and 4 minutes at 60°C. After DNA purification, sequencing was performed using an ABI310 sequencer.

Transfection of constructs containing the LB-ADIR-1F epitope

Constructs from donor and patient cDNA containing the *ADIR* gene were generated using 3 different forward primers and 1 reverse primer and ligated in

pCR3.1 expression vector (Invitrogen) as described previously.[31] A mixture of 100 ng plasmid and 0.8 µL Lipofectamine (Invitrogen) was incubated for 30 minutes at room temperature and used to transiently transfect 2×10^4 Hela/A2 cells. After 5 hours of incubation at 37°C, 50 µL IMDM/10% FBS was added. After 24 hours, 10^4 RDR2 cells were added and again after 24 hours 50 µL of supernatant was harvested and tested for IFN-γ secretion in ELISA.

Ex vivo detection of LB-ADIR-1F specific T cells and T-cell receptor-β variable chain analysis

Recombinant biotinylated HLA-A*02:01 monomers were folded with both *ADIR* peptide variants. Streptavidin-APC tetramers were produced as described previously.[43] Tetramer-positive events in thawed patient samples were sorted by fluorescence-activated cell sorting (FACS) to single cells per well, expanded, and tested for cytotoxicity as described previously.[21] T-cell receptor-β variable chain (TCRBV) expression of cytotoxic clones and tetramer-positive T cells in patient material was determined by staining with FITC-conjugated mAb to TCRBV7 (Beckman Coulter, Mijdrecht, The Netherlands). Sequences of the TCRBV were determined as described previously,[36] and TCR chains were named in accordance with the nomenclature described by Arden et al.[44]

Quantitative real-time-PCR to analyze gene expression

Quantitative real-time PCR analysis was performed as described previously.[45] To normalize for variations in the procedures for RNA isolation and cDNA synthesis, 1% mouse spleen cells were added to each cell sample. Quantitative real-time PCR was performed on an ABI/PRISM 7700 Sequence Detector System (Applied Biosystems) using qPCR-Core Kit (Eurogentec, Seraing, Belgium). Human *ADIR* and *PBGD* results were normalized using the murine *Gapdh* expression. For each sample, relative gene expression was calculated using the results obtained from a serial diluted EBV-LCL. EBV-LCL expression was set to 100. Primers for human *ADIR* were designed spanning exons 1 to 3:
forward 5′-GACGACTGTGACGAGGACGA-3′
reverse 5′-CAAATGCTGGCCATGCAG-3′
and probe 5′-(TET)-CTGGGCTGGCGCCTTCCTCTGT-(TAMRA)-3′.
Primers and probe for human *PBGD* were as follows:
forward 5′-GCAATGCGGCTGCAA-3′,
reverse 5′-GGGTACCCACGCGAATG-3′,
and probe 5′-(TET)-CTCATCTTTGGGCTGTTTTCTTCCGCC-(TAMRA)-3′.
Primers and probe for murine *Gapdh* were as follows:

forward 5′-GGGCTCATGACCACAGTCCA-3′
reverse 5′-ATACTTGGCAGGTTTCTCCAGG-3′
and probe 5′-(TET)-TCCTACCCCCAATGTGTCCGTCGT-(TAMRA)-3′.

Results

Isolation of the HLA-A2–restricted CD8 CTL clone RDR2 recognizing a frequently expressed MiHA

We previously described the isolation of various CTL clones from a female patient who was successfully treated with DLI after relapsed MM.[36] CTL clones were generated by direct cloning of IFN-γ–producing cells upon stimulation by irradiated bone marrow cells harvested from the patient prior to SCT. Panel studies using unrelated EBV-LCLs and blocking studies with HLA allele–specific mAb showed that recognition by the most dominant CTL clones was restricted by HLA-A2. Extensive panel studies using PHA blasts and EBV-LCLs of unrelated sibling pairs demonstrated that most HLA-A2–restricted CTL clones designated RDR2 displayed an identical recognition pattern and lysed 57% of targets from all HLA-A2 individuals tested. All RDR2 clones were found to express an identical TCR-BV7S1, N region, and BJ1S4, illustrating that they were derived from the same clonal T cell.[36] Because RDR2 was isolated from a MM patient, we investigated the sensitivity of her MM cells to lysis by the T-cell clone. The CFSE-based cytotoxicity assay was performed to allow quantitative measurement of lysis of MM cells that were present in relatively low frequencies within the heterogeneous bone marrow samples.[40] Figure 1A shows dot plots of a quantitative CFSE-based cytotoxicity assay performed on thawed bone marrow cells from a MiHA-positive patient containing 10% MM cells. Lysis was measured after incubation with a control CMV-pp65-A2–specific CTL clone or with CTL RDR2. CD138 was used as a marker for the malignant MM cells. A strong decrease of the number of $CD138^+$ cells was observed. In a sample containing 1% MM cells that was taken from the patient from which CTL RDR2 was isolated, lysis was measured of bone marrow cells using CD138 as a marker for the malignant MM cells and CD3 as a marker for nonmalignant patient-derived T cells. Figure 1B illustrates that MM cells from the patient were strongly lysed whereas lysis of normal unstimulated T cells was low. Activated T cells (PHA blasts) and EBV-LCLs from the patient were strongly recognized. Donor-derived PHA blasts and EBV-LCLs were not recognized (data not shown). To further study susceptibility of normal hematopoietic cells to lysis by RDR2, PBMC subpopulations from HLA-A2–positive MiHA-positive donors were tested for recognition by RDR2 and an allo-A2 clone. Whereas similar recognition of PHA blasts and EBV-LCLs by the allo-A2 clone and RDR2 was

observed, RDR2-mediated lysis of normal B cells and T cells was lower as compared with allo-A2–mediated lysis (Figure 1C). In addition, PBMCs were

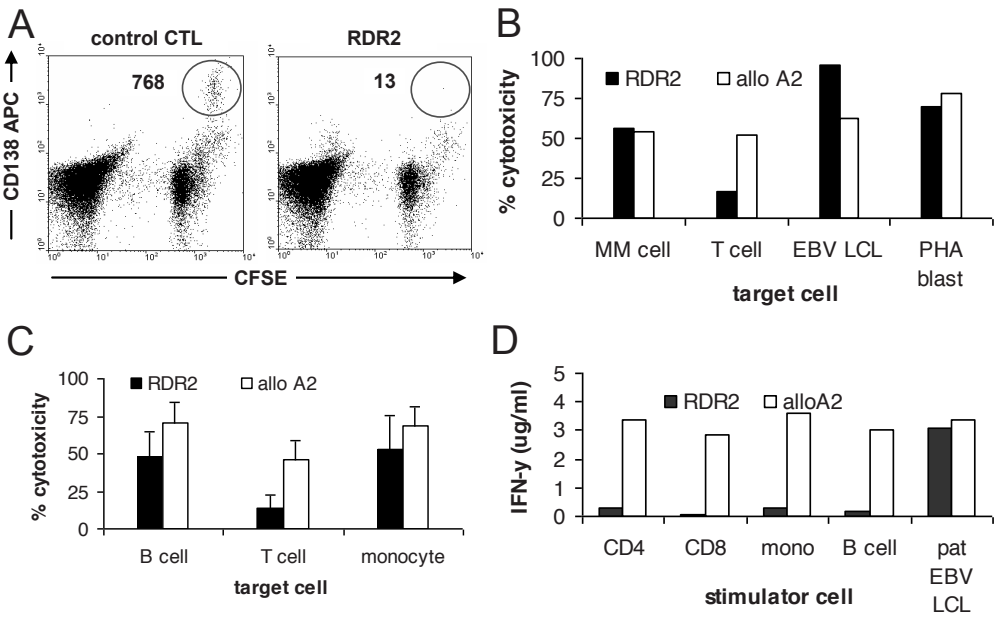

Figure 1. Recognition pattern of CTL clone RDR2 on MM cells and normal hematopoietic cells. (A) Recognition of MM cells by RDR2 in a heterogeneous bone marrow sample of an MiHA-positive patient was determined in CFSE-based cytotoxicity assays after incubation with a control CMV-pp65-A2–specific CTL clone (left dot plot) or with CTL RDR2 (right dot plot) in 1:1 effector-target (E/T) ratios. CFSE-negative cells represent the CTL clones. Strong specific recognition of CFSE-positive CD138 APC-counterstained MM cells was observed. (B) Recognition by RDR2 (closed bars) and control allo-A2 CTL (open bars) was tested in CFSE-based cytotoxicity assays and by IFN-γ secretion. Heterogeneous cell samples were incubated with CTLs at a 1:1 E/T ratio for 4 hours. Patient bone marrow cells were counterstained with CD138 mAb for detection of MM cells or with CD3 mAb for detection of T cells. Patient-derived MM cells were strongly lysed by RDR2 whereas T cells were weakly recognized. Both EBV-LCLs and PHA blasts were strongly lysed. (C) PBMCs from 3 healthy MiHA-positive donors were counterstained with different lineage-specific markers. Lysis by RDR2 was significantly diminished in both B cells ($P = .02$) and T cells ($P < .001$) as compared with lysis by allo-A2 CTL (shown as mean value ± SD). (D) Stimulation of CTLs was measured by INF-γ release after 24 hours of coculture. RDR2 stimulation by resting PBMC subpopulations was low as compared with allo-A2 CTL stimulation whereas activated B cells (EBV-LCL) induced similar IFN-γ release in both CTLs.

separated by magnetic bead cell sorting into CD4 T cells, CD8 T cells, monocytes, and B cells and were used to stimulate RDR2 and allo-A2 CTL in IFN-γ release assays. Whereas all stimulator cell subpopulations were equally able to induce IFN-γ secretion by allo-A2 CTL, stimulation of RDR2 by normal hematopoietic cells was more than 10-fold lower as compared with stimulation by EBV-LCLs (Figure 1D). In conclusion, RDR2 recognized an HLA-A2 restricted epitope causing strong lysis of MM cells and activated T cells and B cells. In contrast, reactivity with normal non-activated hematopoietic cells was relatively low as measured both by direct cytotoxicity and by IFN-γ secretion.

Purification and mass spectrometric identification of the peptide

To identify the epitope that was recognized by CTL clone RDR2, EBV-LCL cells expressing the antigen were lysed, and peptide-HLA complexes were affinity purified using HLA-A2 specific BB7.2 mAb. After elution of peptides, size centrifugation was performed to separate peptides from HLA monomers and β2-microglobulin. After freeze drying, the peptide mixture was subjected to RP HPLC using isopropanol as organic solvent and fractions were collected. ^{51}Cr-labeled T2 cells were loaded with a small sample of each fraction. RDR2 was added, and a single positive fraction could be detected. This fraction was subsequently subjected to RP HPLC with acetonitrile as organic solvent and fractionated. Fractions were tested for reactivity, and again a positive fraction was found. To determine the most abundant masses present in this fraction, part of the fraction was injected on a nano–liquid chromatography system directly coupled to a Q-TOF1 mass spectrometer. Abundantly present masses were fragmented by collision-activated dissociation on an HCT^{plus} mass spectrometer. Analysis of obtained fragmentation patterns led to the sequence of several candidate peptides that were subsequently synthesized. RDR2 recognized an $[M+2H]^{++}$ candidate peptide with mass/charge of 528.8 and sequence SVAPALALFPA at levels as low as 10 pM (data not shown). Furthermore, the fragmentation patterns of the synthetic peptide and the natural eluted peptide were identical (data not shown).

Identification of a polymorphic gene responsible for RDR2 recognition

A basic local alignment sequence tool (BLAST) search of sequence SVAPALALFPA against a 6-frame translation of the European Molecular Biology Laboratory (EMBL) nucleotide database revealed 100% identity to amino acids 13 to 23 from an alternative open reading frame (ORF) of the *ADIR* gene, also known as *TOR3A*[37] (National Center for Biotechnology Information [NCBI] mRNA Reference Sequence [RefSeq] NM_022371). A known single nucleotide polymorphism (SNP) in *ADIR* at nucleotide 78 from C>T (NCBI dbSNP cluster ID rs2296377) results in an amino acid change in an alternative transcript from serine (S) to phenylalanine (F) at position 21, corresponding to position 9 of the eluted peptide (Figure 2A). Both peptides were synthesized and loaded on T2 cells. Patient-type peptide SVAPALAL-F-PA but not donor-type peptide SVAPALAL-S-PA was recognized by RDR2 (Figure 2B). Sequence analysis of the *ADIR* PCR product flanking the SNP revealed that the donor was CC homozygous and the patient CT heterozygous. To demonstrate that patient-type T but not donor-type C of this gene was responsible for recognition by RDR2, constructs were generated from both patient and donor cDNA. Because RDR2 recognized a peptide arising from an alterative ORF controlled by a start codon 5′ upstream from the normal start codon, 3 different forward primers were composed. The first primer was chosen at the normal start codon, thus lacking the alternative start codon. The second primer was chosen at the start of the transcript, thus providing both start codons. The third primer was chosen at the alternative start codon and also contained the normal start codon. The constructs were transiently transfected into Hela/A2 cells. Only patient-derived constructs induced IFN-γ release by RDR2 CTL. Transfection of constructs containing only the normal ORF start codon and lacking the alternative ORF start codon showed a strong decrease of CTL recognition (Figure 2C). All donor-derived constructs failed to induce IFN-γ by RDR2 (data not shown). Next, a panel of 74 unrelated HLA-A2–positive individuals was analyzed by sequencing for determination of the polymorphism and susceptibility to lysis of PHA blasts by RDR2. A 100% correlation between presence of this specific SNP and CTL reactivity proved that this SNP from C>T in the *ADIR* gene generates the MiHA epitope SVAPALALFPA that is recognized by RDR2 (Table 1). The MiHA was designated LB-ADIR-1F.

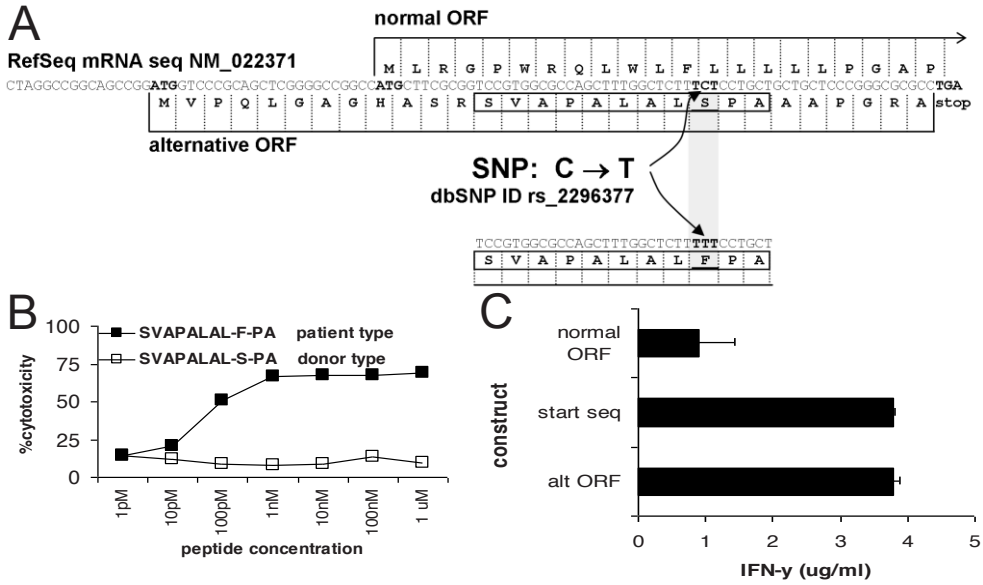

Figure 2. Identification of *ADIR* as the polymorphic gene responsible for RDR2 recognition. (A) BLAST searching of SVAPALALFPA against a translated EMBL database revealed 100% identity to amino acids 13 to 23 from an alternative ORF of the *ADIR* gene. A known SNP at nucleotide 78 results in an amino acid change from S>F. (B) Both patient-type peptide SVAPALAL-F-PA (closed symbols) and donor-type peptide SVAPALAL-S-PA (open symbols) were synthesized and tested for RDR2 reactivity on T2 cells in a ^{51}Cr release assay. Only cells loaded with the patient-type peptide but not cells loaded with the donor-type peptide were lysed. (C) Constructs containing patient-derived DNA were generated. The start of each construct was varied to obtain translation at the start of the transcript, resulting in both the normal and the alternative ORF or specific translation of only the normal or alternative ORF. Constructs were transiently transfected into Hela/A2 cells. RDR2 was cocultured for 24 hours, and IFN-γ release in supernatants was measured by ELISA. RDR2 stimulation was observed in all cases. Stimulation by constructs containing only the normal ORF start codon and lacking the alternative ORF start codon showed strongly diminished CTL recognition. Similar constructs containing the donor-derived DNA were not recognized by RDR2 (data not shown).

Table 1. Correlation of SNP and CTL reactivity in 76 individuals

ADIR nt 78*	No. of individuals	No. of lysed PHA blasts	Frequency, %
CC	33	0	43
CT	36	36	47
TT	7	7	9

* Sequence analysis of nucleotides 1 to 327 of the ADIR gene in PBMCs. Nucleotide 78 represents the SNP.

Tetramer staining and FACS of LB-ADIR-1F–specific CTLs

Both peptide LB-ADIR-1F and peptide SVAPALAL-S-PA were able to bind to recombinant HLA-A*02:01 molecules, and tetrameric complexes were produced. RDR2 specifically bound the LB-ADIR-1F tetramer whereas SVAPALAL-S-PA tetramers and irrelevant HA-1 tetramers were negative (data not shown). LB-ADIR-1F tetramers were used to analyze a series of peripheral blood samples that were taken from the patient before and after DLI. Serum paraprotein levels were analyzed as a marker for disease activity. Whereas LB-ADIR-1F–specific T cells were not detectable prior to DLI, at 7 weeks after DLI high numbers of LB-ADIR-1F–specific CD8 T cells could be detected (Figure 3A). The appearance of LB-ADIR-1F–specific T cells coincided with development of acute GvHD grade II and resulted in complete remission. GvHD was treated successfully with a short course of 1 mg prednisone per kilogram of body weight and cyclosporin. Tetramer-positive T cells were clonally isolated by FACS and expanded. All tetramer-positive CTL clones were able to lyse both patient EBV-LCLs and LB-ADIR-1F–pulsed donor EBV-LCLs (data not shown). TCR characterization of RDR2 showed usage of V-beta BV7S1, J-region BJ1S4, and identical CDR3 regions in 43 of 44 growing clones analyzed. One clone, however, expressed TCRBV6S4 (Figure 3B). Analysis of the patient sample at 7 weeks after DLI revealed that a low percentage of LB-ADIR-1F–positive T cells did not stain with mAb directed against TCR-BV7 (Figure 3C). Functional comparison of the original RDR2, newly isolated identical TCR-BV7S1 clones, and TCR-BV6S4–expressing clones was performed. Whereas cytotoxicity of the original RDR2 and newly isolated BV7S1 clones was similar (data not shown), the BV6S4 clone showed diminished recognition of peptide-loaded T2 cells (Figure 3D). When HLA-A2–positive and LB-ADIR-1F–expressing EBV-LCL cells were used as target cells, again the BV6S4 clone displayed lower cytotoxicity (Figures 3E). Similar results were obtained using HLA-A2–positive and LB-ADIR-1F–expressing PHA blasts (data not shown). To further investigate differential recognition, reactivity of both TCR BV7S1 and TCR BV6S4 was tested on EBV-LCL–expressing HLA-A*0205 and HLA-A*0220 subtypes. Lysis of LB-ADIR-1F–positive HLA-A*0205 EBV-LCLs was absent whereas identical strong lysis between both TCR types was observed of HLA-A*0220 EBV-LCLs. Pulsing of HLA-A*0205 EBV-LCLs with synthetic peptide could only partially restore the recognition to identical levels by both TCR types (data not shown).

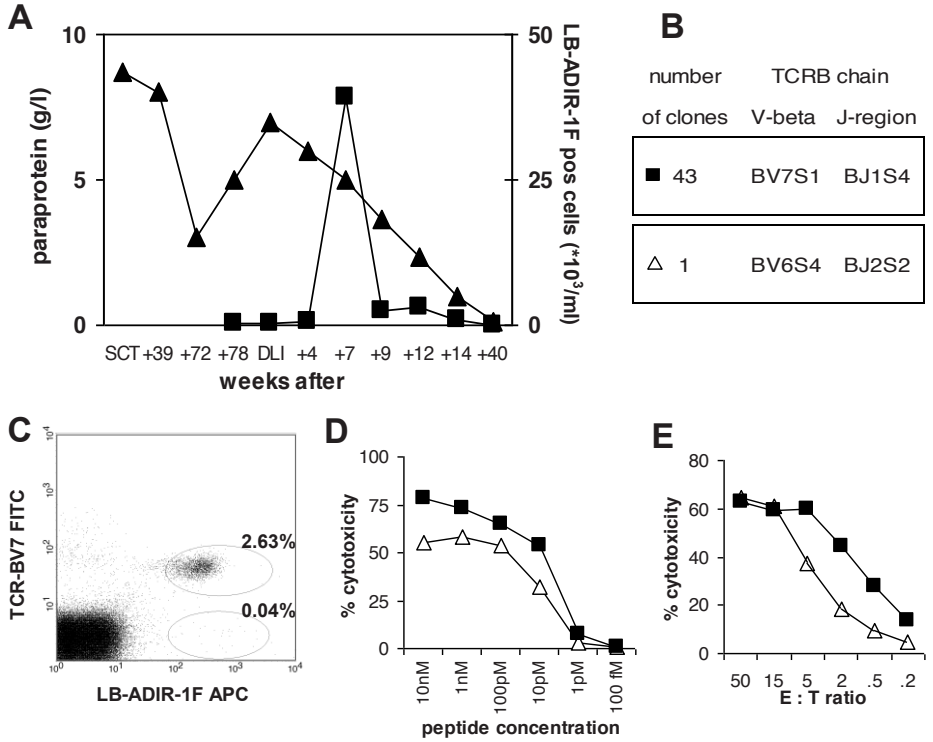

Figure 3. Tetramer staining and clonal analysis of LB-ADIR-1F–specific CTLs in the patient. (A) PBMCs from the patient taken at several time points after SCT and DLI were stained with LB-ADIR-1F tetramers (squares), and serum paraprotein levels were measured (triangles). (B) LB-ADIR-1F tetramer-positive cells present at week 7 after DLI were single-well sorted and expanded. TCRBV sequence analysis was performed on 44 reactive clones revealing TCRBV7S1 in 43 clones and TCRBV6S4 in 1 clone.(C) Reanalysis of the patient sample was performed using counterstaining with TCRBV7 confirming a low percentage of TCRBV7-negative cells in the LB-ADIR-1F–positive population (C). Reactivity of TCRBV7S1- (squares) and TCRBV6S4- (triangles) expressing clones was determined using ^{51}Cr release assays on peptide-pulsed T2 cells (D) and EBV-LCL cells (E), demonstrating that TCRBV6S4-expressing T cells displayed lower cytotoxicity.

Modulation of recognition by IFN-α and *ADIR* gene expression

Because previous studies on the *ADIR* gene indicated that IFN-α could enhance gene expression[37] and because the patient was also treated with IFN-α, we studied the effect of IFN-α on LB-ADIR-1F recognition by RDR2 using MNCs of LB-ADIR-1F–expressing donors. MNCs were precultured for 48 hours in the absence or presence of 1000 IU/mL IFN-α prior to addition of CTL RDR2 at a 1:1 ratio. Maximal recognition was determined by testing MNCs pulsed with

saturating concentrations of synthetic peptide. Cytotoxicity was measured in a 4-hour CFSE assay (Figure 4A), and IFN-γ release was measured after 24 hours (Figure 4B). A nonsignificant increase of recognition was observed in all cases, whereas peptide-pulsed MNCs were recognized at levels comparable to EBV-LCL recognition (data not shown). Because recognition of LB-ADIR-1F–positive cells significantly differed between freshly isolated MNCs and activated cells such as EBV-LCLs and PHA blasts, we measured gene expression by performing quantitative real-time PCR on freshly isolated MNCs and MNCs cultured for 24 and 48 hours in medium containing 10% FBS alone or supplemented with either 1000 IU/mL IFN-α or 0.8 μg/mL PHA. To each cell sample a fixed percentage of 1% murine spleen cells was added prior to RNA isolation and cDNA synthesis. Each sample was assayed for expression of *ADIR*, *PBGD*, and murine *Gapdh*. To exclude variation in mRNA isolation and cDNA synthesis, both *ADIR* and *PBGD* expression levels were normalized to the murine *Gapdh* expression level, which maximally resulted in 2-fold higher or lower outcome. Whereas expression of the household gene *PBGD* was only influenced by PHA, *ADIR* expression already increased upon culturing of the cells, irrespective of the presence of IFN-α. High *ADIR* expression levels in EBV-LCLs and in PHA-stimulated MNCs were observed (Table 2).

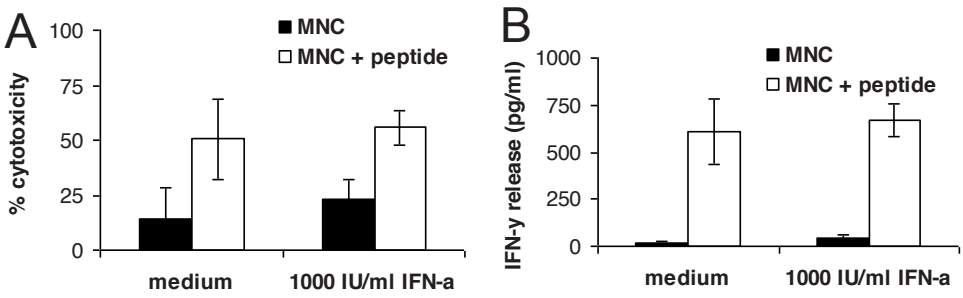

Figure 4. Modulation of LB-ADIR-1F–specific recognition by IFN-α. Recognition of MNCs from 3 different LB-ADIR-1F–positive donors was measured by (A) direct cytotoxicity in 4-hour CFSE assays and by (B) 24-hour IFN-γ release following preincubation in medium alone or in medium containing 1000 IU/mL IFN-α for 48 hours (shown as mean value ± SD). Recognition of MNCs (closed bars) and MNCs exogeneously pulsed with saturating concentrations of synthetic peptide (open bars) was measured at an E/T ratio of 1:1. Only minimal up-regulation of recognition in IFN-α precultured MNCs was observed.

Table 2. Modulation of *ADIR* relative gene expression in PBMCs

incubation (hours)	*ADIR*			*PBGD*		
	control	IFN	PHA	control	IFN	PHA
0	9 ± 3	9 ± 3	9 ± 3	5 ± 1	5 ± 1	5 ± 1
24	24 ± 4	19 ± 6	48 ± 22	5 ± 2	3 ± 1	60 ± 53
48	36 ± 17	23 ± 10	238 ± 91	8 ± 2	5 ± 2	178 ± 153

PBMCs were incubated for the indicated times in the absence or presence of 1000 IU/mL INF-α or 0.8 μg/mL PHA. Prior to mRNA isolation and cDNA synthesis, 1% mouse spleen cells were added to each sample. Data were normalized to murine *Gapdh* expression. Expression of EBV-LCLs was set to 100.

Recognition of normal non-hematopoietic cells by LB-ADIR-1F specific CTLs

Recognition of non-hematopoietic normal tissues was studied using BECs and MSCs expressing the LB-ADIR-1F SNP variant in both direct cytotoxicity assays and stimulation assays. Whereas direct cytotoxicity against patient EBV-LCLs was strong, lysis of MSCs (Figure 5A) and BECs (Figure 5B) was low. LB-ADIR-1F peptide pulsing of MSCs and BECs did not significantly increase lysis, suggesting overall low susceptibility of these target cells to direct cytotoxicity. When MSCs (Figure 5C) and BECs (Figure 5D) were used as stimulator cells in IFN-γ release assays again, low stimulation by both non-hematopoietic cell types was observed. LB-ADIR-1F peptide pulsing of the stimulator cells increased stimulatory capacity of both MSCs and BECs to levels comparable to stimulation by patient EBV-LCLs.

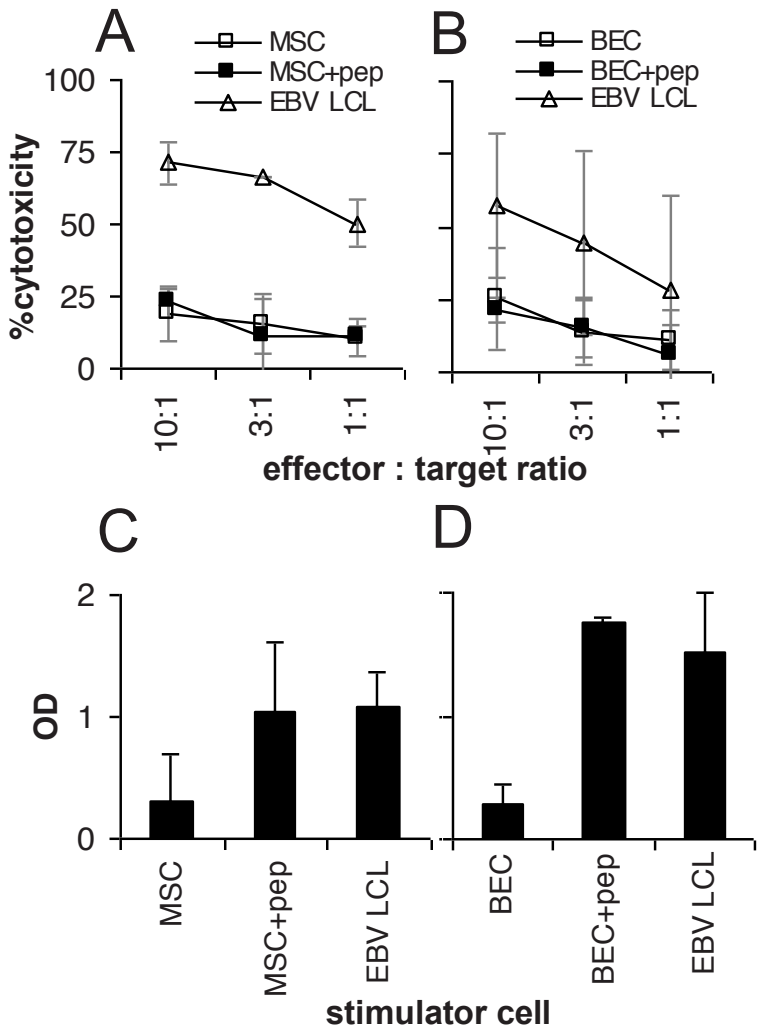

Figure 5. Recognition of normal non-hematopoietic cells by LB-ADIR-1F specific CTLs. Recognition of LB-ADIR-1F–expressing HLA-A2–positive MSCs and BECs by CTL RDR2 was studied in 3 separate 4-hour ^{51}Cr release assays and in 3 separate IFN-γ secretion assays. (A,B) Lysis of untreated target cells (open squares) and target cells exogenously pulsed with saturating concentrations of synthetic peptide (closed squares) was compared with lysis of patient EBV-LCLs (open triangles). (C,D) IFN-γ secretion by CTL RDR2 was measured after 24 hours of co-cultivating CTL RDR2 at a 3:1 responder-stimulator ratio with untreated cells, peptide-pulsed cells, and patient EBV-LCLs. Data are shown as mean values ± SD.

Recognition of malignant hematopoietic cells and solid tumor cell lines

To investigate susceptibility to lysis by LB-ADIR-1F specific CTLs, a panel of HLA-A2 positive malignant hematopoietic cells was subjected to sequence analysis on the LB-ADIR-1F polymorphism and lysis by CTL RDR2 and allo-A2 CTL. Prominent recognition was found of MM cells. Additionally, leukemic cells expressing the LB-ADIR-1F polymorphism were susceptible to lysis by RDR2 (Figure 6A). Furthermore, when a panel of LB-ADIR-1F positive HLA-A2 expressing solid tumor lines was tested for recognition by RDR2, lysis at levels comparable to lysis by the allo-A2 CTL was observed (Figure 6B).

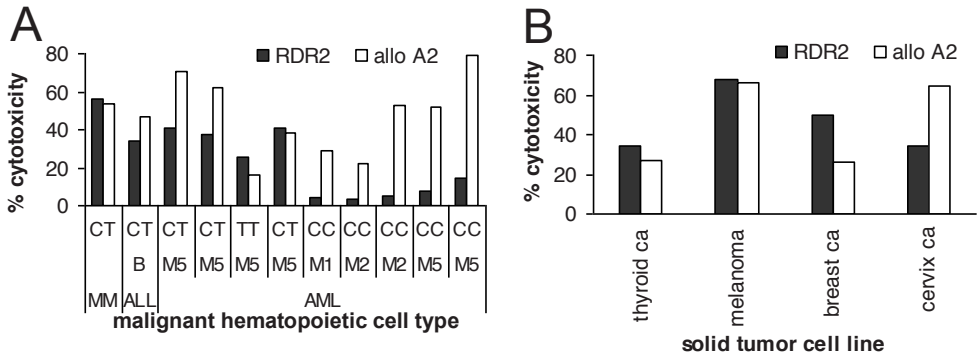

Figure 6. Recognition of malignant hematopoietic cells and solid tumor cell lines.
(A) Lysis of LB-ADIR-1F expressing MM cells in heterogeneous bone marrow samples was measured using the CFSE assay at a 1:1 E/T ratio and leukemic blast cell populations using 4-hour ^{51}Cr release assays at an E/T ratio of 20:1. Recognition by RDR2 is shown by closed bars and allo-A2 control CTLs by open bars. On the x-axis the malignant cell type and the SNP at nucleotide 78 of the ADIR gene are depicted. MM and leukemic cells expressing the LB-ADIR-1F epitope (CT or TT) were recognized by both CTLs whereas LB-ADIR-1F negative (CC) targets were only lysed by control allo-A2 CTLs. (B) A panel of HLA-A2 positive LB-ADIR-1F expressing solid tumor cell lines was tested for lysis by RDR2 (closed bars) and control allo-A2 CTL (open bars) using 4-hour ^{51}Cr release assays at an E/T ratio of 20:1. All tumor cell lines were lysed by LB-ADIR-1F specific CTLs.

Discussion

In this study, we identified the ATP-dependent interferon-responsive gene to encode an activation-induced minor histocompatibility antigen that was recognized by MM-reactive T cells. A dominant HLA-A2–restricted CD8 CTL clone designated RDR2 was isolated from a patient with relapsed MM after allogeneic SCT responding to DLI, resulting in a long-lasting complete remission. Biochemical analysis of peptides eluted from HLA molecules revealed that this CTL clone recognized the peptide SVAPALALFPA, which was encoded by an alternative ORF of the human *ADIR* gene. This MiHA, designated LB-ADIR-1F, differed only by an S>F substitution from the donor-type allelic counterpart. A population study with 76 HLA-A2 individuals revealed a 100% correlation between SNP occurrence and recognition of target cells, confirming that the CTL clone was directed against this *ADIR*-derived epitope, which had a population frequency of 57%.

The function of the *ADIR* gene is unknown. When T-cell recognition of target cells and expression levels of *ADIR* mRNA were analyzed in various cell types, we demonstrated a relatively low expression of the gene in resting cells corresponding with low recognition. When proliferation was induced in PB T cells and B cells, strong gene up-regulation was observed, resulting in high recognition by the LB-ADIR-1F specific T cells. Although concurrent expression of normally and alternatively transcribed *ADIR* proteins cannot be directly determined in the cell, the strong correlation between the *ADIR* SNP and susceptibility to lysis illustrates the absence of specific expression regulation of the alternatively translated protein.

Because the patient was treated with IFN-α and it has previously been described that *ADIR* gene expression could be up-regulated by interferons,[37] we examined gene expression and LB-ADIR-1F specific recognition of PBMCs that were preincubated with IFN-α. A minor increase in *ADIR* gene expression was found after *in vitro* culture in serum irrespective of IFN-α preincubation. In addition, in the presence of IFN-α only a minimal increase in susceptibility to lysis and stimulatory capacity was observed.

Not only MM cells but also other hematologic malignancies and non-hematopoietic solid tumor cell lines were recognized by LB-ADIR-1F specific T cells. MSCs and BECs, which may represent possible target tissues of GvHD,

displayed low recognition by the ADIR-specific T cells. Our results illustrate broad antitumor reactivity of LB-ADIR-1F specific T cells as shown by the lysis of malignant hematopoietic cells as well as solid tumor cell lines combined with limited recognition of non-activated tissues.

Kinetic studies of PB samples isolated from the patient before and after DLI using tetramers recognizing various MiHA differentially expressed in donor and recipient illustrated a strong correlation between the clinical response and the occurrence of not only a high frequency of LB-ADIR-1Fspecific T cells but also of HA-1 and LB-ECGF-1H specific T cells as studied previously.[21,28,31,36] The LB-ADIR-1F specific T cells were the most dominant, comprising almost 3% of the CD8 T cells. Approximately 1% LB-ECGF-1H specific T cells and 0.9% HA1-specific T cells were found with similar kinetics. These *in vivo* circulating MiHA-specific T cells represent 80% of activated PB CD8 T cells as identified by co-expression of HLA-DR. Furthermore, because HA-1, ECGF, and ADIR proteins are highly expressed in MM cells and their relevant polymorphism can be recognized by T cells, we hypothesize that the 3 combined strong reactivities are likely to have caused the clinical course of the major response to DLI resulting in a complete remission lasting now for more than 6 years.

In conclusion, we characterized a novel MiHA encoded by a frequently occurring SNP in the *ADIR* gene that is recognized by tumor-reactive CTLs. We hypothesize that the balance between GvT responses and GvHD is not only determined by the specificity of T cells but also by the activation state of GvHD target tissues.[33–35,46] Up-regulated tissue susceptibility to MiHA-specific T cells may be caused by the cytokine storm shortly after stem cell transplantation due to conditioning regimens resulting in tissue damage and the presence of high numbers of recipient-originated antigen-presenting cells. Homeostatic proliferation of these T cells during the lymphopenic phase may further amplify the magnitude of such responses. This state may result in severe GvHD if these T cells are administered as part of the stem cell graft. Immunotherapy of tumor cells by postponed adoptive transfer of LB-ADIR-1F specific T cells following initially T-cell depleted transplantation may result in a strong GvT response with limited risk of GvHD.

Acknowledgments

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Chapter 3

High-throughput characterization of 10 new minor histocompatibility antigens by whole genome association scanning

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Abstract

Patients with malignant diseases can be effectively treated with allogeneic hematopoietic stem cell transplantation (allo-SCT). Polymorphic peptides presented in HLA molecules, the so-called minor histocompatibility antigens (MiHA), play a crucial role in antitumor immunity as targets for alloreactive donor T cells. Identification of multiple MiHA is essential to understand and manipulate the development of clinical responses after allo-SCT. In this study, CD8 T-cell clones were isolated from leukemia patients who entered complete remission after allo-SCT, and MiHA-specific T-cell clones were efficiently selected for analysis of recognition of a panel of EBV-transformed B cells positive for the HLA restriction elements of the selected T-cell clones. One million single nucleotide polymorphisms (SNPs) were determined in the panel cell lines and investigated for matching with the T-cell recognition data by whole genome association scanning (WGAs). Significant association with 12 genomic regions was found, and detailed analysis of genes located within these genomic regions revealed SNP disparities encoding polymorphic peptides in 10 cases. Differential recognition of patient-type, but not donor-type, peptides validated the identification of these MiHA. Using tetramers, distinct populations of MiHA-specific CD8 T cells were detected, demonstrating that our WGAs strategy allows high-throughput discovery of relevant targets in antitumor immunity after allo-SCT.

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-SCT) is a curative treatment for malignant diseases[1]. In case of relapse, persistent disease, or incomplete donor chimerism after transplantation, donor lymphocytes can be administered to initiate or enhance graft-*versus*-tumor (GvT) reactivity[2]. Following HLA-matched allo-SCT, minor histocompatibility antigens (MiHA) are the target structures for donor-derived T cells. Presented in the context of HLA molecules, MiHA are peptides derived from polymorphic proteins that differ between individuals due to genomic single nucleotide polymorphisms (SNPs)[3–6]. T-cell responses directed against MiHA have been monitored in detail in patients responding to donor lymphocyte infusion (DLI)[7, 8]. In these patients, detection of circulating MiHA-specific T cells coincided with rapid disappearance of malignant cells, indicating the *in vivo* efficacy of MiHA-specific T cells. Whether donor T cells mediate beneficial GvT effects or harmful graft-*versus*-host disease (GvHD) is likely to depend on the tissue distribution of the target antigen. Exclusive MiHA expression on malignant cells may result in a selective GvT effect, whereas T cells recognizing broadly expressed MiHA may lead to detrimental GvHD[9].

Initially, biochemical identification of peptides eluted from HLA class I molecules recognized by MiHA-specific T cells was used for MiHA discovery[10–15]. In addition, cDNA libraries were generated and screened for T-cell recognition to identify the MiHA-encoding gene transcript[8, 16–21]. Finally, genetic linkage analysis has been applied to define the genomic region encoding the MiHA[22, 23]. Irrespective of the MiHA identification method, ultimate validation of each MiHA was provided by the strict correlation between a specific SNP genotype and recognition by the T-cell clone.

SNPs between patient and donor may lead to MiHA expression by different mechanisms. The majority of MiHA are encoded by “missense” SNPs that directly encode an amino acid polymorphism in the protein. In addition, SNPs “synonymous” in the normal open reading frame (ORF) have been shown to encode polymorphic amino acids in MiHA epitopes upon translation of alternative reading frames (ARF), as previously described for LB-ECGF-1H[8] and LB-ADIR-1F[15]. MiHA can also be generated by protein splicing, as illustrated for HwA-9, which is spliced together in a noncontiguous order by the proteasome[18]. Furthermore, MiHA can be encoded by gene transcripts that

differ between patient and donor due to insertion or deletion of a single nucleotide, thereby inducing a shift in the translational reading frame, as shown by the LRH-1 MiHA[23]. Finally, MiHA may be encoded by gene transcripts that are homozygously deleted in the donor, as shown for MiHA encoded by the *UGT2B17* gene[17], or by intronic SNPs altering gene expression by RNA splicing, as shown for ACC-6[19].

Although various MiHA identification approaches have led to the successful discovery of 20 HLA class I restricted MiHA, their laboriousness and low efficiency hampered identification of the majority of MiHA recognized by T-cell clones. Development of advanced array-based SNP genotyping, and the fact that MiHA are encoded by polymorphic genomic regions containing specific SNPs, enabled application of whole genome association scanning (WGAs) as an alternative approach for MiHA discovery[24]. Association analysis may identify associating SNPs that directly encode the amino acid polymorphism or may serve as markers for MiHA-encoding SNPs that are not measured by the array. These markers are in linkage disequilibrium with MiHA-encoding SNPs and therefore associate with T-cell recognition. The feasibility of WGAs as an approach to identify new MiHA has been shown by the discovery of individual MiHA in BCL2A1[24], CD19[25], SLC1A5[26], UGT2B17[26], SLC19A1[27], P2RX7[28], and DPH1[28].

To understand the development of GvT reactivity and GvHD, characterization of large numbers of MiHA is essential. High-throughput identification and expression analysis of MiHA in patients with various clinical responses after allo-SCT may provide insight into the fundamental difference and may allow manipulation of the balance between GvT reactivity and GvHD. Here, we report the successful application of WGAs for high-throughput characterization of MiHA in GvT reactivity. T-cell clones were generated from two patients who received an HLA-matched allograft and responded to DLI for recurrence of the malignant disease. A panel of 80 EBV-transformed B-cell lines (EBV-LCL) was tested for recognition by each T-cell clone. More than 1 million SNPs were measured in the EBV-LCL and subsequently searched for association with the EBV-LCL recognition pattern of each T-cell clone. Strong association with a genomic region was found for 12 of the 17 T-cell clones evaluated. For 10 of these 12 T-cell clones, novel MiHA with balanced population frequencies presented in the common HLA-A*02:01 or B*07:02 molecules could be identified, illustrating the value and efficiency of WGAs for MiHA discovery.

Using tetramer analysis, the kinetics of MiHA-specific T-cell responses in the patients were shown to coincide with the clinical responses that were observed after DLI.

