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A Novel Method for Anti-HLA Antibody Detection Using Personalized Peptide Arrays

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Background. HLA mismatches are the primary cause of alloantibody-mediated rejection (AMR) in organ transplantation. To delineate antigenic and immunogenic potentials among individual HLA mismatches, information regarding antibody specificity at the epitope level, instead of the allelic level, is needed. **Methods.** This study explores a direct screening method for HLA linear epitopes in kidney transplant patients. We custom synthesized a large panel of 15-residue HLA peptides in an array format and measured alloantibody reactivity to these peptides from the sera of post and/or pretransplant patients. Two design concepts for the arrays were followed: a standard array of a fixed panel of peptides or personalized arrays. The standard array contains 420 peptides derived from a predetermined set of HLA-DQ allelic antigens based on templates also used in the single-antigen beads assay. **Results.** The array detected distinct antiserum patterns among transplant subjects and revealed epitope levels of specificity largely in accordance with the single-antigen results. Two personalized arrays that each included donor-derived peptides of HLA-A, -B, -C, -DQ, and -DR sequences were separately designed for 2 transplant subjects. The personalized arrays detected de novo antibodies following transplantation. The new method also showed superior sensitivity to a single-antigen assay in one of the cases whose pathological diagnosis of AMR occurred before single-antigen assay could detect antibodies. **Conclusions.** This pilot study proved the feasibility of using personalized peptide arrays to achieve detection of alloantibodies for linear HLA epitopes associated with distinct donor-recipient mismatches. Single or multiple reactive epitopes may occur on an individual HLA molecule, and donor-specific HLA-DQ-reactivity among 5 kidney transplant subjects revealed patterns of shared epitopes.

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HLA molecules are highly polymorphic cell receptors, posing a major obstacle to the success of organ

transplantation (tx). The allorecognition of mismatched donor HLA directly contributes to chronic rejection.^{1,2} DNA typing for HLA is widely used in the clinic, and one of the most important challenges is to determine which mismatched transplants will fare well and which should be avoided.^{3,4} The reality is that on the one hand, modern genetic tests of DNA utilizing high-resolution typing (by sequence-specific primers) and sequence-based typing (SBT) methods provide increasingly accurate allele sequences,⁵ whereas on the other hand, technologies for unambiguously determining alloantibody reactivity to amino acid (aa) epitopes are lagging far behind.⁶⁻⁸ There is an urgent need for new methods that can distinctively detect antibodies elicited by donor residues.

Considerable effort has been dedicated to methodologies for detecting HLA epitopes.⁹ Two major strategies have made significant progress. The first one, known as the absorption and elution method, was developed by Terasaki's group using recombinant HLA standards individually expressed on cell surfaces to capture antibodies expected to react only to a single antigen. The eluate was then tested in a solid phase Luminex single-antigen (LSA) assay against a panel of homologous alleles. Cross-reactive antigens as a group were analyzed using sequence comparison tools to delineate amino acid positions that most likely constituted the epitope, which was then assigned an identity in TerEps (referred to as Terasaki's epitopes).¹⁰

Following a different strategy, Duquesnoy¹¹ developed a computerized method to find sequence and structural

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features of HLA polymorphism predicted to constitute epitopes. Initially, the program, termed HLA-Matchmaker, sought amino acid triplets in a linear motif that distinguish donor from recipient HLA molecules. By applying additional interlocus and intralocus subtraction, potentially immunogenic triplets were identified. Considering a cluster of triplets in structural proximity (defined as within 3 Å), albeit from discontinuous peptide segments, might together constitute an epitope, the software was updated accordingly to detect such conformational features, termed eplets. To better categorize epitopes that have been experimentally confirmed, a Web-based epitope register was recently established (<http://www.epregistry.com.br>).¹² Although a few eplets have been shown to recognize the amino acid sequence that purportedly defines the epitope,¹³ most remain theoretical.¹⁴

Solid-phase single-antigen beads assay performed on Luminex (referred to as Luminex Single-Antigen beads assay or LSA by One Lambda) is very sensitive and specific to detect preformed or de novo formed antibodies. However, instead of using a donor's own HLA antigens,

the LSA assay uses a fixed panel of allelic antigens, and it remains challenging to make a reliable estimation of rejection risk.^{15–17} Even in the case of a donor allele being present in the LSA panel and showing reactivity to alloantibodies, information regarding which mismatching amino acid(s) constitutes the antigen epitope is still lacking. Conceptually, if mature technologies for mapping epitope positions were available for screening of a large cohort of alloantibodies, retrospective studies would collectively reveal high-risk antigenic positions in HLA molecules. Consequently, when high-resolution sequences of the proposed donor's alleles are provided, clinical decisions may also consider whether certain mismatches occurring at these high-risk positions should be avoided. However, existing epitope-mapping methods all have their own limitations that rely on either empirical antibody and HLA antigen standards, such as TerEps, or arbitrary parameters, such as HLA-Matchmaker, to deduce epitope positions. Here, we developed a direct method for personalized mapping of donor epitopes using peptide arrays, a method adapted from vaccine and antiviral antibody studies.^{18,19}

