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Minor histocompatibility antigens to predict, monitor or manipulate GvL and GvHD after allogeneic hematopoietic cell transplantation

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ABSTRACT

Allogeneic hematopoietic cell transplantation (alloHCT) provides a potential curative treatment for haematological malignancies. The therapeutic Graft-versus-Leukaemia (GvL) effect is induced by donor T cells attacking patient hematopoietic (malignant) cells. However, if healthy non-hematopoietic tissues are targeted, Graft-versus-Disease (GvHD) may develop. After HLA-matched alloHCT, GvL and GvHD are induced by donor T cells recognizing polymorphic peptides presented by HLA on patient cells, so-called minor histocompatibility antigens (MiHAs). The balance between GvL and GvHD depends on the tissue distribution of MiHAs and T-cell frequencies targeting these MiHAs. T cells against broadly expressed MiHAs induce GvL and GvHD, whereas those targeting MiHAs with hematopoietic-restricted expression induce GvL without GvHD. Recently, the MiHA repertoire identified in natural immune responses after alloHCT was expanded to 159 total HLA-I-restricted MiHAs, including 14 hematopoietic-restricted MiHAs. This review explores their potential relevance to predict, monitor, and manipulate GvL and GvHD for improving clinical outcome after HLA-matched alloHCT.

1. Introduction

Allogeneic hematopoietic cell transplantation (alloHCT) provides a potential curative treatment for haematological malignancies [1]. After alloHCT, the patient's blood-forming stem cells are replaced with healthy donor hematopoietic stem cells, providing normal haematopoiesis. The therapeutic Graft-versus-Leukaemia (GvL) effect is induced by mature donor T cells, initiating an attack against patient hematopoietic cells including malignant cells. However, donor T cells may also target healthy non-hematopoietic tissues, provoking potentially life-threatening Graft-versus-Host Disease (GvHD).

To mitigate severe GvHD in unmanipulated grafts, patients require immunosuppressive treatment, which is gradually tapered after alloHCT to achieve a balance between GvL and GvHD. To reduce GvHD risk, donor T cells can be depleted from the graft *ex vivo* or *in vivo* with agents like anti-thymocyte globulin or cyclophosphamide [2]. As T-cell depletion also diminishes GvL reactivity, patients may need donor lymphocyte infusions (DLIs) to establish anti-tumour immunity after alloHCT [3]. T-cell depletion followed by DLI lowers GvHD risk due to reduced patient antigen-presenting cells (APC) at the time of DLI. This facilitates an immune response maintaining GvL reactivity, but minimizing GvHD [4,5].

Since transplantation with HLA-mismatched donors is associated with profound allo-HLA immune responses causing severe GvHD,

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Abbreviations

alloHCT	allogeneic hematopoietic cell transplantation
AML:	acute myeloid leukaemia
APC	antigen-presenting cell
DC	dendritic cell
DLI	donor lymphocyte infusion
EBV-LCL:	EBV-B lymphoblastoid cell line
GTEx	Genotype-Tissue Expression database
GvHD	Graft-versus-Host Disease
GvL:	Graft-versus-Leukaemia
GWAS	genome-wide association study
HPA	Human Protein Atlas
HSPVdb	Human Short Peptide Variation Database
MiHA	minor histocompatibility antigen
pMHC	peptide-MHC
SNP	single nucleotide polymorphism
TCGA	The Cancer Genome Atlas

HLA-matched donors are preferred. After HLA-matched alloHCT, GvL and GvHD responses are induced by donor T cells recognizing minor histocompatibility antigens (MiHAs) [6,7]. MiHAs are polymorphic peptides presented by HLA molecules. The genetic basis for MiHAs lies in differences in single nucleotide polymorphisms (SNPs) between patient and donor, producing peptides with polymorphic amino acids. The balance between GvL and GvHD after HLA-matched alloHCT depends, among other factors, on the tissue distribution of MiHAs on patient cells and donor T-cell frequencies targeting these MiHAs. Most MiHAs are broadly expressed on hematopoietic and non-hematopoietic cells. Donor T cells targeting these MiHAs likely induce both GvL and GvHD. However, a small subset of MiHAs are mainly expressed on hematopoietic cells. T cells targeting these hematopoietic-restricted MiHAs are expected to induce GvL responses without GvHD [8,9].

During treatment with alloHCT and DLI, most patients have no or low detectable tumour cells. Donor T-cell responses after alloHCT are therefore evoked by patient APCs and primarily target healthy patient cells. To evaluate the effectiveness of immune responses, patients are screened for hematopoietic cells of patient and donor origin. Conversion from mixed to full donor chimerism indicates an effective immune response and serves as surrogate for a GvL response.

Between 1995 and 2019, 70 HLA-I-restricted MiHAs were identified using T-cell clones isolated from natural immune responses after alloHCT. These antigens were identified as peptides eluted from HLA-I by mass spectrometry or screening cDNA libraries, but mainly genome-wide association studies (GWAS) [6,7,10]. In GWAS, reactivity of T-cell clones for unknown MiHAs are tested against a panel of SNP-genotyped EBV-B lymphoblastoid cell lines (EBV-LCLs) expressing the HLA restriction alleles of the MiHAs. Based on T-cell recognition, EBV-LCLs are categorized into antigen-positive and -negative groups, and this recognition pattern is analysed for association with SNPs. Associated SNPs are investigated to encode peptides with predicted binding to the relevant HLAs, which are validated as MiHAs if recognized by T-cell clones. In 2020, MiHA-identification by GWAS was optimized using a large panel of EBV-LCLs, which were genotyped in the 1000 Genomes Project [11]. Using this GWAS approach, the repertoire of HLA-I-restricted MiHAs was recently expanded with 89 new antigens to 159 total MiHAs [11,12].

Additionally, a limited number of HLA-II-restricted MiHAs were identified [7,13,14]. These MiHAs are also targeted in immune responses, but their specific impact after alloHCT is beyond the scope of this review.

