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Experimental Data on PIRCHE and T-Cell Reactivity: HLA-DPB1-Derived Peptides Identified by PIRCHE-I Show Binding to HLA-A*02:01 in vitro and T-Cell Activation in vivo

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Keywords
Human leukocyte antigen-DPB1 · Hematopoietic stem cell transplantation · Graft-versus-host disease · Predicted Indirectly ReCognizable HLA Epitopes-I · T-cell epitopes

Abstract
Introduction: Human leukocyte antigen (HLA)-DPB1 mismatches during hematopoietic stem cell transplantation (HSCT) with an unrelated donor result in an increased risk for the development of graft-versus-host disease (GvHD). The number of $CD8^+$ T-cell epitopes available for indirect allorecognition as predicted by the PIRCHE algorithm has been shown to be associated with GvHD development. As a proof of principle, PIRCHE-I predictions for HLA-DPB1 mismatches were validated in vitro and in vivo. ***Methods:*** PIRCHE-I analysis was performed to identify HLA-DPB1-derived peptides that could theoretically bind to HLA-A*02:01. PIRCHE-I predictions for HLA-DPB1 mismatches were validated in vitro by investigating binding affinities of HLA-DPB1-derived peptides to the HLA-A*02:01 in a competition-based binding assay. To investigate the capacity of HLA-DPB1-derived peptides to elicit a T-cell response in vivo, mice were immunized with these peptides. T-cell alloreactivity was subsequently evaluated using an interferon-gamma ELISpot assay. ***Results:*** The PIRCHE-I algorithm identified five HLA-DPB1-derived peptides (RMCRHNYEL, YIYNREEFV, YIYNREELV, YIYNREEYA, and YIYNRQEYA) to be presented by HLA-A*02:01. Binding of these peptides to HLA-A*02:01 was confirmed in a competition-based peptide binding assay, all showing an IC_{50} value of 21 μM or lower. The peptides elicited an interferon-gamma response in vivo. ***Conclusion:*** Our results indicate that the PIRCHE-I algorithm can identify potential immunogenic HLA-DPB1-derived peptides present in recipients of an HLA-DPB1-mismatched donor. These combined in vitro and in vivo observations strengthen the validity of the PIRCHE-I algorithm to identify HLA-DPB1 mismatch-related GvHD development upon HSCT.

Introduction

Human leukocyte antigen (HLA) mismatches between donor and recipient are known to increase the risk for acute graft-versus-host disease (GvHD) during hematopoietic stem cell transplantation (HSCT) [1–5]. The risk for GvHD during HSCT is decreased in patients who receive a transplant from an 10/10 matched unrelated donor compared to patients receiving a 9/10 matched unrelated donor [1]; however, additional matching for the HLA-DPB1 allele can further decrease the risk for GvHD

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[6–15]. For example, Shaw et al. [8] showed that the risk for mortality and GvHD was significantly higher in HLA-DPB1-mismatched individuals among 5,929 patients who received HSCT from a 10/10 matched unrelated donor.

HLA-DPB1 mismatches can be recognized by alloreactive donor T cells via the direct or indirect pathway of allorecognition. Direct T-cell allorecognition is defined by alloreactive donor T cells that directly interact with intact allogeneic HLA-DPB1 molecules of the recipient [4, 5]. In contrast, indirect T-cell allorecognition during HSCT is characterized by donor T cells recognizing mismatched HLA-DPB1-derived peptides from the recipient presented on non-allogeneic HLA molecules of donor antigen-presenting cells (APCs) [4, 5]. Indirect T-cell allorecognition of T-cell epitopes (TCEs) derived from mismatched HLA molecules can be estimated by the Predicted Indirectly ReCognizable HLA Epitopes (PIRCHE) algorithm. This model indicates which peptides derived from mismatched recipient HLA can be presented to donor T cells by HLA class I (PIRCHE-I) or class II (PIRCHE-II) molecules [4, 5, 16], where PIRCHE-I epitopes should theoretically lead to a $CD8^+$ T-cell response and PIRCHE-II to a $CD4^+$ T-helper cell response. PIRCHE-I and PIRCHE-II scores pointed out to be a good indicator for GvHD development, transplantation outcome, and overall survival during HSCT [12, 15, 17–19]. For example, HLA-C mismatches with a higher number of PIRCHE-I or -II correlated with acute GvHD development in a cohort of 20 patients transplanted with an unrelated 9/10 HLA-matched donor, containing a single mismatch for the HLA-C allele [17].

The PIRCHE algorithm could also provide an estimate for transplantation outcome regarding HLA-DPB1 mismatches. We previously showed that recipients receiving an HSCT from a 10/10 matched unrelated donor containing a mismatch for the HLA-DPB1 allele with PIRCHE-I had an increased hazard of acute GvHD compared to recipients without PIRCHE-I [12]. Furthermore, in a limited cohort of pediatric 10/10 HSCT recipients with one or two HLA-DPB1 mismatches, patients with grade II-IV GvHD had higher PIRCHE-I scores than patients with grade I or no GvHD [15]. These observations suggest that the PIRCHE algorithm could potentially serve as a risk indicator for alloreactivity caused by HLA-DPB1 mismatches. However, experimental data about the immunogenicity of HLA-DPB1-derived peptides predicted by PIRCHE still largely lack; the binding affinity values, reported as an IC_{50} value, are theoretically determined [20]. As a proof of principle, the present study aims to validate the value of PIRCHE-I predictions for HLA-DPB1 mismatches by investigating binding affinities of HLA-DPB1-derived peptides to HLA-A*02:01. In addition, the capacity of HLA-DPB1-derived peptides to elicit a T-cell response in vivo was assessed.

