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A proposed algorithm predictive for cytotoxic T cell alloreactivity

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ABSTRACT

Previously we showed that with increasing number of amino acid (AA) differences in single HLA class I mismatched molecules the probability of T cell (CTL) alloreactivity decreases. It is unlikely that every AA difference will affect CTL alloreactivity in a similar way; we hypothesized that the effect of an AA difference may be dependent on its position and/or physicochemical properties. We selected 131 donor-recipient pairs with either a single HLA-A or -C mismatch in the graft-versus-host (GvH) direction and that were compatible for HLA-B, -DRB1 and -DQB1. The alloreactive cytotoxic T lymphocyte precursor (CTLp) frequency was determined and was associated with the AA differences between the single HLA class I mismatches. In the β sheet only AAs that are non-compatible in their physicochemical properties affect CTL alloreactivity, while in the α helices both compatible and non-compatible AAs affect the CTLp assay. Positions 62, 63, 73, 76, 77, 80, 99, 116, 138, 144, 147 and 163 were bivariately associated with the CTLp assay, irrespective of the total number of AA differences. In multivariate analysis positions 62, 63, 73, 80, 116, 138, 144 and 163 were found to be most predictive for a negative CTLp assay. These results formed the basis for a weighted predictive mismatch score; pairs with the highest mismatch scores are estimated to be 13 times more likely to have a negative CTLp. This new algorithm may be a tool in donor selection for hematopoietic stem cell transplantation.

INTRODUCTION

One of the major issues in hematopoietic stem cell transplantation (HSCT) is selection of a suitable donor when a fully human leukocyte antigen (HLA) matched donor is not available. A significant number of patients have to be transplanted with hematopoietic stem cells from a mismatched donor. The immunogenicity of MHC molecules may differ; therefore the challenge is to select a donor for whom the HLA mismatch of the recipient is not very immunogenic. This is feasible as confrontation with foreign MHC will not always lead to an alloreactive immune response.[124] Previously we have shown that a low cytotoxic T lymphocyte precursor (CTLp) frequency of the donor against the HLA mismatch of the recipient is associated with good clinical results of HLA class I mismatched HSCT.[37] Some MHC class I mismatches are indeed associated with low or undetectable CTLp frequencies, whereas high CTLp frequencies can be seen in other HLA class I matched donor-recipient pairs.[124-126]

Previously we investigated whether CTL alloreactivity could be predicted by the number of AA differences on the α helices and the β sheet of single MHC class I mismatched molecules in 74 donor-recipient pairs.[127] Quantification of the allogeneic CTL response *in vitro* was obtained by the CTLp assay,[36] because it has been proven to be clinically relevant in predicting allogeneic HSCT outcome.[128-134] These studies focused on the AA differences on those parts of MHC class I molecules that are important for T cell receptor (TCR) contact and/or peptide binding. Motifs relevant for MHC-TCR interaction are predominantly located in the α helices and peptides binding residues are predominantly located in the β sheet.[33,34,135-138] The preliminary results showed that CTL alloreactivity could be predicted to certain extend. Single HLA-C mismatches with five or more AA differences on the α helices and five or more AA differences on the β sheet did not lead to T cell mediated alloreactivity *in vitro*. So, with increasing number of AA differences the probability of a negative CTLp assay increased significantly. In that pilot study the group of single HLA-A, and -B mismatches was too small and included too many mismatches with few AA differences to enable generalization of these findings to all MHC class I molecules.

The humoral alloimmune response to HLA class I mismatches can be successfully predicted by the HLAMatchmaker algorithm.[39,139] As HLAMatchmaker takes only antibody accessible sites of the HLA molecules into consideration, it is not a suitable tool to predict CTL alloreactivity.[140] Previous attempts to associate predictive levels of cellular alloimmunity with HSCT outcome have been unsuccessful.[42,141,142] So far, the actual outcome of the CTLp assay is used for donor selection in our center. The real challenge is to predict cellular alloimmune responses, especially because the CTLp assay is a complicated and time consuming test. In the present study we investigated a larger population of single HLA class I mismatched pairs and attempt to extend our previous findings to other HLA class I molecules.

