ANTIBODY REPERTOIRE AND GRAFT OUTCOME FOLLOWING SOLID ORGAN TRANSPLANTATION

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ANTIBODY REPERTOIRE AND GRAFT OUTCOME FOLLOWING SOLID ORGAN TRANSPLANTATION

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The first real major breakthrough that laid the basis of HLA antibody detection in the field of solid organ transplantation, came with the introduction of the complement dependent cytotoxicity (CDC) test in 1964 by Terasaki and McClelland. Since then, methods for antibody detection have evolved remarkably from conventional cell-based assays to the current advanced solid phase systems on the Luminex platform, with increasing degree of sensitivity and specificity. The latter have been indispensable for more accurate identification of donor specific HLA antibodies in broadly reactive allo antisera, and to guide donor selection and kidney paired exchange programs through virtual crossmatching, in addition to serving as excellent tools for initiating pre-transplant desensitization and post- transplant antibody monitoring. Consensus is evolving on the optimal routine employment of these methods in donor selection strategies along with an understanding of the clinical relevance of antibodies detected by each of them.

The immunoassays based on the Luminex platform and flow cytometric beads are however unable to discriminate complement fixing from non-complement fixing HLA antibodies. This is important because the former are considered clinically more pertinent in the peri-transplant period. The C1q assay which is a modification of the solid phase assay based on Luminex single antigen beads, which can be used effectively to monitor high dose IVIG desensitization is essentially a surrogate complement fixing assay, retaining the exquisite sensitivity and specificity of the Luminex platform. Currently, information obtained from these assays is preliminary and much needs to be done to standardize technologies and set a consensus 'MFI cut off' for antibody positivity.

Besides the overriding influence of anti-HLA antibodies on overall solid organ graft survival, immune response to non-HLA antigens has become a topic of substantial interest in recent years. An ever expanding list of non-HLA antigens has been implicated in graft rejection for various organs, of which the most noted are the Major Histocompatibility Complex class I chain-related molecule A (MICA), Vimentin, Myosin, Angiotensin II type 1 receptor (AT1R), Tubulin and Collagen. MICA is one of the most polymorphic and extensively studied non-HLA antigenic targets especially in renal transplantation. Although there are clear indications of MICA antibodies being associated with adverse graft outcome, to date a definitive consensus on this relationship has not been agreed. Because MICA molecules are not expressed constitutively on immunocompetent cells such as T and B lymphocytes, it is of utmost importance to address the impact of MICA donor specific antibodies (DSA) as compared to those that are non- donor specific (NDSA) on graft outcome.

The soluble isoform of MICA molecule (sMICA) that is derived from the proteolytic shedding of membrane bound molecules has the potential to engage the NK-cell activating receptor NKG2D and down-regulate its expression. Consequent to the interaction of NKG2D by sMICA, the receptor ligand complex is endocytosed and degraded and thus suppresses NKG2D mediated lysis of the target by NK cells. Thus interaction between NKG2D and sMICA leads to expansion of immunosuppressive/anergic T cells thereby resulting in suppression of NKG2D mediated host innate immunity. These concept support the possible involvement of an immunosuppressive role for sMICA during allotransplantation as shown recently for heart transplantation.

This research topic focusses on the clinical utility of investigating the complete antibody repertoire in solid organ transplantation.

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Editorial: Antibody Repertoire and Graft Outcome following Solid Organ Transplantation

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The Editorial on the Research Topic

Antibody Repertoire and Graft Outcome following Solid Organ Transplantation

In recent years, there has been vast improvement in the survival of solid organ grafts. Improved immunosuppression has resulted in efficient control of cell-mediated immune responses and has also mitigated the effects of HLA mismatching between the recipient and the grafted organ. Antibody-mediated rejection (AMR) however still remains a clinical challenge and the identification of donor-specific HLA antibodies pretransplant are a mandatory requirement for successful grafting. Accurate and sensitive HLA antibody identification has been made possible by the introduction of solid phase assays such as the Luminex bead based platform for antibody testing.

Despite this advancement in detecting and understanding the role of HLA antibodies in rejection, many questions still require answers in order to maximize the outcome in solid organ transplantation. Many of these aspects are dealt with efficiently in this excellent series of papers addressing the role of antibodies in solid organ rejection.

This collection of reviews, some including new findings, by world leaders in their particular subspecialty, represents a compendium of the most recent and exciting developments of special interest to those who are involved in understanding the role of antibodies in organ rejection.

The technical aspects of antibody detection have been addressed in detail by Tait (Melbourne). While solid phase and, in particular, the Luminex bead assays have revolutionized the way antibody screening is now conducted, they have produced their own particular challenge. The failure to discriminate complement from non-complement fixing antibodies by solid phase assays poses a problem with respect to the significance of a positive result, which has been partially addressed by modification of the Luminex assay to measure complement fixation.

Data from clinical studies suggest that non-complement fixing antibodies may compromise graft outcome, albeit not to the same extent as complement-fixing HLA antibodies. The presence of antibodies directed at denatured HLA and detected by the Luminex platform, although not clinically important can also complicate the analysis of antibody specificity determination. Issues such as the importance of the mean fluorescence index on graft outcome and the value of the virtual cross match are also discussed in this review.

The functional aspects and clinical impact of alloantibodies and autoantibodies are discussed in several excellent reviews.

Despite the best efforts of avoiding pretransplant HLA antibodies, there is no way of predicting which patients will develop *de novo* antibodies. Mangiola and co-workers (Pittsburgh) discuss the role of both pretransplant and *de novo* HLA antibodies in pediatric and adult heart transplant recipients. They stress that the treatment for early and late rejection are different, which raises the

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important question of the optimal protocol for posttransplant screening in order to maximize timely detection of antibodies prior to organ damage.

In addition to HLA antibodies, the role of non-HLA antibodies is discussed by several author groups. Matsuda and Sarwal (San Francisco) emphasize the point that antibody-mediated chronic rejection remains the biggest unresolved issue. Chronic AMR has been shown in cases involving HLA identical recipients and donors suggesting a role for non-HLA antibodies. This review is a "tour de force" covering mechanisms underlying antigen recognition, the role of both HLA and non-HLA antibodies in rejection and immunosuppressive approaches.

Nayak et al. (Phoenix) studied both auto- and alloantibodies in lung and pancreatic islet cell transplantation, which are more susceptible to rejection by a combination of antibodies than other forms of transplants. Islet cell transplant patients are often exposed to multiple islet cell infusions and as a result are exposed to many HLA class 1 and 2 mismatches. In addition to HLA immunity, there is evidence that autoantibodies to GAD65 and autoreactive T cells participate in the rejection process. It appears that once tolerance is broken to the self-antigens, then immunosuppression becomes ineffective. In lung transplantation, HLA antibodies are associated with obstructive airway disease while the appearance of MICA antibodies after HLA donor-specific immunization is associated with bronchiolitis obliterans syndrome.

Zhang and Reinsmoen (Los Angeles) have submitted a comprehensive review on the role of non-HLA antibodies in kidney and heart transplant rejection. Antibody specificities discussed include those to myosin, vimentin, Kα1 tubulin, collagen, and angiotensin II type 1 receptor. There appears to be synergy between HLA and non-HLA antibodies. In some cases, development of HLA antibodies precedes the development of antibodies to non-HLA antigens. It is suggested that the damage inflicted on the graft by HLA antibodies exposes cryptic antigens on non-HLA molecules that result in antibody formation by the recipient. There is also evidence that the presence of non-HLA antibodies may predispose the recipient to the formation of HLA antibodies. Given this close relationship between HLA and non-HLA antibodies, the authors stress the point that it is imperative that both types of antibodies be measured in transplant recipients.

Baranwal and Mehra (New Delhi) discuss the importance of MICA antibodies on graft outcome in a thorough review of the literature and demonstrate that MICA antibodies are detrimental to the outcome of solid organ transplantation, but soluble MICA appears to have an inverse relationship to rejection. The mechanism behind this observation appears to be the interaction of the soluble MICA with the MICA ligand NKG2D, thus blocking the activation of NK cells. Amino acid position 129 appears to be pivotal in the induction of immunity in renal transplant patients. Recipients who are homozygous for methionine at this position have a higher incidence of rejection than those with valine homozygosity. The mechanism underlying this association is not understood.

Valenzuela et al. (Los Angeles) discuss the impact of IgG subclass on solid organ rejection. The histology and clinical profiles surrounding both HLA and non-HLA donor-specific antibodies is very heterogeneous with limited understanding of the various roles that the IgG antibody subclasses play. The authors describe in detail the various functions that IgG subclasses subserve, the data for which is largely derived from infectious disease studies and human cancers, but make the point that in clinical transplantation there is little known. Analyzing all known data suggests that IgG3 is predominantly associated with complement-mediated rejection while IgG4 is associated with memory, and subclinical and chronic rejection.

One of the complicating issues is that most patients have mixtures of two or more isotypes, which makes analysis of single isotype function difficult. Newer assays are needed that could measure single antibody isotypes, permitting correlations to be established between antibody isotypes and the various features of complex rejection histology.

An intriguing piece of original research by Geneugelijk et al. (Basel) focuses on nature's allograft, the fetus. They demonstrate that a previous miscarriage prior to a successful second pregnancy produces a lower rate of immunization to paternal HLA than a previous full-term pregnancy. Examination of the number or epitope differences between the mother and fetus revealed the intriguing observation that patients with a previous miscarriage actually had a lower rate of HLA incompatibility. This observation is not readily explained, although the authors have put forward several hypotheses that require further exploration. Suffice to say the mechanism underlying this observation may have some relevance to immunization mechanisms in the clinical allograft situation.

Rajalingam (San Francisco) discusses the role of NK cells in organ rejection. At first sight, this review does not seem central to the theme of the series. However, the link is made by virtue of the fact that the presence of HLA antibody bound to graft endothelium can activate NK cells *via* the antibody-dependent cell-mediated cytotoxicity (ADCC) pathway. Infiltration of grafts with NK cells have been shown in renal, cardiac, liver, and lung transplants indicating a key role for these cells in the rejection process, and NK cell transcripts have been demonstrated in kidney biopsies undergoing AMR.

Morath et al. (Heidelberg) provide an excellent review of ABO incompatible (ABOi) renal transplants with data from the Collaborative Transplant Study (CTS) established by Gerhard Opelz in Heidelberg in 1982. This has been an invaluable resource over four decades comprising 400 participating transplant centers from 42 countries, providing data on kidney, heart, lung, liver, and pancreas transplants.

The concept of a major ABOi renal transplant was an absolute contraindication. However, over the last 25 years, the need to expand living donor options for some patients led investigators into the possibility that with ABO antibody reduction pretransplant, it was possible to transplant across the major ABO barrier. If the ABO titer can be reduced to below 1:32 by plasmapheresis, membrane filtration or immunoadsorption, as measured by the tube method, then although there is a rebound effect posttransplant, it is non-damaging to the graft, a process termed accommodation. Antibody ablation is accompanied by other procedures designed to blunt the rebound response. These include the use of IVIG to modulate the recipient's immune system and anti-CD20 (rituximab) therapy to reduce the B cell pool. A new approach has been the use of eculizumab, a hybrid monoclonal that is a terminal complement blocker, in this case designed to block the damaging effect of

the ABO antibodies bound to the graft endothelium. Results to date however are inconclusive with respect to the efficacy of this approach. A CTS study of 1420 ABOi renal transplants from multiple transplant centers has revealed that while graft survival of ABOi are comparable to ABO compatible grafts, early rejection and the complications of early infection are increased, with one additional patient per 100 dying from this complication in the ABOi.

Interestingly within the ABOi group, the use of anti-CD20 appears to have a significant beneficial effect. Although the use of ABOi transplants has greatly increased the living donor choices for many patients, the authors feel that caution has to be exercised in the use of ABOi due to the complications of infection.

The functional role of B cells has been elegantly addressed by Karahan et al. (Leiden) who stress the point that B cells play other roles in addition to their antibody-producing function. They discuss in detail the mechanisms underlying the role B cells play as cytokine producers and as antigen-producing cells as well as their ability to organize tertiary lymphoid tissue. B cells also invoke T cell immunity and of course regulatory B cells are central to control the immune response. Their pivotal message is that ablation of B cells can be detrimental as well as beneficial. Understanding how the different populations of B cells interact could lead to more rational and targeted immunosuppression.

Wu et al. (Sichuan) discuss the role of IL-21, which is produced by CD4+ T cells and their interaction with both plasma and memory B cells. As with the Karahan et al. paper, the possibility arises that these findings could be used to establish novel immunosuppressive approaches.

Two reviews discuss both historical aspects of HLA matching in solid organ transplantation and the evolution in our understanding of the epitopes recognized by HLA-specific antibodies. In the first such paper, Zachary and Lefell (Baltimore) provide an insight into the different features of HLA matching, focusing on the decreasing effect of matching influence over recent decades. A more sophisticated approach to matching is now employed, which does not treat all mismatches as equal, but rather considers epitope mismatches in the context of the patients' HLA immunological profile including desensitization procedures. When HLA mismatches present in a first donor and are repeated in a patient having a second transplant (repeat mismatches), it appears that they are only associated with increased risk of graft loss in patients who are HLA sensitized or those recipients who underwent nephrectomy of the first failed graft. It appears that reexposure to HLA class 2 is more damaging than class 1.

Rene Duquesnoy (Pittsburgh) summarizes the historical aspects surrounding the development of the epitope and eplet concept of HLA antibody recognition, for which he has been the preeminent pioneer. The original concept of treating each HLA serologically defined antigen as a single entity was overturned by the demonstration using sequence data, that each HLA molecule consists of multiple epitopes, some unique, and some shared with other antigens. The approach that arose from this realization was the use of epitope data in the selection of organ donors, particularly for sensitized patients but also recognizing permissible HLA mismatches in non-sensitized patients. In this excellent review, we are taken on a journey from the initial serological demonstration of antibodies, which broadly recognized HLA antigens, to the study of these antibodies at the molecular level, and the demonstration that there are polymorphic triplets of amino acids which are the signature of the epitope and are referred to as eplets. As this concept gains wider acceptance among transplant units, HLA epitope matching will become the method of choice, replacing the historical method of HLA antigen matching currently in use in many centers.

The final contribution, despite being tangential to the topic under discussion, provides some insight into allograft tolerance in a mouse model. Shen and coworkers (Nanjing, China) utilized a mouse cardiac transplant model to generate T regulatory cells and then transfer them to an intestinal transplant model where they observed prolonged survival. The use of T regulatory cells with costimulation blockade appeared to be a successful tolerance model.

In summary, although great progress has been made in the detection of HLA and non-HLA antibodies and their relevance to the outcome of solid organ transplants, there are still challenges ahead. The definition of a hierarchy of alloresponsiveness to HLA epitopes, which is dependent on the HLA molecules expressed by the recipient, may be revealed by the full sequencing of both recipients and donors made possible by next-generation sequencing. Such an approach used in a large collaborative study could make possible immunogenicity grading of epitopes interpreted in the context of individual recipients' HLA genotypes.

The expansion of antibody screening bead technology to include both HLA and non-HLA epitopes will allow further study of the detrimental or otherwise effect of various classes of antibodies. Although many of the non-HLA antibodies are considered auto in nature, an extensive study of the sequences of these genes in populations may reveal epitopes, which include polymorphic residues thus creating the possibility of using this information to select the most appropriate donor for each recipient.

Finally, many transplant recipients display mixtures of IgG isotypes, both complement and non-complement fixing. The mechanism by which mixtures of these antibodies influence rejection *via* both complement binding mechanisms and by ADCC and how these relate to both acute and chronic antibody rejection requires further study to fully understand the subtleties of these antibody interactions.

We recommend that all those interested in the role of antibodies in their many forms on the outcome and survival of a range of allograft types take advantage of this free online series of papers. They are contributed by leaders in the field of clinical transplantation and represent an up to date summary of the current state of play. Both HLA and non-HLA antibodies are discussed in the context of AMR and the mechanisms, whereby these antibodies compromise the outcome of the graft that are discussed.

The editors are pleased to be able to present this collection of papers to you as a series and feel that it represents an important contribution to the current literature on this topic.

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REVIEW
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Detection of HLA Antibodies in Organ Transplant Recipients – Triumphs and Challenges of the Solid Phase Bead Assay

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This review outlines the development of human leukocyte antigen (HLA) antibody detection assays and their use in organ transplantation in both antibody screening and crossmatching. The development of sensitive solid phase assays such as the enzyme-linked immunosorbent assay technique, and in particular the bead-based technology has revolutionized this field over the last 10–15 years. This revolution however has created a new paradigm in clinical decision making with respect to the detection of low level pretransplant HLA sensitization and its clinical relevance. The relative sensitivities of the assays used are discussed and the relevance of conflicting inter-assay results. Each assay has its advantages and disadvantages and these are discussed. Over the last decade, the bead-based assay utilizing the Luminex® fluorocytometer instrument has become established as the "gold standard" for HLA antibody testing. However, there are still unresolved issues surrounding this technique, such as the presence of denatured HLA molecules on the beads which reveal cryptic epitopes and the issue of appropriate fluorescence cut off values for positivity. The assay has been modified to detect complement binding (CB) in addition to non-complement binding (NCB) HLA antibodies although the clinical relevance of the CB and NCB IgG isotypes is not fully resolved. The increase sensitivity of the Luminex® bead assay over the complement-dependent cytotoxicity crossmatch has permitted the concept of the "virtual crossmatch" whereby the crossmatch is predicted to a high degree of accuracy based on the HLA antibody specificities detected by the solid phase assay. Dialog between clinicians and laboratory staff on an individual patient basis is essential for correct clinical decision making based on HLA antibody results obtained by the various techniques.

Keywords: HLA antibody, CDC, ELISA, Luminex, beads, transplantation

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INTRODUCTION

Rejection of solid organ allotransplants can be cellular or antibody mediated. In the majority of cases the rejection reaction is directed at human leukocyte antigens (HLAs) expressed on the cells of the transplanted organ. While there is no routine test which can be applied to determine the cellular immune status of potential transplant recipients, the detection of HLA antibodies, particularly those directed at the HLAs of the donor has been at the forefront of donor–recipient histocompatibility testing since transplantation became a clinical reality.

The determination of antibody status is one of the most important investigations that is undertaken in potential organ transplant recipients. While levels of HLA incompatibility can be tolerated due to the quality of immunosuppressive drugs that are now available, the presence of antibodies in the recipient specific for HLA incompatibilities present in the donor can be devastating to the graft.

The first organ transplanted on a routine clinical basis was the kidney and a great deal of lessons we have learned about the impact of HLA antibodies on transplanted organs was learnt during the formative years of clinical renal transplantation.

The pretransplant crossmatch which involves testing the recipient's serum for cytotoxicity against the donor cells (lymphocytes) was introduced into the testing algorithm in the 1960s in the early days of renal transplantation (1, 2). The test which relies on the detection of complement-dependent cytotoxicity (CDC) is performed in small microtiter trays. The patient's serum and donor cells are mixed together, rabbit serum as a source of complement is added and lysis due to antibodies in the recipient specific for the donor cells is detected. The crossmatch test is still an essential component of immediate pretransplant testing for all organ transplants and is known as the microlymphocytotoxicity test. A modified form of this test was also used to screen patients' sera for HLA antibodies and to determine specificity. This method with modifications was the basis of HLA antibody screening for nearly three decades but has been replaced in recent years with more sensitive and reproducible assays of antibody activity. The evolution of HLA antibody testing and the associated laboratory and clinical issues that have arisen with the use of this new technology forms the basis of this review. Although renal transplantation is the basis for many of the lessons we have learned using the new methods of antibody detection, they apply equally to other forms of solid organ transplantation.

HLA ANTIBODY DETECTION ASSAYS

Complement-Dependent Cytotoxicity

The clinical importance of the pretransplant crossmatch and the technology for performing the test was described by Terasaki and colleagues (1, 2) and became known as the microlymphocytotoxicity assay or CDC. Essentially, the test consists of incubating patient serum with potential donor lymphocytes to establish if the recipient has donor-specific HLA antibodies (HLA-DSA). Rabbit serum as a source of complement is added and if HLA-DSA are present lysis of the cells occurs. This lysis can be detected by the original method of dye exclusion or by later developments which included fluorescence. It was quickly appreciated that renal transplant patients with DSA had early hyperacute rejection (3, 4). This test was quickly established as an essential and non-negotiable pretransplant test, a negative result enabling renal transplantation to proceed.

Modifications to the test were made to make the test more sensitive such as prolonged incubation and the use of a second antibody such as an anti-IgG reagent (5) but there remain several technical problems with the test. The assay relies heavily on the viability of the donor cells and in the case of deceased donors optimal viability is not always achievable. In addition to IgG antibodies, the test detects IgM as well as auto antibodies. The latter can be overcome to some extent with the use of 1,4-dithiothreitol (DTT) (6, 7), although this can result in the loss of some IgG antibody (8).

In its original form, the assay was performed using unseparated lymphocytes from peripheral blood, lymph node, or spleen obtained by a gradient separation technique (9). This resulted in the detection of both HLA class I antibodies which react with B and T lymphocytes and also with HLA class II antibodies which react with the class II expressing B cells.

The introduction of cell separation techniques such as ficoll gradient separation (9) with subsequent rosetting T lymphocytes with sheep red blood cells (10) and then later the use of magnetic beads specific for each cell population (11) enabled the distinction to be made between HLA class I and class II antibodies. Other approaches which had varying success were also used.

The main issue with the CDC assay is its sensitivity. The development of more sensitive solid phase assays for antibody detection has basically replaced the CDC approach, but because it is the only functional assay it is still used in many centers as a final test of pretransplant compatibility in the form of the CDC crossmatch. However, even this test is slowly being replaced by the "virtual" crossmatch (see later section).

The CDC assay was modified as an antibody screening technique by using a panel of HLA typed cells and testing each patient's serum against this panel. The technique is essentially identical to the crossmatch procedure but by using a panel of cells it is possible to determine the HLA specificity of antibodies present. By testing against both T (which express HLA class I) and B lymphocytes (which express both HLA class I and II) it is possible to characterize both class I and class II antibodies when they occur together. An added step of absorbing sera with platelets, which express HLA class I but not class II, prior to testing enables the determination of class II antibody specificity without the added complicating factor of co-occurring class I antibodies (12).

By using a panel of accurate HLA phenotyped cells, it is possible to express the result as a panel reactive antibody (PRA) percentage (i.e., the percentage of cells in the panel giving a positive result) in addition to determining HLA antibody specificities. The PRA is a useful indicator of the probability of a patient giving a negative crossmatch with an unrelated donor.

Flow Cytometry

The flow cytometry crossmatch (FCXM) was introduced into clinical practise by Garavoy et al. (13). The principle of the test involves incubating donor cells with recipient serum and then adding a fluorescein-labeled second anti-human immunoglobulin antibody that binds to patient antibody bound to the donor cells. The test is read on a flow cytometer, and the degree of positivity is expressed as a channel shift. The main advantage of the FCXM is its sensitivity for antibody detection over the conventional CDC crossmatch (13, 14). In cases where the second antibody is anti-human IgG, it is not possible to discriminate between complement binding (CB) and non-complement binding (NCB) HLA antibodies. However, if that additional information is required, it is possible to use second antibodies to the IgG isotypes and also IgM (15, 16). It is also possible to detect antibodies to both class

I and class II HLA antibodies by using markers to differentiate T and B lymphocytes (17, 18).

With the advent of solid phase and in particular bead technology, and the interpretation of weak antibodies detected by those methods the flow crossmatch is used in many centers to assist in clinical decision making. For example, if the flow crossmatch is positive in the case where a weak HLA antibody is detected by the bead assay (19), a decision may be made to invoke a desensitization protocol or to not proceed with the proposed transplant depending on the patient's transplant and sensitization history. Alternatively, a weak HLA antibody detected by the bead assay in the presence of a negative flow crossmatch may result in the transplant proceeding, but again the patient's immunological history would be a part of the decision making in such a case.

Solid Phase Antibody Detection Assays

Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) was initially used in the HLA field for detecting levels of HLA both cell bound and free but was adapted for detection of HLA antibodies in serum in 1995 (20).

In the modified assay HLA glycoproteins are immune-precipitated usually from EBV transformed cell lines, and immobilized in the wells of microtiter trays. Sera to be tested are added to the wells and antibodies specific for the HLA molecules bind to the relevant epitope. After washing an anti-human IgG labeled with a reporter molecule such as alkaline phosphatase is then added which binds to the primary anti-HLA antibody molecule. After repeated washing to remove any unbound secondary antibody, a substrate is added which is dephosphorylated by the alkaline phosphatase resulting in a color change.

Two levels of testing are achievable using the ELISA technique. One involves the use of a pool of different class I and class II molecules, which essentially gives a positive or negative result, and the second utilizes HLA molecules derived from single individuals which can be used to determine antibody specificity.

The ELISA technique is more sensitive than CDC in detecting HLA antibodies (21, 22) but has the potential drawback of not distinguishing between complement-fixing and non-complement-fixing antibodies. This assay however has been used as a very effective method for detecting pre- and postsensitization in solid organ transplants (23–25) but has been somewhat superseded by the introduction of fluorescently labeled beads to which HLA molecules have been attached.

Luminex® Bead Technology

The introduction of fluorescently labeled beads revolutionized HLA antibody testing during the 1990s. Commercial kits are available (One Lambda, Immucor) which consist of beads impregnated with differing ratios of two fluorochromes resulting in a unique signal for each bead and which have one or several types of HLA molecules attached.

The assay involves first the incubation of a patient's serum with the beads. If the patient has HLA antibodies the serum will react with the bead expressing the appropriate HLA molecule. After washing, the beads are incubated with a secondary antibody, usually with a phycoerythrin (PE)-labeled anti-human IgG (**Figures 1** and **2**).

Three levels of testing are possible depending on requirements. The first level provides a positive/negative result with respect to a patient's antibody status. In this instance, the beads are bound with a large number of class 1 or class 2 molecules derived from lymphoblastoid cell lines. Beads used in second level testing are bound with molecules derived from a single cell line and hence express two HLA molecules for each of the HLA loci (HLA-A, -B, -C for class I and HLA-DR, -DQ and -DP for class II). This testing is essentially analogous to testing with a panel of cells, and therefore, the result can be expressed as a PRA percentage. The third level of testing involves the use of beads bound with single HLA molecules produced by recombinant technology, so called single antigen beads (SAB). These beads provide a real advantage of this technology as complex mixtures of antibodies can be characterized and HLA specificities accurately determined. This technology is now considered essential for the pretransplant testing of sensitized patients.

There are two common methods for the readout. The first method involves conventional flow cytometry and measuring the channel shift associated with antibody binding. The second which has become the most popular approach is the use of the Luminex® fluorocytometer which utilizes two lasers, one of which excites the fluorochrome in the bead and the other laser excites the PE bound to the detection antibody (**Figure 3**). The first readout therefore identifies the unique signal of the bead and hence the specificity of the bound HLA molecule, while the second readout indicates whether or not antibody is bound to the specific HLA molecule.

The degree of fluorescence is expressed as a mean fluorescence intensity (MFI), which is normalized by taking into account the degree of fluorescence observed with an antibody negative serum and with beads to which no HLA molecule is attached. A positive control consists of beads bound with PE-labeled human IgG.

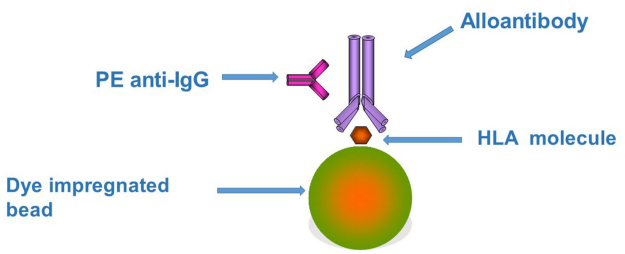

FIGURE 1 | The figure represents the principles underlying the Luminex bead assay. Each bead has one or more different types of human leukocyte antigen (HLA) molecules attached depending on the level of testing being performed. If the test serum contains an HLA antibody it will bind to the appropriate HLA molecule. This binding can be detected by the use of a second phycoerythrin (PE)-labeled anti-human IgG. Each bead gives a specific signal when excited by one of the lasers built into the Luminex instrument due to the unique intensity of fluorophore embedded in the bead. A second laser detects the fluorescent excitation produced by the PE on the second antibody. The combination of the two signals indicates first the presence (PE fluorescence) and second the specificity (bead fluorescence) of the HLA antibody in the test serum.

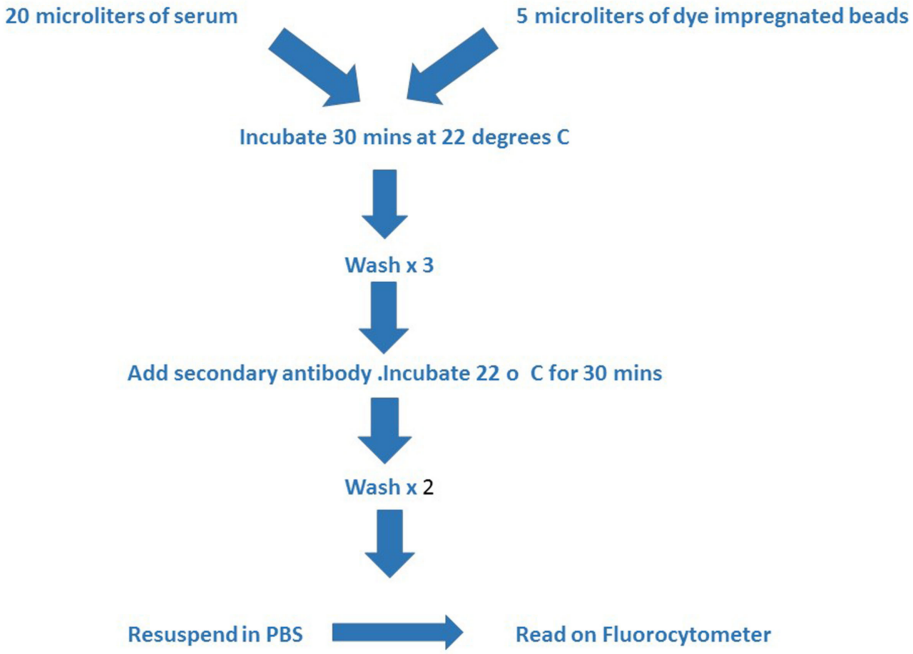

FIGURE 2 | **The figure outlines the technical steps involved in the assay**. The test serum and beads are incubated together at room temperature for 30 min and then washed three times with buffer prior to adding the second antibody. A second incubation period of 30 min at room temperature is followed by two further washes with buffer and then the mixture is resuspended in phosphate buffered saline for reading in the Luminex instrument.

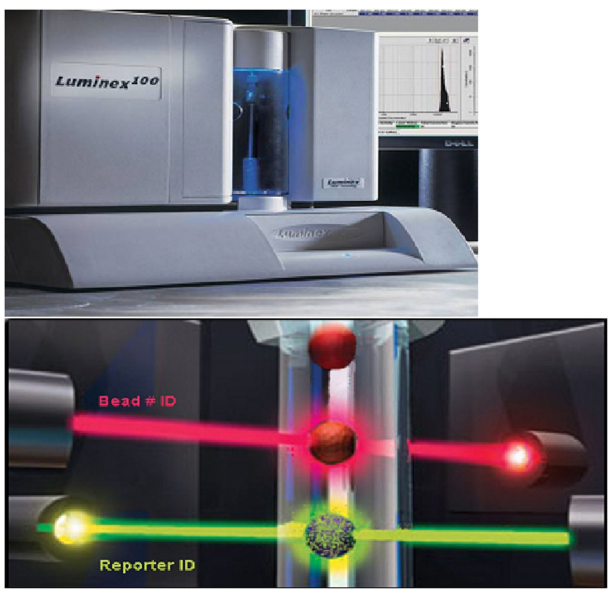

FIGURE 3 | **The top panel shows the Luminex instrument**. There are two lasers in the Luminex instrument (bottom panel). The red laser excites the fluorophore in the bead which provides a unique signal thereby identifying the HLA molecule attached. The green laser excites the phycoerythrin bound to the second anti-human IgG antibody indicating IgG antibody in the test serum has bound to the appropriate HLA molecule attached to the bead. (Modified from a figure provided by Serologicals Corporation.)

SUMMARY COMPARISON OF TECHNIQUES

The advantage of the CDC assay is that it is a functional test involving antibody containing serum and cells. As a crossmatch test it has proved invaluable over the years as a method of avoiding hyperacute rejection due to the presence of HLA-DSA in the recipient (3, 4). As an assay for screening patients for HLA antibodies it has drawbacks. First, it lacks the sensitivity of the other assays described, and second, the assignment of positive and negative reactions can be compromised by viability of the cells used. It also detects both IgG and IgM HLA antibodies in addition to autoantibodies and non-HLA antibodies against other cell surface determinants which have no relevance in organ transplantation.

In the context of organ transplantation, however, it does have the advantage of only detecting CB antibodies. The rationale for replacing this assay with the solid phase assays was driven primarily by the sensitivity issue and the realization that HLA antibodies positive by the solid phase assays but negative by CDC in some cases were clinically relevant (24, 26–29).

Before the introduction of the solid phase assays, the cell-based flow cytometry assay was introduced into clinical practice as a means of providing a more sensitive assay for detection of recipient presensitization to donor-specific HLA. The flow crossmatch however was not amenable to rapid turnaround times and therefore was used primarily in the

living related and living unrelated donor situation rather than for cadaveric donors.

The issue of whether the flow assay was as sensitive as the solid phase assays was the subject of debate for some time but the general consensus is that the bead assays are the most sensitive assay for detecting HLA antibodies albeit with their own unique problems (30).

With respect to the two main solid phase assays the fluorescent bead assay has become the gold standard for HLA antibody detection and is now used in most transplant testing laboratories. The remainder of the review will concentrate on the advantages of this technique, and the challenges facing both laboratory workers and transplant clinicians in interpreting the data generated by this assay.

ADVANTAGES OF THE LUMINEX® BEAD ASSAY

The Luminex® bead assay is a sensitive method for detection of HLA antibodies and represents the current highpoint in the evolution of HLA antibody detection assays. The additional sensitivity provided by this method has enabled the detection of HLA antibodies in potential transplant patients which are not detectable by other means, particularly CDC (24, 26–29). This increased sensitivity has enabled improvement in the success rate of retransplant patients due to the detection of HLA presensitization as a result of previous grafts and the subsequent avoidance of the relevant HLA specificities on second grafts particularly for DP specificities that are not detected by other methods (31).

The development of SAB has enabled the dissection and specificity determination of complex mixtures of HLA antibodies which is not possible with other techniques. This fine level discrimination coupled with the Matchmaker program (32) has enabled the description of epitope sequences to which HLA antibodies are directed (33–35). Armed with this information consideration of sequences of all alleles regardless of whether or not they are represented on the bead panel allows the identification of all HLA alleles to which a patient is immunized.

Obtaining HLA allele information on potential transplant recipients has permitted the identification of antibodies to alleles within the same antigen group. For example, an A*0301 antibody identified in an A*0302 renal transplant patient (36) and an A*2402 antibody in an A*2403 patient (37). The only coding sequence differences between A*2402 and A*2403 are located at positions 166 and 167 which are the unique substitutions within the epitope recognized by the A*2403 patient. Historically, the presence of A*2403 in a donor would have been considered an antigen match for an A*2402 patient yet it clearly represents a potential immunizing situation.

Identification of antibodies to DQA1, DQB1, DRB3, -4, -5, and DPB1 which is not possible using other antibody screening assays has been enabled by the use of beads containing these molecules. As a result it has become evident that antibodies to DQ and DP (38–42) in addition to DR coexist in organ transplant recipients and have been implicated in negative graft outcomes.

INTERPRETIVE CHALLENGES ASSOCIATED WITH THE LUMINEX® BEAD ASSAY

Many of the challenges in interpretation are described in a 2013 report of Consensus Guidelines by an expert Committee under the guidance of The Transplantation Society (43). In addition, reviews have appeared subsequently, which have further contributed to this topic based on more recent data (44, 45). Much of the data had been generated in renal transplantation but the technical issues apply equally to other forms of organ transplantation. The following outlines some of the major issues which require consideration when interpreting bead assay data.

Sensitivity

Although the bead assay represents the most sensitive method for HLA antibody detection one of the main challenges facing clinicians and laboratory scientists is the interpretation of positive results in the context of a negative CDC crossmatch and/or a negative flow crossmatch, and no indication of presensitization by any other screening technique. The question of the clinical relevance of these HLA antibodies in rejection has been reported in renal, heart, and liver transplantation with mixed results (46–52). Many factors impact on the clinical relevance of these detectable low-level antibodies, one being the MFI cut off for positivity used by the reporting center.

Mean Fluorescence Intensity

There is no recommended "cut off" value for MFI positivity. Most laboratories set their "cut off" level for positivity based on levels obtained with relevant controls and also on experience gained from clinical results obtained. A useful approach, particularly for multiparous females or previously grafted patients, is to consider each patient on an individual basis. For example, if a previously grafted patient has an MFI level for a particular HLA specificity to which they were exposed on the first graft and the MFI is above the negative values but below the "cut off" level established in the laboratory, this result should be treated with caution. It may indicate a state of presensitization with very low levels of antibody, the production of which can be reactivated on repeat exposure with a second graft bearing that antigen. Such a result may be interpreted differently in a patient with no history of potential HLA preimmunization.

Other factors such as the variable amount of target HLA present on the bead which can be locus and allele specific (53, 54) can result in variation of the MFI obtained. In the absence of an agreed standard for the performance of the Luminex® bead assay, it is incumbent on each testing laboratory to establish their own MFI "cut off" levels in consultation with their clinical colleagues.

Antibodies to Denatured HLA

The SAB are coated with HLA molecules produced by recombinant technology while the screening beads are coated with HLA molecules immune-precipitated from cell lines. As a result, the SAB express denatured molecules in combination with native molecules. The denatured molecules can express

cryptic epitopes not normally accessible by antibody molecules, and it is not possible to distinguish between these two types of antibodies. It would appear intuitive that since the antibody does not have access to the cryptic epitope that these antibodies will not be clinically relevant. However, a need existed for a means of distinguishing between antibodies to denatured and native epitopes. One manufacturer responded by introducing ibeads which are SAB expressing largely native HLA molecules. These beads however were removed from the market in 2014 and the manufacturer recommends as an alternative using an acid wash procedure.

Antibodies to these exposed cryptic epitopes on denatured molecules have been detected in individuals including non-transfused males (55, 56). Studies comparing antibodies to both denatured and native epitopes have demonstrated that the antibodies to denatured epitopes have no clinical impact in renal or heart transplantation (57, 58). Why antibodies to denatured epitopes are found in individuals, particularly non-transfused normal males, is a subject of debate. The concept of cross reactivity with environmental agents such as pathogens or ingested food has been suggested (56).

Complement-Fixing and Non-Complement-Fixing HLA Antibodies

Unlike the CDC assay which by definition only detects CB HLA antibodies, the bead assay is designed to detect both CB and NCB antibodies. This created debate concerning the NCB antibodies detected by the bead assay, and the concern that many patients were being denied a transplant on the basis of donor-specific NCB antibodies, the clinical significance of which was not established.

Several modifications have been made to the assay to distinguish between CB and NCB HLA antibodies. Using anti-IgG2 and anti-IgG 4 antibodies to detect NCB antibodies Arnold et al. (59) were able to show that up to 40% of re-transplant patients on the waiting list had either HLA class 1 or II NCB antibodies. Wahrmann et al. (60) modified the flow-based bead assay by adding normal serum as a source of complement and anti-C4d as a second antibody and found similar incidence results to Arnold (61).

Modification to the Luminex® method of detection was first described by Chin and colleagues (62). Their approach involved heating the test serum to denature complement and then to add purified human C1q to the serum prior to incubation with the beads. CB antibody binds the C1q and then is detected using a secondary PE-labeled anti-human C1q antibody. This is now the method most commonly used by testing laboratories to distinguish between CB and NCB HLA antibodies. A commercial C1q kit is now available which can detect CB antibodies using either beads in the Luminex system or cells, or for use with cell-based flow cytometry.

The historical association of CDC positive crossmatches with hyperacute or acute rejection led many to believe that CB antibodies detected by the C1q assay would be associated with rejection while NCB antibodies would not. The reality however is that the associations are not so clear cut. Recent studies examining the clinical associations of antibody-mediated rejection with C1q CB and NCB HLA antibodies have yielded some interesting observations.

Calp-Inal et al. (63) showed that the incidence of both acute and chronic rejection was increased in those with CB DSA HLA antibodies pretransplant compared with patients whose DSA were NCB antibodies. Guidicelli et al. (64) demonstrated that while *de novo* CB HLA antibodies were associated with rejection shortly after their appearance NCB antibodies were also associated with rejection in the long term. Piazza et al. (65) also demonstrated a strong association of CB antibodies detected in the C1q assay with inferior graft survival and also demonstrated that the incidence of CB antibodies was increased among those patients with HLA class II antibodies, particularly DQ.

By contrast, Thammanichanond et al. (66) were unable to show a significant effect of CB antibodies with rejection, albeit it in a relatively small cohort of patients. They did however show that the CB antibodies had higher levels of MFI than NCB antibodies.

Likewise, Taylor et al. (67) claim the interpretation of the C1q assay is confounded by the level of antibody, the amount of denatured HLA on the beads and the interference of complement. They further question the justification of its use given the uncertainty in interpretation and the additional cost involved.

There are points to be made with respect to these studies. First, when pretransplant sensitization is involved, in the overwhelming majority of cases the CDC donor crossmatch is negative and therefore lower strength CB antibodies are being selected, which will impact on the overall clinical impact. Second, it is known that CB IgM antibodies which are not detected in the conventional SAB assay can convert to IgG3 CB antibodies posttransplant and are detrimental to the graft (68), which can have a confounding effect on the data analysis when pretransplant antibodies are analyzed. Finally, it appears that NCB antibodies may impact to a degree on graft survival at least in the long term (49, 64).

Recently, a C3d assay has been described (69), similar in principle to the C1q assay, which measures C3d deposition by the addition of human serum to the single bead antigen/antibody complex, followed by the addition of an anti-C3D antibody labeled with PE. Sicard et al. (69) were able to demonstrate in a group of renal transplant patients tested at the time of diagnosis for rejection, patients testing positive for C3d had a higher risk of graft failure. Interestingly, the C1q assay failed to reach statistical significance as a predictor of graft failure.

Comoli et al. (70) in a recent paper presented results of posttransplant testing monitoring for the appearance of *de novo* donor-specific antibodies. Positivity in the C3d assay did not predict graft rejection at the first appearance of *de novo* DSA but at the time of rejection there was a strong correlation. They also demonstrated that conversion within a single antibody from NCB to CB, as demonstrated by C3d positivity, was associated with an increasing MFI. The apparent greater association of rejection with C3d than with C1q may be a reflection of the stage of the complement cascade at which these assays are focused. As indicated by Comoli et al. (70), the presence of C1q does not predict whether the complement cascade will proceed, or just result in C4 deposition on the cell surface. The downstream production of C3d may more accurately predict complete complement activation.

The Prozone Effect

One of the technical challenges of using SAB for HLA antibody detection is the prozone effect whereby a diluted serum gives a higher MFI than the undiluted serum, suggesting an inhibitory effect which can be abrogated by dilution. One explanation for the inhibitory effect is the presence of IgM antibody of the same HLA specificity blocking the binding of the IgG isotype (71). Since IgM antibodies tend to be a lower titer than IgG the dilution effect was consistent with this interpretation. However, recent research suggests that the effect is due to the inhibitory effect of the C3 component of complement, which is produced as a breakdown product of C1. C3 binds to the beads and inhibits the binding of IgG antibody present in the test serum (72). This problem can be overcome by pre-heating of the test serum to destroy any complement activity or by the addition of ethylenediaminetetraacetic acid (EDTA) to the wash buffer (73) or by the use of dithiothreitol (71) which is also used to eliminate IgM antibodies by disruption of disulfide bonds. The possible confounding effect of prozone should be always considered when interpreting results obtained from using variations of the SAB assay for detection of complement-fixing antibodies.

THE DONOR-SPECIFIC LUMINEX CROSSMATCH

In 2008, Billen et al. (74) reported on the use of commercially available beads for donor-specific crossmatching. The beads are coated with one of two mouse antibodies with specificity for a non-polymorphic sequence on either the class 1 or class 2 molecules. Using lysates of donor cells the beads are able to capture the class 1 and class 2 molecules of the donor which can then be reacted with recipient sera and the bound antibody, if present, labeled with a fluorescently labeled second antibody and read as per the conventional bead assay. Billen et al. compared the results obtained in a group of renal patients with the cell-based FCXM results. They demonstrated a sensitivity using the bead crossmatch of 89% for class 1 and 68% for class 2. Interestingly, they failed to detect antibodies to HLA-DQ and -DP antigens by the bead crossmatch which compromised the value of the LUXM as an alternative B cell crossmatch technique.

Billen et al. (75) further reported on a group of 165 CDC crossmatch negative patients, 32 of whom had bead positive crossmatches. There was no difference in acute rejection free survival when the CDC-bead + crossmatch group were compared with the CDC-bead − crossmatch group. However, the group of patients with bead positive crossmatches due to class 1 antibodies had an inferior long-term 5 years survival (41% compared with 70% for the crossmatch negative group). Positivity for class 2 antibodies had no effect.

Guillaume et al. (76) demonstrated that the LUXM can detect class 1 antibodies with an MFI as low as 2,300 in the SAB technique and 1,300 for class 2. They confirmed the failure to detect HLA-DP antibodies and in addition reported on the failure to detect HLA-C.

Recently, Chaidaroglou et al. (77) reported on a comparison of SAB, FCXM, and LUXM for detection of DSA in a group of heart transplant recipients. They found that there was good agreement between SAB and FCXM but not between LUXM and the other two techniques. They questioned the value of LUXM as a technique for prediction or monitoring.

It would seem based on published data to date that the LUXM cannot be recommended as a stand-alone method for organ allocation.

THE VIRTUAL CROSSMATCH

The introduction of solid phase assays and the realization that there were a number of patients whose antibodies were detectable by these methods but were negative by the CDC crossmatch cast some doubt on the complete reliance on the CDC crossmatch as a final test of recipient/donor compatibility. Since antibodies with MFI values between 10,000 and 20,000 are required to obtain positive T cell CDC crossmatches in approximately 90% of cases (78), there are clearly some cases where clinically relevant HLA antibodies which are not detected by the conventional crossmatch. The use of specific and sensitive methods for antibody detection and in particular the HLA SAB allowed for the first time a complete picture of the HLA immunization status of individual patients. From this the concept of a "virtual crossmatch" (VXM) was established (79). The VXM takes into account the HLA antibody profile of a patient and predicts which donors will be crossmatch negative. This approach has been used successfully in renal transplantation. Johnson et al. (80) have reported on a large cohort of patients where the final decision to transplant was based on SAB results rather the FCXM result. When analyzed for clinical outcome based on whether the FCXM was positive or negative, despite the fact the FCXM positive group were more "at risk" than the FCXM negative group, the transplant outcomes were comparable, justifying the use of the VXM in preference to the results obtained by FCXM.

Eby et al. (81) have demonstrated the value of the VXM in pancreas transplants as part of the United Network of Organ Sharing in the USA. Pancreata imported from Networks 3 and 4 and transplanted on the basis of VXM had a cold ischemia 5 h shorter than the cases where a FCXM was performed prior to transplant without any compromise in rejection or graft survival incidences.

More centers are expected to rely on the VXM as a prospective guide to the suitability of transplantation as data on the reliability of this procedure is accumulated.

USE OF THE ANTIGEN BEAD ASSAY – LESSONS LEARNED

The introduction of solid phase HLA antibody detection methods and in particular the bead assays have revolutionized the clinical management of sensitized patients. However, the introduction of this new technology has posed questions concerning the interpretation of generated data which still require resolution.

The "cut off" MFI values used for assigning positivity vary greatly between laboratories. How much this is due to technical variation and how much is based on correlation with clinical experiences of the local transplant center is difficult to ascertain.

Every testing laboratory must determine based on the local performance of the assay and from clinical experience a "cut off" that reflects the level at which antibodies are deemed to be clinically relevant. However in this context, it is imperative to consider the immunological history of the patient. Borderline values or values obtained with antibodies below the "cut off" may be reflective of an increased risk of rejection in patients who have been previously exposed to the particular antigen to which the antibody is directed either by pregnancy or previous grafts. One group of patients of particular interest are those who have undergone desensitization protocols. "Cut off" values therefore should be a guide and not rigidly enforced without careful consideration of the patients' histories.

The interpretation of HLA antibody results obtained with CB fixing assays requires careful interpretation. The prozone effect needs to be considered in patients who are known to be sensitized but test negative in the CB antibody assays. These patients should in addition be tested at a dilution or treated with EDTA or DTT prior to testing. HLA antibodies which test negative with the CB binding assays should not automatically be dismissed as clinically irrelevant. Although the data suggest in renal transplantation that NCB HLA antibodies are not as damaging to the graft as CB HLA antibodies, and tend to have lower MFI values, there are data which indicate that they do have a lower but nevertheless significant association with rejection. In the absence of convincing clinical data for NCB HLA antibodies in other solid organ transplants, they should also be treated with caution.

The relationship of positive bead assay antibody results with other assays is an important aspect of interpretation. There is universal agreement that an HLA antibody detectable by the bead assay which results in a positive CDC crossmatch is a contraindication to renal transplantation. However, a negative CDC crossmatch in such a situation is less clear cut. The MFI cut off for such a scenario is of the order of 10,000 below which the CDC crossmatch may be negative, but this may vary from laboratory to laboratory based on the sensitivity of the CDC crossmatch and the performance of the SAB under local conditions. Some centers have reported an increased risk of rejection in such patients, others have observed no effect. However, group analyses of such patient groups can mask individual patients whose negative outcome has been influenced by bead + CDC negative antibodies. Some centers recommend the use of a flow crossmatch in such patients. A negative flow and CDC crossmatch may be an indicator that transplantation can proceed. In most cases, this occurs in the presence of a low MFI by the SAB assay. However, the point must again be stressed that the interpretation of multiple assay results must occur in the context of the patient's history.

In some centers the CDC crossmatch has been replaced with the VXM. The inherent risk in this approach is that some patients will be denied a transplant in what would be a negative CDC situation and with HLA antibodies which may ultimately prove to be not graft damaging. The confounding factors such as prozone and denatured HLA on beads need careful consideration and analysis when relying on virtual crossmatching as the final pretransplant determinant of compatibility.

Published results using the LUXM crossmatch technique to date suggest that there are too many unresolved issues to recommend this technique as a stand-alone method for solid organ transplant allocation.

FUTURE DIRECTIONS

The introduction of solid phase assays ushered in a new era in antibody detection both in pre- and posttransplant patients. However, the question of B cell presensitization in patients whose primary antigenic stimulus was years previously and no longer have detectable circulating antibodies still represents a challenge. This problem was referred to briefly in the section on MFI where levels of antibody below the accepted level for positivity in previously grafted patients or multiparous females should be flagged as a potential problem as it may indicate a state of presensitization. Knowing the HLA genotype of previous transplant donors or the biological father of the multiparous patients' children can be useful in this regard.

Cardiac grafts in multiparous females with a negative CDC crossmatch have a higher incidence of rejection if the donor shares an HLA with the father of the patient's children (82) even when the primary immunizing pregnancy was up to 30 years previously, demonstrating the long-term effect of memory T and B cells. A comparable study considering HLA epitopes rather than broad antigens has not been reported.

More recently, Mulder et al. (83) used HLA tetramers to study the peptide dependency of HLA antibodies. This technology was utilized by Zachary et al. (84) to investigate the incidence of HLA class I memory B cells in patients with a history of HLA antibodies, but shown to be HLA antibody negative when testing current sera samples. Using tetramers labeled with PE and a labeled CD19 antibody to identify B cells, flow cytometry identified a percentage of cells which bound to the HLA tetramers. The incidence of bound tetramers was significantly greater in previously HLA immunized compared with non-immunized individuals indicating the presence of memory B cells.

A recent report using the ELISPOT assay and HLA class II biotinylated molecules has described the detection of HLA class II-specific memory cells (85) indicating this assay is a useful tool for identifying presensitization in the absence of circulating antibodies (86).

In addition to previously grafted patients and multiparous patients, the use of this technology has potential in monitoring patients who have undergone desensitization protocols prior to transplantation. Depending on the type of desensitization procedure used, it is useful to establish if memory B cells can be detected in patients whose circulating HLA antibody is no longer detected after treatment.

CONCLUSION

The introduction of solid phase and in particular bead-based assays, for detection of HLA antibodies has revolutionized clinical management of organ transplant patients. For the first time, laboratory scientists and clinicians are in a position to fully

reveal a patients immunological status. This technological breakthrough coupled with HLA sequencing data, which permits the identification of epitopes to which HLA antibodies are directed provides a unique opportunity to maximize the transplant success rate. This new found enthusiasm however is tempered by the fact that there are still areas of both technical and clinical contention which require resolution, such as the role of NCB HLA antibodies and the detection of antibodies to denatured HLA antibodies and their role if any in graft rejection. With this rapid rate of evolution of HLA antibody testing technology, it is imperative that laboratory-based scientists and clinicians communicate on an individual patient basis. Having regard to the immunological history of the patient when interpreting HLA antibody results is critical in maximizing the positive clinical impact of this technology.

AUTHOR CONTRIBUTIONS

The sole author was totally responsible for the design and writing of the review.

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MINI REVIEW
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Significance of Anti-HLA Antibodies on Adult and Pediatric Heart Allograft Outcomes

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As methods for human leukocyte antigens (HLA) antibody detection have evolved and newer solid phase assays are much more sensitive, the last 15 years has seen a renewed focus on the importance of HLA antibodies in solid organ transplant rejection. However, there is still much controversy regarding the clinical significance of antibody level as depicted by the mean fluorescence intensity of a patient's neat serum. Emerging techniques, including those that identify antibody level and function, show promise for the detection of individuals at risk of allograft rejection, determination of the effectiveness of desensitization prior to transplant, and for monitoring treatment of rejection. Here, we review current publications regarding the relevance of donor-specific HLA antibodies (DSA) in adult and pediatric heart transplantation (HT) with graft survival, development of antibody-mediated rejection and cardiac allograft vasculopathy (CAV). The negative impact of DSA on patient and allograft survival is evident in adult and pediatric HT recipients. Many questions remain regarding the most appropriate frequency of assessment of pre- and posttransplant DSA as well as the phenotype of DSA memory vs. true *de novo* antibody using large multicenter adult and pediatric cohorts and state-of-the-art methodologies for DSA detection and characterization.

Keywords: AMR, heart transplantation, donor-specific antibodies, allograft vasculopathy, graft failure

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INTRODUCTION

Heart transplantation (HT) has become an accepted therapy for adult and pediatric patients with end-stage heart failure. Despite improved immunosuppression regimens, rejection remains the most common cause of death in the first 5 years after HT. Both cellular and humoral immune-mediated processes that can damage the allograft are primarily directed against human leukocyte antigens (HLA). Antibodies against HLA can be found in patients prior to transplantation after exposure to foreign HLA through blood transfusion, pregnancy, previous transplant, and use of homograft tissue during surgery for some congenital heart defects. Ventricular assist devices (VAD) have also been implicated in the development of HLA antibodies, termed allosensitization. Exposure to donor HLA after HT may also induce *de novo* production of donor-specific HLA antibodies (DSA). The impact of circulating HLA antibodies on heart allografts has been the focus of many investigations and reviews. The introduction of solid phase assays (SPA) based on the luminex single antigen bead assay (SAB) has improved the

sensitivity and specificity of HLA antibody detection; however, it also introduced new challenges for assay interpretation and determining its clinical relevance (1).

Identification of DSA enables the clinician to make informed decisions regarding acceptance of the organ and the choice of immunosuppression (2). Presence of DSA is not always considered a contraindication but rather a risk factor for organ transplantation success (3). Optimizing transplantation of allosensitized candidates is challenging and program specific. The main challenge with the new SPA technology is decision-making regarding donor organ acceptance based solely on antibody strength determined by mean fluorescence intensity (MFI) (2–5). The threshold for accepting a donor for a sensitized patient may vary depending on the patient's clinical status, antibody level, and protocols available for antibody removal therapy. Considering the SPA modification to detect complement-fixing antibodies (C1q-SAB) has reduced the estimated incompatible donor pool in highly sensitized patients (6). Optimizing transplantation of allosensitized candidates using SAB and C1q-SAB methodology to prioritize the assignment of unacceptable antigens has allowed transplantation of highly allosensitized patients across the DSA barrier with survival rates comparable to DSA− heart transplant recipients (5).

Titration of sera prior to SAB testing has emerged as a more accurate way to assess the true level of DSA as compared to MFI value of undiluted sera (7). Furthermore, titration studies provide better estimates of responsiveness to antibody removal therapies (8).

Recognition that some preformed antibodies are against denatured HLA antigens with very little clinical relevance may also impact the search for an acceptable donor (4, 9). The assignment of unacceptable antigens has been greatly improved also by incorporating patterns of epitope reactivity and history of sensitizing events. Recognizing the limitations and advantages of current available methods for antibody determination, quantitation and function has facilitated the introduction of the virtual crossmatch (VXM) in thoracic transplantation. Previously, the need for prospective crossmatch (XM) in sensitized patients was associated with longer waitlist duration and increased mortality (10). Although VXM is widely used for organ allocation, its validity highly depends on how accurate and current is the information on patient sensitization events and comprehensive DNA-based HLA typing of prospective donors as antibodies can be made against every possible polymorphic HLA target antigen (2–5).

RELEVANCE OF DSA ON OUTCOMES

In this report, we focus on a short review of the current state-of-the-art regarding the role of DSA in adult and pediatric HT as determined by the following outcome measures: graft survival (GS), development of antibody-mediated rejection (AMR), and cardiac allograft vasculopathy (CAV) (**Tables 1** and **2**). Although we limit this review to the last 6 years, the retrospective nature of some studies may influence the relevance of DSA on clinical outcomes due to the use of less sensitive testing methods. Furthermore, we considered separately the role of DSA on adult and pediatric clinical outcomes to highlight potential similarities and differences in the two cohorts.

ADULT HT

Graft Survival

The prevalence of allosensitization in heart transplant candidates increased with the introduction of SPA for screening for HLA antibodies (11) (**Table 1**). Nevertheless, the risk for poor GS has remained a significant finding even in the more sensitive SPA testing era (11, 12). The presence of non-cytotoxic HLA antibodies identified by SAB was associated with high risk of death, early graft failure, and late cellular- and antibody-mediated rejection; these findings underscore the need for using sensitive Luminex platform SPA to accurately determine the presence of circulating HLA antibodies (12). Detection of Class I DSA pretransplant was a predictor of short-term but not long-term survival as compared to non-DSA (13). In this study limiting the testing on pretransplantation, the authors could not identify the impact of persistent vs. transient DSA and of *de novo* DSA on clinical outcomes.

De novo antibody production and its role in cardiac allograft survival has been described in several studies (14–16). In a retrospective adult cohort, *de novo* DSA was associated with poor patient survival (HR = 3.198), while *de novo* and persistent DSA was worst (HR = 4.351) (14). Similarly, patients with persistent *de novo*, mostly Class II DQ-specific DSA, had worse survival (15). The 15-year survival was highest in patients who never developed DSA vs. those that developed DSA posttransplantation (70 vs. 47%), and patients with late *de novo* DSA appearing more than 1 year post transplantation had poorest survival (16). Thus, determining the presence of DSA pretransplant for risk assessment and monitoring for persistent and *de novo* DSA posttransplant provide the most comprehensive information for clinical management.

Antibody-Mediated Rejection

The challenges of AMR diagnosis post HT have been addressed by many single-center studies and consensus conferences. In the current era, diagnosis of this clinically important entity has been improved by standardized classification of histologic and immunologic changes in endomyocardial biopsies (EMB) (17) and by advances in the detection of HLA antibodies. Although not required for diagnosis of pathologic AMR (pAMR), the detection of HLA antibodies pre- and post-transplantation has been helpful for risk stratification for the development of AMR and for guiding treatment strategies (18). Patients with positive VXM defined in the presence of DSA >1,500 MFI by SAB had a higher incidence of AMR and cell-mediated rejection. Similar outcomes were observed with positive flow crossmatch (FXM) suggesting that SAB MFI >1,500 can be used as surrogate for FXM (19). Increased risk for a positive complement-dependent cytotoxicity (CDC) XM and early AMR was observed in patients with persistent C1q+ DSA (20). However, patients who had DSA but lost the C1q reactivity posttransplant did not develop early AMR, and the strength of neat sera on SAB did not predict C1q reactivity. In contrast, high titer DSA (>1:16) has been associated with

TABLE 1 | Cited publications from the last 6 years (2010–present) showing the impact of HLA antibody on heart transplantation in adult recipients.

Reference	Number of patients (study period)	Method	DSA	GS	AMR	CAV	Comments
Gandhi et al. (19)	85 (August 2006–January 2010)	CDC-AHG PRA/XM, Flow XM, SAB	All CDC XM–; DSA+ (MFI >1,500), *n* = 11 (13%): Class I = 2, Class II = 6, Class I + II = 3		(*n* = 80 for biopsy) AMR: 7/11 DSA+		CMR ≥ 1R/1A: 9/11 DSA+ vs. 48/69 DSA–/weak; DSA MFI >1,500 associated with increased incidence of AMR and CMR
Smith et al. (14)	243 (October 1995–July 2004)	SAB (8.8 ± 2.5)	57 dnDSA Class II = 48 (42/48 DQ)	Poor GS $p = 0.0001$ (HR = 4.35)		29% 5 y; 55% 10 y	DnDSA risk for poor GS and CAV
Ho et al. (16)	950 (January 1995–December 2009)	CDC T and B, SAB (mean number of sera tested per patient = 24 ± 9)	221 dnDSA 1 y, 118 dnDSA >1 y, 460 no HLA-Ab	GS 52%, $p < 0.005$; GS 48%, $p < 0.001$; GS 70%	23		DSA and non-DSA increased in rejection
Loupy et al. (29)	196 (1985–2009)	SAB	20 very late rejection (VLR >7 y)			CAV grade VLR, 2.06 vs. 0.76 in control	VLR associated with severe CAV
Hodges et al. (15)	762 (November 2005–August 2011)	Luminex Screen, SAB	15 AMR (14/15 dnDSA)	1.8 y mean survival after AMR treatment	15		Late cardiac AMR with dnDSA
Zeevi et al. (20)	15 (8 pediatric, 7 adult)	SAB, SAB-C1q	35 DSA in 14 patients: Class I = 4, Class II = 2, Class I + II = 8		1st month post-Tx: 7/7 cAMR+ are DSA+/C1q+; 4 cAMR-free, DSA+/C1q– ($p < 0.005$)		Persistent C1q+ DSA post-Tx associated with early clinical AMR
Potena et al. (11)	173 (2000–2005)	CDC/PRA, Luminex Screen	Pre-Tx 32 Ab+ Class I = 28, Class II = 16, Class I + II = 12	Survival 65% for Ab+ 82% for Ab–	9/37 with biopsy were HLA-Ab+, pAMR >2		
Raess et al. (13)	272 (1989–2010)	CDC-PRA/XM, Luminex screen, SAB, SAB-C1q	DSA 26 (9.6%), Class I = 14, Class II = 5, Class I + II = 7, C1q+ DSA = 2	Overall survival: 80% (1 y), 68% (5 y) SAB Class I DSA+: 62% (1 y), 50% (5 y) SAB Class I DSA–: 87% (1 y), 73% (5 y)	Fatal pAMR = 6, all ≤1 month post-Tx	(*n* = 245) CAV-free survival: 96% (1 y), 86% (5 y)	ACR-free survival: 38% (1 y), 30% (5 y); pre-Tx HLA Ab status affected short-term survival but had no effect on long-term survival/rejection
Topilsky et al. (27)	51 (January 2004–December 2009)	SAB; Flow XM for 30 patients	All CDC-XM–; DSA+ 17 (33%): Class I = 4, Class II = 11, Class I + II = 2			36 (71%) with Grade 1 CAV	CAV analysis done for patients with only Class II DSA; pre-Tx Class II DSA may give higher risk of accelerated CAV: DSA+ 100% vs. DSA– 64.2% at 4 y
Tible et al. (22)	111 (October 2009–September 2010)	SAB, 150 paired DSA and EMB	47/150 DSA+, Class I = 40.4%, Class II = 40.4%, Class I + II = 19.2%		37		MI and CD68 associated with DSA+
Frank et al. (28)	109 (February 1996–June 2011)	SAB, 330 paired DSA and EMB	51/112, Class I = 5, Class II = 26, Class I + II = 20			24 (22%): 40% DSA+, 13% DSA–	33% with CAV pre-Tx DSA+; Class II DSA, IF C4d+, and MI high risk for failed allograft with CAV

(*Continued*)

TABLE 1 | **Continued**

Reference	Number of patients (study period)	Method	DSA	GS	AMR	CAV	Comments
Coutance et al. (24)	20 (November 2006–February 2013)	Luminex Screen, SAB	19/20 tested were dnDSA+	50% after 1 y	Late AMR (>1 y post-Tx)		Prognosis for late AMR poor despite aggressive therapy
O'Connor et al. (12)	12,858 (June 2004–March 2013); UNOS database	CDC-PRA, Flow-PRA	PRA ≥ 10%, Class I: CDC+ = 227, Flow+ = 2,243, Class II: CDC+ = 126, Flow+ = 2,218	PRA ≥ 10%: HR = 1.24 (95% CI 1.12–1.36)			Percent Ab+ patients increased from 2005 to 2011 as use of flow increased; pre-Tx PRA ≥ 10% by Flow associated with increased risk of graft loss
Svobodova et al. (21)	264 (April 2005–December 2012; mean follow-up 39 months, range 19–66)	CDC-PRA/XM; SAB, SAB-C1q	DSA = 28 (11%): Class I = 18, Class II = 3, Class I + II = 7, C1q+ DSA = 4	90% (1 y), 79% (5 y)	19 (7%)	31 (12%)	74 patients (28%) with 83 instances of ACR grade ≥ Banff 2; pre-Tx DSA and elevated peak CDC-PRA were strongest predictors of AMR
Frank et al. (23)	44 (2005–2011)	SAB-C1q paired with EMB C4d stain	C1q+ DSA in 82% with graft dysfunction	18/44 died or retransplanted	16/17 C4d+ IF had C1q+ DSA; 24 C1q+ DSA were C4d-IF		Better concordance of C4d+ IF with C1q DSA as compared to IgG DSA
Loupy et al. (25)	40, failing grafts	SAB			AMR = 19		
Clerkin et al. (26)	689 (January 2004–December 2013, follow-up through October 2015)	Luminex SAB and/or CDC screen	Overall: $n = 29$ (42.6%); early AMR: $n = 22$ (51.1%); late AMR: $n = 7$ (28.0%)	Decreased post-AMR survival in patients with late vs. early AMR: 80 vs. 93%, 1 y; 51 vs. 73%, 5 y ($p < 0.05$)	$n = 68$ (9.9%): 43 early (<1 y post-Tx), 25 late (>1 y post-Tx)	No difference in prevalence early AMR vs. late AMR ($p = 0.51$); accelerated *de novo* CAV in late AMR + graft dysfunction (50% at 1 y, HR = 5.42, $p = 0.009$)	Graft dysfunction increased in late AMR group (56.0 vs. 25.6%, $p = 0.01$)

Ab, antibody; ACR, acute cellular rejection; AHG, anti-human globulin; AMR, antibody-mediated rejection; C1q, complement component 1q; C4d, complement component 4d; CAV, cardiac allograft vasculopathy; CDC, complement-dependent cytotoxicity; CMR, cell-mediated rejection; XM, crossmatch; DSA, donor-specific HLA antibodies; dnDSA, de novo donor-specific HLA antibody; EMB, endomyocardial biopsies; GS, graft survival; HR, hazard ratio; IF, immunofluorescence; MFI, mean fluorescence intensity; MI, microcirculation inflammation; pAMR, pathologic AMR; post-Tx, posttransplant; PRA, panel-reactive antibodies; pre-Tx, pretransplant; SAB, Luminex single antigen bead assay; VLR, very late rejection; y, year(s); HLA, human leukocyte antigens.

TABLE 2 | Cited publications from the last 6 years (2010–present) showing the impact of HLA antibody on heart transplantation in pediatric recipients.

Reference	Number of patients (study period)	Method	DSA	GS	AMR	CAV	Comments
Rossano et al. (31)	3,534 (October 1987–May 2004, follow-up through May 2008), UNOS database	CDC-PRA/XM most commonly used	PRA >10% = 387 (11%); 9% XM+	Median graft survival PRA >10% = 7.1 y PRA 1–10% = 9.6 y PRA 0% = 9.8 y			Decreased long-term GS in patients with PRA >10%
Irving et al. (38)	59, mean post-Tx follow-up 5.1 y (range 0.7–18.5 y)	Luminex screen/SAB	*N* = 4 (7%): 1 transient Class I, 3 persistent Class II	DSA+: 1/4 functioning, 2/4 retransplanted, and 1/4 died (7 y post-Tx)	DSA+: 2/4 (50%); non-DSA+: 1/15 (7%); no Ab: 5/40 (13%)	DSA+: 3/4 (75%); non-DSA+: 1/15 (7%); no Ab: 3/40 (7.5%)	Severe cellular rejection (≥3R) *n* = 3 (5.1%), all DSA–
Chin et al. (42)	18 (June 2007–February 2009)	CDC-XM, SAB, SAB-C1q, Flow CXM	SAB-IgG DSA: Pre-Tx 61.1%, Post-Tx 55.5%; SAB-C1q DSA: Pre-Tx 21.4%, post-Tx 35.7%	94% (1 y), 82% (2 y)	Within 1st month: *n* = 5 (27.7%), all post-Tx SAB-C1q+ DSA		SAB-C1q assay may better predict early AMR
Mahle et al. (32)	1,904 (January 1993–December 2008) Pediatric Heart Transplant Study Group	CDC-PRA most commonly used	PRA ≥ 10% = 397 (15.8%); PRA ≥ 50% = 189 (7.6%)	1 y patient survival: PRA ≥ 50%, 73 vs. 90% for PRA <10%		No CAV association with pre-Tx Ab	No association of PRA with time to 1st rejection or CAV
Ho et al. (16)	108 (January 2000–December 2009)	CDC-PRA, SAB	PRA >10% Class I = 9% Class II = 14%	87% GS in CDC– vs. 33% CDC+ after 7 y			Correlation between AMR and presence of CDC- or SPA-detected DSA
Scott et al. (34)	101 (2004–2008)	CDC-PRA, FLOW		PRA >25% decreased GS vs. patients with PRA <25%	*n* = 12: 33% with PRA >80% vs. 13% with PRA <80%		
Peng et al. (44)	60 (October 2005–January 2011)	FLOW-PRA, SAB, 183 paired DSA and C4d			6 (3/6 XM+)		Correlation between C4d+ in EMB and DSA >6,000 MFI
Daly et al. (58)	134 (January 1998–January 2011)	CDC-AHG PRA, Luminex SAB; XM+ patients received preoperative plasmapheresis + IVIG	12 XM+ (9%) T+/B+ = 8 T–/B+ = 2 T+/B not tested = 2	No significant difference in GS for XM+ (*n* = 3, 25%) vs. XM– (*n* = 12, 10%)	1 yr post-Tx: XM+ = 6 (50%), XM– = 2 (2%) ($p < 0.001$)		Serious infection higher in XM+ vs. XM– (50 vs. 16%, $p = 0.005$); shorter time to 1st infection in XM+ ($p = 0.001$)
Asante-Korang et al. (35)	70 (January 2005–July 2013)	Luminex PRA, SAB, Flow-XM; desensitization performed in patients with PRA >10%	PRA >10% = 14 (20%)	Overall patient survival: 92.9% in sensitized group vs. 80.4% in non-sensitized	Freedom from AMR or rejection grade ≥2R/3A: 71.4% in sensitized vs. 64% in non-sensitized	Freedom from CAV: 93% for sensitized vs. 91% in non-sensitized	12/14 high PRA patients had reduced Ab levels following desensitization; no significant differences in outcomes between desensitized patients and those with no Ab

(*Continued*)

TABLE 2 | Continued

Reference	Number of patients (study period)	Method	DSA	GS	AMR	CAV	Comments
Chen et al. (36)	25 (January 2008–June 2010)	PRA and SAB, 195 samples	12/25 dnDSA	No impact short-term survival			Majority of dnDSA within 1 y
Irving et al. (47)	108 (1996–2009)	SAB, 691 samples	43 DSA (58% persistent) Class I = 30% Class II = 47% Class I + II = 23%	9/14 with graft loss had persistent DSA		9/10 with CAV DSA+; 6/9 DSA persistent	Persistent DSA associated with poor outcome and CAV
Godown et al. (39)	121 (1987–2014), mean follow-up 4.1 y	Flow, Luminex, all were XM–	dnDSA: 40 (33%) Class I = 24% Class II = 50% Class I + II = 26%				Multiple factors influence DSA development; DSA seen more frequently in patients with prior sensitizing events
Ware et al. (43)	66 (January 2009–September 2013)	SAB	27 DSA+ (4 XM+)	No impact	DSA level associated with pAMR2, 3	No impact	Negative predictive value of DSA testing for absence of pAMR
Tran et al. (37)	105 (January 2002–December 2012, follow-up 0.13–10.8 y)	SAB (5 times first year and yearly after)	45 (43%) DSA Class I = 20% Class II = 62.2% Class I + II = 17.8%	5 y GS 72.4% DSA– vs. 21% DSA+		CAV 36% DSA+ vs. 13% DSA–	DSA+ had 2.5 times more rejection events per year compared to DSA–
Thrush et al. (40)	1,596 (January 2010–December 2014), Pediatric Heart Transplant Study database	Unknown		33 deaths (16%) post-AMR development; short-term patient/GS lower for patients with treated AMR ($p = 0.004$, $p = 0.001$, respectively); patient survival post-AMR diagnosis: 88% 1 y, 77% 3 y	179 (11%), freedom from AMR: 88% 1 y, 82% 3 y		AMR often concurrent with ACR

Ab, antibody; ACR, acute cellular rejection; AHG, anti-human globulin; AMR, antibody-mediated rejection; C1q, complement component 1q; C4d, complement component 4d; cAMR, clinical AMR; CAV, cardiac allograft vasculopathy; CDC, complement-dependent cytotoxicity; XM, crossmatch; DSA, donor-specific HLA antibodies; dnDSA, de novo donor-specific HLA antibody; EMB, endomyocardial biopsies; GS, graft survival; HR, hazard ratio; IF, immunofluorescence; pAMR, pathologic AMR; post-Tx, posttransplant; PRA, panel-reactive antibodies; pre-Tx, pretransplant; SAB, Luminex single antigen bead assay; SPA, solid phase assays; y, year(s); MFI, mean fluorescence intensity; HLA, human leukocyte antigens.

complement-fixing reactivity (7, 20) and has been used to determine unacceptable HLA antigens for sensitized candidates (5). DSA determination by SPA and elevated peak panel-reactive antibodies (PRA) were independent predictors of pAMR in an adult cohort of heart transplant recipients (21). In this study focusing on pretransplant samples, increasing numbers of DSAs and the mean cumulative MFI of DSAs were associated with risk of AMR, and the subset of C1q-reactive DSAs were less informative (21). Pathologic classification of AMR in 37 EMB correlated with circulating DSA and endothelial activation (22). The proportion of DSA+ EMB varied according to pAMR grade, and pAMR2 was associated with 100% DSA positivity (22). The clinical significance of DSA level as depicted only by MFI of neat serum is still controversial, and currently multiple approaches are proposed to capture the DSA level and function, including serum titration and complement-binding assays. A better concordance was observed between C1q+DSA and C4d immunofluorescence (IF)+ staining in EMB as compared with total IgG DSAs and C4d IF+ in EMB among 44 recipients (40 vs. 24%, $p = 0.02$) (23). A majority (82%) of patients with graft dysfunction had circulating C1q+ DSAs (23). However, not all patients with circulating C1q+DSA had C4d IF+ staining on EMB, suggesting that the presence of C1q+DSA may precede the development of pAMR or be due to the low sensitivity of C4d IF staining (23).