Materials and Methods

Peptide Identification

HLA-DPB1-derived peptides that can theoretically be presented by HLA-A*02:01 were identified by running the PIRCHE-I algorithm version 3.3.68 (PIRCHE AG, Berlin, Germany, available via www.pirche.com) [4, 16]. To this end, HLA-DPB1 alleles with an allele frequency over 0.1% in the Deutsche Knochenmarkspenderdatei (DKMS) cohort (n = 3,456,066, accessed on September 27, 2023 via allelefrequencies.net) were evaluated [21]. Accordingly, nineteen different HLA-DPB1 alleles were defined and used as donor and patient input for the algorithm (shown in Fig. 1a). Together with these HLA-DPB1 alleles, HLA genotypes consisting of frequently occurring HLA-A~C~B~DRB1~DQB1 haplotypes containing HLA-A*02:01 were used. Multiple haplotypes were evaluated, since overlapping peptide sequences between the HLA-DPB1 alleles and the used HLA-A, -B, -C, -DRB1, and -DQB1 typing could have resulted in missing HLA-DPB1-derived peptides in this analysis. As such, the PIRCHE analysis was performed using the five most frequently occurring HLA-A~C~B~DRB1~DQB1 haplotypes containing HLA-A*02:01. Frequently occurring haplotypes were identified using data from the National Bone Marrow Program [22]. For each frequently occurring haplotype, the PIRCHE input consisted of nineteen different genotypes as the patient input, each coupled to all nineteen donor HLA genotypes only differing in their HLA-DPB1 allele. Figure 1b shows the PIRCHE input of the first patient for one of the tested frequently occurring haplotypes. Only peptides with a predicted IC50 <500 nM were included in the PIRCHE-I analysis. The HLA-DPB1-derived peptides that were predicted by the PIRCHE algorithm to bind to HLA-A2*02:01 were subsequently analyzed in NetMHCpan4.1 to predict their binding affinity for HLA-A*02:01 [20] before performing the competition-based binding assay.

Cells

For the competition-based peptide binding assay, the Epstein-Barr virus-transformed lymphoblastoid cell line type 48 (EBV-LCL48) from the CEPH HapMap project with cell surface HLA-A*02:01 expression was used [23]. To ensure only HLA-A*02:01-specific binding of reference and test peptides, binding interference of other HLA alleles expressed by EBL-LCL48 was checked using NetMHCpan4.1 binding affinity predictions (shown in online suppl. Tables 1, 2; for all online suppl. material, see https://doi.org/10.1159/000537789) [20, 23]. Cells were cultured in RPMI-1640 medium supplemented with GlutaMAXTM (Gibco, NY, USA), 10% fetal calf serum (FCS) (Sigma-Aldrich, Missouri, USA), and 1% streptomycin/penicillin antibiotics. HLA-A*02:01 expression on the surface of EBV-LCL48 was confirmed by flow cytometric analysis using fluorescein isothiocyanate-conjugated human HLA-A/B/C monoclonal antibody clone W6/32 (Biolegend, San Diego, USA, Cat. No. 311403) and allophycocyanin-conjugated human HLA-A2 monoclonal antibody clone BB7.2 (Biolegend, San Diego, USA, Cat. No. 343307) [23].

Peptides Synthesis

Five HLA-A*02:01-binding HLA-DPB1-derived test peptides identified by PIRCHE-I and negative and positive control peptides (shown in Table 1) were synthetized by J.W. Drijfhout at the Leiden University Medical Center as previous described [20, 24, 25]. The minor histocompatibility antigen HA-1-derived VLHDDLLEA peptide that has been shown to bind to HLA-A*02:01 [26] was used as a positive control. As a negative control, the HLA-DPB1-derived KVNVSPSKK peptide was used, which is predicted by NetMHCpan to not bind to HLA-A*02:01 [20].

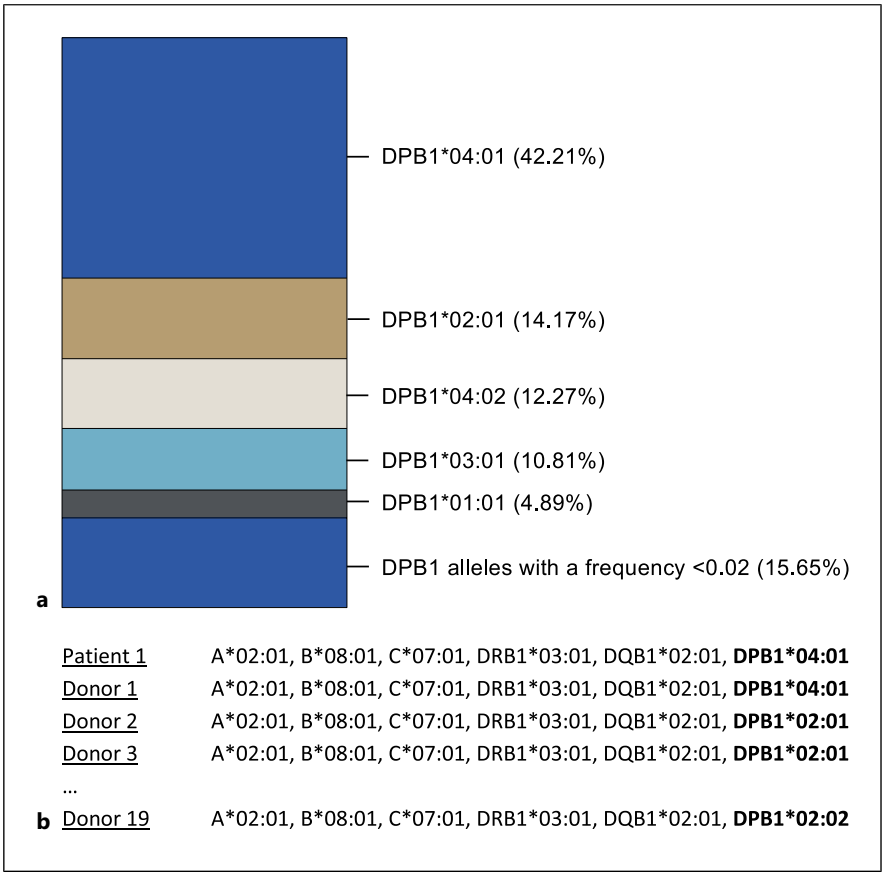


Fig. 1. Overview of HLA types used as inputs for the PIRCHE algorithm. **a** HLA-DPB1 allele frequencies in the DKMS cohort (20). DPB1 alleles with an allele frequency <0.02 (15.65%) include the following HLA-DPB1 alleles with an allele frequency higher than 0.1%: DPB1*05:01, DPB1*13:01, DPB1*17:01, DPB1*10:01, DPB1*14:01, DPB1*11:01, DPB1*06:01, DPB1*19:01, DPB1*09:01, DPB1*15:01, DPB1*23:01, DPB1*16:01, DPB1*20:01, and DPB1*02:02. **b** Example of the input for the PIRCHE algorithm. Depicted is the first patient genotype, which included the HLA-DPB1*04:01 allele, combined with 19 different donors with the same HLA-A, -B, -C, -DRB1, and -DQB1 alleles, but differing in their HLA-DPB1 allele. The other 18 patient genotypes consisted of the same HLA-A, -B, -C, -DRB1, and -DQB1 typing, but differed in their HLA-DPB1 allele (not shown).