It is logical to assume that not every AA difference between MHC class I molecules may affect CTL alloreactivity in a similar way. Both position (specific positions, α helices or β sheet)[143] and physicochemical properties (size, polarity and charge) of the AAs

involved may play a role.[144-146] We aim to develop an algorithm in which we incorporate all aspects of the AA differences to predict CTL alloreactivity against single MHC class I mismatches and to use this as a tool for single HLA class I mismatched unrelated or related donor selection in HSCT.

PATIENTS AND METHODS

Donor-recipient pairs

In total 164 donor-recipient pairs registered by the Europdonor Foundation from 1990 to 2008, for whom successful CTLp assays were performed, were available for this study. The patients were treated at Dutch hematopoietic stem cell transplantation centers. The 150 unrelated donors originated from national or international donor registries and 14 donors were related. All donor-recipient pairs had a single HLA-A, -B or -C mismatch in the GvH direction and were compatible for HLA-DRB1 and -DQB1. Donor-recipient pairs with single HLA class I mismatches without AA differences at the previously specified positions in the α helices or the β sheet [HLA-C*03:03-03:04 (n=11), HLA-C*07:01-07:18 (n=1) and HLA-B*35:02-35:04 (n=1)] were excluded from analysis. The reason for this is that the AA differences in these combinations do not affect T cell recognition, as they are located on parts of the molecule that are not involved in the interaction with the TCR.

This study included only HLA-A (n=55) and -C (n=76) mismatched pairs, because single HLA-B (n=20) mismatched pairs were rare and distinctive in their number and position of AA differences. Finally, 131 single MHC class I mismatched donor-recipient pairs were included in the analysis; the specific HLA mismatches are shown in Table 1.

HLA genotyping and AA sequencing

All donors and patients were typed at high resolution for HLA-A, -B, -C, -DRB1, -DQB1 as described previously.[125] Briefly, polymerase chain reaction sequence specific primer (PCR-SSP) for high resolution allele typing and sequence based typing (SBT), for part of the HLA-C alleles, were used. AA sequences were obtained by using the European Bioinformatics Institute website.[22] MHC class I mismatches were examined for AA differences in the α1/2 domain, with specific interest in positions 50-85 and 138-179 in the α helices and positions 4-12, 21-28, 32-37, 94-102, 112-118 and 123-126 in the β sheet.[127]

CTLp assay

The CTLp assays were performed as described by Zhang *et al,*[36] with minor modifications as described by Oudshoorn *et al.*[125] CTLp assays were performed in the graft-versus-host (GvH) direction. A negative CTLp assay was defined as ≤ 1 recipient specific CTL per 10^6 peripheral blood lymphocytes.

Physicochemical properties of AAs

We divided the AAs into five groups according to their physicochemical properties (as listed in Table 2). All AA differences of the single MHC class I mismatched pairs

