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Detection of antibodies against HLA-C epitopes in patients with rejected kidney transplants

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ABSTRACT

Background: Although HLA-C matching is not considered in kidney transplantation, several reports have shown that anti-HLA-C antibodies are associated with rejection and graft failure. DNA-based typing methods can now accurately determine HLA-C compatibility and sensitive assays such as Luminex with single alleles can identify HLA-C antibodies. HLA-C displays considerable amino acid polymorphism that can be translated into a structurally defined epitope repertoire.

Methods: We have analyzed post-allograft nephrectomy sera from 45 HLA-C mismatched cases submitted by 15 laboratories worldwide participating in the 15th International Histocompatibility Workshop. All of them had HLA class I antibodies detected by a Luminex-based solid phase method using single-allele beads. This study addressed the determination of antibodies against donor HLA-C mismatches. Analysis of antibody reactivity patterns was performed using HLAMatchmaker, a structurally based matching program that considers 56 HLA-C eplets to define antibody-reactive epitopes. Many eplets shared by groups of HLA-C antigens, whereas others are also shared with HLA-A and/or HLA-B antigens.

Results: Twenty-seven patients (60%) had donor-specific HLA-C antibodies, significantly less than the donor-specific antibodies induced by HLA-A and HLA-B mismatches. HLA-C antibody responses correlated with the eplet loads of the HLA-C mismatches. There were 352 instances whereby a donor HLA-C eplet was mismatched and for 84 (24%) of them there was antibody reactivity with a particular eplet (69 instances) or an eplet pair (15 instances). The latter generally consisted of mismatched eplets paired with self-eplets shared between the immunizing HLA-C alleles and HLA alleles of the patient. Several HLA-C eplets exhibited a relatively high immunogenicity as evidenced by their frequencies of specific antibodies.

Conclusion: These findings demonstrate the importance of HLA-C mismatching in humoral sensitization and that HLA-C epitopes can induce specific antibodies. They illustrate the usefulness of HLAMatchmaker in understanding donor-specific antibody reactivity patterns and the determination of HLA mismatch acceptability when transplantation is considered.

1. Introduction

The role of HLA-C in solid organ transplantation has not been thoroughly investigated. During recent years, most studies have addressed the relationship between HLA-C and KIR polymorphisms. HLA-C alleles have been allocated into two groups, termed HLA-C1 and HLA-C2, based on their KIR specificity. [1,2] HLA-C2 interactions tend to inhibit NK cell activation but the donor HLA-C2 type may [3,4] or may not [5] improve organ transplant outcome.

There is also evidence for HLA-C specific T-cell alloreactivity [6–9] and that HLA-C affects cellular rejection [10]. HLA-C mismatching decreases solid organ transplant survival [11,12] and hematopoietic stem cell transplantation [13]. A case report describes a hyperacute rejection associated with an anti-HLA-C antibody [14]. Moreover, anti-HLA-C antibodies affect the refractory state of alloimmunized thrombocytopenic patients requiring compatible platelet transfusions [15].

Serological methods originally used for HLA-C typing are unreliable due to the lack of antisera for all antigens. In contrast, molecular typing techniques allow consistent HLA-C types. Lymphocytotoxicity-based screening for HLA-C antibodies is also technically difficult with HLA-phenotyped panels because other class I antibodies may interfere and the cell surface expression of HLA-C is often lower. Solid-phase techniques using the Luminex platform and microbeads coated with single HLA class I alleles allow a more consistent identification of HLA-C antibodies. Even with these more sensitive methods, the frequency of HLA-C reactive antibody detection is lower than that for HLA-A or HLA-B antibodies [16,17].

Although most clinical laboratories analyze serum reactivity in terms of antibody specificity against HLA antigens, it has become apparent that such HLA antibodies are specific for epitopes rather than antigens.

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Mismatched epitopes presented by the immunizing antigens may induce specific antibodies that react with other antigens if they have the same epitopes. Many epitopes are shared between antigens controlled by the same locus but there are also many cases whereby the epitope is shared between antigens encoded by two or three loci.

This study addresses the detection of donor-specific HLA-C epitopes in patients who had class I antibodies after removal of a failed kidney transplant. One goal was to determine the incidence and strength of HLA-C antibodies in relation to the HLA-A and HLA-B antibodies and how this correlates with the number of mismatched eplets, i.e. the eplet loads of the HLA-C mismatches. The other goal was to identify eplet-defined epitopes reacting with HLA-C mismatch induced antibodies. From the frequencies of specific antibodies we have assessed the relative immunogenicity of mismatched HLA-C epitopes.

2. Materials and methods

2.1. Patients

This study was part of a multi-laboratory collaborative project on HLA epitope immunogenicity conducted under auspices of the 15th International Histocompatibility Workshop. We selected 45 cases contributed by 15 laboratories whereby patients and donors had been typed for HLA-C and the donor mismatches included one or two HLA-C antigen mismatches (Table 1). Class I and class II HLA typing was performed for patients and donors in the respective laboratories by standard serological and/or molecular methods. All cases were primary kidney transplants that had failed and the grafts had been surgically removed. For most patients, no information was available on the cause of graft failure or transplant pathology. Pre-transplant PRA values (determined according to each laboratory's standard testing practices) were not available for 15 patients (33%), but the PRA value for the other 30 patients (67%) ranged from 0 to 8%. All transplants were performed from crossmatch-negative donors; such testing was done according to standard practices employed in the laboratory at the time of transplant.

Table 1
15th International Workshop participants who contributed post-allograft nephrectomy sera for the study of HLA-C eplets.