Table 1. Overview of test peptides, control peptides, and the Fl-peptide used in the competition-based binding assay

Peptide sequence	Used as	Derived from	Predicted binding affinity for HLA-A*02:01 by NetMHCpan4.1 (nM) [20]
RMCRHNYEL	Test	HLA-DPB1	124.4
YIYNREEFV	Test	HLA-DPB1	9.0
YIYNREELV	Test	HLA-DPB1	22.6
YIYNREEYA	Test	HLA-DPB1	59.9
YIYNRQEYA	Test	HLA-DPB1	41.0
KVNVSPSKK	Negative control	HLA-DPB1	$38.6 \cdot 10^3$
VLHDDLLEA	Positive control	HA-1	28.0
FLPSDXFPSV	Fl-peptide	–	5.0

Binding affinities for all peptides were predicted by the NetMHCpan4.1 algorithm for all peptides [22].

Cys-fluorescein-labeled HLA-A*02:01 reference peptide (Fl-peptide) FLPSDXFPSV was kindly provided by J.W. Drijfhout [24, 25]. Peptide purity >95% was ensured by using reverse-phase high-pressure liquid chromatography for all peptides. Test and control peptides were dissolved in 100% dimethyl-sulfoxide and further diluted in phosphate-buffered saline (PBS) to a concentration of 1 mM.

Competition-Based Peptide Binding Assay

In vitro binding affinities for HLA-A*02:01 of all five identified HLA-DPB1-derived peptides were determined in triplicate in a competition-based peptide binding assay using EBV-LCL48 as described before [24]. In short, cells were first treated with an acid elution buffer (pH 3.1) to strip already bound peptides from the HLA molecules. Next, an eleven twofold serial dilution ranging from 600 till 0.5 μM was made for all test and control peptides. The EBV-LCLs were incubated in triplicate for 24 h with 150 nM Fl-peptide together with the test and control peptides in a 96-well V-bottom plate. Subsequently, cells were washed and resuspended with PBS/1% paraformaldehyde following flow cytometric measurement of the Fl-peptide signal using the BD FACS Canto II (BD Biosciences, NJ, USA).

Immunization of HLA-A2 Transgenic Mice

To investigate the ability of the HLA-DPB1-derived peptides to stimulate T cells, mice expressing the HLA-A*02:01 allele were immunized with the PIRCHE-identified HLA-DPB1-derived

peptides, synthesized by J.W. Drijfhout as described under *Peptide synthesis*. HLA-A*02:01 transgenic mice (The Jackson Laboratory, Bar Harbor, ME) were injected with 100 μg of RMCRHNYEL, YIYNREEFV, YIYNREELV, or YIYNRQEYA peptide together with 50 μg CpG oligonucleotides 1826 as described before [27]. Ethical approval was obtained as described under Statement of Ethics. After 103 days, mice were sacrificed, and spleen material was isolated through dissection. Cell samples were obtained by passing the spleen material through a 70-μm cell strainer followed by a 3-min incubation with 1 mL NH_4Cl (0.16 M) dilution for red blood cell lysis. Subsequently, the cell suspension was centrifugated after adding 49 mL ice-cold PBS and the cell pellet was diluted in RPMI/10% FCS for the enzyme-linked immunosorbent spot (ELISpot) assay. Splenocytes that were not used for the ELISpot were stored at −80°C after in RPMI/10% FCS/10% dimethyl sulfoxide dilution.

IFN-γ ELISpot Assay

T-cell alloreactivity of $CD8^+$ T cells from mice previous immunized with peptides mentioned was detected using an interferon-γ (IFN-γ) ELISpot assay. For this assay, the commercially available murine IFN-γ ELISpot set (Diaclone, Besançon, France) was used. For each well, 50,000 TAP-deficient T2 cells expressing HLA-A*02:01 (T2A2) were incubated for 3 h at 37°C with 0.5 μL of 1 mg/mL test peptide dilution (RMCRHNYEL, YIYNREELV, YIYNRQEYA, or YIYNREEFV) in a total volume of 50 μL to ensure peptide loading on HLA-A*02:01 alleles of these stimulator cells. As a negative control, the nonimmunized LLLSGALAL peptide, which is predicted to bind to HLA-A*02:01 with an IC_{50} of 14.14 [20], was loaded on T2A2 cells. To determine maximal IFN-γ production by T cells, RPMI/10% FCS with phytohemagglutinin was used instead of stimulator cell suspension (final concentration 1 μg/mL). The 96-well polyvinylidene difluoride plates were first treated with 100 μL blocker buffer. After washing with PBS, 50,000 stimulator cells or 50 μL phytohemagglutinin solution per well was added. Subsequently, 100,000 mouse splenocytes were added to each well up to a total volume of 100 μL. Following incubation at 37°C for 10–15 h, the wells were washed with PBS/0.05% Tween and treated with detection antibody and streptavidin-AP conjugate according to the manufacturer's instructions. Following the addition of 100 μL BCIP/NBT buffer, the wells were incubated for 5–15 min at room temperature. Spot-forming cells were counted using an ELVIS reader as a measure for IFN-γ producing cells.