Patients

Patient H (HLA-A*02:01, B*07:02, B*44:02, C*05:01, C*07:02, DRB1*04, DRB1*15, DRB4*01, DRB5*01, DQB1*03, DQB1*06, DPB1*04) had myelodysplastic syndrome refractory anemia with excess of blasts type 2. She was transplanted with a T-cell depleted peripheral blood stem cell graft from a male HLA-matched unrelated donor. Chimerism analysis showed a decrease in the percentage of donor cells to 86% during the first 6 months after transplantation; therefore, the patient was treated with DLI. After DLI, the patient developed GvHD limited to skin and mouth, which coincided with a rapid and sustained conversion to 100% donor chimerism. Patient Z (HLA-A*01:01, A*02:01, B*07:02, B*44:02, C*05:01, C*07:02, DRB1*11, DRB1*15, DQB1*03, DQB1*06, DPB1*04, DPB1*14) is a patient with chronic myelogenous leukemia (CML) transplanted with a T-cell depleted peripheral blood stem cell graft from his HLA-identical sibling. More than 1 year after transplantation, the patient was successfully treated with DLI for cytogenetic relapse of CML. The patient did not develop GvHD.

Materials and methods

Cell collection, preparation, and EBV-LCL culture

Peripheral blood and bone marrow samples were collected from the patients, their donors, and third-party 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 gradient centrifugation and cryopreserved. Stable EBV-LCL were generated by *in vitro* transformation of thawed mononuclear cells of selected HLA-A*02:01–positive and B*07:02-positive individuals using EBV supernatant[29] followed by culture in Iscove's modified Dulbecco's medium (IMDM, Lonza) with 10% fetal bovine serum (FBS, Lonza).

Isolation, expansion, and selection of T-cell clones

Peripheral blood mononuclear cells (PBMC) obtained after DLI were thawed for isolation of T-cell clones. Monocytes were depleted using magnetic CD14 beads (Miltenyi Biotec) according to the manufacturer's instructions. CD14-negative cells were stained with allophycocyanin (APC)-conjugated anti-CD8 antibody (Invitrogen) and phycoerythrin (PE)-conjugated anti–HLA-DR antibody (Invitrogen). Propidium iodide (Sigma-Aldrich) was added to exclude dead cells,

and viable HLA-DR$^+$ CD8 cells were single-cell sorted by flow cytometry. Isolated T cells were collected in 96-well U-bottomed plates (Corning) in IMDM with 5% pooled human serum, 5% FBS, interleukin 2 (IL-2; 100 IU/mL), phytohemagglutinin (0.8 µg/mL, Murex Biotec Limited), and 5×10^4 irradiated allogeneic PBMC as feeder cells. Proliferating T-cell clones were restimulated every 10 to 14 days under identical stimulatory conditions at 2×10^5 T cells and 1×10^6 feeder cells per milliliter of culture medium. T-cell (5×10^3 per well) recognition of patient- and donor-derived EBV-LCL as well as third-party EBV-LCL (3×10^4 per well) were tested in 96-well U-bottomed plates in 100 to 150 µL IMDM with 5% pooled human serum, 5% FBS, and 20 IU/mL of IL-2. After overnight coincubation, release of IFN-γ was measured in culture supernatants by ELISA according to the manufacturer's instructions (Sanquin). For blocking studies, EBV-LCL were preincubated with saturating concentrations of antibodies against HLA-A*02 (BB7.2) and HLA-B*07 (VTM3A1) for 30 minutes at room temperature before addition of T cells.

T-cell receptor-β variable chain analysis

T-cell receptor-β variable chain (TCRBV) usage was investigated by flow cytometry using specific monoclonal antibodies as included in the TCRBV repertoire kit (Beckman Coulter). T-cell clones with identical TCRBVs were analyzed by DNA sequencing of the CDR3 region of the TCRBV after amplification by PCR using specific forward and reverse primers. TCRBV products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using the dye terminator cycle sequencing kit (ABI-PRISM, Perkin-Elmer), according to the manufacturer's instructions.

Whole genome association scanning

A panel of 80 HLA-A*02:01 and B*07:02-expressing EBV-LCL was selected for WGAs. To allow simultaneous analysis of all EBV-LCL for recognition by T-cell clones, triplicate samples of EBV-LCL were dispensed at 6×10^4 cells in 50 µL per well in 96-well polypropylene U-bottomed plates (Greiner Bio-One). After addition of 50 µL medium containing 20% DMSO, the panel plates were sealed, frozen, and stored at −140°C. To test the recognition of the EBV-LCL panel by each individual T-cell clone, plates were thawed, washed, and incubated for 2 days, and 5×10^3 MiHA-specific T cells were added to each well. After 24 hours, supernatants were used for IFN-γ ELISA. Of each EBV-LCL, genomic DNA from 5×10^6 cells was isolated using Gentra Puregene Cell Kit (Qiagen). According to the manufacturer's instructions, PCR-free whole genome

amplification was performed. The DNA samples were subsequently fragmented, purified, and hybridized to Illumina Human1M-duo arrays containing probes for 1.1 million SNPs (Illumina). After hybridization, the bead arrays were stained and fluorescence intensities were quantified on an Illumina Bead Array 500GX Scanning Station. Raw data were analyzed using Illumina Genome Studio software, and SNP genotype reports were generated allowing WGAs analysis using the Plink software (http://pngu.mgh.harvard.edu/~purcell/plink, version 1.03[30]). For WGAs, categorization of the test results in two separate groups is required. Therefore, EBV-LCL were divided into MiHA^{pos} and MiHA^{neg} groups using five times the level of IFN-γ production in the absence of EBV-LCL as a threshold for recognition. WGAs was performed by combining T-cell recognition with SNP genotyping data. The level of matching between both patterns was calculated according to Fisher's exact test.

PCR amplification and gene sequencing

RNA from the patients and their donors was isolated from EBV-LCL using Trizol (Invitrogen) and transcribed into cDNA by reverse transcriptase (Invitrogen) using oligo-dT primers (Roche Diagnostics). Genes that were located in associating genomic regions were amplified by PCR using various forward and reverse primers that were selected to comprise entire gene transcript or specific SNP-containing regions (see also Table S1). PCR products were purified and analyzed by DNA sequencing, and the obtained patient and donor sequences were aligned to identify SNP disparities.

Peptide prediction and analysis

For each SNP that was identified by WGAs and gene sequencing, gene fragments comprising the polymorphic patient-type nucleotide flanked by 30 upstream and downstream nucleotides were translated into different reading frames. The online algorithm of NetMHC (31) was used to search for peptides with predicted binding to the appropriate HLA molecule. Peptide candidates were synthesized and dissolved in DMSO. Dilutions were prepared in IMDM and added to MiHA^{neg} donor EBV-LCL (3×10^4 per well) in 96-well U-bottom plates for 2 hours at 37°C. T cells (5×10^3 per well) were added, and after 24 hours, the culture supernatant was used for IFN-γ ELISA.

Tetramer production and staining

Tetramers were constructed by folding peptides in appropriate biotinylated MHC-monomers followed by multimerization using streptavidin conjugated to PE or APC as previously described with minor modifications[32]. Patient samples were thawed and stained with FITC-conjugated CD4 and CD14 antibodies to exclude nonrelevant cells in flow cytometric analysis. MiHA-specific T cells were visualized using PE- or APC-conjugated tetramers and PE-Cy7–labeled anti-CD8. On average, 5×10^5 viable cells were analyzed per staining using a Becton Dickinson FACS CantoII device and DIVA software.

Results

Selection of MiHA-specific CD8 T-cell clones eligible for WGAs

CD8 T cells were isolated from PBMCs obtained 5 or 6 weeks, and 8 weeks after DLI from patient H and patient Z, respectively. Single activated CD8 T cells were isolated using flow cytometry based on expression of HLA-DR. Percentages of CD8 T cells expressing HLA-DR were 11% (week 5) and 19% (week 6) for patient H and 3% (week 8) for patient Z (data not shown). Clonal expansion was observed for 195 T-cell clones from patient H and 37 T-cell clones from patient Z. All 232 clones were tested for recognition of patient- and donor-derived EBV-LCL by IFN-γ ELISA. The strategy followed for selection of MiHA-specific T-cell clones eligible for WGAs is depicted in Figure 1A. In total, 62 T-cell clones from patient H and 16 T-cell clones from patient Z differentially recognized patient, but not donor, EBV-LCL, indicating recognition of MiHA. All 78 patient-specific T-cell clones were investigated for HLA restriction using specific HLA class I, HLA-A*02, and B*07 blocking antibodies. Restriction to HLA-A*02 or B*07 was observed for 43 T-cell clones from patient H and 9 T-cell clones from patient Z. To identify whether T-cell clones recognized the same MiHA, we tested a panel of 30 HLA-A*02 and B*07 expressing EBV-LCL. In addition, TCRBV diversity was determined by flow cytometric analysis using specific TCRBV monoclonal antibodies and by DNA sequencing of the TCRBV CDR3 regions[33]. This led to identification of 20 unique MiHA recognition patterns, including 13 MiHA recognized by single T-cell clones and 7 MiHA recognized by groups of T-cell clones (Table 1). TCRBV diversity was observed in five of the seven groups, indicating the presence of polyclonal T-cell responses targeting these MiHA. Application of WGAs requires a balanced distribution between $MiHA^{pos}$ and $MiHA^{neg}$ test samples. Recognition of 30 EBV-LCL was used to determine this parameter, resulting in exclusion of three clones recognizing rare (clones H11 and H15) or common (clone H9) MiHA. Cross-reactivity to nonself HLA molecules was excluded by the lack of recognition of a panel of 25 HLA-A*02 and B*07 negative EBV-LCL (data not shown).

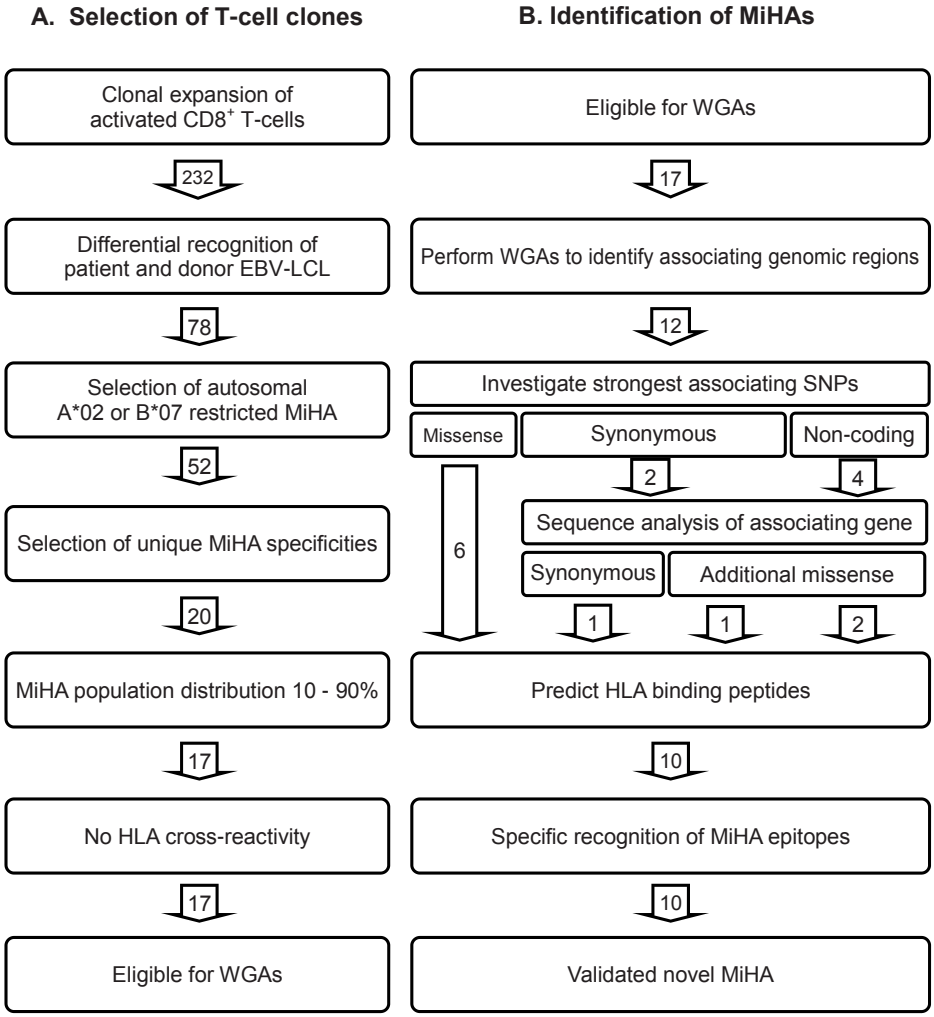


Figure 1. T-cell clone selection and WGAs strategy for MiHA identification.
Strategy outline for selection of CD8 T-cell clones (A) and MiHA identification by WGAs (B). The digits in the arrows depict the number of clones that were selected at each step.

Table 1. Isolation of CD8 T-cell clones recognizing 20 unique MiHA

Patient	Clone type	T-cell clone characteristics*			
		HLA restriction	No. of clones	TCRBV diversity	MiHA frequency (%)
H	H 1	A*02	2	2	50
	H 2	A*02	1	1	41
	H 3	A*02	1	1	23
	H 4	A*02	14	3	50
	H 5	A*02	1	1	97
	H 6	A*02	1	1	52
	H 7	A*02	1	1	0
	H 8	B*07	11	1	67
	H 9	B*07	4	2	37
	H 10	B*07	1	1	60
	H 11	B*07	2	2	53
	H 12	B*07	1	1	39
	H 13	B*07	1	1	13
	H 14	B*07	1	1	10
	H 15	B*07	1	1	0
Z	Z 1	B*07	1	1	47
	Z 2	B*07	1	1	37
	Z 3	B*07	4	2	72
	Z 4	B*07	1	1	53
	Z 5	B*07	2	nt	34

Abbreviation: nt, not tested.

*T-cell clones were isolated from patient H and patient Z after DLI at 5 or 6 weeks, and at 8 weeks, respectively, based on HLA-DR expression.

Identification of associating genomic regions by WGAs

A panel of 80 HLA-A*02:01 and B*07:02 expressing EBV-LCL was generated to identify MiHA-encoding genes by WGAs. Genomic DNA was isolated from each individual EBV-LCL for SNP genotype analysis using Illumina Human 1M-duo bead chips. To estimate the quality of the array data, we extracted SNP genotypes for known MiHA. For 16 of the 27 known MiHA, probes were included on the Illumina Human 1M-duo bead array, and all SNP genotypes were accurately measured with call rates >95% and allele frequencies comparable with dbSNP data (see also Table S2). Next, recognition of all EBV-LCL by each WGAs-eligible T-cell clone was tested using IFN-γ ELISA. Figure 2 shows the recognition of all EBV-LCL for the selected T-cell clones. Clear clustering of $MiHA^{pos}$ and $MiHA^{neg}$ EBV-LCL cells was observed for the majority of the T-cell clones. For clones H6 and H12, $MiHA^{pos}$ EBV-LCL could not be clearly discriminated from $MiHA^{neg}$ EBV-LCL due to the large number of EBV-LCL that were intermediately recognized by the T cells. WGAs was performed by comparing each T-cell recognition pattern with all SNP genotypes. The level of matching between both patterns was calculated according to Fisher's exact test. By selecting SNP genotypes that significantly associated with a T-cell recognition pattern with *P* values $<10^{-9}$, single genomic regions could be identified for 12 T-cell clones. The identified regions varied in length from 1 bp up to 100 kb, comprising a maximum number of three genes and containing 1 to 17 associating SNPs (see also Figure S1 and Table S3). Lack of association with a genomic region was observed for five T-cell clones, including clones H6 and H12, which produced intermediate levels of IFN-γ upon co-incubation with a number of EBV-LCL. Clones H13 and H14, which also failed to show association with a genomic region, recognized MiHA with low population frequencies of 10% and 11%, respectively, suggesting that the power of WGAs was not sufficient to identify these MiHA.

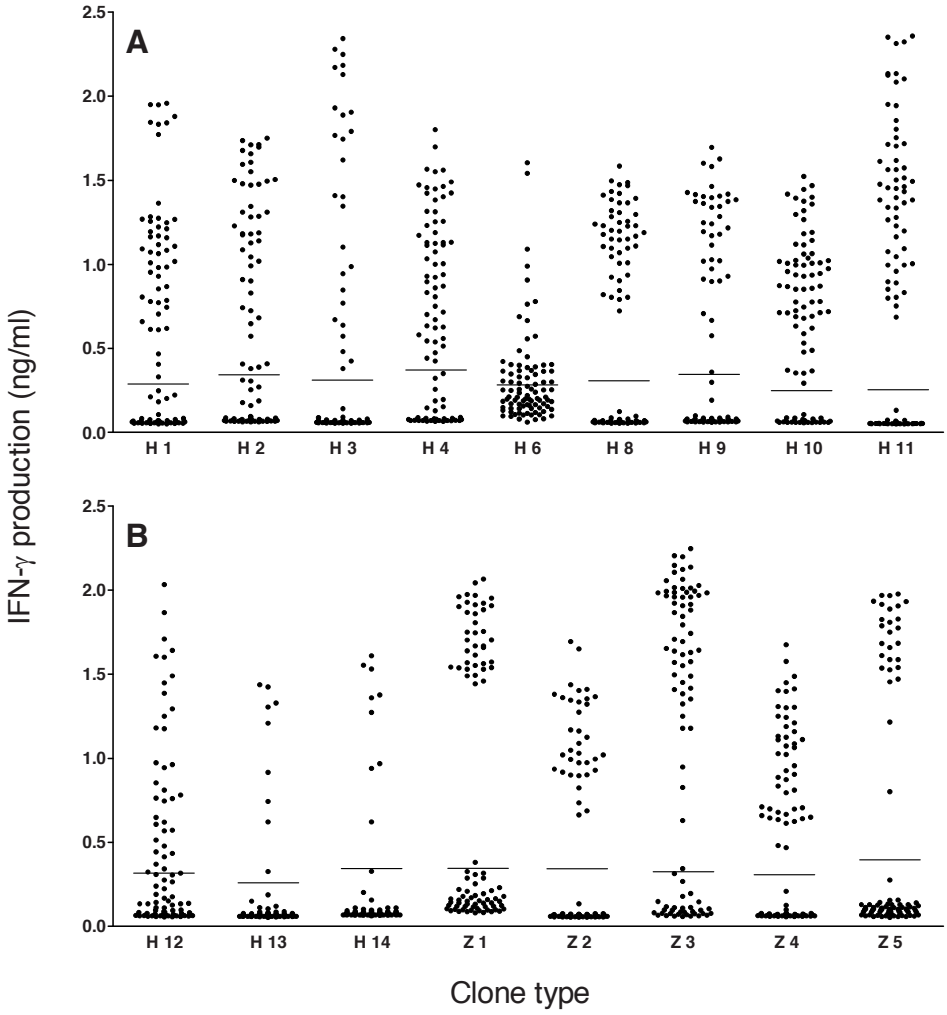


Figure 2. Recognition of a panel of 80 EBV-LCL by MiHA-specific T-cell clones. (A,B) HLA-A*02:01 and B*07:02 restricted T-cell clones recognizing MiHA with population frequencies between 10% and 90% were tested for recognition of a panel of 80 HLA-A*02:01 and B*07:02 EBV-LCL. Each dot represents the mean of triplicate IFN-γ production (ng/mL) as measured by ELISA after overnight co-incubation of the T-cell clone (indicated on the X axis) with each EBV-LCL. Lines indicate the threshold of five times background IFN-γ production to segregate $MiHA^{pos}$ from $MiHA^{neg}$ EBV-LCL.

Identification of MiHA-encoding SNPs

The strongest associating SNPs within each region were further examined, and associating SNPs were found in coding and noncoding gene regions (Table 2). Associating missense SNPs, encoding amino acid polymorphisms in the normal ORF, were found in the genes *WNK1, SSR1, PRCP, PDCD11, EBI3*, and *APOBEC3B*. These SNPs may directly encode the MiHA. Clone H1 and its associating SNP rs12828016 located in exon 21 of the *WNK1* gene is shown as a representative example in Figure 3A. Associating synonymous SNPs were found in the genes *ARHGDIB* and *GEMIN4*, and associating SNPs in noncoding regions were found in the genes *TTK, ERAP1, BCAT2*, and *ERGIC1*. Associating synonymous SNPs and SNPs in noncoding regions may serve as marker SNPs for missense SNPs, which are not measured by the bead array. To search for these disparate missense SNPs, all genes comprising associating synonymous SNPs and SNPs in noncoding regions as identified by WGAs were investigated by analyzing cDNA derived from both patient and donor. Sequence analysis of gene transcripts revealed the presence of disparate missense SNPs in the genes *ERAP1, BCAT2*, and *GEMIN4*. Figure 3B shows clone H8 and its associating SNP rs26654 located in intron 1 of the *ERAP1* gene as a representative example of a marker SNP, which is in linkage disequilibrium with SNP rs26654 located in exon 2 identified after gene sequencing. Sequence analysis revealed that *ARHGDIB* gene transcripts were completely identical between patient and donor, except for the single associating synonymous SNP rs4703 as identified by WGAs. These data suggest that this SNP directly encodes the MiHA in an ARF, as previously reported for other MiHA[8, 15]. The *TTK* and *ERGIC1* genes, identified by strong association of SNPs in noncoding regions, did not show any SNP disparity between patient and donor in coding regions. In conclusion, WGAs in combination with gene sequencing led to identification of disparate missense SNPs for 10 of the 12 genes (Table 2).

Table 2. Identification of MiHA-encoding SNPs

clone type	identified candidate SNPs			SNP function	SNP genotype		gene	
	by association	*P*	by sequencing*		pat	don	name	location
H 1	rs12828016	1.1×10^{-16}		missense	AC	CC	*WNK1*	exon 21
H 2	rs1886568	7.8×10^{-15}		—	GG	AA	*SSR1*	intron 1
	rs10004	7.8×10^{-15}		missense	GG	AA	*SSR1*	exon 2
	rs3778333	7.8×10^{-15}		—	GG	AA	*SSR1*	intron 2
H 3	rs2298668	1.1×10^{-12}		missense	AC	AA	*PRCP*	exon 4
H 4	rs608962	3.6×10^{-20}	not found	—	GG	AA	*TTK*	intron 20
H 8	rs26654	1.2×10^{-19}		—	AG	AA	*ERAP1*	intron 1
			rs26653	missense	CC	GG	*ERAP1*	exon 2
H 9	rs736170	1.3×10^{-16}		—	AG	AA	*BCAT2*	intron 1
	rs4801775	1.3×10^{-16}		—	AG	AA	*BCAT2*	intron 3
	rs4802507	1.3×10^{-16}		—	AC	AA	*BCAT2*	intron 3
			rs11548193	missense	CG	CC	*BCAT2*	exon 6
H 10	rs4703	5.5×10^{-16}		synonymous	CG	GG	*ARHGDIB*	exon 6
H 11	rs2986014	4.2×10^{-15}		missense	AG	GG	*PDCD11*	exon 24
	rs2281860	4.2×10^{-15}		—	AG	GG	*PDCD11*	intron 30
	rs6580	4.2×10^{-15}		—	AG	AA	*CALHM2*	3' UTR
Z 1	rs1045481	9.1×10^{-15}		synonymous	AG	GG	*GEMIN4*	exon 2
	rs1045491	9.1×10^{-15}		—	AG	GG	*GEMIN4*	3' UTR
			rs4968104	missense	AT	AA	*GEMIN4*	exon 2
	rs1064245	9.1×10^{-15}		—	AC	AA	*FAM57A*	3' UTR
Z 2	rs4740	3.4×10^{-14}		missense	AG	GG	*EBI3*	exon 5
	rs4905	3.4×10^{-14}		synonymous	AG	AA	*EBI3*	exon 5
Z 3	rs2076109	8.8×10^{-17}		missense	AG	GG	*APOBEC3B*	exon 3
Z4	rs564349	1.6×10^{-15}	not found	—	GG	AA	*ERGIC1*	intron 3

*Additional SNP disparities in coding regions identified after gene sequencing.

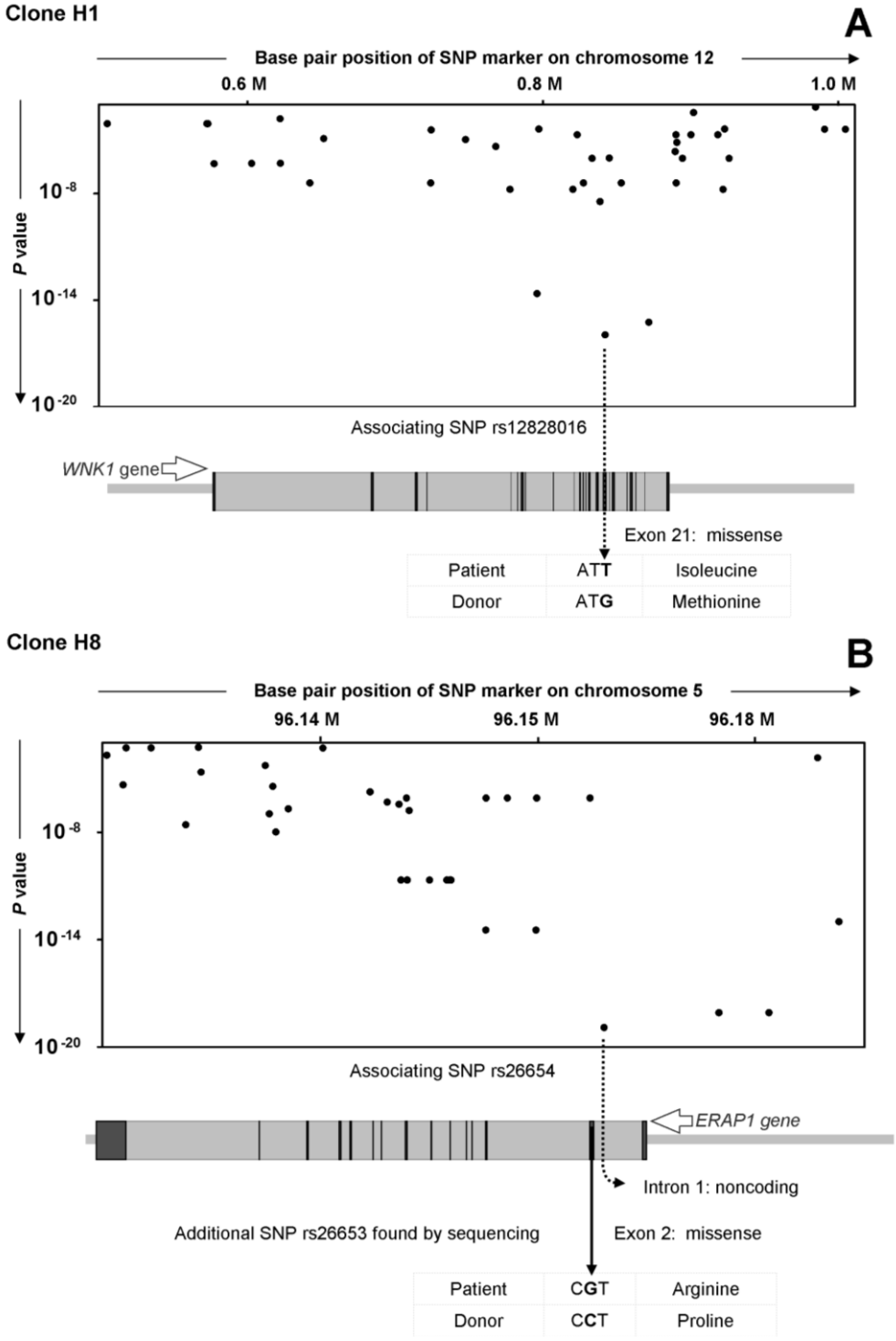

Clone H1
A
Base pair position of SNP marker on chromosome 12
0.6 M
0.8 M
1.0 M
P value
10-8
10-14
10-20
Associating SNP rs12828016
WNK1 gene
Exon 21: missense
Patient ATT Isoleucine
Donor ATG Methionine
Clone H8
B
Base pair position of SNP marker on chromosome 5
96.14 M
96.15 M
96.18 M
P value
10-8
10-14
10-20
Associating SNP rs26654
ERAP1 gene
Intron 1: noncoding
Additional SNP rs26653 found by sequencing
Exon 2: missense
Patient CGT Arginine
Donor CCT Proline

Figure 3 (previous page). Identification of candidate genes and MiHA-encoding SNPs by WGAs. Association results and selection of candidate MiHA-encoding SNPs are shown for *WNK1* (A) and *ERAP1* (B) genes as representative examples. Significance of association for each individual array SNP as expressed by *P*-value is depicted. Each dot represents the array SNP relative to its physical position on the genome. The image below each plot shows the gene aligned to the chromosomal position as indicated on the *X* axis of the plot. Exons and introns are represented by dark and light boxes, respectively. A. For clone H1, the strongest associating SNP is located in exon 21 of the *WNK1* gene and encodes an amino acid polymorphism. B. The strongest associating SNP for clone H8 is located in intron 1 of the *ERAP1* gene. Sequence analysis revealed a disparate missense SNP in exon 2 of the *ERAP1* gene.

Prediction and recognition of MiHA epitopes

Gene sequences containing missense and synonymous SNPs identified by WGAs and DNA sequencing were translated in the normal ORF and in all ARFs, respectively, and searched for peptides with predicted binding to HLA-A*02:01 and B*07:02 using the online algorithm of NetMHC (31). All peptide variants with predicted binding to HLA-A*02:01 and B*07:02 are shown in Table 3. Peptides of 9 to 11 amino acids with predicted binding to HLA-A*02:01 or B*07:02 were synthesized, exogenously pulsed on MiHA^{neg} donor EBV-LCL, and tested for T-cell recognition. For nine polymorphic peptides, strong recognition of a single predicted candidate peptide or variable levels of recognition for candidate peptides of various lengths was observed (Table 3). The SSR1-derived HLA-A*02:01 binding candidate peptide, however, was not recognized. Longer 14-mer and 21-mer SSR1-derived peptides spanning the identified patient-type amino acid polymorphism were clearly recognized, albeit at high peptide concentrations of 50 μmol/L (data not shown), thereby confirming that the missense SNP encodes the MiHA epitope, but leaving the precise composition of the epitope unknown. For all identified MiHA epitopes as shown in Table 3, as well as for the longer SSR1 peptides, no recognition of donor-type peptides was observed. This validated the identification of 10 novel MiHA encoded by SNPs as identified by our approach of WGAs (Figure 1B).

Table 3. Prediction and recognition of MiHA epitopes

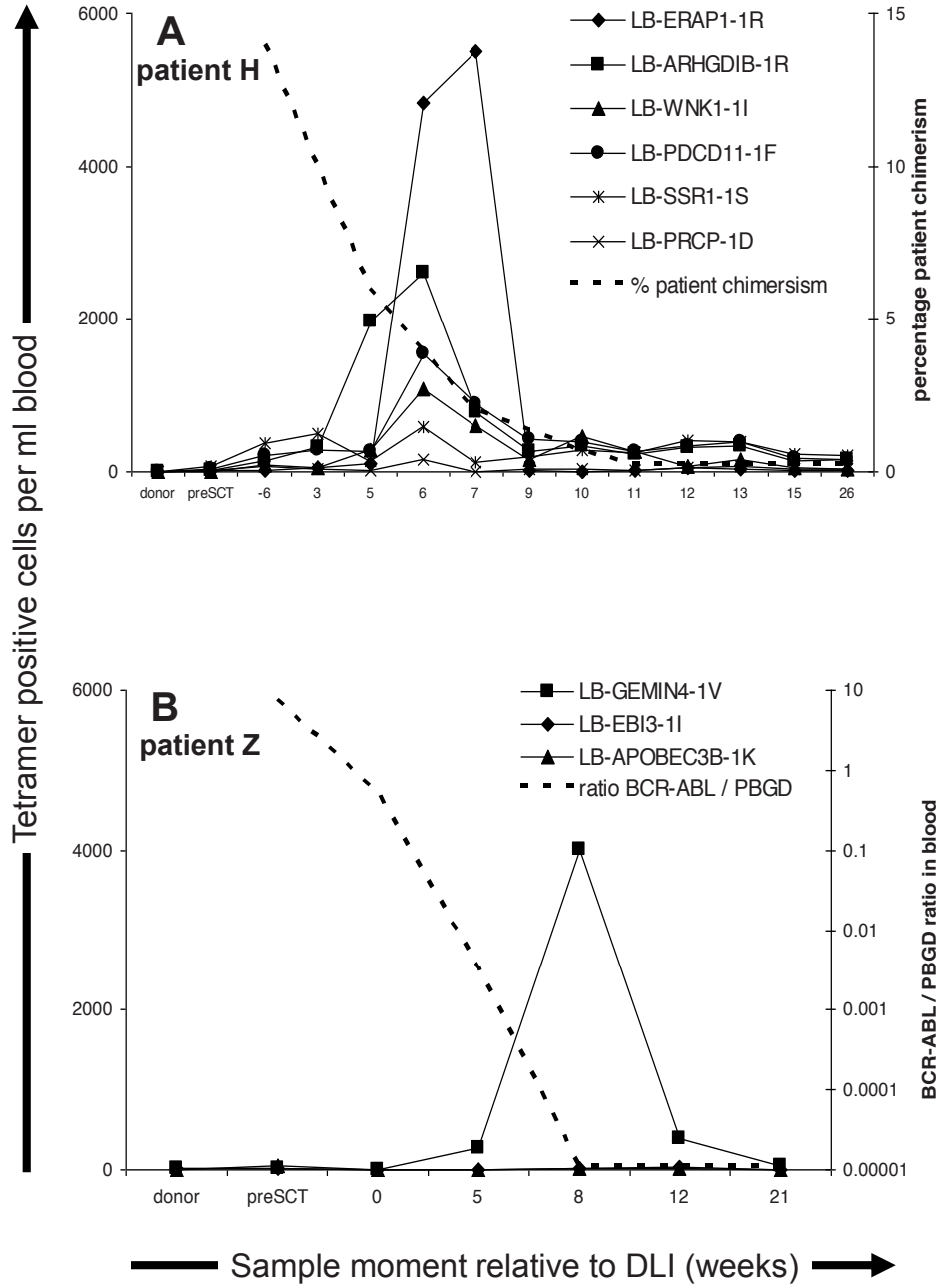
Clone type	MiHA name	SNP	Amino acid polymorphism*	Restriction	Predicted HLA binding peptides*	Measured EC_{50} peptide recognition (nmol/L)
H 1	LB-WNK1-1I	rs12828016	Ile/Met	A*02:01	RTLSPEIITV	4
					TLSPEIITV	>5,000
H 2	LB-SSR1-1S	rs10004	Ser/Leu	A*02:01	SLAVAQDLT	>5,000
H 3	LB-PRCP-1D	rs2298668	Asp/Glu	A*02:01	FMWDVAEDLKA	1
					FMWDVAEDL	17
H 8	LB-ERAP1-1R	rs26653	Arg/Pro	B*07:02	HPRQEQIALLA	20
					HPRQEQIALL	nt
					HPRQEQIAL	nt
H 9	LB-BCAT2-1R	rs11548193	Arg/Thr	B*07:02	QPRRALLFVIL	6
					QPRRALLFVI	195
					QPRRALLFV	>5,000
					GVSQPRRALL	>5,000
					GVSQPRRAL	nt
H 10	LB-ARHGDIB-1R	rs4703	Arg/Pro†	B*07:02	LPRACWREA	4
					LPRACWREAR	45
					LPRACWREART	249
H 11	LB-PDCD11-1F	rs2986014	Phe/Leu	B*07:02	GPDSSKTFLCL	28
					GPDSSKTFL	>5,000
Z 1	LB-GEMIN4-1V	rs4968104	Val/Glu	B*07:02	FPALRFVEV	3
Z 2	LB-EBI3-1I	rs4740	Ile/Val	B*07:02	RPRARYYIQVA	480
					RPRARYYIQV	13
					RPRARYYIQ	nt
					AVRPRARYYI	nt
Z 3	LB-APOBEC3B-1K	rs2076109	Lys/Glu	B*07:02	KPQYHAEMCFL	4,447
					KPQYHAEMCF	285
					KPQYHAEMC	1,604

Abbreviation: nt, not tested. *Patient-type amino acid residues are underlined.
†Amino acid polymorphism after translation in an ARF.

Detection of MiHA-specific T-cell responses

To analyze the kinetics of the T-cell responses specific for the novel MiHA as identified by WGAs, donor samples as well as sequentially obtained peripheral blood samples from the patients were stained with tetramers and analyzed by flow cytometry. Except for LB-BCAT2-1R, we could construct stable tetramers that successfully stained the respective T-cell clones for all novel MiHA. Tetramer analysis revealed the presence of T cells specific for the MiHA in patient H, and for one MiHA in patient Z (Figure 4). The frequencies of MiHA-specific T cells peaked at day 41 and 62 in patients H and Z, respectively, coinciding with conversion to full donor chimerism in patient H and disappearance of the BCR-ABL fusion product as a marker for disease in patient Z. No significant tetramer-positive events were detected using tetramers specific for LB-APOBEC3B-1K and for LB-EBI3-1I in patient Z.

Figure 4 (facing page). MiHA-specific T-cell responses in patients H and Z. Tetramers were constructed to measure MiHA-specific CD8 T cells in peripheral blood samples obtained at several time points, as indicated on the X axis. On the primary Y axis, the absolute number of tetramer-positive cells per milliliter of blood is depicted. The secondary Y axis depicts donor chimerism in patient H (A) and disease status in patient Z (B).


A
patient H
LB-ERAP1-1R
LB-ARHGDIB-1R
LB-WNK1-1I
LB-PDCD11-1F
LB-SSR1-1S
LB-PRCP-1D
% patient chimersism
percentage patient chimerism
6000
4000
2000
0
15
10
5
0
donor
preSCT
-6
3
5
6
7
9
10
11
12
13
15
26
B
patient Z
LB-GEMIN4-1V
LB-EBI3-1I
LB-APOBEC3B-1K
ratio BCR-ABL / PBGD
BCR-ABL / PBGD ratio in blood
10
1
0.1
0.01
0.001
0.0001
0.00001
donor
preSCT
0
5
8
12
21
Tetramer positive cells per ml blood
Sample moment relative to DLI (weeks)

Discussion

In this study, we developed an efficient strategy for T-cell selection and MiHA identification by WGAs. This WGAs-based strategy allowed detailed investigation of the specificity and diversity of antitumor T-cell responses in two patients successfully treated with HLA-matched allo-SCT and DLI. The strategy is based on efficient selection of MiHA-specific T-cell clones and a panel of 80 third-party EBV-LCL for simultaneous measurement of T-cell recognition and 1.1 million SNP genotypes. From the 17 selected T-cell specificities, 10 novel MiHA were identified, illustrating the value of this WGAs-based strategy for broad characterization of MiHA in clinical immune responses.

For 5 of the 17 selected T-cell specificities, we failed to show significant association with SNP genotypes by WGAs. Two T-cell clones recognized a limited number of EBV-LCL, suggesting that the power of the WGAs strategy may not be sufficient to identify MiHA with low population frequencies. Two other T-cell clones for which we failed to show association with SNP genotypes by WGAs released intermediate levels of IFN-γ upon coincubation with a number of EBV-LCL, thereby hampering separation of EBV-LCL in $MiHA^{pos}$ and $MiHA^{neg}$ groups. Finally, for one T-cell clone, a balanced segregation of $MiHA^{pos}$ and $MiHA^{neg}$ EBV-LCL was observed; however, no significant association with any SNP genotype was detected. For this T-cell clone, association may be found by measuring more SNP genotypes, for example, by testing EBV-LCL from the International HapMap project, which have been genotyped for more than 4 million SNPs and recently shown to represent a valuable source for MiHA discovery[25–27].

Our WGAs-based strategy revealed a number of SNPs that associated with T-cell recognition with significant *P*-values. We separated the missense SNPs from the synonymous SNPs and SNPs located in noncoding regions. Missense SNPs were directly analyzed for HLA binding prediction and identification of the MiHA peptide epitopes. All six missense SNPs were found to encode the MiHA epitope. Synonymous SNPs and SNPs in noncoding regions may serve as markers for MiHA-encoding SNPs that are not measured by the bead array. Therefore, genes comprising marker SNPs were first analyzed by gene sequencing to reveal all SNP disparities between patient and donor. For two T-cell clones, associating synonymous SNPs were found, of which one was shown to serve as a marker for a MiHA-encoding missense SNP and the other

directly encoded the MiHA epitope translated in an ARF, as previously described for LB-ECGF-1H[8] and LB-ADIR-1F[15]. Finally, for four T-cell clones, associating SNPs were located in noncoding regions. In two cases, intron SNPs were in linkage disequilibrium with MiHA-encoding missense SNPs, whereas no SNP disparities in coding gene regions were found for the other two T-cell clones.

For two T-cell clones showing strong association with intron SNPs, without SNP disparities in the coding regions of the associating genes of patient and donor, we explored the possibility that a monomorphic epitope is recognized, which may be under differential control of a SNP located in a regulatory region. The entire coding regions of the *TTK* and *ERGIC1* genes were cloned into an expression vector and introduced into MiHA-negative cells, but no T-cell recognition of the transfected genes was observed (data not shown). Therefore, we consider it likely that these MiHA are encoded by SNPs located outside known protein coding regions of the candidate genes, for example, by an intron SNP of an alternative mRNA splice variant, as previously described for ACC-6[19], and we speculate that these MiHA may be most difficult to identify by WGAs.

As a final step in the discovery of MiHA, specific recognition of patient-type peptide was established. All identified MiHA epitopes were accurately predicted using the binding algorithm of NetMHC[31], as shown by T-cell recognition of donor EBV-LCL pulsed with the predicted patient-type, but not donor-type, peptide variants. Weak, but patient-specific, recognition was observed for peptides derived from APO*BEC3B* and *SSR1*. The *SSR1* epitope is a nonconventional peptide of 14 amino acids. Although recognition was weak, the T-cell clone differentially recognized patient-type, but not donor-type, 14-mer peptides demonstrating T-cell specificity for this polymorphism. Weak recognition of pulsed synthetic peptides may indicate posttranslational modification of endogenous epitopes, as previously described for the *SMCY* antigen[34]. Another explanation may be that the T-cell clone expresses a T-cell receptor with a relatively low avidity, which is, however, sufficiently high to recognize MiHA that are abundantly expressed at the cell surface of patient cells.

The MiHA described in this study were identified as targets for T cells isolated from patients receiving DLI for incomplete donor chimerism or recurrent disease

after allo-SCT. Specific tetramer staining of CD8 T cells was shown for the majority of the novel MiHA, and peak frequencies coincided with the development of clinical responses in the patients. Despite staining of the respective T-cell clones (data not shown), no tetramer-positive T cells were detected in the samples of patient Z using LB-APOBEC3B-1K and LB-EBI3-1I tetramers. Intermediate TCR avidity, as expressed by the intermediate EC_{50} value for recognition of *APOBEC3B*-derived peptides, may cause the lack of detection of the LB-APOBEC3B-1K–specific T cells. The absence of tetramer-positive T cells for LB-EBI3-1I may be explained by a low frequency of circulating T cells. Whereas the individual contribution of each MiHA-directed T-cell response cannot be determined, the induction of strong GvT reactivity and the absence of severe GvHD in both patients suggest involvement of one or more MiHA with selective or predominant expression in malignant hematopoietic cells.

In conclusion, we present an efficient WGAs-based strategy for broad characterization of MiHA. Our WGAs approach is valuable as a high-throughput method for detailed analysis of the specificity and diversity of T-cell responses in patients with different clinical responses as well as for selective identification of clinically relevant MiHA with hematopoietic restricted expression.