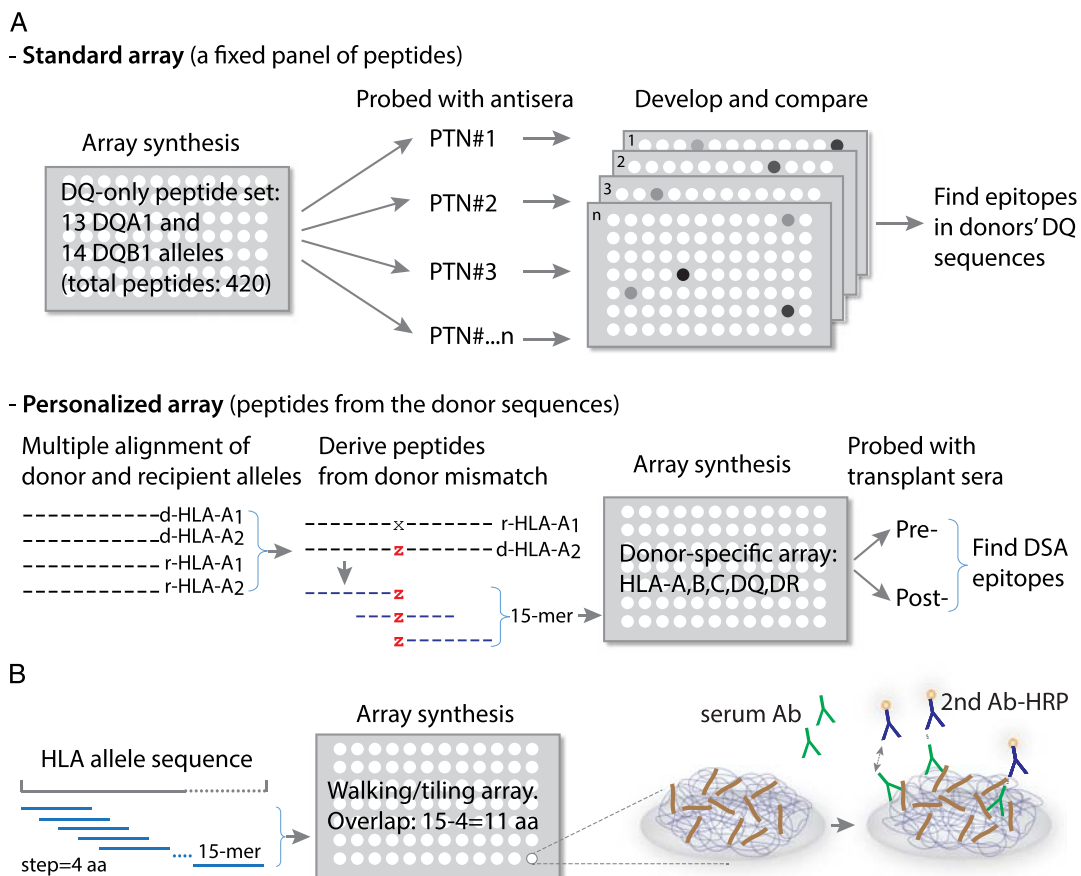


FIGURE 1. The schematic of antibody screening using HLA peptide arrays. **A**, Two different designs of the array. In the standard array, a fixed set of 420 peptides was assembled onto the array. These 15-mer peptides were derived from the extracellular domains of HLA-DQA1 and -DQB1 that comprise the LSA HLA-DQ antigens. The same array was used to probe and reprobe serum samples from different patients (PTN1-n). By contrast, the personalized arrays only comprised donor-specific HLA sequences. First, donor-specific residues (mismatches) were identified through multiple alignment analysis of the corresponding HLA molecular sequences: Two donor (d-) HLA-A alleles and 2 recipients (r-) HLA-A alleles are shown as illustrative examples (“x” and “z” represent recipient and donor-specific residues respectively—these x and z could be any residues). Overlapping 15-mer peptides containing the donor-specific residue “z” were derived. A personal array was made with these donor-specific peptides from HLA-A, -B, -C, -DQA1, -DQB1, -DRB1. This array was used to probe, and then reprobated with posttransplant and pretransplant sera of the same patient to detect de novo antibodies. **B**, In general, the peptides were derived from HLA sequences followed a walking or tiling strategy, so that any continuous epitopes 11 aa in length or shorter were represented by at least 1 peptide in the series. The synthetic peptides (shown as short strings in brown color) were made as a peptide-cellulose (thin threads) conjugate through a covalent spacer/linker (not shown). Antibody (shown in green) binding to the peptide antigens were visualized by using a secondary anti-human IgG HRP reagent.

MATERIALS AND METHODS

Peptide Array Synthesis

The arrays were composed of 15-mer peptides of custom sequence based on select HLA templates. A nonredundant set of serial peptides from donor sequences was consecutively derived and synthesized on the arrays. Peptide synthesis was performed by a robotic instrument known as the Cellu-Spot system (Intavis AG, Köln, Germany).^{20,21} The membrane that holds up to 600 distinct peptides was uniformly derivatized with a simple spacer/linker peptide onto which custom peptide sequences were synthesized in situ after a programmed synthesis cycle.²² This production method has several advantages. First, the local concentration of each individual peptide across the entire array is constant, as determined by the density of the spacer molecule on the membrane. Second, the synthesized peptides are immobilized to the membrane via covalent linkages to the spacer. Therefore, the peptides can withstand harsh treatments to the membrane in a procedure to remove bound antibodies, following which the membrane can be re-probed again without losing performance. Additional methods are in **Materials and Methods, SDC**, <http://links.lww.com/TXD/A29>.

RESULTS

Two Design Concepts for HLA Peptide Arrays

We conducted pilot studies following 2 design concepts for the peptide arrays (Figure 1A). The first concept followed a one-format-fits-all design intended to have an invariable “standard array” for all transplant patients, similar to the LSA platform using a predetermined set of allelic antigens. The second design was completely individualized, with each array tailored to cover discrete donor-recipient mismatches. Arrays were separately probed with pretransplant and post-transplant sera. In order for the peptides to adequately represent an HLA template, we adopted an array layout commonly known as the “walking” pattern, in which a series of overlapping sequences is synthesized.²⁰ With each peptide 15 aa in length and a “walking” step size of 4 aa from the N- to the C-terminus of an HLA sequence, there was an overlap of $15 - 4 = 11$ aa between 2 neighboring peptides (Figure 1B). We reasoned that these 11 aa would be sufficient to cover the typical length of a linear epitope of 4 to 9 residues, so the possibility to have only partial sequences in the series was avoided.

Serum Samples Analyzed by the HLA-DQ Peptide Array

HLA-DQ is recognized as a key target of de novo donor-specific antibody (DSA) and is also associated with unfavorable tx outcomes.²³ However, eplet analysis using HLAMatchmaker noted an up to 10-fold difference in LSA antibody intensity values for alleles that were supposed to share eplets.²⁴ To address this discrepancy, we sought an HLA-DQ peptide array to probe for antiserum reactivity against linear epitopes. To be able to directly relate peptide results to the protein antigen results from LSA assays, the custom peptides were derived from the same HLA-DQ set used in LSA, which has a total of 27 alleles including 13 DQA1 and 14 DQB1 alleles (**Table S1, SDC**, <http://links.lww.com/TXD/A29>). These 27 HLA-DQ sequences were first sequentially parsed into 15-mer peptides following the aforementioned walking scheme. The high degree of shared sequence homology resulted in most peptides individually

derived from multiple DQ alleles. When this occurred, we kept only 1 copy of identical sequences for the synthesis of a non-redundant set of 420 DQ peptides (**Table S2, SDC**, <http://links.lww.com/TXD/A29>) in an array format (hereinafter individual peptides are referred to by their x- and y-axis coordinates on the array). It should be noted that after the removal of the redundant copies, neighboring peptides no longer maintain their serial order. We retrieved the repository sera of three unrelated kidney transplant patients, whose DQ-reactive LSAs and respective organ donors' HLA types were known (**Table 1 and Table S3, SDC**, <http://links.lww.com/TXD/A29>). These 3 antisera were separately probed by the same standard array. Each individual serum produced drastically differing antigen patterns from the others (**Figure 2 and Figure S1, SDC**, <http://links.lww.com/TXD/A29>), demonstrating that the method can capture distinct antibody specificity. To comprehensively evaluate the new assay, we next sought to determine whether the linear peptide results correlate with donor-recipient mismatches and HLA allelic reactivity as determined by LSA, as well as whether linear antigen responses detected by the arrays correlate with graft conditions.

