Allogeneic haematopoietic stem cell donation and transplantation across the MHC class I barrier: "Faster is better than more. More is better than less".

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Allogeneic MHC class I molecules with numerous sequence differences do not elicit a CTL response.

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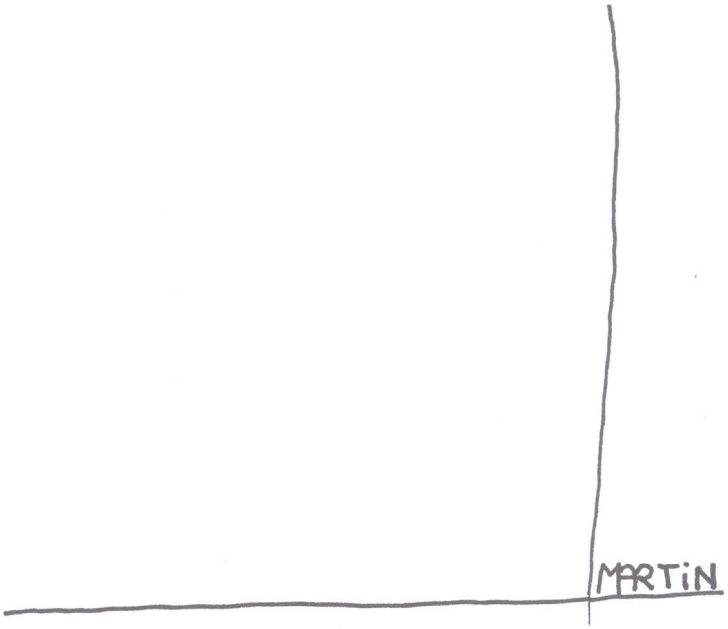

Very very foreign MHC molecules

Abstract

CD8+ T cell-mediated alloreactivity is generally believed to involve recognition of the α1/α2 domains of donor-type class I MHC molecules as well as the peptides they bind. Using the CTLp assay outcome as a parameter for the induction of alloreactivity, we have retrospectively surveyed 80 haematopoietic stem cell donor/patient pairs that feature a range of allelic differences at single HLA-A, -B, and -C loci in an attempt to probe the predictive value of such mismatches. In contrast to the expectation that greater degree of allelic disparity would lead to more alloreactivity, we found that in a substantial number of cases, class I MHC molecules with numerous sequence differences did not elicit an allogeneic CTL response. We propose that in generating a T cell repertoire with a sufficiently narrow responsive for self-MHC, positive thymic selection limits the capacity to recognize allogeneic MHC molecules whose structure and sequence have diverged extensively. These findings are important for donor and patient MHC matching strategies and our understanding of T cell-MHC interaction after haematopoietic stem cell transplantation.

Introduction

An immune response against allogeneic major histocompatibility complex (MHC) antigens can have a detrimental effect on clinical transplantation outcome, whether it results in graft rejection or graft versus host disease (in the case of haematopoietic stem cell transplantation). Confrontation with foreign MHC will not lead to an alloreactive immune response in all cases. Progress in understanding the humoral response resulted in a method to predict which mismatched human leukocyte antigens (HLA) will lead to the production of alloreactive antibodies. This algorithm, HLAMatchmaker, converts each MHC class I allele into a linear string of amino acid triplets, which are accessible to alloantibodies and then determines, by intralocus and interlocus comparison, which allogeneic amino acid triplets on the MHC molecules are not shared with the recipient.[1] Earlier we showed a strong positive correlation between the number of triplet mismatches and the induction of alloantibodies in transplant patients and multiparous women.[2]

In contrast to the humoral response there is no reliable thesis that predicts which MHC class I differences trigger a cellular alloimmune response.[3,4] Unexplained exceptions still occur. On the one hand confrontation with allogeneic tissue without MHC class I incompatibilities can lead to T cell alloreactivity, while on the other hand there is an absence of T cell alloreactivity against some MHC class I differences.[3] Likewise, HLAMatchmaker was proven unsuitable for forecasting the T cell mediated immune response in vitro against single MHC class I incompatibilities.[5] The T cell alloimmune response was quantified in a cytotoxic T-lymphocyte precursor (CTLp) assay. This assay mainly measures direct allorecognition of MHC class I presented on allogeneic peripheral blood leukocytes (PBL) by CD8+ cytotoxic T cells.[6] T cells interact differently with foreign MHC than B cells, which probably implies that T cells will recognize different allogeneic motifs on foreign MHC class I.