Acknowledgments

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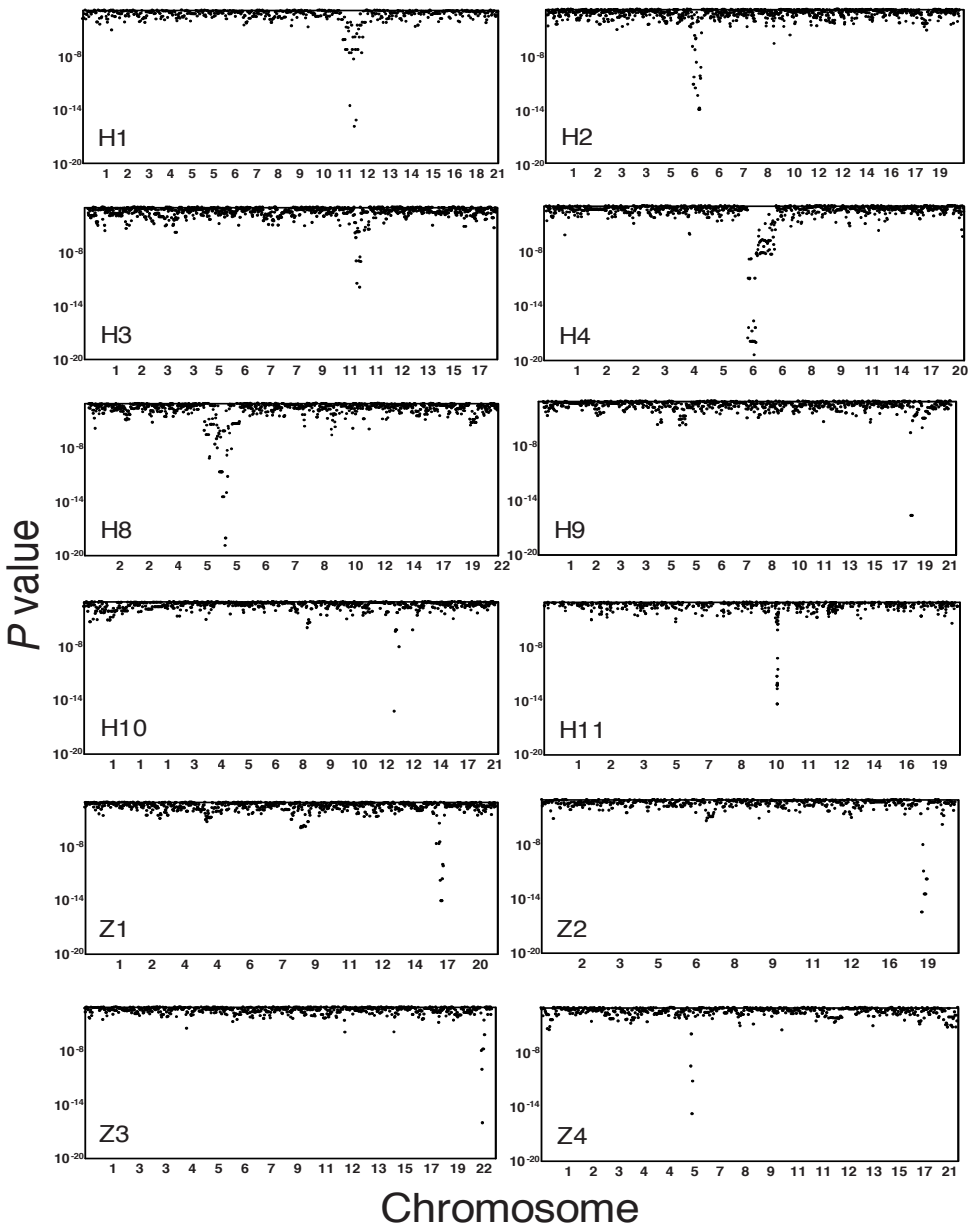

Figure S1. Whole genome association scanning results data

WGAs results for 12 T-cell clones showing significant association with SNP-genotypes in single genomic regions are given. Individual graphs represent the result of the indicated T-cell clone. The *p* value of array SNPs depicted as dots are shown relative to their physical position in the genome, with chromosome numbers plotted on the X-axis. *P* values depicted on the Y-axis indicate the significance of association of each array SNP.

Table S1. Primer sequences

Name	Accession NM id	Length	CDS	PCR product			
				Start	Stop	Remark*	Primer sequence†
ARHGDIB	1175	1216	105-710	69	701	BamH1	For CGCGGATCCGGACAGAGACGTGAAGC
						EcoR1	Rev CCGGAATTCACCACTCCTTCTTAATCGACAG
				510	1216	BamH1	For CGCGGATCCACCATGGTGGATAAAGCAACATTTATGG
						XhoI	Rev CCGCTCGAGCTCAGCCAGAACACACACAG
GEMIN4	15721	3757	120-3296	854	2420	BamH1	For CGCGGATCCACCATGCTGACTGTGTTTGCGC
						EcoR1	Rev CCGGAATTCAGTCTAGCTGTTCTAACTTG
				2287	3655	BamH1	For CGCGGATCCACCATGGATAGGAAGGATCTAGCGA
						EcoR1	Rev CCGGAATTCAGTTATGAACATGTTCCTCC
TTK	1166691	3019	124-2694	124	1515	BamH1	For CGCGGATCCACCATGGAATCCGAGGATTTAAGTG
						XhoI	Rev CCGCTCGAGTCAGCTCATGTAATCATCCAAG
				1343	2215	BamH1	For CGCGGATCCACCATGCAGATTCCGGAGTTAGCC
						EcoR1	Rev CCGGAATTCACATATCTTTGATTGCTTCTGG
				2132	2963	BamH1	For CGCGGATCCACCATGCAACCAGATACAACAAGTG
						XhoI	Rev CCGCTCGAGCTTGCTATCCACCCACTATTC
ERAP1	1040458	5085	348-3173	140	816	-	For GTTTACCCTTTCCCCAGCTC
						-	Rev CCGAAAGATTGCCAGCATAG
				669	1290	-	For AGGAAGGGAGCTGGAGAGAG
						-	Rev GAAAGTCGGGAATAGCAGCA
				1268	1973	-	For TCTTGCTGCTATTCCCGACT
						-	Rev TACATTCCTCCCCCTCACTG
				1890	2583	-	For GTGGATGTGAAAACCATGATGA
						-	Rev TCTGTACGCACGGCTGATAG
BCAT2	1190	1616	38-1216	510	972	-	For ATCGAAGTGGACAAGGACTG
						-	Rev GAGCCATGTCCAGTAGACTC
				918	1472	-	For CTGAATGGTGTTATCCTGCCTG
						-	Rev GCACGACAAGGAGTAATGGG
ERGIC1	1031711	2949	195-1067	191	1439	BamH1	For CGCGGATCCACGATGCCCTTTGACTTCA
						EcoR1	Rev CCGGAATTCGCGAGATTGTCGAAGCCTG
				1395	2910	BamH1	For CGCGGATCCAGAATGCATATCGATCAGC
						EcoR1	Rev CGGGAATTCTAGTTGAACCCAACGAGTG

*Restriction endonuclease cleavage sites were included for cloning of PCR products.

†Primers were selected to completely overlap the transcript, or transcript regions that contained SNPs.

Table S2. Detection of known MiHA by Illumina1M array

MiHA	SNP		Frequency immunogenic allele			Call rate†
	Identifier	Function	Nucleotide	Measured	dbSNP	
ACC-1	rs1138357	missense	G*	0,69	0,75	100
ACC-2	rs3826007	missense	G	0,69	0,75	100
HA-1	rs1801284	missense	G	0,62	0,67	97
HA-3	rs7162168	missense	T	0,37	0,33	98
HA-8	rs2173904	missense	G	0,53	0,53	99
HB-1H/Y	rs161557	missense	C/T*	0,8	0,86	100
HEATR1	rs2275687	missense	A	0,32	0,33	100
HwA-10	rs5758511	missense	C	0,72	0,74	100
HwA-9	rs1365776	protein splicing	G	0,45	0,38	100
LB-ADIR-1F	rs2296377	alternative RF	T	0,27	0,21	99
LB-LY75-1K	rs12692566	missense	A	0,17	0,23	100
LB-MR1-1R	rs2236410	missense	A	0,92	0,82	95
LB-MTHFD1-1Q	rs2236225	missense	G	0,64	0,54	100
LB-PTK2B-1T	rs751019	missense	A	0,55	0,49	99
SLC19A1	rs1051266	missense	A	0,34	0,19	99
ACC-4 ACC-5	rs2289702	missense				
ACC-6	rs9945924	RNA splicing				
CD19^{L}	rs2904880	missense				
DPH1	rs35394823	missense				
HA-2	rs61739531	missense				
HwA-11	rs3745526	missense	Not included in Illumina 1M array			
LB-ECGF-1H	not in dbSNP	alternative RF				
LB-PI4K2B-1S	rs313549	missense				
LRH-1	rs5818907	frameshift				
SLC1A5	rs3027956	missense				
UGT2B17	rs10034124	gene deletion				

* Both alleles of this polymorphism encode an immunogenic epitope.

† Call rate expresses the percentage of successful SNP measurements by the Illumina 1M array after analysis of 80 DNA samples obtained from EBV-LCL.

Table S3. Identification of associating genomic regions

Clone type	Associating genomic region*		
	# SNP $P<10^{-9}$	Location	Length (kbp)
H 1	3	12p13.3	38
H 2	12	6p24.3	99
H 3	5	11q14	62
H 4	17	6q13	76
H 6	0	-	-
H 8	14	5q15	90
H 9	3	19q13	9
H 10	1	12p12.3	<1
H 11	13	10q24.33	46
H 12	0	-	-
H 13	0	-	-
H 14	0	-	-
Z 1	7	17p13	49
Z 2	8	19p13.3	31
Z 3	2	22q13.1	7
Z 4	3	5q35.1	8
Z 5	0	-	-

*For each T-cell clone, associating genomic regions were defined by the number of SNPs with $P<10^{-9}$ as calculated by Fisher's exact test.

Chapter 4

Identification of 4 novel HLA-B*40:01 restricted minor histocompatibility antigens and their potential as targets for graft-*versus*-leukemia reactivity

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Abstract

Patients with hematologic malignancies can be successfully treated with donor lymphocyte infusion after HLA-matched allogeneic hematopoietic stem cell transplantation. The effect of donor lymphocyte infusion is mediated by donor T cells recognizing minor histocompatibility antigens. T cells recognizing hematopoietic restricted minor histocompatibility antigens may induce selective graft-*versus*-leukemia reactivity, whereas broadly expressed antigens may be targeted in graft-*versus*-host disease. We analyzed in detail CD8 T-cell immunity in a patient with relapsed chronic myelogenous leukemia who responded to donor lymphocyte infusion with minimal graft-*versus*-host disease of the skin. CD8 T-cell clones specific for 4 HLA-B*40:01 restricted minor histocompatibility antigens were isolated which were identified by screening a plasmid cDNA library and whole genome association scanning. Detailed T-cell reactivity and monitoring experiments were performed to estimate the clinical and therapeutic relevance of the novel antigens. Three antigens were demonstrated to be expressed on primary leukemic cells of various origins as well as on subtypes of non-malignant hematopoietic cells, whereas one antigen was selectively recognized on malignant hematopoietic cells with antigen presenting cell phenotype. Skin derived fibroblasts were only recognized after pre-treatment with IFN-γ by two T-cell clones. Our data show evidence for different roles of the HLA-B*40:01 restricted minor histocompatibility antigens in the onset and execution of the anti-tumor response. All antigens may have contributed to a graft-*versus*-leukemia effect, and one minor histocompatibility antigen (LB-SWAP70-1Q) has specific therapeutic value based on its *in vivo* immuno-dominance and strong presentation on leukemic cells of various origins, but absence of expression on cytokine-treated fibroblasts.

Introduction

Patients with hematologic malignancies can be successfully treated with HLA-matched allogeneic hematopoietic stem cell transplantation (alloSCT).[1] To reduce the development of graft-*versus*-host disease (GvHD), donor T cells can be depleted from the stem cell graft, and re-administered after alloSCT pre-emptively in case of persistent mixed chimerism or upon detection of relapsed or residual malignant cells.[2] The risk and severity of GvHD after this 2-step procedure of T-cell depleted alloSCT and donor lymphocyte infusion (DLI) is reduced compared to non-T-cell depleted alloSCT.

The anti-tumor effect of DLI after HLA-matched alloSCT is mediated by donor T cells recognizing minor histocompatibility antigens (MiHA) in the context of HLA molecules.[3–7] MiHA are peptides which are differentially expressed at the cell surface of patient and donor cells due to amino acid differences encoded by single nucleotide polymorphisms (SNPs). Donor T cells recognizing MiHA with restricted expression to hematopoietic cells may selectively induce graft-*versus*-leukemia (GvL) reactivity, whereas donor T cells specific for MiHA with broad tissue distribution patterns may also mediate GvHD.

Although various HLA class I associated MiHA have been identified in the last decades,[3–7] the number of MiHA with therapeutic relevance is still limited. We and others recently demonstrated that Whole Genome Association scanning (WGAs) is an efficient method for high throughput identification of MiHA,[8–12] illustrated by the discovery of 10 novel MiHA as targets for CD8 T cells in 2 different patients with leukemia who responded to DLI after HLA-matched alloSCT.[12] Since these MiHA were all presented in HLA-A*02:01 and B*07:02, we selected 80 third-party EBV-transformed B cell lines (EBV-LCL) for co-expression of these HLA molecules, and genotyped all cell lines for more than one million SNPs. MiHA were identified by analysis of association between T-cell recognition of these EBV-LCL and individual SNP genotypes.

In this report, we identified 4 novel HLA-B*40:01 restricted MiHA as targets for CD8 T cells in a patient who developed strong GvL reactivity with minimal GvHD of the skin after treatment with DLI for relapsed chronic myelogenous leukemia (CML) more than one year after HLA-matched alloSCT. One MiHA was identified by screening a plasmid cDNA expression library, whereas the other 3 MiHA were identified by WGAs. The data illustrate that our method of WGAs using a panel of SNP-genotyped EBV-LCL after retroviral transduction of

the HLA restriction allele allows identification of MiHA. Detailed T-cell reactivity and monitoring experiments were performed to estimate the clinical and therapeutic relevance of the HLA-B*40:01 restricted MiHA. The data showed that all MiHA may have contributed to a GvL effect, and that one MiHA (LB-SWAP70-1Q) has specific therapeutic value based on its *in vivo* immuno-dominance and strong presentation on leukemic cells of various origins, but absence of expression on cytokine-treated fibroblasts.

Patient

A 47-year old woman with chronic phase CML was transplanted with hematopoietic stem cells from her HLA-identical sister (HLA-A*02:01, A*24:02, B*08:01, B*40:01, C*03:04, C*07:01)[13]. Nine months after alloSCT, BCR/ABL transcripts were detected, indicating a cytogenetic relapse. Retrospective molecular analysis of stored samples showed the presence of BCR/ABL transcripts at three and six months after alloSCT. In preparation for DLI, she was treated with 3×10^6 units of α-IFN daily s.c. Four weeks later the CML had progressed to a hematologic relapse, and 10^7 donor mononuclear cells per kg body weight were administered. After DLI she converted to 100% donor chimerism. The DLI was complicated by grade I GvHD of the skin and mouth for which no systemic immunosuppressive treatment was necessary. Currently, more than 12 years after DLI, she is still in good clinical condition without GvHD.

Materials and methods

Patient and donor samples

Peripheral blood (PB) and bone marrow (BM) samples and skin biopsies from patients with CML, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and multiple myeloma (MM), and PB and BM samples from healthy individuals were obtained after receiving approval from the LUMC Institutional Review Board and informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated by Ficoll-Isopaque gradient centrifugation and cryopreserved.

Isolation and culture of T-cell clones

Peripheral blood mononuclear cells (PBMC) obtained six weeks after DLI were stimulated overnight with irradiated BM cells obtained from the patient prior to alloSCT, and single IFN-γ producing CD8 T cells were isolated by flowcytometry after staining with PE-conjugated antibody against IFN-γ (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In addition, PBMC were stained with FITC-labeled anti-HLA-DR and APC-conjugated anti-CD8 antibodies (BD Biosciences, Breda, The Netherlands), and *in vivo* activated HLA-DR$^+$ CD8 T cells were single-cell sorted by flowcytometry. T-cell clones were cultured as previously described.[12]

Cell culture

EBV-transformed B cells (EBV-LCL) and COS-7 cells were cultured in IMDM with 10% FCS. PHA-T blasts were generated by stimulating PBMC with IL-2 and PHA similar as described for T-cell clones. Primary fibroblasts (FB) and keratinocytes (KC) were cultured from skin biopsies in DMEM with low glucose (Cambrex) and 10% FCS supplemented with and without IFN-γ (100 IU/mL; Immukine; Boehringer Ingelheim, Alkmaar, The Netherlands) for four days. Proximal tubular epithelial cells (PTEC) cultured with and without IFN-γ (100 IU/mL) were kindly provided by Dr C van Kooten (Dept. Nephrology, LUMC, Leiden, The Netherlands).

Results

Isolation of CD8 T-cell clones for minor histocompatibility antigens

A detailed analysis of CD8 T-cell immunity was made in a patient who developed a strong GvL response with only limited GvHD after treatment with DLI for relapsed CML more than one year after alloSCT. In previous experiments, the GvL response in this patient was shown to coincide with a peak response in numbers of CD8 T cells specific for hematopoietic restricted MiHA HA-1 and HA-2 between 4–8 weeks after DLI.[13] To investigate whether, in addition to HA-1 and HA-2, other MiHA were targeted in this GvL response, single CD8 T cells were isolated by flowcytometry from patient PBMC obtained six weeks after DLI. T cells were isolated based on specific production of IFN-γ after overnight stimulation with irradiated BM cells obtained from the patient prior to alloSCT,[14] and based on expression of activation marker HLA-DR as previously described.[12,15] CD8 T-cell clones showing specific lysis and recognition of patient, but not donor, EBV-LCL in 4 h ^{51}Cr-release assays and IFN-γ ELISA (Figure 1A) were selected and tested against a panel of EBV-LCL sharing one or more HLA class I alleles with the patient. The data demonstrated that the T-cell clones (ZRZ16, ZRZ25, 12A2 and 3H1) were specific for 4 unknown MiHA in HLA-B*40:01, as confirmed by retroviral transfer of the HLA restriction allele (Figure 1B).

Identification of minor histocompatibility antigens by screening a plasmid cDNA expression library

To identify the unknown MiHA, a plasmid cDNA expression library was constructed from patient derived EBV-LCL as previously described.[16] COS-7 cells stably expressing HLA-B*40:01 were transfected with pools of 50 different cDNAs, and screened for T-cell recognition in IFN-γ ELISA using different mixtures of the MiHA specific CD8 T-cell clones. By screening COS-7 cells, one positive cDNA pool was recognized by clone ZRZ16. In a second round of screening of single plasmids, a cDNA was isolated encompassing the 1102 – 2029 bp region of the gene encoding thyroid hormone receptor interactor protein 10 (TRIP10). By cloning different cDNA regions of the *TRIP10* gene, the MiHA was shown to be encoded within the 1574–1810 bp region that contains the stop codon of the TRIP10 coding sequence at 1704 bp *(*See also Figure S1*).* This region was sequenced in patient and donor cDNA, and 3 SNP differences were demonstrated in the 3′ untranslated region (UTR) of the gene

at 1761 bp (rs1049229), 1763 bp (rs1049230) and 1775 bp (rs1049232). Long 19–22 AA peptides comprising protein sequences in 3 different reading frames were synthesized, pulsed on donor EBV-LCL, and tested for T-cell recognition in IFN-γ ELISA. One of the long peptides (ARF1) comprising AA sequences in a reading frame different from the upstream encoded TRIP10 protein was recognized. The minimal epitope LB-TRIP10-1EPC was identified as a 9-mer peptide comprising 3 AA encoded by SNP differences (G**EP**QDL**C**TL), of which the E and C most significantly contributed to T-cell recognition, as determined by analysis of patient type peptides containing single AA of donor origin (See also Figure S1).

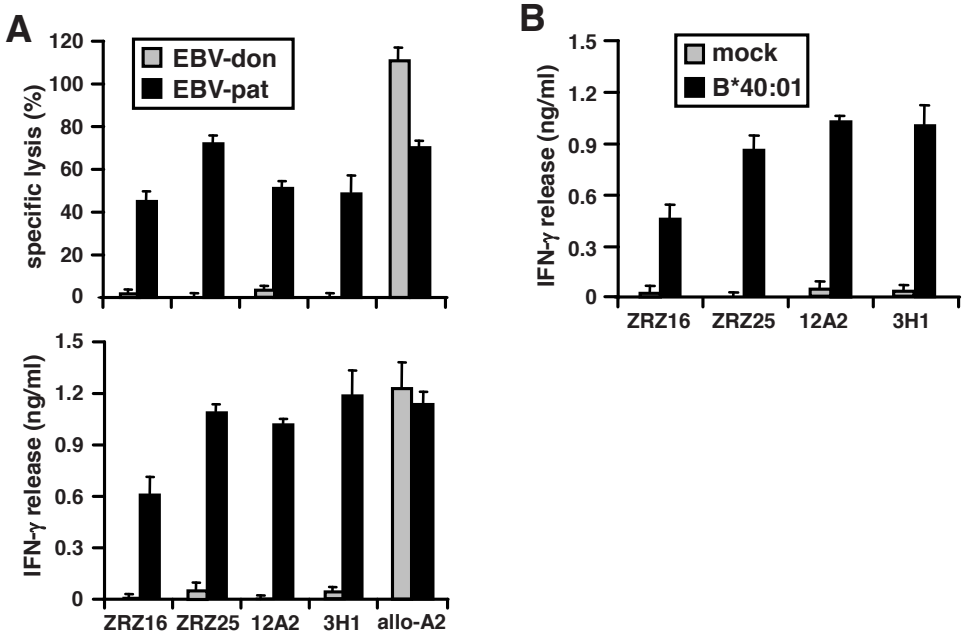


Figure 1. Isolation of CD8 T-cell clones specific for HLA-B*40:01 restricted MiHA. (A) A number of selected CD8 T-cell clones showed reactivity against patient, but not donor, EBV-LCL. Mean percentage of specific lysis in triplicate wells is shown at E:T ratios of 10:1 in 4 h ^{51}Cr-release assays (upper) and of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells in ELISA (lower). Reactivity against patient and donor EBV-LCL by an allo-HLA-A*02:01 reactive T-cell clone is shown as control. (B) Selected CD8 T-cell clones were specific for MiHA in HLA-B*40:01, as demonstrated by specific recognition of MiHA$^+$ EBV-LCL after retroviral transfer of MP71-HLA-B*40:01-IRES-NGFR, but not mock MP71 vector, in IFN-γ ELISA. Specific production of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells in ELISA is shown.

Identification of minor histocompatibility antigens by whole genome association scanning

In addition to screening a plasmid cDNA expression library, WGAs was performed to identify the unknown MiHA in HLA-B*40:01. We retrovirally transduced third party EBV-LCL, which we previously genotyped for more than one million SNPs,[12] with MP71-HLA-B*40:01-IRES-NGFR. A total number of 60 SNP-genotyped EBV-B cell lines expressing HLA-B*40:01 endogenously or after retroviral transduction were analyzed for recognition by T-cell clones ZRZ25, 12A2 and 3H1 in IFN-γ ELISA (Figure 2A). For each T-cell clone, SNP-genotyped EBV-LCL were separated into MiHA positive and negative groups using a threshold of recognition of 5-fold background production of IFN-γ. T-cell recognition patterns of EBV-LCL were subsequently analyzed for association with all individual SNP genotypes as previously described,[12] and the level of matching between both patterns was calculated according to Fisher's exact test using Plink statistical analysis package (http://pngu.mgh.harvard.edu/purcell/plink/version1.03). Table 1 shows the strongest associating SNPs ($P<10^{-9}$) for all T-cell clones. Associating missense SNPs in the genes encoding SON DNA-binding protein (*SON*), SWAP switching B-cell complex 70 kDa subunit (*SWAP70*) and nucleoporin 133 kDa (*NUP133*) were measured for T-cell clones ZRZ25, 12A2, and 3H1, respectively.

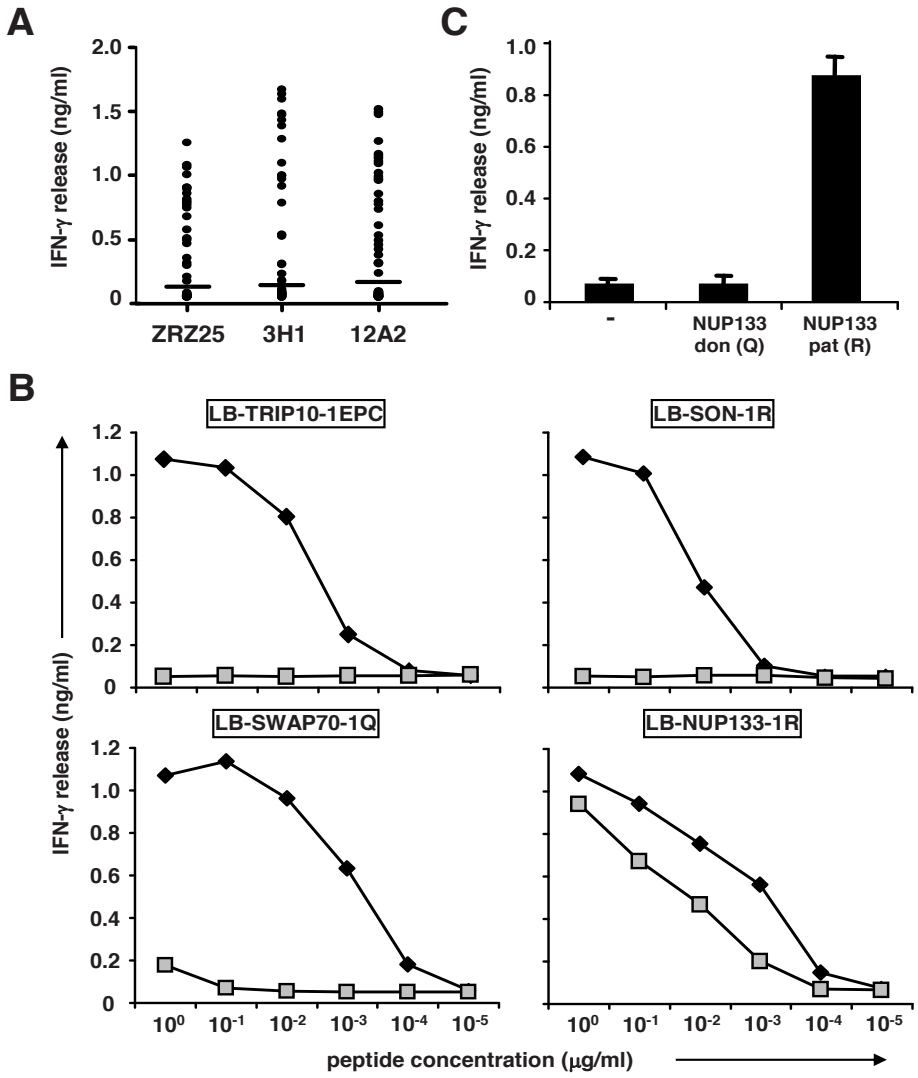
Table 1: Associating SNPs encoding candidate MiHA

T-cell clone	#	gene	SNP id	location	pat/don	p [1]	predicted HLA-binding peptide
ZRZ25	21	*SON*	rs7279549	intron 1		$1.28*10^{-10}$	SETKQR(C)TVL
			rs13047599	exon 3	CT/TT	$1.28*10^{-10}$	
			rs11088256	3' near gene		$1.28*10^{-10}$	
			rs7283856	3' near gene		$1.28*10^{-10}$	
12A2	11	*SWAP70*	rs415895	exon 10	GC/CC	$3.04*10^{-11}$	MEQLEQ(E)LEL
3H1	1	*NUP133*	rs1065674	exon 10	GA/AA	$0.41*10^{-10}$	SEDLILCR(Q)L

[1] *P* value as calculated using Fisher's exact test indicates the strength of association between the indicated SNP genotype and recognition of a panel of ~60 EBV-LCL expressing HLA-B*40:01 endogeneously or after retroviral transduction by the indicated T-cell clone.

To identify the minimal MiHA epitopes, amino acid sequences comprising the associating missense SNPs were searched for peptides with predicted binding to HLA-B*40:01 using the algorithm of NetMHC (http://www.cbs.dtu.dk/ services /NetMHC). For all associating missense SNPs, 9-mer peptides with predicted binding to HLA-B*40:01 were synthesized, pulsed on donor EBV-LCL, and shown to be recognized by the T-cell clones. This validated the identification of 3 HLA-B*40:01 restricted MiHA encoded by the genes for *SON* (LB-SON-1R; SETKQ**R**TVL), *SWAP70* (LB-SWAP70-1Q; MEQLE**Q**LEL) and *NUP133* (LB-NUP133-1R; SEDLILC**R**L) recognized by T-cell clones ZRZ25, 12A2 and 3H1, respectively (Figure 2B). Donor type peptide variants were not recognized, except for the donor peptide encoded by the *NUP133* gene. We amplified the *NUP133* genes from patient and donor derived cDNA by PCR, and cloned the genes into expression vector pcDNA-3. Cells transfected with the patient derived *NUP133* gene encoding the R at position 406 were strongly recognized by clone 3H1, whereas cells transfected with the donor derived *NUP133* gene encoding the Q at this position were not recognized (Figure 2C). These data demonstrated that differential recognition of patient and donor derived EBV-LCL by clone 3H1 is mediated by differences between LB-NUP133-1R and its donor variant in intracellular processing and presentation, and not by differential recognition by the T-cell receptor (TCR). In conclusion, by screening a plasmid cDNA expression library and WGAs, we identified 4 novel MiHA in HLA-B*40:01. The population frequencies of the HLA-B*40:01 associated MiHA are 96% (LB-TRIP10-1EPC; rs1049229, rs1049230, rs1049232), 48% (LB-SON-1R; rs13047599), 69% (LB-SWAP70-1Q; rs415895) and 48% (LB-NUP133-1R; rs1065674) calculated based on allele frequencies of 79%, 28%, 45% and 28% as reported in the SNP database (www.ncbi.nlm.nih.gov/snp), respectively.

Figure 2 (facing page). Identification of MiHA by whole genome association scanning. (A) A panel of approx. 60 SNP-genotyped EBV-LCL expressing HLA-B*40:01 endogenously or after retroviral transfer of MP71-HLA-B*40:01-IRES-NGFR with more than 20% of marker gene positive cells were tested for recognition by T-cell clones ZRZ25, 3H1 and 12A2 in IFN-γ ELISA. EBV-LCL were divided into $MiHA^{pos}$ and $MiHA^{neg}$ groups based on a threshold of 5-fold the background production of IFN-γ (horizontal bars). Mean release of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells is shown. (B) Peptides comprising patient (filled symbols) type amino acids with predicted binding to HLA-B*40:01 as well as donor type peptide variants (gray symbols) were pulsed on donor EBV-LCL and tested for T-cell recognition in IFN-γ ELISA. LB-TRIP10-1EPC (G**EP**QDL**C**TL) contains 3 patient type amino acids and has been identified by cDNA library screening. LB-SON-1R (SETKQ**R**TVL), LB-SWAP70-1Q (MEQLE**Q**LEL)



and LB-NUP133-1R (SEDLILC**R**L) contain single patient type amino acids encoded by exon SNPs identified by WGAs based on significant association with T-cell recognition. Donor type peptides SETK**Q**CTVL and MEQLE**E**LEL encoded by the genes for SON and SWAP70, respectively, were not recognized by the T-cell clones, whereas donor type peptide SEDLILCQL encoded by the *NUP133* gene was similarly recognized as LB-NUP133-1R. Mean production of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells at various peptide concentrations (µg/mL) is shown. (C) *NUP133* genes were isolated from patient and donor derived cDNA and cloned into expression vector pcDNA-3. Hela cells stably expressing HLA-B*40:01 were transiently transfected with patient and donor derived *NUP133* genes encoding the R and Q at position 406, respectively, and incubated with T-cell clone 3H1. Mean production of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells is shown.

T-cell recognition of non-malignant hematopoietic and non-hematopoietic cells

To investigate the cell type specificity in more detail, all MiHA specific CD8 T-cell clones were tested for reactivity against patient derived EBV-LCL and PHA-T cells as well as primary B cells, T cells and monocytes isolated from patient PBMC pior to alloSCT by flowcytometry based on expression of CD19, CD3 and CD14, respectively (Figure 3A). The data showed that all MiHA specific CD8 T-cell clones recognized specific types of primary hematopoietic cells, except for the T-cell clone for LB-TRIP10-1EPC, which showed reactivity against EBV-LCL, but failed to recognize all other non-malignant hematopoietic cells tested. The T-cell clone for LB-SWAP70-1Q showed clear recognition of hematopoietic cells of B-cell origin, whereas no or only weak reactivity was observed against T cells and monocytes.

In addition to hematopoietic cells, we tested T-cell recognition of non-hematopoietic FB cultured from a skin biopsy from the patient. FB were treated with IFN-γ to mimic the pro-inflammatory cytokine milieu of the early post transplantation period. In the absence of IFN-γ, FB were only recognized by control T-cell clone (4D8) recognizing an unknown MiHA in HLA-B*08:01 (Figure 3B). After pre-treatment with IFN-γ, reactivity against FB was measured for the T-cell clones for LB-SON-1R and LB-NUP133-1R, whereas no or only weak recognition of cytokine-treated FB was observed for the T-cell clones for LB-TRIP10-1EPC and LB-SWAP70-1Q. We, therefore, concluded that LB-TRIP10-1EPC and LB-SWAP70-1Q are MiHA with potential therapeutic value based on lack of T-cell recognition of cytokine-treated FB.

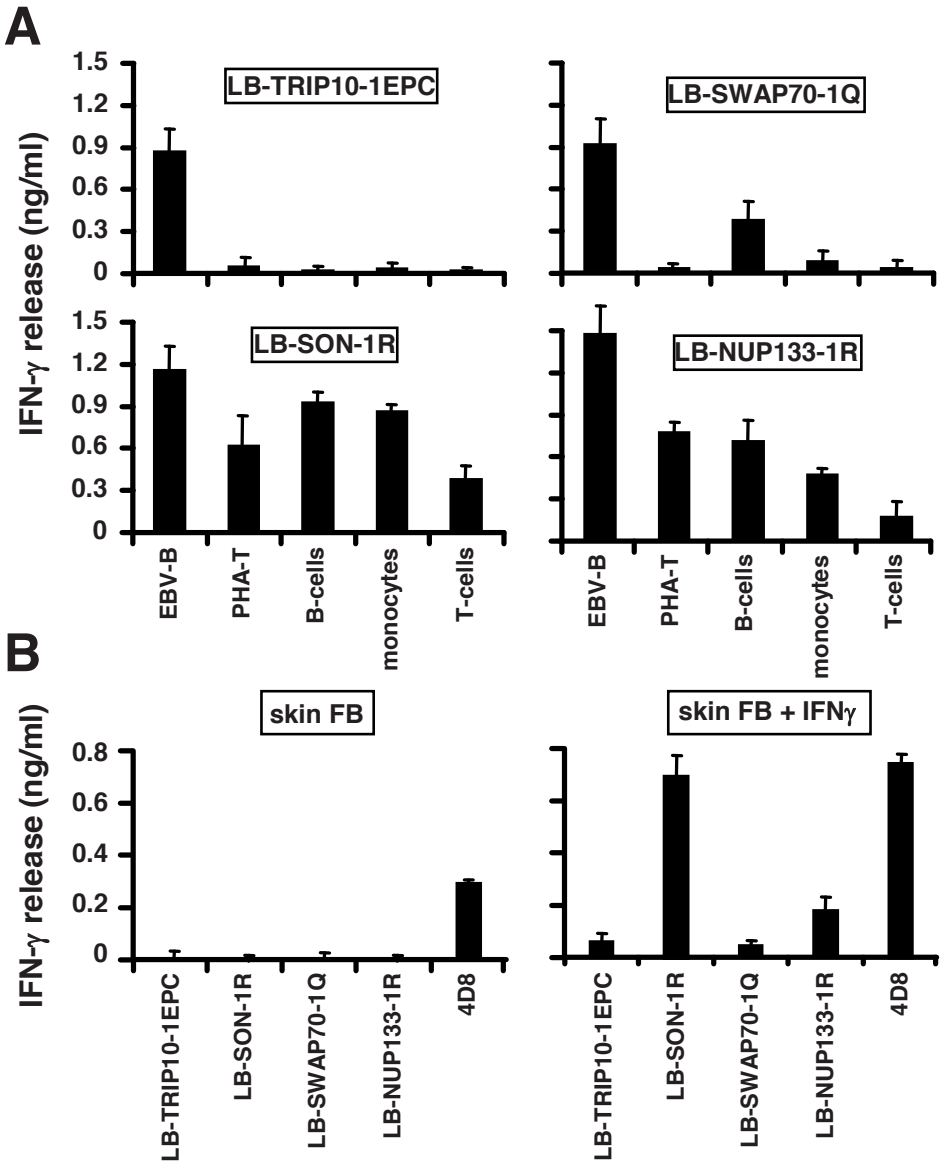
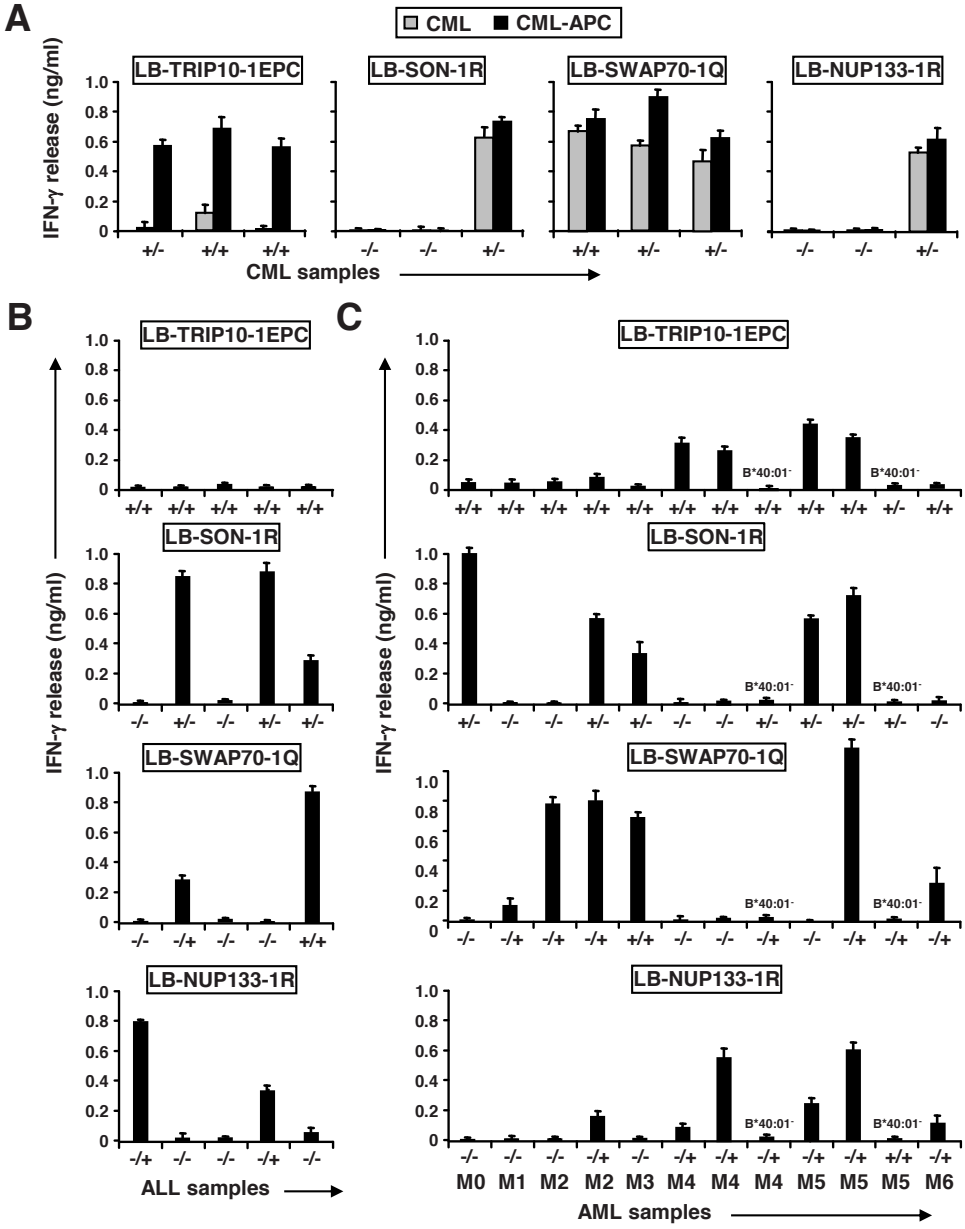


Figure 3. T-cell recognition of non-malignant hematopoietic and non-hematopoietic cells. CD8 T-cell clones specific for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R were tested for recognition of non-malignant hematopoietic and non-hematopoietic cells in IFN-γ ELISA. (A) T-cell clones were tested against patient derived EBV-LCL and PHA-T blasts, as well as primary B cells, T cells and monocytes isolated from patient PBMC prior to alloSCT. (B) T-cell clones were tested against FB cultured from a skin biopsy obtained from the patient after alloSCT. FB were cultured with and without IFN-γ (100 IU/mL) for four days. Reactivity of T-cell clone 4D8 recognizing an unknown MiHA in HLA-B*08:01 is shown as control. Mean production of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells is shown.

T-cell recognition of leukemic cells of different origins

Since the HLA-B*40:01 restricted MiHA were identified as targets for CD8 T cells in a patient with CML, we tested the capacity of the T-cell clones to recognize $CD34^+$ CML cells in IFN-γ ELISA. $CD34^+$ CML cells were isolated from BM samples from 3 HLA-B*40:01-positive patients, and tested for T-cell recognition directly after isolation as well as after *in vitro* culture with growth factors to generate CML cells with professional antigen presenting cell phenotype (CML-APC). All T-cell clones recognized freshly isolated CML cells as well as *in vitro* cultured CML-APC, except for the T-cell clone for LB-TRIP10-1EPC. This T-cell clone showed strong reactivity against CML-APC, whereas freshly isolated CML cells were weakly recognized or not recognized at all (Figure 4A). A similar difference in recognition was observed between monocytes and monocyte-derived dendritic cells (DC) (data not shown). The data demonstrated that LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R are expressed at the surface of CML cells suggesting that these MiHA served as direct targets for CD8 T cells in the GvL response after DLI. The data also showed that LB-TRIP10-1EPC is selectively expressed on professional APC, suggesting that T cells specific for this MiHA were induced at the onset of the immune response but did not contribute to direct lysis of leukemic cells in the execution phase of the response. In addition to CML cells, we analyzed T-cell recognition of ALL and AML cells of different subtypes (M0–M6). As shown in Figure 4B and C, the T-cell clone for LB-TRIP10-1EPC selectively recognized AML cells of monocytic (M4/M5) origin, whereas it failed to recognize ALL and AML cells of other subtypes. The T-cell clones for LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R recognized HLA-B*40:01 and MiHA positive ALL and AML cells irrespective of their subtype origin. All T-cell clones were capable of mediating cytolysis of primary leukemic blasts in ^{51}Cr-release assays (See also Figure S2).

Figure 4 (facing page). T-cell recognition of leukemic cells of different origins. CD8 T-cell clones specific for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R were tested for recognition of malignant hematopoietic cells in IFN-γ ELISA. (A) T-cell clones were tested against $CD34^+$ CML cells directly after flowcytometric isolation from BM cells from 3 HLA-B*40:01 positive patients (gray bars) as well as after *in vitro* culture with growth factors to generate CML-APC (filled bars). (B) T-cell clones were tested against ALL cells from PB and BM samples from 5 HLA-B*40:01 positive patients directly after isolation by flowcytometry based on expression of CD19. (C) T-cell clones were tested against PB and BM samples from 10 HLA-B*40:01 positive patients with more than 40% $CD33^+$ AML cells of different subtypes (M0–M6). Two HLA-B*40:01



negative AML-M4 and M5 samples were included as negative controls. The MiHA status of the samples is shown as +/+, +/− and −/−. Mean production of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells is depicted. Unpaired Student's t-test showed a significant difference in T-cell recognition between MiHA^{pos} (+/− or +/+) and MiHA^{neg} (−/−) CML, ALL and AML samples for LB-SON-1R (p=5.6×10^{-5}), LB-SWAP70-1Q (p=1.2×10^{-4}), and LB-NUP133-1R (p=4.0×10^{-3}). A significant difference for LB-TRIP10-1EPC (p=3.7×10^{-3}) was demonstrated between MiHA positive (+/− and +/+) AML-M4/M5 and MiHA positive (+/+ and +/−) CML, ALL and AML samples of other subtypes.