Correlation Between Peptide Array Results and Donor-Recipient Mismatches

We analyzed peptide sequences associated with the strongest antibody signals (pointed by arrows in Figure 2). A total of 10 sequences were examined with an emphasis on residues not shared by the respective recipients (residues highlighted in red in Figure 2). Patient 1 had his strongest antibody signals at spots b14 and g10 from the donor's DQA1 alleles of *01:02 and *05:01, respectively (highlighted in red in Figure 2; marked in **Table S2, SDC**, <http://links.lww.com/TXD/A29>, and **Table 1**). Neither peptide shares sequences with the recipient's DQA1*02:01 and 03:01 alleles, and as expected, none of the recipient's peptides on the entire array reacted to antibodies. These results were consistent with the notion that certain donor-specific residues in the context of their surrounding amino acid sequences can elicit alloantibody responses.

The array was subsequently used to probe patient 2 who had a previous transplant that ended in graft loss, but her new graft was stable at the time of data collection (**Table 1**). Among the 3 spots (c15, j5, and k12) associated with the strongest antibody signals, none of them are from the current donor, which is consistent with her stable graft condition (**Figure 2**).

In the subsequent reprobing of the third transplant antiserum, a greater number of peptides showed reactivity, including 2 spots both shared with each of the previous blots (**Figure 2**, in dotted circles; and **Figure S1, SDC**, <http://links.lww.com/TXD/A29>). Among the 5 strongest spots (a23, a27, e24, k10, and k12), 4 are from the donor's sequence (in red). It is encouraging to note that unlike patient 2 who had a stable kidney graft and no antibodies against the donor's peptides, both patients 1 and 3, who expressed antibodies against donor-derived peptides, also shared their clinical diagnosis of ongoing antibody-mediated rejection (AMR) (**Table 1**). Lastly, we used the membrane to reprobe a nontransplant serum standard (pooled sera) as the negative control. As expected, we observed only background levels of low antibody intensity.

TABLE 1.**Medical history, and Luminex and HLA typing results of kidney transplant subjects 1-5**

ID	Sex	Primary disease	Pre-TX Luminex	Post-TX Luminex Antigens (+)	Donor HLA	Recipient HLA	HLAMatchmaker	Graft condition
PTN1	M	HTN, DM	N.A.	<u>DQA1*05:01/DQB1*02:01</u> DQA1*04:01/DQB1*04:02	<u>DQA1*01:02, 05:01/DQB1*03:01,</u> 05:01; <u>A2,11; B55:62; C1,9;</u> DR11,15; DR51,52)	DQA1*03:01/DQB1*04:02; DQA1*02:01/DQB1*02:02 (A2,33; B50,60; C6,8; DR4,7; DR53)	DQA41GR DQB55PP	AMR
PTN2	F	GF & reTX	N.A.	DQA1*05:01/DQB1*02:01 DQA1*01:01/DQB1*05:01	DQA1*01:02/DQB1*05:02 (A24,33; B14,50; DR16,7; DR51,53)	DQA1*05:01/DQB1*03:01; DQA1*01:03/DQB1*06:01 (A2,11; B35,51; C4; DR11,15; DR51)	DQB77DR	Stable
PTN3	F	SLE	N.A.	D07DQA1*05:03:05:05/ <u>DQB1*03:01</u> D08(<u>DQA1*03:01/DQB1*03:02</u>)	<u>DQA1*03:01,</u> 05:01/ <u>DQB1*03:01,</u> 03:02; (A3,31; B51; C15; DR4,11)	DQA1*05:01/DQB1*02:01 (A2,68; B18,58; C2,5; DR11)	DQB52L	AMR
PTN4	M	IDDM	No DSA	No DSA (Note: 6 mo later strong-moderate: <u>DQA1*05:01/DQB1*02:01</u>)	<u>DQA1*05:01/DQB1*02:01,</u> 03:01; (A3,23; B52,15;10; C*03:04, 16; DR*03:01,13:03)	DQA1*01:01, 04:01/DQB1*04:02,05:01; DR*01:01,08:03, A3,24; B7,39; C7,8;	N.A.	AMR (C4d+)
PTN5	M	DM	No DSA	Moderate: <u>B*52:01</u> (a non-donor allele nonetheless sharing epitope with <u>B*51:01</u>)	DQA1*4a,8a/ <u>DQB1*4b,</u> 8b; (<u>A2,31; B35,51;</u> C4,15; DR4,8)	DQA*03:01,05:01/DQB1*03:01,03:02; DR*04:07,11:04; A1a,31; B18,35; C4,12;	N.A.	Stable

Highlighted (underlined) HLA alleles, including Luminex positive alleles and donor alleles, contain peptides also showing intense signals on the arrays; the peptide results in patients 1, 3, 4, 5 were consistent with Luminex detections of the corresponding alleles. A complete report of HLA laboratory results is in supplementary Table S3.

HTN, hypertension; DM, diabetic mellitus; GF, graft failure (in a previous kidney transplant); reTX, retransplantation; SLE, systemic lupus erythematosus; IDDM, insulin-dependent diabetes mellitus; N.A., not available.