During T cell development, thymocytes generate a vast array of clonally expressed $\alpha\beta$ T cell receptors (TCR) that mediate the recognition of foreign peptides in the context of self-MHC. Prior to selection the TCR repertoire has an extensive potential diversity up to ~10^{14} different structures.[7] Selection processes in the thymus and peripheral lymphoid organs, however, shape the functional TCR repertoire. T cell must bind to self-peptide-MHC (pMHC) complexes with sufficient affinity for positive selection and peripheral survival. If these affinities are too high, the T cells will be deleted by negative selection[8,9], a process that has been estimated to operate on 20-30% of the thymocytes.[10] The window of binding affinities that separate positive and negative selection appears to be very narrow, differing by as little as threefold.[11] In principle, this means that individual TCR repertoires are based on self-pMHC, resulting in an inimitable and unique TCR repertoire. This is a primary cause for the unpredictability of T cell mediated alloreactivity against allogeneic MHC molecules.

Most important for direct allorecognition are thought to be differences in amino acid motifs in the $\alpha1$ and $\alpha2$ domain of MHC class I that are either relevant for MHC-TCR interaction,

or selection and conformation of peptides presented by MHC. The motifs involved in MHC-TCR association are predominantly located in the α helices of the α1 and α2 domain.[12] The motifs involved in peptide selection and conformation are located within the peptide binding groove in the β sheet and some in the α helices.[13,14] A quantitative and/or qualitative distinction can be made between MHC differences. MHC has many polymorphic amino acid positions. Some MHC molecules differ by only a few amino acids while others have many differences. Certain motifs, however, may be more important for peptide selection, peptide conformation or TCR contacts than others. Furthermore, some amino acids have functional similarities, while other amino acids have very distinct properties.
The question is whether T cell alloreactivity can be predicted by the amino acid sequence of the α1 and α2 domain of the MHC class I molecule. In our center, the CTLp assay is routinely used as a tool to select the most suitable stem cell donor for a given patient. In the present study we retrospectively analyzed the relation between T cell alloreactivity in vitro and MHC class I allele incompatibilities in 80 allogeneic haematopoietic stem cell donor/patient pairs. All donor/patient pairs have a single MHC class I (HLA-A, -B, or -C) allele difference in the graft-versus-host direction and are compatible for the determinants of the HLA-DRB1 and -DQB1 loci. This implies that in a CTLp assay the responder T cells could recognize only one allogeneic MHC class I molecule on the stimulator and target cells.

Materials and Methods

Donor/recipient pairs.

This analysis concerned 80 donor/patient pairs registered by the Europdonor foundation from 1990 to 2003. The 80 patients were from Dutch haematopoietic stem cell transplantation centers. The donors originated from the national donor registry of the Europdonor foundation, International donor registries and related donors. All pairs had a single HLA-A, -B or -C antigen/allele mismatch in the graft-versus-host direction; 26 are mismatched for HLA-A (20 antigen mismatches / 6 allele mismatches), 9 for HLA-B (1 antigen mismatch / 8 allele mismatches) and 45 for HLA-C (39 antigen mismatches / 6 allele mismatches). The specific HLA allele mismatches of all donor-recipient combinations tested are shown in Table 1. All pairs are compatible for the HLA-DRB1 and -DQB1 alleles. We did not exclude the 49 pairs, which were HLA-DPB1 mismatched, as the power of the analysis would become too low.
Seven additional CTLp assays with selected pairs were performed to extend and to confirm the obtained results. All seven pairs were deliberately mismatched for a single HLA-C molecule. The HLA-C molecules differed by at least five amino acids in the β sheet plus at least five amino acids in the α helices.

HLA-Cw*0303/HLA-Cw*0304 mismatched pairs.
All CTLp assays performed for donor/patient pairs with an HLA-Cw*0303/HLA-Cw*0304 mismatch (n=6), containing one amino acid substitution on position 91, resulted in an undetectable CTLp frequency. Position 91 lies outside the α helices, β sheet, and other known functional groups of the molecule and is thus not relevant for T cell recognition.[3] This mismatch is therefore distinctively different from the other HLA-C mismatches that have substitutions on functional parts of the molecule. These pairs were therefore excluded from the analysis.

HLA genotyping and amino acid sequencing.
All donors and patients were typed at high resolution for HLA-A, -B, -C, DRB1, DQB1 and DPB1 as described before.[3] The following techniques were used: polymerase chain reaction sequence specific primer for high resolution allele typing, and sequence based typing for a part of the HLA-C alleles. The amino acid sequences were obtained from the website of the European Bioinformatics Institute.[15] The mismatched HLA molecules were examined for amino acid differences (substitutions) on the α1 and α2 domain (positions 1-182). The α helices of HLA-A, -B and -C consist of positions 50-85 and 138-179 and the β sheet of HLA-A, -B and -C is determined by positions 4-12, 21-28, 32-37, 94-102, 112-118 and 123-126.

