SENSITIZATION AND DESENSITIZATION IN ORGAN TRANSPLANTATION

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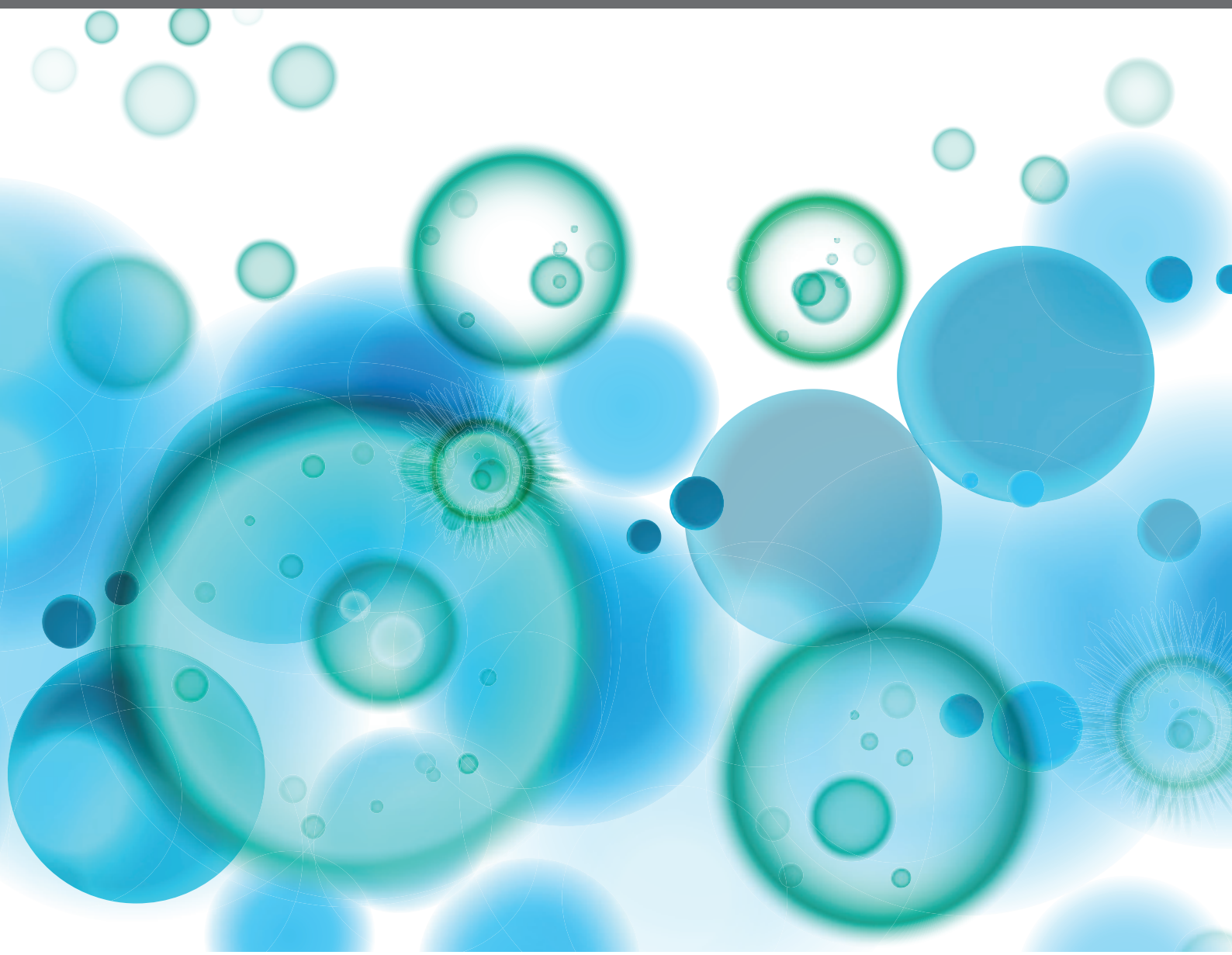

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SENSITIZATION AND DESENSITIZATION IN ORGAN TRANSPLANTATION

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Table of Contents

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EDITORIAL
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Editorial: Sensitization and Desensitization in Organ Transplantation

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Keywords: sensitization, desensitization, transplantation, donor specific antibodies, antibody-mediated rejection (AMR)

Editorial on the Research Topic

Sensitization and Desensitization in Organ Transplantation

Transplantation is the gold standard treatment for end-stage organ failure. Sensitization, the prior exposure to non-self human leukocyte antigen (HLA), is a major impediment to transplantation as it reduces the chances of finding a suitable donor and places individuals at heightened immunological risk, resulting in worse transplant outcomes. As such, there has been great interest in understanding the immunologic nature of sensitization and developing therapies to desensitize these patients and improve outcomes. This Research Topic, encompassing 14 contributions from several groups, aims at providing updates on various topics related to sensitization – current organ-specific practices regarding management of the sensitized patient, etiology of sensitization, desensitization approaches, and new tools available to study sensitization. The consequences and management of sensitization across transplant organ type varies dramatically and comprehensive reviews of the current management of sensitized patients in heart (Habal), lung (Young et al.), and vascularized composite allotransplantation (Moris and Cendales) are included in this special Research Topic edition.

Sensitization occurs through a variety of different modalities, namely prior transplantation, blood transfusions, and pregnancies. While those modalities lead to similar consequences, exposing individuals to non-self HLA, it is unknown whether the immunologic responses related to those exposures are similar across modalities. Nguyen et al. demonstrated, in a retrospective cohort of sensitized heart transplant candidates, desensitization protocols were less efficacious in women, particularly women sensitized due to prior pregnancies. This suggests that not every sensitization event is equal; rather, there exists key differences in the immunologic response to non-self HLA exposure, leading to heterogenous outcomes regarding desensitization. The unique "immunologic paradox" of pregnancy as a sensitization event is thoroughly reviewed by Nellore et al. with an emphasis on memory B cell formation and its correlation with pregnancy-related sensitization.

Screening for circulating HLA antibodies is standard of care prior to transplantation as part of the risk assessment process to optimize donor selection and transplant outcomes. HLA-specific Luminex assays permit sensitive antibody screening across hundreds of HLA alleles. However, these assays are seldom used in research given their prohibitive cost and unavailability for MHC molecules relevant to common animal transplant models. Song et al. described a novel set of techniques that allow for specific measurements of major histocompatibility complex (MHC)

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sensitization across various species. These tools offer the promise of in-depth characterization of MHC mismatches in animal models commonly used to study sensitization in transplantation.

Various approaches have been proposed to improve organ availability and outcomes for sensitized patients. Donor allocation policies have been modified in recent years to reduce the gap between transplantation rates between non-sensitized and sensitized individuals. The Kidney Allocation System (KAS) was implemented in the United States in 2014 to facilitate kidney transplantation in highly sensitized individuals by increasing the pool of donors and giving priority to highly sensitized individuals, resulting in increased transplant rates. The Eurotransplant Acceptable Match (AM) program was founded in 1989 with the goal of increasing transplantation rates in highly sensitized patients in the region. Their matching algorithm takes into account the recipient's HLA antigens in combination with pre-defined acceptable antigen mismatches. Heidt et al. summarized the AM program experience and showed high organ offer (80%) and similar outcomes compared to non-sensitized patients regarding rejection and long-term graft survival. The use of predefined acceptable antigen mismatches when matching sensitized individuals is thus a promising approach to increase transplant rate in allocation systems.

Despite efforts to optimize allocation systems, the most highly sensitized individuals (CPRA > 99.9%) remain on the waitlist longer than non-sensitized patients and experience greater mortality and morbidity. Desensitization has long been advocated as a mean to achieve transplantation in this high-risk population. Schinstock et al. provided thoughtful guidance regarding which patients might benefit from desensitization prior to kidney transplantation in the current KAS era.

A myriad of pharmacologic and non-pharmacologic treatments have been brought forward as desensitization regimens and were thoroughly reviewed in Choi et al.. However, no desensitization therapy has yet solved the barriers to transplantation faced by highly sensitized patients. Kumar and Locke succinctly describe recent advances in the desensitization field, including novel promising pharmacologic approaches and development of novel assays that allow for precise HLA epitope matching. Plasmapheresis remains the mainstay of many protocols aiming at removing circulating donor-specific antibodies (DSA). Manook et al. investigate modulation of the neonatal Fc receptor (FcRn) as a pharmacological alternative to plasmapheresis. Inhibition of FcRn successfully lowers donor-specific antibody (DSA) titers, as shown by reduced T-cell and B-cell crossmatch, in a sensitized non-human primate model. However, the therapy did not prevent rapid DSA rise post kidney transplant or prolong graft survival. Nevertheless, inhibition of FcRn does show potential to reduce DSA in a pre-transplant setting with fewer side effects compared to plasmapheresis.

Plasma cells, the main producers of antibodies, are an obvious target to manage the humoral response in sensitized patients. However, conventional therapies fail to deplete plasma cells given their lack of CD20 expression. CD38-targeting immunotherapies have played an increasingly important role as plasma cell-depletion agents in myeloma treatments. Joher et al. provided a thorough review of those immunotherapies and their possible application to organ transplantation. In this review, it was astutely noted that anti-CD38 therapies induce immune deviation, a phenomenon thought to enhance host-anti-tumor immune responses but with possible deleterious consequences in organ transplantation. Depletion of CD38+ immunosuppressive cells across multiple lineages may be responsible for the elevated antiviral protection as well as elevated alloreactive functional responses.

Sensitized individuals are at a higher risk of developing antibody-mediated rejection (AMR) compared to non-sensitized patients. Histopathology is central to the diagnosis of AMR and the Banff 2019 classification system is commonly used to diagnose AMR. Despite its widespread use, this classification system contains several limitations such as lack of reproducibility and is based on biopsy findings without incorporating clinical features. In her review article, Dr. Cornell elegantly reviewed the most recent Banff classification for AMR, focusing on how it relates to patients undergoing desensitization or undergoing incompatible transplants and offered a possible framework for considering allograft injury associated with DSA as a continuum.

Finally, strategies to induce tolerance would minimize sensitization and abrogate the need for desensitization therapies and life-long immunosuppression. Cellular therapies have been explored since the 1950s to induce non-responsiveness to transplanted allografts. Despite various trials, the balance between efficacy and safety has limited broader implementation. Shaw et al. thoroughly reviewed the history of cellular therapies as they pertain to solid organ transplantation and contrast their salutary effects with their potential to induce sensitization.

To summarize, the collection of review and research articles presented under this Research Topic provides a comprehensive set of information on advances in sensitization and desensitization in solid organ transplantation. Further understandings of the etiology, mechanisms, and consequences of sensitization should provide chances to develop novel therapies to facilitate transplantation in the highly sensitized population.

AUTHOR CONTRIBUTIONS

IA, AJ, JL, and JK participated in writing and reviewing the manuscript.

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REVIEW
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Current Approaches to Desensitization in Solid Organ Transplantation

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Major advancements in the development of HLA antibody detection techniques and our understanding of the outcomes of solid organ transplant in the context of HLA antibody have occurred since the relevance of sensitization was first recognized nearly 50 years ago. Additionally, kidney paired donation programs (KPD) have become widespread, deceased donor allocation policies have changed, and several new therapeutic options have become available with promise to reduce HLA antibody. In this overview we aim to provide thoughtful guidance about when desensitization in kidney transplantation should be considered taking into account the outcomes of HLA incompatible transplantation. Novel therapeutics, desensitization endpoints, and strategies for future study will also be discussed. While most of our understanding about desensitization comes from studying kidney transplant candidates and recipients, many of the concepts discussed can be easily applied to desensitization in all of solid organ transplantation.

Keywords: desensitization, sensitization, kidney transplantation, donor specific antibody (DSA), crossmatch, single antigen bead assays (SAB), antibody mediated rejection, kidney paired donation

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INTRODUCTION

Our understanding and perspectives surrounding desensitization in kidney transplant candidates has advanced in the last 2 decades as HLA antibody detection and measurement techniques have improved, leading to better clarity about who is likely to benefit from aggressive desensitization approaches. In this overview we aim to provide guidance about when desensitization should be considered. Novel therapeutics, desensitization endpoints, and strategies for future study will also be discussed. While most of our understanding about desensitization comes from studying kidney transplant candidates and recipients, many of the concepts discussed can be easily applied to desensitization in all of solid organ transplantation.

Abbreviations: DSA, donor specific antibody; HLA, human leukocyte antigen; PRA, panel reactive antibody; SAB, single antigen bead; cPRA, calculated panel reactive antibody; KPD, kidney paired donation; ABMR, antibody mediated rejection; MFI, mean fluorescence intensity.

WHAT IS SENSITIZATION AND HOW IS IT MEASURED?

The significance of alloantibody was made evident early in transplantation when Dr. Terasaki observed the correlation between early allograft loss and *in vitro* lysing of donor cells after application of recipient serum (e.g. a positive crossmatch) (1). Other notable observations from this landmark study were that patients with prior exposure to alloimmune sensitization including females with a history of pregnancy or recipients of prior transplants, had a higher incidence of immediate failure (1). At that time it was believed that alloantibody was only relevant in the early post-transplant period, but eventually it was acknowledged that donor-specific alloantibody (DSA) towards HLA (human leukocyte antigen) was a major contributor to long term allograft loss through chronic active antibody mediated rejection (2), and thus DSA to HLA is avoided if possible. Recently there has been interest in better understanding the contribution of non-HLA antibody to rejection and graft loss. This is an evolving area of study with many unanswered questions. It also remains unclear whether the presence of non-HLA antibody leads to reduced access to transplantation, thus this review will be focused on sensitization in the context of HLA antibody only.

Detecting HLA antibody in a transplant candidate's serum and measuring sensitization to determine which HLA antigens must be avoided at transplant is a critical first step to avoid DSA. Historically, cell-based panel reactive antibody (PRA) testing was performed to assess sensitization. Attempts were made to use cell panels representing the donor pool in order to estimate the proportion of the population to which the candidate would likely have preexisting DSA, however precise identification of individual antibody specificities was difficult. Techniques to detect and measure HLA-alloantibody have substantially improved. Currently sensitive single antigen bead (SAB) solid phase assays comprised of broad panels of fluorescent HLA-coated microbeads are available that allow for specific HLA alloantibody determination and semi-quantitative measurement. The results of these tests can be used to determine the calculated panel-reactive antibody (cPRA) and establish the breadth of sensitization to predict the probability of finding a donor against whom the recipient has no antibody (3). The cPRA ranges from 0-100% and can be easily calculated with a readily available online calculator that now provides the detailed cPRA (e.g. cPRA 99.555999%) (3). In the United States, the cPRA is used for determining deceased donor allocation priority.

Importantly, the cPRA is dependent upon which antigens are considered unacceptable by a specific transplant center based on the solid phase assay results. For example, a center with minimal risk tolerance for preformed DSA may exclude antigens when the corresponding MFI (mean fluorescence intensity) is very low (e.g. >500), while other centers with more tolerance for antibody mediated rejection (ABMR) risk may use a higher MFI cutoff.

Solid phase assays are almost universally used to measure sensitization clinically, but it is essential to understand that these tests have inherent limitations and do not measure immunologic memory. This is a critical point as the potential of immunologic memory response to the transplant organ has clinical relevance. Immunologic memory is defined as the robust response from the immune system when a foreign antigen that was previously encountered by the immune system is reintroduced, leading to reactivation of memory T and B cells. Immunologic memory can occur as a result of sensitizing events such as pregnancy, prior kidney transplant, blood transfusion, and implants such as homografts. In other words, ABMR remains possible, albeit at low risk, even when solid phase and lymphocyte crossmatch testing using current serum samples are completely negative. Therefore, the results of solid phase assays must be interpreted in the context of a patient's sensitization history and historic results if available. A variety of techniques are available to study antigen-specific B-cell responses in the research setting, but none have been validated to be used routinely in the clinical setting (4).

Terms such as *highly sensitized* are routinely used in the field of transplantation without a universal meaning. Historically even patients with a cPRA of low as 30 percent may have been considered *highly sensitized* prior to the widespread use of kidney paired donation (KPD) programs and prioritization of sensitized patients in deceased donor allocation schemes. In the current era, it is best to avoid terms such as *highly sensitized* and instead report the cPRA and mode of sensitization which is more informative in terms of a patient's allocation priority, probability of receiving an organ offer, and risk of antibody mediated rejection (ABMR).

WHO DERIVES THE MOST BENEFIT FROM DESENSITIZATION IN THE CURRENT ERA?

Desensitization protocols are generally used for the following two reasons: 1) to increase transplant candidates' access to transplantation by decreasing HLA antibody and the number of unacceptable antigens for listing (e.g. reduction in cPRA), or 2) to decrease known DSA prior to a planned positive crossmatch transplant to reduce the risk of immediate graft loss from catastrophic hyperacute rejection. The term desensitization has often been used loosely to refer to any treatment given in the context of known donor specific antibody or positive crossmatch even if the treatment was not expected to decrease alloantibody (e.g. complement inhibitors).

In the last two decades, the need for desensitization or HLA incompatible transplantation has evolved. Before widespread kidney paired donation and changes in donor allocation schemes to prioritize sensitized patients, there was a great need for effective desensitization therapy and positive crossmatch transplantation because of the increasing number of sensitized patients on the waiting list with prolonged waiting times and disproportionately low rates of transplantation particularly for patients with a cPRA > 95% (5). In an attempt to overcome the humeral barriers to transplantation aggressive desensitization strategies were employed. While these treatments did allow some

patients to get transplanted, their effectiveness was variable and graft failure from long term chronic active ABMR was common (6, 7).

In the background of aggressive desensitization strategies came a rising in the utility of KPD (8, 9). Kidney Paired Donation was first introduced in the late 1980s in South Korea (10), but did not gain traction in the United States until 2007 when the legality of this practice was clarified with the Charlie Norwood Living Organ Donation Act. Since that time, kidney paired donation has become widespread. In 2019, over 1100 living donor kidney transplants were facilitated through KPD constituting about 16% of all living donor transplants (11). While there was initial enthusiasm that kidney paired donation would eliminate the need for desensitization and positive crossmatch transplant, it was soon realized that KPD was not sufficient and KPD pools became saturated with sensitized patients (12). Although these programs increase the number of potential donors for sensitized individuals, patients with antibodies against a wide variety of HLA antigens may still not be able to find a crossmatch-negative donor, even if the donor pool is very large. Data from the 3-Mayo site kidney paired donation program found that having a cPRA of > 98% was a risk factor for waiting in kidney paired donation greater than 3 months (13). Data from the National Kidney Registry is comparable (12). Because some sensitized patients may not be able to find an HLA compatible donor, many programs combine KPD with positive crossmatch transplantation to find a donor with a more favorable crossmatch and DSA profile (14, 15).

Given the realization that desensitization and KPD were ineffective strategies for getting many sensitized patients to transplant, deceased donor allocation schemes were revised. In December of 2014, a new kidney allocation system came into effect in the United States that gave tremendous priority to the sensitized patient (16, 17). In the system used previously, all candidates with a cPRA of >80% were given an additional 4 allocation points. Currently, additional allocation points are given on a sliding scale starting with a cPRA as low as 20%. Major priority is given to candidates with a cPRA ≥98%. Candidates with a cPRA of 98%, 99%, and 100% get an additional 24.4, 50.09, and 202.1 additional allocation points, respectively (17). Within months of the implementation of this allocation system, hundreds of transplant patients with cPRA ranging from 90-100% were fortunate to receive a deceased donor transplant after years of waiting (18). However, even with these allocation changes, patients with a cPRA of > than 99.9% continue to have very low rates of kidney transplantation **Figure 1** (19). Candidates with this degree of sensitization are not rare. 70% of patients with a cPRA of 100% on the UNOS

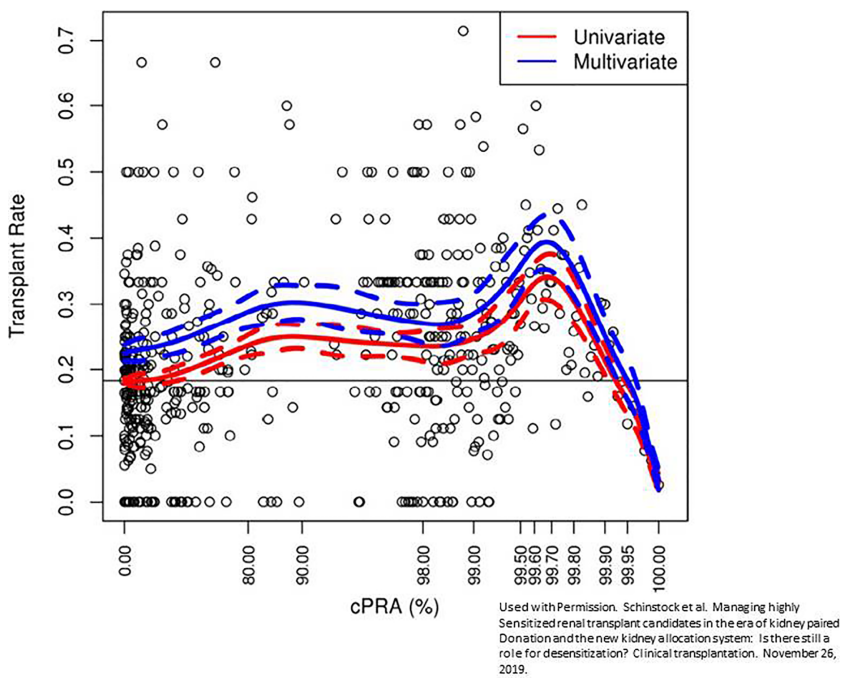

FIGURE 1 | Reduced transplantation rate among patients with cPRA > 99.9%. Multivariate fit of transplantation rate versus calculated panel reactive antibody (CPRA). A fit of transplant rate versus cPRA using a restricted cubic spline with 95% confidence interval controlling for time on the waitlist, age, gender, blood type, waitlist region, and ethnicity. The red line is univariate, while the blue line indicates the multivariate fit corresponding to a candidate with male gender, blood type A, waitlist region 5, Caucasian ethnicity, and waiting time of 2.5 y. Markers represent the observed transplant rate within each window of CPRA of width 0.01%. This prevalent cohort was active as of June 1, 2016, and followed for change in status through June 1, 2017. Used with permission. Schinstock et al. Managing highly sensitized renal transplant candidates in the era of kidney paired donation and the new kidney allocation system: Is there still a role for desensitization? Clinical transplantation. November 26, 2019 (19).

waiting list have a cPRA > 99.9% (19). Not only are these candidates disadvantaged from a transplant perspective, they are at a higher risk for waitlist mortality (20, 21). The problem is that there are simply not enough organs available with unique HLA antigens to be compatible with these candidates, thus further expansion in KPD or changes in allocation policy will not adequately solve this problem (22). Even a small decrease in cPRA among these highly sensitized patients with desensitization has the potential to markedly improve access to transplantation.

It is also worth mentioning that as sensitization increases, the rate of living kidney donor transplantation decreases (19). Analysis of UNOS data has shown that approximately 26% of patients with a cPRA of less than 80% received a living donor kidney transplant compared to 6.5% of candidates with a cPRA > 80% (19). Furthermore, only 2.5% of candidates with 100% cPRA received a living donor transplant and only about half of these were facilitated through KPD (19). These data suggest that there may be a role for desensitization in select cases to facilitate living kidney transplantation given the clear benefits of living versus deceased donor kidney transplantation.

Although the needs for desensitization in kidney transplant have changed in the last two decades, it remains clear that a select group of transplant candidates could derive benefit from effective desensitization. Kidney transplant candidates with a cPRA of > 99.9% have the greatest need for desensitization. One could also argue that select patients with a cPRA < 98% with a incompatible approved living donor or who have been active on the waiting list for several years may also derive benefit from desensitization considering the patient survival benefit of getting off of dialysis (23, 24). These are patients who should be targeted for clinical trials **Table 1**.

KIDNEY TRANSPLANT OUTCOMES FOLLOWING DESENSITIZATION

In addition to understanding *who* would most benefit from desensitization, it is key to understand the outcomes following transplant with DSA to make personalized clinical decisions weighing the risks of incompatible transplantation versus remaining on the waiting list. Despite the patient survival advantage of HLA incompatible transplant; HLA incompatible transplants are associated with reduced allograft survival (6, 25), increased expense (26), and increased hospital readmission rates (26). Understanding the immunological risk specific to a particular donor/recipient pair is a core principle in the transplantation of sensitized patients. In general, the quantity of antibody at the time of transplant correlates with risk, and the level of antibody can be semi-quantitatively determined with a combination of a variety of tests including the solid phase assay SAB mean fluorescence intensity (MFI), titers, cytotoxic and flow cytometric crossmatches, and C1q antibody positivity (27).

In the modern era, kidney transplantation is rarely performed in the setting of high levels of DSA as defined by a positive cytotoxic crossmatch and DSA MFI > 10,000 to avoid immediate allograft loss from hyperacute rejection. In patients with pre-transplant positive cytotoxic crossmatch, the risk of hyperacute rejection and aggressive early ABMR can be as high as 20% and 70% respectively. By 1 year, nearly 50% of those grafts fail (28).

The outcomes after HLA incompatible kidney transplantation with negative cytotoxic crossmatch varies and is largely based on single center retrospective studies with heterogeneous immunosuppression and desensitization protocols. The reported incidence of early acute active ABMR associated with the memory response is as low as 1% among kidney transplant recipients with DSA based on single antigen bead positivity only to up to nearly 40% among patients with DSA positivity based single antigen beads and a high positive flow cytometric crossmatch (7, 29–32). In a large retrospective series among a French cohort, the incidence of early active ABMR was 36.4% with a baseline DSA MFI of 3001-6000 and 51.3% with a baseline DSA MFI of > 6000^{7}. A key message is that transplantation in the context of low level DSA results in acceptable outcomes when other options are not available.

Even when early allograft loss within the first year post-transplant is avoided, chronic active ABMR remains a major problem. At 5 years post-transplant over 50% of surveillance biopsies have features of chronic active ABMR among patients transplanted with DSA and a high positive B cell flow cytometric crossmatch (defined as mean channel shift of 250 with < 106 considered negative). Renal allograft survival following incompatible transplantation also correlates with the amount of DSA at the time of transplantation (25, 30, 33). A multicenter observational study of living donor transplants performed at 22 centers in the United States showed that the 1 and 5 year unadjusted all-cause graft loss was 3.9% and 16.6% among patients without DSA at transplant, 3.8% and 20.2% when SAB were positive for DSA but the flow crossmatch was negative, 6.9% and 28.8% when the flow cytometric crossmatch was positive, and 19.4% and 39.9% when the cytotoxic crossmatch was positive (25).

EMERGING THERAPEUTICS FOR DESENSITIZATION

Unfortunately, desensitization studies are retrospective single center experiences that include heterogeneous candidates with

TABLE 1 | Kidney transplant candidates who may benefit from Desensitization.

1. All kidney transplant candidates with cPRA > 99.9%. These candidates have reduced transplantation rates on the deceased donor list and less likely to benefit from kidney paired donation.
2. Kidney transplant candidates with cPRA > 98% and > 5 years of waiting time. These are candidates who have not benefited from their allocation priority.
3. Kidney transplant candidates with an approved living donor and cPRA >98% but have not had a compatible offer through kidney paired donation.

varied levels of baseline sensitization and a lack of standard endpoints, thus it is difficult to compare the efficacy of the various protocols. Most desensitization protocols include plasmapheresis to reduce circulating HLA antibody and intravenous immunoglobulin for its immunomodulatory effects and to prevent hypogammaglobinemia, but many other therapies have been added or used alone **Table 2**.

Anti-CD 20 Monoclonal Antibodies

Many desensitization regimens also include the chimeric anti-CD 20 antibody rituximab aimed to deplete B cells and minimize the memory response (31, 36, 49, 50). Rituximab has been shown to reduce the PRA, increase the rate of transplantation, and decrease the pretransplant flow cytometric crossmatch mean channel shift (50); but even after treatment, nearly 50% of patients had ABMR within 30 days post-transplant (50). More recently Obinutuzumab has been studied in desensitization (38). This 3rd generation anti CD 20 monoclonal antibody has been associated with a more profound depletion of B cells and is used outside of transplant as a second line agent for hematologic malignancies refractory to rituximab. Similar to rituximab, Obinutuzumab is associated with depletion of peripheral and lymph node B cells, but its effect on MFI, number of unacceptable antigens, and cPRA has been shown to be limited and does not appear to be clinically meaningful (38).

Proteasome Inhibitors

Many therapies that have been studied in desensitization were first used in multiple myeloma because long lived CD38 positive plasma cells that reside in the bone marrow and constitutively secrete alloantibody are the target for both indications. Bortezomib, a reversible proteasome inhibitor, has been shown in *in vitro* models to deplete bone marrow derived plasma cells (51). In clinical studies, this therapy led to modest reductions in alloantibody, but was not well tolerated (39, 40). Similarly, the irreversible proteasome inhibitor carfilzomib has been shown to deplete plasma cells and decrease HLA antibody, but its effects were transient and antibody levels returned to baseline in less than 6 months (41).

Anti-CD38 Monoclonal Antibodies

Daratumumab, an anti CD38 monoclonal antibody, has been studied for desensitization in a nonhuman primate model. This treatment was associated with reduced DSA and prolonged renal survival but was also followed by a rebound in DSA and a severe combined antibody and T cell mediated rejection leading to graft

TABLE 2 | Desensitization therapies in kidney transplantation.

Drug class	Name	Mechanism of Action	Previous and ongoing studies	Key Features
Plasmapheresis	NA	Removal of circulating immunoglobulin	Stegall et al. (28)	
Intravenous Immunoglobulin	NA	Exact mechanism unknown. Multiple Immunomodulatory mechanisms.	Glotz et al. (34) Jordan et al. (35) Stegall et al. (28)	
Anti-CD 20 monoclonal antibodies	Rituximab	Depletes B cells	Jordan et al. (36) Vo et al. (31) Jackson et al. (37)	
	Obinutuzumab		Redfield et al. (38)	3rd generation anti-CD20 dependent on ADCC. Used in for relapsed hematologic malignancies.
Proteosome inhibitors	Bortezomib	Accumulation of unwanted cellular protein and apoptosis.	Woodle et al. (39) Moreno Gonzalez et al. (40)	Reversible proteasome inhibitor
	Carfilzomib		Tremblay et al. (41)	Irreversible proteasome inhibitor. Less neurotoxicity than bortezomib.
	Ixazomib		Ongoing ClinicalTrials.gov Identifier: NCT03213158	First oral proteasome inhibitor
Anti-CD38 monoclonal antibodies	Daratumumab	Depletes plasma cells	Kwun et al. (42)	Studied in nonhuman primate model and was associated with increased in T cell mediated rejection.
	Isatuximab		Ongoing ClinicalTrials.gov Identifier: NCT04294459	
Cysteine protease	Imlifidase	Cleaves heavy chains of human IgG (all subclasses) and eliminates IgG effector functions	Jordan et al. (43) Jordan et al. (44)	Rebound of DSA at Day 7. Retreatment with imlifidase often ineffective because of the development of neutralizing antibodies.
Interleukin-6 Blockade	Tocilizumab	IL-6 receptor inhibitor	Vo et al. (45)	
Complement inhibitors*	Eculizumab	Terminal complement blockade to protect against antibody mediated rejection.	Stegall et al. (46) Marks et al. (47) Glotz et al. (48)	

**Does not deplete antibody and therefore not a "desensitization" agent.*

loss (42). The concomitant T cell mediated rejection was likely from depleting regulatory cell populations (42). Daratumumab has also been used to successfully desensitize a heart transplant candidate. The cPRA dropped from 80% to 62% after daratumumab and the heart candidate received an organ with HLA that included two antigens that were considered unacceptable before treatment (42). A similar but more potent anti CD38 monoclonal antibody, isatuximab, is currently being studied in a phase 1b/2 trial to evaluate the safety, pharmacokinetics, and efficacy for desensitization in kidney transplant candidates [ClinicalTrials.gov Identifier: NCT04294459].

Interleukin-6 Blockade

Interleukin 6 is a proinflammatory cytokine that is central to the acute inflammatory response. It has multiple roles in mediating innate and adaptive immune responses including activation of T helper 17 cells and inhibiting regulatory T cells. Particularly relevant to sensitization, IL-6 is critical for maintaining long lived plasma cells in their niche. A small phase I/II nonrandomized study of the IL-6 inhibitor tocilizumab was used for desensitization among 8 patients who were considered refractory to rituximab and IVIG (45). The use of tocilizumab was associated with reduction in an immunodominant DSA score based on MFI (45). Further studies of IL-6 inhibitors have not been published for pretransplant desensitization; but a randomized multicenter randomized clinical trial using clazakizumab, a soluble IL-6 inhibitor, for chronic active ABMR is currently enrolling patients [ClinicalTrials.gov Identifier: NCT03744910].

Cysteine Protease

A novel agent that has shown promise in desensitization is Imlifidase. This endopeptidase rapidly cleaves all IgG into F(ab') and Fc fragments to impair the effector function from all circulating IgG. In both phase 1 and 2 desensitization trials, this agent led to a precipitous drop in DSA within hours, and therefore is a valuable tool for deceased donor positive crossmatch transplantation to avoid hyperacute rejection (44, 52). The main limitation is that this drug will likely need to be part of a combination therapy regimen because it only cleaves circulating antibody and antibody levels begin to have a brisk rebound within 3-7 days (44, 52). In the future, it may be used instead of pretransplant plasmapheresis to rapidly reduce circulating DSA.

Complement Inhibitors

Eculizumab is a terminal complement inhibitor that does not decrease antibody but has been added to desensitization regimens to minimize the effect of a high level of DSA on the allograft. It has been shown to decrease the incidence of early active ABMR in a small single center cohort from nearly 41.2% in the historical control group compared to 7.7% in the treatment arm (46). Larger multicenter studies in living and deceased donor populations have not confirmed these results, but also included patients at lower risk for ABMR at baseline (47, 48). Regardless, eculizumab has not been shown to improve long term allograft survival when added to desensitization (53, 54). While the long term studies of terminal complement blockade have been disappointing, there may be a role for adding eculizumab to novel high-risk desensitization protocols in the future to minimize the risk of early allograft failure.

NOVEL APPROACHES TO CLINICAL TRIAL DESIGN IN DESENSITIZATION

Use of cPRA and Antibody Titer for Trial Endpoints

Recurrent themes in the desensitization field are the small heterogeneous study populations with varied baseline sensitization, differing access to living donors, and unique desensitization endpoints. The heterogeneous endpoints range from the change in MFI of HLA antibody based on SAB results to the rate of transplantation. Often the endpoints used are subject to laboratory variability and differences in center transplantation practices. Standardizing endpoints and requiring minimal standards for laboratory reporting would be a major advancement in this field.

Of the various endpoints for desensitization, cPRA is advantageous because it can be applied to candidates with and without a living donor and eliminates the bias that occur from varied deceased donor acceptance, availability of a living donor, or access to kidney paired donation. It is easy to measure and directly related to a candidate's probability of receiving a kidney transplant but could be also used in heart and/or lung desensitization studies. The main drawback of cPRA is that is often based on the MFI from undiluted serum samples, and it is well known that MFI results are impacted by inherent assay limitations and intra-laboratory variability particularly in the sensitized patient. Another weakness is that the cPRA can be an insensitive measure of desensitization if the antibody is not decreased enough to change the number of unacceptable antigens. Stepwise dilution of the serum will gradually eliminate antibody positivity and decrease the cPRA, thus determining the *cPRA per titer* can overcome these limitations.

We evaluated cPRA reduction per titer among 20 sensitized patients with a cPRA > 99.9% **Figure 2**. We found that titer determination in a central laboratory using same lot reagents and batch testing leads to reproducible results and that the cPRA per dilution remains constant within approximately 1 titer if serum samples are obtained within 1 year (55). Transplant candidates with a cPRA of > 99.9% have vastly different quantities of antibody (55). While the cPRA began to drop after only 1-2 dilutions for some candidates, other candidates remained at cPRA >99.9% even when their serum was diluted 1:4096. Presumably those candidates whose cPRA decreased with fewer dilutions would be easier to desensitize, thus cPRA per titer could also be used to develop the inclusion criteria for a clinical trial or risk stratification.

The concept of determining the antibody titer has applications beyond the clinical trial including the appropriate assignment of unacceptable antigens, evaluating the efficacy of desensitization, quantifying the change in DSA post-transplant,

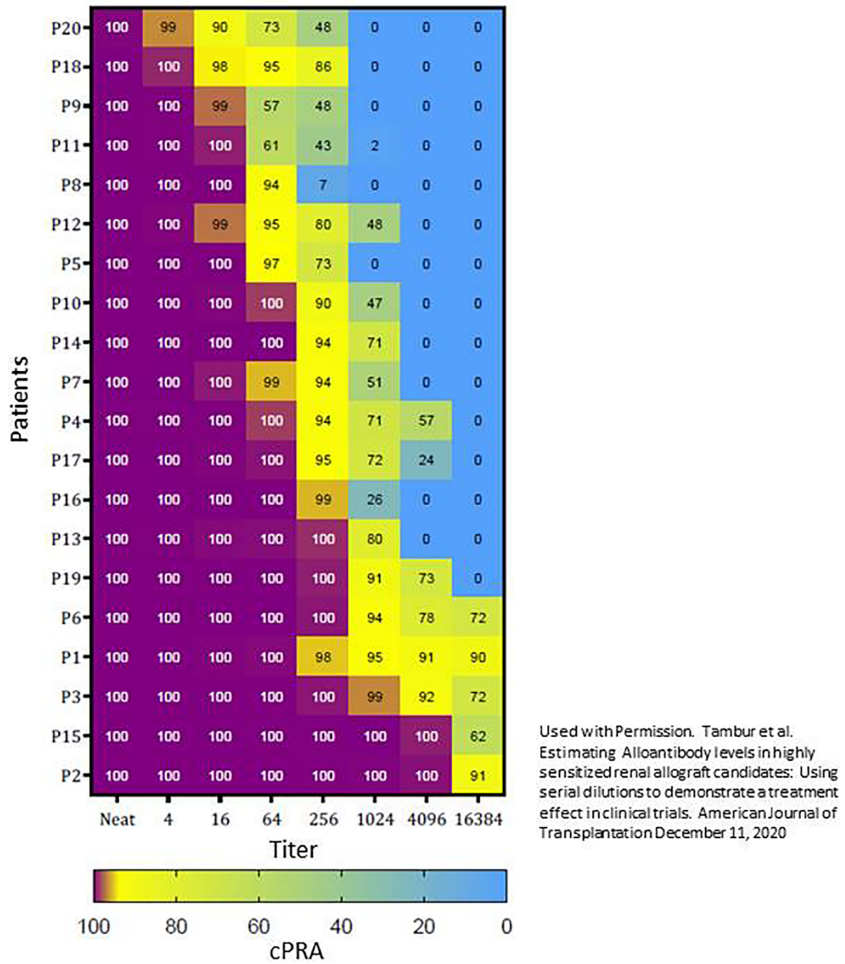

FIGURE 2 | Using titer to stratify patients with cPRA > 99.9%. This stratification was based on the first replicate from the baseline sample. The heat map shows the cPRA obtained from positive antibody specificities per titer for each of the 20 patients. The patients were ordered to show patients who would be the most likely to respond to desensitization (top) to least likely to respond to desensitization (bottom). For example, P2 continued to have a 100% cPRA when serum was diluted 1:4096, and thus it may be extremely difficult to remove enough antibodies to render this patient transplantable. Used with Permission. Tambur et al. Estimating Alloantibody levels in highly sensitized renal allograft candidates: Using serial dilutions to demonstrate a treatment effect in clinical trials. American Journal of Transplantation December 11, 2020 (55).

or evaluating the response to ABMR therapies. We recognize the time and expense to do serum dilutions and titers, but it is important to recognize that multiple serial dilutions are only rarely needed. The number of dilutions tested truly depends on the purpose of testing. For example, if you are willing to attempt desensitization therapy if the antibody is < 1:8, you may only test a 1:8 dilution. In other cases, you may choose to start with testing one dilution (e.g. 1:64) and decide on further testing based on those results. The key is to acknowledge the limitations of the MFI and know when and how to use the titer measurement in practice.

Novel Clinical Trial Designs

With the expansion of the therapeutic options now available for desensitization combined with the probable need for combination therapy comes the opportunity to adapt new strategies for evaluating the safety and efficacy of desensitization protocols. It is simply not feasible to conduct multiple randomized controlled trials to efficiently evaluate novel desensitization strategies. Resources and patients eligible to participate in these trials are finite. Novel adaptive trial designs can be utilized to efficiently study small heterogeneous populations with combination therapeutic regimens and address issues with suboptimal enrollment (56). Adaptive clinical trials adapt depending on predefined outcomes generally based on Bayesian probabilities. The goal of these designs is to learn quickly what does or does not work and halt the study of a therapeutic agent or combination early if futile or unsafe. These designs

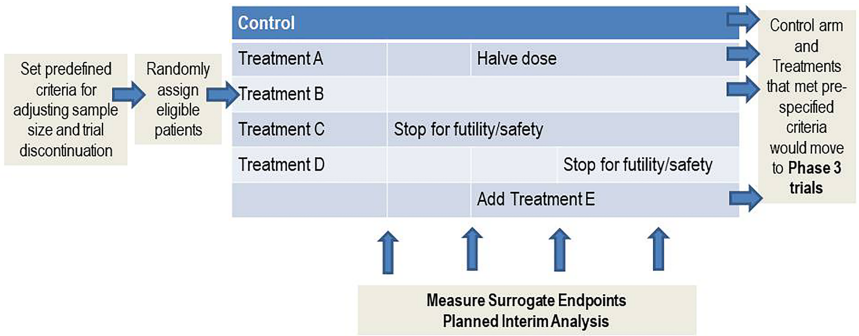

FIGURE 3 | Platform master protocol design. A *platform* master protocol allows for the study of multiple therapies for a single disease perpetually, and therapies can enter or leave the trial of the basis of predefined criteria. This master protocol is ideal for studying small heterogeneous populations.

are most efficient if validated surrogate endpoints such as cPRA are used.

The foundation of these designs is a master protocol (57). Examples of master protocols include *umbrella, basket*, and *platform*. *Umbrella* master protocols are used to study multiple targeted therapies for a single disease while a *basket* protocol would be used to study a single therapy for multiple diseases. A *platform* master protocol is essentially an extension of the umbrella design, but multiple therapies are studied for a single disease perpetually and therapies can enter or leave the trial of the basis of predefined criteria **Figure 3**. The control arm and therapies that meet pre-specified criteria can move onto a phase 3 clinical trial. This platform design would be ideal for desensitization because of the relatively small patient population and multiple different desensitization combinations that need rigorous study. In fact, sensitized kidney, heart, and lung candidates could be studied in the same trial if an endpoint such as cPRA was used.

CONCLUSION

In summary, there remains an unmet need for desensitization for candidates with the highest degree of sensitization who have not benefited from KPD or organ allocation policy changes. Many new therapeutic options are available, and we are hopeful that the use of new endpoints and clinical trial designs in this field will lead to effective desensitization approaches in the future to increase access to transplantation to patients in the most need.

AUTHOR CONTRIBUTIONS

CS, AT, and MS all contributed equally in the design of the manuscript, literature review, writing of manuscript, and revision of manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

MINI REVIEW
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HLA Desensitization in Solid Organ Transplantation: Anti-CD38 to Across the Immunological Barriers

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The presence of anti-human leucocyte antigen (HLA) antibodies in the potential solid organ transplant recipient's blood is one of the main barriers to access to a transplantation. The HLA sensitization is associated with longer waitlist time, antibody mediated rejection and transplant lost leading to increased recipient's morbidity and mortality. However, solid organ transplantation across the HLA immunological barriers have been reported in recipients who were highly sensitized to HLA using desensitization protocols. These desensitization regimens are focused on the reduction of circulating HLA antibodies. Despite those strategies improve rates of transplantation, it remains several limitations including persistent high rejection rate and worse long-term outcomes when compare with non-sensitized recipient population. Currently, interest is growing in the development of new desensitization approaches which, beyond targeting antibodies, would be based on the modulation of alloimmune pathways. Plasma cells appears as an interesting target given their critical role in antibody production. In the last decade, CD38-targeting immunotherapies, such as daratumumab, have been recognized as a key component in the treatment of myeloma by inducing an important plasma cell depletion. This review focuses on an emerging concept based on targeting CD38 to desensitize in the field of transplantation.

Keywords: anti-CD38, daratumumab, HLA desensitization, DSA, solid organ transplantation

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INTRODUCTION

HLA Sensitization and Antibody-Mediated Rejection

Solid organ transplantation (SOT) has become the best therapeutic option for end-stage organ disease but faces two major issues: the limited transplant supply and the poor long-term transplants outcome which have not improved over the past 30 years (1–3). This observation is related to the occurrence of antibody-mediated rejection (ABMR) which remains the death-censored leading cause of transplant loss across all solid organ transplants (3, 4). ABMR is defined on the association of histologic lesions (microvascular inflammation), histologic evidence of alloantibodies–endothelium interaction (c4d staining) and circulating donor-specific antibodies mostly directed against human leucocyte antigens (HLA) (3–10). Following blood transfusion, pregnancy or previous graft failure, candidates for organ

transplantation can become sensitized against HLA and produce circulating anti-HLA antibodies (11, 12). In particular, pending on their properties donor-specific anti-HLA antibodies (DSA), are responsible for ABMR leading to allograft dysfunction and graft loss (13–18). Currently, immunomotoring of the transplant candidate's is routinely performed in order to stratify the immunological risk by determining the presence and specificity of anti-HLA antibodies and potential DSA (11, 16). The highly sensitized patients have longer waitlist times with significant adverse effect on both quality and quantity of life (1, 2). Several strategies are applied to limit the time on the waiting list of highly immunized patients such as prioritization in transplant's access, promotion of transplantation from living-donor allografts, development of kidney paired donation and desensitization.

Desensitization and Solid Organ Transplantation's Outcome

Current desensitization strategies have been developed in kidney transplantation and extended to other solid organ transplantation (17–21). The goal of desensitization regimens in presensitized transplant candidates is twofold including the reduction of anti-HLA level to allow transplantation and the improvement of transplantation outcome through the prevention of ABMR (22). A stepwise approach is commonly used to desensitize including, (i) either high-dose intravenous immunoglobulin (IVIG) or low dose IVIG in association with plasmapheresis to remove antibodies and, (ii) anti-CD20 targeting agent, such as rituximab, to prevent rebound antibodies development by B cell depletion (23–27). Regarding the kidney transplantation field, despite the desensitizing effect, the subsequent transplantation is associated with higher rate of rejection and higher rate of hospital readmission after transplantation (28–30). However, long term outcomes for patient and graft survival have been reported to be similar to that of non-sensitized patients (31). Furthermore, the benefit of desensitization compared to remaining on the transplant waiting list has been evaluated only in few large studies and their results remain controversial (32, 33). Montgomery et al. and Orandi et al. reported a survival benefit at five years after kidney transplantation in 211 and 1025 desensitized patients respectively compared to patients remaining on the waiting list (34, 35). Interestingly, in a study performed on 213 desensitized recipients of living donor transplants, Manook et al. showed that desensitization was not associated with a survival benefit compared to matched sensitized control patients who were waitlisted (36). On the other hand, keeping patients a long time on dialysis represent a considerable financial burden while decreasing the quality and length of life for affected patients (32, 33).Thus, it appear as necessary to develop novel therapeutic approaches in order to prevent ABMR and improve long-term survival of transplanted organs in highly immunized recipient.

Desensitization Regimens Targeting Plasma Cells

The available therapeutic tools to manage the humoral response appears modestly successful in the context of SOT and alloimmunity. Indeed, antibody rebound due to plasma cells (PC), which do not express CD20, limit the efficacy of the most commonly used strategy combining IGIV, plasmapheresis and B cell depletion by anti-CD20 depleting agent. Targeting PC with new pharmacological tool from autoimmunity and cancer research could allow a better management of the humoral response in desensitization protocols (37). In the germinal center, after the enhancement of alloantigen responses by T follicular helper (Tfh), activated B cells develop into memory-B cells, progress to plasmablasts and ultimately to antibody-producing PC (38, 39). These PC are the long-lived mediators of lasting humoral immunity and persist in medullary niche where they can secrete high-affinity complement-activating DSAs (38, 40). Several emerging strategies aim to deplete PCs compartment in order to prevent ABMR (37, 41). First, Interleukin 6 (IL-6) is a cytokine promoting Tfh and enhancing the progression of B cells to high-affinity antibodies producing PC (42). Tocilizumab, a first-in-class humanized monoclonal antibody (mAb) with specificity for IL-6R, reduce inflammation within the allograft during ABMR in heart and kidney transplantation (43) and induce circulating DSA reduction (44). Clazakizumab is a humanized IgG1 mAb with specificity for IL6 which can also induce circulating DSA reduction (45). Both Tocilizumab and Clazakizumab are pharmacological agents with major interest in the development of desensitization strategies targeting PC (37, 46). On another hand, proteasome inhibitors represent one of the most promising solution to deplete PC in the setting of desensitization, targeting more selectively PCs population. Bortezomib and carfilzomib have been evaluated in desensitization trials, lacking control group, leading to controversial results (47, 48). Both induce significant PCs depletion whereas DSA level did not significantly decrease or rebound occurred rapidly. In fact, targeting PC may lead to rapid germinal center activation by deleting the negative feedback usually provided by PC and rebound humoral immunity and compensation (49). Therefore, dual targeting approach (combining PCs depletion with proteasome inhibitors and costimulation blockade) may silence the germinal center and prevent humoral compensation. This strategy has been recently evaluated using carfilzomib and belatacept as desensitization in highly sensitized non-human primate model with a reduction of bone marrow PC, DSA levels reduction, and prolongation of allograft survival. Most animals experienced ABMR with humoral-response rebound, suggesting desensitization must be maintained after transplantation using ongoing suppression of the B cell response (50, 51). An emerging therapy to induce DSA reduction and to prevent rebound DSA development is the use of antiplasma cell therapies such as anti-CD38, anti-CD19 or bispecific anti-CD3/anti-BCMA (B cell maturation antigen). In this review, we propose to focus on anti-CD38 as a desensitization regimen in SOT.