Statistical Analysis

Peptide frequencies of identified HLA-DPB1-derived peptides were determined using allele frequencies in the DKMS cohort for all HLA-DPB1 alleles that contain the identified peptides according to the amino acid sequences from the IPD-IMGT/HLA-database version 3.52 [21, 28]. The exact peptide frequencies were subsequently calculated using the Hardy-Weinberg equation [29]. For the competition-based peptide binding assay, inhibition percentages of test and control peptides were calculated based on mean fluorescence intensity values for the 488 nm laser as described previously [24]. The calculated inhibition percentages were analyzed using GraphPad Prism version 9.9 (GraphPad Software, San Diego, CA, USA) through modeling of log[dose]-response curves from 0 to 100% with nonlinear regression analysis. ELISpot data were analyzed by correcting the counted spot-forming cells of the immunized peptides for the mean background signal from a nonimmunized peptide (LLLSGALAL). If the number of spot-forming cells was lower than those of the negative controls, the number of spot-forming cells was set to 0. One-sample *t*-tests were performed for all twelve mice using GraphPad Prism version 9.9. p values ≤0.05 were considered to be statistically significant.

Results

*Five HLA-DPB1-Derived Peptides Are Predicted by PIRCHE-I to Bind to HLA-A*02:01*

To find HLA-DPB1-derived peptides that can be presented by HLA-A*02:01 that could potentially lead to GvHD in the context of HSCT, PIRCHE-I analyses were performed. As the PIRCHE input, matching HLA types containing the HLA-A*02:01 allele only differing in the nineteen different HLA-DPB1 alleles with an allele frequency of 0.1% or higher [21] were used. Five nonameric peptide sequences (RMCRHNYEL, YIYNREEFV, YIYNREELV, YIYNRQEYA, and YIYNREEYA) were predicted to bind the HLA-A*02:01 allele with an IC_{50} value of 500 nM or lower (shown in Table 2), where a lower IC_{50} value indicates a higher binding affinity. The RMCRHNYEL peptide is located at the amino acid positions 75–83 of the HLA-DPB1 protein in twelve different HLA-DPB1 alleles. The other four peptides beginning with the YIYNR sequence are positioned at positions 28–36 of the HLA-DPB1 amino acid sequences of eighteen different HLA-DPB1 alleles. Three of these five identified peptides were predicted to be strong binders for HLA-A*02:01 according to the NetMHCpan4.1 algorithm, *i.e.*, the predicted binding affinities of these peptides belong to the top 1% predicted scores obtained from random natural peptides [20].

*PIRCHE-I Identified Peptides Derived from HLA-DPB1 Mismatches Show High Affinities for HLA-A*02:01 in vitro*

The binding of the five identified peptides to the HLA-A*02:01 allele in vitro was examined using a competition-based peptide binding assay as previously described [24]. All five identified peptides showed binding to HLA-A*02:01 with a half maximal inhibitory concentration (IC_{50}) value of 21 μM or less under competition with the Fl-peptide (shown in Fig. 2). Although all five HLA-DPB1 derived peptides showed binding to HLA-A*02:01 under competition with the Fl-peptide, some variation in binding affinity between the peptides was observed, with YIYNREEFV and YIYNREELV showing the lowest IC_{50} values (4.9 μM and 3.9 μM, respectively) (shown in Fig. 2a).

*PIRCHE-I Identified Peptides Derived from HLA-DPB1 Mismatches Bound to HLA-A*02:01-Expressing Cells Lead to T-Cell Responses in vivo*

The capacity of YIYNREELV, YIYNRQEYA, RMCRHNYEL, and YIYNREEFV peptides to induce T-cell alloreactivity was investigated in vivo by assessing IFN-γ

Table 2. Overview of recipient HLA-DPB1-derived peptides presented by donor HLA-A*02:01 as predicted by PIRCHE-I [4, 16] that could potentially result in the development of GvHD in HSCT patients

Peptide sequence	Position in peptide	Present in HLA-DPB1[a]	Predicted binding affinity according to NetMHCpan4.1 (nM) [20]	Peptide phenotype frequency	Disparity rate[b]
RMCRHNYEL	75–83	04:01, 02:01, 04:02, 05:01, 17:01, 11:01, 06:01, 15:01, 23:01, 16:01, 20:01, 02:02	124.4 (WB)	0.95	0.048
YIYNREEFV	28–36	02:01, 04:02, 03:01, 17:01, 10:01, 14:01, 06:01, 19:01, 09:01, 23:01, 16:01, 20:01	9.0 (SB)	0.71	0.21
YIYNREELV	28–36	05:01, 02:02	22.6 (SB)	0.045	0.043
YIYNREEYA	28–36	01:01, 13:01	59.9 (WB)	0.13	0.11
YIYNRQEYA	28–36	11:01, 15:01	41.0 (SB)	0.040	0.038