Prognosis after late AMR (defined as AMR >1 year post-transplant) was poor in 20 recipients despite aggressive treatment with immunosuppression, and fulminant CAV was a common condition (24). DSA was present in all tested patients ($n = 19$) with a median cumulative MFI at diagnosis >10,000; most of the patients had *de novo* DSA (24). Antibody-mediated injury and immune-mediated coronary arteriosclerosis were the causes of late graft failure in a recent study of 40 explanted heart allografts (25). AMR was observed in 47.5% failing heart allografts, including 40% of patients in whom unrecognized previous episodes of subclinical AMR occurred years before allograft loss. Among the 19 patients with AMR, 15 were tested for DSA, and 93% had circulating DSA at the time of allograft failure. The immunodominant DSA was Class II in 11/14 DSA+, and the median DSA MFI was 5,000 (25). In contrast, only 37% of patients without AMR features at the time of allograft failure had circulating DSA as compared to the AMR group ($p < 0.001$), and the median DSA MFI was 1,250 (25). In a retrospective cohort study spanning over 10 years, the timing of AMR (early vs. late) was associated with GS and CAV (26). Patients were tested at the time of biopsy for circulating DSA either by CDC (before 2010) or SAB (post 2010). Graft dysfunction was less frequent in early AMR, while late AMR with graft dysfunction showed rapid development of *de novo* CAV despite aggressive treatment and also increased risk of death (26).

Cardiac Vasculopathy

Cardiac allograft vasculopathy continues to remain a limiting factor in long-term survival of heart transplant recipients, and there is increasing evidence of the negative impact of circulating DSA on the development and severity of CAV. Patients with DSA had significantly higher rates and a shorter mean time to CAV and increased severity of CAV as compared to patients without DSA (27, 28). Patients with very late rejection and circulating DSA with evidence of intravascular macrophages had an increased risk of severe CAV as compared to patients without DSA (29).

PEDIATRIC HT

Graft Survival

Allosensitization and GS in pediatric HT recipients have been evaluated in large single- (30) and multicenter datasets (31, 32) (**Table 2**). Pediatric patients with PRA >10% had earlier-onset graft vasculopathy (30) and worse graft and patient survival than did patients with PRA <10% (31, 32). Elevated PRA was an independent risk factor for worse long-term GS (31). Furthermore, significant allosensitization (PRA >50%) at listing was associated with a more than twofold increased risk of death within the first transplant year (32). These large patient cohorts that were transplanted over a period of 18 years may have underestimated the rate of allosensitization because the methodology for PRA screening evolved from a less sensitive cell-based method to the more sensitive SPA. In addition, the SPA may have also increased the need for prospective XM due to an increased use of VXM (33).

In a more recent study patients with PRA >25% had significantly ($p = 0.004$) decreased survival compared to those with PRA <25% (34). In contrast, the outcome of allosensitized pediatric patients with PRA >10% who were desensitized was not different than non-sensitized recipients (35).

Assessments of GS in the presence of DSA show somewhat mixed findings, perhaps related to the duration of follow-up. Although short-term GS was not impacted by the presence of DSA in one pediatric study (36), the 5-year survival was significantly better in patients without DSA in another pediatric cohort (72 vs. 21%) (37). While uncommon, the presence of *de novo* DSA posttransplantation, especially toward Class II HLA, was associated with increased graft loss (38).

Multiple factors appear to play a role in development of *de novo* DSA in pediatric HT including prior sensitizing events, older age, African-American race, and donor death from gunshot wound (39). Knowledge of risk factors for the development of *de novo* DSA in pediatric recipients is likely to be important to guide the frequency of monitoring for HLA antibodies (39).

Antibody-Mediated Rejection

Current understanding of AMR after HT is largely derived from adult studies. Using the Pediatric Heart Transplant Study database, the reported incidence of AMR was 11% (among 1,596 recipients), and patient and GS were lower for those with AMR (40). Risk factors associated with AMR included PRA >10% at HT, a positive CDC XM, and congenital heart disease, suggesting allosensitization related to the use of homografts (40).

The proportion of AMR-free patients was much higher among patients with only solid phase-detected DSA vs. those with CDC-detected DSA (41). Similarly, using the C1q assay, which detects only complement-fixing antibodies, the presence of C1q fixing DSA prior to or early after HT had a positive predictive value of

100%, while absence of C1q fixing DSA had a negative predictive value of 100% for AMR (42). In another analysis, the presence of circulating DSA had 93% sensitivity, 62% specificity, 24% positive predictive value, and 99% negative predictive value for biopsy diagnosis of AMR in pediatric recipients (43). In addition, higher levels of circulating DSA measured by MFI correlated with pAMR severity (43). The authors proposed that DSA monitoring provides a non-invasive tool to tailor the frequency of biopsy surveillance (43). Others have used an institution-specific MFI threshold value for DSA of >6,000 that strongly correlated with C4d deposition on EMB with high negative predictive value (97%) and specificity (95%) (44). Both studies emphasized the advantage of following DSA in asymptomatic pediatric patients, given the value of early detection of AMR (43, 44).

Similar to findings for renal transplantation (45), sensitized recipients with persistent posttransplant DSA with complement-fixing ability appear to be at high risk for AMR (20, 42, 46).

Cardiac Vasculopathy

Overall, DSA+ patients (preformed or *de novo*) had significantly higher rates of CAV compared with DSA− patients. By 5 years, the rate of CAV-free survival was 25% for DSA− vs. 0% for DSA+ (37). Persistent DSA was associated with poor outcome and development of CAV (47).

TREATMENT

Desensitization is aimed to increase the donor pool by either reducing or eliminating HLA allosensitization or by facilitating transplant by reducing the DSA burden. Desensitization treatment targets critical components of the humoral response to either achieve a negative crossmatch pretransplant or to reduce the impact of DSA in positive crossmatch transplants. At low titer, antibody reduction can be achieved with plasma exchange and IVIG. The use of B cell suppression agents (rituximab), plasma cell depletion agents (bortezomib), or inhibitors of complement activation (eculizumab) is usually limited to highly sensitized patients. The current literature in adults is not abundant, mostly observational, with small cohorts, short follow-up, and with inconsistent treatment methodologies (48–53). In 21 highly sensitized patients, the use of plasmapheresis (PP), IVIG, rituximab, and cyclophosphamide resulted in comparable long-term survival when compared to the low sensitized and unsensitized cohorts (53). A recent experience with bortezomib and PP showed that about 50% of the patients had a calculated PRA reduction and were transplanted with a negative crossmatch (48, 52). One year follow-up showed 100% survival and 74% freedom from rejection (48, 52). In a smaller cohort of patients transplanted across a positive crossmatch and treated with eculizumab and ATG, 1-year survival was 89%, and freedom from rejection was 75% (52). In patients treated for AMR, Class I HLA antibodies demonstrated a statistically significant response to bortezomib, whereas Class II responded poorly (51).

In pediatric HT, requiring a negative prospective crossmatch increases the waiting time and more importantly the waitlist mortality (10). Allosensitization is most significant among children with certain forms of congenital heart disease due to the use of homograft during prior surgeries. Also, blood transfusions and VAD use are common causes of allosensitization. Current literature for pediatric heart transplant desensitization is even more limited than in the adult cohort (54–58). Desensitization was carried out successfully with bortezomib and PP in a pediatric setting (54). Furthermore, in a single-center retrospective study in a large cohort of patients, all sensitized patients received PP or plasma exchange preoperatively. If the cytotoxic XM was positive, PP was continued. Patients with negative XM did not receive additional PP and IVIG posttransplant (58). Hemodynamically significant AMR occurred in 50% of patients transplanted across a positive XM vs. 2% of the XM-negative cohort (58). Additionally, incidence of serious infection was higher in patients transplanted across a positive crossmatch (58). Antibody depletion therapies were also used in management of AMR in pediatric patients. Decreased DSA MFI in 21 patients treated with PP correlated with good clinical outcome (55). In another small study, addition of bortezomib to PP and rituximab treatment resulted in a rapid decline in DSA and reversal of AMR without significant side effects (56).

SUMMARY

The negative impact of DSA on patient and allograft survival is evident in adult and pediatric HT recipients. Allosensitization depicted by PRA >10% using cell based (prior era) or SPA (current era) is associated with poor outcome in both cohorts. Furthermore, similar risk factors were identified in adults and pediatric recipients for the development of posttransplant DSA including sensitizing events pretransplant, ECMO, need for mechanical support, non-compliance, and African-American race. In adults, but not in pediatrics, female gender (prior pregnancies) was also associated with a higher risk for development of *de novo* DSA. In children, exposure to homografts as part of surgical repair for some forms of congenital heart disease increase their risk for allosensitization and AMR.

Many questions remain regarding the most appropriate frequency of assessment of pre- and posttransplant DSA as well as the phenotype of DSA memory vs. true *de novo* antibody using large multicenter adult and pediatric cohorts and state-of-the-art methodologies for DSA detection and characterization. The observation that early vs. late AMR in HT may have different prognosis and responses to treatment emphasizes the need to assess the risk of sensitization pretransplantation and to follow by routine monitoring of DSA posttransplant.

The ongoing multicenter clinical collaborative studies supported by National Institute of Health in adult and pediatric HT will hopefully provide answers to many remaining questions regarding the impact of preformed and *de novo* DSA on clinical outcomes and the efficacy of various modalities for desensitization and treatment of AMR.

AUTHOR CONTRIBUTIONS

This is an invited review on impact of HLA donor-specific antibody in cardiac allograft outcome. All authors contributed equally to literature review, summary, and manuscript preparation.

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REVIEW
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Unraveling the Role of Allo-Antibodies and Transplant Injury

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Alloimmunity driving rejection in the context of solid organ transplantation can be grossly divided into mechanisms predominantly driven by either T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR), though the co-existence of both types of rejections can be seen in a variable number of sampled grafts. Acute TCMR can generally be well controlled by the establishment of effective immunosuppression (1, 2). Acute ABMR is a low frequency finding in the current era of blood group and HLA donor/recipient matching and the avoidance of engraftment in the context of high-titer, preformed donor-specific antibodies. However, chronic ABMR remains a major complication resulting in the untimely loss of transplanted organs (3–10). The close relationship between donor-specific antibodies and ABMR has been revealed by the highly sensitive detection of human leukocyte antigen (HLA) antibodies (7, 11–15). Injury to transplanted organs by activation of humoral immune reaction in the context of HLA identical transplants and the absence of donor specific antibodies (17–24), strongly suggest the participation of non-HLA (nHLA) antibodies in ABMR (25). In this review, we discuss the genesis of ABMR in the context of HLA and nHLA antibodies and summarize strategies for ABMR management.

Keywords: HLA antibody, donor-specific HLA antibody, non-HLA antibody, antibody-mediated rejection, humoral immune system, *in vitro* B cell assay

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INTRODUCTION

Organ transplantation improves the quality of life of patients with terminal dysfunction of organs, such as the kidney and pancreas, and is the most effective life support treatment for patients with heart, lung, and liver failure.

Although short-term prognoses for transplanted organs have improved significantly, long-term prognosis after 5–10 years remains insufficient, and reportedly reflects injury from chronic, indolent injury from sub-clinical antibody-mediated rejection (ABMR) (3–5, 15). Acute ABMR is a declining problem in organ transplantation as donor/recipient matching has improved (7, 16) and early acute ABMR is seen usually only in the context of ABO incompatible organ transplants (17, 18), and transplantation in highly sensitized patients with preformed donor-specific HLA antibodies (DSAs). Accordingly, preformed DSA are more likely to be produced before transplantation with histories of complications, such as pregnancy, previous transplant, blood transfusion, and prior organ transplantation (7, 19, 20). Hyper acute rejection, which can occur in the presence of preformed DSA, can be controlled using recently developed desensitization therapies (7).

Rejection due to *de novo* DSAs remains a major cause of transplanted organ loss, in the context of sub-clinical, chronic ABMR (21–24). Moreover, ABMR has also been reported in the absence of DSAs,

leading to the discovery of specific non-HLA (nHLA) antigens that activate humoral immune responses in the graft. Potentially, nHLA antibody-mediated humoral immune responses develop acutely and chronically following transplantation and these antibodies may influence prognoses by participating in the onset and sequelae of rejection (16–18, 25–33). Although graft rejection has been reported among patients with nHLA antigens, one of challenges has been the discovery of the identity of these novel nHLA antigens and to correlate their presence and titers with ensuing mechanisms of transplant rejection.

MOLECULAR PATHOPHYSIOLOGY

During ABMR, antibodies for donor antigens are produced following activation of humoral immune responses, involving activated T cells and complement pathways.

As shown in **Figure 1**, naïve B cells differentiate into DSA-specific plasma cells (PCs) via germinal centers following exposure to antigens. This process involves initial uptake and surface presentation of donor antigens on antigen-presenting cells (APC) in response to an encounter of donor antigens, leading to activation of $CD4^+$ effector T cells (34) and successive promotion of class-switching of naïve B cells and differentiation of memory B cells into PCs (35). Transmission of $CD4^+$ effector T cell signals to B cells primarily involves association of major histocompatibility complex 1 (MHC-I) with T cell receptors (36). In addition, subordinate signaling pathways are activated by binding of CTLA4 (CD152), CD28, and CD40 ligand (CD40L) on T cell surfaces to the B7 (CD80/86) complex and CD40 on B-cell surfaces. Although CTLA-4 binds to B7, it reportedly downregulates T cell activity by binding to B7 with much greater affinity than CD28 (37–40). Intracellular CTLA-4 was closely related to the suppressor function of regulatory T cells (41–43) and reported the close relationship with autoimmune disease, including Graves' disease, type 1 diabetes mellitus (DM) (44–48).

CD28 is expressed on $CD4^+$ effector T cells and naive T cells (47), and promotes interleukin (IL)-2 production from B cells following binding to B7 complexes (48), leading to sustained

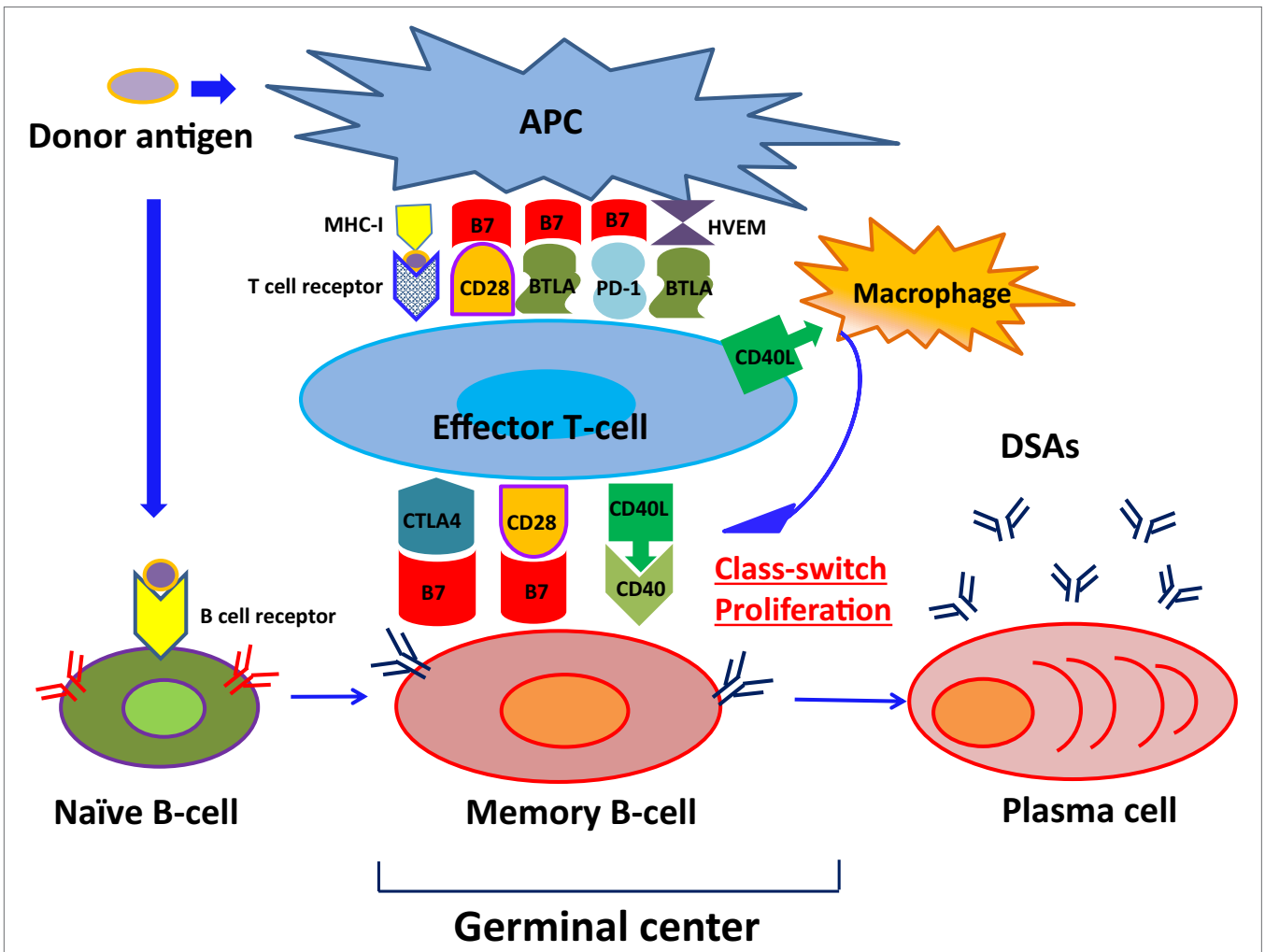

FIGURE 1 | The pathway of naïve B-cell differentiation into DSA-specific PCs. Naïve B cells differentiate into DSA-specific plasma cells (PCs) via germinal centers following exposure to antigens, herpes virus entry mediator; HVEM.

naïve B cell differentiation into memory B cells (49). Conversely, CD40L mediates the class-switch of B cells in the germinal center by binding to CD40 expressing B cells (50) and support $CD4^+$ effector T cells to help B cell differentiation (51, 52). Previous studies by Ettinger et al. (53) also showed that IL-21 induced PC phenotypes of human naïve and memory B cells following stimulation through B cell receptor (BCR) and CD40. Therefore, DSA-specific PCs developed and produced DSAs.

THE ROLE OF COSTIMULATORY PATHWAY IN THE CLINICAL FIELD

CTLA-4Ig (immunoglobulin) binds to B7 and then suppresses the engagement of CD28. CTLA-4Ig can suppress the function of activated T cells through regulatory T cells, which may help suppress established chronic inflammatory disease (54).

In the field of transplantation, Belatacept, which links to the extracellular domain of CTLA-4, has been approved for the treatment of acute kidney rejection. In addition, an important problem in the field is to control antigen-specific memory B cells differentiation into PCs. The infusion of Belatacept might suppress DSAs development in a T-cell-independent manner because it has been reported that the infusion of CTLA-4Ig 1 week or more after transplantation could prevent DSAs development in a fully mismatched mouse cardiac transplant model but did not affect T-cell function (55). In addition, in the recent clinical BENEFIT trial of Belatacept induction by Vincenti et al. (56), there was a significant lower incidence of DSA development with Belatacept induction, when compared to the standard CNI arm, despite the higher incidence of acute rejection seen early with Belatacept induction.

The results might indicate that CTLA-4Ig could inhibit the growth and survival of DSA-specific memory B cells or PCs in a human model. About the other suppressive receptors related to CD28, PD-1 (programed death-1) has been reported to be expressed on the surface of T cells, and B and T lymphocyte attenuator (BTLA) has been reported to be expressed on the surface of both B and T cells, both of which have also been attracting attention as targets for treating autoimmune diseases and cancer (57).

In the field of autoimmune disease, the involvement of signaling through CD40–CD40L interaction in autoimmune diseases has been reported and dysregulation of CD40 may induce macrophage-mediated coronary artery disease (CAD); the blockade of CD40L may, thus, be an attractive therapeutic target to improve CAD (58). Recent studies have also implicated altered regulation of the CD40 axis and generation of pathogenic activating anti-CD40 antibody for the generation of podocyte injury in focal segmental glomerulosclerosis (FSGS) recurrence after kidney transplantation (59, 60). Further research is needed to better elucidate how the CD40 axis may help control other autoimmune diseases.

HUMAN LEUKOCYTE ANTIGEN ANTIBODIES

Histocompatibility analyses using cross-match, human leukocyte antigen (HLA) typing, and antibody tests are widely performed prior to transplantation in many laboratories, and are an accepted approach for limiting organ rejection. Recent developments in laboratory procedures, survey equipment, and technologies have led to highly sensitive detection of HLA antibodies.

Therefore, we could detect a very small amount of HLA antibodies and determine these antibody specificities; trace quantities of HLA antibodies recently provided useful prognostic information for ABMR and transplanted organ outcome and a judgment of transplant evaluation (61–65).

Major histocompatibility complex 1 class 1 (HLA-A, -B, and -C) and MHC class 2 (HLA-DR, -DP, and -DQ) have been identified as HLA antigens, and HLA antibodies can be detected in sera using FlowPRA® Class I & II Screening Tests (One Lamda) to identify Class I or/and Class II HLA antibodies. In further analyses, positive cases should be identified using HLA LAB Screen HLA Class I or/and Class II single antigen beads (One Lambda) with Luminex technology, which determines antibody profiles against HLA Class I or Class II and indicates the presence or absence of DSAs.

THE ROLE OF PREFORMED DSA IN THE PATHOGENICITY OF GRAFT INJURY

Donor-specific HLA antibodies that cause ABMR have been classified as those that are present before transplantation as well as those *de novo* that are produced after transplantation. Previous studies on kidney, heart, lung, and liver transplantation indicate that poor-prognosis is associated with the presence of DSAs before transplantation. We will next discuss the role of preformed DSAs in each organ transplant. With regard to kidney transplants, preformed DSAs have been recognized as one reason of hyper acute rejection. DSAs with high threshold MFI and DSAs with cross match-positive could predict ABMR onset after transplantation (7). With regard to pancreas transplants, we found a report describing that preformed DSAs did not affect graft prognosis (66) but DSAs could be detected from the sera with significantly higher probability than in recipients without a history of preformed DSAs after transplant. As a result, recipients sensitized by DSA before transplant had a history of DM more than 10 years after the transplant, so we should pay more attention to postoperative management, including blood sugar management (67).

With regard to liver, heart, and lung transplants, it is already known that preformed DSAs could affect graft outcome and patient mortality. In addition, preformed C1q binding DSAs have been reported to affect graft prognosis in liver and heart transplants and preformed DSAs with MFI $\geq$5000 and IgG3 DSAs could be risk factors for ABMR onset in liver transplant cases (19, 68, 69). Indeed, additional risk factors for ABMR in these patients include ABO incompatible and cross match-positive status, cases with a history of previous transplants, pregnancy, and blood transfusions (19, 70, 71). With regard to lung transplants, preformed DSAs have been reported to promote *de novo* DSA development early after transplant and patient survival (72–75). Therefore, these data on clinical correlations of DSA and rejection in different organ transplants suggests that improved screening and therapies, such as desensitization before transplant, may be

of benefit across different types of solid organ transplants to limit subsequent postoperative complications (76).

Mechanisms of Onset of ABMR by Preformed DSA

Antibody-mediated rejection caused by preformed DSAs manifests as hyper acute rejection immediately after transplantation, leading to failure of the transplanted organ within several hours. In these cases, DSAs immediately bind to all capillary endothelium surfaces in the transplanted organ, and concomitant complement activation leads to the formation of fibrin clots and acceleration of blood coagulation. Subsequently, rapid peripheral circulation incompetence causes necrosis of vascular walls, intense bleeding of the transplant, and necrosis in neighboring tissues. Finally, inflammatory cells, such as neutrophils, infiltrate capillary endothelial surfaces, and further undermine the transplant (6, 77).

Management of ABMR by Preformed DSA

Improvements in desensitization therapy have enabled management of high risk recipients, such as those with cross match-positive phenotypes and high organ transplantation sensitivity; as a result, the prevalence of severe hyper acute rejection by preformed DSAs has decreased significantly (7). Accordingly, Ng et al. summarized desensitization protocols and complications using rituximab, bortezomib, eculizumab, and alemtuzumab, and reported promising graft survival in patients across various institutes (78). However, complications included anemia and thrombocytopenia, likely reflecting myelosuppression by these agents. In addition, various infections in some cases were detected, including cytomegalovirus (CMV), BK virus, and Epstein–Barr virus (EBV), indicating that desensitization therapy disposes patients to an increased risk of opportunistic viral infections. In addition, it was reported that induction with T-cell depleting agents (anti-thymocyte globulin) was closely associated with CMV, EBV, and BK polyomavirus (BKV) infections in comparison with IL-2a receptor antagonists (anti-CD25) (79). Therefore, the use of T-cell depleting agents should be avoided as an immunosuppressive reagent or induction. If possible, the use of IL-2a receptor antagonists or no induction should be considered (79, 80). Additionally, it was expected that these virus infection may contribute to the activation of immune responses in transplanted organs, and dose reductions of immunosuppressive agents may activate immune reactions to graft antigens.

To address this issue, prediction and early detection of viral infections is critical, and could be used to inform doses reductions of immunosuppressive agents. Concomitant administration of preventive and therapeutic antiviral agents is also critical in the management of these patients.

Desensitization Therapy

Prior to the introduction of rituximab, plasmapheresis and splenectomy were long recommended as desensitization therapies for patients with ABO incompatible kidney transplants. Subsequently, rituximab was shown to inhibit the onset of ABMR without splenectomy. Rituximab is a monoclonal antibody (mAb) against the protein CD20, which is expressed in immature and mature B cells. However, because CD20 is not expressed on PCs, rituximab may not inhibit the production of DSAs by PCs. In addition, recent studies show varying effects of rituximab on B cell phenotypes, with higher sensitivity of naïve B cells than memory B cells (81). Thus, although rituximab suppresses immune activation and may not provide protection from infection, memory B cells may remain viable.

In addition, posterior reversible encephalopathy syndrome (PRES) and acute respiratory distress syndrome (ARDS) was reportedly increased in patients treated with rituximab as severe adversity effect (82). These data warrant further clarification of the depletion mechanisms of rituximab in B cells.

Bortezomib is a proteasome inhibitor that was developed as a treatment for multiple myeloma, and the effectiveness of this agent against transplant rejection was reported in 2008. These studies showed downregulated immune responses to donor antigens, recovery of graft function, and long-term suppression of serum antibody levels. However, inhibition of the proteasome by bortezomib may be detrimental to healthy cells (83–88).

As an alternative, eculizumab is a recombinant humanized monoclonal IgG2/4 antibody that suppresses complement activation and inhibits production of C5, which is the final product of the complement pathway and activates inflammatory responses and ultimately results in apoptosis of infected cells (89). Accordingly, treatments with this agent led to severe infectious diseases, including meningitis (90).

Finally, alemtuzumab is a recombinant DNA-derived humanized IgG1 kappa mAb that is directed toward CD52 and is used is used to treat B-cell chronic lymphocytic leukemia (B-CLL) and multiple sclerosis patients, warranting consideration for the treatment of ABMR. As adverse effect, it has been associated with infusion-related events (91, 92).

Infection as a Trigger of Rejection

Cytomegalovirus Infection as a Trigger of Rejection

Cytomegalovirus is among the most common infections after solid-organ transplantation, and results in significant morbidity, graft loss, and adversity. Although numbers of CMV-seronegative (R^-) cases have increased recently in healthy subjects, those with organ transplants from CMV-seropositive donors (D^+) are at the highest risk of primary CMV disease, which can easily become serious causing the reactivation of latent virus transmitted in the allograft (93, 94). Additionally, a close relationship between CMV infection and allograft rejection has been reported in CMV D^+/R^- liver and kidney transplant patients (93, 95).

Laboratory Diagnosis of CMV

Nucleic Acid Testing. Nucleic acid testing (NAT) is widely used to detect and quantify CMV RNA and DNA.

Serology. Serological analyzes allow risk stratification of patients during the pre-transplant screening phase on the basis of tests for

CMV IgG antibodies in both donors and recipients, and can indicate the presence of latent infection.

Antigenemia. The antigenemia assay detects the CMV pp65 antigen in infected leukocytes from peripheral blood, and has been used for rapid diagnosis of CMV infections in transplant recipients (96).

Treatment of CMV

In a previous study, valganciclovir was found to be more effective than oral ganciclovir at preventing CMV disease in solid organ transplant recipients (97), suggesting that extension of valganciclovir prophylaxis to 200 days may benefit high risk (D^+/R^-) kidney recipients. Following transplantation, CMV disease is predominantly treated using intravenous (IV) ganciclovir (5 mg/kg every 12 h) and oral valganciclovir (900 mg twice daily) (98).

BK Polyomavirus Infection as a Trigger of Rejection

More than 90% of healthy subjects become infected with BKV (99, 100), which is the major cause of polyomavirus-associated nephropathy (Py-VAN) and presents a 1–15% risk of allograft failure in kidney transplant patients (101–106). And it has been reported that BKV-activated antibody reactivity in recipients at the onset of immunosuppression (107). However, although number of BKV-seronegative (R^-) cases has increased recently in healthy subjects, these patients are the most susceptible to BKV disease following transplantation from BKV seropositive donors (D^+) (108, 109).

Laboratory Diagnosis

Screening for BKV replication should be performed at least every 3 months during the first 2 years after transplantation, and then annually until the fifth year.

Nucleic Acid Testing. Nucleic acid testing in polymerase chain reaction (PCR) is used to detect amplifications of BK DNA.

Urine Cytology. Urine cytology is sufficient to detect decoy cells, which are associated with BKV induced organ failure.

Treatment of BKV

First, reduction of immunosuppression should be considered (110, 111). In patients with sustained high-level plasma BKV loads despite dose reductions of immunosuppression agents, administration of antiviral agents (*Cidofovir)*, and a replacement for mycophenolic acid (*Leflunomide)*, intravenous immunoglobulin (IVIG), and anti-mycotic agents (*Fluoroquinolones*) should be considered.

Epstein–Barr Virus Infection as a Trigger of Rejection

Epstein–Barr virus contributes to the pathogenesis of post-transplant lymphoproliferative disease (PTLD) occurring cases early after transplantation in more than 90% of the cases, and small intestine transplantations are associated with higher risks than heart, lung, and liver transplantations (112, 113). The close relationship between EBV infection and ABMR has been reported in heart transplantation (114).

Laboratory Diagnosis

Nucleic Acid Testing. Epstein–Barr virus DNA monitoring for EBV D^+/R^- recipients should be recommended, with continued EBV load screening every 3–6 months until 2–3 years after transplantation. This monitoring is particularly important for EBV-seropositive recipients with intestinal transplants, and monitoring of EBV DNA every 2–4 weeks in the first 3 months should be performed, monthly until 6 months post-transplantation, and then every 3 months until the end of the first year.

Treatment of EBV

Antiviral prophylaxis for high risk patients (EBV D^+/R^-) is considered in some centers (99).

Treatment with acyclovir, ganciclovir, and IVIGs has shown some benefits in the prevention of PTLD among EBV-seronegative recipients who their donors are EBV-seropositive (113).

THE ROLE OF *DE NOVO* DSA IN THE PATHOGENICITY OF GRAFT INJURY

Recent reports show that DSAs play an important role in ABMR onset, and this has been shown by highly sensitive monitoring of HLA antibodies in the sera (11–13). However, DSAs may be absorbed into transplanted organs during the early phases of antibody production (115) (**Figure 2**). Accordingly, in the Guidelines of the Transplantation Society (TTS), post-kidney transplant DSA monitoring is not recommended for all patients beyond the first year (116). Hence, to avoid the influence of absorption, antibody production from PCs has been analyzed *in vitro*, because these antibodies may not be influenced by the absorption and provide us with further detailed illustrations that are available to clarify how these antibodies are produced in organ recipients. However, PCs are seldom found in blood and predominate in bone marrow and secondary lymphoid tissues, techniques for differentiating B cells into PCs, are required to investigate antibody production (117–119).

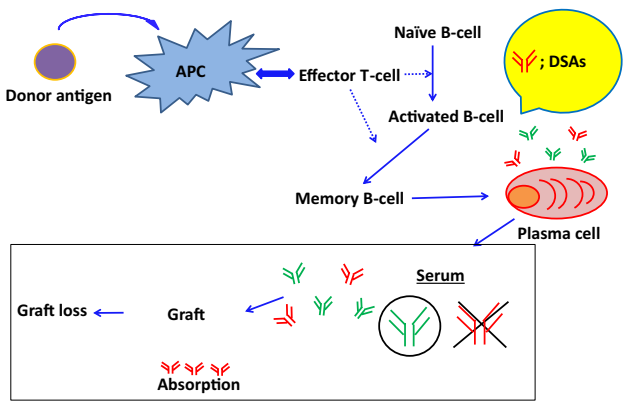

FIGURE 2 | The absorption of DSAs into graft and the development of ABMR. DSAs may be absorbed into transplanted organs during the early phases of antibody production.

In Vitro B Cell Assays

Determination of HLA antibodies in supernatants of cultured B cells can better inform ABMR management than those in sera. Therefore, some researchers have suggested that if peripheral B cells could be differentiated into PCs *in vitro*, then the *in vitro* differentiation of peripheral B cells into PCs may facilitate the control of ABMR. However, unlike *in vitro* T cell assays that have long been used to control T cell-mediated rejection (TCMR), primary cultures of B cells are difficult to maintain and *in vitro* B cells assays have not long been established. Although memory B cells were reportedly differentiated into APC *in vitro* (120), these short comings require further improvements in the ease and convenience of B cell culture, and subsequent assay development that can be used to detect HLA antibodies in B cell culture supernatants to ultimately prevent transplant rejection.

Moreover, there are important points to detect HLA antibodies from the B cell culture supernatant. Peripheral B cells include naïve B cells and memory B cells (121, 122); and these B cells derived from PCs survive for varying durations and produce antibodies. However, HLA antibodies that cause ABMR are mainly produced by memory B cells (123–125), warranting establishment of *in vitro* B cell assays in which memory B cells are selectively differentiated into PCs *in vitro* and are used to produce antibodies. In addition, many reports have showed that long-lived PCs, which produce antibodies in the bone marrow for long periods, play an important role in ABMR (126–129). Therefore, to monitor the progression of antibody-mediated diseases, *in vitro* culture systems, in which B cells are differentiated to their terminal stage (long-lived PCs), are urgently required. On using a clinical specimen, the volume of B cells in peripheral blood is very low following immunosuppression or particularly when we could collect B cells from recipients who experienced desensitization therapy.

However, feeder cells can strongly activate human B cells to proliferate and differentiate in a cell–cell contact-dependent manner in these cases. Thus, *in vitro* B cell assays of HLA antibodies from the cultured supernatants may lead to drug sensitivity tests that are similar to those for T cells, may contribute to clinical applications of personalized immunosuppression, and the development of new immunosuppressant agents that control ABMR.

In Vitro Memory B Cell Assays

We found a report about immunosuppressive agent susceptibility for the differentiation of human B ($CD19^+$) cells *in vitro* with a combination of IL-21, phosphorothioate CpG-oligodeoxynucleotide (CpG-ODN), histidine-tagged soluble recombinant human CD40 L and anti-polyhistidine mAb (130). IL-21 is produced by follicular helper T cells (131), which synergistically induce maximum Blimp-1 upregulation and optimal PC differentiation with CD40 L (132). TLR9 agonist CpG-ODN activates B cells proliferation and promotes PCs differentiation (133). This culture system induced IgG production but could not sustain the survival of PCs for a long period. It might indicate that the other cytokines play an important role in human B cells differentiation into mature PCs *in vitro*, because other groups have reported that APRIL and the B cell activating factor (BAFF) would support the survival of PBs and PCs recently (134–137). In addition, a previous report has shown that $CD27^+$ memory B cells could be differentiated into long-lived PCs with supernatants from bone marrow stromal cell line M2-10B4 (138), which support the long-term culture of human bone marrow stem cells. The mechanisms by which M2-10B4 cells contribute to PCs survival has yet to be revealed, but it is suggested that $CD27^+$ memory B cells demand well-balanced support from stromal cells (139–142). In addition, different environments or signal transmission might be required for the differentiation of $CD27^-$ naïve and $CD27^+$ memory B cells into mature PCs. Therefore, we should improve the *in vitro* B cell assay to sustain $CD27^+$ memory B cell-derived PC survival for a long-term selectively. For example, we should examine how any humoral factors, including growth factor or any cytokines from activated T cells could affect $CD27^+$ memory B cell growth and survival *in vitro*, while referring to the reports that helper T-cells may mediate $CD27^+$ memory B cell differentiation into PCs *in vivo* (143).

Risk Factors of ABMR from *De Novo* DSA

Not all DSAs participate in ABMR and transplanted organ prognosis (7), and although C1q binding DSAs are reported risk factors for ABMR onset, further studies of DSA characteristics are required to identify those with prognostic value. In addition, various other factors influence transplanted organ prognoses (ABMR onset, graft survival) and require further investigation. About the risk factors for graft loss, thrombotic microangiopathy (TMA), glomerulopathy, C4d deposition, and chronic injury change in histopathological diagnoses were reported.

As other factors besides histopathological findings, a history of subclinical ABMR and TCMR and a decline of graft function could be risk factors. This might indicate that a graft would fail with high probability when the humoral immune response toward a donor-specific antigen has proceeded to an irreversible stage.

C1q Binding DSA

C1q appear in the beginning of the classical complement pathway, and C1q binds directly to antigens and initiates classical complement pathway activation. Subsequent C1q-activated reactions include (i) antigen binding, (ii) binding to C-reactive protein, and (iii) binding to antigen–antibody complexes, and can lead to the activation of C3 convertase and the degradation of C3 to C3b and C3a (144, 145). Of these, C3b is the main effector of the complement pathway, while C3a activates inflammatory responses. Indeed, C1q may play important roles in the activation of inflammatory reactions against grafts. Accordingly, C1q binding to DSAs reportedly influences the frequency of ABMR onset and glomerulopathy in solid organ transplants, leading to increased chances of graft failure. Thus, binding of C1q to DSAs may be highly predictive of graft prognosis, warranting the development of interventions that decrease the presence of C1q binding DSAs. The C1qScreen™ (One Lamda) is a reliable tool for distinguishing complement-binding antibodies from non-complement-binding ones, and is widely applied using Luminex-based LABScan™ 100 flow fluorescence analyzers to determine relative amounts of C1q binding antibodies. The C1qScreen™ in combination with the Luminex-based LABScan™ can indicate the relative amount

of C1q bound to DSAs and provides us with useful information from the sera (146). In addition, in C1q-positive cases despite being DSA-negative, graft survival is poor. It suggested that C1q could affect the transplanted organ prognosis by itself.

DSA Characteristics

In this study, we tabulated previously reported factors that participate in ABMR and graft loss (**Table 1**). Reports show that DSAs with higher mean fluorescent intensities (MFI) of ≥15,000 cause ABMR with higher probability than those with MFI of ≤5000, and higher level of DSAs may activate humoral immune reactions to donor antigens. In addition, many papers indicated that class II DSAs should be considered as a risk factor, particularly at the onset of ABMR. However, DSA specificities that activate humoral immune response to donor antigen may depend on the type of transplanted organ, and the further recognition about detailed

TABLE 1 | Various other factors influence transplanted organ prognoses and require further investigation.

		Risk factors	Out come	
Study size	**Organ**	**ABMR**	**Graft loss**	**Reference**
226	Kidney	Highly sensitized patients DSA relative intensity scores greater than 17 Presence of both class I and II DSAs at transplant	ABMR-positive Thrombotic microangiopathy (TMA) positive Induction with intravenous immunoglobulin and rituximab	(147)
62		C1q-positive	C1q-positive Both of DSA- and C1q-positive Transplant glomerulopathy Decline of eGFR	(148)
1016		Complement-binding DSA DSA-positive	Complement-binding DSA DSA-positive	(149)
1307			Subclinical ABMR Subclinical TCMR	(150)
1365		TCMR	TCMR diagnosed after the first year post-transplant Chronic histological injury Transplant glomerulopathy	(151)
67 (grafts)			Late aABMR	(152)
885			Capillary C4d-positive	(153)
1054		TCMR	Higher glomerulitis scores Higher C4d staining scores	(154)
1			Plasma cell-rich rejection (PCRR) with ABMR	(155)
237		DSA-positive preformed DSA-positive	DSA-positive AMR DSA-positive/CXM-positive	(7)
234			Microcirculation inflammation	(4)
274			C1q-fixing DSAs	(140)
152	Pancreas-kidney		*De novo* DSA-positive	(67)
439	Pancreas		Elevated DSA Preformed DSA-positive	(156)
2631	Liver	Preformed class II DSAs positive MFI ≧5000		(19)
1270		Preformed C1q-fixing class II DSA	IgG3 DSA-positive *De novo* IgG3 DSA	(157)
749			*De novo* DSA development	(158)
15	Heart	SAB-C1q-positive DSA CDC-XM-positive		(9)
243			*De novo* DSA-positive Persistent DSA	(159) (160)
44 60	Lung	DSA-positive	HLA-DQ DSA (>10,000)	(71)
546			Early anti-HLA class II DSA Pre-operative HLA antibodies Retransplantation Postoperative PGD	(72)
79	Intestine		*De novo* DSA development early after transplant	(161)
291		DSA-positive	DSA-positive	(162)

association between DSAs and graft outcome is required in each solid organ transplantation.

With regard to kidney transplants, DSAs with high threshold MFI and C1q binding DSAs have been reported to be closely related to TMA, glomerulopathy, microangiopathy, C4d deposition, extensive interstitial fibrosis, and tubular atrophy and these factors could affect graft prognosis in the long term (147–154). With regard to pancreatic transplants, elevated DSAs could affect graft prognosis (67, 156). With regard to liver transplants, IgG3 DSAs and C1qbinding DSAs were related to graft survival and class II DSAs were shown to be closely related to acute rejection early after a transplant (157). In addition, DSAs could affect graft outcome and reduce graft survival 1 year or more after a transplant by itself (158). With regard to cardiac transplants, C1q binding DSAs and cross match positivity could be risk factors for ABMR and *de novo* DSA development and persistent DSA were found to be closely related to graft loss (9, 159, 160). With regard to lung transplants, DSA has been related to ABMR, cellular rejection, and bronchiolitis obliterans and could significantly reduce postoperative survival 3 years later compared with that in DSA-negative recipients. In addition, *de novo* DSA (along with HLA-DR mismatch) development has been reported to reduce postoperative survival (71, 159). With regard to intestine transplants, *de novo* DSA development early after transplant could affect graft prognosis and might be effective for screening of acute rejection because DSA measurement has been shown to be closely related to histological findings (161, 162). The characteristics of DSA that could affect graft prognosis vary among the different types of organ transplant; we should, thus, understand these features well and make use of them for the postoperative management of transplant recipients.

Onset Mechanisms of ABMR from *De Novo* DSA

Antibody-mediated rejection caused by *de novo* DSAs typically appears several weeks to months after transplantation, but can develop at any time as far as a graft engrafts afterward.

Following the absorption of HLA antibodies onto capillary endothelial donor antigens (mainly HLA antigen), activation of pro-complement solidification and accumulation of inflammatory cytokines, macrophages, and neutrophils are caused successively. Therefore, it leads to microangiopathy and gradual annual declines in graft function (52, 163–165). Under these conditions, ABMR-mediated microangiopathy is chronic and sustained, although moderate inflammatory activities result in slow and irreversible disease progression.

Management Strategies of ABMR by *De Novo* DSA

In a previous section, we suggested that immunosuppressive therapy limits differentiation of naïve B cells to germinal center B cells by controlling $CD4^+$ effector T cell stimulation. However, stronger immunosuppressive therapy is required to control B cell growth and survival after differentiation of naive B cells to memory B cells in germinal centers. As a result, chronic use of immunosuppressive agents after differentiation of naïve B cells into memory B cells corresponding to HLA antibodies may no longer affect B cells in germinal centers.

Thus, further attention should be paid to pancytopenia, anemia, and viral infection as well as to those concerning B cell differentiation, because more strong immunosuppressive therapy might be necessary to inhibit memory B cell growth and survival in comparison with naïve B cells. In particular, rituximab administration induces $CD20^+$ memory B cell apoptosis (166), bortezomib therapy inhibits the production of DSAs production from PCs (167), and IVIG can be used to reduce circulating DSAs (168) (**Figure 3**). Hence, the development of new immunosuppressive agents that inhibit memory B cell growth and survival is warranted.

In addition, the development of a diagnostic method for predicting the development of DSA specific memory B cells as soon as possible has been required.

Therefore, the control of ABMR is very difficult; the disease state progresses irreversibly and severely and is unresponsive to increasing immunosuppression following diagnosis using currently available methods. Although graft tissue biopsies are the most reliable diagnostic method for ABMR, it was hard to perform frequently because it is very invasive. Therefore, less invasive diagnostic approaches are urgently required to predict the development of DSA-specific memory B cells.

Histological studies of ABMR following solid organ transplantation show that the classical complement pathway is activated after adhesion of DSAs to capillary endothelia, and that C4d produced and deposited as the final product of this pathway is an important ABMR diagnostic factor before (169). In late years, the number of reported cases of C4d-negative ABMR has recently increased (15) and some DSA-related mechanisms that are independent of the classical complement pathway have been identified. In each organ transplantation, these observations necessitate revision of histologic diagnostic criteria for all organ transplant patients, and improved the understanding of ABMR (170–172).

Banff Score for Diagnosis of ABMR

Pathological diagnoses play important clinical roles, and diagnostic criteria have been revised for all organ transplants. In particular, Banff score are widely used as histologic methods for kidney transplantations, although diagnostic criteria were substantively revised in 2013; indeed, the roles of ABMR, DSAs, and C4d deposition in grafts received greater emphasis in the previous diagnostic criteria before the meeting in 2013. In the 2013 revised edition (**Table 2**), C4d-negative ABMR became the diagnostic focus especially, reflecting on the increased numbers of reported cases. Thus, we listed the important diagnostic criteria for ABMR in the revised edition in 2013, including confirmation of microangiopathy, evidence for DSA-capillary endothelial reactions, and detection of DSA in the serum. In particular, diagnosis of DSA-capillary endothelial reactions requires at least one of the following observations; (i) C4d deposition in peritubular capillaries (PTC), (ii) evidence of more than moderate microangiopathy

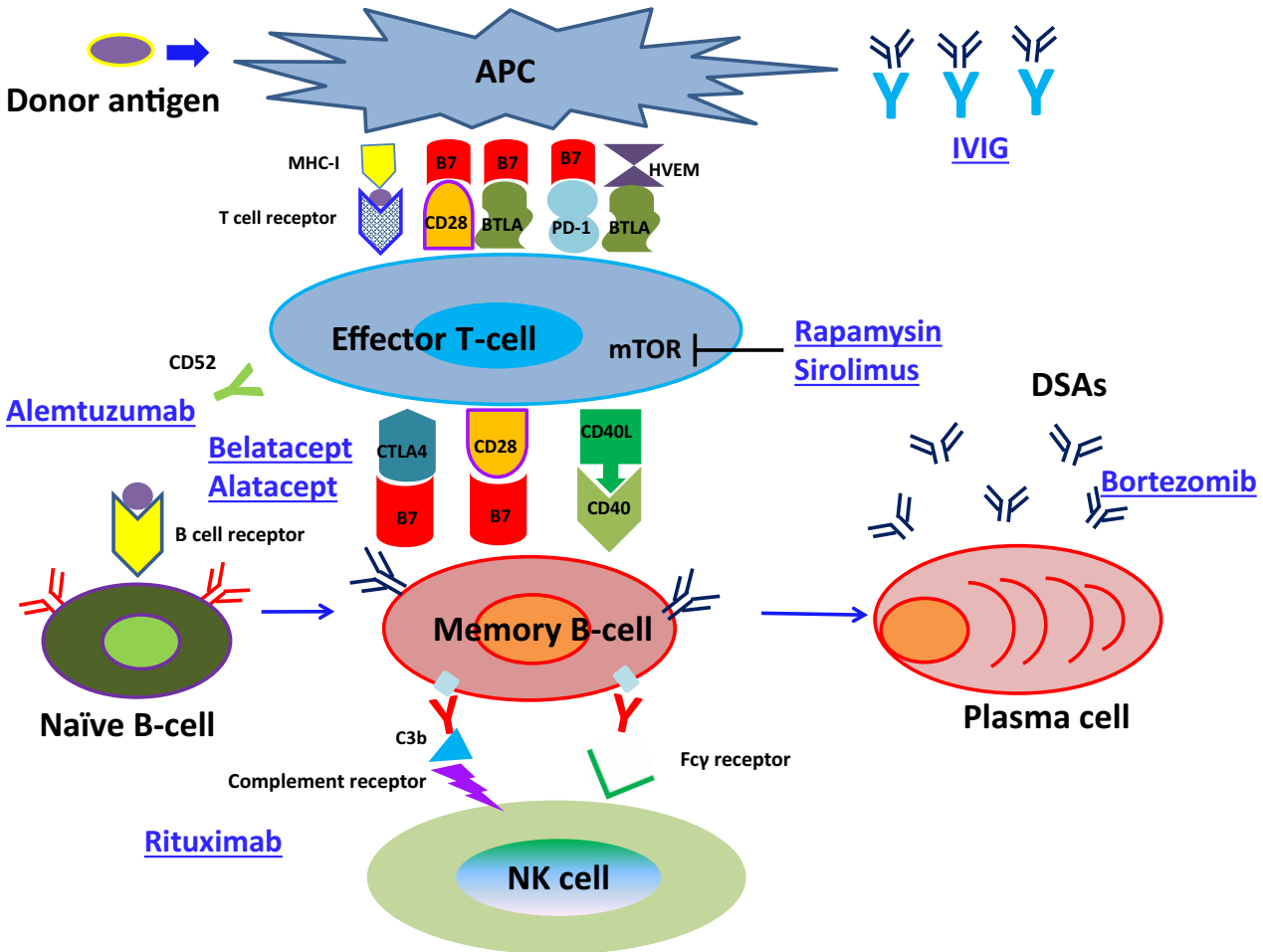

FIGURE 3 | The pathway of naïve B-cell differentiation into DSAs specific PCs and how immunosuppressive reagents suppress the development of ABMR.

TABLE 2 | Revised classification of antibody-mediated rejection.

Acute/active ABMR	
1	Evidence of acute tissue injury, including one or more of the following
	Microvascular inflammation (g > 0 and/or ptc > 0)
	Intimal or transmural arteritis (v > 0)
	Acute thrombotic microangiopathy, in the absence of any other cause
	Acute tubular injury, in the absence of any other cause
2	Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following:
	Linear C4d staining in ptc
	Moderate microvascular inflammation(g + ptc ≧ 2)
	Increased expression of gene transcripts indicative of endothelial injury
3	Serologic evidence of DSAs
Chronic/active ABMR	
1	Evidence of chronic tissue injury, including one or more of the following
	Transplant glomerulopathy(cg > 0)
	Severe ptc basement membrane multilayering (requires EM)
	Arterial intimal fibrosis of new onset, excluding other causes
2	Evidence of current/recent antibody interaction with vascular endothelium, including at least one of the following
	Linear C4d staining in ptc
	moderate microvascular inflammation(g + ptc ≧ 2)
	Increased expression of gene transcripts indicative of endothelial injury
3	Serologic evidence of DSAs

The bold font showed the most important factor to diagnose ABMR (Acute and Chronic).
DSAs, donor-specific HLA antibodies; EM, electron microscopy.
Furthermore, in the revised criteria, ABMR phenotypes have been classified as acute/active; chronic/active corresponding to the diagnostic criteria, which have been listed in detail.

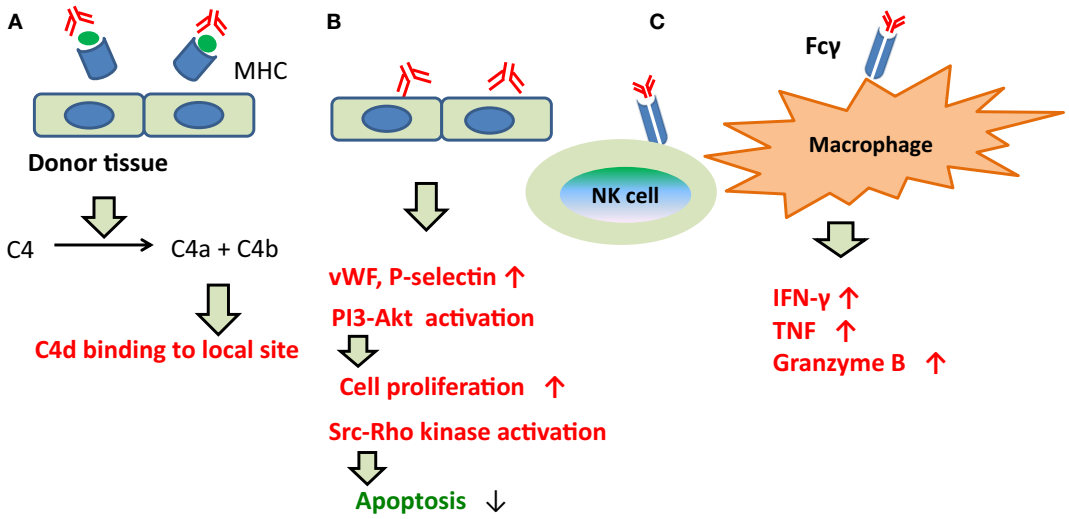

FIGURE 4 | **The development of ABMR caused by DSAs**. **(A)** Indirect injury via complement fixation or recruitment. C1q was the assumed trigger of the classical complement pathway following binding of DSAs to capillary endothelia. Although C4d is the final product. **(B)** Direct injury to the capillary endothelium. DSAs may directly promote vascular endothelial cell growth and proliferation, and inhibit apoptosis in capillary endothelia. **(C)** Recruitment of inflammatory cells with Fc receptors. DSAs have been shown to bind with Fcγ on the cell membrane surfaces of macrophages, natural killer cells, and neutrophils, and to induce inflammatory cytokine production and microangiopathy.

and microvascular inflammation (MVI; g + ptc ≥ 2), and (iii) expression of endothelial activation (ENDAT) and injury transcripts.

Microvascular inflammation scores and C4d deposition in PTC are currently the most commonly used diagnostic criteria. However, according to this standard, ABMR diagnoses are recommended in the presence of strong MVI, even in specimens that are C4d-negative.

Thus, C4d deposition has not been necessary for ABMR diagnoses after the meeting in 2013.

By contrast, prior to 2013, ABMR was considered reflective of injury to capillary endothelia (165, 173), and C1q was the assumed trigger of the classical complement pathway following binding of DSAs to capillary endothelia. Although C4d is the final product (**Figure 4**), this classical pathway was not necessarily activated during ABMR in C4d-negative patients.

About the other pathways, DSAs may directly promote vascular endothelial cell (EC) growth and proliferation, and inhibit apoptosis in capillary endothelia (**Figure 4**); DSAs have been shown to bind with Fcγ on the cell membrane surfaces of macrophages, natural killer cells, and neutrophils, and to induce inflammatory cytokine production and microangiopathy (**Figure 4**).

Furthermore, in the revised criteria, ABMR phenotypes have been classified as acute/active, chronic/active corresponding to the diagnostic criteria, which have been listed in detail.

Among these, evidence of acute tissue injury as the diagnostic criteria of acute/active ABMR, and morphologic evidence of chronic tissue injury as the diagnostic criteria of chronic/active ABMR, is considered central. Therefore, effective management of ABMR entails varied treatments for differing levels of pathological progress, and these diagnostic criteria identify ABMR phenotypes with sufficient accuracy to inform treatments.

THE ROLE OF NON-HLA IN THE PATHOGENICITY OF GRAFT INJURY

Rejection by nHLAs was previously recognized as an unexpected hyper acute rejection of HLA identical transplants (174–177). Recently, it has been accepted that nHLA antibodies play an important role in acute and chronic rejection (178–184).

Moreover, in a report from 1997, antibodies against nHLA antigens were shown to activate humoral immune responses to graft antigens and cause graft injury (185). Subsequently, graft loss due to immunological factors occurred in 56% of cases, and 38% of factors were nHLA. Thus, because the probability of graft loss due to nHLA factors was shown to be greater than that due to HLA factors (186), in late years, more attention has been paid to nHLA factors in the transplantation field. For instance, the former have received increased attention in the transplantation field with the anti-MHC class I chain-related gene A (MICA) antibodies and nHLA antibodies to ECs in the presence of complement, as identified in numerous recent reports. Thus, it was expected that humoral response toward nHLA antigens is primarily activated to donor antigen on ECs.

However, while MICA and ECs was not expressed on lymphocyte membranes and was undetectable using cross-match studies (10), the HLA antibody tests LAB Screen Mixed Class I & II and LAB Screen MICA Single Antigen have been successfully used to detect anti-MICA antibodies in sera.

In addition, there are many reports on the other nHLAs that were associated with ABMR.

These reports showed that the type of nHLA antigens differed between patients with hyper acute rejection, acute rejection, and rejection due to chronic allograft injury (CAI), and it may predict graft success and that management plans could be informed by

TABLE 3 | A list of selected nHLA antibodies and gene in transplantation.

nHLA antibody (nHLA-ab)	Organ	Associated factors	Reference
Anti-protein kinase C zeta (PKCf) ab	Kidney	Graft loss Steroid-resistant rejection and the hypertension Mononuclear cell infiltrate of acute rejection	(188)
Anti-MHC I-related chain A (MICA) ab	Kidney Kidney Kidney Kidney Kidney Heart Heart Liver	Poor graft survival with only MICA and significantly poor with both antibodies($MICA^+/HLA^+$) Preformed MICA antibodies contributes to increasing frequency of graft loss Chronic rejection, poor graft survival Graft rejection, poor 1-year graft survival Poor graft survival The incidence of transplant coronary artery disease No negative effect on graft survival Late graft rejection	(189–197)
Anti-angiotensin II type I receptor (AT1R) ab	Kidney Kidney Kidney heart	Refractory vascular rejection Cronic kidney disease Graft injury, graft loss Cellular and Ab-mediated rejection and early onset of microvasculopathy	(198–201)
Anti-endothelial antibodies (AECA)	Kidney Kidney Kidney Kidney Kidney Heart	Cellular rejection Hyperacute rejection Graft rejection Acute rejection Microvascular damage Early acute rejection	(177, 202–206)
Anti-endothelial-1 type A receptor (ETAR) ab	Kidney Kidney Kidney Heart	Hyperacute rejection Poor graft function early after transplant, hyperacute rejection Graft injury, graft loss Cellular and Ab-mediated rejection and early onset of microvasculopathy	(199, 207)
Anti-peroxisomal-trans-2-enoyl-coA-reductase (PECR) ab	Kidney	Transplant glomerulopathy	(79, 208)
Anti-PRKRIP1ab	Kidney	Cronic kidney disease	(32, 208)
Antivimentin ab	Heart	It did not correlate with early post-transplant rejection or graft survival	(32)
Non-HLA pigmy ab	Heart	Mortality	(209)
Antibodies against Endoglin Epidermal growth factor (EGF)-like repeats Discoidin I-like domains 3 Intercellular adhesion molecule 4 FMS-like tyrosine kinase-3 ligand	Kidney	Acute ABMR	(210)
Antibodies against MIG ITAC IFN-c Glial-derived neurotrophic factor (GDNF)	Kidney	Chronic allograft injury (CAI)	(26)
Collagen type V, K-α1-tubulin	Lung	Graft disfunction, bronchiolitis obliterans syndrome	(207)
nHLA gene			
FN-γ, IL-1B, IL-1RN, IL-2, IL-6, IL-7, IL-17, CCR9, ESR1, FAS Stem cell		GVHD ↑	(206)
IL-10, NOD2, toll-like receptors		GVHD ↑ or GVHD ↓	
VDR		GVHD ↑, mortality ↑	
CTLA4		Acute GVHD ↑, survival ↑	
IL-7R, CXCL10		Transplant-related mortality ↑	
IL-18		Transplant-related mortality ↓	
Il-23R		Acute GVHD ↓	
HLA-E		Chronic GVHD ↓	
IL-1A		Chronic and acute GVHD ↑, transplant-related mortality ↑	
CCl-2		Over roll survival ↓, transplant-related mortality ↑	
CXCL12		Hematological recovery ↑	
TGFβ		Acute GVHD ↓, over roll survival ↓	
HMGB1		Relapse ↑, relapse-related mortality ↑, transplant-related mortality ↑, over roll survival ↑, acute GVHD ↓, chronic GVHD ↑	
MICA		GVHD ↑	

mapping nHLA antigens in recipient sera before transplantation, further indicating the utility of serum nHLA determinations in the diagnosis and management of ABMR.

Management Strategies of ABMR by Non-HLA

Previous studies have reported the detection of nHLA antibodies using ELISA and FACS. However, the clinical utility of these assays remains unclear, because they fail to distinguish antibodies from autoantibodies. Additionally, nHLA antibodies may be detectable in sera from patients with failed grafts but no immunological factors (26, 187). Thus, specific detection of nHLA antibodies that activate humoral immune responses to grafts requires more sensitive methods. For example, by using high-density protein array platforms (26), serum nHLA antibodies in transplant recipients may have up to 9000 different target proteins/antigens and these antibodies were screened immediately, indicating the importance of high throughput screening. In addition, nHLA genotyping of donor and recipient to estimate the risk of ABMR in recipients, such as HLA may be required; the specificities of these antibodies to nHLA should be identified in more details. For example, we should examine if nHLA has the capacity to bound complement or not and if these antibodies could activate humoral immune response toward the donor antigen by using donor-specific ECs.

Mechanism of participation of nHLA antibodies in ABMR and graft loss has not been investigated sufficiently. However, we could find reports that C4d deposition is related to ABMR causing from nHLA antibodies with high probability, indicating that this type of ABMR was caused by the activation of the complement classical pathway. Moreover, further studies are warranted to establish effective immunosuppressive therapies thereby clarifying the mechanism.

In addition, we summarized the association between the representative nHLA antibodies and graft prognosis (**Table 3**).

CONCLUSION

Absorption of DSAs has been regarded as the main cause of ABMR. However, numerous recent studies have characterized the involvement of nHLA antibodies, and have shown that DSA- and nHLA-mediated ABMR phenotypes likely require different management strategies.

Specifically, hyper acute rejections due to preformed DSAs may be avoided with improved desensitization therapy, while *de novo* DSA-mediated ABMR remains difficult to diagnose without invasive graft tissue biopsies prior to critical disease progression. Although the roles of nHLA antibodies have been identified in ABMR, ensuing that mechanisms remain insufficiently understood to inform improvements in management strategies. Moreover, because physiological nHLA antibodies are indistinguishable from those that are closely related to humoral immune reactions against graft antigens, highly sensitive methods that distinguish ABMR-relevant nHLAs are required for clinical diagnoses and management planning for organ transplant patients.

AUTHOR CONTRIBUTIONS

YM and MS designed and wrote the paper. MS provided excellent advices.

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REVIEW
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Autologous and Allogenous Antibodies in Lung and Islet Cell Transplantation

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The field of organ transplantation has undoubtedly made great strides in recent years. Despite the advances in donor–recipient histocompatibility testing, improvement in transplantation procedures, and development of aggressive immunosuppressive regimens, graft-directed immune responses still pose a major problem to the long-term success of organ transplantation. Elicitation of immune responses detected as antibodies to mismatched donor antigens (alloantibodies) and tissue-restricted self-antigens (autoantibodies) are two major risk factors for the development of graft rejection that ultimately lead to graft failure. In this review, we describe current understanding on genesis and pathogenesis of antibodies in two important clinical scenarios: lung transplantation and transplantation of islet of Langerhans. It is evident that when compared to any other clinical solid organ or cellular transplant, lung and islet transplants are more susceptible to rejection by combination of allo- and autoimmune responses.

Keywords: lung, islet of Langerhans, transplantation, antibody, graft rejection

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INTRODUCTION

Solid organ transplantation is increasingly used as a clinical intervention to compensate for functional loss of an organ and to maintain metabolic homeostasis. This palliative treatment option can extend the lifespan and improve the quality of life for recipients, but outcomes vary depending on which organ is transplanted. Lung transplantation (LTx) can be a life-saving measure for patients with many severe chronic lung diseases, including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, alpha-1 antitrypsin deficiency, pulmonary hypertension, interstitial lung disease, and bronchiectasis. Unfortunately, LTx currently has the lowest long-term survival rate compared with survival rates associated with transplantation of other solid organs, its half-life ($t_{1/2}$) being just 5 years. The primary reason for this low survival rate is chronic rejection, the clinical diagnosis for which is bronchiolitis obliterans syndrome (BOS).

Transplantation is not limited to solid organs. Allogeneic endocrine cell transplantation of islets of Langerhans is a treatment option for patients with autoimmune diabetes mellitus [i.e., type 1 diabetes (T1D)]. Transplantation of these cells is a replacement therapy that augments production of endogenous insulin. Islet cells are often isolated from multiple cadaveric donors and recipients require a continuous immunosuppressive therapy. However, long-term sustained normoglycemia is exceedingly difficult to achieve from islet transplantation; the mechanisms that lead to destruction of the islet allografts will be discussed in this review. Autologous islet transplantation—which

also aims to restore endocrine pancreatic function after total pancreatectomy—is not as susceptible to rejection and therefore will not be discussed in this review. Because transplantation depends upon available donor organs and cells, genetic mismatches between donor and recipient become the focal points of the recipient's immunologic responses. Resultant T cell and antibody (Ab) responses have been known to influence, often negatively, both short- and long-term functioning of transplanted allografts, and such incidences frequently portend the unfavorable consequences of graft loss (i.e., rejection).

In this review, we describe the spectrum of Ab responses observed in LTx and in transplantation of islets of Langerhans. LTx, whether unilateral or bilateral, involves surgical replacement of a diseased organ with normal and functioning lungs procured from a cadaveric donor. By contrast, allogeneic islet transplantation involves intrahepatic delivery of donor-isolated islet cells that supplement existing islet cells and insulin production resulting in normoglycemia. With recent increases in the prevalence of chronic obstructive pulmonary disease, cystic fibrosis, and T1D, the demand for transplantable lungs and pancreatic islets has increased. Since 2014, nearly 60,000 LTx procedures have been performed worldwide (both lungs-only and combined heart–lung transplantation; International Society for Heart and Lung Transplantation,[1] accessed on July 25, 2016). As of 2012, approximately 1,400 islet cell transplantations have been recorded (Collaborative Islet Transplant Registry,[2] accessed on July 25, 2016). Additionally, combined transplantation of lungs and pancreatic islets is an effective treatment option to restore respiratory and pancreatic insufficiency in terminal cystic fibrosis (1). The global need for organ transplantation is rising steadily with more prospective transplant recipients added to active waitlists (both first-time transplantation and re-transplantation due to graft failure). Concurrently, an acute shortage persists on the availability of transplantable organs (Organ Procurement and Transplantation Network[3]).

ANTIBODIES IN LTx

Histocompatibility studies originated from the need to decipher mechanisms of graft rejection; these studies ultimately led to the identification of major histocompatibility complex (MHC) proteins. The human counterpart of MHC is the human leukocyte antigen (HLA) system. From an immunologic perspective, the revelation of HLA was critical to the understanding of immune responses—not only as they affect graft rejection, but also how they impact infectious disease, autoimmunity, and tumor biology. Notwithstanding the antigen presentation *via* peptide–MHC complexes that is central to T cell immune recognition and responses, MHC represents the bulk of steady state expression of surface proteins (up to 70,000 molecules per cell) (2). Class I MHC is ubiquitously expressed on every nucleated cell, whereas class II MHC is preferentially expressed on professional antigen-presenting cells (e.g., dendritic cells, macrophages, and B cells).

With more than 200 loci identified, the polygenic nature of HLA combined with high allelic polymorphism (>14,000 alleles for HLA class I and II combined,[4] assessed on November 17, 2016) confers great diversity to HLA molecules (3–6). Furthermore, codominant expression of HLA allows for simultaneous expression of both paternal and maternal HLA haplotypes, which further increases the diversity of the HLA repertoire expressed in a given individual. Because of the high preponderance of HLA class I on every type of cell (i.e., ciliated, non-ciliated, and secretory epithelial cells; endothelial cells; basal cells; and connective tissue) and HLA class II on resident antigen-presenting cells (i.e., lung-resident macrophages and dendritic cells) and B cells, mismatched donor HLA molecules are easily recognized and quickly targeted by the recipient's immune system after transplantation.

Although graft failure was long suspected to be a result of immunological complications, the host-adaptive immune response to MHC antigens wasn't confirmed until 1956, when immunization of malignant cells in mice induced *de novo* Abs against MHC molecules (7). In a clinical setting, the association of preexisting HLA Abs with graft failure was witnessed when a large number of kidney transplant recipients who experienced acute graft rejection had donor HLA Abs (i.e., positive crossmatch), whereas recipients who lacked anti-HLA (i.e., negative crossmatch) had significantly higher graft survival (8, 9). Since these landmark studies, preexisting and *de novo* donor-specific antibodies (DSA) to mismatched HLA have generated a tremendous amount of clinical interest and have been widely applied in the study of all solid organ transplantation (10). The posttransplant development of *de novo* DSA was first documented following LTx in 2002 (11). Since then, a strong clinical association of *de novo* DSA with acute and chronic lung allograft rejection has been confirmed by many independent studies (12–20). Significantly, an association between the extents of donor–recipient HLA mismatches and incidence of chronic rejection (i.e., BOS) has been established (21) indicating a role for anti-HLA immune responses in the post-LTx acceptance and performance of lung allografts.

The pathogenicity of MHC Abs has been demonstrated in our laboratory using a mouse model of obliterative airway disease (OAD), in which ligation of MHC by antibodies led to OAD and lung-restricted autoimmunity (22, 23). In this model, exogenous delivery of anti-MHC class I or anti-MHC class II to the lung microenvironment induced small airway occlusion and fibrosis, creating pathologic lesions similar to those observed in humans with chronic lung graft rejection. While the Ab repertoire associated with lung graft rejection is not fully characterized, *de novo* anti-HLA class I and II titers, even when non-persistent, significantly predispose to chronic rejection (11, 15, 17, 19, 24–28). The alloimmune priming of HLA reactive B cells is believed to trigger loss of self-tolerance and development of cellular and humoral autoimmunity (26, 29). Owing to clinical significance, a number of transplant centers now routinely screen prospective LTx

[1] www.ishlt.org/registries/.
[2] www.citregistry.org/.
[3] https://optn.transplant.hrsa.gov.
[4] https://www.ebi.ac.uk/ipd/imgt/hla/stats.html.

recipients for preexisting DSA for an immediate pretransplant desensitization and monitor for *de novo* DSA during post-transplant period.

In addition to HLA, several non-HLA molecules have been targeted by immune responses after allogeneic transplantation, which can influence post-LTx outcomes. Abs to MHC class I chain A (MICA) were reported to develop after DSA and were significantly correlated with BOS development (30). Abs to mismatched HLA or MICA are suspected to induce immune responses to various tissue-restricted self-antigens (26, 30). Development of Abs to filamentous self-proteins such as Collagen V (Col V) and K-alpha1 tubulin (Kα1T) have been studied in LTx recipients with great interest (31), and in experimental mice with OAD (22, 23). Col V forms the core component of the fibrillar extracellular matrix (ECM) in the lungs, and Kα1T is a cytoskeletal protein involved in intracellular locomotion. Preexisting anti-Col V and anti-Kα1T have also been associated with primary lung graft dysfunction (32, 33), which predisposes LTx recipients to development of both acute rejection (34) and BOS (35). Furthermore, role of anti-Col V and anti-Kα1T has been demonstrated in murine orthotopic LTx model where exogenous Ab administration disrupted an established lung graft tolerance resulting in fibrotic lesions in small airways and elicited lung-directed cellular autoimmunity (36). Despite the current focus on these two tissue-associated self-antigens (i.e., Col V and Kα1T) as a measure of lung-restricted autoimmunity, it is likely that a larger antigenic repertoire participates in lung graft-directed immune responses and rejection. Further analysis of these putative antigens may help delineate the pathogenic processes and facilitate development of new therapeutic strategies.

Intricacy of B Cell Targets

Terasaki proposed a "humoral theory" to explain the basis of Abs influencing allograft rejection (37). This theory, formulated after in-depth analysis of kidney, heart, lung, and liver transplants, states that detection of graft-specific Abs is a reliable measure of humoral sensitization and an early indicator of graft rejection. The humoral theory gained credence when anti-donor humoral response was established to be the major factor in hyperacute and chronic graft rejection (10, 38). B cell sensitization against mismatched donor HLA may readily occur as they are non-self proteins and, by virtue of their cell-surface expression, are amenable to B cells. Further, an indirect antigen presentation pathway has been established in which a recipient's antigen-presenting cells acquire the donor antigens, activating antigen-specific CD4 T cells that provide necessary costimulation for B cell priming (39). An intercellular antigen transfer has also been described in LTx, wherein recipient's antigen-presenting cells acquire and cross-present donor antigens *via* a "semidirect" pathway (40).

The generation of Ab to sub-surface non-HLA antigens (including various tissue-restricted self-antigens) is poorly understood. Col V is an important component of heterotopic collagen fibers, as it initiates the fibril assembly by serving as a nucleator to Collagen I and regulates the number and length of fibrils (41). Col V is a minor component (constituting nearly 2–5% of total collagen in most tissue) and remains buried in the healthy collagen fibers, while Kα1T is a polymerized cytoskeletal protein of the microtubule. Given their intracellular sequestration, it is intriguing that Col V and Kα1T become targets and driving forces for immunopathogenesis of lung allograft rejection. In order for this self-antigen-directed reactivity to proceed, two significant immunologic requisites must be fulfilled: (1) Col V and Kα1T must be available to the immune system since B cell receptors can recognize native or linear epitopes, and the buried or "cryptic" antigens (i.e., Col V and Kα1T) must be accessible to the circulating B cells during post-LTx sensitization; and (2) the repertoire of Col V and Kα1T specific B cells must be intact and functional.