CD38-TARGETING STRATEGIES

CD38 and CD38-Targeting Antibodies

The protein CD38 is a type II transmembrane glycoprotein known as a multifunctional molecule. CD38 play dual roles as receptors and ectoenzymes (52). The CD38/CD31 interactions

are crucial to leukocyte adhesion and transmigration through the endothelium (53). CD38 is also an enzyme that catalyzes several reactions leading to the regulation of cytoplasmic calcium fluxes and a wide range of others physiological functions such as cellular metabolism (52). CD38, found throughout the immune system especially natural killer and PC, is highly expressed in multiple myeloma cells (54). Altogether, this has triggered the development of several CD38 antibodies to treat multiple myeloma (54–56). Daratumumab (DARZALEX®, Janssen), fully human IgG1-kappa, was the first CD38 antibody that was recognized as an emerging therapy against myeloma in the last decade (57). Daratumumab have multiple effects including Fc-dependent immune-effector mechanisms and direct effects. The Fc-dependent immune-effector mechanisms include antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity (54, 55). Direct effects include induction of apoptosis, as well as inhibition of CD38 ectoenzyme function, which may lead to disruption of the PCs niche. Those Fc-dependent effects and direct effects are associated with deep and sustained $CD38^+$ cells depletion, mostly PC and NK cells (54, 55, 58). The ability of daratumumab to efficacy deplete PCs compartment permit to use it as an new agent in therapeutic armamentarium for multiple myeloma (56). Large clinical trials have demonstrated significant improvements in the outcome of patients with relapsed multiple myeloma with use of daratumumab and it has been recently approved in front-line regimens (56–60). Isatuximab (SARCLISA®, Sanofi) is a chimeric IgG1-kappa which has stronger direct effects than daratumumab but lower ability to induce Fc-dependent immune-effector mechanisms, while it remains unknown whether these functional differences observed between different CD38 antibodies affect their therapeutic utility (55, 61). Many other strategies targeting CD38 are under development and a selection is listed in **Table 1**. The CD38-targeting antibodies generally represent a safe treatment. Indeed, the most reported toxicity is infusion related reactions which remain successfully controlled by premedication and infusion rate management with low frequency of recurrence during subsequent injections (62). A higher rate of viral infections in patients treated with daratumumab has been reported in some studies leading to a recommended administration of valaciclovir during the administration of anti-CD38 antibodies (62).

Immunomodulatory Effects of CD38-Targeting Antibodies

CD38-targeting antibodies have immunomodulatory effects such as improving the host-anti-tumor immune response (63). Krejcik et al. showed that daratumumab monotherapy against myeloma was associated with both CD4+ and CD8+ T cell expansion (64). This increase in T-helper cells and cytotoxic T-cell was associated with functional modification including elevated antiviral and alloreactive functional responses, and significantly greater increases in T-cell clonality as measured by T-cell receptor sequencing (63, 64). These modifications are associated with depletion of $CD38^+$ immunosuppressive cells including regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells. It is well known that such regulatory cells inhibit the host-anti-tumor immune response in the context of several malignancies including multiple myeloma (65–67). Altogether, this immunomodulatory activity of CD38 antibodies may be essential to their therapeutic efficacy. Indeed, it

TABLE 1 | Selection of therapeutical regimens targeting CD38.

Anti-CD38 strategies	Nature and mechanism	Statut	NCT number
Daratumumab *Janssen*	Fully human IgG1-kappa anti-CD38 mAb	Approved	X
Isatuximab *Sanofi*	Chimeric IgG1-kappa anti-CD38 mAb	Approved	X
Felzartamab - MOR202 *MorphoSys AG*	Fully human IgG1-lambda anti-CD38 mAb	Ongoing in auto-immune field	NCT04733040 NCT04145440
Mezagitamab - TAK-079 *Takeda*	Fully human IgG1-lambda anti-CD38 mAb	Ongoing in hemato-oncology	NCT03439280
CID-103 *CASI Pharmaceuticals*	Fully human IgG1 anti-CD38 mAb	Ongoing in hemato-oncology	NCT04758767
ISB 1342 *Glennmark Phamaceuticals*	CD3xCD38 bispecific antibody to redirect cytotoxic potential of T cells to $CD38^+$ cells	Ongoing in hemato-oncology	NCT03309111
TAK-169 *Takeda*	Antibody drugs conjugates: anti-CD38 Ab fragment combined to a Shiga-like toxin (payload: ribosome inactivation)	Ongoing in hemato-oncology	NCT04017130
TAK-573 *Takeda*	Antibody drugs conjugates: humanized IgG4 anti-CD38 mAb combined to interferon α (payload: anti-proliferative effects)	Ongoing in hemato-oncology	NCT03215030
^{211}At-OKT10-B10 *Fred Hutchinson Cancer Research Center*	Antibody drugs conjugates: anti-CD38 mAb combined to radioactive Astatine ^{211}At (payload: radiation)	Ongoing in hemato-oncology	NCT04579523 NCT04466475
STI-6129 *Sorrento Therapeutics*	Antibody drugs conjugates: anti-CD38 mAb combined to Duostatin5 (payload: tubulin inhibition)	Ongoing in hemato-oncology	NCT04316442
KP1237 *Kleo Pharmaceuticals*	Endogenous-antibodies recruiting molecule targeting CD38 in order to enhance antibody-dependent destruction mechanism	Ongoing in hemato-oncology	NCT04634435
Anti-CD38 CAR-T Cells *Sorrento Therapeutics*	Imunne cell therapy based on autologous T cells modified into anti-C38 CAR-T cells	Ongoing in hemato-oncology	NCT03464916

has been highlighted in clinical trials showing that expansion of effector T-cells and eradication of immune suppressors cells by daratumumab used against refractory and newly diagnosed multiple myeloma was correlated to a marked improvement in response and progression-free survival (57, 59, 63, 67). It might be hypothesized that these immunomodulatory abilities have important implication for sustained control of the tumor and further deepening of response (63). As a result of these pleiotropic immune modulation, CD38 antibodies also enhance anti-tumor activity of others anti-cancer drugs with several studies highlighting that CD38-targeting antibodies have strong synergistic activity, such as combination to lenalidomide as well as to PD1/PD-L1 inhibitors (56, 68). Besides effect on immune cells, CD38 antibodies may also modulate immunometabolic pathway. Indeed, CD38-targeting agent's exposure could lead to lower adenosine level in tumoral microenvironment, which is known as immunosuppressive metabolite (69, 70). All these properties enhancing the anti-tumoral response are of major interest in the field of oncology while it could be problematic in immunosuppressive strategies such as autoimmune diseases treatment or desensitization and SOT's context.

CD38 ANTIBODIES IN SOLID ORGAN TRANSPLANTATION

CD38 Antibodies in Non Tumoral Context

In the last decade, several strategies to handle with autoimmune or alloimmune pathologic situations include CD38 antibodies (71–73). Indeed, long-lived plasma cells, which produce pathogenic antibodies, are unresponsive to standard immunosuppression. Besides PC depletion and immunomodulatory effect, CD38 expression on PCs from patients with autoimmune condition (74) and reduction of auto-antibodies in patients exposed to daratumumab (75) support the evaluation of daratumumab in patients with autoantibody-dependent disorders and, in extension, to alloimmune situation such as SOT. Available evidence about CD38 antibodies efficacy in these situations are mostly cases reports of daratumumab use against immune cytopenia. Daratumumab were used to treat warm autoimmune hemolytic anemia post-hematopoietic stem cell transplant (76), refractory cold agglutinin disease (77), Evans syndrome (78) and pure red cell aplasia (79) with improvement in the majority of cases. Regarding other autoimmune disease, the administration of daratumumab in two patients with refractory lupus was recently described exhibiting clinical responses associated with significant depletion of long-lived plasma cells and modulation of effector T-cell responses (80). As regard as autoimmune encephalitis, targeting CD38 was achieved with daratumumab in one case of life-threatening anti-NMDA receptor encephalitis and in one case of refractory anti-CASPR2 encephalitis with improvements of neurological sequelae (81, 82). In the last case, severe septicemia leading to patient death highlight an unmet need of rigorous clinical investigation to determine the efficacy and tolerance of CD38-targeting agent in autoimmune disease.

CD38 Antibodies and ABMR Treatment

In antibody-mediated non-neoplastic diseases, alloimmune situation such as SOT represent a field where targeting CD38 is promising as shown in **Figure 1**. As alloantibody-producing

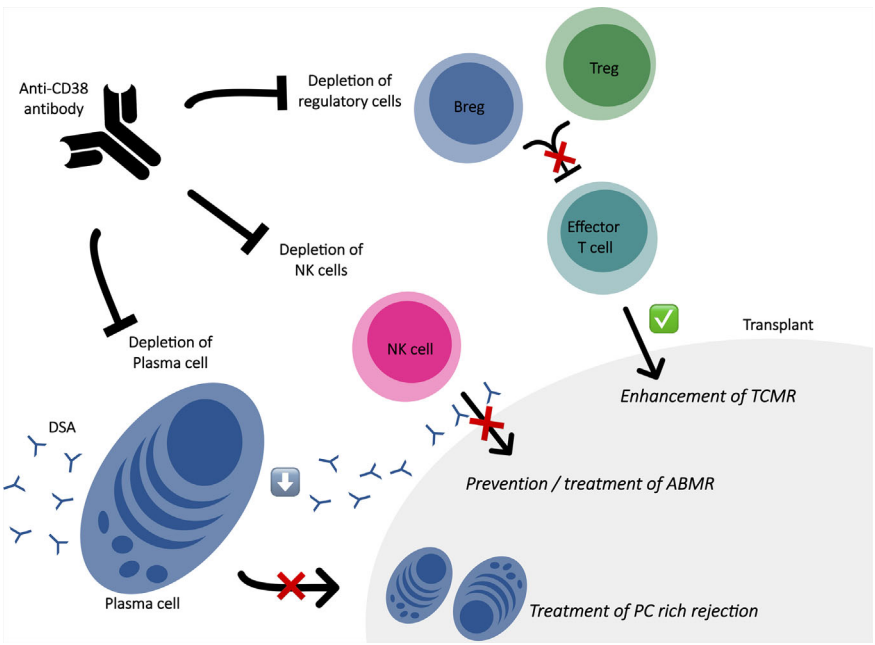

FIGURE 1 | Immune effects of anti-CD38 antibody in the context of solid organ transplantation. ABMR, antibody mediated rejection; Breg, regulatory B cell; DSA, donor specific antibodies; PC, plasma cell; TCMR, T cell mediated rejection; Treg, regulatory T cell.

TABLE 2 | CD38 antibody use in solid organ transplantation.

ABMR Treatment

Réf.	Transplant	Sensitization	IS strategy	Immune event	Treatment	AntiCD38 use	Evolution	Observation
(72)	Heart + Kidney	Immunized: Preformed DSA	- Induction: ATG -Maintenance: + Tacrolimus + MMF + Steroid	*-Delay post-Tx:* 17 months *-Clinical findings:* Cardiogenic shock and acute kidney injury requiring dialysis *-Anti-HLA: de novo* DSA and one preformed DSA *-Histology:* TCMR and ABMR with PC-predominant infiltration in both transplants	Steroid pulses + ATG + Plasmapheresis + IVIG + Rituximab + Eculizumab	Daratumumab: - 16 mg/kg - 8 weekly infusions	*-Clinical:* Heart allograft function returned to baseline + no more need of dialysis *-Anti-HLA:* Dramatic decline of MFI for majority of DSA at 3 months *-Histology:* Significant improvement in acute lesions and the PC infiltrate significantly decreased	-20 weeks after: recurrent acute PC-rich rejection on kidney biopsy -Significant reascension of the MFI of two class 2 DSAs -New series of Daratumumab infusions with kidney allograft function improvement
(73)	Kidney	Immunized: Preformed DSA	- Induction: ? -Maintenance: + Tacrolimus + MMF + Steroid	*-Delay post-Tx:* 13 years *-Clinical findings:* Progressive graft dysfunction and proteinuria in the context of newly diagnosed myeloma *-Anti-HLA:* 1 DSA *-Histology:* chronic active ABMR	None other treatment	Daratumumab: - 16 mg/kg - 8 weekly infusions + 8 fortnightly infusions + 1 monthly infusion thereafter for 9 months	*-Clinical:* Stabilization of renal function and proteinuria *-Anti-HLA:* DSA levels became undetectable after 14 weeks *-Histology:* Abrogation of microvascular inflammation with a decrease of intragraft NK cells densities	-3 months after: subclinical borderline rejection - High-grade tubulitis and mild interstitial infiltrates which were dominated by T-cells -Improvement with high-dose intravenous steroid.
(85)	Kidney	Immunized: ABOi (Anti-A)	- Induction: + Basiliximab + Rituximab -Maintenance: + Tacrolimus + MMF + Steroid	*-Delay post-Tx*: 30 days *-Clinical findings:* acute kidney failure *-Antibodies:* rise in Anti-A titers *-Histology:* ABMR	Steroid pulses + ATG + Immunoadsorption + Eculizumab	Daratumumab: - 16 mg/kg - 6 weekly infusions	*-Clinical:* Recovering of kidney function at baseline *-Anti-A:* Reduction in Anti-A titers leading to discontinuation of immunoadsorption *-Histology:* No lesion	
(86)	Heart	Immunized: History ABMR Preformed DSA	- Induction: ? -Maintenance: + Tacrolimus + MMF + Steroid	*-Delay post-Tx:* 13 years *-Clinical findings:* congestive heart failure *-Anti-HLA:* increase of DSA titers *-Histology:* ABMR	Steroid pulses + Immunoadsorption	Daratumumab: - 16 mg/kg - 8 weekly infusions + 8 fortnightly infusions + 1 monthly infusion thereafter for 9 months	*-Clinical:* Renal and cardiac improvement in 4 weeks *-Anti-HLA:* DSA titers are only slightly reduced *-Histology:* No lesions	

(Continued)

TABLE 2 | Continued

Réf.	Status	Transplantation	AntiCD38 use	Other treatment	Efficacy	AE	IS strategy	Observation
Desensitization								
(72)	Preclinical: NHP	Kidney	Daratumumab: -16 mg/kg -4 weekly infusions (8 weeks before Tx)	Plerixafor (anti-CXCR4): -0.24 mg/kg -same frequency	Significant reduction of DSA levels and prolonged graft survival	None	Induction: anti-CD4 + anti-CD8 Maintenance: Tacrolimus + MMF + Steroid	-Delayed ABMR -DSA rebound -TCMR -Reduction of Breg and Treg -Emergence of activated T cells after kidney transplantation in the desensitization group
(72)	Clinical	Heart	Daratumumab: -16 mg/kg -8 weekly infusions	Plasmapheresis + high-dose IVIG + Rituximab	Significant and persistent reduction of DSA levels and heart transplant access at 6 months	None	NA	Died from surgical complication

ABMR, antibody mediated rejection; ATG, anti-human thymocytes globulins; DSA, donor specific antibodies; IVIG, intravenous immunoglobulins; MMF, mycophenolate mofetil; NHP, nonhuman primate; PC, plasma cells; Ref., reference; TCMR, T cell mediated rejection; Tx, transplantation.

PC express CD38 at a higher level than other $CD38^+$ hematopoietic cells and CD38 antibodies induce a profound depletion of $CD38^+$ PC, CD38 appears as a rational target to handle with harmful alloantibodies such as DSA (83, 84). Currently, only few studies have been published regarding the use of CD38 antibodies for desensitization in patients awaiting transplantation or for treatment of ABMR as shown in **Table 2**. Concerning treatment of ABMR, the first report was in a patient with refractory early active ABMR caused by anti-A isohemagglutinins after kidney transplantation from his ABO-incompatible sister (85). Based on the efficacy of daratumumab in the treatment of pure red cell aplasia following ABO-incompatible hematopoietic stem cell (79) and non-response of several therapies; daratumumab were tested as a rescue solution leading to a significant decrease of the pathogenic isohemagglutinins and resolution of tissue damage in the kidney biopsy. Kwun and colleagues also published a case report of daratumumab as a therapeutic strategy for refractory heart and kidney rejection in a patient who received heart and kidney transplants due to systemic lupus (72). Both transplant biopsy showed T cell–mediated rejection, ABMR and diffuse PC infiltration associated to the presence of several DSA. To face refractory cardiogenic shock and acute kidney failure dependent to dialysis, a compassionate use of daratumumab lead to the resolution of both allograft function, improvement in acute kidney lesions with decreased PCs infiltrate and dramatic decline for the majority of DSA. A recurrent acute PC-rich rejection on kidney biopsy and significant ascension of DSA were successfully managed with daratumumab. Recently, two others cases were reported: one refractory ABMR after a heart transplant successfully treated with daratumumab and one chronic active ABMR in a kidney allograft recipient diagnosed with myeloma exposed to daratumumab (73, 86). In the last one, the exhaustive immuno-monitoring showed that the main mode of action seems to be based on PC depletion, with profound PCs reduction in the bone marrow and peripheral blood and the abrogation of *in vitro* alloantibody production by PC enriched from bone marrow aspirates, leading to significant reduction in DSA levels (73). Another observation is that daratumumab led to depletion of NK cells infiltrating the allograft and circulating NK cells, which is major interest knowing the potential role of NK cells in microvasculature inflammation through engagement of their Fc gamma receptor IIIA with endothelium-bound DSA (87). Interestingly, while follow up biopsy showed resolution of humoral activity, it was observed tubulointerstitial inflammation which prompted steroid treatment. The author highlighted that the molecular signature of this infiltrate was not similar to signature of T-cell mediated rejection leading to question the trigger of this infiltrate not associated with graft dysfunction. Indeed, daratumumab may trigger T-cell alloresponse, even if circulating regulatory T cells were not reduced in the patient's blood which is not necessarily correlated to the modification of immune cell populations at a tissue level. Moreover, the authors recently reported long term data of this case without evidence of ABMR rebound after daratumumab discontinuation (88). Although it is difficult to decipher the role of a rescue with

daratumumab added to a complex antirejection therapy, a drug that specifically deplete PC with a favorable safety profile could represent a step forward in the field.

CD38 Antibodies and Desensitization

The ability of CD38 to desensitize has been evaluated in both preclinical and clinical contexts and published in the same study (72). The preclinical study was based on the use of daratumumab in a non-human primate model which has the most biological similarity to humans for solid organ transplant biology (41, 89). The authors paired donors and recipients for maximal HLA mismatching and practiced, for allosensitization, two serial skin grafts before transplantation with a kidney from paired skin graft donor (72). Daratumumab and plerixafor (anti-CXCR4), known to induce mobilization of PC from bone marrow to peripheral blood, were given as desensitization therapy with an initiation 8-12 weeks after sensitization and 8 weeks before kidney transplantation. Animals received for induction anti-CD4 and anti-CD8 antibodies and for maintenance immunosuppression tacrolimus, mycophenolate mofetil and a methylprednisolone taper. This desensitization regimen reduced significantly preformed DSA, with more than 50% reduction compared with the pretreatment time point, and prolonged graft survival with a depletion of PC without altering the germinal center response since the Tfh population was not eliminated (72). However, desensitized monkeys showed delayed ABMR associated to DSA rebound and T cell–mediated rejection perhaps due to immune deviation. Indeed, the authors observed a reduction of regulatory B and T cells after desensitization with rapid emergence of activated T cells after kidney transplantation. This observation could be related to immunomodulatory effects of daratumumab but CXCR4 inhibition, due to plerixafor, is also known to limit regulatory compartment and to promote effector cells with a potential role in these cell-mediated rejection (90). Thus, in transplant recipients following desensitization with daratumumab, it would be interesting to elaborate new strategies than current immunosuppressive regimens in order to manage these DSA rebounds and the risk of T cell–mediated rejection. Concerning the clinical setting, the authors used daratumumab in a heart transplant candidate remaining highly sensitized after multiple courses of plasmapheresis, high-dose IVIG, and rituximab. It was observed a significant and persistent decrease of allosensitization allowing a heart transplantation six months after daratumumab infusion (72). Currently, based on these promising results, daratumumab are under investigation for desensitization in patients awaiting solid-organ transplantation in two clinical trial, one ruled by the nephrology department of Henri Mondor Hospital (Créteil, France) and another one directed by Stanford University [ClinicalTrials.gov, NCT04204980 and NCT04088903 (91, 92)]. Regarding the trial in kidney transplantation, sensitized patients with calculated panel reactive antibodies (cPRA) > 95% awaiting on the French National kidney allograft waiting-list for at least three years are eligible for the study and are randomly assigned to one of the two steps: (step 1) dose-escalation with 4 mg/kg of daratumumab weekly for four weeks, then with 8 mg/kg weekly for four weeks and then 16 mg/kg weekly for four weeks; (step 2) expansion cohort with eight weekly doses of 16 mg/kg. The primary outcomes are defined as: adverse events, intra-patient variation of cPRA and anti-HLA levels. Several other outcomes are also of interest such as percentage of patients engrafted, and intra-patient variation of ABO antibody titers (91).

CONCLUSION

Therapeutic improvement is required for both prevention and treatment of humoral alloresponse in solid organ transplantation. CD38 antibodies are a promising solution to profoundly deplete high affinity anti-HLA producing plasma cells. Preclinical and clinical experimental results suggests that daratumumab is a potentially therapeutic strategy to reduce DSA production and prevent and/or treat antibody-mediated rejection. However, CD38-targeting agent induce immune deviation which could be deleterious for solid organ transplants enhancing cellular-mediated rejection. Clinical studies are now needed to clarify the indications and efficacy of these promising therapeutic strategies.

AUTHOR CONTRIBUTIONS

NJ, MM, and PG designed the review, collected and interpreted data from literature, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Lung Transplantation and the Era of the Sensitized Patient

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Long term outcomes in lung transplant are limited by the development of chronic lung allograft dysfunction (CLAD). Within the past several decades, antibody-mediated rejection (AMR) has been recognized as a risk factor for CLAD. The presence of HLA antibodies in lung transplant candidates, "sensitized patients" may predispose patients to AMR, CLAD, and higher mortality after transplant. This review will discuss issues surrounding the sensitized patient, including mechanisms of sensitization, implications within lung transplant, and management strategies.

Keywords: immunosuppression, allograft dysfunction, antibodies, rejection, lung transplant

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INTRODUCTION

Lung transplantation has become the definitive treatment for many patients with end-stage lung disease. While there have been advances in short term survival, long-term outcomes after lung transplant are limited by the development of chronic lung allograft dysfunction (CLAD) (1). The International Society of Heart and Lung Transplant (ISHLT) defines CLAD as a substantial and persistent decline (≥20%) in measured forced expiratory volume in one second (FEV1) value from the reference (baseline) value, which is the mean of the best two postoperative FEV1 measurements (taken >3 weeks apart) (2). CLAD can present in a variety of clinical phenotypes. The two most common CLAD phenotypes include an obstructive phenotype called bronchiolitis obliterans syndrome (BOS), defined by a drop in FEV1 but initially preserved functional vital capacity (FVC) and/or preserved total lung capacity (TLC) and a restrictive phenotype called restrictive CLAD/restrictive allograft syndrome (rCLAD/RAS) which is characterized by decline in FVC and/or TLC in addition to the FEV1 decline (2). Overall, the development of CLAD portends a poor prognosis and contributes to worse survival after transplantation with the median survival being 6.5 years in the most recent era (3, 4).

With the poor prognosis of CLAD, lung transplant research has focused on mechanisms, prevention, and treatment of CLAD. One of the strongest and earliest identified risk factors for CLAD is the severity and number of acute cellular rejection episodes (5). Within the past decade, antibody-mediated rejection (AMR) or activation of humoral immunity is being recognized as a separate risk factor for poor long-term outcomes in solid organ transplantation and is considered a risk factor for CLAD in lung transplant recipients specifically (3, 4, 6, 7).

Despite early reports of patients with antibody mediated graft dysfunction, pulmonary AMR lacked a uniform definition making diagnosis and cross-center collaborative studies difficult. Therefore in 2016, ISHLT convened a working group to define pulmonary AMR (8). Alongside developing a definition and classification/grading system for AMR, the group also addressed the

unique challenges of lung transplant candidates with evidence of detectable antibodies to non-self or "the sensitized" pre-transplant patient (8). This review builds on that initial report and will discuss the implications, challenges, and strategies surrounding the sensitized patient before and after lung transplant.

OVERVIEW OF AMR MECHANISM IN LUNG TRANSPLANT

In the early 1990s, the phenomenon of antibody-mediated rejection (AMR) was first described in kidney transplant recipients (9, 10). In addition to histological changes on graft biopsy, donor-specific antibodies (DSA) were described and closely associated with graft dysfunction. The best characterized donor antibodies are specific to human leukocytes antigens (HLA) and divided into two classes (HLA Class I and II), based on their structure and function (8). Despite the wide ability to detect HLA antibodies after transplant, solid organ transplant communities have defined and responded to AMR quite differently (4, 8).

AMR in lung transplant was historically limited to hyperacute rejection, which is thought to occur when preformed DSAs bind to HLA in the donor lung. In these instances, significant and often fatal graft failure occurred within minutes to hours of transplantation and was characterized by hemorrhagic pulmonary edema, severe gas exchange limitation, and diffuse pulmonary infiltrates on imaging studies (4, 11). Subsequent identification of HLA antibodies pre-transplant and avoidance of these antigens in the donor has greatly decreased the risk of hyperacute rejection.

AMR related immune activation in the lung includes allospecific B-cells and plasma cells that produce DSAs directed against HLA on the vascular endothelium in the lung allograft. The resulting antigen-antibody complex leads to an amplified immune response or recruitment of immune cells, *via* both complement-dependent and independent pathways, and subsequent lung tissue pathology and graft dysfunction. Complement is a multifunctional system of receptors, regulators and effector molecules that may amplify both innate and adaptive immunity contributions to AMR (4, 8, 12). Notably, pulmonary AMR is different than other solid organ transplant AMR (4, 8). For instance, the lung allograft may regulate humoral responses locally (independent of secondary lymphoid organs), as well as peripherally which is contrast to other solid organs which regulate the humoral response peripherally (13).

Preliminary work in pulmonary AMR indicate complement-binding DSA are associated with worse outcomes than non complement-binding DSAs (7). DSA associated complement-independent mechanisms of allograft injury include activation of signaling cascades that leads to endothelial and smooth muscle cell proliferation, release of inflammatory cytokines/chemokines, and platelet activation. These findings suggest DSA may play a role in CLAD (4, 8). Of note, lung transplant recipients who develop DSA have a higher risk of developing chronic rejection than individuals who did not develop DSA and worse survival (3, 14). One of the strongest risk factors for post-transplant DSA is pre-transplant detectable HLA antibodies, also called allosensitization. In recent years, data from multiple centers confirmed that allosensitization prior to transplant likely increases the risk of AMR (14, 15).

PRE-TRANSPLANT DETECTION OF HLA ANTIBODIES- TECHNIQUES, REPORTING, AND INCIDENCE

Several studies have demonstrated pre-transplant sensitization with anti-HLA antibodies are associated with decreased waitlist survival and survival after transplantation, increased ventilator days following lung transplant, higher rates of cellular rejection, development of donor-specific HLA antibodies, and bronchiolitis obliterans syndrome (BOS) (3, 16–18), however, this is not a universal finding (19). Various pre-transplant management approaches have been undertaken by the lung transplant community and largely remain institution specific (20). In addition to the potential post-transplant complications, lung transplant candidates with a high-calculated panel reactive antibody (cPRA) often have a longer waitlist time and higher risk of waitlist mortality compared with non-sensitized patients (21). To combat both the pre and post-transplant concerns, centers have employed several therapeutic approaches in an effort to lower or "desensitize" HLA antibody positive individuals prior to transplant (18).

However, many programs will decline highly sensitized lung transplant candidates (21). In a recent survey of lung transplant programs, 21.1% of programs considered a high cPRA as a contraindication to transplant, while 56.1% of programs declined offers for listed candidates who are highly sensitized on the basis of HLA antibodies to donor HLA. A minority of programs (14%) accepted offers regardless of positive virtual crossmatch or actual crossmatch (20). This variability between institutions underscores the need to better understand the effects of allosensitization on transplant related outcomes in an effort to minimize pre and post-transplant morbidity and mortality.

CONSIDERATIONS FOR POLICY CHANGES

Among the 3,500 transplants performed worldwide annually, approximately 60% of donors are allocated by the Lung Allocation Score (LAS) or a similar severity of disease score with a focus on maximizing transplant recipient benefit by balancing predicted mortality on the waiting list and one year survival (22, 23). While some countries have national wait lists, other countries participate in supranational allocation systems (e.g. Eurotransplant) (22). Although not accounted for in many lung allograft allocation systems, allosensitization is recognized as a barrier to transplant (21, 24).

Given the longer waitlist time and thus risk of death on the waiting list, the question has been raised on whether or not allosensitization should be weighted within the LAS or other allocation systems, though this is controversial (25). A single-center study found those with any degree of allosensitization were

less likely to undergo transplant than those without HLA antibodies (17). A separate single-center study considering allosensitization as a continuous variable found allosensitization prolongs the median waiting time and significantly decreases the likelihood of transplant. This study demonstrated a direct relationship between the breadth of allosensitization (as estimated by cPRA) and waiting time, as well as an inverse relationship with the likelihood of lung transplant (21). Given these findings, consideration of allosensitization in organ allocation policies may mitigate the risk of death on the waiting list (21); but with conflicting data regarding whether or not this subset of patients experience higher mortality and complications following transplant (3, 16–19). Thus, it remains difficult to determine whether extra consideration or exceptions for sensitized patients should be made available (26). In 2022, the United States allocation through the Organ Procurement and Transplantation Network will establish a continuous distribution allocation framework and incorporate allosensitization within the new system (27).

PRE-TRANSPLANT SENSITIZATION AND POST-TRANSPLANT OUTCOME

As noted above, the pre-transplant sensitization is associated with variable post-transplant outcomes. As the most of these are single center, retrospective studies, they should be interpreted with caution (4). On one end of the spectrum, Bosanquet et al. showed that pre-transplant allosensitization does not adversely affect outcomes after lung transplantation such as the development of ACR, lymphocytic bronchiolitis, DSA development, CLAD, graft failure, or mortality when potentially reactive HLA are avoided in the donor by a virtual crossmatch with the recipient (19) and similar findings in CLAD were echoed by Zazueta and colleagues (28). On the other end of the spectrum, several other studies reported significant increases in mortality, acute rejection, BOS, and AMR, as well as increased post-transplant ventilator days (3, 16–18, 29). Of important note, HLA class II antibodies, especially HLA-DQ antibodies are associated with worse outcomes and may warrant special management considerations (30, 31). With the concern that allosensitized lung transplant recipients may have more acute and chronic complications after transplant, it has become a great interest to optimally manage these patients (32).

MANAGEMENT STRATEGIES: WAITLIST AND ALLOCATION CONSIDERATIONS AND THERAPY

Early referral is one of the most important considerations for highly sensitized patients. Multiple societies recommend early referral to a transplant center for progressive lung disease that has a projected poor prognosis which also allows modifiable barriers to transplant (such as sensitization) to be addressed proactively to optimize candidacy or allow for early listing (33, 34). Often times lung transplant centers face the conundrum of balancing transplant urgency, which can arise as a result of late referral, with pre-transplant immunologic risk. Unfortunately, there is very little literature to guide this decision making process and is often center specific.

For sensitized patients, some centers are using strategies to potentially increase the donor pool with varying success rates. One of the first approaches is to geographically expand the donor pool using a virtual crossmatch. Rather than relying only on a prospective crossmatch for sensitized patients, which is cumbersome as it requires donor cells sent to the recipient center, a virtual crossmatch matching the donor antigens and recipient antibodies was implemented. All recipients still had a laboratory cross match, but after the transplant. Within this group of sensitized patients, the use of virtual crossmatch was associated with decreased number of days on the waitlist and decreased waitlist mortality and replicated the laboratory cross match findings (35).

Many of these management strategies aim to avoid subsequent DSA development in the recipient, as their development has been linked to adverse outcomes (3, 36). In one large single center study, DQ mismatching was an independent risk factor for the *de novo* DSA development (37). Based off this, a management strategy could consider specifically avoiding a DQ mismatch between donor and recipient, but this may not realistic based on the allocation system and recipient medical condition.

Some transplant centers have employed therapeutic approaches in an effort to lower antibodies or "desensitize" individuals prior to transplant or perioperatively (18, 38). These targeted desensitization therapies are based on antibody mediated rejection therapies. An early prospective single center study found recipients with post-transplant development of DSA who received either IVIG or combination IVIG/rituximab did not have increased risk for acute cellular rejection, lymphocytic bronchiolitis, BOS or increased mortality (39). With these encouraging results post-transplant results, several programs adopted either a pre-transplant approach or a peri-transplant desentization protocol (18, 38).

Pre-Transplant Desensitization Approach in Lung

Using renal transplant experience, lung transplant centers have tried to desensitize pre-transplant patients (40). Appel et al. retrospectively evaluated efficacy of a peri-transplant desensitization regimen utilizing IVIG and extracorporeal immunoadsorption (ECI) in sensitized lung transplant (n=34) which found a significant reduction in ACR, however no significant difference in development of BOS or mortality following transplant (41).

Peri-Transplant Approach

A relatively large single center study of 146 patients with known DSA or cPRA≥30% were treated with a peri-transplant desensitization protocol that included plasmapheresis, IVIG, antithymocyte globulin, and mycophenolic acid. Compared to 194 unsensitized patients, the treated sensitized recipients had significantly lower rates of acute rejection and no significant difference in spirometry, development of CLAD or 1-year graft survival (38).

In a single center study of highly sensitized patients (cPRA≥80%), 18 pre-transplant patients had an aggressive protocol that included plasmapheresis, methylprednisolone, bortezomib, rituximab, and IVIG. While there was a significant decrease in HLA antibody when measured by MFI, there was no difference in cPRA or mortality (18). Eight of 18 patients completed the protocol and went on to be transplanted.

In a recent single center report, a small group (n=5) of highly sensitized patients (cPRA≥80%) underwent a peri-transplant multimodal desensitization protocol which included plasmapheresis, rituximab, IVIG, antithymocyte globulin, carfilzomib, and belatacept. With a median follow up time of 427 days, all patients were alive with CLAD-free survival and no episodes of ACR or AMR. Treated patients had a significantly decreased waitlist time compared with historical controls. Of note, several infectious complications were noted (42).

Of recent interest in other solid organ transplants (kidney and heart) is the use of belatacept, a high affinity variant of CTLA4-IG which binds to CD80 and CD86 on antigen presenting cells thereby preventing CD28 mediated signaling critical for T cell activation and proliferation, T follicular helper cell differentiation, and cognate T/B cell interactions. Belatacept was approved for use in renal transplant recipients on the basis of two randomized controlled trials (43, 44). Several centers have reported lower incidence of DSA and superiority in constraining preexisting HLA antibody responses compared with calcineurin inhibitor-based immunosuppression (45, 46). When use in combination with desensitization strategies that focus on eliminating antibodies (plasmapheresis) and/or precursor cells responsible for antibody production (proteasome inhibitors and anti-CD20 antibodies) the hope is a more effective, durable "immune modulating" strategy that reliably and sustainably reduces HLA antibodies both before and after transplant (45–47).

Indeed there has been some promising data with the addition of belatacept to desensitization regimens in other solid organ transplant patients (47). Alishetti et al. looked at 4 highly sensitized heart patients (cPRA >99%) that underwent a multimodal desensitization protocol with a proteasome inhibitor, dexamethasome, and belatacept +/- plasmapheresis prior to heart transplant. In all four patients, desensitization with this regimen decreased the average MFI of class I and II antibodies and in most cases this response was sustained between cycles and after cessation of the proteasome inhibitor. Additionally, the chances of finding a donor to whom the recipient did not have high MFI antibodies increased markedly after desensitization (based on calculated likelihood ratios of cPRA). Of note, two infectious complications were reported which resolved with treatment (47). To date, the small cohort described in lung candidate above is the only known use of CD28 co-stimulation blockade in multimodal lung transplant desensitization protocols and larger scale trials are needed (42). The higher immunosuppressive effects are attractive in highly allosensitized patients, however safety and efficacy data are yet to be seen (48).

While some centers have reported success in regards to desensitization and transplant related outcomes, the varied protocols, heterogeneous patient populations, and relatively small sample size makes it difficult to draw conclusions regarding the role and impact of desensitization protocols especially in regards to what patients may benefit. Unfortunately, there are not randomized controlled trials that compare the clinical efficacy of different desensitization strategies.

From a logistical standpoint there is a benefit to a pre-transplant protocols that can be scheduled as opposed to peri-transplant which requires on-demand resources. However, the timing of pre-transplant may hinder the impact of protocols if the transplant occurs considerably later. Additional considerations are the off-label use of therapies which may present substantial cost to the patient or health system and can therefore be a limiting factor to accessing therapies. From a safety standpoint, potential side effects related to certain therapies should be considered in the context of the patient.

DISCUSSION

Pre-transplant allosensitization remains a considerable barrier in the ability to receive a transplant and avoid complications after transplant. Sensitization limits the number of donors available, thereby extending waitlist time and mortality. Efforts to expand the donor pool often includes intensive therapies that may increase the risk of morbidity and mortality. Even if a suitable donor is found, pre-transplant allosensitization increases the risk for AMR and potentially other complications including CLAD after transplant. Further research is needed in order to best manage the pre and post transplant concerns of allosensitization.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the manuscript and editing of the manuscript. Primary writing was done by KY. All authors contributed to the article and approved the submitted version.

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Measuring the Impact of Targeting FcRn-Mediated IgG Recycling on Donor-Specific Alloantibodies in a Sensitized NHP Model

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Background: In transplantation, plasmapheresis and IVIg provide the mainstay of treatment directed at reducing or removing circulating donor-specific antibody (DSA), yet both have limitations. We sought to test the efficacy of targeting the IgG recycling mechanism of the neonatal Fc receptor (FcRn) using anti-FcRn mAb therapy in a sensitized non-human primate (NHP) model, as a pharmacological means of lowering DSA.

Methods: Six (6) rhesus macaque monkeys, previously sensitized by skin transplantation, received a single dose of 30mg/kg anti-RhFcRn IV, and effects on total IgG, as well as DSA IgG, were measured, in addition to IgM and protective immunity. Subsequently, 60mg/kg IV was given in the setting of kidney transplantation from skin graft donors. Kidney transplant recipients received RhATG, and tacrolimus, MMF, and steroid for maintenance immunosuppression.

Results: Circulating total IgG was reduced from a baseline 100% on D0 to 32.0% (mean, SD ± 10.6) on d4 post infusion ($p<0.05$), while using a DSA assay. T-cell flow cross match (TFXM) was reduced to 40.6±12.5% of baseline, and B-cell FXCM to 52.2±19.3%. Circulating total IgM and DSA IgM were unaffected by treatment. Pathogen-specific antibodies (anti-gB and anti-tetanus toxin IgG) were significantly reduced for 14d post infusion. Post-transplant, circulating IgG responded to anti-FcRn mAb treatment, but DSA increased rapidly.

Conclusion: Targeting the FcRn-mediated recycling of IgG is an effective means of lowering circulating donor-specific IgG in the sensitized recipient, although in the setting of organ transplantation mechanisms of rapid antibody rise post-transplant remains unaffected.

Keywords: FcRn, IgG, alloantibody, sensitization, non-human primate (NHP)

INTRODUCTION

In transplantation, plasmapheresis (plasma exchange, PEX, or double filtration plasmapheresis, DFPP) has been one of the mainstays of many protocols aiming to remove circulating donor-specific antibody (DSA). For sensitized patients, indications for therapy are prophylactic removal of DSA perioperatively to facilitate transplantation (1, 2), or as treatment for antibody-mediated rejection (AMR) (3). Despite wide utility, this treatment is limited by efficacy (4), and although major complications are rare, the therapy is non-specific and risks off-target effects, such as removal of circulating soluble coagulation factors thus increasing bleeding risk (5), as well as concurrent pharmacological therapies (6), necessitating additional treatment.

In the clinical setting of HLA-incompatible transplantation, the introduction of IdeS (imlifidase) has permitted highly sensitized patients to receive a deceased donor kidney against whom they have a positive initial crossmatch by cleaving soluble IgG at the lower hinge region, thus generating F(ab) and Fc fragments and swiftly reducing levels of circulating DSA shortly before transplantation to a negative crossmatch, in the absence of the deleterious effects of plasmapheresis (7, 8). In addition, further evidence suggests that IdeS is also effective in cleaving IgG when cell bound to the IgG subtype BCR (B cell receptor) complex, resulting in an inhibitory effect on antibody-secreting cells (ASCs) (9). Despite this success, the utility of this treatment may be limited by the emergence of anti-drug antibodies (ADA), which would reduce efficacy and therefore prevent repeated use of IdeS (10).

An alternative biologic mechanism to reduce levels of circulating antibody is to target the neonatal Fc receptor (FcRn). This receptor is expressed in most tissues, but is reliably found in endothelial cells, and antigen presenting cells (APCs) such as macrophages/monocytes, dendritic cells, and B cells (11, 12). Related to the MHC structure, but incapable of itself presenting IgG to T cells, the neonatal FcRn is so called as it is necessary for the passive transfer of maternal antibody to the neonate. Despite the infant name, this receptor persists throughout life and remains an important mechanism by which circulating IgG levels are maintained. IgG captured on the cell surface by the FcRn is taken up by intracellular vesicles under mildly acidic conditions to be subsequently re-released into serum (13, 14). A high affinity monoclonal antibody that targets the FcRn, Rozanolixizumab, has been developed and tested in cynomolgus monkeys, as well as humans (15, 16). Therapeutically, blocking the FcRn is actively being investigated for its role in the treatment of IgG-mediated diseases, such as myasthenia gravis, and primary immune thrombocytopenia (17), and has been reported as a potential agent in the setting of AMR in transplantation both for its direct effect on lowering circulating antibody levels, and as a mechanism to be exploited with respect to maintaining circulating drug levels of IgG-based therapeutics (18, 19). However, it has not been tested in rhesus macaque models of sensitization or IgG-mediated pathology. In the present study, we hypothesized that rhesus specific anti-FcRn mAb could provide a safe and effective pharmacological method to reduce circulating DSA, as well as total IgG levels, and thereby facilitate kidney transplantation in allosensitized recipients.

Abbreviations: ADA, Anti-drug Antibody; APC, Antigen Presenting Cell; AMR, Antibody Mediated Rejection; BCR, B-cell Receptor; BFXM, B-cell Flow Crossmatch; CFCA, Calibration-free Concentration Analysis; DFPP, Double Filtration Plasmapheresis; DSA, Donor Specific Antibody; ESRD, End Stage Renal Disease; FcRn, Neonatal Fc Receptor; FXM, Flow Crossmatch; HLA, Human Leucocyte Antigen; IdeS, Imlifidase; PEX, Plasmapheresis; POD, Post-operation Day; MHC, Major Histocompatibility Antigen; NHP, Non-Human Primate; PBMC, Peripheral Blood Mononuclear Cell; PBS-T, Phosphate-buffered saline with Tween; TFXM, T-cell Flow Crossmatch; TMA, Thrombotic microangiopathy.

MATERIALS AND METHODS

Animals, Surgical Procedures, and Drug Treatments

All animal care and procedures were conducted in accordance with National Institutes of Health (NIH) guidelines and were approved by the Duke University Institutional Animal Care and Use Committee (Duke IACUC# A153-18-06). Six juvenile (3–5 year old) male NHPs (*Macaca mulatta*) were obtained from Alphagenesis (Yemassee, SC). Donor and recipient pairs are created between animals with maximal MHC class-1 and -2 mismatching. Donor-recipient NHP pairs were sensitized to each other with two sequential full-thickness skin transplants performed as previously described (20). A month after skin transplantation, when the anti-donor response is stabilizing following a peak. six animals were treated with a single 'test-dose' of 30mg/kg of anti-RhFcRn mAb (anti-FcRn [RozR1LALA], a LALA-mutated rhesus IgG1 chimeric of Rozanolixizumab, NIH Nonhuman Primate Reagent Resource Cat# PR-0001, RRID: AB_2888630). Peripheral blood sampling was performed to monitor full recapitulation of circulating antibody response at 0, 1, 4, 7, 13, 20 and 27 days post-test dose. The administration of non-native therapeutic monoclonal antibodies (mAbs) can induce anti-drug antibody (ADA) responses in humans (21) and nonhuman primates (NHP) (22) which can affect drug pharmacokinetics and efficacy, limiting the informative value of NHP studies. Therefore, to mitigate the impact of ADA in NHP experiments, the nonhuman primate reagent resource (nhperagents.org) engineered a 'primatized' mAb against FcRn (anti-FcRn [RozR1LALA], a LALA-mutated rhesus IgG1 chimeric of Rozanolixizumab).

A donor-recipient pair of animals (n = 2) received swopping kidney transplantation from their maximally mismatched skin donor, with bilateral native nephrectomies at six weeks after test dosing as previously described (23). For kidney transplantation, anti-RhFcRn mAb was administered at 60mg/kg IV on day −5, 0, and +5 day of kidney transplantation. All transplanted NHPs received induction therapy with 20 mg/kg IV rhesus ATG (Anti-rhesus thymocyte [rhATG7] - Lot 7, rhATG#7, NIH NHP Reagent Resource Cat # PR-1077, RRID: AB_2819339) in 5 divided doses (POD 0 to 4, 4mg/kg daily). Maintenance immunosuppression after kidney transplant consisted of intramuscular (IM) tacrolimus (Astellas Pharma, Northbrook,

IL) twice daily with the dose adjusted to maintain trough levels at 8–12 ng/ml, 30 mg/kg by mouth of mycophenolate mofetil (MMF) for oral suspension (Genentech, San Francisco, CA) twice daily, and methylprednisolone (Pfizer, New York, NY) tapered from 15 mg/kg on day of transplant and then a halved dose daily until a maintenance dose of 0.5 mg/kg was reached and continued until the end point. A subcutaneous dose of 6 mg/kg of ganciclovir (Fresenius Kabi, Lake Zurich, IL) was administered daily as rhesus cytomegalovirus (rhCMV) reactivation prophylaxis.

Comparison in survival and anticipated DSA levels were made between these anti-FcRn mAb treated animals, and previously established historic controls who received the same induction and maintenance immunosuppression, but no desensitization therapy prior to kidney transplantation (24).

In addition, evidence of circulating RhATG levels in Rh anti-FcRn mAb receiving a kidney transplant were compared with time matched (d5 after first ATG dose) animals who received either 20 mg/kg IV rhesus ATG (RhATG#5, n = 4 + RhATG#6, n = 4, NIH NHP Reagent Resource) in 5 divided doses without kidney transplantation (POD 0 to 4, 4mg/kg daily, or rabbit ATG as given to humans (rATG, n= 4), (25).

Anti-FcRn Monoclonal Antibody Quantitation by ELISA

The presence of the anti-FcRn in sera was quantitated *via* ELISA. Purified recombinant rhesus FcRn soluble protein was used to coat plates overnight at 1 μg/μL in PBS (pH 8.0, 4°C). ELISA plates were then washed three times with PBST and blocked with 300uL of non-fat dry milk in PBS (pH 8.0) for 1 h at 37°C. The standard curves were generated with the recombinant anti-FcRn antibody starting at a concentration of 10 μg/μL. Sample dilutions were done in PBS (pH8.0), 5% non-fat dry milk, and 10% plasma with a total volume of 100 uL to each well. Serum samples with unknown amounts of anti-FcRn were serially diluted 2-fold with an initial dilution of 1:10, then added to wells and incubated for 1 h at 37°C. ELISA plates were washed three times with PBST and 100 μL of HRP conjugated anti-human kappa (Southern Biotech) was added to each well and incubated for 1 h at 37°C. The ELISA plate was then washed three times with PBST and developed by adding 100 uL of TMB substrate at room temperature for 2–3 min, stopped and read at 450 nm.

Quantitative Measurement of Anti-Drug Antibody (ADA) Response in Serum

Anti-drug antibody (ADA) responses were evaluated by ELISA against the recombinant anti-FcRn antibody (1 μg/μL coated overnight at 4°C in PBS pH 7.4). ELISA plates were then washed three times with PBST and blocked with 300uL of non-fat dry milk in PBS (pH 7.4) for 1 h at 37°C. Sample dilutions were done in PBS (pH 7.4), 5% non-fat dry milk with a total volume of 100 uL to each well. Serum samples were serially diluted 2-fold with an initial dilution of 1:10, then added to wells and incubated for 1 h at 37°C. ELISA plates were washed three times with PBST and 100 μL HRP conjugated anti-lambda (Southern Biotech) was added to each well and incubated for 1 h at 37°C. ELISA was then washed three times with PBST, developed with TMB, and read at 450 nm. Samples were considered ADA-positive if the absorbance of the sample was 2 times higher than the pretreatment samples.

