Detection of donor-specific antibodies in kidney transplantation

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Abstract

Introduction: Precise and timely detection of human leukocyte antigen (HLA) donor-specific antibodies (DSAs) is vital for evaluating humoral immune status of patients pre- and post-transplantation.

Source of data: Clinically relevant articles on theory, development, methodology and application of HLA-DSA testing in kidney transplantation.

Areas of agreement and controversy: The availability of solid phase HLA-antibody testing revolutionized our ability to detect HLA-DSA and to appreciate their significance in kidney transplant outcome. The best approach to determine the strength, immunogenicity and pathogenicity of HLA antibodies still remains controversial.

Growing points: Assays to identify complement-binding antibodies were developed. Their clinical utilization, pre- and post-transplantation, is currently under investigation. Appreciation of the complexity of HLA-DQ antibodies should lead to better assignment of unacceptable antibodies and cPRA calculation.

Areas timely for developing research: Characterization of HLA-antibody epitopes, and utilization of epitope matching to better define compatible donors could contribute to better transplant outcomes.

Key words: C1q, complement-binding antibodies, donor-specific antibodies, HLA-antibody epitope, HLA-DQ antibodies, prozone, solid-phase assays

Introduction

The presence of pre-transplant antibodies against antigens encoded by the HLA (human leukocyte antigen) complex, and specifically those antigens that are expressed by the organ donor [donor specific antibodies (DSA)] in kidney recipients, is strongly associated with hyperacute and accelerated acute rejection.[1–3] Likewise, the production of *de novo* or anamnestic DSA in kidney recipients post-transplant is often indicative of the onset of antibody-mediated rejection (AMR).[4] Ample clinical data support the importance of HLA-DSA monitoring for risk assessment in recipients pre- and post-transplant.[5,6] The current review addresses new immunologic tools and technologies that allow for more sensitive and accurate DSA reporting.

Over the past 15 years, DSA testing has advanced beyond the so-called 'gold standard' complement-dependent-cytotoxicity (CDC) antibody assay, introducing techniques that no longer required the use of live cells, but rather utilizing solubilized HLA antigens attached to solid-phase matrices [solid-phase assays (SPAs)]. Combined with new immunologic research and latest clinical data, the increased sensitivity and specificity of these assays allows for more nuanced interpretation of DSA impact in kidney recipients pre-transplant.[7] Accurate assignment of a patient's antibody signature allows for a standardized approach to determine the frequency of incompatible donors in a particular population by establishing cPRA (calculated panel reactive antibody levels).[8] Moreover, using this information in non-invasive virtual cross-matching (VXM) one could provide reasonable prediction of the likelihood of a positive cross-match and of AMR risk stratification.[8–10] Furthermore, pre-transplant DSA information can be utilized to formulate desensitization plans based on the strength and type of DSA present in recipients.[11,12] Systematic DSA monitoring post-transplant can allow for timely detection of such antibodies, providing additional tools to individualize approaches to rejection treatment, as well as gauging their effectiveness.[5,13,14]

This review will cover three general topics: (i) SPAs for the detection of HLA-DSA; (ii) hurdles and solutions in DSA assay analysis and interpretation and (iii) state-of-the-art developments in antibody analysis and their utilization in clinical practice.

SPAs for detection of HLA-DSA

The gold standard of DSA detection was based on incubating recipient serum with donor lymphocytes to observe complement-mediated donor cell lysis. While these assays are cost-effective and initial data showed significant correlation with transplant outcome,[2,15] the limited sensitivity and specificity of this approach became apparent with advancements in (molecular) HLA typing. For example, it was realized that a single serologic HLA specificity might in fact represent a family of many HLA alleles previously not recognized. It was also clear that manipulation of the assay could increase its sensitivity, thus revealing antibodies that were not detected before. Moreover, clinical appreciation of chronic rejection, vascular rejection and the acceptable anticipated transplant half-life increased over the last decade.[16] Lastly, many rejections previously categorized as cellular rejection were found to have an antibody-mediated component.[17]