Mechanisms and modality of how the sequestered lung-restricted antigens may become bioavailable has generated significant clinical interests. Metalloproteases produced during transplant-related ischemic reperfusion injury to the lungs structurally impair fiber integrity and strip the collagen fibers, thereby exposing the core Col V fibers (42, 43). In addition, fragments of Col V are released into bronchoalveolar lavage fluid after allogeneic LTx (44). It is possible that during this immunologic assault (which may result in cell death), cytoskeletal components may become available for B cell priming. Nevertheless, the notion of a circulating pool of Col V and Kα1T reactive B cells requires thoughtful analysis and experimentation. Notwithstanding the ubiquitous expression of Col V and Kα1T, lack of a deletional tolerance may introduce risk of autoimmunity. The possible escape of Col V- and Kα1T-reactive B cells may be due to an incomplete clonal deletion, or the immune response directed toward altered/neo-epitopes on Col V and Kα1T generated by posttranscriptional modifications. Recent study strongly suggests that the breakdown of peripheral tolerance *via* T regulatory cell populations may affect the development of immune responses to these fibrillar proteins (45). Nonetheless, clonal tolerance has been successfully achieved in a rat LTx model, in which oral administration of Col V induced protection from chronic rejection (46, 47).

While several possible routes may exist by which donor antigens become available to immune priming, our laboratory and others' have recently demonstrated a long-term persistence of donor-derived alveolar macrophage (AM) in transplanted lungs (48, 49). The donor AMs act as a reservoir of donor HLA and are available for stimulating graft-infiltrating B cells. We have also shown that mismatched MHC present on AM is sufficient to elicit anti-donor T cells and Abs. Therefore, the donor AM may initiate and/or contribute to the post-LTx DSA responses. At the instance of a matched HLA class II allele between donor and recipient, donor AMs can participate in direct presentation of endogenous antigens (donor-derived) to the recipient's CD4 T cells.

In order to define the spread of alloantigenic immune responses in to tissue-restricted autoimmune response, we recently characterized exosomes isolated from serum samples from LTx recipients (50). Exosomes are membrane-bound nano-vesicles involved in cell-to-cell communication. In our study, exosomes from patients with acute rejection and with BOS contained donor HLA, Col V and Kα1T, and various immunostimulatory microRNAs. Exosomes from stable LTx recipients, however, contained neither Col V nor Kα1T and featured a profile of

immunoregulatoy microRNAs. It is hypothesized that exosomes in patients with graft rejection are immunogenic and that these exosomes can essentially traffic and deliver their antigenic cargo toward priming of allogeneic donor HLA and lung-restricted autoantigen specific of CD4 T cells (50). The role of exosomes has also been recognized in other solid organ transplantations (51). Whether or not exosomes participate in the antigen presentation by semidirect pathway has not been established, and their contribution to the indirect and direct pathways of antigen presentations remains to be validated.

Analysis of ECM, as a potential source of lung-restricted self-antigens, in small airway inflammation and fibrosis associated with BOS is unconventional. Biochemically, ECM is a complex adduct of glycoproteins, collagens, and polysaccharides and is responsible for homeostatic maintenance of the lungs, including their development, maturation, and post-injury tissue repair (52, 53). In patients with fibrotic lung diseases or who develop BOS after LTx, aberrant ECM deposition leads to permanent tissue scarring. An alloimmune reaction directed at the lung graft generates "redox hotspots" with surplus reactive oxygen species. Redox reactions in the lungs are known to modulate signaling and composition of ECM (54), pericellular localization, and extracellular focal plaque formation (55). The inflammatory nature of ECM, particularly with regards to ECM-infiltrating immune cells and composition of the fibrotic scars, may indicate the role of ECM as an antigen reservoir and initiator of inflammation that prompts lung-directed autoimmune responses.

With regards to small airway epithelial cells, club cells may play an important role in the elicitation and/or amplification of lung graft-directed immune responses. Club cell secretory protein (CCSP) is an important component of the pulmonary surfactant that has an anti-inflammatory function (56–60). A significant reduction in bronchoalveolar CCSP and club cells has been reported in LTx recipients who develop BOS compared to stable LTx recipients (61). These results suggest that declining CCSP may augment both the innate and adaptive immune responses that lead to lung allograft rejection.

Pathogenesis

The tenets of Terasaki's humoral theory (10, 37, 38) on the primary role of HLA Abs in solid organ rejection remain generally undisputed, and the development of Abs and their correlation with lung graft rejection has been increasingly reported across transplant centers. Because adaptive cellular and humoral effectors work in tandem in the elicitation of graft-directed immune responses, it is difficult to ascribe a dominant role for one over the other. With respect to immunologic factors associated with allograft rejection, Terasaki and Cai suggested that: (1) Abs play a causative role in the pathogenesis of graft rejection and (2) acute cellular rejection (ACR) can be of humoral origin (38).

Antibody-mediated rejection (AMR) of a lung graft results in three primary, interdependent manifestations: (1) hyperacute rejection, (2) acute humoral rejection, and (3) chronic lung allograft dysfunction (CLAD). Although AMR lesions are well defined in renal and cardiac allografts, the criteria for assessment of lung AMR are continuously changing based on varying immunopathology observed in pulmonary biopsies. The recent International Society for Heart and Lung Transplantation grading criteria for evaluating lung graft rejection define pulmonary AMR as presence of donor-HLA-specific Abs and characteristic lung histology that may or may not be accompanied by complement deposition in the graft (62). Furthermore, AMR may persist subclinically—that is, without being detected. Occurrence of preformed, sometimes-low-titer Abs to donor-HLA, Col V or Kα1T pre-LTx have been shown to increase risk of graft rejection (33, 63). Delivery of exogenous anti-Col V and anti-Kα1T produced AMR in experimental murine LTx (32, 64). ACR is a common but reversible immune reaction, with characteristic perivascular or peribronchiolar mononuclear infiltrations. AMR, on the other hand, is difficult to diagnose and may accompany local activation of complements caused by DSA (65–67).

Higher frequency and high grade of AMR are risk factors for development of HLA Abs and BOS (34, 68), but AMR exhibits a strong association with *de novo* development of anti-HLA, significantly increasing the risk of BOS (24). BOS continues to be the major cause of posttransplant morbidity and mortality, affecting approximately 50% of patients with transplanted lungs within 5 years of LTx (69). It clinically manifests with progressive, irreversible loss of respiratory function (>20% of baseline) that is unresponsive to any immunosuppressive regimen (70). A large body of work has established a strong humoral link that predisposes for development of BOS after LTx. DSA directed to MHC class I and II proteins, even when detected only transiently, poses significant and independent risks for BOS development and influences its onset kinetics, severity, and mortality (11, 15, 17, 19, 24–28, 71).

In addition to BOS, restrictive allograft syndrome (RAS) has been recently described as another form of CLAD after human LTx (72). RAS is an airway-restrictive phenotype and is more aggressive than BOS, with median survival of just 6–18 months after diagnosis (73–76). The alloimmune priming of donor-HLA reactive B cells is believed to trigger loss of self-tolerance and intermolecular epitope spreading, eliciting cellular and humoral responses directed to Col V and Kα1T. Therefore, a functional interplay has been proposed in which donor-directed alloimmunity leads to development of tissue-restricted autoimmunity to self-antigens (26, 29, 77).

The immunodominant role of DSA in chronic rejection has been recognized by three distinct observations: (1) *de novo* DSA is associated with recurrent and high-grade cellular rejection and lymphocytic bronchiolitis (11, 15), (2) development of DSA often precedes *de novo* Col V- and Kα1T-specific Abs (26), and (3) depletion of the circulating Abs by Ab-directed therapy offers protection from BOS with a lower hazard ratio and enhanced pulmonary function (25, 27, 78–80). Preexisting Abs to Col V and Kα1T have been found in different terminal lung diseases pre-LTx, and such pretransplant autoantibodies were significantly correlated with poor outcomes, including development of DSA and BOS (33, 81). Moreover, the absence of preexisting Abs to lung self-antigens was correlated with freedom from *de novo* MHC class I and class II DSA and with lower incidence of BOS (33). Preformed antibodies to lung-restricted self-antigens without measurable DSA have also been associated with BOS development after human LTx (24). In summary, the current consensus is

that both DSA and Abs to lung-restricted self-antigens (whether preformed or *de novo*) are significant risk factors for all forms of lung allograft rejection and limit both short- and long-term success of LTx.

Diagnosis

Sensitive detection and measurement of graft-specific Abs have been closely linked with the evolution of allogeneic organ transplantation, often serving as a designation of success in solid organ transplantation. With outcomes being dependent on the extent of antigenic mismatches between donor and recipient, optimization of matching strategies has been the subject of much discussion. A consensus guideline has been formulated for Ab testing in transplantation (82). Crossmatching was an early test in which recipient's serum was mixed with donor's cells to detect presence of anti-donor Abs. A negative crossmatch—first applied in renal transplantation—was effective in minimizing graft failure (9). The development of the complement-dependent cytotoxicity test utilized complement fixation and mixing of donor lymphocytes with recipient's serum in the presence of complement. This assay was further refined when antiglobulin was added to augment the reaction (83).

More recently, however, flow cytometry has been the test of choice to define crossmatch compatibility, given its high sensitivity. A shortcoming of crossmatching is the amount of time it takes—it was difficult to perform prospectively and it increased cold ischemic time of the organ. Currently, a virtual crossmatch is preferred, especially for lung and heart transplantation procedures. The development of the "HLA/Teraski plate" was a crucial step in the commercialization of assay reagents, as it enabled testing for presence and specificity of panel-reactive antibodies (PRAs). Recently, solid-phase immune assays such as FlowPRA (OneLambda, Canoga Park, CA, USA), LABScreen (OneLambda, Canoga Park, CA, USA) and Lifecodes LifeScreen (Immucor, Peachtree Corners, GA, USA) were developed. FlowPRA is a flow cytometry-based immunoassay for rapid detection of HLA class I and class II Abs in human serum. Using a unique set of recombinant HLA protein adsorbed fluorescent microbeads binding of specific Abs in serum is detected by flow cytometry. LABScreen and LifeScreen, on the other hand, allow precise determination of allelic HLA and/or MICA on a Luminex (Luminex Corporation, Austin, TX, USA) platform. Currently, assays such as LABscreen and FlowPRA are standard and are routinely conducted for testing of PRAs at HLA laboratories. These assays can successfully assign antigenic specificities to perform virtual crossmatching.

In stark contrast to the advancements in immunologic detection of HLA Abs, progression of methods to identify and characterize Abs to tissue-restricted self-antigens is in its infancy. The diversity of tissue-restricted self-antigens involved in allograft rejection is still being investigated, and there are no validated kits commercially available for detecting immune responses to self-antigens. In addition, a possibility of low titer circulating Abs (below detection limit) as the graft undergoing rejection may already sequester them further complicates the detection issue. A solid-phase protein microarray has been employed to screen targets of humoral autoimmunity following LTx (84). Given the nature of nuanced B cell antigenic determinants (i.e., conformational vs linear; continuous vs discontinuous), cellular localization (i.e., surface-bound, cytosolic, or nuclear), and posttranscriptional modifications, the results obtained from solid-phase assays are limited in their capacity to gauge the spread of humoral autoimmunity. This prevents full realization of the Ab repertoire and limits researchers' ability to identify novel targets. Currently, home-grown enzyme-linked immunosorbent assays are the most commonly used procedures for detecting Abs to various tissue-restricted self-antigens (31, 32).

CLINICAL PANCREATIC ISLET TRANSPLANTATION

Transplantation of whole pancreas or isolated pancreatic islets are two effective treatment options for brittle (i.e., severe) T1D patients, as both procedures replace the depleted β-cell mass lost due to autoimmunity. Islet transplantation has the advantage of being a minimally invasive procedure compared to transplantation of pancreas. Most of T1D patients receive exogenous insulin therapy to control blood glucose levels, but this often results in severe and recurrent of hypoglycemia (85). Many patients who undergo islet transplantation achieve normoglycemia and experience freedom from the life-threatening consequences of severe hypoglycemic episodes. Despite significant advances in the islet isolation protocol, islets isolated from more than one donor pancreas are often required to achieve insulin-independent status (86), and a majority of islet transplant recipients return to some form of exogenous insulin usage within a few years of transplantation due to chronic rejection (87).

According to the Collaborative Islet Transplant Registry (See text footnote 2) data collected from the islet transplant centers around the world indicate 1- and 5-year insulin-independence rates after final islet infusion at 50% and 23%, respectively. Majority of the procedures performed in North America are islet transplant alone where as a higher number of procedures performed at European centers are simultaneous islet-kidney allograft (SIK) transplantation or islet after kidney allograft (IAK) transplantation. The SIK/IAK rate in North America is 10% were as that in European centers are >35%. A small number of islets after LTx have been successfully performed at the GRAGIL (Groupe Rhin-Rhône-Alpes-Genève pour la Transplantation d'Ilots de Langerhans) consortium (1). This combined transplantation was particularly beneficial for patients with end-stage cystic fibrosis and severe cystic fibrosis-related diabetes. Long-term follow-up on transplant recipients resulted in persistent improvement of glycemic control with normalized glycated hemoglobin (HbA_{1c}) in conjunction with significant (50%) reduction in the daily exogenous insulin requirement. The normoglycemia was durable and was preserved for a durable period of 15 years in these lung-islet combined transplant recipients.

Multiple factors contribute to loss of islet grafts, including poor islet quality, posttransplant inflammation, immunosuppressive drug-induced toxicity, recurrent autoimmunity, β-cell exhaustion, and alloimmune responses (88). Primary causes of islet allograft rejection are thought to be incidences of β-cell

directed autoimmunity in combination with the alloimmune response to multiple mismatched HLA antigens that significantly impact long-term islet cell function.

Alloimmunity against Islet Grafts

Introduction of allogeneic tissue into the body during solid organ or cellular transplantation is known to produce *de novo* anti-HLA, which plays a major role in acute and/or chronic graft failure. Rejection due to alloimmunity after islet transplantation is mainly due to the Abs against donor-specific HLA class I and II molecules (89, 90). Moreover, the requirement of multiple islet infusions to achieve insulin independence exposes transplant recipients to an unusually high number of HLA mismatches, resulting in elevated risk for a broader spectrum of donor-specific HLA Abs (88). Our group researched a possible role for HLA Abs in the rejection of islet allografts (91). This report was followed by a comprehensive analysis of both cellular and humoral responses against donor-specific antigens in seven islet transplant recipients, and a clear association of flow cytometry-detected and immunospot-detected T cells with islet graft failure was revealed (92). Of these seven patients, three with positive donor-specific responses rejected islet allografts, either acutely or chronically. A concurrent report by Rickels et al. reported islet graft failure in two of six patients with detectable HLA class I and II antibodies (93). Cardani et al. analyzed HLA sensitization in 66 patients who underwent islet transplantation at a single center between 1985 and 2006 and reported no significant correlation between positive PRA and islet graft transplantation outcome (94). However, loss of islet graft function was associated with positive PRA after immunosuppression tapering or infection. The study by Cardani et al. also revealed that 24% patients developed PRA after immunosuppression was discontinued.

The Edmonton group revealed its findings on the role of anti-donor HLA in the islet allograft rejection in a large patient cohort, screening posttransplant HLA Abs in 98 patients. In their cohort, 29 patients (30%) developed DSA, including 23 recipients (23.5%) who developed DSA while still on immunosuppression. Ten of the fourteen patients (71%) who discontinued immunosuppression developed extensive amounts of PRA. This report documented posttransplant HLA sensitization among patients who had negative PRA prior to transplantation. Importantly, the fasting C-peptide, which is an indicator of islet graft function, was significantly lower in sensitized recipients compared to non-sensitized recipients (95). Another study showed that pretransplant detection of PRA to MHC class I and II (>15%) among recipients was associated with increased need for insulin post-transplant (96).

The Collaborative Islet Transplant Registry offered a comprehensive analysis of 308 patients who received islet transplantation at different centers between 1999 and 2008. They found that HLA class I sensitization both pre- and posttransplant was correlated with islet graft failure. Unlike any other type of organ transplantation, allogeneic islet recipients could be exposed to a total of 9–25 HLA class I and II antigen mismatches. This extraordinarily high number of mismatches was likely due to the multiple HLA-mismatched pancreas donors used for islet isolation, and also possibly the result of increased risk of patients receiving any future transplants (88). A significant increase in the level of HLA Abs was seen, even when patients adhered to an immunosuppression regimen. It would be beneficial if islet transplants could be performed with single-donor islet infusion, as it would minimize the risk of broad sensitization.

Despite clinical studies reporting a possible association between HLA sensitization and islet graft failure, causality has still not been definitively established. Previous *in vivo* studies have indicated an association between DSA and islet transplantation survival. Using a congenic rat islet transplant model, Bittscheidt and colleagues have reported that graft survival was significantly influenced by the degree of donor-recipient MHC matching as well as recipient presensitization (97).

Other reports have shown that the presence of pretransplant autoreactive T cells and *de novo* donor-specific cytotoxic CD4 T cells resulted in poor outcomes after islet transplantation (92, 98, 99). In fact, the preformed auto- and alloreactive Abs were considered to be negative indicators for survival of the islet graft (96, 98, 100). Thus, the adaptive immune system plays essential role in long-term islet graft survival and influences clinical outcomes. The allosensitization triggered by donor antigens likely elicits the interferon-γ- and interleukin (IL)-2-mediated T helper1 response, which is destructive for the function of the transplanted islets (92). An *in vitro* study of mixed islet leukocyte reaction by Bouwman et al. showed induction of direct T cell response with augmented responses in cases with two or more mismatches of HLA class II (101).

A recent contrasting report showed that 11/18 islet transplant recipients (61.1%) had preexisting anti-HLA, including 6 patients (33%) who developed *de novo* DSA against the HLA of the transplanted islets (90). Remarkably, no significant association existed between the newly developed DSA after islet transplantation and clinical characteristics such as recipient gender, age, number of post-transplant infections, HLA class I/II eplet mismatch, and immunosuppression protocol. Furthermore, the *de novo* DSA was not associated with reduced graft survival or function. Interestingly, the newly formed posttransplant anti-HLA class II Abs were related to the Predicted Indirectly Recognizable HLA Epitopes (PIRCHE-II[5]), specific to the T helper epitopes.

Long-term survival of an allogeneic islet transplant faces a greater challenge than solid organ or other cellular transplants due to the preexisting islet-specific autoimmunity and the likely scenario of multiple HLA-mismatched islet infusions. In a recent analysis of 59 consecutive islet transplant recipients, 39 (66%) experienced *de novo* titer increase in both DSA and autoantibodies (96). Patients with increased Ab titers had significantly lower graft survival. Furthermore, newly developed DSA was associated with MHC class II (HLA-DR) mismatches and preexisting PRA (96).

The expression of MHC class II in the endocrine tissue of freshly isolated pancreatic islets is nearly undetectable. However,

[5]http://company.pirche.org/.

posttransplant infection or the presence of proinflammatory cytokines can induce the expression of MHC class II. Such induced expression of MHC class II has been demonstrated *in vitro* and has been associated with development of donor-specific HLA class II Ab production after islet transplantation (91).

Autoimmunity against Islet Grafts

Because pancreatic islet transplantation is often performed in patients with T1D, the existence of preformed autoantibodies against major islet-specific antigens is an inherent issue for most recipients. Autoantibodies in the circulation are formed during the pathogenesis of T1D prior to islet transplantation. Autoantibodies against pancreatic islets that are deemed clinically significant include anti-glutamate decarboxylase 65 (GAD65), anti-insulin autoantibody, anti-zinc transporter ZnT8, anti-islet cell autoantibody, and anti-tyrosine phosphatase autoantibody (IA-2) (102). Some of the earlier reports of posttransplant immune responses in islet transplant recipients suggested that no correlation existed between preexisting autoantibodies and islet graft failure (96, 98). Similar claims were made about the role of autoantibodies in clinical outcomes of whole pancreas transplants (103). However, preformed autoantibodies in islet recipients have indeed been associated with graft failure compared with recipients who did not have autoantibodies (104). Compared to preformed autoantibodies, the elevated recurrences of autoantibodies specific to ZnT8A, GAD65, and IA-2 in the posttransplant period were predictive of graft failure in islet transplantation (96) and foretold an 80% chance of graft failure in transplanted whole pancreas (105). In spite of the early notion of a non-association between preexisting autoantibodies and graft loss, analysis of these autoantibodies may be clinically useful in helping health practitioners anticipate recurrences of autoimmune graft rejection. A number of factors may affect reappearance of autoantibody, for example, suboptimal immunosuppression protocols that include CD25 antagonism or inhibition of mechanistic target of rapamycin (mTOR) by rapamycin. Antithymocyte globulin (ATG) and mycophenolate mofetil (MMF), meanwhile, decrease the risk of autoantibody recurrence (96).

An early study considered seven islet allograft recipients to determine the significance of autoreactivity in islet transplant outcomes (106). In this study, three out of seven recipients under ATG induction immunosuppression retained comprehensive islet function more than 1 year post-transplant, with less autoreactivity and with no alloreactivities. The remaining four patients received no ATG in their immunosuppression treatment. Of these, three patients lost islet function within 3 weeks, and one patient demonstrated hyper-autoreactivity without alloreactivity and experienced a delayed loss of islet function around 33 weeks, with recurrence of autoimmunity (106). This group later studied the incidence of allo- and autoimmunity in 29 islet transplant recipients 1 year after islet transplantation. Repeat analysis included modification in the immunosuppression regimen. ATG was a part of induction, and tacrolimus with MMF were included in the maintenance immunosuppression protocol. The outcomes of this study demonstrated that pre- and posttransplant autoimmunity were associated with delayed insulin non-requirement and autoimmunity was directly proportional to the recipients' circulating C-peptide 1 year post-transplant. Moreover, seven out of eight recipients who had no history of pretransplant autoreactivity achieved insulin independence while none of the four recipients who had preformed autoreactivity, predominantly against GAD and IA-2, achieved insulin independence (107). These analyses suggest that including ATG in the induction immunosuppression may help control autoreactivity and improve islet graft function.

The association between a recipient's pre- and posttransplant autoreactivity and clinical outcomes are often highly variable from center to center. A previous report (106) asserted that no correlation exists between recipients' autoimmunity and graft function posttransplant, but others have stated that 60% of islet transplantation recipients with fewer autoreactive circulating GAD65 T cells achieved long-term insulin independence, whereas the 40% of patients with elevated levels of autoreactive GAD65-specific T cells producing proinflammatory cytokines and did not achieve long-term insulin independence (108). The findings of Chujo et al. are supported by another report, in which pretransplant GAD65 and IA-2 autoreactive T cells affected the 1-year insulin-independence rate of alloislet transplant recipients (107).

Allogeneic graft rejection and autoimmune recurrence make a critical contribution to long-term outcomes after islet transplantation, and CD4+ and CD8+ T cells are vital in the pathogenesis of graft rejection. Moreover, T1D recipients have an increase in autoreactive memory T cells (109). Donor-specific MHC molecules likewise play an important role in activation of immunogenic T cells in the recipients, affecting islet graft outcomes. Huurman et al. analyzed islet transplantation recipients' cellular responses to donor-specific MHC class II antigens and measured the expansion of alloreactive CD4+ T cells by ^{3}H-thymidine incorporation (110). They also measured release of pro- and anti-inflammatory cytokines in the culture supernatant *in vitro*. Recipients who achieved long-term insulin independence expressed greater IL-10 release and regulatory T cells compared to recipients with failed allografts, who showed more IL-2 release in the supernatant (110).

Autoreactive CD8+ T cells are thought to play an active role in the destruction of alloislet graft. Memory autoreactive T cells have a longer half-life, are preserved in the circulation for a long time and expand after exposure to the specific autoantigens posttransplantation. Velthuis et al. analyzed frequency of CD8+ T cells in T1D patients and islet transplant recipients and demonstrated that T cells reacting to insulin and pre-proinsulin epitopes increased after transplantation (111). Further, the presence of some alloreactive CD8+ T cells possibly due to donor/recipient MHC mismatch induced expansion of cytotoxic T cells causing acute islet graft rejection (99).

Immunosuppression in Islet Transplantation

Understanding the role of islet-specific alloimmune responses and autoimmunity in islet graft function has been useful in designing effective immunosuppression regimens that can control graft failure. Most immunosuppression drugs commonly used

in whole organ transplantation are either lethal to transplanted islets or induce diabetes in the recipients. For recipients of islet transplantations, azathioprine, cyclosporine, and corticosteroids are usually included in the immunosuppression regimen in the immediate posttransplant period. Most islet transplants are performed after renal or simultaneously with renal transplantation. Application of these immunosuppression regimens has produced variable posttransplant outcomes in islet transplantation (112–114). Cyclosporine sparing immunosuppression is preferred based on several *in vitro* studies that have documented its β-cell toxicity (112, 115, 116).

The outstanding posttransplant result achieved with the Edmonton protocol (i.e., seven out of seven patients became insulin-independent) was partly due to a modified immunosuppression regimen, which included no steroids, and included induction with daclizumab and maintenance with low-dose tacrolimus and sirolimus (117). Impaired graft function and β-cell proliferation were reported with sirolimus (118, 119); however, no clinically significant adverse effects or decrease in islet engraftment were reported (120). In spite of its diabetogenic property, inclusion of low-dose tacrolimus in the immunosuppression maintenance protocol effectively improved short-term islet graft function (121). For long-term islet graft function, however, the Edmonton protocol was not effective. Among 47 patients who achieved insulin independence, only four (8.5%) retained the insulin-independent status at 5 years (122).

Recent improvement in the immunosuppression protocol has significantly enhanced the clinical islet transplant outcomes as illustrated in **Table 1**. Long-term islet transplant outcomes were improved with the use of humanized anti-CD3 Ab (OKT3γ1 Ala-Ala) for induction that depletes mature T cells followed by administration of calcineurin and mTOR inhibitors. The same group later effectively used T cell depletion with ATG along with antitumor necrosis factor α in the induction regimen and achieved superior long-term insulin independence in 50% (4/8) of the recipients (123, 124).

The Clinical Islet Transplantation Consortium[6] recently reported encouraging results in 48 patients who underwent islet transplantation. All 48 T1D patients experienced severe hypoglycemic episodes and impaired awareness of hypoglycemia pretransplant, and all maintained a regimen of ATG or basiliximab for induction and sirolimus with low-dose tacrolimus for immunosuppressive maintenance (85). In this study, the primary end point fixed was the accomplishment of $HbA_{1c} < 7.0\%$ (53 mmol/mol) at 1 year and freedom from severe hypoglycemic episodes from day 28 to day 365 after initial transplant. The primary end point was effectively met by 42/48 patients (87.5%) at one and by 34/48 patients (71%) at two posttransplant years. Hypoglycemia awareness was significantly restored ($p > 0.0001$), with marked improvement in Clarke and HYPO scores in all patients in the study (85).

A major obstacle to successful islet engraftment is the activation of an innate immune response, called "instant blood-mediated inflammatory response" (IBMIR). This reaction is characterized by release of proinflammatory cytokines, infiltration of innate immune cells, and activation of coagulation pathways (130). IBMIR has been documented in all three forms of islet transplantation (i.e., autologous, allogenous, and xenogenous). It is estimated that roughly 50% of transplanted islets are irreversibly damaged during the peri-transplant period, usually within hours to days. Following islet transplantation, release of proinflammatory cytokines and chemokines has been reported (131). These soluble molecules include tissue factor, high-mobility group protein B1, cytokines and chemokines such as chemokine (C-C motif) ligand 2, chemokine (C-X-C motif) ligand (CXCL) 12, tumor necrosis factor (TNF) α, IL-1β, IL-6, and CXCL8/IL-8. Introduction of two anti-inflammatory compounds that inhibit TNF-α (etanercept), IL-1β (anakinra) has been shown to improve islet allograft function (126). However, a direct link between the control of IBMIR and development autoimmune and alloimmune responses has

[6]www.citisletstudy.org.

TABLE 1 | Immunosuppression strategies in clinical pancreatic islet transplantation.

Induction IS	Maintenance IS	No. of recipients	Type of transplant	II achieved	Year/reference
ATG + Bela	Sir + MMF	5	ITA	5	2010/(125)
ATG + Efa/	Sir + MMF	5	ITA	5	2010/(125)
ATG + ETA + Ana/	Tac/MMF	3	ITA	3	2011/(126)
Dac/	Tac/Sir	3	ITA	3	2011/(126)
ATG + Tep or ATG + TCDAb + TNFi	Tac or CsA/Sir or CsA/Sir or Eve	29	ITA	15	2012/(124)
TCDAb + TNFi	Tac or CsA/Sir or Eve	20	ITA	10	2012/(124)
TCDAb	Tac or CsA/Sir or Eve	43	ITA	N/A	2012/(124)
Dac	Tac or CsA/Sir or Eve	177	ITA	35	2012/(124)
ATG	Sir	12	ITA	5	2014/(127)
ATG	Tac + MMF	48	ITA	N/A	2014/(128)
ATG or Dac or Bas	Tac or Sir	38	SIK/IAK	4	2015/(129)
ATG or Bas	Sir + Tac	48	ITA	25	2016/(85)
ATG or Bas	Ste or Tac + Aza	18	ITA/SIK/IAL/SILL	9	2016/(90)

Ale, alemtuzumab; Ana, anakinra; ATG, antithymocyte globulin; Aza, azathioprine; Bas, basiliximab; Bela, belatacept; CsA, cyclosporine A; Dac, daclizumab; Efa, efalizumab; Eta, etanercept; Eve, everolimus; Exe, exenatide; IAK, islet after kidney transplantation; IAL, islet after lung or liver transplantation; II, insulin independence achieved in no. of patients; Inf, infliximab; IS, immunosuppression; ITA, islet transplantation alone; MMF, mycophenolate mofetil; N/A, not available; SIK, simultaneous islet-kidney transplantation; SILL, simultaneous islet-liver-lung transplantation; Sir, sirolimus; Ste, steroids; Tac, tacrolimus; TCDAb, T cell depleting antibodies; TNFi, tumor necrosis factor-α inhibition.

not been established in allogenic islet transplantation and will undoubtedly be the focus of future studies.

CONCLUSION

In this review, we have synthesized the published reports on immune responses to alloantigens encoded by HLA loci and non-HLA tissue-restricted self-antigens in the pathogenesis of graft rejection after human lung and islet transplantation. Much work has been done to determine the role of HLA Abs in allograft rejection, but consideration of immune responses to non-HLA antigens (including tissue-restricted self-antigens) is still a new territory. Many recent studies have suggested that tissue-restricted self-antigens direct immune responses and play a meaningful role in allograft rejection. Nevertheless, it remains unclear how these tissue-restricted immune responses initiate and perpetuate graft rejection, how tolerance to these tissue-restricted self-antigens are broken, and what role alloimmunity plays in the pathogenesis of chronic rejection despite immunosuppressive regimens.

Current work would suggest a crosstalk between allo- and autoimmunity in both lung and islet transplantation. It is possible that, once initiated, immune responses to tissue-restricted self-antigens are not suppressed by the immunosuppressive regimen, and this smoldering immune reaction contributes to the onset and progression of chronic allograft dysfunction. In experimental models, there is evidence that Abs to both MHC and to tissue-restricted self-antigens can break established tolerance, suggesting that Abs are the driving force in the induction of chronic allograft dysfunction. Therefore, there is an urgent need to develop new diagnostic and/or therapeutic approaches to prevent Abs and to treat chronic allograft dysfunction.

AUTHOR CONTRIBUTIONS

DKN, PBS, BN, and TM conceptualized the review; DKN and PBS wrote the manuscript; SB participated in discussion; BN and TM critically revised the manuscript.

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MINI REVIEW
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Impact of Non-Human Leukocyte Antigen-Specific Antibodies in Kidney and Heart Transplantation

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The presence of donor human leukocyte antigen (HLA)-specific antibodies has been shown to be associated with graft loss and decreased patient survival, but it is not uncommon that donor-specific HLA antibodies are absent in patients with biopsy-proven antibody-mediated rejection. In this review, we focus on the latest findings on antibodies against non-HLA antigens in kidney and heart transplantation. These non-HLA antigens include myosin, vimentin, Kα1 tubulin, collagen, and angiotensin II type 1 receptor. It is suggested that the detrimental effects of HLA antibodies and non-HLA antibodies synergize together to impact graft outcome. Injury of graft by HLA antibodies can cause the exposure of neo-antigens which in turn stimulate the production of antibodies against non-HLA antigens. On the other hand, the presence of non-HLA antibodies may increase the risk for a patient to develop HLA-specific antibodies. These findings indicate it is imperative to stratify the patient's immunologic risk by assessing both HLA and non-HLA antibodies.

Keywords: human leukocyte antigen, non-human leukocyte antigen antibody, kidney transplant, heart transplant, angiotensin II type 1 receptor antibody

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INTRODUCTION

Despite the advancement of improved immunosuppression regimens and optimized patient management, antibody-mediated rejection remains a major obstacle for long-term graft survival (1). Initial emphasis has been on the identification of human leukocyte antigen (HLA)-specific antibodies directed against the donor HLA class I and/or class II antigens. With the introduction of commercially available solid phase assay reagents, a greater understanding of the specificity, strength, and function of these antibodies has been made possible. Further, the wide acceptance of these reagents in the clinical transplant testing setting, along with proficiency testing programs has allowed for increased consistency in the definition of the antibodies detected. Correlating the antibody information obtained together with the clinical graft outcome has revealed that patients with antibodies against donor HLA are at a higher risk of antibody-mediated rejection and poorer graft outcome (2, 3). However, for many patients who have allograft dysfunction and show histological

Abbreviations: HLA, human leukocyte antigen; MICA, major histocompatibility complex class I chain-related gene A; AT_1R, angiotensin II type 1 receptor; ET_AR, endothelin type A receptor; NK, natural killer; GPCR, G protein-coupled receptor; SNPs, single-nucleotide polymorphisms; PLC, phospholipase C; cAMP, cyclic adenosine monophosphate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FLT3, Fms-like tyrosine kinase-3 ligand; EDIL3, EGF-215-like repeats and discoidin I-like domains 3; ICAM4, intercellular adhesion molecule 4.

characteristics of antibody-mediated rejection on biopsy, no donor HLA-specific antibodies are detected (2, 4). In heart transplant, it has been shown that 40% of patients diagnosed with biopsy-proven antibody-mediated rejection did not show donor HLA-specific antibodies in the peripheral blood (5). These studies initiated investigation into non-HLA-specific antigens, many of which are expressed by the vascular endothelium and often revealed after stress or graft injury. Even though the detection of many of these non-HLA antibodies remains elusive, antibodies to the following non-HLA antigens have been shown to be associated with graft dysfunction or rejection: major histocompatibility complex class I chain-related gene A (MICA), angiotensin II type 1 receptor (AT_1R), endothelin type A receptor (ET_AR), heat shock protein, phospholipid, K-α-tubulin, vimentin, and endothelial cell antigens (6). Recently, commercial reagents have become available to define antibodies to AT_1R, ET_AR, MICA, and endothelial cell antigens. These results have been used in the clinical testing setting to investigate their impact on graft outcome. Studies of antibodies to other non-HLA antigens have been largely single center results. The results for anti-HLA and non-HLA-specific antibodies indicate an interplay between alloimmunity and autoimmunity, impacting graft outcome. This review focuses on the contribution on antibodies against these non-HLA antigens in kidney and heart transplantation. For lung transplant outcomes, please refer to the comprehensive review by Dr. Mohanakumar in this same issue.

ANTIBODIES TO MICA

Major histocompatibility complex class I chain-related gene A gene is localized in the HLA gene cluster. The MICA protein shares similar structure with HLA class I but does not associate with β2 microglobulin at the cell surface and cannot bind peptides. MICA can activate natural killer (NK) cells *via* interaction with activating immunoreceptor NKG2D (7). Of the non-HLA antigens listed above, MICA is highly polymorphic with around 100 alleles identified as of July 2016. Similar to HLA molecules, the recipient and donor may carry different MICA alleles. The recipient's immune system may develop antibodies against the donor-specific MICA allele (8). It has been reported that 5–9% of renal recipients display MICA antibodies (9). The contribution of MICA antibody to pathogenesis of antibody-mediated rejection was first demonstrated in kidney transplantation (10) and was later found to be associated with rejection in pancreas and heart transplant (11, 12). Further, the patient with antibodies against donor-specific MICA is at higher risk of antibody-mediated rejection (5). The expression of MICA is not detectable on the quiescent endothelial cells which lie at the interface of the allograft and recipient blood and are directly targeted by immune response. The expression of MICA can be induced by stress and cytokines, such as TNF-α (13). The development of MICA antibodies may indicate an underlying inflammatory status which exists in these conditions.

Studies have demonstrated that expression of MICA in tumor cells leads to the activation of NK cells *via* MICA/NKG2D interaction, which in turn release cytotoxic proteins and INF-γ (7). Binding of MICA antibody on endothelial cells may block interaction between MICA and NKG2D, and thereby dampen NKG2D-mediated cytotoxicity. However, NK cells may still be activated mainly through Fc receptor-dependent cytotoxicity.

Antibodies against G Protein-Coupled Receptors (GPCRs)

AT_1R and ET_AR belong to the family of GPCRs which have seven transmembrane domains. Antibodies to the GPCRs, AT_1R, and ET_AR, may be relevant due to their endothelial cell surface expression and extracellular regions accessible to antibodies. Some of these antibodies, such as those to AT_1R, have been shown to be involved in the pathophysiology of pregnancy preeclampsia and autoimmune diseases, including systemic sclerosis (14–16). There are several possible mechanisms relevant to explain how patients without an autoimmune disease may develop these autoantibodies. One plausible reason is the immune suppression or an underlying inflammatory process may break the self-tolerance. Also, a shearing process induced by mechanical circulatory systems or dialysis may cause proteins, such as von Willebrand factor, cleaved into smaller peptides (17). It is possible that the extracellular loop of AT_1R may be clipped off the cell surface by shear stress and thereby exposing a neoantigen. The cell surface density of these GPCRs is influenced by polymorphisms and different mRNA processing mechanisms. The severity of injury by AT_1R antibodies may also be influenced by the expression level of these different AT_1R isotypes on the allograft. These antibodies may not only target the allograft but may also have a global effect. The impact of anti-AT_1R antibodies in a clinical setting was first identified in a group of kidney-transplant recipients with malignant hypertension (18), suggesting that binding of AT_1R antibodies, similar to the ligation of AT_1R with angiotensin II, can also promote vasoconstriction, water intake, and sodium retention and increase blood pressure (19). Similar to HLA antibodies, AT_1R antibodies may have detrimental effect on the graft survival. The presence of AT_1R antibodies is associated with antibody-mediated rejection, but not cellular-mediated rejection in kidney transplant (20). In heart transplant, increased levels of AT_1R have been shown to be associated with antibody-mediated rejection, cellular-mediated rejection, and early onset of microvasculopathy at 1 year posttransplant (21). In the same study, high levels of antibodies against another GPCR, ET_AR, also has been reported to be associated with antibody or cell-mediated rejection. The associations observed with either antibody-mediated rejection or cellular rejection are dependent on the current pathological definition of these types of rejection in each organ group.

AT_1R antibodies can synergize with HLA antibodies to predispose the graft to rejection. The presence of both strong binding AT_1R antibody and HLA class II donor-specific antibodies has been found to be associated with accelerated rejection, hypertensive encephalopathy, and worse graft survival in kidney transplant (20, 22, 23). In the absence of donor-specific HLA or MICA antibodies, strong binding AT_1R antibodies have been detected in patients with antibody-mediated rejection. Furthermore, transplant candidates with strong AT_1R antibodies pretransplant are at a higher risk of early antibody-mediated rejection and long-term graft loss.

Activation of AT_1R by its native ligand angiotensin II stimulates phospholipase C, production of cyclic adenosine monophosphate, and activation of mitogen-activated protein kinase, which promotes vasoconstriction and hypertension (24). Association between AT_1R antibody and rejection was first reported in patients with malignant hypertension by Dragun and her colleagues. They found that AT_1R antibodies bind to the extracellular loop 2 of AT_1R and stimulate phosphorylation of extracellular signal-regulated kinase (ERK) (18). However, studies indicate that the angiotensin II binding pocket of AT_1R is composed of all seven transmembrane helices and both extracellular loops 1 and 2 (25, 26), which is distinct from the antibody-binding site. It is not clear how AT_1R antibody stimulates similar signals as native ligand angiotensin II. Several other studies have shown the association of AT_1R antibodies with rejection, but none of these studies reported hypertension in their cohort or if these antibodies could activate signaling to stimulate hypertension (20, 21, 27–30). AT_1R antibodies have been found to be the IgG1 and IgG3 subclasses which have the capacity to activate the complement cascade (18). Interestingly, detrimental effect of AT_1R antibodies on the graft does not need complement activation. Reinsmoen et al. observed C4d-positive biopsy in only one out of six patients who had strong AT_1R antibodies and were diagnosed as antibody-mediated rejection (20). In consistent with this observation, Fuss et al. reported that AT_1R antibodies, but not donor-specific HLA antibodies, were detected in 11 cases of biopsy-proven, C4d-negative acute antibody-mediated rejection according to Banff 2013 (29). Nevertheless, AT_1R antibodies may still be detrimental to the allograft through antibody-dependent cell-mediated cytotoxicity.

AT_1R also plays an important role in glucose metabolism (31). It is suggested that higher expression of AT_1R is associated with increased risk for diabetic nephropathy, and blockade of AT_1R signaling is effective in the treatment of diabetic nephropathy (31, 32). AT_1R antibody can also activate AT_1R signaling. The presence of AT_1R antibody in renal transplant candidate might predispose development of diabetic nephropathy. Further study on the role of AT_1R antibody in diabetic nephropathy is warranted.

A commercially available test reagent provides detection of AT_1R antibody in a relatively easier and standardized manner compared to other non-HLA antibodies. However, this assay is enzyme-linked immunosorbent assay based and is sensitive to interference by intravenous immunoglobulin and rituximab treatment. The linear range of results obtained from this assay is also rather narrow. Improvement of the AT_1R-testing reagent will promote more transplant programs to adopt the AT_1R antibody test.

ANTIBODIES TO VIMENTIN

Vimentin is a subunit of an intermediate filament. As a cytoskeletal element, vimentin is important for stabilizing the architecture of the cytoplasm. However, vimentin is also found to be secreted by macrophages, endothelial cells, vascular smooth muscle cells, activated platelets, apoptotic T cells, and neutrophils (33). Secretion is increased by the pro-inflammatory cytokine TNF-α, but inhibited by the anti-inflammatory cytokine IL10, suggesting that vimentin may be involved in the immune response (34). Antibodies against citrullinated vimentin have been detected in sera of patients with rheumatoid arthritis (35). Vimentin antibodies also exist in pretransplant sera of patients with end-stage kidney disease (36). In this study, titers of IgM antibodies against vimentin increased every year posttransplant compared with pretransplant titers, but no difference was found in patients with interstitial fibrosis and tubular atrophy compared with a kidney recipient control group. By contrast, patients diagnosed with interstitial fibrosis and tubular atrophy have higher levels of IgG antibodies against vimentin. These results suggest that IgG, but not IgM, antibodies against vimentin may play a role in the pathology of interstitial fibrosis and tubular atrophy (37). Similarly, in heart transplant, patients with vimentin antibodies within the first 2 years of transplantation are at higher risk for cardiac allograft vasculopathy at 5 years posttransplant (38). The presence of vimentin antibodies is rather common in heart transplant recipients. Young et al. have shown 34% of patients display vimentin antibodies, but no difference in the rates of early rejection and graft survival was observed (39).

ANTI-MYOSIN ANTIBODIES

Myosins are a large family of proteins which bind actin cytoskeleton and move cargo proteins through ATP hydrolysis. The thymus of mice and humans does not express myosin heavy chain proteins, thus CD4+ T cells are not negatively selected for myosin in the maturation process (40). The autoantibodies frequently associated with autoimmune myocarditis may be the result of this mechanism (40, 41). The presence of myosin antibodies has been associated with antibody-mediated rejection and development of chronic allograft vasculopathy in heart transplantation (42, 43). Three hundred single-nucleotide polymorphisms have been identified in the myosin motor domain of cardiac myosin (44), but it is not yet known whether myosin antibodies detected in the patients are donor specific. The impact of cardiac myosin antibodies on heart transplantation was confirmed by a swine minor antigen-mismatch model, in which animals with strongly binding myosin antibodies after immunization of myosin pretransplant rejected grafts acutely, while animals with low and transient binding myosin antibodies had long-term allograft survival (45). The presence of donor HLA-specific antibodies in patients with antibody-mediated rejection and cardiac allograft vasculopathy precedes the detection of myosin and vimentin antibodies (43), thus again suggesting the interplay of allo- and autoimmune responses.

ANTI-PERLECAN ANTIBODIES

Perlecan is a critical component of the endothelial basement membrane and serves as a barrier between the circulating blood and the surrounding tissue (46). Perlecan is a large, extracellular matrix proteoglycan with many functions. Perlecan can act as a coreceptor for fibroblast growth factor 2 to stimulate cell proliferation as demonstrated in a rat transplant model (47).

TABLE 1 | Non-human leukocyte antigen (HLA) antibodies in solid organ transplantation.

Antibody	Organ	Reference	Number of patients	Major findings
Major histocompatibility complex class I chain-related gene A (MICA)	Kidney	(10)	1,910	The presence of MICA antibodies pretransplant is associated with an increased rate of graft loss
MICA	Heart	(11)	44	The prevalence of MICA antibodies is higher in patients with acute rejection. The appearance of MICA antibodies posttransplant precedes acute rejection
MICA	Heart	(12)	95	Development of MICA antibodies is associated with antibody-mediated rejection
Angiotensin II type 1 receptor (AT_1R)	kidney	(18)	33	Out of 33 patients with refractory vascular rejection, AT_1R antibodies detected in 16 patients with malignant hypertension, but without HLA antibodies. Passive transfer of AT_1R antibodies induces vasculopathy and hypertension in a rat kidney-transplantation model
AT_1R	Kidney	(65)	599	Patients with AT_1R antibodies >10 U had a 2.6-fold higher risk of graft failure 3 years posttransplant and a 1.9-fold higher risk of acute rejection within the first 4 months posttransplant
AT_1R	Kidney	(20)	97	The presence of strong AT_1R antibodies (>17 U) is associated with antibody-mediated rejection, but not cell-mediated rejection
AT_1R	Heart	(66)	200	Pretransplant AT_1R antibodies alone are not associated with antibody-mediated rejection and cell-mediated rejection, but the presence of both AT_1R and *de novo* donor-specific HLA antibodies increases the rate of antibody-mediated rejection and cell-mediated rejection
Endothelin-1 type A (ET_AR)	Heart	(21)	30	Increased levels of ET_AR and AT_1R are associated with cell-mediated rejection and antibody-mediated rejection
Vimentin	Kidney	(37)	70	Levels of pretransplant vimentin IgG, but not IgM, are elevated in patient with interstitial fibrosis and tubular atrophy
Vimentin	Heart	(38)	109	The presence of vimentin antibodies predicts transplant-associated coronary artery disease
Myosin	Heart	(42)	72	Myosin reactive T cell or anti-myosin antibodies are associated with development of chronic allograft vasculopathy
LG3 (perlecan)	Kidney	(51)	60	Antibodies against the LG3 domain of perlecan are present in patients with acute vascular rejection
LG3 (perlecan)	Kidney	(53)	172	Pretransplant LG3 antibodies are associated with delayed graft function
Other non-HLA endothelial cell antigens	Kidney	(59)	150	The presence of antibodies against endoglin, Fms-like tyrosine kinase-3 ligand, EGF-like repeats and discoidin I-like domains 3, and intercellular adhesion molecule 4, is associated with the production of posttransplant donor-specific HLA antibodies, antibody-mediated rejection, and early transplant glomerulopathy

Injection of LG3, a C-terminal fragment of perlecan, increases cell migration and accumulation of recipient-derived α smooth muscle actin-positive cells in neointima in a MHC-mismatched allogeneic aortic segment transplant mouse model (48). Elevated levels of LG3 have been found in the sera of kidney-transplant recipients with acute vascular rejection (49). Studies have shown that vascular injury prompts release of apoptotic exosome-like vesicles which trigger the production of antibodies to LG3 (50). Pre or posttransplant, higher levels of LG3 antibodies have been found to be associated with acute vascular rejection in kidney-transplant recipients (51). Anti-perlecan antibodies have been detected in the sera of transplant patients with glomerulopathy (52). Pretransplant LG3 antibodies have also been shown to be associated with an increased risk of delayed graft function. Passive transfer of LG3 antibodies can cause microvascular injury in a kidney ischemia-reperfusion injury mouse model (53).

Other Non-HLA Antibodies

The endothelium lines the interface between the graft and recipient tissue and antigens expressed by these cells are the first line targets of the recipient's immune system. Antibodies against four non-HLA endothelial antigens: endoglin, Fms-like tyrosine kinase-3 ligand (FLT3), EGF-like repeats and discoidin I-like domains 3 (EDIL3), and intercellular adhesion molecule 4 (ICAM4) have been identified in the sera of renal transplant patients. Endoglin is a membrane glycoprotein primarily expressed on vascular endothelium. It regulates angiogenesis and revascularization (54). FLT3 is a receptor tyrosine kinase regulating cell differentiation, survival, and proliferation (55). It is suggested that activation of FLT3 signaling promotes angiogenesis of multiple myeloma (56). EDIL3 is secreted by endothelial cells and associates with extracellular matrix. The expression of EIL3 inhibits leukocyte–endothelial adhesion (57). ICAM4, also known as the Landsteiner–Wiener blood group antigen, is a single-spanning transmembrane protein. ICAM4 mediates binding of leukocytes *via* its interaction with integrin (58). The presence of these antibodies is associated with the production of posttransplant donor-specific HLA antibodies, antibody-mediated rejection, and early transplant glomerulopathay (59). In heart transplantation, the presence of anti-endothelial cell

antibodies is also associated with increased risk of early acute rejection. Nevertheless, long-term outcome and patient survival are not affected by these antibodies (60).

Renal disease can also result from autoimmune injury, such as systemic lupus erythematosus. Anti DNA/histone antibodies found in the kidney with lupus nephritis can promote pathogenesis by stimulating cytokine production and inflammation (61, 62). Since DNA/histone are only exposed after cell breakdown as happened in cell apoptosis or necrosis, these antibodies are unlikely to be the initiating autoantibodies in lupus nephritis. However, the presence of pretransplant antibodies to apoptotic cells has been shown to correlate with allograft loss in kidney transplantation (63). Mycophenolate mofetil plus corticosteroids which is commonly used in organ transplantation is the standard-of-care treatment for lupus nephritis (64).

CONCLUSION

Vigorous efforts to investigate non-HLA antibodies, their impact on graft outcome, and influence on the alloimmune and autoimmune processes are still ongoing. With the availability of commercial reagents, the study of several of these antibodies to non-HLA antigens has moved to the clinical setting. As more studies populate the literature, there are common themes that appear consistently. The antibodies to these non-HLA-specific antigens do appear to be part of the overall graft dysfunction response (**Table 1**). These non-HLA antibodies are often checked when antibody-mediated rejection is suspected, but no donor-specific HLA antibody is detected. The presence of non-HLA antibodies may also be tested for patients who are deemed to have a high risk of antibody-mediated rejection. Commercial available Luminex bead-based reagents, which can provide detection of non-HLA antibodies in a standardized manner, may promote more transplant programs to evaluate non-HLA antibodies. Some antigens targeted by non-HLA antibodies are not polymorphic at the amino acid sequence. These antibodies may also bind the recipient's tissue and have a global effect in the recipient as happened in patients with AT_1R antibodies. It is unclear if other non-HLA antibodies also have a broader effect or if the effect is limited to the allograft because the graft expresses these antigens at the higher level. The presence of non-HLA antibodies may predispose patients to an increased risk to develop donor-specific HLA antibodies and rejection. Whether the antibodies to non-HLA antigens appear before the donor HLA-specific antibodies or the reverse is unknown at this time. The presence of these non-HLA antibodies may indicate underlying immune status. Lupus patients who usually display autoantibodies have higher level pro-inflammatory cytokines, which may promote immune response to allo-antigens. Autoantibodies may also cause allograft injury through complement-dependent cytotoxicity or antibody-dependent cell-mediated cytotoxicity. Donor HLA antigens released from injured cells may be captured by the recipient dendritic cells and trigger donor-specific HLA antibody production. Alternately, tissue injury caused by donor-specific HLA antibodies may expose neoantigens which lead to auto-antibody production. Certainly each scenario may be part of different overall immune responses in different organs, with different immunosuppressive regimes, and in different genetically disparate settings. The identity of some non-HLA antigens targeted by these antibodies remains elusive. Nonetheless, a continuing theme of these studies is that both alloimmune and autoimmune mechanisms impact graft outcome and may be synergistic to each other.

AUTHOR CONTRIBUTIONS

XZ and NLR participated in literature review and writing.

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Major Histocompatibility Complex Class I Chain-Related A (MICA) Molecules: Relevance in Solid Organ Transplantation

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An ever growing number of reports on graft rejection and/or failure even with good HLA matches have highlighted an important role of non-HLA antigens in influencing allograft immunity. The list of non-HLA antigens that have been implicated in graft rejection in different types of organ transplantation has already grown long. Of these, the Major Histocompatibility Complex class I chain-related molecule A (MICA) is one of the most polymorphic and extensively studied non-HLA antigenic targets especially in the kidney transplantation. Humoral response to MICA antigens has repeatedly been associated with lower graft survival and an increased risk of acute and chronic rejection following kidney and liver transplantation with few studies showing conflicting results. Although there are clear indications of MICA antibodies being associated with adverse graft outcome, a definitive consensus on this relationship has not been arrived yet. Furthermore, only a few studies have dealt with the impact of MICA donor-specific antibodies as compared to those that are not donor specific on graft outcome. In addition to the membrane bound form, a soluble isoform of MICA (sMICA), which has the potential to engage the natural killer cell-activating receptor NKG2D resulting in endocytosis and degradation of receptor–ligand interaction complex leading to suppression of NKG2D-mediated host innate immunity, has been a subject of intense discussion. Most studies on sMICA have been directed toward understanding their influence on tumor growth, with limited literature focusing its role in transplant biology. Furthermore, a unique dimorphism (methionine to valine) at position 129 in the α2 domain categorizes MICA alleles into strong (MICA-129 met) and weak (MICA-129 val) binders of NKG2D receptor depending on whether they have methionine or valine at this position. Although the implications of MICA 129 dimorphism have been highlighted in hematopoietic stem cell transplantation, its role in solid organ transplantation is yet to be explored. This review summarizes the currently available information on MICA antibodies, soluble MICA, and MICA-129 dimorphism in a setting of solid organ transplantation.

Keywords: MICA antibodies, soluble MICA, MICA-129 dimorphism, solid organ transplantation, graft rejection

INTRODUCTION

The Major Histocompatibility Complex (MHC) class I-related chain genes A and B (MICA and MICB) are a new family of proteins encoded within the human HLA class I genes, first described in 1994 by two independent groups of researchers (1, 2). While the latter group referred to them as Perth beta block transcript 11, Bahram and coworkers named them as MIC, a terminology that was later adopted by the World Health Organization nomenclature committee for factors of the HLA system. Unlike the classical HLA molecules, these proteins are not involved in antigen presentation to T cells. Instead they act as ligands for the activating C-type lectin-like receptor, referred to as natural killer (NK) group 2, member D (NKG2D) which is expressed on NK cells, γδ T cells, and CD8+ αβ T cells. Interaction of MICA with NKG2D leads to activation of antigen-specific cytotoxic T-lymphocyte-mediated cytotoxicity, NK cell responses, and cytokine production (3). Besides, polymorphic MICA antigens are capable of inducing antibodies that may kill target cells in the presence of complement (4). Hence MICA is unique to the extent that it plays a key role in linking the innate and adaptive immune responses in organ transplantation.

GENETIC ASPECTS AND BIOCHEMICAL STRUCTURE

MIC genes are located within the MHC class I region of chromosome 6 p21.3. A total of seven genes, designated as MICA to MICG, have so far been described, of which MICA and MICB are the only functional genes, while MICC to MICG are essentially pseudogenes (5, 6). MICA gene is located centromeric to HLA-B locus at a distance of 46.4 kb, and this close proximity results in a very strong linkage disequilibrium effect between the two (**Figure 1**).

The domain structure of MICA is much like that of the classical HLA class I molecules with 30% sequence homology and three extracellular domains. Of these, the α1 domain is encoded by exon 2, α2 by exon 3, and α3 by exon 4. The transmembrane (TM) region is encoded by exon 5, while the carboxy-terminal cytoplasmic tail is encoded by exon 6. There are five introns of which the first is the largest intron (7). The gene spans 11.7 kb region and is transcribed into an mRNA of 1,382 bp, which gives rise to 383-amino acid polypeptides of 43 kDa including the leader peptide.

Unlike the HLA class I molecules, the MICA does not bind β_2-microglobulin (β_2-m) (**Figure 2**). Though the structure of MICA looks very similar to its classical class I counterpart, its α-2 helix which is one of the groove defining helices, is disordered and flexible making it unsuitable for peptide binding. Furthermore, as opposed to the HLA class I molecules, the platform formed by the α1 and α2 regions of the MICA molecule points downwards toward the cell membrane thus exposing its underside to the intercellular space. However, when MICA interacts with its receptor NKG2D, the flexible α2 helix becomes ordered by a further two alpha-helical turn and the α1 and α2 domains flip back 96° (8).

EXPRESSION PROFILE OF MIC PROTEINS

Unlike the ubiquitous expression of classical HLA class I molecules, MIC proteins have limited tissue distribution being expressed constitutively on epithelial cells especially in the gastrointestinal tract (9), endothelial cells, fibroblasts, monocytes, keratinocytes (10), and dendritic cells (11). Zwirner and colleagues demonstrated that MIC molecules are not expressed on resting T or B lymphocytes, and unlike the HLA class I antigens, are not upregulated by INF-γ. Nevertheless, the expression of MICA can be induced on activated CD4+ T cells through release of IL-2 that powerfully induces MICA through calcineurin and other pathways in cooperation with CD3 engagement. Using confocal microscopy, these investigators found low levels of MICA expression on the surface of activated CD4 T cells and stated that this might indicate a protective mechanism of T-cell-dependent NK cell attack (12). MICA through engagement of NK cells helps to accomplish the removal of activated T-cells once the final phase of immune response is completed.

In a study involving total body tissue scan of both MICA and MICB transcription using Northern blot assay, Schrambach et al. reported that both the genes are transcribed in virtually all body tissues except the central nervous system (13). The surface expression of MICA is enhanced under stress conditions such as autoimmune diseases (14), DNA damage (15), ischemia-reperfusion injury (16), viral infections (17), and inflammation (18). Since MICA antigens are also frequently found on tumor cells (19), it implies that they are cell stress markers and their tissular expression is a signal for destruction by NK cells.

MICA POLYMORPHISM

MICA is the most polymorphic non-classical class I gene known so far with 105 alleles having already been reported and new alleles being continuously identified.[1] This polymorphism differs from that of the HLA genes in various aspects. First, the magnitude of polymorphism is far less than that seen in the HLA system. Second, in contrast to the HLA class I molecules, where the polymorphism is located predominently in the proximity of antigen binding groove, the MICA polymorphism is dispersed to all the three extracellular domains with the greatest variability in the α2 domain, encoded by exon 3. Another interesting aspect of the polymorphism of MICA is the observed variations in the TM region for several MICA alleles despite having identical extracellular domains. Therefore, it is essential to study polymorphism in the TM region to avoid typing ambiguities (20). Moreover, unlike the polymorphic positions of HLA that typically consists of several amino acids, MICA polymorphism is generated mainly by single amino acid substitutions (except positions 90 and 91) resulting in dimorphism (except residues 156 and 251).

In contrast to MICA, the genetic polymorphism of MICB is limited with a total of 45 alleles reported so far.[2] There is no concrete evidence to indicate its relevance in transplant outcome.

[1] http://hla.alleles.org/nomenclature/stats.html, April 14, 2016.
[2] http://hla.alleles.org/nomenclature/stats.html, April 14, 2016.

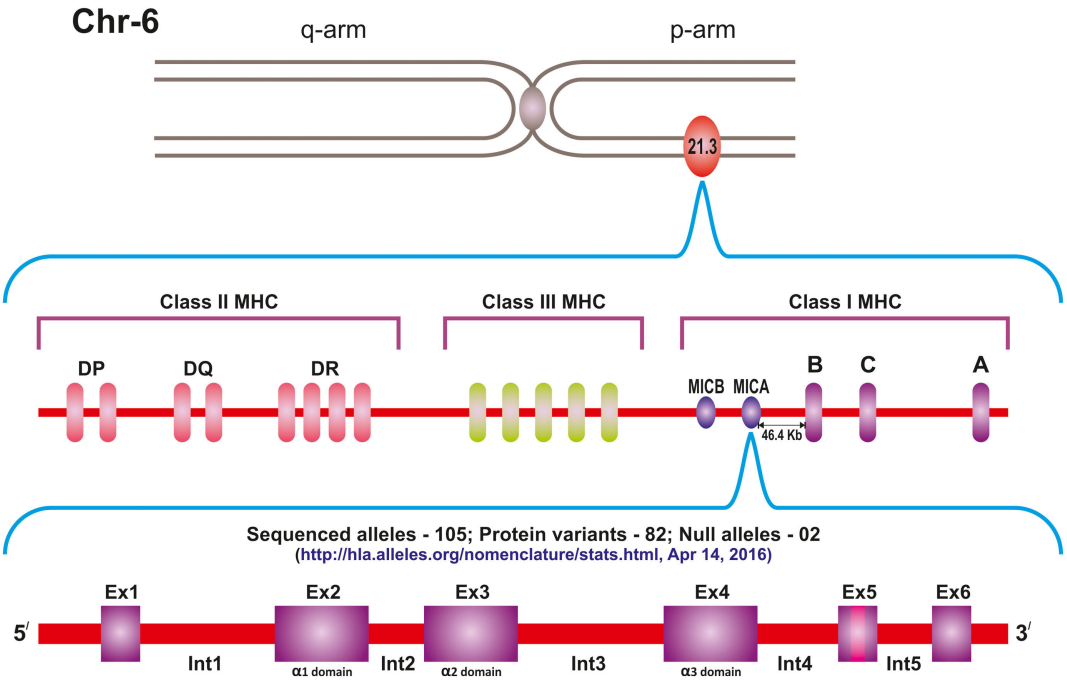

FIGURE 1 | Location of the MICA gene on the short arm of chromosome 6, centromeric to HLA-B locus. Currently 105 sequenced alleles and 82 protein variants of the gene are known.

MICA-129 DIMORPHISM

Despite the highly polymorphic nature of MICA genes, only one functional site has been identified that appears to affect the binding of MICA ligands to its receptor NKG2D. Accordingly, a non-synonymous Methionine to Valine change at position 129 of the α2 domain categorizes MICA alleles into "MICA-129 met," which is a strong binder of NKG2D receptor and "MICA-129 val" having weak binding ability. This dimorphism is identified on a single SNP rs1051792 A>G polymorphism at position 454 in exon 3 of MICA gene, corresponding to amino acid position 129 of the MICA protein. It has been shown that MICA-129 met has a 10- to 50-fold greater capacity to complex NKG2D than those with MICA-129 val (21). The functional consequence of this dimorphism has recently been studied in great details by the group led by Ralf Dressel in Germany (22) who demonstrated that MICA-129 met isoforms are able to induce stronger and faster NKG2D signaling leading to higher degree of NK cell-mediated cytotoxicity and release of INF-γ. This variant was also found to mediate faster co-stimulation and activation of CD8+ T cells. However, such effects were not sustained because the MICA-129 variant was able to induce rapid downregulation of the NKG2D receptor (22). Furthermore, the same group of investigators showed that MICA-129 met isoform is less efficiently expressed on the cell surface as compared to the MICA-129 val variant. This could be due to the intracellular retention of the former and its increased shedding from the cell surface (23). Similarly, like their NKG2D receptor counterparts that according to the polymorphism in the NKC region can be categorized into high NK cell cytotoxicity and low overall cytotoxicity, MICA-129 variants can also associate differently in pathological conditions requiring NK cell-mediated cytotoxicity.

Several studies have shown an association of met/val dimorphism with various diseases which include inflammatory bowel disease (24), nasopharyngeal cancer (25), and latent autoimmune diabetes (26). **Table 1** summarizes all such studies in various pathological conditions involving different ethnic groups as per literature reports. Although only a limited literature is available on the role of met/val dimorphism in transplantation settings, a study by Boukouaci et al. (27) reported a strong association of MICA *val/val* genotype with increased risk of chronic graft-versus-host disease development in patients undergoing hematopoietic stem cell transplantation (HSCT). Furthermore, the same study revealed that the serum levels of soluble MICA isoform and the presence of antibodies to MICA were associated with cGvHD, which is a major complication following HSCT (27). Recently, Isernhagen et al., in a cohort of 452 patients who underwent HSCT, showed that MICA-129 met tends to increase the risk of acute GVHD (aGVHD). Presence of even one MICA-129 met allele reduced the probability of developing severe or fatal aGVHD (22). The increased risk of aGVHD was explained on the fact that the MICA-129 met variant leads to faster and more robust NKG2D signaling while the rapid downregulation of NKG2D on alloreactive CD8+ T cells explains the reduced severity of aGVHD. This effect was even more evident in patients carrying homozygous MICA-129 met alleles receiving ATG. In addition, a higher relapse rate was observed in patients with MICA-129 met as compared to those with MICA-129 val/val genotype because

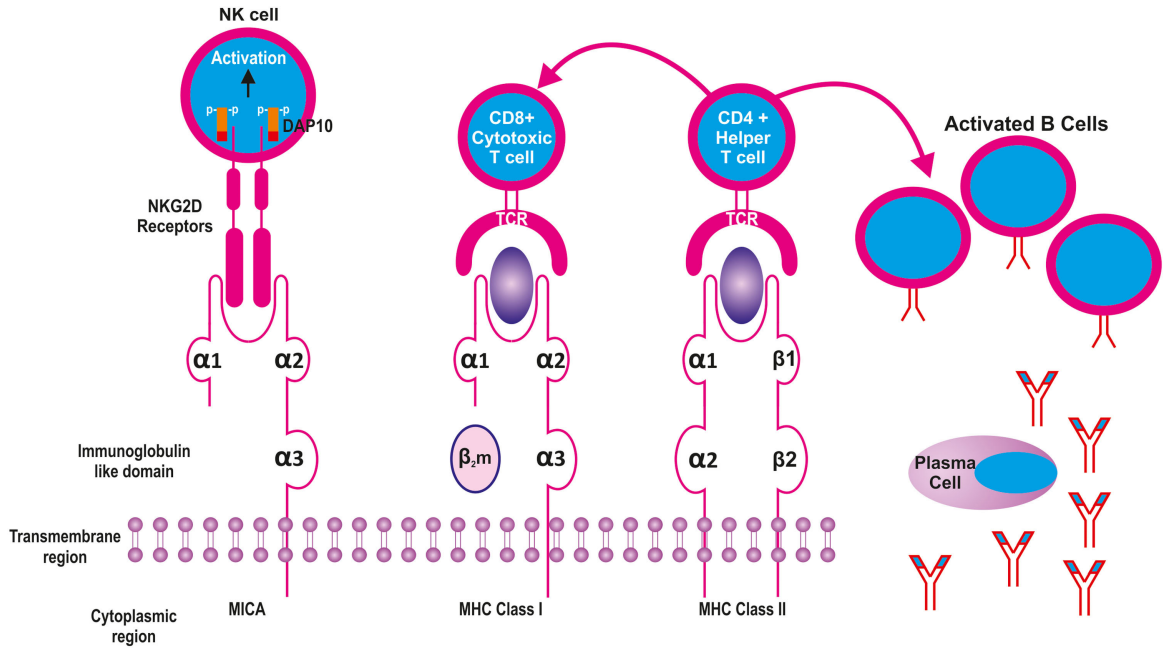

FIGURE 2 | Structural similarities between Major Histocompatibility Complex (MHC) class I and II molecules with MICA. The latter is equivalent to the heavy chain of MHC class I molecule without the β2 microglobulin. While the MHC I and II present peptides to CD8 and CD4 cells, respectively, the MICA recognizes NKG2D receptors on the surface of natural killer (NK) cells.

TABLE 1 | Summary of MICA-129 dimorphism studies reported to be associated with various disease conditions in different ethnic groups.

MICA-129 dimorphism	Year	No. of patients	Ethnicity	Disease	Association	Reference
Met/met	2005	129	Algerian	Juvenile ankylosing spondylitis	Positive	Amroun et al. (28)
Val/val	2009	130	Tunisian	Nasopharyngeal carcinoma	Positive	Douik et al. (25)
Val/val	2009	211	French	Chronic GVHD	Positive	Boukouaci et al. (27)
Met/met	2010	88	Spanish	Ulcerative colitis	Positive	Lopez-HernÂndez et al. (24)
Val/val	2011	272	Chinese	Ulcerative colitis	Positive	Zhao et al. (29)
Met/met	2011	716	Japanese	Systemic lupus erythematosus	Positive	Yoshida et al. (30)
Val/val	2012	73	Algerian	Type1 diabetes	Positive	Raache et al. (26)
Met/met	2013	340	Canadian	Cutaneous Psoriasis	Positive	Pollock et al. (31)
Met/met	2013	552	Vietanamese	Hepatocellular carcinoma	Positive	Tong et al. (32)
Met/met	2015	189	Brazil	Severity of chronic chagas disease	Positive	Ayo et al. (33)
Met/met	2015	452	Germany	Acute GVHD	Positive	Isernhagen et al. (22)

of reduced graft versus leukemia effect of NK and CD8+ cells consequent to downregulation of NKG2D by MICA-129 met variants. As a corollary to this, it is reasonable to hypothesize that the inflammatory processes-related abovementioned MICA features might also influence complications that occur during renal allograft rejection. Although immunologically MICA-129 dimorphism has the potential to affect graft outcome following solid organ transplantation, unlike HSCT, there is no published literature highlighting its role for the same. This certainly opens up a new area of research in renal allograft outcome.

IMMUNE RESPONSE TO MICA

The first indication that MICA could act as a new polymorphic alloantigen was provided by Zwirner et al. (34) who reported the presence of anti MICA antibodies in the sera of solid organ transplant recipients. Later, similar antibodies were reported in mice immunized with recombinant MICA (4). These investigators also demonstrated MICA as a target for complement-dependent cytotoxicity. Few years later, the landmark study by Zhang and Stastny (35) demonstrated that immunization of mice with recombinant MICA*001 having all the three extracellular domains, could elicit responses in both T and B cells. While the former showed the predominance of CD4+ T-cells, proliferating CD8+ T cells were also present and the stimulated CD8+ T cells were able to kill target cells pulsed with MICA by cell-mediated cytotoxicity. Furthermore, MICA stimulated CD4+ T cells were Th2 skewed, secreting high levels of IL-4 and correspondingly low levels of INF-γ. Thus these cells seem to provide a powerful aid to responding B cells.

Although MHC class II or class I antibodies are able to inhibit the proliferation of CD4+ and CD8+ T cells, respectively, the same is not the case with blocking of the NKG2D receptor. This led to the conclusion that T-cell response induced by MICA is confined to classical MHC molecules, which is in accordance with the indirect allorecognition of MICA peptides presented by host MHC antigens. In order to explain an efficient immune response elicited by MICA antigens despite the restricted polymorphism and much less amount of MICA on the cell surface as compared to HLA, Stastny and his group (36) proposed that "in addition to the adaptive immune response of T and B cells against an alloantigen, MICA also is capable of setting in motion the mechanisms of innate immunity. Co-stimulation by engagement of NK cells might have the effect of potentiating the T and B cell response. Another possibility might be that MICA is in itself rather immunogenic and capable of eliciting a response from a large repertoire of cells through any of a variety of mechanisms. This could result from cross-reactivity with unidentified microorganisms, expansion of the repertoire of responding immune cells, other genetic factors that might determine the magnitude of the specific immune response, or perhaps structural features of the MICA molecules that make them immunogenic." Taken together, these findings support the concept that MICA antigens play a role in human allograft rejection by activating both humoral as well as cellular mechanisms. Furthermore, upregulation of NKG2D by interleukins, NK cell activation (in case of inflammatory conditions), NK cell-induced dendritic cell maturation, and subsequent activation of alloreactive T cells as well as NKG2D-mediated decrease in regulatory T cells could contribute to graft rejection and graft loss in transplantation (3). It may be mentioned that cellular stress-induced expression of MICA such as on renal tubules could either augment NKG2D-mediated co-stimulation of cytotoxic T cells or direct activation of alloreactive CD8+ T cells through TCR independent mechanism (3, 37). At the same time, an ever increasing amount of data has highlighted a possible association between anti-MICA antibodies and graft rejection (38). The possible mechanisms for MIC-mediated allograft rejection include development of anti-MICA antibodies, recognition of MIC on allografts, and NKG2D-mediated cytotoxicity.

MICA ANTIBODIES: RELEVANCE IN SOLID ORGAN TRANSPLANTATION

Following the first demonstration of the expression of MICA antigens on endothelial cells (10), attention was directed toward investigating the possibility of these molecules being a target for graft destruction in solid organ transplantation. Soon, the same group of investigators showed that antibodies in patient's serum could specifically react with different recombinant MICA molecules (34). Others also demonstrated the expression of MICA on renal and pancreatic allograft biopsies (39) and confirmed it to be a target for complement-dependent cytotoxicity using both mouse MICA monoclonal antibodies as well as human alloantibodies (4). Similarly, in a study on 139 renal allograft recipients, Sumitran-Holgersson et al. (40) showed a significant correlation of MICA antibodies with graft loss. Thus, the year 2002 was an important milestone in providing evidence that MICA expression in graft tissues could lead to antibody-mediated lysis and that MICA antibodies could have an important role in precipitating antibody-mediated rejection (AMR). This was followed by a lull period of 3 years before Mizutani and coworkers (41) published a retrospective study of "serial ten-year follow up of HLA and MICA antibody production prior to kidney graft failure" providing evidence that patients who had both antibodies rejected their grafts more frequently than those who did not have either of these antibodies. Another study by the same authors indicated that MICA antibodies detected at pretransplant period could have a role in the development of AMR (42). The above mentioned studies along with the development of more reliable and convenient Luminex bead based assay system opened the floodgates for several studies investigating the relevance and impact of MICA antibodies on allograft outcome. Hence, the year 2007 witnessed a surge in studies investigating the effect of MICA antibodies on graft outcome in solid organ transplants, more so in kidney and heart transplants. **Table 2** summarizes relevant studies on the influence of MICA antibodies on graft outcome in various solid organ transplantations.