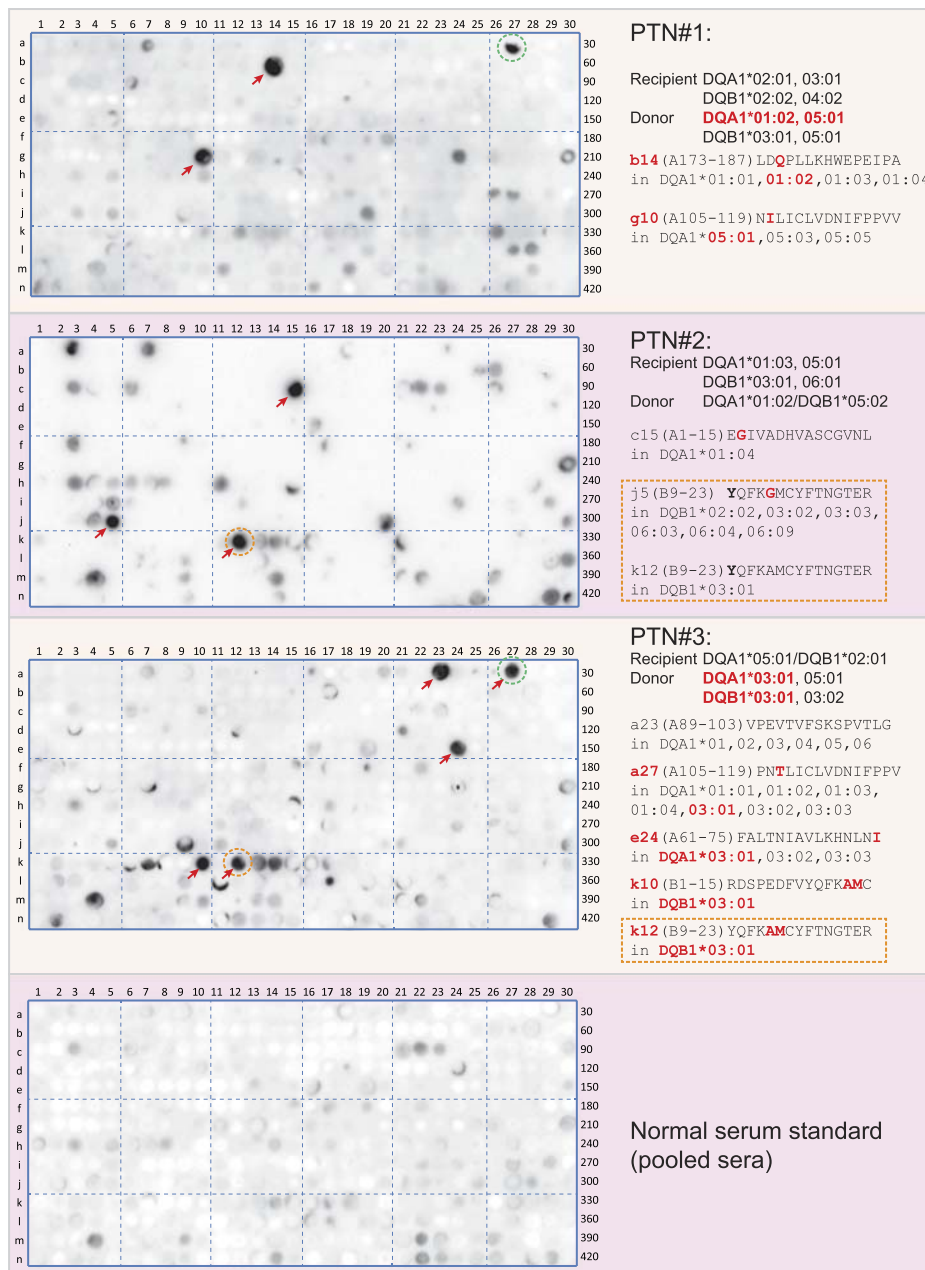


FIGURE 2. Antibody screening of 3 transplant patients using the same standard HLA-DQ array. A standard array was assembled with a nonredundant set of 420 peptides derived from 27 HLA-DQ sequences (peptide sequences in Table S2, SDC, <http://links.lww.com/TXD/A29>). These peptides were synthesized in a 30 × 14 (x axis: 1 to 30; y axis: a-n) format on the membrane. The array was used to probe sera from 3 transplant patients (PTNs) 1–3 and a normal serum standard. Antibody-reactive peptides were identified by their x-y coordinates on the array (Red arrows: spots selected for annotation and analysis). On the right, the recipients' and his or her donors' DQA1 and DQB1 types are separately listed, followed by information about the antibody positive peptides (such as b14 and g10 in the first blot) with regards to aa start to end positions (A for DQA1; B for DQB1) and sequences, and the names of DQ alleles that share these peptides. Nonrecipient residues (mismatches) are highlighted with red letters (single-letter amino acid codes). If a positive peptide matches the donor's but not the recipient's sequence, the name of this donor allele is highlighted in red as well: b14, g10 are associated with donor 1's DQA1*01:02 and 05:01 respectively; a27 and e24 are associated with donor 3's DQA1*03:01 and k10 and k12 are with DQB1*03:01. Two peptides each reacted to more than 1 serum: k12 to patients 2 and 3 (marked by orange circles and corresponding boxes); a27 to patients 1 and 3 (green circles). A pseudo-color overlay of the three blot images for patients 1 to 3 is presented in Figure S1, SDC (<http://links.lww.com/TXD/A29>). No antibody signal was observed from the array probed with normal serum standard (bottom).

High-Resolution Peptide Array Results Correlate With Low-Resolution LSA Specificity

Next, we sought to determine whether antibody specificity for linear peptides/epitopes correlates with single-antigen reactivity measured by LSA, despite that most alloantibodies

are expected to target conformational as opposed to linear epitopes. Patient 1's top positive LSA (with the highest mean fluorescent intensity value) was DQ2(DQA1*05:01/DQB1*02:01) (Table 1), of which a distinct DQA1*05 peptide at g10, as discussed before, had a strong antibody signal

(Figure 2 PTN1 and Table 1). Interestingly, although the patient 2 serum also reacted to DQ2(DQA1*05:01/DQB1*02:01), patients 1 and 2 do not share reactive peptides, suggesting different epitopes might have separately accounted for reactivity against the same DQ2 LSA. HLAMatchmaker identified 2 eplets for patient 1 (DQA41GR and DQB55PP) and 1 different eplet for patient 2 (DQB77DR). There was a total of 16 peptides on the array that contained these software-identified eplets; however, none of them showed a positive signal from the patients' sera (Table S2, SDC, <http://links.lww.com/TXD/A29>: eplet-containing peptides shaded in grey; and Figure S2A, SDC, <http://links.lww.com/TXD/A29>).

Patient 3 had her strongest antibody titer (>1:1024) for DQ7 (DQA1*05:03,05:05/DQB1*03:01) in LSA, followed by DQ8 (DQA1*03:01/DQB1*03:02) (titer = 1:512). We noted that several positive spots on the array formed a cluster including k10, k12–14 that were also derived from DQB1*03:01 (k10, k12, k13), which is a donor allele (Table 1). By contrast, HLAMatchmaker predicted a DQB52PL eplet. However, neither of the 10 peptides on the array that contain this P⁵²LG motif, such as in spots k20–k22, l18–l20, l27, and m5–m7, were reactive to the patient 3 antiserum (Figure 2, PTN3; and in Figure S2B and Table S2, SDC, <http://links.lww.com/TXD/A29>—red letters). Collectively, 5 out of the top 6 reactive peptides in patients 1 and 3, with the only exception of b14, who share AMR diagnosis were derived from the alleles associated with the highest Luminex signals (highlighted in Table 1 and in Figure 2), indicating that it is possible that the epitope reactivity identified by the array directly contributed to LSA readings. However, all three patients, the epitopes mapped by the peptide array did not match the eplets predicted by HLAMatchmaker.