SPA can utilize different platforms ranging from ELISA assays through the more sensitive flow-cytometry micro-spheres and the most popular assay multiplex-bead array that uses luminex technology. In all of these approaches, isolated HLA antigens serve as target, eliminating the non-specific responses observed previously due to binding of autoantibodies or antibodies to targets that are not relevant for transplant outcome. There are three levels of resolution using SPA, as illustrated in Figure 1. [1] The screening assay, in which solubilized HLA antigens isolated from multiple cells are bound to the solid-phase matrix, as run by ELISA or luminex, provides a qualitative result (yes/no for the presence of HLA class I or II antibodies). A screening assay run by flow cytometry can provide actual %PRA (Fig. 1A). [2] The phenotype beads, or single donor beads, provide 'cell-like' reagents by virtue of having six HLA class I or class II antigens (two alleles of each locus HLA-A, -B and -C; or HLA-DR, -DQ and -DP)
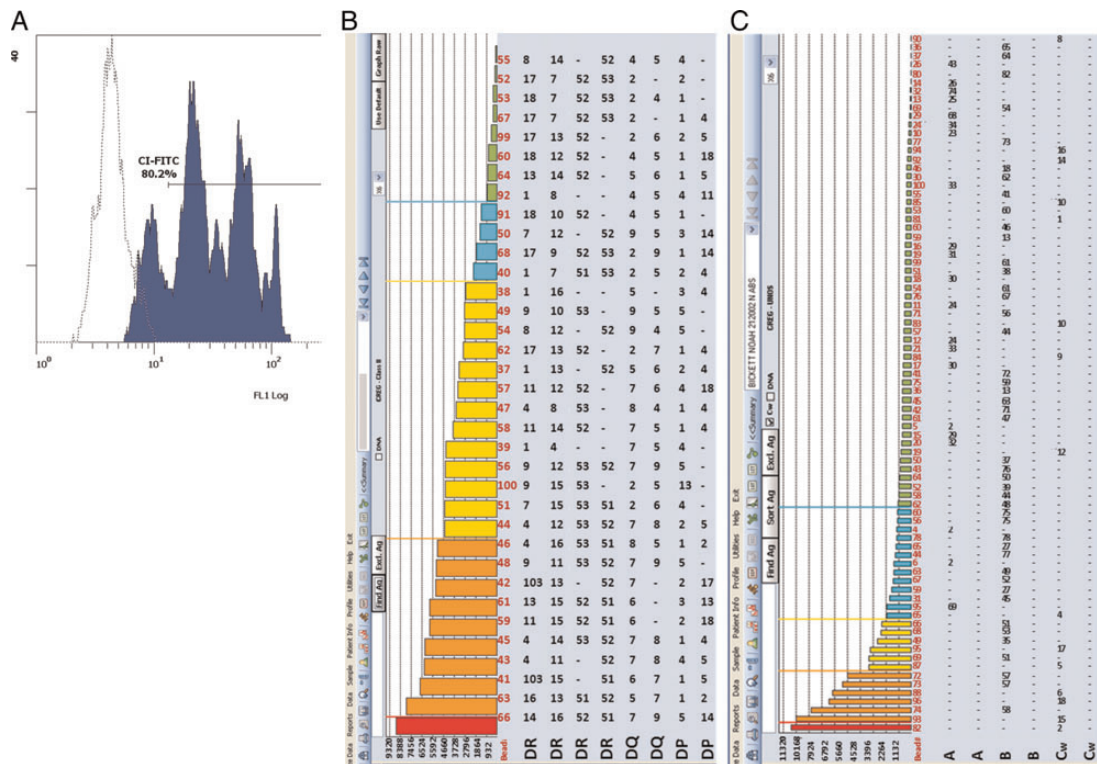
Fig. 1 Solid matrix-bound-solubilized HLA antigens can be used to interrogate the presence of HLA antibodies in three levels of resolution. (**A**) PRA (screening) assay uses a pool of 30 beads coated with a panel of either HLA Class I or Class II purified antigens. The beads are incubated with patient serum, followed by FITC-labeled anti-human IgG antibody that will bind only to HLA antigen–antibody complexes. As depicted, the resultant fluorescent shift (dark histogram) provides a qualitative assessment of the % of panel reactive HLA antibodies (%PRA)—80.2% in this case, in comparison with the negative control (dashed unfilled histogram). (**B**) Phenotype or single donor beads are each coated with six HLA antigens (in this example, HLA class II: HLA-DR, -DQ, -DP) solubilized from a single cell and attached to unique beads. Each of these beads can be identified by their unique internal fluorescence signature. As in the previous assay, patient serum is incubated with the beads, and HLA-antigen antibody binding is identified by the use of a fluorescently labeled secondary antibody, and can be presented as bar graphs. In the case of highly sensitized patients, as shown in this example, complete and accurate assignment of antibody specificity is difficult and may be impossible. (**C**) SA beads are coated with multiple copies of one particular HLA allele and thus allow for a more accurate identification of antibody specificity. The number of molecules per bead is higher than for the phenotype beads, thus increasing the sensitivity of this assay. Semi-quantification of antibody strength is depicted on the y-axis of the bar graph as MFI values. MFI cutoffs to determine the strength of response are subjectively defined by individual laboratories. As presented in this example, specific cutoff points may sometimes be difficult to assign as there is no clear difference in slowly escalating values between negative and weakly positive responses. An arbitrary cutoff was placed at 1000 MFI units (noted by dividing line between beads 91 and 92 in panel B and beads 62 and 60 in panel C), however, there is very little difference between antibody specificities that are slightly below or slightly above this mark. Additional information needs to be incorporated for meaningful clinical determination of cutoff in this case.