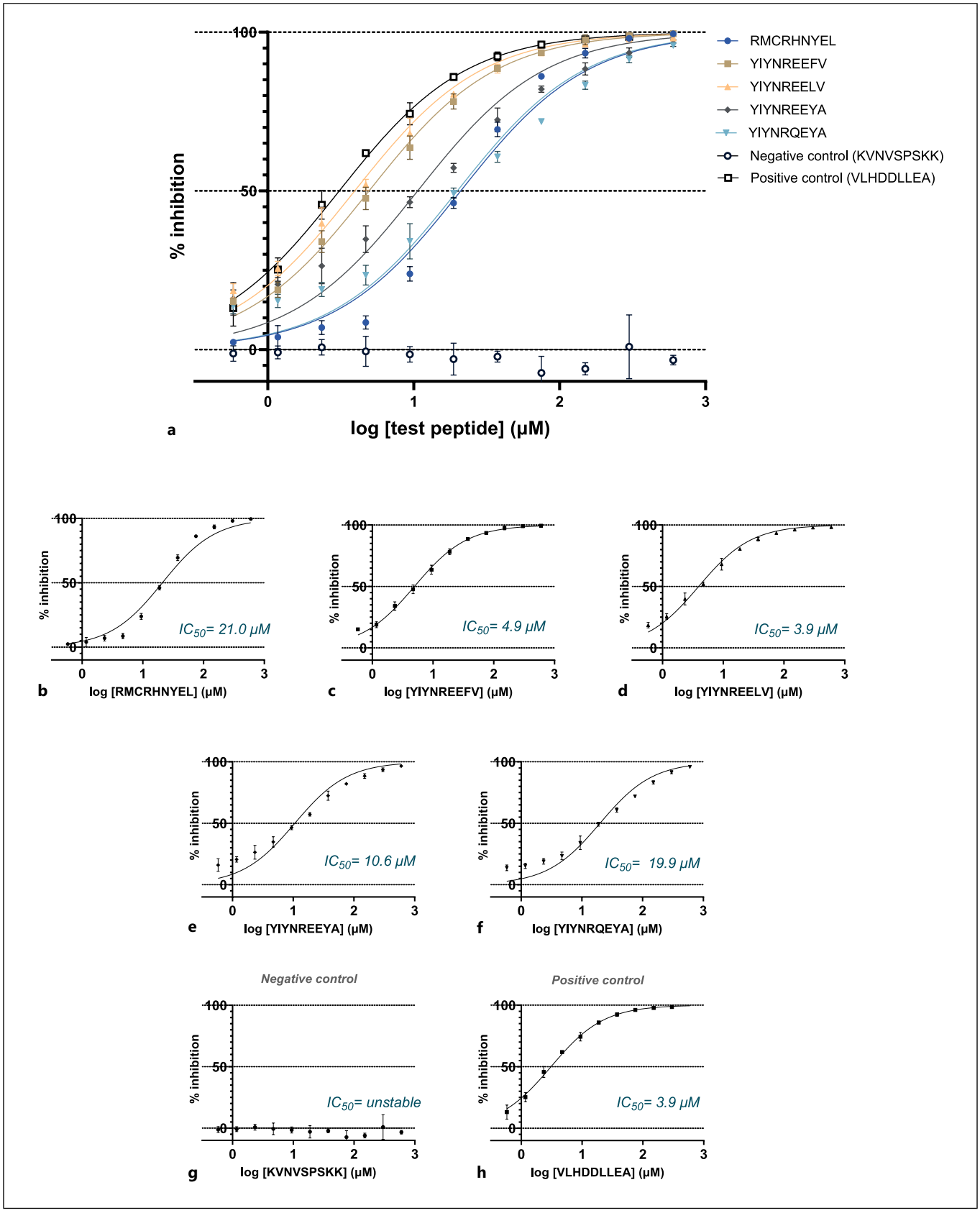
Predicted binding affinities were determined using NetMHCpan4.1 [20]. The listed peptide phenotype frequencies were calculated based on IPD-IMGT/HLA-database version 3.52 and German DKMS cohort allele frequencies as described previously [21, 28, 29]. IC_{50} values were calculated by performing nonlinear regression analysis on inhibition data of the performed competition-based binding assay [24]. [a]Only alleles with an allele frequency over 0.1% in the Deutsche Knochenmarkspenderdatei (DKMS) cohort [21]. [b]Disparity rates were calculated as: peptide phenotype frequency * (1 − peptide phenotype frequency).

production following immunization of HLA-A*02:01 transgenic mice with these peptides. All four peptides elicited a T-cell response, although differences between mice were observed (shown in Fig. 3). The YIYNRQEYA peptide showed a significant IFN-γ response in all three YIYNRQEYA-immunized mice ($p < 0.0001$; $p = 0.04$; $p = 0.006$). In addition, a slight but significant T-cell response after T2A2-peptide pulsing was also detected in both YIYNREEFV-immunized mice samples ($p = 0.03$; $p = 0.05$). A slight increase in IFN-γ production was detected for the YIYNREELV and RMCRHNYEL peptides, although these were not statistically significant.

Discussion

Matching for HLA-DPB1 decreases the risk of GvHD in HSCT recipients receiving a 10/10 matched unrelated donor [6–15]. More specifically, studies have shown that the PIRCHE-I score, as a measure for indirect $CD8^+$ T-cell allorecognition, is associated with the risk of GvHD in HLA-DPB1-mismatched 10/10 HSCT recipients [12, 15, 30]. However, since experimental data validating the PIRCHE algorithm still largely lack, we here investigated as a proof of principle whether HLA-DPB1-derived peptides predicted by PIRCHE-I could bind to HLA-A*02:01 in vitro and could elicit a T-cell response in vivo. The 19 most frequently occurring HLA-DPB1 alleles were analyzed to predict which HLA-DPB1-derived peptides could theoretically bind to HLA-A*02:01. All five different peptides identified by the PIRCHE algorithm – RMCRHNYEL, YIYNREEFV, YIYNREELV, YIYNREEYA, and YIYNRQEYA – were able to bind to the HLA-A*02:01 allele with an IC_{50} value of 21 μM or lower. It should be noted that the obtained IC_{50} values are difficult to compare with the predicted IC_{50} values, since (1) IC_{50} values depend on the reference peptide used, and (2) predicted values are calculated using a neural network trained with experimental data; the main aim of these in silico predictors is to classify peptides as strong, weak, or nonbinders. The peptides loaded on HLA-A*02:01-expressing T2A2 cells elicited a T-cell response in HLA-A*02:01-transgenic mice, where specifically immunization with the YIYNRQEFA peptide resulted in a clear T-cell response. Thus, we show that the identified HLA-DPB1-derived peptides can indeed bind to HLA-A*02:01 and are able to induce IFN-γ production in vivo, supporting previous studies showing that indirectly recognized HLA-DPB1 mismatches might be a risk for GvHD development during HSCT [12, 15, 30].