Participating laboratory	Institution	Location	Nr of cases
Wil Allebes	University Medical Center, St. Radboud	Nijmegen, the Netherlands	1
Patricia Campbell	University of Alberta Hospitals	Edmonton Canada	5
Vaughan Carter	National Blood Service	Newcastle upon Tyne, UK	3
Silvia Chrenova	Slovak Center for Organ Transplantation	Bratislava, Slovakia	5
Ilias Doxiadis	Leiden University Medical Center	Leiden, the Netherlands	2
Aliki Iniotaki	National Tissue Typing center	Athens, Greece	2
Andres Jaramillo	Gift of Hope Organ & Tissue Network	Elmhurts, IL, USA	1
Ed Kaminski	Derriford Combined Laboratories	Plymouth, UK	1
Andrew lobashevsky	Indianapolis Transplant Center	Indianapolis, IN, USA	1
James McCluskey	Australian Red Cross Blood Services	Adelaide, Australia	1
Sandra Nehlsen Cannarella	Detroit Medical Center	Detroit, MI, USA	4
Marilyn Pollack	University of Texas Health Science Center	San Antonio, TX, USA	5
Tsuyoshi sato	Sapporo Hokuyu Hospital	Sapporo, Japan	1
Constanze Schönemann	Univ Clin Charite Campus Virchow Klinikum	Berlin, Germany	5
Adriana Zeevi	University of Pittsburgh Medical Center	Pittsburgh, PA, USA	8
			TOTAL 45

2.2. Antibody screening for HLA class I antibodies

Serum samples were drawn between 1 and 11 months post-allograft nephrectomy, and were tested with Luminex microbead assays using HLA-ABC single-allele kits from two vendors (One Lambda Inc., Canoga Park, CA, and Gen-Probe Transplant Diagnostics, Stamford, CT), according to the manufacturers' instructions. Briefly, small volumes of patient serum (30 μl for OneLambda, 10 μl for Gen-Probe) were incubated with a mixture of Luminex microspheres, each coated with a single HLA class I allele, in 96-well plates. After washing to remove unbound antibody, the mixtures were incubated with phycoerythrin-conjugated anti-human IgG. After incubation, the wells were diluted and analyzed on a Luminex 100 instrument (Luminex, Austin, TX); median fluorescence intensity (MFI) values were determined using the manufacturer's software. Reactivity was determined by comparing the MFI values of the beads possessing individual mismatched antigens to those of positive and negative controls, as well as to the average MFI of beads possessing the patients' self HLA antigens.

2.3. HLAMatchmaker analysis of serum reactivity

We analyzed the antibody reactivity patterns with the Luminex panels with HLAMatchmaker, a structurally based matching program that considers each HLA antigen as a string of epitopes represented by "eplets" identified from molecular modeling of small patches of exposed polymorphic amino acid residues within a radius of 3 Ångstroms [18]. Eplets are described by their sequence positions and standard amino acid single letter codes.

This study focuses on eplets on HLA-C molecules determined from alleles in the Luminex panels. Table 2 lists 56 such eplets and 17 (30%) are unique for single or groups of HLA-C alleles; several eplets (e.g., 184H and 69KRQ) are present on very large groups of HLA-C alleles. Thirty-nine eplets (70%) are shared with HLA-A and/or HLA-B alleles involving single alleles such as A*80:01 and (especially) B*46:01, or groups of alleles; eplets such as 9F and 156LA are shared by very large groups of alleles and they display a considerable degree of interlocus sharing. Table 2 shows also 19 eplets (34%) that have been verified experimentally so far with specific antibodies as described elsewhere. Most of them correspond to epitopes reported by Terasaki's group [19–21]; we refer to them as TerEps. Others have been identified with human monoclonal antibodies or informative allosera [22,23].

The HLAMatchmaker antibody analysis program has worksheets to enter the HLA types of the antibody producer, the immunizer and the allele panel as well as the MFI values that can be readily copied from csv files of the manufacturer's Luminex computer programs. This program is posted on the website http://www.HLAMatchmaker.net.

An understanding of an antibody reactivity pattern requires information about the immunogenetic basis of the sensitizing event leading to the formation of specific antibodies. HLAMatchmaker can determine on the immunizing antigen which eplets are mismatched for the antibody producer. This analysis addresses the detection of antibodies reacting with donor class I epitopes. The first step is to record into the program those alleles that give negative reactions with antibody. Such alleles can be expected to have epitopes that are not recognized by patient's antibodies and the program automatically removes these epitopes displayed on all alleles in the panel. From the remaining eplets on the reactive alleles we can then determine which ones are shared with the immunizing alleles and might therefore be reacting with donor-specific antibodies.

Table 2
Repertoire of 57 HLA-C eplets and their presence on HLA-A and HLA-B antigens.