coating the bead, thus maintaining the ratio of expression similar to that observed on the cell surface. This type of assay is a closer physiologic representation of actual cells with the added sensitivity and specificity inherent to the luminex assay. This assay can be utilized to assess changes in breadth and strength of HLA antibodies.[18,19] However, it is not always sufficient for accurate and complete assignment of HLA-antibody specificities in patients that are highly sensitized (Fig. 1B). To this aim, the third type of assays [3] provides micro-particle beads that are covered with multiple copies of a single HLA allele—hence referred to as single antigen (SA) beads (Fig. 1C). In this modality, color-coded polystyrene

beads are conjugated with purified HLA class I or II molecules. Beads are distinguished based on unique dye ratio of two-dye, fluorescent coding system in which they are infused (Fig. 2A). Each bead population is conjugated with distinct HLA molecules. When these beads are incubated with recipient serum, HLA antibodies will bind to their specific HLA target and this binding can be detected by emission of fluorescence, reported as mean fluorescence intensity (MFI; Fig. 2B and 1C). A variant of this assay is also available in an ELISA format.

SPA kits are widely available commercially, increasing timesaving and reproducibility within a laboratory. The ability of SPA to detect low levels of HLA antibodies greatly increases the accuracy and efficiency of the DSA detection process. Moreover, SPAs are not limited to detecting complement-fixing antibody. While it is beyond the scope of this paper to provide thorough review of the correlation between DSA detection using SPA and renal transplant outcome, it should be noted that early reports presented more controversial views, whereas later studies are more unanimous in supporting the superior value of DSA detection using Luminex-based assays.[20–24] We argue that the convergence of data confirming the significance of DSA detection by SPA over the past few years is a result of better understanding of the intricacies of data interpretation of these assays and growing experience rather than a real shift in their role in graft outcome.