We also investigated whether the MiHA specific T-cell clones were capable of lysing hematopoietic progenitor cells in colony forming cell (CFC) assays. For this purpose, a BM sample obtained from the patient from whom the T-cell clones were isolated was selected at the time of relapse of the CML after alloSCT prior to DLI. The T-cell clones for the HLA-B*40:01 restricted MiHA failed to lyse the CML progenitor cells, whereas substantial inhibition in numbers of CFU-GM and erythroid colonies was observed for the T-cell clone for HA-1 (See also Figure S3). The cytolytic capacity of the T-cell clone for LB-SWAP70-1Q was also investigated in a BM sample from a patient with transformed juvenile CML and BM samples from 2 healthy individuals. The T-cell clone failed to lyse hematopoietic progenitor cells from healthy individuals, whereas substantial inhibition in numbers of CFU-GM was observed when the T-cell clone for LB-SWAP70-1Q was pre-incubated with the juvenile CML progenitor cells (See also Figure S3). Outgrowth of erythroid colonies was not observed, compatible with the malignant transformed nature of the juvenile CML progenitor cells. In conclusion, the data showed that LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R are relevant targets in GvL reactivity against CML, AML and ALL, whereas LB-TRIP10-1EPC may only be relevant as a direct target for AML-M4/M5. Furthermore, the data suggest that the HLA-B*40:01 restricted MiHA may not have played a dominant role in sustaining long-term clinical remission of the patient with CML from whom the respective T-cell clones were isolated based on lack of cytolysis of the malignant hematopoietic progenitor cells. Finally, we conclude that LB-SWAP70-1Q is a MiHA with therapeutic potential based on strong presentation on leukemic cells of different origins and lack of T-cell recognition of cytokine treated FB.

Expression patterns of the genes encoding the HLA-B*40:01 restricted MiHA

The expression patterns of the *TRIP10*, *SON*, *NUP133* and *SWAP70* genes were investigated by microarray gene expression analysis using a panel of selected (malignant) hematopoietic and (cytokine treated) non-hematopoietic cell samples. For each malignancy (CML, AML, ALL, CLL and MM), malignant cells were isolated by flowcytometry from 5 different patients. Non-malignant hematopoietic cells (PBMC, B cells, T cells, monocytes, immature and mature DC, HSC) were isolated by flowcytometry from 3 different healthy individuals. Non-hematopoietic FB, KC and PTEC were derived from 3 healthy individuals. These cells were cultured with and without IFN-γ to mimic the pro-inflammatory cytokine milieu of the early post transplantation period. The data showed broad

expression patterns and significant expression of all genes in cytokine treated non-hematopoietic cell types (See also Figure S4). The data, therefore, demonstrate that the threshold for T-cell recognition of the HLA-B*40:01 restricted MiHA, and in particular of LB-SWAP70-1Q, the MiHA with selective GvL effect, is not only determined by gene expression, but rather by intracellular processing and presentation mechanisms and/or surface expression of adhesion and co-stimulatory molecules.

***In vivo* monitoring for MiHA specific T cells**

We investigated the *in vivo* immuno-dominance of the HLA-B*40:01 restricted MiHA in the patient with CML from whom the T-cell clones were isolated. We previously measured peak responses of HA-1 and HA-2 tetramer positive T cells in this patient, and 2 PB samples obtained during this peak response at six and seven weeks after treatment with DLI were selected for tetramer analysis. The data showed dominance for LB-SWAP70-1Q and LB-NUP133-1R in the immune response with 0.94% and 0.42% of tetramer positive cells within the PBMC population gated for negative expression of CD4, CD14, CD19 and CD56 at week 6 after DLI, respectively (See also Figure S5). These frequencies exceeded the T-cell frequencies for HA-2 (0.33%) and HA-1 (0.13%) as measured at weeks 6 and 7, respectively. In addition to LB-SWAP70-1Q and LB-NUP133-1R, a low frequency of T cells specific for LB-SON-1R (0.03%) was detected at week 7 after DLI. T cells specific for LB-TRIP10-1EPC could not be detected at significant frequencies. The *in vivo* immunogenicity of LB-SWAP70-1Q, the MiHA with selective GvL effect, was also evaluated in another HLA-B*40:01 and MiHA positive patient transplanted with an HLA-matched MiHA negative donor. The frequency of HLA-B*40:01 in our transplanted patient and donor population is 12.5%, which is higher than the 2–9% reported for various European countries (www.allelefrequencies.net). We screened 1,069 genomic DNA samples for the SNP encoding LB-SWAP70-1Q. A total number of 612 individuals were positive for LB-SWAP70-1Q, indicating a frequency of 57% in our patient and donor population. DNA samples from 398 patient-donor pairs included 297 (75%) transplantations with sibling donors and 101 (25%) transplantations with matched unrelated donors (MUD). Of the 398 patient-donor pairs, 56 LB-SWAP70-1Q positive patients were transplanted with MiHA negative donors, including 37 (12%) sibling and 19 (19%) MUD donors. Since HLA-B*40:01 is expressed in 12.5% of the human population, our data indicate a chance for mismatching for LB-SWAP70-1Q of 1.5% and 2.3% after sibling and MUD transplantation, respectively. Of the 56 LB-SWAP70-1Q positive

patients transplanted with MiHA negative donors, 7 patient-donor pairs were positive for HLA-B*40:01. From 4 patients, PB and BM samples were available for T-cell monitoring. Three of these patients, however, were not treated with DLI and no obvious immune responses were ongoing at the available time points after alloSCT. One patient with CLL was successfully treated with DLI, and PB samples pre-DLI and six and eight weeks post-DLI were available for T-cell monitoring. These samples were screened for the presence of T cells for LB-SWAP70-1Q directly *ex vivo* as well as after one week of *in vitro* stimulation with peptide, but no tetramer positive T cells could be detected (data not shown). In conclusion, the data show that LB-SWAP70-1Q, the MiHA with selective GvL effect, was dominant in the immune response in the patient with CML from whom the T-cell clones were isolated. Since only one other patient could be monitored for T cells specific for LB-SWAP70-1Q, conclusions as to whether LB-SWAP70-1Q is immunogenic in a broader patient population cannot be drawn.

Discussion

Although an increasing number of MiHA in different HLA molecules have been identified in the last decades, the number of MiHA with therapeutic relevance is still limited.[3–7] We previously demonstrated induction of CD8 T cells specific for hematopoietic restricted MiHA HA-1 and HA-2 in a patient who developed a strong GvL response with limited GvHD of the skin after treatment with DLI for relapsed CML more than one year after HLA-matched alloSCT.[13] We also isolated $CD4^+$ T cells from this patient, and showed recognition of 6 HLA class II associated MiHA, of which 5 MiHA have been identified by screening a recombinant bacteria cDNA library.[15,17] In this report, we demonstrated that in addition to HA-1 and HA-2, which are both presented by HLA-A*02:01, CD8 T cells were induced against 4 MiHA in HLA-B*40:01 and one MiHA in HLA-B*08:01, illustrating the diversity of MiHA targeted in this GvL response. The HLA-B*40:01 restricted MiHA were identified by screening a plasmid cDNA expression library and WGAs, and detailed T-cell reactivity and monitoring experiments were performed to estimate the clinical and therapeutic relevance of the MiHA.

T cells specific for LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R were shown to recognize CML, ALL and AML cells, illustrating the relevance of these MiHA as direct targets in GvL reactivity. LB-TRIP10-1EPC, however, was expressed on AML-M4/M5 of monocytic origin, but not on ALL and AML cells of other subtypes. Surface expression of LB-TRIP10-1EPC was also demonstrated for professional APC, including *in vitro* cultured CML-APC and monocyte derived DC, but expression could not be measured on freshly isolated CML progenitor cells and monocytes, nor on (specific types of) peripheral blood cells (B cells, T cells or monocytes). Based on these T-cell recognition data, we speculate that T cells specific for LB-TRIP10-1EPC were induced by professional leukemic APC at the onset of the immune response, but that these T cells did not mediate direct cytolysis of CML cells in the execution phase of the anti-tumor response. Although all T-cell clones for the HLA-B*40:01 restricted MiHA required similar amounts of peptide to be activated, surface expression of endogenous LB-TRIP10-1EPC in HLA-B*40:01 may be low, and the affinity of the T-cell receptor as expressed on the T-cell clone may not be sufficiently high to recognize endogenous LB-TRIP10-1EPC in the absence of high expression of costimulatory and/or adhesion molecules. Significant expression of the co-stimulatory molecule B7-2 (CD86) has been

reported for AML-M4/M5,[18] and may contribute to recognition of these leukemic subtypes by T cells specific for LB-TRIP10-1EPC.

Despite clear recognition and lysis of primary leukemic cells in IFN-γ ELISA and ^{51}Cr-release assays, all T-cell clones for the HLA-B*40:01 restricted MiHA failed to lyse the malignant progenitor cells of the patient with CML from whom the T-cell clones were isolated in CFC assays. Our data, therefore, suggest that the HLA-B*40:01 restricted MiHA are relevant targets in GvL reactivity, but that they may not have played a dominant role in sustaining long-term clinical remission of the patient with CML.

All T cells specific for the novel HLA-B*40:01 restricted MiHA were tested for recognition of skin-derived FB. These cells were cultured with and without IFN-γ to mimic the pro-inflammatory cytokine milieu of the early post transplantation period.[19] In the absence of IFN-γ, all T cells failed to recognize FB. After treatment with IFN-γ, the only T cells that failed to recognize FB were specific for LB-SWAP70-1Q and LB-TRIP10-1EPC. Lack of T-cell reactivity against FB and normal hematopoietic progenitor cells cannot merely be explained by gene expression and, therefore, the immunological threshold for T-cell recognition of the HLA-B*40:01 restricted MiHA rather seems to be determined by intracellular antigen processing and presentation mechanisms and/or surface expression of adhesion and costimulatory molecules.

In vivo monitoring of the patient with CML from whom the T-cell clones were isolated demonstrated that 2 HLA-B*40:01 restricted MiHA (LB-SWAP70-1Q and LB-NUP133-1R) were dominant in the immune response, and exceeded the T-cell frequencies for the well-known hematopoietic restricted MiHA HA-1 and HA-2. One of these MiHA (LB-SWAP70-1Q) has been shown to be recognized on leukemic cells of various origins, but not on cytokine treated non-hematopoietic FB, illustrating the therapeutic potential of this MiHA as target for GvL reactivity. However, although the T-cell clone for LB-SWAP70-1Q as described in this study failed to recognize cytokine treated FB, various non-hematopoietic cells were shown to express the *SWAP70* gene at significant levels. It can not, therefore, be completely excluded that *in vitro* or *in vivo* strategies to induce LB-SWAP70-1Q specific T cells with high avidities may have the potential to mediate GvHD due to reactivity towards non-hematopoietic tissues. Expression analysis also demonstrated overexpression of the *SWAP70* gene in B cells, and its protein product has been described to exert relevant

functions in nuclear and signal transduction events in activated B cells.[20–22] The SWAP70 protein has been found to be a component of a complex that promotes recombination between DNA regions during heavy chain class switching,[20,21] and it transduces signals from tyrosine kinase receptors to Rac in a Ras-independent manner.[22]

Recently, the first clinical study on adoptive transfer of MiHA specific CD8 T cells to patients with acute leukemia who relapsed after alloSCT has been reported by Warren *et al.*[23] The clinical results are encouraging since several patients achieved complete remissions, although at the price of significant GvHD. The MiHA specific CD8 T cells as administered in the clinical study were selected for cytolytic activity against patient, but not donor derived EBV-LCL, and lack of reactivity to patient derived dermal FB, but only in the absence of cytokines. Our data, however, demonstrated that 2 HLA-B*40:01 restricted MiHA were presented on patient derived FB after pre-treatment with IFN-γ. We, therefore, support a more stringent selection strategy of MiHA as targets for T-cell therapies for selective stimulation of GvL reactivity without GvHD.

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Supplementary Materials and Methods

Retroviral transduction

EBV-LCL were transduced with a retroviral vector encoding HLA-B*40:01 (MP71-HLA-B*40:01-IRES-NGFR) as previously described[1]. EBV-LCL with transduction efficiencies more than 20% based on marker gene expression were included in the panel for WGAs. Cells with less than 20% transduction efficiencies were enriched for nerve growth factor receptor (NGFR) marker gene expression after staining with PE- or APC-labeled anti-NGFR antibodies (BD Biosciences) by flowcytometry or magnetic beads (Miltenyi Biotec). For each T-cell clone, a total number of 60 EBV-LCL expressing HLA-B*40:01 endogenously or after retroviral transduction were included for WGAs.

Isolation and culture of primary hematopoietic cells

Patient PBMC obtained prior to alloSCT were stained with FITC-conjugated anti-CD14, PE-conjugated anti-CD3 and APC-conjugated anti-CD19 antibodies (BD Biosciences), and monocytes, T cells and B cells were isolated by flowcytometry based on expression of CD14, CD3 and CD19, respectively. In addition, PB and BM samples from patients with CML, AML, ALL, CLL and MM were stained with APC-conjugated anti-CD34, anti-CD33 or anti-CD19, and PE-conjugated anti-CD5 or anti-CD38 antibodies (BD Biosciences). CD34+ CML, CD33+ AML, CD19+ ALL, CD19+CD5+ CLL and CD38+ MM cells were subsequently isolated by flowcytometry. Isolated CD34+ CML progenitor cells were modified into leukemic APC[2] and isolated monocytes were cultured to immature and mature dendritic cells (DC)[3] as previously described.

Enzyme linked immunosorbent assay

Stimulator cells (3×10^4 cells/well) were co-incubated with CD8 T-cell clones (5×10^3 cells/well) overnight at 37°C in U-bottom 96-well plates. Peptide pulsing was performed by incubating donor EBV-LCL (1×10^6 cell/mL) for 2 h with synthetic peptides in IMDM with 2% FBS. Peptide-pulsed donor EBV-LCL were washed twice and subsequently used as stimulator cells. Release of IFN-γ was measured in 50 µL culture supernatants by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sanquin, Amsterdam, The Netherlands).

Chromium release assay

Target cells were labeled for 1 h at 37°C with 100 µCi (3.7 MBq) $Na_2{}^{51}CrO_4$ (Amersham, Uppsala, Sweden). After washing, target cells (1×10^3 cells/well) were incubated with CD8 T-cell clones at different effector-to-target (E:T) ratios for 4 or 10 h. Release of ^{51}Cr was analyzed in 25 µL supernatants. The percentage of specific lysis was calculated with the following formula:
[experimental release (cpm) – spontaneous release (cpm)]/[maximal release (cpm) – spontaneous release (cpm)] x 100%. Maximal release was induced by 1% Triton (Sigma-Aldrich).

Colony forming cell assay

BM samples were incubated with irradiated (20 Gy) CD8 T-cell clones at E:T ratios of 3:1. After overnight pre-incubation, single-cell suspensions were cultured at 2×10^4 target cells/mL in 30 mm culture dishes containing IMDM with methylcellulose supplemented with growth factors (GM-CSF, stem cell factor, IL-3, erythropoietin and other supplements; MethoCult, Stemcell Technologies SARL, Grenoble, France). As controls, single-cell suspensions containing 2×10^4 target cells/mL and irradiated T cells at E:T ratios of 3:1 were seeded without pre-incubation. After 14 days of culture, numbers of colony forming units (CFU) for granulocyte/monocyte (CFU-GM) and erythroid lineages were scored.

Construction and screening of a plasmid cDNA expression library

A cDNA library was constructed as previously described[4]. Poly(A)+ mRNA was isolated from patient EBV-LCL, and converted to cDNA using an oligo-d(T) primer. The cDNA was size-fractionated using column chromatography and ligated into pCR3.1 (Invitrogen, Breda, The Netherlands). Ligation products were transformed into E. Coli Top10 bacteria, and ampicillin resistant clones were divided into pools of approx. 50 different cDNA. Pools of cDNA were transfected into COS-7 cells stably expressing HLA-B*40:01. Transfection and screening of the cDNA library was performed using different mixtures of MiHA specific T-cell clones.

Whole genome association scanning

WGAs was performed as previously described[5]. Briefly, a panel of 60 EBV-B cell lines stably expressing HLA-B*40:01 endogenously or after retroviral transduction (>20% marker gene expression) was tested for T-cell recognition in IFN-γ ELISA. For each T-cell clone, EBV-B cell lines were divided into MiHA positive and negative groups based on a threshold of 5-fold the background

production of IFN-γ. All EBV-LCL included in the panel were genotyped for more than 1.1 million SNPs by Human 1M-duo arrays (Illumina, Inc., San Diego, CA, USA). WGAs analysis was performed by combining T-cell recognition and SNP genotyping data using Plink software (http://pngu.mgh.harvard.edu/purcell/plink/ version 1.03), and the significance of association between both patterns was calculated using Fisher's exact test.

Genotyping for single nucleotide polymorphisms

Genomic DNA was isolated by the Gentra Systems PureGene genomic isolation kit (Biocompare, San Francisco, CA, USA). SNP rs1049232 in the *TRIP10* gene was analyzed using forward and reverse primers for amplification and two TaqMan MGB probes labeled with VIC and FAM dyes to detect the different alleles (Applied Biosystems, Foster City, CA, USA). Genotyping for SNP rs1049229 (*TRIP10*), rs13047599 (*SON*), rs415895 (*SWAP70*) and rs1065674 (*NUP133*) was performed using allele-specific primers labeled with VIC and FAM dyes according to the manufacturer's instructions (KBioScience, Hoddesdon, UK).

Microarray gene expression analysis

Malignant cells were isolated from PB and BM samples from patients with CML, AML, ALL, CLL and MM by flowcytometry based on expression of CD34, CD33, CD19, CD19/CD5 and CD38, respectively. Non-malignant B cells, T cells and monocytes were isolated from PBMC based on expression of CD19, CD3 and CD14, respectively. Hematopoietic stem cells (HSC) were isolated from G-CSF mobilized PB based on expression of CD34. Non-hematopoietic cells included skin-derived FB and KC, and PTEC cultured with and without IFN-γ (100 IU/mL). Total RNA was isolated using small and micro scale RNAqueous isolation kits (Ambion, Inc., Austin, TX, USA), and amplified using the TotalPrep RNA amplification kit (Ambion). After preparation using the whole-genome gene expression direct hybridization assay (Illumina), cRNA samples were dispensed onto Human HT-12 v3 Expression BeadChips (Illumina). Hybridization was performed in the Illumina hybridization oven for 17 h at 58°C. Microarray gene expression data were analyzed using Rosetta Resolver 7.2 software.

***In vivo* T-cell monitoring**

PB samples were stained with a mixture of FITC-conjugated antibodies against CD4, CD14, CD19 and CD56 as well as a mixture of APC- and PE-conjugated tetramers for the HLAB*40:01 restricted MiHA or a mixture of an APC-

conjugated HA-2 tetramer and PE-conjugated HA-1 tetramer. For each tetramer, a minimum of 5×10^5 PBMC were analyzed. Tetramers were constructed as previously described[6] with minor modifications.

References Supplementary Materials and Methods

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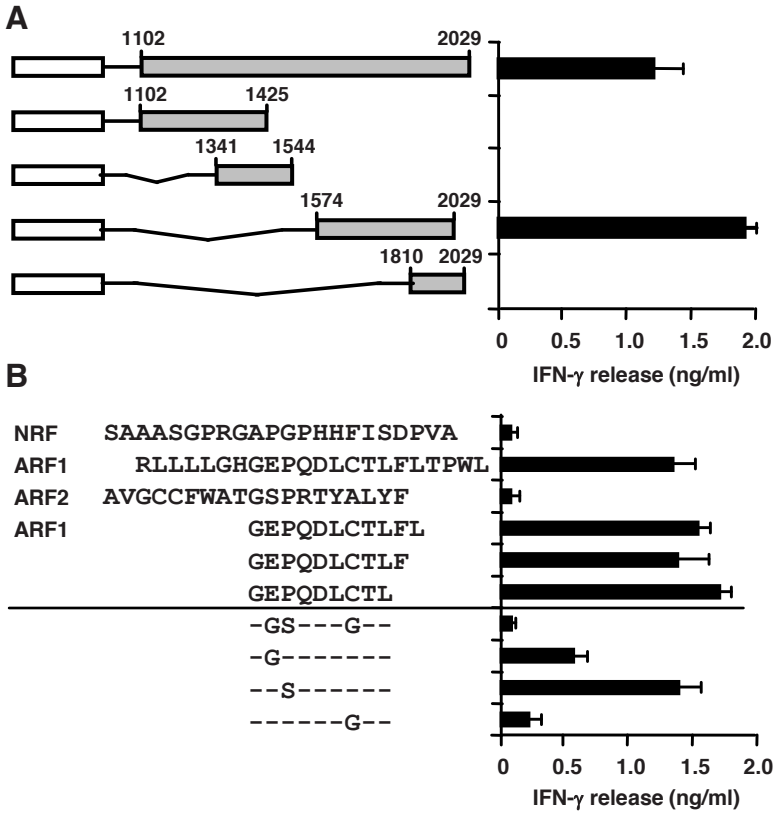


Figure S1. Identification of LB-TRIP10-1EPC by screening a plasmid cDNA library. Transfection and screening of a cDNA plasmid library generated from mRNA from patient derived EBV-LCL in COS-7 cells expressing HLA-B*40:01 (COS-B*40:01) revealed isolation of a 1102 – 2029 bp *TRIP10* cDNA recognized by T-cell clone ZRZ16. (A) Deletion variants of the 1102 – 2029 bp *TRIP10* cDNA were constructed and tested for T-cell recognition after transfection into COS-B*40:01 cells in IFN-γ ELISA. Indicated is the mean release of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells. The results show that the MiHA is encoded by a 1574 – 1810 bp region which contains the stop codon of the TRIP10 coding sequence at 1704 bp. (B) Peptides comprising 3 patient type AA encoded by SNP rs1049229, rs1049230, and rs1049232 that are predicted to bind to HLA-B*40:01 were synthesized and tested for T-cell recognition in IFN-γ ELISA. The mean release of IFN-γ (ng/mL) in 50 µL culture supernatants of duplicate wells is indicated. A 9-mer peptide (GEPQDLCTL) encoded in the 3' untranslated region (3' UTR) in an alternative reading frame (ARF1) of the upstream encoded TRIP10 protein was recognized by clone ZRZ16. The 9-mer donor type peptide (GGSQDLGTL) was not recognized. Analysis of patient type peptides containing single AA of donor origin demonstrated that the E and C most significantly contributed to T-cell recognition.

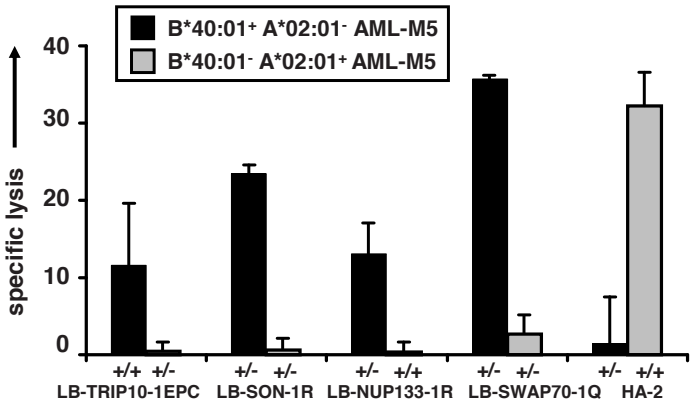
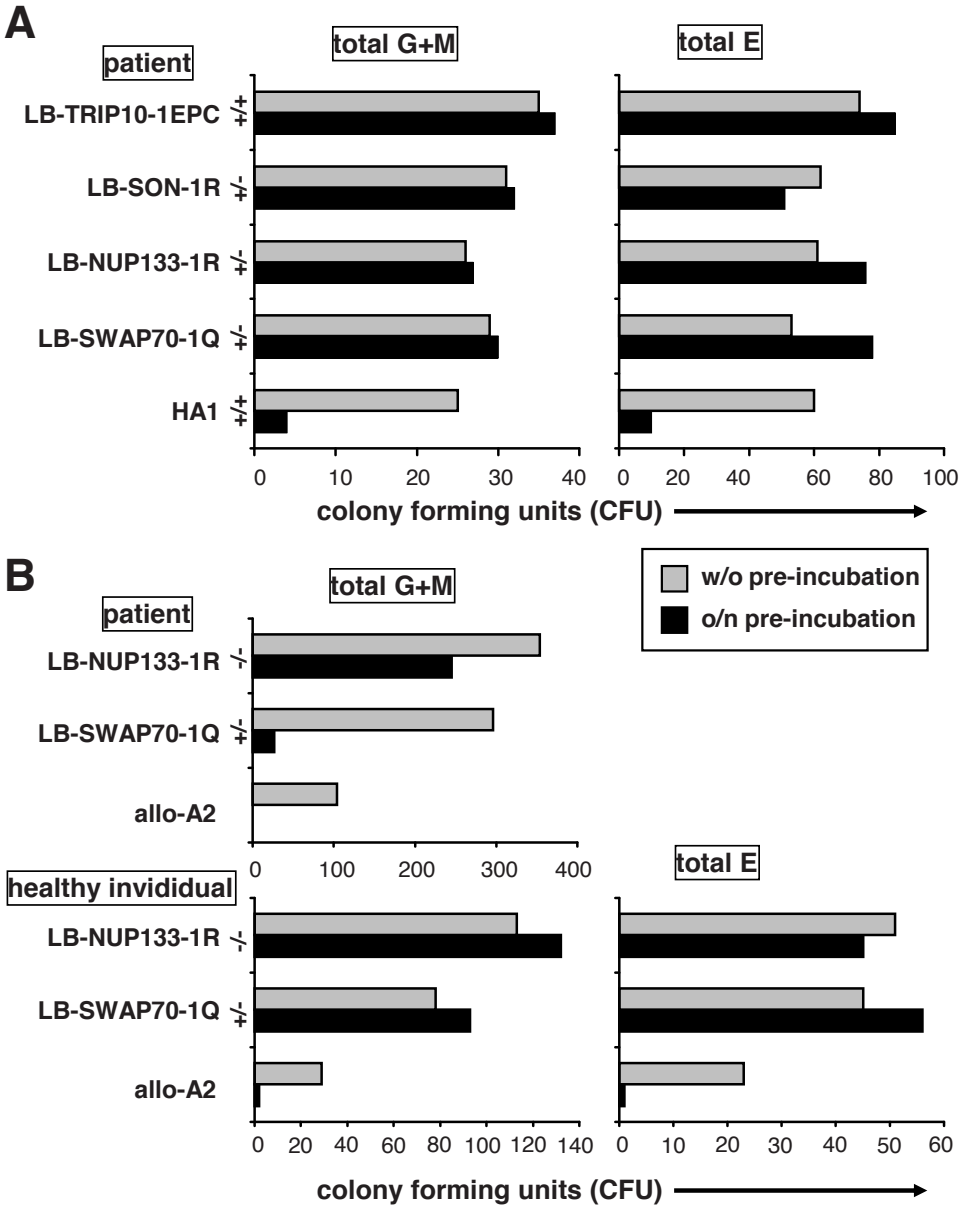


Figure S2. T-cell mediated lysis of primary leukemic blasts. CD8 T-cell clones specific for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R were investigated for cytolytic activity against primary leukemic blasts in 10 h 51Cr-release assays at E:T ratios of 30:1. Mean specific lysis of triplicate wells is shown for an HLA-B*40:01 positive and A*02:01 negative AML-M5 sample positive for all 4 HLA-B*40:01 restricted MiHA, and an HLAB*40:01 negative and A*02:01 positive AML-M5 control sample.

Figure S3 (next page). T-cell mediated lysis of hematopoietic progenitor cells. Lysis of hematopoietic progenitor cells was measured in CFC assays. BM cells ($2x10^4$ cells/mL) were seeded as single-cell suspensions in medium with methylcellulose supplemented with growth factors after overnight pre-incubation with irradiated T-cell clones at E:T ratios of 3:1. (A) The T-cell clones for LB-TRIP10-1EPC, LB-SON-1R, LB-SWAP70-1Q and LB-NUP133-1R and a T-cell clone for HA-1 were analyzed for specific lysis of the malignant hematopoietic progenitor cells from the patient with CML from whom the T-cell clones were isolated. For this purpose, a BM sample was selected at the time of relapse after alloSCT prior to DLI. (B) The T-cell clone for LB-SWAP70-1Q and an HLA-A*02:01 specific allo-reactive T-cell clone were tested for lysis of hematopoietic progenitor cells from an HLA-B*40:01 and LBSWAP70-1Q positive patient with transformed juvenile CML (upper graph) and 2 HLA-B*40:01 and LBSWAP70-1Q positive healthy individuals. Representative data are shown for one healthy individual (lower graph). Indicated are the numbers of CFU for granulocytes and monocytes (CFU-GM) and erythroid cells as scored after 14 days of culture of BM cells after overnight pre-incubation with the irradiated T-cell clones (black bars) and, as controls, for BM cells and irradiated T-cells seeded as single-cell suspensions without overnight pre-incubation (gray bars).

Figure S3. T-cell mediated lysis of hematopoietic progenitor cells



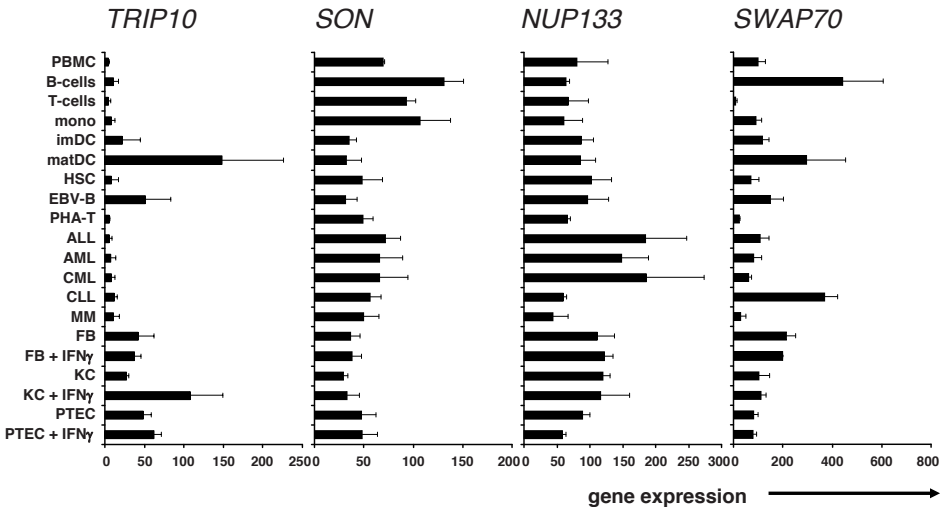
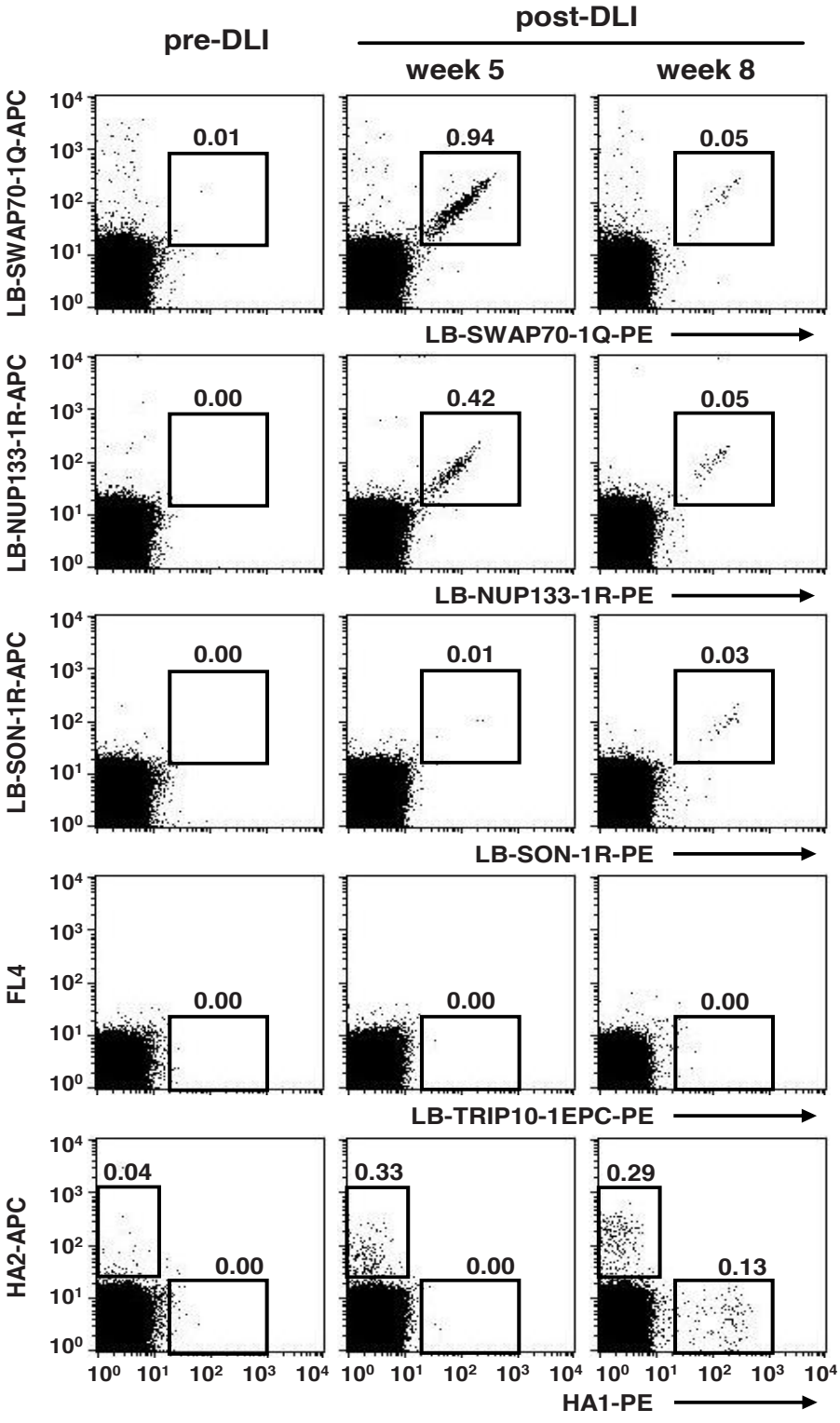


Figure S4. Expression patterns of the genes encoding the HLA-B*40:01 restricted MiHA. Expression as measured by microarray gene expression analysis is shown for the genes encoding *TRIP10, NUP133, SON*, and *SWAP70* in non-malignant hematopoietic cells (PBMC, B cells, T cells, monocytes, immature and mature DC and hematopoietic stem cells), malignant hematopoietic samples (CML, ALL, AML, CLL and MM), and non-hematopoietic cell types (FB, KC and PTEC pre-treated with and without IFN-γ).

Figure S5 (next page). In vivo monitoring for MiHA specific T cells. Screening for MiHA specific T cells was performed for the patient with CML from whom the T-cell clones were isolated. PB samples pre-DLI and 6 and 7 weeks post-DLI were selected for *in vivo* T-cell monitoring. PBMC were stained with a mixture of FITC-conjugated anti-CD4, CD14, CD19 and CD56 antibodies and a mixture of PE- and APC-conjugated tetramers containing LB-SWAP70-1Q, LBNUP133-1R, LB-SON-1R or LB-TRIP10-1EPC, or a mixture of PE-conjugated HA-1 and APC-conjugated HA-2 tetramers. Cells were gated for negative expression of CD4, CD14, CD19 and CD56, and mean fluorescence intensities are shown for PE- and APC-conjugated tetramers. Percentages are shown of gated cells that are double positive for PE- and APC-conjugated LB-SWAP70-1Q, LB-NUP133-1R, and LB-SON-1R tetramers, single positive for PE-conjugated LB-TRIP10-1EPC and HA-1 tetramers, and single positive for the APC-conjugated HA-2 tetramer.


pre-DLI
post-DLI
week 5
week 8
LB-SWAP70-1Q-APC
0.01
0.94
0.05
LB-SWAP70-1Q-PE
LB-NUP133-1R-APC
0.00
0.42
0.05
LB-NUP133-1R-PE
LB-SON-1R-APC
0.00
0.01
0.03
LB-SON-1R-PE
FL4
0.00
0.00
0.00
LB-TRIP10-1EPC-PE
HA2-APC
0.04
0.00
0.33
0.00
0.29
0.13
HA1-PE

Chapter 5

Durable remission of renal cell carcinoma in conjuncture with graft-*versus*-host disease following allogeneic stem cell transplantation and donor lymphocyte infusion: rule or exception?

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Abstract

Allogeneic stem cell transplantation (alloSCT) followed by donor lymphocyte infusion (DLI) can be applied as immunotherapeutic intervention to treat malignant diseases. Here, we describe a patient with progressive metastatic clear cell renal cell carcinoma (RCC) who was treated with T-cell depleted non-myeloablative alloSCT and DLI resulting in disease regression accompanied by extensive graft-*versus*-host disease (GvHD). We characterized the specificity of this immune response, and detected a dominant T-cell population recognizing a novel minor histocompatibility antigen (MiHA) designated LB-FUCA2-1V. T cells specific for LB-FUCA2-1V were shown to recognize RCC cell lines, supporting a dominant role in the graft-*versus*-tumor (GvT) reaction. However, coinciding with the gradual disappearance of chronic GvHD, the anti-tumor effect declined and 3 years after alloSCT the metastases became progressive again. To re-initiate the GvT reaction, escalating doses of DLI were given, but no immune response could be induced and the patient died of progressive disease 8.5 years after alloSCT. Gene expression studies illustrated that only a minimal number of genes shared expression between RCC and professional antigen presenting cells but were not expressed by non-malignant healthy tissues, indicating that in patients suffering from RCC, GvT reactivity after alloSCT may be unavoidably linked to GvHD.

Introduction

Allogeneic stem cell transplantation (alloSCT) is a highly effective treatment for many hematological malignancies[1]. Following HLA-matched alloSCT, the curative graft-*versus*-tumor (GvT) reactivity is mediated by donor-derived T cells recognizing minor histocompatibility antigens (MiHA) expressed by the malignant patient cells. MiHA are polymorphic peptides presented by HLA-molecules and are the result of genomic single nucleotide polymorphisms (SNPs) that are disparate between patient and donor. The repertoire of patient specific MiHA can act as non-self antigens to infused donor T cells[2]. If MiHA are co-expressed by malignant cells and normal non-hematopoietic tissues, alloreactive donor T cells may induce both GvT reactivity and graft-*versus*-host disease (GvHD). Donor T cells recognizing MiHA exclusively expressed by normal and malignant hematopoietic cells from the patient can mediate GvT reactivity in the absence of GvHD. Since hematopoiesis after alloSCT is of donor origin, complete elimination of patient hematopoiesis does not impair normal hematopoiesis and immunological function. T-cell depletion of the graft reduces the risk of GvHD, but increases relapse rates by abrogating therapeutic GvT reactivity. Postponed donor lymphocyte infusion (DLI) can be applied to prevent or treat disease recurrence[2,3].

Clinical beneficial effects of alloSCT for treatment of non-hematopoietic tumors were mainly observed in patients with metastatic renal cell cancer (RCC)[4,5] and metastatic breast cancer[6]. In RCC, alloSCT resulted in an overall response rate ranging between 20-40%[7]. In the majority of these cases, however, GvT reactivity was associated with development of clinically significant GvHD. The concurrence of GvT reactivity and GvHD indicates that tumor controlling donor T cells often recognize MiHA that are co-expressed by tumor cells and by normal tissue cells. Specific GvT reactivity and concurrent prevention of GvHD by replacement of the normal patient counterpart by donor cells, comparable to achievement of full donor chimerism in bone marrow and peripheral blood of hematological patients after alloSCT, is obviously not possible in patients with solid tumors.

For development and expansion of a primary donor-derived immune response after DLI, it may be essential that MiHA are presented by recipient-derived dendritic cells (DC)[8]. DC of patient origin can present both endogenously derived MiHA, and cross-present antigens that are generated from proteins

taken up from surrounding damaged tissue cells. In patients with hematological malignancies, the hematopoietic origin of DC may explain relative skewing of the T-cell response towards hematopoietic cells, and targeting of hematopoiesis restricted MiHA can result in GvT reactivity in the absence of GvHD[9,10]. Solid tumor cells and DC however, originate from different lineages and successful targeting of these malignancies may often involve MiHA that are broadly expressed not only on DC and malignant cells, but also on the normal counterpart of tumor cells.

In this study, we describe a patient with clear cell RCC who showed tumor regression and prolonged survival after alloSCT followed by DLI. Extensive chronic GvHD coincided with durable disease control but the disease became progressive when GvHD resolved. Subsequent administration of escalating doses of DLI could not re-induce the GvT reaction. We identified a strong T-cell response targeting a novel MiHA (LB-FUCA2-1V) presented by HLA-B*07:02, and induction of LB-FUCA2-1V specific T cells coincided with tumor control and GvHD. Broad recognition of GvHD target tissues by LB-FUCA2-1V specific T cells correlated with a broad expression profile of the *FUCA2* gene. Gene expression profile studies showed that, in contrast to leukemic cells, only a limited number of genes are selectively co-expressed by RCC and DC, and not by cells representing normal tissue cells. GvT reactivity may therefore be unavoidably correlated with GvHD after alloSCT and DLI for treatment of RCC.

Materials and methods

Sample collection and preservation

Peripheral blood samples and skin biopsies were collected from patient, donor, and third party individuals after approval by the Leiden University Medical Center institutional review board according to the Declaration of Helsinki. Written informed consent was given by patient and donor, and by 3rd party individuals to investigate materials and to publish data and case details. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque separation and cryopreserved. Skin biopsies were immediately processed.

Generation and culture of cell lines

EBV-transformed B-lymphoblastic cell lines (EBV-LCL) were generated in-house from PBMC from patient, donor and third party individuals. EBV-LCL were generated and cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) with 10% FBS (Lonza)[11]. To obtain fibroblast and keratinocyte cell lines, single cell suspensions were generated from skin biopsies by mechanical and enzymatic dissociation. Fibroblasts were obtained by culturing in Dulbecco's modified Eagle's medium (DMEM) with low glucose (Lonza) with 10% FBS and keratinocytes by culturing in keratinocyte serum-free medium supplemented with 30 µg/ml of bovine pituitary extract and 2 ng/ml of epithelial growth factor (Invitrogen, Carlsbad, CA). RCC and melanoma cell lines were previously established in Leiden or kindly provided by Prof. A. Knuth (University of Zürich, Zürich, Switzerland) (RCC Mz1774 and RCC Mz1851) and Prof. P Straten (Danish Cancer Society, Copenhagen, Denmark, MEL SK23) and were cultured in DMEM with 8 % FBS. Immature dendritic cells were derived from monocytes isolated from PBMC using MACS CD14 MicroBead isolation (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and cultured for 2 days in IMDM with 10% FBS with 100 ng/ml GM-CSF (Novartis, Basel, Switzerland) and 500 IU/ml IL-4 (Schering-Plough, Bloomfield, NJ). DC were subsequently matured for 2 days by adding 10 ng/ml TNF-α (R&D Systems, Abingdon, UK), 10 ng/ml IL-1β (Immunex, Seattle, WA), 10 ng/ml IL-6 (Cellgenix, Freiburg, Germany), 1 µg/ml PGE2 (Sigma-Aldrich, Zwijndrecht, The Netherlands), and 500 IU/ml IFN-γ (BoehringerIngelheim, Ingelheim am Rhein, Germany). In selected EBV-LCL and RCC, transductions with retroviral vector pLZRS containing HLA-B*07:02 and the truncated NGFR marker gene were performed as previously described[12.]

SNP genotyping

SNPs encoding known MiHA were determined in patient and donor-derived genomic DNA extracted with Gentra Puregene Blood Kit (Qiagen, Venlo, The Netherlands). For LB-APOBEC3B-1K, LB-ARHGDIB-1R, LB-BCAT2-1R, LB-ECGF-1R, LB-MR1-1H and LRH-1, 10 ng DNA was amplified with allele specific primers using the KASPar SNP genotyping system (KBioscience, Herts, UK). For LB-EBI3-1I, LB-ERAP1-1R, LB-GEMIN4-1V, LB-MTHFD1-1Q, LB-PDCD11-1F, HwA-9 and HwA-10, 10 ng DNA was amplified in the presence of allele specific probes using Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA). After amplification, fluorescent signals were analyzed on a 7900HT device running with SDS software (Applied Biosystems). Allele specific primers and probes were selected according to the manufacturer's instructions (See also Supplemental Table S1: MiHA disparities between donor and patient).