Cytotoxic T-Lymphocyte precursor assay.
The CTLp assays were performed for the selection of the best allogeneic haematopoietic stem cell donor for the patients. The CTLp assay was performed as described by Zhang *et al*[16], with minor modifications as described by Oudshoorn *et al*.[3] The target cells were labeled with ^{51}Cr. The reproducibility of this assay has been described previously.[16] In each assay, donor cells were used as the responder and the patient cells as the stimulator and target (graft-versus-host direction). In this study an undetectable CTLp frequency was defined as CTLp ≤ 1 per 10^6 PBL) and a detectable CTLp frequency was defined as CTLp > 5 per 10^6 PBL. We thus selected all pairs with CTLp ≤ 1 per 10^6 PBL and CTLp > 5 per 10^6 PBL in order to get two distinct groups. Twenty-eight pairs with a CTLp frequency between two per 10^6 PBL and five per 10^6 PBL have been excluded; 7 out of the 28 pairs are mismatched for HLA-A, 3 for HLA-B and 18 for HLA-C.

Cytotoxic T-Lymphocyte precursor assay and natural killer cells.
Besides cytotoxic T cells, natural killer (NK) cells are thought to be possibly involved in killing of the host cells in the CTLp assay. NK cell reactivity can be inhibited by specific inhibitory KIR ligands.[17] HLA-C molecules bearing the Ser77 and Asn80 motif (C1) are ligands for the inhibitory KIR2DL2 and KIR2DL3 while HLA-C molecules bearing the

Asn77 and Lys80 motif (C2) are ligands for the inhibitory KIR2DL1.[18,19] NK cell mediated alloreactivity can occur when the target cells do not have the same inhibitory motifs as the responder.[20]

Statistical analysis.
The Mann-Whitney *U* test was used to determine differences in number of amino acid substitutions between the pairs with an undetectable and detectable CTLp frequency. To analyze the effect of interaction between variables on T cell reactivity the products of the variables were used. This interaction term is based on the idea that if both variables of the product are high the product is higher than when only one variable is high, even when the sum of variables is the same. The Chi-Square test, or if mentioned the Fisher's exact test, was used to compare frequencies.

Results

Total number of amino acid substitutions on the α1/α2 domain of class I MHC.
The CTLp assays performed on donor/patient pairs with a single HLA-A, -B or -C allele difference showed no relation between the absolute number of amino acid substitutions on the α1 and α2 chain of the HLA molecule and CTLp frequency (Figure 1). There was no relation found between additional HLA-DPB1 mismatches and CTLp frequencies. With respect to HLA-C mismatched pairs, there was no relation between inhibitory killer immunoglobulin-like receptor (KIR) ligand differences and CTLp frequency. Of the 33 KIR compatible pairs, 12 had a detectable CTLp frequency, while 3 out of 12 pairs with such an incompatibility had a detectable CTLp frequency.

Amino acid substitutions on specific positions.
Neither the group with an HLA-A, or –B incompatibility nor the group with an HLA–C incompatibility showed a clear relation between dissimilarities on specific positions on the α1 and α2 domain and CTLp frequencies. Also differences on positions 114 and 116 on the β sheet (involved in MHC peptide binding), which have been described to increase the risk on acute graft versus host disease and transplant related mortality[21] did not correlate with T cell alloreactivity in vitro (HLA-A or –B differences: $p = 0.76$ and HLA-C differences: $p = 0.77$; Figure 2). A clear relation between the kind of amino-acid substitution (positively to negatively charged, charged to neutral or polar) and the CTLp outcome was also not observed.