Table 1. HLA class I mismatches (GvH direction) in the study population

HLA-A (n=55)		
01:01-03:01	02:01-34:02	24:02-31:01
01:01-24:02	02:01-68:01 (n=3)*	24:03-24:02
01:01-68:01 (n=2)*	02:03-02:07	25:01-26:01
02:01-01:01	02:05-02:01 (n=2)*	25:01-34:02
02:01-02:05 (n=3)*	02:05-24:02	26:01-25:01
02:01-02:06 (n=5)*	03:01-11:01	31:01-02:01
02:01-02:11	11:01-03:01	31:01-30:01 (n=2)*
02:01-03:01	11:01-26:01	32:01-01:01
02:01-11:01	11:01-68:01	32:01-32:02
02:01-23:01	11:04-11:01	32:01-68:01
02:01-26:01 (n=2)*	11:04-68:02	68:01-32:01
02:01-31:01	24:02-02:01	68:01-69:01
02:01-32:01 (n=2)*	24:02-23:01	68:02-68:01
02:01-33:01	24:02-24:03 (n=2)*	
HLA-C (n=76)		
01:02-02:02 (n=3)*	03:04-06:02	07:02-05:01
01:02-03:03 (n=2)*	03:04-07:01	07:02-15:02
01:02-03:04 (n=2)*	04:01-02:02	07:04-05:01 (n=2)*
01:02-07:02	04:01-16:01 (n=3)*	08:02-08:01
01:02-14:02	05:01-01:02	12:03-07:01 (n=2)*
02:02-01:02 (n=2)*	05:01-03:04	14:02-15:02 (n=4)*
02:02-03:03 (n=2)*	05:01-14:02	15:02-02:02
02:02-03:04	05:01-15:02	15:02-03:04 (n=2)*
02:02-05:01 (n=2)*	05:01-16:04	15:02-04:01 (n=2)*
02:02-07:01	07:01-02:02	15:02-14:02
02:02-07:02	07:01-03:03	15:02-16:02
02:02-14:02	07:01-05:01 (n=2)*	16:01-04:01
02:02-15:02 (n=4)*	07:01-07:02	16:01-05:01
03:03-04:01 (n=4)*	07:01-12:03 (n=2)*	16:01-07:01
03:03-07:02	07:01-15:02	16:02-07:01
03:04-01:02	07:02-01:02	16:04-05:01
03:04-04:01	07:02-02:02 (n=2)*	17:01-06:02

*Of the 65 multiple donor-recipient pairs, 14 (21.5%) had aberrant CTLp assays.

were categorized into compatible (within group) or non-compatible (between group) AA differences. We hypothesized that compatible AA differences may have a different influence on CTL alloreactivity than non-compatible AA differences.

Statistical analysis

We investigated the association of the number of all, non-compatible and compatible AA differences on the α helices and β sheet (combined and separately) with the occurrence of a negative CTLp assay. The AA difference variables were categorized into

Table 2. Five groups of AAs according to their physicochemical properties

Negatively charged	Asp (D), Glu (E)
Positively charged	Lys (K), His (H), Arg (R)
Polar / Neutral	Gly (G), Thr (T), Ser (S), Gln (Q), Asn (N)
Apolar	Leu (L), Ile (I), Val (V), Met (M), Cys (C), Ala (A), Pro (P)
Large / Apolar	Trp (W), Phe (F), Tyr (Y)

three groups (tertiles) based on the distribution over the HLA mismatches. We chose to use tertiles to ensure objectivity and even sized categories. In all variables the group with the lowest number of AA differences was set as reference. Differences in discrete variables (all AA difference variables (in tertiles), HLA-DPB1 mismatching (yes/no) and killer-cell immunoglobulin-like receptor (KIR) ligand mismatching (yes/no) between positive and negative CTLp assay were examined with Pearson's Chi Square test.

Odds ratios (OR), 95% confidence intervals (95% CI) and p-values were obtained using logistic regression analysis to quantify the association between the number of AA differences and negative CTLp assay. In addition we analyzed each specific different AA position (yes/no) separately to test for association with negative CTLp assay. All AA positions with a significance level below p-value ≤ .200 were tested in bivariate analysis correcting for the total number of AA differences. Those that remained associated with negative CTLp assay at a significant level of p-value ≤ .200 were selected for multivariate analysis.

Multivariate analysis using a backward stepwise approach (based on the likelihood ratio test) was conducted to identify the most predictive model for negative CTLp assay. Under this approach, we start with fitting a model with all the AA positions of interest (identified in univariate and bivariate analysis). Then the least significant AA position is dropped. We continue by successively re-fitting reduced models and then re-considering all dropped AA positions for re-introduction into the model. This means that two separate significance levels must be chosen for deletion from the model and for adding to the model (the entry p-value is set at .050 and the removal p-value at .100).