Cloning and testing of T cells recognizing known MiHA

Tetramers were constructed by folding peptides in biotinylated HLA-B*07:02 monomers followed by multimerization using streptavidin conjugated to PE as previously described with minor modifications[13]. MiHA specific T cells were visualized using PE-conjugated tetramers and PE-Cy7 labeled anti-CD8 antibodies (BD Biosciences, Breda, The Netherlands). Tetramer$^+$ T cells were single cell per well sorted on a FACS Aria device (BD) in 96-wells U-bottom plates (Corning, Amsterdam, The Netherlands) containing T cell medium (TCM, IMDM with 5% pooled human serum, 5% FBS and IL-2 (100 IU/ml, Chiron, Amsterdam, The Netherlands)), and stimulated with phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford, UK) and $5x10^4$ irradiated allogeneic PBMC. Growing T-cell clones were restimulated every 10 days in TCM at a concentration of $2x10^5$/ml with $1x10^6$/ml irradiated allogeneic PBMC and PHA. TCR β-chain analysis was performed using the TCRBV repertoire kit (Beckman Coulter, Mijdrecht, The Netherlands). The reactivity of T-cell clones was measured after 24h co-incubation with 3-fold stimulator cells and release of IFN-γ in culture supernatants was measured by ELISA (Sanquin, Amsterdam, Netherlands). In selected experiments, IFN-γ pretreatment (100 U/ml) of stimulator cells was performed for 24h at 37^{o}C and prior to co-incubation, these cells were thoroughly washed to remove IFN-γ.

Isolation and characterization of T cells recognizing novel MiHA

Post-DLI PBMC were stimulated with irradiated (30Gy) pre-alloSCT patient derived PBMC. The next day, T cells were purified using pan T cell isolation (Miltenyi) and stained with HLA-DR-FITC (BD). HLA-DR-expressing T cells were single cell sorted and expanded as described above. Recognition of EBV-LCL was blocked with 10µg/ml specific monoclonal antibodies for 30 min at 37^{o}C prior to T cell addition. Whole genome association (WGAs) was performed as described previously[10,14]. Briefly, T-cell recognition of a panel of 80 EBV-LCL was mapped to a SNP genotype database containing 1.1 million SNPs of each cell in the EBV-LCL test panel. The level of matching was calculated using Fisher's exact test using 'Plink' software[15]. For candidate gene *FUCA2* (NM_032020) sequencing, mRNA from patient and donor was isolated from EBV-LCL using Trizol (Invitrogen) and transcribed into cDNA by reverse transcriptase (Invitrogen) using oligo-dT primers (Roche Diagnostics, Almere, The Netherlands). *FUCA2* gene transcripts were amplified by PCR using forward (5'-GAATATTGGGCCCACACTAGA-3') and reverse (5'-CATTTGCTT TCTCCATGTGC-3') primers covering the region of interest. PCR products were analyzed by DNA sequencing, and patient and donor sequences were aligned to detect disparities. For the SNPs that were identified by WGAs and gene sequencing, amino acid sequences spanning the SNP were analyzed using the online algorithm of NetMHC to search for sequences with predicted binding to HLA B*07:02[16]. Candidate peptides were synthesized, dissolved in DMSO, diluted in IMDM and added to donor EBV-LCL (2x10^{4}/well) in 96-well U-bottom plates for 2h at 37^{o}C. T cells (2x10^{4}/well) were added, and after overnight incubation supernatants were tested for IFN-γ production by ELISA.

Microarray gene expression analysis

Lineage specific hematopoietic cells were purified from 3rd party donor PBMC by flowcytometric sorting based on expression of CD19, CD3, and CD14. Purified malignant hematopoietic cells were obtained by flowcytometric sorting from leukemic samples for CD19^{+} cells from 2 different B-ALL patients and for CD33^{+}/CD14^{-} cells from an AML-M4 and an AML-M5 patient. Non-hematopoietic normal cell lines included skin-derived fibroblasts, keratinocytes and proximal tubular epithelial cells cultured with and without IFN-γ (100 IU/ml, 2 days). Non-hematological malignant cells included renal cell carcinoma (RCC 90.03 and RCC 92.11) and melanoma (MEL SK23 and MEL 136.2). Total RNA was isolated using small and micro scale RNAqueous isolation kits (Ambion,

Austin, TX, USA), and amplified using the TotalPrep RNA amplification kit (Ambion). After preparation using the whole-genome gene expression direct hybridization assay (Illumina), complementary RNA samples were dispensed onto Human HT-12 v3 Expression BeadChips (Illumina). Hybridization was performed for 17h at 58°C and mean fluorescence intensities (MFI) were quantified using a BeadArray 500GX device. Microarray gene expression data were analysed after quantile normalization in R 2.15 (R Project).

Results

Clinical course

A 51 year old female patient with progressive metastatic clear cell RCC and multiple lung metastases was treated with non-myeloablative alloSCT. Prior to stem cell transplantation, the patient received a conditioning regimen consisting of Fludarabine (6x30 mg/m^2), Busulphan (2x3.2 mg/kg), Cyclophosphamide (2x750 mg/m^2) and horse anti-thymocyte globulin (Lymphoglobulin, 4x10 mg/kg). T cells were depleted from the peripheral blood stem cell graft derived from her HLA-identical brother by incubation with 20 mg of Alemtuzumab 'in the bag'[17]. Engraftment was obtained and XY-FISH analysis of PBMC showed full donor chimerism one month after alloSCT. However, incomplete donor chimerism (84%, 80%, 95% and 93%) was detected after 2, 3, 5 and 7 months, respectively (Figure 1). GvHD did not occur after alloSCT, and no change in the tumor status was observed. Seven months after alloSCT postponed DLI was

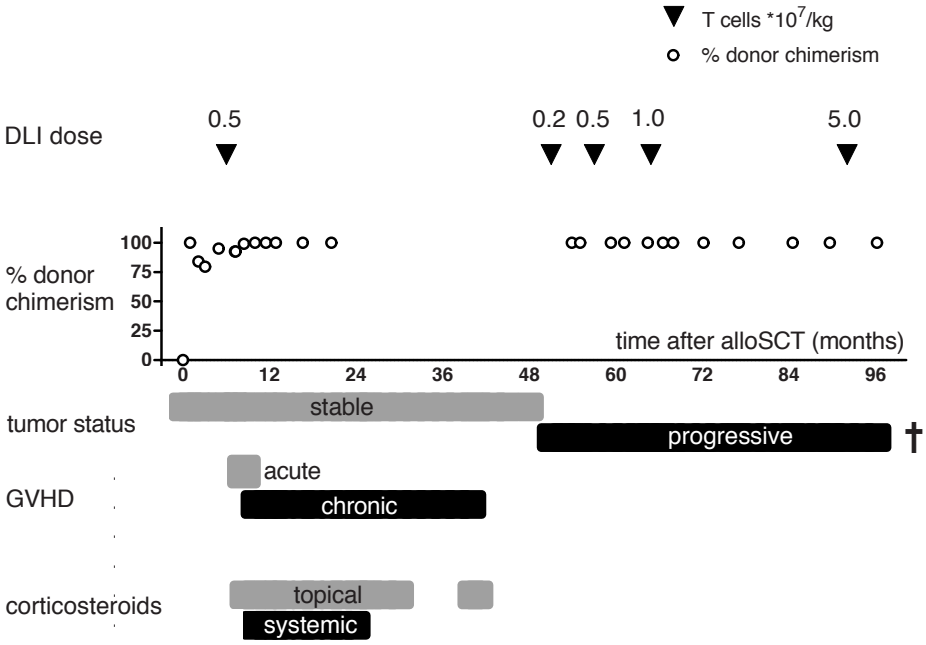

Figure 1. Clinical course. DLI doses, donor chimerism and the clinical course following DLI are depicted during time after allo-SCT (months, x-axis). The infused dose of T cells (filled triangles) and chimerism status (% of donor cells as measured by XY-FISH in PBMC, open circles) are shown in the upper part of the graph. Rectangles in the lower part of the graph indicate tumor status, GvHD state and GvHD treatment.

administered at a single dose of $5x10^6$ T cells/kg, resulting in conversion to full donor chimerism, which persisted during the following years. Severe acute skin GvHD occurred 30 days after DLI and developed into persistent extensive chronic skin GvHD in the following years. Skin GvHD gradually resolved after prolonged topical and systemic treatment with corticosteroids (Figure 1). GvHD was accompanied with 50% reduction in size of the measurable lung metastasis and stable disease (according to RECIST criteria) for 2 years. Nearly 2 years after alloSCT a new lesion developed in the remaining kidney. The gradual resolution of chronic GvHD was accompanied by diminished GvT reactivity and growth of lung metastases 4 years after alloSCT. In an attempt to re-initiate GvT reactivity, escalating DLI doses of $2x10^6$, $5x10^6$, $1x10^7$ and $5x10^7$ T cells/kg were given at 51, 57, 64 and 92 months after alloSCT, respectively. No GvHD developed but also no GvT reactivity could be achieved and the patient died of progressive disease 8.5 years after alloSCT.

Detection of T cells specific for known MiHA

To characterize the specificity of the immune response, we first measured SNP encoding known MiHA to detect disparities between patient and donor. Given the HLA-type of the patient, 13 MiHA were selected and analyzed by SNP genotyping assays (See also Supplemental Table S1). The only MiHA expressed in the patient but absent in the donor, and therefore potentially allowing a donor-derived T-cell response targeting patient cells, was the previously identified MiHA LRH-1, encoded by a single nucleotide deletion in the P2RX5 gene causing a frame shift. Using LRH-1 tetramers, T cells specific for LRH-1 were detected in peripheral blood at the onset of GvHD at a frequency of 0.14% of CD8 T cells (data not shown). Single LRH-1 tetramer positive T cells were subsequently isolated using flowcytometry, expanded, and tested for recognition of various normal and malignant cells (data included in Figure 3A). Patient derived EBV-LCL strongly stimulated LRH-1 specific T cells, as measured by the production of IFN-γ. Recognition of patient-derived skin fibroblasts was very weak and could only be observed after pretreatment with IFN-γ. Dendritic cells (DC) and keratinocytes were not recognized. No recognition of LRH-1 positive RCC cell lines, tested either directly or after pre-incubation with IFN-γ, was observed, indicating that additional T-cell responses targeting other MiHA than LRH-1 must have been involved in the immune response.

Isolation of T-cell clones recognizing the novel MiHA LB-FUCA2-1V

To further identify T-cell responses targeting unknown MiHA in this patient, we incubated peripheral blood taken 37 days after the first DLI at the time that GvHD was apparent with pre-transplant PBMC and isolated activated T cells. Clonal expansion of CD8 T cells expressing HLA-DR initially resulted in the generation of 7 MiHA-specific T-cell clones (data not shown). Two T-cell clones could sufficiently be expanded to allow further characterization. One T-cell clone was demonstrated to be restricted to HLA-B*38:01, as determined by using a panel of partly HLA-matched EBV-LCL (data not shown). Another T-cell clone was restricted to HLA-B*07:02 (Figure 2A), allowing characterization of the MiHA by WGAs using our panel of SNP-genotyped HLA-B*07:02 positive EBV-LCL[10]. T cell recognition of this panel separated MiHA^{pos} and MiHA^{neg} EBV-LCL, and association between the recognition pattern and a detailed SNP genotype map of the tested EBV-LCL identified significantly associating SNPs located on chromosome 6 in a genomic region spanning three genes (Figure 2B). The majority of the associating SNPs was located in non-coding regions except for rs3762001 and rs3762002, which both encoded amino acid polymorphisms in the *FUCA2* protein (Figure 2C). Predicted binding of polymorphic peptides in HLA-B*07:02 was only found for rs3762002, which encoded a valine to methionine substitution at position 356 of the FUCA2 protein (NP_114409). DNA sequencing of rs3762002 demonstrated the presence of the valine encoding SNP in the patient, but not in the donor (Figure 2C). Specific recognition of patient type peptide (RLRQ***V***GSWL) at nanomolar concentrations confirmed that SNP rs3762002 encoded the novel MiHA, which was designated LB-FUCA2-1V (Figure 2D). Tetramers were produced, and staining of T cells in a PBMC sample collected at the onset of acute GvHD 35 days after the first DLI, revealed 1.5% of circulating LB-FUCA2-1V specific T cells (Figure 2E). In samples taken shortly thereafter, frequencies of tetramer positive T cells strongly decreased, and became undetectable at 6 months after DLI. In line with the absence of any clinical effect following administration of escalating doses of donor lymphocytes between 4 and 8 years after alloSCT, LB-FUCA2-1V specific T cells remained undetectable. In order to detect low numbers of MiHA-specific T cells, PBMC samples were stimulated with donor-derived monocytes pulsed with LB-FUCA2-1V or LRH-1 peptide and cultured for 7 days prior to tetramer staining. LB-FUCA2-1V specific T cells were expanded to 2.64% and 0.64% of CD8 T cells in samples taken 83 and 128 days after the first DLI, respectively. LRH-1 specific T cells were present at low frequencies, but could

not be expanded by *in vitro* stimulation. Interestingly, LB-FUCA2-1V specific T cells were induced in an aliquot of the 5th DLI, illustrating that a low precursor frequency of LB-FUCA2-1V specific T-cells were present in the donor, but still remained undetectable in patient PBMC taken 90 days after this DLI (See also Figure S2).

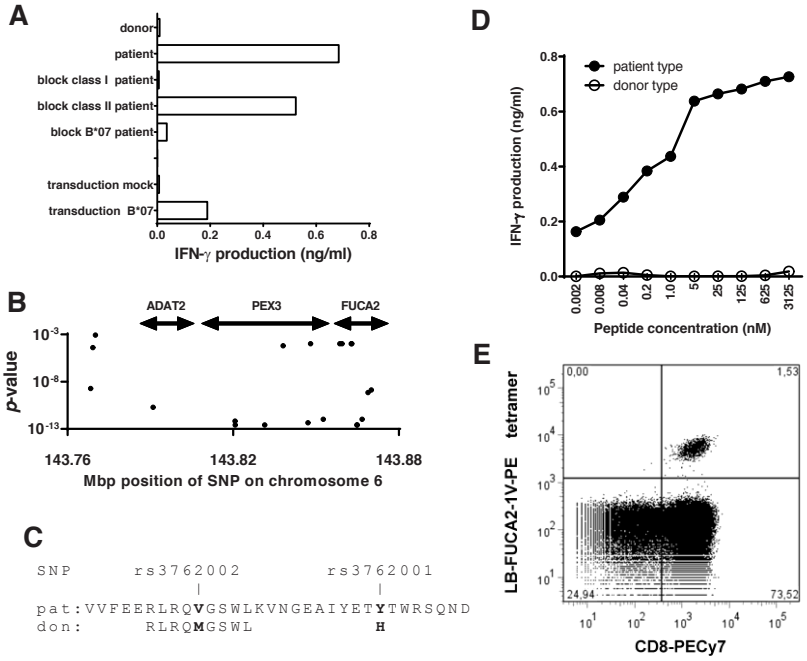

Figure 2. *FUCA2* encodes a novel MiHA presented by HLA-B*07:02. (A) HLA-restricted reactivity of a T-cell clone with unknown specificity was determined by testing recognition of patient EBV-LCL pre-incubated with monoclonal antibodies against HLA class-I, HLA class-II and HLA-B*07 prior to addition of T cells. In addition, mock transduced and pLZRS-NGFR-HLA-B*07:02-transduced third party EBV-LCL were used as test cells. Reactivity was measured by Elisa and is depicted as the concentration of IFN-γ (ng/ml) in the supernatant after 24 h of co-cultivation. (B) WGAs identified a region on chromosome 6 associated with T cell recognition. Each dot represents a SNP relative to its position on chromosome 6 and the significance of association is expressed by *P*-value. Double-headed arrows locate the genes *ADAT2*, *PEX3* and *FUCA2*. (C) The *FUCA2* gene contains 2 associating non-synonymous SNPs. The amino acid sequence containing these SNPs was investigated for potential peptide binding to HLA-B*07:02, resulting in 1 candidate peptide sequence spanning rs3762002. (D) Synthetic peptides containing the patient specific valine residue (closed circles) and donor specific methionine residue (open circles) were loaded on donor EBV-LCL and tested for recognition by the T-cell clone. (E) LB-FUCA2-1V tetramers were used to stain a patient sample collected at the onset of GvHD.

Tissue distribution of LRH-1 and LB-FUCA2-1V

The isolated LB-FUCA2-1V specific T cells were tested for recognition of normal and malignant cells. In contrast to the isolated LRH-1 specific T cells, LB-FUCA2-1V specific T cells broadly recognized tested target cells, including mature monocyte derived DC's (monoDC) (Figure 3A). Furthermore, specific recognition of MiHA^{pos} RCC cell lines was observed, indicating a dominant role for LB-FUCA2-1V specific T cells in tumor control. Recognition of fibroblasts and keratinocytes was measured after pretreatment with IFN-γ, suggesting a role in development of GvHD. Next, we analyzed mRNA expression levels of the *P2RX5* and *FUCA2* genes, encoding the LRH-1 and LB-FUCA2-1V MiHAs, respectively. The analysis confirmed B-cell specific expression of the LRH-1 encoding gene *P2RX5*, in the absence of significant gene expression in other cell types. Substantial expression of the *FUCA2* gene was measured in RCC and in proximal tubular epithelial cells (PTEC), but also in fibroblasts and to a lesser extent in keratinocytes (Figure 3B). In addition, *FUCA2* mRNA was detectable in the majority of hematopoiesis-derived cells, which is in line with broad recognition of these cell types by the LB-FUCA2-1V specific T cells.

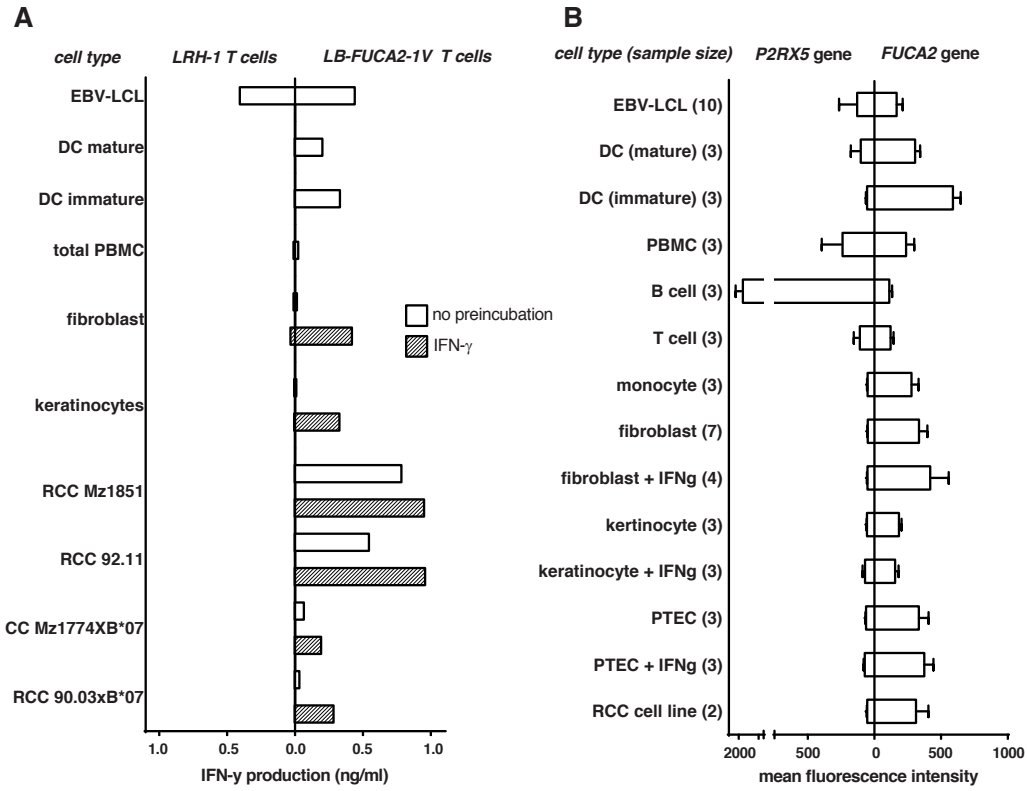

Figure 3. LRH-1 and LB-FUCA2-1V recognition and gene expression of *P2RX5* and *FUCA2*. (A) LB-FUCA2-1V and LRH-1 specific T cells were tested against patient-derived cells (EBV-LCL and fibroblasts) and a panel of 3rd party cells expressing HLA-B*07:02 and the LB-FUCA2-1V and/or LRH-1 MiHA. Cell lines RCC 90.03 and RCC Mz1774 were retrovirally transduced to express B*07:02. Fibroblasts, keratinocytes and RCC cell lines were tested after 24h pre-incubation in the absence (open bars) or presence (hatched bars) of 100 IU/ml of IFN-γ. Reactivity was measured by Elisa and is depicted as the concentration of IFN-γ (ng/ml) in the supernatant after 24 h of co-cultivation. (B) Expression patterns of the MiHA encoding genes *P2RX5* (LRH-1) and *FUCA2* (LB-FUCA2-1V) were determined by quantifying mRNA levels using microarray analysis. Expression, depicted as mean fluorescence intensity (MFI), is shown in hematopoietic cells (PBMC, B cells, T cells, monocytes, immature and mature DC and EBV-LCL), non-hematopoietic cells (fibroblasts, keratinocytes and PTEC pretreated with and without IFN-γ) and RCC cell lines. Numbers between brackets indicate the number of analyzed individual samples.

Identification of genes specifically expressed by RCC

To estimate the likelihood that a GvT reaction targeting RCC can be induced in the absence of GvHD after alloSCT and DLI, we compared gene expression profiles of RCC cell lines representing the GvT target cells, and skin-derived fibroblasts and keratinocytes representing the non-intended GvHD target cells. In addition, since the induction of effective immune responses depends on proper stimulation by professional antigen presenting cells, we also included gene expression profiles of mature monoDC. Genes with a desired expression profile were selected based on significant over-expression in both RCC cell lines and monoDC as compared to fibroblasts and keratinocytes. By setting the cutoff value for over-expression at 10-fold, 17 genes were shown to be over-expressed in RCC and monoDC (Figure 4 and Supplemental Table S3). To compare these data with another non-hematopoietic malignancy, and with leukemic cells that can be targeted in the absence of GvHD, we performed similar comparisons as described above using melanoma cell lines, and ALL or AML samples instead of RCC cell lines. A similar number of genes (28 genes) was over-expressed by both melanoma cell lines and monoDC as compared to fibroblasts and keratinocytes. In contrast, the number of genes selectively over-expressed in AML and monoDC (135 genes) and ALL and monoDC (89 genes) was significantly higher (Figure 4). In conclusion, microarray gene expression analysis demonstrated that the *a priori* chance for beneficial GvT reactivity without GvHD in patients with solid tumors is significantly lower than in patients with hematological malignancies.

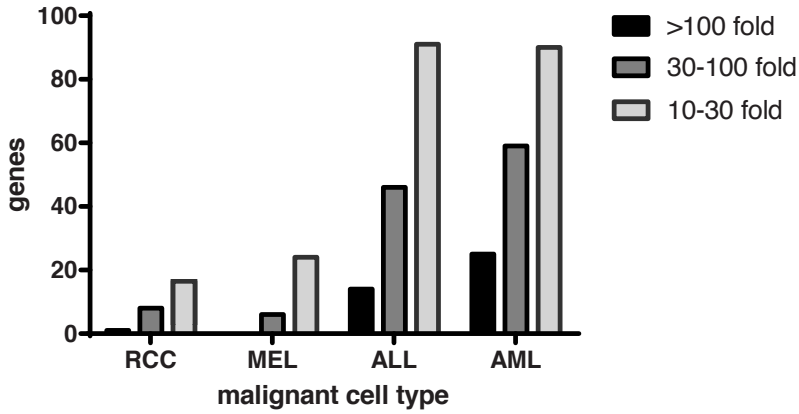

Figure 4. Genes over-expressed in both malignant cells and monoDC as compared to healthy tissue cells. Mature monocyte derived DC (monoDC), RCC cell lines, melanoma (MEL) cell lines, ALL and AML were investigated for gene expression using microarray techniques. For each cell type, 2 different samples were analyzed. Indicated are the numbers of genes showing more than 10, 30 or 100-fold over-expression in both the malignant cells and monoDC as compared to fibroblasts and keratinocytes. Fold over-expression was calculated from the difference in mean log-transformed values, and only genes with a significant difference ($p<0.05$) in mean log-transformed values as measured by a standard Student's t-test were selected.

Discussion

We analyzed T-cell responses elicited after alloSCT and DLI in a patient suffering from progressive clear cell RCC. Tumor regression and stable disease was induced coinciding with severe GvHD requiring long term immune suppression which not only reduced GvHD, but also GvT reactivity. High frequencies of T cells recognizing the novel MiHA LB-FUCA2-1V were identified that strongly recognized various cell types, including RCC cells and normal tissue cells, demonstrating a dominant role for LB-FUCA2-1V specific T cells in GvT reactivity and GvHD. Broad recognition of LB-FUCA2-1V corresponded with a broad mRNA expression pattern of the encoding *FUCA2* gene. Using mRNA expression profiles, only a limited number of genes was found to be selectively expressed by RCC, but not by normal tissue cells explaining why after alloSCT and DLI for treatment of RCC, effective GvT reactivity may unavoidably be associated with GvHD.

Based on SNP genotyping for previously characterized MiHAs, we first investigated LRH-1 as a potential target for a donor-derived GvT reaction. We detected low frequencies LRH-1 specific T cells in patient samples obtained after DLI, which showed selective reactivity to patient-derived EBV-LCL, but not to other cell types. This was in line with the absence of *P2RX5* transcripts encoding the LRH-1 MiHA in the non-recognized cell types. Although it has been reported that various carcinoma and melanoma cell lines may be susceptible to LRH-1 specific lysis[18,19], we could not demonstrate recognition of MiHA^{pos} genotyped RCC cells by the isolated LRH-1 specific T-cell clones. We therefore concluded that LRH-1 specific T cells may have been induced *in vivo* by residual patient-derived B cells at the time of the first DLI, but that the LRH-1 specific T cells were not likely to be mediators of the GvT reactivity or GvHD.

By analyzing *in vivo* activated CD8 T cells, we identified by WGAs high frequencies of HLA-B*07 restricted T cells recognizing a novel MiHA which we designated LB-FUCA2-1V. LB-FUCA2-1V specific T cells strongly recognized a variety of target cells including RCC, DC and normal skin-derived fibroblasts and keratinocytes. The broad recognition pattern, together with high levels of circulating LB-FUCA2-1V specific cells, indicated a dominant role for LB-FUCA2-1V in the GvT reaction, but also in development of severe GvHD. The *FUCA2* gene is located on chromosome 6 and encodes an enzyme called alpha-L-fucosidase that catalyzes hydrolytic cleavage of terminal fucose

residues of glycoproteins[20]. Interestingly, *FUCA2* has been implicated as a factor associated with several neoplastic diseases including endometrial, oral, gastric, and hepatocellular carcinoma[21-24]. In vitro recognition of fibroblasts and keratinocytes by LB-FUCA2-1V specific T cells only occurred after pre-treatment with IFN-γ, whereas no difference in gene expression levels was detected. This illustrates that the immunological threshold for T-cell recognition is not exclusively determined by gene expression, but also by other factors increasing the avidity of the T-cell : target-cell interaction. After the first DLI, residual or repopulating hematopoietic cells and DC of patient origin may have initiated the T-cell response resulting in an inflammatory environment. This may have up-regulated adhesion molecules like CD54 on fibroblasts rendering them susceptible to T-cell induced cell lysis[25,26]. Subsequent destruction of tissue cells amplifies the inflammation potentially resulting in the cascade leading to persistent GvHD. Re-induction of GvT reactivity by administration of escalating doses of DLI 4-8 years after the initial DLI failed. This may be due to impaired antigenic stimulation by tumor cells in the absence of patient derived APC, resulting in T cell tolerance. Furthermore, sustained signalling of T cell co-inhibitory molecules by tumor cells may have induced an exhausted T cell phenotype, resulting in a lost or reduced capacity of the tumor-specific T cells to expand *in vivo*[27].

In hematological malignancies, alloSCT followed by postponed DLI can result in clinical remissions in the absence of GvHD[28]. We therefore explored whether GvT reactivity in solid tumors might be separable from GvHD in a similar way as graft-*versus*-leukemia can be separated from GvHD. In analogy to hematopoiesis restricted expression of MiHA that represent potentially specific targets on leukemic cells, the separation of GvT reactivity from GvHD requires targeting of MiHAs that are overexpressed in RCC as compared to normal counterpart tissue cells. Effort has been made to identify such MiHA that are selectively expressed on malignant solid tumor cells, but only a few MiHA, including C19orf48[29] and ZAPHIR[18], and a tumor associated antigen encoded by human endogenous retrovirus type E (HERV-E)[30] have been identified as targets for allo-reactive T cells in patients with RCC after treatment with alloSCT. Since it has been reported that DC are essential for the development of GvT reactivity after alloSCT, we assumed that specific GvT reactivity will occur only if the targeted genes are expressed by both DC and the malignant cell population, in the absence of expression in GvHD target tissues. Thus, the

likelihood that a specific GvT reaction will occur in patients with solid tumors is determined by the number of genes that is expressed by both DC and the malignant cell population but not by normal non-hematopoietic tissues from the patient. As illustrated in Figure 4, in RCC tumors only 17 genes fulfilled these criteria, whereas in AML and ALL, 133 and 92 genes respectively, were highly expressed by both DC and the malignant cells, but not in non-hematopoietic tissues, and therefore are candidate targets for a specific graft-*versus*-leukemia response. These results indicate that the likelihood of developing a tumor specific allo-immune response after alloSCT is low in patients with solid tumors, whereas in patients with hematological malignancies a significant number of targets may be identified for a leukemia specific allo-immune response. In conclusion, our results indicate that development of GvT reactivity without GvHD in patients with solid tumors is unlikely to occur, and that clinically effective T-cell mediated tumor control after alloSCT and DLI in the treatment of RCC may be unavoidably linked to GvHD.

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Table S1: MiHA disparities between donor and patient

MiHA	HLA restriction	Gene ID	SNP ID	SNP genomic context	Platform	Donor status	Patient status
HwA-9	A*03	*SP110*	rs1365776	CTCTCATCTTACCTC[C/T]TGGGAGGCTTTTTTT	Taqman	pos	pos
HwA-10		*CENPM*	rs5758511	GCAAGTCCCACACTC[A/G]GCCCATGCACTTGCT	Taqman	pos	pos
LB-APOBEC3B-1K		*APOBEC3B*	rs2076109	TCCCAGGTGTATTTC[A/G]AGCCTCAGTACCACG	KASPar	neg	neg
LB-ARHGDIB-1R		*ARHGDIB*	rs4703	CAAGGGCATGCTGGC[C/G]CGAGGCACGTACCAC	KASPar	pos	pos
LB-BCAT2-1R		*BCAT2*	rs11548193	AACAGGAGCGCGCGC[C/G]TGGGCTGGCTGACAC	KASPar	neg	neg
LB-EBI3-1I		*EBI3*	rs4740	CGAGCCAGGTACTAC[A/G]TCCAAGTGGCGGCTC	Taqman	pos	pos
LB-ECGF-1R	B*07	*TYMP*	rs112723255	GTACTCTCCGACCGC[A/G]CGCCATTCGCCGCCC	KASPar	neg	neg
LB-ERAP1-1R		*ERAP1*	rs26653	GCAATTTGCTCCTGA[C/G]GGGGGTGTTCCAGGA	Taqman	pos	pos
LB-GEMIN4-1V		*GEMIN4*	rs4968104	GAATTGGGACCCTGC[A/T]CTTCCACAAACCTAA	Taqman	pos	neg
LB-PDCD11-1F		*PDCD11*	rs2986014	GATTCCTCCAAGACC[C/T]TCTTATGTCTGTCCC	Taqman	pos	neg
LRH-1		*P2RX5*	rs3215407	GTTCTGCCGCTGGTT[/G]GGGGTCACAATCAGG	KASPar	neg	pos
LB-MTHFD1-1Q	DRB1*03	*MTHFD1*	rs2236225	CCATCATTGCAGACC[A/G]GATCGCACTCAAGCT	Taqman	pos	pos
LB-MR1-1H	DRB3*02	*MR1*	rs2236410	AATTCAGGGACCCCA[C/T]GGATGGGATCCGAAA	KASPar	neg	neg

The MiHA status of donor and patient was determined by performing DNA SNP genotyping using KASPar and Taqman assays.

Table S2. Genes over-expressed by RCC and monoDC as compared to fibroblasts and keratinocytes

Gene ID	mRNA transcript ID	RCC 92.11	RCC 90.03	monoDC1	monoDC2	FB1	FB2	KC1	KC2
SPP1	NM_000582.2	10535	14291	2728	4355	121	55	54	118
	NM_001040058.1	12810	12568	1140	6482	128	50	50	101
BIRC3	NM_001165.3	487	1020	14037	16088	60	77	64	85
	NM_182962.1	442	936	9879	18151	232	221	194	366
SRGN	NM_002727.2	14629	110	27015	19122	179	46	111	255
	NM_002727.2	2922	56	12753	5399	64	52	54	142
DOCK2	NM_004946.1	299	1793	1502	2334	126	60	47	69
RARRES3	NM_004585.3	1095	774	16580	1603	157	179	109	152
CD70	NM_001252.3	1285	3336	641	165	49	54	90	52
HRASLS3	NM_007069.2	1584	845	9180	2489	576	491	59	50
MARCKSL1	NM_023009.4	582	587	14545	13561	167	148	182	419
UCP2	NM_003355.2	47	700	2464	3850	51	55	46	71
BATF3	NM_018664.1	281	406	3473	1013	62	63	60	54
ITGAX	NM_000887.3	124	86	3260	2438	40	44	39	50
SLC15A3	NM_016582.1	564	809	13141	21098	506	444	126	239
TBXAS1	NM_001061.2	167	623	762	1017	47	43	44	49
C15orf48	NM_032413.2	264	121	4197	1224	60	56	46	56
KYNU	NM_003937.2	378	431	1682	2196	44	45	70	314
	NM_001032998.1	260	702	1277	1434	49	49	66	193
ALDH1A1	NM_000689.3	55	2449	630	1164	56	59	48	55
SEMA4D	NM_006378.2	85	378	1829	1297	56	58	45	47

Gene expression levels were measured on beadchip arrays and are expressed as mean fluorescence intensity. By using a cut-off value of 10-fold, genes overexpressed by both RCC and monoDC as compared to fibroblasts (FB1, FB2) and keratinocytes (KC1 KC2) were selected.

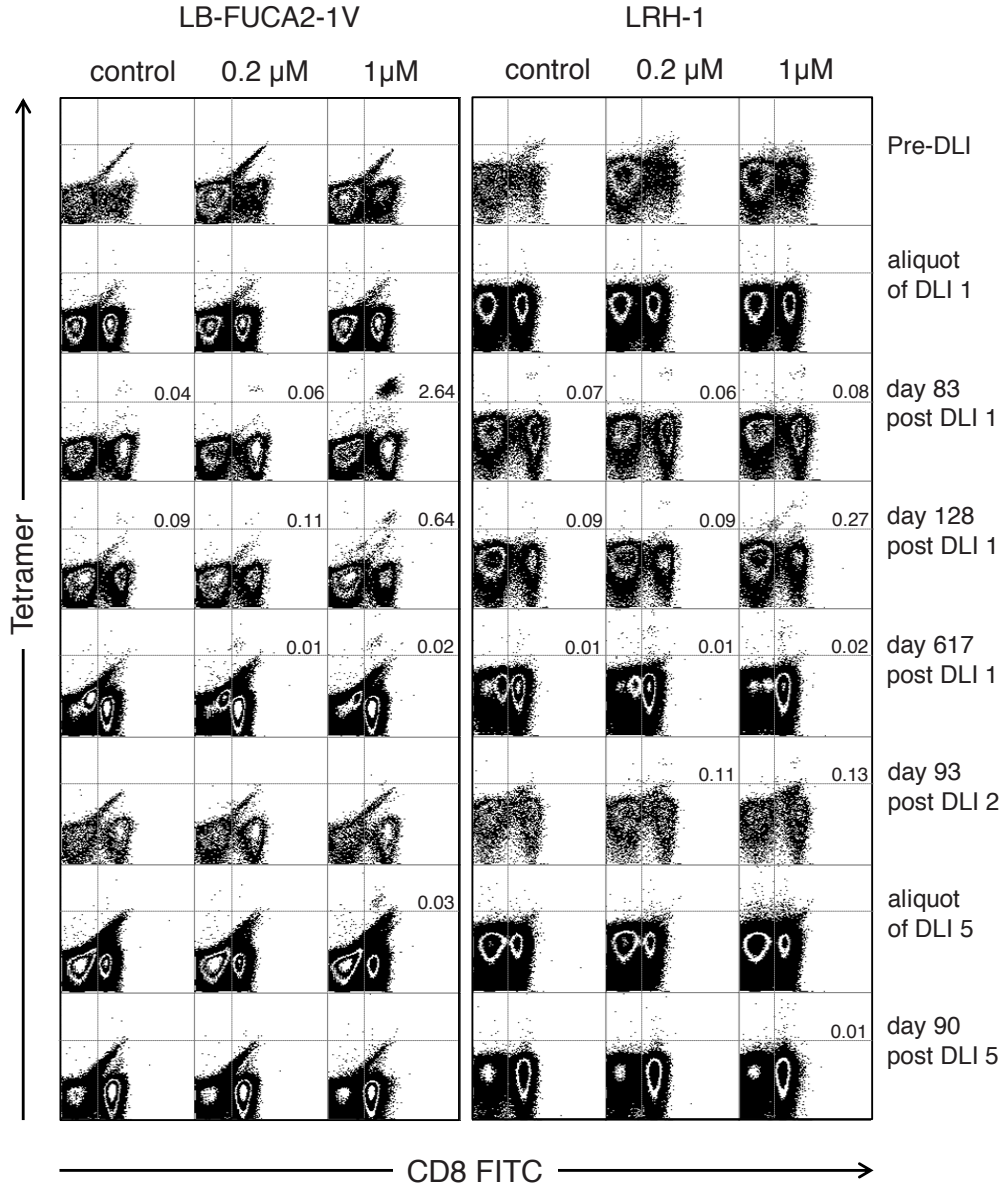

Figure S2. Frequency of circulating MiHA-specific T cells after *in-vitro* peptide stimulation. Donor derived monocytes were isolated using CD14 microbeads and pulsed overnight with 0.2 µM or 1 µM of LB-FUCA2-1V or LRH-1 peptide or medium alone. Aliquots of donor lymphocytes used for DLI and samples of patient PBMC taken before and after DLI were co-cultured with peptide-pulsed monocytes in the presence of 30 IU/mL IL-2. After 7 days, MiHA-specific T cells were analyzed using CD8-FITC and LB-FUCA2-1V-PE (left panels) and LRH-1-APC (right panels) tetramers, respectively. In samples containing discrete populations of tetramer^{pos} cells, the percentage of tetramer^{pos} events calculated as a percentage of CD8 T cells is depicted.

Chapter 6

T-cell response magnitude, diversity and specificity separate graft-*versus*-leukemia reactivity from graft-*versus*-host disease after allogeneic stem cell transplantation and donor lymphocyte infusion.

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Abstract

Patients with leukemia who receive a T-cell depleted allogeneic stem cell graft followed by postponed donor lymphocyte infusion (DLI) can experience graft-*versus*-leukemia (GvL) reactivity with a lower risk of graft-*versus*-host disease (GvHD). We investigated magnitude, diversity and specificity of alloreactive CD8 T-cells in patients without or with GvHD after DLI. We found both higher magnitudes and diversities of minor histocompatibility antigen (MiHA) specific CD8 T cells in patients with GvHD. Whereas strength of MiHA recognition on hematopoietic cells was similar, non-hematopoietic cells were stronger recognized by T-cell clones from patients with GvHD, especially after activation by inflammatory cytokines. Investigation of MiHA-encoding genes showed that similar types of antigens were recognized, however, relative skewing towards targeting of broadly expressed MiHA in patients with GvHD was observed. An inflammatory environment can render tissue cells susceptible to T-cell recognition, thereby amplifying the alloreactive response resulting in GvHD.

Introduction

The curative effect of allogeneic stem cell transplantation (alloSCT) as treatment modality for hematological malignancies is based on the capacity of donor T cells to elicit an effective immune response against malignant hematopoietic cells from the patient[1-3]. Alloreactive donor-derived T cells may however also target normal non-hematopoietic tissue cells, resulting in potentially life-threatening graft-*versus*-host disease (GvHD)[4]. T-cell depletion of the graft can prevent GvHD but also impairs development of the associated graft-*versus*-leukemia (GvL) effect[5,6]. Persistent or recurring malignant hematopoietic cells after alloSCT will then require elimination by subsequent donor lymphocyte infusion (DLI)[7-11]. The observation that T-cell depletion followed by postponed DLI reduces development of GvHD after alloSCT can be explained by the timing of the DLI, when pre-alloSCT chemotherapy induced tissue damage and infections have largely been resolved, the 'cytokine storm' has subsided, and immune response provoking patient-derived antigen presenting cell (APC) are gradually being replaced by donor APC[3,12]. Although T-cell depletion of the graft followed by DLI reduces incidence and severity of GvHD, this complication remains a serious risk for morbidity and mortality[13,14].

Alloreactive T cells recognize non-self antigens on patient cells encoded by patient-specific genomic polymorphisms[15]. In fully HLA-matched alloSCT, target antigens are minor histocompatibility antigens (MiHA), which are polymorphic peptides presented in self-HLA encoded by single nucleotide polymorphisms (SNPs)[16,17]. Genomic disparities between donor and patient therefore determine the potential antigen repertoire that can be targeted by donor T cells. The tissue distribution of HLA class I restricted MiHA is a relevant factor that determines the clinical effect of donor CD8 T cells after alloSCT. Donor T cells recognizing MiHA with restricted expression on hematopoietic cells including the malignant cells of the patient are expected to induce the selective GvL effect. Donor T cells recognizing MiHA with ubiquitous expression on both hematopoietic and non-hematopoietic tissues are more likely to mediate both the GvL effect and GvHD[18].

In the last decade, increasing numbers of MiHA have been characterized mainly due to application of whole genome association scanning (WGAs). We identified multiple MiHA by WGAs, and demonstrated in several patients that at least 3-8 different HLA class I restricted MiHA were targeted by donor CD8 T

cells after HLA-matched alloSCT and DLI[19-21]. Whereas severe GvHD frequently coincided with development of T-cell responses against ubiquitously expressed MiHA, relatively selective GvL reactivity was not always associated with T-cell responses recognizing MiHA selectively expressed by hematopoietic cells. Apparently, other factors also determine the balance between GvL reactivity and GvHD. We hypothesized that in addition to tissue specificity, magnitude and diversity of the immune response will influence this balance. Moreover, the effects of immune responses are subject to environmental factors, such as inflammatory cytokines and chemokines, which are influenced by infections, tissue damage and exogenous immune regulatory drugs. Inflammatory cytokines may up-regulate surface expression of HLA, costimulatory and adhesion molecules and modify antigen processing and presentation resulting in differential clinical effects of donor T cells capable of recognizing MiHA on various tissues from the patient[22].

In this study, we characterized frequency, diversity and specificity of CD8 T-cell responses in 11 patients who entered complete remission and/or full donor chimerism after DLI. Our data show that both frequencies and diversity of MiHA-specific CD8 T cells were higher in patients who developed GvHD as compared to patients without GvHD. In patients without GvHD, immune responses were skewed towards T cells that were unable to recognize patient non-hematopoietic fibroblasts. We identified 19 MiHA, including 13 novel MiHA and 6 previously published MiHA and illustrate that tissue distribution of MiHA-encoding genes is not the main determinant separating GvL reactivity from GvHD, but that also inflammatory environmental circumstances can contribute to overcoming the threshold for GvHD development.

Materials and methods

Blood samples and skin biopsies

Peripheral blood samples and skin biopsies were collected from patients, donors, and third party individuals after approval by the Leiden University Medical Center institutional review board according to the Declaration of Helsinki. Written informed consent was given by all patients, donors and by third party individuals to investigate materials and to publish data and case details.