Donor-specific Single Allele Study

Having shown the consensus between peptide and LSA results, we investigated the sensitivity of the peptide assay using

allelic templates that showed no LSA reactivity. We produced 2 new arrays that each covered only a single allele from the donor of either subject 1 or subject 3. For patient 1, we selected HLA-A11 as the donor template for the peptide array (Table S4, SDC, <http://links.lww.com/TXD/A29>). Probing the patient 1 antiserum revealed only a single spot (at position o23 in Figure 3, upper panel) that contained 2 nonrecipient residues (“D” and “I” in red letters). Similarly, for patient 3 we selected HLA-DR4 to assemble the peptide array (Table S3, SDC, <http://links.lww.com/TXD/A29>). The strongest reactive peptide t26 contains nonrecipient residue Thr²³⁵(T235) (Figure 3, bottom panel). Although the clinical significance of array-detected antibodies remains to be determined, we performed an exploratory study on difficult cases, having a biopsy diagnosis of AMR but no LSA antibodies.

Longitudinal Studies of Pretransplant and Posttransplant Sera Using Full-Panel Personalized Arrays

Next, we sought to use the arrays to understand immunogenicity in 2 transplant cases exhibiting opposing kidney-allograft conditions, with patient 4 displaying clinical signs of rejection also supported by immunohistologic (C4d) evidence of AMR²⁵ and with patient 5 whose graft condition was stable (Table 1). Both patients were tested negative for DSA by LSA. We investigated these 2 patients each with their personalized arrays spotted with peptides that covered their respective donors' HLA-A, -B, -C, -DQA1, -DQB1 and -DRB1 alleles. To do that, we performed a sequence alignment of donor vs. recipient HLA sequences to locate mismatches (highlighted in Figure S3, SDC, <http://links.lww.com/TXD/A29>), and generated templates for the synthesis of donor-derived peptides that encompassed all mismatched residues. Accordingly, 204 and 117 peptides were derived for patients 4 and 5 respectively to assemble 2 arrays (Figure 4 and 5, and Table S5 and S6, SDC, <http://links.lww.com/TXD/A29>). These personal arrays were used to

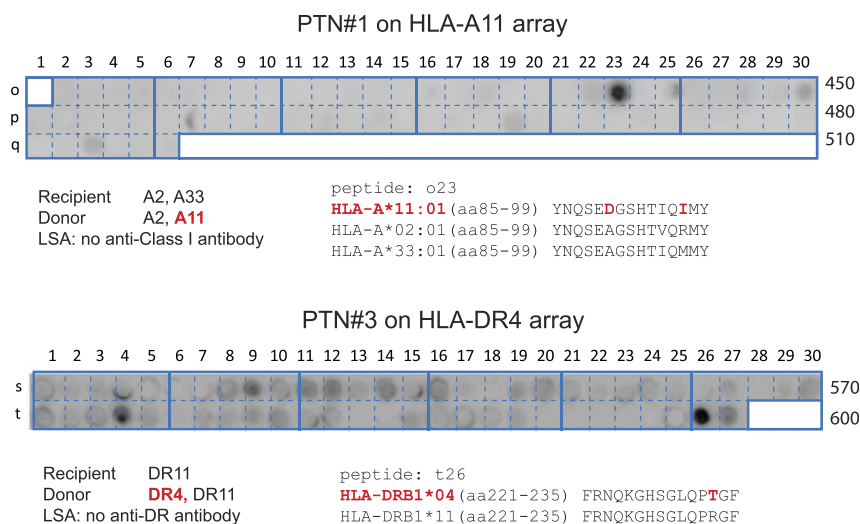
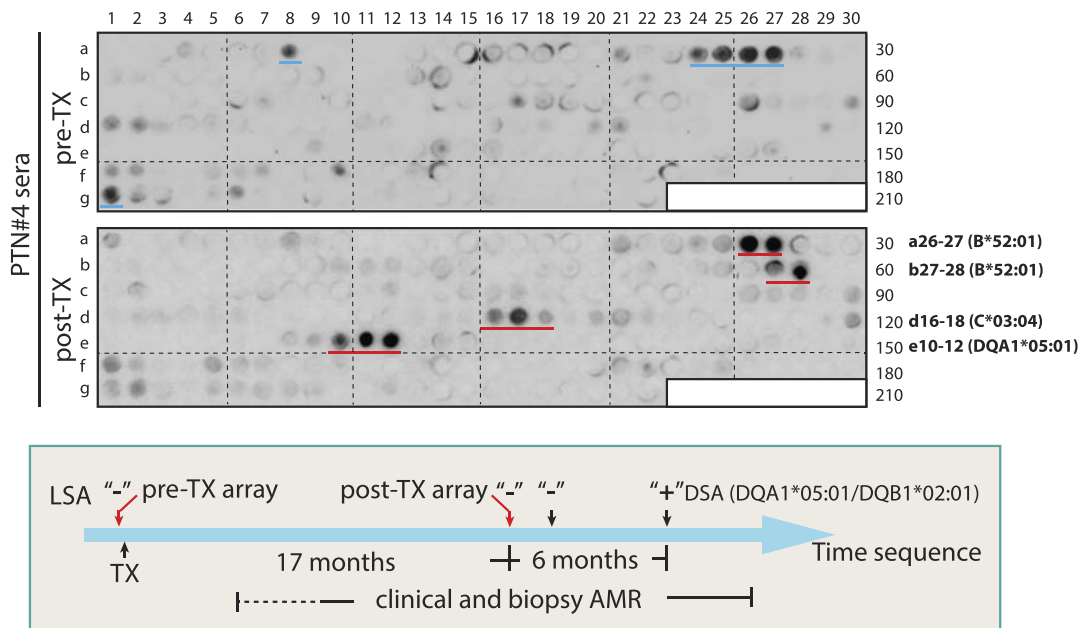