The β estimates (regression coefficients) of the AA positions remaining in the final prediction model were used to define a weighted predictive AA 'mismatch score'. This mismatch score was also categorized into three groups (tertiles) based on the distribution over the donor-recipient pairs and the group with the lowest scores was set as reference. For both the multivariate prediction and the final model we included model evaluation statistics and Somer's D and c-statistics as a measure of association.

See for statistical decision making Figure 1. Two sided p-values ≤ .050 were considered statistically significant and all analyses were performed using SPSS 17.0 for Windows.

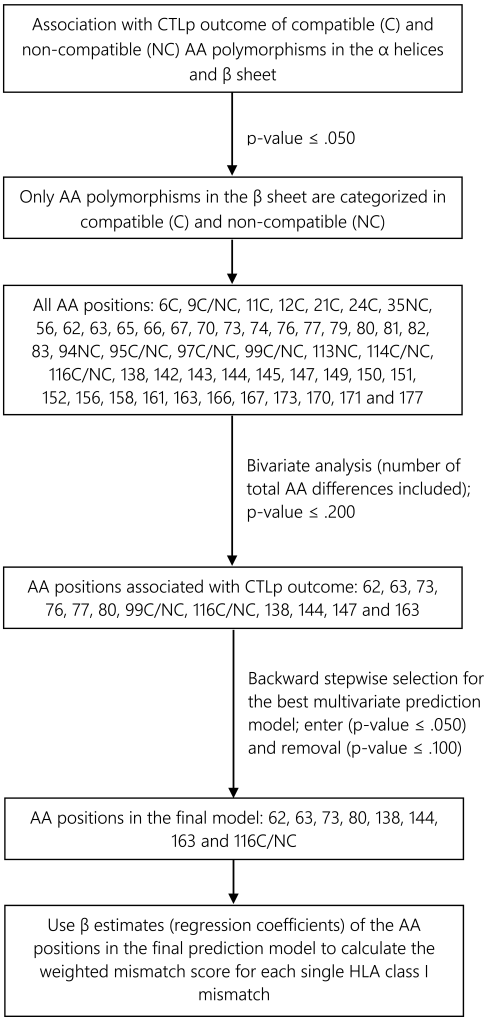

Figure 1. Statistical decision making

RESULTS

We tested whether CTL alloreactivity is dependent on position and/or physicochemical properties of AA differences in mismatched HLA class I molecules. The study population consisted of 55 donor-recipient combination mismatched for HLA-A and 76 for HLA-C, of which 79 had a positive and 52 had a negative CTLp assay. The polymorphic AA positions are very different among these mismatched molecules. HLA-A molecules are more polymorphic in the α helices and the HLA-C molecules are more polymorphic in the β sheet. Also the total number of AA differences varies between the mismatches [median (range): 11 (1-22)].

Of the 131 donor-recipient pairs, 97 had either one (n=66) or two (n=31) mismatched HLA-DPB1 allele(s) in the GvH direction. For 3 donor-recipient pairs the HLA-DPB1 match grade was unclear due to missing typing of the donor. Of the 76 HLA-C mismatched donor-recipient pairs, 19 had a killer-cell immunoglobulin-like receptor (KIR) ligand mismatch in the GvH direction and 57 were matched for their KIR ligands. Neither HLA-DPB1 nor KIR ligand mismatching was associated with the CTLp assay (data not shown).