Hurdles and solutions in DSA assay analysis and interpretation

The use of multiplex-bead arrays by laboratories around the world became widespread in the past 10 years and its infancy was fraught with technical complications. Issues such as the concentration of antigens on the beads, appropriate conformation, lot-to-lot variations, etc. steered many laboratories to devise center-specific modifications of the assay and its interpretation in order to 'improve' correlation between laboratory test results and clinical outcome.[25,26] While many of the vendor issues have been improved, center-specific protocols remain in use. As the literature is reviewed, it is important to remember how much was learned in recent years.
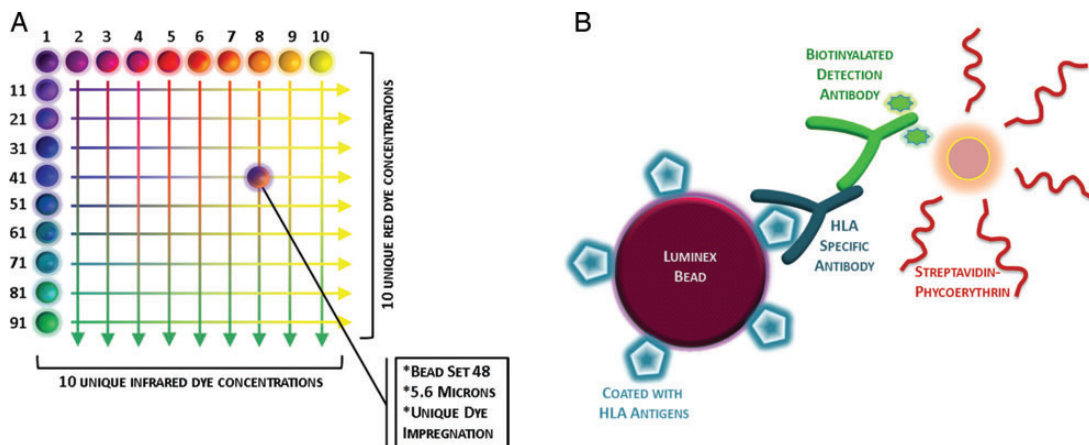
Fig. 2 HLA-antibody detection using SA assays. (**A**) A luminex SPA contains 100 unique sets of 5.6-μm beads, each identified through a unique combination of two fluorescent dyes (red and infrared) impregnated into each bead. In the illustrated example, bead 48 has 4 units of the red dye and 8 units of the infrared dye. (**B**) Each bead within the multiplex bead array is coated with multiple copies of an individual, soluble, HLA antigen. When incubated with patient serum, the HLA molecules can bind their cognate HLA-antigen specific antibodies. Once such complexes are formed, they can bind a biotinylated detection antibody conjugated with streptavidin, and the complex will emit fluorescence upon exposure to laser light. This fluorescence is detected on a luminex platform and antibody reactivity is presented as mean fluorescence intensity (MFI; examples in Fig. 1B and C).

Hurdles and solutions to DSA detection and translation of data for clinical application can be considered from two perspectives: (i) technical variability and (ii) interpretive variability between transplant centers.

Standardization of multiplex bead antibody DSA detection assays

Since SPA kits become widely available and more commonly used, variations between data reported by different transplant laboratories have been noted. Specific concerns were centered on the lack of a national standard operating procedure (SOP), differences between the amount of antigen attached to individual beads within a kit, differences between kits produced by different manufacturers and lot-to-lot variations in commercial kits. A multi-center study launched by seven core laboratories of the Clinical Trials in Organ Transplantation (CTOT) project, was specifically designed to address the aforementioned factors.[27] Specifically, reagents corresponding to the three levels of resolution (screening, phenotyping and SA), of two different lots, manufactured by the two most commonly utilized vendors: one Lambda and the other Immucore (Gen-Probe) were tested by the seven laboratories using 20 well-characterized sera. The critical component of this study was the use of a specific SOP adopted across the seven centers to standardize the assays. While MFI values did vary between the laboratories, antibody assignments were consistent between the different manufacturers, kits types and lots. Importantly, as part of that study, results of proficiency testing performed by 5/7 laboratories, using shared sera but lab-specific SOP, were also compared. The CTOT study showed that rigid adherence to a common SOP across all centers resulted in a 37% decrease in data output (MFI) variation when compared with each laboratory utilizing its own in-house protocol. This is a very significant finding as many centers are 'improving' their ability to detect low levels of HLA antibodies by 'minor modification' to the manufacturer's recommended SOP. As the CTOT study showed, these 'center-specific minor modifications' can translate into 'major differences' in MFI values and affect the ability to

compare results between centers. Global normalization for data comparison can also be applied to reduce variation across centers.[27]

Variations in MFI interpretation hamper consensus in reporting correlation between DSA levels and clinical outcome data

As previously mentioned, data output for multiplex bead arrays are commonly collected as MFI values. These values can be further categorized into ranges of strength (i.e. strong, moderate or weak), or simply deemed as positive or negative. While many studies, including the CTOT study, point to a positive cutoff >1000 MFI for SPA data, there is no consensus regarding cutoff values and, to date, each laboratory adopts its own individual cutoff points to determine at what MFI antibody detection should be considered positive. While a standard range of antibody detection MFI cutoffs would undoubtedly help facilitate data comparison across multiple laboratories and studies, the nature of antibody–antigen interactions complicate a straightforward assessment (Fig. 1C, legend). As antibody titers provide some evidence of affinity and avidity of antibody binding to cognate antigens, titration studies have also been suggested as factors to consider in standardization of antibody strength determination. Several factors contributing to the complexity of immune interactions are discussed below. These should be part of the multifactorial consideration used for final determination of antibody strength and risk stratification for transplantation.