FIGURE 3. Donor-specific single allele study using peptide array. Two donor-specific alleles were selected as templates for peptide antigens: HLA-A11(A*11:01) for patient 1 and HLA-DR4(DRB1*04:01) for patient 3. In each array, serial peptides were derived to cover the template allele (peptide sequences in Table S1, SDC, <http://links.lww.com/TXD/A29>). On the A11 array, patient 1 serum reacted only to peptide o23, which was derived from aa 85 to 99 of A*11:01 of the donor. This peptide, as compared to the corresponding sequences of recipient's A2 and A33 (represented by A*02:01 and A*33:01, respectively), contained 2 mismatched residues of Asp⁹⁰(D90) and Ile⁹⁷(I97) (mismatch in red letter “D” and “I” in the alignment). Similarly, the DR4 array was made to probe serum 3 with the strongest signal at t26. The t26 peptide contained 1 mismatched residue Thr²³⁴(T234) (red letter “T”), as compared with the self DR11 sequence.



pos (Donor allele)	Peptide sequence	Reactivity
a25 (B*52:01)	PRTEPRAPWIEQEGP	-
a26 (B*52:01) (E87)	APWIEQEGPEYWDRE	++++
a27 (B*52:01)	EQEGPEYWDRETQIS	++++
a28 (B*52:01)	PEYWDRETQISKTNT	-
b27 (B*52:01)	LRWEPSSQSTIPIVG	+
b28 (B*52:01) (I306)	PSSQSTIPIVGIVAG	++++
b29 (B*52:01)	STIPIVGIVAGLAVL	-
d16 (C*03:04)	ALGFYPAEITLTWQW	+
d17 (C*03:04) (W243)	YPAEITLTWQWDGED	+++
d18 (C*03:04)	ITLTWQWDGEDQTQD	+
e9 (DQA1*05:01)	ESYDCKVEHWGLDKP	-
e10 (DQA1*05:01)	CKVEHWGLDKPLLKH	++
e11 (DQA1*05:01) (K197)	HWGLDKPLLKHWEPE	++++
e12 (DQA1*05:01)	DKPLLKHWEPEIPAP	++++
e13 (DQB1*02:01)	MLSMLSTPVAEGRDS	-

FIGURE 4. Donor-specific HLA-A, -B, -C, -DQ, -DR array study of mismatched epitopes in patient 4. Serial peptides were derived from the donor's sequences to cover mismatched residues (peptide layout in [Table S5, SDC](#), <http://links.lww.com/TXD/A29>). The array was used to probe the posttransplant serum (lower blot: post-TX) and subsequently the pretransplant serum (upper blot: pre-TX) from the same patient. The 4 sets of strong spots from posttransplant probing are marked by red lines (in lower blot), whereas 2 medium intensity spots that were only associated with pretransplant serum are marked by blue lines (in upper blot). Clinical time sequence for the subject is shown: Two red arrows indicate the time points when pre and posttransplant sera on the array were taken (17 months apart). Additional blood draws are indicated by the black arrows. All serum samples were analyzed by LSA: all were negative for DSA with the exception of the last sample taken 6 months after the array analysis. This last sample was positive for DQ2(DQA1*05:01/DQB1*02:01) rated as strong-moderate. This patient exhibited clinical signs of graft rejection with multiple biopsies showing pathological features of AMR including C4d positivity. The bottom table shows peptide sequences and their reactivity to the posttransplant serum. Donor-specific (mismatched) residues of E87, I306, W243, and K197 are in red letters and peptides showing strong antibody reactivity are in bold fonts.

probe the pretransplant and posttransplant sera of the intended patients.

On array patient 4, positive signals were detected in both probing rounds, with several clusters of spots, such as b27–28, d16–18, and e10–12, being exclusively associated with the posttransplant serum. The analysis of each series pinpoints the exact mismatching residue as in Figure 4. The observed pattern of cross-reactivity between these partially overlapping peptides indicated shared epitopes within overlapping sequences. Among the preexisting intensities, while a8, a24, 25, and g1 signals were reduced in the posttransplant probing round (Figure 4 and [Figure S3, SDC](#), <http://links.lww.com/TXD/A29>) possibly due to the effects of immunosuppressants, b26, 27 signals were greatly elevated in the posttransplant serum. Collectively, it could be speculated that a total of 4 mismatch-associated linear epitopes (Figure 4, bottom table). It is remarkable to note that in a new blood draw taken 6 months later, LSA detected emerging DSA to DQ2 (DQA1*05:01/DQB1*02:01) (also noted in Table 1) that matches the peptide results in e10–12 for DQA1*05:01.

In parallel, we studied patient 5 whose graft function was stable with a moderate LSA reading for HLA-B*52:01, a nondonor allele (Table 1). We detected peptides associated with distinct posttransplant signals (red underlines with details