With increasing number of AA differences in both the α helices and β sheet the probability of a negative CTLp assay increased statistically significant. In the α helices all AA differences contribute to this effect, while in the β sheet only AA differences with non-compatible physicochemical properties do (Figure 2). Therefore, only AA differences in the β sheet are categorized in compatible and non-compatible according to their physicochemical properties. Next, we combined the number of all AA differences in the α helices and only the number of non-compatible AA differences in the β sheet; the

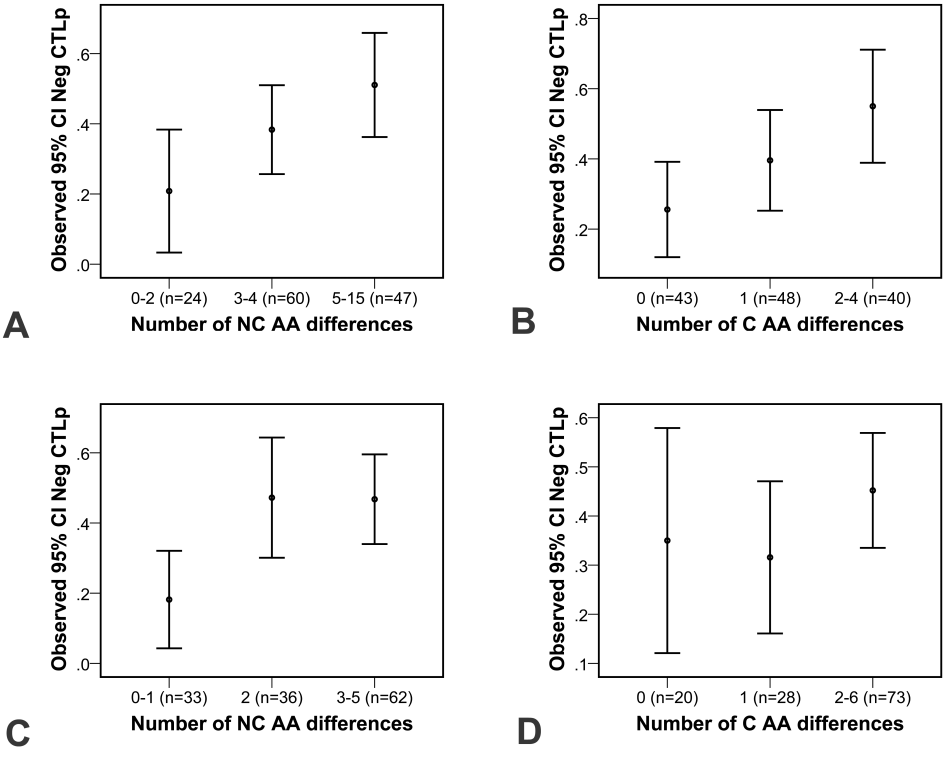

Figure 2. Observed association between the number of noncompatible (NC) (A: Chi square = 6.151; p = 0.046) and compatible (C) (B: Chi square = 7.493; p = 0.024) AA differences on the α helices and the probability of a negative CTLp assay. Observed association between the number of noncompatible (NC) (C: Chi square = 8.530; p = 0.046) and compatible (C) (D: Chi square = 2.156; p = 0.340) AA differences on the β sheet and the probability of a negative CTLp assay.

odds that the CTLp assay is negative is almost five times larger for mismatches with ≥9 AA differences compared to mismatches with 0-5 AA differences (Table 3 and Figure 3).

Twelve AA positions (62, 63, 73, 76, 77, 80, 99, 116, 138, 144 and 147) in both α helices and β sheet were identified to be associated with a negative CTLp assay, even after correction for the total number of AA differences (data not shown). Eight AA positions (62, 63, 73, 80, 138, 144, 163 and 116) remained in the final model (after backward stepwise selection) and are therefore most predictive for a negative CTLp assay (Table 4).

Table 3. Association between the number of all AA difference on the α helices combined with the number of non-compatible AA differences on the β sheet and the probability of a negative CTLp assay (univariate analysis)

	n	OR (95% CI)*	p-value
α helices (all) + β sheet (non-compatible)			.016
0-5	27	1.00 (ref)	
6-8	44	2.51 (0.80-7.93)	.116
9-19	60	4.70 (1.57-14.06)	.006

*Odds ratios and 95% confidence intervals.