Measurement of DSA

As described previously, DSA levels were determined by flow cytometric crossmatching using donor splenocytes or PBMCs, incubated with recipient serum from serial blood draws (26). DSA titer measurement was made on serum samples which were stored at –80°C and batched for analysis. For IgG measurement, samples were serially diluted (1:50) in each run, while for IgM measurements, neat sera were used. No serum, 'naïve' (pre-sensitization) as well as 'peak' (2 weeks following second skin transplantation) sensitization samples were run as negative and positive controls. Donor PBMCs or splenocytes were incubated with recipient serum, washed, and then stained with FITC-labeled anti-monkey IgG (Sera Care, 5210-0216), or FITC-labelled anti-monkey IgM (Sera Care, 5230-0423), anti-CD20 mAb (2H7), anti-CD3 mAb (SP34–2) (both BD Bioscience), and Live/Dead Fixable Blue staining (Life Technologies, Carlsbad, CA). The mean fluorescence intensity (MFI) of anti-monkey IgG or IgM on T or B cells was measured on BD LSRFortessa (BD Biosciences) and analyzed using FlowJo software version 9 or 10 (Tree Star). Results were expressed as either MFI, or MFI fold-change from the pre-sensitized (naïve) time point. A MFI value lower than the neat naive sample was considered negative.

Total IgG Measurements

Measurement of IgG in sera was done by surface plasmon resonance (SPR) using a protein G chip and calibration-free concentration analysis (CFCA) (27). Concisely, the serum samples were initially diluted 1:10000 in HEPES-Buffered Saline (HBS) and then serially diluted 2-fold in HBS and loaded into a the Biacore T200 SPR equipment. CFCA calculations were done using software using an average molecular weight of 150 kDa.

Total IgM Measurements

Measurement of IgM in sera was measured by Human IgM ELISA Kit (Bethyl Laboratories, Inc. E88-100). The kit contains an ELISA plate pre-coated with anti-human IgM antibodies. The 8 standard solutions were prepared by reconstituting a vial of 750 ng of human IgM in 1.0 ml of 1X Dilution Buffer B and serially diluting by 3-fold. The sera samples were prepared by diluting 10 μL of sera in 990 μL of 1X Dilution Buffer B, and repeating the dilution again to yield a total of 1:10000 dilution. Wells in the ELISA plate were loaded with 100 μL of standard solutions or diluted sera samples. The plate was incubated at room temperature (20–25°C) for 1 h. ELISA plate was then washed four times with 1X Wash Buffer (300μL/well) and 100 μL of biotinylated detection antibody was added to each well, to be incubated at room temperature for 1 h. The ELISA plate was then washed four times with 1X Wash Buffer and the plate was incubated for 30 min at room temperature after adding 100 μL of streptavidin-conjugated horseradish peroxidase (SA-HRP) in

the wells. Plates were then washed four times with 1X Wash Buffer (300μL/well) and the plate was developed for 30 min at room temperature in the dark after adding 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) in each well. After the reaction was stopped with 100 μL of Stop Solution, the plate was read using a plate reader at 450 nm.

ATG Quantitation by ELISA

The presence of ATG antibodies in sera was measured by ELISA using rabbit-specific antibodies. ELISA plates were coated overnight at 4°C with 100 μL at 1 μg/μL of Goat anti-Rabbit IgG Antibody (EMD Millipore) in PBS (pH 7.4). ELISA plates were then washed three times with PBST and blocked with 300 uL of non-fat dry milk in PBS for 1 h at 37°C. A standard curve was generated with rhesus ATG starting at a concentration of 10 μg/μL and doing a 2-fold serial dilution. Successively, on the same plate, serial dilutions of 2-fold was done on rhesus serum in PBS with 5% non-fat dry milk. ELISA plates were washed three times with PBST and 100 μL of HRP-conjugated goat anti-rabbit IgG (H+L) (Jackson Immuno Research) was incubated on each well for 1 h at 37°C. ELISA plates were then washed three times with PBST and developed by adding 100 uL of TMB substrate at room temperature for 2–3 min. After the reaction was stopped, the plates were read using a spectrophotometer at 450 nm.

Measuring Tetanus Toxoid-Specific and Rhesus gB-Specific IgG

The magnitude of tetanus toxoid-specific and rhesus gB-specific IgG responses were measured by ELISA. First, 384-well plates were coated with either 2 μg/ml of Clostridium Tetanus Toxoid (Creative Diagnostics) or 1.5 μg/ml of RhCMV gB, diluted in 0.1 M sodium bicarbonate buffer, pH 9.6. Coated plates were incubated at 4°C overnight. Plates were then washed one time with wash buffer (95% DI water, 4% 25X PBS, 1% Tween-20), blocked with 40 μl blocking buffer per well (4% whey, 15% goat serum, 0.5% tween 20, 80.5% 1X PBS), and incubated at 4°C overnight. Samples were diluted in blocking buffer at 1:5 for tetanus toxoid ELISA and 1:30 for Rhesus gB ELISA and serially diluted 1:3 in a 96-well plate (CSL Behring). A known seropositive sample was used as a positive control for the gB ELISA and the WHO tetanus standard was used as a positive control for the tetanus toxoid ELISA. After blocking, plates were washed one time and 10 μl of diluted sample were added to the plate in duplicate. Plates were incubated for 2 hours at RT. Secondary antibodies (Mouse anti-Monkey IgG-HRP, Southern Biotech; Goat anti-Human IgG-HRP, Jackson ImmunoResearch) were prepared at a 1:5000 dilution in blocking buffer. After the incubation, plates were washed two times and 10 μl of diluted secondary antibody were added to each well. Plates were sealed and incubated for 1 hour at RT. Plates were then washed four times and 20 μl of room temperature SureBlue Reserve TMB Substrate (VWR) was added to each well. Plates were incubated for 10 min while shielded from light. After incubation, 20 μl of TMB Stop Solution (VWR) was added to each well. Plates were immediately read at 450 nm on the SpectroMax using the SoftMax software interface. Data are reported as area under the curve (AUC) because full sigmoidal curves were not achieved by all samples.

Histology, Immunohistologic Analysis, and Pathologic Grading

At euthanasia, graft specimens were harvested and fixed in 10% buffered formalin before being paraffin embedded for sectioning, and staining with hematoxylin & eosin (H&E), Schiff (Periodic Acid–Schiff) or polyclonal anti-human C4d (American Research Products, Waltham, MA) prior to histologic grading. Histology specimens were evaluated in a blinded fashion by a transplant pathologist (A.B.F.) and scored based on the current Banff criteria of renal allograft pathology (28–31).

Statistical Analyses

Statistical analyses were performed using Prism 8.0 (GraphPad Software, San Diego, CA). Data are expressed as mean±SD (error bar), and p values of less than 0.05 were considered to be statistically significant. Normally distributed data within the same treatment group but at different time points were evaluated using a two-tailed paired t test, while statistical comparisons between different groups were performed with two-tailed unpaired t test for normally distributed data or the Mann–Whitney U test for categorical data.

RESULTS

Anti-Rhesus FcRn mAb and Anti-Drug Response in Sensitized NHPs

Maximally MAMU mismatched rhesus macaque pairs underwent swapping skin transplantation as a sensitization event. One month following skin transplantation, once serum DSA had declined from a peak, 6 animals were given a single dose of 30mg/kg of anti-Rh FcRn mAb IV. Infusions were well tolerated and did not result in adverse reactions. Anti-Rh FcRn mAb was detected in serum on 1d post infusion, but was rapidly undetectable thereafter (**Figure 1A**). ADA (anti-anti-FcRn antibody) was detectable following a single dose, with maximal development at 21d post infusion (**Figure 1B**). Interestingly, ADA responses varied considerably by animal with pre- and peri-transplant dosing (**Supplemental Figures 1** and **3**).

Effect of Anti-Rhesus FcRn mAb on Total Circulating IgG and IgM

The total IgG was reduced for all animals, which was most maximally evident on post infusion day (PID) 4 with a mean reduction of 68±10.6% from baseline which was significant ($p<0.05$), although the likely nadir may have been on PID 5 (**Figure 2A**). By PID 28, the total IgG level had recapitulated back to baseline (103± 23.7% of baseline, **Figure 2A**). In contrast, while both CD3 (T cell FXCM) and CD20 (B cell FXCM) reduced significantly on PID 4 (TFXM 40.6% of baseline SD ± 12.5, p = <0.05 BFXM 52.8% of baseline SD ± 19.3, p = <0.05), the reduction was maintained at PID 28 (TFXM IgG 46.2% of baseline SD ± 13.8, p = <0.05, BFXM IgG 58.8% of baseline SD ±

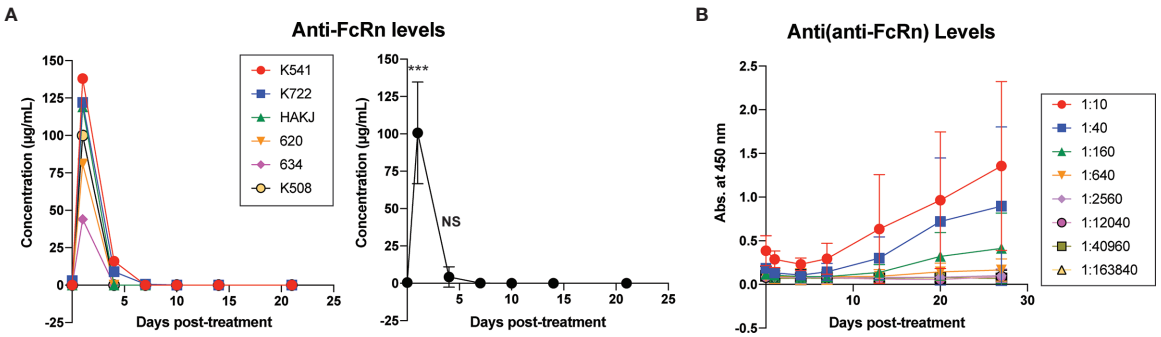

FIGURE 1 | Kinetics of anti-FcRn mAb and anti-drug antibody (ADA) levels following a single dose of rhesus anti-FcRn mAb (30mg/kg IV, n=6). **(A)** Anti-FcRn mAb concentration detected by ELISA in serum post-infusion over time demonstrating individual and collective values and rapid fall in circulating mAb present. **(B)** Generation of anti-drug antibodies ADAs over time detected by ELISA. ***, < 0.001, ns, not significant.

11.2, p = <0.05, **Figure 2B**), suggesting a more sustained effect on DSA in contrast to circulating total IgG levels. Typically, in this model, a gradual decay of circulating DSA is observed over time. The sustained reduction of DSA observed in anti-Rh FcRn treated animals was, however, greater, compared to historical untreated controls at the same experimental time interval (see **Supplemental Figure 1**). Interestingly, the second skin transplant had the same effect in boosting serum DSA for anti-Rh FcRn treated animals, suggesting that there was no continuing effect reducing circulating DSA IgG, and that the humoral apparatus to generate DSA remained intact (see **Supplemental Figure 2**).

Total IgM and DSA IgM showed no evidence of significant reduction following anti-RhFcRn mAb treatment on PID 4, compared to PID 0 (TFXM IgM 68.5% of baseline SD ± 35.99, p = 0.06, BFXM 78.62% of baseline SD ± 30.8, p = 0.12, **Figure 3B**),

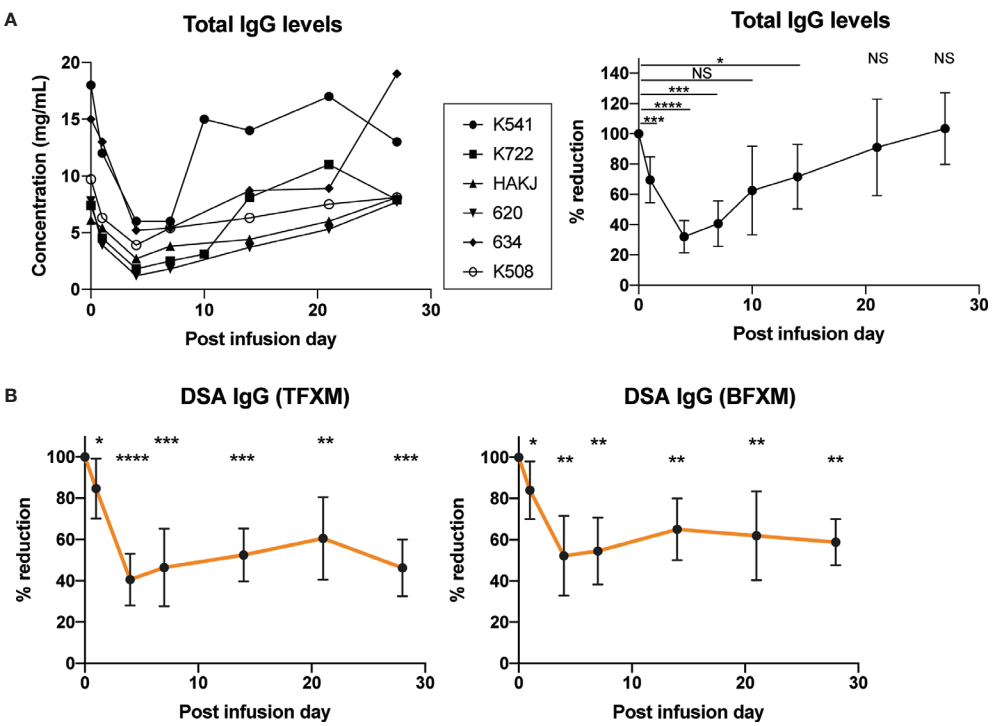

FIGURE 2 | Effect of a single dose of rhesus anti-FcRn mAb (30mg/kg IV, n=6) on circulating total IgG and IgG donor-specific antibody (DSA) levels. **(A)** Total serum IgG concentration for individual animals (left). Total IgG level was reduced after anti-FcRb mAb treatment. **(B)** Percent reduction of circulating DSA IgG with TFXM and BFXM post-infusion demonstrating maximal reduction on post infusion day (POD) 4 to POD7, and persistent significant reduction to 1 month post infusion. * indicates p<0.05; ** indicates p<0.001; *** indicates p<0.005; NS indicates no statistical significance.

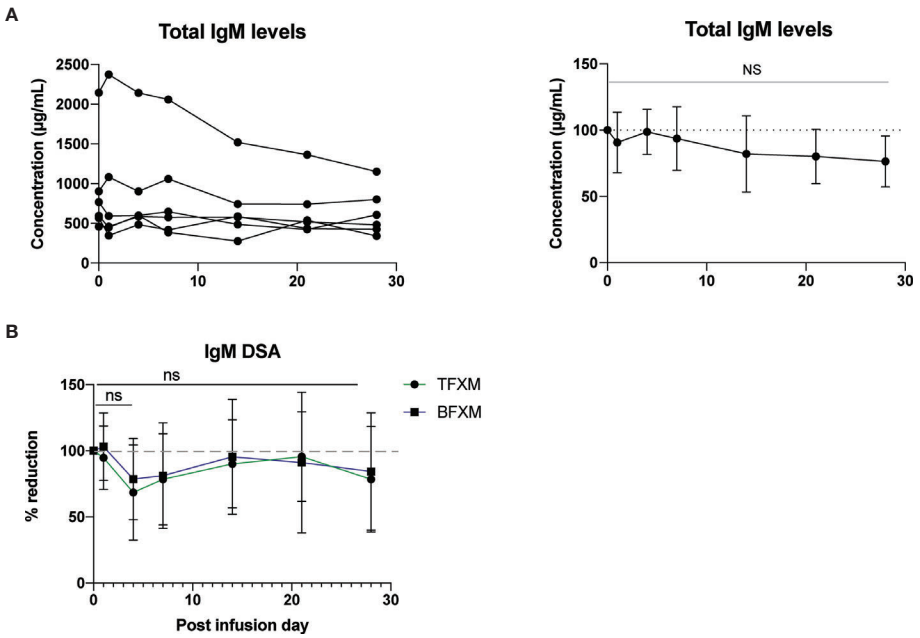

FIGURE 3 | Effect of a single test dose of rhesus anti-FcRn (30mg/kg IV, n = 6) on circulating IgM. **(A)** Total serum IgM concentration by animal. Reduction of total serum IgM post infusion. **(B)** Percent reduction of circulating donor-specific antibody (DSA) IgM for CD3 (TFXM) & CD20 (BFXM) post infusion demonstrating no significant reduction for one month following infusion. NS indicates no statistical significance.

and largely remained static over the course of the post infusion period **(Figures 3A, B).** Together, these data suggest that a single dose (30mg/kg) of anti-FcRn mAb tentatively reduces total circulating IgG and DSA IgG but is incapable of reducing the IgM isotype of DSA.

Effect of Anti-Rhesus FcRn mAb on Pathogen-Specific IgG

Since all rhesus macaques are vaccinated against tetanus toxoid and acquire rhesus cytomegalovirus (rhCMV) exposure in life, we measured the effect of a single dose of Rh-anti-FcRn treatment on protective immunity by measuring antibodies against CMV envelope glycoprotein B (gB) and tetanus toxoid (26). Unsurprisingly, as with the total and DSA IgG, a significant reduction in circulating pathogen-specific antibody was measured which reached nadir at PID 7 for anti-gB (rhesus CMV) antibody, and PID 4 for anti-tetanus toxoid antibody, **Figures 4A, B**. These data demonstrate the non-selective nature of anti-FcRn mAb in blocking the recycle of IgG antibodies, regardless of their specificity.

Anti-FcRn as Antibody Removal Treatment in the Context of Sensitized Kidney Transplantation

Given the efficacy of a single dose of Rh anti-FcRn, we tested a higher dose (60mg/kg, IV) in the setting of allosensitization kidney transplant in order to test whether a more profound reduction of DSA could be achieved. Two animals received Rh anti-FcRn 5 days prior to kidney transplantation, and again on the day of kidney transplantation, following initiation of the first dose of RhATG, as well as on POD5 (**Figure 5A**). Compared to previously reported control animals treated with the same induction and maintenance regimen who received no desensitization (24), there was no prolongation in survival with rh anti-FcRn mAb (**Figure 5B**). Histopathology of the kidney allograft demonstrated evidence of acute AMR in both anti-FcRn treated animals, with C4d deposition, and glomerulitis and peritubular capillaritis (**Table 1**). One animal also had evidence of acute cellular rejection, in addition to TMA-like features with glomerular fibrin deposition and fibrinoid arterial necrosis (**Figure 5C**). Serum anti-FcRn mAb levels indicated the presence of drug, although, as previously, circulating levels fell rapidly after 24h, and therefore were not captured following the initial administration of anti-FcRn 5 days before kidney transplantation (**Figure 5D**). Total circulating IgG levels reduced in response to treatment prior to kidney transplantation. However, despite a brief rise in circulating IgG on the first post-operative day, following repeated administration of anti-FcRn on POD 0 and POD 5, there was a reduction compared to pre-transplant baseline (**Figure 5E**). With respect to circulating DSA, there was an initial reduction from baseline levels, but after POD 4, and the increase from baseline was exponential (**Figure 5F**). IgM DSA showed a similar trajectory, albeit with a more limited reduction prior to transplantation (**Figure 5G**).

Given the repeated administration of Rh anti-FcRn, both following the earlier 30mg/kg single infusion, as well as the subsequent repeat dosing of 60mg/kg IV, ADA were measured by ELISA. One of the animals demonstrated evidence of ADA in

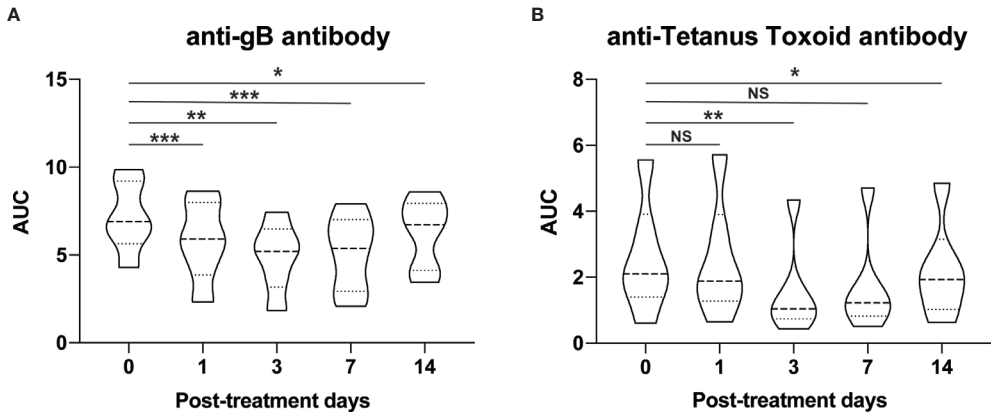

FIGURE 4 | Pathogen-specific antibody changes after anti-FcRn mAb treatment. Effect of single dose of rhesus anti-FcRn mAb (30mg/kg IV, n=6) on circulating antibodies against protective immunity **(A)** CMV envelope glycoprotein B (gB) and **(B)** tetanus toxoid. * indicates p<0.05; ** indicates p<0.001; *** indicates p<0.005; NS indicates no statistical significance.

greater concentration from POD 1 (**Supplemental Figure 3**), although the functional consequence of this antibody is unclear given the similar pattern of short survival and allograft AMR. To investigate the relative lack of efficacy of Rh anti-FcRn we first investigated whether the presence of Rh anti-FcRn mAb could be interfering with the concurrent RhATG therapy. We compared serum RhATG levels measured on d4/5 post-administration of RhATG in anti-FcRn treated animals (n=2) to animals who received Rhesus (n=7) or rabbit ATG (n=4) as part of a different experiment (In submission). There was no apparent difference in circulating RhATG concentration on POD4/5 (**Supplemental Figure 4**). Taken together, perioperative administration of anti-FcRn mAb successfully interfered with total circulating IgG maintenance *via* FcRn-mediated IgG recycling. However, it did not result in the reduction of circulating DSA (**Figure 5F**) compared to controls without anti-FcRn mAb treatment (**Supplemental Figure 5A**). This points to anti-RhFcRn being insufficient to reduce alloantibody produced by newly generated plasma cells as a consequence of rapid differentiation from the memory B cell compartment.

DISCUSSION

In this study, we demonstrate the first application of anti-FcRn in a sensitized rhesus macaque model to evaluate circulating DSA. Targeting the FcRn-mediated recycling mechanism in transplantation is extremely attractive, since an effective treatment has the potential to reduce the requirement for both plasmapheresis and high-dose IvIg (18, 32). As expected, anti-FcRn mAb treatment (30mg/kg) promoted a significant reduction of total IgG level as well as donor-specific IgG **(Figure 2)**. Circulating anti-FcRn mAb was only detectable on the PID 1, indicating rapid uptake and efficacy, which is in keeping with previous studies (15, 16). Interestingly, although the reduction of IgG DSA was less than total IgG at nadir (POD4: BFXM -47.8% ± 19.8, TFXM -59.4 ± 12.5, *vs.* 68% ± 10.6), it took longer for IgG DSA to return to baseline level. Given the natural decay of DSA after skin transplantation, it is uncertain whether the observed phenomenon truly represents an increased sensitivity of IgG DSA with respect to inhibition of the FcRn-mediated recycling or not, although compared to time-matched controls, the reduction of DSA at PID 28 was significant (**Supplemental Figure 1**).

As our results demonstrate, post-transplant there was incomplete reduction of the circulating the DSA IgG response. In part, this may relate to the construct of the Rh anti-FcRn mAb itself. The IgG1 isotype might be less effective at targeting the FcRn mechanism compared to rozanoliziumab, which is constructed with an IgG4 isotype. However, it should be considered that the direct relationship between NHP IgG isotypes with respect to their human IgG subtype corollary as well as their pathogenicity is unclear. However, it should be noted that an increased dose (60mg/kg, compared to the single dose of 30mg/kg) did not lead to a greater reduction of either IgG or DSA IgG from baseline, suggesting saturation. In the meantime, anti-FcRn mAb treatment showed the absence of any significant effect of circulating total IgM or DSA IgM **(Figure 3)**. IgM DSA is rarely measured in the clinical setting and is largely present at lower circulating levels, although since it is a more potent activator of complement, high levels of IgM DSA have been linked to aggressive AMR resistant to complement therapy in both humans and NHPs ((33) Schmitz et al, under revision). As evidenced by this data, FcRn is an IgG-targeting mechanism, and therefore offers no protection in that setting.

Furthermore, given the non-specific nature of targeting the FcRn, as demonstrated with the observed decrease in CMV and anti-tetanus toxoid antibody responses **(Figure 4)**, the off-target effect with respect to protective immunity likely precludes prolonged usage. Even for patients with an otherwise intact immune system, such as those highly sensitized patients consideration should be given to the negative consequences of

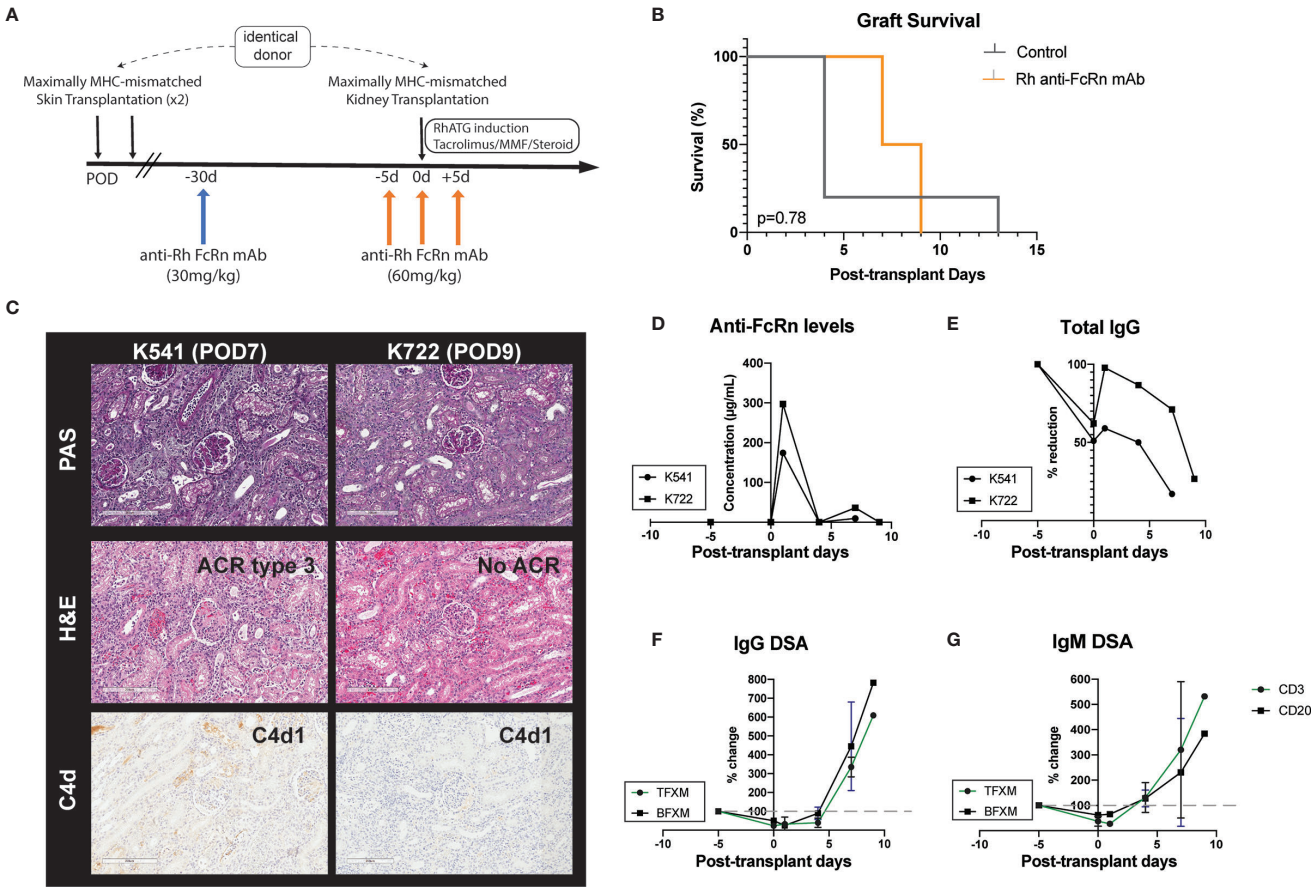

FIGURE 5 | Desensitization with anti-FcRn mAb for kidney transplantation in a highly sensitized non-human primate (NHP) model. **(A)** Schematic representation of dosing strategy and immunosuppression regimen indicating single test dose (30mg/kg IV), followed by administration of 60mg/kg IV in the peri-transplant setting. **(B)** Graft survival of transplanted animals treated with anti-FcRn mAb compared to control (no desensitization). **(C)** Kidney allograft histopathology (PAS, H&E, and C4d staining) from anti-FcRn mAb-treated animals demonstrating evidence of capillaritis, glomerulitis, and C4d deposition. K541 also demonstrated evidence of acute cellular rejection. **(D)** Measurement of circulating anti-FcRn mAb detectable in serum. **(E)** Reduction of total serum IgG following administration of anti-FcRn mAb. **(F)** Percentage change pre-transplant (d–5) of circulating DSA IgG with TFXM and BFXM. **(G)** Percentage change from pre-transplant (d–5) of circulating DSA IgM with TFXM and BFXM post-infusion.

decreasing circulating protective antibody for any prolonged period of time. Alternatively, combining anti-FcRn treatment with administration of high dose IvIg may have the dual benefit of permitting the exogenous passive transfer of protective antibody, while additionally providing homeostatic pressure to reduce the rapid recapitulation of the recipients own alloantibody response.

Our results indicate that, in fact, the efficacy of the treatment, with respect to reduction of circulating total IgG, was maintained with repeated dosing post-transplant. However the paradoxical increase in DSA IgG post-transplant, whilst receiving repeated anti-FcRn treatment, potentially indicates that, in the sensitized recipient, the response to allograft itself drives a rapid DSA production likely from memory B cells (anamnestic response), which can easily overwhelm the effects of the anti-FcRn. Although it has been modelled that in states of greater IgG production, such as autoimmune disease, the efficacy of anti-FcRn therapy is likely increased (34) our speculation is plausible, since targeting FcRn-mediated IgG recycling results in the accelerated removal of IgG, but does not interfere with the production of IgG. In other words, blockade of FcRn is likely rendered ineffective in the setting of rapid or ongoing antibody production, such as is the case in active AMR.

Interference with other antibody-based therapeutics should be considered as another downside of using an anti-FcRn approach in transplantation. The widespread use of Thymoglobulin (ATG) and other monoclonal antibody–based treatments, such as rituximab or alemtuzumab, in addition to belatacept-based maintenance regimes, raise the concern that targeting the FcRn receptor would reduce the availability of circulating therapeutic antibody-based treatment, although it should be noted that our transplant data lack conclusivity with respect to any reduction of depletional effect of RhATG induction (**Supplemental Figure 5**), although early cellular rejection hints at an incomplete lymphocyte depletion at induction (**Figure 5**). Comparison of circulating RhATG levels

TABLE 1 | Banff Scoring of rejected kidney allografts.

	t	v	i	ti	i-IFTA	g	ci	ct	cg	mm	cv	ah	ptc	Tubular Injury	Interstitial Plasma cells	Interstitial Neutrophils	Interstitial Eosinophils	Edema	Inclusions	PTC fibrin	Glomerular fibrin	Arteriole fibrin	Interstitial Hemorrhage	C4d (N.A. = not available)	Diagnosis [ACR = acute cellular rejection, AMR = antibody-mediated rejection, TMA = thrombotic microangiopathy]
K541	2	3	2	2	2	3	1	0	1b	3	0	0	3	1	0	0	0	1	0	0	1	1	1	1	**ACR, type 3**, with fibrinoid arterial necrosis & endarteritis. Findings also suspicious for **CAMR** (transplant glomerulopathy, peritubular capillaritis, glomerulitis, TMA-type findings with glomerular fibrin, fibrinoid arterial necrosis)
K722	1	0	2	2	2	3	1	1	1b	1	0	0	3	1	0	0	0	1	0	0	0	0	1	1	"Borderline changes" "suspicious" for ACR, Findings also suspicious for AMR (glomerulitis, peritubular capillaritis, & possible early transplant glomerulopathy)

in anti-FcRn treated animals and animals part of an another project receiving RhATG indicate no significant lowering of circulating RhATG levels on POD 4/5 (manuscript submitted, **Supplemental Figure 4**).

Despite this, targeting FcRn-mediated IgG recycling remains an attractive alternative to plasmapheresis with which to reduce pre-formed DSA, particularly when considering the many side effects from plasmapheresis—particularly cardiovascular and coagulation related—that serve to limit its utility for all transplant candidates. The question of how and how such a drug might be used in transplantation is therefore clearly of interest to the field. The most obvious possible use for anti-FcRn targeting is, therefore, potentially for sensitized patients awaiting a kidney allograft; however, the potential for a vigorous anamnestic response should be considered with respect to organ acceptance and immunosuppression options, while as described earlier, combination therapy with IvIg administration may serve to suppress the repopulation of the immunoglobulin compartment with DSA. Anti-FcRn treatment may also be of utility when combined with agents targeting Ab-producing cells, such as proteasome inhibitors (i.e. bortezomib, carfilzomib, etc.). In this context, giving anti-FcRn could rapidly reduce DSA in circulation, while proteasome inhibition would target and limit the source of additional and rapid DSA production which might otherwise overwhelm the efficacy of anti-FcRn.

To our knowledge, this is the first attempt to test the efficacy of anti-FcRn mAb on DSA in the transplantation setting. Our data clearly demonstrated potential of anti-FcRn mAb in reducing DSA, while also demonstrating its limitations, including a lack of ability to control DSA when accompanied by new production of IgG. Further investigation in how to use it in the clinical setting is required for translation of this agent in transplantation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Duke University Institutional Animal Care and Use Committee (Duke IACUC# A153-18-06).

AUTHOR CONTRIBUTIONS

MM participated in research design, conduct of experiments, acquisition of data, analysis of data, and writing of the manuscript. WF, JY, and YB conducted experiments and acquired data. RS, ZF, and BS participated in surgical

procedures, AK participated in research design and analysis of data. MH and SP participated in measuring anti-TT and anti-gB antibody level, ABF participated in conducting experiments and acquiring data (histology and histological grading). DM provided research product (anti-FcRn mAb), participated in research design and conduct of experiments. JK and SK participated in research design, conduct of experiments, analysis of data and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.660900/full#supplementary-material

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REVIEW
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Emerging New Approaches in Desensitization: Targeted Therapies for HLA Sensitization

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There is an urgent need for therapeutic interventions for desensitization and antibody-mediated rejection (AMR) in sensitized patients with preformed or *de novo* donor-specific HLA antibodies (DSA). The risk of AMR and allograft loss in sensitized patients is increased due to preformed DSA detected at time of transplant or the reactivation of HLA memory after transplantation, causing acute and chronic AMR. Alternatively, *de novo* DSA that develops post-transplant due to inadequate immunosuppression and again may lead to acute and chronic AMR or even allograft loss. Circulating antibody, the final product of the humoral immune response, has been the primary target of desensitization and AMR treatment. However, in many cases these protocols fail to achieve efficient removal of all DSA and long-term outcomes of patients with persistent DSA are far worse when compared to non-sensitized patients. We believe that targeting multiple components of humoral immunity will lead to improved outcomes for such patients. In this review, we will briefly discuss conventional desensitization methods targeting antibody or B cell removal and then present a mechanistically designed desensitization regimen targeting plasma cells and the humoral response.

Keywords: sensitization, desensitization, alloantibody, plasma cells, germinal center

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INTRODUCTION

Desensitization treatment within the field of transplantation refers to the process of antibody removal (1), specifically preformed donor-specific HLA antibody (DSA). DSA as a barrier to successful transplantation was first described in early reports of kidney hyperacute rejection in the context of positive complement dependent cytotoxicity (CDC) by Ramon & Terasaki et al. (1), Improvements in histocompatibility testing have removed the risk of hyperacute rejection across all organs, yet acute and chronic AMR remain a major contributor to poor transplant outcomes (2, 3).

The Current Status of HLA Sensitization

For many patients awaiting transplantation, blood transfusion, prior transplantation, and pregnancy are the sources of sensitization (4, 5). There is evidence that the primary source of HLA sensitization is important. Transplantation appears consistently as the strongest sensitizing event inducing both class I and II HLA antibody (6, 7); however, there is some evidence that post-transplant, those with pregnancy induced HLA-antibody respond more rapidly (5). Waiting times

for highly sensitized patients, calculated Panel Reactive Antibody (cPRA)> 80% (8) are longer, leading to increased morbidity and mortality. Compared to patients with absent or low cPRA, the highly sensitized candidates could expect to wait twice as long for a compatible transplant in both the USA (9) & UK (10). In Europe, the acceptable mismatch program has long been advocated to reduce waiting times for highly sensitized patients (11). Recently, in the USA the new kidney allocation scheme (KAS) (12), was specifically designed to improve the transplantation rates for sensitized patients by providing more allocation points and mandating regional and national sharing for those with the highest CPRA. Early KAS reports suggest that this has largely been successful (13, 14).

For sensitized patients with an incompatible living kidney donor, to whom they have DSA, the decision is whether to use a kidney paired donation (KPD) program to obtain a compatible match, await a compatible deceased donor offer, or proceed with a HLA-incompatible, positive cross match transplant (HLA-i) which requires desensitization prior to transplantation. Simulation of how KPD programs might run were initially optimistic (15), but over time many struggle with a pool enriched with highly sensitized patients unless specific matching interventions are made (16), or a combination of both desensitization and KPD is utilised (17).

The Current Standing of Desensitization

In the USA, multicenter data demonstrates a clear survival benefit (18) in proceeding with an HLAi; however, in the UK the picture is more nuanced with no survival benefit demonstrated, although for the patients awaiting a compatible transplant, around 40% remain untransplanted at 5 years post-listing (19). Desensitization then remains a guaranteed route for a highly sensitized recipient to obtain transplant, albeit with greater immunological risk. To date, desensitization therapies have largely relied upon physical methods of antibody removal in the form of repeated plasmapheresis, in conjunction with additional agents. In this review, we will outline the currently used regimen for desensitization, as well as describing new potential approaches.

THE CURRENT DESENSITIZATION TREATMENT STRATEGY

In highly sensitized patients removal of circulating anti-HLA antibody or lowering cPRA is an important and fundamental strategy for expanding donor options and successfully transplanting across DSA barriers. However, durable inhibition of HLA antibody production is the "holy grail" for successful kidney transplantation (KT) in sensitized patients.

Plasmapheresis or Immunoadsorption

Plasmapheresis (PP) has been used for several decades as a method for lowering circulating antibody in various immune diseases (20, 21). Plasmapheresis physically removes large molecular weight substances from the plasma, including antibodies, complement components immune complexes and coagulation factors (22). Using the double filtration plasmapheresis (DFPP) system, a cascade of filtration traps larger molecules, and thus allow lower molecular weight components to pass back to the patient (23).Together with IVIg, it has been used to effect successful transplantation for positive crossmatch patients, and for many units, is the mainstay of desensitization prior to transplantation (24–26). In Europe and Australia immunoadsorption (IA) using staphylococcal protein A column has been applied in eliminating antibodies (27–29). The kinetics of antibody removal by PP are predictable within limited periods compared with other treatment modalities since plasma proteins are reliably removed (30). Therefore, PP or IA can be used as an effective treatment modality in the setting of planned transplantation across a positive HLA cross-match in living donor KT. PP or IA has a limitation of antibody rebound after the completion of treatment sessions.

Intravenous Immunoglobulin

IVIG has been widely used in inflammatory and autoimmune conditions (31). IVIG also has a role in AMR treatment in kidney transplantation (32). Although widely used as part of desensitization regimens for many decades, the precise mechanism of action is unknown as a result of its broad spectrum of effects. Many potential mechanisms of action of IVIG in transplantation have been proposed. The main mechanisms are considered to be neutralization of circulating anti-HLA antibodies with anti-idiotypic antibodies (31), the inhibition of complement activation (33, 34), and binding to Fc receptors on immune cells (35, 36). It is also postulated that IvIg following plasmapheresis prevents rebound of DSA, by providing an abundant quantity of circulating IgG (37, 38). IVIG has been used in various doses according to protocol from 100mg/kg to 2.0g/kg in desensitization prior to living donor KT or for deceased donor KT of patients with high PRA.

Although various combinations of IVIG or PP with rituximab have been proposed, two protocols have been widely accepted and used (39).

PP With Low-Dose IVIG vs. High-Dose IVIG Alone

Using PP with low-dose IVIG, many centers report transplant outcomes with acute AMR rates of 12-43% when used in combination with various induction agents, anti-thymocyte globulin, anti-IL-2Rc antibody or OTK3 (40–43). The NIH IGO2 study, a controlled clinical, multi-center, double blinded trial of IVIG (2g/kg, monthly 4 times) versus placebo in sensitized patients, HLA antibody levels were reduced further, and the transplantation rate was higher in the IVIG group than in the placebo group (44). Glotz et al. reported results of high-dose IVIG desensitization with anti-thymocyte globulin induction in cross-match positive patients (45). Jordan et al. reported successful transplantation outcomes with two doses of 2mg/kg IVIG on day 0 and day 30 with rituximab in 20 patients (46). In this study, 16 patients among 20 could receive KT within 6 months. In their subsequent series, they used high-dose IVIG

2g/kg, 3 times on day 1, day 30 and at the time of transplantation with rituximab (47). Among 76 patients with PRA ≥30%, 31 patients received living donor KT, and 45 patients received deceased donor KT with reduced waiting time of 4.2 ± 4.5 months.

Anti-CD20 Antibody (Rituximab)

Rituximab is an anti-CD20 monoclonal antibody that binds to CD20 expressed on immature and mature B-lymphocytes, inducing apoptosis *via* antibody-dependent cytotoxicity, complement-dependent cytotoxicity or direct apoptosis. Originally, anti-CD 20 antibody was used to treat B-cell lymphoma. In transplantation, rituximab was introduced to deplete B cells with the goal of reducing donor-specific antibody (DSA) production (48). Rituximab has been used as an additional therapy as part of desensitization treatments, in conjunction with plasmapheresis & IvIg (46, 49). The half-life of rituximab in patients with end-stage renal disease is known to be 9-14 days (50). Rituximab administration can maintain durable B-cell depletion for at least six months, but rituximab does not bind to plasma cells as they do not express CD20 (51).

Unmet Need For Sensitized Patients

Because no randomized controlled clinical trial has compared the two main protocols described above, and the study populations and the criteria for transplantation vary, it is difficult to evaluate which protocol is best. Desensitization protocols using high-dose IVIG or low-dose IVIG + PP with rituximab have relative advantages and disadvantages. A PP-based protocol with low-dose IVIG, within limited periods, is more effective and predictable for lowering antibody levels. On the other hand, in spite of possible non-response, high-dose IVIG has the advantage in patients with high PRA on the waiting list of being less invasive given the unpredictable time to transplantation. However, both current desensitization protocols have limitations. Regardless of whether high-dose IVIG or low-dose IVIG with PP were used, acute AMR rate as well as acute cellular rejection rates were higher in desensitized patients than in non-sensitized patients (52). In a study that included surveillance biopsy of desensitized KT recipients, the subclinical AMR rate was 31% at 3 months post-transplantation, and patients with subclinical AMR at 3 months post-transplantation had higher C4d, ptc and arteriosclerosis scores post-transplantation at 1 year than the patients without subclinical AMR at 3 months post-transplantation (53). Transplant glomerulopathy was reported at a rate of 44% at a mean of 18 months post-transplantation (54). After desensitization, long-term outcomes of KT seems to be worse than for unsensitized patients (42).

SENSITIZATION IN THORACIC ORGAN TRANSPLANT RECIPIENTS: SIMILARITIES AND DIFFERENCES

Thoracic transplantation shares similar immunologic challenges as HLA sensitization in kidney recipients. However, no alternative organ replacement modalities support life in end-stage lung disease as dialysis in end-stage renal disease. While left ventricular assist devices (LVAD) have emerged as a viable alternative to heart transplant, it is not without significant risks and complications that limit access to therapy. As such, thoracic transplantation faces a greater urgency and waitlist mortality, and desensitization regimens must take into account these temporal challenges of sensitized patients. 1 in 7 adult heart transplant candidates are sensitized, a number that has doubled in the past two decades (55). A rising incidence is anticipated due to the expanding use of LVADs as a bridge to transplantation, advanced congenital heart disease surgery leading to more patients surviving to require transplant, and, to a smaller extent, an increase in re-transplantation (55). On the contrary, the true burden of sensitization in lung transplantation is unknown. National and international registries lack robust DSA or PRA/cPRA data for lung transplants. In the ISHLT registry, women, who are known to have greater sensitization secondary to pregnancy, comprise 60% of the waitlist but receive only 43% of the transplants, with a median time to transplantation of 233 days compared to 86 for men (56). To better understand and quantify this issue, comprehensive cPRA reporting in lung transplantation registries is required. Many lung transplant programs currently practice avoidance of DSA at the time of organ allocation, significantly limiting sensitized candidates' access to transplant (57).

A common sequela of AMR in heart transplantation is cardiac allograft vasculopathy (CAV), and in lungs, chronic lung allograft dysfunction (CLAD), both of which result in significant mortality and morbidity within 5 years of transplantation (57–63). The primary goals of desensitization in thoracic transplantation are to increase access to transplantation through expansion of the donor organ pool and to prevent AMR and its subsequent morbidity and mortality. No approach has demonstrated significant and sustainable reductions in HLA antibody prior to transplant, and patients with elevated PRA continue to be at higher risk for rejection and reduced survival (64).

Shifting Toward Sensitization

As mentioned, use of mechanical support as bridge to transplantation has been steadily increasing, reaching 50% of patients on the waiting list for heart transplant in 2013. Particular attention to the immunologic challenges associated with LVADs to target interventions is necessary. While many studies suggest that LVAD-associated allosensitization limits sensitized candidates' access to transplant, they fail to show that it leads to rejection or increased mortality after receiving a transplant (65). Notably, most of the evidence implicating such findings has been gathered from studies that examined pulsatile-flow LVADs and pre-dates the use of current generation continuous-flow LVAD (65–67). In a more recent study by Ko et al, 23% of patients became newly sensitized after continuous-flow LVAD implant (68). Compared with patients without new sensitization or those already sensitized at baseline, these patients had an increased risk of ACR and AMR, but comparable survival 5 years post-transplant, consistent with an earlier study (69). This suggests that even if the alloantibody levels were decreased before transplant by conventional methods, such as IVIg and

plasmapheresis, maintenance immunosuppression targeting memory B cells and plasma cells is critical to prevent rebound DSA. In addition, the patients who were newly sensitized after LVAD implant and did not reach transplantation had a higher level of allosensitization (27.9% vs. 10.2%) and a high mortality of 39.5% during follow-up. This is consistent with a study by Alba et al. that also found an association between high PRA and lower transplant probability that likely drives the high mortality observed (70). A key concern with LVAD is the requirement of blood transfusions that result in the generation of new anti-HLA antibodies; however, our understanding of the mechanism by which patients on LVAD support develop allosensitization is largely unchanged since 1999 (71). It is also known that platelets and fibrinogen can adhere to the surface of LVAD coated with polyurethane membrane and form a fibrin matrix which traps other cells (72). The trapped cells could provide subsequent excessive activation signals *via* cytokines and costimulation to T cells. During this aberrant state of T cell activation, LVAD patients are believed to develop B cell hyper-reactivity with subsequent allosensitization. By way of a CD95-dependent pathway, these activated T cells then undergo apoptosis (73).

Current Desensitization Strategy in Thoracic Organ Transplantation

Despite these challenges, efforts to desensitize patients on the waitlist have generated limited success. The current research is centered around renal transplant experience with application in thoracic transplantation limited by several factors. In both heart and lung transplantation, there are requirements for donor-recipient size matching and transplant urgency is comparatively greater. Consequently, patients do not survive to begin clinical trials and the unpredictable nature of donor availability significantly limits the use of desensitization treatments prior to transplantation as prolonged period of treatment may confer more risks than benefits. Progress has been made from using IVIg and PP alone to using a variety of targeted therapies, although evidence in thoracic transplantation remains scarce. No large cohort desensitization strategy has been described in thoracic transplantation.

NEW PHARMACOLOGIC STRATEGIES FOR DESENSITIZATION

Targeting Antibodies

IgG Endopeptidases

More recently, attempts have been made to fundamentally alter the structure of preformed antibody, using IgG endopeptidase (IdeS) which is a bacterial enzyme produced by *S. pyogenes* that cleaves all four human IgG subclasses into F(ab) & F (c) fragments, thus inhibiting both complement-dependent cytotoxicity and antibody-dependent cytotoxicity (74). IdeS has additional effects by cleaving the IgG present in the B-cell receptor complex (BCR), thus switching off B-cell memory as a downstream effect (75). Jordan et al. recently completed a trial of IdeS in 25 highly sensitized patients prior to HLA-incompatible kidney transplantation (76). All patients had near-complete or complete reductions of anti-HLA antibodies and donor-specific antibodies at 24 hours post-transplant, which allowed successful transplantation in 24/25 (96%). However, in 1-2 weeks the levels of these antibodies rebounded. Ultimately, one patient had graft loss from hyperacute rejection, while 10/25 (40%) had evidence of antibody-mediated rejection in the early post-transplant period. These findings suggest that IdeS has strong, albeit transient, ability to reduce DSA that may make this therapy useful in combination with strategies that allow for longer-term control of DSA rebound.