The YIYNREEYA peptide, which was also predicted by PIRCHE-I as an HLA-DPB1-derived peptide that could bind to HLA-A*02:01, demonstrated binding to the HLA-A*02:01 allele with an IC_{50} value of 11 μM, indicating that it has potential to be presented by HLA-A*02:01 on APCs to T cells. However, this peptide was not tested in vivo, since the amino acid sequence YIYNREEYV is present in the genome of the *Mus musculus*. Because these two sequences differ only at the amino acid located at the C-terminal residue of the peptide, which binds in the F-binding pocket [31], and because valine and alanine are very similar, it is likely that no T-cell alloreactivity can be seen in vivo. Therefore, this peptide needs to be validated in a human context. It is important to note that in humans, various



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HLA-DQB1 alleles contain the YIYNREEYA peptide as well, including DQB1*03:01 and DQB1*06:02. Together, these alleles have a combined allele frequency of 50.9% in a European Caucasian population according to the NMDP frequency register [22], suggesting that the YIYNREEYA peptide is likely considered as self in the majority of the population and will not lead to T-cell alloreactivity. Alternatively, this peptide may evoke immune responses in specific HLA-DPB1-mismatched settings and spread its response to the alternative HLA-DQB1 mismatches and vice versa. Such a scenario may for instance be relevant in the context of multiple transplantations. Clinical evaluation of the relevance of the YIYNREEYA is therefore less straightforward.

The PIRCHE algorithm addresses protein processing by selecting peptides with a proteasomal processing probability of 0.5 or greater according to the NetChop3.1 algorithm [5], indicating that the identified peptides have a high probability to be generated by the proteasome in APCs [32]. In the present study, in vitro validation of antigen processing was not executed. Future research should investigate protein processing of the identified HLA-DPB1 peptides. Such studies may include proteasomal cleavage analyses and/or immunization experiments with longer peptides. In addition, interindividual variability between mice was observed. As this observation could be a consequence of different percentages of T cells in the splenocytes, additional experiments with T cells instead of splenocytes could be performed to further investigate the T-cell response in vivo.

We demonstrate that the HLA-DPB1-derived peptides can lead to a response in vivo. With respect to clinical relevance, investigating T-cell alloreactivity against these HLA-DPB1-derived peptides in a human setting is of importance. Validation of alloreactive T cells in a human setting could be performed by using HLA-peptide tetramers to assess whether T cells specific for HLA-DPB1-derived peptides can be present in the blood of HSCT patients with GvHD. Based on population frequencies, HLA-A*02:01-YIYNREEFV tetramers could identify alloreactive T cells specific for the YIYNREEFV peptide. This peptide is the most frequently occurring peptide mismatch of the peptides we investigated with a disparity rate of 21% (shown in Table 2). Since approximately 50% of the European Caucasian population expresses HLA-A*02:01 [22], this peptide mismatch could be relevant in an estimated 10% of the total Western population. Besides using tetramers to investigate the presence of such T cells in individual patients, a retrospective clinical study could assess the effect of the identified HLA-DPB1 peptide mismatches on the development of GvHD development in a bigger cohort of patients receiving HSCT from a 10/10 matched donor. In this way, the clinical relevance of the indirect allorecognition of HLA-DPB1 mismatches in patients receiving HSCT can be addressed.

As an alternative to indirect recognition of T cells, direct recognition of HLA-DPB1 mismatches has been shown to play a role in the development of GvHD [6, 9, 30, 33]. Several biological models have been developed to predict the immunogenicity of an HLA-DPB1 mismatch, including a model that evaluates the cell surface expression of HLA-DPB1 molecules based on a single-nucleotide polymorphism [33] and the TCE model [6, 9]. The TCE model defines permissive and nonpermissive HLA-DPB1 mismatches based on the alloreactivity of structural TCEs shared between HLA-DPB1 alleles [6, 9]. Compared to permissive mismatches, nonpermissive mismatches were associated with an increased risk of overall mortality [14]. Interestingly, the YIYNREEFV peptide is present in all HLA-DPB1 alleles belonging to the most immunogenic TCE4 groups 1 and 2. This observation could potentially support the finding by Thus et al. [12], who showed that pairs with TCE-predicted graft-versus-host nonpermissive mismatches had significantly higher PIRCHE-I numbers compared to both permissive and host-versus-graft nonpermissive mismatches. Further research is warranted to assess the potential synergy or overlap of PIRCHE-I with other models such as the TCE model. In this context, HLA-DPB1-derived peptides that can be presented by other HLA class I alleles should be considered as well.

Although HLA-DPB1 mismatches have been reported to increase the risk for GvHD in multiple studies [6–10, 15], these mismatches can also decrease the hazard of disease relapse [7, 8, 10, 13, 15, 34]. Kawase et al. [34] identified various HLA-DPB1 mismatches that were significantly associated with a lower risk of relapse, of which the majority was not associated with a higher

Fig. 2. Log dose-inhibition curves with indicated IC_{50} values from the inhibition data of all test peptides (**b–f**) and negative and positive control peptides (**g**, **h**) for the HLA-A*02:01 allele. IC_{50} values for binding to HLA-A*02:01 were obtained by performing nonlinear regression analysis on the inhibition data of all peptides. Error bars indicate standard deviations. **a** Overview of the log dose-inhibition curves of all tested peptides including negative and positive control peptides shows variation in binding affinities between the five tested HLA-DPB1-derived peptides. **b–f** Log-inhibition curves for HLA-A*02:01 binding of RMCRHNYEL (**b**), YIYNREEFV (**c**), YIYNREELV (**d**), YIYNREEYA (**e**), and YIYNRQEYA (**f**) peptides having IC_{50} values of 21.0 μM, 4.9 μM, 3.9 μM, 10.6 μM, and 19.9 μM, respectively. **g**, **h** Log-inhibition curves for HLA-A*02:01 binding of negative (KVNVSPSKK) (**g**) and positive (VLHDDLLEA) (**h**) control peptides. The negative control peptide did not show any binding to the HLA-A*02:01 allele and the positive control peptide showed binding to HLA-A*02:01 with an IC_{50} value of 3.9 μM.