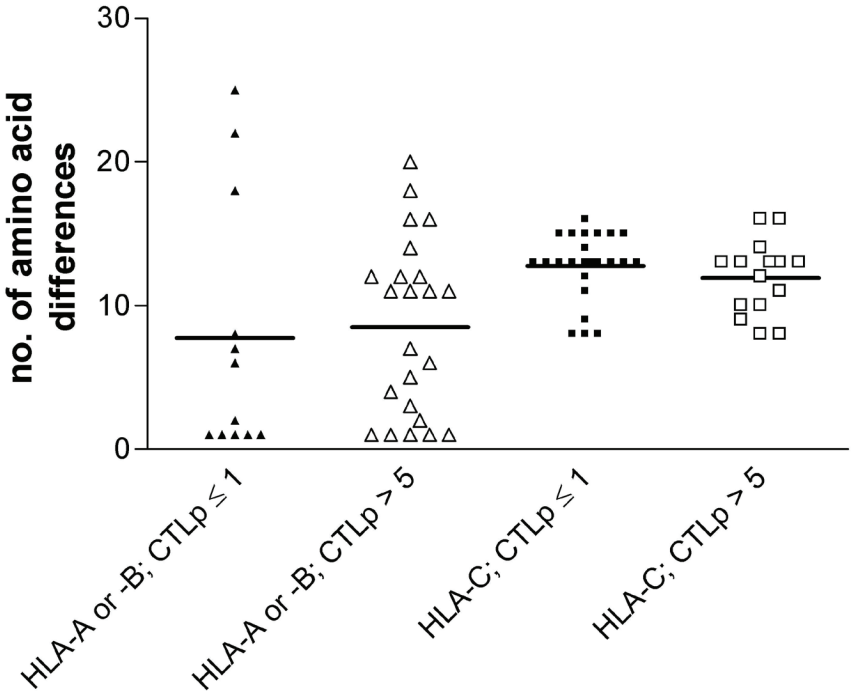

Figure 1: Number of amino acid differences of single HLA class I incompatibilities versus T cell alloreactivity in vitro (CTLp/106 PBL). *The number of pairs in each group: single HLA-A or –B difference: n = 12 (CTLp ≤ 1) and n = 23 (CTLp > 5); single HLA-C difference: n = 24 (CTLp ≤ 1) and n = 15 (CTLp > 5). Horizontal lines indicate the mean of each group. No statistical difference was found for pairs with a HLA-A or –B difference (p = 0.46) or pairs with a HLA-C difference (p = 0.29).*

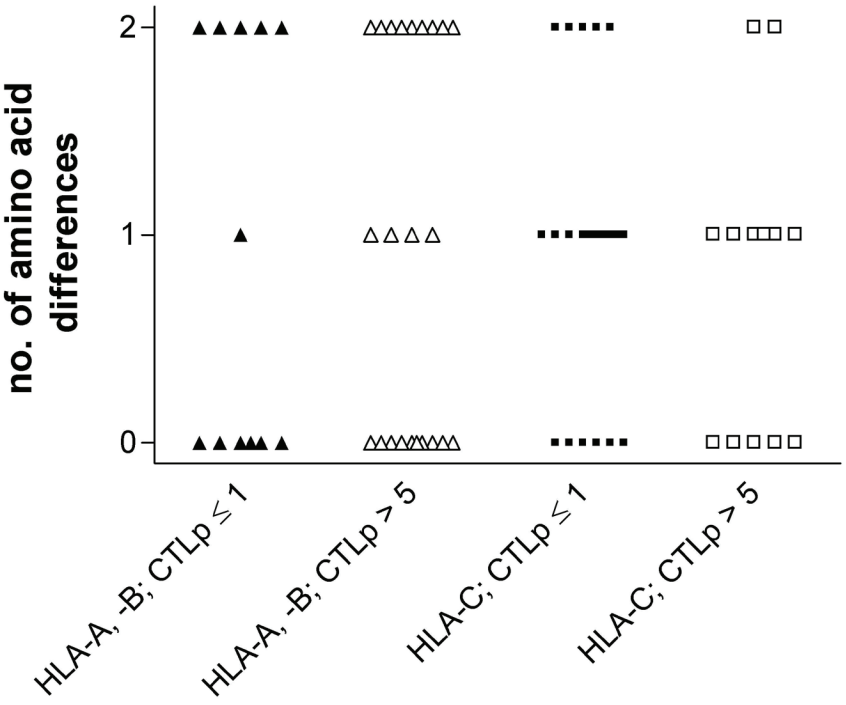

Figure 2: Amino acid differences on positions 114 and 116 in the β sheet of MHC class I versus T cell alloreactivity in vitro (CTLp/106 PBL). *The number of pairs in each group: single HLA-A or –B difference: n = 12 (CTLp ≤ 1) and n = 23 (CTLp > 5); single HLA-C difference: n = 24 (CTLp ≤ 1) and n = 15 (CTLp > 5). No clear relation was found between amino acid differences on these positions and T cell alloreactivity in vitro, neither for HLA-A or –B differences (p = 0.76) nor in case of HLA-C differences (p = 0.77).*

Amino acid substitutions in the α helices and β sheet of MHC class I.

There was a relation between the number of substitutions in the α helices of HLA-C and CTLp frequency (Figure 3). Pairs with no detectable CTLp frequency have on average a higher number of incompatibilities in the α helices than those with a detectable CTLp frequency (p = 0.03). This relation was not found in the group of donor/patient pairs with a single HLA-A or –B difference. No relation was found between the number of substitutions in the β sheet and CTLp frequency (Figure 4).