RENAL TRANSPLANTATION

Impact of Pretransplant MICA Antibodies

Exact mechanisms by which individuals develop antibodies to MICA are largely unknown. Although, pregnancy *per se* and previous transplants can sensitize the patient leading to the development of anti-MICA antibodies (34), the role of blood transfusions in their induction is not fully clear (43, 44).

The first major study to evaluate the potential association of MICA antibodies with overall allograft survival was conducted by Zou et al. (43). It was an international collaborative study involving 20 centers in 13 countries with pre transplant serum samples obtained from 1,910 patients. The experiment was performed blindly by testing MICA antibodies without any knowledge of the clinical course. The results showed that at least 217 of the 1,910 patients (11.4%) had MICA antibodies and their 1-year graft survival (GS) was 88.3% as compared to 93% in the group without MICA antibodies ($p = 0.01$). Among patients of primary renal grafts, survival was even lower (87.8%) compared with 93.5% for those who did not have MICA antibodies ($p = 0.005$). Interestingly the association of MICA sensitization with GS was observed in patients well matched for HLA. Independent analysis of 326 patients with 0 or 1 HLA-A, -B, or DR mismatches also showed that recipients with MICA antibodies had poorer GS of 83.2% compared to 95.1% of those without MICA antibodies. However, the study did not investigate the impact of possible confounding factors that are likely to influence graft loss.

Subsequently, Lemy and colleagues (44) studied for the presence of MICA antibody in sera from 494 healthy controls and 597 patients with chronic kidney disease (CKD) stage V and reported threefold higher prevalence of MICA antibodies in patients with CKD when compared with controls. Using logistic regression analysis involving subsets of patients free of transfusions and transplantation also revealed at least twofold higher prevalence

TABLE 2 | Presence of MICA antibodies and their effect on allograft outcome in solid organ transplantation.

Organ	Detection time	Year	Number of patients	Transplant (DD/LD)	Follow-up (duration)	Reference	Outcome
Kidney	Pre-tx	2002	139	DD	3 months	Sumitran-Holgersson et al. (40)	↑AMR
		2007	1,910	DD	1 year	Zou et al. (43)	↑AMR, ↓GS
		2010	425	NS	1, 5, and 10 years	Lemy et al. (44)	↔
		2012	40	LD	1 year	Solgi et al. (45)	↔
		2013	727	DD + LD	3, 6, 12, and 24 months	Sánchez-Zapardiel et al. (46)	↑AMR
	Post-tx	2005	145	DD + LD	10 years	Mizutani et al. (41)	↓GS
		2007	185	LD		Panigrahi et al. (47)	↑AMR
		2007	1,921	DD + LD	4 years	Terasaki et al. (48)	↓GS
		2009	284	DD	3 years	Suarez-Alvarez et al. (49)	↑AMR
		2011	442	DD + LD	5.9 years (mean)	Cox et al. (50)	↑CR
		2012	779	DD + LD	4 years	Lemy et al. (51)	↔
		2012	147	DD + LD	6 months	Seyhun et al. (52)	↔
Heart	Pre and post-tx	2007	44	DD	1 year	Suarez-Alvarez et al. (53)	↑AMR
	Pre-tx	2009	491	DD	1 and 5 years	Smith et al. (54)	↔AMR/CAV ↑GS
	Pre-tx	2010	63	DD	6 months	Pavlova et al. (55)	↔
	Post-tx	2010	95	DD	1.8 and 8.9 years (mean)	Nath et al. (56)	↑AMR, ↑CAV
	Post-tx	2011	168	DD	2 years (median)	Zhang et al. (57)	↑AMR
		2015	05	Animal experiments (rat-to-mouse cardiac transplantation model)		Yu et al. (58)	↑AR
Liver		2008	84	NS	2 years	Uzunel et al. (59)	↔
		2013	123	NS	7 years	Ciszek et al. (60)	↔

MICA, major histocompatibility complex class I chain-related molecule A; AT1R, angiotensin II type 1 receptor; AECA, anti-endothelial cell antibody; Col V, collagen V; KA1T, k-α1 tubulin; LSECs, liver sinusoidal endothelial cells; AMR, antibody-mediated rejection; GS, graft survival; AVR, acute vascular rejection; CAV, cardiac allograft vasculopathy; BOS, bronchiolitis obliterans syndrome; DD, deceased donor; LD, live donor; NS, not specified; AR, acute rejection; ↔, no adverse effect.

of MICA antibodies in CKD patients when compared to healthy controls. It is intriguing that these antibodies were more frequent in males rather than females in the cohort as a whole (14 versus 7%) and also among individual groups despite pregnancy being an independent risk factor for their development. Thus factors remaining significantly associated with MICA antibodies after logistic regression analysis were blood transfusions, previous transplantation, and females with two or more pregnancies. The finding of blood transfusion as a significant sensitizing event was in sharp contrast with the findings of Zou although five transfusions were required for categorization as "transfused" compared to only one in Zou's study (43). Another very interesting finding of this study was that no sensitizing events could be identified in a third of the patients with MICA antibodies and CKD stage V, implicating other possible mechanisms for MICA sensitization. Additionally, 20% of CKD patients had MICA antibodies that were auto-reactive, a rare finding with HLA antibodies (61).

It is important here to debate on the results of two important studies—one carried out by Zou et al. (43) and the other by Lemy and colleagues (44). Even though the broad design of the two studies has been similar with pretransplant testing for MICA antibodies, the outcomes were dissimilar in terms of GS. Furthermore, there were differences in the number of patients included in the two studies, but the latter group of investigators found better survival in patients positive for MICA antibodies, albeit insignificantly. However, an analysis of immunosuppression protocols between the two studies showed that Lemy's group of patients were more heavily immunosuppressed and that could make an effect on the incidence and impact of MICA antibodies. Others also failed to show significantly higher rejection rates in patients expressing MICA antibodies as compared to those who did not express (62). Similarly, Solgi et al. (45) did not find significant difference in rejection episodes on comparing patients with or without the presence of anti-MICA antibodies. A retrospective study involving 727 renal allograft recipients published by Sánchez-Zapardiel et al. (46) revealed a 7.15% prevalence of MICA antibodies in patients waiting for a renal transplant. They reported that preformed anti-MICA antibodies significantly increased the risk for allograft rejection particularly early after transplantation and that this effect was independent of the presence of anti-HLA antibodies. However, no significant difference was noticed in allograft survival and rejection rates at 2-year follow-up. Moreover, no significant epidemiological or clinical differences were observed between MICA antibody positive and negative groups. The study did not define the donor specificity of anti-MICA antibodies. The same group of authors further demonstrated that presence of anti-MICA antibodies at pretransplant periods can bind native MICA molecules on the cell membrane and was able to mediate cell death by fixing and activating the complement cascade by using both the C1q single-antigen beads assay and complement-dependent cytotoxicity (63).

Our experience with live donor renal transplantation (64) is very similar to that of others (65, 66) suggesting that presence of pretransplant MICA antibodies especially those against donor antigens with MFI in the range of 10,000–20,000 are capable of causing hyperacute and acute rejection (AR). Clearly, there are gap areas and lack of consensus on the epidemiology and specificity of MICA antibodies on the one hand and their impact on AR and GS on the other.

Impact of Posttransplant MICA Antibodies

The issue of *de novo* occurrence of MICA antibodies posttransplantation has been a subject of intense investigation. In a study involving 185 consecutive live related donor renal transplant patients, we analyzed posttransplant serum samples at varying time periods and reported a significant decline in 2-year GS if both HLA and MICA antibodies were detected (47). The survival was only 17% compared to 89% of those with no antibodies. Furthermore, patients with either MICA or HLA antibodies alone also had a significantly reduced GS of 71% as compared to the no antibody group.

Simultaneously, a large collaborative international study coordinated by Paul Terasaki tested sera for both HLA and MICA antibody production from 1,329 recipients of renal grafts (964 from deceased donors and 365 living donors) from 21 participating centers as a part of the 13th International Histocompatibility and Immunogenetics Workshop Conference (IHIWC) and 22 centers as a part of the 14th IHIWC (48). Only those recipients who did not produce HLA antibodies pretransplant (pretransplant testing for MICA antibodies was not performed) and who survived for more than 6 months were included in this study. HLA antibodies were detected with CDC, ELISA, or Luminex techniques, while MICA antibodies were detected using eight different recombinant MICA molecules produced in HMY2. C1R cells, isolated and coated on Luminex beads. In the 13th workshop, the 4-year deceased donor GS among 806 patients who were negative for HLA antibodies was 81% as compared to 58% for 158 recipients with HLA antibodies ($p < 0.0001$) and 72% for 69 patients with the presence of MICA antibodies ($p = 0.02$). Among those with living donor grafts, 4-year GS was 78% for 275 patients without HLA antibodies, 62% for 90 patients with HLA antibodies ($p = 0.0008$), and 80% for 21 patients with MICA antibodies ($p =$ NS). In the 14th workshop, 1-year GS for deceased donor recipients without MICA antibodies was 96.8% as compared to 82.7% for 33 patients with MICA antibodies alone ($p = 0.0005$). However, the same was not observed with living donor recipients as 19 patients with MICA antibodies had 100% 1-year GS. Multivariate analysis at both time points revealed that MICA antibodies were significantly and independently associated with reduced GS in deceased donor grafts, providing strong evidence for the involvement of these antibodies with graft rejection. It may be mentioned that these studies did not consider previous AR episodes or other confounding factors that are also likely to influence graft loss. The explanation for the lack of significance of MICA antibodies in living donor transplants was attributed to the limited number of patients in the study group.

Up until around 2009, the specificity of MICA antibodies and epitopes recognized by them had received very little attention. Gautier et al. (67) performed MICA typing of 43 recipient–donor pairs of patients undergoing third renal transplant and also evaluated MICA antibody using the LABScreen SAB Luminex method (One Lambda). The antibody screening was done on the day of transplant and after 1 year. They observed greater frequency of patients with two MICA mismatches among those who developed rejection; whereas all patients with graft losses had 0 or 1 MICA mismatch. Antibodies specific to donor MICA antigens (MICA-DSA) were found to be equally associated with functional and failed grafts. In this study, although MICA genotyping was attempted on all patients and donors including those positive for MICA antibodies, the authors did not examine the nature of mismatches between those who produced *de novo* MICA antibodies and those that did not.

At around the same time, Suarez-Alvarez and colleagues (49) combined a clinical study of MICA antibody production in deceased donor renal transplantation along with MICA epitope analysis. Posttransplant sera of 284 patients were tested for MICA antibodies using Luminex technology, and patients were followed for up to 3 years. The results revealed presence of MICA antibodies alone without the presence of anti-HLA antibodies in 30 (10.6%) patients. Furthermore, 29.6% of patients who developed AR had MICA antibodies as compared to 13.3% of the antibody negative group ($p < 0.05$). Using epitope mapping with a synthesized library of overlapping peptides from the extracellular domains of MICA molecules, the investigators determined nine antigenic regions reactive with MICA antibodies in patient's serum. Four of these regions were mapped to variable sites in the molecule with polymorphic amino acids while five antigenic regions located in constant region had shared epitopes found in all MICA alleles.

Others used a novel technique to detect *de novo* HLA and MICA antibodies in 15 patients following renal transplantation (68). Pre- and posttransplant sera were profiled using the Invitrogen Protoarray Human Protein Microarray platform containing 5,056 non-redundant human proteins, purified from insect cells. For the purpose of analysis, three main clinical phenotypes with five patients each were considered (i) the first group comprised of patients positive for C4d and undergoing AR, (ii) the second group with cellular rejection were negative for both donor-specific antibodies (DSA) and C4d, and (iii) the third group consisted of patients with stable graft function without any rejection episodes. The results revealed *de novo* occurrence of MICA antibodies in 11 of the 15 patients with the mean antibody signal intensity being higher in those with C4d+ AR as compared to those with C4d− AR. Additionally, integrative genomics predicted localization of MICA antigen to the glomerulus in the normal kidney. Immunohistochemistry confirmed the finding that MICA antigens preferentially localized to glomerular podocytes. MICA expression in normal kidney podocytes may actually be a means to resist NK cell-mediated cytotoxicity. These investigators also showed the induced expression of MICA *in vivo* on infiltrating lymphocytes during rejection episodes suggesting that MICA antibody-mediated immune responses occurred irrespective of graft rejection and that antibody levels increase during AMR but not cellular rejection. Therefore, keeping in view the significant rise in antibody titers prior to and during humoral rejection, serial measurement of MICA antibody levels rather than checking cross-sectionally at the time of rejection may be more useful. Besides the observed correlation between C4d+ AR and high MICA levels, the latter were also significantly associated with MHC class II-specific circulating DSA. Since an association of HLA-DSA class II with development of chronic glomerular injury is already established, it is possible that anti-MICA antibodies may be playing a potentiating role in the pathogenesis of chronic transplant glomerulopathy.

Whether there is an influence of MICA allele mismatching on antibody production and graft rejection is not fully clear? Cox and coworkers (50) screened 442 renal transplant recipients for MICA antibodies using three different Luminex-based single antigen kits—One Lambda, Gen-Probe, and an "in-house" assay. Mean time for testing of antibodies was 7 months after transplantation and the mean follow-up period was 5.9 years. MICA antibody specificities were considered positive only if confirmed by at least two different kits. In 227 of the above recipient-donor pairs, MICA allele typing was performed by DNA sequencing. At least 17 recipients (7.5%) developed MICA antibodies of which 10 had *de novo* DSA. Moreover, eight of these MICA+ recipients and seven of those who had *de novo* DSA had no HLA antibodies. On multivariate analysis, MICA mismatching was found to be an independent significant factor associated with the development of MICA antibodies. Also the presence of both MICA and HLA antibodies together significantly correlated with ACR but not AMR, although occurrence of MICA antibodies alone failed to show an association with either of these events. Nevertheless, recipients with MICA DSA alone showed a significant association with graft dysfunction (↓ eGFR) 2 years following transplantation, as were those with HLA-DSA alone who showed significantly reduced eGFR after 3 years. Thus the kinetics of antibody response in this study pointed toward an accelerated graft dysfunction in the presence of MICA antibodies.

In a retrospective study performed by Lemy et al. (51) on 1-year posttransplant sera from 779 renal transplant recipients, a 5.4% prevalence of MICA antibodies was observed. MICA+ patients were more frequently HLA sensitized and had to undergo re-engraftment. There was no significant difference in 4-year death-censored GS between MICA positive and negative patients (97 versus 94%, $p = 0.28$). By Cox multivariate analysis, graft loss was found to be independently associated with the number of HLA-DR mismatches, AR within the first year posttransplantation, 1-year serum creatinine ≥1.5 mg/dl, and the presence of HLA antibodies at 1 year, but not the presence of MICA antibodies. Another study comprising of 84 renal allograft recipients with a follow-up of 4 years reported that more than one-third of the recipients developed antibodies to HLA and/or MICA and the percentage of recipients who developed *de novo* antibodies increased with time after transplantation elapsed. Recipients positive for these antibodies had higher serum creatinine levels and worse allograft function than those without antibodies (69).

HEART TRANSPLANTATION

A number of studies have shed light on the correlation of MICA antibodies to cardiac allograft rejection episodes. Suarez-Alvarez (70) demonstrated significant correlation between the presence of anti-MICA antibodies detected by CDC using recombinant cell lines and AR following heart transplantation. A year later, the same group performed another study to investigate a possible relationship between MICA antibody production and heart allograft rejection in 44 recipients (53). This time, MICA antibodies were detected using both MICA transfected cell lines in a CDC assay and a commercial assay using Luminex beads. While a quarter of the patients were antibody positive by the CDC technique, only seven (15.9%) showed MICA antibody by the Luminex assay. Nine patients had rejection and a majority of them (60%) were positive for MICA antibodies by the CDC method as compared to five patients (14.3%) without rejection ($p = 0.0038$). Analysis by Luminex revealed 55.5% of AR patients as compared to only 6% without rejection had MICA antibodies ($p = 0.0020$). They also performed MICA allele DNA-typing for donors and recipients where the recipient was positive for MICA antibodies. All patients with MICA antibodies and AR had MICA-DSA although five patients also had autoantibodies. This study was limited by the small number of patients; nevertheless it was the first study to show a possible correlation between MICA-DSA and AR. Additionally, they determined MICA mRNA levels in endomyocardial biopsies obtained from 10 cardiac transplant recipients and found these to be higher in biopsies showing rejection than those without it. In majority of the cases, MICA expression was higher immediately following transplantation, independent of the rejection event, suggesting an upregulated antigen expression due possibly to cellular stress.

In contrast to the above, Smith and coworkers (54) in their study on 491 heart transplant recipients did not find any significant correlation of pre- or posttransplant MICA antibodies or MICA-DSA with cardiac allograft survival, AR episodes, or cardiac allograft vasculopathy (CAV). Similar observations were made by Pavlova et al. (55) who reported from their study of 68 heart transplant recipients that patients with pretransplant MICA antibodies did not significantly associate with AMR or ACR, although a trend was observed with AMR ($p = 0.06$). Others however demonstrated a significant association of anti-MICA antibodies with AR and CAV (56). These investigators also showed that development of HLA-DSA preceded the detection of MICA antibodies. An apparent explanation given by the authors was that this could be because of binding of HLA-DSA to the allograft giving rise to inflammatory cascade, which may result in upregulation of MICA antigens, alloreactivity, and sensitization. Another study found a significant correlation between the presence of MICA-DSA with AMR, while anti-MICA antibodies that were not donor specific (NDSA) did not correlate (57). In this study, 10% of the patients developed autoantibodies to MICA, but these did not associate with the development of AMR. Using an allogeneic animal model system involving rat-to-mouse cardiac transplants, Yu and coworkers (58) reported high MICA expression in recipients' heart and provided evidence to show that anti-MICA antibodies in their sera were associated with high risk of AR.

LIVER TRANSPLANTATION

There are only limited studies defining the role of MICA antibodies in liver transplantation. A study of MICA antibody production in liver allograft recipients did not reveal an association with allograft rejection (59). Histological analysis revealed that MICA is not normally expressed on liver cells and its expression is not induced during rejection episodes. Ciszek and coworkers (60) analyzed the impact of anti-HLA and anti-MICA antibodies in 123 ABO compatible liver transplant recipients with a follow-up

of 7 years. They reported that neither the presence of anti-HLA nor anti-MICA antibodies correlated with acute graft rejection or GS.

SOLUBLE MICA (sMICA): ROLE IN SOLID ORGAN TRANSPLANTATION

In addition to the membrane bound form, a soluble isoform of MICA (sMICA) derived from the proteolytic shedding of membrane bound molecule appears in the serum. MICA, a ligand for NKG2D receptors, forms a complex with ERp5, a disulfide isomerase/chaperone and induces a conformational change enabling proteolytic cleavage of MICA by ADAM proteases. The interaction of NKG2D by the sMICA results in the endocytosis and degradation of receptor–ligand complex and thus suppresses NKG2D-mediated host innate immunity (**Figure 3**).

Most studies on soluble MICA release in the serum have been directed toward understanding their influence on tumor growth, with very little literature available on the associated biology. The intricate nexus between the science behind sMICA role in cancers and transplant rejection has been highlighted through a few studies. For example, Suarez-Alvarez et al. (70), while evaluating the role of MICA on heart graft acceptance, demonstrated an inverse relationship between sMICA levels and AR. The study was conducted on 31 heart transplant recipients with a follow-up of 1 year, of which 8 patients suffered AR while the remaining 23 patients did not develop AR. Further analysis showed that 17 out of 23 patients without AR had detectable levels of sMICA as compared to two patients in the rejected group ($p < 0.03$). On combined analysis of MICA antibodies and sMICA, the authors found tendency for MICA antibodies to occur in the absence of sMICA in the AR group of patients. Conversely, the sMICA levels were detected in patients without MICA antibodies and in absence of AR. These authors published another paper in the same year, where they monitored sMICA levels in pretransplant serum samples and at 15 days, 3 months and 1 year posttransplantation, in 34 heart transplant recipients (71). sMICA was practically absent in the pretransplant sera, while it was detected in 21 patients at 15 days posttransplantation. Interestingly, 20 of these 21 patients did not develop AR ($p = 0.0001$), whereas 9 of the 13 patients, in whose serum sMICA was not detected, developed AR. These observations are in conformity with the previous study suggesting that presence of sMICA contributes to better graft acceptance. Recent experiment conducted on an animal model (rat-to-mouse cardiac transplantation) also demonstrated a negative association of sMICA with AR. The investigators reported that xenografts having anti-MICA antibodies and experiencing AR tended to

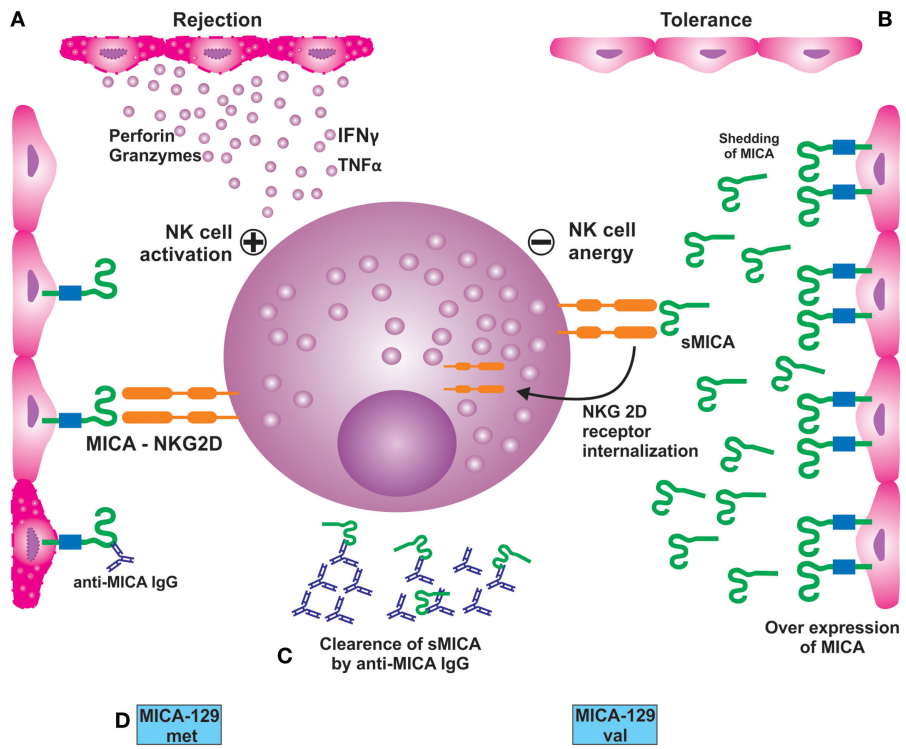

FIGURE 3 | **(A)** Interaction of NKG2D receptor with its ligand MICA, expressed on endothelial cells, results in activation of NK cells leading to their damage. **(B)** Over expression of MICA on endothelial cells membrane and its proteolytic shedding from the surface leading to the formation of sMICA. The latter interacts with the NKG2D receptor, resulting in its internalization and subsequent suppression or impairment of NKG2D-mediated immune response; **(C)** preformed or *de novo*-developed MICA antibodies recognize MICA antigens present on the allograft leading to acute or chronic rejection; **(D)** potential impact of MICA-129 met/val dimorphism on renal graft outcome. Patients expressing met/met in homozygous state are prone to rejection episodes than those with val/val genotype.

develop in the absence of sMICA (58). Assadiasl and coworkers (72) in their study on 30 each patients of coronary artery disease and transplant recipients with stable grafts and 15 healthy controls did not find any significant difference in the presence and amount of soluble MICA between the three groups.

Zou et al. (73), in an attempt to assess the effect of sMICA on the outcome of liver transplantation, evaluated levels in pre- and posttransplant sera from 133 consecutive primary liver transplant patients and in sera from 88 healthy volunteers using sandwich ELISA. The study revealed that 37.6% of recipients had significantly higher pretransplant sMICA than the healthy population, while recipients with decreased posttransplant sMICA following liver transplantation had a lower incidence rate of biliary cast syndrome (BCS) than those with sustained high levels of sMICA after transplantation (10.5 versus 38.7%, $p = 0.0302$) suggesting that dynamic changes in these levels are associated with BCS development.

Clearly, there is a general dearth of published literature evaluating a possible correlation between circulating levels of sMICA and graft outcome in solid organ transplantation. Studies involving larger cohorts and diverse ethnic groups are needed to determine the applicability of sMICA as a potential biomarker of prognostic importance in solid organ transplantation.

CONCLUSION

Despite clear indications of MICA antibodies impacting graft outcome adversely, a definitive consensus on this relationship is yet to be arrived. Furthermore, only a few studies have dealt with the impact of MICA-DSA as compared to those that are NDSA on graft outcome. Two factors are important while analyzing the role of MICA antibodies: (i) currently employed pretransplant crossmatch procedures are not sensitive enough to detect MICA DSA and (ii) the currently used immunosuppressants for induction and maintenance may not be effective in suppressing the immune response against MICA antigens because they are all directed at suppression of T cell response albeit through different mechanisms. Data so far suggest that circulating levels of soluble MICA could well prove to be a potential biomarker of prognostic importance in the assessment of patients after renal transplantation. At the present moment, there is scarcity of published literature evaluating a possible correlation between production of sMICA and their titers with graft outcome in renal transplantation. Further studies involving larger cohorts and diverse ethnic groups could help to reinforce the current findings. Our data on MICA-129 dimorphism adds another dimension in defining its exact role and influence following solid organ transplantation.

AUTHOR CONTRIBUTIONS

AKB and NKM designed and wrote the paper. NKM provided excellent inputs and advice.

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REVIEW
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Antibody Subclass Repertoire and Graft Outcome Following Solid Organ Transplantation

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Long-term outcomes in solid organ transplantation are constrained by the development of donor-specific alloantibodies (DSA) against human leukocyte antigen (HLA) and other targets, which elicit antibody-mediated rejection (ABMR). However, antibody-mediated graft injury represents a broad continuum, from extensive complement activation and tissue damage compromising the function of the transplanted organ, to histological manifestations of endothelial cell injury and mononuclear cell infiltration but without concurrent allograft dysfunction. In addition, while transplant recipients with DSA as a whole fare worse than those without, a substantial minority of patients with DSA do not experience poorer graft outcome. Taken together, these observations suggest that not all DSA are equally pathogenic. Antibody effector functions are controlled by a number of factors, including antibody concentration, antigen availability, and antibody isotype/subclass. Antibody isotype is specified by many integrated signals, including the antigen itself as well as from antigen-presenting cells or helper T cells. To date, a number of studies have described the repertoire of IgG subclasses directed against HLA in pre-transplant patients and evaluated the clinical impact of different DSA IgG subclasses on allograft outcome. This review will summarize what is known about the repertoire of antibodies to HLA and non-HLA targets in transplantation, focusing on the distribution of IgG subclasses, as well as the general biology, etiology, and mechanisms of injury of different humoral factors.

Keywords: IgG subclass, HLA donor-specific antibodies, transplant

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INTRODUCTION

The clinical significance of antibody-mediated rejection (ABMR) to transplant outcome is now widely established across solid organ transplants. The recent introduction of solid-phase single antigen antibody testing by Luminex enabled detection of donor-specific antibodies with significantly greater sensitivity than had been previously available. Advances in T cell immunosuppression have significantly reduced T cell-mediated rejection as a cause of graft loss in medication adherent patients; consequently, antibody-mediated rejection has emerged as the leading cause of allograft failure (1).

Transplant recipients may be transplanted with either preformed donor-specific antibodies (DSA) that were generated by prior allosensitization events, such as pregnancy, transfusion, or transplantation, or develop DSA *de novo* after transplantation. Wiebe and colleagues reported (2) that low-risk renal transplant recipients develop *de novo* DSA at a rate of about 2% per year, appearing usually

around 2 years post-transplant. By 12 years post-transplant, the final incidence of DSA was 27%. Similar rates of *de novo* DSA were reported by Everly et al., wherein 25% of patients had DSA by 10 years post-transplant (3). Pediatric and adult heart transplant recipients developed *de novo* DSA with an incidence of about 30–40% by 10 years post-transplant (4–6). Liver (7–9), lung (10, 11), pancreas (12, 13), and bowel (14, 15) transplant recipients also develop donor-specific human leukocyte antigen (HLA) antibodies.

Overall, DSA are seen in ~20% of solid organ transplant recipients and are a significant clinical factor in transplant outcomes. Diagnostic criteria for ABMR vary slightly across solid organs, although endothelial cell injury, complement deposition, and mononuclear cell infiltration are recurrent manifestations. In renal transplants, acute ABMR is defined by histological evidence of tissue injury, such as microvascular inflammation (MVI) or arteritis, with or without complement C4d staining, and serological evidence of DSA (16, 17). Chronic rejection of renal allografts may also be triggered by donor-specific antibodies and is characterized by transplant glomerulopathy, capillary basement membrane duplication or fibrosis, and MVI (17). In cardiac allografts, histologic changes, including endothelial cell activation and intravascular $CD68^+$ macrophages, as well as complement activation detected by C4d or C3d deposition, are included in the diagnosis of pathologic ABMR (18, 19). ABMR in lung (20), pancreas (21), and liver (22) allografts also include a combination of C4d staining, mononuclear cell infiltration, and histological assessment of microvessel endothelial cell activation.

Transplant recipients developing DSA to polymorphic HLAs exhibit significantly worse graft survival and rejection rates. Allograft loss was higher in renal transplant patients who developed *de novo* DSA compared with patients who did not and had no dysfunction, and interestingly, patients could be further stratified by concurrent clinical ABMR at the time of DSA appearance. Those with subclinical DSA fared worse in the long-term than those without any DSA, but significantly better than those who had clinical ABMR at the detection of DSA, all of who lost their allografts by 8 years after the appearance of DSA. While non-adherence and delayed graft function (DGF) were significant predictors of graft loss, the strength or MFI of DSA was not itself a strong predictor (2, 23). Pediatric heart transplant recipients with DSA have higher incidences of cardiac allograft vasculopathy (CAV), also called transplant coronary artery disease (TCAD), compared to those without DSA, more rejection episodes, and lower graft survival at 5 years (5, 6). In adult heart transplant recipients, DSA is also an independent predictor of patient survival (4). Many studies have also demonstrated a clear decrement in outcome and graft survival among patients with antibody to non-HLA targets, such as major histocompatibility complex class I chain-related gene A (MICA) (24–27).

In spite of overwhelming evidence that patients with DSA tend to fare worse as a group than those without, these same studies have consistently shown that up to half (range 20–50%) of patients with HLA DSA do *not* experience poorer graft outcomes, including rejection incidence and graft loss, compared to their DSA^- counterparts, at least to the endpoints reported (5, 6, 28–30). Indeed, even among DSA^+ patients with adverse outcomes, there is a spectrum from subclinical, "indolent" antibody-mediated graft injury to clinically manifested acute antibody-mediated rejection to devastating hyperacute rejection. This has led to the hypothesis that not all antibodies are equally pathogenic and that identification of antibody features controlling graft injury might enable further stratification of patients with DSA who are at risk for rejection and allograft failure. Certainly, the titer of antibody is important for the degree of graft injury. The affinity of an antibody for its antigen and the effector functions an antibody can engage are also likely to be relevant to its "pathogenicity" in antibody-mediated allograft injury. Such diversity in antibody function is controlled in large part by the antibody's isotype and subclass. This review will discuss the function of different IgG subclasses relevant to graft injury, the subclass repertoire of HLA and non-HLA antibodies found in transplantation, and the generation of antibodies of various isotypes and subclasses.

DIVERSITY OF EFFECTOR FUNCTIONS AMONG IgG SUBCLASSES

The heavy chain constant regions of human IgG are more than 90% homologous, with variation mostly localizing to the CH2 domain and hinge regions. Not coincidentally, these are the locations for Fc-mediated effector engagement, chiefly binding to FcγRs and complement C1q. Immunoglobulin effector functions are engaged by the Fc portion of IgG and include complement-dependent cytotoxicity (CDC), opsonization, antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis. IgG subclasses vary in the length and flexibility of their hinge region, with IgG2 and IgG3 representing opposite ends of the spectrum. The hinge region is the shortest and most rigid in IgG2, while IgG3 has a uniquely long hinge region that is quite flexible, promoting increased availability to the binding sites for FcγR and C1q. Studies optimizing effector functions of therapeutic antibodies have also pinpointed key amino acid residues, which control affinity of the Fc region for FcγRs and for complement components.

IgG4 has been called an "odd antibody" due to its unique structural properties (31). IgG4 can form Fab arm monomers (half-molecules) that are monovalent. This feature is thought to represent a self-limiting process of the humoral immune response, as these monomers can block better effector subclasses from binding to antigen. Another consequence of reduced stability in the IgG4 Fc tail is its reported ability to exchange Fab arms and form bispecific antibody molecules.

Affinity, Persistence, and Localization

Some evidence points to differences in IgG subclass affinity for antigens. Berkowska et al. found that in peripheral blood B cells, transcripts of IGHG2 and IGHG4 contained higher loads of somatic hypermutation in the variable regions compared with IGHG1 and IGHG3 (32). Thus, "indirect" sequential switching to IgG2 and IgG4 might indicate more germinal center reactions and longer activity of the enzyme activation-induced cytidine deaminase (AID). It might be surmised from this work that later subclasses IgG2 and IgG4 result from more extensive affinity

maturation compared with upstream IgG1 and IgG3 (32), as was suggested by early work evaluating IgG subclass response and affinity in human KLH immunization (33). These results are similar to findings that mutation loads were highest in antigen-specific IgG4 compared with other subclasses after secondary immunization with *Meningococcus* (34). Studies of murine IgG subclasses reveal similar differences in affinity. One study put forth the hypothesis that the constant region itself affected affinity for antigen, where IgG of different subclasses, but with identical variable regions, displayed different affinity for antigen (35–37). Antibody affinity (i.e., variable region variants) for antigen appears to be important for its ability to provoke Fc-mediated effector functions such as ADCC, discussed below (38, 39).

IgG3 has the shortest half-life, of about a week, while the other subclasses have a half-life of about 3 weeks. This is due to an arginine at amino acid position 435 of IgG3, rather than a histidine which is present in other subclasses, significantly increasing its affinity for the neonatal Fc salvage receptor (FcRn) (40). Interestingly, although many allotypes of immunoglobulin have been identified to date (41), only polymorphisms in IgG3 which revert this position to histidine demonstrate any change in function, increasing the half-life of these alleles of IgG3 (42). The affinity of IgG subclasses for FcRn is of relevance to antibody-mediated diseases of the fetus and newborn. In addition to variation in placental transport, the isotypes of antibodies also differ in their ability to diffuse into the host's own tissues, which serves to compartmentalize the humoral response. IgM has poor diffusion due to its size, whereas IgA is found in secretions and at epithelial surface. IgG is predominantly in circulation but also can diffuse into the tissues. Traditionally, IgG3 was described to have poor transport across the placenta due to its low affinity for FcRn; however, it was recently emphasized that the majority of IgG3 alleles represented in Western study populations were R435, in contrast to the H435 found frequently in other groups. Therefore, ethnic differences in IgG allotypes are also important to antibody effector functions.

Agonism

A key function of antibodies is to block their target, neutralizing it as with viruses. IgG, being bivalent, can also dimerize its target antigen and stimulate inhibitory or agonistic signaling depending on the target. Our group and others have shown that antibodies to HLA class I molecules also activate intracellular signaling cascades in vascular endothelial and smooth muscle cells, resulting in increased cell proliferation, migration, and recruitment of leukocytes (43–54) (**Figure 1A**). Less is known about agonistic signaling of antibodies against HLA II molecules. In antigen-presenting cells, HLA-DR ligation by antibodies mimics TCR engagement, and induces cell activation and proliferation (55–59). Endothelial cells expressing HLA-DR also respond to anti-HLA-DR antibodies by increased allostimulation of T cells (60, 61). Transcriptome studies of renal transplant biopsies undergoing ABMR have revealed an enriched endothelial-specific signature, paralleling these *in vitro* studies (62). Antibodies to the angiotensin II type 1 receptor (AT1R) agonistically activate AT1R signaling and induce detrimental effects on vascular endothelial phenotype and function (63, 64) (**Figure 1B**). AT1R antibodies are implicated in systemic sclerosis (SSc), preeclampsia (65), hypertension (66), and allograft dysfunction (67, 68). Auto-antibodies to endothelial cells [anti-endothelial cell antibodies (AECA)], against yet mostly unidentified antigens, activate endothelia to express a variety of adhesion molecules (69).

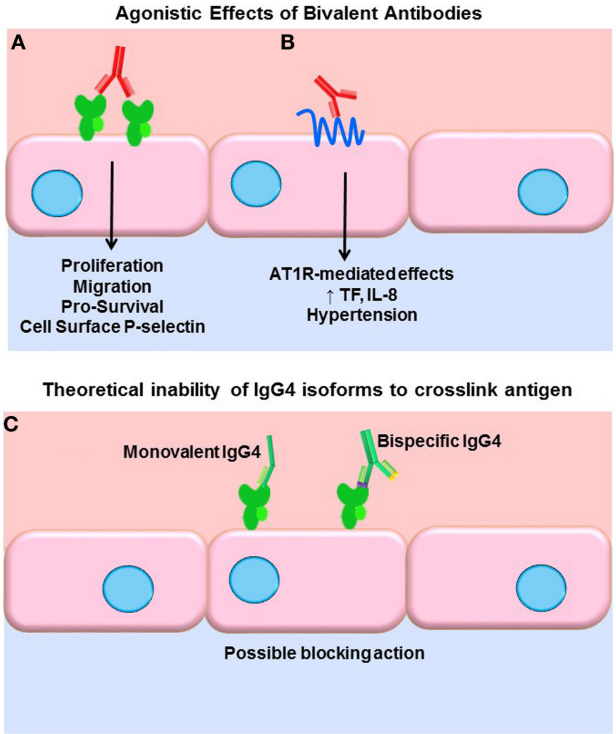

FIGURE 1 | Agonistic signaling by antibodies. (A) Bivalent IgG of any subclass may dimerize or crosslink target antigens, such as HLA molecules. Many studies have demonstrated that HLA ligation on endothelial cells, vascular smooth muscle cells, and antigen-presenting cells induces intracellular signaling *via* tyrosine kinases. HLA signaling promotes cell proliferation, migration, and increased expression of survival proteins such as Bcl-2 and Bcl-XL. In addition, HLA cross-linking triggers exocytosis of endothelial vesicles called Weibel–Palade bodies, which contain vasoactive mediators and the adhesion molecule P-selectin. Increased cell surface P-selectin in turn supports increased adherence of leukocytes. **(B)** Anti-AT1R antibodies act agonistically, binding to an epitope on this multi-pass transmembrane receptor and stimulating increased IL-8 expression and tissue factor production. AT1R agonism is also implicated in malignant hypertension in a variety of diseases as well as transplantation. **(C)** Some molecules of human IgG4 have been shown to form monovalent Fab arms that may cross-dimerize with other clones of IgG4 to create bispecific antibodies. Although not experimentally demonstrated, in theory, such monovalent and bispecific IgG4 molecules would be incapable of cross-linking HLA and might in fact block other subclasses from binding.

Functional target agonist activity is presumably independent of the Fc portion of the antibody, as stimulation of endothelium with the $F(ab')_2$ fragment still elicits these functional changes. Interestingly, Stein et al. demonstrated that a chimeric anti-HLA-DR hIgG4 (engineered to stay bivalent, not form half-molecules), while not able to induce Fc-mediated functions such as complement activation or ADCC of tumor B cells, significantly increased intracellular Akt signaling, suppressed proliferation, and induced apoptosis at a comparable level as the parental anti-HLA-DR (70).

So, while generally incapable of eliciting Fc-mediated effector functions, if bivalent, IgG4 can still cross-link HLA molecules and provoke intracellular signaling cascades and target cell phenotype changes. Therefore, it could be surmised that signaling of HLA and non-HLA targets in the allograft can be induced by all subclasses of immunoglobulin, with the possible exception of monovalent or bispecific forms of IgG4 (**Figure 1C**). The differential ability of IgG subclasses to induce agonistic signaling remains to be explored.

Complement-Dependent Cytotoxicity and Inflammation

Arguably, the most studied effector function of antibodies in transplantation is the ability to activate complement. Histological detection of the complement split product C4d within allograft vasculature has been central to the diagnostic criteria of ABMR for decades.

The complement system is ancient part of the innate immune system culminating in activation of terminal split products that are highly inflammatory and can cause target cell death (whether mammalian or pathogen). Three arms of the complement system, lectin, alternative, and classical pathways are triggered by different stimuli, but all converge on the central regulator C3. Canonically, the classical pathway is activated by immunoglobulin. The details of the classical complement pathway have been excellently reviewed elsewhere (71); a brief summary can be found in **Figure 2**.

Activation of complement is balanced by complement regulatory proteins, both soluble and cell surface, and self-limiting due to cleavage of active mediators. Such regulatory proteins, including decay-accelerating factor (DAF, CD55), MCP (CD46), and CD59, expressed on endothelial and other cells, and serum C1-INH and Factor H, limit inflammation and confer protection of host cells during complement activation. DAF acts to inhibit C3 activation downstream of C4 and C2 and upstream of C3 and C5. Anti-EGFR IgG3 promoted deposition of C1q and C3b on target tumor cells, as well as generation of upstream C4a, but failed to result in the generation of C3a or C5a due to high expression of CD55 on the tumor cells (72), demonstrating the resistance of cells to terminal complement activation when they express complement regulatory proteins. Indeed, deficiency of the complement regulatory protein DAF abolishes protection of corneal allografts from alloimmune destruction (73) and exacerbates rejection of cardiac allografts in mice (74, 75). Interestingly, among human cardiac transplant recipients with active $C4d^+$ antibody-mediated rejection, those without concurrent dysfunction exhibited increased expression of DAF compared to patients who had ABMR and allograft dysfunction (76). These studies underscore the protective role of complement regulatory proteins in transplantation.

The four subclasses of IgG vary in their affinity for C1q (**Table 1**). IgG3 is a potent stimulator of complement activation, with IgG1 following closely behind. IgG2, although typically cited as non-complement fixing, in fact can fairly efficiently bind to C1 and activate complement under conditions of high antigen density and/or high antibody titer. IgG4, in contrast, has nearly no detectable complement-activating properties. Experiments using subclass switch variants carrying the same variable regions have shown that IgG4 is a poor activator of human CDC compared with IgG1 and IgG3, with IgG2 having intermediate complement activity at higher antibody concentration (70, 77). Interesting murine models of ABMR showed that non-complement-fixing subclasses of antidonor IgG were able to augment complement activation by the stronger complement-fixing subclasses (78).

It is worthwhile to note that the CDC cross-match assay used in transplantation employs rabbit complement rather than human complement. Due to heterophilic antibodies and interspecies interactions between human IgG subclasses and rabbit complement proteins, the CDC is not necessarily reflective of true complement-activating capacity of human antibodies. Indeed, human IgG2, while not a good activator of the classical human complement cascade, effectively triggers activity of rabbit complement (79). Nevertheless, the cytotoxic cross-match generally does reveal antibodies with very high titer and correlates well with clinical outcomes (80).

Terminal complement activation ultimately triggers cell death, and this outcome has been a focus early in transplantation due to its dramatic and devastating injury to the allograft (81) (**Figure 2A**). Given the protection of the graft endothelium by constitutive expression of complement regulatory proteins, it is conceivable that only very high titers of strongly complement-fixing antibodies can overcome inhibition to cause endothelial cytolysis. Hyperacute rejection is now a rare event due to improved sensitivity of antibody and cross-match tests and general avoidance of strong donor-specific antibodies. Consequently, there has been increased interest in the upstream mediators of the complement system, and the role of "complement-dependent inflammation." Anaphylatoxins and opsonins C4a, C3a, C3b, and C5a are all critically important in regulating innate inflammation, as well as modulation of adaptive immunity. C4a, C3a, and C5a are chemoattractant for neutrophils and monocytes (82, 83) (**Figure 2B**). Endothelial cells respond to C5a by releasing intracellular vesicles containing adhesion molecules and vasoactive mediators (45, 84), and to sublytic deposition of membrane attack complex (MAC) by activation of non-canonical NFκB and upregulation of inflammatory genes including VCAM-1 and E-selectin (85, 86) (**Figure 2C**). Complement may also be implicated in transplant vasculopathy, although arteriopathy also develops in C3-deficient mice (87, 88).

The regulation of adaptive immunity by complement, particularly complement C3 components, has been revealed by several studies. When B cells encounter opsonized antigen coated with C3d, coligation of the BCR with complement receptors CD21 and CD35 lowers the threshold for B cell activation and enhances humoral immunity [reviewed in Ref. (89)]. Heeger and colleagues have expanded our understanding of how complement modulates T cell responses [reviewed in Ref. (90, 91)]. For example, C3a and C5a enhance T cell proliferation and activation, as well as antigen-presenting cell activation.

It Is Unclear Whether *In Vitro* Complement Fixation Is a Reliable Predictor of Rejection or Graft Loss

Investigators have also utilized modifications of the HLA solid-phase assays to infer the ability of antibodies to activate

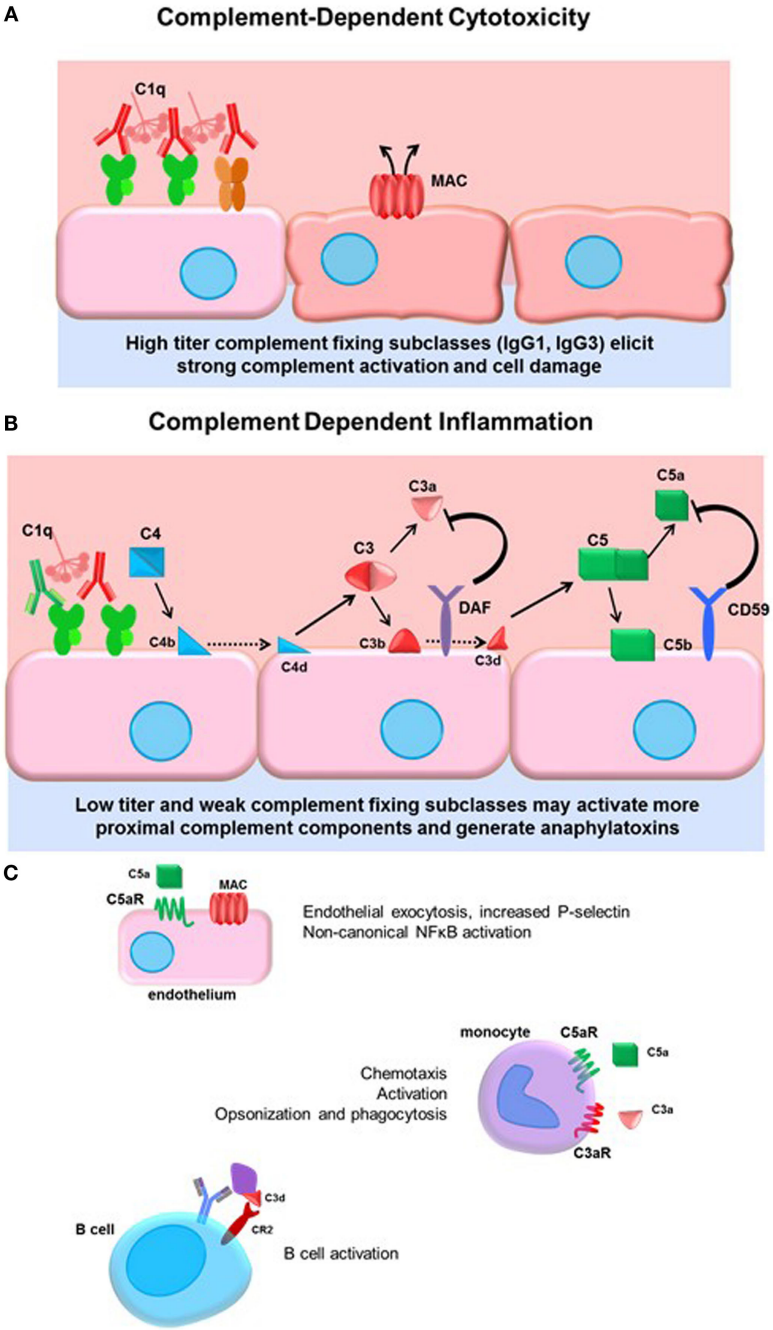

FIGURE 2 | **Complement-dependent cytotoxicity and inflammation**. **(A)** High titers of antidonor HLA antibodies, particularly of the efficient complement-fixing subclasses IgG1 and IgG3, may promote terminal classical complement pathway activation. Complement activation must overcome the regulatory factors and push complement activation to terminal MAC formation and cell damage. **(B)** Lower titers of antibody or less efficient complement-fixing subclasses, such as IgG2, may result in truncated complement activation, with upstream anaphylatoxin release and opsonin deposition. The initiator C1 complex, composed of globular C1q, embedded with catalytically active C1r and C1s, recognizes the Fc portion of IgM and most of the IgG subclasses, triggering a conformational change in the hexameric shape of the C1 complex. This activates the autocatalytic cleavage of C1r, which then activates C1s. C4 and C2 are cleaved by C1s, forming C4a and C2a split products that generate the C3 convertase. C3 convertase cleaves C3 protein into C3a, a soluble inflammatory protein, and C3b, which is covalently bound to the cell surface. C3b may be further cleaved to C3d or form the C5 convertase. Terminal activation of the C5 convertase cleaves C5 protein, generating the potent anaphylatoxin C5a and the membrane-bound C5b. C5b recruits C6–9 proteins to form the membrane attack complex (MAC), disrupting membrane integrity. Complement regulatory proteins DAF and CD59 at the host cell surface restrain activation of the complement cascade at the two key amplification steps, C3 cleavage and C5 cleavage. **(C)** Many cells express receptors for the soluble and membrane-bound complement split products. Endothelial cells respond to C5a by upregulating P-selectin, and to sublytic concentrations of MAC by activation of non-canonical NFκB pathways, adhesion molecule, and cytokine upregulation. Monocytes and neutrophils express C3a and C5a receptors, which participate in chemotaxis of myeloid cells. Complement receptor 2 (CR2) is a component of the BCR that binds to opsonized, C3d-coated antigen. CR2 signaling enhances the BCR signal and lowers the threshold for B cell activation.

TABLE 1 | Summary of IgG subclass effector functions.

	ADCC (NK)	Complement activation	Antigen recognized	Binding to FcγR alleles							
				FcγRI CD64	FcγRII CD32a-H	FcγRII CD32A-R	FcγRII CD32b	FcγRIII CD16a-V	FcγRIII CD16a-F	FcγRIII CD16b-NA1	FcγRIII CD16b-NA2
IgG1	+++	+++	T-dep protein	+++	++	+++	+	+++	++	+++	+++
IgG2	+	+[b]	Carbohydrate and T-dep protein	–	++	+	–	+	–	–	–
IgG3	+++	+++	T-dep protein	+++	++	+++	++	+++	+++	+++	+++
IgG4	[a]	–	T-dep protein	+++	+	+	±	++	–	–	–

[a]*ADCC elicited by IgG4 depends on the glycosylation pattern of the Fc region.*

[b]*Complement activation by IgG2 depends on the titer of antibody and the density of antigen.*

complement, measuring binding of complement components C1q (30, 92–95), C4d (96–100), or C3d (96, 101, 102) to single antigen beads and to cells. However, reports conflict as to whether such assays provide better resolution of antibody pathogenicity and indeed ability to initiate the complement cascade in these *in vitro* assays seems to still be tied to antibody titer. Certainly, the strength or titer of antibody seems to be linked with its pathogenicity. For example, as recognized early in solid organ transplantation, very high titers of HLA DSA (that can cause a positive CDC cross-match) can trigger hyperacute rejection and early graft dysfunction. Increasing strength of DSA is associated with lower graft function in renal transplant recipients (103). Zeevi et al. found that high titers of HLA DSA were able to fix C1q and were associated with early ABMR in heart transplant patients (104). In contrast, Smith et al. found that both non-complement-fixing and complement-fixing DSA (measured by C4d deposition on single antigen beads) were associated with reduced heart transplant patient survival (4) and did not conclude that complement activation *in vitro* was a useful predictor of more pathogenic DSA. In studies of kidney transplantation, Crespo et al. reported that C1q-fixing DSA had higher MFIs but that C1q positivity did not correlate with outcome in renal transplant recipients, whereas other groups have uncovered an added predictive value of C1q-positive DSA in renal allograft survival (30, 94, 105–107). In conclusion, there is no clear consensus on whether donor-specific antibodies which fix complement in *in vitro* assays better discriminate those that are detrimental to allograft survival; it does appear, though, that antibodies which do not bind to complement in these assays are still relevant to graft outcome.

FcγR-Mediated Functions

Antibodies can engage Fc receptors present on most hematopoietic cells. FcγRs are highly selective in their affinity for IgG subclasses (**Table 1**) [reviewed in Ref. (108, 109)]. The receptors for IgG, the FcγR family, are expressed on myeloid and NK cells as well as B cells. All myeloid cells express activating FcγRs, and some coexpress an inhibitory FcγR. There are three major classes of human FcγRs, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), which are differentially distributed on innate and adaptive effector cells. FcγRI is an activating receptor with high affinity for both monomeric and complexed IgG and is present on the major population of monocytes, as well as macrophages and activated neutrophils. FcγRII has two subtypes, FcγRIIa, expressed on monocytes, macrophages, and highly on neutrophils, and the inhibitory FcγRIIb. FcγRII has comparably lower affinity than FcγRI for monomeric IgG but binds efficiently to high avidity ligands of complexed or immobilized IgG. FcγRIIIa is expressed on NK cells and a minor population of monocytes, while FcγRIIIb is expressed on neutrophils.

Polymorphisms in the FcγR system shape individual susceptibility to infectious disease, autoimmunity, and response to antibody-based therapeutics (110–115). While FcγRI has no known polymorphism, FcγRIIa, FcγRIIIa, and FcγRIIIb are each dimorphic, with two alleles having different affinities for IgG (116). For example, the FcγRIIa alleles H131 and R131 differ in their affinity for IgG1, with H131 having higher affinity and the ability to bind to IgG2. Taken together, it is probable that the transplant recipient's own constellation of FcγR alleles offset or augment the effects of the subclass repertoire during FcγR-mediated injury.

Opsonization and Phagocytosis

Antibodies act in concert with complement activation to mark target cells and microbes for uptake by phagocytes, a process called opsonization. FcγRs work with complement receptors to elicit phagocytosis. IgG3 most potently induced opsonization of meningococci (chimeric antibodies + human complement) and respiratory burst in PMN induced, with IgG1 slightly less potent and very activity little with IgG2 or IgG4 (77). Therefore, IgG3 and IgG1 are typically thought of as the most potent opsonizing antibodies.

Antibody-Dependent Cell-Mediated Cytotoxicity

Engagement of FcγRs activates ADCC (**Figure 3**). NK cells express CD16A and CD16C. In NK cells, FcγR cross-linking initiates intracellular signaling leading to polarized release of perforin and granzyme, causing death of the antibody-coated target cell. While myeloid cells, such as macrophages, also carry out ADCC, the mechanisms are less clear. Typically, IgG1 and IgG3 are the most efficient activators of NK cell-mediated ADCC due to the higher affinity of FcγRIIIa for these subclasses (**Table 1**). Not only is IgG4 ineffective at eliciting ADCC but also has been shown to actively block monocyte-mediated antitumor ADCC when present in equal concentrations with IgG1, through competitive binding to FcγRI (117).

There is limited evidence directly demonstrating that ADCC is actually occurring within allografts during antibody-mediated rejection. How ADCC might manifest histologically is unclear. Early work attempted to prove that alloantibodies were able to induce ADCC against allogeneic endothelial cells, using *in vitro* assays. The authors showed that sera of only a few post-transplant patients were able to induce lysis of cultured endothelial cells by NK cells (118). A follow-up study suggested that patients whose serum was capable of inducing ADCC against cultured endothelial cells had more vascular rejection and graft loss than patients without ADCC activity (119). Experimental models have shown that NK cells are critical for chronic antibody-mediated rejection in the mouse [reviewed in Ref. (120, 121)]. Hidalgo et al. identified NK transcript signatures in renal transplant biopsies from patients with rejection, particularly late antibody-mediated rejection (122, 123). Taken together, these results point to a role for NK cells in antibody-mediated rejection beyond ADCC.

FcγR Signaling

We and others have shown that FcγR signaling in monocytes and neutrophils participates in the leukocyte recruitment cascade (124, 125). Concurrent engagement of FcγRs and adhesion molecules augments firm adhesion of myeloid cells through increased activation of integrins, enabling increased capture of FcγR-bearing leukocytes by antibody-coated endothelium (**Figure 3**). FcγR signaling also influences macrophage differentiation, dendritic cell maturation, and enables prolonged antigen presentation. Thus, subclasses that more effectively engage FcγRs on macrophages, neutrophils, and dendritic cells should be better capable of inducing FcγR signaling in these cells.

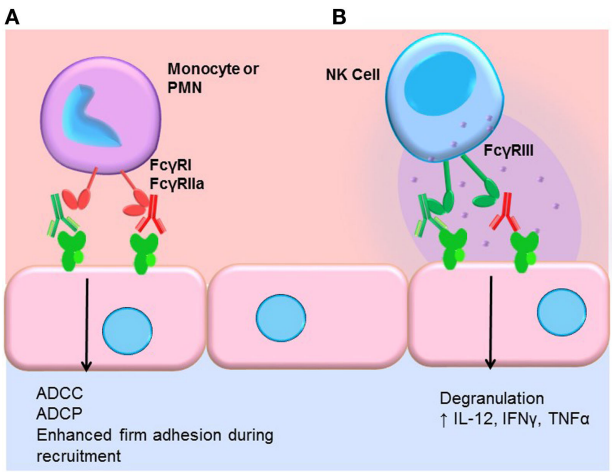

FIGURE 3 | FcγR-mediated effector functions. (A) Monocytes and neutrophils express FcγRs, which bind preferentially to different IgG subclasses. FcγR cross-linking by myeloid cells induces activation and mediates antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis (ADCP), and augments recruitment of leukocytes from the blood. In general, IgG1 and IgG3 efficiently bind to most FcγR isoforms expressed by monocytes and neutrophils. IgG4 can bind quite well to FcγRI (CD64), and IgG2 is bound by an allelic variant of FcγRIIa (CD32a, H131). **(B)** NK cells express FcγRIII (CD16). Engagement of FcγRIII by antibody-coated target cells induces ADCC *via* degranulation and release of perforin/granzyme. Cross-linking of FcγRIII on NK cells also upregulates the inflammatory cytokines IL-12, IFNγ, and TNFα.

ANTIBODY SUBCLASSES IN TRANSPLANTATION

Different routes of allosensitization trigger distinct patterns of IgG subclasses directed against HLA, which supports the paradigm that the inflammatory milieu upon antigen exposure, as well as the antigen itself, controls selection of subclass. Intriguingly, memory formation also appears to differ after various allosensitization events, pointing to immunologically distinct mechanisms of immunization against HLA and MICA through transfusion, pregnancy, and transplantation.

Assays to Identify HLA Antibodies

Cell-based and solid-phase testing for HLA antibodies classically identify antibodies of the IgG isotype as donor-specific IgG HLA antibodies. The classical complement-dependent cytotoxic assay identifies strong anti-HLA IgG antibodies that bind HLA on the surface of the target cell and initiate the complement cascade culminating in the formation of the MAC complex and cell death identified by fluorescent microscopy. The T and B flow cross-match identifies anti-HLA IgG binding to the surface of T or B cells and quantitates the median channel shift over cells incubated with a negative control serum. In comparison to the CDC, the T and B flow cross-match is more sensitive and quantitative allowing for the identification of antibodies that are weak/moderate in strength. Solid-phase assays identify HLA antibody that bind HLA antigen bound to a plate (ELISA) or bead (single antigen bead) in a cell-free environment. The single antigen bead assay currently allows the most quantitative and sensitive measurement of HLA IgG antibodies identified by Luminex technology.

Antibody Subclasses in Allosensitization (Pretransplant)

Significant effort has been contributed to determining the IgG subclass repertoire in pre- and post-transplant patients (**Table 2**). To achieve these goals, laboratories have developed protocols that modify the traditional single antigen bead assay by replacing the IgG detection antibody with subclass-specific clones for IgG1–4 (126–130). The data are consistent in showing IgG1 as the predominant Ig subclass in pre- and post-transplant sera, and have attempted to associate specific subclasses with sensitizing events, allograft pathology, and loss (see below) (126–130).

Allosensitization can occur following immunizing events such as pregnancy, transfusion, or transplant. In a study of sensitization to HLA antigens using subclass-specific IgG1–4 reporter antibodies in the single antigen bead assay, Lowe and colleagues showed that the subclass repertoire following unequivocal immunization to HLA antigens through pregnancy and transplantation is heterogeneous and dominated by the IgG1 subclass in 38 patients (128). IgG2 secondarily dominant following blood transfusion and failed transplant, while IgG3 was secondarily dominant following pregnancy. Generally, blood transfusion stimulated a restricted, IgG1-dominated response to HLA antigens.

TABLE 2 | Summary of different methods for detecting IgG subclasses against HLA and non-HLA targets.

Reference	Organ/ target	Determinants of positive cutoff	Antibody source
Lefaucheur et al. (130)	Renal/HLA	For each IgG subclass antibody and each single antigen bead, the mean MFI values from 4 non-sensitized healthy male controls +5 SD, and if that value was <500 MFI; the IgG 1–4 reactivity was considered positive if the normalized MFI was ≥500	Southern Biotech
Khovanova et al. (129)	Renal/HLA	>5× the greater than the average negative control bead MFI in the single antigen bead assay of all tests for each subclass. IgG1: 120.6, IgG2: 72.0, IgG3: 62.7, IgG4:17.2	Southern Biotech
Lowe et al. (128)	Renal/HLA	>5× the greater than the average negative control bead MFI in the single antigen bead assay of all tests for each subclass. IgG1: 120.6, IgG2: 72.0, IgG3: 62.7, IgG4:17.2	Southern Biotech
Kaneku et al. (127)	Liver/HLA	Normalized trimmed MFI higher than 500 was defined as positive on the basis of binding patterns after validation and dilution experiments	Southern Biotech
Honger (126)	Renal/HLA	A positive result was defined by a MFI value above a cutoff that was generated for each IgG subclass and for every individual bead by using four negative control sera from healthy non-sensitized and HLA antibody-negative men: cutoff MFI = mean NC1–4 + 3 SDs NC1–4. To determine the amount of IgG subclasses, we used the ratio above the corresponding cutoff (i.e., ratio = MFI IgG subclass divided by MFI cutoff)	Southern Biotech
Jackson et al. (131)	Endothelial cells	Median fluorescence values for IgG subclasses identified in test serum were normalized to values obtained when cells were incubated with normal control AB serum (Atlanta Biologicals, Norcross, GA, USA). IgG subclass analysis of HLA antibodies was assessed in the same manner using 10 ECP donors and pooled sera from high calculated panel reactive antibody (cPRA) transplant candidates	Southern Biotech
Griffiths et al. (132)	Renal/HLA	For each IgG subclass and for every individual bead by using four negative control sera (NC1–4) from healthy, non-sensitized, and HLA antibody-negative cutoff MFI mean NC1–4 + 3 SDs NC1–4. To determine the amount of IgG subclasses, we used the ratio above the corresponding cutoff	Sigma

The strength of pretransplant DSA is associated with ABMR and graft loss. As shown by Lefaucheur and colleagues using the traditional single antigen bead assay, the relative risk for ABMR increased significantly as the strength of preformed DSA increased (133). Patients with MFI of 3000–6000 or >6000 had greater than 60-fold or 100-fold risk, respectively, of developing AMR. Graft survival at 8 years after transplant among patients with preformed DSA >3000 MFI was only 60.6%, as compared to 78.4% for patients with preformed DSA strength of ~500–3000 MFI and 82.5% for patients negative for preformed DSA (133).

Donor-Specific HLA Antibody Subclasses: Post-Transplant

Preliminary work, using subclass-specific IgG1–4 antibodies in flow cross-match or ELISA platforms, suggested that predominant expression of IgG1 in pretransplant sera was associated with acute rejection (134) and graft loss (132, 135). In a small study evaluating the IgG subclass of DSA using flow cytometry on donor spleen cells in kidney and liver recipients (136), one patient who lost the graft due to hyperacute rejection had high IgG3 DSA pretransplant despite a negative CDC-XM, suggesting that high titers of this subclass are potent mediators of complement-dependent rejection.

More recent data, using the subclass-specific modification of the single antigen assay, support the predominance of the IgG1 subclass in transplant patient sera (126, 127). IgG2 and IgG4 do not typically constitute a large proportion of HLA DSA (137). Several groups have attempted to further define the various subclasses as predictive biomarkers of graft pathology and outcome (127, 129, 130). Lobashevsky et al. analyzed the specificities as well as the subclasses of DSA pretransplant and post-transplant in three renal recipients using SAB modification with subclass secondaries in each patient, the proportion of different IgG subclasses were different against different antigens (138). One patient experienced rejection early post-transplant and had two DSA, both of which were about equal mixture of IgG1 and IgG2; the other two patients had good outcomes: one had IgG1, IgG2, and IgG4 DSA, whereas the other had predominantly IgG1. It is important to note that this paper highlights that each antigen can be recognized by multiple subclasses.

A cohort of post-liver transplant patients with chronic rejection and a group of patients without rejection were studied to determine if the presence of IgG subclass-specific DSA correlated with clinical state (127). The data showed that chronic rejection in liver transplant patients is correlated with the presence of DSAs of multiple subclasses, while normal graft function in the presence of DSA is correlated with DSA isolated to the IgG1 subclass. Furthermore, DSA of the IgG3 subclass was more closely associated with graft loss than DSA to other subclasses or no DSA. Everly et al. also found that the presence of IgG3 HLA DSA, particularly concurrent with IgM DSA, was predictive of allograft failure in renal transplant recipients (139).

Gao et al. first observed that IgG4 was increased in most recipients post-transplant (136). In pre- and post-transplant sera from 80 sensitized renal transplant patients, pretransplant IgG4 levels were predictive of acute ABMR in the first 30 days post-transplant, while preformed IgG4 and post-transplant day 30 IgG3 were associated with graft loss (129). In another study, sera from 125 consecutive renal transplant patients with DSA detected within the first year post-transplant evaluated for subclasses of IgG showed that IgG3 is associated with AMR, while IgG4 was associated with subclinical ABMR in protocol biopsies and late allograft injury (130). These data regarding the potential pathogenesis of IgG4 in renal transplant patients in the early post-transplant period are interesting as this subclass has been classically considered a marker of chronic antigen exposure produced due to "hyperimmunization" (140). The comment by Schaub et al. (141)

is right on point – the presence of later subclasses IgG2 and IgG4 is suggestive of a more advanced humoral response with active T cell help. Donor-specific IgG4, despite its inability to activate complement, is correlated with poor graft outcome. Nevertheless, it is at this time difficult to dissect whether a predominance of IgG4 in any inflammatory disease, including transplantation, is due to the mechanism/pathogenicity of this subclass or is reflective of extensive antigen exposure and immune memory.

Given that most patients exhibit a mixture of IgG subclasses directed to HLA, several attempts have been made to evaluate whether grouping subclasses by presumed capacity to activate effector functions is able to further stratify risk. HLA DSA in sera from pre-kidney transplant patients were classified according to the presence of preformed DSA that are strong complement fixing (IgG1 and IgG3; $n = 21/74$ patients), weak/non-complement fixing (IgG2 and IgG4; $n = 4/74$ patients), or a mixture of both (containing a mixture of IgG1–4; $n = 46/74$) (126). While a trend was observed implying that patients with exclusively weak/non-complement-fixing DSA had lower incidence of ABMR at 6 months post-transplant, the incidence and histologic phenotypes of ABMR in patients displaying strong complement-fixing DSA was not significantly different from those that displayed a mixture of weak/non-complement-binding and strong complement-binding DSA. In a similar approach, Arnold et al. described the IgG subclass patterns of *de novo* DSA in adult renal transplant recipients by grouping subclasses together based on presumed complement activity. They observed that a majority of patients had exclusively "complement-fixing" IgG1 and IgG3, while the remainder had a mixture of complement-fixing and non-complement-fixing subclasses, with a very small percentage having IgG2 and IgG4 alone. ABMR was more often observed in patients with a mixture of subclasses than with IgG1/IgG3 only; however, there was no difference in graft survival between these groups (142). Interestingly, the DSA in patients with only IgG2 and IgG4 were directed exclusively against HLA class II antigens. Finally, Freitas et al. found that most patients exhibit a mixture of IgG subclass directed against donor HLA-DQ antigens, and there was no significant difference in incidence of rejection comparing patients who had IgG1 and IgG3 compared with those with a preponderance of IgG2 and IgG4 (95).

Non-HLA Antibodies

Antibodies to non-HLA antigens on the surface of the endothelium or epithelium, aka, non-HLA antibodies, or AECA, have been identified with specificity to alloantigens, such as MICA or major histocompatibility complex class I chain-related gene B (MICB) (143, 144), or autoantigens, such as vimentin (145), cardiac myosin (CM), collagen V (ColV), agrin (146), endoglin, EGF-like repeats, Fms-like tyrosine kinase-3 ligand, ICAM-4 (69), and AT1R (67). Currently, there are only a few tests in clinical laboratories for the identification of non-HLA antibodies for transplant patients. Non-HLA antibodies to AT1R are measured by ELISA. Antibodies to MICA are measured using a MICA single antigen test (27). Donor-specific anti-endothelial cells can be measured by flow cytometry-based XM-ONE, which detects binding of IgG and IgM to peripheral blood endothelial cell precursors (147). While the isotypes and subclasses of antibodies to MICA are yet to be characterized, they do not appear to be predominantly complement fixing, at least in *in vitro* assays (148). Contradictorily, antibodies to MICA are found in both $C4d^+$ and $C4d^-$ ABMR (149). Seminal work by Dragun et al. (67) showed that AT1R antibodies were present in renal transplant recipients with refractory vascular rejection but with no HLA DSA [substantiated by Reinsmoen et al. (150)], and the same group went on to show that these antibodies were predominantly IgG1 and IgG3 subclasses (151). Interestingly, however, histological manifestations of AT1R-mediated graft dysfunction do not typically include positive C4d staining, suggesting that injury *via* anti-AT1R antibodies might be complement independent, despite a predominance of strongly complement-fixing subclasses. As mentioned above, bivalent antibodies may act agonistically; and clinically AT1R-mediated graft dysfunction can present with hypertension and histologically with MVI (152). Using XM-ONE, patients with positive endothelial progenitor cross-match experienced increased rejection (majority were $C4D^-$ and classified as cellular rejection) and higher serum creatinine (153). AECA that bind ECP were found to be present in about 60% of patients tested (HLA DSA^-) and were primarily of the IgG2 and IgG4 subtypes (69, 131, 154).

Limitations

Several caveats to the modification of single antigen testing to detect IgG subclasses warrant discussion. First, the IgG 1 and 2 subclass-specific antibodies exhibit non-specific binding to single antigen beads coated with the alternate antigen. For example, the IgG1 subclass-specific antibody cross-reacts with the IgG2 antigen at an MFI that is about 4.42% of the value observed when it specifically binds its target on an IgG1 coated bead (128). Cross-reactivity is observed between the IgG2 subclass-specific antibody and the IgG1 antigen ranging from 3 to 15%; however, the IgG3 and 4 antibodies appear to be less cross-reactive (126, 128). Second, the IgG1–4 antibodies bind single antigen beads coated with their cognate antigen with different strengths (IgG1 > IgG2 > IgG3 > IgG4), suggesting that the antibodies have different affinity for their target antigens (128). Third, the concentration of the different IgG subclasses cannot be directly compared to infer relative abundance of each subclass. Fourth, the sensitivities of the traditional single antigen assay and modified subclass-specific assay are different. Notable is that a minor proportion of antibodies detected using total IgG were not detected with any four subclass reagents. Antibodies <2000 MFI in the traditional single antigen bead assay can be negative in the subclass-specific assay (126, 129). Finally, a review of the literature shows that the methods for defining the threshold for positivity are vastly different (**Table 2**). While not diminishing the findings, these limitations, as well as the inherent semi-quantitative nature of the Luminex assay, do restrict the analyses to the presence or absence of IgG1–4 against a specific HLA antigen and currently do not reflect the titer or concentration of any subclass.

Another notable consideration is the variability of induction therapy among studies of IgG subclass distribution of HLA DSA, including basiliximab (129, 130), ATG (130), OKT3, thymoglobulin, or daclizumab (155). The impact of different

immunosuppression and induction therapies on isotype switching has not been thoroughly evaluated. What little is known is discussed below and suggests that both maintenance immunosuppression and induction treatments influence B cell class switching.

B CELL DIFFERENTIATION AND CLASS SWITCHING

IgG Subclasses in the Context of Protective Immunity

Exposure to different types of antigens stimulates dramatic skewing of IgG subclasses. Protein antigen leads to T-cell-dependent isotype switching to IgG1 and IgG3 that tend to dominate responses to viral and bacterial protein antigens. The importance of the diversity of Ig isotypes and subclasses is apparent from phenotypes of humans with selective subclass deficiencies, monoclonal gammopathy, and multiple myeloma. IgG3 or IgG1 deficiency increases susceptibility to bacterial respiratory tract infections. By the classical paradigm, bacterial and yeast polysaccharide antigens stimulate T-independent IgM responses and IgG2 production. Other evidence suggests that antibody responses to glycolipids and glycoproteins may obtain some help from the T cell compartment. IgG2 is predominantly produced in response to pneumococcal polysaccharides and encapsulated antigens (156, 157). The most infectious complications are seen in individuals with IgG2 deficiency, who demonstrate heightened susceptibility to respiratory bacterial infections, due to impaired responses to polysaccharides of encapsulated bacteria. IgG4 is often produced in settings of non-infectious immunity, such as allergy; immune therapy in beekeepers and allergic individuals promoted switching to IgG4 and relieved symptoms of allergy (158, 159). IgG4 is also produced in response to helminth and filariasis parasitic infections (which, like allergy, also elicit IgE) (160, 161). The clinical significance of deficiency of IgG4 is unclear.

Mechanisms of Class Switching

Immunoglobulins are tetrameric proteins composed of two heavy chains and two light chains connected by disulfide bonds. The IgG subclasses are numbered in order of abundance in circulation rather than order on the genome; in the human germline, the genes encoding the constant regions are ordered μ, δ, γ3, γ1, α1, γ2, γ4, ε, and α2. With the exception of membrane IgM and IgD in mature naïve B cells, a single B cell can only express one isotype of immunoglobulin at a time.

