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Title: Characterization and recognition of minor histocompatibility antigens
Issue Date: 2016-01-13
Chapter 7

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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$^{\text{pos}}$ 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$^{\text{neg}}$ from MiHA$^{\text{pos}}$ 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
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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\textsuperscript{pos} but not in MiHA\textsuperscript{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<sup>pos</sup> 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
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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.
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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 leukemia 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.
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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. However, T cells have also been demonstrated to ‘snatch’ HLA class II from nearby APC through trogocytosis. 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\textsuperscript{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 \textit{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 \textit{in vivo} immune response.

\textit{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, \textit{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. 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. 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. 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 or tumor antigens that result from somatic mutations or overexpression. 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\textsuperscript{pos} and MiHA\textsuperscript{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\textsuperscript{pos} and MiHA\textsuperscript{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
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‘1000-Genomes’ project are available which contain the complete SNP landscape of the human genome. EBV-LCL used for the 1000-Genomes project are available and can thus be used to assemble new panels for WGAs.

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. 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 peptides.
splice variants that encode immunogenic peptides. To facilitate high throughput identification of SNP involved in RNA splicing, all transcripts as expressed by MiHA\textsuperscript{neg} and MiHA\textsuperscript{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\textsuperscript{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\textsuperscript{neg} and MiHA\textsuperscript{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 \textit{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\textsuperscript{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\textsuperscript{17,18}. Confirmation of MiHA immunogenicity requires detection of high-avidity T cells in MiHA\textsuperscript{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\textsuperscript{19}. Due to these technical advances, this ‘reverse’ approach has become increasingly attractive as a tool for MiHA discovery.

MiHA tissue distribution

The \textit{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\textsuperscript{20} and LB-TRIP10-1EPC\textsuperscript{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\textsuperscript{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. 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. 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.
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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. 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.

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. Preclinical models demonstrated that purified CD4 cells are indeed capable to control tumor growth. Human studies also confirm that CD8 depleted DLI after HLA-matched alloSCT can induce GvL reactivity in the absence of severe GvHD. 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. 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. 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\textsuperscript{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\textsuperscript{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\textsuperscript{pos} sibling donor for a MiHA\textsuperscript{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\textsuperscript{33}.

In this thesis, we identified two MiHA (LB-SWAP70-1Q, LB-ARGHDIB-1R\textsuperscript{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 \textit{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.

\textbf{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 \textit{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\textsuperscript{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\textsuperscript{36}. If non-polymorphic antigens with selective expression on RCC can be targeted, these therapies may be preferable above alloSCT.
Summary and General discussion

References


Chapter 7