However, there seems to be an interaction between the number of amino acid differences in the α helices and in the β sheet and their effect on T cell alloreactivity. This was more apparent for HLA-C than for HLA-A and –B mismatched couples. As shown in the upper right quadrant of Figure 5, HLA-C differences with five or more substitutions in the α helices and at least five substitutions in the β sheet did not lead to detectable CTLp frequencies (HLA

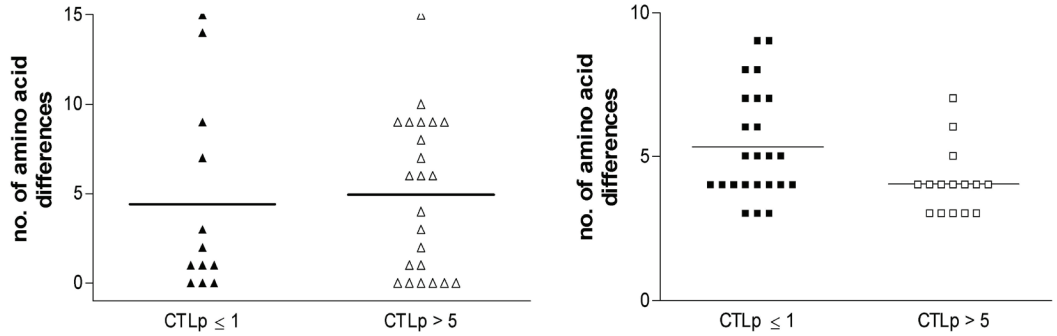

Figure 3: Number of amino acid differences in the α helices of MHC class I versus T cell alloreactivity in vitro (CTLp/10⁶ PBL). *The number of pairs in each group: single HLA-A or –B difference: n = 12 (CTLp ≤ 1) and n = 23 (CTLp > 5); single HLA-C difference: n = 24 (CTLp ≤ 1) and n = 15 (CTLp > 5). Horizontal lines indicate the mean of each group. A statistical difference was found; HLA-C mismatched pairs with a undetectable CTLp frequency had on average a higher number of substitutions in the α helices than the pairs with a detectable CTLp frequency (p = 0.03). No difference was found for HLA-A and -B differences (p = 0.67).*

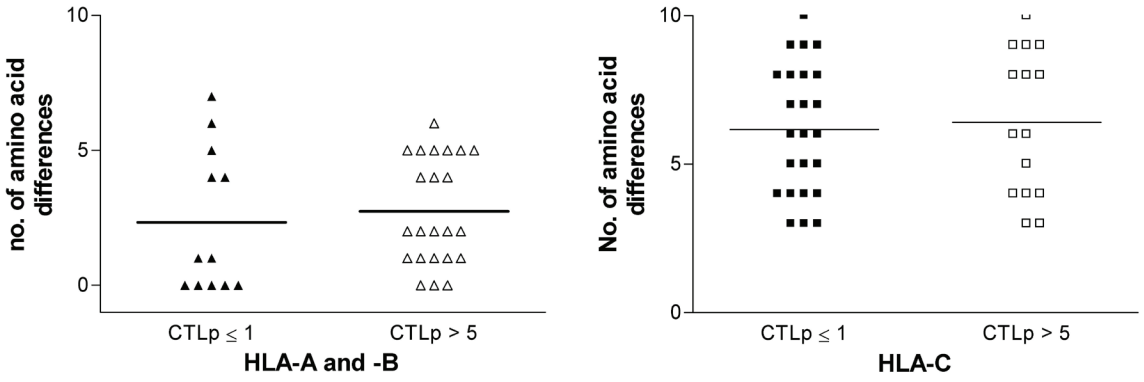

Figure 4: Number of amino acid differences in the β sheet of MHC class I versus T cell alloreactivity in vitro (CTLp/10⁶ PBL). *The number of pairs in each group: single HLA-A or –B difference: n = 12 (CTLp ≤ 1) and n = 23 (CTLp > 5); single HLA-C difference: n = 24 (CTLp ≤ 1) and n = 15 (CTLp > 5). Horizontal lines indicate the mean of each group. No statistical difference was found (HLA-A and –B: p = 0.37; HLA-C: p = 0.75).*

mismatches are shown in Table 1). The mean interaction term (as described in Methods) was higher in the group of the undetectable CTLp frequencies, 32 compared to 25 (p = 0.04). Due to small numbers, the data on HLA-A and -B is not conclusive (p = 0.57). In the upper right quadrant of Figure 6, there are three pairs with numerous amino acid differences in the α helices and in the β sheet that did not lead to detectable CTLp frequencies. However, there were many pairs with few amino acid differences that did lead to either detectable or undetectable CTLp frequencies.