Eplet	Epitope	HLA-C alleles	HLA-A alleles	HLA-B alleles
1CK	TerEp#5032	C*01		
9D		C*06/C*07/C*18		B*08
9F		C*01	A*01/A*02:01/A*02:02/A*02:03/A*03/A*32/A*36/A*74/A*80	
9S		C*04:01/C*14	A*23/A*24/A*30	
9Y		C*02/C*03/C*04:03/C*05/C*08/C*12/C*15/C*16/C*17	A*02:06/A*11/A*25/A*26/A*34//A*43/A*66/A*68/A*69	B*07/B*13/B*14/B*15/B17/B22/B*35/B*38/B*39/B*42/B*44 B*46/B*47/B*48/B*51/B*52/B*53/B*59/B*67/B*78/B*81/B*82/
12AVR		C*05/C*06/C*07/C*08/C*12/C*16/C*17/C*18		B*14:02/B*14:05/B*14:06
14WR		C*04:01		
21H	TerEp#39	C*02/C*03/C*04:03/C*15		
35Q		C*05/C*08	A*80	
63REN		C*07:01/C*15		B*15:16
69KRQ		C*01/C*02/C*03/C*04/C*05/C*06/C*07:02/C*07:04/C*08/C*12/C*14/C*16/C*17/C*18		B*46
69RA		C*04/C*06/C*07/C*12/C*17/C*18		
69RT		C*01/C*02/C*03/C*05/C*08/C*14/C*15/C*16		B*46
71AT		C*01/C*02/C*03/C*05/C*08/C*14/C*15/C*16		B*07:02/B*15:16/B17/B22/B*27/B*42/B*46/B*67/B*73/B*81/B*82
73AN	TerEp#5037	C*04/C*06/C*17/C*18		
73AS		C*07/C*12		
73TN		C*02/C*05/C*15	A*01/A*23/A*24/A*26/A*29/A*30:02/A*36/A*43/A*80	B*13/B*15:13/B*15:16/B17/B*38/B*44/B*49/B*51/B*52/B*53/B*59
73TS		C*01/C*03/C*08/C*14/C*16	A*25/A*32	Bw6
76TVN		C*02/C*05/C*15		
77TVS	TerEp#421, WK4C11	C*01/C*03/C*08/C*14/C*16		B*46
77VSN		C*01/C*03/C*07/C*08/C*12/C*14/C*16		B*46
79RK	TerEp#244	C*02/C*04/C*05/C*06/C*15/C*17/C*18		
79VRN	TerEp#246	C*01/C*03/C*07/C*08/C*12/C*14/C*16		B*46/B*73
80SRN	OUW4F11	C*01/C*03/C*07/C*08/C*12/C*14/C*16		Bw6 (not B*73)
82RNR	KAM3H9	C*01/C*03/C*07/C*08/C*12/C*14/C*16		Bw6
90D		C*04/C*06/C*07/C*18	A*01/A*11/A*25/A*26/A*34/A*36A*43/A*66:01/A*80	B*73
103L		C*01/C*02/C*04/C*05/C*06/C*07/C*08/C*12/C*14/C*15/C*16/C*17/C*18		B*13/B*15:16/B21/B22/B*35/B*45/B*53/B*58/B*59/B*82
113YD		C*01/C*02/C*03/C*06/C*07/C*12/C*14/C*16		B*15:02/B*15:13/B*44
113YN		C*04/C*05/C*08/C*17/C*18		B*14/B*37/B*45/B*49/B*50/B*73
116F		C*04/C*05/C*07:04/C*08/C*17/C*18		B*14/B*37/B*38/B*39/B*67/B*73
116L		C*15		B*13/B*45/B*49/B*50/B*54/B*55/B*56/B*59/B*82
116S		C*02/03:02/C*06/C*07:01/C*07:02/C*12/C*14/C*16		B*15/B*18/B*35/B*46/B*53/B*57:01/B*58
116Y		C*01/C*03:03/C*03:04	A*02/A*23/A*24/A*68:02/A*69	B*07/B*08/B*15:10/B*40/B*41/B*42/B*48/B*51/B*52/B*57:03/B*78/B*81
138K		C*05/C*08:02		
1421S		C*17		B*40:01/B*48/B*81
147L		C*07/C*17		B*40:01/B*48/B*81
151ARA		C*07/C*16		
151ARE		C*01/C*02/C*03/C*04/C*05/C*06/C*08:02/C*12/C*14/C*15/C*17/C*18		B*07/B*14/B*15/B*46/B*49/B*50/B*51/B*52/B*55/B*78
151ART		C*08:01		
156DA	156DA ref (22)	C*07:04		B*08/B*37/B*41/B*42/B*44:02/B*45/B*82
156LA		C*03/C*07:01/C*07:02/C*08:01/C*15/C*17	A*02:01/A*02:06/A*03/A*23:01/A*29/A*30/A*31/A*32/A*33/A*34:02/A*69/A*74/A*80	B5/B*13/B*14/B*15:02/B*15:03/B*15:10/B*15:13/B*15:16/B*15:18/B17/B*18/B21/B22/B*27/B*35:01/B*40/B*44:03/B*47/B*48/B*53/B*59/B*73/B*78/B*81
156RA		C*01/C*04/C*05/C*08:02/C*14/C*18		B*07/B*35:08
156WA		C*02/C*06/C*12	A*02:02/A*02:03/A*02:05/A*23:02/A*25/A*26/A*34:01/A*43/A*66/A*68	B*15:01/B*15:11/B*15:12/B*46
163EW	TerEp#222	C*02/C*17	A*66:02	B*07/B*13/B*27/B*40/B*47/B*48/B*73/B*81
163LW	TerEp#245, IND2D12	C*03		B*15(not B*15:12)/B*35/B*46/B*49/B*50/B*51/B*52/B*53/B*56/B*57/B*58/B*78
163TW		C*01/C*04/C*05/C*06/C*07/C*08/C*12/C*14/C*15/C*16/C*18/	A*02/A*03/A*24:03/A*29/A*30/A*31/A*32/A*33/A*34/A*36/A*68/A*69/A*74	B*08/B*14/B*18/B*37/B*38/B*39/B*41/B*42/B*54/B*55/B*59/B*67

(continued on next page)

Table 2 (*continued*)

Eplet	Epitope	HLA-C alleles	HLA-A alleles	HLA-B alleles
166LE		C*03		B5/B*15(not B*1512)/B17/B21/B*35/B*44/B*45/B*46/B*53/B*56/B*78/B82
173K	TerEp#5081	C*03		
177KT	TerEp#40	C*05/07:04/C*08		
184H		C*01/C*02/C*03/C*04/C*05/C*06/C*08/C*12/C*14/C*15/C*16/C*18		
184R		C*17		
193PL	TerEp#37	C*07		
193PV		C*01/C*02/C*03/C*04/C*05/C*06/C*08/C*12/C*14/C*15/C*17/C*18		B*35/B*51/B*52/B*53/B*58/B*78
219W	TerEp#5057, TRA2G9	C*01/C*03/C*04/C*14/C*18		
253Q	TerEp#38	C*07/C*17	A*02/A*25/A*26/A*29/A*31/A*32/A*33/A*34/A*43/A*66/A*68/A*69/A*74	B*73
267QE	TerEp#41	C*07/C*17		B*73
270C		C*17		B*73

2.4. Single-allele absorption–elution studies

To further determine the presence of HLA-C epitope-specific antibodies, we selected some sera for absorption–elution analysis. This was done with microbeads coated with a single HLA class I allele that was different from the mismatched donor allele, but that shared the eplet thought to be recognized by antibody. Briefly, 25 µl of sera was incubated at room temperature with 25 µl of allele-coated microbeads and the absorbed serum removed. The antibody coated beads were washed and then incubated at room temperature with 25 µl Pierce IgG elution buffer. Normal pH was reconstituted with 0.75 µl high pH TRIS buffer. The eluate was separated from the beads and was tested for antibodies in the routine Luminex assay using class I single-allele kits. All reagents were kindly provided by Gen-Probe (protocol from personal communication, Justin Mostecki, Gen-Probe).

3. Results

3.1. Antibody screening results

Among the 45 patients in this study, 34 (76%) received a renal transplant from a donor with a single HLA-C mismatch, while 11 patients (24%) had been exposed to two mismatched HLA-C antigens, for a total of 56 mismatches. Our assessment of antibody reactivity took into account the variability among the 15 participating laboratories regarding the use of different Luminex kits and the MFI values of controls. Our comparison of the Luminex results considered the reactivity with self-alleles as a negative control and we have applied the following formula to determine the level of reactivity towards a single mismatched allele:

$$\%MFI = \frac{MFI(Allele) - Average\,MFI(Self\ Alleles)}{MFI(Positive\,Control) - Average\,MFI(Self\ Alleles)} \times 100\%$$

Note: if the positive controls had lower MFI values than reactive alleles, we substituted them with the median values of the five most reactive alleles.