Anti-FcRn Approach

Brambell et al. identified FcRn, a neonatal IgG receptor that is closely related to the MHC Class I receptor, which is involved in a variety of critical biological and immunological functions, most notably regulating serum IgG levels and the recycling and transcytosis process that results in an increased half-life of IgG and albumin in human serum (77–80). Strategies that block the IgG-FcRn interaction are hypothesized to promote IgG degradation and decrease pathogenic autoantibodies and alloantibodies (81, 82). IVIG was one of the first therapies to decrease anti-HLA antibodies and treat antibody-mediated autoimmune diseases through blocking the IgG-FcRn pathway, leading to saturation of FcRn receptors and degradation of IgG molecules (78, 83, 84). Since then, multiple therapies targeting FcRn or the IgG-FcRn interaction have been developed as treatment for autoimmune and infectious diseases, with promising benefits as therapeutic agents in reducing AMR in transplantation. Several monoclonal antibodies against FcRn such as M281, SYNT001, Rozanolixizumab, RVT-1401, and ABY-039 are in various clinical development stages. M281, a deglycosylated IgG anti-FcRn mAb, was well tolerated and achieved reduction of serum IgG levels of 80% from baseline in a phase I clinical trial (85). Rozanolixizumab (UCB7665) is a high affinity anti-human neonatal FcRn mAb that reduced plasma IgG concentrations in cynomologus monkeys by up to 85% (86). This led to a Phase I clinical trial of Rozanolixizumab in healthy human subjects that demonstrated therapeutic potential with sustained dose-dependent reductions in serum IgG concentrations when administered IV or SC (87). Phase II clinical trials of Rozanolixizumab were recently completed in patients with immune thrombocytopenia (NCT02718716) and myasthenia gravis (NCT03052751) (86). Seijsing et al. found that an engineered alternative scaffold protein [affibody molecule (Z_{FcRn})] effectively blocked the IgG-FcRn interaction when repeated injections of Z_{FcRn} and Z_{FcRn} fused to an albumin binding domain (ABD) in mouse models led to a 40% reduction of IgG in serum (88). ABY-039 is a molecule similar to Z_{FcRn} -ABD undergoing phase I trial (NCT03502954). Additional studies in animal models that inhibit IgG-FcRn binding include an anti-FcRn directed mAb, 1G3, that accelerated endogenous serum IgG clearance and reduced the severity of myasthenia gravis in rat models (89). Abdegs, an engineered antibody that inhibited FcRn recycling and enhanced IgG degradation, was efficacious in a murine model of arthritis (90). Synthetic FcRn-binding peptides (FcBP), small molecule

FcRn antagonists, and other molecules that interact with the Fc binding site may also block IgG-FcRn interactions (78). Our group also tested anti-rhesus FcRn mAb in a skin-sensitized NHP model with kidney transplantation (*manuscript in submission*). Treatment with aFcRn prior to transplantation significantly reduced the levels of total and donor-specific alloantibody. However, in the context of renal transplantation, anti-FcRn treatment did not block the synthesis of DSA, such that transient reduction in DSA was followed by robust DSA increase and antibody-mediated rejection (*manuscript in submission*). The anti-FcRn approach demonstrated promising applications in lowering alloantibody levels in transplantation; however potential limitations and complexity of using the agent require further investigation in transplantation.

Targeting Plasma Cells

Following the discovery that alloantibody secreting cells predominantly exist as long-lived plasma cells (LLPC) in the bone marrow compartment, along with the identification of these cells as being $CD138^{+}CD20$ (91), bortezomib was used to lower alloantibody (92). Bortezomib, a proteasome inhibitor (PI) which depletes non-malignant plasma cells, was proposed to reduce anti-donor HLA antibody. While some groups have demonstrated efficacy of bortezemib to desensitize transplant recipients, the drug was used in combination with conventional therapies (93). Now several biologics targeting plasma cells are available and are being considered.

Targeting Plasma cells with Proteasome Inhibition

Bortezomib (Velcade®) is a 1st generation, reversible inhibitor of the 26S proteasomal subunit. This drug is a potent inhibitor of plasma cells, which rely on rapid protein turnover to continually secrete antibodies, and succumb to oxidative stress and apoptosis when cellular recycling mechanisms are rendered nonfunctional. For this reason, bortezomib is approved for usage in multiple myeloma, a malignancy of plasma cells (94). Everly et al. first described its use as effective treatment of AMR and ACR as well as reduction in DSA in kidney transplant recipients (95), and Mulder et al. showed that proteosome inhibitors bortezomib, carfilzomib, oprozomib (ONX 0912), and immunoproteasome inhibitor ONX 0914 (previously PR-957) reduced B-cell proliferation, immunoglobulin production, and induced apoptosis of activated B-cells (96). Following some success for usage in refractory antibody-mediated rejection after kidney transplantation (97, 98), several groups have used bortezomib in the context of desensitization. Woodle et al. in the first trial with bortezomib variably combined with plasmapheresis and rituximab showed modest success with a reduction in the immunodominant DSA of 38/44 (86%) highly sensitized patients, successful transplantation of 19/44 (43.2%), and 17/19 (89.5%) of grafts functional at a median follow-up of 436 days (92). Jeong et al. used a combination of high dose IVIG, rituximab, and bortezomib and demonstrated a small reduction in the MFI value of class I PRA, and an increased rate of deceased donor kidney transplantation (8/19 or 42.1% of desensitized patients vs. 4/17 or 23.5% of controls, $p = 0.004$) with no graft loss in the desensitized group at a median follow-up of 23 months (93). The interpretation of these early favorable outcomes was limited by the small, non-randomized nature of the studies, and the confounding nature of its combination with conventional desensitization methods. Studies using bortezomib as monotherapy for desensitization have shown less promising results with poor reduction of anti-HLA antibodies and significant toxicity with longer courses of the drug (99, 100) that have caused enthusiasm for its use in new desensitization regimens to wane.

Carfilzomib (Kyprolis®) is a 2nd generation, irreversible inhibitor of the 20S proteasomal subunit. Studies in patients with multiple myeloma suggest that this drug may be more efficacious and better tolerated than its predecessor bortezomib (101). A current clinical trial of carfilzomib for desensitization is underway (NCT02442648). Most recently, carfilzomib was studied as desensitization monotherapy yielding 72.8% median reduction in HLA antibodies and a 69.2% reduction in bone marrow plasma cells with acceptable drug safety and toxicity (102). Another second generation PI, ixazomib, warrants further testing. Ixazomib is an oral-form peptide boronic acid proteasome inhibitor distinct from bortezomib and recently had a successful phase III trial (TOURMALINE-MM1) in multiple myeloma (103–106). Other PI's including marizomib, delanzomib, and oprozomib are being studied as anti-cancer and autoimmune therapies. PI's have notably been studied most recently as desensitization therapy and additional studies in utilizing PI as maintenance immunosuppressive treatment are needed.

Immunoproteasome Inhibitors

Conventional PIs are broad spectrum PIs with various dose-dependent adverse effects. An attractive alternative would be to solely target the proteasome of immune cells. Hematopoietic origin cells display proteasomes with distinct catalytic subunits and the complex is referred to as the immunoproteasome (107). Interestingly, immunoproteasome is also expressed in nonhematopoietic cells exposed to pro-inflammatory mediators such as IFN-γ and TNF-α (108). Therefore, inhibition of the immunoproteasome allows for both the targeting of immune-specific cells but also cells actively involved in the inflammatory response. In kidney transplantation, it was found that patients with chronic AMR have up-regulated immunoproteasome activity (109). Newly developed immunoproteasome inhibitors (IPI) could selectively inhibit proteasomes of cells involved in graft rejection after transplantation, such as B and T lymphocytes and APC's, and regulate pro-inflammatory cytokines and the differentiation of helper T cells (110, 111). But similar to conventional PIs, PC population would be more sensitive on IPIs. Current work in animal models has found that IPI is superior to PI in suppressing the cellular and humoral immune response, preventing chronic AMR, and prolonging survival (110–112). ONX-0914, formerly known as PR-957, is an LMP7-selective immunoproteasome inhibitor that is undergoing clinical studies in the treatment of autoimmune diseases and has potential applications in transplantation (110, 111). ONX-0194 and bortezomib combined suppressed DSA production, B cells and plasma cells after kidney transplant,

inhibited IgG, complement, and proinflammatory cytokines IFN-γ and IL-17, and reduced chronic allograft nephropathy. In mismatched mouse cardiac transplantation, IPI treatment with a noncovalent LMP-7 inhibitor, DPLG3, combined with CTLA4-Ig led to decreased effector T cells and T cell exhaustion (113). Other IPI's such as Ipsi-001 and PR-924 are currently under investigation as potential anti-cancer agents. IPI is particularly attractive due to its specificity on immune cells which shows larger safety margin compared to conventional immunoproteasome inhibitors (114, 115). This may allow the continuous (or long-term) treatment of IPI after transplantation in sensitized recipients.

Outside of multiple myeloma therapies, there is still a range of opportunities to target alloantibody reduction. Building on the success of PIs, there has been focus on inhibiting protein degradation *via* inhibition of initial ubiquitin binding rather than the downstream proteasome complex (116). Another promising avenue is modulating the endoplasmic reticulum (ER). Inositol-requiring enzyme 1 (IRE1) inhibitors are currently under development and may be available in the near future (117, 118).

Monoclonal Antibodies for Targeting Plasma Cells

Inhibiting proteasome activity with PI should affect more than plasma cell population since all eukaryotic cells utilize proteasome to maintain their homeostasis. Even IPI should have broad impact on immune cells. Therefore, monoclonal antibody targeting of plasma cell population is very attractive.

CD38 is expressed at high levels by B lineage progenitors in bone marrow, B-lymphocytes in germinal centers, and terminally differentiated plasma cells (119, 120). Conversely, mature naive and memory B cells express low levels of the molecule (121, 122). Plasma cells (PC) actively producing allo-antibodies should express high levels of CD38, thus resulting in a reasonable target for PC depletion in desensitization therapy (123) or deletion of plasma cells during active AMR (124). Daratumumab is a human IgGκ monoclonal antibody that targets CD38 and induces apoptosis of PC (122, 125) *via* Fcγ receptor-mediated cross-linking (126) and macrophage-mediated phagocytosis (127). In addition to depleting $CD38^+$ cells, daratumumab also promotes expansion of memory and naïve T-cells (122), and is approved as monotherapy in patients with multiple myeloma (MM) (122, 125, 128, 129). Isatuximab, is an anti-CD38 mAb also used in the treatment of MM. It induces apoptosis of $CD38^+$ cells through Fc-dependent and Fc-independent mechanisms (130), depletes B-lymphocyte precursors (131), and depletes NK cells through direct activation and crosslinking of CD38 and CD16 on NK cells (130). Elotuzumab is an IgG_1 mAb that targets signaling lymphocytic activation molecule F7 (SLAMF7), also known as CD319, which is highly expressed on MM, NK and other immune cells (132). Elotuzumab was found to activate NK cells and induce apoptosis of $SLAMF7^+$ cells *via* both CD16-dependent and CD16-independent mechanisms (132, 133).

There are only anecdotal cases evaluating monoclonal antibodies targeting PC in organ transplantation to prevent or treat antibody-mediated rejection. Daratumumab showed effective desensitization and reversed acute/chronic antibody-mediated rejection (134, 135). In our sensitized NHP model, we reported the effectiveness of daratumumab in combination with an anti-CXCR4 antagonist, plerixafor which mobilizes PC from bone marrow to peripheral blood (135). However, we also reported a possible off-target effect of daratumumab which result in depletion of other CD38 expressing regulatory cells including Treg, Breg, MDSC etc. This feature makes daratumumab attractive for multiple myeloma (122), but could trigger alloimmune responses in transplantation patients. Daratumumab and eculizumab combined therapy reduced *dn*DSAs, improved heart and kidney graft function, and resulted in undetectable circulating PCs. However, class II DSA returned after discontinuing daratumumab therapy (135). The second patient was a highly sensitized recipient who received daratumumab desensitization therapy prior to heart transplantation. After eight weeks, there was found to be reduction in cPRA (98% vs 62%) and class 1 anti-HLA antibodies (35 vs 14) (135). Currently, there is a phase 1 clinical trial to evaluate daratumumab in decreasing circulating antibodies in sensitized recipients awaiting heart transplantation (ClinicalTrials.gov, NCT04088903). There is also a clinical trial to evaluate the safety and efficacy of isatuximab as desensitization therapy in patients awaiting kidney transplantation (ClinicalTrials.gov, NCT04294459). If applied to transplant, these therapies from myeloma field need be carefully evaluated on their off-target effect in a transplantation setting.

Costimulation Blockade

Rebound of DSA after short-term PI has been reported (136–138). This repletion of PC and DSA would be partially due to an intra-marrow PC repopulation which might be related to PC populations resistant to PI treatment. In the meantime, PC population can expand outside of bone marrow. We observed that the depletion of PC with bortezomib initiated germinal center activation (138). This is probably due to the tightly intertwined network among humoral components. PC may provide a negative feedback loop to Tfh cells (or GC response) since these cell populations compete for similar cytokines/survival factors. Therefore, once one population, in this case PC, disappear then the other cell population (Tfh) is promoted (138). For this reason, targeting T cell help for B cell activation could be a potential strategy for desensitization, especially since the impact of costimulation blockade on humoral responses has been shown in multiple studies (139–141). We and others have reviewed this topic (142–145). It is notable that targeting PC together with costimulation signals successfully prevented the rapid rebound of DSA seen with PI monotherapy (146–149). This suggests that targeting a single humoral component might not be effective in controlling preformed or on-going allo-humoral responses.

Targeting Mediators/Survival Factor

Interleukin-6 Receptor Inhibition

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by many different cell lineages. The membrane-bound IL-6 receptor (IL-6R) is expressed only on hepatocytes and some immune cells

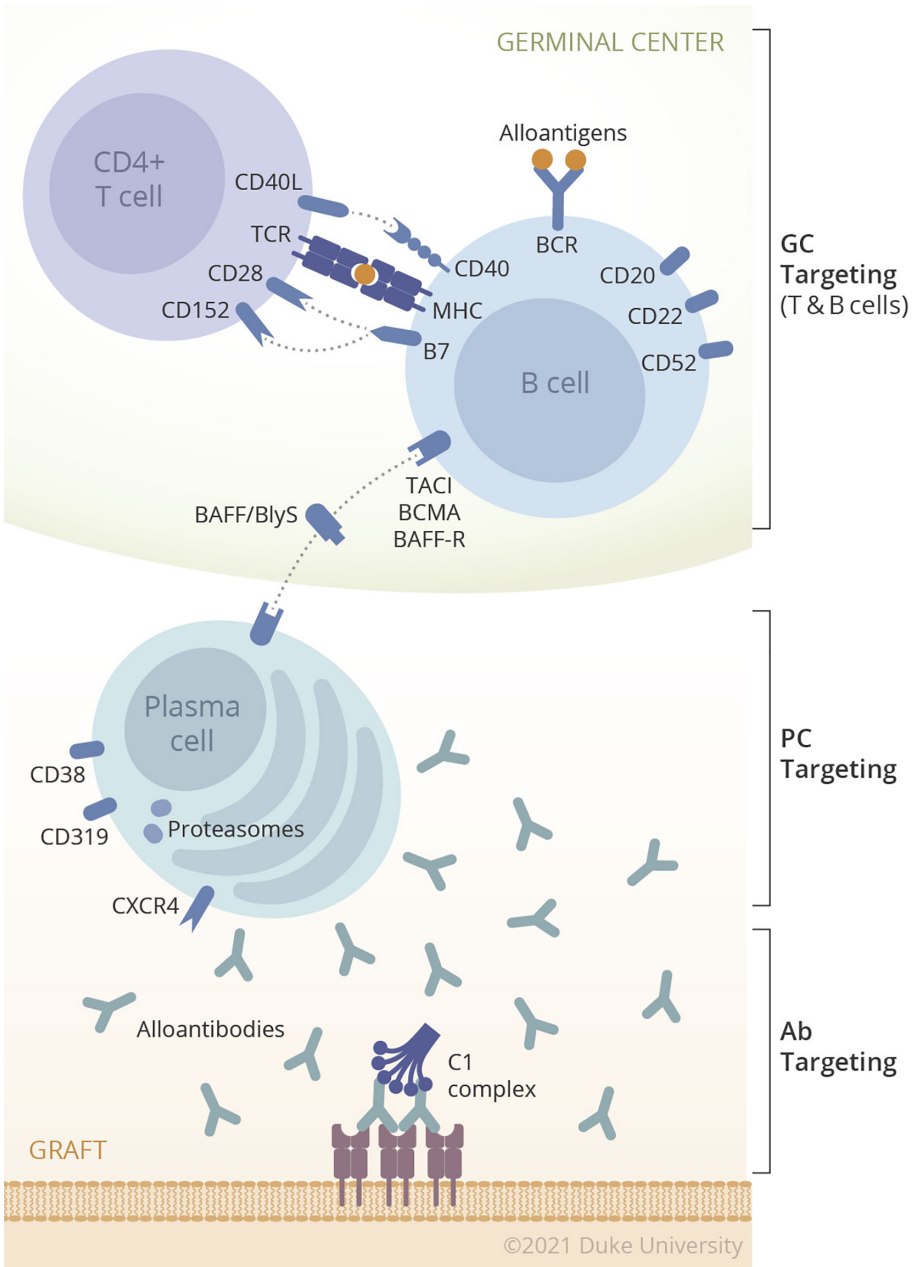

FIGURE 1 | Multiple components of humoral immunity in organ transplantation.

(150), but a soluble IL-6R also exists that can bind IL-6 and together this complex can signal through the transmembrane cytokine receptor gp130 (trans-signaling) expressed on nearly all cell types (151). IL-6 is critical for many inflammatory pathways and has a key role in the induction of follicular helper T cells, which direct naïve B cells in the germinal center to differentiate to memory B cells and high-affinity, IgG-secreting plasma cells (152). Accordingly, dysregulated production of IL-6 has been associated with chronic diseases such as diabetes, systemic lupus erythematosus, rheumatoid arthritis, cancer, end-stage renal disease, crescentic glomerulonephritis, and graft versus host disease (153–158). IL-6 has also been associated with deviation of T cells towards a Th17 phenotype, reduction of the proportion of Treg cells, and potentiation of allograft rejection in kidney transplantation (159).

Tocilizumab (Actemra®) is a humanized monoclonal antibody with activity against both the membrane and soluble forms of IL-6R approved to treat moderate to severe rheumatoid arthritis, systemic juvenile idiopathic arthritis, polyarticular juvenile idiopathic arthritis, and Castleman's disease (151).

TABLE 1 | Broad Overview of Possible Therapeutics for New Desensitization Regimens.

Drug	Target	Development	Reference
B Cells			
Ofatumumab	Anti-CD20	FDA approval for CLL	(166, 167)
Ocrelizumab	Anti-CD20	FDA approval for primary progressive multiple sclerosis	(168)
Ocaratuzumab	Anti-CD20	Clinical trials	(169, 170)
Obinutuzumab	Anti-CD20	FDA approved for CLL	(171)
Blisibimod	Anti-BAFF	Clinical trials	(165)
Tabalumab	Anti-BAFF	Clinical trials	(172–175)
Atacicpet	Anti-APRIL & Anti-BAFF	Clinical trials	(176)
BR3-Fc	Anti-BAFF	Clinical trials	(177, 178)
Belimumab	Anti-BAFF	FDA approval for SLE	(179)
hAPRIL.03A & hAPRIL.01A	Anti-APRIL	Pre-clinical	(180)
Epratuzumab	Anti-CD22	Clinical trials	(181, 182)
Lucatumumab	Anti-CD40	Clinical trials	(172, 183)
Dacetuzumab	Anti-CD40	Clinical trials	(172, 184)
Galiximab	anti-CD80	Clinical trials	(185, 186)
Plasma Cells			
Indatuximab ravtansine	anti-CD138	Clinical trials	(172, 187)
Isatuximab	Anti- CD38	Clinical trials	(172, 188, 189)
Moxetumomab	anti-CD22 immunotoxin	Clinical trials	(190)
Siltuximab	IL-6 inhibitors	FDA approval for multicentric Castleman's disease	(172, 191, 192)
Daratumumab	Anti-CD38	FDA approval for multiple myeloma	(172, 191)
MOR202	Anti-CD38	Clinical trials	(172, 191)
Elotuzumab	Anti-CS1	FDA approval for multiple myeloma	(193)
Milatuzumab	Anti-CD74	Clinical trials	(172)
T Follicular Cells			
Pembrolizumab	PD-1 inhibitor	FDA approval for unresectable or metastatic solid tumor	(191, 194)
Nivolumab	PD-1 inhibitor	FDA approval for inoperable or metastatic melanoma	(172, 191, 195–198)
Pidilizumab	PD-1 and DLL1 Inhibitor	Clinical trials	(191)
BGB-A317	PD-1 inhibitor	Clinical trials	(199)
Durvalumab	PD-L1	FDA approval for locally advanced or metastatic urothelial carcinoma	(200–202)
Ubiquitin-Proteasome Inhibitors			
IPP-201101	Spliceosomal peptide	Clinical trials	(203, 204)
Marizomib	Proteasome inhibitor	Clinical trials	(203, 205–209)
Delanzomib	Proteasome Inhibitor	Clinical trials	(203, 210)
Oprozomib	Proteasome Inhibitor	Clinical trials	(203)
IPSI-001	Immunoproteasome	Pre-clinical	(115, 203, 211)
ONX-0914	Immunoproteasome	Pre-clinical	(203, 212)
PR-924	Immunoproteasome	Pre-clinical	(203, 213)
RO5045337	Ubiquitin E3 ligase	Clinical trials	(203)
RO5503781	Ubiquitin E3 ligase	Clinical trials	(203)
LCL161	Ubiquitin E3 ligase	Clinical trials	(203, 214)
AEG 35156	Ubiquitin E3 ligase	Clinical trials	(203, 215, 216)
Lenalidomide	Ubiquitin E3 ligase	FDA approval for multiple myeloma and myelodysplastic syndromes	(203)
Pomalidomide	Ubiquitin E3 ligase	FDA approval for relapsed and refractory multiple myeloma	(203)
Ubistatins	19S proteasome	Pre-clinical	(203, 217)
b-AP15	19S *DUBs	Pre-clinical	(203)
P5091	DUBs	Pre-clinical	(203)
P22077	DUBs	Pre-clinical	(203)
WP-1130	DUBs	Pre-clinical	(203)

**DUB - Proteasome-associated deubiquitinases.*

Pharmacologic inhibition of IL-6 signaling is attractive in the context of desensitization strategies, as animal models have shown that this therapy reduces alloantibody responses by inhibition of bone marrow plasma cells and induction of Treg cells (160). Vo et al. recently examined the efficacy of high dose IVIG + tocilizumab in 10 highly sensitized patients who were poorly responsive to high dose IVIG + rituximab (161). This regimen was associated with reduced donor specific antibody number and strength, decreased wait list time, and increased rate of transplantation. No transplanted patients had evidence of antibody-mediated rejection on protocol biopsies. Larger, randomized control trials will be helpful in determining the ultimate value of this treatment given these promising preliminary results.

Anti-BAFF Agents

B cell activating factor (BAFF) is a homotrimer and member of the tumor necrosis factor (TNF) family that is found on the cell surface as a transmembrane protein or released in soluble form after cleavage (162). BAFF is secreted by multiple cell types,

binds to three separate receptors, and is critical for the maturation of B cells (163). BAFF also acts as a potent B cell activator and is important in B cell proliferation and differentiation. Therefore, blocking this molecule may be essential when targeting allo-B cell response. A monoclonal antibody against BAFF, belimumab (Benlysta®), was the first targeted biologic approved for the treatment of systemic lupus erythematosus (164). Belimumab monotherapy was tested for desensitization in kidney transplantation (NCT01025193), but this trial was closed early due to a reported lack of efficacy. Blisibimod is a second anti-Baff agent developed for SLE. It is a fusion protein consisting of four BAFF binding domains. This anti-BAFF agent completed Phase II testing and currently being tested in a Phase 3 trial, CHABLIS-SC1 [(165), NCT01162681]. While considerable progress has been made in the field of desensitization, many potential and untested therapies remain. Other anti-BAFF agents including tabalumab, atacicept, and blisibimod have not been evaluated for desensitization in human trials.

A MULTI-MODAL APPROACH TO DESENSITIZATION

The concept of desensitization has been expanded from only targeting alloantibody (IVIG/IA/plasmapheresis) to instead targeting the upstream sources of antibody such as B cells (Rituximab) and PC (proteasome inhibitor). The conventional desensitization concept, removal of preformed antibody, may prevent hyperacute rejection or acute AMR but without long-lasting impact on humoral alloimmunity. While many desensitization therapies have been tried alone or in combination in animal models and human trials, none yet have solved the barriers to transplantation faced by highly sensitized patients with high titer HLA antibodies. The answer to desensitization may lie in novel therapies not yet tested or those outside the field of transplantation. Therefore, upcoming therapies targeting plasma cells are potentially very attractive. However, considering the previous sensitization events to HLA, allograft could trigger the memory response in sensitized patients. For this reason, targeting each component of humoral response such as alloantibody, B cells, or PC, would tentatively reduce the steady state level of DSA, this would not promote long-term control of humoral response after transplantation. Due to its compensatory mechanism, it would more logical that we develop strategies to desensitizing patients that target multiple steps of DSA production. Fortunately, there are many agents targeting each step of the humoral response as shown in **Figure 1** and **Table 1**. Unfortunately, there will also be too many potential combinations of biologics to permit exhaustive evaluation of each possible combination. Therefore, rational approaches merit testing in a preclinical model before being translated into the clinic.

AUTHOR CONTRIBUTIONS

AC, MM, and DO participated in literature search and wrote the manuscript. BE, JP, and KF participated in writing the manuscript. AJ critically reviewed the manuscript. JK and SK participated in writing and reviewing the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Highly Sensitized Patients Are Well Served by Receiving a Compatible Organ Offer Based on Acceptable Mismatches

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Highly sensitized kidney patients accrue on the transplant waiting list due to their broad immunization against non-self Human Leucocyte Antigens (HLA). Although challenging, the best option for highly sensitized patients is transplantation with a crossmatch negative donor without any additional therapeutic intervention. The Eurotransplant Acceptable Mismatch (AM) program was initiated more than 30 years ago with the intention to increase the chance for highly sensitized patients to be transplanted with such a compatible donor. The AM program allows for enhanced transplantation to this difficult to transplant patient group by allocating deceased donor kidneys on the basis of a match with the recipient's own HLA antigens in combination with predefined acceptable antigens. Acceptable antigens are those HLA antigens towards which the patients has never formed antibodies, as determined by extensive laboratory testing. By using this extended HLA phenotype for allocation and giving priority whenever a compatible donor organ becomes available, organ offers are made for roughly 80% of patients in this program. Up till now, more than 1700 highly sensitized patients have been transplanted through the AM program. Recent studies have shown that the concept of acceptable mismatches being truly immunologically acceptable holds true for both rejection rates and long-term graft survival. Patients that were transplanted through the AM program had a similar rejection incidence and long-term graft survival rates identical to non-sensitized patients transplanted through regular allocation. However, a subset of patients included in the AM program does not receive an organ offer within a reasonable time frame. As these are often patients with a rare HLA phenotype in comparison to the Eurotransplant donor population, extension of the donor pool for these specific patients through further European collaboration would significantly increase their chances of being transplanted. For those patients that will not benefit from such strategy, desensitization is the ultimate solution.

Keywords: donor specific antibodies, donor specific antibody (DSA), kidney transplanation, histocompatibility, desensitization, HLA, acceptable antigen, organ allocation

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INTRODUCTION

Sensitization against Human Leucocyte Antigens (HLA) occurs through pregnancy, blood transfusions or organ transplants. Highly sensitized patients awaiting a renal transplant are disadvantaged since their broad immunization status results in positive (virtual) crossmatches with almost all organ donors (1). Such broad immunization status precludes timely transplantation through regular deceased donor allocation schemes, which are based on the exclusion of donors carrying HLA to which the antibodies are directed (unacceptable antigens) (2). In addition, for highly sensitized patients the chance of finding a related or unrelated living donor to which they don't harbor HLA-specific antibodies is also extremely slim, further reducing their options (3). Highly sensitized patients accrue on the transplant waiting list. Within Eurotransplant, the percentage of patients awaiting a kidney transplant with a Panel Reactive Antibody (PRA) level of ≥85% increased from 2.0% to 5.6% from 2011 to 2019).

One strategy for transplanting highly sensitized patients is to temporarily remove circulating antibodies and/or antibody production by desensitization treatment, creating a window of opportunity for transplantation of either a deceased or living donor organ in the presence of a negative crossmatch (4). While this is a successful procedure for a proportion of highly sensitized patients, it is still hindered by antibody rebound and relatively high acute antibody-mediated, as well as chronic rejection rates (5, 6). In addition, the added burden of immunosuppression involved in such procedures puts the patient at increased risk for infectious complications (pneumonia, BK nephropathy and CMV disease) and malignancies (mainly skin cancer) (6). Finally, these procedures are very costly and resource intensive (7). The survival benefit for patients undergoing desensitization prior to kidney transplantation is not unequivocally clear, since contrasting results have been published (8, 9).

Ideally, one would like to timely transplant highly sensitized patients without administering additional immunosuppressive drugs beyond the standard immunosuppressive protocols. However, this is not possible if the allocation is based on unacceptable antigens. This was realized already in the Netherlands in 1985 when the first Dutch study on developing an alternative program for highly sensitized patients was initiated, which formed the foundation for what we now know as the Eurotransplant Acceptable Mismatch (AM) program (10). Subsequently, the Eurotransplant AM program was officially launched in 1989 with the goal to increase the transplantation rate of highly sensitized patients in the Eurotransplant region (11).

The rationale for the AM program is that by actively defining the acceptable antigens, a negative crossmatch can be predicted. The increased chance for patients to be transplanted in the AM program comes from the addition of the acceptable antigens to the HLA phenotype of the patient, thereby creating an 'extended' HLA type on the basis of which allocation takes place, in combination with mandatory shipment of compatible donor organs to the AM patient (12). Patients are transplanted using standard immunosuppressive protocols without additional desensitization treatment. This strategy did not only result in favorable outcomes as discussed below, but is also a cost-effective strategy to transplant highly sensitized patients. Nguyen et al. determined the effect of the AM approach on quality adjusted life years and healthcare costs and showed an overall lifetime gain of 0.034 quality-adjusted life-years and savings of over $4,000 per highly sensitized patient (13). These data imply that the AM approach would be feasible in developing countries as well.

The new Kidney Allocation System (KAS) in the United States, introduced in 2014, was also accompanied with priority for highly sensitized patients within the regular allocation scheme (14). Although 3-year graft survival data of highly sensitized patients transplanted through KAS look promising (15), it remains to be seen if priority without allocation based on acceptable antigens is accompanied by acceptable long-term survival rates. For the Eurotransplant population is has previously been shown that highly sensitized patients that were included in the AM program, but transplanted through regular allocation (exclusion of unacceptable antigens only), had a markedly inferior graft survival compared to highly sensitized patients transplanted through the AM program (16).Therefore, from March this year, patients included in the AM program will only receive organ offers through AM program allocation.

DEFINING ACCEPTABLE ANTIGENS

Through the years, the AM program has seen many adaptations and updates as technical advances in the field of histocompatibility testing emerged. When the AM program started in the late 1980's, HLA typing was mainly done by serology, and HLA antibody specificities were determined by using complement dependent cytotoxicity (CDC) assays (17). In those days, acceptable antigens were defined through negative reactions in regular CDC screening assays, but more importantly, by using patient-specific cell panels with single HLA antigen mismatched donor cells for HLA-A and -B, and later, also HLA-DR (18). Again, negative reactions indicated the absence of antibodies against this particular HLA antigen and indicated that a subsequent crossmatch with a donor organ carrying this specific mismatch would be negative. Subsequently, off-the-shelve target cells were generated in the form of Single Antigen Lines (SALs), which are K562 cells transfected with single HLA class I specificities (19). The major advantage of this approach compared to using peripheral blood mononuclear cells or isolated lymphocyte subsets as target cells is that no interference of other HLA alleles is present, but still reactivity to natively expressed HLA antigens is determined (20). More recently, the introduction of solid phase assays, and more specifically the luminex Single Antigen Bead (SAB) assays have

Abbreviations: AM, acceptable mismatch; CDC, complement dependent cytotoxicity; HLA, Human Leucocyte Antigen; ETKAS, Eurotransplant Kidney Allocation System; ETRL, Eurotransplant Reference Laboratory; KAS, kidney allocation system; PRA, panel reactive antibody; SAB, single antigen bead; AL, single antigen line; STAMP, Scandiatransplant Acceptable Mismatch Program.

facilitated an even more thorough identification of acceptable antigens, especially for HLA class II, for which reliable reagents were historically scarce. Whereas the increased sensitivity, and especially specificity of the SAB assays are useful in this respect, the results of these assays are always interpreted in light of the immunization history of the patient for inclusion in the AM program. Classifying all positive reactions in SAB assays as unacceptable antigens without taking into account specific reaction patterns, the immunization history, the CDC reactions, as well as a thorough risk-benefit analysis for the individual patient, adds to the problem of high sensitization rates rather than solving it (21). Therefore, all applications for the AM program are reviewed by the Eurotransplant Reference Laboratory (ETRL), ensuring equal and transparent inclusion criteria for all patients.

With the field of histocompatibility testing for renal transplantation gradually moving towards HLA epitope matching, the application of epitope analysis for highly sensitized patients was already described before. Computer programs such as HLAMatchmaker allow to extend the repertoire of acceptable antigens through analysis of HLA antigens that do not have epitope mismatches with the total epitope repertoire of the patient's self HLA (22, 23), and have been used for defining HLA class I acceptable antigens since 2004. Increased knowledge on the relevant epitopes for HLA class II will allow for extending these analyses for HLA class II in the near future (24).

TRANSPLANT RATE OF AM PROGRAM PATIENTS

Since the start of the AM program in 1989 up till the end of 2020, a total of 2992 highly sensitized patients have been included in the program and 1790 transplants were performed (**Figure 1A**). All countries within Eurotransplant contribute to the AM waiting list and effectuated transplants, with the vast majority coming from Germany and the Netherlands (**Figure 1B**). The numbers for the Netherlands are relatively high because the program started as a Dutch National program and was subsequently extended to the whole of Eurotransplant (12). Of all deceased donor kidney transplants within the Eurotransplant region, on average 3.3% are allocated through the AM program (**Figure 1C**).

It was previously shown that the waiting time for highly sensitized patients to be transplanted within the AM program is shorter when compared to highly sensitized patients receiving an organ through regular allocation in the Eurotransplant Kidney Allocation System (ETKAS) (16, 22). Since organ offer rates and transplant rates are not necessarily identical, we here analyzed both the organ offer rate and the transplant rate within the AM program. When analyzing the organ offer rate of patients within the AM program it is clear that 50% of patients listed on the AM waiting list receive an offer within the first 7 months of listing. Thereafter, the slope gradually decreases with a plateau of around 80% of patients

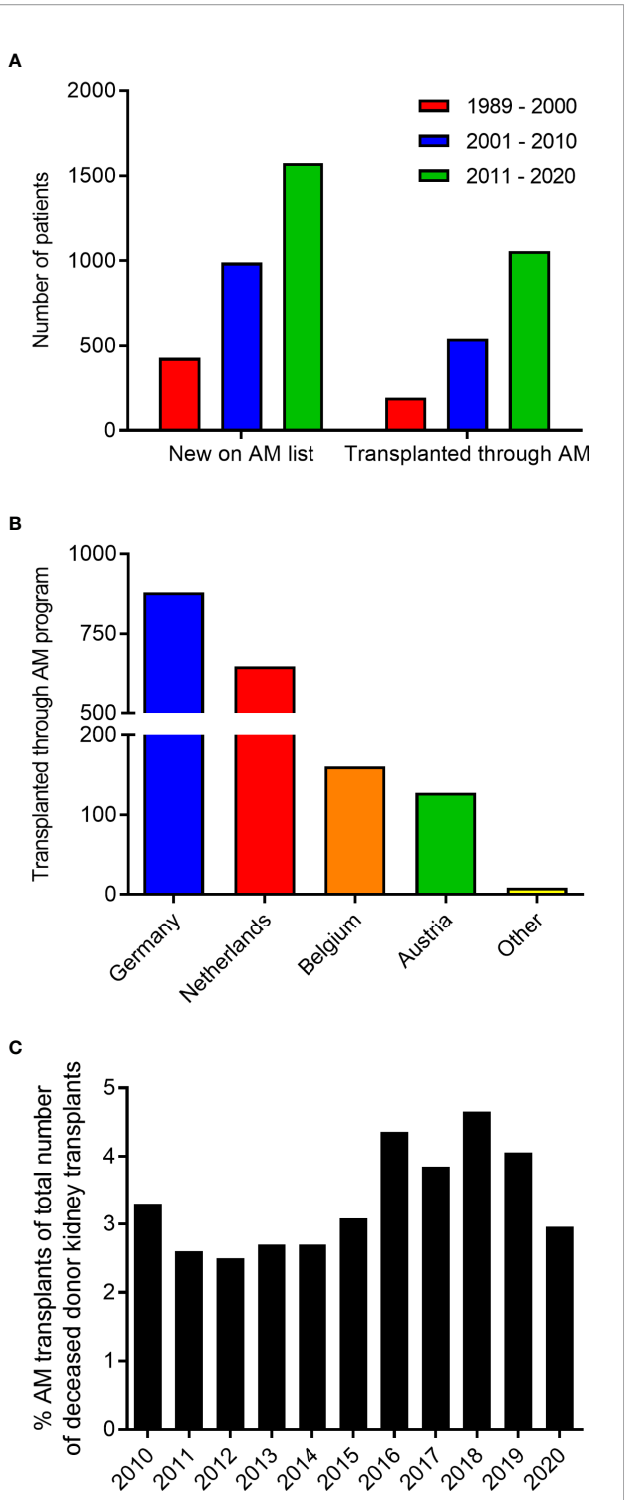


FIGURE 1 | Characteristics of patients enrolled in the AM program. **(A)** The number of patients included in the AM program and transplanted through the AM program from 1989 to 2020. **(B)** Country of origin of patients transplanted through the AM program. **(C)** Percentage of transplants through the AM program within all renal transplants from deceased donors within Eurotransplant in the last 10 years.

receiving an offer at 52 months (**Figure 2A**). The rate of organ offers is clearly related to the donor frequency within the AM program, which can be calculated for each AM patient, based on blood group compatibility and the own HLA type plus acceptable antigens (https://etrl.org/FreqAM.aspx) (**Figure 2B**). From these data it is apparent that meticulously defining acceptable antigens contributes to the chance of the individual patients to receive an organ offer within the AM program. Upon analysis of transplants effectuated through the AM program, it is clear that 50% of patients listed receive a transplant within 20 months of listing. After 20 months, AM patients continue to be transplanted with no clear plateau being reached within 67 months (**Figure 2C**). Similar to organ offers, it is clear that patients with the lowest chance as indicated by the donor frequency within the AM program have the slowest rate of transplantation (**Figure 2D**). The disparity between AM offers and effectuated transplants could be due to several reasons, such as patients not being fit for transplant at the time of offer, or the organ not being deemed suitable for the patient involved. It is obvious that immunological reasons, such as institutional minimal match criteria for HLA matching should not result in declining an AM offer, since all mismatches within the AM program are acceptable mismatches, which do not affect graft survival (12, 16).

The data on organ offers and transplantation show that even within priority programs such as the AM program, a subset of patients will not receive any organ offers, especially those in the group of the lowest chance as indicated by the donor frequency within the AM program. These are often patients with a relatively uncommon HLA phenotype in comparison to the available donor population. Here, the rationale that led Professor Jon J. van Rood to initiate the foundation of Eurotransplant, namely the larger the donor population, the higher the chance of finding a compatible donor, still rings true (25). In the proof-of-concept EU-FP7 study entitled 'A Europe-wide strategy to enhance transplantation of highly sensitized patients on basis of Acceptable HLA mismatches: EUROSTAM' it was recently shown that up to 27% of highly sensitized patients with neglectable chances of receiving an organ offer within their own donor population had an increased chance of receiving a compatible organ offer from another European allocation system (26). This simulation only included 4 additional donor populations, suggesting an even higher benefit when more organizations would be included. These data advocate the development of an AM program that unites several allocation systems only for those patients that will otherwise not be transplanted.

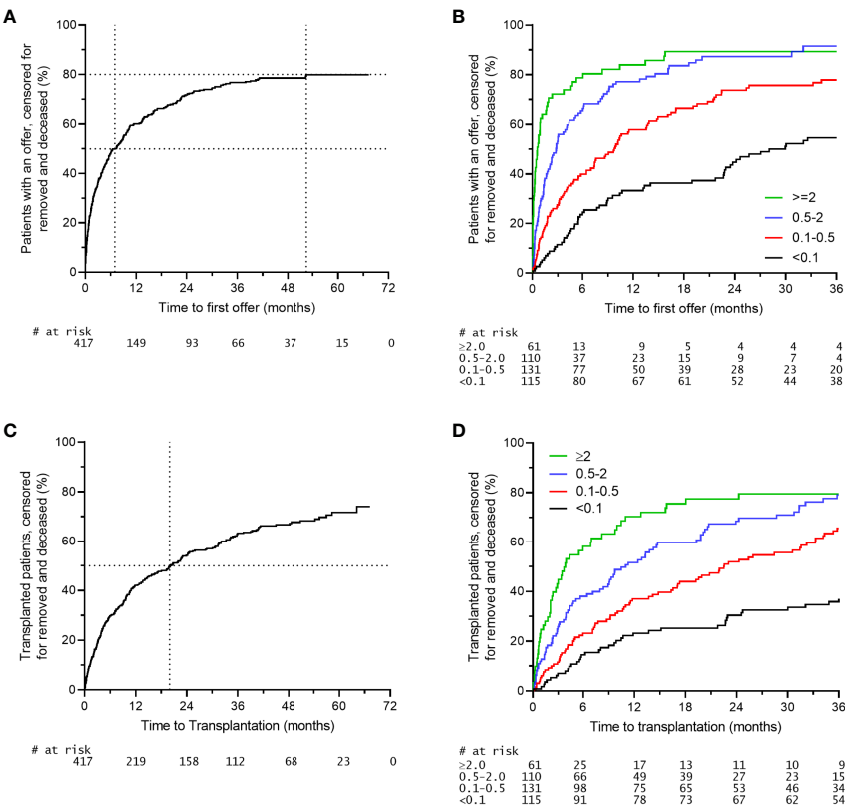


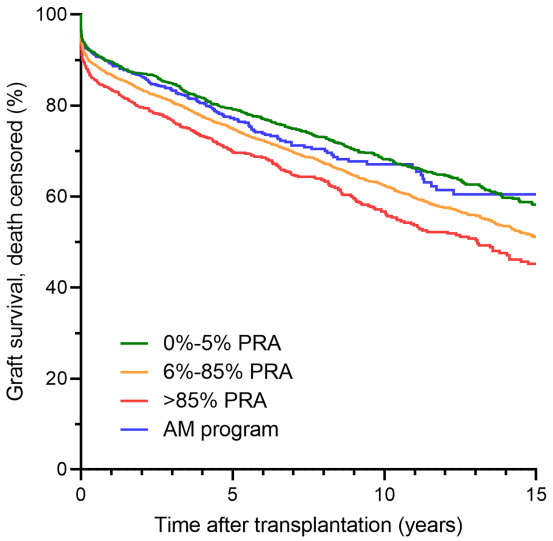
FIGURE 2 | Organ offers and effectuated transplants in the AM program. A time period of 01-01-2015 to 31-21-2016 was selected for inclusion of AM patients (n = 417). **(A)** Rate of first organ offer to patients on the AM waiting list. **(B)** Rate of first organ offer to patients on the AM waiting list stratified for the chance of an organ offer within the AM program. **(C)** Rate of transplantation of patients on the AM waiting list. **(D)** Rate of transplantation of patients on the AM waiting list stratified for the chance of an organ offer within the AM program.

OUTCOME OF AM PROGRAM TRANSPLANTS

When the HLA mismatches used for the allocation in the AM program are indeed acceptable, one would expect that there is no effect of these HLA mismatches on graft survival. Indeed, it has been shown that, in contrast to regular allocation, there is no effect of the number of HLA mismatches, either on the broad or split antigen level, on 10-year death-censored graft survival in patients transplanted through the AM program (12, 16). Early studies showed that the 2-year graft survival rates of AM patients were similar to those of non-sensitized patients (22). More recent data with higher patient numbers and longer follow-up showed that patients transplanted through the AM program had similar 10-year death-censored graft survival rates compared to non-sensitized patients transplanted through regular allocation (16). An updated analysis shows that also the 15-year death-censored graft survival is similar when comparing highly sensitized patients transplanted through the AM program and non-sensitized patients transplanted through regular allocation, and significantly better than that of highly sensitized patients transplanted outside the AM program (**Figure 3**). Similar observations regarding short-term graft survival were made in the relatively young Scandiatransplant Acceptable Mismatch Program (STAMP) (27). In their analysis of 96 patients, the authors did observe a non-significant trend towards higher rejection rates in STAMP as compared to control patients. Contrastingly, an analysis of all Dutch patients transplanted through the Eurotransplant AM program (n=113) showed a comparable cumulative rejection incidence to non-sensitized patients, both at 6-months and at 5-years follow-up, whereas highly sensitized patients transplanted through regular allocation had a significantly higher rejection incidence (28). The difference between the outcomes of these two studies could be attributed to the minimal match criterium used in the Eurotransplant AM program and not in the STAMP program, or the fact that the Eurotransplant AM program makes use of a central reference laboratory for strict inclusion into the AM program and acceptable antigen definition, whereas in the Scandinavian program the acceptable antigen definition is performed locally, likely resulting in a less uniform patient population. It has indeed been shown that acceptable mismatch definition by individual centers based on the same serum samples results in a huge variability in what antigens are regarded as being acceptable (29). Other differences between the two programs are that the STAMP program has a minimum waiting time of 1 year before acceptance, whereas the AM program currently has 2 years waiting time as criterium. The minimum PRA for acceptance in STAMP is 80% and currently in the AM program is 85%.

THE FUTURE OF THE AM PROGRAM

As mentioned before, the AM program is continuously adapted to the state-of-the-art knowledge in the field of histocompatibility testing. Some major changes in the AM program are underway that will be discussed here.



# at risk				
0%-5% PRA	2357	1061	580	204
6%-85% PRA	4575	2105	1121	443
>85% PRA	1295	436	218	83
AM program	1017	299	96	35

FIGURE 3 | The 15-year death censored graft survival of AM patients is similar to that of unsensitized patients. Selection was based on criteria described prior (16) and included: transplantation from 1996 onwards (start ETKAS allocation), minimum 1 HLA mismatch, kidney only, repeat transplants (since the vast majority of AM patients are repeat transplant candidates). Patients transplanted through ETKAS are subdivided into 0-5% PRA (non-sensitized), 6-85% PRA (intermediately sensitized), >85% PRA (highly sensitized, transplanted outside the AM program).

AM Program Inclusion Criteria

The current waiting time before a patient can be submitted to enter the AM program is a generic waiting time (from initiation of dialysis) of 2 years. However, the waiting time for a kidney graft is hugely variable within individual Eurotransplant countries, creating a disbalance between waiting time of highly sensitized patients and all other patients within individual countries, especially those countries with a very long waiting time for regular allocation. Therefore, in the future the median waiting time of a country will be used as minimal time that a patient from that country must be on dialysis before the patient is eligible to enter the AM program. A similar, but separate cut-off will be used per country for pediatric patients. Only for patients with a chance within regular allocation of lower than 0.01% (based on unacceptable antigens fulfilling the criteria below) the waiting time criterium will be omitted.

Traditionally, the AM program was reserved for those patients with a CDC-based PRA of at least 85%. This was later extended to specificities found in luminex, provided that these specificities could be attributed to an immunizing event. With the recent introduction of the vPRA within Eurotransplant the actual chance of receiving an organ offer within regular allocation can be used as inclusion criterium. This chance includes the blood group, as well as the vPRA, based on the HLA phenotype of 10.000 actual organ donors with Eurotransplant. From **Figures 2B, D** it is clear that there is a

subgroup of patients that get transplanted within the AM program almost instantly after listing. These patients likely are not that difficult to transplant, warranting stricter inclusion criteria for the AM program. In several publications it has been shown that the patient group that is particularly disadvantaged in regular allocation is the group with a chance of receiving a compatible organ offer of 2% or lower (26, 30). Therefore, the inclusion criterium for the AM program will change to a chance of receiving a compatible organ offer through regular allocation lower than 2%. The unacceptable antigens underlying this value must comply to the ETRL specifications:

-Minimum of one unacceptable antigen must be detectable by CDC.

-The additional unacceptable antigens, defined by Luminex reactivity only, must be attributable to a defined immunizing event.

-In case one of the abovementioned unacceptable antigens results in a clear epitope reactivity pattern, additional antigens carrying this epitope will be included for AM eligibility. The epitopes that will be considered are those that have been indisputably antibody verified, as defined by a list to be published by the ETRL.

-The total list of unacceptable antigens fulfilling the criteria above, together with the blood group must result in an ETKAS chance of <2% for acceptance in the AM program.

Extension of AM Program Allocation With HLA-C and HLA-DQ

It is clear that allocation to AM patients based on acceptable antigens is superior to allocation to AM patients based on exclusion of unacceptable antigens only (16). However, acceptable antigens currently used for allocation to AM patients are only defined for HLA-A, -B and -DR antigens. With the introduction of solid phase HLA-specific antibody detection techniques it is possible to accurately define acceptable antigens for HLA-C and HLA-DQ as well. In fact, for the vast majority of AM patients, acceptable antigens for HLA-C and -DQ have already been defined but are currently not used for AM allocation. In the near future, allocation will include acceptable HLA-C and -DQ antigens. It is to be expected that outcomes after transplantation will further improve when selection of donors in the AM program is extended to using acceptable antigens for HLA-C and -DQ in addition to HLA-A, -B and -DR.