The results on HLA-C differences were further substantiated with seven additionally selected pairs (Table 1) having a single HLA-C difference that differs by at least five amino acids in the β sheet and at least five in the α helices; only one of these lead to a detectable CTLp frequency. This results in a significant association for the total group (p = 0.003; Table 2).

Table 1: Specific HLA mismatches of all donor-recipient pairs studied with the CTLp assay.

HLA-A mismatches (n=26)		
0101-1101	0201-3201	1101-6801
0101-2402	0201-3402	1104-6802
0201-0206 (n=2)	0201-6801 (n=2)	2402-2403
0201-0205	0301-1101	2501-2601
0201-0301	0301-3101	3201-3202
0201-1101	1101-1104	6801-6802
0201-2402	1101-2601	6801-6901
0201-3101 (n=2)	1101-2601	
HLA-B mismatches (n=9)		
1401-1402	3501-3503 (n=3)	5703-5801
2702-2705	3502-3504	
2703-2705	4402-4403	
HLA-C mismatches (n=45)		
0102-0202 (n=6)	0303-0304 (n=6) [A]	0501-0702 [B]
0102-0304 (n=2)	0304-0501 [B]	0501-0704 [B]
0102-0702 (n=2)	0304-0602 [B]	0701-1203
0202-0303 (n=2)	0304-0701 [B]	0701-1502 [B]
0202-0701	0304-1502	0701-1601 (n=2)
0202-0702 (n=2)	0401-1502 (n=2)	0701-1602
0202-1402	0401-1601 [B]	1402-1502 (n=4)
0202-1502 (n=2)	0501-0701 (n=2) [B]	1502-1602
Additional couples with HLA-C mismatches (n=7)		
0303-0401 [B]	0304-0704 [B]	
0304-0401 [B]	0501-0704 (n=4) [B]	

[A] These pairs were excluded from the analysis (as described in Methods).

[b] Mismatches with five or more substitutions in the α helices and at least five substitutions in the β sheet.

Table 2: Observed frequency of T cell mediated alloreactivity in vitro related to the number amino acid differences in the α helices and in the β sheet of single HLA-C differences.

	CTLp frequency ≤ 1	CTLp frequency > 5
Group 1 [A]	15	1
Group 2 [B]	15	15

[A] Group 1 contains the pairs with five or more amino acid differences in the α helices and five or more in the β sheet. [B] Group 2 is the remaining group. Group 1 has an insignificant number of cases with a detectable CTLp frequency compared to group 2 (fisher's exact test: p = 0.003).

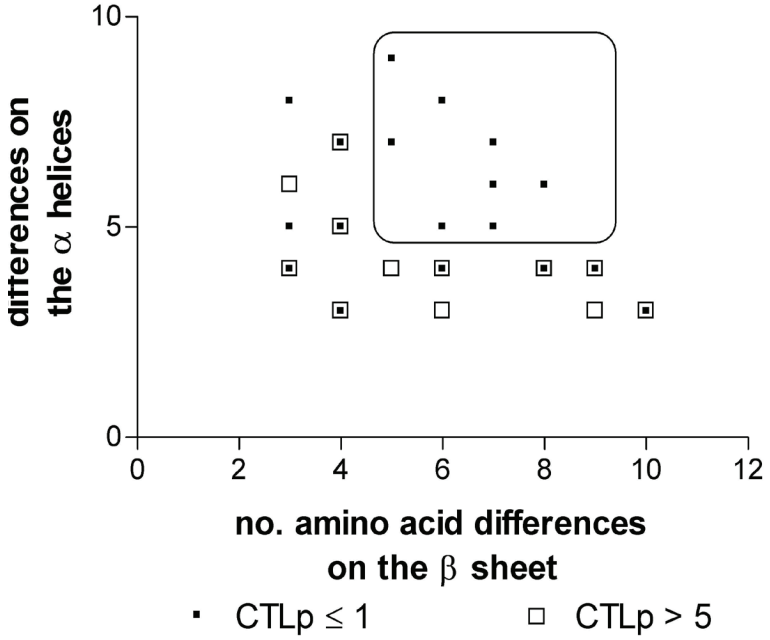

Figure 5: The number of amino acid differences in the α helices and β sheet of HLA-C versus T cell alloreactivity in vitro (CTLp/10^6 PBL). *The number of pairs in each group: 24 within the group with an undetectable CTLp frequency and 15 within the group with detectable CTLp frequency. Five or more differences in the α helices and at least five differences in the β sheet lead to undetectable CTLp frequencies. Please note that certain pairs have similar numbers of amino acid differences on both α helices and β sheet and thus overlap each other in this figure.*