Intrinsic and extrinsic factors can interfere with SPA multiplex result interpretation

SPA interpretation can be confounded by problematic serum samples, which are commonly found among renal patients. Specifically, dialysis patients were noted to have abnormally high levels of immune complexes or IgM resulting in low positive and/or high negative control values, which can greatly interfere with the detection of antibody specificities and strength. These issues can be corrected with Euglobulin IgM depletion, EDTA or DTT treatment of the sera.[25,28–31] False-positive reactivity may also be due

to antibodies against denatured or non-native conformation of the antigens.[7]

A limitation of a multiplex assay is the fact that a particular target can be present on multiple beads. Specifically, if a patient's serum exhibits low-level antibodies to an antigenic target, but this target is present on multiple beads (shared epitopes), it is possible that the net reactivity observed per each individual bead is lower than if only one bead would have expressed that target. In theory, a 'dilution effect' is generated due to the multiplex nature of the assay. This limitation can be somewhat circumvented by implementing rigorous analysis of specificity patterns (for example, analyzing results by known CREGs, presumed epitopes, etc.). If the antigenic target is unique to one or a small number of HLA targets, this dilution effect is not a concern.

Common humanized or antibody-based therapeutics such as intravenous Ig (IVIg) and thymoglobulin can cross-react with targets in SPAs, creating artificially high background or false results. It is therefore critical for the laboratory to obtain a complete treatment history for the patient and perform careful evaluation of potential interfering factors for thorough and thoughtful evaluation of each patient's antibody signature.

'Prozone' effect can mask identification of HLA antibodies in SA assays

Highly sensitized patients are prone to inhibition or masking of the presence of strong antibodies. This phenomenon, although not fully understood, is referred to in jargon as a 'prozone' effect. This inhibition effect can manifest itself as an 'unexpected' (strong) positive cross-match in the 'absence' of detectable DSA in SA assays. Obviously, serum samples with such inhibition effect cannot support accurate VXM assessment. However, once such sera are diluted, the 'masking' factors are diluted as well, and the true breadth and strength of its antibody signature is revealed. Figure 3 provides an example of antibody signature of a serum exhibiting prozone effect and how the MFI values change with titration. In this example, some specificities increase in MFI strength as the serum is diluted with each titer, as opposed to decreasing in MFI strength as expected.

No clear data are available regarding the frequency of inhibition effects in serum samples of patients pre- or post-organ transplantation; however, in our single center experience up to 10% of overall patient population may exhibit such inhibition effects at some time. Studies suggested that freeze/thaw cycles, heat inactivation, DTT or EDTA treatment may resolve 'prozone'.[28,29,31] Our center prefers to use actual titration of complicated serum samples, as additional information can be gained from this method. In a recent study of highly sensitized transplant patients (n = 34) we compared neat SA assays (neat MFI values), C1q assays and titration studies. This highly sensitized cohort demonstrated a 30% instance of prozone in undiluted serum samples. Interestingly, these same strong antibody specificities were also detected by the C1q assay. Remarkably, we observed that samples with similar MFI values could have different titer values. Specifically, in 19/34 patients who exhibited strong MFI values (>10 000), different ranges of titers ranging between 1:32- >1:1024 were observed. Based on correlation between titer information and number of desensitization cycles in our center (data not published) we believe that titer information provide a more accurate measure of antibody strength than numerical MFI values.

Incomplete representation of donor antigens within the SPA reagents can lead to false VXM assignment