Isolation and expansion of CD8 T-cell clones

PBMC samples were thawed, washed and CD4 T cells and monocytes were depleted using magnetic CD4 and CD14 microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8 T-cell enriched samples were stained using fluorescein isothyocyanat (FITC) conjugated CD8 (BD Biosciences, Breda, The Netherlands) and phycoerythrin (PE) conjugated anti HLA-DR (BD). Single cells were sorted using a BD FACS Aria device with BD FACSDiva software and collected in 96-well round-bottom TC-plates (Corning, Amsterdam, The Netherlands) pre-filled with 100 µl T-cell culture medium (TCM) consisting of Iscove's modified Dulbecco's medium (Lonza, Verviers, Belgium) with 5% pooled human serum (Sanquin, Amsterdam, The Netherlands) 5% FBS (Lonza), 120 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands), 2 ng/ml IL-7 and 2 ng/ml IL-15 (Myltenyi). T cells were stimulated with phytohemagglutinin (0.8 µg/ml, Murex Biotec Limited, Dartford, UK) and $5x10^4$ irradiated (50Gy) allogeneic PBMC. Growing T-cell clones were transferred to 24-well TC-plates and remaining negative wells were restimulated under identical conditions. Alternatively, CD8 T-cell clones were expanded after bulk-sorting of $HLA\text{-}DR^{pos}$ CD8 T cells followed by manual limiting dilution in 384-well TC-plates prefilled with 30 µl TCM with 0.8 µg/ml PHA and $2.5x10^4$ irradiated allogeneic PBMC per well. At day 7, T cells were restimulated with 0.8 µg/ml PHA and $5x10^4$ irradiated allogeneic PBMC in 50 µl TCM. From day 10 onward, growing T-cell clones were collected in flat-bottom 96-well TC-plates. Expansion of selected T-cell clones by restimulation was repeated every 14 days in TCM at $2x10^5$ T cells per ml with 0.8 µg/ml PHA and $1x10^6$ allogeneic feeder cells per ml. TCM was added twice weekly.

Generation and culture of test cells

EBV-transformed B lymphoblastic cell lines (EBV-LCL) were generated from PBMC from patient, donor and third party individuals and cultured in IMDM with 10% FBS (Lonza)[23]. Monocyte derived dendritic cells (monoDC) were generated from PBMC after CD14 enrichment using microbeads (Miltenyi) by culturing for 2 days in IMDM with 10% FBS, 100 ng/ml GM-CSF (Novartis, Basel, Switzerland) and 500 IU/ml IL-4 (Schering-Plough, Bloomfield, NJ) and maturation for 2 days with 10 ng/ml TNF-α (R&D Systems, Abingdon, UK), 10 ng/ml IL-1β (Immunex, Seattle, WA), 10 ng/ml IL-6 (Cellgenix, Freiburg, Germany), 1 μg/ml PGE2 (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 500 IU/ml IFN-γ (Boehringer Ingelheim, Ingelheim am Rhein, Germany). CD40L B cells were generated from PBMNC after CD19 enrichment using microbeads (Miltenyi) and stimulated with irradiated (70 Gy) murine fibroblasts expressing CD40L in IMDM with 10% FBS and 100 IU/ml IL-4 (Schering-Plough). Expanding CD40L B-cell cultures were restimulated every 2 weeks[24]. Fibroblast cell lines (FB) were obtained by seeding mechanically dissociated skin biopsies in low-glucose Dulbecco's modified Eagle's medium (DMEM, Lonza) with 10% FBS. Confluent FB cultures were trypsinized, diluted and re-seeded. From patient 4716 no skin biopsy could be obtained, but instead a bone marrow-derived stromal cell line was generated. For stimulation experiments, confluently grown FB cultures were maintained in culture medium alone (steady-state FB), or were stimulated with 200 IU/ml IFN-γ for 4 days (activated FB). Prior to testing, FB were harvested by trypsinization and vigorously washed 3 times to remove IFN-γ.

Testing of T-cell reactivity

For initial screening of growing T-cell clones, resuspended culture aliquots were taken, washed in IMDM and diluted in 0.2 ml IMDM with 5% HS, 5% FBS and 10 IU/ml IL-2. Two thousand T cells were dispended in 20 μl in 384-well TC-plates. EBV-LCL and CD40L B cells ($2x10^4$), monoDC ($5x10^3$) and FB ($7.5 x10^3$) were added in a volume of 20μl. For HLA blocking experiments, frequency analysis and WGAs, cultures of T-cell clones were washed, diluted to $2x10^3$ T-cells per 20 μl and added to $7.5 x10^3$ EBV-LCL in 20 μl. After overnight incubation, IFN-γ production was measured in 10 μl aliquots by ELISA according to the manufacturer's instructions, adapted to 384-well format (Sanquin). Recognition was defined as more than 3 times IFN-γ production in the absence of stimulator cells. For measuring tissue specificity, stimulator:T-cell ratios ranging from 9 to 0.1 were obtained by coculturing $7.5 x10^3$, $2.5 x10^3$,

8.3 x10^2 and 2.8 x10^2 EBV-LCL or FB with 8.3x10^3 or 2.5x10^2 T cells. ELISA was performed on undiluted, 3-fold and 10-fold diluted supernatants and the amount of IFN-γ produced per single T cell was calculated.

Tetramer staining and TCRvβ identification

HY-antigen specific tetramers were constructed by folding peptides into biotinylated HLA monomers and multimerization using streptavidin conjugated to PE as previously described[25]. T-cell receptor-β variable chain usage was determined using IO Test (Beckmann Coulter). Data were acquired on a FACS Calibur device (BD).

HLA restriction and MiHA frequency analysis

HLA restriction of MiHA-specific T-cell clones was determined by blocking of recognition of patient EBV-LCL that were pre-incubated for 30 minutes with antibodies specific for HLA-class I (W6.32), HLA-BC (B1.23.2), Bw4/A*24 (MUS4H4), Bw6 (KAM3H9), A*02 (BB7.2), B*13 (JOK3H5) or B*35 (IND2D12). In addition, EBV-LCL were transduced with potential HLA restriction molecules using a retroviral LZRS vector, containing the truncated nerve growth factor receptor (ΔNGFR) marker[26,27]. Retroviral supernatants were generated of HLA-A*11:01, A*24:02, B*13:02, B*35:01, B*44:02, B*44:03, B*51:01, C*05:01, C*14:02 and C*16:01. Non-treated 96-well plates (Greiner) were coated with Retronectin CH-296 (Clontech) and blocked with 2% human albumin (Sanquin). Viral supernatants were diluted 3 times in IMDM with 10% FBS and aliquots of 50µl per well were spun at 3000G for 30'. Supernatants were discarded and 5x10^4 EBV-LCL were transferred to each well. EBV-LCLs showed a retroviral transduction efficiency ranging from 20% to 70% based on staining with anti ΔNGFR-PE (BD).

MiHA identification

Whole genome association scanning (WGAs) was performed as previously described[20]. Briefly, a panel of 80 EBV-LCL was genotyped for 1.1 million SNPs using micro-arrays. T-cell recognition of the panel was measured and recognition patterns were compared to the SNP genotype data. The level of matching between the patterns of recognition and SNP genotypes was calculated by Fisher's exact test using 'Plink' software[28]. MiHA-encoding genes were amplified and Sanger sequenced from both patient- and donor-derived EBV-LCL using cDNA that was obtained by reverse transcription of Trizol-isolated (Invitrogen) RNA with oligo-dT primers (Roche Diagnostics, Almere,

The Netherlands). See also Supplemental experimental procedures for gene specific primer sequences. Sequences were searched for donor-to-patient disparities using alignment software (Geneious, Auckland, New Zealand). Protein sequences spanning SNP-encoded amino acid polymorphisms were analyzed by NetMHC (Buus et al., 2003) to identify peptides with predicted HLA binding. Candidate peptides were synthesized, dissolved in DMSO, diluted in IMDM and incubated with $2x10^4$ donor EBV-LCL cells in 20 µl in 384-well TC-plates for 2 hours at 37°C. Thereafter, $2x10^3$ T cells were added to each well in 20 µl, and after overnight incubation, supernatants were tested for IFN-γ production by ELISA.

Gene expression profiles

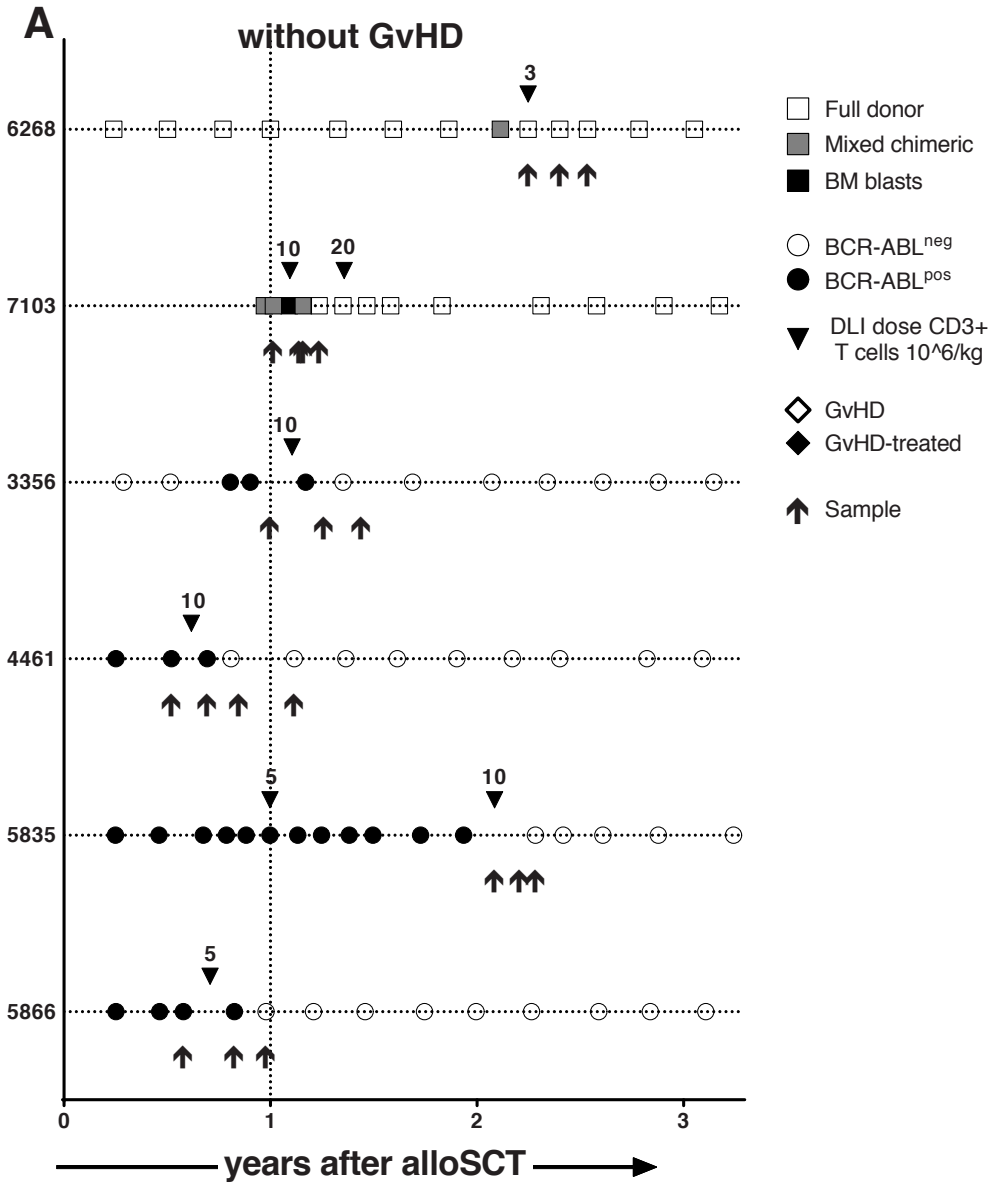
Total RNA was isolated from EBV-LCL and FB using RNAqueous (Ambion, Austin, TX, USA), and amplified using the TotalPrep RNA (Ambion). After preparation using the whole-genome gene expression direct hybridization assay (Illumina), samples were dispensed onto Human HT-12 v3 Expression BeadChips (Illumina) and hybridized for 17 h at 58°C. Mean fluorescence intensities (MFI) were quantified using a BeadArray 500GX device. Raw data were analysed after quantile normalization in R 2.15[29].

Results

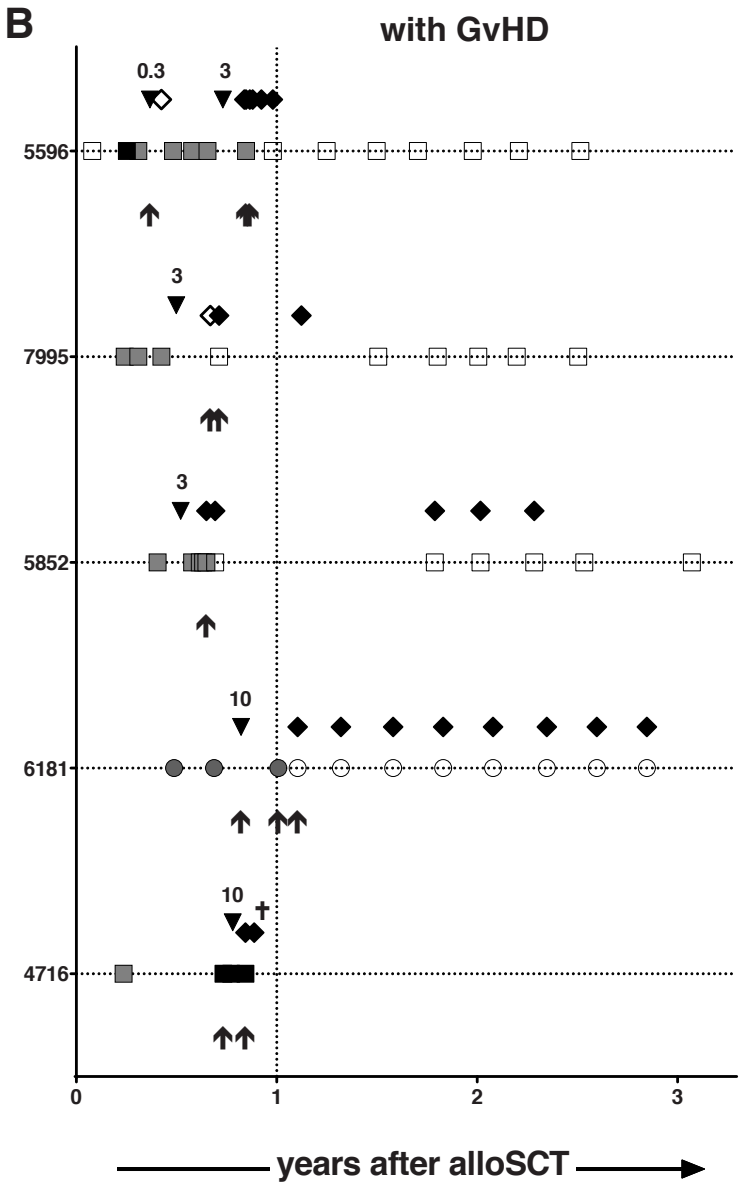
Patient selection

To compare CD8 T-cell responses in GvL reactivity and GvHD after DLI, 11 patients were selected who were treated for chronic myeloid leukemia in chronic phase (CML-CP), acute myeloid leukemia (AML) or high risk myelodysplastic syndrome (MDS) between 2000 and 2010 with a T-cell depleted stem cell graft from a fully HLA-matched sibling or unrelated donor. Pre-transplant conditioning was either myeloablative (cyclophosphamide and total body irradiation) or nonmyeloablative (fludarabin, busulphan and *in vivo* T-cell depletion using Alemtuzumab combined with horse ATG in case of an unrelated donor). Peripheral blood stem cells were the main stem cell source. After engraftment and development of no or limited grade I GvHD, patients with mixed chimerism or with residual or relapsed disease were treated with escalating doses of donor lymphocytes starting at 6 months with $3*10^6$ CD3+ T cells/kg until a response was observed. Patients with unrelated donors received half this dose. With the exception of patient 7103, no cytoreductive treatment was administered prior to DLI (See also Table S1). Samples after DLI were taken at routine follow-up time points or when GvHD was observed. GvL reactivity was defined by conversion to full donor chimerism or disappearance of leukemic cells indicating molecular remission. Of the 11 patients, 10 patients were evaluable for GvL reactivity and 6 patients remained free of GvHD (Figure 1A). Five patients developed GvHD and received systemic immunosuppression (Figure 1B, see also Table S1). Four of 6 patients without GvHD were treated with DLI in the second year after alloSCT whereas all 5 patients with GvHD received DLI in the first year after alloSCT (p=0.06, Fisher's exact test). One patient died 74 days after DLI due to complications related to grade IV GvHD and could not be evaluated for GvL reactivity. All other 10 patients are alive between 5 and 15 years post alloSCT.

Figure 1. Clinical course of selected patients. Interventions and follow-up are depicted per patient on horizontally dotted lines. Regular monitoring post-alloSCT included donor chimerism (squares) or BCR-ABL (circles) by PCR and the presence of blasts in bone marrow aspirates by morphology. Open symbols indicate full donor chimerism or absence of disease. Filled squares indicate disease recurrence as detected by bone marrow blasts (black squares), detectable BCR-ABL transcripts (black circles) or mixed chimerism (grey squares).



DLI was performed (filled triangles, dose given in 10^6 CD3+ T cells/kg) and patients remained free of GvHD (n=6, A) or were diagnosed with GvHD (n=5, open diamonds, B) requiring systemic immunosuppressive treatment (filled diamonds). Arrows indicate collected samples. A vertical dotted line is plotted at 1 year post-DLI. Patient 4716 died 74 days after DLI. For detailed patient characteristics, see also Table S1.



Selection of alloreactive T cells

We hypothesized that activated CD8 T cells in post-DLI immune responses express HLA-DR. We analyzed frequencies of circulating HLA-DR^{pos} CD8 T cells in samples taken before and after DLI by flowcytometry (Figure 2). A significant increase in HLA-DR^{pos} CD8 T cells was observed from 10.3% ± 5.3 % (mean ± SD) in pre-DLI samples to 33.2% ± 19.1% (p=0.002, Wilcoxon matched-paired signed rank test) in post-DLI samples, which was irrespective of the presence of GvHD. To investigate in which compartment alloreactive T cells resided, we separated HLA-DR^{pos} and HLA-DR^{neg} T cells from selected post-DLI samples of patients 4461, 5866 and 6181. T-cell clones were generated from sorted single cells and were tested for alloreactivity as defined by recognition of patient, but not donor EBV-LCL. Of 261 T-cell clones obtained from sorted HLA-DR^{pos} CD8 T cells, 41 T-cell clones were alloreactive, whereas only one alloreactive T-cell clone was identified in 268 T-cell clones from HLA-DR^{neg} cells. In contrast, levels of EBV-LCL reactive T-cell clones, as defined by recognition of both patient and donor EBV-LCL, were similar between HLA-DR^{pos} and HLA-DR^{neg} compartments (data not shown). Tetramer staining of EBV-LCL reactive T-cell clones from patient 5866 revealed that 56% of EBV-LCL reactive T-cell clones recognized validated EBV epitopes derived from viral proteins BRLF1, BMFL1 EBNA3B or BZLF1 (data not shown). In conclusion, our results confirmed that alloreactive T cells during clinical immune responses after DLI specifically reside in the HLA-DR^{pos} CD8 T-cell compartment.

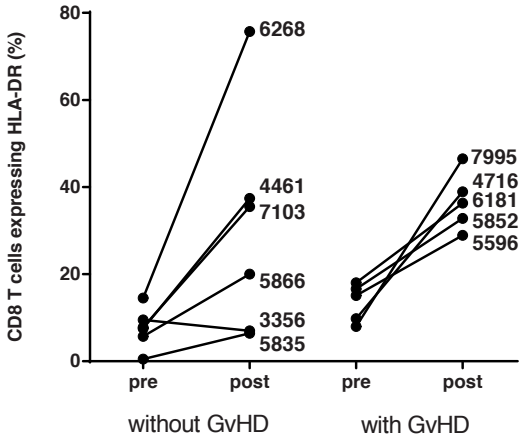
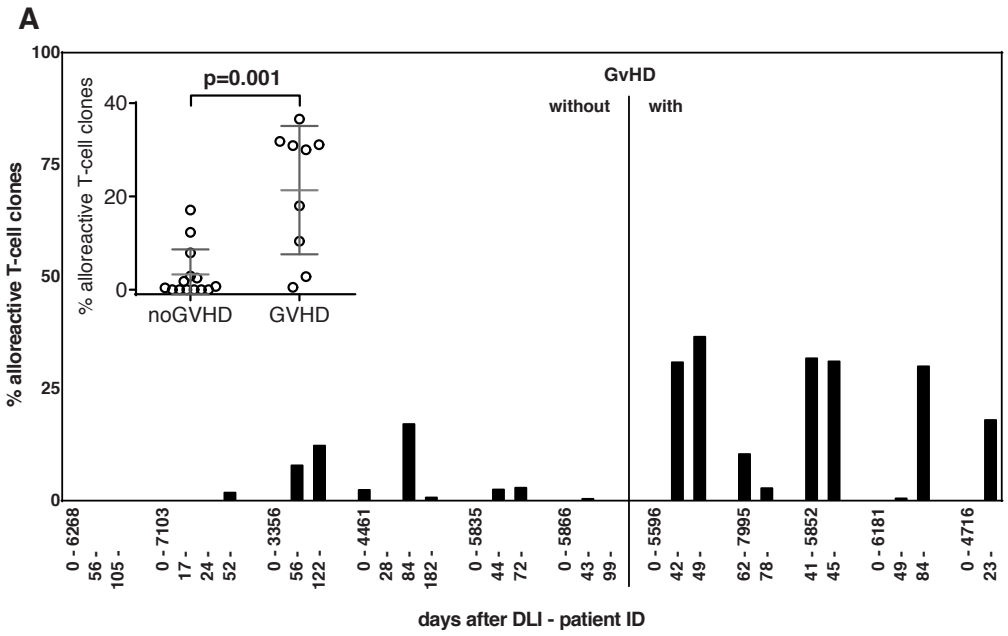


Figure 2. HLA-DR expression of CD8 T cells. In samples taken pre- and post-DLI, HLA-DR^{pos} CD8 T cells were detected by flowcytometry. Percentages of CD8 T cells that expressed HLA-DR^{pos} within the total CD8 T-cell population are depicted. Lines connect pre- and post-DLI samples for each patient (patient ID's are depicted in the graph).

Frequencies of the alloreactive T-cell response

To compare magnitudes of alloreactive responses between patients without and with GvHD, we selected 20 samples from patients without GvHD and 12 samples from patients with GvHD. HLA-DR^{pos} CD8 T cells were single-cell sorted and T-cell clones were generated. T-cell clone reactivity was tested using patient and donor EBV-LCL. In addition, if sufficient patient pre-alloSCT cells were available, monocyte-derived dendritic cells (monoDC) and B cells that were expanded using CD40L expressing mouse fibroblasts (CD40L B cells) were tested (See also Table S2). Alloreactive T-cell clones were defined by selective recognition of patient cells in the absence of recognition of donor cells. Per sample, alloreactive T-cell clones are expressed as a percentage of total growing T-cell clones (Figure 3A). The median percentage of alloreactive T-cell clones in post-DLI samples was significantly lower in patients without GvHD (0.6% range 0%-17.1%) as compared to patients with GvHD (30% range 0.5%-36.6%, p=0.001 Mann-Whitney test). In addition to alloreactive T-cell clones, T-cell clones were detected that recognized EBV-LCL of both patient and donor. These T-cell clones do therefore not target allo-antigens but recognize antigens that are expressed by B cells after EBV transformation. In 4 of 6 patients without GvHD and 3 of 5 patients with GvHD, more than 50% of the T-cell clones were EBV-LCL reactive, illustrating that patients in both groups were equally capable to elicit anti-viral immune responses and that the low frequencies of alloreactive T cells in patients without GvHD cannot be explained by general suppression of the immune system (Figure 3B). To exclude the possibility that we missed alloreactive T-cell clones during screening by testing solely with hematopoietic cells, we tested all growing T-cell clones from 3 patients with GvHD against fibroblasts (See also Table S2). To obtain maximal recognition, FB were activated for 4 days in the presence of IFN-γ (200 IU/ml) prior to testing. No T-cell clones that recognized FB in the absence of recognition of EBV-LCL were identified (data not shown). In conclusion, our data show that patients with GvHD after DLI have higher frequencies of circulating alloreactive CD8 T cells than patients without GvHD.


A
p=0.001
% alloreactive T-cell clones
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0 - 6268
56 - 105 -
0 - 7103
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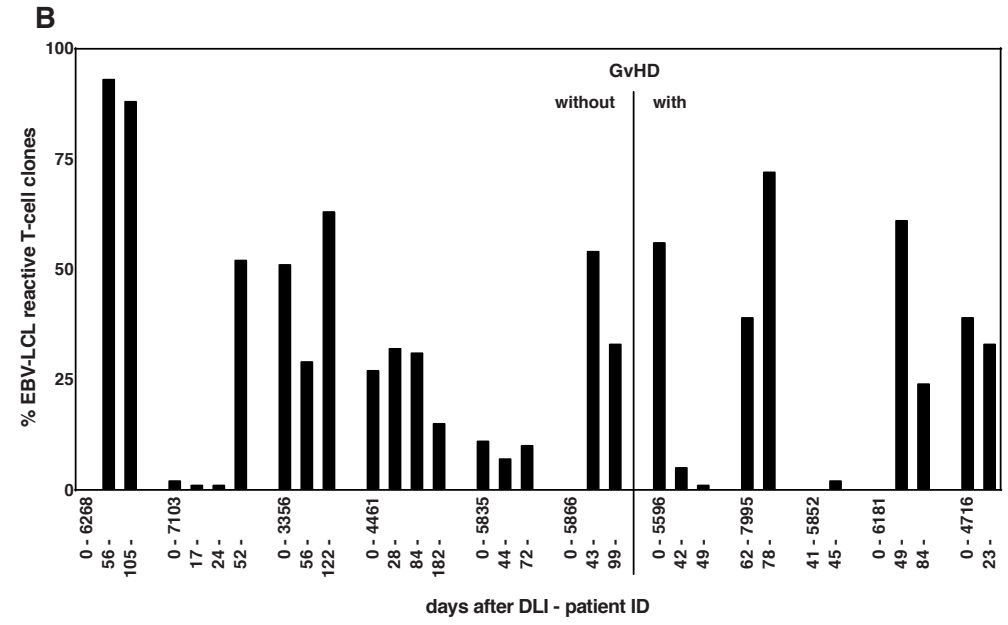
0 - 3356
56 - 122 -
0 - 4461
28 - 84 - 182 -
0 - 5835
44 - 72 -
0 - 5866
43 - 99 -
0 - 5596
42 - 49 -
62 - 7995
78 -
41 - 5852
45 -
0 - 6181
49 - 84 -
0 - 4716
23 -
days after DLI - patient ID


B
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0 - 7103
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0 - 4461
28 - 84 - 182 -
0 - 5835
44 - 72 -
0 - 5866
43 - 99 -
0 - 5596
42 - 49 -
62 - 7995
78 -
41 - 5852
45 -
0 - 6181
49 - 84 -
0 - 4716
23 -
days after DLI - patient ID

Figure 3 (facing page). Frequencies of the alloreactive T-cell response. HLA-DR^{pos} CD8 T cells were single-cell sorted and expanded from samples taken prior (day 0) and after DLI. Patients are stratified by development of GvHD and time points of sample collection are indicated on the x-axes per patient. Y-axes depict percentages of tested T-cell clones that were alloreactive (A) or EBV-LCL reactive (B).
(A) Alloreactive T-cell clones are defined by recognition of patient, but not donor test cells. Percentages of alloreactive T-cell clones in post-DLI samples were compared between patients without GvHD and patients with GvHD using Mann Whitney unpaired test (insert).
(B) In the same samples, T-cell clones recognizing EBV-LCL from both patient and donor were quantified and depicted as the percentage EBV-LCL reactive T-cell clones. No differences in percentages of EBV-LCL reactive T-cell clones were detected between patients without GvHD and patients with GvHD by Mann Whitney unpaired test. For sorting details and tested stimulator cells, see also Table S2.

Diversity of alloreactive T cells

To identify the diversity of targeted MiHA by 158 isolated T-cell clones, we determined HLA restriction, MiHA frequency and TCR clonality. HLA restriction was determined in most cases by using broad and allele-specific HLA blocking antibodies. In the remaining cases, additional retroviral HLA transduction experiments were performed resolving HLA restriction for in total 154 T-cell clones (Table 1, see also Table S3).

Table 1. Response diversity

patient ID	HLA-A		HLA-B		HLA-C		unknown	total
	1st allele	2nd allele	1st allele	2nd allele	1st allele	2nd allele		
7103	1							1
3356	2	1	5	2				10
4461	2		1	1				4
5835			1		2			3
5866			1					1
5596	2	5	3 (5)	1			1	12 (14)
7995	1 (2)		2		1 (2)			4 (6)
5852	7 (9)		7 (9)	2	3		1	20 (24)
6181	1 (3)		1	3 (4)				5 (8)
4716	3 (6)	1	3	3			2	12 (15)

Numbers respresent MiHA diversity per HLA allele as indicated in the table heading.
In case multiple TCR were found to recognize the same MiHA, the total number of different TCR is given between brackets.
For HLA-restriction and TCR-vβ usage per T-cell clone, see also Table S3.

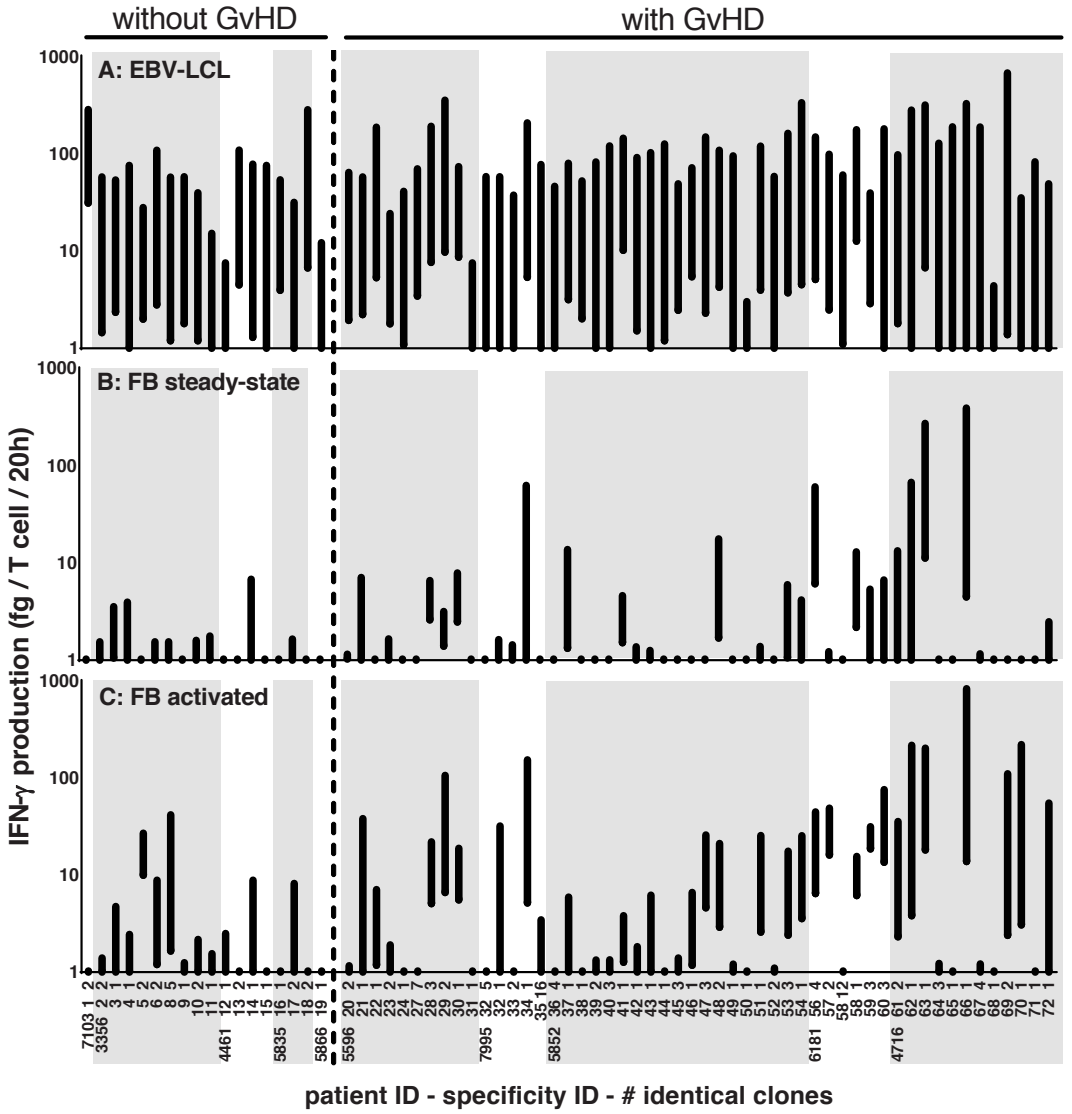
To detect T-cell clones that targeted identical MiHA, recognition was tested of 24 EBV-LCL that endogenously expressed HLA-A*02 and B*07. Recognition of MiHA restricted to other HLA molecules was tested after retroviral transduction of the relevant HLA molecule. Obtained recognition patterns were used to group identical T-cell clones which resulted in identification of 19 MiHA targeted in patients without GvHD and 53 MiHA in patients with GvHD (Table 1). The average number of MiHA targeted in patients without GvHD (3.8 ± 1.7) was significantly lower than in patients with GvHD. (10.6 ± 2.9, mean ± SD, p=0.04, Mann Whitney test). Of the 72 MiHA targeted by CD8 T cells from 10 patients, 33 MiHA were recognized by 2 or more T-cell clones. For these T-cell clones, we determined whether identical TCRvβ were expressed using an antibody panel covering 60% of all different TCRvβ. We found that T-cell clones specific for the same MiHA isolated from patients without GvHD expressed 1 TCR-vβ whereas T-cell clones for the same MiHA from patients with GvHD expressed 2 or more TCR-vβ in at least 13 cases (Table 1). The data show that repertoires of targeted MiHA as well as diversities of the T-cell responses for each MiHA are larger in patients with GvHD than in patients without GvHD.

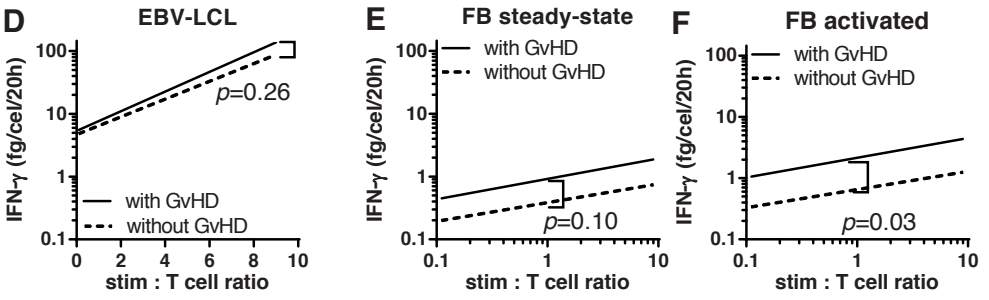
Tissue specificity of alloreactive T cells

We next investigated whether MiHA targeted in patients without GvHD were expressed on different cell types compared to MiHA targeted in patients with GvHD. T-cell specificities for the entire repertoire of targeted MiHA in patients without or with GvHD were tested against titrated numbers of hematopoietic cells (EBV-LCL, Figure 4A). As non-hematopoietic cells, FB obtained from skin biopsies were tested. FB were cultured in the absence of cytokines (steady-state FB, Figure 4B), or in the presence of IFN-γ (200 IU/ml) for 4 days to mimic inflammatory conditions (activated FB, Figure 4C). Under inflammatory conditions, higher expression of HLA, molecules involved in antigen processing, costimulatory ligands and adhesion molecules leads to a lower threshold for T-cell recognition[4,12]. We quantified T-cell recognition by calculating the absolute amount of IFN-γ produced per single T cell during overnight stimulation. Despite activation, recognition of FB was absent or lower as compared to recognition of EBV-LCL by most T-cell clones. Comparison of stimulation including the entire range of stimulator-to-T cell ratios was performed by application of mixed models (See also: Supplemental experimental procedures: Statistical analyses).

This showed comparable IFN-γ production after EBV-LCL recognition between T-cell clones from patients without or with GvHD (at ratio 9:1, without GvHD 87.8 fg IFN-γ/T cell/20h, with GvHD 137.0 fg IFN-γ/T cell/20h, *p*=0.26, Figure 4D.). Stimulation by steady-state FB revealed a trend towards higher IFN-γ production by T-cell clones from patients with GvHD (at ratio 1:1, without GvHD 0.7 fg IFN-γ/T cell/20h, with GvHD 1.9, fg IFN-γ/T cell/20h, p=0.10, Figure 4E). Activation of FB resulted in significantly stronger stimulation (at ratio 1:1, without GvHD 1.3 fg IFN-γ/T cell/20h, with GvHD 4.4 fg IFN-γ/T cell/20h, *p*=0.03, Figure 4F). This shows that T-cell clones that broadly recognize MiHA were more frequently found in patients with GvHD. In contrast, MiHA-specific T-cell clones with selective recognition of hematopoietic EBV-LCL, but not non-hematopoietic FB, were more often found in patients without GvHD.

Figure 4 (next page). Tissue specificity of alloreactive T cells. T-cell clones representing all specificities targeted in patients without GvHD (left) and patients with GvHD (right) were incubated at 833 and 2500 T cells per well with 0.1, 0.3, 1, 3 and 9-fold excess of stimulator cells. On the x-axis, T-cell specificities are plotted per patient, including the number of isolated T-cell clones with identical specificity (see also Table S3). As stimulator cells were used: EBV-LCL (A), FB cultured under steady-state conditions (B) and activated FB cultured for 4 days with 200 IU/ml IFN-γ (C). Bars represent the dose-response range between lowest and highest ratio of stimulator cells as measured by production of IFN-γ in femtogram (fg) per single T cell per 20 h. Mixed models were applied to compare dose-response ranges between T-cell clones from patients without GvHD (dashed line) and with GvHD (solid line) after stimulation with EBV-LCL (D), steady-state FB (E), or activated FB (F). X-axes of E and F are in log scale since inclusion of log(ratio) instead of ratio led to a better model fit for FB outcomes. *P*-values refer to differences in outcome between both patient groups at stimulator:T cell ratios of 9:1 for EBV-LCL (D) and 1:1 for FB (E and F). See also Supplemental experimental procedures: Statistical analyses.


without GvHD
with GvHD
A: EBV-LCL
B: FB steady-state
C: FB activated
IFN-γ production (fg / T cell / 20h)
patient ID - specificity ID - # identical clones


D
EBV-LCL
p=0.26
with GvHD
without GvHD
IFN-γ (fg/cel/20h)
stim : T cell ratio
E
FB steady-state
p=0.10
F
FB activated
p=0.03

Antigen specificity

To molecularly characterize the MiHA that were targeted by the isolated T-cell clones, we applied whole genome association scanning (WGAs) to detect MiHA-encoding single nucleotide polymorphisms (SNPs). T-cell clones restricted to HLA-A*02 or B*07 were tested on an extensive panel of SNP-genotyped EBV-LCL that endogenously expressed these HLA molecules[20]. T-cell clones restricted to other HLA class I molecules were tested against the same EBV-LCL panel after retroviral transduction of the relevant HLA molecule. From a total of 72 tested T-cell clones, 3 T-cell clones selectively recognized male EBV-LCL, indicating recognition of Y-chromosome encoded antigens which was confirmed in 1 case by staining with *KDM5D*-A*02 tetramers (Table 2). For the remaining 69 T-cell clones, 23 associating SNPs could be found. From patients 5569 and 4716, T-cell clones associated with an identical SNP in the *NDC80* gene. From the 22 unique associations, 13 SNPs were located in coding sequences of known genes and 9 SNPs were located in noncoding regions. For the latter 9 SNPs, sequencing revealed additional donor-to-patient SNP disparities in 6 cases, of which 5 were located in known genes and 1 was located in a genomic region that was not near any known gene but that encoded an amino acid polymorphism in a 255 bp open reading frame. For 3 associating SNPs in noncoding gene regions of *POLE*, *RWDD4A* and *TTK*, no disparities between patient and donor transcripts were found, hampering further characterization of the antigenic peptide . Peptide sequences spanning the 19 identified amino acid polymorphisms were searched for T-cell epitopes by the prediction algorithm NetCTLpan[30]. T-cell specificity for predicted patient-type epitopes but not donor-type controls was tested after loading of titrated synthetic peptides on donor EBV-LCL. Predicted epitopes were recognized at IC_{50} levels ranging from $5x10^{-6}$ M to $1x10^{-10}$ M, whereas donor variants were recognized at $2x10^{2}$ to $1x10^{5}$ fold higher concentrations, which validated the identification of 19 MiHA (Table 2). In conclusion, our strategy identified 19 MiHA targeted by alloreactive CD8 T-cell clones isolated from 9 patients responding to DLI. Of these 19 MiHA, 6 MiHA have previously been published[20,31] and 13 MiHA are novel T-cell epitopes.