Reduction of the Minimal Match Criteria

Currently, within the AM program, minimal match criteria of two HLA-DR or one HLA-DR and one HLA-B (split level) are adhered to. For patients with the chance of an organ offer within the AM program lower than 0.1%, these minimal match criteria are reduced to one HLA-DR match at the broad antigen level. A recent analysis from the ETRL showed that the 10-year graft survival of patients who are transplanted through the AM program according to the minimal match criteria is comparable to those transplanted with reduced minimal match criteria (31). In a second analysis, it was shown that the 6-month cumulative rejection incidence was similar in patients transplanted according to the minimal match criteria and the reduced minimal match criteria (28). These data indicate that acceptable mismatches are truly acceptable and are not detrimental. In a timespan of 2 years, 417 organ offers for AM patients were denied an offer based on the fact that the MMC were not met. Since no effect of the minimal match criteria is found, they will in the future be reduced to one HLA-DR match on the broad antigen level for all AM patients. For patients with the chance of a kidney within the AM program lower than 0.1%, the minimal match criteria will be abandoned altogether.

CONCLUDING REMARKS

Allocation of kidneys to highly sensitized patients remains a challenging task. Over the years, the Eurotransplant AM program has proven to be an efficient way to both prioritize highly sensitized patients, and also maximize transplant longevity. With the fields of histocompatibility and organ allocation evolving, the AM program has been adapted to novel insights and will continue to be updated on basis of the most current insights. Regardless of its success, there is a subset of patients that will not be transplanted through the AM program due to their extremely broad sensitization status in combination with their uncommon HLA phenotypes. For these patients, alternative options must be explored. This could either be looking for compatible donors outside the own donor pool, but could also be by clever integration of living donor programs with priority for highly sensitized patients (32). The latter can include desensitization programs, which represent a valid last resort for those that can otherwise not be transplanted. These include the widely used plasmapheresis and IVIg with or without rituximab, or possibly the more recently introduced complement inhibitor eculizumab, anti-CD20 obintuzumab, or IgG cleaving enzyme imlifidase [reviewed in (33)].

AUTHOR CONTRIBUTIONS

SH: wrote the manuscript. GH: performed analyses, co-wrote manuscript. ML-vO: performed analyses, co-wrote manuscript. FC: co-wrote manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Memory B Cells in Pregnancy Sensitization

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Memory B cells play an important role in immunity to pathogens as these cells are poised to rapidly differentiate into antibody-secreting cells upon antigen re-encounter. Memory B cells also develop over the course of HLA-sensitization during pregnancy and transplantation. In this review, we discuss the potential contribution of memory B cells to pregnancy sensitization as well as the impact of these cells on transplant candidacy and outcomes. We start by summarizing how B cell subsets are altered in pregnancy and discuss what is known about HLA-specific B cell responses given our current understanding of fetal antigen availability in maternal secondary lymphoid tissues. We then review the molecular mechanisms governing the generation and maintenance of memory B cells during infection – including the role of T follicular helper cells - and discuss the experimental evidence for the development of these cells during pregnancy. Finally, we discuss how memory B cells impact access to transplantation and transplant outcomes for a range of transplant recipients.

Keywords: memory B cell, pregnancy, sensitization, HLA, antibody

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INTRODUCTION

Pregnancy represents the most common alloimmune exposure in humans. Exposure to non-self antigens of paternal origin can prime maternal B cells to generate antibodies, and maternal production of antibody against fetal antigens can occur after immunization with either minor (i.e. blood group antigens) or major antigens [i.e. human leukocyte antigen (HLA)]. Rh alloimmunization occurs when anti-D antibodies are produced in response to immunization with fetal blood (1). While this maternal anti-D antibody can cross the placenta and cause hemolytic disease of the newborn, this review will focus on the generation and consequences of antibody against the major alloantigen - HLA. As HLA alloantigens expressed by the semi-allogeneic fetus can be re-encountered on a transplanted organ from either a living or deceased donor, alloimmunization from pregnancy has particular impact for female transplant candidates and recipients. The maternal immune response to fetal alloantigens thus sets the stage for what is to come later in life and influences both access to transplantation as well as post-transplantation outcome. Despite the prevalence of pregnancy alloimmunization, the immunologic consequences of this event are very poorly understood, as pregnancy represents a unique "immunologic paradox" (2) that differs significantly from other types of immunization contexts. In this review, we discuss our

current understanding of pregnancy alloimmunization with a particular focus on the generation of anti-HLA antibody and B cell memory. Herein, we use the term *pregnancy alloimmunization* to describe the response of any maternal adaptive immune subset to fetal antigen during pregnancy, whereas the term *pregnancy sensitization* refers specifically to the generation of alloantibody. In this regard, a parous woman has been alloimmunized by prior pregnancy even if she has not been sensitized (i.e. has detectable alloantibody).

CLINICAL IMPACT OF PREGNANCY SENSITIZATION

The clinical significance of pregnancy sensitization was first appreciated in the 1950s when JJ van Rood and colleagues were studying transfusion reactions in peripartum women (3). Although these investigators did not understand the structure or the etiology of the soluble factor(s) mediating cellular agglutination in their assays, this "factor" was later identified as anti-HLA antibody. This critical discovery by van Rood and colleagues impacted not only the emerging fields of transfusion medicine and organ transplantation but also allowed the development of the first reagents that were used to HLA type human tissue as well as the methodology (i.e. cytotoxicity assays). Subsequent studies which relied on cytotoxic assays later determined the prevalence and timing of pregnancy-induced anti-HLA antibody (4–6). The advent of single-antigen bead technology has greatly improved detection methods and revealed that pregnancy elicits a paternal HLA-reactive antibody response in 50-84% of mothers in the first year after pregnancy that may have HLA epitope bias (6–12). As in other types of immune exposures, the number of times that a woman is exposed to fetal alloantigen matters, as multiparous females have a higher incidence of paternal HLA-reactive antibodies (7) with strong binding to HLA epitopes (13). These anti-HLA antibodies make it more difficult for multiparous women with end organ disease to find appropriate organ donors and therefore contribute to sex-based disparities in organ transplantation (14–16). Even when transplantation in women with high levels of pre-existing anti-HLA antibody is avoided, the existence of low-level alloantibody or memory T and B cells generated by pregnancy alloimmunization may negatively impact transplant outcomes, although the specific impact of this alloimmunization event has been difficult to enumerate among other factors that influence graft outcomes (17–22). In particular, it is difficult to attribute post-transplant outcome to changes in anti-HLA antibody quantity over time as longitudinal investigation of pregnancy-induced anti-HLA antibody titers has not been performed in post-transplant recipients. Although the current available literature does not suggest that pregnancy alloimmunization promotes poor transplant outcomes *per se*, the available data sets are highly confounded. As discussion of this important topic is beyond the scope of this review, interested readers are referred to a review of pregnancy alloimmunization that discusses these data sets in detail (15). Altogether, these clinical observations underscore the importance of understanding how alloreactive B cells and antibody-secreting cells form and function during pregnancy.

OVERVIEW OF THE ANATOMY OF PREGNANCY SENSITIZATION AND THE AVAILABILITY OF FETAL ALLOANTIGEN

To understand the mechanisms of pregnancy sensitization, it is useful to first consider the locations where maternal immune cells may encounter fetal antigen. Given our present understanding of the anatomy of pregnancy and immunity, there are several potential locations. First, immune cells in the maternal blood contact the embryo-derived trophoblast of the placenta. However, the encounter of maternal blood with placental trophoblast is not thought to significantly prime maternal B cells to produce anti-HLA antibody because human trophoblast does not express HLA-A, HLA-B, HLA-DR, HLA-DP or HLA-DQ (23–26). Nevertheless, pregnancy sensitization might still occur locally at the fetomaternal interface as maternal T and B cells encounter conceptus-derived antigens in the uterine tissue that are presented by decidual macrophages or dendritic cells. Indeed, unsupervised high dimensional flow cytometric analyses identify B cell phenotypes in uterine tissue. While immunohistochemistry demonstrates that these decidual B cells are positioned next to T cells, ectopic lymphoid follicular structures in the gravid uterus have not been formally identified. Given the importance of follicular structures in the genesis of antibody-secreting cells (discussed further below), these data suggest that anti-HLA antibody from antibody-secreting cells is unlikely to be generated in the uterus. Furthermore, *in vitro* analyses of decidual B cells *versus* peripheral B cells demonstrate an augmented ability to produce the immunoregulatory cytokine, IL-10, in the presence or absence of co-stimulatory signals (27). These data therefore suggest that the uterus may comprise a specialized tissue-resident B cell population with an as yet undetermined interaction with uterine T cells. These studies also imply IL-10 producing decidual B cell subsets arise from naïve B cell precursors locally and are maintained in an antigen-selected manner as memory after interaction with fetal trophoblast cells. In support of the latter hypothesis, *in vitro* co-culture experiments demonstrate that fetal trophoblast cells induce IL-10 production in B cells (28). Altogether, these data suggest that B cell populations in the uterus are unique and may not participate in the generation of alloantibody. This conclusion is additionally supported by emerging data suggesting that B cells play an important role in determining pregnancy outcome. For example, in murine models of pregnancy, mice that inherently lack B cells (i.e. μMT), give birth to fetuses that were smaller than wild type and with fewer regulatory T cells (29). Moreover, recurrent miscarriage has been associated with phenotypic abnormalities in the B cell compartment (30). Altogether, these data suggest that B cells in the uterus are unlikely to contribute to a large degree to the total pool of HLA antibody that is produced during pregnancy, as B cells are much more frequent in locations

outside the uterus that promote the differentiation of antibody-secreting cells. We further discuss antigen availability outside the uterus below as well as our current understanding of B cell differentiation into both memory B cells and antibody-secreting cells.

In light of the findings discussed above, it is likely that the majority of pregnancy alloimmunization occurs at sites distant from the fetomaternal interface (**Figure 1**). It is indeed now well established in both mice and humans that conceptus-derived cells, proteins, exosomes, RNA, and DNA disseminate broadly in the maternal circulation and can deposit in maternal tissues (31–36). Importantly, disseminated protein antigens are detectable in the maternal spleen and other secondary lymphoid organs in mouse models of pregnancy (37) where these antigens can prime both maternal T cells (37–45) and B cells (46). Although fetal microchimerism clearly occurs, resulting in seeding of maternal tissues that may persist for decades, its impact on humoral immunity remains undefined (32, 36). In mothers, the development of regulatory T cells may be influenced by the presence of fetal microchimerism, and this T cell literature is excellently reviewed by Kinder et al. (47) However, the persistence of circulating anti-HLA antibodies with specificity for fetal HLA alleles and their vigorous recall response following transplant suggest that the B cell arm of the adaptive response is not similarly skewed towards a regulatory phenotype (48).

Although it is possible that antigen presenting cells from the uterus may traffic to the uterine draining lymph node and prime maternal T and B cells as well, mouse studies suggest that such egress of maternal antigen presenting cells from the uterus is impaired during pregnancy (49). Collectively, these data suggest that pregnancy alloimmunization primarily occurs in secondary lymphoid organs such as the spleen and lymph nodes, perhaps after uptake of circulating antigen by antigen presenting cells within these sites. However, many knowledge gaps remain. It is important to note that the majority of these data have yet to be validated in humans owing to the extreme polymorphism of HLA and the lack of HLA-specific reagents. Moreover, it is unclear how effective B cell help is provided by maternal T cells given that pregnancy alloimmunization often propels the expansion of hypofunctional and/or suppressive regulatory T cell populations.

GENERATION OF MEMORY B CELLS AND ANTIBODIES

To understand B cells responses in pregnancy, it is first useful to review what is known about B cell activation and differentiation in the context of infection and immunization, where B cell biology has been better studied. In this section, we review the foundation of humoral responses by outlining the pathways of B cell activation and the subsequent production of memory B cells and antibody-secreting cells.

B Cell Activation Pathways

Naïve B cells circulate through the secondary lymphoid organs in search of their cognate antigen in either soluble or membrane-bound form (50). While naïve B cells may be activated outside of secondary lymphoid organs in ectopic lymphoid structures (51) or in mice lacking organized lymph nodes or spleens (52), secondary lymphoid organs represent the chief location for initial antigen encounter and B cell activation (53). Although B

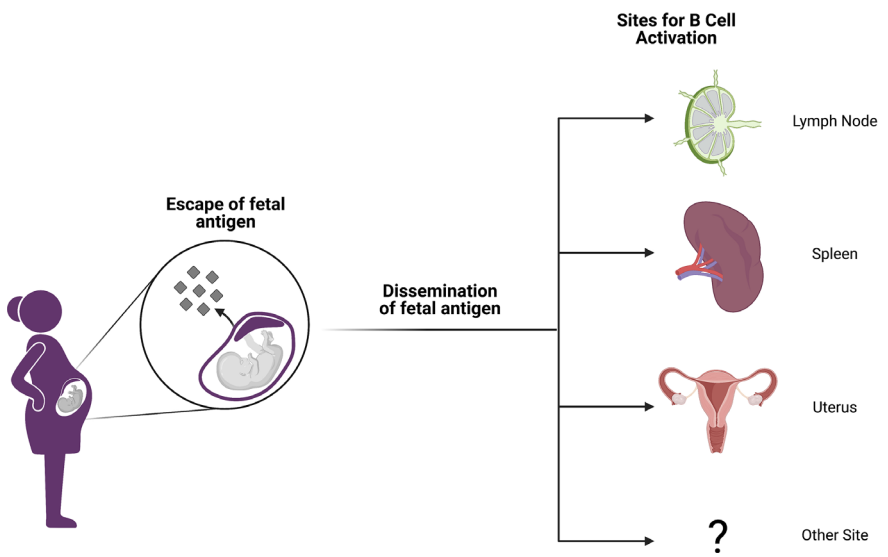

FIGURE 1 | Anatomic sites of alloimmunization in pregnancy. Although fetal antigen is known to disseminate widely, it is not well understood at which sites B and T cell priming take place in allosensitization. Candidate locations include secondary lymphoid organs (SLOs) like lymph nodes and spleen. However, it is also possible that immune structures within the gravid uterus may host alloantigen encounters or perhaps other anatomic sites are involved. Figure created with BioRender.com.

cell receptors are able to recognize intact, soluble antigen, multiple mechanisms exist to concentrate and present antigen for B cells. Afferent lymph enters a lymph node at the subcapsular sinus, where subcapsular sinus macrophages capture and present complement-opsonized antigen to B cells (54). Non-opsonized antigen may reach the medulla of the lymph node where it is bound by medullary macrophages or dendritic cells, phagocytosed, and presented or transported to the follicle (55). Within the follicle, follicular dendritic cells are specialized for the task of presenting antigen to B cells, capturing opsonized antigen and recycling it in a non-degradative endosomal compartment that facilitates long-term antigen presentation within a secondary follicle's germinal center (GC) response (56). How antigen is captured and presented in the context of pregnancy is unknown.

Following the naïve B cell's initial encounter with its cognate antigen, the B cell becomes activated and follows one of three major pathways, as indicated by the i, ii, iii numeric identifiers below (**Figure 2**). The B cell follows chemokine and oxysterol gradients to migrate to the interface of the B cell follicle and the T cell zone (T:B border), upregulating the receptors Epstein-Barr virus-induced molecule 2 (EBI2) (57) and C-C chemokine receptor 7 (CCR7) while maintaining C-X-C motif chemokine receptor 5 (CXCR5) expression (58). Some B cells then enter the germinal center (GC) while others remain in the extrafollicular (EF) region. In the germinal center (also known as the secondary follicle), somatic hypermutation and clonal selection produce high-affinity memory B cells and long-lived antibody-secreting cells in a T-dependent process (i). In the extrafollicular region, naïve B cells differentiate into memory B cells or antibody-secreting cells *via* T cell-dependent (ii) or T-independent processes (iii) (59). Although the spectrum of antigen that might prime B cell responses in pregnancy is unknown, fragments of HLA proteins are likely to be involved in the priming of anti-HLA antibody. We thus will focus on the first two pathways (i, ii) given that B cell priming by protein antigens is a T-dependent process (60, 61), and anti-HLA antibody is known to persist for decades in some women after pregnancy sensitization, thus implying the differentiation of long-lived antibody-secreting cells.

At the T:B border, B cells present their antigen and receive $CD4^+$ T cell help from developing T follicular helper cells (Tfhs). Tfhs, whose primary purpose is to help activated B cells become memory B cells or long-lived antibody-secreting cells, differentiate from naïve $CD4^+$ T cells after being primed by dendritic cells in the lymph node or spleen (62). They express the Tfh lineage-defining transcription factor *Bcl6* and the chemokine receptor CXCR5 and then migrate to the T:B border to interact with activated B cells (63). Here, B cells provide the key second step in Tfh differentiation by expressing ICOS ligand (ICOSL) and presenting the Tfh's cognate antigen *via* major histocompatibility complex class II (MHC class II) proteins (64). At the same time, the Tfh provides the B cell with critical support. Tfh expression of CD40 ligand (CD40L) delivers the second signal of B cell activation following ligation of the B cell receptor (65), and cytokines like IL-21 and IL-4 facilitate both T-dependent extrafollicular and germinal cell responses (66). From the T:B border, Tfhs then enter the germinal center, becoming germinal center Tfhs (GC-Tfhs) (67). Of note, others use the nomenclature *pre-Tfh* or *extrafollicular mantle Tfh* to refer to

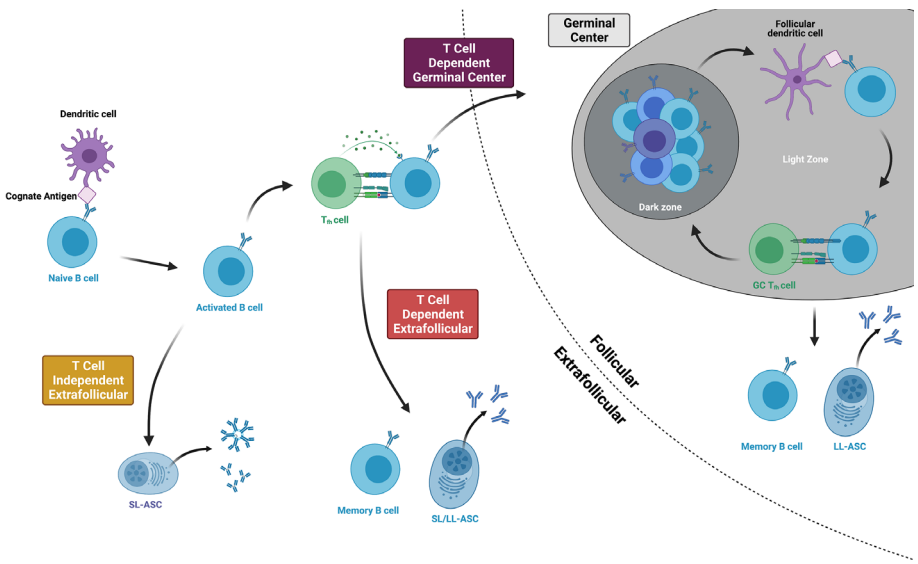

FIGURE 2 | Three main pathways of B cell activation and differentiation. Protein antigens promote T cell dependent responses, culminating in either germinal center or extrafollicular pathways that produce long-lived memory B cells and antibody-secreting cells (ASCs). Within the germinal center, B cells cycle between the dark and light zones, proliferating, mutating, and undergoing affinity maturation. T cell independent antigens include mitogens and highly repetitive polysaccharides that can stimulate B cell responses without T cell help. Figure created with BioRender.com.

BCL-6$^+$ CD4$^+$ T cells outside of the germinal center and simply *Tfh* for those within a germinal center structure (67, 68).

Following these events at the T:B border, B cells briefly proliferate in the outer follicle before making a fate decision to enter the germinal center or follow an extrafollicular pathway (69). The affinity of the B cell receptor, cytokine milieu, Tfh interactions, and innate sensors all contribute to this decision (70). High affinity B cell clones experiencing B cell receptor crosslinking may favor an extrafollicular response (71). Although B cells must meet a relative affinity threshold in order to effectively capture antigen and engage Tfhs at the T:B border (72), this threshold may be sufficiently low that it does not provide a considerable barrier to germinal center entrance among naïve antigen-specific clones (73). Tfhs assist activated B cells by indirectly assessing affinity and secreting IL-21 (66). and pathogen- or damage-associated patterns that signal through toll-like receptor 9 (TLR9) promote rapid antibody production by inducing the differentiation of antibody-secreting cells while decreasing B cell antigen presentation and thus, the effectiveness of B cell and T cell cooperation (74). Overall, these fate decisions correlate with EBI2 expression, whose loss guides the activated B cell into the germinal center and whose persistence corresponds with an extrafollicular response (75).

Germinal Center Responses

Activated B cells that enter the germinal center participate in the immunological equivalent of natural selection, resulting in a population of memory B cells and long-lived antibody-secreting cells that maintain long-lasting memory. Germinal centers feature two histologic compartments: a light zone where follicular dendritic cells present antigen for B cells (76) and Tfhs positively select higher-affinity B cell clones and a dark zone where B cells proliferate and acquire immunoglobulin gene somatic mutations (77). In the light zone, cognate B cells and Tfhs become "entangled" *via* multiple cell surface receptor interactions (78) with Tfhs providing B cell help *via* CD40L signaling and cytokines including IL-4, IL-21, and BAFF (79). B cells then migrate to the dark zone, where expression of the enzyme Activation-induced Cytidine Deaminase (AID) generates uracil bases which may be replaced with thymine bases leading to somatic hypermutation (80). These newly mutated germinal center B cells proliferate and return to the light zone to compete for antigen from follicular dendritic cells and re-engage Tfhs, with higher affinity B cells receiving continued Tfh help (79). It should be noted that several traditionally-held views about germinal centers have been challenged by new research. One such view, which holds that class-switch recombination occurs within the germinal center has been challenged by data showing that the majority of naïve B cells undergo class-switch recombination prior to germinal center entry and that class-switch recombination may in fact be repressed within the germinal center (81). Another view, which holds that somatic hypermutation and memory develop solely within the germinal center is addressed in the following paragraph, noting the existence of these processes in extrafollicular responses. Work continues to elucidate the factors that control the fate decision of a germinal center B cell to become a memory B cell or a long-lived antibody-secreting cell. Lower affinity B cell receptors may favor memory B cell development, as B cells receiving less Tfh help maintain high expression of the transcriptional repressor *Bach2* and subsequently become memory B cells (82). In addition to affinity, timing may also play a role in fate decisions, with the early germinal center response predominantly yielding memory B cells and the late germinal center response producing long-lived antibody-secreting cells (83). Overall, these fate decisions are consistent with observations that memory B cells may overall be more broadly reactive and of lower affinity than long-lived antibody-secreting cells (84, 85).

Extrafollicular Responses

Some activated B cells do not enter the germinal center reaction and instead proliferate and differentiate *via* an extrafollicular pathway. Recent work utilizing a semiallogeneic mouse model of pregnancy suggests that the maternal B cell response to fetal antigens may proceed in a fashion independent of the germinal center, which may implicate the extrafollicular pathway in the development of maternal sensitization (46). In an excellent review, Elsner et al. distinguish between canonical B cell responses, in which a brief extrafollicular phase precedes the germinal center response, and non-canonical ones, which have extended extrafollicular phases (86). Although the factors which skew a response towards the non-canonical extrafollicular pathway are an area of active investigation, it appears that both pathogen and host factors play important roles (**Figure 3**). *Salmonella Typhimurium, Borrelia Burgdorferi, and Ehrlichia Muris* promote the development of a non-canonical extrafollicular response *via* lipopolysaccharide (LPS)- and tumor necrosis factor-α (TNF-α)-mediated collapse of traditional lymph node and splenic architecture (87, 88). Innate sensors and cytokines play a role in extrafollicular responses as well, as studies of systemic lupus erythematosus have shown that B cell TLR7 hyperresponsiveness and IL-21 signaling synergize to generate autoreactive antibody-secreting cells outside of germinal centers (89). Although it appears that only germinal centers can generate IgG-secreting long-lived antibody-secreting cells (90, 91), mice lacking germinal centers are still capable of generating IgM-secreting long-lived antibody-secreting cells that home to the spleen as opposed to the bone marrow (92). Aside from these differences in the output of antibody-secreting cells, extrafollicular responses are still capable of inducing AID expression, yielding somatic hypermutation and class-switch recombination, and generating antigen-specific memory B cells (87, 93, 94). Consequently, it will be important in future work studying pregnancy sensitization in both humans and mice to determine the isotype of the fetal-specific or anti-HLA antibodies which are detected. This information will allow us to draw inferences about whether B cell responses originate within the follicle (i.e. germinal center) or outside the follicle (i.e. extrafollicular response). Discrimination between these two pathways has significant implications for multiple facets of pregnancy sensitization, including the durability of the antibody that is generated.

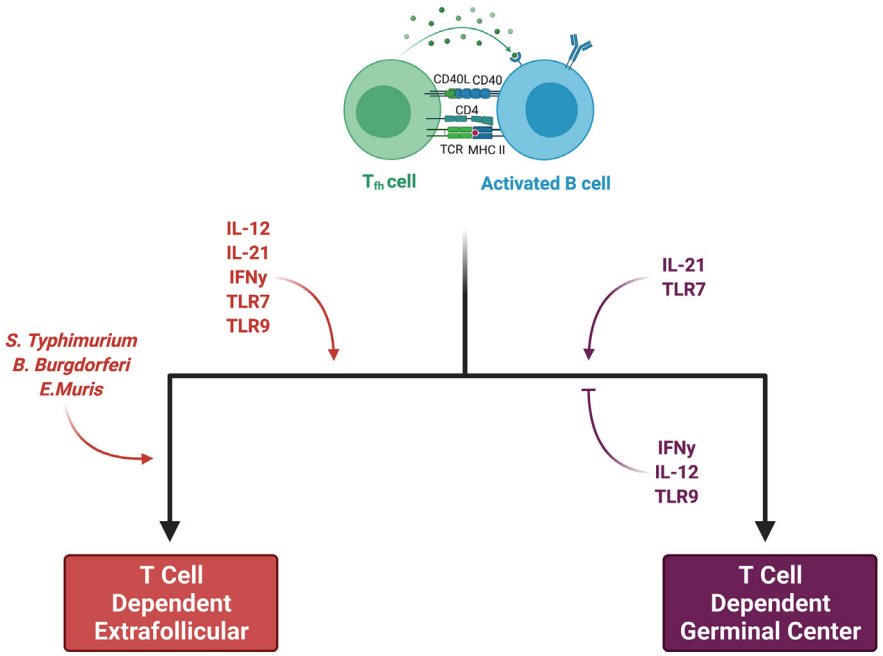

FIGURE 3 | Factors skewing responses toward either germinal center or extrafollicular pathways. Both pathogen and host factors contribute to a B cell's decision between an extrafollicular (EF) and germinal center (GC) response. Certain bacteria promote an EF response through collapse of secondary lymphoid organ (SLO) architecture. While IL-21 and TLR7 may promote both EF and GC responses, IL-12, IFN-y, and TLR9 all favor EF responses. IL-12 suppresses Tfh development while favoring short-lived ASC production. INF-y may have the same effects on Tfh suppression. TLR9 clearly stimulates EF responses, whereas it may inhibit GC responses, at least in autoimmune diseases. Figure created with BioRender.com.

FUNCTION OF MEMORY B CELL AND ANTIBODY-SECRETING CELL RESPONSES

Memory B cells provide the host protection from subsequent pathogen challenge through mechanisms that largely complement long-lived antibody-secreting cells. Importantly, multiple studies have shown that the reactivities of memory B cells and antibody-secreting cells do not overlap perfectly (85, 95). For example, memory B cells produced in response to certain viral infections are more broadly reactive than the antibodies produced by antibody-secreting cells, and these memory B cells may cross-react with different epitopes on other viral strains, affording protection from variants that are not neutralized by the antibodies produced by antibody-secreting cells (84, 85, 96). Memory B cell recall responses are facilitated by B cell receptor reactivity that is broader than the reactivity of long-lived antibody-secreting cells but is still antigen-specific, and memory B cells can rapidly respond to a second infection by differentiating into antibody-secreting cells or by entering germinal centers where they may affinity-mature, switch isotypes, and emerge as new memory B cells or high-affinity antibody-secreting cells (97). Although a mouse model with homologous boosting showed that memory B cells infrequently reentered germinal centers (98), humans respond to heterologous boosting with broadly reactive memory B cells that rapidly differentiate into antibody-secreting cells and reenter germinal centers (99). In addition to B cell receptor affinity, multiple other factors regulate the memory B cell recall response. Circulating antibodies (generated by long-lived antibody-secreting cells) may bind epitopes that memory B cells would otherwise recognize, effectively inhibiting a memory B cell recall response against those bound, cognate epitopes while promoting a response against exposed epitopes (100). The isotype of the memory B cells also appears to indicate its fate preference during a recall response, as both mouse and human IgM+ memory B cells show a propensity to reenter germinal centers, whereas IgG + memory B cells are more likely to differentiate into antibody-secreting cells (101, 102). It is not clear whether the isotype of the B cell receptor mediates this effect through properties related to intracellular domains of the B cell receptor or as a result of cell-intrinsic properties generated at the time of memory B cell formation.

HLA-REACTIVE B CELLS

HLA-reactive B cells have been studied following both pregnancy and transplant, revealing insights into their frequency, persistence, and specificity. Three techniques have been used in humans to study HLA-reactive B cells: 1) ELISPOT, 2) polyclonal activation, and 3) fluorochrome-labeled HLA

tetramers. Following pregnancy or transplant, HLA-reactive memory B cells are detectable by ELISPOT at frequencies of approximately 1 in 1,000 to 1 in 40,000 B cells, and these cells are notably absent from unsensitized individuals (103–106). Studies that polyclonally activated circulating HLA-reactive memory B cells and studied their supernatants have shown a restricted number of specificities as compared to circulating anti-HLA antibodies. However, "hidden sensitization," which is the presence of memory B cells which target HLA specificities that are not represented among circulating anti-HLA antibodies, is estimated to exist in perhaps 40% of sensitized patients (107, 108). Like ELISPOT, HLA tetramers have been used to estimate frequencies of HLA-reactive B cells following a sensitizing event. However, in light of data showing that up to 6% of B cells in sensitized humans and 1% of B cells in unsensitized humans bind such tetramers, the assay may lack specificity (109, 110). Notably, other investigators who have studied women sensitized through pregnancy have shown significantly lower frequencies, estimating that roughly 1 in 10,000 memory B cells is HLA-reactive (13). Concerns about the specificity of tetramer-binding B cells are likely justified, given that fewer than 30% of antibodies expressed from these tetramer-positive B cells bind the HLA molecule of interest, a finding consistent for both HLA class I and class II tetramers (13, 111). While these data therefore showcase how HLA-reactive memory B cells in humans can be captured and characterized, it is clear that available assays provide a limited view of prevalence and specificity. Given that the detection of HLA-reactive B cells in transplant patients carries prognostic significance (112), it is critical that we better understand the biology of these cells and close the significant gaps that remain in our understanding of how and where these cells differentiate, the signals governing their differentiation, and the molecular and epigenetic programs that guide this differentiation in humans. In summary, we conclude that although HLA-reactive B cells can be detected using current assays, their prevalence and the breadth of their anti-HLA repertoire may be underestimated given the use of small volume peripheral blood samples. Future work that pairs the above techniques with more advanced sampling, phenotyping, and antibody cloning approaches from vaccine (99, 113) and infectious disease studies (114) will enhance our understanding of HLA-reactive memory B cells and may provide insights that allow for better diagnosis and treatment of anti-HLA responses in transplant.

Murine models of pregnancy and allotransplantation have been used to overcome some of these limitations, and mouse investigations have thus revealed several insights into the origins and functions of alloreactive B cells (46, 115). Pertinent to this review, a recent study used peptide:MHC tetramers to examine alloreactive T and B cells concordantly in mice alloimmunized by either pregnancy or transplantation. The authors mated congenic mice and then performed allogeneic heart transplantation with and without co-stimulation blockade on the postpartum female partner to assess the role of pregnancy-induced alloimmunity on transplant outcome. The authors found that postpartum mice developed fetal alloreactive B and T cell responses and that the alloreactive T cell response remained tolerogenic after secondary heart allotransplantation. Next, the authors performed experiments on animals that lacked circulating immunoglobulins (sIgKO) or animals that lacked immunoglobulins and B cells (μMT) to dissect the relative roles of pregnancy-induced alloreactive memory B cells *versus* antibody-secreting cells in precipitating allograft rejection. Postpartum μMT animals were able to spontaneously accept allogenic heart transplants long-term without any immunosuppressive medication, suggesting that the alloreactive humoral immune arm that develops after pregnancy is critical to mediating subsequent organ transplant rejection. This spontaneous acceptance was lost when postpartum sIgKO were given allogeneic heart transplants, suggesting that even in the absence of circulating fetal specific antibody, a subset of alloreactive memory B cells elicited by pregnancy and recalled after transplant, was sufficient to abrogate tolerance. What subset of memory B cells mediates this break in tolerance and the mechanisms by which this occurs remain to be elucidated.

LESSONS FROM MEMORY B CELLS IN OTHER IMMUNE STATES

Although the authors do not perform molecular characterization of the alloreactive memory B cell compartment that develops in these postpartum mice and is recalled after subsequent allotransplantation, the authors perform limited immuno phenotyping. Despite the presence of circulating fetal-specific antibody, the alloreactive B cells of postpartum animals were found to lack classic germinal center markers in uterine-draining lymph nodes. Only after subsequent heart allotransplantation did the alloreactive B cells of postpartum animals express classical germinal center markers. Thus, the alloreactive B cells in postpartum animals after primary pregnancy appear to arise in extrafollicular reactions and have memory properties as they can be recalled after allotransplantation, present antigen to donor-specific T cells and form secondary antibody-secreting cells in germinal center reactions. These data are interesting in the context of recent insights into the functional diversity of antigen-specific memory B cells from the autoimmune (89) and immunization literature (116, 117) which have identified certain transcriptionally distinct antigen-specific memory B cells which are poised to directly form antibody-secreting cells in extrafollicular reactions (effectors) *versus* others that are recalled into germinal center reactions in lymphoid tissues to undergo affinity maturation and produce daughter antibody-secreting cells (effector memory) and daughter memory B cells (central memory). It will be important for future studies of the pregnancy-induced alloreactive immune response to clarify the transcriptional and functional diversity of this response in order to understand if particular alloreactive memory B cell subsets that develop after pregnancy sensitization are more or less important to mediating subsequent allograft rejection.

CONCLUSIONS

While our knowledge of B cell responses in pregnancy is underdeveloped, recent work suggests that fundamental mechanisms underlying B cell responses in infection are generalizable to the pregnancy setting. The further development of reagents that can track antigen-specific B cell responses in humans will be critical to improve our understanding of B cell differentiation and fate after pregnancy alloimmunization, including the generation of memory B cells, as well as short-lived and long-lived antibody-secreting cells. An improved understanding of these cell types and the mechanisms by which they arise will be critical to improve transplant access and outcomes among female transplant recipients and alleviate the significant sex disparity that exists in organ transplantation.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Impact of Sex in the Efficacy of Perioperative Desensitization Procedures in Heart Transplantation: A Retrospective Cohort Study

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Background: Sensitized patients, i.e. recipients with preformed donor-specific HLA antibodies (pfDSA), are at high-risk of developing antibody-mediated rejections (AMR) and dying after heart transplantation (HTx). Perioperative desensitization procedures are associated with better outcomes but can cause sensitization, which may influence their efficacy.

Methods: In sensitized patients (pfDSA>1000 mean immunofluorescence (MFI) units), we assessed the effect of perioperative desensitization by comparing treated patients to a historical control cohort. Multivariable survival analyses were performed on the time to main outcome, a composite of death and biopsy-proven AMR with 5-year follow-up.

Results: The study included 68 patients: 31 control and 37 treated patients. There was no difference in preoperative variables between the two groups, including cumulative pfDSA [4026 (1788;8725) *vs* 4560 (3162;13392) MFI units, p=0.28]. The cause of sensitization was pregnancy in 24/68, 35.3%, transfusion in 61/68, 89.7%, and previous HTx in 4/68, 5.9% patients. Multivariable analysis yielded significant protective association between desensitization and events (adjusted (adj.) hazard ratio (HR)=0.44 (95% confidence interval (95CI)=0.25-0.79), p=0.006) and deleterious association between cumulative pfDSA and events [per 1000-MFI increase, adj.HR=1.028 (1.002-1.053), p=0.031]. There was a sex-difference in the efficacy of desensitization: in men (n=35), the benefit was significant [unadj.HR=0.33 (95CI=0.14-0.78); p=0.01], but not in women (n=33) [unadj.HR=0.52 (0.23-1.17), p=0.11]. In terms of the number of patients

treated, in men, 2.1 of patients that were treated prevented 1 event, while in women, 3.1 required treatment to prevent 1 event.

Conclusion: Perioperative desensitization was associated with fewer AMR and deaths after HTx, and efficacy was more pronounced in men than women.

Keywords: heart transplant, antibody mediated allograft rejection, desensitization, sex influence, gender inequalities

INTRODUCTION

Heart transplantation (HTx) is the only curative treatment for advanced heart failure (1). Immunological sensitization affects cardiac allograft longevity; and recipients at high immunological risk present an increased risk of early postoperative cardiac graft dysfunction, antibody-mediated rejection (AMR), and death (2).

Sensitization refers to a state, in which HLA antibodies are circulating in the blood of recipients prior to transplantation. The means of sensitization include pregnancy, blood transfusions, previous heart transplantations, and mechanical assistance devices such as left ventricular assistance devices (3).

The presence of preformed donor-specific antibodies (pfDSA) in a recipient patient characterizes sensitization and needs to be accounted for when considering perioperative immunological induction strategies, as well as postoperative desensitization (3–8). Indeed, exclusive postoperative desensitization procedure after HTx seemed to benefit sensitized patients, as they presented similar overall survival as compared to contemporary patients without pfDSA (9).

Several elements point towards a higher immunological risk of pregnancy-induced sensitization as compared to other causes of sensitization (4). The present work aimed to assess whether the cause of sensitization affected the efficacy of perioperative desensitization procedures. In a historical cohort comparison analysis in sensitized patients, we compared those who benefited from desensitization (after it was implemented), to those with similar immunological risk who did not (before the protocol was implemented). Subgroup analyses then assessed between-group differences based on gender and pregnancy.

METHODS

This is a retrospective analysis comparing two cohorts of HTx recipients. We included all consecutive sensitized HTx recipients, three years around the date of implementation of desensitization procedures (01/2007), in a high-volume heart transplantation center. Patients were considered sensitized when presenting with pfDSA on the day of HTx, with mean fluorescence intensity above 1000 units. We excluded patients with combined transplantation procedures (i.e. kidney and heart, or liver and heart).

Our institutional review board approved the protocol, informed consent was obtained at listing, and data were collected as part of the HEARTS registry (clinicaltrials.org identifier NCT03393793). The study was in strict compliance with the International Society for Heart and Lung Transplantation (ISHLT) ethics statement.

Study Outcomes and Definitions

The main study endpoint was a composite of death and biopsy-proven antibody-mediated rejection (AMR) up to 5-years follow-up. Furthermore, subgroup analyses were performed based on gender and cause of sensitization. Secondary analyses included the analysis of the composite endpoint at 1-year follow-up, and analyses on death and AMR separately at 5-years follow-up. Sensitivity analyses included postoperative 30-days mortality as endpoint.

Following current recommendations, diagnosis of graft rejection required histological confirmation on endomyocardial biopsy (EMB) specimens. Routine EMB protocol remained similar during all the study period and consisted of 3 biopsies per month starting on day 15 until day 65 after HTx, then once every 20 days until four months, then monthly until six months, then once every 45 days until year 1. After year 1, they were performed every four months until year 3 and then every eight months until year 5. An additional EMB was performed when in presence of indirect signs of rejection (decrease in left ventricular ejection fraction, acute arrhythmia, or clinical indication). All biopsy specimens were processed and examined according to current standards, requiring retrospective analyses, from frozen samples in some cases (10, 11). Standard serial sections were cut from formalin-fixed paraffin-embedded EMB specimens and stained with hematoxylin-eosin-saffron for diagnosis. Immunofluorescence for C4d was performed on all specimens (frozen section; C4d monoclonal 1/100; Quidel Corporation; Polyclonal rabbit anti-mouse fluorescein isothiocyanate; Dako). Only capillary staining for C4d was assessed.

Standard Immunosuppressive Protocol

Except for postoperative desensitization, the standard immunosuppressive regimen did not change during the study period (2004-2010). As previously described (12), in the postoperative period it included thymoglobulin induction therapy from day 0 to day 4 (rabbit ATG, Thymoglobuline, Genzyme, Lyon, France), methylprednisolone bolus infusion on day 1, ciclosporine after day 1, and mycophenolate mofetil (MMF) after day 4. Corticosteroids were converted to oral form starting on day 4, with a dosage of 1 mg/kg initially. They were progressively lowered to reach 20 mg/day at 2 months and 5 mg/day after the anniversary date of HTx. Ciclosporine was the only calcineurin-inhibitor used at the time, and trough targets depended on delay since transplantation.

Intervention: Perioperative Desensitization Procedure Around HTx

Starting in 2007, a dedicated protocol was performed in sensitized patients, i.e. transplanted with pfDSA with MFI

above 1000. It included plasmaphereses, with 1 session immediately before HTx and 4 after, for a total of 5 sessions (with a 1:1 fresh frozen plasma/albumin ratio), then IVIg (2 g/kg over a four-day period starting on day 5).

Anti-HLA Antibodies Detection

The detection of anti-HLA antibodies was based on Luminex Single Antigen beads technology (One Lambda, Canoga Park, CA). The fluorescence of each bead is detected by a reader and recorded as the normalized mean fluorescence intensity (MFI) (13). Retrospective cross matches were performed by complement-dependent cytotoxicity (CDC) assay on T- B- donor lymphocytes with historical and current sera. HLA typing of heart recipients was performed by low-resolution class I HLA-A, -B, and class II HLA-DR, -DQ PCR-SSO (LABType, One Lambda). Donor HLA-A, B, DR, DQ typing was performed by CDC (One lambda tissue-typing trays) and controlled by molecular biology. Because this technology was routinely deployed after the date of HTx of some patients, it was performed retrospectively on samples that had been stored to that effect, guaranteeing the homogeneity of pfDSA assessment.

In the present study, pFDSA were considered positive (i.e. recipient patient was sensitized), only if they presented a mean fluorescence intensity (MFI) above 1000. Cumulative DSA (cDSA) was computed as the sum of MFI of all DSA. Only patients with positive pfDSA on the day of HTx, confirmed with retrospective analyses, were included in the present study.

Standard AMR Treatment

AMR was treated with high-dose intravenous corticosteroids (1 g/day for 3 days) concomitantly to plasmaphereses sessions over 5 days then intravenous immunoglobulins (IVIg) (2 g/kg over a 4-day period) were administered. Thereafter, oral corticosteroids were then increased to 1 mg/kg for 10 days, with progressive weaning afterward.

Statistical Analyses

Continuous variables are described as median (interquartile range) and categorical variables as number (percentage). Fischer's exact tests were performed to compare proportions between groups. Cumulative survival curves for the time-to-event analyses were constructed according to the Kaplan-Meier method. Cox regression was used to assess the association between treatment and clinical outcomes. The alpha risk was set to 5%. All calculations were performed using SPSS v22.0 (IBM, Armonk, USA).

RESULTS

Overall, 68 sensitized patients were included with 37 patients in the desensitized group and 31 patients in the historic control group. They were 48.4 (interquartile range: 36.2;54.9) years-old, with 33/68, 48.5% women and 12/68, 17.6% under preoperative extracorporeal life support (ECLS). The cause of sensitization was pregnancy in 24/68, 35.3%, transfusion in 61/68, 89.7%, and previous HTx in 4/68, 5.9% (overlaps are presented in **Figure 1**). Baseline characteristics are presented in **Table 1**.

There were no significant differences in baseline characteristics between the two groups. In particular, patients in the treated group were under similar ECLS support, as compared to those in the historic control group (9/37, 24.3% *vs.* 3/31, 9.7%, p=0.20). There was no significant difference in women proportion (20/37, 54.1% *vs.* 13/31, 41.9%, p=0.34), nor pregnancy-induced sensitization (16/37, 43.2% *vs* 8/31, 25.8%, p=0.20). In desensitized women, pregnancy-induced sensitization proportion was similar as in women in the historic control group (16/20, 80% *vs* 8/13, 61%, p=0.42). Causes of sensitization were similar in the two groups (p=0.45). Cumulative pfDSA were similar between the two groups [4026 (1788;8725) *vs* 4560 (3162;13392) MFI units, p=0.28]. DSA included class II anti-HLA antibodies in the same proportion in the two groups (19/31, 61.3% *vs* 26/37, 70.3%, p=0.45).

Cumulative preformed DSA were higher in women than in men [8576 (3972;16450) *vs* 3004 (1600;4984) MFI units, p<0.0001]. In women (n=33), cumulative preformed DSA were higher in those sensitized because of pregnancy than other women [4978 (4027;11183) *vs* 11231 (3896;20727), p<0.0001].

Effect of Desensitization on Clinical Outcomes, in the Overall Cohort

Desensitization was associated with a significant reduction in the incidence of the main composite outcome (death or AMR) with 29/31, 93.5% *vs*. 20/37, 54.1% of patients during the five-year follow-up; corresponding in survival analyses to an unadjusted (unadj.) hazard-ratio (HR) of 0.42 (0.23-0.74), p=0.003 (see **Figure 2A**). Multivariable Cox survival analyses (accounting for cumulative DSA, age, desensitization intervention, and sex) confirmed desensitization was independently associated with fewer events with an adjusted (adj.) HR=0.44 (0.25-0.79), p=0.006; the other independent variable being cumulative DSA (per 1000 MFI-increase, adj. HR=1.031 (1.005-1.057), p=0.018).

Secondary analyses showed the benefit of desensitization against death, with a significant protective association (7/37, 18.9% *vs* 15/31, 48.4%, unadj.HR=0.34 (0.14-0.85), p=0.02) (see **Figure 3**), and a trend against AMR (16/37, 43.2% *vs* 17/31, 54.8%; unadj.HR=0.51 (0.26-1.03), p=0.06). Multivariable analyses accounting for cumulative DSA yielded similar results.

Effect of Desensitization: Comparing Men and Women

Subgroup analyses were performed (see **Figure 4**). In men, desensitization was associated with fewer events [8/17, 47.1% *vs*. 17/18, 94.4%; unadj.HR=0.33 (0.14-0.78); p=0.01]. In women, desensitization was not significantly associated with fewer events [12/20, 60% *vs*. 12/13, 92.3%; unadj.HR=0.52 (0.23-1.17), p=0.11]. In terms of the number of subjects to treat, in men, 2.1 patients treated prevented one event, while in women, 3.1 need to be treated.

Effect of Desensitization: Comparing Pregnancy-Induced to Other Causes of Sensitization

In women patients who are sensitized because of pregnancy (n=24), desensitization was not significantly associated with fewer events [11/16, 68.8% *vs*. 8/8, 100%, unadj.HR=0.51 (0.20-1.28), p=0.15].

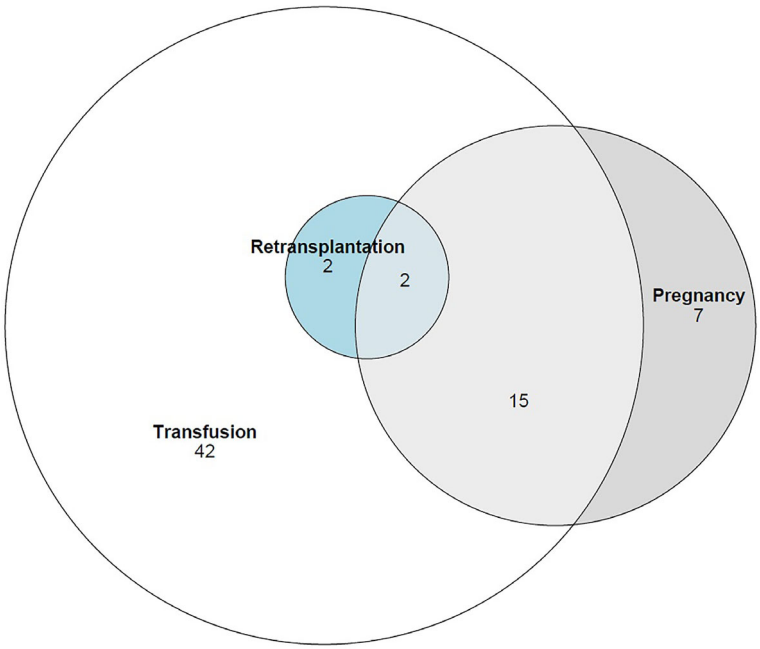

FIGURE 1 | Causes of sensitization in the overall cohort.

TABLE 1 | Baseline characteristics.

3	Overall (n=68)	Control (n=31)	Treated (n=37)	*p-value*
Women	33 (48.5%)	13 (41.9%)	20 (54.1%)	0.34
Age, median, [IQR], in years	48.4 [36.2;54.9]	50.4 [38.5;55.6]	45 [35.4;54.4]	0.45
Height, median, [IQR], in cm	167.5 [162;175]	171 [162;175]	167 [163;172]	0.41
Weight, median, [IQR], in kg	65 [58.5;78.5]	68 [59;82]	63 [56;78]	0.17
Cause of heart failure				0.54
Dilated cardiomyopathy	34 (50.0%)	17 (54.8%)	17 (45.9%)	
Ischemic cardiopathy	19 (27.9%)	9 (29.0%)	10 (27.0%)	
Other	15 (22.1%)	5 (16.1%)	10 (27.0%)	
LVAD or TAH	12 (17.6%)	5 (16.1%)	7 (18.9%)	1.0
Preoperative ECMO	12 (17.9%)	3 (9.7%)	9 (24.3%)	0.11
Cause of sensitization				0.45
pregnancy	24 (35.3%)	8 (25.8%)	16 (43.2%)	
transfusion subgroup	61 (89.7%)	28 (90.3%)	33 (89.2%)	
previous HTx	4 (5.9%)	1 (3.2%)	3 (8.1%)	
Cumulative DSA MFI, [IQR], in u	4031 [2074;12083]	4026 [1788;8725]	4560 [3162;13392]	0.28
pregnancy-related subgroup	11231 [3894;20727]	13372 [4643;33728]	10233 [3896;16063]	0.42
transfusion subgroup	4026 [1943;9035]	3515 [1787;8651]	4034 [2724;11183]	0.35
previous HTx subgroup	15960 [12023;19054]	8576 [na]	16450 [15470;21657]	0.50

ECMO, extracorporeal membranous oxygenation; HTx, heart transplantation; IQR, interquartile range; LVAD, left ventricular assistance device; TAH, total artificial heart.