The genes encoding the various isotypes and subclasses are flanked by switch sites, each with its own promoter. The promoters for the switch regions contain binding sites for cytokine-responsive transcription factors, bridging exogenous signaling with isotype selection. Many cytokines, mostly Th2-associated, have been implicated in isotype and subclass specification, including IL-4, IL-13, IL-21, and IL-6 (162–165). However, no dedicated and unique switch factor has yet been identified for any given immunoglobulin heavy chain. Transcription of these switch regions produces a sterile germline RNA transcript (GLT). Subsequently, the variable region is joined with a downstream CH segment encoding a different Ig isotype to generate a new heavy chain (**Figure 4**).

Mature naïve B cells express membrane IgM and IgD. B cell activation is step-wise, and a simple schematic is shown in **Figure 5**. Formation of a synapse between T and B cells facilitates CD40–CD40L (CD154) interactions that prime the B cell. Cytokines signal the B cell to switch the isotype and subclass of immunoglobulin, and to secrete Ig. To isotype switch, the B cell must rearrange its DNA, linking the functional variable (VDJ) region of the heavy chain to one constant region heavy chain gene in a process called class switch recombination (CSR).

The first isotype of immunoglobulin that is produced is membrane-associated IgM (mIgM). IgM is also the first to be secreted upon B cell activation, as the switch from mIgM to secreted IgM requires only a change in mRNA splicing of the μ transcript to exclude the CH4 transmembrane domain. Upon primary exposure to an antigen, B cells will secrete IgM within 4–5 days, peaking by about 1–2 weeks. Expression of IgG, IgA, and IgE, however, usually require further, division-linked, and genomic DNA rearrangement and do not appear until about a week after initial exposure. With a few exceptions, CSR is CD4 T cell dependent.

Seminal work by Lechler and others demonstrated that immune recognition of allogeneic proteins occurs through three major pathways: direct, indirect, and semidirect allorecognition. The details of these pathways have been excellently reviewed elsewhere (166, 167). The direct pathway of allorecognition represents and important, although apparently transient (168, 169), mechanism of T cell response to solid organ allografts. Current paradigm holds that CD4 T cells recognize MHC class I alloantigens *via* the indirect pathway and are indispensable for alloantibody-mediated rejection (170–174). Shed alloantigens, including soluble MHC, may be taken up by host antigen-presenting cells, processed, and allopeptides presented in the context of host MHC II. Whole soluble donor MHC stimulates a more robust alloantibody response than immunization with MHC peptides. B cells themselves are involved in the indirect presentation of donor antigen and activation of CD4 T cells (175, 176).

Given what is known about antibody isotype specification, how might alloreactive B cells be driven to form anti-HLA antibodies of a given IgG subclass? The cytokine milieu and availability of costimulation are critical for B cell antibody generation and the environment under which class switching occurs during transplantation has yet to be explored experimentally. As mentioned above, it is conceivable that solid organ transplantation represents an extreme form of chronic antigen stimulation that ultimately results in the formation of IgG4 alloantibodies. One important feature of B cell activation unique to the post-transplant setting is that it occurs under the veil of maintenance immunosuppression. A few experimental studies have attempted to address the impact of immunosuppressive drugs on the mechanisms of class switching.

Effects of Immunosuppression

The frequency of DSA in patients with medication non-adherence is much higher than in those without – ~70% at 12 years (1, 2, 177) – suggesting that current immunosuppressive regimens impact humoral allosensitization.

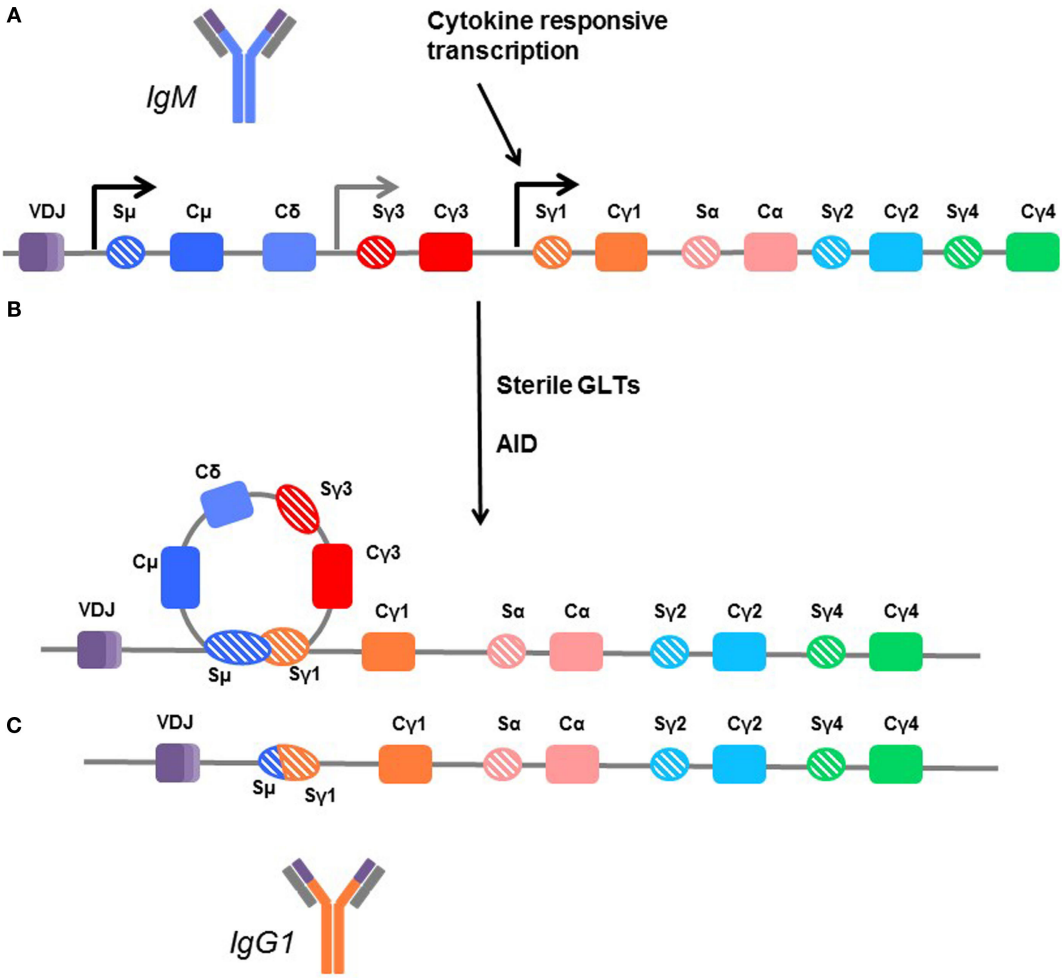

FIGURE 4 | **Mechanisms of class switching**. **(A)** In unswitched human B cells, the germline arrangement of heavy chain immunoglobulin genes is ordered by the variable region (VDJ) followed by the constant regions for IgM (Cμ), IgD (Cδ), IgG3 (Cγ3), IgG1 (Cγ1), IgA1 (Cα), IgG2 (Cγ2), and IgG4 (γ4). Each is flanked by a switch region (e.g., Sγ3). These regions are sites of transcription initiation and produce sterile germline transcripts (GLTs). **(B)** Transcription from the flanking switch regions is thought to make the DNA accessible to enzymes, such as activation-induced cytidine deaminase (AID), which facilitates recombination between switch regions, looping out the interior constant region genes. **(C)** In this example, the B cell is switching directly from IgM (dark blue) to IgG1, and the Cμ, Cδ, and Cγ3 genes are removed so that the variable region can be directly fused with the Cγ1 region to form the IgG1 molecule (orange). Thus, B cells which are isotype switched can only further isotype switch the remaining subclasses downstream. Cγ4 is terminal.

In rodents, mycophenolate mofetil (MMF) treatment significantly altered IgG subclass distribution and reduced autoantibody production and development of systemic autoimmunity (178, 179). In an *in vitro* study with human T and B cells, cyclosporine, mycophenolic acid (MPA), rapamycin, and, to a lesser extent, tacrolimus inhibited T cell proliferation; however, activation of T cells, as measured by CD25 and CD69, was unaffected. All of these drugs slightly dampened CD154 (CD40L) expression but significantly reduced Tfh cell differentiation and suppressed cytokines implicated in B cell help (180, 181). However, if T cells were first activated and then subsequently exposed to immunosuppression, only rapamycin and MPA prevented IgM production by B cells. Therefore, memory T cells may still be capable of stimulating B cell responses even under suppression by tacrolimus (180).

In mice, costimulation of T cells through B7-1 (CD80) and B7-2 (CD86) are critical for antibody responses and particularly for IgG isotype switching *via* a non-redundant role with CD40 (182). Belatacept is a CTLA-4 fusion protein that blocks T cell costimulation *via* B7. In a non-human primate transplant model, costimulatory blockade with CTLA-4 fusion proteins reduces *de novo* alloantibody production in mice and non-human primate models (183–185). In human renal transplant recipients, the BENEFIT trial revealed that lowly HLA-sensitized patients treated with Belatacept had lower reduced frequency of *de novo* donor-specific antibody production compared with the control cyclosporine arm (186, 187). The B cell compartment in Belatacept-treated recipients was skewed toward naïve and transitional phenotypes (188). It is notable that patients in these trials were either DSA-free or low risk for preformed HLA DSA (186, 187). The impact of Belatacept on B cell activation and isotype switching in allosensitized patients might be less efficacious due to reduced

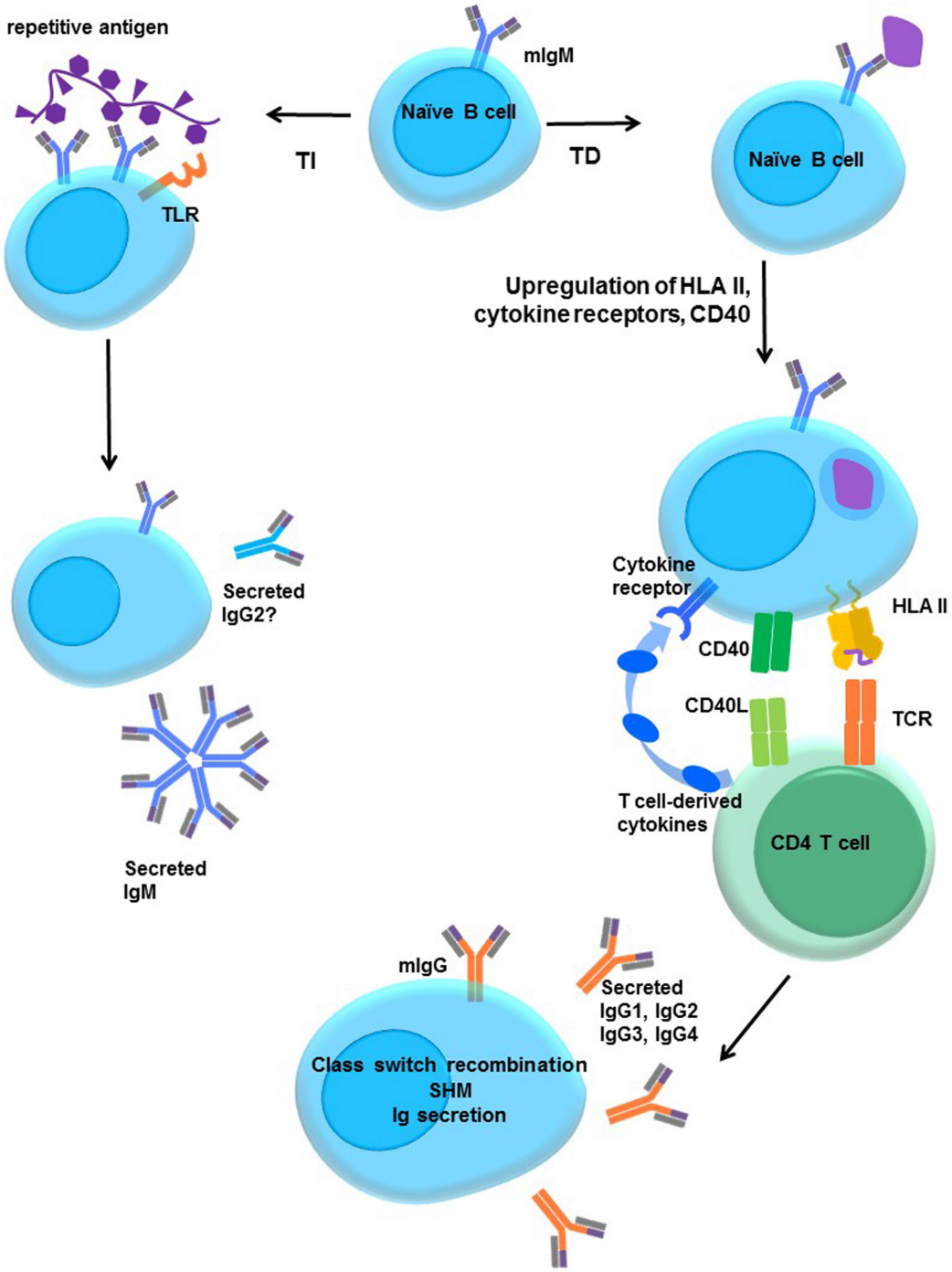

FIGURE 5 | **Activation of naïve B cells**. Upon encountering cognate antigen through the membrane-associated IgM BCR, engagement of the BCR triggers intracellular signaling. On its own, moderate BCR cross-linking is not sufficient to induce proliferation, but cells do upregulate costimulatory molecules, such as CD40 and B7 (CD80/CD86), and increase antigen processing and presentation. The B cell may internalize the antigen into endosomal vesicles (and downregulates surface IgM), enabling it to process and present the antigen in HLA. B cells upregulate HLA II, cytokine receptors, costimulatory molecules CD40, B7-1, and B7-2, enter the cell cycle, and increase expression of prosurvival genes, and protein translation machinery. T-dependent protein antigens can be internalized and processed into peptides for presentation to CD4 T helper cells in HLA molecules. CD4 T cells provide additional costimulation *via* CD40L and expression of cytokines, such as IL-21, IL-10, IL-13, or IL-4. Integration of the BCR, CD40, and cytokine signaling stimulates class switch recombination, and the B cell ultimately switches from production of only membrane IgM and IgD to secretion of other isotypes such as IgG. T-independent antigens, such as those with repetitive motifs, extensively cross-link the BCR but are difficult to internalize. Glycolipid or polysaccharide antigens cannot be presented in classical HLA molecules for $CD4^+$ T cell help. Concurrent signals may derive from Toll-like receptor (TLR) stimulation by antigens, NKT or αβ T cell help, leading to enhanced B cell activation and secretion of IgM or IgG2.

costimulation requirements of memory immune cells [discussed in Ref. (189)].

There is also evidence that immunosuppressive drugs might alter isotype switching *via* direct effects on B cells. Prior depletion of B cells using rituximab reduced IgM and IgG1 responses after vaccination early after treatment, with a durable inhibition of IgM seen 6–10 months after vaccination (190). mTOR is central to the ability of B cells to proliferate and regulates CSR, and rapamycin has an impact on IgG production and plasmablast differentiation (191). Leflunomide is an immunosuppressant often used off-label in transplant recipients with viremia, such as with CMV or BK virus. Leflunomide acts on both T and B cells through inhibition of the JAK/STAT pathway critical for B cell signaling. Leflunomide inhibits IgG production in rodents, including reduction of donor-specific antibodies in transplant models (192, 193).

In summary, multiple immunosuppressive agents have been demonstrated to impact isotype switching by B cells in *in vitro* and animal models. However, definitive evidence from systematic trials in humans is lacking to demonstrate differential effects on donor-specific IgG subclass production.

CLOSING REMARKS

Clinical experience confirms that all donor-specific antibodies are *not* created equal. ABMR is a wide spectrum of graft injury from complement-mediated hyperacute rejection, to histological injury without graft dysfunction, to fibrotic chronic rejection and vasculopathy. Whether further discrimination of pathogenic DSA can be provided by complement fixation *in vitro* or by identifying the subclass(es) present remains to be determined. A majority of individuals pre- and post-transplant exhibit antibodies against HLA that are a mixture of IgG subclasses. Cumulatively, studies to date indicate that donor-specific IgG3 may be most relevant to acute antibody-mediated injury, while IgG4 DSA might signify alloimmune memory and correlate with subclinical and chronic rejection. However, IgG1 is also found in nearly all cases, indicating that heterogeneous subclass responses are the norm. These studies highlight the complexity of the alloimmune response and underscore the constraints on interpreting the relevance of DSA subclass repertoire in graft outcome, since exclusive skewing of donor-specific antibodies to one single subclass is rarely observed.

Three challenges must be overcome in order to identify characteristics of pathogenic DSA. First, laboratories are faced with the task of developing reliable, informative, and cost-effective assays that can detect differences in effector functions or other features of HLA antibodies. Second, the mechanisms of graft injury by different subclasses of HLA antibodies should be confirmed in experimental transplant models and *in situ* in allografts. To date, we can only postulate that in the setting of transplantation, anti-HLA IgG3 and IgG1 might elicit extensive complement activation and ADCC, while IgG2 and/or IgG4 may induce only HLA signaling in the allograft with little complement activation or FcγR-mediated functions. Much of the knowledge of antibody effector functions is derived from infectious disease and autoimmune and cancer research, but little work has evaluated the capacity of different human HLA IgG subclasses to elicit inflammation and injury during ABMR. Finally, few interventions exist for the treatment of ABMR, and their impact on class switching of alloreactive B cells is mostly uncharacterized. Therapies might be designed to manipulate the humoral alloimmune response to produce one subclass rather than another, but more effort is needed to understand the details of isotype specification by cytokines and other signals.

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NV and MH contributed to the outline and writing of the manuscript. ER contributed to the outline and critical review of the manuscript.

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MINI REVIEW
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ABO-Incompatible Kidney Transplantation

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ABO-incompatible (ABOi) kidney transplantation has long been considered a contraindication to successful kidney transplantation. During the last 25 years, increasing organ shortage enforced the development of strategies to overcome the ABO antibody barrier. In the meantime, ABOi kidney transplantation has become a routine procedure with death-censored graft survival rates comparable to the rates in compatible transplantations. Desensitization is usually achieved by apheresis and B cell-depleting therapies that are accompanied by powerful immunosuppression. Anti-A/B antibodies are aimed to be below a certain threshold at the time of ABOi kidney transplantation and during the first 2 weeks after surgery. Thereafter, even a rebound of anti-A/B antibodies does not appear to harm the kidney transplant, a phenomenon that is called accommodation, but is poorly understood. There is still concern, however, that infectious complications such as viral disease, *Pneumocystis jirovecii* pneumonia, and severe urinary tract infections are increased after ABOi transplantations. Recent data from the Collaborative Transplant Study show that during the first year after kidney transplantation, one additional patient death from an infectious complication occurs in 100 ABOi kidney transplant recipients. Herein, we review the recent evidence on ABOi kidney transplantation with a focus on desensitization strategies and respective outcomes.

Keywords: kidney transplantation, ABO incompatible, survival, desensitization, antibodies

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INTRODUCTION

In an earlier publication from 1955, Hume et al. expressed their skepticism on the feasibility of ABO-incompatible (ABOi) kidney transplantations: "… *we do not feel that renal transplantation in the presence of blood incompatibility is wise*" (1). Since then, major ABO incompatibility has been considered a contraindication to kidney transplantation. A major breakthrough came in 1982, with the first large study on ABOi kidney transplantation by Alexandre et al. from Belgium (2, 3). Successful desensitization was achieved by repeated plasmapheresis (PP), splenectomy, donor thrombocyte transfusion, and infusion of A or B trisaccharide, together with intensified immunosuppression. One-year graft survival in this study was a remarkable 75%. This led to a wider utilization of ABOi kidney transplantations, first in Japan from the late 1980s, in the US from the mid 1990s, and in Europe from the early 2000s. While, even today, kidney transplantation is best performed in the absence of (major) ABO incompatibility, a large end-stage kidney disease population and an increasing organ shortage result in waiting times for a deceased donor kidney transplant exceeding 5 years in some countries such as Germany. One possibility to reduce the waiting time is the transplantation across ABO antibody barriers. Theoretically, the number of kidney transplantations from living donors can be increased by up to 30% when patients are transplanted across the ABO antibody barrier. With currently existing protocols, as many as 90% of patients with an ABOi living donor may effectively

be desensitized and transplanted. The aim of desensitization protocols is the reduction and maintenance of anti-A/B antibodies (isoagglutinins) during the first 2 weeks after transplantation below a threshold that is considered to be safe (e.g., <1:32 in tube technique). Thereafter, even when anti-A/B antibodies recur at high levels they will not harm the kidney transplant, a phenomenon that is called accommodation. In recent years, graft survival rates after ABOi kidney transplantation nearly equaled those after ABO-compatible (ABOc) procedures.

Herein, we review the latest efforts and results in kidney transplantation across the ABO antibody barrier.

BLOOD GROUP ANTIGENS AND ANTIBODIES

The ABO antigen system consists of oligosaccharides that are predominantly expressed on red blood cells and are also found on endothelial cells, tubuli, and glomeruli making the ABO antigen system important for kidney transplantation. Patients with different blood groups differ with respect to their antigen density on erythrocytes. Compared to blood group A1 and blood group B individuals, blood group A2 recipients, who make up 20% of all Caucasian individuals with blood group A, have a low expression of blood group antigen molecules (30–50%) on the surface of erythrocytes, which is believed to be responsible for the lower immunogenicity of organs from blood group A2 donors (4, 5). ABOi kidney transplantation with A2 organs has been accomplished with standard immunosuppressive therapy without any additional measures (6). Of interest, anti-A/B antibodies are formed upon contact with gut bacteria during early infancy. Naturally occurring anti-A/B antibodies are predominantly of the IgM class but especially in blood group O individuals they also consist of IgG and IgA class (7). While the pathogenic importance of anti-A/B antibodies in solid organ transplantation is well known, the relative contribution of the different immunoglobulin isotypes and their subclasses to organ rejection remains to be elucidated.

DESENSITIZATION FOR ABOi KIDNEY TRANSPLANTATION

Despite the absence of a generally accepted desensitization protocol for the transplantation across the ABO antibody barrier, all currently proposed strategies share some common principles (**Figure 1A**).

These include together with a powerful maintenance immunosuppression one or more of the following:

(1) Anti-A/B antibody depletion at the time of transplantation using PP, double-filtration PP/membrane filtration, or selective or unselective immunoadsorption (IA)
(2) Modulation of the recipient's immune system by the use of intravenous immunoglobulins (IVIgs)
(3) Reduction of the B lymphocyte pool by splenectomy, or more recently by the anti-CD20 antibody rituximab
(4) Prevention of the deleterious consequences of complement activation upon anti-A/B antibody binding to the graft endothelium.

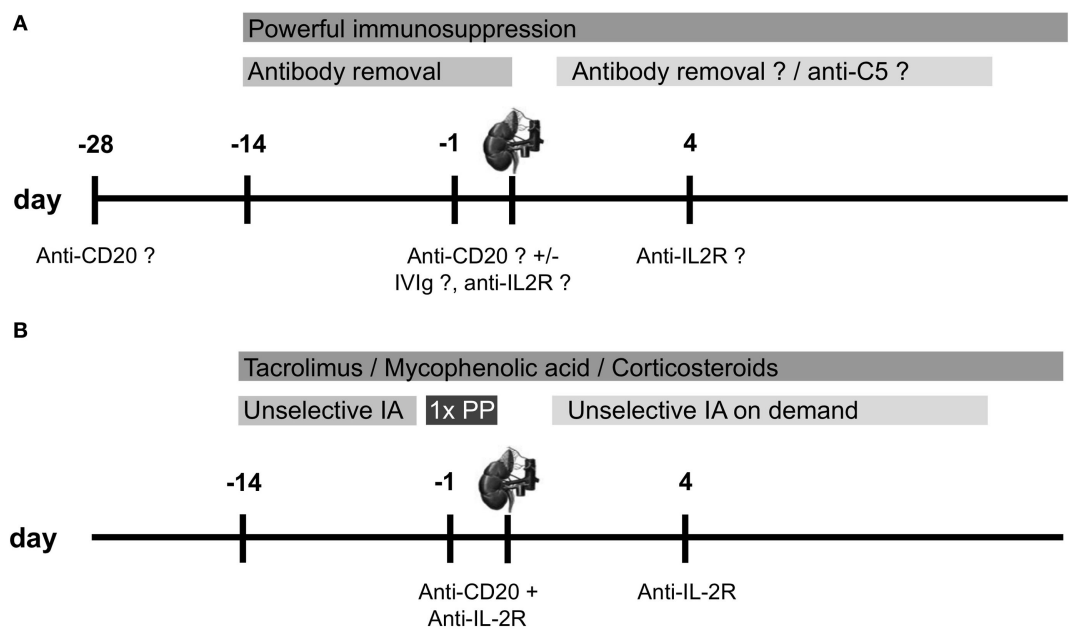

FIGURE 1 | **Overview of desensitization protocols for ABO-incompatible (ABOi) living donor kidney transplantation**. **(A)** Scheme for a standard desensitization protocol performed by the majority of centers with modifications in the utilization of desensitization devices, and **(B)** desensitization protocol for ABOi living donor kidney transplantation at the University of Heidelberg. Anti-CD20 therapy is usually performed with rituximab 375 mg/m^2, anti-IL-2R therapy is performed with basiliximab 20 mg [modified from Ref. (8)]. IA, immunoadsorption; IVIg, intravenous immunoglobulin; PP, plasmapheresis.

Antibody Depletion by Extracorporal Treatment

Antibody removal strategies may be divided into methods that completely remove plasma proteins such as PP, methods that remove only a specific fraction of the plasma proteins including the immunoglobulins (such as membrane separation), and more specific methods such as unselective or selective IA. While PP is the preferred antibody removal strategy in the US, membrane separation is popular in Japan. Unselective and selective IAs are often used in Europe.

Selective anti-A/B antibody removal is feasible by the Glycosorb columns that contain synthetic terminal trisaccharide A or B blood group antigen linked to a sepharose matrix. In addition, they may also reduce total IgG as well as IgG against polysaccharide antigens such as anti-*Pneumococcus* IgG (9). In a recent analysis, Wahrmann et al. found single treatments with unselective IA to be more effective than with selective anti-A/B antibody columns in removing anti-A/B IgG (median reduction to 28 versus 59% of baseline, $P < 0.001$) (10). In contrast, unselective IA was less effective in the removal of anti-A/B antibodies of the IgM (74 versus 30%, $P < 0.001$) and IgG3 subclass (72 versus 42%, $P < 0.05$). The same group found that a combination of membrane separation and unselective IA effectively removed IgG and IgM antibodies and effector molecules such as complement C1q component (11). No significant differences were found in clinical studies that compared the impact of different IA strategies on clinical outcomes, including anti-A/B antibody reduction, survival, kidney function, rejection episodes, or complications (12).

Intravenous Immunoglobulins

Intravenous immunoglobulins are given by many centers before ABOi kidney transplantation to prevent the anti-A/B antibody rebound in the early phase after transplantation. In addition, IVIg infusion is believed to reduce infectious complications by substituting depleted immunoglobulins. As a note of caution, IVIg preparations contain IgG antibodies directed against A/B antigens and can effectively increase anti-A/B antibody titers upon administration (12, 13).

B-Cell Depletion by Splenectomy or Rituximab

Before the introduction of pharmacological anti-B cell therapies, splenectomy was an integral component for the reduction of the B lymphocyte pool prior to ABOi kidney transplantation. Due to the surgical risk and increased risk of sepsis, splenectomy was gradually substituted by the anti-CD20 antibody rituximab. More recently, several groups completely abandoned anti-B cell therapies from their protocols. Flint et al. reported on 37 patients from Melbourne who underwent ABOi kidney transplantation after antibody removal by PP (14). Transplantation was accomplished by the use of standard immunosuppressive therapy without rituximab when the patient had reached an anti-A/B antibody titer of less than 1:32 (tube method). Patient and graft survival in this cohort was 100% after a median of 26 months after transplantation. Two antibody-mediated rejection episodes were successfully reversed. We observed in the Collaborative Transplant Study (CTS) a higher rate of death-censored graft loss in ABOi kidney transplant recipients when rituximab was omitted (see below) (15).

Inhibition of Complement Activation

An emerging new concept in the transplantation across ABO antibody barriers is the inhibition of complement activation upon binding of antibodies to the allograft endothelium. Biglarnia et al. described an intentional simultaneous ABOi kidney pancreas transplantation (16). Severe antibody-mediated rejection in this patient during anti-A/B antibody rebound was successfully treated by inhibiting the assembly of the membrane attack complex by eculizumab. Stegall et al. performed a single center study to evaluate the efficacy of eculizumab added to conventional therapy in the prevention of antibody-mediated rejection after ABOi living donor kidney transplantation (unpublished, NCT01095887). Patients received eculizumab at the time of transplantation, on day 1 after surgery and weekly thereafter for 4 weeks. The study was terminated after only six patients due to poor enrollment. Two of the six patients reached the primary study endpoint of antibody-mediated rejection after 3 months and two allografts had to be removed during the study period. Therefore, results on the use of eculizumab after ABOi kidney transplantation are inconclusive.

DESENSITIZATION PROTOCOLS AND SURVIVAL AFTER ABOi LIVING DONOR KIDNEY TRANSPLANTATION

Table S1 in Supplementary Material gives an overview over studies on ABOi kidney transplantation. The largest cohort of patients after ABOi kidney transplantation with the longest follow-up of more than 20 years is reported from Japan. Most patients were desensitized by double-filtration PP, and splenectomy was more recently replaced by the anti-CD20 antibody rituximab. For the most recent area from 2001 to 2010, patient and graft survival rates for the 1,427 analyzed patients were an excellent 98 and 96% for the first year, and 91 and 83% after 9 years, respectively (17). Data from the Scientific Registry of Transplant Recipients on the outcomes of 738 ABOi kidney transplantations that were performed between 1995 and 2010 in the US have recently been published (18). Most patients were desensitized by PP and low-dose IVIg. The cumulative incidence of graft loss during the first year after transplantation was 5.9% in ABOi as compared to only 2.9% in ABOc transplantations and occurred mainly during the first 2 weeks after surgery due to rejection. In 2003, Tydén et al. from Sweden published a protocol for ABOi transplantation that is based on recipient desensitization by selective IA using Glycosorb columns (19). In addition, splenectomy was replaced by the anti-CD20 antibody rituximab. This protocol led to a renaissance of ABOi kidney transplantation in Europe. Recently, Genberg et al. published their extended experience with this protocol (20). Of 45 patients desensitized for ABOi kidney transplantation, 43 were eventually transplanted between September 2001 and May 2010 (96%). Overall patient and graft survival after a mean follow-up

of 4.5 years was 93 and 91%, respectively. None of the patients experienced early acute antibody-mediated rejection that could be linked to anti-A/B antibodies. Recently, the Freiburg group from Germany compared the results of 100 ABOi kidney transplantations performed between April 1, 2004, and October 28, 2014, with the results of 248 ABOc transplantations performed during the same time period (21). Using the Stockholm protocol, they achieved in recipients of ABOi transplants a 10-year patient and death-censored graft survival of 99 and 94%, respectively, which did not differ significantly from the 80 and 88% survival rates, respectively, in recipients of ABOc transplants. The rates for antibody- and T-cell-mediated rejections were also not significantly different. A study from the UK showed similar death-censored graft survival in 62 patients 3 years after ABOi kidney transplantation when compared to ABOc controls (22). However, patient survival in ABOi transplant recipients was reduced due to infectious complications, mostly *Pneumocystis jirovecii* pneumonia.

Since 2006, at our center in Heidelberg, we have been using a protocol for desensitization of ABOi kidney transplant candidates that is very similar to the Swedish protocol (**Figure 1B**) (8, 12). The major difference is the use of unselective instead of selective IA, allowing also the desensitization for HLA-incompatible living donor kidney transplantation. Further differences are the omission of IVIg application and a variable number of IA treatments depending on the strength of anti-A/B antibody. To remove pathogenically relevant anti-A/B antibodies of the IgM class more efficiently, at least one additional PP treatment was performed in all patients the day before surgery as of August 2012 (23). An early analysis of ABOi kidney transplantations showed successful desensitization of 12 patients after a median of six IA treatments (12). Anti-A/B titer reduction with unselective IA was comparable to that of a historical control group that received selective IA. In a more recent analysis, we compared 34 ABOi kidney transplant recipients who were desensitized with unselective IA to 68 matched, standard risk living donor kidney recipients (23). After a median postoperative follow-up of 22 months, graft survival in ABOi kidney transplant recipients was insignificantly lower compared to standard risk recipients ($P = 0.05$). One of the two patient deaths in the ABOi kidney transplant recipients was due to *P. jirovecii* pneumonia at postoperative day 169. This patient death may be attributable to intensified immunosuppression that was applied during desensitization including rituximab. Other important differences between ABOi and standard risk kidney recipients were a higher incidence of BK virus replication ($>10^4$ copies/mL plasma, 21 versus 6%, $P = 0.04$) and BK virus nephropathy (SV 40 positive in biopsy, 12 versus 0%, $P = 0.01$) and a higher prevalence of colonization with multidrug-resistant bacteria (15 versus 1%, $P = 0.02$).

RESULTS FROM THE CTS

We recently published 3-year outcomes of 1,420 ABOi kidney transplant recipients who were transplanted at 101 different centers between 2005 and 2012 (15). Patients were compared to a matched group of ABOc kidney transplant recipients and to all ABOc kidney transplant recipients from centers that performed at least five ABOi procedures. Overall graft, death-censored graft, and patient survival were not statistically significant different between the groups. Early patient survival was reduced in ABOi kidney transplant recipients due to a higher rate of early infection-associated death ($P = 0.037$ versus matched controls and $P < 0.001$ versus center controls). Specifically, one additional death per 100 patients occurred in the first year after ABOi kidney transplantation from an infectious complication. **Figure 2** provides the updated results for this cohort of 1,420 ABOi kidney transplant recipients (15). Of note, a trend toward better 3-year death-censored graft survival in patients receiving anti-CD20 therapy ($P = 0.081$) in the meantime has become statistically significant after longer follow-up ($P = 0.009$, **Figure 2C**), suggesting a need for anti-B cell therapies in the setting of ABO incompatibility.

COMPLICATIONS AND HURDLES OF ABOi KIDNEY TRANSPLANTATION

Accomodation versus Rejection

In contrast to transplantation in the HLA-sensitized patient, accommodation appears to be a frequent phenomenon after ABOi kidney transplantations and is often associated with C4d deposition in peritubular capillaries of allograft biopsies. An accommodation phenotype may be achieved by the controlled anti-A/B antibody exposure to antigens in the early phase after kidney transplantation. About 2 weeks after successful transplantation, accommodation is established and even high anti-A/B antibody exposure does not harm the kidney transplant. Local upregulation of complement regulatory proteins, like CD45, CD55, and CD59, as a consequence of anti-A/B antibody-dependent inactivation of ERK1/2 signaling pathway are discussed as one possible mechanism (24).

Infection and Malignancy

There are conflicting results on infectious complications after ABOi kidney transplantation in the literature. A higher frequency of viral infections such as CMV, HSV, VZV, and BK virus, as well as *P. jirovecii* pneumonia, wound, and severe urinary tract infections have been described (22, 25, 26). In the CTS and the Heidelberg cohort, an increased risk for early severe infections was observed, resulting in approximately one additional patient death in 100 ABOi kidney transplant recipients during the first year after surgery (15, 23). We and others also observed a higher incidence of BK virus replication and BK virus-associated nephropathy (23). Of note, in a study by Sharif et al., the rate of BK virus nephropathy was about three times higher in ABOi patients compared to patients with HLA antibodies, despite comparable immunosuppressive therapy (27). Bentall et al. hypothesized that different blood group antigens may influence binding of viral pathogen receptors to sialic acid on renal tubular cells (28).

Hall et al. found no increased cancer risk when comparing 318 ABOi kidney transplant recipients to matched ABOc controls (29). The analysis of 1,420 ABOi transplantations from the CTS study also did not show an increased risk of malignancy in ABOi compared to ABOc patients (15).

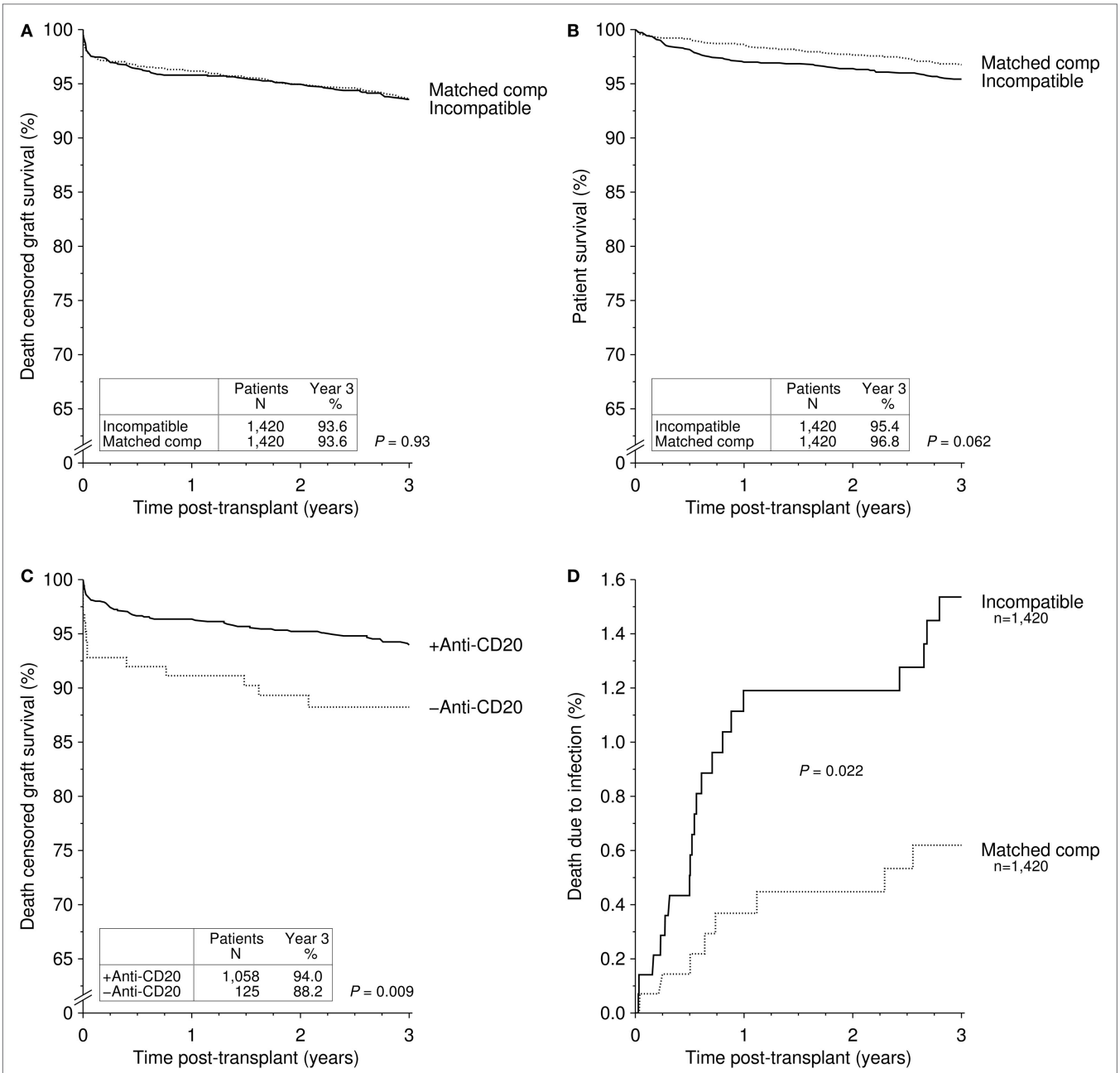

FIGURE 2 | Cumulative incidence of (A) death-censored graft survival and (B) patient survival in recipients of an ABO-incompatible (ABOi) living donor graft and matched controls receiving an ABO-compatible (ABOc) living donor graft [updated Figure 1 of Ref. (15)]. **(C)** Cumulative incidence of death-censored graft survival in recipients of an ABOi living donor graft with and without anti-CD20 antibody treatment [updated Figure 4 of Ref. (15)]. **(D)** Cumulative incidence of death due to infection in recipients of an ABOi living donor graft and matched controls receiving an ABOc living donor graft [updated Figure 3 of Ref. (15)].

Additional Observations

A study from the US Renal Data System registry found a two times higher risk of early hemorrhage in 119 ABOi kidney transplant recipients when compared to ABOc controls (adjusted HR, 1.96, $P < 0.05$) (26). A higher bleeding risk was also found in a cohort of pediatric kidney transplant recipients with two major bleeding episodes in three patients, which was attributed to the unspecific binding of coagulation factors during repeated IA (30). This assumption is supported by the findings of de Weerd et al. who found a significant correlation between the number of pre-transplant apheresis treatments and the peri- and posttransplant bleeding risk (31).

Some authors observed an increased rate of surgical complications after ABOi kidney transplantation, which were attributed to early intensified immunosuppression with mycophenolic acid and removal of coagulation factors by apheresis. The Freiburg group reported a significantly higher number of lymphoceles in ABOi patients than in ABOc controls (33 versus 15%; $P = 0.003$) that required surgical revisions in 20 and 8% ($P = 0.013$) of patients, respectively (21). Also, the overall need for surgical revision was significantly higher in ABOi patients compared to ABOc controls (38 versus 24%, $P = 0.032$).

FUTURE PERSPECTIVES

A new strategy that may come into clinics in the future is the reduction of blood group antigen levels in the allograft by *ex vivo* infusion of endo-beta-galactosidase (32).

Another approach is the complete avoidance of the ABO antibody barrier by kidney exchange programs. However, despite the usage of large kidney exchange programs, including the utilization of altruistic donors, the blood group O recipients accumulated on the waiting list in different studies (33). Desensitization for ABOi kidney transplantation was the only way to transplant these patients within a reasonable period of time.

CONCLUSION

In recent years, ABOi kidney transplantation has become a routine procedure. By this approach, about 30% of living donors who were refused in the past can now donate their kidneys and thereby significantly expand the living donor pool. Transplantation in the presence of major ABO incompatibility, however, places the patient at a somewhat higher risk of early rejection, infection, and infection-associated death. Therefore, whenever possible, ABOc procedures should be preferred.

AUTHOR CONTRIBUTIONS

All authors substantially contributed to preparation of the manuscript and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

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A Previous Miscarriage and a Previous Successful Pregnancy Have a Different Impact on HLA Antibody Formation during a Subsequent Successful Pregnancy

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Inherited paternal HLA antigens from the semi-allogeneic fetus may trigger maternal immune responses during pregnancy, leading to the production of child-specific HLA antibodies. The prevalence of these HLA antibodies increases with the number of successful pregnancies. In the present study, we investigated the effect of a single prior miscarriage on HLA antibody formation during a subsequent successful pregnancy. Women with a successful pregnancy with one or more prior miscarriages (n = 229) and women with a successful pregnancy without a prior miscarriage (n = 58), and their children were HLA typed. HLA antibody analyses were performed in these women to identify whether HLA antibodies were formed against mismatched HLA class-I antigens of the last child. The percentage of immunogenic antigens was significantly lower after a single successful pregnancy that was preceded by a single miscarriage (n = 18 women) compared to a successful pregnancy that was preceded by a first successful pregnancy (n = 62 women). Thus, our data suggest that a previous miscarriage has a different impact on child-specific HLA antibody formation during a subsequent successful pregnancy than a previous successful pregnancy. The lower immunogenicity in these women cannot be explained by reduced numbers of immunogenic B-cell and T-cell epitopes. In conclusion, our observations indicate that increasing gravidity is not related to an increased prevalence of HLA antibodies in a single successful pregnancy that was preceded by a single prior miscarriage.

Keywords: allo-sensitization, HLA antibodies, pregnancy, miscarriage, PIRCHE-II

Abbreviations: IPA, inherited paternal HLA antigens; MFI, mean fluorescence intensity; PIRCHE-II, Predicted Indirectly ReCognizable HLA Epitopes presented by HLA class-II.

INTRODUCTION

A successful pregnancy requires an optimal interplay between the maternal immune system and the semi-allogeneic fetus. Breakdown of the maternal immune tolerance may result in fetal rejection. Thus, the maternal tolerance toward the fetus has to be maintained both locally at the fetal-maternal interface and systemically, since bidirectional trafficking of cells and soluble HLA between the mother and the fetus takes place (1–3). As early as 4 weeks of gestation, semi-allogeneic fetal DNA can be detected in the maternal circulation (2) and the presence of this fetal microchimerism can persist for decades after delivery (4).

Inherited paternal HLA antigens (IPA) of fetal origin are able to prime maternal immune responses at the fetal-maternal interface as well as in the maternal circulation (5, 6). These immune responses may lead to the production of child-specific HLA antibodies (7–9). The maternal production child-specific HLA antibodies of the IgG isotype requires interaction between activated B-cells and primed T-helper cells. First, B-cell activation occurs upon antigenic uptake of IPA by the B-cell receptor (10). Subsequently, upon T-cell recognition of degraded IPA presented on HLA class-II molecules, T-helper cells provide co-stimulation *via* CD40–CD40L interaction and secrete cytokines (10, 11). These signals drive proliferation and differentiation of naive B cells into memory cells and plasma cells and induce IgM to IgG isotype switching (10, 11). Thus, the maternal production of child-specific IgG HLA antibodies requires the activation of B cells by T-helper cells where both B cells and T-helper cells respond to the same antigen, a phenomenon called linked recognition (12).

Despite abundant allogeneic fetal contact, only 10–40% of the mothers develop child-specific HLA antibodies (8, 9). The exact mechanism behind HLA antibody formation is currently unclear. Increasing gravidity (8, 13) and the fetal and maternal HLA phenotype combination (14) may be important determinants in the immunogenicity toward IPA. We previously showed that HLA antibody formation during a successful pregnancy without prior miscarriages is related to the number of predicted HLA-derived T-helper epitopes as determined by the PIRCHE-II model (Predicted Indirectly ReCognizable HLA Epitopes) (15). This model identifies the number of mismatched HLA-derived peptides that can be presented by HLA class-II molecules, designated as PIRCHE-II (16).

HLA antibodies play an important role in organ transplantation; the presence of pre-transplantation donor-specific HLA antibodies is associated with antibody-mediated rejection and an impaired graft survival (17–20). Therefore, more insight into the immunogenicity of mismatched HLA after pregnancy may have has implications in the transplantation field. In contrast to transplantation, the effect of IPA-specific HLA antibodies on the fetus is presumably rather harmless, as the prevalence of IPA-specific HLA antibodies is relatively high in normal pregnancies. However, both beneficial and harmful effects of HLA antibodies on pregnancy outcome have been described, indicating that the role of IPA-specific HLA antibodies on pregnancy outcome is debatable (21). Most of these studies focused on HLA antibody formation in (recurrent) miscarriage(s), whereas studies about the effect of a prior miscarriage on HLA antibody formation during a subsequent successful pregnancy are limited. In the present study, we investigate for the first time the effect of a single previous miscarriage on HLA antibody formation during a subsequent first successful pregnancy.

MATERIALS AND METHODS

Population and Sample Collection

We included in this study 301 mothers who gave birth between September 2009 and April 2011 at the University Hospital Basel, Switzerland. All women included had either their first full-term pregnancy or gave birth to children from the same partner before. Fully HLA class-I matched mother–child pairs ($n = 3$) were excluded from the analyses. In some mother–child pairs, the child was homozygous for a HLA class-I IPA for which the mother was heterozygous ($n = 8$). These mother–child pairs were also excluded from analyses, as these HLA class-I IPA was identical to the mother and thus not immunogenic. From all participating women, blood transfusions and previous miscarriages were documented. Three women had previous blood transfusions, and these mother–child pairs were excluded from further analysis. From the remaining 287 mother–child pairs, a total of 58 women had one or more prior miscarriages. These women with one of more prior miscarriages were used to study the effect of a prior miscarriage on HLA antibody formation during a subsequent successful pregnancy.

After obtaining informed consent from all the participating women, blood samples were taken from the mother 1–4 days after delivery. Cord blood of the child was sampled directly after delivery. HLA antibody analysis was performed on the maternal blood samples, and HLA typing was performed on blood samples that were obtained from both the mother and the cord blood. This study was approved by the local ethics committee (EKBB; reference number 23/09).

HLA Typing

High-resolution HLA typing was performed on maternal blood samples and cord blood samples using either sequence-based typing (www.histogenetics.com) or SSO DNA typing (LABType HD; One Lambda). Identification of mismatched IPA was based on two-field resolution HLA typing of both mother and child.

HLA Antibody Analysis

Maternal post-delivery blood samples were analyzed for the presence of HLA antibodies using single HLA class I-antigen beads according to the instructions of the manufacturer (iBeads Lot 1; One Lambda) as described previously (9). For the analyses presented in this paper, we consider mean fluorescence intensity >1,000 as positive. Mismatched HLA class-I IPA against which the mother had developed HLA-specific antibodies were classified as immunogenic HLA, whereas mismatched HLA class-I IPA against which the mother had not developed HLA-specific antibodies were classified as non-immunogenic HLA. The percentage of immunogenic antigens was calculated for individual groups by

dividing the number of immunogenic HLA by the total number of HLA class-I IPA mismatches multiplied by 100%.

Identification of HLA Class-I-Derived PIRCHE-II

The numbers of HLA class-I derived epitopes from the child presented by maternal HLA class-II molecules, PIRCHE-II, were determined as described previously (15). Briefly, for all mismatched HLA-A, HLA-B, and HLA-C antigens of the child, we used the netMHCIIpan-3.0 algorithm to predict how mismatched HLA-derived peptide may align in the binding groove of maternal HLA-DRB1 [algorithm available *via* http://www.cbs.dtu.dk/services/NetMHCIIpan-3.0/ (22)]. Subsequently, the binding affinity of this peptide to maternal HLA-DRB1 was predicted by the algorithm, considering binding affinities with an IC_{50} of <1,000 nM as relevant HLA-DRB1 binders. HLA-DRB1 binders were designated as a PIRCHE-II when the predicted binders differed at least one amino acid with the maternal HLA amino acid sequence. Only unique child-specific epitope-HLA complexes were counted as a PIRCHE-II. The PIRCHE algorithm is available *via* http://www.pirche.org.

HLAMatchmaker

HLAMatchmaker version 2.1 was used to determine the number of HLAMatchmaker eplets for all mismatched HLA class-I molecules of the child. Eplets that were present in HLA of the child and absent in the mother's HLA-A, HLA-B, HLA-C, and HLA-DRB1 locus were counted as mismatched eplets. The HLAMatchmaker software is available *via* http://www.epitopes.net (23).

Statistical Analysis

We used the GraphPad Prism software version 6.02 (GraphPad Software, Inc., La Jolla, CA, USA) and the SPSS Statistics software version 20 (IBM SPSS Software) for the statistical analyses. Pearson's chi-square tests were used to analyze differences in percentage of immunogenic antigens between different groups. Mann–Whitney *U* tests were used to analyze differences in the number of mismatched eplets and PIRCHE-II between different groups. *p*-values <0.05 were assumed to indicate statistical significance.

RESULTS

Population Characteristics

Table 1 summarizes the characteristics of the study population. Of all 287 women, the majority of the women (79.8%) did not have any prior miscarriage. A total of 58 women had one or more prior miscarriages. The majority of these women with a prior miscarriage had a single prior miscarriage. In all 287 women, 738 HLA-class I IPA mismatches were identified. **Table 2** summarizes the number of mismatched IPA for pregnancies with and without prior miscarriage(s) and the percentage of immunogenic HLA per locus. The percentage of immunogenic IPA between these groups did not significantly differ ($p = 0.72$, $p = 0.64$, and $p = 0.08$ for HLA-A, HLA-B, and HLA-C, respectively, in Pearson's chi-square tests with Yates' correction).

First Pregnancy and First Miscarriage Have a Different Impact on HLA Antibody Formation during a Subsequent Successful Pregnancy

Multiple successful pregnancies and prior miscarriages may have a differential effect on HLA immunization during a subsequent successful pregnancy. To investigate the effect of a first pregnancy and a first miscarriage on HLA antibody formation during a subsequent successful pregnancy, we compared secundigravidae without a prior miscarriage (i.e., these women had two successful pregnancies without a prior miscarriage; $n = 65$ women) with secundigravidae with a prior miscarriage (i.e., these women had a single successful pregnancy that was preceded by a single miscarriage; $n = 18$ women) (**Figure 1**). The secundigravidae without a prior miscarriage group had a total of 162 HLA class I mismatched IPA, whereas the secundigravidae with a prior miscarriage had 44 HLA class I-mismatched IPA. The percentage of immunogenic antigens was higher for secundigravidae without a prior miscarriage (21%) compared to secundigravidae with a prior miscarriage (2.3%) (**Figure 1**; $p = 0.003$). For the secundigravidae with a prior miscarriage, only a single HLA was immunogenic (HLA-C*01:02), while the other 43 mismatched HLA were non-immunogenic. When using a lower fluorescence intensity cutoff (>500), the percentage of immunogenic antigens for secundigravidae with a prior miscarriage increased marginally (4.5%). These observations indicate that the HLA immunogenicity is significantly lower during a subsequent successful pregnancy in women who experienced a prior miscarriage compared to women who had a prior successful pregnancy. The percentage of immunogenic

TABLE 2 | Number of mismatched inherited paternal HLA antigens (IPA) per locus; *n* (% immunogenic IPA per locus).

	HLA-A	HLA-B	HLA-C
Pregnancies without prior miscarriage	234 (16%)	259 (17%)	245 (6%)
Pregnancies with prior miscarriage(s)	40 (20%)	54 (20%)	48 (15%)

TABLE 1 | Population characteristics.

	Without prior miscarriage, *n* (%)	With prior miscarriage(s), *n* (%)		
		1 prior miscarriage	2 prior miscarriages	≥3 prior miscarriages
First full-term pregnancy	154 (53.7)	18 (6.3)	7 (2.4)	4 (1.4)
Second full-term pregnancy	65 (22.6)	15 (5.2)	5 (1.7)	2 (0.7)
Third or more full-term pregnancy	10 (3.5)	4 (1.4)	2 (0.7)	1 (0.3)

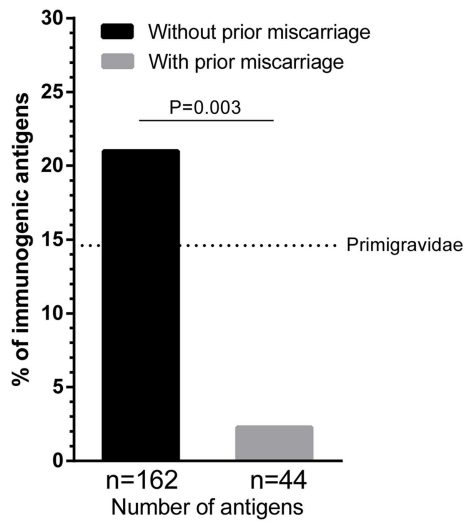

FIGURE 1 | The effect of first pregnancy and first miscarriage on subsequent successful pregnancy. The percentage of immunogenic antigens is higher for secundigravidae without a prior miscarriage (black bar) than secundigravidae with a prior miscarriage (gray bar). The dotted line represents the percentage of immunogenic antigens for primigravidae (women with a single successful full-term pregnancy; 14.6%). For each group, *n* represents the number of mismatched antigens. The *p* value is derived from Pearson's chi-square test.

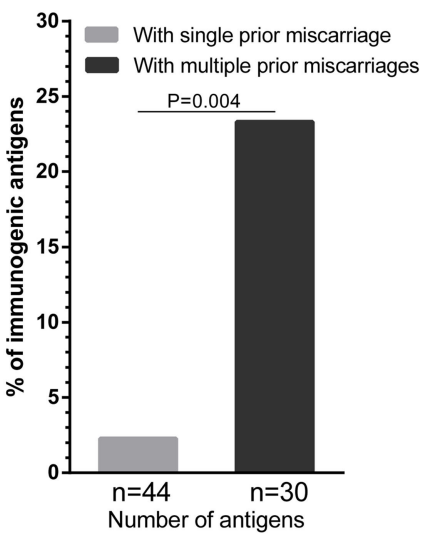

FIGURE 2 | The effect of the number of prior miscarriage on HLA sensitization during a subsequent successful pregnancy. HLA sensitization in women with a single successful pregnancy that was preceded by a single prior miscarriage was compared with HLA sensitization in women with a single successful pregnancy that was preceded by multiple prior miscarriages. The percentage of immunogenic antigens is higher for women with multiple a prior miscarriages (dark gray bar) than for women with a single prior miscarriage (light gray bar). For each group, *n* represents the number of mismatched antigens. The *p* value is derived from Pearson's chi-square test.

antigens in the secundigravidae with a prior miscarriage group was also lower than the percentage of immunogenic antigens in the primigravidae group (i.e., these women had a single successful pregnancy without a history of prior miscarriages) (dotted line in **Figure 1**; 14.6%; primigravidae versus secundigravidae with a prior miscarriage: $p = 0.02$), indicating that the immunization pattern observed in secundigravidae with a prior miscarriage is not similar to the immunization pattern observed in primigravidae.

Next, we investigated the effect of the number of prior miscarriages on HLA sensitization during a subsequent successful pregnancy. We compared the percentage of immunogenic antigens between women with a single successful pregnancy that was preceded by a single prior miscarriage (i.e., secundigravidae with a prior miscarriage) and women with a single successful pregnancy that was preceded by multiple prior miscarriages (**Figure 2**). For women with multiple prior miscarriages, the percentage of immunogenic HLA was higher (23.3%) compared to women with a single prior miscarriage (2.3%) (**Figure 2**; $p = 0.004$), indicating that the number of prior miscarriages may influence HLA sensitization during a subsequent successful pregnancy.

We previously showed that the probability of HLA antibody formation increases with the number of PIRCHE-II in successful pregnancies without a prior miscarriage (15). Thus, we showed that in these pregnancies, including secundigravidae without a prior miscarriage, a higher number of PIRCHE-II was related to a higher percentage of immunogenic antigens. Therefore, one could hypothesize that the single immunogenic HLA-C*01:02 in the secundigravidae with prior miscarriage group has a higher number of PIRCHE-II compared to the other non-immunogenic HLA. To investigate this aspect in the secundigravidae with a prior miscarriage group, the PIRCHE-II numbers for the mismatched antigens were divided into quintiles (i.e., five equal groups) (**Figure 3**). For each of these quintiles, we plotted the percentage of immunogenic antigens, and we investigated in which quintile the single immunogenic HLA-C*01:02 of secundigravidae with a prior miscarriage was present. The single immunogenic HLA-C*01:02 is not an outlier, as it was present in the central quintile (12–16 PIRCHE-II). This observation indicates that the lower percentage of immunogenic HLA in the secundigravidae with a prior miscarriage group cannot be explained by having an increased or a reduced number of PIRCHE-II compared to non-immunogenic HLA.

The Lower Immunogenicity in Secundigravidae with a Prior Miscarriage Is Likely Not Due to Lower Numbers of Immunogenic B Cell and T-Helper Cell Epitopes

The ability to develop HLA antibodies against child-specific HLA mismatches is determined by allo-epitopes that are present on mismatched HLA. The HLAMatchmaker algorithm identifies

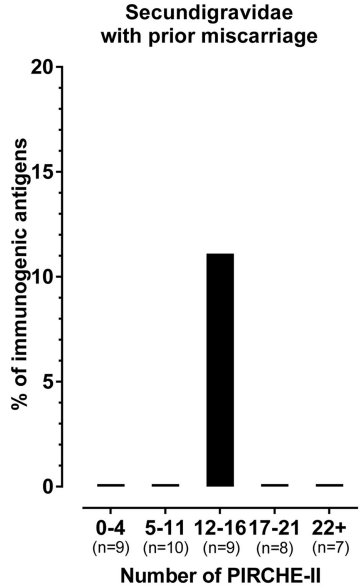

FIGURE 3 | The single immunogenic HLA in the secundigravidae with a prior miscarriage group has a median number of PIRCHE-II. The number of PIRCHE-II was divided into quintiles (0–4 PIRCHE-II, 5–11 PIRCHE-II, 12–16 PIRCHE-II, 17–21 PIRCHE-II, and 22+ PIRCHE-II). For each individual quintile, the percentage of immunogenic antigens was plotted. All HLA mismatches present in the 0–4 PIRCHE-II, 5–11 PIRCHE-II, 17–21 PIRCHE-II, and the 22+ PIRCHE-II quintiles were non-immunogenic, resulting in 0% immunogenic antigens. The single immunogenic HLA-C*01:02 is present in the 12–16 PIRCHE-II quintile, whereas the other HLA mismatches present in this PIRCHE-II quintile were non-immunogenic, resulting in 11.1% immunogenic antigens. For each group, *n* represents the number of mismatched antigens.

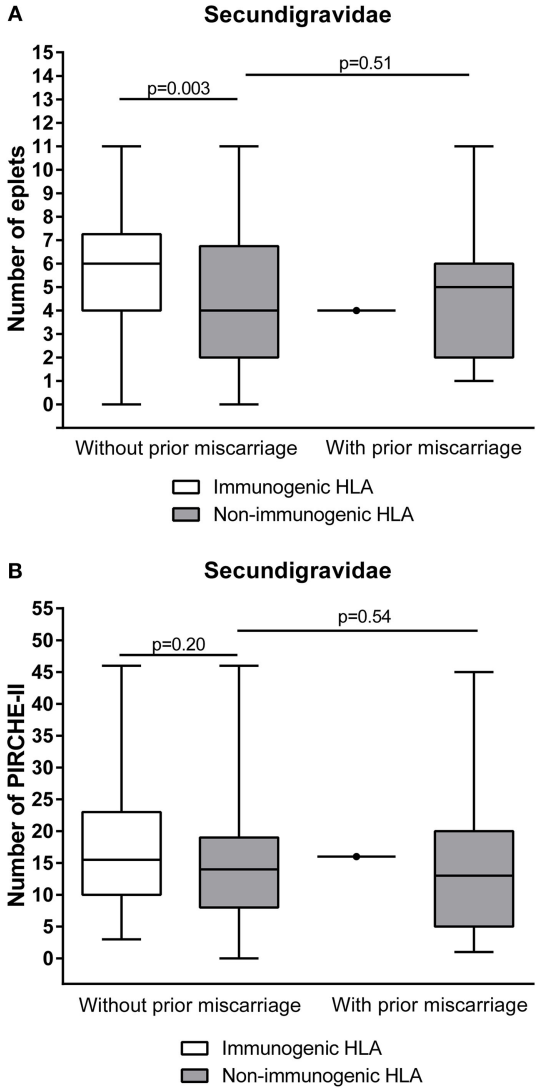

FIGURE 4 | Comparison of the number of immunogenic factors between secundigravidae without a prior miscarriage and secundigravidae with a prior miscarriage. Non-immunogenic HLA of secundigravidae with a prior miscarriage contains a similar number of mismatched eplets **(A)** and PIRCHE-II **(B)** compared to non-immunogenic HLA of secundigravidae without a prior miscarriage. For the secundigravidae with a prior miscarriage group, the single immunogenic HLA is depicted as a dot. The reported *p*-values are derived from Mann–Whitney *U* tests. The boxes extend from the 25th to 75th percentiles, and the middle line represents the median. The whiskers are drawn from the lowest to the highest PIRCHE-II value.

the number of antibody-accessible allo-epitopes (eplets) on mismatched HLA that are not present on self-HLA. To investigate whether the lower immunogenicity in secundigravidae with a prior miscarriage is due to a lower number of immunogenic B-cell epitopes in this population, we calculated the number of mismatched eplets for secundigravidae with a prior miscarriage and for secundigravidae without a prior miscarriage (**Figure 4A**). Since only a single HLA of the secundigravidae with a prior miscarriage is immunogenic, analyses were performed on the non-immunogenic HLA groups of both populations. The number of eplets did not differ between non-immunogenic HLA of secundigravidae with a prior miscarriage and non-immunogenic HLA of secundigravidae without a prior miscarriage ($p = 0.51$). When analyzing the number of PIRCHE-II (T-helper cell epitopes) in both groups (**Figure 4B**), the number of PIRCHE-II was similar for non-immunogenic HLA of secundigravidae with a prior miscarriage compared to non-immunogenic HLA of secundigravidae without a prior miscarriage ($p = 0.54$). Thus both the eplet and PIRCHE-II numbers are comparable between secundigravidae with a miscarriage and secundigravidae without a miscarriage, indicating that the number of immunogenic factors (i.e., B-cell and T-helper cell epitopes) is not altered in secundigravidae with a prior miscarriage.

DISCUSSION

Maternal immune responses can be formed against IPA of the fetus during pregnancy, leading to IPA-specific antibodies and T cells (5, 7). Despite the clinical relevance of HLA-specific antibodies in transplantation outcome, the clinical relevance of paternal HLA-specific antibodies in pregnancy outcome is currently unclear (21). The present study was initiated to

investigate the effect of a first pregnancy and a first miscarriage on HLA antibody formation during a subsequent first successful pregnancy.

In our cohort of 287 mother–child pairs, we investigated HLA immunization against mismatched IPA of the most recent child in secundigravidae with or without a single prior miscarriage. The percentage of immunogenic HLA was significantly lower in secundigravidae with a prior miscarriage compared to secundigravidae without a prior miscarriage. Several studies have shown that the prevalence of HLA antibodies increases with the number of successful pregnancies (9, 13). Our data show that the relation between increasing gravidity and the prevalence of HLA antibody formation is absent in secundigravidae with a prior miscarriage, indicating that a previous miscarriage behaves differently when compared to a previous successful pregnancy.

Our results suggest that a prior miscarriage has a different immunological impact on a subsequent successful pregnancy than a prior successful pregnancy. The lower immunogenicity observed in secundigravidae with a prior miscarriage cannot be explained by altered numbers of mismatched eplets and PIRCHE-II (**Figure 4**). Alternatively, the lower percentage of immunogenic antigens among secundigravidae with prior miscarriage in our population may result from tolerizing effects of a first short allogeneic interaction during the prior miscarriage. These tolerizing effects may be caused by fetal microchimerism, as the increased occurrence and long-term persistence of fetal microchimerism in the maternal system after or during fetal loss has been described previously (24, 25). Alternatively, the low percentage of immunogenic antigens among secundigravidae with a single prior miscarriage might also be explained by natural selection of a particular HLA genotype during a subsequent pregnancy. The chance of inheriting an alternative paternal haplotype during a subsequent successful pregnancy compared to the previous miscarriage is 50%. However, a previous miscarriage may further stimulate HLA genotype diversity by putting additive selective pressure on a subsequent pregnancy. Either directly or *via* modulating the maternal immune system, the HLA genotype of the miscarried fetus may discriminate against that particular HLA genotype during or shortly after conception (26). If this hypothesis is correct, a previous miscarried fetus facilitates the selection of the HLA genotype of a subsequent child. Such a selection may be achieved *via* a maternal immune response directed against the HLA genotype that is similar to the HLA genotype of the miscarriage itself, resulting in either selective abortion of the fetus or *via* a female alloimmune response against certain HLA genotypes present in seminal fluid, as seminal plasma contains soluble HLA (27) and spermatozoa also express both HLA class-I and class-II (28). However, currently no data are available to support such a natural selection of a particular HLA genotype. To challenge this hypothesis, the HLA typing of the current child should be compared with the HLA typing of the previous miscarried fetus. HLA typing of the miscarried fetus is not available for the current cohort and is in general hard to obtain. Alternatively, inclusion of paternal HLA typing may provide a better insight in this mechanism.

The duration of maternal exposure to allo-epitopes is significantly shorter during a miscarriage compared to a full-term pregnancy. Therefore, one might argue that alloimmunization is negligible in pregnancies that end in a miscarriage and that the alloimmunization pattern of secundigravidae with a prior miscarriage is more comparable to the alloimmunization pattern of primigravidae. In this study, we showed that the percentage of immunogenic antigens for secundigravidae with a prior miscarriage was also lower than the percentage of immunogenic antigens observed for primigravidae (**Figure 1**), demonstrating that the immunization pattern in secundigravidae with a prior miscarriage differs from the immunization pattern that was observed in primigravidae. Thus, despite a shorter duration of maternal allo-exposure during pregnancy loss, the effect of a prior miscarriage on a subsequent pregnancy cannot be neglected in terms of HLA antibody formation.

Although our investigation on the differential effect of a first pregnancy and a first miscarriage on a subsequent successful pregnancy are unprecedented, our observation might be supported by previous reports. For example, Triulzi et al. showed that women with a single pregnancy that ended in a miscarriage had a diminished HLA alloimmunization compared to women with a single pregnancy that ended in a successful delivery (13). Furthermore, Masson et al. reported that the HLA immunization incidence was diminished in women with a single successful pregnancy that was preceded by one or more miscarriages compared to women with a single successful pregnancy that was not preceded by one or more miscarriages (29). However, the latter study did not take the number of prior miscarriages into account. In our population, we observed that women with a single successful pregnancy that was preceded by two or more miscarriages had a higher percentage of immunogenic antigens than women with a single successful pregnancy that was preceded by a single miscarriage (**Figure 2**). This observation indicates that the number of prior miscarriages may have impact on HLA sensitization during a subsequent successful pregnancy.

The probability of HLA antibody formation increases with the number of PIRCHE-II in pregnancies that were not preceded by one or more miscarriages, including secundigravidae without a prior miscarriage (15). In our cohort of secundigravidae with a prior miscarriage, the single immunogenic HLA had a number of PIRCHE-II that was comparable to the other non-immunogenic HLA (**Figure 4**), indicating that the PIRCHE-II effect is absent in pregnancies that were preceded by miscarriages.

Our study has limited details about the miscarried fetus itself, as the paternity, HLA typing, and cause of the miscarriage were not documented. Furthermore, the miscarriages in our cohort were self-reported. Since a majority of the miscarriages are unnoticed (30), it may well be that the number of prior miscarriages is underestimated. Therefore, also in the secundigravidae without prior miscarriage group and in the primigravidae group some women might have previous miscarriages, which may led to underestimation of immunization toward IPA in normal pregnancies. Moreover, serum samples for HLA antibody analysis after the miscarriage are lacking for our cohort. These latter serum samples may answer the question whether the mother had developed HLA antibodies against the miscarried fetus or not and

would provide more insight in the possible mechanisms behind our observations.

In summary, we showed that a previous miscarriage and a previous successful pregnancy have a different impact on HLA antibody formation during a subsequent successful pregnancy. In contrast to successful pregnancies, increasing gravidity is not related to increased child-specific HLA antibody formation in secundigravidae with a prior miscarriage. Further details about the miscarried fetus itself or paternal HLA typing will be required to explain the observed different impact of a previous miscarriage and a previous successful pregnancy on child-specific HLA antibody formation during a subsequent successful pregnancy. These data may help to understand the mechanism of child-specific HLA antibody formation during a successful pregnancy that was preceded by a miscarriage and therefore will have implications in the transplantation field.

AUTHOR CONTRIBUTIONS

All the authors met the authorship criteria as described by *Frontiers in Immunology*. KG, GH, SS, and ES were involved in design of the work and interpretation of the data. HD and IH were involved in acquisition of the data. All the authors were involved in drafting or revising the manuscript and approved the final version. All the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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HLA Mismatching Strategies for Solid Organ Transplantation – A Balancing Act

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HLA matching provides numerous benefits in organ transplantation including better graft function, fewer rejection episodes, longer graft survival, and the possibility of reduced immunosuppression. Mismatches are attended by more frequent rejection episodes that require increased immunosuppression that, in turn, can increase the risk of infection and malignancy. HLA mismatches also incur the risk of sensitization, which can reduce the opportunity and increase waiting time for a subsequent transplant. However, other factors such as donor age, donor type, and immunosuppression protocol, can affect the benefit derived from matching. Furthermore, finding a well-matched donor may not be possible for all patients and usually prolongs waiting time. Strategies to optimize transplantation for patients without a well-matched donor should take into account the immunologic barrier represented by different mismatches: what are the least immunogenic mismatches considering the patient's HLA phenotype; should repeated mismatches be avoided; is the patient sensitized to HLA and, if so, what are the strengths of the patient's antibodies? This information can then be used to define the HLA type of an immunologically optimal donor and the probability of such a donor occurring. A probability that is considered to be too low may require expanding the donor population through paired donation or modifying what is acceptable, which may require employing treatment to overcome immunologic barriers such as increased immunosuppression or desensitization. Thus, transplantation must strike a balance between the risk associated with waiting for the optimal donor and the risk associated with a less than optimal donor.

Keywords: HLA matches, HLA mismatches, immunogenicity, match probability, sensitization, repeated mismatches, donor-specific antibody

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INTRODUCTION

There is overwhelming evidence of the benefits of HLA matching in organ transplantation including better graft function, longer graft and patient survival, and reduced risk of sensitization. However, when a well-matched related donor is not available, the wait for a well-matched unrelated donor can be prolonged, which can reduce quality of life, impede physical and cognitive development in the young, and increase the risk of death. Furthermore, in countries where there is substantial

Abbreviations: CPRA, calculated panel reactive antibody; FCXM, flow cytometric crossmatch; PRA, panel reactive antibody; SPI, solid phase immunoassay; USRDS, United States Renal Data System.

ethnic diversity, allocation of deceased donor organs by HLA match can result in a disparity, among ethnic groups, in access to transplantation. The effects of HLA matching are confounded by many factors that can affect outcome such as sensitization, immunosuppression, recipient ethnicity and age, and donor type and quality. Thus, transplantation is a balancing act between capturing the benefits of a well-matched transplant and diminishing the problems associated with achieving that transplant. Strategies must consider both the benefits and disadvantages of matching, the detrimental effects of mismatching, and what can be done to minimize negative effects of both matching and mismatching.

Here, we will review the impact of HLA matching/mismatching on graft outcomes, other factors that impact the effect of HLA, other consequences of mismatches, and the factors that should be evaluated – HLA antigens, epitopes, and amino acids. We will examine the effect of HLA mismatches on the current transplant and on future transplants as well as HLA matching strategies for the non-sensitized and sensitized patients.