Table 2: Antigen specificity part 1

patient ID	specificity ID	MiHA frequency	HLA restriction	WGAs results			
				SNP	significance of association[1)]	gene ID	SNP function
7103	1	0.56	A*02	rs1801284	$1.1*10^{-12}$	*HMHA1*	missense
3356	2	0.41	A*02	rs1634508	$4.4*10^{-14}$	*CCL4*	near gene
	5	0.69	B*07	rs2071915	$7.3*10^{-12}$	*TMEM8A*	missense
	6	0.25	B*07	rs11541046	$1.1*10^{-9}$	*MOB3A*	missense in ARF[2)]
	9	0.29	B*07	rs305087	$1.1*10^{-14}$	C16ORF	no gene
	10	0.31	B*13	rs1049564	$3.8*10^{-11}$	*PNP*	missense
4416	13	0,13	A*02	rs12292394	$2.4*10^{-8}$	*NCAPD3*	missense
	14	0,88	B*07	rs4918752	$2.0*10^{-10}$	*ZDHHC6*	ARF[2)] in 5'UTR
5835	17	0.61	C*14	rs5745001	$4.6*10^{-11}$	*POLE*	intron
5866	19	0.53	B*35	rs4547833	$5.7*10^{-12}$	*RWDD4A*	intron
5596	22	0.43	A*02	rs7233405	$1.2*10^{-15}$	*NDC80*	intron
	27	0.68	B*08	rs2076109	$1.0*10^{-15}$	*APOBEC3B*	missense
	28	0.68	B*08	rs1695	$2.0*10^{-19}$	*GSTP1*	missense
	29	0.46	B*08	rs1045491	$1.4*10^{-15}$	*GEMIN4*	3' UTR
5852	37	0.50	A*02	rs608962	$3.6*10^{-20}$	*TTK*	intron
	38	0.50	A*02	rs12828016	$1.1*10^{-16}$	*WNK1*	missense
	44	0.67	B*07	rs26654	$1.2*10^{-19}$	*ERAP1*	intron
	45	0.60	B*07	rs4703	$5.5*10^{-16}$	*ARHGDIB*	missense in ARF[2)]
	46	0.37	B*07	rs4801775	$1.3*10^{-16}$	*BCAT2*	intron
	47	0.53	B*07	rs2986014	$4.2*10^{-15}$	*PDCD11*	missense
6181	58	0.51	B*51	rs4801853	$2.2*10^{-14}$	*C19ORF48*	missense
4716	61	0.58	A*02	male associated	na	*KDM5D*	na
	62	0.58	A*24	male associated	na	unknown	na
	63	0.58	B*40	male associated	na	unknown	na
	64	0.43	A*02	rs7233405	$1.2*10^{-15}$	*NDC80*	intron
	66	0.30	B*40	rs238221	$6.2*10^{-18}$	*ZNFX1*	missense

[1)] determined by Fisher's exact test

[2)] alternative reading frame

Table 2: Antigen specificity part 2

patient ID	specificity ID	MiHA identification				
		coding SNP[3]	peptide[4]	IC50 (M)	IC50 (M) donor var	designated
7103	1		VL***H*** *(R)* DDLLEA	$1*10^{-10}$	$1*10^{-5}$	HA-1
3356	2	rs1130371	CADPSE***T*** *(S)* WV	$5*10^{-10}$	$5*10^{-7}$	LB-CCL4-1T
	5		RPRSVT***I*** *(V)* QPLL	$1*10^{-8}$	$5*10^{-5}$	LB-TMEM8A-1I
	6		***C*** *(S)* PRPGTWTC	$5*10^{-8}$	$>1*10^{-4}$	LB-MOB3A-1C
	9		***R*** *(W)* PCPSVGLSFL	$5*10^{-7}$	$>1*10^{-4}$	LB-C16ORF-1R
	10		TQAQIFDY***S*** *(G)* EI	$5*10^{-8}$	$1*10^{-5}$	LB-PNP-1S
4416	13		WL***Q*** *(R)* GVVPVV	$1*10^{-10}$	$1*10^{-6}$	LB-NCAPD3-1Q
	14		RPR***Y*** *(H)* WILLVKI	$1*10^{-8}$	$1*10^{-5}$	LB-ZDHHC6-1Y
5835	17	not found	-	-	-	-
5866	19	not found	-	-	-	-
5596	22	rs9051	HLEEQI***P*** *(A)* KV	$1*10^{-9}$	$>1*10^{-4}$	LB-NDC80-1P
	27		***K*** *(E)* PQYHAEMCF	$5*10^{-6}$	$>1*10^{-4}$	LB-APOBEC3B-2K
	28		DLRCKY***V*** *(I)* SL	$5*10^{-7}$	$>1*10^{-4}$	LB-GSTP1-1V
	29	rs4968104	FPALRFVE***V*** *(E)*	$5*10^{-9}$	$5*10^{-5}$	LB-GEMIN4-2V
5852	37	not found	-	-	-	-
	38		RTLSPE***I*** *(M)* ITV	$4*10^{-9}$	$5*10^{-5}$	LB-WNK1-1I
	44	rs266531	HP***R*** *(P)* QEQIALLA	$2*10^{-8}$	$>1*10^{-4}$	LB-ERAP1-1R
	45		LPRACW***R*** *(P)* EA	$4*10^{-9}$	$>1*10^{-4}$	LB-ARHGDIB-1R
	46	rs11548193	QP***R*** *(T)* RALLFVIL	$6*10^{-9}$	$1*10^{-5}$	LB-BCAT2-1R
	47		GPDSSKT***F*** *(L)* LCL	$3*10^{-8}$	$>1*10^{-4}$	LB-PDCD11-1F
6181	58		TAWPGAP***E*** *(G)* V	$4*10^{-10}$	$5*10^{-6}$	LB-C19ORF48-2E
4716	61		FIDSYICQV[5]	-	-	KDM5D
	62		unknown	-	-	unknown
	63		unknown	-	-	unknown
	64	rs9051	HLEEQI***P*** *(A)* KV	$1*10^{-9}$	$>1*10^{-4}$	LB-NDC80-1P
	66		NEIEDVW***Q*** *(H)* LDL	$3*10^{-7}$	$>1*10^{-4}$	LB-ZNFX1-1Q

[3] if discrepant from associating SNP, detected by Sanger sequencing

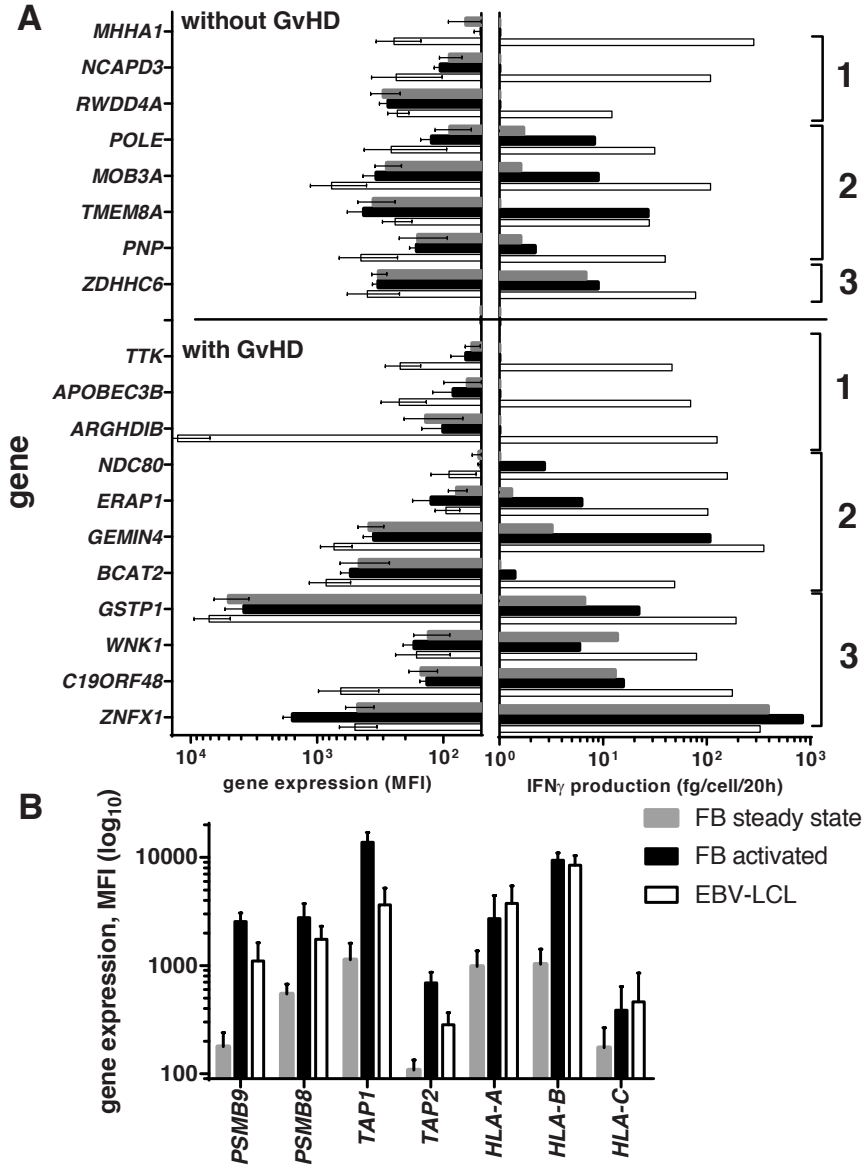
[4] donor variant amino acid is depicted between brackets of patient and donor cDNA (see also Supplemental experimental procedures.)

[5] determined by tetramer staining

Expression of MiHA-encoding genes

To investigate whether mRNA expression profiles of the identified MiHA-encoding genes could explain FB recognition patterns as observed in figure 4, we quantified MiHA gene expression levels of 6 EBV-LCL and 4 FB by micro-array techniques. Of the 22 MiHA-encoding genes, probe fluorescences for *CCL4*, *PDCD11* and *KDM5D* did not exceed background, and no probe was included for the LB-C16ORF-1R encoding transcript. We grouped MiHA according to the level of FB recognition (Figure 5A, right panel). Type 1 MiHA were not recognized on steady-state or activated FB. Type 2 MiHA could be recognized on FB, but only after activation by IFN-γ. Type 3 MiHA were recognized on both steady-state and activated FB. MiHA gene expression levels are depicted adjacent to T-cell recognition bars (Figure 5A, left panel). In both steady-state and activated FB, absence of recognition of type 1 MiHA correlated in 5 of 6 cases with low MiHA-encoding gene expression as compared to EBV-LCL. In steady-state FB, absent or low recognition of type 2 MiHA correlated in 6 of 8 cases with relative low MiHA-gene expression. In activated FB however, increased recognition could not be explained by increased expression of the MiHA-encoding genes. In type 3 MiHA, gene expression levels were comparable between FB and EBV-LCL in 4 of 5 cases. For type 2 MiHA, increased recognition after FB activation was not associated with increased MiHA gene expression levels. We therefore investigated accessory genes involved in peptide generation (*PSMB8, PSMB9*), intracellular translocation (*TAP1, TAP2*) and presentation (*HLA-A*, *HLA-B* and *HLA-C*). We observed strong up-regulation in activated *versus* steady-state FB (Figure 5B). Whereas similar numbers of T-cell clones targeting relative hematopoiesis-restricted MiHA (type 1) and inducible FB recognition (type 2) were isolated from patients without or with GvHD, a relative high contribution of type 3 MiHA was isolated from patients with GvHD. The data indicate that gene expression is required, but is not always sufficient for recognition of FB. In addition, between patients without and with GvHD, no strict distinction in the type of targeted MiHA was observed, but only skewing towards recognition of non-hematopoietic MiHA in patients with GvHD.

Figure 5: mRNA levels of MiHA-encoding and accessory genes (facing page). Gene expression levels in 3rd party cell lines were obtained from 6 EBV-LCL (open bars) and 4 FB cell lines using micro-array analyses. FB were analyzed in steady-state (grey bars) or after activation with 200 IU/ml IFN-γ for 4 days (black bars). (A) Expression levels are depicted per gene as mean fluorescence intensity ± sd (MFI, left panel). Stimulation of T



cells by patient EBV-LCL and FB (steady-state and activated) was measured by IFN-γ Elisa after incubation at stimulator:T-cell ratios of 9:1 (fg/cell/20h, right panel). Specificities are stratified by GvHD status of the patient from who the T-cell clones were isolated (top: without GvHD, bottom: with GvHD). MiHA specificities with comparable recognition patterns are grouped: type 1: no FB recognition, type 2: recognition of activated FB only, type 3: recognition of both steady-state and activated FB. (B) Expression levels of genes involved in MiHA processing and presentation were analyzed and MFI values ± sd are depicted for immunoproteasome subunits *PSMB8* and *PSMB9*, peptide transporters *TAP1* and *TAP2*, and peptide presenting *HLA-A*, *HLA-B* and *HLA-C* molecules.

Discussion

In this study we analyzed alloreactive T-cell responses in 11 patients who responded to DLI after T-cell depleted alloSCT, and compared CD8 T-cell response characteristics between patients without and with GvHD. *In vivo* activated CD8 T cells were isolated, clonally expanded and analyzed for reactivity against hematopoietic and non-hematopoietic cells representing targets of GvL reactivity and GvHD, respectively. We measured higher frequencies of MiHA-specific T cells in patients with GvHD as compared to patients without GvHD. Moreover, alloreactive T-cell responses in patients with GvHD were more diverse by both the repertoire of targeted MiHA and the variety of T-cell receptors targeting each MiHA. Furthermore, MiHA-specific T-cell clones from patients with GvHD displayed stronger reactivity against FB than T-cell clones from patients without GvHD, whereas the T-cell clones responded equally strong to EBV-LCL. We identified 22 SNPs associating with MiHA recognition, and validated 19 MiHA, including 6 previously described and 13 novel MiHA. For MiHA-encoding genes, analysis of mRNA expression and MiHA recognition in EBV-LCL and FB showed relatively frequent targeting of broadly expressed genes in patients with GvHD. Our data demonstrate that both magnitude and diversity of the alloreactive T-cell response in patients with GvHD is higher than in patients without GvHD. In patients without GvHD, the alloreactive T-cell response was sufficiently strong to mediate GvL reactivity, but apparently below the threshold for GvHD development due to skewing towards T cells that efficiently target hematopoietic cells but are relatively unable to target non-hematopoietic cells.

Following DLI, development of clinically appearing acute GvHD marks alloreactivity which enables accurate sampling. In patients with GvL reactivity without GvHD the moment of a clinical response is difficult to precisely determine. However, testing of multiple post-DLI samples identified alloreactive T-cell clones in 5 of 6 patients without GvHD. It is conceivable that in one patient the immune response was missed due to either the interval between sampling or a very low response magnitude. We show that the alloreactive CD8 T-cell repertoire resides in the HLA-DR^{pos} T-cell population indicating an active, ongoing immune response. Detection of alloreactive T-cell clones was based on selective recognition of patient but not donor-derived hematopoietic test cells. During GvHD, responding T cells may however also target non-hematopoietic MiHA. Testing of FB recognition by all growing T-cell clones from 3 patients with

GvHD did not identify additional alloreactive T-cell clones that failed to recognize hematopoietic cells. These results indicate that EBV-LCL present a repertoire of MiHA specific for both various hematopoietic lineages and non-hematopoietic cells that are recognized during GvL reactivity and GvHD responses.

Our analyses show a significant higher magnitude and diversity of CD8 alloreactivity in patients with GvHD. This could not be explained by different capacities to generate immune responses, since patients in both groups were equally able to elicit responses against EBV. Remarkably, all patients with GvHD received DLI within the first year after alloSCT as compared to only 2 of 6 patients without GvHD. It was shown that the probability to survive without GvHD was highest if DLI was given beyond 1 year from alloSCT[32,33]. Early after alloSCT, higher levels of residual patient APC may elicit higher response magnitudes. APC may also persist longer due to the non-myeloablative pre-transplant conditioning that was given to 3 of 5 patients with GvHD as compared to 1 of 6 patients without GvHD. During immune recovery, patients are susceptible to viral infections leading to release of inflammatory cytokines thereby increasing alloreactivity after DLI, as was demonstrated by development of severe GvHD in two cases of CMV reactivation[22]. Whereas genetic donor-to-patient disparities determine the maximal repertoire of MiHA that can be targeted, the presence of residual patient APC and/or inflammatory conditions are likely to contribute to the magnitude of the alloreactive response.

In addition to higher response magnitudes, we observed stronger FB recognition by T-cell clones from patients with GvHD, especially under inflammatory conditions. This was not caused by a lower response capacity of the T cells, since strength of EBV-LCL recognition was comparable. MiHA recognition of EBV-LCL and steady-state FB indicates broad MiHA expression, whereas MiHA that are selectively recognized on EBV-LCL but not on activated FB are more likely to be hematopoiesis specific. FB recognition after activation with IFN-γ may be caused by a direct increase of MiHA gene expression. It may however also be the indirect result of more efficient processing of the antigenic peptide, stronger adhesion and costimulation, and higher HLA expression levels. Following initiation of hematopoiesis-directed alloreactivity, the chance that T cells target non-hematopoietic tissues may depend on the response magnitude, since high numbers of alloreactive T cells may contribute to the

inflammatory environment that increases antigen presentation on non-hematopoietic tissues resulting in GvHD.

For one third of the isolated MiHA specificities, we could identify associating SNPs and genes. Failure to characterize remaining MiHA can be explained by high or low MiHA population frequencies, or by absence of the MiHA-encoding SNP in the dataset we used. In addition to a range of novel MiHA, we isolated T cells recognizing a known MiHA (HA-1), and from two different patients T cells were isolated that both recognized the novel LB-NDC80-1P. Furthermore, the genes *GEMIN4*, *APOBEC3B* and *C19ORF48* were found to encode MiHA restricted to HLA molecules different from those previously reported[20,34]. This suggests that despite countless missense SNP-disparities between donor and patient, the repertoire of immunogenic polymorphic peptides is limited. Further analysis of patient samples and MiHA discovery, as we describe here, will give rise to a panel of MiHA that are clinically relevant both for GvL reactivity and GvHD.

Differential T-cell recognition patterns against steady-state and activated FB enabled clustering of 3 types of MiHA. Type 1 MiHA were not recognized on FB, type 2 MiHA recognition on FB was absent or low but could be induced or increased, respectively. Type 3 MiHA were recognized on FB, irrespective of the FB activation state. Absence of FB recognition in type 1 MiHA could in the majority of cases be explained by low gene expression levels. Strong recognition of type 3 MiHA on FB correlated in the majority of cases with comparable levels of gene expression between FB and EBV-LCL. For type 2 MiHA however, more diverse unpredictable gene expression patterns were observed, including very low gene expression levels in FB for *NDC80*. On FB, type 2 MiHA are probably presented at levels that are insufficient for T-cell recognition. Inflammatory conditions may lead to recognition by enhancing antigen processing and HLA surface expression, and increasing expression of costimulatory and adhesion molecules. We identified T cells specific for all 3 MiHA types irrespective of GvHD development and propose that during initiation of the immune response after DLI, professional APC present all 3 MiHA types. Subsequent skewing towards more broadly expressed MiHA may occur under inflammatory conditions, resulting in GvHD. This implies that GvHD prevention after DLI should focus both on specificity of the response, for example by depletion of donor T cells that recognize broadly expressed immuno-dominant MiHA, and on monitoring and manipulation of *in vivo* circumstances.

In conclusion, our data show that frequencies of alloreactive CD8 T cells are higher in patients with GvHD than in patients without GvHD but that there is no strict separation in the type of MiHA targeted by T cells from both patient groups. However, the alloimmune response in patients with GvHD is skewed towards T cells with stronger FB recognition, especially after treatment of FB with inflammatory cytokines. Identification of MiHA targeted in these patients significantly increased the repertoire of known hematopoiesis-restricted and broadly expressed MiHA. Improvement of the outcome of alloSCT in favor of GvL reactivity and reduced risk of GvHD may be obtained by infusion of selected hematopoiesis-specific T cells and depletion of T cells that target broadly expressed MiHA.

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Supplemental experimental procedures

Statistical analyses

Fisher's exact test was applied to calculate significance of correlation between the time point of DLI after alloSCT and GvHD occurrence (Figure 1), and to detect SNP associating with recognition patterns of EBV-LCL panels (Table 2: Antigen specificity). Wilcoxon's matched-pairs signed rank test was applied to calculate significance of difference of HLA-DR expression in pre-, and post-DLI samples (Figure 2). Mann-Whitney's test was applied to calculate significance of difference in magnitudes of alloreactivity (Figure 3A) and response diversity between patients without and with GvHD (Table 1). To analyse experiments determining recognition strength and tissue specificity, mixed models were applied to calculate differences of recognition of EBV-LCL (Figure 4D), steady-state FB (Figure 4E) and activated FB (Figure 4F) between patients without or with GvHD and its dependence on the stimulator : T-cell ratio, taking into account clustering of patients and T-cell clones. Recognition of EBV-LCL was evaluated in a separate model, while steady-state FB and activated FB recognition were both assessed in a combined model. For all statistical analyses, *p*-values below 0.05 were considered as significant.

Table S1. Patient characteristics

patient ID	age at alloSCT	don→pat gender[1)]	disease	donor[2)]	HLA-A	HLA-B	HLA-C	conditioning regimen[3)]	stem cell source[4)]	state pre DLI[5)]	GvHD	GvHD treatment	follow up (years)
6268	42	f→f	AML-M4	sd	02:01 24:02	35:01 44:02	04:01 05:01	MA cyclophosphamide TBI	PBSC	MC	no	no	8,4
7103	53	m→m	MDS	sd	02:01	07:02 44:02	05:01 07:02	NMA fludarabin busulphan Alemtuzumab	PBSC	BM blasts cytoreduction	no	no	5,6
3356	45	m→f	CML-CP	sd	02:01 24:02	07:02 13:01	06:02 07:02	MA cyclophosphamide TBI	PBSC	BCR-ABL pos	no	no	15,4
4461	23	f→m	CML-CP	sd	02:01	07:02 44:02	05:01 07:02	MA cyclophosphamide TBI	PBSC	BCR-ABL pos	no	no	12,7
5835	50	m→m	CML-CP	sd	29:02	44:03 51:01	14:02 16:01	MA cyclophosphamide TBI	PBSC	BCR-ABL pos	no	no	9,1
5866	55	f→f	CML-CP	sd	02:01 11:01	35:01 51:01	04:01 14:02	MA cyclophosphamide TBI	PBSC	BCR-ABL pos	no	no	9,9
5596	66	m→m	AML	mud	01:01 02:01	08:01 51:01	07:01 15:02	NMA fludarabin busulphan Alemtuzumab horse ATG	PBSC	bm blasts (smouldering)	grade II	dermovate, prednisone, cyclosporine	8,3
7995	63	m→m	AML	sd	01:01	35:02 52:01	04:01 12:02	NMA fludarabin busulphan Alemtuzumab	PBSC	MC	grade II	prednisone, cyclosporin, mycophenolate mofetil	4,9
5852	56	m→f	MDS	mud	02:01	07:02 44:02	05:01 07:02	NMA fludarabin busulphan horse ATG	PBSC	MC	grade II, skin+mouth	dermovate, prednisone, cyclosporin	9,7
6181	48	m→f	CML-CP	sd	11:01 24:02	44:02 51:01	05:01 15:02	MA cyclophosphamide TBI	bone marrow	BCR-ABL pos	grade III, skin+mouth	dermovate, prednisone, cyclosprine	8,6
4716	36	f→m	AML-M1	sd	02:01 24:02	40:01 44:02	03:04 05:01	MA cyclophosphamide TBI	PBSC	bm blasts (smouldering)	grade IV, deceased	prednisone, cyclosporin, mycophenolate mofetil	0,2
												median:	**8,6**

[1)]f=female
m=male

[2)]sd=sibling donor
mud=matched unrelated donor

[3)] MA=myeloablative
NMA=non myeloablative
TBI=total body irradiation

[4)]peripheral blood stem cells

[5)]MC (mixed chimerism) by STR-PCR
BCR-ABL by real time PCR

Table S2. Cloning and selection of HLA-DR^{pos} CD8 T cells and tested stimulator cells

status post DLI	patient ID	days after DLI	isolated cells	well format cloning	% cloning efficiency (growing clones)	tested target cells[2] EBV-LCL pat	EBV-LCL don	monoDC pat	monoDC don	CD40L-B pat	CD40L-B don	FB[3] pat
without GvHD	6268	0	5760	384	3 (157)	■	■					
		56	14976		10 (1534)	■	■					
		105	11520		13 (1462)	■	■					
	7103	0	3072	384	10 (75)	■	■			■		
		17	6604		10 (192)	■	■			■		
		24	6374		11 l(192)	■	■			■		
		52	6604		31 (384)	■	■			■		
	3356	0	480	96	10 (50)	■	■	■	■	■	■	
		56	2080		9 (191)	■	■	■	■	■	■	
		122	864		11 (96)	■	■	■	■	■	■	
	4461	0	923	96	13 (123)	■	■	■	■	■	■	
		28	1212		7 (90)	■	■	■	■	■	■	
		84	960		4 (36)	■	■	■	■	■	■	
		182	1152		11 (131)	■	■	■	■	■	■	
	5835	0 [1]	384	96	20 (78)	■	■	■	■	■	■	
		44 [1]	2016		6 (129)	■	■	■	■	■	■	
		72 [1]	480		16 (78)	■	■	■	■	■	■	
	5866	0	864	96	15 (126)	■	■	■	■	■	■	
		43	3396		8 (272)	■	■	■	■	■	■	
		99	3168		3 (94)	■	■	■	■	■	■	
	cloning efficiency (mean ± SD)				**10.8 ± 6.4 %**							
	number of growing clones (mean ± SD)				**277 ± 432**							
with GvHD	5596	0	480	384	8 (36)	■	■			■	■	■
		42 [1]	4702		5 (237)	■	■			■	■	■
		49 [1]	9310		2 (206)	■	■			■	■	■
	7995	62	16282	384	3 (518)	■	■					■
		78	16282		6 (936)	■	■					■
	5852	45	2000	96	10 (132)	■	■					
	6181	0	384	96	7 (25)	■	■	■	■		■	
		49	1440		14 (202)	■	■	■	■		■	
		84	960		12 (115)	■	■	■	■		■	
	4716	0	480	384	11 (52)	■	■	■	■			■
		23	16454		2 (287)	■	■	■	■			■
	cloning efficiency (mean ± SD)				**8.7 ± 7.2 %**							
	number of growing clones (mean ± SD)				**202 ± 183**							

[1] Indicated are the days after the 2nd DLI.

[2] Shaded boxes indicate that cells in column header were tested.

[3] FB were tested after 4 days activation in the presence of 200 IU/ml IFN-γ.

Table S3. Response diversity

patients without GvHD

patient ID	specificity ID	HLA restriction	MiHA frequency	clones with similar specificity	identified TCRvβ	MiHA diversity
7103	1	A*02	0.54	2	22	1
3356	2	A*02	0.42	2	8	10
	3	A*02	0.04	1	1	
	4	A*24	0.38	1	neg*	
	5	B*07	0.67	2	13.2	
	6	B*07	0.25	2	neg	
	7	B*07	0.71	1	20	
	8	B*07	1.00	5	17	
	9	B*07	0.29	1	neg	
	10	B*13	0.29	2	neg	
	11	B*13	0.04	1	neg	
4461	12	A*02	0.75	1	1	4
	13	A*02	0.13	2	8	
	14	B*07	0.88	1	neg	
	15	B*44	0.29	1	neg	
5835	16	B*44	0.98	1	8	3
	17	C*14	0.63	2	3	
	18	C*14	0.04	2	neg	
5866	19	B*35	0.54	1	5.3	1

Patients with GvHD

patient ID	specificity ID	HLA restriction	MiHA frequency	clones with similar specificity	identified TCRvβ	MiHA diversity
5596	20	A*01	0.67	2	5.1	12
	21	A*01	0.67	1	13.2	
	22	A*02	0.42	1	20	
	23	A*02	0.63	2	neg	
	24	A*02	0.04	1	neg	
	25	A*02	0.29	1	8	
	26	A*02	0.38	1	neg	
	27	B*08	0.67	7	7.1 neg	
	28	B*08	0.67	3	16	
	29	B*08	0.46	2	21.3 1	
	30	B*51	0.96	1	3	
	31	unknown	0.46	1	13.2	

*Not staining with TCRvβ-specific antibodies included in the assay.

Table S3. Response diversity (continued)

Patients with GvHD						
patient ID	specificity ID	HLA restriction	MiHA frequency	clones with similar specificity	identified TCRvβ	MiHA diversity
7995	32	A*01	0.96	6	2 neg*	4
	33	B*35	0.00	2	16	
	34	B*35	0.67	1	neg	
	35	C*04 or C*12	0.96	16	14 neg	
5852	36	A*02	0.50	4	9 17	20
	37	A*02	0.50	1	neg	
	38	A*02	0.21	1	1	
	39	A*02	0.04	2	5.1 20	
	40	A*02	0.13	3	8 neg	
	41	A*02	0.98	1	neg	
	42	A*02	0.08	1	neg	
	43	B*07	0.67	1	1	
	44	B*07	0.58	1	neg	
	45	B*07	0.38	3	neg	
	46	B*07	0.54	1	neg	
	47	B*07	0.13	3	12 16	
	48	B*07	0.04	2	1 neg	
	49	B*07	0.17	1	8	
	50	B*44	0.71	1	12	
	51	B*44	0.04	1	neg	
	52	C*05	0.96	2	23	
	53	C*05 or C*07	0.04	3	9	
	54	C*05 or C*07	0.08	1	17	
	55	unknown	not tested	2	neg	
6181	56	A*11	0.63	4	12 20 21.3	5
	57	B*44	0.88	2	5.1	
	58	B*51	0.50	13	neg	
	59	B*51	0.04	3	1 17	
	60	B*51	0.04	3	1	
4716	61	A*02	0.58	2	12 13.1	12
	62	A*24	0.58	1	neg	
	63	B*40	0.58	1	9	
	64	A*02	0.42	3	14 23 neg	
	65	A*02	0.04	1	neg	
	66	B*40	0.29	1	neg	
	67	B*40	0.83	4	14	
	68	B*44	0.04	1	neg	
	69	B*44	0.88	2	neg	
	70	B*44	0.96	1	neg	
	71	B or C	0.00	1	13,1	
	72	unknown	0.79	1	neg	

*Not staining with TCRvβ antibodies included in the assay.

Supplemental experimental procedures: Primers used for amplification and sequencing of MiHA-encoding genes

WGAs results		NCBI Reference Sequence			PCR product[2]	Primer sequence 5' ► 3'	
SNP ID	gene name	Accession	Length (bp)	CDS[1]		Forward	Reverse
rs1634508	*CCL4*	NM_002984	667	80 ► 358	32 ► 583	TGAGTTCTGCAGCCTCACCTCTGA	ACAGTGACAGTGGACCATCCCCA
rs2071915	*TMEM8A*	NM_021259	3,656	130 ► 2,445	231 ► 1,018	CGGCTACAGCGGGAAGAGCGA	CAGCACTGAAAGCCACTGTCCC
					737 ► 1,409	CCCTCCTCTCCCATCCCAGCTAC	AAGGGCGAGGCAGCATTCACG
rs11541046	*MOB3A*	NM_130807	3,347	319 ► 972	361 ► 919	ACATTCCGCCCCAAGCGCAA	CGATGAGGCCGAACTCCTTGACG
rs305087	*C16ORF99*	mRNA not known, PCR performed on genomic DNA			1 ► 368	ACGCACACAAACACCATCCTGC	ATCTTCCCTGCCCTCCCTCACC
rs1049564	*PNP*	NM_000270	2,438	147 ► 1,016	132 ► 742	GCGTCTGCGAGACCATGGAGAA	TGGGGCCTGCCACCATCACATA
rs12292394	*NCAPD3*	NM_015261	5,661	607 ► 5,103	2,163 ► 2,753	TTCCGAACCCTCAGGGGAGATCA	GCCGAATGTTCCGTGCCAGT
rs4918752	*ZDHHC6*	NM_022494	2,187	425 ► 1,666	744 ► 1,400	ACAAGGCACCACGTTCACATCACTG	ATTCAGAGGGCAGCAGGCACC
rs5745001	*POLE*	NM_006231	8,024	210 ► 7,070	437 ► 1,418	GCGCTTAGGCAGTGCAGTGGA	GATGCACTGGGGCGCCTTGT
					1,373 ► 2322	CTTCCAGAAGGACAGCCAGGGG	GAGCTGGCCCCTCTGGGAACA
					2,312 ► 3,261	GGGGCCAGCTCGGGCCTTTC	ACAGCACGTCCAGCCAGTAGTCA
					2,973 ► 3,954	GTCACCCGCTCAGAGAACAGCA	CAGTCGGCGTGAGGTCCTGG
					3,934 ► 4,637	CCCAGGACCTCACGCCGACT	GAACTGGGCCAGAGAGCGCAT
					4,618 ► 5,539	TGCGCTCTCTGGCCCAGTTCA	CTGGCAGCCTGACCACCCGT
					5,326 ► 6,031	TGGAGTTCGATGACCAAGCCACT	TGGGAGTCTTGCAGTCCACAGTGA
					6,211 ► 7,014	CAGCGTACATCGTGGCCGTGT	ACGACATGCCGTAGTGCTGGG
rs4547833	*RWDD4A*	NM_152682	2,593	227 ► 793	26 ► 754	TCGTAGCCCACAGCCCACTGC	CACAACATCAACCCAGTTCCAGCC
rs7233405	*NDC80*	NM_006101	2,209	183 ► 2,111	15 ► 745	GCGTAATGACGTCAGCGCC	AGCCAAACTAAGGCTGCCAC
					702 ► 1483	GCTCCTCATACATGGCCTCAC	CCTTTGGAATTCTCAGCACCT
					1,400 ► 2,042	AGCGATTGAAACACAATTAGCAGA	CGAGAGATCTTCTGACATGCATT
rs608962	*TTK*	NM_001166691	3,019	124 ► 2694	124 ► 1,515	ATGGAATCCGAGGATTTAAGTG	CAGCTCATGTAATCATCCAAG
					1,343 ► 2,215	GCAGATTCCGGAGTTAGCC	ACATATCTTTGATTGCTTCTGG
					2,132 ► 2,963	ATGCAACCAGATACAACAAGTG	CTTGCTATCCACCCACTATTC
rs26654	*ERAP1*	NM_001040458	5,085	348 ► 3173	140 ► 816	GTTTACCCTTTCCCCAGCTC	CCGAAAGATTGCCAGCATAG
					669 ► 1,290	AGGAAGGGAGCTGGAGAGAG	GAAAGTCGGGAATAGCAGCA
					1,268 ► 1,973	TCTTGCTGCTATTCCCGACT	TACATTCCTCCCCCTCACTG
					1,890 ► 2,583	GTGGATGTGAAAACCATGATGA	TCTGTACGCACGGCTGATAG
rs4703	*ARHGDIB*	NM_001175	1,216	105 ► 710	69 ► 701	CCGGACAGAGACGTGAAGC	CCACTCCTTCTTAATCGACAG
					510 ► 1,216	GTGGATAAAGCAACATTTATGG	CTCAGCCAGAACACACACAG
rs4801775	*BCAT2*	NM_001190	1,616	38 ► 1216	510 ► 972	ATCGAAGTGGACAAGGACTG	GAGCCATGTCCAGTAGACTC
					918 ► 1,472	CTGAATGGTGTTATCCTGCCTG	GCACGACAAGGAGTAATGGG
rs4801853	*C19ORF48*	NM_199250	1,667	898 ► 1,251	776 ► 1,425	CAGCCCACCATGGACAGGGGTA	TCTGGCCAGTGACACCCACAG
rs238199	*ZNFX1-AS1*	NR_003604	1,020	n.a.	9 ► 694	CAGGGTGGAGAGCACGAG	CCCGCATTCATCCTGGTTCA
					458 ► 980	ATTCGTTCTTTCGCGTCTGC	ATGCAGGTAGGCAGTTAGAAAT
rs238221	*ZNFX1*	NM_021035	7,371	248 ► 6,004	2,828 ► 3,300	GCTAAAATGCTTCTGGCCATGAG	GCAATGGTATGGGCCTCAAG

[1] first and last bp of the coding sequence [2] first and last bp of the PCR product

Chapter 7

Summary and General discussion

Summary

Donor alloreactivity contributes to the curative effect of allogeneic stem cell transplantation (alloSCT) and donor lymphocyte infusion (DLI). Characterization of minor histocompatibility antigens (MiHA), the molecular structures targeted by alloreactive donor T cells in the HLA-matched setting, provides insight into the mechanisms of graft-*versus*-leukemia (GvL) reactivity and graft-*versus*-host disease (GVHD) and may identify MiHA that are effective and safe targets for adoptive immunotherapy.

MiHA are polymorphic peptides encoded predominantly by single nucleotide polymorphisms (SNP) that encode amino acid substitutions in proteins. After proteasomal degradation, polymorphic patient-specific peptides that bind to human leukocyte antigens (HLA) are presented to donor T cells which can lead to activation, expansion and targeting $MiHA^{pos}$ patient cells. Discovery of MiHA focuses on isolation and characterization of the antigenic peptide by biochemical techniques or on identification of the MiHA-encoding transcript by cDNA cloning techniques. Whole genome association scanning (WGAs) identifies the MiHA-encoding SNP and is based on separation of $MiHA^{neg}$ from $MiHA^{pos}$ test cells from which detailed SNP genotype data are available.

MiHA expression on target cells of GvL reactivity and GVHD, can be tested directly by T-cell recognition and by quantification of MiHA-encoding gene expression levels. Alloreactive donor T cells targeting MiHA that are exclusively expressed by malignant cells may induce selective GvL reactivity without GvHD.

In ***chapter 2*** we show the biochemical purification and mass spectrometric (MS) characterization of LB-ADIR-1F, a MiHA that was targeted by a dominant alloreactive T-cell response in a multiple myeloma (MM) patient who obtained complete remission after alloSCT and DLI. LB-ADIR-1F specific T cells lysed MM cells *in vitro* but also demonstrated weak recognition of non-hematopoietic cells, which may have caused the GvHD that was observed after DLI. We identified the antigenic peptide that contained an amino acid polymorphism encoded by SNP rs2296377. In a panel of 74 test cells, the presence of this SNP correlated in all cases with T-cell recognition. The amino acid polymorphism occurred in a reading frame that was initiated by an alternative out-of-frame start codon located upstream of the normal start codon. Tetramer

analysis showed high levels of circulating LB-ADIR-1F specific T cells at 7 weeks post-DLI thereby confirming that targeting of this novel MiHA can contribute to GvL reactivity but may also cause GvHD. Despite labor-intense biochemical purification and MS analyses, application of biochemical techniques and MS can lead to identification of antigenic peptides presented by HLA.

Whereas biochemical techniques and MS can robustly identify MiHA, we set out to increase the speed of MiHA discovery by WGAs. ***Chapter 3*** describes implementation and validation of WGAs for MiHA discovery using a panel of 80 EBV-LCL that was high-resolution SNP-genotyped. From 2 patients who responded to DLI, 20 different HLA-A*02 or B*07 restricted MiHA-specific T-cell clones were analyzed. For 12 T-cell clones, WGAs detected SNP that were present in $MiHA^{pos}$ but not in $MiHA^{neg}$ EBV-LCL. To define the MiHA peptides, we performed HLA-binding prediction of peptide sequences that spanned the SNP-encoded amino acid polymorphism. Specific T-cell recognition of patient-type peptides but not donor-type peptides validated the discovery of 10 novel MiHA. Tetramer analysis of blood samples that were collected after DLI showed significant numbers of circulating T cells specific for several of the novel MiHA thereby validating that WGAs enables rapid discovery of clinically relevant MiHA.

In ***chapter 4,*** we applied retroviral transduction of the EBV-LCL used for WGAs to discover MiHA that are restricted to other HLA molecules than HLA-A*02 or B*07. We analyzed 4 HLA-B*40 restricted T-cell clones that were isolated from a patient who was treated with alloSCT and DLI for chronic myelogenous leukemia. WGAs identified SNP in *SON*, *SWAP70* and *NUP133* as potentially MiHA-encoding. For 1 T-cell clone, cDNA library screening was performed which identified a SNP with high population frequency in *TRIP10*. Specific recognition of patient-type peptides validated LB-SON-1R, LB-SWAP70-1Q and LB-TRIP10-1EPC as 3 novel MiHA. For LB-NUP133-1R, also the donor peptide was recognized, in contrast to selective recognition of patient but not donor-derived *NUP133* transcripts. This suggests that LB-NUP133-1R immunogenicity does not result from specific recognition of the patient amino acid polymorphism. It is more likely that the complete LB-NUP133-1R peptide is not presented by donor cells which may be due to differential processing of the parental *NUP133* protein caused by the amino acid polymorphism. The data show that WGAs can support MiHA discovery irrespective of the HLA-restriction

molecule. In regard to population frequency, recognition of various hematological malignant cells and absence of fibroblast recognition, LB-SWAP70-1Q is a promising target for adoptive immunotherapy to treat patients who suffer from hematological malignancies.

Due to better treatment tolerability, T-cell depleted alloSCT and DLI were also applied to elicit graft-*versus*-tumor reactivity in patients with solid tumors. ***Chapter 5*** describes a patient with metastasized renal cell carcinoma (RCC) who, after alloSCT and DLI, suffered from acute GvHD and severe chronic GvHD. This strong response coincided with tumor regression resulting in stable disease for 4 years. A MiHA specific T-cell clone was isolated from blood that was taken at the onset of GvHD. WGAs identified LB-FUCA2-1V as the target of this alloreactive T cell that was detectable at high levels in blood. The *FUCA2* gene was broadly expressed as measured by mRNA analyses. Concordant with this, LB-FUCA2-1V specific T cells strongly recognized RCC cell lines and also fibroblasts that were activated by IFN-γ. We therefore concluded that this T-cell clone was involved in both GvT reactivity and development of GvHD. In patients with solid tumors, alloreactivity was more often accompanied with GvHD than in patients with leukemia. To estimate the chance that GvT can occur without GvHD, we analyzed gene expression profiles of both RCC cell lines as targets of GvT reactivity, and skin-derived cells representing targets of GvHD. Given the prerequisite that efficient T-cell responses are initiated by professional antigen presenting cells (APC), GvT reactivity without GvHD requires targeting of genes that are expressed by both professional APC and RCC cells but not by epithelial cells. Only a limited number of genes fulfilled these criteria. Significantly more genes were found when RCC cells were replaced by leukemic cells in the analysis. This indicates that induction of GvT reactivity to treat patients with solid tumors may unavoidably lead to development of GvHD.

Patient suffering from hematological malignancies can achieve GvL reactivity after alloSCT and DLI without or with development of GvHD. ***Chapter 6*** describes a comparison of both quantitative and qualitative aspects of alloreactive CD8 T-cell responses after DLI between the patient groups. We observed a significant increase in the absolute number of circulating HLA-DR^{pos} CD8 T cells after DLI, independently of development of GVHD. Activated CD8 T cells were clonally expanded and we found both higher magnitudes and diversities of the alloreactive T-cell response in patients with GvHD. Whereas isolated T-cell clones from patients without or with GvHD were equally capable

to respond to hematopoietic cells, recognition of skin-derived fibroblasts (FB), representing targets of GvHD, was stronger by T-cell clones isolated from patients with GvHD. Inflammatory conditions further increased this difference of FB recognition. WGAs was applied to identify the MiHA that were targeted. This allowed analysis of expression levels of MiHA-encoding genes. This showed that increased recognition of MiHA under inflammatory conditions could not be explained by increased expression levels of MiHA-encoding genes. However, higher expression of genes involved in MiHA processing and presentation was observed confirming the prominent role of the micro-environment on recognition of MiHA on non-hematopoietic cells.

In summary, from patients who receive alloSCT and DLI, T-cell clones can be isolated that recognize MiHA that can be selectively expressed by hematopoietic and leukemic cells resulting in GvL reactivity. However, recognition of normal tissue cells can also occur, which is likely to be correlated with development of GvHD. By developing methods for high-throughput discovery of MiHA, we are now able to perform comprehensive analyses of *in vivo* allo-immune T-cell repertoires to gain insight in the biology of GvL/GvT reactivity and GvHD and to identify MiHA that can safely be targeted by adoptive immunotherapy.

General discussion

Treatment with alloSCT and DLI can be considered as cellular immunotherapy, since donor T, B, and NK cells can be immune effector cells capable of inducing curative GvL reactivity. Candidate target antigens for donor T cells mediating GvL reactivity after HLA matched alloSCT are polymorphic peptides that are presented on the tumor cell by HLA surface molecules, the so-called minor histocompatibility antigens (MiHA).

After successful alloSCT, donor stem cells replace patient hematopoiesis. Alloreactive T cells in DLI after alloSCT have the potential to attack all cells of patient origin, which may include leukemic cells (GvL reactivity), healthy non-hematopoietic tissues (GvHD) or residual hematopoietic cells of patient origin in case of mixed chimerism (graft-*versus*-patient hematopoiesis). Hematopoietic cells that originate from the donor stem cells, however, express antigens that are considered as “self” for the donor lymphocytes and will therefore be ignored. In contrast to anti-tumor therapies for non-transplanted patients, T-cell therapies for hematological malignancies after alloSCT do not require targeting of antigens that are specifically expressed on leukemic cells, but may also be directed against MiHA that are expressed by all hematopoietic patient cells. As such, a large repertoire of hematopoiesis-restricted antigens can be suitable targets to treat hematological malignancies after alloSCT.

This thesis describes the role of CD8 T cells in clinical immune responses that are induced in patients after alloSCT and DLI. From these patients, large repertoires of T-cell clones have been isolated representing *in vivo* alloimmune responses. We focused on patients with malignant hematopoietic diseases, but also investigated the response after alloSCT and DLI in a patient who was treated for metastatic renal cell carcinoma (RCC). Different strategies have been followed to identify the molecular targets of donor-derived alloreactive CD8 T cells, which greatly increased our understanding of how genetic polymorphisms encode MiHA.

By comparing alloimmune responses between transplanted patients who responded to DLI without or with GvHD, a correlation between magnitude and diversity of MiHA-specific CD8 T cells and development of GvHD was demonstrated. From these patients, we isolated alloreactive T-cell clones and investigated T-cell recognition of target cells of GvL reactivity (malignant

hematopoietic cells), graft-*versus*-patient hematopoiesis (healthy hematopoietic cells) and cells representing non-hematopoietic tissues that are targeted in GvHD. The data show that T cells can be distinguished by hematopoiesis-restricted recognition or broad reactivity against hematopoietic as well as non-hematopoietic cells. T cells with hematopoiesis-restricted recognition can induce selective GvL-reactivity without GvHD, whereas T cells with broad reactivity that includes non-hematopoietic tissues can mediate both GvL-reactivity and GvHD. As such, T cells with hematopoiesis-restricted recognition are of special interest, since these T cells recognize MiHA that may be suitable targets for adoptive immunotherapy.

In this chapter, we discuss strategies to isolate alloreactive T cells from post DLI samples and different methods that can be used to discover MiHA, including 'reversal' of the MiHA discovery approach. In addition, the tissue distribution of MiHA and the effect of environmental factors on T-cell recognition are considered. Finally, developments in post-alloSCT cellular immunotherapy to induce GvL reactivity without GvHD, including application of alloreactivity to treat solid tumors, are discussed.

Selection of alloreactive T cells

In vivo activated antigen-specific T cells can reach levels in peripheral blood that allow detection and isolation. Within the total number of PBMC, however, antigen-specific T cells do not exceed percentages of 1-5% of CD8 T cells. Clonal analysis of circulating alloreactive T cells therefore requires enrichment by *in vivo* or *in vitro* induced cell surface proteins or secreted proteins. In chapters 3 and 6 of this thesis, *in vivo* activated T cells were directly isolated by surface expression of HLA-DR from post-DLI patient samples. In chapters 2, 4 and 5, post-DLI samples were stimulated *in vitro* with patient cells obtained prior to alloSCT and activated T cells were isolated by secreted IFN-γ (chapters 2 and 4) or up-regulated HLA-DR (chapter 5). Direct post-DLI isolation by HLA-DR surface expression allows quantitative analysis of circulating activated MiHA-specific T cells. Compared to isolation of *in vivo* activated T cells by HLA-DR, more efficient selection of MiHA-specific T cells may be obtained after *in vitro* stimulation with patient cells, but T-cell reactivity is likely to be skewed towards MiHA that are expressed on the cell type used for stimulation.