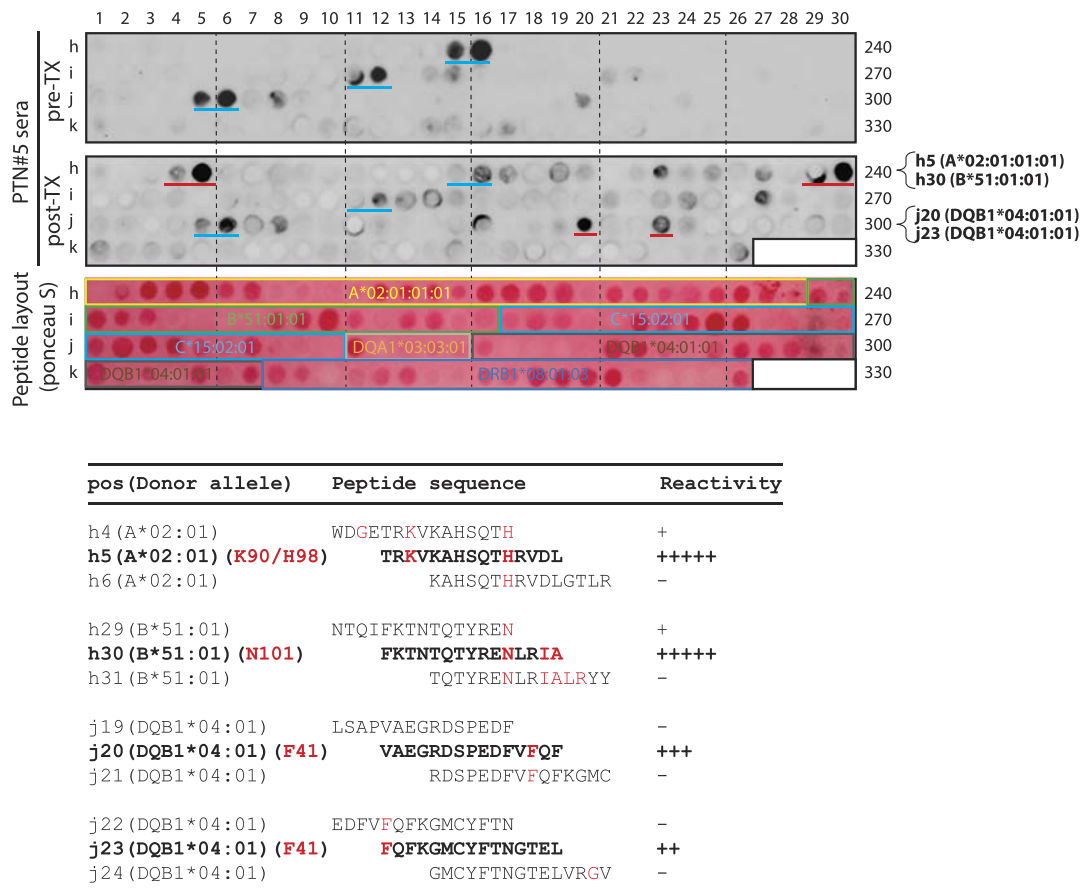


FIGURE 5. Donor-specific HLA-A, -B, -C, -DQ, -DR array study of mismatched epitopes in patient 5. Similar to Figure 4, this donor-specific array was first probed with posttransplant serum and then with pretransplant serum of patient 5. Ponceau S staining illustrated the general layout of the array as an example (peptide spots are stained in varying shades of red). Color intensity was influenced mostly by the amino acid composition of each peptide as opposed to peptide concentration. The color boxes indicate the positions of peptides corresponding to the HLA alleles.

in Figure 5: table below, and highlighted in Figure S4 and Table S6, SDC, <http://links.lww.com/TXD/A29>). Notably, de novo reactivity can be assigned to the donor's HLA-A*02:01, HLA-B*51:01 and DQB1*04:01. Interestingly, the h30 peptide as assigned to B*51:01 (Figure 5) shares an identical sequence with that of the LSA-positive nondonor allele of B*52:01 (as noted in Table 1). However, with this particular patient, the array detected antibodies were nonimmunogenic because the graft was stable. Overall, in the 5 patients, with the exception of patient 2, the LSA-positive alleles also contained peptides that were positively identified on the arrays (highlighted in Table 1), indicating that alloantibodies for linear epitopes to some degree contribute to LSA readings.

Linear Epitopes Mapped to the 3-D Structure of HLA-DQ

Because the array method only detects linear peptide sequences in the absence of folded structures, we wished to determine the spatial locations of these peptides in HLA crystal structures. Because we identified antibody reactive DQ peptides in each of the 5 patients, we chose to model a composite view of all these peptides together in a DQ8 (DQA1*03/DQB1*03:02) co-crystal structure (PDB ID: 1JK8).²⁶ To do that, we created a compilation of a total of 17 positive peptides/epitopes from all 5 patients and mapped

these peptides to their corresponding positions in DQ8 (Figure 6A). There were several interesting observations from this composite 3-D view of the epitopes. First, the majority of the 17 peptides each mapped to a single β -strand, except for e24 from patient 3, which located to the α B helix of DQA1. Three β -strands, β 1, β 6, and β 12, were composed of 15 of the 17 reactive peptides, and each of these 3 β -strands were antibody-reactive in at least 2 patients (Figure 6B). Strikingly, β 1 was the only segment of DQB1 subunit (in DQB1*02,*03,*04 alleles: highlighted in Figure 6B) associated with antibody signals (in patients 2, 3, and 5). The observed antibody reactivity to these β -strands across individual patients, allelic variants, and different HLA genes suggests that highly antigenic structures, or epitope “hotspots,” in HLA-DQ possibly exist, as exemplified in particular by β 1 being the only antibody-reactive segment of DQB1.

DISCUSSION

The advent of high-resolution typing and direct sequencing allows us to genetically define HLA mismatches without ambiguity. The field is now moving quickly towards practical solutions for aa sequence-level and epitope-level determination of antigenicity. In this pilot study of 5 kidney transplant subjects, we focused on the development of a novel methodology for high-resolution linear epitope mapping, intended as