Table 4. Association between specific positions of AA differences and the probability of a negative CTLp assay (multivariate analysis)

	β estimate	OR (95% CI)*	p-value
62	2.232	9.32 (1.52-57.11)	.016
63	-2.111	0.12 (0.01-1.02)	.052
73	1.159	3.19 (1.26-8.08)	.015
80	1.042	2.84 (1.19-6.78)	.019
138	1.149	3.15 (0.84-11.85)	.089
144	-1.622	0.20 (0.03-1.35)	.097
163	1.081	2.95 (1.20-7.26)	.019
116			.035
116 non-compatible	0.449	1.57 (0.65-3.80)	.321
116 compatible	-1.976	0.14 (0.02-1.03)	.054
Overall model evaluation	**Chi Square**		**p-value**
Likelihood ratio test	35.739		.000
Measure of concordance	**Test statistic**		
Somer's D	.582		
C-statistics	.791		

*Odds ratios and 95% confidence intervals. The β estimate (regression coefficient) represents the magnitude of the association with negative CTLp assay. Overall model evaluation statistics examine the predictive improvement of the fitted model compared to the intercept only model. Somer's D and c-statistics represents measures of association; whether predicted probabilities agree with actual outcomes.

Their β estimates (regression coefficients) formed the basis of the 'weighted predictive AA mismatch score' for a negative CTLp assay. Mismatched pairs with the highest mismatch scores are 13 times more likely to have a negative outcome of the CTLp assay (Table 5 and Figure 4). Correction for the total number of AA differences by including this variable in the model did not alter the association.

DISCUSSION

Attempts to predict molecular interactions that lead to CTL alloreactivity have not yet resulted in clear-cut results. We retrospectively analyzed the association between CTL alloreactivity *in vitro* and single HLA-A and -C mismatches in 131 donor-recipient pairs. Our results show that compared to HLA alleles that are more similar to self HLA alleles, divergent HLA alleles more often do not lead to T cell mediated alloreactivity *in vitro*. This is in line with the previously proposed hypothesis that the possibility for TCR-MHC binding decreases if allogeneic MHC differs too much from autologous MHC.[127] Next we looked at AAs that are (non-)compatible in their physicochemical properties. In the β sheet only AAs that are distinctive in their physicochemical properties affect CTL alloreactivity negatively. This suggests that in contrast to compatible AAs, non-

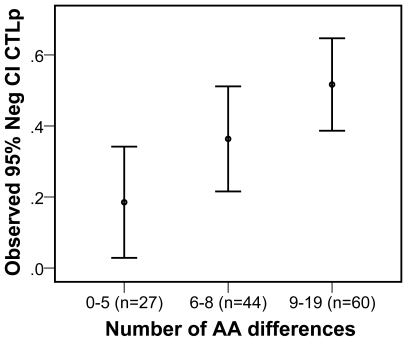

Figure 3. Observed association between the number of all AA (AA) differences on the α helices combined with the number of noncompatible AA differences on the β sheet and the probability of a negative CTLp assay (Chi square = 8.854; p = 0.012).

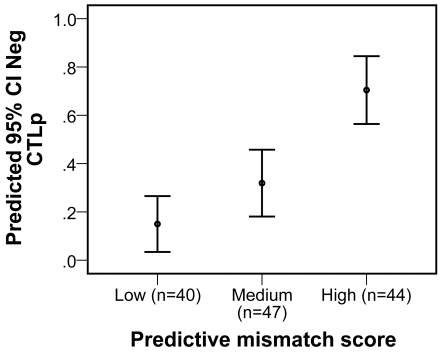

Figure 4. Predicted association between the weighted predictive mismatch score and the probability of a negative CTLp assay (Chi square = 28.770; p = 0.000)