Donor-specific HLA-C antibodies were detected in 27 patients (60%). Fig. 1 shows that the %MFI values for HLA-C mismatches were significantly lower than for HLA-A and HLA-B mismatches. We noted that 29/56 (52%) HLA-C mismatches had induced weak or no antibody reactivity as indicated by their <10%MFI values.

3.2. Eplet loads and antibody responses

For each HLA-C antigen mismatch we assessed the epitope load by determining the number of mismatched eplets. The epitope load of the donor HLA-C antigen mismatch correlates with the incidence of specific antibody production in this group of patients (Fig. 2). Responders with >10%MFI values) had been exposed to a median of 11 mismatched eplets, significantly more than the 7 eplets exposed to patients with <10% MFI values ($p = 0.009$; Mann–Whitney U test).

3.3. Examples of antibody specificity patterns against HLA-C epitopes

We present two cases illustrating how an HLAMatchmaker analysis can determine donor-specific antibodies against HLA-C epitopes. In both of them, we have done absorption–elution studies with informative alleles to further verify an epitope-specific antibody.

Case 24 Serum pf28 (submitted by Patricia Campbell, Edmonton, Alberta, Canada) came from a patient who typed as A2,3; B60,62; Cw9,- and who had rejected a transplant from a deceased, unrelated donor who was mismatched for A11 and Cw4. HLAMatchmaker analyses require 4-digit allele typings, and we converted these HLA antigens to the most likely alleles in the patient's HLA type: A*02:01,A*03:01; B*15:01, B*40:01; C*03:03,- (Table 3). Luminex testing showed antibodies induced by both class I mismatches. C*04:01 has seven mismatched eplets, but three of them (69RA, 90D, 103L) were on non-reactive alleles and we concluded that none of them were specifically recognized by patient's antibodies. Four eplets remained on reactive HLA-C alleles, shown in bold, underlined font. Most informative were C*02:02 and C*15:02 which shared only 79RK with the immunizing C*04:01. This suggested the presence of 79RK-specific antibodies. We absorbed the serum with C*15:02 beads and the eluate reacted with all 79RK-carrying alleles but no other alleles (Table 3). This eplet corresponds to TerEp #244 (Table 2).

This serum showed also antibodies against another HLA-C epitope that corresponded to 156RA on the immunizing C*04:01. Three alleles, C*01:02, C*08:02 and C*14:02, were informative (Table 3) but two 156RA-carrying alleles, B*07:02 and B*35:08 with MFI values of 209 and 259, were considered negative. Based on previous experience [22,24], we postulated that the reactive 156RA-carrying alleles must share another amino acid configuration with the immunizing C*04:01 and that this configuration is absent on the

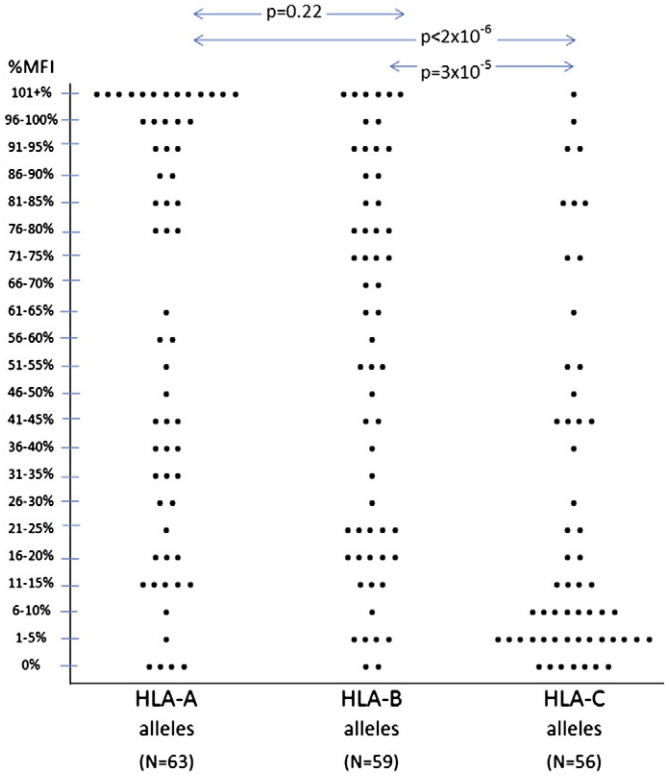

Fig. 1. Reactivity of sera from 45 allograft nephrectomy cases with donor-specific HLA class I alleles in Luminex single-allele assays.

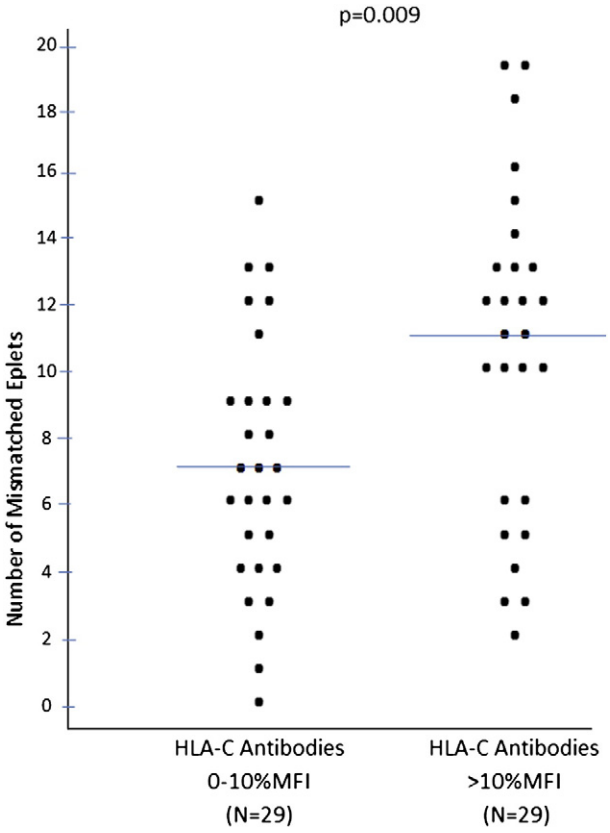

Fig. 2. Association between eplet load of HLA-C mismatches and donor-specific HLA-C antibody reactivity. Sera with >10% MFI values are generally considered positive for antibody.

non-reactive 156RA-carrying alleles. This turned out to be the 69KRQ eplet which is also present on the patient's own C*03:03. In other words, the second HLA-C antibody is specific for an epitope defined by the 156RA + s69KRQ pair (s designates a self-eplet). Interestingly, this serum reacted also with C*08:01 which has 151ART + s69KRQ rather than 156RA + s69KRQ and this might reflect some cross-reactivity between these pairs.