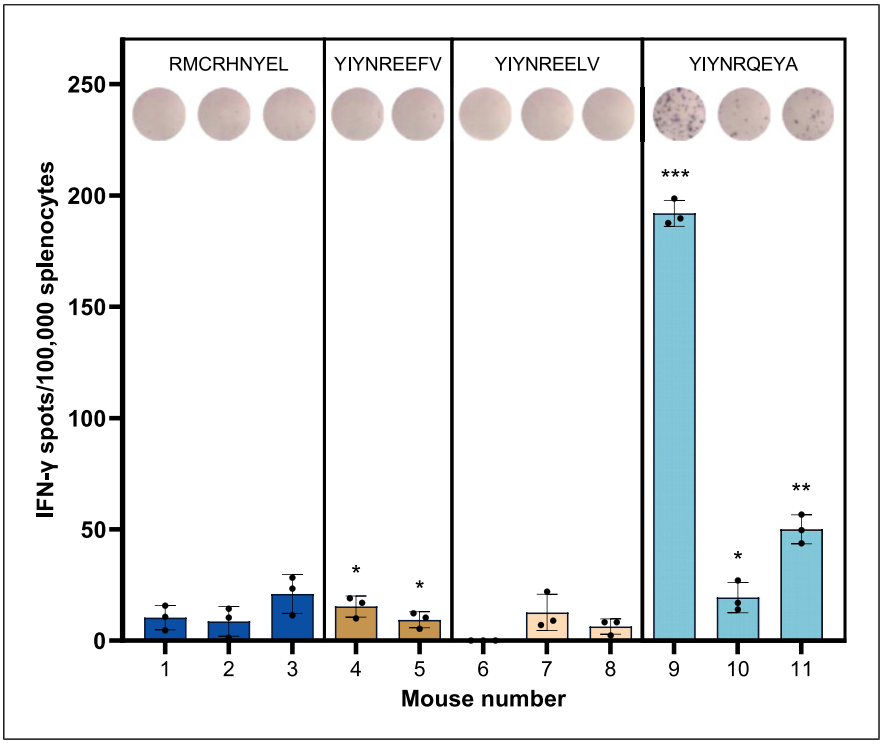

Fig. 3. HLA-DPB1-derived peptides RMCRHNYEL, YIYNREEFV, YIYNREELV, and YIYNRQEYA loaded on T2A2 cells elicited a T-cell response in HLA-A*02:01 transgenic mice. T-cell alloreactivity against immunized HLA-DPB1-derived peptides in HLA-A*02:01 mice was examined using an IFN-γ ELISpot assay with peptide-loaded T2A2 cells. Spot-forming cells for the immunized peptides were corrected for the mean signal from a nonimmunized control peptide (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

occurrence of severe acute GvHD in their previous study [35]. Three of the HLA-DPB1 mismatches they identified correspond with a YIYNREEFV mismatch in graft-versus-host direction [34]. Evaluating the presence or absence of HLA-A*02:01 in these patients may provide further indications on a potential role for indirect recognition of mismatched HLA-DPB1 on relapse.

Overall, we here for the first time structurally evaluated the binding and in vivo immunogenicity of HLA-DPB1-derived peptides in the context of HLA-A*02:01. These PIRCHE-I peptides derived from HLA-DPB1 mismatches can contribute to GvHD development via the indirect pathway of T-cell recognition, which can be predicted by the PIRCHE-I algorithm. Our data suggest that the PIRCHE algorithm is a valid indicator for alloreactivity caused by HLA-DPB1 mismatches. Additionally, our findings provide experimental evidence that HLA-DPB1 mismatches can induce T-cell alloreactivity and may thereby contribute to GvHD development. Eventually, data like presented in the current study could further specify which HLA-DPB1 mismatches are particularly associated with high risk for T-cell alloreactivity following HSCT.

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Statement of Ethics

All mouse experimental procedures were approved by the Institute's Animal Ethics Committee and by the Dutch Central Authority for Scientific Procedures on Animals (2013.III.08.061).

Conflict of Interest Statement

The authors of this manuscript have conflicts of interest to disclose. The UMC Utrecht has filed a patent application on the prediction of an alloimmune response against mismatched HLA. E.S. is listed as an inventor on these patents. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

E.S. and K.G. conceived the experiments; E.T.M.P., A.E.M., L.M.T. and KG conducted the experiments; E.T.M.P., A.E.M., L.M.T., K.G. and E.S. analyzed the results; and E.T.M.P., A.E.M., and E.S. drafted the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary data.