In other causes of sensitization (n=44), desensitization procedure was associated with fewer events [unadj.HR=0.32 (0.14-0.70), p=0.005]. In details, in patients with a history of transfusion (n=61), unadj.HR was 0.40 (0.22-0.74), p=0.003), in patients with previous HTx (n=4), there was no association with the primary endpoint (p=0.62) (see **Figure 3**).

Additional Sensitivity Analyses

The association between desensitization intervention and the incidence of the primary composite endpoint (death and AMR) at one-year follow-up was assessed. These analyses confirmed that there was a significant reduction of deaths and AMR in patients who underwent desensitization as compared to those who did not [unadj.HR=0.49 (0.26-0.89), p=0.02]. Multivariable Cox survival analysis also confirmed the independence of association between desensitization and event reduction [adj.HR=0.49 (0.27-0.91), p=0.023]. The other independent variable associated with the events was cumulative DSA [per 1000-MFI increase, adj.HR=1.028 (1.002-1.053), p=0.031] (see **Figure 2B**).

Focusing on postoperative mortality (assessed by 30-days mortality), there was no significant difference between the two groups (5/37, 13.5% in the treated group *vs* 6/31, 19.4% in the control group, p=0.33). Causes of postoperative deaths were rejection in 2/5, 40.0% in treated patients *versus* 4/6, 66.7% in control patients; and sepsis in 2/5, 40.0% in treated patients *versus* 1/6, 16.7% in control patients (p-value could not be computed due to the limited number of events). Finally, there was one case of fatal hemorrhage in the treated group (*versus* none), and one case of fatal stroke in the control group (*versus* none).

Exploratory Analyses

Association between the presence of class II anti-HLA antibodies and the primary outcome, with a 5-year follow-up, was assessed and did not yield significant association. Interaction analyses between preformed cumulative DSA and desensitization procedures, regarding the primary outcome, with a 5-year follow-up, also did not yield significance, although it may be due to lack of power.

DISCUSSION

Our study yielded three main findings: i) desensitization procedures were associated with fewer deaths and AMR; ii) the benefit of desensitization was not equal between men and women; and, iii) preformed cumulative DSA was independently associated with deaths and AMR, after adjusting on desensitization procedures.

Sensitization is a major challenge because it restricts access to organ transplantation, due to the limited available donor pool and increasing wait time. Post-transplantation outcomes are less favorable in sensitized than in non-sensitized recipient patients, which may topple the risk-benefit balance towards treatment abstention in some of these patients at high immunological risk (3, 10).

Sensitized patients (i.e. patients with pfDSA) present more adverse outcomes after HTx, with more deaths and AMR than patients without pfDSA (7). The presence of pfDSA is mostly dependent on a previous sensitization event, for which main causes are blood transfusion, pregnancy, and previous transplantation (14). Furthermore, we previously reported that pregnancy-induced pfDSA was associated with more AMR than those related to other means of sensitization (4). In a recent statement, the American Heart Association emphasized the need to better characterize patients who would best benefit from desensitization procedures, which we humbly tried to partially address in the present paper (3). Our team previously described the benefit of performing postoperative desensitization (9).

In the present paper, a historical comparison was performed, examining the time of implementation of desensitization procedures in sensitized patients. The results confirmed the efficacy of these procedures, with a reduced incidence in AMR and deaths after HTx in the overall cohort. However, subgroup analyses showed that sensitization due to previous pregnancy was less beneficial for desensitization procedures than sensitization due to other causes (previous heart transplantation and blood transfusions). Indeed, the impact of desensitization procedures appeared to be less significant in this subgroup. Moreover, the efficacy of desensitization was less significant in women as compared to men (in terms of the number of subjects to treat to prevent one event, 3.1 women *vs.* 2.1 men) (15). The reasons for these findings may include the fact that women present more pfDSA than men (14) and that for a given quantity of pfDSA,

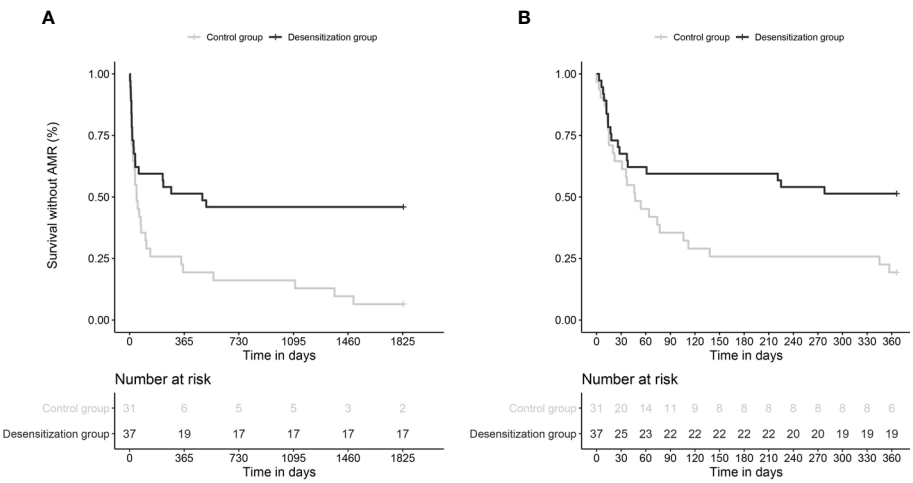

FIGURE 2 | Survival curves comparing the incidence of the main composite endpoint (death and AMR) in desensitized patients and historic control patients, with a 5-year follow-up **(A)** and 1-year follow-up **(B)**. **(A)**. Multivariable Cox survival analyses (with cumulative DSA, age, desensitization intervention, and sex) confirmed desensitization was independently associated with fewer events with an adjusted (adj.) HR = 0.44 (0.25-0.79), p = 0.006; the other independent variable being cumulative DSA [per 1000 MFI-increase, adj. HR = 1.031 (1.005-1.057), p = 0.018]. **(B)**. Multivariable Cox survival analyses (with cumulative DSA, desensitization intervention) yielded a significant association between desensitization and the primary outcome with 1-year follow-up, adj.HR = 0.49 (0.27-0.91), p = 0.023) and cumulative DSA also independently associated with the primary outcome [per 1000-MFI increase, adj.HR=1.028 (1.002-1.053), p = 0.031].

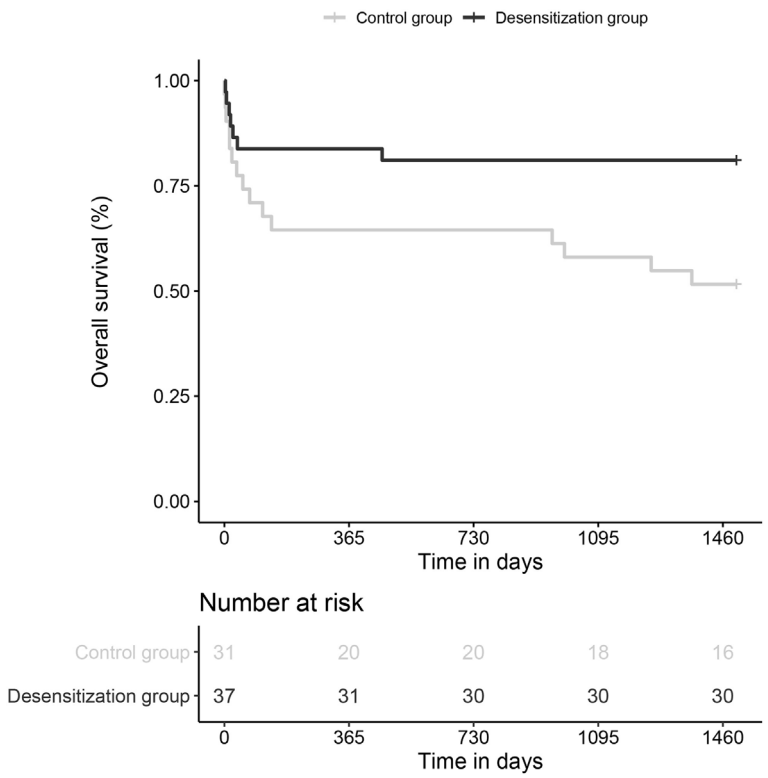

FIGURE 3 | Survival curves comparing the incidence of death in desensitized patients and historic control patients, with a 5-year follow-up.

they are more at risk of developing AMR afterward (4). Gender difference in solid organ transplant recipients regarding de-novo HLA antibodies production is unclear. In a previously described cohort of 463 patients, we did not observe any difference between women and men regarding *de novo* DSA production (4). Yet, in another study, in 47 patients after vaccination, women seemed more exposed to the increase of non-specific HLA antibodies than men (16). Regardless, the hypothetical means to address this issue of gender-difference in desensitization efficacy may rely on intensification of desensitization procedures in sensitized women with previous pregnancies or escalating maintenance immunosuppression (17, 18). In our study, the lack of significant difference of postoperative mortality (13.5% in the treated group *vs* 19.4% in the control group) may be due to the limited sample size.

A strength of this study is that all *sera* were analyzed to assess DSA, even for the study period in which Luminex based analyses were not standard of care, for which, samples had been stored to that effect. Similarly, endomyocardial biopsies were all retrospectively re-analyzed to uphold the most recent standards. We also acknowledge several limitations to the present work.

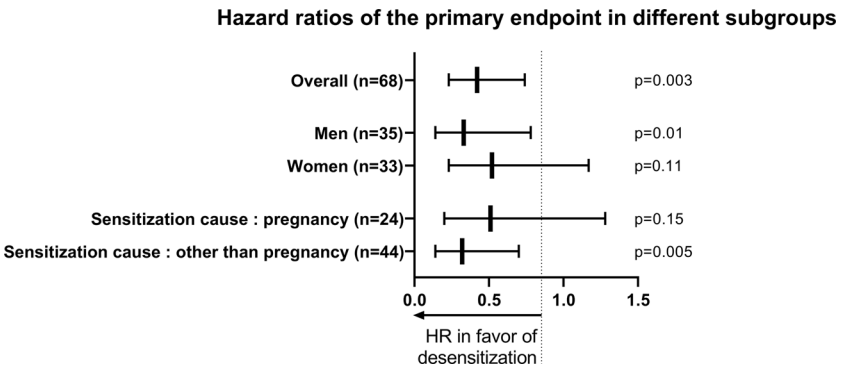

FIGURE 4 | Forest plot comparing the efficacy of desensitization in different subgroups, on the incidence of the main composite endpoint (death and AMR).

The retrospective monocentric design of the study, with historical comparison, means that future studies will need to externally validate the results found. Likewise, the relatively small number of patients demands confirmation. Indeed, proper interaction analyses to assess between-group-effect on treatment-effect could not be performed on relatively few patients (however difficult that may be in the field of heart transplantation). Furthermore, patients could present multiple causes of desensitization, however, the small number of patients could not allow subgroup analyses between overlapping causes, which may be more feasible in larger transplanted cohorts such as those in renal transplantation (14). We could not assess the efficacy of desensitization on DSA after HTx, specifically *de novo* DSA, due to the lack of *sera* in the historical control cohort. Maintenance immunosuppression regimen has been subject to change in the past 15 years with tacrolimus suggested instead of ciclosporin (19) and mammalian target of rapamycin (mTOR) inhibitors in addition to, or in place of, calcineurin inhibitors (12, 20). These may impact the treatment effect observed due to desensitization. However, maintenance immunosuppression is more associated with later outcomes than those occurring before the first year postoperative, whereas desensitization may be associated with a treatment effect that is more important for first-year outcomes than five-year outcomes (as attested by sensitivity analyses). While the difference was not significant, the historic control cohort did not present the same preoperative risk, being less severe compared to the treated cohort; however, the results point towards better outcomes in the treated cohort, comforting the efficacy of desensitization despite worse preoperative odds. Finally, this latter point also emphasizes that patients who would not otherwise have undergone HTx due to high operative and immunological risk did benefit from transplantation thanks to specific desensitization procedure, and did so with acceptable risk in this retrospective analysis. These findings may help alleviate immunological risk in sensitized patients, with dedicated perioperative desensitization procedures.

CONCLUSION

In this retrospective study of sensitized patients at the time of HTx, desensitization protocol was associated with a significant reduction in death and AMR. The cause of sensitization affected the efficacy of the protocol, with a less significant effect in women. These results warrant further research in patients at higher-immunological risk, to promote gender equality after HTx.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Pitié-Salpétrière Comité de Protection des Personnes. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LN wrote the manuscript and performed all analyses. J-ES, M-CB, GC, JA, AB, CS, V-DK, MC, PR, MK, SV, PL, and SS participated to data collection and patient follow-up, and provided critical review to the manuscript. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.659303/full#supplementary-material

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Allo-Specific Humoral Responses: New Methods for Screening Donor-Specific Antibody and Characterization of HLA-Specific Memory B Cells

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Antibody-mediated allograft rejection (AMR) causes more kidney transplant failure than any other single cause. AMR is mediated by antibodies recognizing antigens expressed by the graft, and antibodies generated against major histocompatibility complex (MHC) mismatches are especially problematic. Most research directed towards the management of clinical AMR has focused on identifying and characterizing circulating donor-specific HLA antibody (DSA) and optimizing therapies that reduce B-cell activation and/or block antibody secretion by inhibiting plasmacyte survival. Here we describe a novel set of reagents and techniques to allow more specific measurements of MHC sensitization across different animal transplant models. Additionally, we have used these approaches to isolate and clone individual HLA-specific B cells from patients sensitized by pregnancy or transplantation. We have identified and characterized the phenotypes of individual HLA-specific B cells, determined the V(D)J rearrangements of their paired H and L chains, and generated recombinant antibodies to determine affinity and specificity. Knowledge of the BCR genes of individual HLA-specific B cells will allow identification of clonally related B cells by high-throughput sequence analysis of peripheral blood mononuclear cells and permit us to re-construct the origins of HLA-specific B cells and follow their somatic evolution by mutation and selection.

Keywords: donor specific antibody (DSA), B cell, sensitization, transplantation, antibody mediated rejection (AMR)

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INTRODUCTION

HLA sensitization remains a barrier to transplantation of all organ types (1, 2). The etiology of allo-antibodies stem from exposure to foreign HLA through pregnancy, transfusion, and previous transplantation and is driven by the high polymorphism among HLA genes. Advances in histocompatibility testing have improved the sensitivity and specificity of HLA antibody detection and has revealed broader HLA sensitization among transplant candidates. Transplantation in the presence of donor-specific HLA antibody (DSA) for mismatched HLA

determinants in the donor's phenotype increases the risk of rejection and allograft loss, while locating an HLA compatible donor can increase waiting time and associated mortality (3).

Screening for HLA antibodies is routinely performed prior to transplantation to avoid DSA and the increased risk for AMR and graft failure. In this way, donor selection and transplant outcomes are optimized. HLA single antigen bead (SAB) Luminex® assays allow sensitive screening for antibodies specific for hundreds of HLA alleles and are used widely in clinical practice. These assays, however, are prohibitively expensive for non-clinical studies and unavailable for animal species frequently used to study humoral responses to MHC antigens, notably macaques and swine. This lack of comparable diagnostic tools limits detailed study of the breadth and specificity of MHC-specific humoral responses in non-human transplant model systems. Crossmatch assays using donor lymphocytes have been used to detect the presence or absence of species-specific anti-MHC antibodies in experimental transplantation studies with rhesus macaques (4) and swine (5). In a series of recent studies (6, 7), swine SLA class II single antigen expressing HEK293 cell lines were generated to monitor xeno-reactive antibody responses with human serum samples, albeit, with singleplex capability and limited MHC allele coverage.

Sensitized patients are vulnerable to AMR when transplanted in the presence of circulating DSA, but some may also experience early AMR as a result of post-transplant activation of cryptic HLA-specific B memory. Unfortunately, current methods of assessing AMR risk at time of transplant relies solely on detection of circulating DSA and are unable to detect HLA-primed memory B (Bmem) cells that can rapidly proliferate and differentiate to antibody secreting cells on restimulation. In order to reveal this hidden humoral memory, a number of groups have developed *in vitro* methods to evaluate the presence of HLA-specific B cells capable of alloantibody production on stimulation. Luque et al. have demonstrated "Flourospot" techniques that permit semi-quantitative, multiplex detection of HLA specific B cells that secrete antibody following B cell stimulation (8). Karahan et al. demonstrated a bulk-culture method stimulating PBMC with TLR8/9 ligands and IL-2 to elicit HLA antibody production and screening culture supernatant using a commercially available SAB Luminex® platform (9). These methods demonstrate the ability to detect HLA-specific humoral memory, likely residing in the Bmem cell compartments; yet the exact *in vivo* frequency and phenotype of DSA B cells, and the characteristics of DSA B-cell antigen receptors (BCRs) remain obscure. A recent study by Heidt and colleagues has moved the field a step forward by using HLA tetramers to sort single HLA-specific B cells to allow more accurate characterization of their frequency and BCR structural analysis (10).

Here we present new tools developed to aid in the identification and characterization of B cells sensitized by allo-MHC exposures and to advance research in human and animal transplantation models. In our previous studies with influenza-vaccinated subjects, a single B-cell culture method was established to profile hemagglutinin specific Bmem from PBMC samples (11). This culture system supports the proliferation and differentiation of single naïve and Bmem cells and secretion of clonal antibody into culture supernatants. The amount of antibody secreted is sufficient so that antibody specificities are readily determined with the culture supernatants, while the V(D)J DNA rearrangements encoding BCRs can be recovered from expanded B-cell clones in culture wells for sequencing and re-expression. For antibody screening of the culture supernatants, we developed a cell-based immunoassay platform for the detection of antibody binding to cell-surface expressed antigens in a multiplex way (submitted). In this study, we further adapted this cellular platform and established an economic, versatile, and multiplex reporter cell assay for the detection of antibodies specific for MHC molecules from different mammalian species. Coupled with the established single Bmem cell culture system, we identified and characterized an anti-HLA B memory clone from one allo-sensitized patient.

MATERIALS AND METHODS

Mice, Rhesus Macaques and Human Subjects

Female C57BL/6 mice were obtained from the Jackson Laboratory and maintained under specific-pathogen-free conditions at the Duke University Animal Care Facility. Splenocytes were isolated from one 12-week-old mouse for RNA extraction. All experiments involving animals were approved by the Duke University Institutional Animal Care and Use Committee (IACUC A128-20-06).

Serum samples from rhesus macaques were obtained from animals sensitized by serial skin transplantation as part of an established transplant model (12). Naïve juvenile male rhesus macaques received two swapping skin transplants from their maximally Mamu mismatched donor, at eight-week intervals. Day 0 samples refer to naïve sera, while "Day 54-70 represent 'peak' samples, taken 2 weeks after the second skin transplant (IACUC A153-18-06).

Peripheral blood mononuclear cells (PBMCs) were obtained from one human subject under Duke Institutional Review Board Committee guidelines (IRB Pro00062495) and archived sera from transplant recipients obtained under IRB Pro00104220.

HLA Typing and HLA Antibody Analyses

High-resolution HLA typing was performed by next generation sequencing using the MIA FORA kit (Immucor, Inc., Norcross, GA, USA) on an Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA). HLA specific antibodies were evaluated using HLA single antigen Luminex® beads (SAB) according to manufacturer's instructions and acquired on a LABScreen 200 instrument (One Lambda, Canoga Park, CA). HLA antibody positivity was determined by HLA pattern analysis incorporating serological cross-reactive patterns and HLA matchmaker eplet analysis FUSION version 4.3 (One Lambda, Canoga Park, CA) and HLA Epitope Registry (https://www.epregistry.com.br/). Recombinant antibodies: P3E7 and P3E7-UA were tested at 0.16 mg/ml and P3E7 was also tested at 0.96 mg/ml (~6x) and analyzed as described above. Three-dimensional structures of the

HLA-A molecules were created using Cn3d software (https://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

Original Plasmids and DNA Templates for Gene Cloning

pU6-(BbsI)_CBh-Cas9-T2A-BFP (Addgene plasmid # 64323) was a gift from Ralf Kuehn (13). pLV-EF1a-IRES-Puro (Addgene plasmid # 85132) was a gift from Tobias Meyer (14). pMD2.G (Addgene plasmid # 12259) and psPAX2 (Addgene plasmid # 12260) were gifts from Didier Trono. Human IgG1 expression vector AbVec2.0-IGHG1 (Addgene plasmid # 80795), human IgK expression vector AbVec1.1-IGKC (Addgene plasmid # 80796) and human IgL expression vector AbVec2.1-IGLC2-MscI (Addgene plasmid # 80797) were gifts from Hedda Wardemann (15, 16). The lentiviral transfer vector plasmids pLB-EF1a, pLB-EXIP, pLB-EF1a-IRES-mCD86, pLB-EF1a-EBFP2, pLB-EF1a-mTurquoise2, pLB-EFS-mNeonGreen and pLB-EF1a-mCardinal were generated previously (submitted). Plasmid mNeonGreen-C1 was provided by Allele Biotechnology and Pharmaceuticals Inc. under a license for non-commercial use.

DNA templates coding 14 HLA alleles were synthesized (Gene Universal Inc.) based on amino acid sequences in the IPD-IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/), including HLA-A*01:01, A*02:01, A*03:01, A*24:02, B*07:02, B*15:01, B*27:05, B*35:01, B*44:02, B*57:01, DRA*01:01/DRB1*04:01, DQA1*05:01/DQB1*02:01, DQA1*04:01/DQB1*04:02 and DQA1*05:03/DQB1*03:01. For class II molecules, the coding sequences for α and β chains were linked with coding sequences for a T2A peptide (17), which can induce ribosomal skipping during translation. DNA templates coding following molecules were also synthesized (Gene Universal Inc.) based on amino acid sequences in GenBank database, including mouse IAb (H2-Aa (GenBank Accession NM_010378.3) and H2-Ab1 (NM_207105.3) linked with a T2A peptide), mouse CD74 (NM_010545), human CD74 (NM_001025159.2) and rhesus β2M (NP_001040602.1). Plasmids containing coding sequences for eight class I Mamu alleles were provided by Dave O'Connor (University of Wisconsin-Madison), including Mamu-A1*001:01, A1*002:01, A1*004:01, A1*006:02, A1*023:01, B*001:01, B*012:01 and B*017:01. Coding sequences for mouse H2Kb, β2M and cytoplasmic domain truncated CD86 (amino acid 1-268) were cloned from splenocyte cDNA samples from one C57BL/6 mouse. Coding sequences for human β2M were cloned from PBMC cDNA samples from one healthy donor. Rhesus IgG1 and IgK constant region sequences were cloned from PBMC cDNA samples from a rhesus monkey.

Plasmid Modification and Gene Cloning

Standard molecular cloning procedures were followed for plasmid modification and gene cloning. Endotoxin-free plasmids were prepared (E.Z.N.A.® Endo-free Plasmid DNA Mini Kit II, Omega Bio-tek) for mammalian cell transfection. All plasmids were verified by DNA Sanger sequencing (Duke University DNA Analysis Facility).

CRISPR-Cas9 targeting plasmid pCRISPRpuro was generated by replacing EBFP2 coding sequences in plasmid pU6-(BbsI)_CBh-Cas9-T2A-BFP with puromycin-resistance gene sequences from plasmid pLV-EF1a-IRES-Puro. The empty lentiviral transfer vector plasmids pLB-EXIG and pLB-EXIN were generated by replacing the puromycin-resistance gene sequences in plasmid pLB-EXIP with mNeonGreen coding sequences from plasmid mNeonGreen-C1, or neomycin-resistance gene sequences from plasmid pIRESneo2 (Clontech), respectively. Rhesus IgG1 expression vector pAbVec2.1-RhIgG1 and IgK expression vector pAbVec2.1-RhIgK were generated by replace the human IgL constant region sequences in AbVec2.1-IGLC2-MscI with rhesus IgG1 and IgK constant region sequences, respectively.

Coding sequences for H2Kb alone or H2Kb and mouse β2M linked with a T2A peptide (H2Kb-T2A-mB2M) were cloned into plasmid pLB-EXIG. H2Kb dtSCT cassettes (18) presenting OVA257 peptide (SIINFEKL) and VSV8 peptide (RGYVYQGL) were cloned into plasmid pLB-EXIP. Mouse IAb coding sequences (H2-Aa and H2-Ab1 linked with a T2A peptide) were cloned into plasmid pLB-EF1a. Coding sequences for human CD74, mouse CD74 and a chimeric mouse CD74 (ΔmCD74.Eα52, with the core CLIP peptide KPVSQMRMATPLLMRPM replaced with Eα52 peptide ASFEAQGALANIAVDKA) were cloned into plasmid pLB-EF1a-IRES-mCD86. Coding sequences for class I and class II HLA alleles and class I Mamu alleles were cloned into plasmid pLB-EXIP. Coding sequences for human and rhesus β2M molecules were cloned into plasmid pLB-EXIN.

Culture, Transfection and Transduction of Mammalian Cell Lines

The cell lines used included Raji (ATCC CCL-86), HEK 293T (ATCC CRL-11268), and hybridoma cell lines GAP A3 (ATCC HB-122), W6/32 (ATCC HB-95) and BB7.2 (ATCC HB-82). K530 cell line was generated previously (submitted) from K562 cell line (ATCC CCL-243) by knocking out human *CD32A* gene with standard CRISPR-Cas9 technology. HEK 293T and GAP A3, W6/32 and BB7.2 hybridoma cells were cultured in DMEM medium (Gibco) supplemented with 10% heat-inactivated HyClone FBS (Cytiva), 10 mM HEPES buffer and 55 μM 2-Mercaptoethanol (all Gibco). Raji, K530 and derivative cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% heat-inactivated HyClone FBS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 1× MEM NEAA, 55 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (all Gibco). For all K530 derivative cell lines, unless otherwise indicated, monoclonal cell lines were established by single-cell sorting (see below) and used in binding assays in this study.

Single-guide RNAs (sgRNAs) targeting human *B2M* exon 1 were designed with the online tool (http://crispr.mit.edu). sgRNAs used in this study were sgRNA-hB2M-1 (GGGCCG AGATGTCTCGCTCCG), sgRNA-hB2M-2 (GGAGTAG CGCGAGCACAGCTA) and a negative control sgRNA (TGTCATGCGTCACTTAGTGC). Corresponding DNA oligos were synthesized and cloned into plasmid pCRISPRpuro. K530 cells were transfected with CRISPR-Cas9 targeting plasmids using Lipofectamine 3000 Transfection Reagent (Invitrogen). Twenty-four hours after transfection, puromycin was added (2 μg/ml) for a pulse-selection. Two days later, cells were transferred to

puromycin-free medium and cultured for another seven days before flow cytometry analysis and single-cell sorting (see below).

Lentiviral transfer vector plasmids were co-transfected into HEK 293T cells with packaging plasmids pMD2.G and psPAX2 using Lipofectamine 3000 Transfection Reagent (Invitrogen). Forty-eight hours after transfection, culture supernatants were harvested and filtered through 0.45-μm PVDF membrane filters (Millipore). K530 derivative cell lines were transduced with the filtered supernatants containing lentiviral vectors by spinoculation at 1000× *g* for 45 min at 32°C. For transductions with pLB-EXIP-based vectors, cells were selected with puromycin (Sigma, 2 μg/ml) between 3-7 days after transduction. Seven days after transduction, the cells were harvested for flow cytometry analysis and single-cell sorting (see below).

Antibodies and Staining Reagents

Monoclonal antibodies used in this study included: APC-conjugated anti-human β2M (hβ2M-APC, clone 2M2, BioLegend 316312), HLA-ABC-PE-Cy7 (clone W6/32, BioLegend 311430), HLA-DR/DP/DQ-PE-Cy7 (clone Tü39, BioLegend 361708), hCD74-PE (clone LN2, BioLegend 326808), hβ2M-PE (clone 2M2, BioLegend 316306), H2Kb-AF647 (clone AF6-88.5, BioLegend 116512), H2Kb/H2Db-PE (clone 5041.16.1, ThermoFisher MA5-18000), SIIN-H2Kb-PE (clone 25-D1.16, BioLegend 141604), mCD86-PE (mouse CD86, clone GL1, BioLegend 105007), IAb-PE (clone AF6-120.1, BioLegend 116407), mCD74-AF647 (clone In1/CD74, BioLegend 151003), CLIP-PE (clone cerCLIP.1, Santa Cruz sc-12725-PE), Ea52/IAb-PE (clone YAe, Santa Cruz sc-32247-PE), Anti-mCD86-PE-Vio770 (clone PO3.3, Miltenyi Biotec 130-105-135), mIgG2a-PE (clone RMG2a-62, BioLegend 407108), HLA-DQ-PE (clone HLADQ1, BioLegend 318105), HLA-ABC (clone W6/32, BioLegend 311402), HLA-A2 (clone BB7.2, BioLegend 343302), HLA-A2 (One Lambda 0791HA), HLA-B7 (clone BB7.1, BioLegend 372402), HLA-B27 (clone HLA-ABC-m3, Sigma MAB1285), HLA-DQ/DP/DR (clone Tü39, BioLegend 361702), HLA-DR (clone L243, BioLegend 307602), HLA-DQ (clone Tü169, BioLegend 361502), hCD19-PE-Cy7 (clone HIB19, BioLegend 302216), hCD3-PE-Cy5 (clone UCHT1, BD 555334), hCD14-TriColor (clone TuK4, Invitrogen MHCD1406), hCD16-PE-Cy5 (clone 3G8, BD 555408), hCD24-BV510 (clone ML5, BioLegend 311126), hCD27-BV421 (clone M-T271, BioLegend 356418), hIgM-FITC (clone MHM-88, BioLegend 314506), hIgD-APC-Cy7 (clone IA6-2, BioLegend 348218), hIgG-APC (clone G18-145, BD 550931). Antibody GAP A3 was purified (11) from hybridoma cell line culture supernatants after adaptation into suspension culture in Expi293 Expression Medium (Invitrogen).

Isotype control antibodies included: Mouse IgG1 kappa-PE (clone MOPC-21, BioLegend 400112), Mouse IgG1 kappa-APC (clone MOPC-21, BioLegend 400122), Mouse IgG2a kappa-PE (clone MOPC-173, BioLegend 400212), Mouse IgG2a kappa-PE-Cy7 (clone MOPC-173, BioLegend 400254), Rat IgG2a kappa-PE (clone RTK2758, BioLegend 400508), Rat IgG2b kappa-AF647 (clone RTK4530, BioLegend 400626), Human IgG1 kappa (hIgG1K, Southern Biotech 0151K-01), Human IgG1 lambda (hIgG1L, Southern Biotech 0151L-01), Rhesus Monkey IgG (RhIgG, Southern Biotech 0135-01). Secondary antibodies included: Goat Anti-Human IgG-PE (Southern Biotech 2040-09), Goat Anti-Mouse IgG, Human ads-PE (Southern Biotech 1030-09), Mouse Anti-Monkey IgG-PE (clone SB108a, Southern Biotech 4700-09). Other staining reagents included: 7-AAD (BD 559925), Tetramer-PE (T-Select HLA-A*02:01 HIV gag Tetramer-SLYNTVATL-PE, MBL TB-M027-1).

Cell Surface and Intracellular Staining, Flow Cytometry Analysis and Single-Cell Sorting and Cloning

For cell surface staining, cultures of K530 and derivative cells were harvested, centrifuged at 300× *g* for 2 min at 4°C and resuspended in staining buffer (PBS supplemented with 2% heat-inactivated FBS). After incubation with antibodies at 4°C in the dark for 30 min, cells were washed with staining buffer and resuspended in staining buffer for either secondary staining following the same procedure above or stored on ice for flow cytometry analysis or single-cell sorting. For intracellular staining, Fixation/Permeabilization Solution Kit (BD 554714) was used and manufacturer's instructions were followed.

Flow cytometry analysis was carried out using BD FACSCanto II cytometer (Duke Cancer Institute Flow Cytometry Shared Resource). Single-cell sorting was performed with BD Aria II (The Duke Human Vaccine Institute Research Flow Cytometry Facility). The bulk cell line after transfection or transduction were labeled with corresponding antibodies and single cells expressing FP or antigen of interest were sorted into 96-well flat-bottom plates containing 100 μl/well of the complete RPMI medium above supplemented with 20% heat-inactivated FBS. Nine days after sorting, robustly proliferating cell clones were transferred into 24-well plates for further expansion. Three days later, individual monoclonal cell lines were validated for the expression of FP or antigen of interest and a single clone with a uniform expression level was selected for further engineering or used as a reporter cell line in immunoassays.

Multiplex Reporter Cell Assay

Fluorescence-barcoded reporter cell lines expressing unique MHC antigens were pooled and labeled with reference antibodies, diluted serum samples or single B-cell culture supernatants. After a secondary labeling with PE-conjugated secondary antibody, the binding activities were detected by flow cytometry as described above. To increase the throughput, 96-well V-bottom plates were used for staining and a high-throughput sampler was used during flow cytometer detection.

Single Bmem Cell Sorting and Culture

Human Bmem cells were isolated by flow cytometry and single B-cell cultures were established as described (11). Briefly, frozen-thawed PBMCs were stained with antibody cocktails containing hCD3-PE-Cy5, hCD14-TriColor, hCD16-PE-Cy5, hCD19-PE-Cy7, hCD24-BV510, hCD27-BV421, hIgM-FITC, hIgD-APC-Cy7, hIgG-APC, 7-AAD and HLA-A2 tetramer-PE. Antigen-specific Bmem cells were identified as 7-AAD$^-$ CD3$^-$ CD14$^-$ CD16$^-$ CD19$^+$ CD24^{hi} CD27$^+$ IgM$^-$ IgD$^-$ IgG$^+$ Tetramer$^+$.

Single antigen-specific B-cells were sorted into 96-well culture plates with MS40L^{low} feeder cells (19, 20) and in the presence cytokines, including human IL-2, IL-4, IL-21 and BAFF (all Peprotech). After 25 days' culture, supernatants were harvested for screening the reactivity of clonal IgG Abs. Expanded clonal B cells were frozen for V(D)J sequence analyses. IgG$^+$ culture supernatants were tested for HLA binding activities with HLA-expressing cell lines in a multiplex reporter cell assay.

BCR Rearrangement Amplification and Analysis

Rearranged V(D)J gene sequences for human Bmem cells from single-cell cultures were obtained as described (11). V(D)J rearrangements were identified with IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/) (21). Unmutated ancestor (UA) sequences were inferred with Cloanalyst (22).

Recombinant IgG Expression and Purification

Rhesus IgG1 and human IgG1 versions of W6/32 and human IgG1 version of BB7.2 antibody expression vectors were prepared by cloning the heavy and light chain V(D)J sequences (16) from hybridoma cell line cDNA samples to corresponding expression vectors mentioned above. V(D)J sequences of HLA-specific Bmem cell clone H02P3E7 and the inferred UA (synthesized, Gene Universal Inc.) were cloned into human IgG1 and IgK expression vectors as described (15). Recombinant antibodies were produced and purified as described (11).

Luminex® Assay

Luminex® beads conjugated with anti-human IgG were incubated with serial dilutions of IgG$^+$ culture supernatants or recombinant human IgG1 antibodies. After washing, either PE-conjugated goat anti-human IgG or PE-conjugated HLA-A2 tetramers were added as the detection reagents. The concentrations of bead-bound human IgG1 antibodies were normalized to a commercial isotype control antibody and the antigen binding activities of the bound antibodies were revealed by corresponding MFI values in the tetramer-PE staining group.

RESULTS

Generation of a Versatile Cell Line for Surface Expression of MHC Class I and II Molecules From Different Mammalian Species

We recently developed (submitted) the K530 cell line, a CD32A-deficient derivative of K562 cells, which was subsequently used as the parental cell line for endogenous barcoding by fluorescent proteins (FPs) and cell surface antigen expression. The parental K562 line was derived from a chronic myelogenous leukemia and does not express HLA class I and class II products (23) but is positive for β2M.

Concerned that this endogenous human β2M could interact with transduced MHC class I α chain from non-human species and generate chimeric class I molecules with altered structure and epitopes (24–26), we further modified the K530 cell line by knocking out the human *B2M* gene using a standard CRISPR-Cas9 mediated gene targeting strategy. Briefly, K530 cells were transfected with a CRISPR-Cas9 targeting vector co-expressing Cas9 nuclease and single-guide RNAs towards exon 1 region of human *B2M* gene (**Figure S1A**). After puromycin selection, more than 70% of surviving cells showed decreased intracellular β2M expression comparable to that of K530 cells stained with an isotype control antibody (**Figure S1B**). A monoclonal cell line, K528, was generated by single-cell flow cytometry sorting. The lack of β2M expression in K528 cells was confirmed by intracellular staining (**Figure S1B**), and the inactivation of *B2M* genes validated by genomic DNA sequencing (**Figure S1C**).

The resultant clonal cell line, K528, is negative for HLA class I, class II and β2M but expresses low levels of cytoplasmic CD74 (**Figure 1A**, **Figure S2**). The K528 line can readily be made to express MHC molecules from any mammalian species. To demonstrate this versatility, the mouse class I molecule H2Kb was transduced in either K530 or K528 cells, *i.e.*, in the presence or absence of human β2M, respectively. The β2M-independent H2Kb mAb (clone 5041.16.1), recognizes transduced H2Kb comparably on K530 (human β2M+) cells transduced with H2Kb alone or with co-expressed mouse β2M; on transduced K528 (human β2M–) cells, the 5041.16.1 mAb binds only to cells expressing both H2Kb and mouse β2M (**Figure 1B**). In contrast, the mouse β2M-dependent H2Kb mAb (clone AF6-88.5), recognizes only cells co-transduced with H2Kb and mouse β2M. Significantly, AF6-88.5 mAb binding to co-transduced K530 was significantly reduced by the presence of human β2M, suggesting that the endogenous human β2M efficiently competes with transgenic mouse β2M to generate chimeric class I molecules (compare 5041.16.1 binding to AF6-88.5 binding). Only K528 cells co-expressing mouse β2M supported normal expression of surface H2Kb molecules as determined by the mouse β2M-dependent H2Kb-specific antibody (**Figure 1B**). In the absence of endogenous human β2M, pMHC molecules from other species with fixed peptide can also be expressed by K528 cells as single-chain trimers with disulfide traps (dtSCT) (18) (**Figure 1C**). For a mouse class II molecule IAb, co-expression of mouse CD74 increased the surface expression level dramatically. A chimeric mouse CD74 molecule with the core CLIP peptide replaced with an exogenous antigenic peptide drove the presentation of corresponding peptide by IAb (27), suggesting that the natural class II molecular processing and antigen presentation pathways can be simulated in these cells (**Figure 1D**). As for human class II molecules, co-expression of human CD74 results in much higher surface expression of human DQ molecules (**Figure 1E**). We conclude that K528 is a versatile parental cell line for surface expression of MHC class I and II molecules from different mammalian species.

Multiplex Detection of Anti-HLA Antibodies in Serum Samples From Allo-Sensitized Patients

Using the newly generated K528 cell line, a basic panel of 16 fluorescence-barcoded sub-lines were generated in the same way

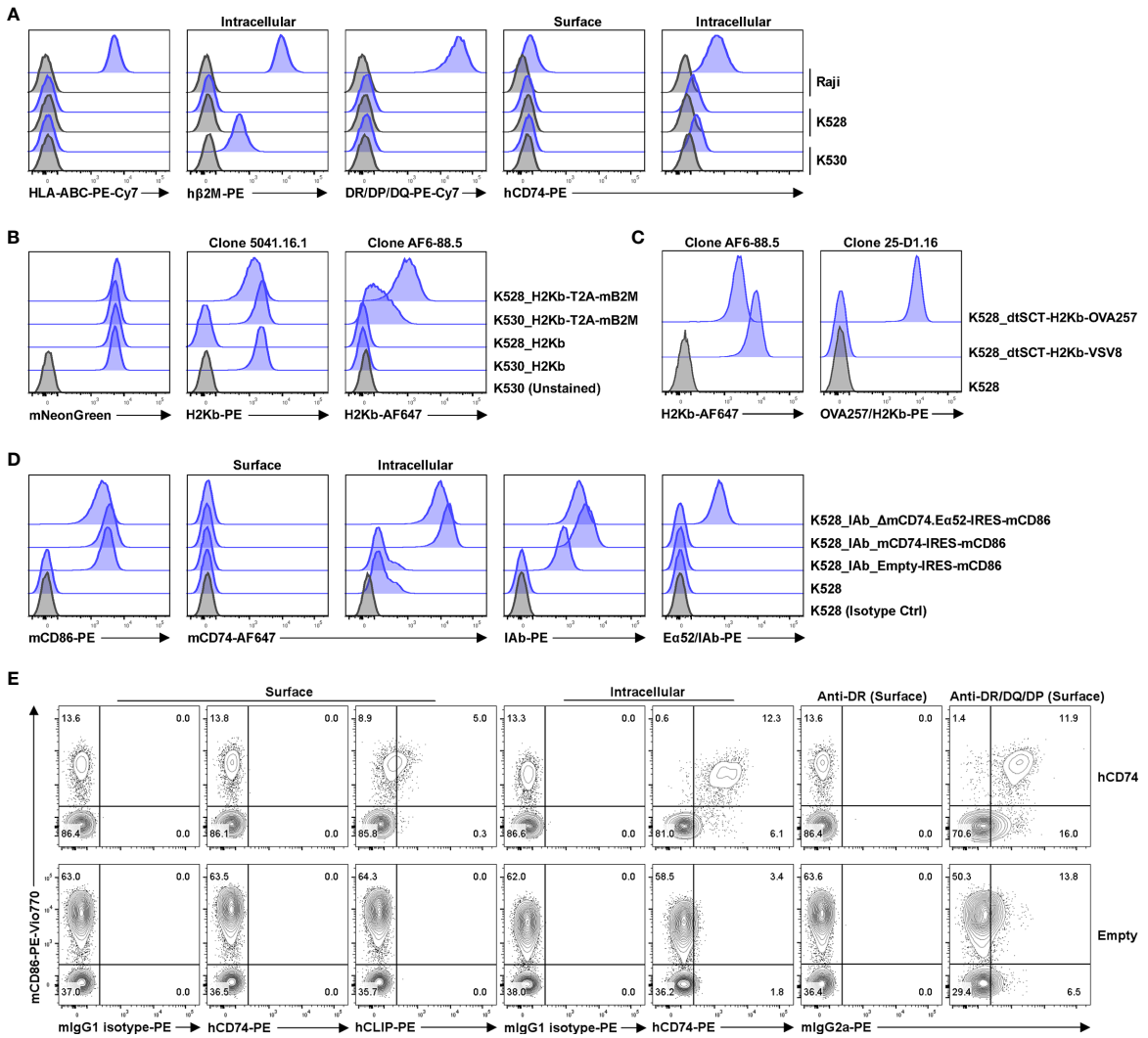


FIGURE 1 | K528 is a versatile cell line for transgenic expression of MHC class I and II molecules from different mammalian species. **(A)** Phenotypic analysis of K528 cell line. K530 and Raji cells were used as controls in straining. The gating strategy is shown in **Figure S2**; similar gating strategies were applied to other panels below. Grey histograms show staining with isotype control antibodies. **(B)** K528 and K530 cells were transduced to express H2Kb with or without co-expressed mouse β2M molecules. The expression vectors co-express mNeonGreen as a selection marker. Monoclonal cell lines with comparable mNeonGreen fluorescence intensities were selected for comparison. Cell surface expression of H2Kb molecules were detected with either mouse β2M-independent (clone 5041.16.1) or -dependent (clone AF6-88.5) antibodies. **(C)** K528 cells were transduced to express single-chain trimer with disulfide trap (dtSCT) structures of H2Kb molecules with either VSV8 or OVA257 peptides. Surface expression of H2Kb and H2Kb molecules presenting OVA257 peptides were detected with relevant antibodies. **(D)** K528 cells were transduced to express IAb molecules. The resultant monoclonal cell line was further transduced with lentiviral vectors with expression cassettes Empty-IRES-mCD86 (K528-IAb), mCD74-IRES-mCD86 (K528-IAb-mCD74) or ΔmCD74.Eα52-IRES-mCD86 (K528-IAb-ΔmCD74.Eα52) in which the core CLIP sequences were replaced with IAb presenting peptide Eα52 from IEd molecule. Monoclonal cell lines expressing comparable levels of mCD86 were selected for analysis. Surface and intracellular mCD74, surface IAb and Eα52/IAb complex were detected by FACS. **(E)** K528 cells expressing HLA-DQA1*05:01/DQB1*02:01 were transduced with lentiviral vectors with expression cassettes hCD74-IRES-mCD86 (hCD74) or Empty-IRES-mCD86 (Empty). Bulk cell lines after transduction were used for detection by FACS.

as for K530 cell line (submitted). Briefly, K528 cells were transduced to express different combinations of four FPs which can be discriminated from each other by flow cytometry, resulting in 16 sub-lines with unique fluorescence barcodes. To establish a general tool for anti-HLA antibody screening, we designed a basic panel consisting of ten HLA class I and four class II molecules which are highly prevalent in our patient population. Class I genes were transduced into barcoded cell lines along with human β2M whereas class II genes were incorporated along with human CD74. The resultant HLA-expressing reporter-cell panel was subsequently validated for specificity using a panel of HLA-specific monoclonal antibodies. All 14 HLA-expression cell lines were pooled along with two internal control cell lines without HLA transgene but express all

or none of the barcoding FPs, respectively. The pooled reporter cells were then labeled with HLA-specific reference antibodies individually followed by a PE-conjugated secondary antibody. The labeled and washed cells were then examined for FP and PE signals by flow cytometry. Individual reporter cell lines were demultiplexed by gating for specific combinations of FP expression (**Figure S3**). Binding activity by each HLA reference antibody is proportional to the median fluorescence intensity (MFI) for each reporter cell line in the PE detection channel. In consequence, the specificity of each reference HLA antibody can be determined within the reporter cell line panel used for screening. In all cases, the binding patterns of each reference antibody matched its published specificity (**Figure 2A**).

This reporter cell panel permitted detection of anti-HLA antibodies present in serum samples from allo-sensitized patients. Ten blinded, serum samples were tested (**Figure 2B**, **Figure S4**) and the FACS MFI readouts compared to corresponding Luminex® readouts from a clinical HLA SAB assay (**Figure 2C**). No HLA antibodies detected by the reporter cell assay were absent in the clinical SAB assay, confirming the high specificity of the reporter cell assay. In contrast, for 59 true positive (TP) readouts in the SAB assay, only 33 were detected in the reporter cell assay. Most (22/26, 85%) of the discrepancies correlated with lower bead MFI values when compared to HLA alleles that tested positive with the reporter cell assay. These discrepant results may reflect lower sensitivity of the reporter cell assay due to the lower density of HLA antigen on the cell surface compared to beads. This lower density would result in lower signals, especially when the relevant antibody is at low concentration in the serum (28). It is also possible that these "false negatives" in the reporter cell assay reflect spurious antibody binding in the SAB assay due to the presence of denatured HLA antigens (29–31). However, for this study and in clinical practice, this type of spurious background binding is mitigated by the use HLA pattern analysis to determine TP SAB reactions. We conclude that the multiplex reporter cell assay likely exhibits lower sensitivity than the clinical SAB assay. If this is the case, its application should be limited to conditions when the concentration of relevant antibody is not limiting, *e.g.*, with monoclonal antibodies or single B-cell culture supernatants. In the case of single B-cell cultures, the clonal antibodies in most (≥ 90%) culture supernatants reach concentrations above 1 μg/ml (11). Under these conditions, the reporter cell assay is an economic and effective alternative to clinical SAB assay in high-throughput screening.