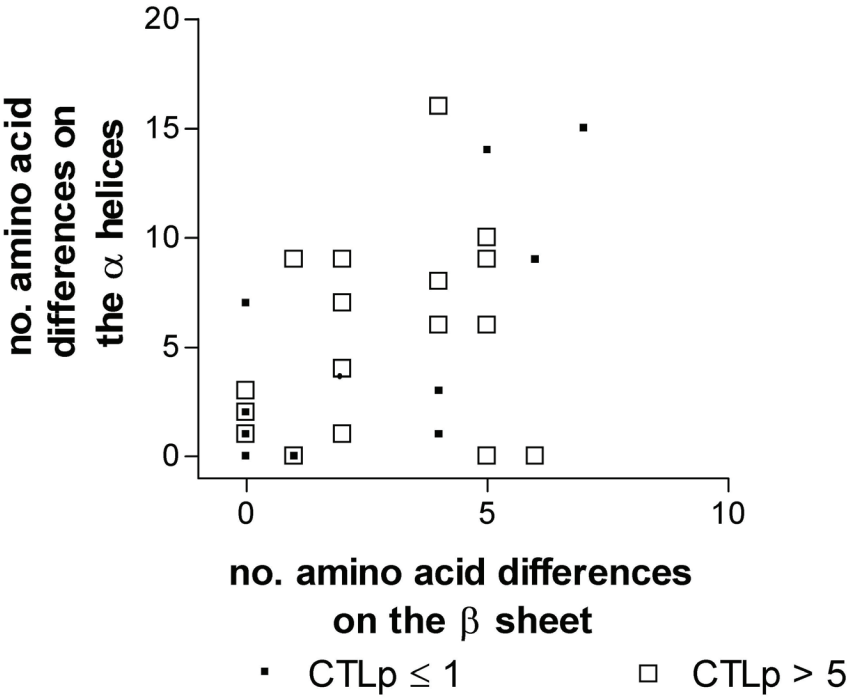

Figure 6: The number of amino acid differences in the α helices and β sheet of HLA-A or -B versus T cell alloreactivity in vitro (CTLp/10^6 PBL). *The number of pairs in each group: 12 within the group with an undetectable CTLp frequency and 23 within the group with detectable CTLp frequency. Please note that certain pairs have similar numbers of amino acid differences on both α helices and β sheet and thus overlap each other in this figure.*

Discussion

In contrast to that observed in humoral alloreactivity against MHC class I differences, our study revealed that the number of amino acid substitutions does not correlate positively with T cell mediated alloreactivity in vitro. Specifically, we find that neither the frequency of amino acid differences nor their position within the class I MHC molecule is predictive of alloreactivity. The positive correlation found between substitutions on position 114 and 116, and T cell mediated alloreactivity in previous studies[3,21] could not be confirmed. In agreement with our previous study, we found no relation between inhibitory KIR ligand mismatches and CTLp frequency.[3] The absence of responses against allogeneic PBL was not due to lack of surface MHC class I expression, as the samples could be serologically typed for the mismatched MHC class I alleles.

A crucial but unexpected finding is that HLA-C mismatches with more than 5 substitutions in the α-helices and more than 5 in the β-sheet did not lead to T cell mediated alloreactivity in

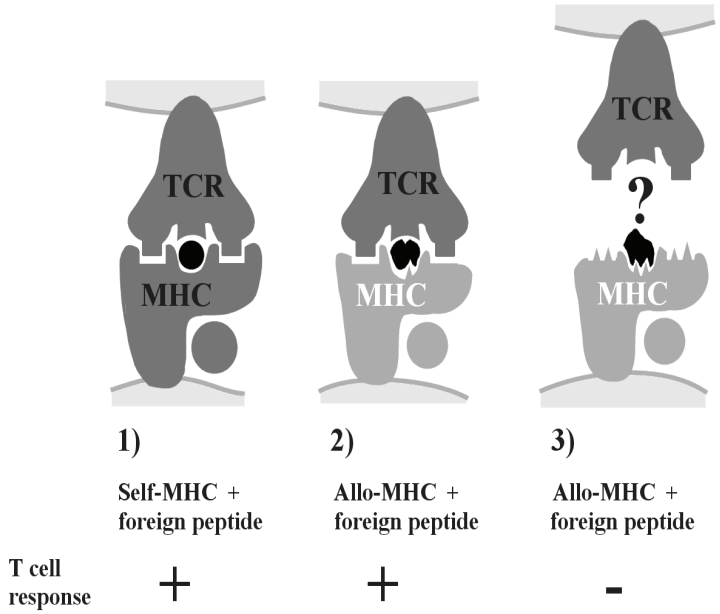

Figure 7: The possibility for TCR-MHC binding decreases if allogeneic MHC differs too much from autologous MHC. *The peptide presented by the MHC molecule is depicted in black. 1) Self-MHC with peptide can bind to self-TCR and may lead to T cell reactivity in case of non-self-peptides being presented by the MHC molecule. 2) Allogeneic MHC with peptide can bind to TCR and may lead to T cell activation based on cross reactivity. 3) Allogeneic MHC with peptide cannot bind to TCR in case of too many amino acid differences in both the* α *helices and* β *sheet between the MHC molecules and therefore no alloreactive T cell response will be induced.*