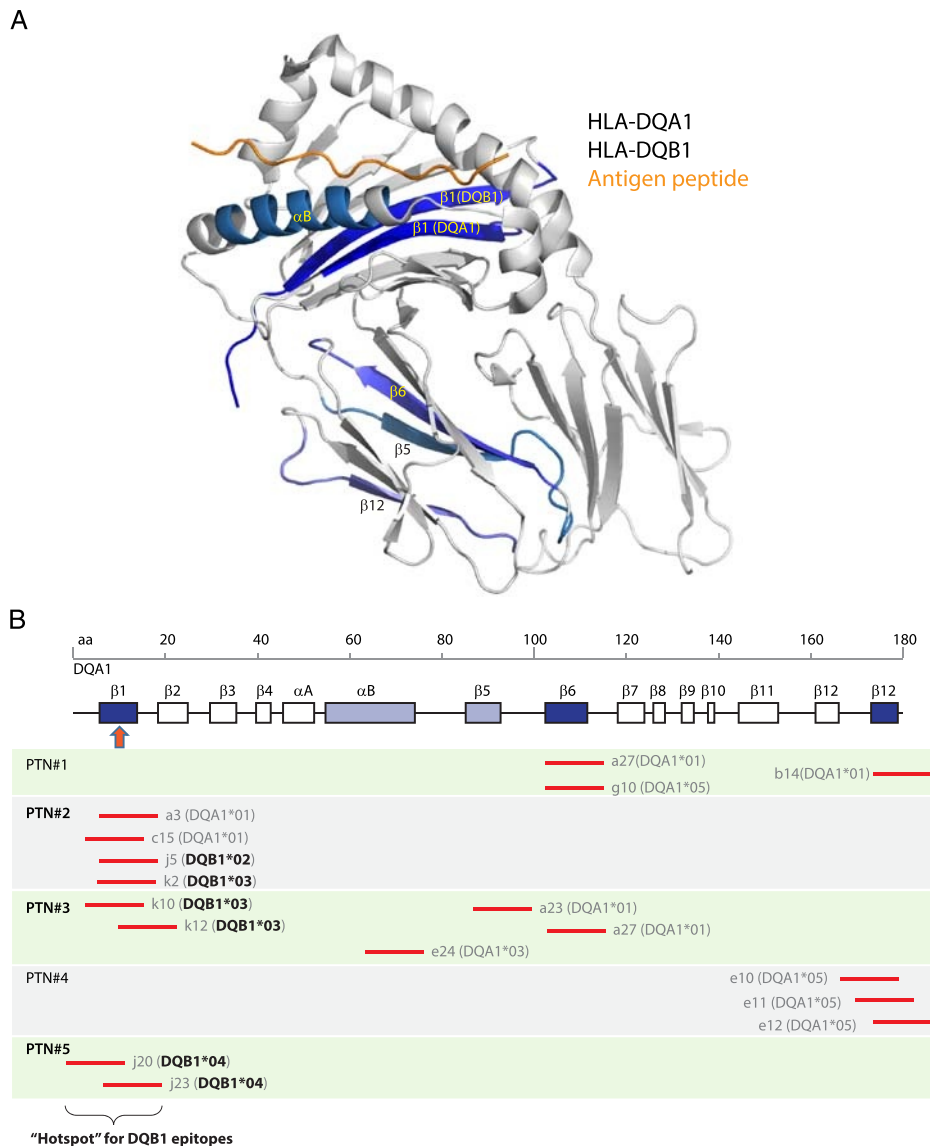


FIGURE 6. Structural locations of HLA-DQ epitopes. Cocystal structure of HLA-DQ8 was used as a template (see details in Methods, SDC, <http://links.lww.com/TXD/A29>). The structure was composed of a DQA1 and a DQB1 subunits together with an antigen peptide (A). Protein secondary structures of the α -helices and β -strands are shown in panel B. DQA1 and DQB1 peptides that reacted with either 1 of the 5 serum were located on the cocystal structure (in A and in B: shaded in blue). Three β -strands, β 1, β 6 and β 12 (dark blue in B), each represented by multiple peptides (short red lines corresponding to the linear aa positions of DQA1), reacted with multiple patients' samples (based on results in Figures 2 to 5). All 6 DQB1 peptides (in bold font) reactive to antibodies in patients 2, 3, 5 are located to the β 1 "hotspot" segment (in B: pointed red arrow).

a future tool for rejection risk stratification based on the locations of the mismatches on HLA molecules. Hundreds of peptides derived exclusively from HLA sequences were custom synthesized onto membrane-based arrays, which were subsequently used to probe pretransplant and posttransplant sera. With both standard and personalized arrays, we demonstrated the excellent performance of the method in detecting peptide-specific antibody reactivity and also revealed antigenic hotspots in HLA-DQ. As compared with the single antigen assay, the array method has 2 main advantages: First, the arrays detect antibody specificities at the amino acid level. Second, the arrays can accommodate personalized design of antigen sets based on the sequences of the donors, whereas LSA probes often do not encompass all high-resolution typing or sequence-based typing sequences of the donor.²⁷

Peptide array is a powerful tool for high throughput screening of antibody epitopes that has been traditionally used in determining viral epitopes to aid vaccine design.^{18,19} The synthesis method using the covalent conjugation of peptides to membrane matrices renders outstanding reproducibility (re-robing study in Figure S5) with epitope-level resolution. It is also encouraging to note that the peptide results were mostly in agreement with the antigen results by LSA (Table 1). The present study was focused on addressing the specificity of the new method in detecting HLA antibodies with individual specificity (as in patients 1-3), and on testing personalized designs (as in patients 4-5) based on DNA typing. Future studies with extended cohorts and more longitudinal time points will provide further insights about the performance of the array and the

pattern of epitope locations. It is also expected that peptide arrays are highly sensitive, even when compared with LSA. This is because the local molar concentration of short peptides would surpass those of LSA recombinant antigens on beads by multiple orders of magnitude. This property of the array could have allowed for detecting DSA sooner than LSA, as in patient 4 (Figure 4). However, the method that sensitively detected antibody signals in each of the 5 patients, including the 2 with stable graft conditions, is also alarming. Future studies will have to focus on determining the clinical relevance of the array results. As a new method, it is important for us to address the caveats and limitations.

First, we have experienced 1 example of an LSA-positive allele (rated as moderate) not having any antibody-reactive peptide (Figure S6, SDC, <http://links.lww.com/TXD/A29>). Additionally, there are 3 LSA positive alleles of patients 1 (QA1*04:01/DQB1*04:02) and 2 (both DQ alleles) with no representative array peptides showing positive signals (Table 1). Conversely, there is also a disparity in LSA signals among alleles that share the same positive peptide on the array. Above all confounding mechanisms, the most important contributing factor is that most antibodies recognize conformational epitopes on LSA, and perhaps only a few target linear sequences, as part of which may not be accessible to antibodies.²⁸ Conformational epitopes are defined in the context of naturally folded HLA molecules. These epitopes, unlike those used in our method in the form of peptides, are compositional entities formed by discontinuous aa sequences within structural proximity of each other.²⁹ Experimental results have identified antibodies that recognize epitopes only in folded, but not in linearized protein antigens, and vice versa.³⁰ However, in other context, antibody actions against linear epitopes are being exploited in viral antigen responses and in vaccine design.^{31,32} We note that most HLA-DQ peptides with strong antibody signals each span a single β -strand in the HLA-DQ crystal structure (Figure 6). Therefore, even in the context of a tertiary DQ fold, these epitopes naturally adapt to a linear configuration. It is therefore conceivable that antibody recognition may still occur in a linear fashion.

Another notable discrepancy in the study is that, in the DQ study of patients 1-3, the peptide epitopes did not match the structural eplets predicted by HLA-Matchmaker. This could possibly be due to the distinction between linear versus conformational epitopes, which may even extend beyond 1 protein given that HLAs are intertwined hetero-dimers and HLA-Matchmaker considers structural proximity. One other caveat in the new method is that there were a few examples of supposed recipient sequences clearly showing intense antibody signals, such as the k12 peptide to patient 2, and a23 and t4 peptides to patient 3. Antibodies reacting to self-alleles are increasingly recognized, mostly observed in LSA tests.^{33,34} However, the mechanism for the development of autoantibodies following tx and the clinical relevance of these antibodies remains to be addressed.³⁵⁻³⁸

Also, for proof-of-concept in this initial study, we only focused on spots associated with the highest antibody signals. We have not attempted to determine whether applying cutoff values for positive peptides will better reflect the state of immunity. As a new test that operates independent of LSA and cell-based absorption-elution assays, 1 of the research applications of the array method is to be performed in conjunction with

other epitope mapping tools such as TerEps and HLA-Matchmaker. Future studies are needed to address the clinical relevance of antibodies that target linear epitopes. The possibility that linear sequences only constitute parts of conformational epitopes, which still require additional structural determinants to promote pathogenesis, must also be explored.

In summary, we have developed a new personalized approach to detect HLA epitopes using a customizable peptide array. We showed the feasibility and the robustness of the workflow to detect transplant alloantibodies. Future studies using a larger sample size would help validate the method, aimed to delineate alloantibody responses to each donor-recipient mismatch, and in a broader context to identify antigenic “hotspots” that can ultimately assist clinical decisions through acceptable mismatch programs.³⁹

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