2. Prediction of GvL and GvHD

Various studies explored the potential of using SNP mismatches in patient-donor pairs to predict GvL and GvHD after HLA-matched alloHCT (Table 1, Fig. 1).

2.1. Predicting GvL and GvHD by SNP mismatches

Martin et al. [15] demonstrated that patients with unrelated donors had twice as many mismatched SNPs as patients with related donors. Each 1% increase in coding SNP mismatches in patients with related donors was associated with a slight increase in GvHD risk. In patients with unrelated donors, however, the GvHD risk was significantly higher, suggesting that increased GvHD risk after HLA-matched alloHCT is primarily due to HLA-DP mismatching [16] rather than an effect of MiHAs. In this study, the total number of coding SNP mismatches were used to measure the degree of relatedness between the patient and donor, and thus the likelihood that MiHAs are targeted on non-hematopoietic tissues. An increased risk of GvHD in patients with unrelated donors compared to related donors does not preclude involvement of MiHAs. In contrast, considering that mismatched HLA-DP alleles are targeted by CD4 T cells [16], increased GvHD risk in patients with unrelated donors may be (partly) attributed to CD8 T-cell responses against HLA-I-restricted MiHAs that are stimulated by helper T cells [17].

Table 1
Overview of studies using potential or confirmed MiHAs to predict clinical outcome.

Study	Year	Investigated individuals/material	Disease	Subject	Methods	Investigated (potential) MiHAs	Association with clinical outcome
Martin et al. [15]	2017	3057 HLA-matched patients with related (n = 1840) or unrelated (n = 1217) donors.	Diverse	SNPs	SNP arrays (Affymetrix 5.0 Human GeneChip, Illumina 1M Quad & Illumina 2.5M BeadArray).	n.a.	Association of each 1% increase in coding SNP mismatches with a slight increase in GvHD risk in patients transplanted with related donors. Significantly higher risk of GvHD in patients transplanted with unrelated donors than in patients transplanted with related donors.
Lansford et al. [19]	2018	101 HLA-matched patients with related (n = 72) or unrelated (n = 30) donors.	AML, CML, MDS, MPN	Predicted MiHAs	SNP arrays (Illumina NS-12 microarrays), predicted HLA-binding by NetMHCpan & tissue expression using bulk RNA-seq data of AML (own data) and healthy tissues (HPA).	On average approximately 800 and 1200 potential HLA-binding MiHAs in patients transplanted with related and unrelated donors, respectively, including approximately 300 and 600 potential GvL-MiHAs and 230 and 460 potential GvHD-MiHAs. GvL-MiHAs defined by gene expression above 50 TPM in AML or bone marrow and below 5 TPM in GvHD target organs (skin, liver, colon); GvHD-MiHAs defined by gene expression above 50 TPM in GvHD target organs.	No association between number of GvL-MiHA mismatches and relapse. No association between number of GvHD-MiHA mismatches and GvHD.
Jadi et al. [22]	2023	2249 HLA-matched patients with unrelated donors.	AML & MDS	Predicted MiHAs	SNP arrays (Illumina Human OmniExpress BeadChip) & predicted HLA-binding by NetMHCpan.	On average 39–40 potential HLA-binding MiHAs per patient.	Association of high number of predicted MiHAs with increased risk of GvHD mortality. Predicted HLA-B*08:01-binding *GSTP1*-encoded MiHA associated with increased GvHD mortality. Predicted HLA-B*40:01-binding *CRISPLD2*-encoded MiHA associated with decreased leukaemia-free survival. Predicted HLA-C*03:04-binding *SERPINF1*-encoded MiHA associated with increased disease-related mortality.
Granados et al. [23]	2014	2 EBV-B cell lines from HLA-identical female siblings.	Healthy	Predicted MiHAs	Whole Exome Sequencing & immunopeptidomics.	10 potential HLA-binding MiHAs in one of two individuals.	n.a.
Hombrink et al. [24]	2013	4 HLA-A*02:01 positive EBV-B cell lines.	Healthy	Predicted MiHAs	Immunopeptidomics & HSPVdb.	23 potential HLA-A*02:01-binding MiHAs.	n.a.

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Table 1 (*continued*)

Study	Year	Investigated individuals/material	Disease	Subject	Methods	Investigated (potential) MiHAs	Association with clinical outcome
Bykova et al. [32]	2018	100 virtual HLA-mismatched patient-donor pairs.	Healthy	Predicted MiHAs	1000 Genomes Project & predicted HLA-binding by NetMHCpan3.0 & MixMHC.	On average 116 and 65 potential HLA-binding MiHAs in virtual patients with unrelated and related donors, respectively. On average 213 potential HLA-binding MiHAs per HLA allele.	n.a.
Granados et al. [25]	2016	13 HLA-A*02:01 and/or B*44:03 positive EBV-B cell lines.	Healthy	Predicted MiHAs	Whole Exome Sequencing, Whole Transcriptome Sequencing, tissue expression using bulk RNA-seq data of AML (TCGA) and healthy tissues (HPA) & immunopeptidomics.	A total of 6773 potential HLA-A*02:01 or B*44:03-binding MiHAs, including 119 frequently mismatched MiHAs, and 39 potential GvL-MiHAs. GvL-MiHAs defined by gene expression above 1 RPKM in AML, at least twofold higher expression in bone marrow than skin and expression below 10 FPKM in 27 other tissues. GvHD-MiHAs defined by gene expression above 10 FPKM in 27 healthy tissues.	n.a.
Olsen et al. [26]	2023	3231 HLA-matched patients with unrelated donors.	AML, ALL & MDS	Predicted MiHAs	SNP arrays (Illumina Human OmniExpress BeadChip), predicted HLA-binding by NetMHCpan, tissue expression using bulk RNA-seq data of AML (TCGA) and healthy tissues (Genotype-Tissue Expression; GTEx), and immunopeptidomics.	On average 1476 potential HLA-binding MiHAs per patient, with 704 potential GvL-MiHAs and 24 potential MiHAs binding to HLA-A*02:01, B*35:01 or C*07:02. GvL-MiHAs defined by gene expression above 50 TPM in AML and below 50 TPM in GvHD target organs (skin, liver, colon); GvHD-MiHAs defined by gene expression below 50 TPM in AML and above 50 TPM in GvHD target organs.	n.a.
Larsen et al. [36]	2010	126 HLA-matched patients with related (n = 70) or unrelated (n = 56) donors.	Diverse	Predicted & known MiHAs	Genotyping for 53 SNPs in 11 genes known to encode at least one MiHA using the GenomeLab SNPstream genotyping system (Beckman Coulter) & predicted HLA-A- and HLA-B-binding by NetMHCpan.	26 SNPs in 6 MiHA-encoding genes mismatched in the GvH direction. A total of 215 potential HLA-A- or HLA-B-binding MiHAs identified in the cohort with a median of 3 MiHAs per patient.	No association between number of SNP mismatches and clinical outcome. Association of more than 3 predicted MiHAs with lower 5-years overall survival and higher treatment-related mortality.
Nie et al. [34]	2021	391 patients with HLA-matched (n = 108) or HLA-mismatched (n = 283) related donors.	Diverse	Predicted & known MiHAs	Targeted Next Generation Sequencing of 35 genes encoding at least one known MiHA.	On average less than one potential HLA-binding MiHA per patient.	No association between (predicted) MiHA mismatches and relapse or GvHD.