References

1 Shaw BE, Arguello R, Garcia-Sepulveda CA, Madrigal JA. The impact of HLA genotyping on survival following unrelated donor haematopoietic stem cell transplantation: review. Br J Haematol. 2010;150(3):251–8.
2 Mangum DS, Caywood E. A clinician's guide to HLA matching in allogeneic hematopoietic stem cell transplant. Hum Immunol. 2022;83(10):687–94.
3 Hornick P, Rose M. Transplantation immunology: methods and protocols. In: Hornick P, Rose M, editors. Methods in molecular biology Humanapress; 2006. p. 161–71.
4 Geneugelijk K, Thus KA, Spierings E. Predicting alloreactivity in transplantation. J Immunol Res. 2014;2014:159479–12.
5 Geneugelijk K, Spierings E. Matching donor and recipient based on predicted indirectly recognizable human leucocyte antigen epitopes. Int J Immunogenet. 2018;45(2):41–53.
6 Crocchiolo R, Zino E, Vago L, Oneto R, Bruno B, Pollichieni S, et al. Nonpermissive HLA-DPB1 disparity is a significant independent risk factor for mortality after unrelated hematopoietic stem cell transplantation. Blood. 2009;114(7):1437–44.
7 Shaw BE, Gooley T, Madrigal JA, Malkki M, Marsh SGE, Petersdorf EW. Clinical importance of HLA-DPB1 in haematopoietic cell transplantation. Tissue Antigens. 2007;69(Suppl 1):36–41.
8 Shaw BE, Gooley TA, Malkki M, Madrigal JA, Begovich AB, Horowitz MM, et al. The importance of HLA-DPB1 in unrelated donor hematopoietic cell transplantation. Blood. 2007;110(13):4560–6.
9 Zino E, Frumento G, Marktel S, Sormani MP, Ficara F, Di Terlizzi S, et al. A T-cell epitope encoded by a subset of HLA-DPB1 alleles determines nonpermissive mismatches for hematologic stem cell transplantation. Blood. 2004;103(4):1417–24.
10 Shaw BE, Potter MN, Mayor NP, Pay AL, Smith C, Goldman JM, et al. The degree of matching at HLA-DPB1 predicts for acute graft-versus-host disease and disease relapse following haematopoietic stem cell transplantation. Bone Marrow Transplant. 2003;31(11):1001–8.
11 Fleischhauer K, Shaw BE. HLA-DP in unrelated hematopoietic cell transplantation revisited: challenges and opportunities. Blood. 2017;130(9):1089–96.
12 Thus KA, Ruizendaal MTA, de Hoop TA, Borst E, van Deutekom HWM, Te Boome L, et al. Refinement of the definition of permissible HLA-DPB1 mismatches with predicted indirectly ReCognizable HLA-DPB1 epitopes. Biol Blood Marrow Transplant. 2014;20(11):1705–10.
13 Shaw BE, Marsh SGE, Mayor NP, Russell NH, Madrigal JA. HLA-DPB1 matching status has significant implications for recipients of unrelated donor stem cell transplants. Blood. 2006;107(3):1220–6.
14 Fleischhauer K, Shaw BE, Gooley T, Malkki M, Bardy P, Bignon JD, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. Lancet Oncol. 2012;13(4):366–74.
15 Stenger W, Künkele A, Niemann M, Todorova K, Pruß A, Schulte JH, et al. Donor selection in a pediatric stem cell transplantation cohort using PIRCHE and HLA-DPB1 typing. Pediatr Blood Cancer. 2020;67(3):e28127.
16 Geneugelijk K, Spierings E. PIRCHE-II: an algorithm to predict indirectly recognizable HLA epitopes in solid organ transplantation. Immunogenetics. 2020;72(1–2):119–29.
17 Thus KA, Te Boome L, Kuball J, Spierings E. Indirectly recognized HLA-C mismatches and their potential role in transplant outcome. Front Immunol. 2014;5(210):210.
18 Geneugelijk K, Thus KA, Van Deutekom HWM, Calis JJA, Borst E, Keşmir C, et al. Exploratory study of predicted indirectly recognizable HLA epitopes in mismatched hematopoietic cell transplantations. Front Immunol. 2019;10(880):880.
19 Ayuk F, Bornhäuser M, Stelljes M, Zabelina T, Wagner EM, Schmid C, et al. Predicted indirectly ReCognizable HLA epitopes (PIRCHE) are associated with poorer outcome after single mismatch unrelated donor stem cell transplantation: a study of the cooperative transplant study group (KTS) of the German group for Bone Marrow and stem cell transplantation (DAG-KBT). Transfus Med Hemother. 2019;46(5):370–5.
20 Reynisson B, Alvarez B, Paul S, Peters B, Nielsen M. NetMHCpan-4.1 and NetMHCIIpan-4.0: improved predictions of MHC antigen presentation by concurrent motif deconvolution and integration of MS MHC eluted ligand data. Nucleic Acids Res. 2020;48(W1):W449–54.
21 Seitz S, Lange V, Norman PJ, Sauter J, Schmidt AH. Estimating HLA haplotype frequencies from homozygous individuals: a Technical Report. Int J Immunogenet. 2021;48(6):490–5.
22 Gragert L, Madbouly A, Freeman J, Maiers M. Six-locus high resolution HLA haplotype frequencies derived from mixed-resolution DNA typing for the entire US donor registry. Hum Immunol. 2013;74(10):1313–20.
23 Dausset J, Cann H, Cohen D, Lathrop M, Lalouel JM, White R. Centre d'etude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome. Genome *Genomics* Published Online. 1990;6(3):575–7.
24 Kessler JH, Benckhuijsen WE, Mutis T, Melief CJM, van der Burg SH, Drijfhout JW. Competition-based cellular peptide binding assay for HLA class I. Curr Protoc Immunol. 2004;Chapter 18(12):Unit 18.12–5.
25 Van Der Burg SH, Ras E, Drijfhout JW, Benckhuijsen WE, Bremers AJ, Melief CJ, et al. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells. Identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. Hum Immunol. 1995;44(4):189–98.
26 den Haan JMM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science. 1998;279(5353):1054–7.
27 Hoppes R, Oostvogels R, Luimstra JJ, Wals K, Toebes M, Bies L, et al. Altered peptide ligands revisited: vaccine design through chemically modified HLA-A2–restricted T cell epitopes. J Immunol. 2014;193(10):4803–13.
28 Barker DJ, Maccari G, Georgiou X, Cooper MA, Flicek P, Robinson J, et al. The IPD-IMGT/HLA database. Nucleic Acids Res. 2023;51(D1):D1053–60.
29 Mayo O. A century of Hardy-Weinberg equilibrium. Twin Res Hum Genet. 2008;11(3):249–56.
30 Buhler S, Baldomero H, Ferrari-Lacraz S, Mamez AC, Masouridi-Levrat S, Heim D, et al. Analysis of biological models to predict clinical outcomes based on HLA-DPB1 disparities in unrelated transplantation. Blood Adv. 2021;5(17):3377–86.
31 Nguyen AT, Szeto C, Gras S. The pockets guide to HLA class I molecules. Biochem Soc Trans. 2021;49(5):2319–31.
32 Nielsen M, Lundegaard C, Lund O, Keşmir C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics. 2005;57(1–2):33–41.
33 Schöne B, Bergmann S, Lang K, Wagner I, Schmidt AH, Petersdorf EW, et al. Predicting an HLA-DPB1 expression marker based on standard DPB1 genotyping: linkage analysis of over 32,000 samples. Hum Immunol. 2018;79(1):20–7.
34 Kawase T, Matsuo K, Kashiwase K, Inoko H, Saji H, Ogawa S, et al. HLA mismatch combinations associated with decreased risk of relapse: implications for the molecular mechanism. Blood. 2009;113(12):2851–8.
35 Kawase T, Morishima Y, Matsuo K, Kashiwase K, Inoko H, Saji H, et al. High-risk HLA allele mismatch combinations responsible for severe acute graft-versus-host disease and implication for its molecular mechanism. Blood. 2007;110(7):2235–41.