Although the reactive HLA-C alleles carried also 14WR (unique for Cw4) and 73AN, none of them were informative enough for determining antibody specificity. This serum had also antibodies induced by the A11 mismatch. It appears that their specificity was primarily against 163RW. Unexpectedly, the serum reacted strongly with B8 which shares no epitopes with A11 or Cw4 and seems likely that this reflects third-party antibodies induced by another sensitizing event. Finally, we noted that A*68:02 (but not A*68:01) and B*54:01 had MFI values above the cutoff point of 1200. Further analyses did not reveal any epitope specificity pattern for this weak reactivity.

Case 28 Serum MA (submitted by Silvia Chrenova, Bratislava, Slovakia) came from a patient who typed as A3,11; B35,51; Cw4, and had rejected a transplant from a related donor mismatched for the A2,B60,Cw12 haplotype. This serum had antibodies against epitopes on all three donor class I mismatches. C*12:03 had five mismatched eplets but there were no antibodies against three of them: 1CK, 79VRN and 156WA. We identified two HLA-C antibody specificities (Table 4). One involved the 77VSN eplet paired with a self-eplet 151ARE (indicated with prefix "s") found on the patient's C*04:01; C*16:01, which has 77VSN paired with 151ARA, did not react with patient's serum (MFI = 146). Although the reactive C*07:01 and C*07:02 have also 77VSN + 151ARA, their reactivity appeared to be due to antibodies specific for another eplet, 73AS, on the immunizing C*12:03. This became evident following absorption with the 77VSN + s151ARE carrying C*08:02. The C*08:02 eluate reacted with all 77VSN + s151ARE carrying alleles but not with the two C*07 alleles. In other words, the serum reactivity with C*07:01 and C*07:02 is due to antibodies against a second epitope presented by the immunizing C*12:03. The reactivity of C*08:01 might be related to its epitope 77VSN + 151ART which may cross-react with 77VSN + s151ARE specific antibodies.

The reactivity with C*17:01 was not caused by C*12:03-induced antibodies but appeared related to antibodies induced by the other two class I mismatches. C*17:01 shares 142IS with the immunizing B*40:01 and 253Q with the immunizing A*02:01. To determine whether the C*17:01 reactivity was related to 142IS-specific antibodies, we absorbed the serum with B*40:01. The eluate reacted especially with alleles that share 163EW + s72TE with B*40:01 but not with C*17:01 (MFI = 188). It seems therefore that the reactivity with C*17:01 was likely due to 253Q-specific antibodies induced by the A*02:01 mismatch; this conclusion is strengthened by the strong reactivity of B*73:01, which shares only 253Q with the immunizing A*02:01.

Table 3
Example of antibody specificity pattern of a patient sensitized by a Cw4 mismatch.

Case	edmpf-28(Edmonton)
Patient	A*02:01, A*03:01; B*40:01; C*03:03,-
Donor	Mismatched eplets
C*04:01	**14WR**, 69RA, **73AN, 79RK**, 90D, 103L, **156RA**
A*11:01	90D, **151AHA**, 156QA, **163RW**

Allele	Eplets on Reactive Alleles	Serum MFI	C*15:02 Eluate MFI
C*04:01(Donor)	14WR, 73AN, 79RK, 156RA + s69KRQ	10474	2432
C*04:03	73AN, 79RK, 156RA + s69KRQ	15418	3799
C*18:01	79RK, 156RA + s69KRQ	10372	13158
C*05:01	73AN, 79RK, 156RA + s69KRQ	13681	11316
C*06:02	73AN, 79RK	5851	3045
C*17:01	73AN, 79RK	2944	3906
C*15:02	79RK	12164	5765
C*02:02	79RK	5255	3292
C*01:02	156RA + s69KRQ	1762	119
C*08:02	156RA + s69KRQ	3015	114
C*14:02	156RA + s69KRQ	1540	167
C*08:01	156RT + s69KRQ	1856	148
A*11:01(Donor)	151AHA, 163RW	2647	49
A*11:02	151AHA, 163RW	3579	100
A*25:01	163RW	2229	41
A*26:01	163RW	3703	81
A*43:01	163RW	2983	94
A*66:01	163RW	3878	36
B*08:01	Third Party Epitope?	8224	99
A*68:02	?positive	2199	46
B*54:01	?positive	1710	68
74 Neg alleles (MFI < 1200)		396 ± 212	88 ± 39
Positive control		12482	17172
Negative control		257	128

The A*02:01 and B*40:01 mismatches induced other antibodies as well (Table 4). There were 23 reactive alleles that shared eplets with A*02:01, whereas 12 reactive alleles shared eplets with B*40:01(data not shown).

3.4. Donor-specific HLA-C eplets on antibody-reactive alleles

The cases with HLA-C antibody reactivity were divided in two groups. Table 5 describes 14 cases whereby reactive alleles had donor-specific single eplets and Table 6 lists 13 cases that included donor-specific eplet pairs on informative, reactive alleles. Each patient had also antibodies against HLA-A and/or HLA-B mismatches but they did not interfere with the HLA-C epitope analysis.

Table 5 shows in each case which donor HLA-C eplets were mismatched and those on reactive alleles are underlined in bold font. The %MFI values indicate the reaction strengths with the immunizing alleles. In most cases, the antibody reactivity patterns were restricted to small numbers of mismatched eplets; this applies particularly to Cases 2, 3, 8, 9, 11, 12, 13 and 14. In cases 1 and 4, the extensive reactivity with HLA-C alleles made it difficult to determine the antibody-specific eplets; the compositions and sizes of the Luminex panels did not always yield informative alleles to distinguish between eplet reactivity patterns. On the other hand, we could clearly identify donor-specific eplets which did not induce antibodies. Although we generally consider %MFI value of 10% as the cutoff for a positive antibody response, and the reactions were weak, we were able to identify antibody reactivity patterns towards eplets on informative alleles for cases 3, 9 and 12, with %MFI values of only 9%, 10% and 8%, respectively.