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Table 1 (*continued*)

Study	Year	Investigated individuals/material	Disease	Subject	Methods	Investigated (potential) MiHAs	Association with clinical outcome
Martin et al. [18]	2021	1868 HLA-matched patients with related donors including 824 HLA-A*02:01 positive patients (discovery cohort); 838 HLA-A*02:01 positive patients with related donors (validation cohort).	Diverse	Predicted & known MiHAs	SNP arrays (Affymetrix 5.0 Human GeneChip, Illumina 1 M Quad & Illumina 2.5 M BeadArray) (discovery cohort), SNP genotyping by targeted Next Generation Sequencing (validation cohort) & predicted HLA-A*02:01-binding by NetMHCpan4.1.	30 SNP mismatches predicted to encode potential HLA-A*02:01-binding MiHAs. 13 SNP mismatches encoding 17 known HLA-A*02:01-binding MiHAs.	No association between (predicted) MiHA mismatches and relapse or GvHD.
Spellman et al. [35]	2009	730 patients with HLA-matched unrelated donors.	AML, ALL, CML, MDS	Known MiHAs	SNP genotyping using a Luminex based, multiplex assay.	5 MiHAs (hematopoietic-restricted HA-1, HA-2, HB-1; broadly-expressed HA-3, HA-8).	No association between MiHA mismatches and relapse or GvHD.
Spierings et al. [37]	2013	849 patients with unrelated (n = 639) or related (n = 210) donors.	Diverse	Known MiHAs	SNP genotyping using a PCR-SSP-based assay.	10 H-Y antigens & 10 MiHAs (hematopoietic-restricted HA-1, HA-2, HB-1, ACC-1, ACC-2, SP110, PANE1; broadly-expressed HA-3, HA-8, UGT2B17).	Association of HA-8 mismatches with GvHD. Association of mismatches for one or more hematopoietic-restricted MiHAs with longer relapse-free survival and better overall survival. Associations only found in patients with GvHD, not in patients without GvHD.
Hobo et al. [38]	2013	327 patients with related (n = 264) or unrelated (n = 63) donors. Patients were positive for at least one HLA-restriction allele for selected MiHAs.	Diverse	Known MiHAs	SNP genotyping using the KASPar assay system (Kbioscience).	3 H-Y antigens & 14 MiHAs (hematopoietic-restricted HA-1, HA-2, ACC-1, ACC-2, PANE1, LRH-1; broadly-expressed HA-3, HA-8; remaining HwA11, SP110, ZAPHIR, HEATR, LB-ECGF, LB-ADIR).	Association of one or more MiHA mismatches with longer relapse-free survival. H-Y mismatches associated with GvHD. Associations more significant in patients with related donors.

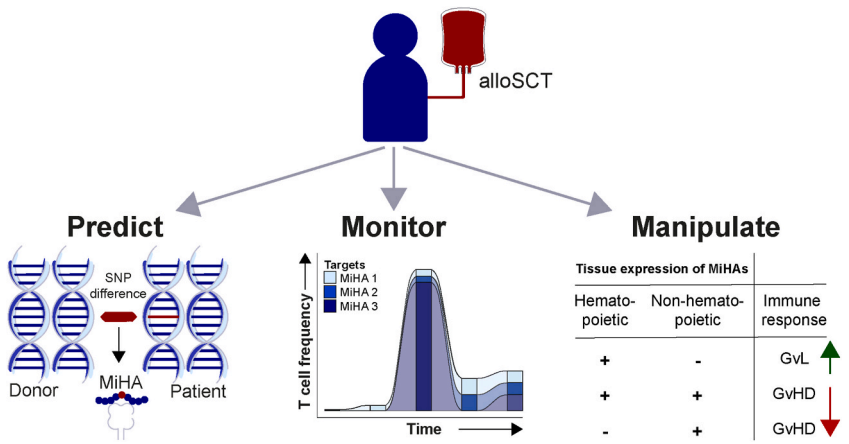

Fig. 1. The potential relevance of HLA-I-restricted MiHAs to predict, monitor or manipulate GvL and GvHD after HLA-matched alloHCT. HLA-I-restricted MiHAs can be used to improve clinical outcome after HLA-matched alloHCT in various ways. First, SNP mismatches encoding predicted or validated MiHAs may be used to predict GvL or GvHD after alloHCT. Second, MiHA-specific T cells can be measured to monitor GvL and GvHD after alloHCT. Finally, immune responses after alloHCT can be manipulated to augment GvL by increasing T-cell frequencies against hematopoietic-restricted MiHAs, or mitigate GvHD by depleting T cells against MiHAs with broad or preferentially non-hematopoietic expression.