The biological function of HLA-DR on T cells is largely unknown. TCR ligation can lead to HLA class II gene expression and cell surface presentation via signaling of CD2 or CD3[1]. However, T cells have also been demonstrated to 'snatch' HLA class II from nearby APC through trogocytosis[2,3]. Non-hematopoietic tissues lack HLA class II, but expression can be up-regulated by inflammation. HLA class II expression on T cells may be similarly up-regulated by IFN-γ produced after antigenic stimulation via autocrine signaling. There may also be a role for HLA-DR expression in amplification of virus specific immune responses. The intimate contact of T cells with virus-infected tissue cells may lead to viral infection of T cells, which subsequently present viral epitopes and amplify the virus-specific immune response. Despite the poorly understood biological function of HLA-DR surface expression on T cells, this thesis clearly shows that in patients with GvHD after DLI, higher frequencies of MiHA-specific T cells are found within the HLA-DR^{pos} compartment than in patients who remained free of GvHD.

Isolation of alloreactive T cells depends on immune response dynamics at the time of sampling. Expression of the *in vivo* activation marker may vary during initiation, expansion, effector, retraction and memory phases of the immune response. Moreover, systemic immunosuppression to treat GvHD may alter phenotypes and expression levels of activation markers of alloreactive T cells. If multiple MiHA are targeted, the dynamics of individual specificities may also vary, as demonstrated by early detection of LB-ARGHDIB-1R as compared to LB-ERAP1-1R specific T cells in chapter 3 and early detection of HA-2 as compared to HA-1 specific T cells in chapter 4. In addition to HA-1 and HA-2, which are both expressed by HLA-A*02:01, T cells for HLA-B*40:01 restricted MiHA also showed different kinetics in the same patient. These data suggest that an order of events may take place in which strong primary responses support development of secondary responses. Since T cells are often isolated from single samples, it is important to realize that the relative T-cell frequencies as detected in the sample of choice may not reflect the actual contribution of the T cells during the overall *in vivo* immune response.

In vitro stimulation of peripheral blood or bone marrow samples using pre-alloSCT patient cells may synchronize T cells that are in different phases of the immune response. This may identify a more comprehensive repertoire of MiHA-specific T cells with naïve and memory phenotypes. Moreover, *in vitro* stimulation with malignant cells may result in isolation of T cells that recognize

MiHA with therapeutic relevance. Using *in vivo* activation marker HLA-DR, we also isolated MiHA specific T-cell clones that produced low levels of IFN-γ in response to patient EBV-LCL. These T cells may have been missed in experiments in which T cells are isolated by secretion of IFN-γ. We previously demonstrated that in addition to IFN-γ secretion, expression of CD137 and CD154 can be used to detect alloreactive T cells[4]. Compared to IFN-γ secretion, these markers are expressed for a prolonged time, which facilitates more efficient T-cell isolation. The relative *in vivo* abundance of MiHA specific T cells may however be lost following *in vitro* stimulation.

It has been suggested in various studies that *ex vivo* analysis of peripheral blood may not reliably represent alloimmune responses that take place *in situ*[5,6]. It is indeed likely that peripheral blood mainly serves to transport immune cells throughout the body, while initiation of immune responses occurs in lymphoid organs and immune cells exert their effector function in other tissues. In a limited number of patients with leukemia, we compared simultaneously taken bone marrow and peripheral blood samples, but did not observe significant discrepancies in frequencies of MiHA-specific T cells between both compartments (data not shown).

MiHA discovery strategies

Genetic polymorphisms are transcribed into mRNA and are subsequently translated into amino acid polymorphisms that can be presented as antigenic peptides by HLA class I or II surface molecules. Different approaches have been developed to discover MiHA. HA-1 and HA-2, which are the first MiHA that have been identified, were discovered by mass-spectrometric analysis of peptide pools after elution from immuno-precipitated HLA molecules and HPLC fractionation. This method has successfully been applied to discover LB-ADIR-1F as described in chapter 2. In contrast to HLA class I restricted antigens, HLA class II restricted antigens are difficult to identify by mass spectrometry probably due to low surface expression of the antigen and the presence of peptide length variants that can be bound by HLA class II molecules. HLA class II restricted MiHA have, however, successfully been identified by screening cDNA expression libraries[7,8]. By this method, pools of plasmids containing cDNA from MiHA^{pos} cells are expressed as protein pools by bacteria. Lysed bacteria are exogenously loaded on HLA class II of MiHA^{neg} EBV-LCL and tested for recognition by the MiHA-specific CD4 T-cell clone. Similarly, for screening of

CD8 T-cell clones, MiHA encoding cDNA can be identified in plasmid pools after transfection in COS-7 cells for endogenous presentation by HLA class I. This method resulted in discovery of several HLA class I MiHA, including LB-TRIP10-1EPC as described in chapter 4. In both mass spectrometric analysis of HPLC peptide pools and cDNA library screening, T-cell clones need to carry high affinity TCR and possess an avidity that is sufficient for recognition of low antigen levels in peptide or cDNA pools. Moreover, large numbers of T cells are required thereby limiting these approaches to T-cell clones that can be expanded to large numbers.

Mass spectrometric analysis of HPLC peptide fractions and cDNA library screening as described above also allow identification non-polymorphic antigens that are recognized by alloreactive T cells in mismatched HLA-molecules[9] or tumor antigens that result from somatic mutations or overexpression[10]. Still, these approaches are very laborious. For high-throughput identification of MiHA, the more global approach of whole genome association scanning (WGAs) proved to be more successful. WGAs identifies the MiHA-encoding SNP by testing T-cell reactivity against a panel of EBV-LCL containing both $MiHA^{pos}$ and $MiHA^{neg}$ cells. In this thesis, we demonstrated the high-throughput capacity of WGAs by discovery of 25 novel HLA class I restricted MiHA (10 in chapter 3, 3 in chapter 4 and 12 MiHA in chapter 6). For WGAs, a panel of 80 HLA-A*02 and B*07 expressing EBV-LCL was assembled, frozen and stored in microtiter plates. Using micro-array techniques, one million SNPs were measured for each EBV-LCL. Reactivity of MiHA specific T-cell clones against the EBV-LCL panel was tested and MiHA-encoding SNPs have been identified by comparing patterns of recognition with patterns of SNP distribution. As WGAs has demonstrated to be an efficient method for MiHA discovery using the endogenously expressed HLA-A*02 and B*07, we expanded the method towards additional HLA class I restriction molecules by batch-wise retroviral transduction as described in chapters 4 and 6.

Detection of MiHA-encoding SNP by WGAs depends on segregation of $MiHA^{pos}$ and $MiHA^{neg}$ test cells. In chapter 3 we observed that despite strong segregation, WGAs fails to detect associating SNP in some cases. It is required that the MiHA-encoding SNP, or a SNP that is in linkage disequilibrium with the MiHA-encoding SNP, is genotyped. Our panel of 80 EBV-LCL was genotyped for one million SNP and therefore still lacks many genotypes of the 38 million SNPs as estimated to be present in human genomes. Currently, data of the

'1000-Genomes' project are available which contain the complete SNP landscape of the human genome[11]. EBV-LCL used for the 1000-Genomes project are available and can thus be used to assemble new panels for WGAs[12].

WGAs may also identify SNP in intron regions that are involved in mRNA splicing. If MiHA are encoded by alternative transcripts, it may be difficult to identify the MiHA peptide. Various reports demonstrated that MiHA can be encoded by alternative transcripts[13,14]. Alternative transcripts may explain why in some cases significant association with SNP in noncoding gene regions has been found, but no MiHA peptide could be resolved (chapters 3 and 6). In one case, we identified an associating SNP in a genomic region that is unknown to contain a functional gene. The associating SNP was located on chromosome 16 at the border of a 500kbp 'gene desert' 26 kbp from the nearest known gene. Translation of the associating SNP in different reading frames revealed a peptide with strong predicted binding to HLA-B*07:02. We demonstrated recognition of this peptide by the T-cell clone and designated the MiHA as LB-C16ORF-1R. These data demonstrate that T cells can recognize polymorphic structures encoded or generated by SNP in alternative transcripts in which intron regions are retained or by SNP in transcripts derived from genomic regions that are unknown to contain a functional gene.

An important factor that influences the power of WGAs is the frequency of the MiHA-encoding SNP in the panel of test cells. The chance to detect SNP by WGAs is optimal if MiHA frequencies range between 10-90%. To identify MiHA with frequencies outside this range, the panel of test cells should be enlarged, thereby enabling discovery of the full repertoire of MiHA as targeted by *in vivo* immune responses in patients after alloSCT (and DLI). For discovery of targets with therapeutic value, however, MiHA with low or high frequencies are less relevant.

MiHA discovery revealed that SNP can generate immunogenic peptides in different ways. First, SNP can be transcribed into mRNA and directly encode amino acid changes in peptides that are recognized by T cells. In addition, SNP as present in primary gene transcripts can be translated in alternative reading frames or encode immunogenic peptides when located in 5' or 3' untranslated regions. Furthermore, SNP can encode amino acid changes in peptides that influence proteasomal cleavage of the parental protein. Finally, SNP can be located in introns and modify RNA processing, thereby generating *de novo*

splice variants that encode immunogenic peptides. To facilitate high throughput identification of SNP involved in RNA splicing, all transcripts as expressed by MiHA^{neg} and MiHA^{pos} test cells can be analyzed in RNA sequence data. RNA sequencing on EBV-LCL from the 1000-Genomes project has been performed by the Geuvadis consortium[15]. Combining these data with whole genome SNP data from the 1000-Genomes project allows analysis of all SNPs present in the human genome by WGAs and analysis of the transcripts in MiHA^{neg} and MiHA^{pos} cells. As such, this combined strategy may provide a powerful tool for future discovery of MiHA.

Reversing the MiHA-discovery approach

Above described 'forward' approaches for MiHA discovery rely on established T-cell clones that have been isolated from *in vivo* immune responses. The presence of the T-cell clone proves both the antigenic capacity of the MiHA and the existence of MiHA-specific T-cells. A disadvantage of the 'forward' approach however, is that many isolated T-cell clones recognize broadly expressed MiHA that thereby lack therapeutic value. As an alternative, MiHA discovery can start by first selecting genes with leukemia- or hematopoiesis-restricted expression. Next, these genes are assessed for the presence of polymorphic amino acids, and peptides spanning these polymorphisms can be tested for HLA-binding, leading to a panel of MiHA candidates[16]. As a validation step for HLA presentation, MiHA candidates can be searched in databases of eluted peptides, which drastically reduces the number of MiHA candidates[17,18]. Confirmation of MiHA immunogenicity requires detection of high-avidity T cells in MiHA^{neg} individuals using HLA-peptide tetramers. Previous experiments showed that tetramers can bind to T-cells with weak functional reactivity against endogenously processed and presented MiHA. This can be overcome by assessing 'structural avidity' which measures the dynamics of dissociation of the HLA-peptide ligand from the TCR, thereby allowing identification of functionally relevant T cells[19]. Due to these technical advances, this 'reverse' approach has become increasingly attractive as a tool for MiHA discovery.

MiHA tissue distribution

The *in vivo* effect of alloreactive T cells is likely to depend on the tissue distribution of each individual MiHA as targeted in the immune response after alloSCT and DLI. The tissue distribution of MiHA can be assessed by measuring T-cell recognition of cells of various tissues. This analysis requires

expression of the relevant HLA-restriction molecule as well as presence of the MiHA-encoding SNP by the test cells. Due to different population distributions of MiHA-encoding SNP, specific panels of tissue cells need to be collected for analysis of each MiHA. As non-hematopoietic cell type, FB can be relatively easily collected and cultured from patient derived skin biopsies. As such, MiHA specific T-cell clones isolated from a patient can be analyzed for reactivity to autologous patient FB. Whereas absence of recognition of skin FB does not exclude T-cell reactivity against non-hematopoietic cells of other tissues, it allows initial separation of hematopoiesis-restricted MiHA from ubiquitously expressed MiHA, as shown in chapters 4, 5 and 6.

In addition to the MiHA expression level on target cells, T-cell recognition may also depend on the affinity of the TCR that is expressed by the T-cell clone that is available for testing. It can be expected that T-cell clones isolated from *in vivo* clinical immune responses express high-affinity TCR. However, recognition of target cells that lack sufficient expression of ligands required for costimulation and adhesion may be low or undetectable. As such, results as to whether a MiHA is targeted during GvHD may be biased by the T-cell clone used for analysis.

An alternative approach to estimate involvement of MiHA in GvL and GvHD is quantification of MiHA-encoding gene transcripts. The main advantage of this approach is that cells from various tissues can be collected and analyzed independently of the presence of specific HLA alleles and MiHA-encoding SNP. Expression of single genes can be quantified by real-time PCR, whereas transcriptome-wide analysis can be performed using micro-array based platforms. In both techniques, expression of MiHA-encoding genes can be compared between malignant cells, normal hematopoietic cells and normal tissue cells. This approach does however not allow comparison of different gene transcripts within one cell type, due to different primer- and probe-binding efficiencies per gene. Furthermore, MiHA may be encoded by (rare) splice variants that are not detected. Although these problems may be solved by RNA sequencing as advanced technique for transcriptome analysis, gene transcripts may still vary in efficiency to encode proteins, and antigens may differ in their processing and presentation behavior. In chapter 6 of this thesis, we compared gene expression of MiHA-encoding genes with T-cell recognition of hematopoietic and non-hematopoietic cells. We found that absence of MiHA recognition on FB correlated with hematopoiesis-restricted MiHA-encoding gene

expression. Strong FB recognition correlated with ubiquitous expression of the MiHA-encoding genes. Interestingly, we also isolated T cells that failed to recognize FB, whereas the MiHA-encoding genes seemed to be ubiquitously expressed. Lack of FB recognition may be explained by expression of intermediate affinity TCR by the T cell combined with low MiHA expression levels and insufficient expression of adhesion and costimulatory molecules. Future experiments are required to investigate whether these TCR and/or MiHA are valuable to induce GvL reactivity without GvHD after alloSCT.

In this thesis we show that post-DLI immune responses can be induced in the presence of low levels of patient hematopoietic cells. These responses are most likely induced by residual patient professional APC, which is supported by the observation that MiHA have been identified that are exclusively expressed by mature DC. T cells for LB-EBI3-1I[20] and LB-TRIP10-1EPC[21] have been isolated from patients suffering from CML, but expression of *EBI3* and *TRIP10* was undetectable in CML cells. In contrast, significant expression of these genes was found in mature DC. Similar to this, in chapter 5, LRH-1 specific T cells were detected in a patient with RCC after HLA-matched alloSCT and DLI[22]. The P2RX5 gene that encodes LRH-1, shows restricted expression in B cells, which is in line with B-cell specific recognition by LRH-1 specific T cells. These cases illustrate that patient residual professional APC and B cells elicited *in vivo* T-cell responses that not directly mediated GvL or GvT reactivity or GvHD. These responses may however have contributed indirectly to the immune response by creating inflammatory conditions that initiated or amplified other T cell reactivities.

As described above, T cells with specific reactivity against mature DC have successfully been isolated based on recognition of patient, but not donor, EBV-LCL. To investigate whether T cells exist that exclusively recognize mature DC and lack reactivity against patient EBV-LCL, we screened all T-cell clones from selected patients against mature monocyte-derived DC and EBV-LCL. In all cases, recognition of mature DC was accompanied with reactivity against EBV-LCL, suggesting that EBV-LCL are equally ‘professional’ in presenting antigens as mature DC (chapter 6). Furthermore, since professional APC can cross-present antigens derived from non-hematopoietic cells, T-cells may be activated that target antigens that are not presented by other hematopoietic cells. Since our strategy to identify MiHA is predominantly based on recognition of hematopoietic cells, we may have missed this type of reactivity. In chapter 6, for

a limited number of patients who developed severe GvHD after DLI, all growing T-cell clones were tested for recognition of fibroblasts (FB). We never observed FB recognition without recognition of hematopoietic test cells. This suggests that targeting of MiHA that are exclusively expressed by tissue cells and not by hematopoietic cells is rare.

Influence of the micro-environment on T-cell recognition of MiHA

During pathogenic infections, tissue damage and inflammation cause maturation of professional APC and presentation pathogen-derived antigens to T and B cells. AlloSCT conditioning regimens also induce tissue damage followed by cytopenia and infections during the early post-alloSCT period. Therefore, to predict the role of MiHA in GvHD, T-cell reactivity to non-hematopoietic cells should be analyzed under similar conditions. This is supported by 2 cases of severe GvHD that occurred after DLI in patients who were treated with HLA-DP mismatched alloSCT[23]. In these patients, CMV infections early after alloSCT induced cytokine release by the T-cells involved in the anti-viral response. These cytokines subsequently induced HLA-DP alloantigen expression on non-hematopoietic cells that enabled targeting by alloreactive donor T cells.

The pro-inflammatory cytokine IFN-γ is known to up-regulate molecules involved in antigen processing (*PSMB8* and *PSMB9*) and peptide transport into the endoplasmic reticulum (*TAP1* and *TAP2*). Furthermore, IFN-γ increases surface expression of HLA and adhesion and costimulatory molecules[24]. As a result, higher MiHA presentation and increased T-cell avidity are expected to lower the threshold for T-cell activation resulting in targeting of MiHA that are not recognized under steady-state. This is demonstrated in chapter 6, where we characterized various MiHA encoded by genes with ubiquitous expression profiles. A substantial number of these MiHA however, were only recognized after FB activation by IFN-γ. Addition of TNF-α and IL-4 in some cases resulted in recognition that was not observed in the presence of IFN-γ alone, suggesting that synergistic effects may further lower the threshold for T-cell recognition (data not shown). In chapter 5, we isolated T cells specific for LB-FUCA-1V from a patient with RCC who suffered from extensive chronic GvHD after HLA matched alloSCT and DLI. Similar as described above, LB-FUCA-1V specific T cells were capable of recognizing FB only after pre-treatment with IFN-γ.

It is evident that T-cell recognition is dependent on multiple factors. Whereas our *in vitro* data may explain some *in vivo* observations, both T cell and stimulator cell variables complicate the translation of single laboratory parameters to clinical effects.

Cellular therapies to treat recurring disease after alloSCT

Clinical observations indicate that delayed infusion of donor lymphocytes after T-cell depleted alloSCT can induce GvL reactivity in the absence of GvHD. This can be explained by the presence of limited numbers of residual patient APC at the time of DLI and a less inflammatory micro-environment. However, in the majority of patients as described in this thesis, GvL reactivity after DLI was accompanied with GvHD. As described above, the occurrence of viral infections early after alloSCT can trigger development of GvHD[23]. This illustrates that environmental factors can strongly influence the balance between GvL reactivity and GvHD. Further investigation should focus on how to manipulate this balance.

In addition to the micro-environment, the specificity of responding T cells is likely to play a role in GvHD development. Various strategies have been developed to separate potentially harmful from beneficial T-cells. One strategy that has been proposed to prevent GvHD is selective transfer of memory T cells. As compared to unseparated DLI, the TCR repertoire of the memory T-cell compartment is limited, which limits the repertoire of alloantigens that can be recognized. However, *in vivo* activation of memory T cells may be obtained at lower antigen presentation levels, which may increase the risk of targeting of non-hematopoietic tissues. It seems therefore unlikely that by selecting a subset of T cells more selective GvL reactivity can be obtained[25].

Another strategy that can be followed to enhance immune reconstitution without GvHD is selective infusion of CD4 T cells from the donor. The rationale for this therapy is that HLA class II is predominantly expressed on normal and malignant hematopoietic cells, but not on non-hematopoietic tissues. Administration of CD4 T-cell DLI may therefore prevent GvHD while preserving GvL[26,27]. Preclinical models demonstrated that purified CD4 cells are indeed capable to control tumor growth[28]. Human studies also confirm that CD8 depleted DLI after HLA-matched alloSCT can induce GvL reactivity in the absence of severe GvHD[27,29]. In our laboratory, we isolated various MiHA-

specific CD4 T-cell clones from patients responding to alloSCT and DLI. *In vitro* studies demonstrated that these CD4 T-cell clones are capable of recognizing leukemic cells, whereas they often fail to react with FB even after HLA class II up-regulation by inflammatory cytokines. These data illustrate that alloreactive CD4 T cells may induce GvL reactivity after alloSCT without GvHD[8]. However, we also demonstrated that CD4 DLI in patients treated with HLA-DP mismatched alloSCT can lead to severe GvHD. A substantial number of CD4 T-cell clones for mismatched HLA-DP molecules from these patients were shown to recognize HLA class II positive FB *in vitro*, illustrating that although the risk may be lower for CD4 DLI than for unmanipulated DLI, GvHD can still develop if T-cell reactivity exceeds a critical threshold.

In addition to phenotypic characteristics, donor T cells for infusion can be selected by functionality. Alloreactive T cells can be selected for reactivity against leukemic cells or other patient-derived hematopoietic cells or, alternatively, T cells can be removed based on reactivity to non-hematopoietic cells. In our department, a clinical study is ongoing in which patients are treated with alloreactive T cells that respond to leukemic cells using activation markers for selection. Although this strategy may augment GvL reactivity, T cells are not selected for lack of reactivity against non-hematopoietic cells and development of GvHD can therefore not be excluded. In another clinical study, patients are treated with alloreactive T cells that have been selected *in vitro* for lack of reactivity against patient FB[30]. Although this strategy may reduce the risk for GvHD, T-cell reactivity against other non-hematopoietic cell types was not tested, which may explain the frequent occurrence of pulmonary toxicity. Moreover, in this clinical study, T-cell reactivity against cytokine pre-treated FB was not tested, and it has been clearly demonstrated in this thesis that MiHA encoded by broadly expressed genes are often recognized on FB only after pre-treatment with IFN-γ.

Separation of GvL reactivity from GvHD by abovementioned strategies skews the repertoire of donor T cells to desired anti-tumor immunity without defining the exact molecular nature of the antigens that are targeted. Therefore, T cells can be infused that recognize well-defined hematopoiesis or leukemia specific MiHA. Various strategies can be followed for *in vitro* production of such donor T cells for adoptive transfer. Donor T cells for hematopoiesis-restricted MiHA can be isolated from the DLI by HLA-peptide multimers. However, frequencies of MiHA-specific T cells in the naïve donor repertoire are extremely low and it

remains therefore difficult to isolate sufficient numbers of high-avidity T cells capable of recognizing leukemic cells for adoptive transfer[31].

Another strategy is gene transfer of TCR recognizing hematopoiesis-restricted MiHA. These TCR can be isolated from established T-cell clones that have been selected for selective and strong reactivity against leukemic cells. In our department, a clinical study has been started in which patients are treated with donor T cells that have been genetically engineered with the TCR for HA-1, which is a well-defined hematopoiesis restricted MiHA[32]. The HA-1 TCR is transferred to EBV- and CMV-specific T cells, which strongly limits the TCR-repertoire that is expressed by the infused donor cells. This reduces the risk for unknown reactivity by pairing of one of the introduced TCR chains with an endogenous TCR chain. Furthermore, EBV and/or CMV reactivation after alloSCT may lead to expansion of the genetically modified donor T cells via triggering of the endogenous TCR, thereby providing sustained anti-tumor immunity. Due to the population distribution of both the HLA-restriction allele and MiHA-encoding SNP, a panel of MiHA to treat all patients by TCR-gene transfer is required. HA-1, for example, is presented by HLA-A*02:01 which is expressed by 50% of Dutch individuals. HA-1 targeted therapy requires the combination of an HA-1 positive patient (~60%) and an HA-1 negative donor (~40%). In unrelated alloSCT, 60% x 40%= 24% of patient-donor pairs meet this criterion resulting in eligibility of only 12% of all transplanted patients. In related alloSCT, the chance to find a $MiHA^{neg}$ sibling donor for a $MiHA^{pos}$ patient is even lower. To cover the majority of individuals in the Caucasian population, it has been suggested that T-cell therapy should focus on targeting MiHA in the 5 most common HLA-A alleles A*01, 02, 03, 11 and 24[33].

In this thesis, we identified two MiHA (LB-SWAP70-1Q, LB-ARGHDIB-1R[34]), which may have therapeutic value to augment GvL reactivity after alloSCT without GvHD. Both MiHA are encoded by hematopoiesis-restricted genes and their specific T cells failed to recognize FB. In addition, we discovered three MiHA with potential therapeutic value (LB-TTK-1D, LB-CCL4-1T and LB-C16ORF-1R). T cells specific for these MiHA showed hematopoiesis-restricted recognition, but expression profiles for the MiHA-encoding genes still need to be established.

We demonstrated that large numbers of T-cell clones could be isolated from *in vivo* immune responses by HLA-DR expression. By screening for reactivity

against test cells derived from different tissue types, T-cell clones have been identified that either recognized hematopoietic MiHA or MiHA that are ubiquitously expressed. For selective identification of therapeutic MiHA, other strategies may be more efficient in which T cells are activated *in vitro* by stimulation with patient-derived normal or malignant hematopoietic cells. Moreover, during first screening of growing T-cell clones, lack of reactivity against patient FB can be tested, thereby increasing the chance to discover MiHA with therapeutic value.

AlloSCT as treatment modality for solid tumors

AlloSCT and DLI for treatment of hematological malignancies demonstrated the potential of donor T-cells to mediate immune reactions resulting in long-term clinical remissions. This initiated several clinical trials that applied alloSCT as treatment of patients with solid, non-hematopoietic tumors. Graft-*versus*-tumor (GvT) reactions were observed in various patients, although at the cost of GvHD in most cases. This was also observed in a patient with RCC as described in chapter 5, who showed tumor regression and long-term stable disease at the cost of severe acute and extensive chronic GvHD. Evidence was obtained that induction of strong alloimmunity in this patient depended on the presence of residual APC or other hematopoietic cells of patient origin as detected by mixed chimerism at the time of the first DLI, whereas escalating doses of DLI in the presence of full donor chimerism failed to elicit an alloimmune response.

Since GvT-reactivity and GvHD are strongly associated in patients with solid tumors and the presence of residual patient APC seems to be crucial for efficient induction of alloimmunity, we investigated in chapter 5 the number of genes with shared expression between professional APC and RCC by micro-array gene expression analysis. This analysis showed that the number of genes with shared expression between APC and RCC are significantly lower than between APC and leukemic cells, illustrating that the chance that T cells are induced with reactivity to RCC is low. Moreover, the majority of genes that are shared between APC and RCC are also expressed in FB, demonstrating that T cells with reactivity to RCC are likely to recognize healthy non-hematopoietic tissues.
These data are in line with clinical observations that GvL reactivity can be separated from GvHD in patients with hematological malignancies, but that

selective GvT reactivity in the absence of GvHD is difficult to achieve in patients with non-hematopoietic malignancies.

The advantage of MiHA directed TCR-gene therapy is that donor T cells acquire their specificity by genetic modification and therefore do not depend on *in vivo* activation by professional patient APC. Broad application of TCR gene therapy to target non-hematopoietic MiHA on solid tumors after alloSCT requires identification of multiple tumor-specific MiHA with balanced population frequencies in common HLA molecules. The chance that sufficient numbers of such MiHA can be identified, however, is low. It may therefore be more attractive to treat RCC using TCR or chimeric antigen receptors (CAR) that target non-polymorphic tumor antigens. In CAR therapy, the antigen-binding site of an antibody that recognizes a cell surface antigen is introduced into T cells by gene transfer. Clinical studies have showed that patients suffering from chemotherapy-refractory chronic lymphocytic leukemia or relapsed B-ALL can effectively be treated with autologous T cells transduced with a CAR for a non-polymorphic CD19 cell surface antigen[35]. Additionally, patients suffering from solid tumors have successfully been treated with autologous T cells that were transduced with a TCR for a non-polymorphic intracellular NY-ESO-1 antigen[36]. If non-polymorphic antigens with selective expression on RCC can be targeted, these therapies may be preferable above alloSCT.

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Nederlandse samenvatting

Allogene stamceltransplantatie

Patiënten met hematologische maligniteiten kunnen effectief behandeld worden met allogene stamceltransplantatie (alloSCT). De intensieve voorbehandeling die erop gericht is maligne cellen te vernietigen brengt ook ernstige schade toe aan de normale bloedvorming waardoor het nodig is om gezonde hematopoëtische stamcellen van een donor te transplanteren. Deze groeien vervolgens uit in de patiënt en nemen de vorming van alle typen bloedcellen over. In het stamceltransplantaat bevinden zich naast de stamcellen echter ook rijpe cellen van het immuunsysteem van de donor, zoals T-lymfocyten (T-cellen). Een ernstige bijwerking van allogene stamceltransplantatie is de graft-*versus*-host (GvH)-ziekte, waarbij normale weefsels worden aangevallen door onder andere T-cellen van de donor. De ontwikkeling van GvH-ziekte gaat meestal ook gepaard met een krachtige graft-*versus*-leukemie (GvL)-reactie van donor T-cellen tegen achtergebleven maligne cellen, en zorgt voor langdurige onderdrukking van de maligniteit. Verwijdering van T-cellen uit het stamceltransplantaat kan de GvH-ziekte voorkomen, maar het vermindert ook de gewenste GvL-reactie. Het is gebleken dat een uitgestelde donor lymfocyten infusie (DLI) enkele maanden na de alloSCT de gewenste GvL-reactie kan veroorzaken zonder dat daarbij altijd ernstige GvH-ziekte optreedt. We nemen aan dat dit komt omdat door alle voorbehandelingen op het moment van de transplantatie de omstandigheden in de patiënt, zoals weefselschade en infecties, zodanig veranderd zijn dat donor T-cellen gemakkelijk geactiveerd worden. Bij de uitgestelde DLI kan de situatie in de patiënt in zekere mate weer genormaliseerd zijn waardoor wel een GvL-reactie ontstaat maar de kans op GvH-ziekte sterk afneemt.

Transplantatie antigenen

Het is bekend dat bij transplantatie van organen de weefseltypering van donor en ontvanger zo veel mogelijk gelijk moet zijn om het risico op afstoting zo klein mogelijk te maken. Het kiezen van een donor die volledig identieke ‘humane leukocyten antigenen’ (HLA) heeft kan echter niet voorkomen dat afstotingsreacties optreden. De oorzaak hiervan zijn ‘minor histocompatibiliteits antigenen’ (minors). Dit zijn fragmenten van eiwitten, ofwel peptiden, die verschillend kunnen zijn tussen individuen ten gevolge van genetische varianten ofwel ‘single nucleotide polymorphisms’ (SNPs). Als peptiden binden

aan HLA-moleculen en worden gepresenteerd aan het immuunsysteem gedragen zij zich als antigenen en kan een afweerreactie van T-cellen op gang komen. Er zijn 2 typen HLA-moleculen te onderscheiden: HLA klasse I dat op alle kernhoudende cellen tot expressie komt en kan worden herkend door CD8 T-cellen, en HLA klasse II dat vooral op hematopoëtische cellen tot expressie komt en kan worden herkend door CD4 T-cellen. Bij alloSCT als behandeling van leukemie kunnen T-cellen van de donor die minors herkennen die op patiënt leukemiecellen tot expressie komen een GvL-reactie geven. Als er echter ook minors herkend worden op normale weefselcellen, kan dat GvH-ziekte veroorzaken.

Immunotherapie

Ons immunologisch onderzoek in het kader van alloSCT en DLI richt zich op de identificatie en karakterisering van minors. Omdat gebleken is dat T-cellen die minors op leukemiecellen herkennen GvL-reactiviteit en dus genezing kunnen geven, wordt cellulaire immunotherapie ontwikkeld met T-cellen die minors herkennen. Het is hiervoor van belang om de mechanismen te begrijpen die ten grondslag liggen aan het verschil tussen GvL-reactiviteit en GvH-ziekte. Het is waarschijnlijk zo dat niet alleen de expressie van de minor zelf, maar ook de omstandigheden waaronder dit plaatsvindt bepalen of zich GvH-ziekte zal ontwikkelen. Minors ontstaan door SNPs en gedragen zich als antigenen omdat ze in het ene individu wel, en in het andere individu niet voorkomen. Daarnaast komt een minor tot expressie in een specifiek HLA molecuul. Zowel SNPs als HLA moleculen hebben een verdeling in de bevolking en binnen een donor-patiënt paar kan een immuunreactie alleen plaatsvinden als de donor minor^{neg} en de patiënt minor^{pos} is. Om cellulaire immunotherapie beschikbaar te maken voor de meeste patiënten is het daarom noodzakelijk om een aantal minors in verschillende HLA moleculen ter beschikking te hebben.

In **hoofdstuk 2** beschrijven we de ontdekking van een minor die herkend werd door een groot aantal CD8 T-celklonen, verkregen uit een patiënte die succesvol werd behandeld voor ziekte van Kahler met alloSCT en DLI. Van een groot aantal gekweekte cellen die herkend werden door deze T-celklonen hebben we de HLA-gebonden peptiden geïsoleerd. Op basis van biochemische eigenschappen werden de peptiden gescheiden. De verkregen peptide-fracties werden getest op hun vermogen om testcellen van de donor, die eerst niet herkend werden door de T-celkloon, gevoelig te maken voor herkenning. Door middel van massaspectrometrie konden we de sequentie van het antigene

peptide oplossen, dat afkomstig bleek te zijn van het ATP-dependent interferon responsive (*ADIR*) gen. Een SNP in dit gen, aanwezig bij de patiënt en afwezig bij de donor, zorgde voor een aminozuurverandering in het peptide waardoor donor T-cellen het antigeen herkenden. Recombinant eiwit technieken maken het mogelijk om peptide-HLA complexen te produceren. Polymeren van deze complexen (tetrameren) kunnen gekoppeld worden aan een fluorescerende stof en worden gebruikt als kleurstof om minor-specifieke T-cellen aan te tonen. Met behulp van tetrameren zagen we ruim 2% LB-ADIR-1F specifieke T-cellen in het bloed van de patiënte op het moment dat aantoonbaar de ziektemarker voor Kahler (paraproteïne) daalde. LB-ADIR-1F specifieke T-cellen vertoonden sterke herkenning van verschillende typen leukemiecellen en ook van cellijnen afkomstig van solide tumoren. Niet-hematopoëtische cellen die positief waren voor de LB-ADIR-1F coderende SNP werden zwak herkend, en ook het niveau van genexpressie was laag. Dit betekent dat LB-ADIR-1F specifieke T-cellen in beperkte mate specifiek zijn voor hematopoëtische cellen, maar onder bepaalde omstandigheden ook GvH-ziekte kunnen veroorzaken.

Het karakteriseren van minors volgens de techniek zoals hierboven beschreven geeft een helder inzicht in het verband tussen de SNP en het ontstaan van de minor. Het is echter zeer bewerkelijk en daardoor niet geschikt voor het stelselmatig oplossen van grote aantallen minors. In **hoofdstuk 3** laten we zien dat whole genome association scanning (WGAs) dit wel mogelijk maakt, door gebruik te maken van een panel van testcellen waarvan 1 miljoen SNPs zijn gemeten. Na het testen van de herkenning van het gehele panel door een T-celkloon die een onbekende minor herkent, kan dit herkenningspatroon vergeleken worden met de patronen van alle SNPs. Een overeenkomend patroon geeft aan dat deze SNP betrokken is bij het ontstaan van de minor. We hebben WGAs toegepast op 17 minor specifieke CD8 T-celklonen die geïsoleerd waren uit bloedmonsters afgenomen na DLI bij 2 patiënten. Met ons panel van testcellen, dat uit 80 cellijnen bestond, vonden we in 12 gevallen sterke associatie met een SNP. Na de bevestiging dat de associërende SNPs in de patiënt aanwezig, en in de donor afwezig waren, hebben we met behulp van online algoritmes die de bindingssterkte van peptiden in HLA moleculen voorspellen, voor 10 T-celklonen kandidaat peptiden gevonden. Alle voorspelde peptiden werden in meer of mindere mate herkend, wat de juistheid van de WGAs resultaten bevestigde. We vonden minors die direct het gevolg waren van een aminozuurverandering door de associërende SNP. Daarnaast vonden we ook associaties waarbij een andere SNP, die blijkbaar genetisch gekoppeld

was aan de associërende SNP, de minor veroorzaakte. Voor verschillende van de nieuw gekarakteriseerde minors konden we in bloedmonsters van patiënten minor specifieke T-cellen aantonen met tetrameren. Dit laat zien dat WGAs een zeer geschikte methode is om snel minors te karakteriseren.

In **hoofdstuk 4** beschrijven we 4 verschillende minor specifieke CD8 T-celklonen die geïsoleerd werden uit een patiënt met chronisch myeloïde leukemie die na alloSCT goed reageerde op DLI. Deze T-celklonen herkenden minors die gepresenteerd werden door HLA-B*40:01. Eén van de minors is gevonden door gebruik te maken van een 'library cDNA' techniek, en 3 minors door het toepassen van WGAs. Om dit te kunnen doen hebben we eerst de testcellen van het WGAs panel genetisch gemodificeerd zodat deze het betreffende HLA-B*40:01 molecuul tot expressie brachten. Van de 4 T-celklonen hebben we bepaald of ze verschillende soorten leukemiecellen konden herkennen. Bij één T-celkloon zagen we uitsluitend herkenning van meer uitgerijpte leukemiecellen, terwijl de andere 3 T-celkonen alle typen leukemiecellen konden herkennen. Om de herkenning van niet-hematopoëtische cellen te testen, werd van de patiënt een huidbiopt afgenomen. Hieruit werden zogenaamde fibroblasten gekweekt die we konden gebruiken om de herkenning door de T-celklonen te testen, als model voor het optreden van GvH-ziekte. Hieruit bleek dat geen van de T-celklonen de fibroblasten kon herkennen. Echter, na voorbehandeling van de fibroblasten met interferon-gamma, een stof die geproduceerd wordt bij immuunreacties, vertoonde 1 kloon zeer sterke herkenning, 1 kloon matige herkenning, en 2 klonen zeer zwakke herkenning. Dit laat zien dat tijdens immuunreacties na DLI, reactiviteit tegen een variëteit aan minors kan ontstaan. De meeste T-celklonen konden de leukemiecellen goed herkennen, maar sommigen herkenden minors die door niet-hematopoëtische cellen tot expressie gebracht worden onder condities die voorkomen bij ontstekingsreacties.

Door het verminderen van de intensiteit van de voorbehandeling bij alloSCT, werden op beperkte schaal patiënten met solide tumoren ook allogeen getransplanteerd, met als doel het opwekken van een 'graft-*versus*-tumor'(GvT) reactie. Het leek er echter op dat bij deze patiënten de GvT-reactiviteit zelden optrad zonder GvH-ziekte. In **hoofdstuk 5** beschrijven we een patiënt die leed aan uitgezaaid niercelcarcinoom en werd behandeld met alloSCT en DLI, en bij wie zich 30 dagen na DLI ernstige GvH-ziekte ontwikkelde. Ondanks

behandeling hiervoor ontwikkelde zich de uitgebreide chronische vorm van GvH-ziekte. Tijdens deze GvH-ziekte werd een afname in de grootte van de uitzaaiingen in de longen vastgesteld, en de tumorgroei stabiliseerde. Toen de chronische GvH-ziekte na ruim 3 jaar verdwenen was, verdween echter ook de GvT-reactie en de patiënt overleed 8 jaar na de alloSCT door progressie van de ziekte. Uit een bloedmonster dat was afgenomen bij het ontstaan van de acute GvH-ziekte, isoleerden we geactiveerde CD8 T-cellen. Van een T-celkloon vonden we met behulp van WGAs een sterke associatie met het *FUCA2* gen. Een SNP in dit gen bij de patiënt veroorzaakte een aminozuurverandering, en dit bleek herkend te worden door de T-celkloon. De minor LB-FUCA2-1V werd herkend in niercelcarcinoom cellijnen, maar ook in fibroblasten, zij het na voorbehandeling van de fibroblasten met interferon-gamma. Dit was in overeenstemming met het patroon van genexpressie van *FUCA2*. Om vervolgens in algemene zin het verband tussen GvT-reactiviteit en GvH-ziekte te bestuderen, hebben we gekeken naar de expressie van 40.000 genen, verkregen met micro-array technieken. Het opwekken van een efficiënte immuunreactie is afhankelijk van presentatie van antigenen door professionele antigeen presenterende cellen (APC). Vervolgens kunnen deze antigenen ook herkend worden op andere cellen, zoals tumorcellen (GvT-reactie) en normale weefselcellen (GvH-ziekte). We hebben daarom eerst alle genen geselecteerd die tot expressie kwamen in zowel APCs als in niercelcarcinoomcellen. Binnen deze set aan genen hebben we bekeken hoeveel genen *niet* tot expressie kwamen in fibroblasten en keratinocyten, waarbij we aannamen dat presentatie van minors door deze huidcellen GvH-ziekte tot gevolg kan hebben. Dit leverde slechts een beperkt aantal genen op, zeker als we dit aantal vergeleken met het aantal genen dat we vonden als we dezelfde selectie maakten uit genen met een gedeelde expressie in APCs en leukemiecellen. Hieruit concluderen we dat na alloSCT en DLI de kans klein is om bij niet-hematopoëtische tumoren een GvT-reactie op te wekken zonder daarbij ook GvH-ziekte te veroorzaken.

GvL-reactiviteit die wordt waargenomen na uitgestelde DLI gaat soms niet en soms wel gepaard met GvH-ziekte. Omdat we aannemen dat dit allebei immunologische reacties zijn die veroorzaakt worden door donor T-cellen, hebben we in **hoofdstuk 6** gezocht naar verschillen in de donor T-cel reactie bij 6 patiënten zonder en 5 patiënten met GvH-ziekte na DLI. We hebben eerst de mate van activatie van CD8 T-cellen in het bloed gemeten aan de hand van de marker HLA-DR, die bij geactiveerde T-cellen op het oppervlak komt. We zagen een sterke toename van het absolute aantal HLA-DR^{pos} CD8 T-cellen rond 60

dagen na de DLI. Er was echter geen verband tussen deze stijging en het optreden van GvH-ziekte. Om te verrijken voor alloreactieve T-cellen in de bloedmonsters, hebben we HLA-DR^{pos} CD8 T-cellen gesorteerd en vervolgens gekloneerd. Uit alle groeiende T-celklonen hebben we alloreactieve T-celklonen geselecteerd die cellen van de patiënt wel, en die van de donor niet herkenden. Deze alloreactieve T-celklonen waren bij patiënten met GvH-ziekte in sterk verhoogde mate aanwezig. Vervolgens hebben we de herkenning van hematopoëtische cellen getest, als model voor GvL-reactiviteit. We vonden geen verschil tussen de T-celklonen die geïsoleerd waren uit het bloed van patiënten zonder en met GvH-ziekte, wat overeenkomt met de waarneming dat in de patiënten uit beide groepen GvL-reactiviteit opgetreden was. Om de herkenning van niet-hematopoëtische fibroblasten te testen, als model voor het optreden van GvH-ziekte, werd van alle patiënten een huidbiopt afgenomen. Enkele T-celklonen uit patiënten zonder GvH-ziekte herkenden de fibroblasten zwak, terwijl we sterkere fibroblasten herkenning zagen door T-celklonen uit patiënten met GvH-ziekte. Voorbehandelen van de fibroblasten met interferon-gamma, versterkte de reactiviteit tegen de fibroblasten vooral door de T-celklonen van patiënten met GvH-ziekte. Deze studie toont aan dat ook in patiënten zonder GvH-ziekte alloreactieve T-cellen aantoonbaar zijn op het moment van de klinische GvL-reactie. Het aantal circulerende alloreactieve T-cellen is echter sterk toegenomen bij patiënten met GvH-ziekte waardoor mogelijk een sterkere algemene activatie van het immuunsysteem plaatsvindt. Hierdoor, en door de aanwezigheid van T-celklonen die ook niet-hematopoëtische cellen kunnen herkennen, kan GvH-ziekte ontstaan.

In dit proefschrift laten we detailleerde studies zien van klinische immuunresponsen die optreden na alloSCT en DLI. We pasten methoden toe om de moleculaire structuur van de antigenen op te helderen. De technieken om dit op grote schaal te doen hebben we ontwikkeld en geoptimaliseerd waardoor we een representatief beeld krijgen van T-cel gemedieerde *in vivo* immuunresponsen. De verschillen die we aangetoond hebben tussen GvL-reactiviteit en GvH-ziekte kunnen uiteindelijk leiden tot het ontwikkelen van therapieën waarbij GvH-ziekte voorkomen kan worden. Hiervoor is het van groot belang dat we minors ontdekken die uitsluitend door leukemiecellen en andere bloedcellen gepresenteerd worden. T-celklonen die deze hematopoiese specifieke minors herkennen kun ingezet worden om zeer gerichte immunotherapie te ontwikkelen.

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Curriculum vitae

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