We noted with Case 1 the presence of 163EW shared between the immunizing C*02:02 and reactive alleles; this suggested the presence of 163EW-specific antibodies (data not shown). This patient was also sensitized by the donor's B*27:05 which also has 163EW and it was not possible to determine whether C*02:02 or B*27:05 (or both) had induced the 163EW-specific antibody response. Therefore 163EW is listed between parentheses in Table 5.

Case 4 has a similar uncertainty about specific HLA-C eplet-induced responses. The immunizing donor's C*03:04 had a mismatched 151ARE present on reactive HLA-C and HLA-B alleles including the donor-specific B*07:02 which had also induced antibodies. This eplet is shown between parentheses in Table 5 because it is unknown whether 151ARE-reactive antibodies had been induced by B*07:02 or C*03:04 or both. Moreover, we put 21H, 173K and 219W between parentheses because each reactive HLA-C allele which carries these eplets has also 151ARE. In other words, we could not rule out that the HLA-C reactivity reflected by these antibodies could have been induced by the B*07:02 mismatch.

There were 13 cases with HLA-C eplet pair-specific antibodies identified by informative alleles in the Luminex panel (Table 6). Eplets that induced only pair-specific antibodies are marked with asterisks and the pairs themselves are shown between parentheses and in italic

Table 4
Example of antibody specificity pattern of a patient sensitized by a Cw12 mismatch.

Case	MA(Bratislava)
Patient	A*03:01, A*11:01; B*35:01, B*51:01; C*04:01
Donor	Mismatched eplets
C*12:03	1CK, **73AS**, **77VSN**, 79VRN, 156WA
A*02:01	**66RKH, 62GE, 65RKV, 70KAH**, 70AHS, **107W, 127K, 142TKH, 145KHA, 151AHV, 184A, 193AV**, 206S, **253Q**
B*40:01	32L, 41T, 44RK, 62REI, 65QIS, **142IS, 163EW, 177DT, 180E**

Allele	Eplets on Reactive Alleles	Serum MFI	C*08:02 Eluate MFI
C*12:02	73AS, 77VSN + S152ARE	1274	1235
C*01:02	77VSN + s151ARE	3605	1577
C*03:03	77VSN + s151ARE	4722	1897
C*03:04	77VSN + s151ARE	2273	1897
C*08:02	77VSN + s151ARE	7786	3130
C*14:02	77VSN + s151ARE	2595	1205
B*46:01	77VSN + s151ARE	3349	595
C*08:01	77VSN + 151ART	4715	2810
C*07:02	73AS	4945	74
C*07:01	73AS	2203	87
C*17:01	142IS, 253Q	1458	133
B*73:01	253Q	7889	74
A*02:01 (Donor)	66RKH, 62GE, 65RKV, 70KAH, 107W, 127K, 142TKH, 145KHA, 151AHV, 184A, 193AV, 253Q	11419	82
A*02:01 eplet-carrying reactive alleles ($n = 23$)		6339 ± 2692	80 ± 41
B*40:01 (Donor)	142IS, 177DT, 180E, 163EW + s73TE	11615	53
B*40:01 eplet-carrying reactive alleles ($n = 12$)		4792 ± 2537	60 ± 18
44 Neg alleles (MFI < 100)		507 ± 200	81 ± 33
Positive control		13524	17111
Negative control		325	80

fonts. We could clearly identify single pairs in eight of these cases, such as 79VRN + s131R in Case 15, and 79RK + s144TQR in Case 17. In the other five cases, we could not distinguish which pair(s) had induced specific antibodies. Examples are Case 16: 77TVS + 156RA, 156RA + s80SRN and/or 77TVS + s151ARE, and Case 19: 77TVS + s156LA and/or 151ARE + s77VSN. Nevertheless, these findings suggested the presence of eplet pair specific antibodies.

The pairs consisted generally of a donor-specific mismatched eplet and a self-eplet (indicated with a prefix "s") shared between the patient and the donor. These findings are analogous to previously reported data on human monoclonal antibodies specific for pairs [24,25]. There was one exception whereby both eplets were donor-specific mismatches: Case 22 showed antibodies reactive with 73AN + 156RA and/or 79RK + 156RA.

3.5. Frequencies of antibody responses to donor HLA-C eplets

This cohort of patients had been exposed to 38 donor HLA-C eplets mismatched a total of 352 times (Table 7). There was antibody reactivity in 84 instances (24%) either with a particular eplet (69 instances) or an eplet pair (15 instances) shared by reactive alleles. The eplets are ranked according to their frequencies of antibody reactivity and their corresponding, experimentally verified epitopes are also shown. These frequencies provide an estimate of the relative immunogenicity of HLA-C eplets. For example, 79RK showed antibody reactivity in 11 of 13 instances (85%), including 4 cases whereby antibodies reacted with 79RK eplet pairs. This eplet is equivalent to TerEp#244. Altogether, seven eplets 79RK, 138K, 76TVN, 173K, 177KT, 14WR and 193PL had 40% or higher antibody frequencies, suggesting that they have relatively high immunogenicity among HLA-C eplets. Twelve eplets had a 20–40% antibody incidence. Ten eplets showed low antibody frequencies in 5–20% range.

In contrast, we could not identify any antibody reactivity with nine mismatched donor eplets in this group of patients. Although more cases need to be tested, it seems that these eplets have, at best, a very low immunogenicity. This might be related to a limited molecular surface exposure and antibody accessibility as noted for 9D, 12AVR, and 113YD. On the other hand, some eplets such as 71AT and 90D are in well-exposed

Table 5
Fourteen allograft nephrectomy cases with antibody reactivity towards donor HLA-C single eplets.