EFFECT OF HLA MATCHING/ MISMATCHING ON OUTCOMES

Assessment of the effects of mismatching has been confounded by variability over time of the ability to determine HLA phenotype accurately; by considering only matched but not mismatched antigens; by evaluating the effect of only some HLA loci; and by the diminished sensitivity and specificity of cell-based tests for HLA antibody. Although numerous early studies reported that increased numbers of matched antigens or decreased numbers of mismatched antigens led to improved graft and patient survival, improved graft function, and fewer rejection episodes, later reports suggested that ongoing improvements in immunosuppression therapies either diminished or eliminated any benefit of matching. However, large studies and more recent reports have reaffirmed the benefits to be derived from matching. Data from the Collaborative Transplant Study showed that with or without cyclosporine use, the renal transplant success rate was 20% higher when there was no mismatch of HLA-B and -DR than when there was a mismatch (1). Similarly, data from the United Network for Organ Sharing showed that long-term graft survival of deceased donor renal transplants with no HLA-A, -B, and -DR mismatch was nearly 20% better than for fully mismatched grafts with a stepwise reduction in survival with each increased degree of mismatch (2). Similar results were observed in a study of more than 150,000 renal transplants in which 10-year graft survival of first deceased donor kidney transplants was 17% higher among the zero HLA-A, -B, and -DR-mismatched patients than among those fully mismatched with an even greater benefit derived in sensitized patients (PRA >50%) (3). When graft survival was examined for deceased donor renal transplants occurring in different eras, it was seen that 5-year graft survival was 11% higher among transplants occurring between 1995 and 2004 compared to those occurring in the 10 years prior (73 vs. 62%) and that the strength of the association with HLA mismatch decreased in the second decade, but was still present. Furthermore, an association between extent of mismatch and treatment for rejection was present in both decades (4). In contrast, Su et al. found a diminishing benefit of HLA matching in deceased donor renal transplants over the period 1995–1998 (5). A single center study showed a dramatic benefit of HLA matching among highly sensitized patients receiving deceased donor kidney transplants (6). One hundred and forty-two patients with CPRA >80%, negative flow cytometric crossmatches (FCXM) with donor T and B lymphocytes, and no detectable donor-specific antibody tested by ELISA, were grouped according to mismatch. For patients with 0–2, 3–4, or 5–6 HLA-A, -B, and -DR mismatches, the incidence of rejection was 4.4, 11.4, and 31.3% and 5-year graft survival was 100, 81, and 74%, respectively. This study found a strong effect of HLA-A, but not -B or -DR mismatch on graft loss. Others have found that mismatches for class I and class II had independent effects on patient survival where 0-DR/2-4AB and 0-1AB/1-2DR mismatches had 10-year patient survival of 86 and 89%, respectively, compared to only 74% for 1-4 AB/1-2DR mismatches. The best survival of 92% was with mismatches limited to 1 A or B antigen. Freedom of graft failure due to immunologic causes was 96.5% for mismatches limited to 1 A or B antigen and no DR mismatch and was 89–91% for all other mismatch groups (7).

Although there had been reports of the role of HLA-A and/or -B mismatch, it eventually became apparent that of the HLA antigens tested routinely, HLA-DR matching contributed the most to graft survival and function. This is of particular importance since there are fewer antigens encoded by the DRB1 locus than by either the A or B loci making it easier to find zero DR mismatches compared to zero A or B mismatches, particularly when dealing with a very HLA heterogeneous population as in the United States. Connolly et al. (8) showed that among 516 primary deceased donor kidney recipients, zero DR-mismatched transplants had significantly better survival than those with even a single DR mismatch at both 1 year (92.8 vs. 84.5%) and 5 years (88.3 vs.73.9%) ($P < 0.0001$). The effect was independent of HLA-A or -B match but diminished if cold ischemia time was more than 26 h. Al-Otaibi et al. (9) found that pediatric renal patients who received fully DR-matched grafts had significantly better graft survival than did those receiving grafts with one or two DR mismatches. However, in this study, there were more living donors in the well-matched group, which most likely contributed to the outcomes. It is not clear why matching for DR would be more important than matching for HLA-A or -B. Perhaps, DR antigens are more immunogenic than are A or B antigens. Perhaps, there is a gene dose effect. There is strong linkage disequilibrium within the HLA complex such that mismatching for DR may also increase the likelihood of mismatching for DQ antigens that have not been included in many evaluations of associations between match and outcome. Additionally, mismatching for DRB1 antigens may also include a mismatch for the antigens encoded by the linked DRB3, 4, and 5 loci that encode DR52, 53, and 51, respectively. **Figure 1** shows that most DR haplotypes are fixed such that DR15 or 16 also have the DRB5 gene that encodes DR51. Haplotypes with DR11, 12, 13, 14, 17, or 18 bear the DRB3 gene that encodes DR52 and haplotypes with DR4, 7, or 9 have the DRB4 gene that encodes DR53. (Note that some very rare DR1 haplotypes also have the DRB5 gene. Also, an exception occurs on haplotypes bearing DR7 and DQ9. These haplotypes

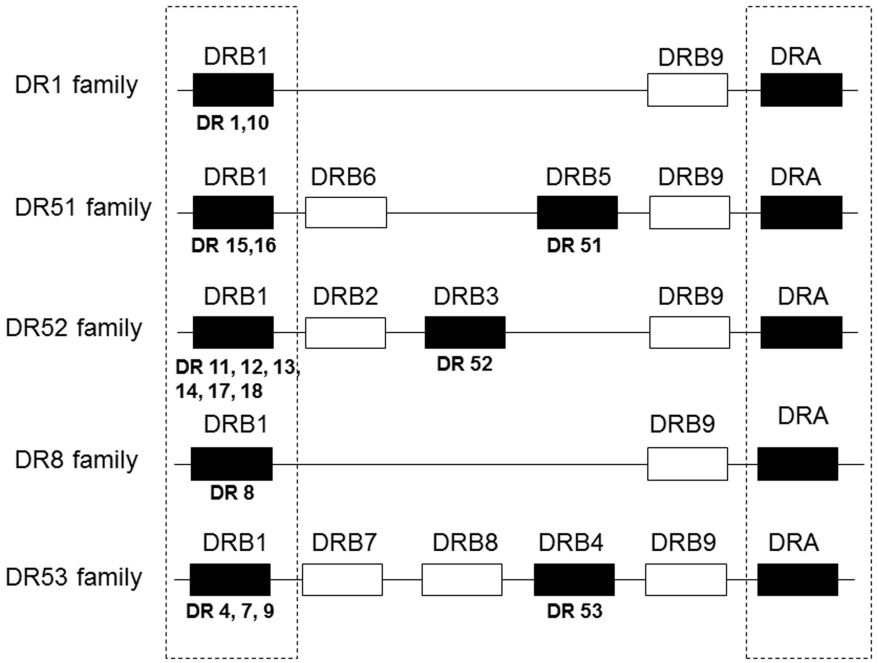

FIGURE 1 | Gene organization of HLA-DR haplotypes. DR haplotypes have varying numbers of genes, some of which encode a polypeptide chain (filled boxes) and others are pseudogenes that have no detectable product (open boxes). DR molecules are comprised of two polypeptide chains, an α chain and a β chain. All DR haplotypes have a DRA gene that encodes the relatively invariant α chain and a DRB1 gene that encodes the β chain of the DR1-DR18 antigens. Some haplotypes carry an additional gene, DRB3, 4, or 5, that encodes the β chain of the DR52, 53, or 51 molecules, respectively. DR haplotypes can be grouped into families defined by the number of DRB genes present as shown in the diagram.

have a null allele at the DRB4 locus and do not express DR53.) So that a patient with a DR1, DR4 phenotype, who is mismatched with a DR11, 12, 13, 14, 17, or 18, is also mismatched for DR52. To complicate matters further, DR52 antigens share an epitope with DR11, 12, 13, 14, 17, and 18 (10) and with DR8 (11) so that in addition to two mismatched antigens, there is a double dose of the shared epitope. Indirect evidence to support the possibility of a gene dose effect was reported by Kim et al. (12) who assessed graft survival in patients mismatched for one or more antigens present in either the heterozygous state (one copy of the antigen) or homozygous state (two copies of the mismatched antigen) with the latter being scored as a single mismatch in most studies. They found that zygosity affected both mean and 10-year survival with worse outcomes occurring with two doses of the mismatched gene (**Table 1**). In the past decade, there have been numerous, additional reports correlating improved outcomes with reduced mismatches of HLA-A, -B, and/or -DR antigens (13–19), and there has been little or no evidence to the contrary.

TABLE 1 | Effect of gene dose of mismatched antigen.

Mismatched antigen	Mean graft survival (years)	
	Heterozygous	Homozygous
1 HLA-B	20.1	6.7
1 HLA-DR	16.9	14.7
1 each HLA-A, -B, -DR	15.0	13.0
	10-year graft survival (%)	
1 HLA-B	85	0
1 HLA-DR	87	67
1 each HLA-A, -B, and -DR	84	70

Homozygosity with two copies of a mismatched antigen, compared to heterozygosity with a single copy, was associated with reduced mean graft survival and 10-year graft survival. Adapted from Kim et al. (12).

Nearly, all studies have examined outcomes vis-a-vis matching or mismatching of HLA-A, -B, and -DRB1-encoded antigens. However, at the time of this writing, there are limited data on matching at other HLA loci (HLA-C, -DRB3, -DRB4, -DRB5, -DQA, -DQB, -DPA, and -DPB). Frohn et al. investigated the impact of HLA-C mismatches on rejection in 104 renal transplants (20). They controlled for HLA-B mismatch to eliminate linkage disequilibrium as a confounding factor. They found that patients with one or two mismatches for an HLA-C antigen had a significantly higher incidence of rejection compared to those with no HLA-C mismatch (54 and 100 vs. 0%) but only when there was also one HLA-B mismatch. Patients with one HLA-B and two HLA-C mismatches also had decreased graft survival that approached statistical significance ($P = 0.055$). In an early study of data from the Southeastern Organ Procurement Foundation on 12,050 first deceased donor transplants, no effect of matching for HLA-DQ was found when other factors affecting outcome were taken into account (21). In contrast, in a recent study, Lim et al. found DQ mismatching to incur a significantly increased risk of rejection that was further increased in the presence of DR mismatches (22). Rosenberg et al. (23) found that DP mismatches

in patients matched for DR and DQ did not impact graft survival or function. Similarly, data from the Collaborative Transplant Study found no deleterious effect of DP mismatches in first deceased donor transplants but did find significantly reduced graft survival in regraft patients (24). The widespread adoption of DNA-based typing methods has facilitated typing for HLA-DQ and -DP. However, unlike other HLA molecules that have one polypeptide chain that is invariant or has limited variability, both polypeptide chains, the α chain encoded by DQA and DPA and the β chain encoded by DQB and DPB, are polymorphic. Both chains may have immunogenic epitopes and there are epitopes comprised of particular combinations of α and β chains (25, 26). Consequentially, studies that consider matching only for DQB and DPB may incorrectly identify a mismatch as a match between a donor and recipient.

The benefits of matching have been seen in all or nearly all types of transplants defined by organ type including heart (27–32), lung (33–36), liver (37–40), and pancreas (41, 42). It should be noted, however, that there are numerous reports of a lack of an effect of HLA matching on outcomes in liver transplantation. The production of large amounts of soluble HLA class I molecules and the dual vasculature of the liver may reduce the susceptibility of this organ to immune attack. Regarding pancreas transplantation, most studies of matching have been of simultaneous kidney–pancreas transplants. The two citations here are of pancreas only transplants.

Other Effects of HLA Mismatches

In separate reports from the Collaborative Transplant Study, it was shown that HLA mismatches were associated with death with functioning graft and with posttransplant lymphoproliferative disease. Increasing numbers of HLA mismatch in renal transplantation were associated with an increased need for antirejection therapy that might account for an increased incidence of death with functioning graft due to infection and cardiovascular disease (43). It was also shown that among 9,209 pediatric kidney transplants, HLA-A, -B, and -DR mismatches were a risk factor for 5-year graft survival, but two DR mismatches appeared to incur an increased risk for non-Hodgkins lymphoma (44). There is a differential cost associated with different degrees of HLA match. Schnitzler et al. looked at Medicare payment information in the United States Renal Data System (USRDS) according to the degree of HLA mismatch. At three years, Medicare payments for zero, 1–3, 4–5, and 6 antigen mismatches were $60,400, $64,000, $71,000, and $81,000, respectively (45). Increased sensitization associated with HLA mismatches and HLA matching strategies are topics requiring more extensive discussion and will be discussed below.

OTHER FACTORS RELATED TO MATCHING AND OUTCOMES

Regrafts

Coupel et al. (46) reported on 233 second renal transplants for which repeated mismatches were permitted when no antibody to the mismatch was present. They found that DR mismatch was a major predictor of graft loss with DR mismatched patients having 5- and 10-year graft survival rates of 73 and 54%, respectively, compared to 82 and 69% for zero DR mismatches. Others have investigated if there was a risk associated with repeated mismatches in regrafts. Cabacungan (47) found no effect of a repeated class I (HLA-A, -B) mismatch, but saw a 5–8% decrease in 1-year graft survival when there was a repeated DR mismatch. This is in contrast to a report of transplants occurring in Southeastern Organ Procurement Foundation member centers, which found a lack of risk associated with repeated mismatch occurring in 158 of 753 regrafts (48). Doxiadis et al. (49) found that repeated DR mismatches, but not repeated A or B mismatches, significantly reduced 1-, 3-, and 5-year graft survival and that the effect was magnified when the survival of the first graft was less than 6 months. Tinckam et al. (50) looked at the effect of a repeated mismatch present in 3,868 of 13,789 regrafts listed in the USRDS. They found that repeated class I or class II mismatches were a risk for graft loss in patients who underwent transplant nephrectomy of the first transplant prior to receiving the second transplant and that the effect was stronger with class II mismatches. Repeated class II mismatches were also a risk factor in sensitized patients. They postulated that sensitization may be a marker for a more aggressive responder type or an indication of undetected low-level antibody to the repeated mismatch. Risk of graft loss was limited to those two subgroups of patients, sensitized patients, and patients who had undergone nephrectomy of the previous transplant. This report is extremely important because avoiding repeated mismatches unnecessarily reduces a patient's chance for transplantation and increases waiting time while increasing immunosuppression in the face of a repeated mismatch may be unnecessary and incur an increase in the attendant side effects. Additional studies could determine further the level of risk associated with repeated mismatches, particularly among patients who are neither sensitized nor have had a nephrectomy of a previous transplant.

Recipient Race

Historically, Black recipients were considered to have poorer survival of renal grafts compared to White recipients. However, there are limited data on potentially combined effects of HLA matching and recipient race. Butkus et al. (51) showed that the effect of HLA mismatching in deceased donor transplantation was comparable in Blacks and Whites but that Blacks had, on average, more antigen mismatches. They found that the poorer graft survival among Black patients was attributable to socioeconomic factors, such as the type of insurance coverage and non-compliance. Smith and Butterly (52) saw a disparity between Black and White recipients of living donor transplants at all levels of match but that the disparity was diminishing over time from 1985 to 2000.

Donor Factors

Donor factors, such as age and type, may exacerbate or reduce the effect of HLA mismatches. Using USRDS data for pediatric renal transplants occurring during 1994–2004, Foster et al. (53) examined the effect of HLA mismatch with consideration of donor age and further categorized transplants by donor type

(deceased or living). Donors were grouped by age in 5-year increments up to the age of 50 with donors older than 50 comprising the last group. They found that, among deceased donor transplants, there was a significant difference in graft survival between the best- and worst-matched transplants with each increasing number of mismatches increasing the risk of graft failure by 4% with donors 35 years old or older. Among the deceased donor transplants, young donor age offset the impact of poorer matches while better matches ameliorated the negative impact of older donor age. Among living donor transplants, they found that HLA mismatch, but not donor age, was relevant to graft survival and that 5-year graft survival was better among poorly matched living donor transplants than among well-matched transplants with deceased donors of any age. Similarly, Terasaki et al. (54) reported that 10-year graft survival was better with five to six mismatched antigens when the donor was young than with zero mismatches and donor age greater than 55 years. A study of risk factors among 1,632 living donor renal transplants found that risk factors for patient survival were donor >65 years old and five to six antigen mismatch, while risk factors for graft survival were donor >65 years old and a mismatch of three antigens or more (55).

Immunosuppression

It was believed that the development and use of cyclosporine and lymphocyte depleting agents would diminish or negate the effect of HLA on outcomes, but the impact was limited. However, there is currently a wide array of therapeutic agents used for induction, maintenance immunosuppression, and/or treatment of rejection, and these may have a more substantial impact on the effect of HLA mismatches. In 2001, Meier-Kriesche et al. (56) compared the effects of mycophenolate mofetil (MMF) and azathioprine on matching in 8,459 and 11,216 first renal transplants. They found that there was less graft loss with MMF than with azathioprine, but that there was still a significantly lower rate of graft loss among zero mismatched (3.5%) compared to six antigen-mismatched transplants (11.3%) and that there was an incremental increase in risk of graft loss with each increase in the number of mismatched antigens. Also, they showed that mismatches of one or two antigens with azathioprine treatment had better graft survival than six antigen mismatches treated with MMF. As noted above, Opelz and Döhler (4) examined the impact of HLA in two different decades (1985–1994 and 1995–2004) and saw that the impact was diminished in the second decade, but was still strong. Martins et al. (57) also saw a diminished impact of HLA matching with triple therapy of MMF, antithymocyte globulin, and tacrolimus. However, from the more current references cited above, it is apparent that HLA matching still provides the benefits of longer graft survival and better graft function.

Opportunity for Transplantation and Allocation of Deceased Donor Organs

Although transplanting well-matched organs is highly desirable, the high degree of polymorphism in the HLA system results in a low likelihood of finding a well-matched, unrelated donor even when only HLA-A, -B, and -DR are considered (58). This is particularly true where there is great HLA heterogeneity, as in the United States. This is born out by the distribution of zero-mismatched deceased donor renal transplants in the United States during the period 1998–2001. The percentage of each group receiving a zero A, B, and DR mismatch was 21.4% of Whites, 7% of Blacks, 14.3% of Hispanics, and 6.6% of Asians (59). Considering only partial matching such as for HLA-DR, Vu et al. (60) determined that the average probability of finding a zero DR mismatch among local donors was 5% and this value was reduced to 2% when ABO compatibility was considered. Of course, the probability of a well-matched donor among first degree relatives is appreciably higher; however, this opportunity is not available to the vast majority of patients who need a kidney transplant. The desire for a well-matched donor should be balanced with a reasonable probability of finding such a donor. Algorithms for calculating the frequency of a donor who is a zero mismatch at a single locus or at any two or more loci are shown below (61). It is necessary to have allele and haplotype frequencies to perform the calculations, which can be found at various web sites. For frequencies for deceased donors where the donor population is comprised of different ethnic groups, the calculations should be done for each group that is a substantial proportion of the population. The values for each group should be weighted according to their proportion in the donor population and the weighted values should be summed to derive the probability of a donor in the total population.

Frequency of a Donor Who Is a Zero Mismatch at a Single Locus

This is a relatively easy calculation but must use allele, not antigen, frequencies. If the patient is homozygous for an antigen then, if i represents the frequency of the patient's allele for the homozygous antigen, the frequency of a donor who is a zero mismatch at that locus is i^2.

If the patient is heterozygous for an antigen and the frequencies of the patient's alleles at that locus are given by i and j, then the frequency of a zero mismatch at one locus is

$$P(\text{zero single locus mm}) = i^2 + 2ij + j^2 = (i+j)^2.$$

Note that there may be several alleles that encode a serologically defined antigen. Then the allele frequency for that antigen would be the sum of all the alleles that encode that antigen and to which the patient does not have antibody.

Frequency of Donor Who Is a Zero Mismatch at Two or More Loci

If the patient is homozygous at all loci under consideration, the probability of a zero mismatch is h^2 where h is the frequency of the haplotype comprised of the loci under consideration.

If the patient is heterozygous at one or more loci then

1. Determine all the haplotypes that can be included in the phenotype of the loci under consideration.

2. Assign a population frequency to each haplotype, represented by *hn*.
3. The phenotype that has no mismatched antigens occurs when any one haplotype is in the homozygous state or with combinations of any of two of the compatible haplotypes.
4. This is given by $h1^2 + h2^2 + h3^2 + \ldots 2h1h2 + 2h1h3 + \ldots$ which reduces to $(h1 + h2 + h3\ldots)^2$

Example: frequency of a zero B, DR mismatch for a patient with the phenotype A1, A2; B8, B44; DR11, DR17. The haplotypes that have no mismatched antigens are B8/DR11, B8/DR17, B44/DR11, and B44/DR17 that will have frequencies represented by *h*1, *h*2, *h*3, and *h*4, respectively. Then,

$$\begin{aligned} P(\text{zero B / DR mismatch}) &= h1^2 + h2^2 + h3^2 + h4^2 + 2h1h2 \\ &\quad + 2h1h3 + 2h1h4 + 2h2h3 \\ &\quad + 2h2h4 + 2h3h4 \\ &= (h1 + h2 + h3 + h4)^2. \end{aligned}$$

The probability can then be used to determine the number of donors needed to achieve a certain probability of finding such a donor. This is determined by

$$P\begin{pmatrix}\text{donor with selected phenotype} \\ \text{among } n \text{ donors}\end{pmatrix} = 1 - (1 - y)^n,$$

where *y* is the probability a donor will have the selected phenotype and *n* is the number of donors,

$$(1 - y)^n = 1 - P\begin{pmatrix}\text{donor with selected phenotype} \\ \text{among } n \text{ donors}\end{pmatrix}$$

$$n \log (1 - y) = \log \left[1 - P\begin{pmatrix}\text{donor with selected} \\ \text{phenotype among } n \text{ donors}\end{pmatrix}\right]$$

$$n = \log \left[1 - P\begin{pmatrix}\text{donor with} \\ \text{selected phenotype} \\ \text{among } n \text{ donors}\end{pmatrix}\right] \Big/ \log(1 - y).$$

For example, when the frequency of donors with the selected phenotype is 0.01, the number of donors, *n*, needed to achieve a 95% probability of such a donor occurring is

$$n = \log\ 0.05 / \log\ 0.99 = 298.$$

When trying only to avoid unacceptable mismatches, the frequency of donors can be obtained from programs such as the UNOS CPRA calculator, which can be found at https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/. The CPRA calculator determines the frequency of donors with unacceptable antigens using allele and haplotype frequencies in the United States donor population. The probability of a donor with no unacceptable antigens among *n* donors is given by

$$P\begin{pmatrix}\text{donor with no unacceptable} \\ \text{antigens among } n \text{ donors}\end{pmatrix} = 1 - \text{CPRA}^n.$$

Following the derivation above,

$$n = \log\left[1 - P\begin{pmatrix}\text{donor with no} \\ \text{unacceptable antigens}\end{pmatrix}\right] \Big/ \log \text{CPRA}.$$

So, for a patient with a CPRA of 0.95, the number of donors needed to have a 95% probability of finding such a donor is

$$n = \log\ 0.05 / \log\ 0.95 = 58.$$

HLA MISMATCHES AND SENSITIZATION

Sensitization to HLA antigens can be provoked by transfusion, pregnancy, or transplantation. Of these, the rate of sensitization and the strength and duration of HLA antibodies is greatest for transplantation where more than 70% of transplantation patients become sensitized compared to approximately 40% of transfused patients and 11–19% of parous females (62). We previously examined the impact of varying degrees of mismatch for HLA-A, -B, -DRB1, -DRB3-5, and DQ, for a possible total of 10 mismatches, among 534 renal transplant patients (63). We found that the rate and extent of sensitization was proportional to the degree of mismatch. There was a substantial increase in extent of sensitization, on average, for patients whose previous transplant involved mismatches of two or more antigens, regardless of the race, gender, or previous sensitization status of the patient. Wait list time is longer for sensitized patients than for non-sensitized patients and this incurs greater costs for dialysis and antibody testing. For patients on the waitlist in 1996 and 1997, we determined that the costs were $297,204, $480,803, and $1,036,078 for patients with PRAs of 0–9, 10–79, and ≥80. These figures are likely to be much higher today with higher dialysis costs and more sensitive antibody tests, even when antibody testing frequency is reduced as a cost-saving measure. Thus, the more mismatches, the greater the risk of sensitization and the higher the cost of a subsequent transplant.

Willicombe et al. (64) looked at the *de novo* development of donor-specific antibody among 505 renal transplant recipients who had no pretransplant donor specific antibody when tested in multianalyte bead assays on the Luminex® platform. They found that 18.2% of patients made donor-specific antibody after transplantation. Of those, 30% were specific only for class I, 45% only for class II, and 25% for both. Interestingly, half were specific only for DQ. The frequency of *de novo* donor-specific antibody among patients matched for 2DR vs. 2DQ antigens was 9.4 and 21%, respectively. In a smaller study, Tagliamacco et al. (65) found an even higher rate of posttransplant antibody following a first transplant in 82 non-sensitized pediatric renal transplant patients. In this study, 29% made donor-specific antibody *de novo*, 83% of which was specific for DQ. Similar to the findings of Lopes, Kosmoliaptsis et al. (66) saw that 67% of previously transplanted patients were sensitized. When examined by class of antigen mismatched, they found that HLA-A mismatches had a greater effect than either HLA-B or -C. For class II antigens, the effect was comparable for DR and DQ but greater than for class I with the resultant antibodies stronger than those for class I. Among patients who

were sensitized prior to the initial transplant, the frequency of sensitization went from 13 to 34% for class I mismatches and from 5 to 22% for class II mismatches. Meier-Kriesch et al. (67) looked at sensitization among nearly 16,000 patients who were relisted after the loss of a first graft. They found that increases in PRA and the odds of being newly sensitized were proportional to the number of previously mismatched HLA-A, -B, and -DR antigens. They saw a strong effect of mismatches of HLA-A and -B with two HLA-A mismatches producing a greater increase in PRA than did two HLA-B mismatches (23 vs. 13%). Among patients with no HLA-A, -B mismatches, only 10% were newly sensitized upon being relisted but that increased to 50% for mismatched patients. Furthermore, they saw a greater increase in sensitization in Blacks compared to Whites (18.3 vs. 13.9%). This has implications for changes in United States deceased donor allocation policies as eliminating points for HLA-B matches has resulted in an increase in the percentage (48.1–52%) of deceased donor transplants going to Blacks with a concurrent increase in the frequency of two HLA-B antigens mismatched (46–72%) (68). In turn, this increased level of mismatch may drastically decrease the opportunity for a subsequent transplant. Evidence for this was presented in a report by Gralla et al. (69) who looked at data for nearly 12,000 pediatric renal transplant patients, 2,704 of whom experienced graft failure and were listed for another transplant. There were 1,847 who were retransplanted. Among patients who were retransplanted, the mean PRA had increased from 6 to 45% while among the 857 who did not receive another transplant, the mean PRA increased from 8 to 76%. The ability to obtain a subsequent transplant was inversely correlated with the number of previous mismatches. Eighty percent of patients whose first graft was mismatched for two or fewer antigens were retransplanted. The percentage dropped to 56% for more than three previous mismatches. In a similar study of 8,433 pediatric patients, Foster et al. (70) also saw a declension in the likelihood of a second transplant with increased numbers of mismatched antigens in a first graft.

The conundrum created by these data is that on the one hand, higher numbers of mismatches not only reduce graft life and function but also increase the risk of sensitization with a resultant decreased opportunity for a future transplant, which may have a greater impact on pediatric patients who will most likely need more than one transplant. On the other hand, the opportunity for finding a well-matched unrelated donor is small for the majority of patients and extended time waiting incurs increased morbidity and mortality. A compromise may be to select donors with mismatches that have a low probability of inducing a humoral response. It has been proposed that antibody response to a transplant correlates with epitope load presented by the donor HLA antigens or the number of amino acid differences in the membrane distal portions of donor and recipient HLA antigens and great interest has been generated in "epitope matching" (71–74). Epitope matching may be the answer to reduce the humoral response to mismatched grafts, however, greater elucidation of the antibody binding and immunogenic properties of proposed epitopes is needed before widespread clinical application is possible. The portion of the HLA molecule seen by the patient's immune system is comprised of its two membrane distal domains. Epitopes may reside on the α helix of one or the other domain or may be formed by interaction of the two α helices. This was demonstrated clearly in exon shuffling experiments (75–77). The properties, such as electrostatic potential and hydrophobicity, of the amino acids that comprise an epitope affect the identity and immunogenicity of the epitope and must also be taken into consideration (78, 79). Improvements in HLA antibody identification have permitted serologic confirmation of several proposed epitopes. However, the sera of most patients contains a mixture of antibodies and it is often difficult to define epitope specificity precisely.

We investigated antibody specificity in 703 patients who developed antibody to donor HLA following the transplantation (80). This was not a study of the frequency of antibody development. The hypothesis was that if all HLA antigens are equally immunogenic, newly developed antibody should be specific for all the mismatched donor antigens. We found that the frequency of antibody response varied both among loci and among the different antigens at each locus (**Table 2**). We also found that the presence of an antigen in the patient's phenotype that was cross-reactive with the mismatched donor antigen reduced the response to that antigen. The strength of the effect of a cross-reactive antigen in the patient's phenotype varied among antigen pairs. An HLA-A1 in a patient diminished the response to an HLA-A3 mismatch by 44%, but an HLA-A11 in the patient diminished the response to HLA-A3 by only 19%. Interestingly, not only was there was a variability in the effect of cross-reactivity but also there was a directionality. For example, an HLA-A2 in the patient reduced the response to HLA-B57 by 83%, but an HLA-B57 in the patient reduced the response to HLA-A2 by only 8%. Antibody response was not affected by the total number of mismatches, the number of mismatched DR antigens, nor the DR phenotype of the patient. These data could be used to identify the donor who would be the least likely to provoke a humoral response when several donors are available. Two groups of patients in particular may derive a greater benefit

TABLE 2 | Frequency of response to mismatched HLA antigens.

Response[a]	HLA-mismatched antigen			
	A	B	DR	DQB
Mean overall	53.2	42.4	52.6	59.0
Range[b]	30.8–76.2	15.0–66.1	40.0–73.0	47.4–90.0
Mean with no cross-reactive antigen in patient	60.2	52.0	61.0	71.3
Mean with cross-reactive antigen in patient	49.7	35.5	43.0	45.5

Frequencies of antibodies, defined by multiplexed bead assays, to mismatched HLA antigens were determined from 703 renal transplant patients who had no detectable donor-specific antibody before transplantation. The impact of patients having antigens in their phenotype that were cross-reactive with the mismatched antigen was also assessed. Adapted from Lucas et al. (80).

[a]*Percentage of mismatched patients who made antibody.*

[b]*The range of antibody response to different antigens within the locus.*

from the consideration of the immunogenicity of donor antigens. Pediatric patients are likely to need more than one transplant in their lifetime and when well-matched donors are not available, it would be possible to use these data to limit the sensitization to the first transplant. Black patients have higher rates of sensitization than do White patients. As of 2013, the percentage of different groups of patients on the United States wait list who are sensitized was 43, 35, and 33% for Blacks, Whites, and Hispanics, respectively (81). We also found a higher antibody response in Black vs. Whites in our study of immunogenicity, although the difference was not statistically significant. The racial disparity in deceased donor transplants in the United States has been attributed to the use of HLA matching in allocation schemes. However, this disparity is also due in part to a higher rate of sensitization among Black patients and to differences in the ABO distribution between the donor population and Black patients (82). Unfortunately, the utility of data on immunogenicity would be limited with deceased donor allocation schemes that are driven primarily by wait time.

HLA-MATCHING STRATEGIES FOR SENSITIZED PATIENTS

Although it may be desirable to avoid all donor-specific antibodies, this will prevent many patients from being transplanted. Several strategies have been developed to deal with sensitization. It is important to correctly identify all the HLA antibodies of a patient, assess the level of risk associated with those antibodies, determine the level of risk that is acceptable, and determine the likelihood of finding a suitable donor, that is a donor who meets the risk specifications.

Solid phase immunoassays (SPI) and, in particular, the multianalyte bead assay performed on the Luminex® platform provide outstanding sensitivity and specificity in HLA antibody detection and characterization. These assays have provided improved detection of antibodies specific for HLA-C, -DQ, -DP, and subtypes of serologic antigens defined by alleles within an antigen group. They are essential to the identification of epitopes and reveal low-level sensitization not detected in cell-based assays. Utilization of SPIs is essential for safe transplantation of the sensitized patient, but accurate interpretation of results requires substantial experience and expertise. The high degree of sensitivity of these assays make them subject to interference from IgM, complement, and immune complexes (83), and the presence of distorted HLA molecules in the assay may lead to incorrect positive or negative results (84). SPIs are semiquantitative and should be used in conjunction with cell based assays (85) and the results correlated with crossmatch test results (86, 87). Tambur et al. (88) have shown that titrating sera in the multianalyte bead assay provides a good indication of antibody strength and is very useful when cell-based assays cannot be performed either because of lack of donor cells or because of the presence of therapeutic cell depleting agents in the serum. One of the most difficult problems is determining a threshold for positivity – that is, knowing when an antibody is really present. Although manufacturers have greatly reduced lot-to-lot variability in sensitivity, the high sensitivity of these assays makes them susceptible to run-to-run and operator variability. Furthermore, there is bead-to-bead variability due to varying amounts of misformed molecules on different beads and greater antigen concentration on beads bearing HLA-C, -DQ, and -DP antigens. We have found that these problems are diminished somewhat with phenotype panels, but these panels are not sufficiently informative for broadly reacting sera. Using cutoffs for positivity that are too low may deprive some patients of safe transplants while cutoffs that are too high can represent an unrecognized risk.

There is a great deal of information about antibodies to HLA-A, -B, and -DR and data about antibodies to other HLA antigens are increasingly available. SPIs have shown that antibodies to DQ are inordinately common following transplantation and their complexity is being increasingly appreciated (25, 26). As noted earlier, because both polypeptide chains of DQ molecules are polymorphic analysis of DQ reactive antibody must take into account both the DQA and DQB alleles. Antibodies to a unique combination of DQA and DQB are most readily recognized when a patient's antibody reacts with a molecule bearing the patient's own DQB but a different DQA and does not react with other DQ molecules bearing the same DQA or when the antibody reacts with only one molecule bearing a particular DQA and DQB but with no other molecules bearing either that DQA or DQB. Less is known about antibodies to HLA-C and DP. As early as 1986, hyperacute rejection of a renal allograft due to antibody to an HLA-C antigen was reported (89). More recently, Bachelet et al. (90) reported on loss of a renal graft they attributed to antibodies to two donor Cw antigens. Although the flow cytometric crossmatch was positive, the mean fluorescence intensity (MFI) values were moderately low (6,931 and 8,920). The patient also had antibodies to donor DP antigens. Ling et al. (91) reported on eight patients with antibody to donor Cw antigens, one of which had a positive FCXM while the crossmatch tests of the other seven were negative. The patients were followed for 3–24 months during which there was no antibody-mediated rejection and no graft loss. While exceptional cases of acute rejection mediated by antibodies to HLA-C may occur, the inherently low expression of these antigens suggests that they may be more involved in chronic rejection (92).

There is complexity with DP antibodies that is the result of cross-reactivity between certain HLA-DR and certain HLA-DP antigens due to shared epitopes (93). Two sequence dimorphisms of DPB1 define the immunodominant serologic epitopes of HLA-DP. Callender et al. (94) showed that while 42% of 650 patients on a renal waiting list had DP antibody, nearly 80% had antibody to the cross-reactive DR antigens. The strengths of most of the antibodies was low with only 3 of the 271 sera yielding a positive cytotoxicity crossmatch. Furthermore, 40% of patients with DP antibody had not been previously transplanted. These data suggest that much of DP reactivity may be cross-reactivity with DR which may account, to some extent, for the reduced graft function and survival associated with DR mismatching. What needs to be determined is the extent to which DP antibody alone is pathogenic. Jolly et al. (95) reported two cases of antibody-mediated rejection and graft failure due to antibody to donor DP. In neither case was there antibody to other donor antigens, nor did the donors have DR antigens cross-reactive with the DP

antibodies, suggesting that the graft failure was attributable to the DP antibodies. Redondo-Pachon et al. (96) observed higher rates of acute rejection and of delayed graft function when donor-specific antibodies included specificity for DP. Goral et al. (97) also reported antibody-mediated rejection in two patients who received kidneys from donors mismatched only for DP and who had flow cytometric positive crossmatches positive with donor B cells and negative with autologous B cells. Collectively, the data indicate that patients should be tested for antibodies to all expressed HLA loci – A, -B, -C, -DRB1, -DRB3-5, -DQA, -DQB, -DPB and most likely -DPA and that both donors and recipients should be typed for these loci.

Economic pressures have forced many transplant programs to reduce the amount of antibody testing performed especially for patients who are likely to wait a long time to transplantation. HLA antibodies can be transient, particularly those produced in response to transfusion or pregnancy. In the absence of a complete screening history, it is possible that sensitization would not be recognized. The amount of risk associated with donor-specific antibody that is present historically but not currently has not been clearly resolved. Some reports indicate that renal transplantation could be performed safely in the face of an historic positive, current negative crossmatch (98, 99). Lyne et al. (100) reported on 47 patients with positive historic, current negative crossmatches. However, only 18 of the 47 crossmatches remained positive after treatment of the serum with dithiothreitol, indicating that 29 of the positive crossmatch results were due to IgM antibody. Overall graft survival rates for patients with apparent IgG antibody were not significantly different from those with apparent IgM antibody. In contrast, Leavey et al. (101) saw increased early acute rejection among patients with historic IgM (42%) or IgG T cell positive crossmatches (57%) compared to patients with historic B cell only positive crossmatches (32%) and the IgG-positive group also had reduced 1-year graft survival (71%) compared to the other two groups (95%). Using a method developed in our laboratory to enumerate HLA-specific B cells by staining B cells with HLA tetramers, we found that patients with an increased level of B cells specific for HLA-A2, -A24, or -B7 who did not have antibody to those specificities at the time of transplant made antibody specific for the HLA antigen for which they had increased level of B cells even if the transplant was not mismatched for those antigens. Patients without increased numbers of HLA-specific B cells did not make the antibody (102). One patient with elevated B cells specific for HLA-B7 was mismatched for the antigen. That patient made IgG antibody to B7 within 48 h of transplantation and experienced severe antibody-mediated rejection. The timing of the antibody appearance indicates an anamnestic response. Donor-specific antibody of the IgG class that appears within the first posttransplant week reflects an anamnestic response that indicates a risk for patients with cryptic sensitization. A possible explanation for the apparently conflicting results cited above is that in some cases, the disappearance of antibody reflects a senescence of the immune response, while in others, it indicates an active suppression. Another possibility is that certain immunosuppression agents abrogate an anamnestic response. We studied the effect of rituximab treatment on posttransplant antibody responses in 26 patients who had elevated HLA-specific B cells, but no antibody specific for the tested antigen at the time of transplant. Of patients treated with rituximab, 0 of 10 made antibody after transplantation while 13 of 16 who were not treated with rituximab did make antibody (103). These data suggest that a positive historic crossmatch or known previous sensitization represents a manageable risk that does not require avoiding those antigens to which a patient was previously sensitized.

Strategies for transplanting sensitized patients include avoiding mismatches to which the patient currently has antibody, overcoming low-level donor-specific antibody with more intense immunosuppression, or eliminating or reducing donor-specific antibody to an acceptable level *via* desensitization applied prior to or at the time of transplantation. It is likely that no one approach is optimal for all patients and that transplanting sensitized patients in a timely and safe manner may require programs to utilize all three strategies, customized to the immune status and medical condition of each patient.

Finding donors to which a sensitized patient does not have antibodies has been greatly enhanced by kidney paired donation programs. These programs are directed toward patients who have a willing, but incompatible living donor. By transporting donor kidneys, recipients and donors can undergo surgery at their home institution. Another approach, the acceptable mismatch program, has significantly increased transplantation rates for patients awaiting a deceased donor transplant. This program was pioneered by Claas and colleagues in the Netherlands in the late 1980s (104). As initially implemented, the strategy was the determination of HLA-A and -B mismatches to which the patient had not formed alloantibodies. Successful implementation in Eurotransplant involved extensive antibody screening for HLA-A and -B specific antibodies coupled with sharing of sera among participating centers for crossmatching of all ABO-compatible donors. Allocation within Eurotransplant for the acceptable mismatch program affords highly sensitized patients the highest priority when a donor becomes available who is compatible with the patient's antibody profile (105). Since its implementation in Eurotransplant, waiting time among highly sensitized patients has been significantly reduced while both short- and long-term graft survival comparable to non-sensitized patients has been achieved (105, 106). Use of current SPIs for definition of HLA-specific antibodies, coupled with a molecularly based algorithm for determination of acceptable antigen mismatches has added to the potential application of acceptable mismatch programs (107, 108). In a cost-benefit analysis among patients on the deceased donor wait list in Australia, an acceptable mismatch allocation model was found to be an equitable approach to improve access for highly sensitized transplant candidates without compromising the benefits to other patients on the wait list (109). Acceptable mismatch programs have the advantages of being lower in cost and non-invasive compared to desensitization protocols; however, a compatible donor may not be found for up to 40% of patients who may have rare HLA phenotypes and/or be very broadly sensitized (105, 110). The degree of HLA heterogeneity among the patient population compared to the donor pool is a factor in the United States with large numbers of Black transplant candidates, as the degree of HLA phenotype

heterogeneity is significantly higher among Blacks than among other ethnicities (58). Therefore, it has been recognized that for successful transplantation of highly sensitized patients, both acceptable mismatch programs and desensitization should be considered (110).

Low-level, donor-specific antibody is associated with an increased frequency of antibody-mediated rejection (111–113) and subclinical rejection (114) and reduced long-term graft survival (115, 116). However, despite efforts to avoid mismatches with a donor to whom a patient has antibodies, there remain patients for whom such a donor cannot be found. Dialysis reduces quality of life, is attended by numerous health issues particularly in young patients, and limits many activities enjoyed by healthy individuals. Ameliorating or delaying the effects of donor-specific antibody can be achieved with various therapeutic agents and procedures such as lymphocyte and plasma cell-depleting agents, plasmapheresis, and intravenous immunoglobulin. It has been clearly demonstrated both in a single center (117) and a multicenter study (118) that desensitization provides a significant survival benefit over patients receiving a compatible deceased donor transplant or patients who remain on a wait list. Eliminating unacceptable antigens or antigens to avoid can be done by raising the threshold for what is unacceptable, without consideration of specificity or the breadth of sensitization. In an Australian kidney paired donation program with a registry of 53 donor–recipient pairs and two altruistic donors, no matches were found using a cutoff of 2,000 MFI for acceptability. When the threshold was raised to 8,000, matches were found for 70% of the patients (119). The threshold for unacceptable antigens could be changed according to the correlation with crossmatch. That is, the threshold could be raised to just below what would yield a positive flow cytometric crossmatch. This may be more difficult to assess for donors to whom a patient has multiple antibodies as the collective strength of the antibodies is difficult to assess from SPIs. One may choose to eliminate unacceptable antigens by specificity or by source of sensitization. For example, one may choose to keep as unacceptable, antigens that were previous transplant mismatches and to which the patient has antibody at a low level. Ferrari et al. (87) have recommended raising unacceptable thresholds only if desensitization treatment is available and if the antigens are not rare in the donor population. Using allele and haplotype frequencies or programs such as the CPRA calculator, one can determine the impact on the likelihood of finding a donor when unacceptable antigens are eliminated (shown above). **Table 3** provides the number of unrelated donors needed for a 95 or 99% probability of finding a donor for different levels of CPRA.

One may eliminate unacceptable antigens by either class I or class II based on the differential expression of these antigens. Muczynski et al. (120) reported that class II antigens were expressed constitutively in the endothelium of renal peritubular and glomerular capillaries. However, McDouall et al. (121) demonstrated that class II was not expressed constitutively on large vessels. Several others have reported that cultured endothelial cells do not express class II constitutively (122–124). It is difficult to know if cultured cells are representative of the *in vivo* situation or if cells obtained *via* biopsy have been provoked to express class II. Our experience with desensitization indicated that patients with persistent DR or DQ antibody at a level below flow cytometric crossmatch had only a slightly increased frequency of antibody-mediated rejection compared to patients with no detectable donor-specific antibody and there was no increased rejection in patients with persistent antibody to DR51, 52, or 53 (125). In fact, 10-year graft survival occurred with one patient who had persistent antibody to donor DR52 at the level of cytotoxicity. Antibody-mediated rejection occurred only when the patient was treated with thyroxine, an agent known to stimulate class II expression. Another patient had graft survival of at least 5 years with a DQ7 antibody that had spiked to a very high titer in the cytotoxicity assay following an anaphylactic reaction. These data and examples suggest that if HLA class II antigens are expressed constitutively on vascular endothelium, it is at low levels.

TABLE 3 | Number of donors needed for different CPRA levels.

CPRA[a]	Number of donors required	
	95% probability of a donor[b]	99% probability of a donor[b]
0.9999	29,956	46,049
0.9990	2,994	4,602
0.9900	298	458
0.9500	58	90
0.9000	22	44
0.8500	18	28
0.8000	13	21
0.7500	10	16

The numbers (n) of donors required for 95 and 99% probability (P) of finding a compatible donor at different CPRA levels are shown. The numbers were derived by the following algorithm: n = log[1 − P(donor with no unacceptable antigens)]/log CPRA.
[a]This is also 1- frequency of donors with no unacceptable antigens (see text).
[b]Number of donors, rounded to nearest whole number, needed to have a 95 or 99% probability of an acceptable donor.

Thus, patients with levels of antibody that are naturally low or have been reduced by desensitization are able to be transplanted with reasonably good graft function and survival. However, these patients should be monitored frequently in the early posttransplant period and periodically for the life of the graft for changes in antibody level. Pro-inflammatory events, such as infection, trauma (such as surgery), an allergic reaction, and blood transfusion, can all stimulate non-specific activation of memory B cells leading to an increase in donor-specific antibody (126, 127). Finally, although not the topic of this review, it is worthwhile to mention that a very reasonable approach to transplanting the sensitized patient is with a donor who is well matched for HLA, but is ABO incompatible. This may be particularly beneficial to the pediatric transplant candidate (128).

SUMMARY

We have reviewed data here that are summarized as follows:

- All HLA mismatches are associated with some degree of risk of reduced graft function and survival and the risk is proportional to the number of mismatched antigens.

- Good HLA matches with unrelated donors are uncommon, and the desire to achieve a good match should be balanced against the risk associated with prolonged time on dialysis.
- Repeated mismatches represent an increased risk only in sensitized patients or in patients who underwent nephrectomy of a previous graft.
- There are other deleterious effects of mismatching, one of the most serious being sensitization, which is most problematic for patients who will need another transplant.
- Balancing risk of sensitization and wait time may be achieved by favoring less immunogenic mismatches.
- Matching strategies for sensitized patients may be to avoid donor antigens to which a patient has antibody or to reduce antibody strength to an acceptable level and/or utilize more intense immunosuppression.
- Matching strategies should be customized to both the patient and to the transplant program's resources.

AUTHOR CONTRIBUTIONS

AZ and ML together developed the outline for this paper, reviewed all relevant literature, wrote the paper, and prepared the figure and all tables.

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Reflections on HLA Epitope-Based Matching for Transplantation

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HLA antibodies are primary causes of transplant rejection; they recognize epitopes that can be structurally defined by eplets. There are many reviews about HLA epitope-based matching in transplantation. This article describes some personal reflections about epitopes including a historical perspective of HLA typing at the antigen and allele levels, the repertoires of antibody-verified HLA epitopes, the use of HLAMatchmaker in determining the specificities of antibodies tested in different assays, and, finally, possible strategies to control HLA antibody responses.

Keywords: HLA, epitope, eplet, antibody, mismatch acceptability

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INTRODUCTION

It is now universally accepted that HLA matching affects transplant outcome and that HLA antibodies are primary causes of transplant rejection. HLA antibodies recognize epitopes, which can be structurally defined by eplets, i.e., small configurations of polymorphic amino acid configurations on the HLA molecular surface. This article addresses the concept that HLA matching can be determined at the epitope level. It is not intended as an extensive review of the literature; the www.HLAMatchmaker.net website has numerous epitope-related publications, and there are several review articles (1–4). Rather, this paper offers some recent reflections about the role of HLA epitopes in histocompatibility.

EPITOPES AND A HISTORICAL PERSPECTIVE OF SEROLOGICAL HLA TYPING

HLA emerged from observations by a few investigators including Rose Payne, Jon van Rood, and Jean Dausset who during the early 1960s studied sera with leukocyte antibodies in patients with non-hemolytic transfusion reactions and in women after pregnancies (5). Most reactivity patterns with leukocyte panels were uninterpretable, until international HLA workshops were organized whereby collaborating laboratories adapted the so-called microdrop complement-dependent lymphocytotoxicity technique developed by Terasaki and McClelland (6). Sera could be grouped into non-overlapping clusters with highly correlated reactivity patterns, and this permitted assignments of specificities such as HLA-A1, A2, B5, and B7. Such clusters served as reference standards for serological HLA typing reagents. Later on, subclusters of sera identified the so-called splits such as A10 was split into A25 and A26, and B16 was split into B38 and B39. Continued workshop efforts led to a set of HLA-class I specificities also called antigens that could be identified serologically with the complement-dependent lymphocytotoxicity technique. Most HLA antigens were defined with the so-called monospecific sera, but many others could be only identified from reactivity patterns of selected sera on typing trays.

Yunis and Amos first proposed the HLA-D locus from cellular assays based on mixed lymphocyte reactivity (7). Specific Dw determinants were later identified with HLA-D homozygous typing

cells and primed alloreactive lymphocytes. Certain sera had antibodies with blocking effects on lymphocyte reactivity and with complement-dependent cytotoxicity assays using B-cells, and it was possible to identify serum clusters specific for serologically defined Dw related or DR antigens now referred to as DR1, DR2, DR3, etc. (8). Subclusters of sera also identified "splits" such as DR11 and DR12 of DR5.

HLA workshop studies during the 1980s identified clusters of sera specific for the DRB3/4/5-encoded antigens DR51, DR52, and DR53 and the DQB antigens DQ1, DQ2, DQ3, and DQ4 and again subclusters of sera demonstrated "splits" such as the DQ5 and DQ6 splits of DQ1.

As noted above, serological typing was primarily based on reactivities of specific antisera with antigens. Since it is now recognized that HLA antibodies are specific for epitopes rather than antigens, it seems obvious that HLA-typing sera must recognize distinct epitopes uniquely present on serologically defined antigens. The HLAMatchmaker analysis has shown that many HLA antigens detected by the so-called monospecific or duospecific sera have unique eplets, and almost all of them are recorded in the International HLA Epitope Registry (http://www.epregistry.com.br) as experimentally verified with informative antibodies (**Table 1**). In other words, anti-A1 antibodies actually recognize the 163RG eplet, which is only found on A1, anti-B7 antibodies are specific for 177DK uniquely present on B7, and anti-Cw1 antibodies recognize an epitope defined by 6K. Several serological splits can be explained by eplets paired with other residue configurations. For instance, A10 corresponds to 149TAH, whereas the A25 and A26 splits represent 149TAH + 80I and 149TAH + 80N, respectively. Similarly, the B38 and B39 splits of B16 are defined by antibodies specific for epitopes defined by the 158T + 80I and 158T + 80N pairs, respectively. Also, **Table 1** illustrates that most serologically defined DR and DQ antigens have uniquely distinct eplets. The cellularly defined DP specificities [originally called SB (9)] do not have unique eplets, and this explains why they cannot be readily determined serologically with monospecific sera.

The information in **Table 1** is based on molecular structure and amino acid sequence information of HLA antigens which did not emerge until after the late 1980s. Before that, the specificities of serum clusters in the early workshops could only be designated with an arbitrary notation system of serologically defined antigens, although we can now readily see that they reflect the recognition of distinct epitopes. Accordingly, the HLA antigen-matching effect on transplant outcome can be reinterpreted as actually demonstrating the influence of matching for epitopes, albeit limited numbers were considered in these association analyses.

EPITOPES AND SEROLOGICALLY CROSS-REACTING GROUPS

Many sera with HLA antibodies exhibit complex reactivity patterns that prohibit their use in serological typing. Early studies identified sera that reacted with the so-called cross-reacting groups (CREGs) of HLA antigens; the A2-CREG and B7-CREG are common examples (10, 11). Each CREG has the so-called public determinants shared between certain

TABLE 1 | Specificities of commonly used serological typing reagents and their corresponding eplets.

Serological specificity	Corresponding eplet	Serological specificity	Corresponding eplet	Serological specificity	Corresponding eplet
Al	163RG	B18	30G	DR7 + 9	$78V_2$
A1 + A36	$44KM_2$	B18 + B35	44RT	DR8	25YRF
A2	66RKH	B27	71KA	DR8 + 12	16Y
A2 + A28	142MT	B40	44RK	DR9	13FEY
A2 + A69	107W	B44	199V	DR10	$40YD_2$
A3	161D	B48	245TA	DR11	57DE
A9	66GKH	Bw4	82LR	DR12	37L
A10	149TAH	Bw6	80ERN	DR13	71DEA
A11	151AHA	Cwl	6K	DR14	57AA
A25 + A32	76ESI	Cw2	211T	DR15	71A
A29	62LQ	Cw3	173K	DR17	26TYD
A30	152RW	Cw4	17WR	DR51	$96EN_3$
A30 + A31	56R	Cw5 + 8	138K	DR52	98Q
A31 + A33	73ID	Cw7	193PL	DR53	$48YQ_6$
A68	245VA	DR1	$12LKF_2$	DQ1	$52PQ_2$
B5 + B35	193PV	DR1 + 10	13FEL	DQ2	$45GE_3$
B7	177DK	DR1 + 51	96EV	DQ3	55PP
B8 + Cw7	9D	DR2	$142M_2$	DQ4	$56L_2$
B12	167ES	DR3	74R	DQ5	$74SR_3$
B13	144QL	DR4	$96Y_2$	DQ6	125G
B15	163LW	DR3 + 6	31YYFH	DQ7	45EV
B16	158T	DR7	$25Q_3$	DQ8	56PPA
B17	71SA			DQ7 + 9	56PPD

Eplet descriptions are according to HLAMatchmaker (www.epitopes.net). The number represents the sequence location of one of the polymorphic residues annotated with standard single letters. Some eplets have subscripted numbers indicating additional residue configurations in other locations.

groups of antigens and so-called private determinants limited to a given serologically defined antigen within the CREG (12). Many public determinants correspond to structurally defined epitopes. As an example, the A2-CREG, which includes A2, A23, A24, A68, A69, and B17, has several epitopes corresponding to public determinants including the 127K eplet on A2 + A23 + A24 + A68 + A69, 144TKH on A2 + A68 + A69, 107W on A2 + A69, and 62GE on A2 + B17, and all of them have been antibody verified. It has become evident that all HLA class I antigens have public determinants or shared epitopes that can be recognized by antibodies.

Although CREG matching has been applied to the identification of compatible platelet donors for refractory alloimmunized thrombocytopenic patients (13), no beneficial effect could be convincingly demonstrated on transplant outcome largely because these studies did not consider individual public epitopes. Most highly sensitized patients have antibodies against public epitopes that can now be defined structurally.

EPITOPE-BASED MATCHING REQUIRES DNA-BASED HLA TYPING AT THE ALLELE LEVEL

Serological HLA typing had always accuracy problems because of the lack of reagents and technical limitations. After elucidation of the HLA molecular structure and nucleic acid sequencing of HLA antigens during the late 1980s, the application of DNA-based technologies permitted accurate HLA antigen typing results. Very soon, many antigens were identified with amino acid differences, and this led to assignments of alleles to be annotated with a colon (:) followed by two and later on three digits after more than 100 alleles corresponding to the 2-digit antigen had been identified (Examples are A*02:01 and A*02:101). Certain amino acid differences affect the expression of eplets. For instance, A*24:02 has the antibody-verified 166DG eplet shared with A*01:01, A*23:01, A*80:01, and B*15:12, whereas A*24:03 has 166EW shared with A*02:01, A*03:01, etc. This example illustrates the difficulty of matching at the antigen level, in this case A24 if the patient has antibodies against 166DG or 166EW. It is now recognized that HLA compatibility is better determined at the allele than at the antigen level (14).

There are thousands of HLA alleles and the question arises which ones should be typed for in the clinical transplant setting. One might focus on alleles present in the patient and donor population of a given transplant program. Although rare alleles might be excluded it is now apparent that because of the increasing racial and ethnic heterogeneity of most populations such alleles occur more frequently as mismatches. Tissue typing techniques are moving forward very fast in the clinical setting, and each HLA allele has precise information about its eplet repertoire. The degree of eplet mismatching of a donor allele depends on the HLA phenotype of the recipient, which has its own repertoire of self-eplets. Such determinations can be readily made with specifically designed computer programs such as HLAMatchmaker.

ANTIBODY REACTIVITY WITH HLA EPITOPES

Epitope-based HLA compatibility determination requires a basic understanding of how antibodies interact with epitopes. Antibody reactivity testing with HLA panels can be done with different techniques from immunoglobulin and complement component C1q binding to isolated HLA molecules attached to microbeads to flow cytometric binding on lymphocytes and complement-dependent lymphocytotoxicity. These methods may give different results which make clinical relevance assessments difficult. Epitope specificity analyses of antibodies may clarify some of these issues.

Antibody binding to protein epitopes occurs through six complementarity-determining region (CDR) loops, three of them H1, H2, and H3 are on the immunoglobulin heavy chain and L1, L2, and L3 are on the light chain. Each loop interacts with a small set of amino acid residues in the so-called structural epitope and CDR-H3 which binds to the so-called functional epitope (or hot spot) in a central location has a dominant role in determining antibody specificity. Eplets are considered to be equivalent to functional epitopes. **Figure 1** shows models of eplets in three different locations on class I molecules and in context with corresponding structural epitopes and hypothetical contact sites for the CDRs of antibody. Two eplets are readily antibody accessible on the upper domains (**Figures 1A,B**), but antibodies to eplets on the membrane-proximal domain (**Figure 1C**) might interact with only solubilized but not with lymphocyte membrane-bound HLA molecules because these epitopes may not be readily antibody accessible. Would such epitopes be clinically significant?

Antibodies through their CDR-H3 recognize specific eplets centrally located in the structural epitope. Other eplets in the same sequence location but with different residue compositions are generally non-reactive, but there can be exceptions if the eplet has a high degree of residue homology with the eplet that had induced the antibody. As an example, the 82LR eplet, which describes the well-known Bw4 epitope, has two closely nearby residues 80I and 80T. Many 80I + 82LR-induced antibodies recognize also 80T + 82LR and binding strengths can vary from high to low. This is an example of the so-called Landsteiner type of serological cross-reactivity, whereby different but structurally related epitopes react with the same antibody. It should be noted that certain 80I + 82LR-induced antibodies never react with 80T + 82LR; they can be designated as specific for only 80I. This epitope-based analysis seems helpful in the assessment of the mismatch acceptability of 82LR (or Bw4) when 80T + 82LR-carrying alleles have no or very low reactivity with patient's serum.

As illustrated in **Figure 1**, the structural epitope has other amino acid configurations that interact with the remaining CDRs of antibody. As described in many immunology textbooks, an important consideration is the so-called affinity maturation process during the antibody response, whereby DNA regions corresponding to CDRs undergo mutations which increase antibody affinity with the structural epitope. As an example, let us just consider for **Figure 1A** one CDR-loop L2, which as a result of affinity maturation, has increased binding with a given amino acid

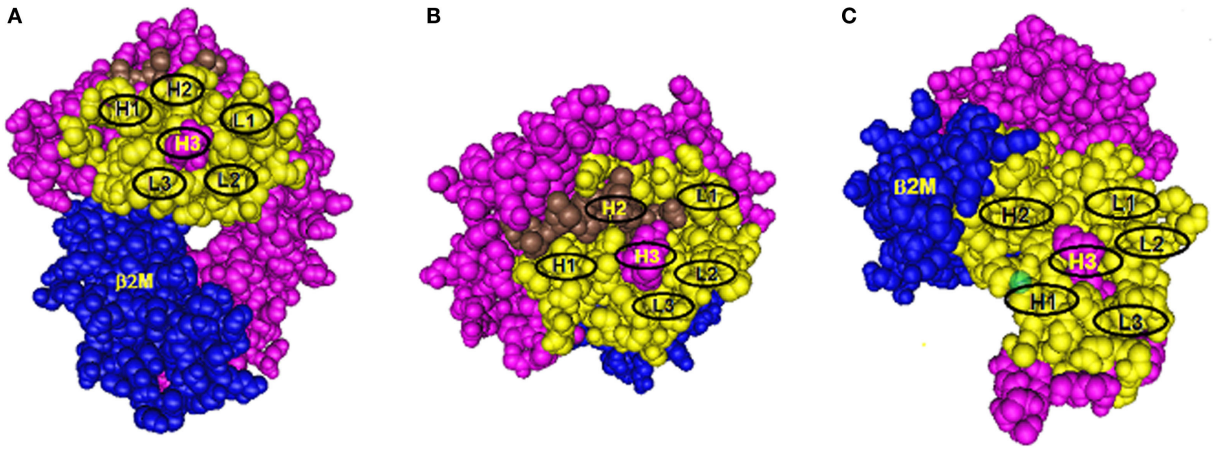

FIGURE 1 | Three models of structural HLA class I epitope. The HLA molecule has three components: HLA chain (pink), β2-microglobulin (blue), and the bound peptide (green). The centrally located eplet (in pink) interacts with CDR-H3. Residues within a 15-Å radius are colored yellow and include configurations (in oval circles) that make contact with other CDRs on heavy chain (H1 and H2) and light chain (L1, L2, and L3). **(A–C)** reflect three different epitope locations on the molecule.

configuration in the structural epitope of the immunizing allele. Two possible configurations can be considered. One comprises only monomorphic residues shared between all alleles in the panel, and the epitope recognized by antibody corresponds solely to an eplet. Second, this configuration has polymorphic residues and only alleles that share such residues with the immunizing allele are antibody reactive. Such residues represent critical contact sites for antibody. Accordingly, these epitopes can be defined by eplets paired with distinct residue configurations (including eplets) in other sequence locations within a 15-Å radius, a presumed dimension of a structural epitope. As an example, there are antibodies specific for 82LR paired with 145RA; this epitope is present on all 82LR-carrying alleles except A25 and B13 that have 82LR + 145RT and 82LR + 145LA, respectively. It should be noted that the critical contact residue configurations are almost uniformly present on at least one allele of the antibody producer, and this suggests an autoimmune component of the antibody response to a mismatched eplet.

As illustrated in **Figure 1B**, some CDRs can make contact with peptides bound to the groove, and it is possible that peptides serve as critical contact sites for a CDR such as H2. Indeed, it has been reported that certain HLA antibodies are peptide dependent (15–17).

For many protein antigen–antibody complexes, there is a certain level of permissiveness for residue substitutions in critical contact areas, and this applies also for HLA epitopes. Certain residue substitutions in the structural epitope have a moderate effect on an allele's reactivity with antibody, whereas others are inhibitory to the level of weak reactivity or non-reactivity of specific eplet-carrying alleles. The latter might be considered as epitope-based acceptable mismatches.

The same rules apply to epitope descriptions of class II alleles which more often solely correspond to eplets. Only few DRB epitopes correspond to eplet pairs, and this might be due to monomorphic residue nature of the DRA chain. Several immunogenic DRB1 eplets are also on alleles encoded by DRB3, DRB4, and/or DRB5. Each of them has distinct antibody-verified eplets, and it seems that epitope matching for DRB3/4/5 affects the class II antibody response.

DQ and DP encode for heterodimers of A and B chains which are both polymorphic and have distinct eplets many of which have been antibody verified. DQ-specific antibodies appear most prevalent among antibodies induced by class II mismatches. Such antibodies are specific for eplets on DQA and DQB chains, and there is emerging evidence that some DQ epitopes are defined by eplet pairs involving both chains (18, 19). This suggests that epitope-based matching should consider the DQ heterodimer rather than the individual chains alone. DP mismatching involves generally fewer epitopes on DPB and especially on DPA; immunogenic eplets, such as 84DEAV and 55DE, are present on large groups of DPB alleles.

REPERTOIRES OF ANTIBODY-VERIFIED HLA EPITOPES

Eplets are small configurations of amino acid residues that play dominant roles in HLA epitopes reactive with antibodies. Such configurations are theoretical considerations based on residue differences in polymorphic sequence locations, but we must raise the question how many of them are actually recognized by specific antibodies. One would expect the clinical relevance of epitope-based matching to apply only to epitopes that have been experimentally verified with informative antibodies. The HLA Epitope Registry has a list of antibody-verified epitopes recorded this far for each locus, but the repertoire is still incomplete. Very recently, the website includes a downloadable PDF file "EpiPedia of HLA" that describes the antibody verifications of HLA epitopes in detail. With the help of participating HLA laboratories that might have interesting serum antibody reactivity patterns, we will continue our analyses to identify new epitopes. The HLAMatchmaker website www.epitopes.net (formerly www.HLAMatchmaker.net) has now a downloadable

Excel document "Five Maps of HLA Epitopia," which describes the sequence locations of antibody-verified eplets and polymorphic residues as potential candidates defining additional epitopes. These maps can be used in navigating the continents of HLA Epitopia while searching for newly antibody-defined epitopes (20).

TECHNIQUE-DEPENDENT EPITOPE SPECIFICITIES OF HLA ANTIBODIES

The use of C1q-binding and complement-dependent lymphocytotoxicity assays has added other dimensions of the complexity of the antibody response to a mismatch. These tests are based on sequential events following the formation of the antibody–epitope complex. C1q binding requires a conformational change in the antibody molecule, thereby exposing the C1q receptor on the Fc part, and complement-dependent cytotoxicity is initiated by the activation of antibody-bound C1qrs complex as the first step of the classical complement pathway. Both processes require free energy released during antibody–epitope complex formation, and the amount depends on the binding strength between all CDRs with the structural epitope. Some antibodies react only with the immunizing epitope in Ig-binding assays; this means that the amount of free energy release is insufficient for the activation of complement-dependent mechanisms that contribute to inflammatory responses (21, 22). A given antibody can react with the immunizing eplet and certain eplet-sharing alleles in the panel in all three assays. Other eplet-carrying alleles react only in Ig-binding assays, because they lack certain critical residues in the corresponding structural epitopes required for strong binding with antibody required for the initiation of the inflammatory process. Are such alleles acceptable or unacceptable mismatches?

PRETRANSPLANT SERUM TESTING FOR EPITOPE SPECIFICITIES OF HLA ANTIBODIES

The above interpretations about antibody reactivity and epitope specificity are more readily made with monospecific sera. However, most sera from sensitized patients have mixtures of antibodies, and although the reactivity patterns are generally limited to a few specificities, there are additional features that can make epitope-based interpretations often quite challenging. They include unexpected reactivities of certain panel alleles due to "natural" antibodies or non-specific blocking factors including a prozone effect; sera may also have competing antibodies with different characteristics including Ig subtypes. Many sera from highly sensitized patients have antibodies reacting with high-frequency (i.e., >80%) epitopes, which make detections of antibodies against lower frequency epitopes more difficult unless these antibodies are separated through absorption–elution studies with selected alleles.

Technique dependencies of serum reactivity may also affect the interpretation of epitope specificities, especially for highly sensitized patients who have several antibody populations in different concentrations and affinities that affect their reactivity with HLA panels. Again, absorption–elution studies with selected alleles might dissect these serum reactivity patterns, so that an epitope analysis can be more readily done.

The HLAMatchmaker website has three downloadable antibody analysis programs in Excel format: HLA-ABC, HLA-DRDQDP, and MICA. The latest 02 versions focus on antibody-verified epitopes recorded so far in the HLA Epitope Registry. All of them correspond to single eplets or eplets paired with other residue configurations uniquely shared by a group of antibody-reactive alleles. The antibody analysis programs also include "other" theoretical eplets, which might become experimentally verified if informative antibodies are identified. The HLAMatchmaker website has a downloadable instruction manual for the epitope analysis of HLA antibodies tested in assays with single alleles.

The 02 versions require entering of the HLA information of the panel, the MFI values of each allele and the allelic HLA type of the patient and preferably the immunizer (e.g., a previous transplant or in case of a pregnancy, the paternal allele of a child). A calculation of the MFI for the self alleles offers a basis for determining a cutoff value be entered, and the program then determines which epitopes are shared between reactive alleles.

In the clinical setting, the primary purpose of the serum HLA antibody analysis of transplant candidates is to identify potential donors whose mismatched HLA alleles are acceptable. This approach is useful not only for organ transplantation but also for platelet transfusions of allosensitized thrombocytopenic patients. Eurotransplant has incorporated HLAMatchmaker in the Acceptable Mismatch Program to identify donors for highly sensitized patients (23, 24).

Epitope specificity analyses might also be useful in desensitization protocols to remove donor-specific antibodies (25). Such protocols are not always uniformly successful but for some patients they may remove some epitope-specific antibodies, thereby opening new windows of opportunity regarding the identification of selected allelic mismatches.

POSTTRANSPLANT MONITORING OF HLA ANTIBODIES

Many posttransplant studies have shown associations between the appearance of donor-specific antibodies with allograft rejection and failure. Most studies have cases whereby the transplant continues to function quite well in the presence of donor-specific antibodies, and this raises the question which antibodies are clinically significant in transplantation. By definition, such antibodies must be absorbed by the allograft where they recognize epitopes expressed on the vascular endothelium and other tissues and initiate inflammatory processes leading to rejection. Accordingly, testing for circulating HLA antibodies in the presence of a transplant has its limitations, and this becomes apparent with the increased serum reactivity often seen after allograft nephrectomy (26–28), and the identification of donor-specific antibodies in eluates from surgically removed transplants (29–33). The question about epitope specificities of clinically important antibodies can be studied in comparative analyses of pre- and post-allograft nephrectomy sera and better yet by analyzing antibody reactivity patterns of allograft eluates.

CONTROL OF ANTIBODY RESPONSES TO HLA EPITOPES

HLA matching at the epitope level also benefits transplant outcome in non-sensitized patients who have no donor-specific HLA antibodies before transplantation. Numerous studies have demonstrated significant correlations between eplet loads of HLA mismatches and the development of donor-specific class I and class II antibodies as well as rejection incidence and allograft outcome (34–48). These findings are clinically useful in two ways. First, for each transplant, the eplet load can be readily determined with special matching programs that can be downloaded from the HLAMatchmaker website. The amount of a mismatched epitope load can be considered as a risk factor during the posttransplant monitoring for antibody-mediated rejection. This information seems also useful in clinical protocols to achieve immunological tolerance (25).

Second, eplet loads can be used to develop new donor selection strategies for non-sensitized recipients especially younger patients. Some transplant programs have already begun to implement this approach (49–51). HLA mismatches with low eplet loads can be expected to improve transplant outcome. Even if the first allograft rejected, retransplant candidates might become less highly sensitized, thereby making it easier to find acceptable mismatches.

The relative immunogenicity of HLA eplets plays an important role in mismatch permissibility of HLA alleles. This can be determined empirically by analyzing the frequencies of antibody responses to mismatched donor eplets which depend on the HLA phenotypes of the recipient and their specificities would be for epitopes defined by eplets or eplet pairs (52). Such studies do not consider the mechanisms of the antibody response which involves the activation of B-cells with epitope-specific Ig-receptors, their subsequent proliferation induced by cytokines from helper T-cells and the differentiation including affinity maturation into antibody-producing plasma cells.

Three recent concepts have begun to address these mechanisms. First, the non-self–self paradigm of HLA epitope immunogenicity is based on the hypothesis that HLA antibodies originate from B-cells with low-avidity, self-HLA epitope-specific Ig-type receptors that can interact with non-self eplets to generate a signal for B-cell activation. Such non-self eplets would be recognized by the specificity-determining CDR-H3, and in the context of the corresponding structural epitope they must be surrounded by self-residues that contact the other CDRs of antibody (53–56). Second, Kosmoliaptsis et al. have proposed that the relative antigenicity of an eplet can be predicted from the physiochemical properties of its amino acid residues (57–59). Accordingly, the electrostatic difference between a non-self eplet and a self-eplet might provide the trigger for B-cell activation (60). Third, activated B-cells need T-cell help for their proliferation and differentiation into antibody-producing plasma cells. The group of Eric Spierings has proposed the so-called PIRCHE-II concept (i.e., Predicted Indirectly ReCognizable HLA Epitopes presented by HLA-DRB1), whereby helper T-cells release cytokines upon recognition of DRB-presented mismatched HLA peptides generated after processing of Ig receptor–allele complexes taken up by activated B-cells (61, 62).

These are reasonable theoretical concepts, although no direct experimental methods are available to analyze the very early phases of the antibody response (63). At present, we can only use indirect approaches, such as serum antibody specificity analysis, molecular assessments of matching, and structural analysis of HLA-antigen–antibody complexes, to study the immunogenicity of HLA epitopes. It is important to know a complete repertoire of antibody-verified epitopes. Such investigations could lead to a better understanding of HLA epitope immunogenicity, and how this can be applied to permissible mismatch strategies.

CONCLUSION

Epitope-based HLA matching has become a new concept in the clinical transplant setting. It relies on HLA typing at the allele level and can be used to identify acceptable mismatches for sensitized patients and to develop permissible mismatch strategies for non-sensitized patients. Our understanding of HLA epitopes is still in progress, and more studies are needed to identify antibody-verified epitopes to be recorded in the HLA Epitope Registry. Also, histocompatibility testing laboratories will have opportunities to sort out complex serum reactivity patterns and interpret technique-dependent epitope specificities of HLA antibodies and their clinical relevance. Sooner or later, there will be new epitope-based HLA-matching approaches with increased benefits to transplant patients.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Donor-Specific Regulatory T Cells Acquired from Tolerant Mice Bearing Cardiac Allograft Promote Mixed Chimerism and Prolong Intestinal Allograft Survival

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The induction of donor-specific transplant tolerance has always been a central problem for small bowel transplantation (SBT), which is thought to be the best therapy for end-stage bowel failure. With the development of new tolerance-inducing strategies, mixed chimerism induced by co-stimulation blockade has become most potent for tolerance of allografts, such as skin, kidney, and heart. However, a lack of clinically available co-stimulation blockers has hindered efficient application in humans. Furthermore, unlike those for other types of solid organ transplantation, strategies to induce robust mixed chimerism for intestinal allografts have not been fully developed. To improve current mixed chimerism induction protocols for future clinical application, we developed a new protocol using donor-specific regulatory T (Treg) cells from mice with heart allograft tolerance, immunosuppressive drugs which could be used clinically and low doses of irradiation. Our results demonstrated that donor-specific Treg cells acquired from tolerant mice after *in vitro* expansion generate stable chimerism and lead to acceptance of intestinal allograft. Increased intragraft Treg cells and clonal deletion contribute to the development of SBT tolerance.