For complete and accurate VXM assignment it is imperative to verify that all donor antigens are indeed represented in the panel utilized for testing. The presence of antibodies to some but not all alleles of an antigen group has been well documented. For example, patients typed as B*44:02 can produce antibodies to B*44:03.[32] Some of the less common HLA specificities are not even represented among the available reagents (i.e. A*02:07 or, DRB1*04:07). In some instances, intermediate-to-high resolution typing of the donor may be necessary to ascertain that the true DSAs were tested. This last point is especially crucial for DQα/β alleles that are both polymorphic and can pair together with variable antibody-binding consequences as will be discussed later.
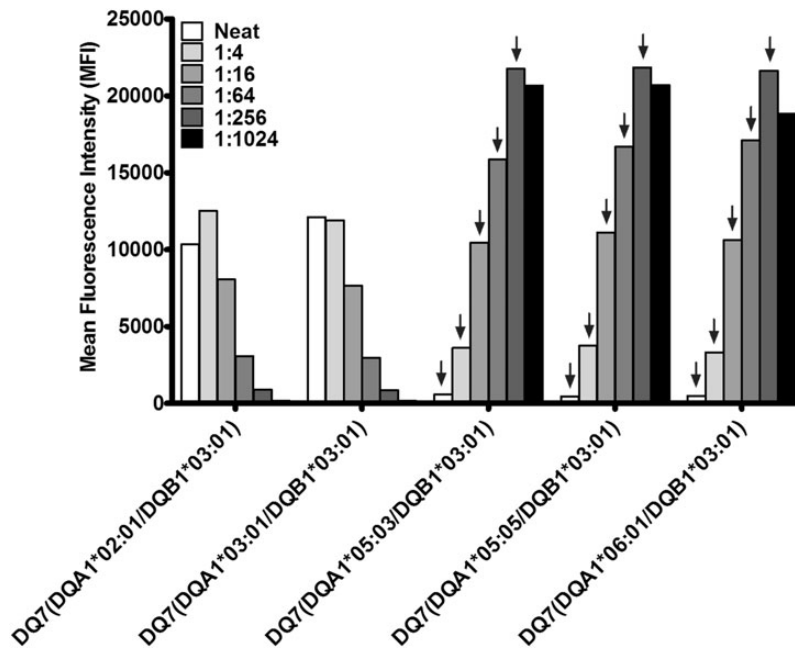
Fig. 3 'Prozone' effect, as revealed by serum titration, is undetectable in neat sera SA testing. A serum sample from a highly sensitized patient was run using titration studies; five dilutions have been performed as indicated (neat—1:1024). For simplicity, the results of only five beads representing all alleles of the serologic HLA-DQ7 specificity are presented. The two beads to the left are examples of positive antibodies, both with MFI values of >10 000 in the neat sample. As the serum is diluted (color code in inset), the MFI values decreased as expected. However, the three sets of results to the right show what can be interpreted as negative antibodies, with MFI values of ~200 in the neat (undiluted) sample. As the serum sample is diluted, an increase in MFI values is observed, reaching a peak at ~20 000 MFI only after the sample has been diluted 1:256. Relying on the results of the neat sample could have led to complete misinterpretation of the strength of these antibodies.

State-of-the-art developments in antibody analysis and its utilization in clinical practice

Complement-binding antibody assays

The complement system encompasses an extensive network of molecules designed to facilitate immunologic opsonization, lysis and eventual clearance of bacteria and/or foreign or unwanted cells from the body. IgG1 and IgG3 are considered the IgG antibody species that are able to fix or bind complement and initiate the complement cascade leading to antibody-bound target cell lysis and clearance.

In the late 1980s it was recognized that the complement effector molecule C4b binds at the sight of complement fixing DSA to propagate complement cascades, leaving another component of the complement, C4d, as an inactivated product that remains covalently bound to the cell surface acting as a durable *in situ* marker of complement activation.[13,33,34] The correlation between C4d deposition, DSA detection and graft loss implicated complement fixing antibodies in the progression of AMR and graft failure. This served as the impetus for assessing C4d fixing antibodies using the conventional SPAs.[35,36]

Recently, an additional modification of the SPA was developed to detect antibodies capable of binding the C1q complement component. C1q is the first step in the activation of the classic complement

cascade and is therefore considered reliable indicator of the cytotoxic potential of antibodies.[37–39] Initial reports, in small cohort studies, suggested that the presence of C1q-binding antibodies pretransplantation and those generated *de novo* post-transplant are associated with worse graft outcome in renal transplant recipients.[40,41] Loupy *et al.*[42], in a large cohort study, demonstrated that the presence of *de novo* C1q-binding HLA-DSA, detected in the first year post-transplant, was an independent predictor of worse 5-year allograft survival. Correlation was also reported with AMR in the first year post-transplant, with more microvascular inflammation and injury, more C4d deposition in the peritubular capillaries of the graft and a lower estimated glomerular filtration rate (GFR) at 1 year.