Case	Location	Patient	Recipient HLA	Donor HLA-C	% MFI	Eplet mismatches (eplets on reactive alleles are underlined in bold large font)
1	Pittsburgh	FF	A*01:01,-;B*08:01,-;C*07:01,-	C*02:02	94%	9Y,**21H**,**69KRQ**,**69RT**,71AT,**76TVN,79RK**,**151ARE**,156WA, **(163EW)**,**184H**,**193PV**
2	Pittsburgh	WW	A*25:01,A*68:01;B*35:01,B*53:01;C*04:01, C*06:02	C*03:03	65%	21H,69RT,71AT,**77TVS**,77VSN,79VRN,116Y,**173K**
3	Detroit	ZA	A*02:05,A*32:01;B*07:06,B*40:02;C*02:02, C*15:05	C*03:03	9%	77TVS,77VSN,79VRN,163LW,166LE,**173K**,219W
4	Nijmegen	112179	A*02:01,-;B*40:01,B*48:01;C*07:02	C*03:04	24%	**(21H)**,69RT,71AT,77TVS,**(151ARE),163LW,166LE,(173K),184H,193PV, (219W)**
5	Berlin	ND11	A*24:08,A*30:01;B*08:01,B*44:02;C*07:01,-	C*04:01	54%	**14WR**,69KRQ,**73AN**,**79RK**,113YN,116F,151ARE,156RA,184H,193PV,219W
6	Newcastle	N8VH	A*01:01,A*26:01;B*44:03;C*01:02,C*07:03	C*05:01	72%	35Q,12AVR,**76TVN**,**79RK**,113YN,116F,**138K**,177KT
7	Pittsburgh	AA	A*24:02,A*32:01;B*08:01,B*40:01;C*03:04, C*07:01	C*05:01	134%	35Q,**76TVN**,**79RK**,113YN,116F,**138K**,156RA,**177KT**
8	Leiden	156684	A*29:02,-;B*45:01,B*50:01;C*06:02,-	C*05:01	81%	9Y,35Q,69RT,71AT,76TVN,116F,**138K**,156RA,**177KT**
9	Pittsburgh	NN	A*01:01,-;B*08:01,-;C*07:01,-	C*06:02	10%	69KRQ,**73AN**,**79RK**,151ARE,156WA,184H,193PV
				C*07:02	0%	69KRQ,
10	Plymouth	Pt2	A*32:01,A*68:02;B*35:01,B*53:01;C*04:01,-	C*07:02	42%	9D,1CK,12AVR,**73AS**,77VSN,79VRN,113YD,147L,**151ARA,193PL,267QE**
11	Adelaide	ADEL-1	A*02:01,-;B*51:01,B*40:01;C*03:04,-	C*07:02	15%	9D,1CK,12AVR,69RA,73AS,90D,103L,116S,151ARA,**193PL**,267QE
12	San Antonio	POL7157	A*02:01,-;B*35:01,B*45:01;C*04:01,C*06:02	C*07:02	8%	73AS,77VSN,79VRN,**147L**,151ARA,**193PL**,267QE
13	Pittsburgh	MM	A*02:01,A*33:01;B*08:01,B*15:03,C*02:02, C*03:03	C*07:02	27%	12AVR,69RA,73AS,90D,**147L**,151ARA,**193PL**,267QE,
14	Detroit	JK	A*01:01,A*02:06;B*37:01,B*55:02;C*01:02, C*06:02	C*08:02	51%	35Q,**138K**,**177KT**

Table 6
Thirteen allograft nephrectomy cases with antibody reactivity towards donor HLA-C eplets and eplet pairs.

Case	Location	Patient	Recipient HLA	Donor HLA-C	% MFI	Eplet mismatches(eplets on reactive alleles are underlined in bold large font)
15	Athens	T-34	A*24:02,A*31:01;B*44:03,B*35:01; C*04:01,-	C*01:02	98%	9f,1CK,69RT,71AT,**77TVS**,77VSN,**79VRN*** *(*79VRN +s131R)*
16	Berlin	ND12	A*02:01,-;B*18:01,B*27:05;C*02:02, C*07:02	C*01:02 C*03:03	15% 42%	**77TVS***,**156RA***,219W **77TVS***,163LW,166LE,**173K**,219W *(*77TVS +156RA,156RA +s80SRN,77TVS +s151ARE)*
17	Edmonton	rm29	A*29:02,A*31:01;B*44:02,B*51:01; C*07:04,-	C*02:02 C*03:03	9% 44%	**21H**, 69RT,71AT,**76TVN**,**79RK***,116S,156WA,163EW,184H 21H,69RT,71AT,73TS,77TVS,173K,184H,219W, *(*79RK +s144TQR)*
18	Detroit	ZS	A*02:01,A*23:01,B*44:02,B*49:01; C*05:01,C*07:01	C*02:02	73%	21H,**156WA***,163EW *(*156WA +s69KRQ)*
19	Bratislava	BM	A*01:01,-;B*08:01,-;C*07:01,-	C*03:04	85%	9Y,21H,69KRQ,69RT,71AT,**77TVS***,**151ARE***,163LW,166LE,173K,184H,193PV,219W *(*77TVS +s156LA,151ARE +s77VSN)*
20	Detroit	TG	A*30:02,A*74:01;B*44:03,B*35:01; C*04:01,C*14:02	C*03:04 C*06:02	93% 25%	**21H**,116Y,**173K** 9D,12AVR,**156WA*** *(*156WA +s73AN,156WA +s90D)*
21	San Antonio	POL8790	A*02:01,A*68:01;B*18:01,B*51:01; C*05;01,C*08:02	C*03:04 C*07:01	14% 38%	21H,113YD,**173K**,219W 9D,63REN,**69RA***,73AS,90D,113YD,147L,151ARA,**193PL**,267QE *(*69RA +s156LA)*
22	Edmonton	hb20	A*01:01,A*02:01;B*08:01,B*39:01; C*07:01,-	C*04:01	18%	9S,**14WR**,69KRQ,**73AN***,**79RK***,113YN,151ARE,**156RA***,184H,193PV,219W *(*73AN +156RA,79RK +156RA)*
23	Edmonton	pf28	A*02:01,A*03:01;B*40:01,B*15:01; C*03:02,-	C*04:01	84%	9S,**14WR**,69RA,73AN,79RK,90D,103L,113YN,116F,**156RA*** *(*156RA +s69KRQ)*
24	Berlin	ND9	A*02:01,A*68:01;B*52:01,B*07:02; C*07:02,-	C*05:01	42%	**35Q**,69RT,**76TVN**,**79RK***,113YN,116F,**138K**,**177KT**,184H *(*79RK +s90A)*
25	Pittsburgh	PM	A*24:03,A*25:01;B*18:01,B*40:02; C*03:03,-	C*05:01 C*07:03	49% NA	35Q,12AVR,**76TVN**,**79RK***,103L,113YN,116F,**138K**,156RA,177KT 69RA,73AS,103L,151ARA,166LE,193PL,267QE *(*79RK +s90A)*
26	Chicago	10559	A*33:01,A*30:02;B*08;01,-; C*07:01,-	C*07:02 C*08:02	17% 23%	**69KRQ*** 9Y,35Q,**69KRQ***,69RT,71AT,77TVS,113YN,116 F,138K,151ARE,156RA,177KT,184 H,193PV *(*69KRQ +s77VSN,69KRQ +s73AS,69KRQ +s156LA)*
27	Bratislava	MA	A*03:01,A*11:01;B*51:01,B*35:01; C*04:01,C*15:02	C*12:03	7%	12AVR,**73AS**,**77VSN***,79VRN,113YD,156WA *(*77VSN +s151ARE)*

*Eplet has corresponding pair on reactive alleles.

sequence positions. These data must be considered preliminary and more studies are needed to assess HLA-C eplet immunogenicity.