Other studies investigated whether SNP mismatches for predicted MiHAs were associated with clinical outcome. Comparing HLA-A*02:01-positive and -negative patients, Martin et al. [18] identified 30 predicted HLA-A*02:01-binding MiHAs. However, association of these potential MiHAs with clinical outcome was not confirmed in a validation cohort. Lansford et al. [19] demonstrated that patients transplanted with unrelated donors had twice as many predicted HLA-I-binding MiHAs than patients with related donors, corresponding to a twofold difference in coding SNP mismatches [15,20]. Predicted MiHAs were then assigned as GvL-MiHAs or GvHD-MiHAs based on gene expression in acute myeloid leukaemia (AML), bone marrow, and non-hematopoietic tissues in the Human Protein Atlas (HPA). However, no association was found between numbers of GvL/GvHD-MiHAs and clinical outcome. Since numbers of predicted MiHAs varied across HLA-I alleles, HLA type could potentially serve as estimate for total MiHAs. However, there was no association between cumulative predicted peptide-binding frequencies of HLA alleles and GvL or GvHD [21]. Jadi et al. [22] demonstrated that patients with high numbers of predicted MiHAs faced an increased risk of GvHD mortality. Among 516 SNPs analysed, one antigen showed association with increased GvHD mortality, another with decreased leukaemia-free survival and a third with increased disease-related mortality. The latter two peptides were proposed to suppress immune responses by competing out MiHAs, which seems less plausible, given that MiHAs are recognized on both homozygous and heterozygous SNP-positive EBV-LCLs in GWAS.

Based on these studies, it can be concluded that GvL and GvHD cannot be predicted by coding SNP mismatches and predicted MiHAs. Probably, potential clinical effects of MiHAs in these studies have been masked by numerous false positives. This is supported by studies showing that not every predicted MiHA is processed, presented or targeted. Granados et al. [23] demonstrated that only 10 polymorphic peptides identified by mass spectrometry (0.22% of all HLA-I-binding peptides) were mismatched in a patient-donor pair. Hombrink et al. [24] eluted peptides from HLA-I and identified polymorphic peptides by matching peptides to a database of 7-14-mer peptides encoded by known variants in Human Short Peptide Variation Database (HSPVdb) in normal or alternative reading frames. A total of 23 peptides were validated as HLA-A*02:01-binding peptides. Although low-abundant peptides fall below the detection limit of mass spectrometry, these studies revealed that the number of presented polymorphic peptides is limited. Furthermore, as described in section 4, not every MiHA is targeted in immune responses after alloHCT. In conclusion, since only a small proportion of predicted MiHAs are processed, presented and targeted by donor T cells, GvL and GvHD cannot be accurately predicted by coding SNP mismatches or predicted MiHAs.

2.2. Predicting hematopoietic MiHAs

SNP mismatches were also investigated to encode hematopoietic-restricted MiHA candidates with potential therapeutic relevance. Granados et al. [25] eluted peptides from EBV-LCLs, and identified polymorphic peptides by mass spectrometry. Genes encoding these peptides were analysed for expression in AML (The Cancer Genome Atlas, TCGA) and healthy tissues (HPA). Hematopoietic MiHA candidates were defined by at least twofold higher expression in bone marrow than skin, detectable expression in AML and expression $<$10 FPKM in 27 other tissues. Olsen et al. [26] performed SNP genotyping for more than 700,000 SNPs and predicted MiHAs in 3231 patients treated with alloHCT. GvL-MiHAs were defined by expression $>$50 TPM in AML (TCGA) and $<$50 TPM in skin, liver and colon (Genotype-Tissue Expression database, GTEx). On average, 704 GvL-MiHAs were identified for each patient, and 25 frequently mismatched GvL-MiHAs candidates were validated as HLA-I-binding peptides by mass spectrometry.

In above studies, none of the hematopoietic MiHA candidates were identified as natural targets in immune responses after HLA-matched alloHCT, except for LB-NDC80-1P, which showed expression in colon and rectum by single-cell RNA-seq [12]. However, although the hematopoietic MiHA candidates do not seem immunogenic *in vivo*, they can still be therapeutically relevant to stimulate GvL after HLA-matched alloHCT.

3. The repertoire of HLA-I-restricted MiHAs

In recent studies aimed at discovering new MiHAs, T-cell clones were isolated from 39 patients who responded to DLI after HLA-matched alloHCT with GvL and varying degrees of GvHD. Optimized GWAS revealed 89 new MiHAs, expanding the total repertoire of MiHAs identified as natural targets in immune responses after HLA-matched alloHCT to 159 HLA-I-restricted MiHAs [11,12].

Optimized GWAS was specifically designed to identify MiHAs in seven common HLA-I alleles (A*01:01, A*02:01, A*03:01, B*07:02, B*08:01, C*07:01, C*07:02), each occurring in 15–50% of the European population with lower frequencies in other populations [11,12]. Most MiHAs (n = 151) bind to HLA-A or HLA-B alleles, suggesting that HLA-C-restricted MiHAs (n = 8) play a smaller role in immune responses, potentially caused by low HLA-C surface expression [27]. Notably, a significant number of MiHAs (n = 49) were identified for HLA-B*07:02. The reason is unknown, but may be explained by low constraints on the C-terminal anchor residue [28], thereby allowing more peptide length variants with variable C-terminal residues [12], increasing the likelihood that polymorphic peptides are presented.