Keywords: donor-specific regulatory T cells, mixed chimerism, transplantation tolerance, small bowel transplantation, bone marrow transplantation

INTRODUCTION

For patients with end-stage bowel failure, small bowel transplantation (SBT) is recognized as a definitive therapy (1). However, the intestine carries the largest population of lymphoid cells of any transplanted solid organ, which are the least tolerogenic cells in any organ and they have the potential risk of inducing graft-versus-host reaction (2). Therefore, both acute and chronic rejection after SBT is still a great challenge to overcome, which leads to the inferior overall outcome of SBT when compared

Abbreviations: (DS) Treg, (donor-specific) regulatory T cells; APC, antigen-presenting cell; BMC, bone marrow cells; BMDCs, bone marrow-derived dendritic cells; CFSE, carboxyflurescein diacetate succinmidyl ester; CTLA-4, cytotoxic T lymphocyte antigen-4; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICOS, inducible co-stimulator; IFN-γ, interferon-γ; IL, interleukin; MLR, mixed lymphocytes reaction; SBT, small bowel transplantation; TNF-α, tumor necrosis factor-α.

to that of other transplanted organs (3). Mixed hematopoietic chimerism, in which both donor and host stem cells contribute to hematopoiesis, could help to achieve potent donor-specific tolerance across full MHC barriers (4). Establishment of mixed chimerism through transplantation of donor bone marrow into recipients is one of the most promising strategies for inducing transplantation tolerance (5). Most of the chimerism-inducing protocols require the use of co-stimulation blockade agents (6). Recent results from experimental mouse studies based on co-stimulation blockade induction of stable mixed chimerism are encouraging. However, translation of tolerance protocols from preclinical animal studies to the clinic is still a major challenge due to the lack of clinically available co-stimulation blockers (6). Regulatory T (Treg) cells have long been recognized to play a critical role in self-tolerance, but administration of Treg cells on their own does not induce robust immune tolerance across MHC barriers in immunocompetent hosts (7). Combining Treg cell therapy with co-stimulation blockade and rapamycin has been tested to promote full MHC-mismatched mixed chimerism, and the results are encouraging (8, 9). Furthermore, recipient donor-specific Treg (DSTreg) cells are thought to be the most potent to promote mixed chimerism among all types of Treg cells (9). However, whether recipient DSTreg cells could lead to intestinal allograft acceptance after establishment of mixed chimerism has not been fully elucidated.

Our previous work (10) demonstrated that allograft acceptance can be established by donor-specific transfusion with complete blockade of inducible co-stimulator (ICOS)/B7h signaling. Furthermore, this allograft acceptance was transferable and maintained by $CD4^+CD25^+$ T cells from recipient mice with long-term allograft survival, and these Treg cells could be expanded *in vitro* and exert donor-specific immune negative regulation. In the present study, a non-myeloablative protocol of combined transfusion of DSTreg cells and donor bone marrow, together with cytotoxic T lymphocyte antigen CTLA4Ig (abatacept, clinically available co-stimulation blocker) and rapamycin, was developed to establish mixed chimerism in lightly irradiated mice. We evaluated the possibility of mixed chimerism to induce murine SBT tolerance and tried to develop new methods for clinical use.

MATERIALS AND METHODS

Animals

Male mice of inbred strains BALB/c (H-2^d), C57BL/6 (B6, H-2^b), and C3H/HeJ (C3H, H-2^k) aged 6–8 weeks were obtained from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). All the animal experiments were carried out following the Guidelines for the Care and Use of Laboratory Animals of the Fourth Military Medical University and were approved by the ethical review committee of the Fourth Military Medical University.

Bone Marrow Preparation and Treatment Regimens

Age-matched male mice received 3 Gy total body irradiation (Day −1), co-stimulation blockade with abatacept (0.5 mg/mouse, Day 2) (Orencia, Bristol-Myers Squibb Pharmaceuticals, Princeton, NJ, USA), and three doses of rapamycin (0.1mg/mouse, Days −1, 0, and 1) (LC Laboratories, Woburn, MA, USA) were injected intravenously on day 0 with 2.0×10^7 unseparated bone marrow cells (BMCs) harvested from MHC-full mismatched BALB/c donors (8–12 weeks old), with or without expanded fresh Treg cells or expanded DSTreg cells (3×10^6 per mouse). The preparation of BM of BALB/c mice was performed as previously described (11).

SBT and Histological Graft Assessment

Heterotopic SBT was performed using a modified technique of Guo et al. (11). Briefly, about 5 cm of ileum was removed from donor mice on a vascular pedicle consisting of the superior mesenteric artery, abdominal aorta, and portal vein. The donor abdominal aorta was anastomosed end-to-side to the recipient infrarenal aorta and the donor portal vein to the recipient inferior vena cava. The proximal and distal ends of the intestinal graft were exteriorized stomas. Intestinal allografts were scored according to the following definitions: 0, no rejection; 1, scattered apoptotic crypt cells; 2, focal crypt destruction; and 3, mucosal ulceration with or without transmural necrosis (11).

Skin Grafting

Full-thickness tail skin from donor (BALB/c) and fully mismatched third-party (C3H) mice was grafted 100 days after SBT and visually inspected at short intervals thereafter. Grafts were considered to be rejected when <10% remained viable (12).

Isolation of $CD4^+CD25^+$ Treg Cells

$CD4^+CD25^+$ Treg cells were isolated as previously described (8). Fresh or DSTreg cells were isolated from spleen of naïve or tolerant B6 mice. $CD4^+CD25^+$ cells were purified by magnetic bead separation using negative selection for $CD4^+$ and subsequent positive selection for $CD25^+$ by incubating $CD4^+$ enriched cells with phycoerythrin (PE)-conjugated α-CD25 (PC61) followed by α-PE microbeads ($CD4^+CD25^+$ Regulatory T-cell Isolation Kit; MiltenyiBiotec, Bergisch Gladbach, Germany) (13). The purity of separated cells was >90%.

Generation of Dendritic Cells

Bone marrow-derived dendritic cells (BMDCs) were induced in the medium of 4 ml complete RPMI 1640, by adding 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). On days 3 and 5, the culture medium was replaced by fresh medium with GM-CSF (20 ng/ml). On day 6, cells were additionally treated with 1 μg/ml lipopolysaccharide for 24 h to further induce the maturation of DC. Loosely adherent cells and those in the culture supernatant were harvested by gentle washing with PBS for further use.

Treg-Cell Proliferation Assay

Sorted $CD4^+CD8^-CD25^+$ T cells (5×10^4) from naïve and tolerant B6 mice bearing cardiac grafts were cultured for 14 days at 37°C in 5% CO_2 with 2×10^5 BALB/c BMDCs in the presence or absence of interleukin (IL)-2 (1000 U/ml) and rapamycin (100 nM), alone

or in combination. In some experiments, T cells were prelabeled with a solution of 5 mM carboxyflurescein diacetate succinmidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA), followed by culture for 7 days with various stimuli. CFSE dilution was analyzed on a Beckman Coulter Epics XL.

$CD4^+CD25^+$ Treg Cell Immunosuppression Assay

Mixed lymphocytes reaction (MLR) was used to assess the suppressive activity of $CD4^+$ $CD25^+$ Treg cells as previously described (14, 15). Briefly, $CD4^+CD8^-CD25^+$ and $CD4^+CD8^-CD25^-$ T cells with ratios ranged from 1:1 to 1:16 were cultured for 72 h in 96-well flat-bottomed plates with anti-CD3 (5 μg/ml) and irradiated splenocytes (APCs). After 3 days in culture, ^{3}H-thymidine was added to each well for an additional 18 h. ^{3}H-thymidine incorporation was measured on a β-scintillation counter (15).

MLR in Mixed Chimeras

MLRs in chimeras were performed as described previously (12, 16). Briefly, 4×10^5 responder splenocytes were incubated in triplicate with 4×10^5 irradiated (30 Gy) stimulator cells of either B6 (recipient), BALB/c (donor), or C3H (third party) origin or with medium only. After 72 h incubation, cells were pulsed with [3H]-thymidine (Amersham Biosciences, Little Chalfont, Bucks, UK) for 18 h. Incorporated radioactivity was measured using scintillation fluid in a β counter. Stimulation indices were calculated in relation to medium controls.

Isolation of Lamina Propria Lymphocytes in the Small Intestine

Isolation of lamina propria cells from the small intestine was performed as previously described (17). The whole transplanted small intestine was cut into pieces 0.5 cm in length and shaken twice at 250 rpm for 30 min at 37°C in Hanks' Balanced Salt Solution (Life Technologies) supplemented with 5% (v/v) fetal bovine serum (CellGro) containing 2 mM EDTA. The remaining intestinal tissues were washed and shaken for 30 min at 37°C in RPMI 1640 plus 5% (v/v) fetal bovine serum and type IV collagenase (1 mg/ml; Sigma). Cell suspensions were enriched by centrifugation at room temperature at 500 *g* for 20 min in 40%/70% Percoll (GE Healthcare) in RPMI 1640. The interface layer cells were used for further analysis.

Flow Cytometry, Monoclonal Antibodies, and Reagents

Peripheral blood was collected, the red cells were lysed, and the remaining cells washed with a whole blood lysis kit (R&D Systems, Minneapolis, MN, USA). Peripheral blood leukocytes were stained with fluorochrome-conjugated anti-CD3, anti-CD11b, anti-GR1, anti-B220, anti-H-2Kb, anti-Vb11, anti-H-2Kd, anti-Vb8.1/8.2, anti-Vb5.1/5.2 (PharMingen, San Diego, CA, USA), anti-CD4, anti-CD8 (Caltag, Burlingame, CA, USA), or immunoglobulin isotype controls (PharMingen, Caltag). Donor chimerism was expressed as a percentage that was calculated using the following formula: ($H\text{-}2K^{d+}$ cells/total gated cells) × 100 (11).

The following monoclonal antibodies (mAbs) were purchased from BD Biosciences PharMingen: anti-mCD4-APC-CY7, anti-mCD45RB-PE, anti-mCD44-PE, anti-mCD62L-PE, anti-mCTLA4-PE, anti-mGITR-PE. Anti-mCD4-FITC, anti-mCD8-PE, anti-mCD4-APC, anti-mFoxp3-PE, and anti-mCD25-PE-CY5 were purchased from eBioscience.

ELISA for Intestinal Inflammatory Cytokines

Small intestine cytokines were measured with a mouse-specific cytokines ELISA kit (eBioscience, San Diego, CA, USA). Tissues were homogenized in ice-cold RIPA buffer [150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, and 1% Triton-X (pH 7.4)], and samples processed for mouse-specific ELISA kits.

Statistical Analysis

Survival data were analyzed using the Kaplan–Meier method with the log-rank test to verify the significance of the difference in survival between the groups. Data are presented as mean ± SD. Student's unpaired *t*-test for comparison of means was used to compare groups. $P < 0.05$ was considered to be of significant difference.

RESULTS

Expansion of Fresh and Donor-Specific $CD4^+CD25^+$ Treg Cells

Previously, we have shown that DSTreg cells from tolerant B6 mice bearing cardiac grafts induce cardiac graft acceptance in irradiated B6 mice (**Figure 1A**) (10). To expand $CD4^+CD25^+$ Treg cells, we first purified single splenocytes from naïve or tolerant B6 mice using a $CD4^+CD25^+$ Regulatory T-cell Isolation Kit (MiltenyiBiotec) (18). The purity of separated cells was confirmed to be >90% (**Figure 1C**). Numerous *in vitro* and *in vivo* studies have suggested the critical role of rapamycin in expanding naturally occurring $CD4^+CD25^+Foxp3^+$ Treg cells that are normally found in the naïve splenic $CD4^+$ T-cell compartment, as well as in maintaining their suppressive function *in vitro* (19–21). We used similar culture conditions with some modification. After 14 days stimulation, we found that DSTreg and fresh Treg cells could be expanded stably *in vitro*, and the combination of rapamycin plus IL-2 resulted in greatest expansion of fresh $CD4^+CD25^+$ Treg cells (23.7 ± 1.8-fold) and DSTreg cells (31.5 ± 2.1-fold) (**Figure 1B**). The purity of the expanded cells was >95% (**Figure 1C**). Furthermore, DSTreg cells proliferated more efficiently than fresh Treg cells in response to IL-2 and rapamycin (**Figure 1B**). When analyzing the expression of surface markers by flow cytometry, DSTreg cells from tolerant mice expressed equal levels of CD62L, CD44, CTLA-4, and GITR as fresh Treg cells derived from naïve mice did (data not shown). *Ex vivo* fresh Treg and DSTreg cells were also assessed for suppression in MLR assays. Expanded DSTreg cells displayed a more powerful inhibitory function than fresh Treg, expanded fresh Treg, and DSTreg cells (**Figure 1D**). We also confirmed that the enhanced suppressive function of expanded DSTreg cells was donor specific (**Figure 1E**). Therefore, we established a method that could expand DSTreg cells *in vitro*.

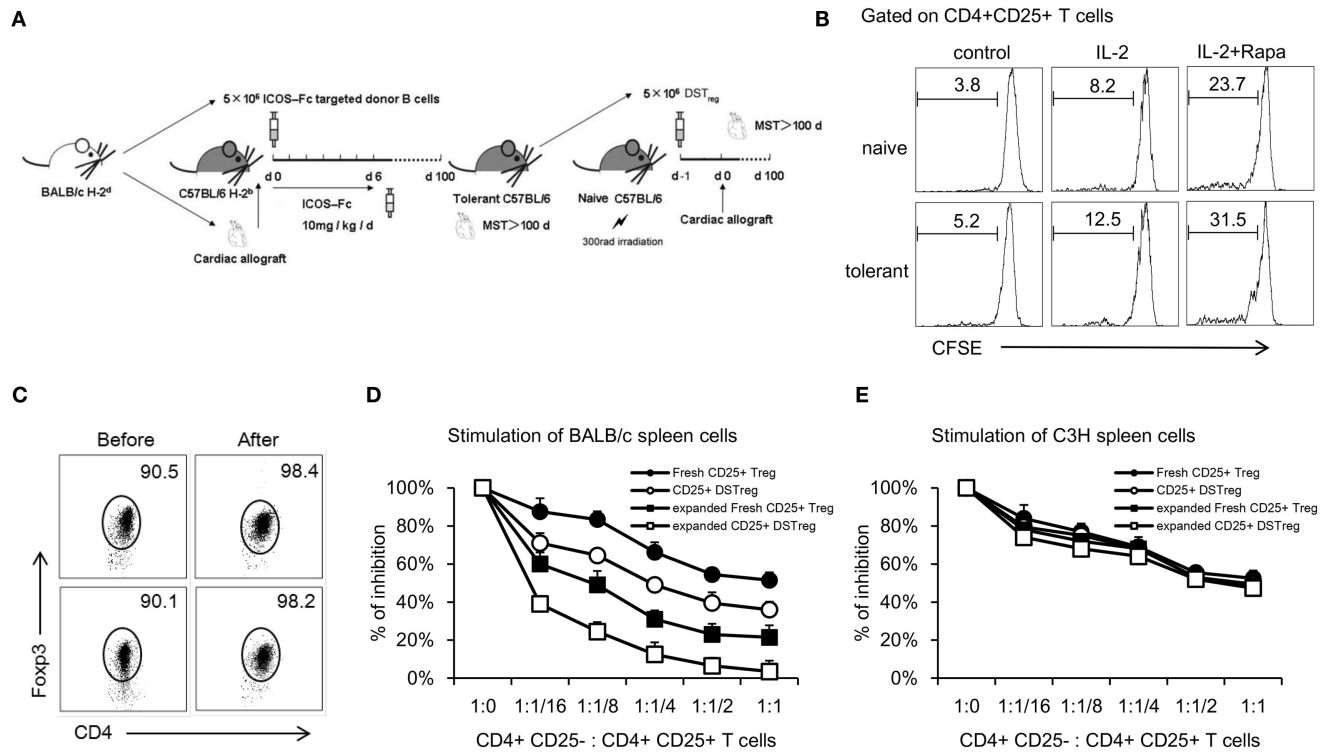

FIGURE 1 | ***In vitro*** **expansion of CD4⁺CD25⁺ Treg cells and comparison with immunosuppressive function of expanded Treg cells**. **(A)** Schematic drawing of the protocol to acquire DSTreg cells from tolerant mice bearing heart allografts. **(B)** Fresh CD4⁺CD25⁺ T cells and DSTreg cells (5×10^4) were separated by magnetic bead sorting and cultivated in the presence or absence of IL-2 (1000 U/ml), splenic donor APCs (10^5 cells), and rapamycin (100 nM), or in combination for 7 days at 37°C in 5% CO_2. DSTreg cells proliferated more efficiently in response to the indicated stimulation compared with fresh CD4⁺CD25⁺ T cells as determined by CFSE-labeled cell proliferation assay *in vitro*. **(C)** Representative FACS blot depicting Foxp3 expression among CD4⁺ T cells from naïve or tolerant mice after *in vitro* cultivation. **(D)** Fresh CD4⁺CD25⁺ T cells, *ex vivo*-expanded fresh CD4⁺CD25⁺ T cells, DSTreg cells from tolerant mice, and *ex vivo*-expanded DSTreg cells were assayed for suppressive activity in response to BALB/c spleen cells and third-party spleen cells (C3H) **(E)**. All curves are expressed as percentage of control MLR and represent the combined average values of five individual experiments. Data are shown as mean ± SD (n = 3–5 mice/group). One representative of at least two independent experiments is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared among the indicated groups.

Donor-Specific Treg Cell Treatment Leads to Multilineage Mixed Chimerism

Next, we investigated the potency of expanded DSTreg and expanded fresh Treg cells for induction of chimerism and tolerance. Under the cover of co-stimulation blockade with abatacept, a low dose of 3 Gy irradiation and three doses of rapamycin, B6 mice received a conventional dose of fully mismatched BALB/c BM (2×10^7 cells per mouse), together with different numbers of expanded DSTreg or fresh Treg cells (10^5, 5×10^5, 10^6, 2×10^6, or 3×10^6 per mouse). Most recipients treated with Treg cells developed mixed chimerism, whereas no chimerism was detected without Treg cell treatment 4 weeks after BMT (**Figure 2A**). BMT recipients treated with expanded DSTreg cells displayed higher chimerism rates than those with expanded fresh Treg cells, and cell numbers could even be reduced to 5×10^5 cells/mouse (**Figure 2A**). Chimerism levels in recipients treated with expanded DSTreg cells were also significantly higher than those in recipients treated with expanded fresh Treg cells (**Figures 2B,C**). The best induction of stable mixed chimerism was achieved with injection of 3×10^6 cells/mouse (**Figure 2C**); therefore, we used this number of cells for subsequent experiments. We also discovered that BMT recipients treated with expanded DSTreg cells achieved substantial levels of T cells, B cells, granulocytes, and macrophages chimerism at indicated time points post-BMT, while the levels of chimerism in recipients treated with expanded fresh Treg cells were relatively low (**Figure 2D**). Therefore, these results suggest that expanded DSTreg cells were more potent than expanded fresh Treg cells in the induction of hematopoietic chimerism.

Clinical Manifestation and Graft Survival Rates

After the establishment of BM chimerism, we further investigated whether mixed chimerism induced by Treg cells could promote intestinal allograft tolerance. SBT was performed 4 weeks after BMT. The intestinal grafts in the allogeneic group were all rejected within 14 days. Intestinal grafts in recipients that received BM cell infusion showed similar survival rates to those in the allogeneic group (**Figure 3A**), while intestinal grafts in the BM cells + expanded fresh Treg cells group survived for an extended period of 50 days after transplantation (**Figure 3A**). Intestinal grafts in the chimera recipients induced

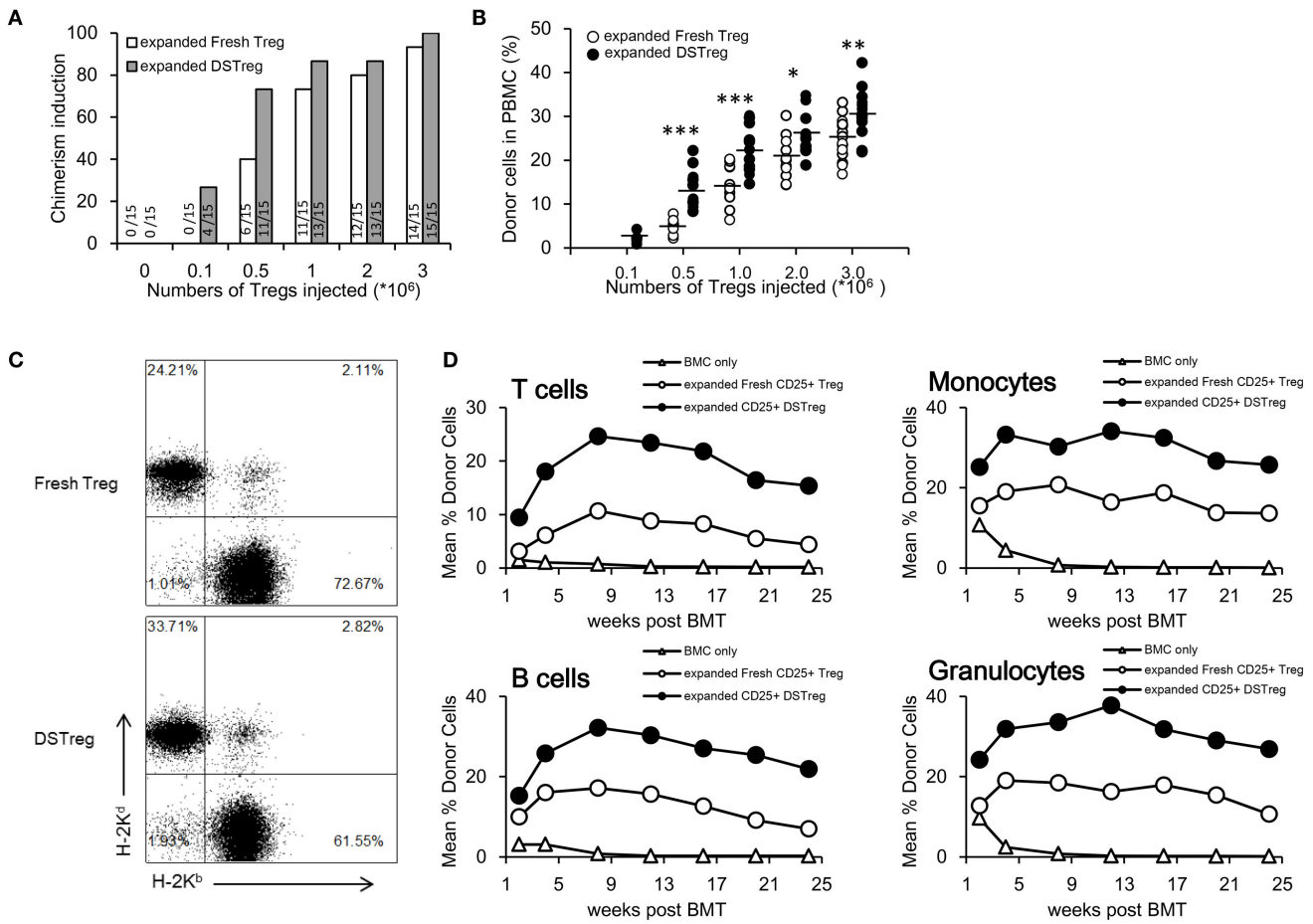

FIGURE 2 | **DSTreg cell treatment together with low-dose irradiation leads to multilineage mixed chimerism**. **(A)** Groups of C57BL/6J mice were transplantated with fully mismatched BALB/c BM cells (2×10^7) under low doses of irradiation (3 Gy total body irradiation at day −1), co-stimulation blockade with abatacept (CTLA-4-Ig) (0.05 mg at day 2), and three doses of rapamycin (0.1 mg at days 1, 0, and 2) were additionally treated with or without different numbers of expanded DSTreg cells, or expanded fresh Treg cells at day 0. Percentages of successfully induced chimeras are shown. Chimerism was considered to be established if donor cells were detectable by flow cytometry within both the myeloid lineage and at least one lymphoid lineage for the duration of follow-up. **(B)** Hematopoietic reconstitution was assessed at 3 weeks after BMT. Values for individual mice are shown; bars indicated means. **(C)** Typical FACS plots of H-2K^b (recipient) versus H-2K^d (donor) staining were carried out 3 weeks after BMT. **(D)** Donor (H-2D^d) chimerism among leukocytes was assessed by flow cytometry of peripheral blood at multiple time points (2, 4, 8, 12, 16, 20, and 24 weeks post-BMT) and is shown as mean percentage. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$ compared among the indicated groups.

by expanded DSTreg cells survived for the longest period, and more than half of the grafts survived for the duration of follow-up, without the presence of both acute and chronic rejection (**Figure 3A**). When comparing the histological results among each group, chimeras induced through DSTreg cell infusion demonstrated viable and best-preserved structure of mucosa and villi (**Figure 3B**). The stoma in this group also had good structure. The relative rejection score was also significantly lower in chimeras induced by BM and DSTreg cell treatment (**Figure 3C**).

The main factors controlling organ rejection are the balance between cellular immune responses mediated by T-helper cells that produce numerous proinflammatory cytokines and inhibitory cytokines. Thus, the grafts concentrations of inflammatory-response-related Th1/Th2 and Th17/Treg cytokines were also assayed with ELISA. The concentrations of tumor necrosis factor (TNF)-α, IL-17A, IL-23, and interferon (IFN)-γ all increased gradually in the allogeneic control group (**Figure 3D**). Compared to the allogeneic control group, the concentrations of IL-23, IFN-γ, and IL-17A in the grafts of chimeras were significantly lower on days 5, 7, and 11 than those in the allogeneic control group (**Figure 3D**). The concentrations of inhibitory cytokines transforming growth factor-β and IL-10 were significantly higher in the chimera group than in the allogeneic group on days 5, 7, and 11 (**Figure 3E**), and the levels of these two inhibitory cytokines were higher in the BM + DSTreg cell group than in the BMC + fresh Treg cells group (**Figure 3E**). These results suggest that recipients treated with BMC + DSTreg cells can better accept small bowel allografts, with low levels of inflammatory response and acute rejection.

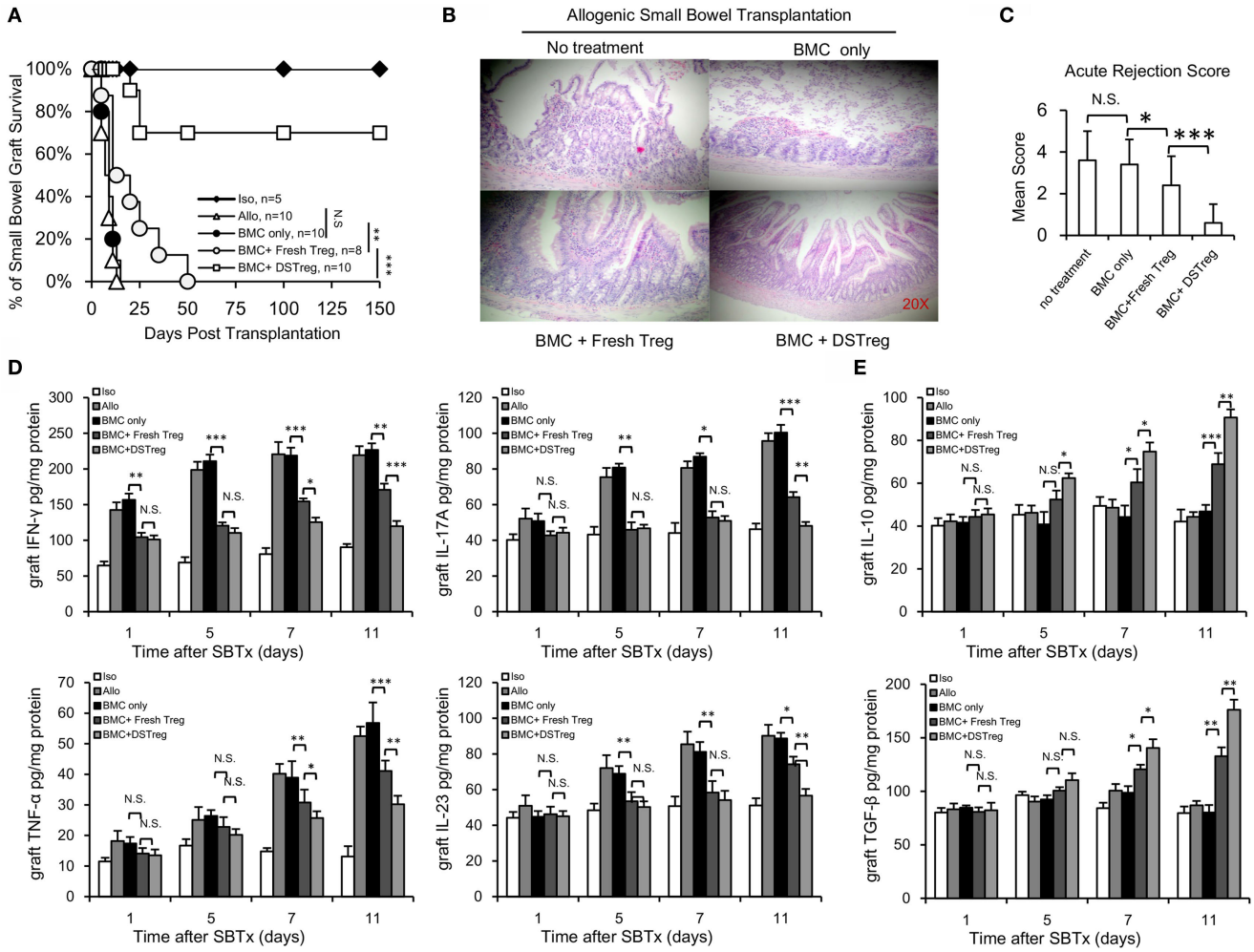

FIGURE 3 | Chimeras induced by DSTreg cells developed full donor-specific intestine allograft tolerance, and tolerant chimeras displayed hypo-inflammatory responses in the intestinal allografts. **(A)** Graft survival of intestinal allografts. **(B)** Representative hematoxylin-and-eosin-stained (original magnification: ×100) sections of intestinal allografts from recipients treated with low-dose irradiation (3 Gy) and BM cells, with or without expanded DSTreg cells, and expanded fresh Treg cells (14 days post-SBT). **(C)** Allografts were assigned an acute rejection score by a blinded pathologist. **(D,E)** Cytokine concentrations in the recipient intestinal allografts were measured by ELISA on days 1, 5, 7, and 11 after SBT. Data are shown as mean ± SD (n = 4–7). One representative of at least two independent experiments is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared among the indicated groups.

Increased Infiltration of CD4^{+}Foxp3^{+} Treg Cells in Small Bowel Grafts and Deletion of Donor-Reactive T Cells of Mixed Chimeras Induced by BM and DSTreg Cell Infusion

To assess tolerance in these fresh Treg and DSTreg induced chimeras, *in vitro* MLR assays were performed to evaluate self-reactivity, donor-reactivity, and third-party reactivity. In chimeras treated with Treg cells, responsiveness toward the donor was almost reduced to the level of self-reactivity (**Figure 4A**), whereas third-party reactivity was preserved (**Figure 4A**). CD4^{+}Foxp3^{+} Treg cells have been implicated to play a crucial role in homeostasis of intestinal immunity (18, 22, 23), and dysregulation of the number and function of Treg cells contributes to the development of intestinal transplantation rejection (24, 25). Previous results have also suggested higher levels of inhibitory cytokines relative to Treg cells in recipients treated with expanded DSTreg cells. Therefore, we obtained intestinal grafts and directly assessed the infiltration of CD4^{+}Foxp3^{+} Treg cells as previously described (17). The percentage of infiltrating CD4^{+}Foxp3^{+} Treg cells was significantly higher in grafts of chimeras treated with BM + expanded DSTreg cells compared to those of chimeras treated with BM cells only or BM + expanded fresh Treg cells (**Figures 4B,C**). Although the percentage of Foxp3^{+} Treg cells in CD4^{+} T cells in the spleen of recipients treated with expanded DSTreg cells was higher than that in recipients treated with expanded fresh Treg cells, no significant difference was discovered (**Figure 4D**). The frequency of CD4^{+}Foxp3^{+} Treg cells in peripheral blood is

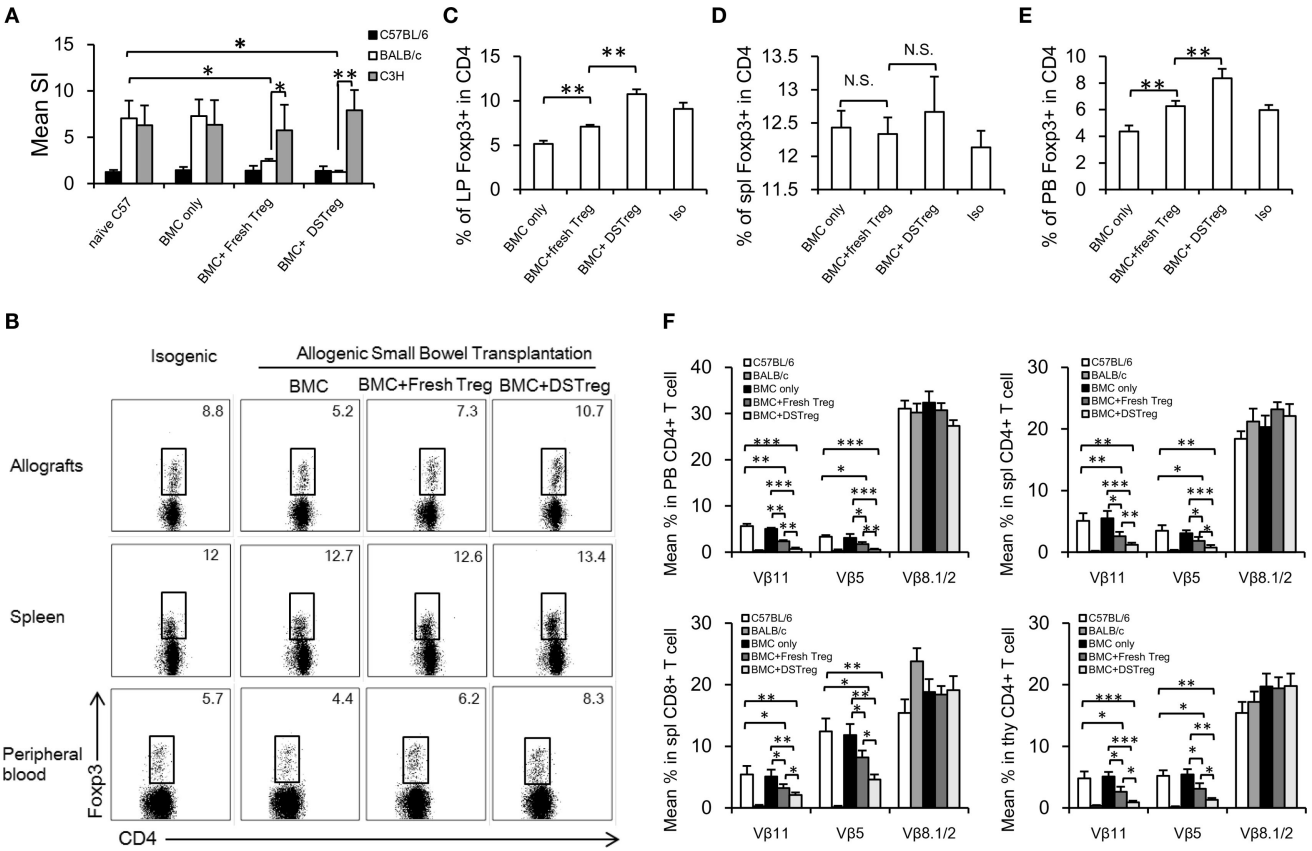

FIGURE 4 | Regulatory mechanism and clonal deletion contribute to intestinal allograft acceptance in chimeras treated with expanded DSTreg cells. **(A)** Donor reactivity was assessed in MLRs at 8 weeks post-BMT. Donor-specific responses and third-party reactivity were measured in each group. Simulation indices were calculated by dividing the mean cpm from responses against recipient (C57BL/6J), donor (BALB/c), or third-party (C3H) stimulator cells by mean background cpm (i.e., cpm with no stimulator population). The percentage of $CD4^+Foxp3^+$ cells in recipient allografts **(B,C)**, spleen **(D)**, and peripheral blood **(E)** was measured by multicolor flow cytometry on day 14 after SBT. **(F)** Deletion of donor-reactive T cells in chimeras was shown by assessing percentages of Vβ11, Vβ5, and Vβ8. Multicolor flow cytometry was used for measurement in selected mice at 8 weeks post-BMT. Chimeras treated with expanded DSTreg cells showed significant peripheral and central clonal deletion among donor-reactive T cells, as measured by percentage of Vβ11 and Vβ5 (but not Vβ8). Data are shown as mean ± SD (n = 4–6). One representative of at least three independent experiments is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared among the indicated groups.

negatively correlated with severity of graft versus host diseases in humans (26) and is also regarded as a biomarker for hematopoietic cell transplantation outcomes (27). Similar results were also obtained in our model (**Figure 4E**).

Mixed chimerism was induced in our model and chimeric recipients displayed hyporesponsiveness to donor antigens; therefore, deletion of intrathymic and peripheral donor-reactive T cells might also underlie the lack of responsiveness to donor antigens. To test this hypothesis, we examined the levels of certain Vβ subunits within the T-cell receptor repertories. The frequencies of $V\beta11^+$ and $V\beta5^+$ peripheral $CD4^+$ T cells were low in chimeras treated with expanded DSTreg cells, which suggested the establishment of peripheral clonal deletion, while it was incomplete in chimeras with BM cells, low-dose irradiation, and expanded fresh Treg cells (**Figure 4F**). No deletion was seen in recipients without Treg cell treatment (**Figure 4F**). Furthermore, significant intrathymic deletion was also achieved in recipients treated with expanded DSTreg cells (**Figure 4F**).

Donor-Specific Skin Graft Acceptance in Mixed Chimeras Receiving BM and DSTreg Cell Infusion, and Maintenance of Normal Immune Response to Third-Party Grafts

To determine whether or not tolerance was donor-specific in recipients treated with DSTreg cells after SBT, skin transplantation was performed 100 days after SBT. Mice receiving DSTreg cells that did not accept small bowel grafts rejected both BALB/c and C3H skin grafts within 14 days, while mice achieving small bowel grafts permanently also accepted BALB/c skin grafts permanently. These mice also rejected C3H skin grafts, which further showed that mice that accepted small bowel grafts achieved donor-specific transplantation tolerance (**Figure 5**).

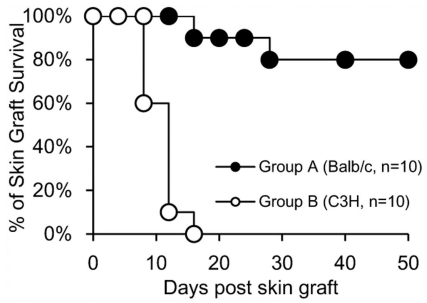

FIGURE 5 | Mixed chimeras of DSTreg cell infusion group received donor-specific skin grafts and retained normal immune response to third-party grafts. Skin transplantation was performed 100 days after SBT in recipients (C57BL/6) treated with DSTreg cells, and survival curve of skin grafts from donor-specific Balb/c mice and C3H mice was shown. Donor-specific Balb/c skin grafts survived in most chimeras, whereas C3H skin was rejected. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared among the indicated groups.

DISCUSSION

We (28) and others (23, 29) have suggested the critical role of Treg cells in maintaining intestinal homeostasis, and better control of the number and function of Treg cells in GALT, especially in the intestine, might be a promising prospect for the acceptance of intestinal allografts. How to maintain long-term DSTreg cells and/or expand them *in vivo* has always been a central problem in immune tolerance, and isolating sufficient numbers of Treg cells for *in vivo* use is also a significant clinical challenge (21). Previously, we have shown that donor-specific transfusion with complete blockade of ICOS/B7h signaling can achieve immune tolerance (10). Furthermore, this allograft acceptance is transferable and mediated by $CD4^+CD25^+Foxp3^+$ T cells from recipient mice. To acquire large numbers of these DSTreg cells for further use, we tried to expand the cells *in vitro*. Mature DCs have been suggested to be the most potent APCs to expand antigen-specific Treg cells in the presence of high-dose IL-2 (30). Since spleen cells are a mixture of many cell types with only 1–1.5% being DCs, among which the majority are in an immature state, we used BMDCs as donor-specific APCs to stimulate Treg cells, with or without rapamycin or IL-2, alone or in combination. After 2 weeks of co-culture, our results clearly showed that BMDCs could expand these DSTreg cells with potent suppressive function *in vitro* in the presence of IL-2 and rapamycin, which is consistent with other *in vitro* and *in vivo* results (19–21). *In vitro* results also demonstrated that these expanded DSTreg cells displayed more powerful immunosuppressive function. However, it has been shown that infusion of DSTreg cells alone only delays $CD4^+$ T-cell-mediated skin graft rejection and $CD8^+$ T-cell-mediated allograft rejection (11, 31, 32), and results from highly immunogenic organ transplantation models are still frustrating (11). We (4) also acquired similar results in SBT, which drove us to seek better strategies for immune tolerance in SBT. Studies have also suggested that rodent models for tolerance through mixed chimerism are among the most robust, which might be the best candidate for clinical trials (8, 33, 34). Recently, long-term stable kidney allograft survival without maintenance immunosuppression was also achieved by infusion of BM cells (35). Therefore, chimerism might be a promising strategy for intestinal transplantation.

Various mixed chimerism protocols have been developed including the use of immunosuppressive drugs, co-stimulation blockade (36, 37), $Foxp3^+$ Treg cell application (8, 38), and T-cell depletion (11, 39). Among all these, strategies based on the use of co-stimulation blockade are the most potent at inducing mixed chimerism (6). However, there are few reports on protocols for promoting stable intestinal allograft acceptance. Guo et al. have conducted a series of studies on biological agents that delay intestinal acute rejection and found that chimerism with anti-CD40L mAb, CTLA4-Ig, donor BM, and busulfan prolong intestinal allograft survival (11). However, their strategy still failed to achieve long-term survival of intestinal allografts with different levels of chimerism and persistence of donor-reactive T cells in recipients. Furthermore, they could not identify Treg cells in chimeric recipients bearing intestinal allografts, which suggest the absence of a regulatory mechanism in this model. Recently, combining Treg therapy with non-cytoreductive BMT has been suggested to promote acceptance of heart grafts in mice (12). Although this kind of Treg-cell-induced strategy achieved immune tolerance, the levels of chimerism observed were low and depletion of donor-reactive T cells was also incomplete, which might not be suitable for application to immunogenic organs like the intestine (8, 12). Furthermore, in addition to tolerization of intrathymic newly developing T-cell repertoire, pre-existing mature donor-reactive T cells also need to be tolerized through peripheral mechanisms in such protocols (9).

In our study, we found that DSTreg cells from tolerant mice stably induced mixed chimerism and further promoted the intestinal allograft acceptance. The percentage of Treg cells in the intestine was also significantly high in the DSTreg-cell-induced chimeras, which might have contributed to the low levels of inflammatory response. Similar results were found in peripheral blood, which strongly suggests achievement of immune tolerance in these recipient mice. Besides, clonal deletion is considered the backbone of tolerance through chimerism (40), and most groups investigating tolerance associated with chimerism-inducing strategies have reported deletion of donor-reactive $CD4^+$ T cells (11). We also observed significant deletion of $V\beta5^+$ and $V\beta11^+$ MMTV-reactive $CD4^+$ and $CD8^+$ T cells in DSTreg-cell-induced chimeras. Therefore, clonal deletion and regulatory mechanisms both contribute to promote allograft acceptance in our study. We also further demonstrated that immune tolerance induced in our model was donor specific.

In conclusion, we have developed a new mixed-chimerism-inducing protocol using the DSTreg cells acquired from tolerant mice bearing heart allografts and have promoted the acceptance of intestinal allografts. These results underlie the clinical potential of Treg-cell-based chimerism and subsequent prevention

of solid organ transplantation rejection that is highly immunogenic. Further studies on determining the origin (donor or recipient) and migration pattern of Treg cells in these tolerant mice bearing intestinal allografts are needed so that they can be used clinically.

AUTHOR CONTRIBUTIONS

J-FD and W-XG conceived and designed the experiments; X-FS and J-PJ performed the experiments; J-JY and W-ZW analyzed the data; J-PJ, X-FS, and J-JY contributed reagents/materials/analysis tools; J-FD and X-FS wrote the paper.

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REVIEW
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B Cell Immunity in Solid Organ Transplantation

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The contribution of B cells to alloimmune responses is gradually being understood in more detail. We now know that B cells can perpetuate alloimmune responses in multiple ways: (i) differentiation into antibody-producing plasma cells; (ii) sustaining long-term humoral immune memory; (iii) serving as antigen-presenting cells; (iv) organizing the formation of tertiary lymphoid organs; and (v) secreting pro- as well as anti-inflammatory cytokines. The cross-talk between B cells and T cells in the course of immune responses forms the basis of these diverse functions. In the setting of organ transplantation, focus has gradually shifted from T cells to B cells, with an increased notion that B cells are more than mere precursors of antibody-producing plasma cells. In this review, we discuss the various roles of B cells in the generation of alloimmune responses beyond antibody production, as well as possibilities to specifically interfere with B cell activation.

Keywords: HLA, donor-specific antibodies, antigen presentation, cognate T–B interactions, memory B cells, rejection

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INTRODUCTION

In the setting of organ transplantation, B cells are primarily known for their ability to differentiate into long-lived plasma cells producing high affinity, class-switched alloantibodies. The detrimental role of pre-existing donor-reactive antibodies at time of transplantation was already described in the 60s of the previous century in the form of hyperacute rejection (1). With the introduction of the complement-dependent cytotoxicity crossmatch assay by Terasaki and colleagues, the problem of hyperacute rejection was largely eliminated (2, 3). In the decades that followed focus shifted toward T cells and the prevention of cellular rejection. As a consequence, many drugs have been developed to successfully keep T cell immunity in check (4). With T cells largely under control, it is now clear that B cells remain important as precursors of antibody-producing plasma cells. However, B cells also give rise to humoral immune memory in the form of memory B cells, process and present alloantigens to T cells, are involved in ectopic lymphoid follicle formation, and modulate T cell responses by secreting cytokines. Reciprocal cognate interactions between T cells and B cells play key roles in the generation of alloimmune responses (5) (**Figure 1**).

In order to understand how B cells contribute to adaptive immune responses, we will first summarize the basics of human B cell development. Afterward, we will focus on the various roles of B cells in the setting of solid organ transplantation by antibody production, alloantigen presentation

Abbreviations: ABMR, antibody-mediated rejection; APC, antigen-presenting cell; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor; BCR, B cell receptor; Bregs, regulatory B cells; CDC, complement-dependent cytotoxicity; CTLA4, cytotoxic T lymphocyte-associated protein 4; DSA, donor-specific antibody; FDC, follicular dendritic cells; GC, germinal center; HEV, high endothelial venules; HLA, human leukocyte antigens; Ig, immunoglobulin; MHC, major histocompatibility complex; MPA, mycophenolic acid; PRA, panel reactive antibodies; SCS, subcapsular sinus; T_{FH}, follicular T helper cells; Tregs, regulatory T cells.

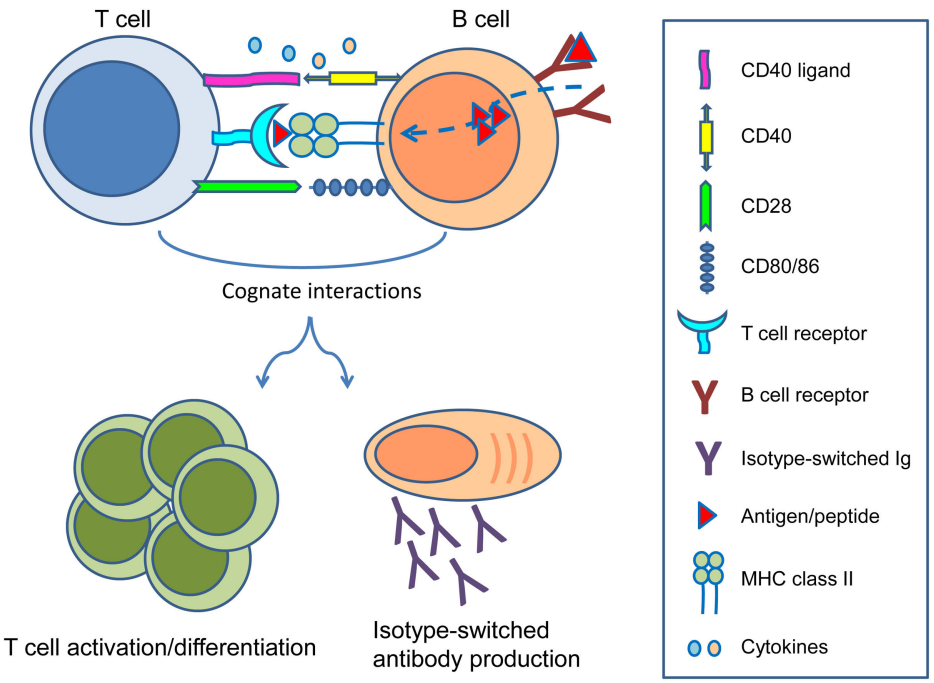

FIGURE 1 | **Reciprocal interactions between T cells and B cells**. Following B cell receptor-mediated uptake of protein antigens, activated B cells process and present antigenic peptides in the context of major histocompatibility complex (MHC) class II on their surface to cognate T cells that recognize the MHC–peptide complex through their T cell receptor. Ligation of CD40 ligand and CD28 on T cells to CD40 and CD80/86 on B cells, as well as production of several cytokines enable differentiation of both B cells and T cells into effector and memory subsets. While B cells can become isotype-switched antibody-producing plasma cells and memory B cells, T cells can become activated as effectors or differentiate into memory T cells to sustain cellular immune responses.

to T cells, intragraft tertiary lymphoid organ formation, as well as immune regulation. Finally, we will discuss new venues in interfering with B cell activation.

GENERATION OF HUMORAL IMMUNE RESPONSES IN SECONDARY LYMPHOID ORGANS

B Cell Development in Bone Marrow

B cells are crucial components of the humoral immune response. They participate in eradication of pathogens by their ability to differentiate into antibody-producing plasma cells, thereby propagating long-term serological immune memory. B cell development encompasses a programed set of events that initiate in primary lymphoid organs, which advances to a functional maturation stage in secondary lymphoid organs. Development and survival of B cells depend on the cell surface expression of a functional antigen receptor, namely, the B cell receptor (BCR), which is a membrane-bound immunoglobulin (Ig) molecule in complex with Ig α/β heterodimer signaling molecules (6). In order to generate a functional BCR capable of recognizing a broad range of antigens but not self, the gene segments encoding the BCR go through rearrangements in the bone marrow, by the assembly of variable (V), diversity (D), and joining (J) gene segments at both Ig heavy and light chain loci *via* DNA recombination (7). Newly formed B cells that express autoreactive BCRs are modified either by receptor editing or deleted by apoptosis. Upon completion of receptor editing, immature B cells with an intact BCR on their cell surface leave the bone marrow as transitional B cells to further continue maturation in the peripheral circulation and secondary lymphoid organs (8).

Modifications of the BCR proceed in germinal centers (GCs) at later stages of B cell differentiation during T cell-dependent immune responses as discussed below. While certain B cell subsets respond to polysaccharide antigens such as non-self blood group antigens by producing natural antibodies independent of T cell help, responses to protein antigens [e.g., human leukocyte antigen (HLA)] develop in the presence of T cell help. Since alloimmune responses are generally directed at protein antigens, we will focus on T cell-dependent follicular B cell responses.

B Cell Activation in Secondary Lymphoid Organs

Secondary lymphoid organs are located at strategic sites throughout the body and provide the proper environment for T and B cells to come into contact with antigen and interact with each other. Both aspects are essential for the generation of antibody responses. In lymph nodes, B cells form follicles in the cortex just beneath the subcapsular sinus (SCS) of the lymphatic vessel, while T cells are localized in the paracortex adjacent to B cell follicles. The paracortex contains high endothelial venules through which lymphocytes and dendritic cells enter the lymph

node (9). Immature naïve B cells continuously circulate through the peripheral blood, lymph, and enter secondary lymphoid organs in order to gain access into B cell follicles where they can complete their maturation and receive further survival signals. These naïve B cells home to secondary lymphoid organs through chemokines secreted by a network of stromal and follicular dendritic cells (FDC) (10–12). If a B cell does not encounter its specific antigen it detaches from FDC, leaves the lymph node *via* efferent lymphatics, and continues to recirculate between peripheral blood and secondary lymphoid organs (13).

Mature naïve B cells can become activated when their BCR engages an intact antigen inside or outside primary B cell follicles. While follicular B cells can recognize antigen presented on the surface of FDC, small soluble antigens can quickly diffuse from SCS into B cell follicles and can directly be recognized by BCRs. Large antigens such as immune complexes and viruses can be transported to B cell follicles by specialized $CD169^+$ macrophages resident at SCS. These macrophages lack phagocytosis ability and can present the antigen in its intact form to B cells (14). The immunological synapse between antigen-presenting cell (APC) and BCR initiates downstream signaling events and rearrangements of the B cell cytoskeleton. Subsequently, B cells that have acquired and processed antigen move toward the boundaries of T and B cell zones to survey for cognate T cell help. $CD4^+$ T cells in interfollicular and paracortical T cell zones initially interact with cognate antigen-presenting dendritic cells and subsequently increase their ability to migrate to B cell follicles.

A mature naïve B cell requires two signals to become activated: the first signal is received through the engagement of its BCR with cognate antigen and the second through cognate interaction with $CD4^+$ T cells, termed as follicular helper T cells (T_{FH}). Upon receiving T cell help at the T–B cell border, B cells can either differentiate into short-lived extrafollicular plasmablasts that produce low-affinity IgM antibodies or can proceed to go through GC reactions.

GC Reactions

Repositioning of antigen-activated T and B cells from the T–B cell zone back to the follicle initiates the GC reaction. During this transient reaction, B cells start to proliferate and consequently trigger the egress of naïve, circulating B cells from the primary follicle. The follicle resolves into light and dark zones harboring B cells at various levels of cell division. Although the exact mechanisms that define the fate of B cells in GC are not entirely understood, signaling through the BCR and interactions with T_{FH} are known to be essential for their survival and differentiation into long-lived plasma cells and memory B cells. B cells present antigen to T_{FH} in GCs for the second time during the course of the humoral immune response. GC B cells with high-affinity BCR appear to be most efficient at antigen uptake, processing, and presentation to T_{FH} cells as well as being more prone to survival than those with low-affinity BCR. Ligation of peptide/major histocompatibility complex (MHC) class II, CD40, and CD80/86 on B cells with the TCR, CD40L, and CD28 on T cells, respectively, in the presence of cytokines such as IL-2, IL-4, IL-5, and IL-21 appear to be crucial (15–17). The activated B cells undergo clonal expansion, class switch recombination from IgM to IgG, IgA, or IgE and acquire somatic hypermutations in the variable region of their BCR (18, 19). Affinity-driven selection enables further proliferation and differentiation of B cells with high-affinity BCR into long-lived plasma cells and memory B cells (20). While long-lived plasma cells preferentially home to the bone marrow, memory B cells remain quiescent until re-encounter with antigen and recirculate between secondary lymphoid organs and the peripheral blood (21, 22). Generation of rapid antibody responses following antigen re-challenge requires efficient antigen presentation by memory B cells to cognate memory T_{FH}. Upon receipt of this T cell help, memory B cells rapidly differentiate into plasma cells and produce high levels of antigen-specific, mainly IgG type of antibodies.

WHY ARE B CELLS IMPORTANT IN SOLID ORGAN TRANSPLANTATION?

Solid organ transplantation is a life-saving treatment option for patients with end-stage organ failure. The level of genetic disparities at HLA class I and II loci between donor and recipient, as well as the ability of the recipient's immune system to respond determine the strength of the immune response to an allograft (23–25). Immune responses directed toward mismatched HLA evoke both the cellular and the humoral arm of the adaptive immune system (26, 27). To prevent immunological rejection of the allograft, patients receive life-long immunosuppressive treatment. Currently available immunosuppressive regimens are centered on T cells and have been successful in curtailing acute cellular rejection. Successful treatment of cellular rejection by targeting T cells with immunosuppressive drugs have reduced acute rejection rates and hence improved short-term graft survival. It is clear that these drugs are insufficient in controlling humoral immune responses since antibody-mediated rejection (ABMR) is the leading cause of chronic allograft failure (28, 29). A growing body of evidence suggests that B cells play essential roles in alloimmunity besides mediating humoral immune responses. Understanding the various functions of B cells and the delicate balance between different B cell subsets may facilitate advances in B cell-targeting immunosuppressive drug development and eventually direct toward understanding the mechanisms involved in allograft tolerance.

SIGNIFICANCE OF ANTIBODY RESPONSES IN SOLID ORGAN TRANSPLANTATION

Antibodies binding to mismatched HLA (or non-HLA) molecules on donor endothelial cells initiate a set of signaling events leading to recruitment of effector cells to the graft endothelium through complement-dependent and -independent pathways. This process results in graft thrombosis and eventually a decline in allograft function. Clinical studies have shown that both pre-existence and *de novo* production of IgG donor-specific antibodies (DSA) are strongly associated with acute and chronic allograft injury in kidney, heart, lung, and to some extent, liver transplantation (29–34). On the contrary, studies on IgM and

IgA DSA did not reveal any isolated effect of these isotypes on allograft outcome unless they were co-existent with IgG antibodies (35, 36). This indicates that the above described GC response needs to be active for pathological antibody response to occur in the setting of organ transplantation.

In accordance with several earlier studies, Loupy et al. found in a large-scale retrospective study on renal transplant recipients that patients developing DSA after transplantation have inferior 5-year graft survival rates compared to those without DSA (37). Among those patients with *de novo* DSA, the capability to fix complement was associated with more severe lesions including microvascular inflammation and C4d deposition. In a recent study, Lefaucheur et al. investigated the role of complement fixation of HLA-DSA in a cohort of 635 kidney transplant recipients (38). The authors categorized patients into three groups: ABMR-free, acute ABMR, and subclinical ABMR. They found that whereas ABMR-free patients most prominently had $IgG1^+$ DSA lacking C1q fixing capacity, patients with acute ABMR most frequently showed $IgG3^+$ DSA, which was associated with microvascular inflammation, C4d deposition in peritubular capillaries, and inferior graft survival. Interestingly, patients classified as having subclinical ABMR showed $IgG2^+$ and $IgG4^+$ DSA and had predominantly chronic lesions. Results from this study highlight the divergence between acute complement-dependent and chronic complement-independent roles for HLA-specific antibodies in mediating different types of allograft injury.

While circulating antibodies are mainly produced by long-lived plasma cells residing in the bone marrow, local alloantibody production within intragraft tertiary lymphoid organs has also been described (39). Thaunat et al. demonstrated the presence of alloantibodies in supernatants of renal cortex tissue cultures, suggestive for local antibody production within the graft. Comparison of HLA antibody specificities and strength of the antibody response revealed differences in serum and supernatant samples from the same patient (39). Several studies have shown the presence of DSA eluted either from core needle biopsy samples or explanted renal tissue of patients with failed allografts, which may be due to absorbance of circulating alloantibodies but may also be pointing toward local production (40–42). Huibers et al. found DSA in lysates of coronary arteries of heart allograft autopsies harboring ectopic lymphoid structures. Interestingly, DSA and non-DSA found in the graft and serum at the time of autopsy were directed only against HLA class II (43). A recent study by Milango et al. showed the presence of DSA in both serum and graft eluates at the time of nephrectomy in the absence of immunosuppressive treatment. Although HLA-C and -DP mismatches between the recipients and donors were not analyzed, 80% of HLA antibody specificities were found to be directed at mismatched donor epitopes both for HLA classes I and II (44).

Currently available methods to detect serum HLA (discussed elsewhere in this issue of *Frontiers in Immunology*) do not provide any information on the magnitude of HLA-specific memory B cells (45). As described above, these memory B cells can rapidly differentiate into antibody-secreting cells upon re-challenge. Memory B cells exert this rapid function upon re-encounter with the immunizing HLA or in response to a non-specific innate stimuli due to their lower activation threshold and constitutive toll-like receptor expression (46–48). Several reports have shown the presence of additional HLA antibody specificities that are not detected in serum but in the culture supernatants of polyclonally activated peripheral blood B cells from kidney transplant recipients with a history of sensitization (49, 50). Therefore, studying donor-specific B cell responses in the transplant setting is certainly of importance, and several recently developed techniques allow to do so (51–57).

A ROLE FOR B CELLS IN ANTIGEN PRESENTATION TO ALLOREACTIVE T CELLS

Expression of high levels of MHC class II and costimulatory molecules on activated B cells, their capacity to take up antigens by their BCR, and ability to clonally expand make B cells also extremely potent APC (58–64). Nonetheless, the APC function of B cells in transplantation setting was initially neglected among others due to murine studies reporting efficient $CD4^+$ T cell priming in B cell-deficient mice transplanted with skin or cardiac allografts (65–67). However, it turned out that the developmental absence of B cells may have triggered non-B cell APC to deviate T cell responses toward a Th1 phenotype, thereby potentiating allograft rejection (68).

In order to assess the role of B cells as APC to alloreactive T cells in the transplant setting, Noorchashm et al. generated bone marrow chimeric mice lacking either MHC class II or the MHC class II peptide loading machinery, specifically in B cells (69). Both of these chimeras showed prolonged cardiac allograft survival compared to wild-type controls, which experienced early T cell-mediated rejection. These results indicate that antigen presentation by B cells is involved in T cell-mediated rejection. However, although the authors observed impaired IgG alloantibody production in addition to a decreased $CD4^+$ T cell division rate, these experiments did not formally answer the question whether B cells are required for T cell differentiation into effector or memory subsets. This question was addressed by Ng et al. in an allogeneic skin transplantation model using B cell-deficient (μMT) mice. Whereas similar numbers of IFN-γ producing $CD4^+$ and $CD8^+$ T cells compared to wild type were found early after transplantation (effector phase), at a later stage (memory phase), μMT mice showed decreased numbers of alloreactive IFN-γ^+ T cells (70). These data suggest that memory T cell development is dependent on the interaction with B cells. While these studies provided evidence for the contribution of B cells to antigen presentation and T cell differentiation, the impact of alloantibodies on transplant outcome was not formally excluded. It appears that both alloantibodies and B cell-dependent T cell activation are important since Burns and colleagues showed that the enhanced T cell-mediated rejection of murine cardiac allografts upon re-challenge is caused by a combined effect of alloantibodies and memory B cell-dependent activation of T cells (71).

In clinical kidney transplantation, the possible role for B cells as APC in T cell-mediated rejection mainly comes from

studies on renal biopsies. A landmark study by Sarwal and colleagues showed dense B cell clusters in biopsies of acute cellular rejections that did not correlate with C4d deposition but were associated with steroid resistance and inferior graft survival (72). Since then, several groups confirmed the correlation of graft-infiltrating $CD20^+$ B cell clusters with steroid-resistant acute cellular rejection and poor graft survival (73–75), whereas other investigators did not find any prognostic significance of these intragraft B cell clusters neither for treatment sensitivity nor for transplant outcome (76–78). Remarkably, $CD20^+$ B cell clusters were mainly present in cases of T cell-mediated rejections without any association to ABMR, which is suggestive for a significant role of B cells other than antibody production (72–75). Indeed, intragraft $CD20^+$ B cells have been shown to display an activated, mature phenotype as shown by CD79a and HLA-DR expression and are often found in close proximity to $CD4^+$ T cells (75). In an elegant study using cell distance mapping, $ICOS^+CXCR4^+$ F_{TH}-like cells were found in close proximity to B cells in both T cell-mediated or mixed cellular rejection, thereby strongly supporting the concept of antigen presentation by these B cells to alloreactive T cells (79).

B CELLS IN TERTIARY LYMPHOID ORGANS OF CHRONICALLY REJECTED ALLOGRAFTS

Ectopic lymphoid organs resemble canonical secondary lymphoid organs regarding their T and B cell compartmentalization and interaction with dendritic cells, as well as the utilization of chemokine-mediated signaling pathways. By contrast, they display impaired lymphatic drainage and therefore trap the antigen leading to continuous exposure of immune cells to the antigen. *De novo* formation of lymphoid-like structures as a result of persistent antigen exposure at sites of chronic infection or inflammation in non-lymphoid organs has been described in both autoimmunity and cancer (80, 81).

Upon organ transplantation, an environment containing persisting antigen similar to an autoreactive milieu is created and as a result can lead to tertiary lymphoid organ formation (82). Kerjaschki et al. demonstrated proliferating T cells (75%) and B cells (25%) in nodular infiltrates in close proximity with lymphatic vessels in explanted kidney allografts (83). Similarly, Thaunat et al. described the presence of lymphoid neogenesis in virtually all allografts explanted due to chronic rejection (39, 84). B cells in these explants were organized into nodules reminiscent of either primary or secondary B cell follicles. Relatively high expression of genes characteristic for GCs were observed in renal secondary B cell follicles indicating a highly activated phenotype for graft-infiltrating B cells (39, 85). Furthermore, local B cell proliferation, a characteristic for the GC response, occurs as shown by Ki67 positivity and clonality of infiltrating B cells (83–85). In tertiary lymphoid organs, graft-infiltrating B cells might be contributing to lymphoid angiogenesis by prominent expression of vascular endothelial growth factor-A (86). Organization of the lymphoid infiltrates in the form of ectopic GCs may lead to containment of the alloimmune response within the graft. The aforementioned absence of DSA in circulation or discrepancies in specificities or strength of locally produced and circulating HLA antibodies supports this hypothesis (39, 84). It is possible that the infiltrates observed during acute T cell-mediated rejection may represent an early stage of tertiary lymphoid organ development.

B CELLS AS IMMUNE REGULATORS

In addition to their roles in immune activation, (subsets of) B cells may also have regulatory function (87). Several groups have reported B cells with regulatory properties in controlling autoimmunity and inflammation (87–90). A complicating factor in studying regulatory B cells (Bregs) is the lack of a unique marker to define these cells. This has resulted in a wide range of B cell subsets to be identified as Bregs with the ability to secrete IL-10, IL-35, or TGF-β (91–93). In mice, a T cell costimulatory molecule termed as T cell Ig domain and mucin domain (TIM1) was found to be useful for identifying IL-10-producing Bregs (94). In humans, two main subsets of B cells enriched for Bregs have been described: $CD24^{hi}CD38^{hi}$ transitional B cells (89) and $CD24^{hi}CD27^+$ B10 cells (95). Whereas IL-10, IL-35, and TGF-β have all been described as effector molecules of Bregs, in the setting of transplantation, the main focus has been on IL-10-producing B cells.

In transplantation, regulatory functions of B cells have mainly been investigated in murine models of allograft tolerance. Ding et al., using a mouse model of islet transplantation, demonstrated that TIM1 may also have functional properties in Breg development. They observed prolonged allograft survival in mice treated with an agonistic anti-TIM1 antibody compared to untreated mice (94). Interestingly, in mice depleted of B cells before transplantation, anti-TIM1 treatment accelerated allograft rejection, indicating an important role for B cells in TIM1-mediated tolerance. Transfer of $TIM1^+$ B cells into untreated recipients of islets led to prolonged allograft survival. This regulatory effect was defective in $TIM1^+$ B cells, showing the dependency of B cells on IL-10 for their regulatory capacity. Shortly after, Lee et al. reported 100% long-term islet allograft survival in mice treated with a combination of anti-CD45RB and TIM1 (96). They demonstrated prompt rejection of islet allografts if regulatory T cells (Tregs) were depleted before transplantation, implying that Bregs require an interaction with Tregs to induce tolerance. Furthermore, Le Texier et al. have shown the presence of intragraft IgM^+ B cells in rats with cardiac allograft tolerance compared to the presence of IgG^+ B cells in allografts showing chronic rejection (97), suggestive for a restriction in B cell activation in the tolerant group. To demonstrate that tolerance was (at least partially) caused by B cells, the authors performed adoptive transfer of splenic B cells from tolerant rats to show allograft tolerance in these secondary mice.

A hint toward a role for B cells in clinical transplantation tolerance came from studies identifying B cell signatures in operationally tolerant kidney transplant recipients who were immunosuppression-free for at least 1 year with stable graft function (98–100). Microarray analyses on peripheral blood revealed

22 B cell-specific genes that were enriched in tolerant patients compared to those with stable graft function. Furthermore, the CD20 transcript was found to be the only marker higher in urine sediments of tolerant patients. Indeed, three genes (*IGKV4-1*, *IGLL1*, and *IGKVID-13*) encoding Ig kappa and lambda light chains in the course of B cell differentiation were shown to be predictive of operationally tolerant patients (98). In an accompanying study, six highly overexpressed genes were identified in tolerant patients (*CD79B*, *TCL1A*, *SH2D1B*, *MS4A1*, *FCRL1*, and *FCRL2*) that were associated with B cell-related pathways (99). Interestingly, expression of *CD79B*, *MS4A1*, and *TCL1A* has been shown to be significantly downregulated in renal transplant recipients with acute rejection (101, 102).

Tolerant patients showed increased peripheral blood B cell numbers and a redistribution of B cell subsets toward a naïve ($IgM^+IgD^+CD27^-$) and transitional ($CD24^{hi}CD38^{hi}$) phenotype with increased expression of IL-10, compared to patients with stable graft function under immunosuppressive treatment (98, 99). The findings on IL-10-competent transitional B cells are in line with the definition of Bregs as described by Blair et al. (89). Pallier and colleagues confirmed the elevated peripheral blood B cell numbers and found that B cells with a memory phenotype ($IgD^-CD38^{-/+}CD27^+$) were increased (103). Whether these are the B10 cells as described by the group of Tedder remains to be established (95). Compared to patients with stable graft function, the majority of the operationally tolerant patients do not have circulating DSA and have a lower frequency of $CD38^+CD138^+$ plasma cells in the peripheral blood (98, 99, 103). In order to determine whether there was a defect in tolerant patients in generating humoral immune responses, Chesneau et al. polyclonally activated purified B cells from operationally tolerant patients *in vitro*. Polyclonally activated B cells proliferated and produced normal levels of IgM and IgG, accompanied by increased levels of IL-10 compared to those with stable graft function (104). In order to asses the inhibitory role of polyclonally activated B cells of tolerant patients on autologous $CD4^+CD25^-$ T cells, Chesneau et al. blocked IL-10, TGF-β, and granzyme B in a T–B cell co-culture system and found that only granzyme inhibitors affected the suppressive effects of B cells (105). However, antigen specificity, a prerequisite for immune regulation, has yet to be demonstrated.

EFFECTS OF IMMUNOSUPPRESSIVE TREATMENTS ON B CELLS

In the current practice of kidney transplantation, standard triple immunosuppressive regimen consists of a calcineurin inhibitor (tacrolimus or cyclosporine), a purine analog (mycophenolic acid-MPA), and corticosteriods as maintenance therapy in addition to a non-depleting anti-CD25 monoclonal antibody as the induction agent (106). Since these agents exert their effects preferentially on T cells, they may abrogate humoral immune responses indirectly by inhibiting the T cell help (107), although some of these also have a direct effect on B cells (108, 109). Drugs specifically interfering with humoral immunity can be classified into several groups: drugs that deplete B cells from the circulation, those that interfere with T–B cell interaction, drugs targeting B cell survival signals, and drugs interfering with antibody production or effector function.

Current therapies for (highly) sensitized patients are primarily focused on removal of antibodies before transplantation by plasmapheresis, intravenous immunoglobulins, or immunoadsorption (110). Addition of rituximab, a humanized murine CD20 antibody which depletes circulating $CD20^+$ B cells, to desensitization protocols resulted in improved outcomes in ABO-incompatible transplantation (111–113). Surprisingly, when rituximab was administered to non-sensitized patients as induction therapy, a higher rate of acute rejection was observed compared to controls (114). In addition to its application in treatment of ABMR (115), administration of rituximab led to successful treatment of steroid-resistant acute cellular rejections (116) and resolution of B cell infiltrates in graft (117–120). However, in patients experiencing chronic allograft dysfunction, rituximab treatment was ineffective in resolution of tertiary lymphoid organs despite the successful depletion of circulating B cells (121). Kamburova et al. showed long-lasting B cell depletion in patients receiving rituximab as induction agent with repopulating B cells mainly consisting of transitional B cells (122). Similar results were obtained when patients were treated with alemtuzumab, an anti-CD52 monoclonal antibody (123, 124). Although polyclonal activation of purified B cells did not reveal a difference in proliferation or IgM-producing cells, a significant decrease in IgG-producing cells was observed (123).

Another way of attenuating B cell responses can be achieved by blocking the critical costimulatory pathways between T and B cells. A recent study by Chen et al. in a mouse model of cardiac allograft transplantation showed that costimulation blockade with a high-affinity CTLA-4Ig (belatacept) inhibited memory B cell responses and DSA formation, thereby leading to prolonged graft survival (125). By blocking both CD28–CD80/86 (belatacept) and CD40–CD40L (2C10R4) pathways in a non-human primate model of ABMR, Kim et al. showed a decrease in clonal B cell expansion in GCs (126). Combined blockade led to reduced IL-21 production and was strongly associated with reduced DSA levels. Importantly, results of a large phase 3 trial confirmed the efficacy of belatacept in the clinical setting (127). This study revealed a reduction of DSA in the belatacept-treated group with a significant reduced risk of graft loss and death compared to the cyclosporine-treated group.

Several studies have shown increased serum levels of B cell-activating factor (BAFF) following treatment with depleting agents in kidney transplant recipients (128, 129), possibly due to a lack of BAFF consuming B cells. BAFF has a critical role in promoting survival, maturation, and activation of B cells, as well as maintaining self-tolerance (130). High levels of BAFF have been described in the setting of autoimmunity, and it is conceivable that high BAFF levels could also influence alloimmunity. Indeed, elevated serum BAFF levels were associated with increased risk of developing DSA and ABMR in the setting of kidney transplantation (131–133). Blockade of BAFF and/or the related molecule called a proliferation-inducing ligand (APRIL) may be an additional tool to downregulate humoral alloimmune responses as was suggested by the prolonged survival of cardiac allografts in BAFF-deficient mice (134). Also, in a non-human

primate ABMR model, BAFF/APRIL blockade (atacicept) was able to prevent *de novo* DSA production (135).

Plasma cells are responsible for the continuous production of antibodies and therefore have a high proteasomal activity. Proteasome inhibitors, such as bortezomib, are effective for the treatment of plasma cell malignancies (136). Bortezomib has been used to treat ABMR and diminish DSA production in sensitized transplant recipients (137–140). However, the inhibitory capacity of proteasome inhibitors is not limited to plasma cells as also naïve and memory B cell proliferation can be affected (141). Therefore, antibody production through plasma cells, as well as the various effects of B cells, may be dampened by proteasome inhibition.

CONCLUSION AND REMARKS

B cells contribute to acute and chronic allograft rejection processes by producing DSA. More recently, other functions have been attributed to B cells that may also influence the alloimmune response, such as antigen presentation to T cells, formation of tertiary lymphoid organs, or secretion of regulatory cytokines.

Considering that one-third of the patients on the kidney waiting lists are sensitized as a result of previous exposure to allogeneic HLA, memory B cells and their effector functions may play central roles in prospective transplantation outcome of these patients. Upon re-challenge, HLA-specific memory B cells generated during primary immune responses can promptly become high-affinity DSA-producing plasma cells and may serve as potent APC by their high expression of HLA-DR and costimulatory molecules. In conclusion, a variety of B cell populations with different functions may affect the alloimmune response after transplantation. Future therapies targeting B cells should take into consideration these different functions and the consequence that a simple depletion of all B cells will also interfere in the beneficial effects of certain B cell subpopulations.