Multiplex Detection of Mamu Antibodies in Serum Samples From Sensitized Rhesus Macaques

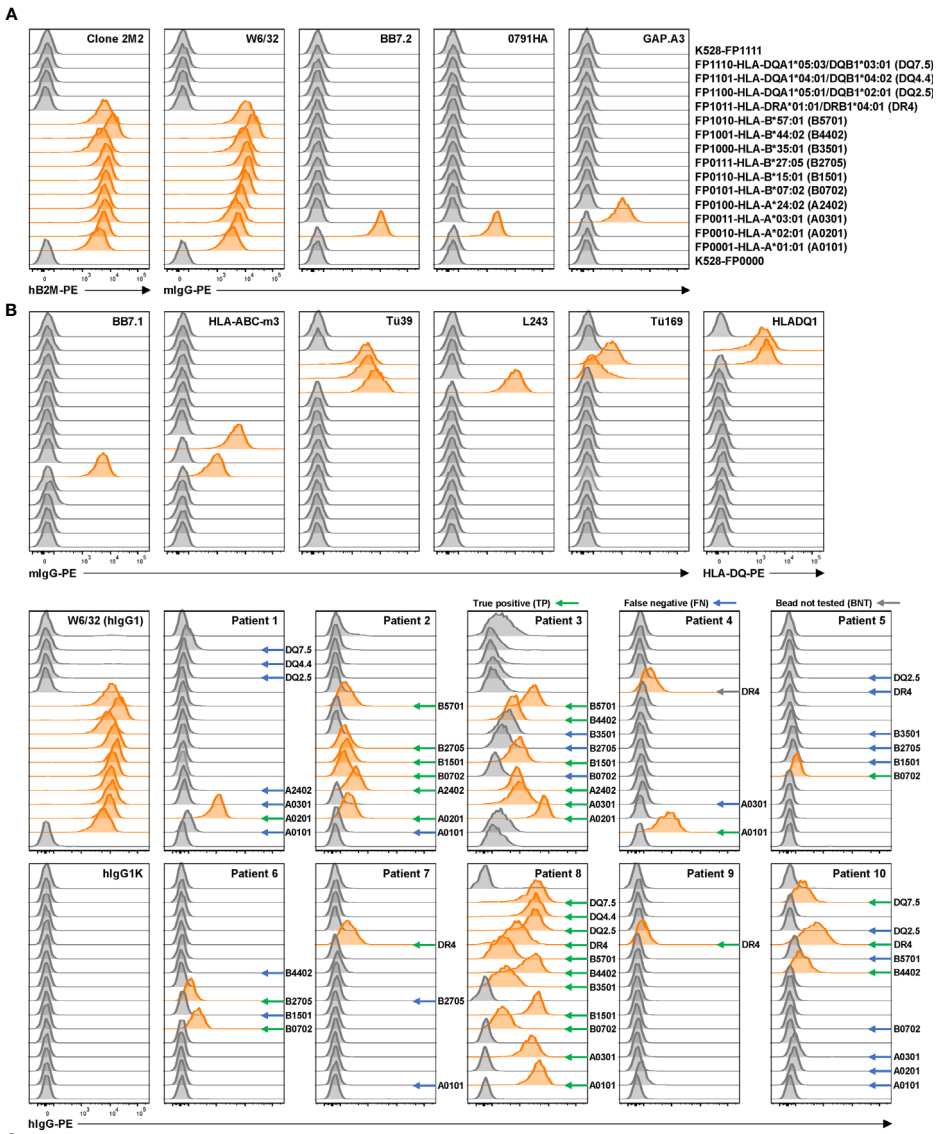
Rhesus macaques (RMs) are a valuable animal model in many pre-clinical studies, including transplantation. However, specific reagents for studies with RMs are limited. To our knowledge, only one allele-specific Mamu antibody has been reported (32) and there is no single Mamu antigen bead panel available commercially. The multiplex reporter cell platform we established for HLA is readily adaptable to Mamu antigen expression. To demonstrate this potential, a panel of class I Mamu molecules and rhesus β2M were expressed in FP-barcoded reporter cell lines by transduction. Robust surface expression of Mamu proteins was confirmed using the cross-reactive antibody, W6/32 (**Figure 3**). To validate the specificity and utility of this reporter cell panel, we tested 15 blinded serum samples from allo-sensitized RMs following two sequential skin transplants (12) for the presence of allo-Mamu antibodies. We predicted the elicited antibody reactivities based on donor Mamu haplotypes that were mismatched with recipient haplotypes, and compared these to the binding activities observed in the reporter cell assay. As shown in **Figure 3** and **Table 1**, except for a single case in RM H73E, we detected binding activities for all the predicted antibody responses (23/24, 96%). This single negative result is either a false negative due, perhaps to the relative low density of Mamu proteins expressed by the reporter cells, or to the non-responsiveness of the particular allo-antigen in this host RM.

On the other hand, 12 binding signals beyond the predicted reactivities were detected. These positive readouts could be false positive results due to expression artifacts in the reporter cells, or – and we believe more likely - the result of cross-reactive epitopes shared between Mamu allo-antigens. Unfortunately, validated reagents to rule out either of these two possibilities do not exist; however, in view of the high specificity of the HLA reporter cell assay above (**Figures 2A, B**), and considering the well documented cross-reactive epitopes shared between HLA molecules (**Figure 2C**), we believe that these positive readouts represent true cross-reactivities in allo-sensitized RMs. The potential identification of novel, cross-reactive Mamu epitopes, highlights the utility of this multiplex reporter cell assay in experimental models where multiplex bead reagents are unavailable or difficult to generate.

Of note, serum samples from RM HADV at Day 0 and from H73E at Days 0 and 70 exhibited high background staining of all reporter cell lines including the internal control lines bearing no MHC molecules; with dilution this polyreactivity became negligible (compare serum dilutions of 1/50 to 1/500). Our records show no significant differences in pre-sampling treatments for these animals and we were able to exclude technical issues in the binding assay. Consequently, we consider this serum polyreactivity to reflect intrinsic properties of the animals or samples. Possible explanations include 1) presence of polyreactive, presumably low affinity, serum IgG, 2) serum IgG antibody specific for an unknown antigen(s) present on the parental K528 cells, and 3) serum components, e.g., complement-decorated antibody/antigen complexes, which mediate non-specific binding of antibodies to the reporter cells.

Evidence for Affinity Maturation in an HLA-Specific Bmem Cell From a Sensitized Patient

We established an antigen-specific single-cell culture system recently (11, 20, 33, 34), which supports robust proliferation of single Bmem cells (to ≈90,000 daughter cells by day 25) followed by plasmacytic differentiation and IgG production, yielding an



												TP=33	FN=26	BNT=1	
	NC	A0101	A0201	A0301	A2402	B0702	B1501	B2705	B3501	B4402	B5701	DR4	DQ2.5	DQ4.4	DQ7.5
Patient 1	244	5338	23242	3676	3990	113	106	115	41	363	306	612	11307	6352	16382
Patient 2	41	1316	8111	0	9849	5904	2035	1840	0	0	8060	311	434	0	0
Patient 3	569	0	22936	17512	17867	2749	5921	5215	9650	9884	23089	0	0	0	14
Patient 4	49	9530	0	2175	192	0	0	210	196	0	448	NT	NT	NT	NT
Patient 5	48	0	30	156	14	9556	2828	4334	1887	323	0	1342	6814	257	836
Patient 6	39	0	0	0	0	15982	1399	10884	103	2443	0	NT	NT	NT	NT
Patient 7	78	2398	37	122	199	393	0	1384	689	8	0	18160	174	0	0
Patient 8	326	23251	0	22179	0	12367	19975	0	11176	8710	11297	20778	22005	22337	23157
Patient 9	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	5432	0	0	593
Patient 10	41	2475	4306	4626	0	946	0	63	0	3260	3810	13047	1155	276	13489

FIGURE 2 | Application of reporter cell line assay in multiplex detection of anti-HLA antibodies in serum samples from allo-sensitized patients. **(A)** K528-derived fluorescence-barcoded reporter cell lines were transduced to express HLA class I molecules along with human β2M or class II molecules along with human CD74. **(A)** The resultant cell lines were pooled, and the surface expression of HLA molecules on individual cell lines were validated with a panel of reference monoclonal antibodies. Example gating strategy is shown in **Figure S3**. Demultiplexed reporter cell lines with MFI values above three-fold of the average MFI value of internal control cell lines (K528-FP0000 and K528-FP1111) were scored as positive and the histograms were highlighted in orange. **(B)** Multiplex detection of anti-HLA antibodies with 10 serum samples from allo-sensitized patients. W6/32 (hIgG1) was used as positive control, and hIgG1κ and hIgG1λ (**Figure S4**) as negative controls in staining. Positive readouts that are also positive in clinical HLA SAB readouts were considered as true positive (TP) and indicated with green arrows. Negative readouts that are positive in bead readouts were considered as false negative (FN) and indicated with blue arrows. Positive readouts that were bead not tested (BNT) in SAB assay are indicated with gray arrows. MFI values are listed in **Table S1**. **(C)** Clinical HLA SAB MFI readouts. The color codes are in accordance with those in B), with TPs in both assays highlighted in green, and TPs in SAB assay while FNs in reporter cell assay in blue. HLA alleles are listed in abbreviations as indicated in parentheses in **(A)**. For class II molecule lines, bulk cell line after antibiotic selection were used.

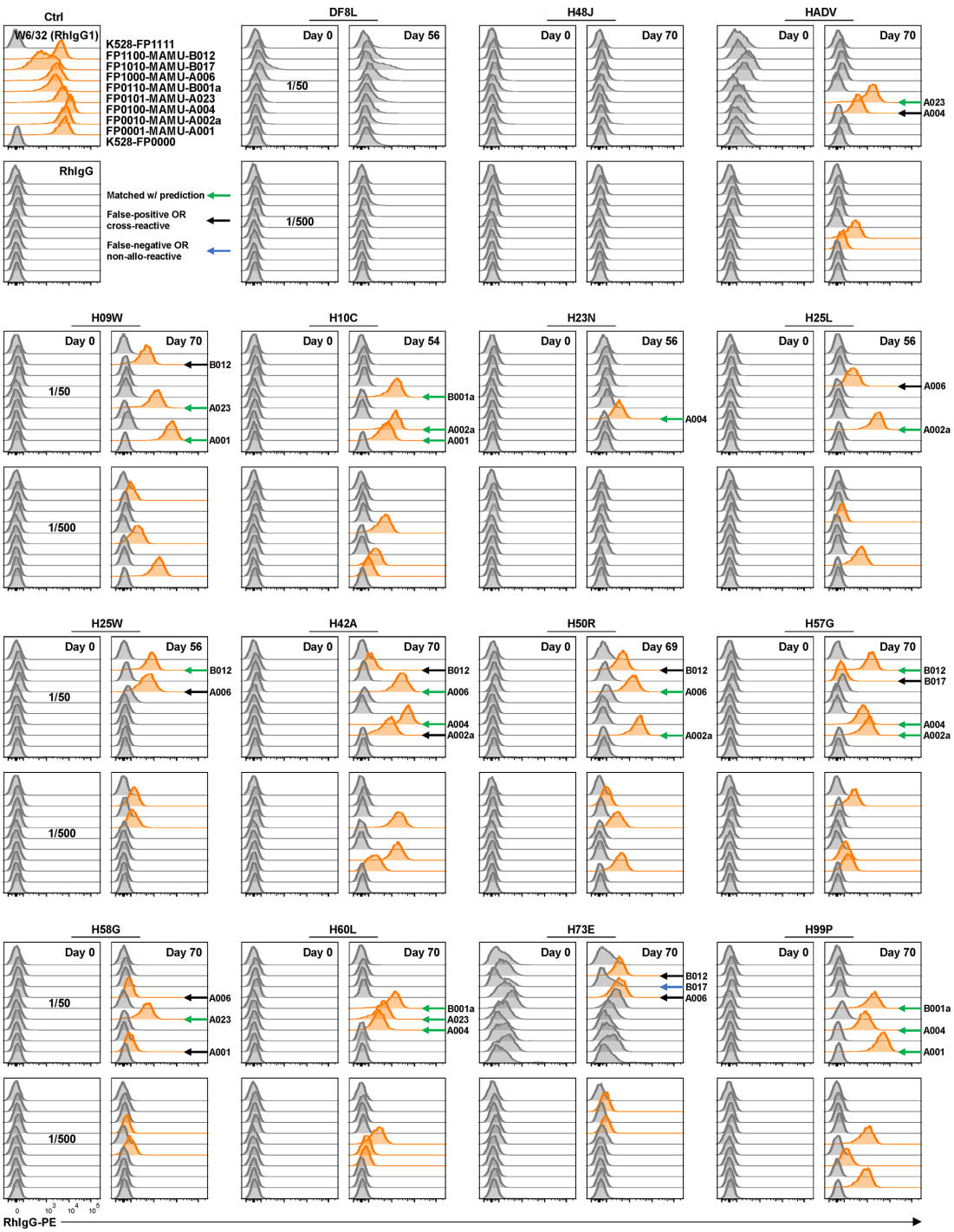


FIGURE 3 | Application of reporter cell line assay in multiplex detection of anti-Mamu antibodies in serum samples from allo-sensitized RMs. K528-derived fluorescence-barcoded reporter cell lines were transduced to express Mamu class I molecules along with rhesus β2M. The resultant monoclonal cell lines were pooled, stained with sera from 15 allo-sensitized RMs at dilutions 1/50 and 1/500. W6/32 (RhIgG1) was used as positive control, and normal rhesus serum IgG (RhIgG) as negative control in staining. Demultiplexed reporter cell lines with MFI values above three-fold of the average MFI value of internal control cell lines (K528-FP0000 and K528-FP1111) were scored as positive and the histograms were highlighted in orange. The specificities were predicted based on donor Mamu haplotypes that mismatch with host ones. Positive readouts matched with prediction were indicated with green arrows. Positive readouts beyond prediction were either false positive detections or due to cross-reactivity and were indicated with black arrows. Predicted but undetected specificities were either false negative detections or due to non-allo-reactivity of the donor Mamu molecules in the corresponding host, and were indicated with blue arrows. B012 stands for B012a or B012b.

TABLE 1 | Mamu haplotypes of recipient and donor RMs and reporter cell line detection results.

RM ID	Recipient haplotype	Donor haplotype	Matched with prediction	False positive OR cross-reactive	False negative OR non-allo-reactive
DF8L	A004/A025/B012b/B017a	A007/A701b/B068/B085	—	—	—
H48J	A004/A023/B002/B043a	A019/A019/B015c/B015c	—	—	—
HADV	A025/A002a/B012b/B001a	A019/**A023**/B015c/B043a	A023	A004	—
H09W	A006/A004/B024a/B015a	**A001**/**A023**/B055/B043a	A001, A023	B012	—
H10C	A004/A025/B012b/B048	**A002a**/**A001**/B012a/**B001a**	A001, A002a, B001a	—	—
H23N	A016/A025/B001a/B012b	**A004**/A019/B056b/B015c	A004	—	—
H25L	A004/A008/B012b/B069b	A018a/**A002a**/B002/B055	A002a	A006	—
H25W	A004/A002a/B028/B055	A008/A008/B048/**B012b**	B012	A006	—
H42A	A001/A023/B055/B043a	**A006**/**A004**/B024a/B015a	A004, A006	A002a, B012	—
H50R	A004/A001/B048/B047a	**A002a**/**A006**/B015a/B055	A002a, A006	B012	—
H57G	A023/A074/B043a/B001a	**A004**/**A002a**/B001a/**B012a**	A002a, A004, B012	A006	—
H58G	A004/A002a/B001a/B012a	**A023**/A074/B043a/B001a	A023	A001, A006	—
H60L	A002a/A105/B012a/B002	**A004**/**A023**/**B001a**/B043a	A004, A023, B001a	—	—
H73E	A001/A004/B001a/B069a	A008/A025/B015b/**B017a**	—	A006, B012	B017
H99P	A002a/A002a/B015a/B012a	**A001**/**A004**/**B001a**/B012b	A001, A004, B001a	—	—

Donor haplotypes mismatching with recipient haplotypes, which had a matched reporter cell lines in the detection panel and would therefore be considered as a predicted positive readout, are highlighted in bold. B012 stands for B012a or B012b.

average 50 μg/ml of clonal antibody in culture supernatants. This robust production of clonal IgG enables high-throughput screening for MHC-specific clones using the reporter cell assay described in this report. With this culture system, the V(D)J rearrangements encoding MHC-specific BCRs can be readily recovered by PCR amplification, sequenced, and expressed as recombinant antibodies (rAbs) for further characterization.

We employed this single-cell culture method to isolate and characterize HLA-specific Bmem cells and to generate HLA rAbs from an allo-sensitized patient. The patient was sensitized by three pregnancies and a history of transfusion. Serological HLA-A*02:01 reactivity was detected by SAB as the immunodominant or strongest antibody as measured by mean fluorescent intensity (MFI). PBMCs from this patient were labeled with A*02:01 tetramer along with surface markers ($CD19^+$ $CD27^+$ $CD24^{hi}$ IgD^- IgM^- IgG^+) to identify Bmem cells. $Tetramer^+$ IgG^+ Bmem cells (**Figure 4A**) were sorted into single B-cell cultures as described (11). After 25 days of culture, a cloning efficiency of 40% (270/672) was determined by screening for secreted IgG in culture supernatants. IgG^+ supernatants were then tested for HLA binding activity using the HLA reporter cell assay. One clone, H02P3E7, exhibited avid binding to A*02:01 but not to A*01:01 (**Figure 4B**).

We sequenced the V(D)J rearrangements present in this HLA-A*02:01 specific clone by amplifying the human IgG heavy- and light-chain genes from the expanded, clonal B-cell population in the corresponding culture (15, 35). The recovered and sequenced BCR genes carried mutations (>1%) in both the VH and VL gene segments (**Figure 4C**), consistent with this IgG^+ Bmem cell clone being derived from a germinal center (GC) response. A recombinant antibody (rAb), P3E7, was generated from the V(D)J sequences present in the cloned Bmem cell H02P3E7 and a second, P3E7-UA, from the inferred, germline V(D)J sequences expressed by the unmutated ancestor [UA, (36)] of the H02P3E7 Bmem cell. The binding of both rAbs to A*02:01 was confirmed in a Luminex® assay using A*02:01 tetramer as the antigen target (**Figure 4D**). The binding avidity of rAb P3E7 was comparable to that of BB7.2, a hybridoma derived A*02:01-specific antibody, and has a calculated Kd value of 68.5 pM. Interestingly, the binding activity of the rAb P3E7-UA to A*02:01 was decreased by 4-logs compared to the mutated rAb P3E7. In a reporter cell assay (**Figure 4E**), we confirmed that both rAbs can bind to cell-surface expressed A*02:01 antigens, with P3E7 showing much more avid binding activity than P3E7-UA does. Moreover, P3E7 also shows binding activity towards B*57:01, suggesting that this antibody binds to a shared epitope in these two alleles. This observation supports the notion that in the sensitized patient, this Bmem cell resulted from HLA-specific affinity maturation in a GC prior to its entry into the Bmem compartment.

In addition, we determined the HLA allele binding patterns of rAbs P3E7 and P3E7-UA using a clinically validated HLA class I 100-plex SAB panel. The threshold for positivity was determined by examining MFI values for the negative controls and the point at which the HLA allele binding pattern was interrupted by non-related HLA-B and -C alleles. Tests of the unmutated P3E7-UA rAb displayed strong reactivity for beads bearing HLA-A*02 and B*57 alleles (23,679 - 19,171 MFI), lower binding to B*15:16 (B63) and B*58:01 (15,685 - 9,377 MFI), and the lowest binding to A*43:01 and A*29 alleles (3,815-1,866 MFI) (**Figure 5A**). Using the HLA Epitope Registry (https://www.epregistry.com.br/), we identified a common HLA amino-acid motif or "eplet" shared across the HLA alleles to which the rAbs bound. The 10 bound HLA alleles share homology within a potential 15 angstrom conformational epitope surrounding an arginine residue at amino acid position 65 defined by HLA eplet 65RA (**Figures 5B, E**). As expected, stronger reactivity was observed for the mutated P3E7 rAb compared to the unmutated P3E7-UA (**Figures 5A, D**) along with an extended binding pattern to include 13 additional HLA alleles also containing HLA eplet 65RA.

Bordering amino acid differences influence antibody binding to HLA conformational epitopes; binding variability, however, may also reflect the quality and density of HLA molecules on individual

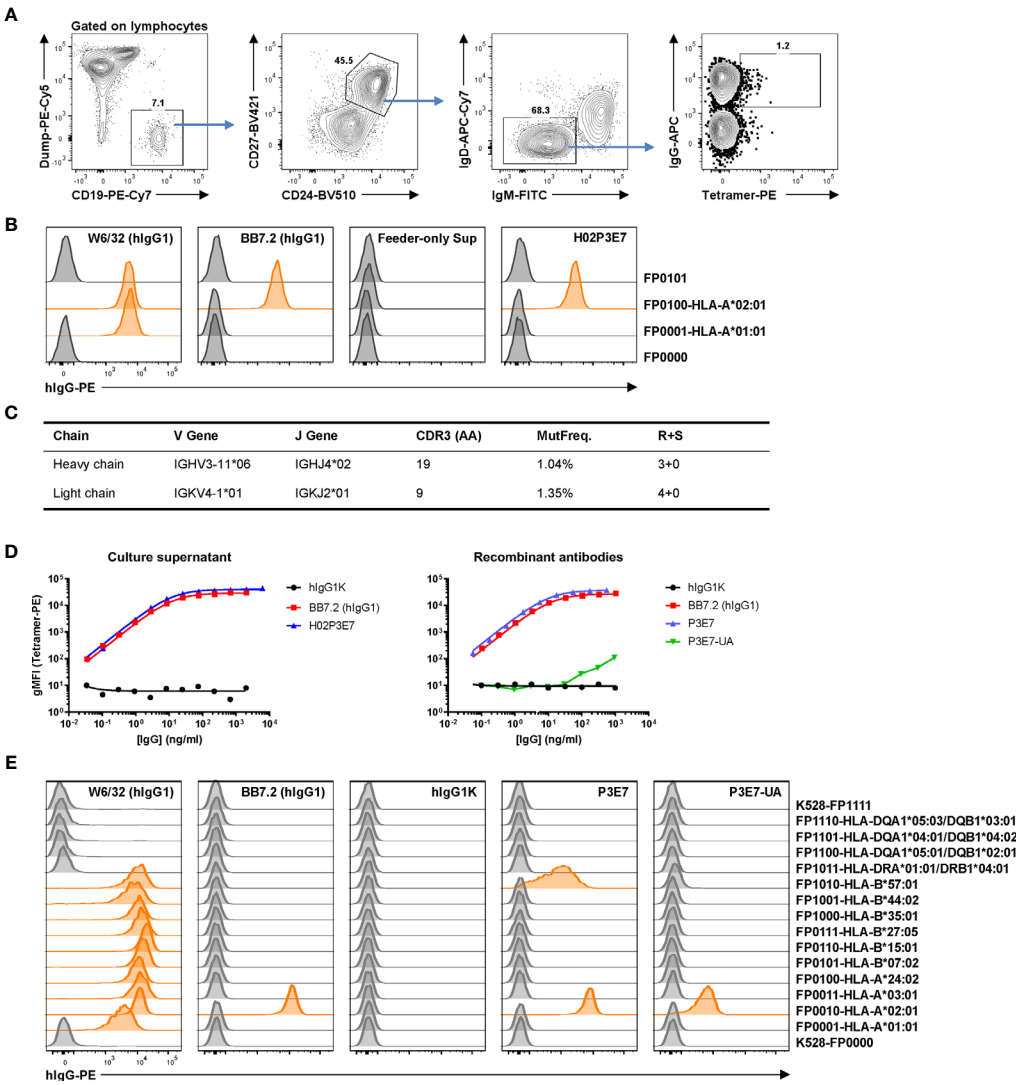
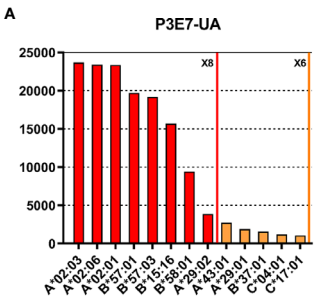
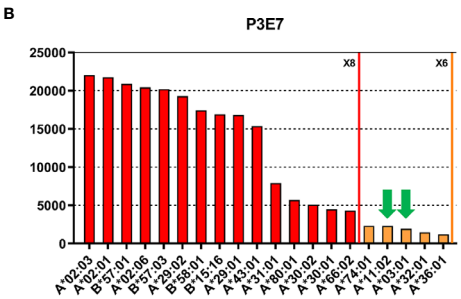


FIGURE 4 | Identification and characterization of an anti-allo HLA Bmem cell clone from a sensitized patient. **(A)** Gating strategy for antigen-specific single B-cell sorting. Frozen-thawed PBMCs from an allo-sensitized patient was stained with HLA-A*02:01 tetramer and surface markers to identify tetramer-binding IgG$^+$ Bmem cells for single B-cell culture. Dump, CD3/CD14/CD16/7-AAD. After 25 days culture, IgG$^+$ supernatants were subjected to a reporter cell assay to screen for HLA-binding clones. **(B)** Example data of reporter cell assay with positive control antibodies, a negative control (feeder-only) supernatant and supernatant from the single HLA-A*02:01-binding clone H02P3E7 identified. In this experiment, two HLA-expressing and two control reporter cell lines were used. W6/32, pan class I HLA specific; BB7.2, HLA-A*02-specific. **(C)** Genetics of the BCR of the identified clone H02P3E7. MutFreq, nucleotide mutation frequency of V genes. R+S, replacement and silent mutations. **(D)** Binding activities of the culture supernatant (left panel) from Bmem clone H02P3E7 and rAbs (right panel) from identified clone (P3E7) and inferred germline clone (P3E7-UA) to HLA-A*02:01 tetramer antigen. hIgG1K, human IgG1 kappa isotype control antibody. **(E)** Reporter cell assay with control antibodies and rAbs P3E7 and P3E7-UA. Demultiplexed reporter cell lines with MFI values above three-fold of the average MFI value of internal control cell lines (K528-FP0000 and K528-FP1111) were scored as positive and the histograms were highlighted in orange.

Luminex® beads. Simultaneous comparison of P3E7 and P3E7-UA binding at identical concentrations allowed us to control for bead differences and examine how BCR mutation affected BCR affinity and specificity. Our analysis revealed that affinity maturation of P3E7 increased binding to non-HLA-A*02 alleles with variable residues at position 45 and 62 increasing MFI values (>15,000) for HLA-B*58, A*29, B*15:16 and A*43 from that observed for the progenitor P3E7-UA antibody (**Figure 5B**). This expanded reactivity also allowed binding to additional HLA alleles bearing the 65RA eplet but with substantial variability about this conformational epitope. Interestingly, in the course of its somatic evolution, the H02P3E7 Bmem cell acquired the capacity to recognize HLA alleles (A*11:01 and A*03:01) present within the patient; this auto-reactivity was not present in the germline P3E7-UA rAb. When P3E7 was tested at a higher concentration (6x) the same reactivity pattern was maintained and extended to include A*68, A*69, A*01, and A*33:01 and correspondingly higher (> 6,000) MFI values were observed for the autologous HLA





C

P3E7-UA

Polymorphic residues within conformational epitope surrounding Eplet 65RA

Allele	Ag	43	44	45	56	62	65	66	71	73	76	77	163	166	167
A*02:03	A2	Q	R	M	G	G	R	K	S	T	V	D	T	E	W
A*02:06	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A*02:01	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*57:01	B57	P	–	–	–	–	–	N	A	–	E	N	L	–	–
B*57:03	B57	P	–	–	–	–	–	N	A	–	E	N	L	–	–
B*15:16	B63	P	–	–	–	R	–	N	A	–	E	N	L	–	–
B*58:01	B58	P	–	T	–	–	–	N	A	–	E	N	L	–	–
A*29:02	A29	–	–	–	–	L	–	N	–	–	A	N	–	–	–
A*43:01	A43	–	–	–	–	L	–	N	–	–	A	N	R	–	–
A*29:01	A29	–	–	–	–	L	–	N	–	–	A	N	–	–	–
R65 containing alleles below X6 cutoff line															
A*66:02	A66	–	–	–	–	R	–	N	–	–	–	–	E	–	–
A*31:01	A31	–	–	–	R	–	–	N	–	I	–	–	–	–	–
A*33:01	A33	–	–	–	–	R	–	N	–	I	–	–	–	–	–
A*32:01	A32	–	–	–	–	–	–	N	–	–	–	S	–	–	–
A*30:01	A30	–	–	–	R	–	–	N	–	–	–	–	–	–	–
A*26:01	A26	–	–	–	–	R	–	N	–	–	A	N	R	–	–
A*69:01	A69	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*01:01	A1	–	K	–	–	Q	–	N	–	I	–	N	R	D	G
A*34:02	A34	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*74:01	A74	–	–	–	–	Q	–	N	–	–	–	–	–	–	–
A*68:02	A68	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*30:02	A30	–	–	–	R	–	–	N	–	–	E	N	–	–	–
A*80:01	A80	–	–	–	E	E	–	N	–	–	A	N	E	D	G
A*11:02	A11	–	–	–	–	Q	–	N	–	–	–	–	R	–	–
A*25:01	A25	–	–	–	–	R	–	N	–	–	E	S	R	–	–
A*66:01	A66	–	–	–	–	R	–	N	–	–	–	–	R	–	–
A*36:01	A36	–	K	–	–	Q	–	N	–	–	A	N	–	–	–
A*03:01	A3	–	–	–	–	Q	–	N	–	–	–	–	–	–	–
A*33:03	A33	–	–	–	–	R	–	N	–	I	–	–	–	–	–
A*68:01	A68	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*11:01	A11	–	–	–	–	Q	–	N	–	–	–	–	R	–	–
A*34:01	A34	–	–	–	–	R	–	–	–	–	–	–	–	–	–

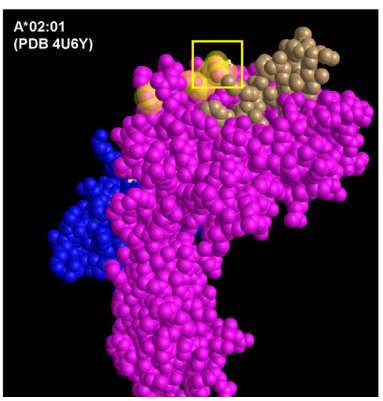
D

P3E7

Polymorphic residues within conformational epitope surrounding Eplet 65RA

Allele	Ag	43	44	45	56	62	65	66	71	73	76	77	163	166	167
A*02:03	A2	Q	R	M	G	G	R	K	S	T	V	D	T	E	W
A*02:01	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*57:01	B57	P	–	–	–	–	–	–	–	–	N	A	–	E	N	L	–	–
A*02:06	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*57:03	B57	P	–	–	–	–	–	N	A	–	E	N	L	–	–
A*29:02	A29	–	–	–	–	–	–	L	–	N	–	–	A	N	–	–	–
B*58:01	B58	P	–	T	–	–	–	N	A	–	E	N	L	–	–
B*15:16	B63	P	–	–	–	R	–	N	A	–	E	N	L	–	–
A*29:01	A29	–	–	–	–	–	–	L	–	N	–	–	A	N	–	–	–
A*43:01	A43	–	–	–	–	L	–	N	–	–	A	N	R	–	–
A*31:01	A31	–	–	–	R	Q	–	N	–	I	–	–	–	–	–
A*80:01	A80	–	–	–	E	E	–	N	–	–	A	N	E	D	G
A*30:02	A30	–	–	–	R	Q	–	N	–	–	E	N	–	–	–
A*30:01	A30	–	–	–	R	Q	–	N	–	–	–	–	–	–	–
A*66:02	A66	–	–	–	–	R	–	N	–	–	–	–	E	–	–
A*74:01	A74	–	–	–	–	Q	–	N	–	–	–	–	–	–	–
A*11:02	A11	–	–	–	–	Q	–	N	–	–	–	–	R	–	–
A*03:01	A3	–	–	–	–	Q	–	N	–	–	–	–	–	–	–
A*32:01	A32	–	–	–	–	G	–	N	–	–	–	S	–	–	–
A*36:01	A36	–	K	–	–	Q	–	N	–	–	A	N	–	–	–
R65 containing alleles below X6 cutoff line															
A*34:02	A34	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*11:01	A11	–	–	–	–	Q	–	N	–	–	–	–	R	–	–
A*33:03	A33	–	–	–	–	R	–	N	–	I	–	–	–	–	–
A*68:01	A68	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*69:01	A69	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*33:01	A33	–	–	–	–	R	–	N	–	I	–	–	–	–	–
A*68:02	A68	–	–	–	–	R	–	N	–	–	–	–	–	–	–
A*66:01	A66	–	–	–	–	R	–	N	–	–	–	–	R	–	–
A*01:01	A1	–	K	–	–	Q	–	N	–	–	I	–	N	R	D	G
A*25:01	A25	–	–	–	–	R	–	N	–	–	E	S	R	–	–
A*26:01	A26	–	–	–	–	R	–	N	–	–	A	N	R	–	–
A*34:01	A34	–	–	–	–	R	–	–	–	–	–	–	–	–	–

E



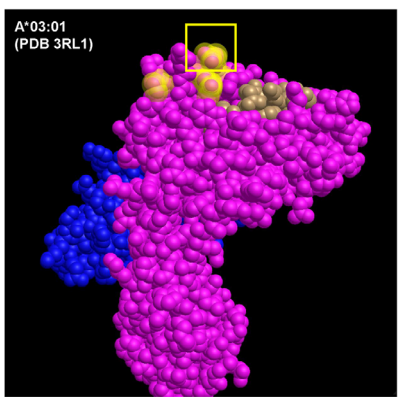


FIGURE 5 | Interrogation of HLA-A*02 specific recombinant antibodies isolated from an allo-sensitized patient. HLA binding specificities of rAbs P3E7-UA **(A)** and P3E7 **(B)** using a Luminex® single HLA antigen bead 100-plex panel. Alleles with MFI values above the X6 threshold (MFI > 1000) are shown. Autologous alleles are indicated with green arrows. **(C, D)** HLA alleles sharing eplet 65RA and the polymorphic amino acids within this potential 15 Å conformational epitope. Alleles are listed in the order of MFI values from high to low. Alleles above the X8 threshold (MFI > 3000) are shaded in dark blue and alleles above the X6 threshold in light blue. Autologous HLA alleles are highlighted in green. **(E)** Three-dimensional structure of allogeneic (A*02:01) and autologous HLA structures from published data with PDB ID indicated. HLA α chain (pink), β2M (blue) and bound peptide (brown) are visualized using Cn3d software. Residues 56, 62, and arginine at position 65 (box) are highlighted yellow.

alleles as well (data not shown). The location of eplet 65RA on allogenic HLA-A*02:01 [Protein Data Bank (PDB) ID 4U6Y (37)] and autologous A*03:01 [PDB ID 3RL1 (38)] structures are shown in (**Figure 5E**). Therefore, the process of affinity maturation in this patient was coupled to the generation of humoral autoreactivity.

DISCUSSION

In our report of new experimental tools for monitoring of MHC-sensitized recipients, we have demonstrated how FP barcoded cells expressing HLA or Mamu proteins can be used to detect and characterize specific antibody and B cells. Our data describe a method for isolation of individual MHC-specific Bmem cells and their characterization by panels of MHC reporter cells. Using this single cell method, we can interrogate HLA antibody-secreting clones for their BCR sequences, infer clonal relationships between mutated Bmem cells and UAs, and demonstrate affinity maturation as a process of clonal evolution. Lastly, by the generation of representative rAbs, we demonstrate the utility of SAB Luminex® panels and HLA eplet analysis to define HLA conformational epitopes recognized by individual B cells and the trajectory of their evolution by somatic mutation.

It is notable that this technology/method can benefit in nonhuman primate (NHP) research since currently there are no available means to evaluate individual Mamu (or Mafa) specific antibodies. Non-human primates offer an important experimental model to develop novel therapies for clinical translation (39). Attempts to use HLA-specific SAB reagents to characterize NHP alloantibody responses have been made (40), albeit with limited resolution of specificity. In most NHP research, therefore, the standard assay for alloantibody detection relies on a donor-cell antibody capture method analogous to the flow cytometric crossmatch used in clinical transplantation (12, 41, 42). Unfortunately, antigen-specific alloantibody quantitation, such as the HLA SAB Luminex® assay provides, has not been realized, despite conserved epitopes permitting detectable positivity (43). Our use of FP barcoded cells expressing Mamu antigens (**Table 1**) allows for multiplex detection of multiple alloantibody specificities within the same sample, with good detection of alloantibody directed against sensitizing antigens. The additional positivity detected in the Mamu reporter assay may reflect false positives but, as the single-cell human rAb data suggest, more likely represents unknown cross-reactive binding across similar epitopes. Since this degree of alloantibody specificity has not been widely available these new tools allow for a greater understanding of shared epitopes, binding patterns, hierarchy among Mamu (or Mafa) antigens.

In human studies, the utility of HLA expressing reporter cells may be limited to that of a screening tool, given the apparent greater sensitivity of the SAB Luminex®; assay. For research purposes, however, SAB testing with commercially available products can be prohibitively expensive. For example, in the case of single-cell cultures in which hundreds or thousands of individual culture wells contain clonal IgG, SAB bead analysis of each well could present a daunting expense. As for the analogous Luminex®; bead assay, the barcoding of each HLA or Mamu expressing cell line with a unique pattern of FPs permits multiplexing of the assay in a single tube, limiting the volume of sample necessary for antibody detection.

In conjunction with our barcoded reporter cell lines, we used a single human B-cell culture system (11, 20, 33, 34) to isolate and characterize HLA-specific Bmem cells from an allo-sensitized patient. In previous studies, we sorted hemagglutinin-binding Bmem cells from vaccinated subjects into single-cell cultures and about half of the cultured Bmem cells produced hemagglutinin-specific IgG (11). In this study, however, from 270 cultures of Bmem cells identified by HLA-A*02:01 tetramer binding only a single clonal culture produced HLA-specific IgG (**Figure 5B**). The low frequency of HLA-specific clones among tetramer-binding B cells is likely the consequence of the low specificity of the antigen-tetramer "hook" and perhaps, the absolute frequency of HLA-specific B cells. As HLA-specific Bmem cells are usually at low frequency in allo-sensitized patients (10, 44), development of high-quality HLA antigen hooks will be critical for efficient isolation of specific Bmem cells.

With the single HLA-specific B cell clone identified, we determined the BCR V(D)J sequences and identified mutation frequencies of about 1% in the V genes of both the heavy- and light chain gene rearrangements (**Figure 4C**). We confirmed the avid binding of the A*02:01 tetramer by secreted clonal IgG with a recombinant antibody prepared from the recovered BCR gene sequences. In addition, the inferred germline progenitor (UA) of the recovered Bmem clone was also generated and comparison of P3E7 and the UA recombinant antibodies provided evidence for an avidity increase of some 10^4-fold as a consequence of somatic hypermutation (**Figure 4D**). These data, and the Bmem phenotype recovered by flow sorting (**Figure 4A**), suggest strongly that this B cell clone was a typical class-switched Bmem cell derived from a GC response after successful rounds of affinity maturation. This is an example, albeit a single one, of HLA-specific Bmem cells in sensitized patients. Moreover, the strong binding avidity of the mature rAb implies that the H02P3E7 Bmem cell would likely be capable of differentiating into a plasma cell (45), and to secrete HLA antibody on rechallenge. Antibody of this affinity would impose a threat of an acute AMR after transplantation even if HLA serum antibody titers were negligible prior to transplant (44). This observation highlights the significance of monitoring of HLA Bmem cells in pre-transplant patients in clinic.

An interesting observation with the HLA Bmem recovered is that its mutated and affinity-matured BCR acquired autoreactivity absent in its germline precursor. Whereas the inferred germline antibody was specific for the products of several allo-HLA-A*02 alleles, the more avid BCR expressed by the Bmem H02P3E7 daughter cell acquired the capacity to bind autologous HLA molecules in the course of its somatic evolution (**Figure 5**). The acquisition of self-reactivity is known to be associated with human Ig-class recombination (35) and, presumably, clonal maturation in GCs. Nonetheless, most current models for GC responses posit negative selection to limit the generation of autoimmunity by AID-mediated

hypermutation (46–49). Indeed, we observed the opposite of what Goodnow and colleagues have named clonal redemption (47, 48). Rather than the recruitment of autoreactive B cells into GCs where they lose autoreactivity in the process of affinity maturation to an exogenous antigen, the naïve P3E7-UA cell was presumably recruited to a GC on exposure to allo-HLA and there its progeny acquired reactivity to self-HLA in conjunction with allo-specific affinity maturation (**Figure 5**). Surprisingly, this *de novo* autoreactivity was not against some rare or cryptic self-antigen but to universally expressed HLA class I determinants. While it is possible that the binding activities to self HLA molecules detected in the SAB assay are not physiologically relevant, it will be interesting to determine if the relevant membrane associated HLA molecules trigger BCR signaling in B cell lines harboring the matured P3E7 BCR. Additional study will be needed to determine whether autoreactivity is a common phenomenon among allo-HLA Bmem cells.

These new cell tools permit multiplex detection of allo-MHC antibody in humans and non-human primates. The utility of this approach is limited only by the number of FP barcoded cell lines and has the potential of multiplexing up to 256 distinct cells for antibody detection in single samples. Additionally, we have demonstrated the application of barcoded reporter cells in screening for HLA-specific antibody production by single-cell cultures of human Bmem cells. In this way, we characterized and expressed rAb derived from a human Bmem cell, demonstrating its somatic evolution by mutation and selection for increasing affinity. We rapidly identified the 65RA eplet as the putative epitope for the Bmem BCR using SAB HLA-Luminex® assays. These combinations of new and established techniques offer increased understanding of humoral responses to allo-MHC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank accessions MZ152901, MZ152902, MZ152903, MZ152904.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Duke Institutional Review Board Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Duke University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SS, SK, and GK contributed to conception and design of the study. SS, MM, JK, AJ performed the experiments described in this study. SK and GK helped to review and trouble-shoot all experiments. SS, MM, JK, and AJ wrote specific sections of the initial manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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New Perspectives on Desensitization in the Current Era - An Overview

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Blood group and tissue incompatibilities remain significant barriers to achieving transplantation. Although no patient should be labeled "un-transplantable" due to blood group or tissue incompatibility, all candidates should be provided with individualized and realistic counseling regarding their anticipated wait times for deceased donor or kidney paired donation matching, with early referral to expert centers for desensitization when needed. Vital is the careful selection of patients whose health status is such that desensitizing treatment is less likely to cause serious harm and whose anti-HLA antibody status is such that treatment is likely to accomplish the goal of increasing organ offers with an acceptable final crossmatch. Exciting new developments have re-energized the interest and scope of desensitization in the times ahead.

Keywords: kidney, desensitization, kidney paired donation, novel therapeutic agents, allocation

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INTRODUCTION

For patients with end-stage renal disease (ESRD), kidney transplantation is the optimal therapeutic option associated with a higher quality of life and lower mortality than chronic hemodialysis. The best results are achieved with a kidney from a healthy ABO and HLA matched living donor. Living donation accounts for a third of all kidney transplants performed in the United States. Unfortunately, not every donor recipient pair is feasible because of human leukocyte antigen (HLA) sensitization or ABO incompatibility (ABOi). To overcome these barriers to transplant, strategies such as desensitization have been developed. Here in we discuss historical and current perspectives on desensitization and reflect on future directions.

DESENSITIZATION: THE HISTORICAL PERSPECTIVE

Sensitization to HLA is observed in 30% of patients on the kidney-transplant waiting list and an additional 11% of donor recipient pairs are ABO incompatible (1). This relegates a significant number of kidney transplant candidates with otherwise suitable living donors to the deceased-donor waiting list because of preformed antibodies to HLA and ABO blood group antigens. Desensitization is the removal of circulating donor specific antibodies to HLA or ABO antigens to prevent graft rejection. Crossing HLA and ABO barriers became an emerging phenomenon starting in the mid 90's and gained significant traction over the next two decades. Use of preconditioning, either with high-dose intravenous immune globulin (IVIg) or with plasmapheresis plus low-dose IVIg, increased transplantation rates, reduced waiting time and had promising short-term outcomes across many single center studies. A survival advantage was

established for patients undergoing desensitization for HLA incompatibility followed by live donor transplantation compared with those waiting for a compatible transplant (2). Similarly, candidates who received an ABOi LDKT had higher cumulative survival at 5 and 10 years (90.0% and 75.4%, respectively) than similar patients who remained on the waitlist or received an ABOc LDKT or ABOc DDKT (81.9% and 68.4%, respectively) (3). However, many of these data were collected before implementation of the Kidney Allocation System (KAS), and patients in the control group were not necessarily enrolled in paired exchange programs. Additionally, regardless of the treatment strategy, highly sensitized patients whose calculated panel reactive antibody (cPRA) is ≥98% remained difficult to transplant as desensitization proved to be challenging in those with high HLA or ABO antibody titers.

DESENSITIZATION – THE PRESENT ERA

Enthusiasm (and need) for desensitization of highly sensitized patients has decreased in recent years. Desensitizing treatments are expensive, resource intensive, and place patients at risk for morbidity associated with higher doses of maintenance immunosuppression. Furthermore, these treatments remove circulating antibody or temporarily inhibit antibody production without significant effect on immunologic memory. Additionally, the survival data are not replicable. The 5-year data from the Mayo Clinic showed significantly worse patient and graft survival in those undergoing desensitization with plasmapheresis and low-dose intravenous immunoglobulin (PP/IVIg) versus HLA compatible transplant, as well as protocol biopsy-detected transplant glomerulopathy in 55% of desensitized versus 7% of HLA-compatible recipients (4). In addition, adjunct therapies such as bortezomib, a potent, reversible proteasome inhibitor that targets terminally differentiated plasma cells, has had limited use secondary to unclear efficacy and adverse side effect profile. The largest study of bortezomib-based desensitization therapy along with plasmapheresis and Rituximab was a prospective trial of 44 sensitized patients conducted in five phases, differing in bortezomib dosing density and plasmapheresis timing, and showed a substantial reduction in immune-dominant anti-HLA DSA. Nineteen of 44 (43%) patients were transplanted during the study period with low acute rejection rates (18.8%) and *de novo* DSA formation (12.5%), demonstrating proteasome inhibitor-based desensitization consistently and durably reduced HLA Ab levels and may be a reasonable alternative to IVIg based plasmapheresis regimens in a select population (5). However, bortezomib as monotherapy in a study of 10 highly sensitized kidney transplant candidates with DSAs against their intended living donor resulted in only a modest reduction in DSAs with no change in CPRA despite use of 32 doses of bortezomib. Not surprisingly, the treatment was not well tolerated due to adverse effects (6). Given these data, the best option would be to avoid HLA-incompatible transplant whenever possible, although not necessarily at the expense of significantly prolonged dialysis exposure while awaiting a compatible offer. Several options in the present era, to include changes in the 2014 KAS for highly sensitized recipients and allocation of A2/A2B donor kidneys to B recipients and kidney paired donation (KPD) allowed for alternative choices for these patients.

KAS prioritizes organ offers for patients with high levels of anti-HLA sensitization providing sensitized patients with a cPRA of 20% and above additional points toward organ allocation priority, and highly sensitized candidates with a cPRA of 100%, 99%, and 98% provided 202.1, 50.09, and 24.4 additional points for allocation priority, with each point being equivalent to 1 year of wait-time (7). In addition, candidates with cPRA of 100% receive priority for kidneys shared nationally. These innovations have reduced the median waiting time for highly sensitized patients with cPRA of 98%–100% from >19 years to 3.2 on the deceased donor waiting list (8). These priority allocation points continue with the new allocation policy that went into effect March 14, 2021.

In the United States, the allocation of deceased donor kidneys is based on ABO matching as opposed to ABO compatibility. The waiting time for blood type B kidney transplant candidates is typically longer than for candidates with other blood types. The blood type B kidney transplant candidate waitlist has the highest proportion of ethno-racial minority candidates, who are less likely to receive a living donor kidney transplant compared to white candidates, exacerbating longer waiting times for patients in these populations. With the goal of improving equity by improving access to transplantation for minority populations in the United States, KAS now allows allocation of type A, non-A1 and type AB, non-A1B (commonly known as A2 and A2B) kidneys to blood type B candidates if anti-A titers are low, with the program's titer threshold defined in written policy.

Finally, KPD or paired kidney exchange has become increasingly utilized as an approach to overcome biologic incompatibility, wherein two or more incompatible donor-recipient pairs exchange kidneys and all recipients benefit by receiving compatible transplants. The scope of KPD has further expanded with introduction of non-directed donors and compatible pairs in the form of multi-way exchanges and kidney donor chains to name a few. This has allowed the practice of KPD in the United States to expand exponentially, from a handful of transplants per year when it first started in 2002 to over 1000 transplants in 2020. An additional novel approach has become the combination of both KPD and desensitization to facilitate compatible and successful transplantation. An HLA sensitized patient pair can be paired with a better immunological match in the KPD pool than the original donor and subsequently desensitized to achieve transplantation establishing KPD and desensitization are not mutually exclusive strategies. Importantly, the development and implementation of KPD programs has been demonstrated to mitigate racial and gender disparities in access to living donor kidney transplantation (9).

While the above options have reduced the need for desensitization, it has not been made redundant. Despite KAS, the most highly sensitized >99.5% PRA candidates on the wait list still do not get their fair share of transplants. Only 9.7%

(213/2204) of candidates with a calculated panel reactive antibody ≥99.9% received a transplant, and the most highly sensitized candidates were less likely to receive a living donor transplant. Among candidates with a CPRA ≥ 99.5% (i.e. 100%), only 2.5% of transplants were from living donors (13 total, 7 from KPD). Nearly 4 years after KAS (6/30/2018), 1791 actively wait-listed candidates had a CPRA of ≥99.9% and 34.6% (620/1791) of these had ≥5 years of waiting time (10). Thus, despite KPD and KAS, the most highly sensitized candidates have not been transplanted even with prolonged waiting time. Candidates with a CPRA ≥ 99.9% and sensitized candidates with an incompatible living donor and prolonged waiting time may benefit from desensitization to improve their ability to receive a transplant.

Despite the changes in KAS for Blood type B recipients, there is still a vast number of Blood type O and B wait list candidates with disproportionately longer wait times facing a high mortality while they wait. Clinical outcomes after ABO antibody reduction in ABO-incompatible living-donor kidney transplant recipients are not much different from those achieved in ABO-compatible control groups and desensitization for ABOi remains an unmet need (11).

DESENSITIZATION: FUTURE DIRECTIONS

Exciting developments in the field of immunosuppression, diagnostics and therapeutic options continue to offer hope to the subset of patients that are considered "un-transplantable" *via* desensitization with and without KPD.