3.1. Recurrent MiHAs

On average, patients were mismatched for 14–15 MiHAs with up to 12 antigens targeted in natural immune responses after alloHCT [12]. Interestingly, two-thirds of isolated T-cell clones were specific for MiHAs targeted in multiple patients, a surprising finding given the substantial number of SNP mismatches in each patient-donor pair. Whole exome sequencing revealed on average 7000 and 12,500 coding SNP mismatches encoding 12,500 and 22,000 peptides with predicted binding to patient's HLA-I alleles in transplantations with related and unrelated donors, respectively [29]. Given that only a handful of antigens are targeted in each patient, the majority of

coding SNP mismatches apparently do not encode polymorphic peptides presented by HLA-I on the cell surface or encode peptides that are not or only weakly immunogenic. The latter possibility postulates that SNP mismatches encode MiHAs with varying surface densities, and that only few MiHAs targeted by high-affinity T cells dominate in immune responses after alloHCT.

3.2. HLA-I-binding features

The observations that only few MiHAs are targeted in each patient and that the same MiHAs can be targeted in multiple patients suggest common features in, for example, gene expression, antigen processing, HLA-I-binding affinity or *in vivo* immunogenicity.

Fuchs et al. [12] investigated predicted HLA-binding of all 159 MiHAs. One quarter of the MiHAs showed at least tenfold stronger predicted HLA-I-binding than their allelic variants. In these MiHAs, the anchor residue is often polymorphic and allelic variants are not able to bind to the HLA-I-restriction allele. Since donor T cells have not been exposed to these allelic variants during thymic selection, the entire polymorphic peptide of the MiHA is "foreign" to the donor-derived immune system. However, most MiHAs display comparable predicted HLA-I binding as their allelic variants. It is possible that allelic variants might not be presented on the cell surface due to intracellular processing. Donor T cells for these MiHAs may display similar reactivity against allelic variants when loaded as exogenous peptides on cells [6]. For the majority of MiHAs, however, donor T cells do not react against allelic variants as exogenous peptides or require significantly higher peptide concentrations [12]. This suggests that most allelic variants are presented by HLA-I on the cell surface, and that high-affinity T cells for allelic variants have been deleted in donors through negative selection in the thymus [30]. This is supported by the discovery of various bi-allelic MiHAs (n = 12; 6 pairs), where both variants are immunogenic [12], and detection of allelic variants in HLA-I peptidomics by mass spectrometry [31]. If most allelic variants are presented by HLA-I on the cell surface, the question may arise why relatively few bi-allelic MiHAs have been identified. This may be explained by a lower immunogenicity of allelic variants or lower probability to be identified. The probability to identify a MiHA is dependent on its mismatch frequency. MiHAs that are frequently mismatched have per definition allelic variants that are less frequently mismatched [32] and are thus less likely to be identified.

Since most MiHAs exhibit similar predicted HLA-I-binding as their allelic variants, *in vivo* immunogenicity of MiHAs does not appear to depend on a difference in HLA-I-binding affinity between both peptide variants. This favours a model in which most MiHAs may be immunogenic due to differences in biochemical features between the polymorphic amino acid variants directly interacting with T-cell receptors or conformational changes induced in the peptide-HLA complex by polymorphic amino acids [33]. As a result, the MiHA is presented to the donor's immune system in another conformation than the allelic variant, enabling T cells to distinguish between both peptides. Strong immunogenicity resulting from a conformational change may also explain why HLA-B*07:02-restricted MiHAs often contain arginine as polymorphic amino acid, while these are not more abundant among HLA-B*07:02-binding polymorphic peptides on the cell surface [12].

3.3. Prediction of GvL and GvHD by confirmed MiHAs

Various studies investigated whether GvL and GvHD can be predicted by confirmed MiHAs, but conflicting results were reported [18,34–38].

Larsen et al. [36] examined 23 SNPs in six genes, each encoding at least one confirmed MiHA. Total mismatches for these 23 SNPs did not associate with clinical outcome, but the degree of mismatching in potential MiHAs with predicted binding to patients' HLA alleles correlated with lower overall survival and higher treatment-related mortality after HLA-matched alloHCT.

Spierings et al. [37] measured SNPs for seven antigens published as hematopoietic-restricted MiHAs, three broadly-expressed MiHAs and 10 H-Y antigens. In 849 patients transplanted with related (n = 639) or unrelated (n = 210) donors, mismatching for HA-8 was associated with GvHD. Additionally, mismatches for one or more hematopoietic-restricted MiHAs were associated with longer relapse-free survival and better overall survival. These associations were found in patients with GvHD, but not in patients without GvHD.

Hobo et al. [38] analysed SNPs for 14 MiHAs and three H-Y antigens in 327 patients transplanted with related (n = 264) or unrelated (n = 63) donors, who were positive for at least one HLA-I restriction allele. Patients transplanted with related donors showed longer relapse-free survival if one or more MiHAs were mismatched, while mismatching for H-Y antigens was associated with GvHD.

These studies suggest that validated MiHAs can be used to predict GvL and GvHD. However, the accuracy of these predictions is currently low, likely because only a limited number of MiHAs have been examined. The recently broadened repertoire, consisting of 159 HLA-I-restricted MiHAs, provides new opportunities to optimize algorithms to predict GvL and GvHD.

4. Tissue expression of MiHAs

To assess the potential impact of MiHAs on GvL and GvHD, all confirmed 159 MiHAs were analysed for tissue expression using single-cell RNA-seq data from the HPA [39]. Tissues often affected by GvHD were selected, and expression was calculated as the ratio between the highest expression values in hematopoietic versus non-hematopoietic cell clusters. MiHA-encoding genes were categorized into three groups based on at least threefold higher expression in hematopoietic cells (n = 29), at least threefold higher expression in non-hematopoietic cells (n = 9), and a group showing comparable expression in hematopoietic and non-hematopoietic cells (n = 85).