vitro in all cases. Unfortunately, the group with an HLA-A or –B difference was too small and included too many allele mismatches with few amino acid differences to be able to generalize these findings to all MHC class I mismatches. The absence of a T cell repertoire able to recognize extensively mismatched MHC class I molecules, might lie in the selection of T-cells in the thymus, and perhaps in the periphery. This would imply that MHC molecules, which have diverged sufficiently from the selecting allele, may be unable to support the initial association between the TCR and its docking sites on the self-MHC class I molecule, shown to be a first step in forming a stable pMHC-TCR complex.[22] Thymocytes capable of binding with such allogeneic pMHC complexes therefore would be expected not to possess sufficient affinity for self-pMHC for positive selection and peripheral survival. Conversely, the post-selection T cell repertoire would be constrained in its recognition of extensively mismatched MHC and would not form a stable pMHC-TCR complex, resulting in the absence of allorecognition (Figure 7).

The results reported here seem to contradict the observation made by Petersdorf and colleagues that an antigen mismatch leads to a stronger allogeneic immune response in haematopoietic stem cell transplantation than an allele mismatch.[23] Alleles encoding MHC molecules that are being recognized by alloantibodies induced by pregnancy or blood transfusions have been identified as MHC antigens. By convention in stem cell transplantation outcome analysis, the term MHC allele mismatch refers to a donor and recipient both having a different MHC allele out of a family of MHC alleles that encodes for the same MHC antigen. [23] But rather than contradicting their observation our finding might explain why a haematopoietic stem cell transplantation with a major antigen mismatch in some cases leads to a successful outcome.

For this analysis we used CTLp assay outcomes from donor searches performed for patients between 1992 on to 2004. Although the CTLp assay may not be the most sensitive assay for measuring T cell alloreactivity, it has been proven a clinically relevant parameter for the assessment of pre-transplant anti-patient T cell alloreactivity in stem cell donors.[4,24-30] This is not the case for alternative assays to assess pre-transplant alloreactivity such as the interferon γ Enzyme-Linked Immunosorbent Spot (ELISPOT) assay. The IFN-γ ELISPOT has been shown to correlate with the occurrence of acute rejection and chronic graft injury in solid organ transplantation.[31] At the moment we are comparing the clinical relevance of the CTLp assay and IFN-γ ELISPOT in order to evaluate whether the latter, less complex and time consuming, can replace the CTLp test as predictive marker for graft versus host disease or patient survival.

As the CTLp assay has been proven a clinically relevant parameter for haematopoietic stem cell transplantation outcome[4,24-30], these results could help in selecting an acceptable donor for patients depending on transplants with haematopoietic stem cells from an incompletely matched donor. This implicates that a graft with a single MHC class I allele mismatch cannot automatically be chosen over a graft with a single antigen mismatch from the very start. Our own observation that between 1997 and 2002 a significant number of Dutch patients (43% of patients from North-West European origin and 87% of the patients from other origin) did not have a matched unrelated donor (compatible for the alleles of HLA-A, -B, -C, -DRB1 and -DQB1), illustrates the importance of a tool able to point out acceptable mismatches.

These results could shed a new light on xenograft transplantation as the allelic diversity of MHC class I molecules is probably larger between species than within a single species. Under this assumption we hypothesize that the affinity of the human TCR repertoire is too low for xenogeneic MHC class I. This could imply that direct recognition of xenogeneic MHC class I is of no importance to the immune response against a xenograft and that the focus should rather be on taking precautions against humoral responses and indirect recognition of xenogeneic peptides presented by self-MHC. Although it can not be excluded that a minor component from a naïve repertoire could be primed by allo/xenografts and

selectively expand to pathogenic levels.
In conclusion, our data show that CD8+ cytotoxic T cell alloreactivity in vitro can be predicted in certain cases. A MHC mismatch with numerous amino acid differences seems to circumvent cytotoxic T cell allorecognition in vitro. Prediction of cytotoxic T cell alloreactivity in vitro that is based on the idea that it correlates positively with the total number of amino acid substitutions or with substitutions on specific positions on the MHC molecule is inaccurate.

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