AUTHOR CONTRIBUTIONS

GK, FC, and SH designed the outline and wrote the manuscript.

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REVIEW
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The Biological Effects of IL-21 Signaling on B-Cell-Mediated Responses in Organ Transplantation

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Antibody-mediated rejection has emerged as one of the major issues limiting the success of organ transplantation. It exerts a highly negative impact on graft function and outcome, and effective treatment is lacking. The triggers for antibody development, and the mechanisms leading to graft dysfunction and failure, are incompletely understood. The production of antibodies is dependent on instructions from various immunocytes including CD4 T-helper cells that secrete interleukin (IL)-21 and interact with antigen-specific B-cells *via* costimulatory molecules. In this article, we discuss the role of IL-21 in the activation and differentiation of B-cells and consider the mechanisms of IL-21 and B-cell interaction. An improved understanding of the biological mechanisms involved in antibody-mediated complications after organ transplantation could lead to the development of novel therapeutic strategies, which control humoral alloreactivity, potentially preventing and treating graft-threatening antibody-mediated rejection.

Keywords: IL-21, IL-21 receptor, JAK/STAT, B-cell, organ transplantation, rejection

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INTRODUCTION

Antibody-mediated rejection remains an important barrier to improving long-term survival after solid organ transplantation (1–3). In cellular rejection, graft injury is due to direct cytotoxic activity of immune cells against graft parenchymal tissue. Antibody-mediated rejection, in contrast, is characterized by graft damage induced by circulating alloantibodies. Alloantibodies are produced by activated B-cells in response to antigen, costimulation, and cytokines such as interleukin (IL)-21 (4, 5).

Interleukin-21 was discovered by Parrish-Novak et al. using a functional cloning approach based on expression of the IL-21 receptor (IL-21R) gene and is located at chromosome 4 on position q26–q27 (6). The common γ-chain (γc) is a component of the IL-21R complex. IL-21 binding to the IL-21R/γc results in signaling *via* the JAK/STAT pathway (6, 7). This cytokine, a four-α-helix bundle,

Abbreviations: AID, activation-induced cytidine deaminase; BAFF, B-cell activating factor; BCL-6, B-cell lymphoma-6; Blimp-1, B lymphocyte-induced maturation protein-1; BMPs, bone morphogenetic proteins; Btk, Bruton's tyrosine kinase; BTB, also named POZ, pox virus and zinc finger; CpG, 5′-C-phosphate-G-3′; CSR, class switch recombination; GrB, granzyme B-cell; Ig, immunoglobulin; IL-21, interleukin-21; IL-21R, IL-21 receptor; LPS, lipopolysaccharide; mAb, monoclonal antibody; RD2, the second repression domain; Tfh, follicular T helper cells; TLO, tertiary lymphoid organ; TLR, toll-like receptor; XBP-1, X-box-binding protein-1; γc, common γ chain.

is a typical family I cytokine with broad pleiotropic actions and is primarily produced by T follicular helper cells (Tfh), Th17, and natural killer T-cells, rather than being generally produced by most tissue cells (6, 8, 9). IL-21 controls the activation, proliferation, differentiation, cytotoxicity, and survival of various target immune cells (10, 11). It is also important for the generation of B-cell responses in germinal centers resulting in isotype switching, affinity maturation, antibody production, and development of B-cells (12, 13). In particular, IL-21-mediated actions by Tfh cells are required for efficient antibody responses. The effectors and immune regulatory functions of IL-21 are mediated by binding to target B-cell surface receptors, which consist of α-chain and the γc that is shared with IL-2, IL-4, IL-7, IL-9, and IL-15 receptors (10, 14, 15).

Antibody-mediated ("humoral") rejection is a key cause of graft dysfunction and failure after organ transplantation (1, 16, 17) with 30–50% of failed allografts affected (18–20). Immunohistochemical and gene expression studies have shown that a large number of B-cells infiltrate the rejected allograft (18, 21–24), contributing to anti-donor responses.

Identifying the role of IL-21-mediated B-cell activation and differentiation pathways is critical for understanding the signaling pathways that underlie antibody-mediated rejection. In this review, we discuss the potential role of IL-21 on B-cells after organ transplantation.

IL-21 SIGNALING PATHWAY IN B-CELLS

The IL-21R is expressed by human naive B-cells, memory B-cells, germinal center B-cells (14), and as shown recently, plasma cells (25). IL-21R is upregulated on human memory B-cells after activation by anti-CD40 mAb (14).

Binding of IL-21 with IL-21R/γc triggers the catalytic activation of JAK1 and JAK3. This causes phosphorylation of tyrosine residues on IL-21R/γc, providing docking sites for STAT proteins and other signaling molecules (26). On recruitment, STATs are phosphorylated and form homodimers or heterodimers, which translocate into the nucleus and modulate expression of the target genes (27), which regulate B-cells, such as B-cell-induced maturation protein-1 (Blimp-1) (28), B-cell lymphoma (BCL)-6 (29), activation-induced cytidine deaminase (AID) (30), granzyme (31), somatic hypermutation (SHM) (32), paired box 5 (Pax5) (33), X-box-binding protein 1 (XBP-1) (34), and Bim (35). IL-21 mediates B-cell proliferation, immunoglobulin (Ig) production, and apoptotic functions mainly through the potent effects of STAT3 and/or STAT1 activation but also, to a lesser extent, through STAT4 and STAT5 (36–39) (**Figure 1**).

B-CELL ACTIVATION AND DIFFERENTIATION

B-cell receptor (BCR) ligation triggers activation of multiple downstream molecules. Burton's tyrosine kinase (Btk), one of the downstream products of the BCR signaling pathway, selectively regulates IL-21-induced STAT1 phosphorylation and translocation in the nucleus. Btk deficiency is associated with arrested cell development at the pre-B-cell stage. In addition, Btk is involved in cytokine-controlled B cell activation. In concert with IL-21, CD40, and B-cell activating factor (BAFF), this kinase mediates the crosstalk with cytokine pathways through regulation of IL-21-induced phosphorylation of STAT1 (25). IL-21 and CD40L collaborate to synergistically promote Blimp-1 activation and plasma cell differentiation (28). CD40L alone has no direct effect on Blimp-1, but it greatly augments the IL-21-triggered JAK-STAT

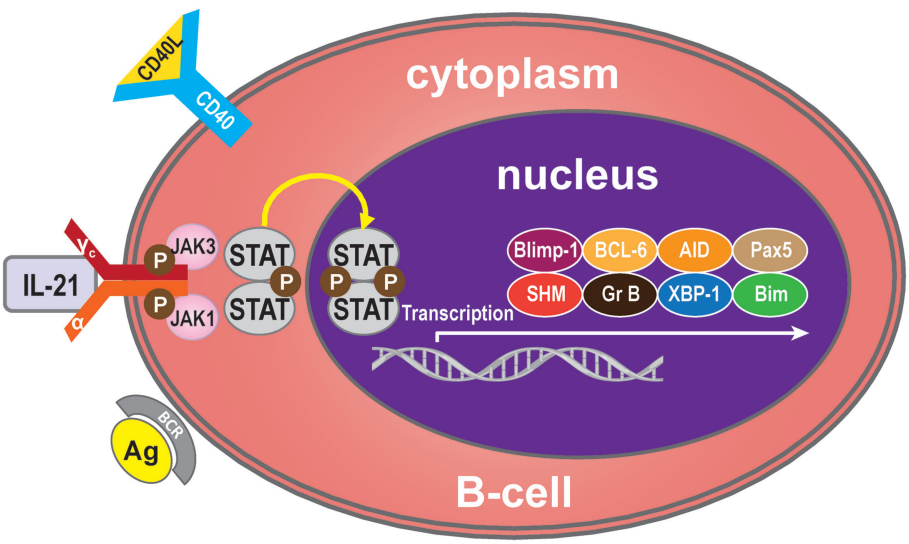

FIGURE 1 | IL-21 signaling pathway. Many molecules participate in the IL-21 signaling pathway in B-cells, but the main molecules are IL-21R, JAK, and STAT to activate transcription of Blimp-1, BCL-6, AID, Pax5, SHM, granzyme B, XBP-1, and Bim. Generally, IL-21 binds with the IL-21R of B-cells to trigger signaling pathways. The JAK and STAT family molecules are activated in turn, while the balance of the transcription factors Blimp-1 and BCL-6 control the maturation B-cell.

signaling. During this phase, STAT3 plays a more significant role than STAT1, because STAT3 mutations dramatically reduce the number of memory B-cells and abolish the ability of differentiation of naive B-cells into plasma cells (10). In contrast, STAT1 deficiency has no effect on memory B-cell formation *in vivo*. Thus, STAT3 is essential for the generation of effector memory B-cells from naive precursors (40). In addition, treatment with CD40L enhances the ability of STAT3 to upregulate Blimp-1 by removing BCL-6, which is a potent inhibitor of Blimp-1 expression. It has been speculated that IL-21 induces Blimp-1 and BCL-6 to regulate isotype-switched B-cells (41). Blimp-1 is a transcription factor and involved in plasma cell formation and maturation (42). Importantly, IL-21 costimulation upregulates expression of Blimp-1 (43). Consistent with this, IL-21-driven plasma cell differentiation from both naive blood B-cells and from memory B-cells are preceded by induction of Blimp-1 upregulation. Blimp-1 initiates plasma cell differentiation by downregulating MHC, CIITA, Pax5, and c-myc expression (33, 44, 45) and by inducing XBP-1 expression (46, 47). Blimp-1 level is very low when BCL-6 is over-expressed in B-cells (48). BCL-6 may block plasma cell differentiation due to downregulation of Blimp-1 (49). BCL-6 also can control B-cell development by BTB and RD2, two molecules that repress distinct functional effects of B-cells during the germinal centers reaction. BTB is required for B-cell survival and proliferation, while RD2 might be important for the prevention of terminal B-cell differentiation (50).

Since IL-21 activates STAT3 in B-cells, this may indicate that activation of STAT3 in human B-cells is pivotal for the induction of Blimp-1 expression and plasma cell differentiation (11, 40). It has been reported that IL-21-dependent CD86 upregulation is reliant on STAT3 phosphorylation and PI3K, revealing unexpected roles for these pathways in IL-21-mediated B-cell responses (51). In addition, IL-21 drives humoral immune responses *via* STAT3-dependent induction of the transcription factors required for plasma cell generation (52). These authors reported that IL-21*via* STAT3 sensitizes B-cells to the stimulatory effects of IL-2. Thus, IL-2 plays an adjunctive role in IL-21-induced B-cell differentiation. An absence of this secondary effect of IL-21 may amplify humoral immunodeficiency in patients with mutations in STAT3 and IL-21R due to impaired responsiveness to IL-21. In concert, IL-21 and BAFF stimulate and may maintain humoral immunity in humans (53). BAFF has the ability to substitute for CD40L activity with regards to IL-21 costimulation and differentiation of memory B-cells present in spleen (53) (**Figure 2**).

IMMUNOGLOBULIN PRODUCTION

Critical sites for the generation of antibody responses are the germinal centers in lymphoid follicles present in lymph nodes that also have been identified in transplanted organs (4, 54) where antigen-primed B-cells interact with T-cells, most of which are Tfh cells secreting IL-21. The B-cells are driven to undergo Ig isotype

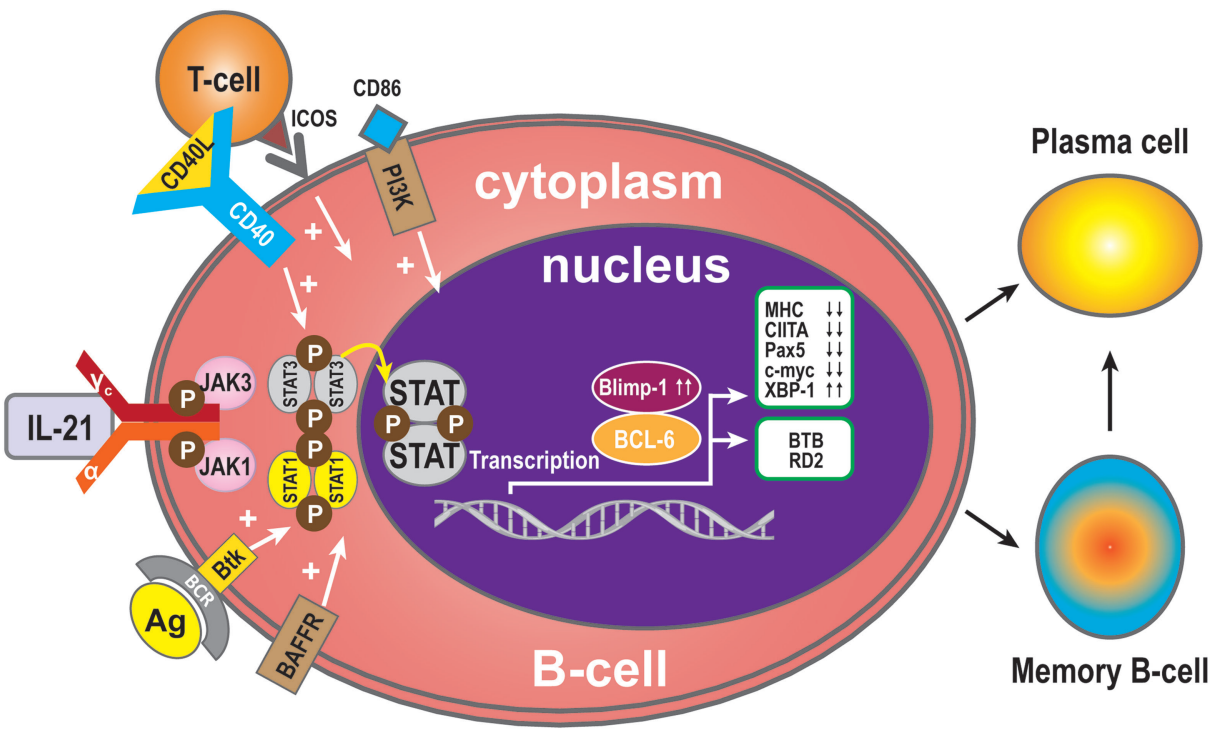

FIGURE 2 | B-cell activation and differentiation. Plasma cells are the main executors of B-cell regulation by the IL-21signal pathway. STAT3 is the dominant member of the STAT family in this respect. Transcription Blimp-1 has a positive role and BCL-6 a negative role in plasma cell maturation. Additionally, CD40L, ICOS, CD86, and BAFF can promote B-cell differentiation to plasma cells, while MHC, CIITA, Pax5, and c-myc are switched off during B-cell differentiation to plasma cells or memory B-cells, and XBP-1 is induced. The transcription factor BCL-6 activates BTB, which is required for cell survival and proliferation, while RD2 prevents terminal differentiation of B-cells.

switching, with SHM and secretion of high affinity antibodies (12, 55–58). Bryant et al. reported that IL-21 stimulates naive B-cells to mainly produce IgM+ B-cells, while low frequencies of IgG and IgA secreted B-cells were also detected (59). When IgG was produced, IL-21 favors naive B-cells to develop into IgG1- and IgG3-secreting B-cells (56, 57, 59–62). It has been reported that IgM-specific Abs targeting BCR and IL-21 costimulation also induce the expression of AID (63, 64). Interestingly, although AID catalyzes both class switch recombination (CSR) and SHM, only CSR is induced in naive human B-cells after stimulation by IL-21 and anti-CD40 (45, 47, 60, 65). The C-terminal of AID is required for CSR but not for SHM (65, 66), and it has been postulated that IL-21 induces AID activity only at the C terminus. Multiple studies have shown that IL-21 causes CSR of CD40-stimulated human naive splenic IgM+ B-cells to IgG1 and IgG3, and CSR of CD40-stimulated cord blood B-cells to IgA (47, 60). As well as the molecules described above, among the group of cytokines called bone morphogenetic proteins (BMPs) (67), BMP-2, -4, -6, and -7 inhibit CD40L/IL-21-induced production of IgM, IgG, and IgA. In memory B-cells, BMP-6 upregulated expression of DNA-binding protein inhibitor genes, but potently inhibited CD40L/IL-21-induced upregulation of the transcription factor XBP-1 (34). This factor is crucial for final stage in plasma cell differentiation (34). As described above, Btk is an efficient propagator of IL-21 signaling, critical for CSR in human B-cells and secretion of Ig (25). Additionally, the outcome of IL-21-mediated Ig secretion depends on the presence of IL-4 and IL-10, which influence the outcome of IL-21-mediated CSR. IL-10 acts synergistically with IL-21 to induce secretion of IgA by CD40L-stimulated human B-cells, whereas IL-4 has an inhibitory effect (47). As shown by the group of Bromberg, IL-10 deficiency in B-cells prevents transplantation tolerance, resulting in decreased follicular immune regulatory CD4+ T-cells, a recently identified T cell subset, and increased IL-21 expression by Tfh cells in the B-cell and T-cell marginal zones (68). This has implications for our understanding of the mechanisms involved in tolerance and show at the same time that B cells play pivotal roles in the induction of this immune phenomenon (68). Interestingly, as with IL-21, IL-10, in combination with toll-like receptor (TLR), signaling also enhances phosphorylation of STAT3, resulting in increased IgG production. Hence, IL-21 and IL-10 increase the activity of the TLR–MyD88–STAT3 pathway in human B-cells by enhancing Ig production stimulated by STAT3 phosphorylation (69) (**Figure 3**).

REGULATORY B (B10) CELLS

Interleukin-21 may also modulate the immune response by immune-dampening regulatory mechanisms. One of these is performed by B10 cells, named for their ability to produce abundant

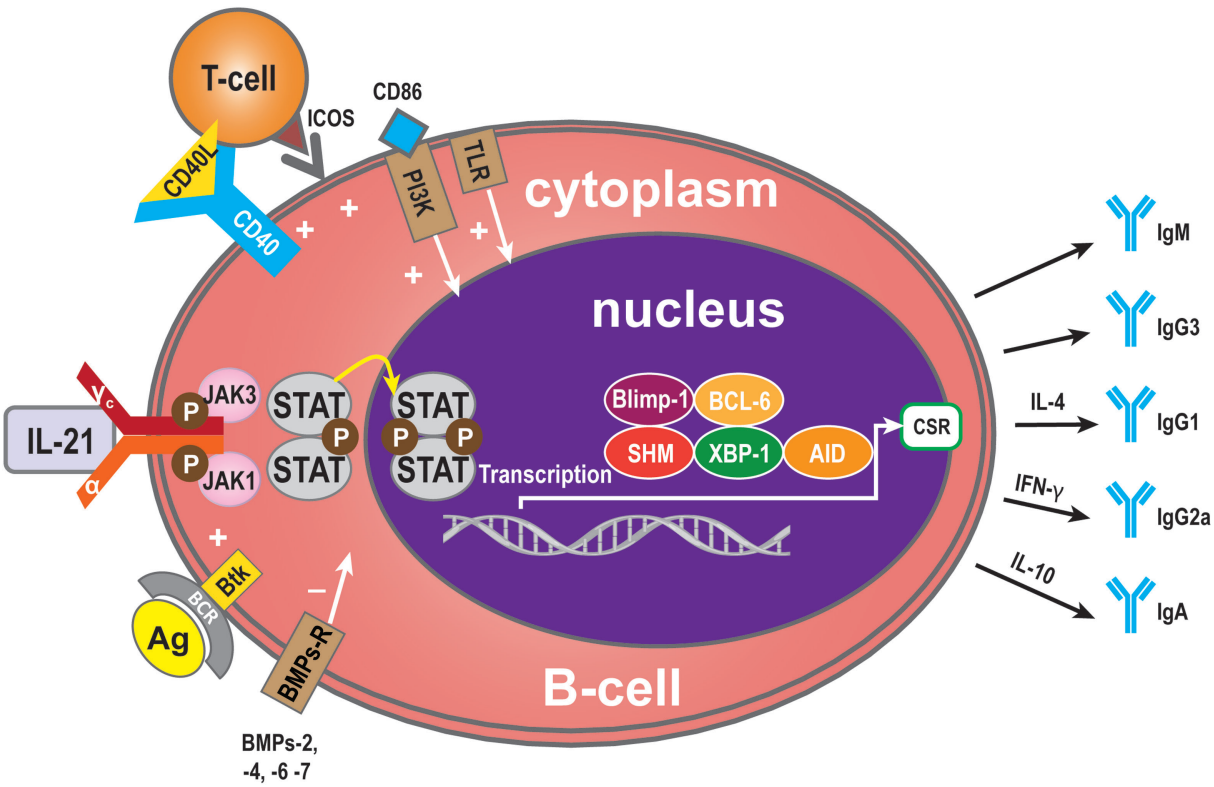

FIGURE 3 | Immunoglobulin (Ig) production. Ig is produced by plasma cells, so the signaling pathway for mediation of Ig production is similar to that for IL-21-mediated plasma cell maturation. Some molecules, however, have a specific role in Ig production: BMP-2, -4, -6, and -7 may exert a negative influence and Btk a positive influence. In addition, some cytokines contribute to Ig CSR. IL-4 can induce to IgG1 formation, IFN-γ to IgG2a, and IL-10 to IgA.

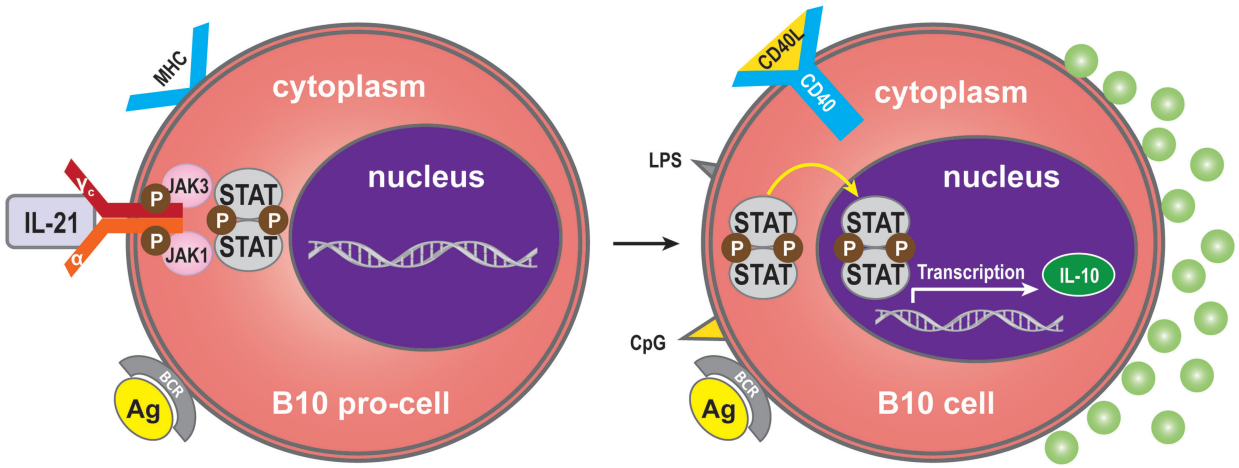

FIGURE 4 | **Regulatory B (B10) cells**. IL-21 binding to IL-21R expressed on B10 pro-cells may trigger B10 pro-cell maturation to B10 cells. Synergistically with MHC-II, LPS, CpG, or CD40 ligation stimulation can induce this cell subset to produce IL-10.

IL-10 (70). Expression of IL-10 is a common characteristic of regulatory immune cells, and B10 cells are thus referred to as regulatory B-cells (71, 72). The B10 cell subset represents <1% of peripheral blood B-cells in humans (73). A high proportion of peripheral B10 cells and progenitor (pro)-B10 are present in the $CD24^{hi}CD27^{+}$ B-cell subset, and approximately 60% also express CD38 (73). B10 progenitors and B10 cells have been identified in human (73). *Ex vivo*, human B10 progenitors can be driven to develop into B10 cells by lipopolysaccharide (LPS) or 5′-C-phosphate-G-3′ (CpG), or by CD40 ligation. *In vitro*, IL-21/CD40-receptor signaling pathways can promote the development and expansion of B10 cells by four million-fold to suppress the immune response. IL-21R signaling, together with major histocompatibility complex class II and CD40 cognate, interacts with $CD4^{+}$ T-cells and although not required for B10 cell development, are necessary for B10 cell effector functions that result in antigen-specific responses. Interestingly, BCR ligation augments human B-cell IL-10 responses to CpG (74). Whether human B10 cells develop into antibody-secreting cells, or enter the memory B10 cell subset, remains to be determined (75). B10 cells may represent a subset, which is similar to regulatory T-cells (76) (**Figure 4**).

B10 cells are able to control the immune response, but an excessive reaction from these cells may also promote tumor cell growth or chronic infection (77). It is possible that regulatory fine tuning by B-cells and IL-21 production by T-cells might be a key factor in maintaining immune tolerance (78). Most investigations of B10 cells have concentrated on autoimmune diseases (79), but a few have assessed their role in transplantation (80). A mouse islet T-cell transplantation study has demonstrated that B10 cells control immune responses (81).

B-CELL APOPTOSIS MEDIATED BY IL-21

The effects of IL-21 on B cells depend on the costimulatory signals that are received. In the absence of signal from a T cell (such as the T cell engaging CD40), BCR activation is required for IL-21-mediated B cell apoptosis (15, 29, 35). The balance between STAT1 and STAT3 is critical for IL-21-induced B-cell apoptosis in the IL-21 signaling pathway. STAT1 mainly acts in cell cycle arrest and apoptotic cell death (45, 47, 82, 83). By contrast, STAT3 mostly exerts an anti-apoptotic effect, especially in numerous malignancies where it is constitutively active (83). In some circumstances, IL-21 can induce apoptosis of B-cells activated *via* signals through the TLR, LPS, CpG, anti-IgM, and IL-4 (11, 15). Complete protection from IL-21-mediated apoptosis was not inhibited by other molecules involved in apoptotic pathways. Functional studies have demonstrated that IL-21 substantially inhibited proliferation and Bim-dependent apoptosis of activated mouse B-cells (47). Hagn et al. reported that CpG together with IL-21 may enhance their apoptosis-inducing and immunogenizing effects (84). It is therefore possible that combining CpG with IL-21 could more effectively induce apoptosis in B-cells than CpG or IL-21 alone. Furthermore, IL-21 can inhibit B-cell proliferation when receiving a strong signal *via* TLR while preventing apoptosis of B-cells *via* upregulation of B-cell leukemia/lymphoma-X linked (BCL-XL), an anti-apoptotic protein of the BCL-2 homology 3 (BH3) family (11, 85) (**Figure 5**). From this viewpoint, IL-21 appears to act as an immunosuppressive cytokine on B-cells. This finding indicates that the apoptotic effects of IL-21 may only be relevant in situations where a humoral immune response is improperly triggered, thereby shutting down at least one arm of the immune system before extensive damage is done (7).

GRANZYME B PRODUCTION BY B-CELLS

Interleukin-21 can induce BCR-stimulated human B-cells to differentiate into granzyme B-expressing cytotoxic cells (GrB) in a STAT3-dependent manner in the absence of a CD40 signal

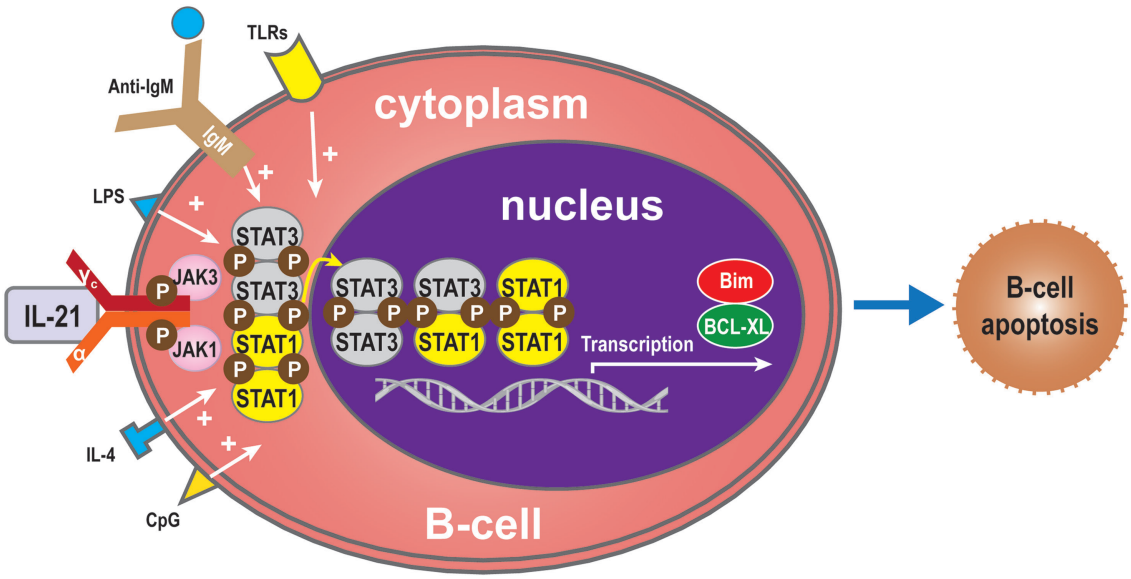

FIGURE 5 | B-cell apoptosis. IL-21 can also induce apoptosis of B-cells when activated by LPS, TLRs, CpG, anti-IgM, or IL-4 in the absence of T-cell signals. In the absence of such molecules, the balance between STAT1 and STAT3 regulates B-cell apoptosis *via* the IL-21 signaling pathway. STAT1 induces cell death, while, conversely, STAT3 exerts an anti-apoptotic effect. Bim also plays an apoptotic role and BCL-XL an anti-apoptotic role.

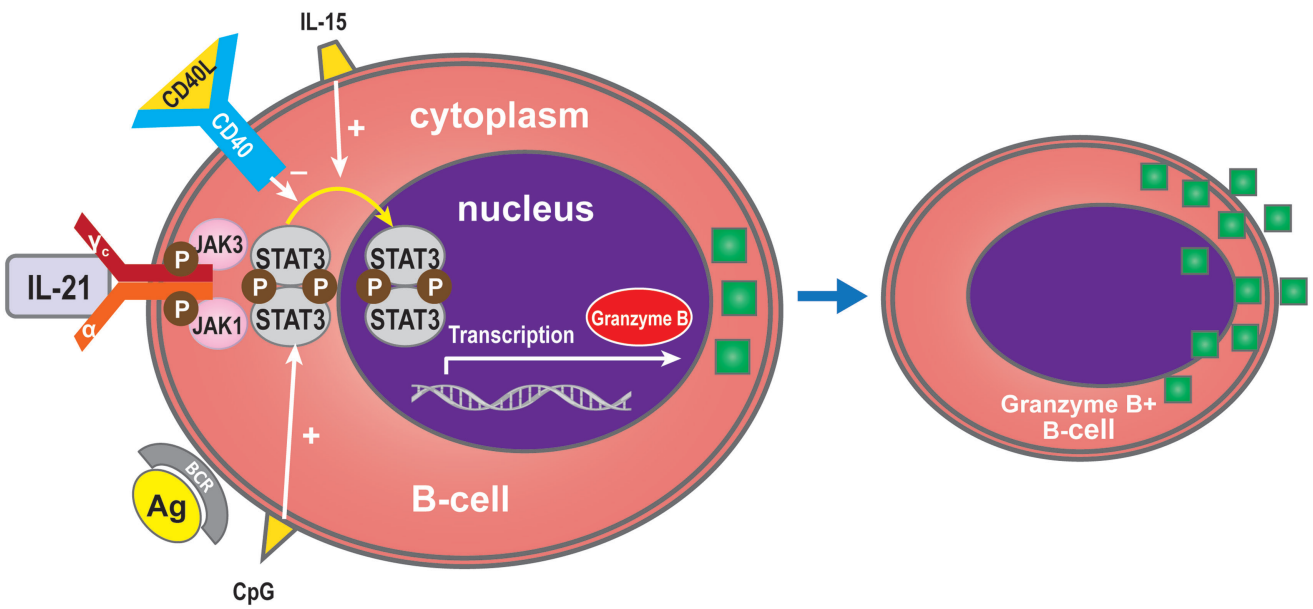

FIGURE 6 | Granzyme B expression by B-cells. IL-21 can induce BCR-stimulated B-cells to differentiate into granzyme B (GrB), an effect which is dependent on STAT3 and which is promoted by IL-15 or CpG. CD40 inhibits differentiation into GrB-cells.

(31, 77, 86–88). GrB$^+$ B-cell numbers are dependent on IL-21 production, and increasing doses of anti-IL-21 decreased the number of GrB-expressing B-cells in co-culture systems (78). The increase in GrB$^+$ B-cells in the circulation of tolerant recipients may be due to a direct effect of IL-21 (78). GrB secreted by B cells may play a key role in the regulation of immune responses (78, 89). Xu et al. showed that IL-21 initially triggers transcription of the GrB gene in B-cells, while STAT3 is required for GrB synthesis in PCs activated by IL-21 and IL-15. The defect in GrB formation in STAT3-deficient B-cells might arise from a lack of cell proliferation and differentiation (88). Recent *in vitro* studies have indicated that CD40 signaling in B-cells inhibits their differentiation into GrB$^+$ cells (31, 77) (**Figure 6**).

IL-21 AS A POSSIBLE PLAYER IN ALLOREACTIVITY AFTER TRANSPLANTATION

Antibody-mediated rejection is a major problem after organ transplantation mediated by anti-HLA antibodies and donor-specific antibodies (DSA). This poorly defined alloimmune response is refractory to treatment with conventional immunosuppression (1). From our recent studies, we know that in this process, B-cells can be activated by IL-21-producing Tfh cells and differentiate into Ig-producing plasma cells. We reported that these Tfh cells as well as B-cells infiltrate the allograft during rejection and colocalize in follicular-like structures in the transplanted kidney (4, 18). These tide clusters of T and B-cells form highly organized lymphoid structures named tertiary lymphoid organs (TLOs). Associations between the presence of these TLO and poor graft outcome have been reported (90–92). In contrast, Xu et al. reported that IL-17, and not IL-21, is responsible for lymphoid neogenesis. Therefore, they suggested that Th17, but not Tfh, cells could play a role in the process of lymphoid neogenesis (93). It is likely that infiltrated and organized T and B-cells contribute to the anti-donor response by antigen presentation of B cells and by help of Tfh cells to the infiltrated B-cells. Besides IL-21, the capacity of Tfh cells to provide help to B-cells depends upon the acquisition of molecules that are known to play functional roles in T-cell–B-cell interactions, such as CD40 ligand, inducible co-stimulator (ICOS), and programed death 1 (PD-1) (18, 33, 94, 95).

In organ transplantation, specifically targeting B-cells to decrease plasma cell differentiation by either IL-21-dependent B cell apoptosis or IL-21R blockade may provide novel approaches for the prevention of the development of *de novo* DSA and treatment of antibody-mediated rejection.

The first approach is speculative and based on the finding that IL-21 induces B-cell apoptosis when costimulation signals are absent (15, 29, 35). At the same time, IL-21 might stimulate the cytolytic functions of alloantigen activated CD8 T cells, the aggressors in acute rejection (96, 97). Therefore, we should be careful with IL-21 cytokine treatment. This strategy should first be tested in experimental animal models by using various concentrations of IL-21 to define if B cell apoptosis and T cell cytotoxicity rely on the same or different concentrations of IL-21. This knowledge is helpful to better understand the role of IL-21 in B-cell-mediated immune processes such as apoptotic cell death.

The second approach could be blockade of the IL-21 pathway proven to affect the production of pathogenic immunoglobulins in animal models of autoimmune diseases. In these studies, blockade of the IL-21R signaling pathway reduced B-cell-mediated diseases (98). Also, in a mouse model of islet transplantation, mIL-21R-Fc combined with CTLA-4-Ig diminished T-cell and B-cell effector functions, and tolerance was induced in a subgroup of treated animals (99). It is critical to determine whether neutralizing the IL-21 function also inhibits production of anti-HLA antibodies and DSA in organ transplant recipients. So far, such studies have not been conducted, but based on the biological functions of IL-21, the promising findings in animal models for autoimmune diseases and *in vitro* studies, targeting the IL-21 pathway could be expected to reduce the incidence of antibody-mediated alloreactivity. Our studies using peripheral T-cells and B-cells derived from kidney transplant patients showed that the interaction between IL-21-producing Tfh cells and B-cells could be inhibited by an IL-21 receptor antagonist. In these co-cultures, B-cell differentiation and IgM and IgG production were diminished (4). We believe that IL-21-producing Tfh cells play a dominant role in alloreactivity and should be targeted by novel immunosuppressive agents.

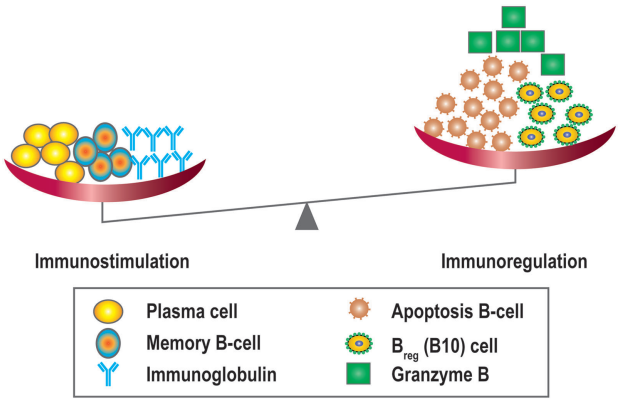

FIGURE 7 | Overview of the immunostimulatory and immunoregulatory effects of IL-21. IL-21 promotes B-cell proliferation, plasma cell differentiation, B-cell memory, and Ig class switching, and is also important for the development of IL-10 + regulatory B-cells (Bregs/B10), B-cell apoptosis, and granzyme B producing cells.

Like many other cytokines, IL-21 has multiple functions. In addition to its actions in B-cell apoptosis and differentiation it also drives regulatory B10 responses. These cells have been shown to suppress T-cell-mediated rejection induced by mismatched MHC molecules and prolong allogeneic islet T-cell survival, suggesting a potential regulatory role for B10 cells in organ transplantation (80, 81). Since IL-21 can promote regulatory B10 cell proliferation, harnessing the anti-inflammatory properties of B10 cells by anti-IL-21 agents could potentially stimulate antibody-mediated rejection and promote a less favorable tolerogeneic environment by modulating the plasma cell/Breg (B10) balance (68) (**Figure 7**). Recently, another type of Bregs was described, which could be inhibited by anti-IL-21 treatment. The number of GrB-producing B-cells with regulatory properties was significantly higher in tolerant patients compared to patients with stable graft function (78). This observation suggests that targeting the IL-21R pathway with immunosuppressive agents may harness this cell population. Data in this area, however, remain sparse.

CONCLUSION

In general, IL-21 promotes humoral immunity, and IL-21 blockade may attenuate B-cell hyperactivity in which also

costimulatory signals are involved. However, B-cells may have a dual effect, acting both as a driver and as a regulator of the immune system (78, 79, 100). In B-cells that recognize Ag and receive T-cell help, IL-21 induces survival, proliferation, isotype switching, and differentiation to Ig-secreting plasma cells or GrB-producing B-cells. B-cells can also cause cell death or, in the form of regulatory B10 cells, can induce autoimmunity if they receive a strong signal *via* BCR, or *via* TLR, and IL-21 costimulation. An equilibrium between effector and suppresser cells is necessary to maintain B-cell homeostasis and the immune balance, especially for the prevention of antibody-mediated transplantation rejection. Future studies should focus on elucidating details of the signaling cascades and downstream changes in gene and protein expression within B-cells in response to IL-21, either alone or in combination with other molecules. This knowledge may ultimately lead to an effective therapeutic strategy to overcome antibody-mediated rejection following transplantation, particularly by targeting the differentiation of B-cells into plasma cells *via* IL-21 signaling pathways.

AUTHOR CONTRIBUTIONS

YW, NB, YS, MH, LW, and CB researched the literature and wrote the review.

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REVIEW
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The Impact of HLA Class I-Specific Killer Cell Immunoglobulin-Like Receptors on Antibody-Dependent Natural Killer Cell-Mediated Cytotoxicity and Organ Allograft Rejection

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Natural killer (NK) cells of the innate immune system are cytotoxic lymphocytes that play an important roles following transplantation of solid organs and hematopoietic stem cells. Recognition of self-human leukocyte antigen (HLA) class I molecules by inhibitory killer cell immunoglobulin-like receptors (KIRs) is involved in the calibration of NK cell effector capacities during the developmental stage, allowing the subsequent recognition and elimination of target cells with decreased expression of self-HLA class I (due to virus infection or tumor transformation) or HLA class I disparities (in the setting of allogeneic transplantation). NK cells expressing an inhibitory KIR-binding self-HLA can be activated when confronted with allografts lacking a ligand for the inhibitory receptor. Following the response of the adaptive immune system, NK cells can further destroy allograft endothelium by antibody-dependent cell-mediated cytotoxicity (ADCC), triggered through cross-linking of the CD16 Fc receptor by donor-specific antibodies bound to allograft. Upon recognizing allogeneic target cells, NK cells also secrete cytokines and chemokines that drive maturation of dendritic cells to promote cellular and humoral adaptive immune responses against the allograft. The cumulative activating and inhibitory signals generated by ligation of the receptors regulates mature NK cell killing of target cells and their production of cytokines and chemokines. This review summarizes the role of NK cells in allograft rejection and proposes mechanistic concepts that indicate a prominent role for KIR–HLA interactions in facilitating NK cells for Fc receptor-mediated ADCC effector function involved in antibody-mediated rejection of solid organ transplants.

Keywords: antibody-mediated rejection, antibody-dependent cell-mediated cytotoxicity, human leukocyte antigen, killer cell immunoglobulin-like receptors, natural killer cells, donor-specific antibodies, solid organ transplantation, transplant rejection

ANTIBODY-MEDIATED REJECTION OF ORGAN ALLOGRAFT

The major hurdle to successful organ transplantation is graft rejection, a process orchestrated by sophisticated cell and antibody-mediated defense mechanisms, which has evolved primarily to combat invading microbes or diseased and damaged cells. The T cell-targeted immunosuppressive regimens (including T cell-specific antibodies, calcineurin inhibitors, mycophenolic acid, rapamycin, and prednisone) have effectively reduced the incidence of cell-mediated transplant rejection and have substantially improved 1-year graft survival to 88% following renal transplantation (1). Nevertheless, alloantibodies mediate a substantial proportion of the remaining graft rejection episodes, contributing to both early and late graft loss, particularly in sensitized populations such as patients with previous transplants and patients who have previously had multiple pregnancies or multiple blood transfusions (2).

Antibody-mediated rejection (ABMR) is recognized to be a key problem in organ transplantation and a major cause of late graft loss (3). Based on time course, the ABMR is classified as hyperacute, acute, or chronic (1). Hyperacute rejection, the first rejection phenotype observed in human organ transplantation, occurs immediately on perfusion of the transplanted organ with the blood of the recipient (4). Preformed donor-specific antibodies (DSAs) in recipient's blood bind to antigens expressed on vascular endothelium of the transplanted allograft [such as human leukocyte antigens (HLAs), ABO blood group antigens, and other endothelial antigens] and trigger a cascade of complement activation, which results in tissue injury involving blood vessel wall damage, hemorrhage, neutrophil infiltration, platelet, and fibrin deposition. Reliable cross-matching methods and screening recipients for preformed circulating HLA antibodies to the prospective donor have almost eliminated the incidence of this devastating phenotype (5, 6).

Acute ABMR occurs at any time from days to years following transplantation, and results from DSA that may be preexisting or develop *de novo* after transplantation (7). At present, acute ABMR is defined by four criteria: clinical evidence of acute graft dysfunction, histologic evidence of acute tissue injury, immunohistologic evidence for the action of DSAs (C4d deposition in peritubular capillaries), and DSAs detected in the serum (8). ABMR occurs in 6.7% of renal transplant patients and is present in approximately one-third of renal transplant patients diagnosed with acute rejection (9–11). Acute ABMR is characterized by a rapid rise in serum creatinine and is resistant to therapy with steroids or T cell-specific reagents.

Chronic ABMR develops over months or years before there are signs of graft dysfunction and is mediated by antibodies that develop *de novo*. The features of chronic ABMR in renal allografts include the following: duplication of the glomerular basement membrane, intimal cell proliferation of arterioles and infiltration with mononuclear cells, and lamination of the peritubular capillary basement membrane, which occurs together with the deposition of C4d in peritubular capillaries and glomeruli. Chronic ABMR is the result of cumulative damage to the kidney and over 50% of recipients develop chronic ABMR at 10 years after transplantation (12). A further category of rejection, subclinical rejection, has recently been recognized, and this refers to pathological injury in the graft that has been caused by antibody and/or T cells, but which has not yet resulted in graft dysfunction.

MECHANISMS INVOLVED IN ANTIBODY-MEDIATED REJECTION OF ORGAN ALLOGRAFT

The Y-shaped structure of IgG antibodies provides a bifunctional capacity to initiate and regulate host defense mechanisms in the following ways: antigen binding through the Fab (antigen-binding fragment) portion of the antibody and the interaction with immune cells and complement proteins (fragment crystallizable or Fc). Following DSA binding to the allograft endothelium, at least four distinct cellular and humoral mechanisms exert significant graft injury and failure (**Figure 1**).

Activation of the Complement Cascade

Complement fixation by antibody is essential for the pathogenesis of acute and hyperacute rejection (13). The binding of DSA to a cell surface antigen expressed on the allograft may trigger

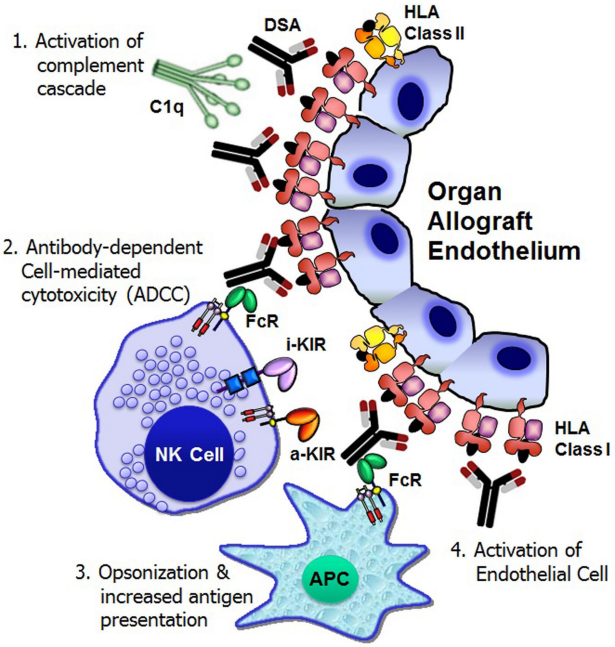

FIGURE 1 | Mechanisms of donor-specific antibody-mediated rejection of renal allografts. Donor-specific HLA antibody binding to the allograft endothelium may trigger four distinct cellular and humoral mechanisms that could result in significant graft injury and failure: (1) activation of complement cascade can cause direct injury to the capillary endothelium, (2) antibody-dependent cell-mediated cytotoxicity by natural killer cells, (3) opsonization and increased antigen presentation, and (4) activation and proliferation of endothelial cell. FcR, Fc receptor; i-KIR, inhibitory KIR; a-KIR, activating KIR; APC, antigen-presenting cell; C1q, a complement complex.

the classical complement pathway, a component of the innate immune system (14). The complement component C1q binds to structures in two or more Fc domains of IgM or IgG, which causes C1q to undergo a conformational change, which allows the enzymatic components C1r and C1s in the collagenous portion of the antibody-bound C1q to cleave C4 molecules (13). This initiation step then leads to the recruitment of other proteins in order to form the C3 convertase protein complex. Activation of C3 leads to the generation of two pro-inflammatory anaphylatoxins, C3a and C5a, and the membrane attack complex that eventually forms a pore in the membrane of the target and induces cell death. The classical pathway is only one of three methods of complement activation, the others being the alternative pathway and the lectin pathway, and all three pathways converge at the point of C3 cleavage (13). C4d is a split product of C4 groups, which remains covalently bound to the tissue and is thereby a durable *in situ* marker of complement activation. Detection of C4d deposition in capillaries has proved to be the most reliable marker of ABMR (15). Although the peritubular capillary C4d detection is important, it is not necessary to diagnosis ABMR, since the presence of DSA has the potential to cause transplant glomerulopathy and graft loss due to complement-independent mechanisms (16).

Antibody-Dependent Cell-Mediated Cytotoxicity

In addition to activating complement-dependent cytotoxicity against the allograft, antibodies can mount immune responses through interacting with Fc receptors (FcγRs), which are widely expressed throughout the hematopoietic system (17). Three different classes of FcγRs, known as FcγRI (CD64), FcγRII (CD32) with A, B, and C isoforms, and FcγRIII (CD16) with A and B isoforms, have been recognized in humans. Except FcγRIIIB that is present mainly on neutrophils, all other FcγRs are activating receptors. Innate immune effector cells, including monocytes, macrophages, dendritic cells (DCs), basophils, and mast cells, coexpress activating and inhibitory FcγRs, whereas B-cells express the inhibitory receptor FcγRIIB (17). Natural killer (NK) cells, particularly those with $CD56^{dim}$ $CD16^{+}$ phenotype express activating low-affinity FcγRIIIA. NK cells are regarded as the key effector cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC) function since NK cells are the only subset that do not coexpress the inhibitory FcγRIIB (18).

Infiltration of recipient NK cells into the renal (19), cardiac (20), lung (21), and liver (22) allografts shortly following transplantation have been observed indicating a role for human NK cells in solid organ transplantation. Direct evidence for the role of NK cells in microcirculation injury during ABMR comes from the findings of NK cells and NK cell transcripts in kidney biopsies from patients with donor-specific HLA antibodies (23, 24). Mechanistic studies confirming the role of DSA-dependent NK cell-mediated cytotoxicity in organ allograft rejections is lacking (25). However, clinical trials with cancer therapeutic antibodies have shown that the induction of NK cell-mediated ADCC have direct bearing on organ allograft rejection. For example, rituximab, a chimeric mouse-human IgG1 monoclonal antibody that recognizes the CD20 antigen expressed on mature B-cells, is used to treat patients with B-cell lymphomas and autoimmune disorders. Both quantitative and qualitative differences in NK cell function are correlated with rituximab clinical activity, suggesting that ADCC performed by NK cells may be a primary mechanism of rituximab activity (26). Furthermore, responses to rituximab may depend on polymorphisms present in the FcRIIIA receptor, a receptor mainly expressed on NK cells (27, 28). Several other antibodies are currently being evaluated in the clinic and, for many of them, their effect seems to be mediated at least in part by NK cell-mediated ADCC (29). In addition to ADCC, on FcγRIIIA stimulation, NK cells produce cytokines and chemokines, including interferon-γ (IFN-γ), which may induce HLA expression on endothelial cells, thus providing more antigenic targets for antibodies and shortening graft survival (30). More understanding of FcγRIIIA-mediated regulation of NK cell function is critical in order to define the role of NK cell transcripts in kidney biopsies from patients with donor-specific HLA antibodies.

Opsonization and Promotion of Antigen Presentation

In addition to their well-defined roles in triggering ADCC by NK cells, FcγRs regulate antigen presentation, immune complex-mediated maturation of DCs, B cell activation, and plasma cell survival, and therefore, FcγRs ultimately regulate the production and specificity of their ligands, antibodies (31). The ligation of Fab of the DSA to the alloantigen attracts phagocytes (neutrophils, monocytes, macrophages, and DCs) to infiltrate into the allograft. The Fc fragment of the antibody binds to an Fc receptor on the phagocyte, facilitating receptor-mediated phagocytosis, which accelerates the kinetics of the phagocytosis process (32). Phagocytosis initiates specific mechanisms that result in trafficking of the antigen–IgG immune complexes into compartments from which the antigens are processed into peptides for HLA class I and class II presentation to $CD8^{+}$ and $CD4^{+}$ T cells, respectively, thereby FcγRs bridge the humoral and cellular branches of the adaptive immune response.

Activation of Endothelial Cells

The *in vitro* experiments of anti-HLA antibody ligation have shown that HLA class I molecules expressed by endothelial cells stimulates endothelial cell activation and proliferation (33, 34). Endothelial cell proliferation may be at least partly causative of arterial intimal thickening that is characteristics of chronic allograft rejection.

NATURAL KILLER CELLS LINK INNATE AND ADAPTIVE IMMUNITY

Natural killer cells are the third population of lymphocytes defined by the $CD3^{-}$ $CD56^{+}$ cell surface phenotype, and they represent 5–25% of the mononuclear cell fraction of normal human peripheral blood (35). NK cells share several features with $CD8^{+}$ cytolytic T-lymphocytes in their development, morphology, cell surface phenotypes, killing mechanism, and

cytokine production (36). NK cells were originally described as innate lymphocytes capable of lysing target cells quickly by direct cytotoxicity in an antigen-independent manner without the "priming" period required by T-cells (37). NK cells are recognized to express a sophisticated repertoire of activating and inhibitory receptors that are calibrated to ensure self-tolerance, while exerting early assaults against virus infection (38) and tumor transformation (39). In addition to cytolytic functions, NK cells produce high levels of IFN-γ and a wide range of pro-inflammatory cytokines and chemokines, which contribute to the shaping of adaptive immune responses (40). Recently, NK cells have been shown to mount antigen-specific immunologic memory, a hallmark characteristic of adaptive immunity (41). Having properties of both innate and adaptive immunity, NK cells spontaneously lyse target cells, as well as function as regulatory cells influencing subsequent antigen-specific T-cell and B-cell responses.

NK CELLS IN SOLID ORGAN TRANSPLANTATION

Experiments with rodent models clearly indicate a role for NK cells in acute and chronic allograft rejection (42–44). The most convincing evidence of NK cell-mediated rejection was observed with the heart allograft missing-self-MHC class I in CD28-deficient recipient mice; in this model, rejection is prevented by depletion of host NK cells (45). NK cells play a crucial role in mediating long-term kidney allograft injury (46). Currently, used clinical regimen of immunosuppressive agents such as cyclosporine A (47), FK506 (48), mycophenolate mofetil (49), azathioprine (50), and rapamycin (51) appears not to abrogate NK cell function. NK cell number and the cytotoxicity function were preserved to a greater extent in a regimen of tacrolimus and mycophenolate mofetil than they were with cyclosporine A and azathioprine 12 months after kidney transplantation (52). Even in the presence of polyclonal anti-thymoglobuline antibody that depleted T and NK cells transiently, the NK cell effector function is preserved after kidney transplantation (53).

NK CELLS USE A COMPLEX RECEPTOR–LIGAND SYSTEM TO DISTINGUISH NON-SELF FROM THE SELF

Natural killer cells use very complex and specific receptor–ligand system that integrates signals triggered by an array of inhibitory and activating receptors, which trigger cytotoxicity and the secretion of chemokines and cytokines (54, 55). Unlike T- and B-lymphocytes, NK cells do not express receptors that require somatic gene rearrangements to generate receptor diversity and specificity. Instead, NK cells express a wide array of conventional germline-encoded receptor families with inhibitory or activating functions that scan for missing-self, induced-self, and altered-self on target cells. The well-characterized NK cell receptor gene families include killer cell immunoglobulin-like receptors (KIR), killer cell lectin-like receptors, leukocyte immunoglobulin-like receptors, and natural cytotoxicity receptors (56–59).

KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTORS AND HLA CLASS I LIGANDS

The KIRs are crucial for human NK cell development and function (56, 58, 60) (**Figure 2**). The *KIR* gene family does not exist in rodents and found only in primates, and therefore *KIR* genes are considered to be originated recently and evolved rapidly (61, 62). The *KIR* gene family consists of 16 highly homologous genes clustered at the leukocyte receptor complex on chromosome 19 (63, 64) (**Figure 3**). Seven of them encode inhibitory KIRs (3DL1–3, 2DL1–3, and 2DL5), six encode activating KIRs (3DS1, 2DS1–2DS5), one encode a KIR that can trigger both inhibitory and activating signals (2DL4), and two are pseudogenes (*2DP1* and *3DP1*) that do not encode a cell surface receptor. By recognizing specific HLA class I ligands, the inhibitory KIRs trigger signals that stop NK cell function, while the ligands for activating KIRs are not elucidated. Genetic association studies suggest the possibility of activating KIRs recognizing cell surface determinants expressed following infection or tumor transformation, or under certain physiological, stress such as transplantation (65).

In general, humans have two copies of each autosomal gene, one per chromosome. However, due to deletion and duplication, the basic diploid rule does not apply to *KIR* gene family. The number and type of *KIR* genes vary substantially between haplotypes, and all *KIR* genes display sequence polymorphism (66) (**Figure 3**). On the basis of gene content, *KIR* haplotypes are broadly classified into two groups (67). Group A haplotypes have a fixed gene content (*KIR3DL3–2DL3–2DP1–2DL1–3DP1–2DL4–3DL1–2DS4–3DL2*) that encode four inhibitory KIRs, 2DL1, 2DL3, 3DL1, and 3DL2, specific for four major HLA class I ligands, C2, C1, Bw4, and A3/A11, respectively, and an activating KIR 2DS4, which is weakly specific for some HLA-C allotypes (C1 or C2 epitope), as well as the HLA-A3/11 epitope (**Figure 2**). In contrast, group B haplotypes are variable both in numbers and combinations of *KIR* genes, and comprising several genes (*2DL2*, *2DL5*, *2DS1*, *2DS2*, *2DS3*, *2DS5*, and *3DS1*) that are not part of the A haplotype (63, 68, 69). Moreover, B haplotypes possess KIRs that have no binding to HLA class I ligands, such as KIR2DL5, 2DS2, 2DS3, and 2DS5. While group A haplotypes contain only *KIR2DS4* as an activating gene, group B haplotypes contain up to five activating *KIR*s – *KIR2DS1*, *2DS2*, *2DS3*, *2DS5*, and *3DS1*. Inheritance of paternal and maternal haplotypes comprising different *KIR* gene contents generates human diversity in *KIR* genotypes (70). For example, homozygotes for group A haplotypes have only seven functional *KIR* genes, while the heterozygotes for group A and certain group B haplotypes may have all 14 functional *KIR* genes. All human populations have both group A and B *KIR* haplotypes, but their incidences vary substantially among populations (71–74). The A and B haplotypes are equally distributed in Africans and Caucasians, while the A haplotype is overrepresented in Northeast Asians (Chinese, Japanese, and Koreans) and the B haplotype occurred most frequently in the indigenous populations of India, Australia, and America (75).

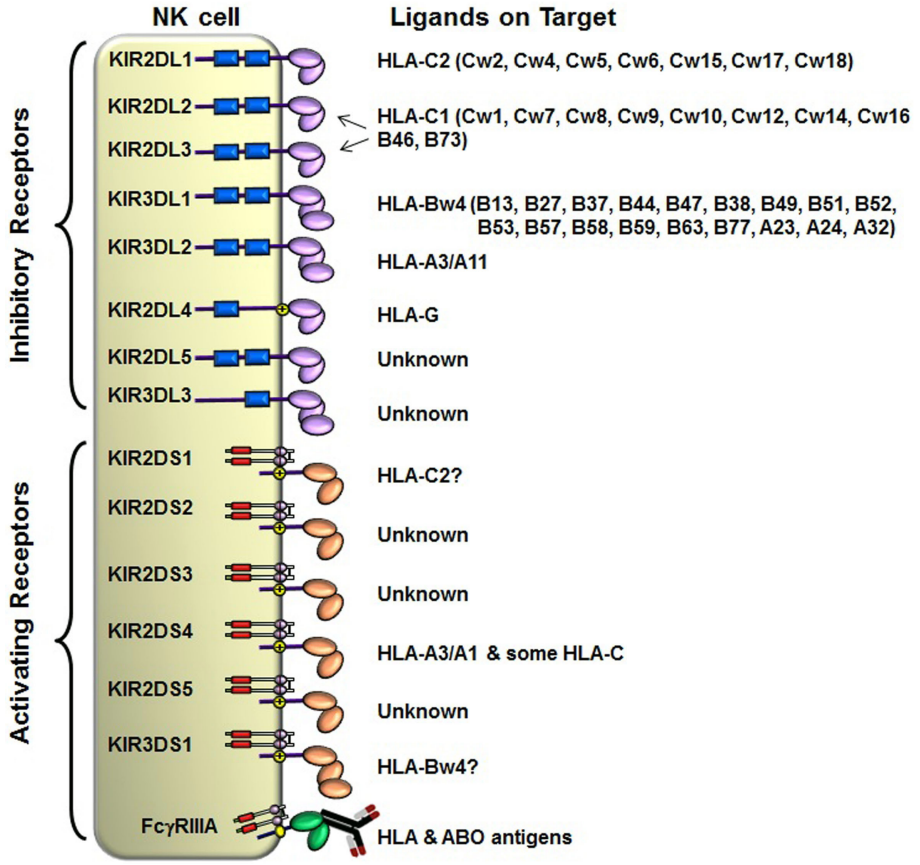

FIGURE 2 | Killer cell immunoglobulin-like receptor (KIR) and human leukocyte antigen (HLA) class I ligands. Fourteen distinct KIRs have been characterized in humans that comprise either 2 or 3 (2D or 3D) Ig-like domains and either a long (L) or short (S) cytoplasmic tail. Six KIRs are activating types and the remaining KIRs are inhibitory types. The cytoplasmic tails of the inhibitory KIRs carry an ITIM motif (shown as blue boxes) that trigger inhibitory signals upon binding to distinct HLA class I ligands. The short-tailed activating KIRs lack ITIM, but carry a positively charged amino acid residue in the transmembrane region (shown by yellow circle with + mark) that allows the interaction with an adopter chain DAP-12. The DAP12 contains ITAM motifs (shown as red boxes), which trigger activating signals upon the short-tailed KIR bound to a relevant ligand.

NK CELLS USE THREE DISTINCT MECHANISMS TO INJURE ALLOGRAFT TISSUE

The recipient NK cells can recognize and respond against the allograft by three possible mechanisms: missing-self recognition, induced-self recognition, and ADCC (**Figure 4**) (76). Because NK cells circulate in a state that can spontaneously deliver effector function, it is critical that they do not attack surrounding healthy cells. To prevent such detrimental autoreactivity, NK cells express an array of inhibitory receptors recognizing self-HLA class I molecules (**Figure 4A**). Expression of four distinct HLA class I molecules (HLA-A, -B, -C, and -E) on normal healthy cells provides ligands for various inhibitory receptors of NK cells and, consequently, are resistant to NK cell attack. Downregulation of HLA class I expression due to certain viral infections, neoplastic transformations, or absence of relevant HLA class I ligands on the allograft at the setting of allogeneic transplantation, alleviates inhibitory signals, permitting NK cells to eliminate these unhealthy or allogeneic target cells, a phenomenon originally described as the "missing-self" hypothesis (77) (**Figure 4B**, i). In addition to the "missing-self" mechanism, the expression of ligands for activating receptors on stressed target cell surface might also contribute to NK cell attack, known as "induced-self" recognition (**Figure 4B**, ii). The activation receptors can directly recognize stress-induced ligands associated with certain physiological conditions, such as infection, tumor transformation, and transplanted allograft (58, 78). The third mechanism is mediated *via* an ADCC (**Figure 4B**, iii), in which NK cells are activated through the low-affinity Fc receptor for IgG FcγRIIIA (CD16) by binding to the Fc portion of DSA. In summary, the NK cells discriminate the stressed unhealthy cells or allograft from the healthy self by gauging the net input of activating and inhibitory signals perceived from the NK cell receptors upon their interactions with target cell ligands.

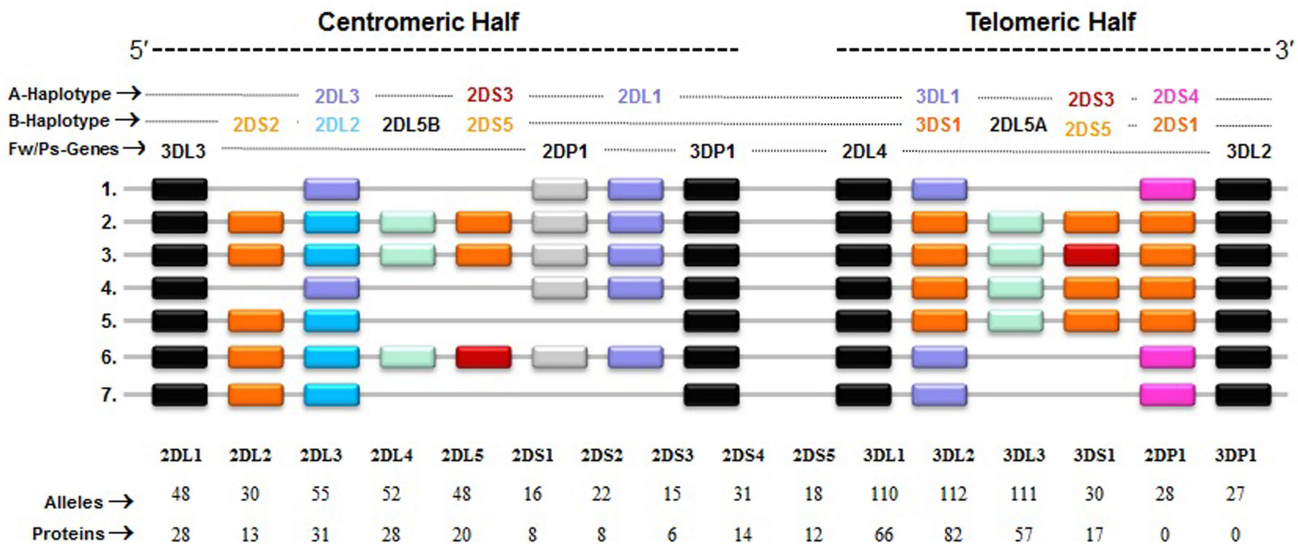

FIGURE 3 | **Killer cell immunoglobulin-like receptor (KIR) haplotypes vary in gene content**. Map of common *KIR* haplotypes in Caucasian populations. Each box represents a *KIR* gene. Haplotype#1 represents group-A *KIR* haplotype and the remainder are group-B haplotypes. The framework (Fw) genes, present in all haplotypes are shown in gray; genes encoding activating KIR are in pink (A haplotype-specific) or red/orange (B haplotype-specific); those for inhibitory receptors are in purple (*A* haplotype-specific) or blue (B haplotype-specific); and Pseudogene (Ps) *2DP1* is in white. All *KIR* genes are polymorphic, and the number of alleles and proteins characterized for each *KIR* gene is indicated.

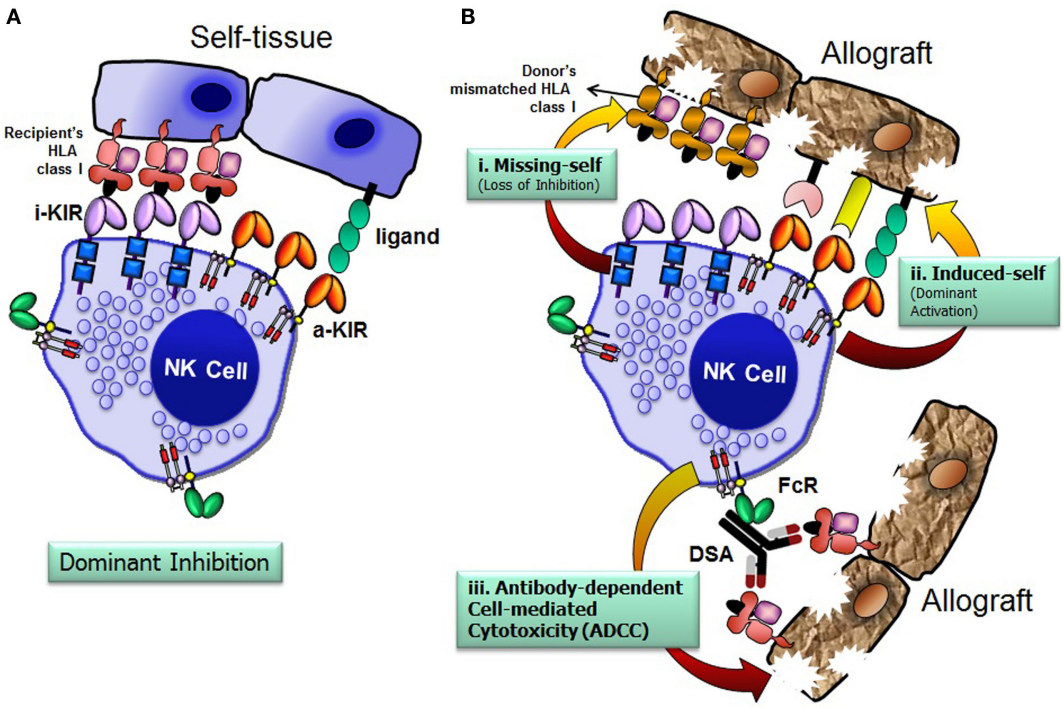

FIGURE 4 | **Natural killer (NK) cells distinguish allograft lacking self-human leukocyte antigen (HLA) class I molecules**. The net signal integrated from the inhibitory and activating receptors determines the effector function of NK cells. NK cells spare healthy cells that express high levels of HLA class I molecules and low levels of ligands for activating receptors **(A)**. Recognition of cognate HLA class I ligands on a healthy cell by inhibitory receptors expressed by NK cells prevents lysis of the healthy cell **(A)**. NK cells recognize and injure allograft that has either disparate HLA class I (i. missing-self; loss of inhibition), express high levels of ligands for activating receptors (ii. induced-self; dominant activation), and/or iii. donor-specific HLA antibody-dependent cell-mediated cytotoxicity **(B)**. FcR, Fc receptor; i-KIR, inhibitory KIR; a-KIR, activating KIR; DSAs, donor-specific antibodies.

CLONAL EXPRESSION OF KIR AND ACQUISITION OF NK CELL TOLERANCE AND RESPONSIVENESS

Similar to T- and B-lymphocytes, NK cells are developed from $CD34^+$ hematopoietic stem cells in the bone marrow and undergo terminal maturation in secondary lymphoid tissues (79–81). A signature feature of KIR is their clonal expression on NK cells, so that each NK cell clone in a person expresses only a portion of the genes within their *KIR* genotype (82–84). Stochastic expression of different combinations of receptors by NK cells results in this repertoire of NK clones with various ligand specificities. Once a given KIR is expressed on an NK cell clone, it is maintained in a stable way in the progeny of the clone. The process that establishes these clonal patterns is based on epigenetic regulation by DNA methylation and histone modifications (85–87).

Because *KIR* and *HLA* genes are located on different chromosomes (*KIR* on chromosome 19 and *HLA* on chromosome 6), *KIR* genes are inherited independently from *HLA* genes, and KIR may be expressed in the absence of their HLA ligands (88). Most, but not all, NK cell clones in peripheral blood express at least one inhibitory receptor for self-HLA class I (82). Only those NK cell clones expressing at least one inhibitory KIR specific for self-HLA class I molecule are "licensed," or functionally active, to eliminate target cells that have downregulated or which are missing the respective HLA class I ligands (74, 78, 89–91). A conceivable explanation for NK cell licensing is that inhibitory KIRs, upon specific interaction with self-HLA class I allotypes, deliver a signal resulting in NK cell maturation and acquisition of effector function. NK cells lacking inhibitory receptors for self-HLA class I molecules are considered to be developmentally immature, "unlicensed," and substantially hyporesponsive to HLA class I-negative targets (74, 89, 92, 93). Therefore, the NK cell responsiveness is most fundamentally distinguished by the presence or lack of inhibitory KIR for self-HLA class I. Licensed NK cells further vary in effector function quantitatively according to the strength of the inhibitory KIR and HLA interactions and the copy number of the corresponding inhibitory *KIR* and *HLA* genes (94–96). In summary, KIR receptor–HLA class I ligand interactions at the developmental stage set the functional threshold for NK cell and regulate NK cell effector function.

KIR–HLA INTERACTIONS CAN MODULATE THE DSA-DEPENDENT NK CELL-MEDIATED CYTOTOXICITY AGAINST ORGAN ALLOGRAFT

Polymorphic variation among the *KIR* and *HLA* class I genes and their resulting impact on the KIR and HLA interaction constitute a major source of variability in NK cell responsiveness (94–96). These differences influence clinical outcomes in diverse settings, including monoclonal antibody therapy for lymphoma (97), transplantation for hematological malignancies (98), kidney transplantation (99, 100), and other settings in which NK cell involvement contributes to disease control and clinical responses (101–105). However, not all KIR^+ licensed NK cells are equivalent, as polymorphic diversity in the *KIR* and *HLA* genes underlie significant variation in binding strength and specificity, which quantitatively influence licensing, inhabitability, and ADCC (104, 106–111).

However, studies supporting a role for licensing in human ADCC are limited. A prominent role for KIR3DL1/HLA-Bw4 interactions in licensing NK cells for CD16-mediated effector function was published recently (112). When individuals expressed both inhibitory KIRs that interact with HLA-C and the corresponding HLA-C ligand, their NK cells exhibited greater general and Fc receptor-mediated effector functions than NK cells from those individuals lacking the relevant HLA-C ligand (74). Similarly, expression of KIR3DL1, an inhibitory KIR that interacts with the HLA-Bw4 public epitope, was associated with higher NK cell cytotoxicity and IFN-γ production upon exposure to HLA class I-deficient target cells when the NK cells were isolated from HLA-Bw4 donors (94, 112). Therefore, the interindividual differences in compound *KIR* and *HLA* class I ligand genotypes associated with differences in NK cell reactivity would impact DSA-mediated NK cell ADCC against the organ allograft. The individualized assessment of the recipient's KIR, FcR, HLA types, HLA antibodies, and the donor's HLA types at the molecular and functional levels have the potential to distinguish between mechanisms that could guide identification of new therapeutic targets for ABMR.

CONCLUDING REMARKS

The complement-independent mechanisms that lead to the ABMR of kidney allografts remain poorly understood. Recent studies finding a link between ABMR and abundance of NK cell molecular signatures in transplant biopsy suggest relevance of NK cells as innate immune cytotoxic effectors to antibodies through ADCC. However, the direct pathogenic role of donor-specific HLA antibody-mediated NK cytotoxicy in transplant rejection remains poorly documented by mechanistic studies. NK cells use very complex and specific germline-encoded KIR receptor and HLA class I ligand system that integrate signals triggered by an array of inhibitory and activating receptors, which set NK cell maturation and acquisition of effector function. Moreover, the FcγRIIIA polymorphisms and expression levels can also modulate NK cell activation against allograft. Future studies that integrate both recipient factors (such as KIR receptors, HLA class I ligands, FcγRIIIA polymorphisms, and donor-specific HLA antibodies) and donor factors (such as HLA class I ligand compatibility with recipient) that establish variable KIR–HLA conditioned NK cell-FcγRIIIA-antibody–antigen interactions will identify potential interindividual variability of humoral alloimmune responses.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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