Discovery of MiHAs with preferential non-hematopoietic expression may seem unexpected given that these antigens have been identified by GWAS using EBV-LCLs. Apparently, despite low relative gene expression, EBV-LCLs sufficiently stimulate T cells for these

MiHAs. Similarly, patient-derived APC may induce *in vivo* immune responses against MiHAs with preferential expression in other cell types than B cells. Nevertheless, the repertoire of MiHAs may be skewed towards antigens expressed on EBV-LCLs. Additionally, non-hematopoietic MiHAs indirectly presented on APCs after exogenous uptake might have been overlooked. To explore the existence of these MiHAs, T-cell clones were isolated from patients with skin GvHD, but none of the T-cell clones showed reactivity against skin fibroblasts [40]. Since these T-cell clones were isolated from peripheral blood during induction of the immune response prior to the onset of skin GvHD, it cannot be excluded that T cells for skin-specific MiHAs may have been absent in peripheral blood. However, Sacirbegovic et al. [41] showed that in early GvHD, there is an influx of MiHA-specific T cells from peripheral blood in affected tissues, where these T cells are maintained by tissue-resident progenitor-like T-cells in late GvHD. Peripheral blood samples at the onset of an immune response may thus provide a snapshot of all MiHAs that are targeted in the patient.

4.1. Hematopoietic-restricted MiHAs

Among 29 genes with preferential hematopoietic expression, 20 genes showed at least fivefold higher expression in hematopoietic cells. Eleven genes were also hematopoietic-restricted in a microarray dataset in which non-hematopoietic cells were cultured with IFN-γ to mimic the pro-inflammatory environment post-alloHCT [12]. These 11 genes encode 14 MiHAs of which three, i.e. HA-2 [42], LB-ITGB2-1 [43], LRH-1 [44], have previously been reported as MiHAs with therapeutic relevance. HA-1 [45] was validated as hematopoietic-restricted, but its encoding gene *ARHGAP45* showed expression in specialized epithelial cells in the lung and small intestine, leading to only a threefold preferential expression in hematopoietic cells [12]. New MiHAs with hematopoietic-restricted gene expression still require validation as therapeutic candidates by demonstrating presentation on malignant cells and absence on non-hematopoietic cells. Preliminary data with T-cell clones for these MiHAs showed no reactivity against (IFN-γ pre-treated) fibroblasts, supporting that these antigens are hematopoietic-restricted.

Of 11 genes encoding hematopoietic-restricted MiHAs, three (*MYO1G*, *ITGB2*, *IL1*0RA) are expressed in most hematopoietic cell types, whereas the other genes are more myeloid- (*LILRB4*, *DOK2*, *F13A1*) or lymphoid-specific (*P2RX5*, *LTA*, *SLAMF1*, *APOBEC3H*, *TXNDC11*), emphasizing the need to validate these MiHAs on malignant cells of diverse hematopoietic origins.

5. Monitoring of MiHA-specific T cells

Monitoring MiHA-specific T cells could be valuable to follow GvL and GvHD after HLA-matched alloHCT (Fig. 1). Confirming GvL responses by measuring MiHA-specific T cells is particularly relevant in patients without GvHD, where no clinical symptoms are apparent to indicate an immune response.

Hobo et al. [38] screened 327 patients and stained T cells directly after thawing and after in vitro peptide stimulation with dual-colour peptide-MHC (pMHC) multimers for 15 MiHAs. T cells for 10 MiHAs were each detected in 10–60% of mismatched patients. No T cells were detected for five MiHAs, whereas these antigens were each mismatched in at least five patients. Importantly, MiHA-specific T-cell responses were associated with improved relapse-free survival. This association was stronger than for MiHA-encoding SNP mismatches, emphasizing the relevance to develop prediction tools for GvL and GvHD where SNP mismatches are weighted for their capacity to elicit *in vivo* T-cell responses.

Similarly, using barcoded pMHC multimers for 147 HLA-I-restricted MiHAs, T-cell responses were detected only in a proportion of mismatched patients [46]. In 16 patients who responded to DLI with no (n = 3), limited (n = 3), or severe GvHD (n = 10), high T-cell frequencies above 10% of CD8 cells were detected for three MiHAs in two patients with severe GvHD. Both patients were transplanted with HLA-DP-mismatched donors, indicating that HLA-DP mismatching does not impede robust immune responses against HLA-I-restricted MiHAs. MiHA-specific T-cell frequencies above 1% of CD8 cells were identified for 13 MiHAs in 8 patients with severe GvHD and two patients with limited GvHD, supporting previous findings that MiHA-specific T-cell frequencies are higher in patients with GvHD [40]. Longitudinal screening revealed high T-cell frequencies typically 6–8 weeks after DLI.

Measuring T-cell responses against a multitude of antigens allows identification of immunodominant MiHAs and analysis of immunodominance after alloHCT. Immunodominance is well-documented in virus-specific T-cell responses as demonstrated by different epitopes dominating during acute and latent infection with EBV or CMV [47,48]. Screening large patient cohorts to identify immunodominant MiHAs and understand immunodominance is essential to develop accurate algorithms to predict GvL and GvHD and design optimal strategies to manipulate immune responses after HLA-matched alloHCT.

6. Immunotherapy after alloHCT

Although hematopoietic-restricted MiHAs are attractive targets for immunotherapy (Fig. 1), clinical trials in which T cells against these antigens are stimulated to augment GvL after alloHCT are still limited.

6.1. Vaccination with MiHAs

Since patient APCs are essential for induction of GvL and GvHD [4], Franssen et al. [49] attempted to boost the effectiveness of DLI after HLA-matched alloHCT by vaccinating patients with donor-derived mature dendritic cells (DCs). Nine multiple myeloma patients with persistent or progressive disease after DLI received an equivalent dose of DLI and vaccination with DC loaded with one or two hematopoietic-restricted MiHAs (UTA2-1, LRH-1, HA-1). Remission was not induced, but four of five patients with MiHA-specific T cells at 0.01–0.7% of CD8 cells remained in stable disease for 6–10 months.

Vaccination with MiHA-loaded DCs proved feasible and safe, but clinical effects were limited possibly due to a suboptimal vaccine, poor persistence or rapid inactivation of MiHA-specific T cells *in vivo*. To increase clinical efficacy, other strategies such as mRNA vaccines, inactivating PD-1 ligands on DCs, combining vaccination with immune checkpoint inhibitors, or vaccination with more hematopoietic-restricted MiHAs might be considered [50,51]. Additionally, vaccination of donors could be explored as strategy to enhance GvL [52].