4. Discussion

This study shows that HLA-C mismatches induced antibodies in 60% of patients who, after removal of a failed kidney transplant, had class I antibodies detectable by Luminex assays using single-allele beads. An HLAMatchmaker analysis of antibody reactivity identified the reactive HLA-C epitopes. Analogous to the recent findings of Bryan et al. [16], we noted that HLA-C antibody frequencies and reactivities are lower than those induced by donor HLA-A and HLA-B mismatches. The incidence of anti-HLA-C antibodies is associated with the degree of mismatching, as measured by the eplet load. This finding is analogous to data reported by investigators in the Netherlands and the United Kingdom who showed that the incidence of anti-donor HLA-A and HLA-B antibodies in transplant patients correlates with the number of mismatched donor triplets or eplets determined by HLAMatchmaker [26,27]. Moreover, an

Table 7
Frequencies of antibody reactivities induced by HLA-C eplet mismatches.

Eplet	Equivalent	Numbers of mismatches	Reactive eplets	Reactive as pairs	Total	Antibody frequency	Eplet	Equivalent	Numbers of mismatches	Reactive eplets	Reactive as pairs	Total	Antibody frequency
79RK	TerEp#244	13	7	4	11	85%	73AS		11	2	0	2	18%
138K		9	7	0	7	78%	69RA		6	0	1	1	17%
76TVN		9	6	0	6	67%	193PV		8	1	0	1	13%
173K	TerEp#5081	11	7	0	7	64%	77VSN		10	0	1	1	10%
177KT	TerEp#40	10	5	0	5	50%	79VRN	TerEp#246	10	0	1	1	10%
14WR		6	3	0	3	50%	184H		11	1	0	1	9%
193PL	TerEp#37	12	5	0	5	42%	267QE	TerEp#41	12	1	0	1	8%
73AN	TerEp#5037	8	3	0	3	38%	151ARA		14	1	0	1	7%
21H	TerEp#39	14	5	0	5	36%	69RT		14	1	0	1	7%
151ARE		6	1	1	2	33%	9Y		4	0	0	0	0%
156RA		10	0	3	3	30%	9D		10	0	0	0	0%
69KRQ		7	1	1	2	29%	90D		9	0	0	0	0%
77TVS	TerEp#421	14	3	1	4	29%	71AT		10	0	0	0	0%
147L		8	2	0	2	25%	12AVR		10	0	0	0	0%
156WA		8	0	2	2	25%	116F		11	0	0	0	0%
163EW	TerEp#222	4	1	0	1	25%	113YN		11	0	0	0	0%
163LW	TerEp#245	4	1	0	1	25%	113YD		7	0	0	0	0%
166LE		4	1	0	1	25%	103L		5	0	0	0	0%
219W	TerEp#5075	13	3	0	3	23%							
35Q		9	1	0	1	11%	Total		352	69	15	84	24%

association has been found between the number of mismatched triplets and flow cytometry cross-match positivity with donor cells [28]. These data support the concept that epitope load of a donor mismatch affects HLA sensitization following a transplant.

For one-fourth of the 352 HLA-C eplet mismatches there was antibody reactivity with a single eplet or an eplet pair shared by reactive alleles (Table 7). The latter are examples showing that a mismatched eplet, the driving force for the antibody response, may require a self amino acid configuration on the molecular surface of the immunizing antigen to make up a specific epitope [24,25]. This configuration is about 6–15 Ångstroms away from the mismatched eplet and would serve as a critical contact site for antibody. Eplet-carrying antigens with other configurations in that surface position would not react with antibody. Thus, mismatched eplets can induce specific antibodies with different reactivity patterns which depend on certain self-configurations. One might raise the question whether eplet-carrying antigens can be considered as acceptable mismatches if they lack the critical self-configuration necessary for antibody reactivity.

This study has shown that eplets shared by HLA-C with HLA-A and/or HLA-B antigens can induce specific antibodies. We have previously reported a sensitization case against the 156DA eplet on the immunizing C*07:04 which is shared with a group of HLA-B alleles including B*08:01, B*37:01, B*41:01, B*42:01, B*44:02, B*45:01 and B*82:01 [23]. Sensitization against 151ARE on a C*02:02 mismatch (Case 1) led to antibodies that reacted with all 151ARE-carrying HLA-B alleles. These findings demonstrate that in some cases, post-transplant sensitization induced by an epitope on a HLA-C mismatch may render certain HLA-A and/or HLA-B antigens to become unacceptable mismatches because they share that epitope although the patient may have never been exposed to these antigens.

Our analysis of this cohort of 45 transplant cases worldwide has yielded a preliminary assessment of the relative immunogenicity of HLA-C eplets determined from the frequencies of antibody reactivity with eplet-carrying alleles in Luminex panels. Several eplets such as 79RK 138K and 76TVN had rather high frequencies of antibody reactivity whereas other eplets were never reactive. Table 7 shows that many experimentally proven epitopes (TerEps) are equivalent to the apparently more immunogenic eplets. It seems likely that the remaining immunogenic eplets which at present, have no epitope equivalents, can be verified with informative antibodies. Thus, it seems possible to define experimentally a complete repertoire of HLA-C epitopes recognized by alloantibodies.

Although these HLA-C antibodies were detected in sera from patients with failed kidney grafts it is not known how many, if any, contributed to these transplant failures. This analysis demonstrates nevertheless that these antibodies are specific for structurally defined epitopes and this type of information seems useful in determining their clinical relevance. Most transplant programs including the United Network for Organ Sharing (UNOS) do not require laboratories to report HLA-C types for transplant candidates and potential donors. This lack of information may especially affect retransplant candidates who have HLA-C antibodies because it may make it more difficult to identify acceptable mismatches and interpret cross-match results [16]. Therefore, organ allocation will benefit from HLA-C typing and antibody determination. The identification of antibody-reactive epitopes may permit a better assessment of unacceptable mismatches for sensitized patients.

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