This study, as well as others,[38,39,41] showed a clear correlation between the ability to bind C1q and the level of DSA as measured by MFI (C1q-binding antibodies were most always those antibodies with high MFI values). We, and others, have shown that C1q-binding capacity correlated with actual antibody strength, as measured by titration studies, bypassing inconsistency that may be associated with prozone effects. These observations beg the question whether the reported correlations between C1q-binding ability and transplant outcome are not due to the presence of stronger antibodies, inferring a more robust immune response. Reports demonstrating that C1q-binding antibodies can revert to non-C1q IgG antibodies and vice versa are quite disconcerting and to our humble opinion, at this point, preclude unequivocal decisions regarding the long-term risk of these antibodies in the context of kidney pre-transplant decision-making.

A new look at HLA-DQ and HLA-DP antibodies (HLA-DQ/DP are the sum of their parts)

Recent studies support strong correlation between the presence of donor-specific HLA-DQ antibodies and increased risk for transplant rejection.[43–45] Moreover, HLA-DQ antibodies are likely the most commonly developed *de novo* DSA post-transplant. Unfortunately, matching criteria used by most transplant societies still consider only antigens in HLA-A, -B and -DR loci.

A critical consideration when investigating antibodies against HLA class II antigens is the fact that unlike class I molecules, HLA class II antigens are composed of two chains (α and β) encoded by two distinct genes (A and B). While the DQ and DP antigens have two polymorphic chains, the α chain of HLA-DR is virtually non-polymorphic and therefore does not contribute to differences between HLA-DR alleles. Given that for many years HLA-DR antigens were the only class II targets that were considered relevant to transplant outcome, common practice was to consider only the β-chain of class II molecules, disregarding the polymorphic nature and the potential contribution of half of the DQ and DP molecules, i.e. the α chain. However, since the α and β chains form a single molecule that is expressed on the cell surface, this long-standing but misleading practice should be altered.

In a series of publications we have demonstrated the added complexity in analyzing antibodies against HLA-DQ when both the α and β chains are considered.[10,46–49] In fact, we have presented cases in which a patient typed as HLA-DQ7 for example, can have antibodies against HLA-DQ7. Of course, the two HLA-DQ7 alleles represent two different species of the serologically equivalent DQ7; in this particular example, DQA1*03:01/DQB1*03:01 and DQA1*05:01/DQB1*03:01. The ramifications on defining unacceptable antigens on utilization of antibody information in performing VXM and on assigning cPRA are evident. In a retrospective, blinded study we assessed the ability to accurately predict flow cytometric cross-match results in a cohort of 1130 consecutive XM assays performed by our OPO laboratory. Using the complete HLA-DQα/β information for donor typing and antibody assignment—we were able to correctly predict negative or positive XM results. More importantly, if serologic assignment of antibody specificity were used for unacceptable antigens determination, 25% of patients with HLA-DQ antibodies would not have even been considered for those XM assays. Referring to the DQ molecule as the combination of its α/β components permitted those XM assay to take place and provided those highly sensitized patients with an opportunity to get a transplant.[49]

The fact that a patient may generate antibodies to some but not all alleles within an antigenic HLA-DQ specificity has an impact on how their level of sensitization should be assessed. The use of cPRA as a measure of predicting the percent of potential donors against whom a patient may have antibodies have significantly improved organ allocation.[48] cPRA is calculated based on the frequency of different HLA phenotypes, or combinations of HLA phenotypes that are incompatible with the patient. For that purpose, HLA-DQ is considered to have seven potential serologic specificities. However, the number of HLA-DQ alleles (as α/β combinations) is much higher. In order to gain some insight into the potential effects of this dichotomy we calculated HLA-DQ allele frequencies in a population of patients awaiting transplantation in our center and their potential living donors. Using these frequency data to re-estimate cPRA demonstrated that patients with limited range of antibodies, to some but not all alleles within a serologic DQ specificity, are currently assigned an artificially high cPRA.[48] A comprehensive analysis of DQ allele frequency in a larger, multi-ethnic, donor pool cohort will help establish a more equitable approach for organ transplantation in patients with HLA-DQ antibodies. A similar approach, although probably to a different degree, is likely applicable for HLA-DP antibodies.