6.2. *Adoptive transfer of MiHA-specific T cells*

Adoptive transfer of MiHA-specific T cells was also explored as strategy to augment GvL. Warren et al. [53] treated seven patients who relapsed after alloHCT with MiHA-specific T-cell clones. These clones were isolated from in vitro cultures generated by stimulating T cells in post-alloHCT samples with patient cells obtained prior to alloHCT. Selected T-cell clones lysing patient EBV-LCLs, but not donor EBV-LCLs and patient fibroblasts, were expanded and infused. Each patient received at least four infusions. Three to five days after infusion, MiHA-specific T cells were detected in the bone marrow at 0.5–18.6% of CD8 cells, but frequencies rapidly declined. Complete remissions were induced in five patients, but responses were not sustained and could have been attributed to prior cytoreductive chemotherapy or immunosuppression withdrawn prior to T-cell infusion. Notably, three patients with persistent leukaemia for more than three weeks after completing chemotherapy achieved clinical remission after T-cell infusion, indicating that MiHA-specific T cells can induce GvL responses. Of note, various patients developed pulmonary toxicity, demonstrating that selection of T-cell clones that fail to react against skin fibroblasts does not preclude toxicity. Testing against fibroblasts cultured with pro-inflammatory cytokines may be more informative, as MiHA-specific T-cell clones often react against fibroblasts after pre-treatment

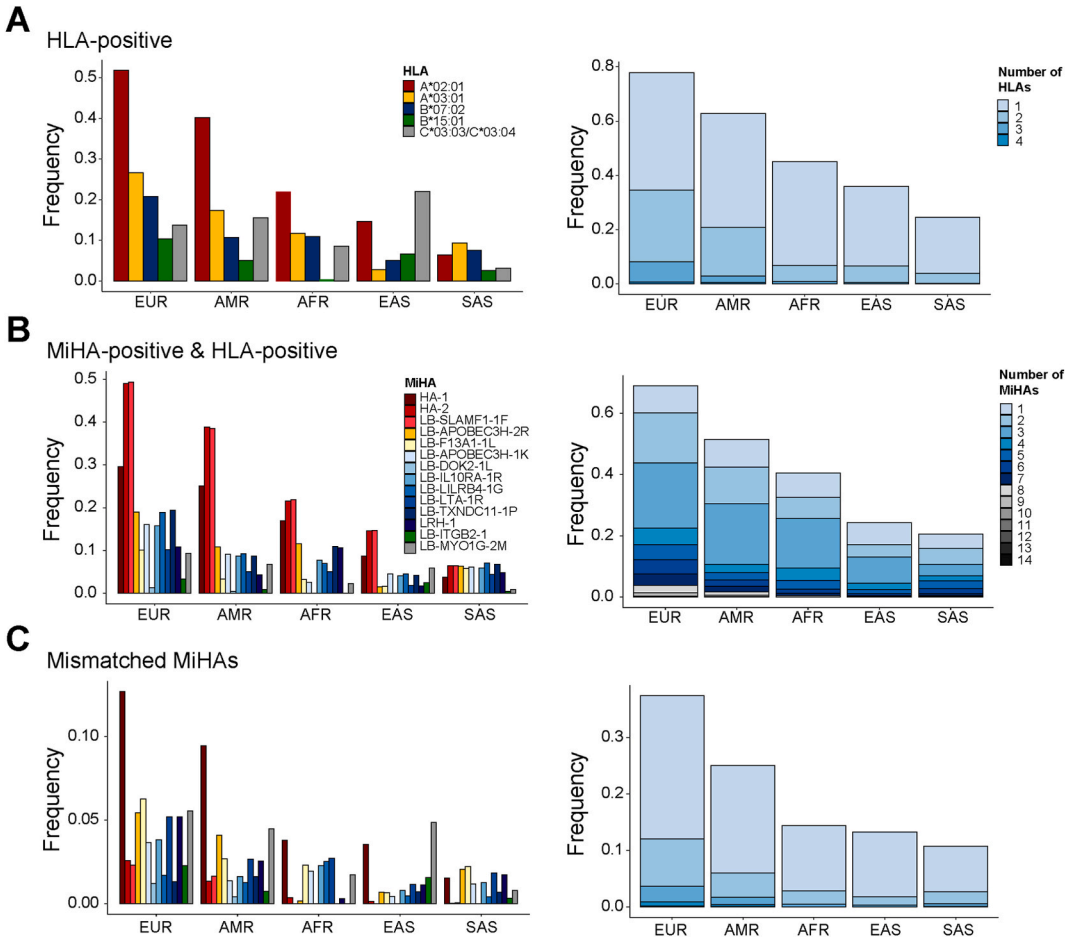

Fig. 2. Population and mismatch frequencies of 14 hematopoietic-restricted MiHAs. A) Frequencies for different populations in the 1000 Genomes Project to be positive for one (left) or more (right) HLA-I restriction alleles for 14 hematopoietic-restricted MiHAs are shown. B) Frequencies for different populations to be positive for one (left) or more (right) hematopoietic-restricted MiHAs and respective HLA-I alleles are shown. Hematopoietic-restricted MiHAs can be targeted in MiHA- and HLA-I-positive patients transplanted with donors who are negative for the respective HLA-I allele. C) Hematopoietic-restricted MiHAs can be targeted in MiHA- and HLA-I-positive patients transplanted with donors who are positive for the respective HLA-I allele, but negative for the MiHA. Frequencies for different populations to be mismatched for one (right) or more (left) MiHAs are displayed.

with IFN-γ, which stimulates antigen processing and surface expression of HLA, adhesion and costimulatory molecules [40].