Table 5. Association between the predictive mismatch score and the probability of a negative CTLp assay (univariate analysis)

	n	Score	OR (95% CI)*	p-value
Score				**.000**
Low	40	< 1.081	1.00 (ref)	
Medium	47	1.081-2.218	2.66 (0.92-7.69)	.072
High	44	> 2.218	13.51 (4.58-39.91)	.000
Overall model evaluation		**Chi Square**		**p-value**
Likelihood ratio test		29.905		.000
Measure of concordance		**Test statistic**		
Somer's D		.509		
C-statistics		.755		

*Odds ratios and 95% confidence intervals. The β estimate (regression coefficient) represents the magnitude of the association with a negative CTLp assay. Overall model evaluation statistics examine the predictive improvement of the fitted model compared to the intercept only model. Somer's D and c-statistics represents measures of association; whether predicted probabilities agree with actual outcomes.

compatible AA differences influence the peptide binding repertoire in such a way that a MHC-peptide complex that is very distinct from self MHC-peptide complexes prevents allorecognition. In the α helices both compatible and non-compatible AAs affect the CTLp assay, implying that in MHC-TCR interaction all AA differences in the α helices have similar effects on allorecognition.

Furthermore, we were able to identify a differential importance of AA differences at specific positions for the T cell mediated alloresponse to HLA class I mismatches.

AAs at position 62, 76 and 163 point up from the antigen binding site and are believed to be in direct contact with the TCR.[33,136] AAs at position 63, 73, 77, 80, 147, 99 and 116 point toward the antigen binding site and are believed to be involved in peptide binding[33]. Position 63 and 99 form part of pocket A and B, 73 of pocket C, 147 of pocket E and 77, 80 and 116 of pocket F[34,147,148] and residue 116 plays a key role in determining the specificity of the F pocket.[148] Some of these positions and specific allele mismatches have been associated with transplant outcome; although not a single position or allele mismatch was consistently identified to be detrimental or favourable.[42-47,149] Differences in study -design and/or -population may be responsible for the inconsistent results.

The association between AA polymorphism and the CTLp assay that we found is not absolute and may be diluted because of the additional impact of non inherited maternal antigens,[150] minor histocompatibility antigens or indirect recognition of a peptide derived from the mismatched HLA molecule in one of the shared HLA molecules. Furthermore, the TCR repertoire of an individual is based on self MHC and is therefore unique while alloreactivity is often based on cross reactivity by virus-specific memory T cells.[151] Together this may explain the unpredictability of T cell responses.

The basis of T cell mediated alloreactivity has been debated extensively and has generally resulted in two possible models, a MHC- and a peptide- driven mechanism. The first proposes that the alloreactive TCR directly recognizes polymorphisms in the allo MHC molecules independently of the bound peptide and thereby adopting novel docking modes.[152] The second suggests that responses to bound peptides that differ in sequence as well as peptides adopting different conformations when bound to the allo or self MHC molecules are most important in triggering alloreactivity.[135,153-155] If molecular mimicry is the basis of the alloimmune response this would explain why increasingly divergent allo MHC molecules are less likely to be targets for T cell mediated cross-reactivity.[35] Probably both mechanisms play a role,[35,156,157] which would explain why the strongest effect is found when combining both the number of AA differences in α helices (TCR contact) and the β sheet (peptide binding region).

In this study we were able to reproduce previous findings[127] and extend them to single mismatched HLA-A molecules. Unfortunately the number of single HLA-B mismatches was too low to show that a similar algorithm applies for HLA-B as well. Due to the relative low numbers we were also not able to stratify for HLA-A and -C. Therefore we could not test whether the mode of interaction with TCRs is similar for these two types of molecules. Although promising, these results need to be prospectively validated in a larger and extended population before the algorithm can be used for single HLA class I mismatched unrelated or related donor selection in HSCT. What these results do clearly show is that an HLA Class I mismatch with few or small AA differences is not necessarily better than an HLA Class I mismatch with numerous or large AA differences. The next step is to evaluate the clinical value of the weighted prediction mismatch score for transplant outcome.

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