Characterization of HLA-antibody epitopes and its use in defining compatible donors

Cross-reactivity between HLA antigens, namely, recognition of several different HLA antigens by a single antibody, was recognized early on.[50–52] While it was already then hypothesized that cross-reactivity is a result of sharing portion of the amino-acid sequence between members of a cross-reactive group (CREG), understanding of the concept required several developments. It was only after the elucidation of the three-dimensional structure of the HLA molecule,[53,54] the understanding of the breadth of allelic polymorphism (gaining high resolution sequence analysis) and availability of tools to closely interrogate antibody specificities (SA assays) that the concept of HLA epitope was fully conceptualized.

HLA-antibody epitope is defined as the specific stretch of amino acid residues that are critical for antibody recognition. In the core of this antibody footprint is an area of one or few polymorphic residues that are unique to one HLA molecule or are shared by a group of HLA alleles. Thus, epitopes that where once defined by serologic cross-reactivity can now be structurally mapped by amino acid sequences. Most HLA antigens have multiple epitopes to which an antibody can bind. Experimental evidence to the presence of HLA epitopes came from absorption/elution studies performed by the Terasaki group. In these experiments serum samples to be investigated were incubated with recombinant cell lines, expressing multiple copies of a single HLA molecule. Following elution of the bound antibodies and using SA assays for testing, many of the eluates reacted against the HLA alleles expressed by the recombinant cells, but also against additional HLA alleles that shared one or a combination of amino acids—defining the epitope recognized by the eluted antibody.[55] Duquesnoy[56] on the other hand developed a computer-based algorithm—HLA-Matchamker, focusing on the structural basis of HLA polymorphism. In later iterations, the structural epitope was defined as patches of one to three amino acids, not necessarily linearly attached, with an overall radius of ~15 A defining the functional epitope.[57]

Soon after, multiple studies investigating the role of epitope load in transplant outcome followed.[58] In a recent publication, Wiebe *et al.*[59] reported that locus-specific epitope mismatches were more numerous in patients who developed HLA-DR or HLA-DQ *de novo* DSA. They also suggested an optimal threshold for epitope mismatching that led to minimal or no DSA formation in their patient population, stating that epitope matching outperforms traditional low-resolution HLA-antigen-based matching. Finally, they asserted that epitope, not antigen, matching is the best predictor of HLA class II *de novo* DSA development and that there is clear inequality in epitope immunogenicity.

Physiochemical modeling studies showed differences in the number and distribution of polar and charged amino acid side chains outside of the

conventional epitope site. Changes that affected the folding and structural composition of different HLA class I or class II antigens were studied.[60–62] Additional studies from our laboratory demonstrated significant differences in antibody reactivity, as demonstrated by MFI values, when a specific antibody recognizes the same epitope in the context of different HLA-DQ alleles. These observations indicate that additional insight is required for refinement of prediction and accurate determination of HLA epitope antigenicity.[63]

The use of epitope antigenicity and immunogenicity information has a significant role not only on accurately predicting VXM but also on increasing the likelihood of finding a compatible donor for the highly sensitized patients as demonstrated by the Eurotransplant group.[64] Moreover, epitope-matching approaches should allow for assigning the most compatible donor such that the potential for *de novo* antibody formation is minimized and thus decreasing the likelihood of patients becoming highly sensitized following a failed transplant.

Discussion

The introduction of SPA for the detection of HLA antibodies in general and DSA in particular revolutionized our understanding of the role and significance of AMR in transplant outcome. As we gather more experience in using these assays, we can provide more nuanced interpretation of the data (HLA-DQ, prozone effect, etc.), providing better guidance for clinical utility in risk stratification and treatment tailoring.

Complement-binding activity of DSA and its impact on transplant outcome is under investigation using SPA approaches. Whether their affects are due to actual activation of the complement system or a result of the sheer strength of the antibodies, the results of these studies should help in further risk stratification and individualization of treatment protocols.

Better characterization of HLA-antibody epitopes, generation of epitope registries, and eventually epitope matching should provide educated tools to find suitable donors for the highly sensitized patients

but more importantly to minimize risk for *de novo* generation of DSA post-transplantation.

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