Meij et al. [54] treated three patients with relapsed leukaemia after HLA-matched alloHCT with HA-1-specific T cells generated in vitro by stimulating donor T cells with peptide-loaded donor-derived DCs. One patient with CML who was treated for a molecular relapse, developed stable disease for three months. The other two patients had relapsed AML and experienced rapid progressive disease. HA-1-specific T cells were only measured in the CML patient for up to eight weeks after infusion, but frequencies were low and only detectable after in vitro stimulation with the HA-1 peptide.

In these two studies, prolonged in vitro culture was needed to obtain sufficient T-cells for infusion. During in vitro culture, T cells undergo progressive differentiation, leading to poor *in vivo* expansion and persistence [55]. T-cell receptor gene transfer allows rapid generation of MiHA-specific T cells. In a study by van Balen et al. [56], an HA-1-specific TCR was introduced into EBV- and CMV-specific T cells, which were isolated from donor peripheral blood by pMHC multimers. Dual virus- and HA-1-specific TCR-T cells were infused to protect patients against relapses of high risk AML and viral reactivations after alloHCT. Five patients received one or two TCR-T-cell infusions 8–14 weeks after alloHCT without additional lymphodepletion. TCR-T cells were detected at low frequencies peaking at 0.6–1.5% of mononuclear cells in two patients. One patient had smouldering disease during the first infusion and died from progressive disease after the second infusion. Antigen loss could be excluded, since relapsed AML cells were recognized by TCR-T cells in vitro.

Similar as the DC vaccination study, adoptive therapy with HA-1-specific TCR-T cells proved feasible and safe, but clinical effects were limited. HA-1-specific TCR-T cells survived, but did not expand *in vivo*, what may be caused by inefficient exposure to HA-1-expressing patient hematopoietic cells. HA-1-specific TCR-T cells may require robust stimulatory signals [57], which may not be provided by AML cells. To improve clinical efficacy, other HA-1-specific TCRs may be introduced [58], or T cells with other phenotypes may be infused, as T cells with naïve or memory stem cell phenotypes have shown superior *in vivo* expansion and anti-tumour responses after infusion [55]. Additionally, since CD4 cells often play a crucial role in anti-tumour responses [17], patients may be infused with CD8 and CD4 cells engineered with the HA-1 TCR along with the CD8 co-receptor [59].

7. Concluding remarks & future perspectives

The expanded repertoire of HLA-I-restricted MiHAs opens up new opportunities to improve the balance between GvL and GvHD after alloHCT (Fig. 1).

Validated MiHAs may be used in algorithms to predict GvL and GvHD after HLA-matched alloHCT. However, accurate algorithms must integrate crucial information on *in vivo* immunogenicity and immunodominance of MiHAs. This requires T-cell monitoring in large patient cohorts treated with different transplantation strategies. Validated MiHAs may also serve as valuable resource for computational modelling to develop prediction tools for patients with HLA-I alleles for which no or few MiHAs have been identified. MiHAs can also be employed in pMHC multimers to monitor immune responses, facilitating personalized treatment with DLI or other immunotherapies. Lastly, MiHAs offer the potential to manipulate immune responses. GvL can be enhanced by promoting T-cell responses against hematopoietic-restricted MiHAs, while GvHD can be suppressed by depleting T cells for MiHAs with broad or preferential non-hematopoietic expression. Identification of 11 new hematopoietic-restricted MiHAs as potential therapeutic targets broadens the scope of treatment for more patients and allows simultaneous targeting of multiple antigens (Fig. 2), resembling natural immune responses after alloHCT. Immune responses can be boosted through vaccination or cellular therapies, and antibodies binding to peptide-HLA complexes may be used as T-cell engagers or as antibody-drug conjugates for selective T-cell depletion [60]. Antibody domains were also employed in CAR-T cells targeting MiHAs [61].

Given the success of TCR-T cells targeting NY-ESO-1 or WT1 in inducing clinical responses in patients with haematological malignancies [62,63], clinical trials with TCR-T cells targeting hematopoietic-restricted MiHAs, such as HA-1 [NCT03326921; NCT05473910], hold promise. Notably, TCR-T cells for hematopoietic-restricted MiHAs may also be relevant in transplantations across HLA barriers [64,65] to treat MiHA-positive patients transplanted with donors negative for HLA-I restriction alleles. MiHAs with high population frequencies, which have limited utility in HLA-matched alloHCT due to scarcity of MiHA-negative donors, may become particularly relevant in HLA-mismatched alloHCT.

In conclusion, the expanded repertoire of HLA-I-restricted MiHAs opens the door to diverse strategies to enhance overall survival of patients with haematological malignancies after alloHCT.

Practice points

- MiHAs are major targets in T-cell responses after HLA-matched alloHCT inducing GvL as well as GvHD.
- Algorithms predicting GvL and GvHD after HLA-matched alloHCT based on SNP mismatches or predicted MiHAs are currently not accurate for clinical applications.
- MiHAs are attractive targets for immunotherapy, but current approaches such as vaccination and adoptive T-cell transfer against hematopoietic MiHAs are limited and need optimization.

Research agenda

- The recently expanded repertoire of HLA-I-restricted MiHAs provides new opportunities to predict, monitor or manipulate GvL and GvHD after alloHCT.

- Information on immunodominance of MiHAs in T-cell responses after alloHCT should be integrated in algorithms predicting GvL and GvHD by SNP mismatches.
- T-cell monitoring of large patient cohorts treated with alloHCT is needed to acquire information on immunodominance of MiHAs in GvL and GvHD.
- More clinical trials are needed to explore the relevance of MiHAs as targets for immunotherapy to establish an optimal balance between GvL and GvHD after alloHCT.
- The recent discovery of multiple hematopoietic-restricted MiHAs provides opportunities to treat more patients with new immunotherapies to stimulate GvL after alloHCT with no or limited GvHD.

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Kyra J. Fuchs: Writing – review & editing, Visualization. **J.H. Frederik Falkenburg:** Writing – review & editing. **Marieke Griffioen:** Writing – review & editing, Writing – original draft, Visualization.

Declaration of competing interest

None.

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