- Imlifidase is a promising new Investigational therapeutic. The IgG-degrading enzyme derived from *Streptococcus pyogenes* (Imlifidase; IdeS) is a recombinant cysteine protease that cleaves all four subclasses of human IgG into F(ab')2 and Fc fragments, inhibiting CDC and antibody-dependent cellular cytotoxicity. The efficacy of Imlifidase as a desensitization strategy was evaluated in two independent phase I/II trials (12). Treatment with Imlifidase produced complete cleavage of IgG into F(ab')2 and Fc fragments within six hours of infusion. Intact IgG remained absent in all patients for at least seven days, and there was a persistent reduction in IgG levels at 28 days after infusion. IVIG and rituximab following Imlifidase was associated with less donor specific antibody (DSA) rebound, with the median fluorescent intensity (MFI) of the immunodominant DSA reported to be 0 at one month. Mean estimated GFR (eGFR) at one to six months posttransplant was 70 mL/min/1.73 m2 in the United States study.
- Obinutuzumab is a third-generation anti-CD20 monoclonal antibody less reliant on complement-dependent cytotoxicity and is mediated primarily through antibody-dependent cellular cytotoxicity with effective B cell depletion not only in the peripheral blood but also in the secondary lymphoid organs and may have more lasting effects on memory B cells and plasma cells than Rituximab.
- Carfilzomib is a second-generation, irreversible proteasome inhibitor that has a more favorable toxicity profile than bortezomib with a pronounced reduction in DSA MFI when used in combination with plasma exchange in a small study of 12 highly sensitized patients (13).
- Tocilizumab is a monoclonal antibody directed against the IL-6 receptor that is being used for treatment of rheumatoid arthritis. In one pilot trial of ten transplant candidates who had failed desensitization with IVIG and Rituximab and were subsequently treated with IVIG and tocilizumab, five were successfully transplanted (14).

An Achilles heel in ABOi transplantation is the variable correlation of traditional anti-A and Anti-B blood group titers detected *via* hemagglutination with graft loss from acute antibody mediated rejection early post-transplantation. In contrast to the hemagglutination assay, the ABO-glycan microarray allows detailed characterization of donor-specific antibodies necessary for effective transplant management, representing a major step forward in precise ABO antibody detection *(*15*)*. Characterization of ABH antigen subtype expression in other organs such as kidney and liver will be valuable for its wider application in ABOi organ transplantation. The glycan microarray may allow reliable assessment of patients for their suitability to receive an ABOi transplant and for appropriate pre-and posttransplant clinical management. Furthermore, by accurately assessing the absence of antibodies specific for graft antigens, unnecessary interventions may be avoided such as antibody removal by plasmapheresis or aggressive immunosuppressive therapies.

An exciting application to this technology is the development underway of silica microparticles functionalized with A and B blood group carbohydrate antigens (A type I, A type II, B type I, and B type II) to enable the detection and monitoring of ABO antigen-specific B cells much like the single antigen bead analysis in HLA testing. This approach therefore comprises a novel, general platform for screening B cell populations for binding to carbohydrate antigens, including, in this case, the human A and B blood group antigens (16).

Patients with DSA and T-cell activation as demonstrated by high levels of soluble CD30 (sCD30) in pretransplant serum have a threefold higher risk of graft loss than patients with DSA but low sCD30 levels *(*17*)*. Using this and other novel biomarkers to follow treatment response in addition to traditional DSA MFI/titer measurement may offer additional guidance into management before and after transplantation. Furthermore, the conventional HLA mismatch has been challenged recently by the concept of HLA epitope matching algorithms that claim to offer a more precise assessment donor recipient HLA compatibility. Molecular mismatch has been proposed as a prognostic biomarker categorizing individual donor recipient pairs into low and high risk where for every 10 eplet mismatches there is a 2-fold increased hazard of developing a DR or DQ antibody (18). When individuals were categorized based on thresholds of eplet mismatch into low or high risk there was a prognostic co-relation for both cellular and antibody mediated rejection (19). Furthermore, patients at low HLA DR/DQ mismatch were able to tolerate less immunosuppression and still had less rejection and *de novo* DSA formation (18). This is

promising for future adoption in the field of desensitization where one could better define acceptable mismatches *via* epitope matching and also try and cross low level epitope mismatches more safely while avoiding the high-risk phenotypes. Rate limiting factors for current adoption include lack of universal high-resolution typing for every donor and recipient pairs and time consuming and labor intensive testing that is often impractical especially in allocation schemes that have a 12 -24 hr turnaround time. HLA epitope matching, however, has a promising role in living donor kidney transplantation and KPD matching.

In non-kidney solid organs transplant candidates with a high wait list mortality where dialysis and living donors is not an option, desensitization for deceased organ donor transplants continues to have a role and lessons learned in desensitization in kidney transplantation paves the way for future innovations.

CONCLUSIONS

When living donors are available, paired exchange should be attempted to avoid the cost and risk associated with desensitizing therapy as well as the posttransplant immune response that will likely translate into worse long-term graft survival. Paired exchange options should be exhausted, and a realistic estimate of wait time taking into account priorities for high cPRA patients under KAS should be considered. Proceeding with desensitization for those highly sensitized patients without living donors, where paired exchange is not possible and expected wait time is considered unacceptable, may be a reasonable consideration. Although no patient should be labeled "un-transplantable" due to blood group type or DSA, all candidates should be provided with individualized and realistic counseling regarding their anticipated wait times for deceased donor or kidney paired donation matching, with early referral to expert centers when needed. Careful patient selection, which involves the identification of individuals who can withstand desensitization treatment and have favorable antibody profiles amenable to successfully overcoming the incompatibility to allow transplantation, remains the cornerstone desensitization. One cannot emphasize enough the importance of careful antibody monitoring throughout the posttransplant period. Lessons learned from the past along with exciting developments in pipeline will pave the way for the future of desensitization.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Sensitization and Desensitization in Vascularized Composite Allotransplantation

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Vascularized composite allotransplantation (VCA) is a field under research and has emerged as an alternative option for the repair of severe disfiguring defects that result from severe tissue loss in a selected group of patients. Lifelong immunosuppressive therapy, immunosuppression associated complications, and the effects of the host immune response in the graft are major concerns in this type of quality-of-life transplant. The initial management of extensive soft tissue injury can lead to the development of anti-HLA antibodies through injury-related factors, transfusion and cadaveric grafting. The role of antibody-mediated rejection, donor-specific antibody (DSA) formation and graft rejection in the context of VCA still remain poorly understood. The most common antigenic target of preexisting alloantibodies are MHC mismatches, though recognition of ABO incompatible antigens, minor histocompatibility complexes and endothelial cells has also been shown to contribute to rejection. Mechanistically, alloantibody-mediated tissue damage occurs primarily through complement fixation as well as through antibody-dependent cellular toxicity. If DSA exist, activation of complement and coagulation cascades can result in vascular thrombosis and infarction and thus rejection and graft loss. Both preexisting DSA but especially *de-novo* DSA are currently considered as main contributors to late allograft injury and graft failure. Desensitization protocols are currently being developed for VCA, mainly including removal of alloantibodies whereas treatment of established antibody-mediated rejection is achieved through high dose intravenous immunoglobulins. The long-term efficacy of such therapies in sensitized VCA recipients is currently unknown. The current evidence base for sensitizing events and outcomes in reconstructive transplantation is limited. However, current data show that VCA transplantation has been performed in the setting of HLA-sensitization.

Keywords: vascular composite allotransplantation, sensitization, desensitization, antibody-mediated rejection, hand transplantation, face transplantation, burns

INTRODUCTION

Vascular composite allotransplantation is an evolving field in organ transplantation since it has emerged as a viable option to repair tissue defects resulting from traumatic or other injuries in selected patients (1). Vascularized composite allografts (VCAs) consist of anatomically distinct tissues such as skin, muscles, connective tissue, bones and neurovascular elements that are transplanted as a single unit (2–4). So far, VCAs have been used in various settings including transplantation of face, upper or lower extremity, abdominal wall and genitourinary organs (4, 5) (1). As with other solid organ grafts, they are limited by immune mediated rejection and a concomitant requirement for immunosuppression (2–4). Also, candidates for VCA are frequently sensitized, making them susceptible for antibody-mediated rejection (AMR).

Sensitization consists of the ability of the immune system to recognize and react to foreign human leukocyte antigens (HLA) by producing antibodies and developing memory cells, which are common risk factors for acute allograft rejection. In VCA, many possible reasons for sensitization have been described, including blood transfusions, previous pregnancies or transplants as well as cadaveric skin allotransplantation that is commonly used to provide temporary coverage in burn patients. Despite initial reports underestimating the role of antibodies in VCA damage, it is currently established that AMR is also an important process affecting graft viability (5, 6). Thus, many patients are currently precluded for a life-enhancing VCA due to sensitization and lack of well-established desensitization protocols.

We hereby provide an overview on current evidence of sensitization in the field of VCA, followed by posttransplant strategies of desensitization and their potential impact on future management of VCA patients.

BACKGROUND OF ALLO-SENSITIZATION AND VCA SPECIFIC CONSIDERATIONS

The exposure of the immune system to non-self HLA may result in the generation of HLA antibodies that happens the settings of transfusion, transplantation or pregnancy. The degree of polymorphism in the HLA system results in a large number of non-self stimuli for antibody development (7). In the setting of transplantation, the presence of donor-specific HLA antibodies (DSA) is well-known to be related to hyperacute rejection (8). Kidney transplant literature supports that both pre-existing DSA and DSA produced *de novo*, which appear in the period after 3 months post transplantation (dnDSA) are harmful, although it seems that AMR patients with preexisting DSA had superior graft survival to patients with dnDSA (9). Similarly, DSAs directed against either class of HLA antigen are harmful but is seems that DSAs directed against HLA class II antigens have been more strongly associated with late-onset AMR, *de novo* antibody production, and reduced graft survival (10).

There is significant body of knowledge coming from the burn literature on the mechanisms of sensitization in VCA (**Table 1**).

TABLE 1 | Sensitizing factors in VCA.

Sensitizing Factors
Burns
Multiple Blood Transfusions
Pregnancy
Previous Transplants
Allogeneic Skin Grafts
Ventricular Assist Devices
Extracorporeal Membrane Oxygenation

Burn patients experience sensitization (development of anti-HLA antibodies), during resuscitation and wound coverage. Of interest, burn patients are at higher risk for sensitization during resuscitation with blood products and VCA (measured by average panel-reactive antibody; PRA) compared to burn patients undergoing blood transfusion only. Importantly, burn patients can develop higher PRA levels compared to trauma (non-burn) patients (11). In the same setting, a recent study showed that almost all burn patients undergoing resuscitation with blood products and skin allotransplantation developed anti-HLA antibodies, of which about 50% had complement-fixing antibodies. Of interest, the majority of these patients (62%) were considered highly sensitized (PRA≥85%). Cryopreserved, but not glycerol-preserved skin allografts, history of pregnancy, and number of blood units were associated with HLA sensitization (12). Similarly, it was shown that burn patients with skin allografts developed lower PRAs when evaluated during the acute phase of trauma compared to burn skin transplant recipients when tested years after transplant (6 ± 12% *vs* 42 ± 33%, P = 0.08). The latter demonstrates that detection of HLA antibody is lower in the acute burn period than months to years after injury thus increasing sensitization may ultimately limit burn patients' candidacy for VCA or decrease success of these procedures (13). Some have proposed emergency VCAs in burn patients as potential strategy for early definitive reconstruction avoiding procedures triggering HLA antibody formation (14). The prevalence of sensitization in patients awaiting VCA is unknown relative to other transplants. Trauma patients waiting for hand or face VCA, without extensive transfusion requirement or prior skin transplant, are mostly healthy, young individuals with a low risk of pre-existing sensitization. According to the literature, more than 80% of the patients who have received reconstructive VCAs (hand or face) are male with an average age of about 30 years (15, 16). On the contrary, the average age for kidney transplantation is above 45 years with more than 60% being males (17).

CHARACTERISTICS OF ANTIBODY-MEDIATED REJECTION

Diagnostic criteria for AMR were first described in the setting of kidney and cardiac allografts (5, 18), and subsequently extended for pancreas, liver and lung (19–21). Characteristics of AMR in small intestine and VCA have also been described (22, 23), but consensus criteria for AMR in these organs are lacking.

Main universal characteristics of acute AMR include serological evidence of antibodies, histological evidence of endothelial cell injury, complement activation, and infiltration of innate immune cells (24). As far as the allograft endothelium is concerned, it seems that it plays an active role in the pathogenesis of rejection due to its phenotypic changes according to the microenvironment conditions created by post-transplant inflammation, alloreactive lymphocytes, DSA and complement activation, that in turn, might lead to promotion of proinflammatory alloresponses favoring the expression of Th1 T cells, M1 macrophages and NK cells (25). In VCA, if preformed antibodies (DSA) exist, activation of complement and coagulation cascades can result in vascular thrombosis and infarction and thus hyperacute rejection and graft loss. This hypothesis was confirmed again by recent evidence of AMR in highly sensitized face transplant recipients (26, 27). On the contrary, the effects of dnDSA on VCAs are largely unknown. Grafts with potentially high immunogenicity such as VCA may increase the development of dnDSA and the majority of studies have reported that the presence of DSA is associated with rejection and graft impairment (28). Histopathologic assessment of VCAs is critical for the early and accurate diagnosis of rejection and timely institution of effective immunotherapeutic regimens. Currently, AMR is not included in the BANFF classification of VCA rejection (29). Supportive data for AMR have been limited to demonstration of C4d deposition in hand transplant recipients, experience that is currently different to the one from solid-organ transplantation where C4d deposition is commonly associated with DSA (~40–60%) and is part of the diagnostic criteria for classic AMR. Reports of VCA recipients with C4d deposition had absence of DSA (6, 30, 31). Findings that were partially confirmed by Petruzzo et al. where detected DSA was not related to C4d deposition. However, there has been one confirmed case of AMR in which C4d deposition was specific to the allograft and occurred in the presence of DSA (32).

PREVENTION AND MANAGEMENT OF AMR IN VCA

Prevention and management of AMR in VCA is a research area requiring further attention. Currently, prevention and desensitization protocols as well as treatment of AMR in VCA recipients is based on those recommended in solid organ transplantation. In general, despite the understandable advantage of reducing maintenance immunosuppression, with or without cell-based therapy, overly aggressive minimization is potentially linked to a higher incidence of acute rejection episodes, an increased risk of chronic rejection and the development of dnDSA (33). Regarding induction therapy in VCA, antithymocyte globulin (ATG) is currently the most commonly used T-cell depleting induction agent. Other alternative approaches include the use of alemtuzumab and basiliximab (34). For maintenance immunosuppression, protocols commonly used are derived from solid-organ experience and mainly consist of triple therapy with tacrolimus, MMF and steroids (4, 35), There have been reports of management of VCA patients with dual maintenance immunosuppression regimen subsequent to ATG induction (36). Tacrolimus is the most commonly used calcineurin inhibitor with early (initial period of 1–5 months after transplantation) trough levels of 10–15 ng/mL and 5–10 ng/mL thereafter. Tacrolimus trough levels<5ng/mL appeared to be associated with a higher risk for acute rejection (37). Most centers taper steroids rapidly in the early post-transplant period with a subsequent maintenance of 5 to 15 mg/d for 6 to 12 months in most patients (38). Experimental and clinical data also support the preventive role of belatacept as centerpiece of immunosuppressant for VCA by providing sufficient protection against rejection (4, 39–43).

Treatment against AMR usually includes steroids, total plasma exchange, IVIG, plasmapheresis, bortezomib and anti-CD20 mabs (44). Both preexisting DSA but especially *de-novo* DSA are currently discussed as main contributors to late allograft injury and graft failure (7). The Innsbruck group reported the first case of a primarily B-cell-driven rejection episode with the development of dnDSA indicative of AMR in a patient after forearm transplantation at 9 years post-transplant, without recent trigger such as surgery or blood transfusion. The patient did not improve with steroid treatment, but administration of rituximab resulted in complete remission of clinical symptoms (45). A possible explanation for the development of dnDSA in VCA patients might be the association between post T-cell mediated rejection dnDSA development and pretransplant sensitizing events which was not specific to the DSA first detected in the early posttransplant period. It could be possible that most DSA reported as *de novo* are actually secreted by memory B cells undergoing clonal expansion triggered by the proinflammatory microenvironment of T-cell mediated rejection (28, 46). Another major finding in these patients is the evidence of lymphoid neogenesis in the dermis of both grafts reminiscent of tertiary lymphoid organs (47). However, diagnosis of AMR remains incompletely described, as staining for C4d and DSA titers has been shown to be unreliable in VCA (5, 48, 49).

DESENSITIZATION STRATEGIES IN VCA

There are no well-established desensitization protocols in VCA literature. Recent literature summarized the potential strategies for desensitization in patients with VCA (50, 51). Immunoadsorption and plasma exchange aims to remove, selectively or not, antibodies for antigens A and B. However, antibody titres bounce back a few weeks after treatment if not combined with another treatment. Another treatment is rituximab that deplete B cells. But it does not target plasma cells due to lack of CD20 receptors. The proteasomal inhibitor bortezomib triggers the apoptosis of plasma cells and reduces the alloantibody production *via* this pathway. Intravenous immunoglobulins (IVIG) neutralize anti-idiotypic antibodies, inhibit the complement cascade and reduce antibody formation by down regulating mechanisms or eliciting apoptosis of B cells (**Figure 1**).

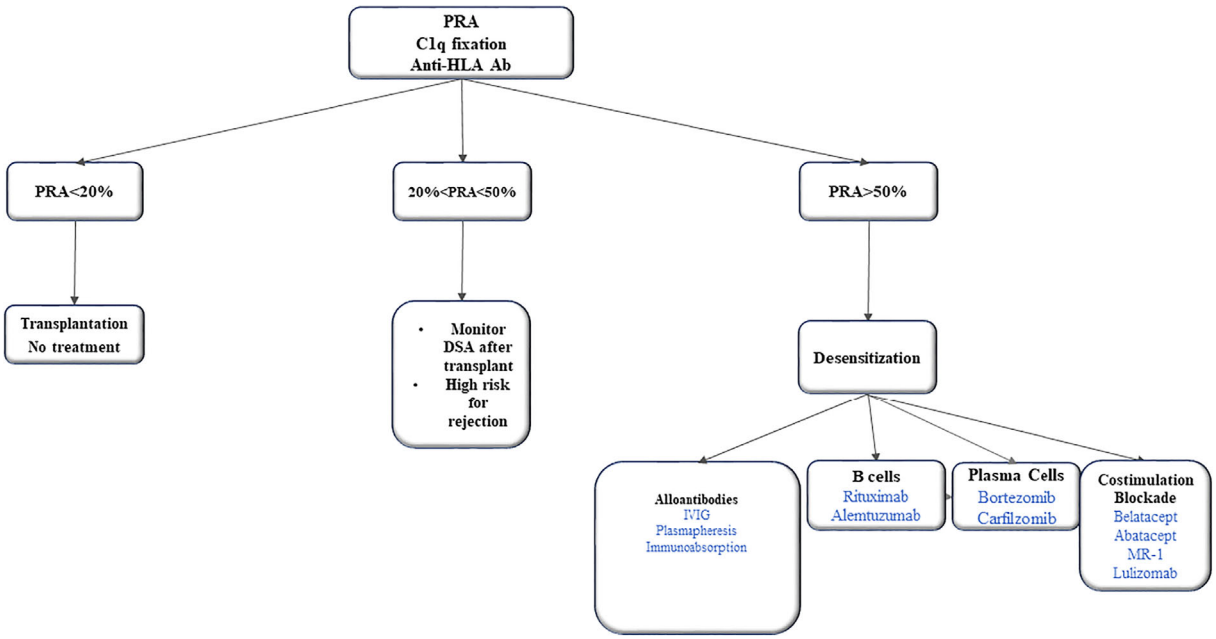

FIGURE 1 | Desensitization strategies in VCA.

Many novel data are emerging from experimental animal models and limited human case series. It was recently shown that hindlimb transplant rats with prior skin transplant sensitization showed prolonged graft survival when desensitized with of total body irradiation, fludarabine, and syngeneic hematopoietic stem cell transplantation, that was related to significantly reduced DSA level as well as no evidence of CD4d deposition at the time of rejection (52). In 2014, Chandraker et al. reported the first experience of a full-face allotransplant in a pre-sensitized burn patient with a positive perioperative crossmatch and high levels of circulating anti-HLA class I and class II antibodies with a calculated PRA score of 98. Despite plasmapheresis in addition to induction with ATG, the recipient developed an AMR with rising DSA titers and evidence of C4d positivity in the biopsy, which showed a Banff grade III rejection. The patient received anti-AMR therapy combining plasmapheresis, eculizumab, bortezomib and alemtuzumab. The DSA levels decreased, clinical condition improved and the histological signs of rejection had resolved by 6 months after the transplantation (30). The long-term efficacy of such therapies in sensitized VCA recipients is currently unknown. Whether desensitization strategies will increase the recipient pool of VCA patients remains to be seen. Since the report, the patient experienced irreversible rejection, graft loss and was re-transplanted in July 2020.

Most of the literature on desensitization protocols emerges from kidney transplantation (30, 53). Emerging data from experimental models showed that multiple factors such as proteasome inhibitors, costimulation blockades, BAFF/APRIL blockades and complement inhibitors significantly prolong graft survival by disorganizing germinal center responses and reducing DSA levels (54–56). The idea of using these protocols might be very promising, but there remain pros and cons with these approaches as they have not been totally effective in solid organ transplants and they are accompanied by side-effects such as increasing risk for severe infections, renal failure, thrombotic events and malignancy (28, 57).

CONCLUSIONS

The prevalence of sensitization in patients awaiting VCA is unknown relative to other transplants. Patients qualifying for skin containing VCAs after severe burns who require aggressive resuscitation with multiple blood products and temporary skin coverage are usually at risk of sensitization. The management of potential VCA patients starts at the time of initial injury. The prevention of sensitization and the possible desensitization strategies to extend VCA survival is an area under research. Currently, there is no well-established desensitization protocol for VCA patients. Emerging knowledge from other solid organ transplants might guide management of sensitized VCA patients in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Current Desensitization Strategies in Heart Transplantation

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Heart transplant candidates sensitized to HLA antigens wait longer for transplant, are at increased risk of dying while waiting, and may not be listed at all. The increasing prevalence of HLA sensitization and limitations of current desensitization strategies underscore the urgent need for a more effective approach. In addition to pregnancy, prior transplant, and transfusions, patients with end-stage heart failure are burdened with unique factors placing them at risk for HLA sensitization. These include homograft material used for congenital heart disease repair and left ventricular assist devices (LVADs). Moreover, these risks are often stacked, forming a seemingly insurmountable barrier in some cases. While desensitization protocols are typically implemented uniformly, irrespective of the mode of sensitization, the heterogeneity in success and post-transplant outcomes argues for a more tailored approach. Achieving this will require progress in our understanding of the immunobiology underlying the innate and adaptive immune response to these varied allosensitizing exposures. Further attention to B cell activation, memory, and plasma cell differentiation is required to establish methods that durably abrogate the anti-HLA antibody response before and after transplant. The contribution of non-HLA antibodies to the net state of sensitization and the potential implications for graft longevity also remain to be comprehensively defined. The aim of this review is to first bring forth select issues unique to the sensitized heart transplant candidate. The current literature on desensitization in heart transplantation will then be summarized providing context within the immune response. Building on this, newer

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Abbreviations: ACR, Acute Cellular Rejection; ADCC, Antibody-Dependent Cellular Cytotoxicity; AMR, Antibody Mediated Rejection; AT1R - angiotensin II type 1 receptor; Bmem, Memory B cell; BMPC, Bone Marrow Plasma Cell; CDC, Complement Dependent Cytotoxicity; CHD, Congenital Heart Disease; cPRA, calculated Panel Reactive Antibody; CTLA4, Cytotoxic T-Lymphocyte-Associated Protein 4; DSA, Donor Specific Antibody; FcRN, Neonatal Fc Receptor; FCXM, Flow cytometric crossmatch; GC, Germinal Center; HLA, Human Leukocyte Antigen; IdeS - IgG-degrading enzyme derived from *Streptococcus pyogenes*; IVIG, Intravenous Immunoglobulin; LLPC, Long Lived Plasma Cell; LN, Lymph Node; LVAD, Left Ventricular Assist Device; MCS, Mechanical Circulatory Support; MFI, Mean Fluorescence Intensity; MICA, Major Histocompatibility Complex Class I Chain-Related Molecule A; PI, Proteasome Inhibitor; PPCM, Peripartum Cardiomyopathy; PRA, Panel Reactive Antibody; SAB, Single Antigen Bead; TCR, T-cell Receptor; UPR, Unfolded Protein Response.

approaches with therapeutic potential will be discussed emphasizing the importance of not only addressing the short-term pathogenic consequences of circulating HLA antibodies, but also the need to modulate alloimmune memory.

Keywords: HLA, sensitization, desensitization, non-HLA antibodies, heart transplantation, cPRA, humoral immune response

INTRODUCTION: SENSITIZATION TO HUMAN LEUKOCYTE ANTIGENS (HLA) IN HEART TRANSPLANTATION

HLA sensitization is a major barrier to heart transplantation, the incidence of which is increasing (1, 2). Sensitized patients wait longer for transplant and are at higher risk of dying on the waiting list or being delisted (3). Post-transplant, there is an increased incidence of adverse outcomes including both cellular and antibody mediated rejection as well as cardiac allograft vasculopathy (4–6). Heart transplant candidates bear the burden of all the same risk factors for sensitization as other transplant candidates, namely pregnancy, prior transplant, and transfusions. However, additional sensitizing events also contribute. These include homografts (7) used for congenital heart disease repair, and mechanical circulatory support (5), most commonly left ventricular assist devices (LVADs). Given that immune 'insults' can accumulate over time, this risk can be quickly magnified.

As is the case across solid organ transplantation, desensitization strategies are limited by variable efficacy and frequently accompanied by brisk rebound. In the case of end-stage heart disease, this is further complicated by tenuous hemodynamics, time sensitivity, and in some cases chronic device infections. In the United States, sensitized transplant candidates do not receive priority status on the waitlist (8). While policy change is one approach, prioritization can be a double-edged sword, resulting in hesitancy to develop robust, mechanistically driven desensitization strategies with the potential to improve post-transplant outcomes (9). Instead, prioritization may favor peri-transplant antibody management approaches that temporize the situation but do not modulate the underlying immune response. Multidrug regimens may more comprehensively address the underlying immune response, particularly for patients in whom years of repeated allosensitizing events result in high titer HLA antibodies with cytotoxic capabilities (10–12). However, the factors driving differences in B cell memory and plasma cell characteristics are incompletely understood, the response to treatment is heterogeneous, and therapeutic options remain limited. Moreover, while emphasis has been placed on therapeutic approaches that directly target humoral alloimmunity, it is important to consider that allosensitization includes T-cell memory and that there is growing appreciation of the complex interaction between innate and adaptive immunity. The objective of this review is to 1) summarize the current state of desensitization in heart transplantation supported by experience in kidney, 2) provide context within the immune response, and 3) building on these findings, introduce rational strategies with the potential to improve long-term outcomes. A brief overview of HLA sensitization, the methods used to detect allosensitization, and considerations unique to heart transplantation will first be provided to contextualize the review.

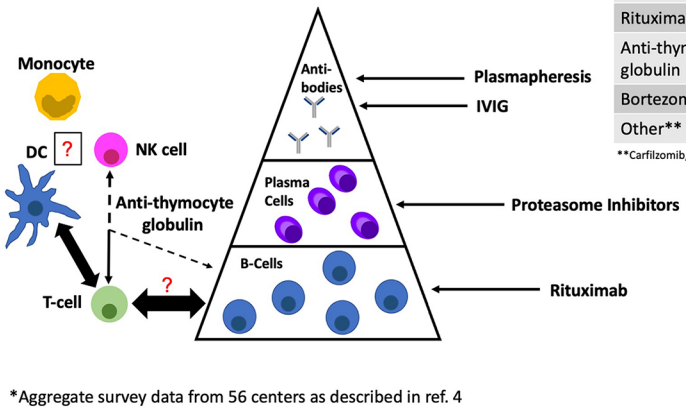
SENSITIZATION AND UNIQUE CONSIDERATIONS FOR THE HEART TRANSPLANT CANDIDATE

Overview of the Immune Response to Foreign HLA

Exposure to foreign HLA can initiate a complex set of immune reactions which may result in a short-term 'effector response', long-lived plasma cells (LLPCs) capable of sustained antibody secretion over decades, and/or the establishment of B and T cell memory [reviewed in (13)]. As a result, HLA allosensitization may either be overt (detectable HLA antibodies) or cryptic (the presence of cellular memory in the absence of detectable HLA antibodies). Although only the former is commonly considered in the context of desensitization, both are associated with increased risk of rejection and/or worse outcomes after transplant (14, 15). Importantly, because HLA antibodies are the product of a T cell-dependent response (16), and because alloreactive effector T cells themselves are potently pathogenic, both T and B cell memory, as well as their ability to sustain reciprocal interactions, should be considered (17). Notably, monocytes, dendritic cells (DCs) and natural killer (NK) cells, which are at the boundary between innate and adaptive immunity, feature prominently in the response to the allograft, yet their importance in the context of desensitization is only beginning to be considered. **Figure 1** provides a schematic overview along with the most common current approaches to desensitization based on a recent international survey (4). The primary and recall humoral response are detailed in several recent reviews and summarized below (13, 18, 19). Alloreactive T cells and innate immunity will also be briefly considered.

The Adaptive Response to HLA Antigens: Humoral Alloimmunity

In brief, during the primary immune response, non-self HLA encountered during pregnancy (paternal), exposure to foreign tissue (transplant, homografts), or blood products (transfusions) is transported to secondary lymphoid organs (e.g., lymph nodes or spleen) which are broadly divided into B cell rich follicles and T cells zones (18). T and B cells first encounter antigen independently in their respective zones. T cells acquire a T



Desensitization Therapy	Frequency of Use in Heart Transplant Desensitization*
IVIG	79%
Plasmapheresis	62%
Rituximab	52%
Anti-thymocyte globulin	19%
Bortezomib	8%
Other**	17%

**Carfilzomib, MMF, cyclophosphamide, immunoadsorption.

FIGURE 1 | Common approaches to desensitization in heart transplantation and their primary site of action. DC, dendritic cell; NK, natural killer cell.

follicular helper (Tfh) like phenotype (upregulating BCL-6, CXCR5, PD1, and ICOS) while B cells downregulate CXCR5 and upregulate CCR7 and EBI2 allowing them to migrate to the T cell-B cell border (20). Here, cognate interactions with T cells occur in the presence of costimulatory signals (CD28, CD40L, and ICOS). At this stage, B cells can either directly differentiate in an *extrafollicular manner* or enter the germinal center. Extrafollicular B cells can differentiate into antibody secreting cells (ASCs) or acquire a memory phenotype (13). Alternatively, B cells can enter the germinal center (GC) which marks another critical collaboration between T and B cells ultimately resulting in selection and clonal proliferation of B cells with a high affinity B cell receptor (BCR). Some of these may migrate to the bone marrow and become plasma cells (BMPCs), while others differentiate into quiescent memory B cells (Bmem). The understanding of these different fates and factors driving them is rapidly evolving and beyond the scope of this review. However, three concepts emerge with particular relevance from the desensitization perspective. Firstly, it is important to consider that both extrafollicular and GC B cells can acquire a memory phenotype with heterogeneity in Ig subclass, antigen affinity (resulting from somatic hypermutation), and longevity (19, 21, 22). Secondly, because BMPCs secrete antibodies with specificities that parallel those found in the blood (23), they represent an important therapeutic target. Establishing the phenotype and characteristics of anti-HLA-secreting BMPCs and the factors driving their differentiation will be critical to designing targeted therapies that ideally eliminate HLA-secreting PCs while leaving protective immunity intact. Thirdly, the opportunity for continual T cell-B cell interactions both within and outside of the GC suggests that strategies targeting these interactions may be beneficial both to dampen the active alloresponse and prevent rebound (24, 25).

The Adaptive Response to HLA Antigens: T-Cell Alloimmunity

Sensitized transplant candidates are at increased risk of cellular rejection highlighting the effect of enhanced T-cell reactivity towards the donor graft independent of the T-dependent antibody response (26, 27). In addition to overt exposure to antigenic HLA, the recipient's history of encounter with environmental antigens can shape the donor reactive response through cross-reactivity or heterologous immunity (28, 29). In kidney transplant recipients, the extent of HLA molecular mismatch also influences alloimmune risk (30). For the sensitized heart transplant candidate, these factors can fine tune the donor reactive response and influence post-transplant outcomes. For the patient without an apparent sensitization history, this implies that absence of overt HLA exposure is not synonymous with low risk. At the epidemiological level, this may contribute to the heterogeneity in reported outcomes amongst HLA sensitized heart transplant recipients.

The Innate-Adaptive Interface in HLA Sensitization

While treatment of HLA sensitization is focused on the aforementioned adaptive immune response, innate immune cells, including monocytes, dendritic cells (DCs), and natural killer (NK) cells play an important role in the response to the allograft. DCs serve as antigen presenting cells (APCs) to activate T cells and are therefore a critical first step in the adaptive response. However, because this can occur through the direct, indirect, and semi-direct pathways [reviewed in (31–33)] activation of both CD4 and CD8 T cell can theoretically occur at any timepoint post-transplant. Monocytes are the precursors to some subsets of DCs and also macrophages the latter featuring prominently in the allograft during AMR, secreting proinflammatory cytokines, recruiting additional immune cells,

present antigen locally, and provide costimulatory signals (34). NK cells are also present in the allograft during antibody mediated rejection (AMR) suggesting a logical link between DSA and graft damage *via* antibody-dependent cellular cytotoxicity (ADCC) (35). However, NK cells can function independent of DSA secreting cytotoxic, proinflammatory molecules in the setting of 'non-self' recognition (36, 37). While the above characterization suggests a pathogenic role for the innate response, tolerogenic potential has also been described (31, 34). Moreover, at least some innate immune cells can develop memory adding a layer of complexity to their role in the allograft over time. This suggests that manipulation, rather than inhibition of the innate immune response may be of benefit. Whether strategies that synergistically modulate innate immunity while abrogating the alloreactive adaptive response can lead to sustained improvement in long-term outcomes for highly sensitized transplant recipients, remains to be established.

Assessment of Allosensitization

In addition to HLA antibody detection, which is most commonly done using the Luminex Single Antigen Bead (SAB) assay, numerous approaches have been developed to detect alloreactive memory T and B cells. **Table 1** provides an overview of some of these assays. It should be noted that, at the present time most of these remain exploratory in nature. For further descriptions, the reader is referred to Sensitization in Transplantation: Assessment of Risk (2017 & 2019) (38, 39) as well as several recent reviews (40, 47–49).

Unique Mechanisms of HLA Sensitization in Heart Transplantation and Their Implications for Desensitization

Congenital Heart Disease

Advances in surgical techniques to repair congenital heart defects have revolutionized the prognosis for children with complex congenital heart disease (CHD) with many surviving to adulthood (50–52). Some will develop end stage disease making CHD the second most common indication for heart transplantation between the ages of 18-39 (53). The risk of HLA sensitization is increased, in many cases driven by homograft material used during previous surgical repair leading to chronic exposure to foreign HLA (54). In the absence of immunosuppression brisk allosensitization ensues, which is often broadly reactive, high titer, and resistant to desensitization (7, 55–58). This is further compounded by multiple sternotomies, blood transfusions, and in some cases left ventricular assist device (LVAD) implantation (59). Given that many such patients may be turned down for transplant, the true scope of the problem is likely underestimated. Whether children, with perhaps greater immune plasticity and a shorter duration of exposure, respond differently is difficult to parse out (60–62).

Pregnancy & Peripartum Cardiomyopathy

Peripartum cardiomyopathy (PPCM) is an important cause of morbidity and mortality globally with a geographically polarized incidence ranging from 1:100 in the developing world to 1:2000-4000 in the US where cases may be on the rise (62–67). While many women will recover, a subset progress to end-stage disease necessitating advanced therapies. In the transplant setting, PPCM is associated with higher PRA, increased risk of rejection and worse survival (68). The divergence between the potential for a tolerogenic T cell state and the development of antibodies against paternal antigens raises important questions in the context of transplantation (69). Whether the 'T-cell centric' focus of our current immunosuppressive regimens, and relative lack of adequate B cell control is particularly detrimental in this setting is unknown. In a mouse model of pregnancy induced allosensitization followed by heart transplant with a semi-allogenic graft, T cell tolerance to the graft was overcome in the presence of pregnancy-sensitized B cells, whether or not DSA was present. B cell depletion (α-CD20) restored allograft acceptance (70). The clinical implications remain to be defined, but may be of particular relevance when transplant occurs in close proximity to the sensitizing event.

LVAD

Nearly half of patients who undergo heart transplantation are supported on an LVAD prior to transplant (71). While LVAD implant is associated with increased risk of developing HLA-reactive antibodies, their significance in terms of post-transplant outcomes is less clear (72). In an early UNOS registry analysis, LVAD recipients waited longer for transplant but there was no difference in 1-year survival or rejection episodes between sensitized and non-sensitized patients, even when highly sensitized (PRA>90%) patients were considered (72). Similarly, Chiu et al. used propensity matching to compare sensitized transplant candidates with and without mechanical circulatory support (MCS) (5). In contrast to non-MCS sensitized candidates who had increased 1-year mortality, patients transplanted from MCS had similar outcomes to non-sensitized transplant recipients.

Both the quality and duration of *de novo* detectable HLA antibody responses following LVAD implant may contribute to the discrepancy in outcomes. Using the Luminex Single Antigen Bead Assay (SAB), Shankar et al. described an increase in cPRA from 20% to 53% (p=0.024) after LVAD implant without evidence of cytotoxicity (defined as a CDC PRA>10%) (73). Similarly, a recent comparison of the HeartMate II and HeartMate 3 (HM3) device found that *de novo* sensitization, defined by SAB, persists with the newer generation HM3 although the development of high MFI (>10,000) class I antibodies was less frequent. Similar to the findings by Shankar et al, cytotoxicity defined by a *de novo* positive CDC PRA >10%, was low (74). In contrast to pregnancy and prior transplant, where high MFI HLA antibodies can persist for decades, the response post-LVAD may decline more quickly. In a cohort of 268 patients, 30 (23%) developed newly detectable HLA sensitization (defined as cPRA>10%) after LVAD implant, which declined over time in 67% of these transplant candidates (cPRA<10%) without desensitization (75). Nonetheless, compared to non-sensitized or previously sensitized transplant recipients, the risk of ACR and AMR was higher suggesting that memory persist.

Potential device-intrinsic effects on cellular and humoral immunity have been investigated (76). Whether the type of

TABLE 1 | Assays to detect & characterize allosensitization (38–46).

Assay	Description	Potential Utility for Desensitization	Limitations
HLA antibodies			
Luminex single-antigen bead (SAB) assay	Fluorochrome labelled beads are coated with specific HLA class I or II alleles and mixed with patient's serum; HLA antibodies bind the bead and a secondary Phycoerythrin (PE)-conjugated anti-IgG antibody permits detection. Result reported as a normalized mean fluorescence intensity (MFI).	Permits detection and identification of the specificity of HLA reactive antibodies in the patient's serum.	Mean fluorescence intensity (MFI) does not necessarily correlate with antibody titer. Inhibition, shared-epitopes, and bead saturation may affect results. This is particularly relevant in the setting of robust HLA sensitization
		Used to 'define' the presence of HLA sensitization. The most commonly used assay to assess baseline and post-treatment response. However, dilutions may be required to assess response to desensitization. Post-transplant monitoring (memory *vs. de novo* response).	Lack of standardization between labs in the United States.
Complement binding assays (e.g. C1q assay)	Same Luminex SAB assay as above. C1q binding is detected using a PE-conjugated anti-C1q secondary antibody.	Detect antibodies capable of activating the complement cascade. Positivity may be a reflection of high titer DSA. Monitor response to desensitization.	C1q binding *in vitro* may not correlate with *in vivo* effects. C1q negative antibodies can still be associated with C4d+ AMR.
IgG subclass	Same Luminex SAB assay as above with detection antibody that recognizes IgG1-4.	Different IgG subclasses may pose greater risk of acute rejection (e.g. IgG1 and IgG3). However, other subclasses are not without risk.	Lack of analytically validated reagents.
		Assess response of different subclasses to different desensitization strategies.	Presence of various subclasses; interpretation is limited in the context of different affinities of the reagents.
Complement Dependent Cytotoxicity (CDC) Assay	Recipient serum is mixed with donor cells (CDC crossmatch) or an established panel of cells for which the HLA specificities are known. Complement is added and cell death is quantified.	A positive CDC crossmatch is associated with hyperacute/accelerated rejection.	*In vitro* response may not be the same as *in vivo*. Less sensitive, more specific for cytotoxic antibodies. Not routinely used to determine individual antibody specificities.
HLA reactive B cells			
HLA tetramers by flow cytometry	Fluorochrome labelled HLA tetramers are used to detect HLA reactive B cells by flow cytometry.	Longitudinally quantify HLA reactive B cells during desensitization. Detect differences in HLA reactive B cell phenotype in response to treatment.	Nonspecific binding (reduced with dual fluorochrome or decoy tetramer). Low sensitivity when limited number of B cells are present. Not all Bmem may be capable of secreting antibody. Does not quantify antibody secretion.
HLA antibody analysis in cultured supernatants following polyclonal B-cell stimulation	Polyclonal stimulation of B cells (6-day culture); detect HLA antibodies in the supernatant on the Luminex platform.	Determine magnitude of Bmem response capable of antibody secretion. Determine Bmem specificities. Improve risk stratification before transplant. Implications for desensitization not well established.	Assumes all Bmem secrete the same amount of antibody. Does not quantify Bmem. Low sensitivity for low frequency Bmem. Unable to characterize Bmem phenotype. Long culture period (6 days) makes it impractical for peri-transplant risk stratification (deceased donors).

(Continued)

TABLE 1 | Continued

Assay	Description	Potential Utility for Desensitization	Limitations
HLA reactive B-cell ELISPOT	Polyclonal B cell stimulation (6-day culture); add HLA multimers to individual wells; detect IgG spots	More precise quantification of HLA reactive B cells with Ab secreting potential.	Need HLA multimer for each specificity of interest. Long culture period (6 days) makes it impractical for peri-transplant risk stratification (deceased donors). Labor intensive and costly.
HLA reactive T cells			
T-cell ELISPOT	Detect HLA reactive T cells using donor or 3rd party inactivated APCs.	Quantify donor reactive T cells. Monitor changes during desensitization.	Most commonly used to measure T cells activated *via* the direct pathway of antigen presentation. Does not differentiate between anergy and deletion post transplantation. Implications in heart transplantation not well established.
Mixed Lymphocyte Reaction (MLR)	Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelled recipient T cells are added to irradiated donor PBMCs; proliferation at day 6 is assessed by flow cytometry.	Assess frequency of donor reactive T cells.	Assay optimization is required to achieve reproducible results. Differential proliferative response between T cell subsets.
Donor reactive T cell repertoire	1. CFSE MLR is performed as above using pretransplant recipient T cells (responders) and irradiated donor PBMCs (stimulators). 2. Sequencing: a. $CFSE^{low}$ T cells are sorted (e.g. CD4 and CD8) and the T cell receptor (TCR) is sequenced. b. unstimulated recipient pretransplant T cells are sorted (CD4, CD8) and sequenced. 3. $CFSE^{low}$ pre-transplant T-cell receptor (TCR) sequences that meet expansion criteria relative to their frequency in the unstimulated sample, are considered alloreactive. 4. Longitudinally follow alloreactive TCRs in the peripheral blood and/or biopsy specimens over time.	Pre-transplant alloreactivity can be followed prospectively during desensitization and post-transplant monitoring. Assess for clonal deletion.	As above. Does not account for donor reactive clones that die prior to the 6-day timepoint during the MLR. Detailed statistical consideration are required for interpretation.

device and generation affects this is still unclear. Early studies in patients bridged to transplant with first-generation LVADs demonstrated diminished T cell responses in mixed lymphocyte reactions while non-TCR mediated activation was less affected (77). Apoptosis was enhanced, especially in the CD4 compartment although the specificity was not established. *In vivo*, the response to intradermal skin challenge with mumps and candida albicans was impaired raising the possibility of T cell dysfunction. Non-HLA humoral sensitization with reactivity to autoantigens has also been described (78, 79). In a cohort of patients bridged to transplant with a second-generation LVAD, this was attributed to natural (polyreactive) antibodies. A potential association with primary graft dysfunction but not rejection was found (80). Collectively, the increased incidence of allosensitization and predisposition to early cellular rejection raises the question of whether selective inhibition of B cell – T cell interactions and/or enhancing regulatory T cell function would be of particular benefit in this population.

Antibodies to Non-HLA Antigens and Their Relevance to the Sensitized Heart Transplant Candidate

Antibodies to non-HLA antigens including the angiotensin II type 1 receptor (AT1R), major histocompatibility complex class I chain-related molecule A (MICA), cardiac myosin, and vimentin have been associated with increased risk of adverse outcomes in heart transplant recipients (81–86). Many of these studies were retrospective, single-center analyses and dissecting the role of pre-transplant non-HLA antibodies *versus* their development post-transplant has also been difficult. In the multicenter CTOT-5 long-term follow up cohort, the persistence at 12-months of anti-cardiac myosin antibodies (but not vimentin) present before transplant was associated with the composite endpoint of death, retransplantation, coronary stent, myocardial infarction, and cardiac allograft vasculopathy, albeit that this association was weak (87). Some but not all studies have described an association between pre-transplant anti-AT1R antibodies and increased risk of ACR, AMR, and CAV (83, 88, 89). Notably, a link between LVAD implantation and the development of anti-AT1R has also been described making this antibody of particular relevance to the heart transplant population (82). Functionally, anti-AT1R can exert agonistic effects on the AT1 receptor, induced ERK kinase signaling in endothelial cells, and promoted vascular changes in a rat kidney transplant model (90) suggesting that a complement/Fc receptor independent mechanism may contribute to its pathogenic effects.

The possibility that non-HLA antibodies synergize with DSA to exert allograft damage is salient to the highly HLA sensitized transplant candidate (83, 91). Non-HLA antibodies may either be directed towards donor polymorphisms or be reactive to autoantigens. Zhang et al, found that transplant recipients who developed anti-MICA antibodies directed at donor polymorphisms (but not against non-donor polymorphisms) were at risk of AMR (84). In a separate study, pre-transplant anti-MICA was associated with inferior kidney allograft survival (92). Thus, in already high-risk HLA sensitized heart transplant candidates, assessment of the non-HLA antibody repertoire and careful donor selection might be warranted. Conversely, in the setting of autoreactive non-HLA antibodies, donor selection would be expected to have less relevance from this perspective.

An important question is whether contemporary desensitization strategies used for HLA antibodies are effective for non-HLA sensitization. This may depend in part on their mechanism of action and the relative importance of antibody concentration/titer to downstream effect. In kidney transplant recipients with malignant AT1R antibodies, plasmapheresis and IVIG, with the addition of an ARB improved allograft survival (90). In the small subset of patients studied, anti-AT1R became undetectable. Furthermore, in highly HLA sensitized heart transplant candidates, bortezomib was associated with a reduction in anti-AT1R (93). The success of using an ARB in conjunction with plasmapheresis/IVIG ± rituximab in anti-AT1R+ heart transplant recipients with allograft dysfunction is not clear. In a recent report, although symptoms were ameliorated, only a fraction of patients experiencing improvement in graft function (94). Further work is needed to establish how anti-AT1R and other non-HLA antibodies should be managed, particularly in highly HLA sensitized heart transplant candidates.

CURRENT APPROACHES TO DESENSITIZATION IN HEART TRANSPLANTATION

Antibody Reduction Strategies

Clinical

The use of IVIG to permit transplantation amongst sensitized transplant candidates was described in the early 1990s as an extension of its observed effects in autoimmune mediated disease (95–99). Today it continues to be included in up to 79% of heart transplant desensitization protocols making it the most commonly used treatment (4). While there have been no randomized controlled trials of IVIG for desensitization in heart transplantation, its efficacy in kidney transplant was formally addressed in the randomized, placebo-controlled IG02 trial (100). Monthly IVIG infusions (2g/kg) led to a modest but significant reduction in PRA and a trend towards an increase in transplantation, albeit after 6-months when the PRA had returned to near baseline.

In a study of 13 sensitized children awaiting heart transplantation, 77% of sensitized patients defined by C1q-single antigen bead (SAB) PRA >18% were successfully desensitized with IVIG and transplanted with 100% 1-year survival (101). Similarly, in 13 children with homograft repair, those receiving higher cumulative doses of IVIG were more likely to respond to treatment although longer follow-up time was a confounding factor (60). IVIG has also been used to desensitize LVAD recipients with newly elevated CDC PRAs after implant. Dowling et al. reported on 4 LVAD recipients that developed an elevated CDC PRA after LVAD implant. All four resolved their CDC PRA to baseline within 6 months of starting IVIG (102). Similarly, John et al. described a cohort of 26 heart transplant candidates supported on an LVAD who were treated with

monthly IVIG and cyclophosphamide. Compared to untreated sensitized candidates (n=45) desensitization reduced the time to transplant and decreased the risk of post-transplant rejection down to that of non-sensitized recipients (103, 104). However, these observations should be considered in the context of the aforementioned points regarding LVAD-related HLA sensitization. Indeed, the magnitude of IVIG's effect has been challenged with the use of the more sensitive SAB assay. Nair et al, described a modest reduction in mean MFI with interindividual variability in response (105). The cPRA was not significantly reduced. A similarly heterogeneous response was observed when both the microcytotoxicity assay (CDC PRA) and Luminex SAB were performed in a small cohort of kidney transplant candidates, albeit that the dose of IVIG was lower (106).

More commonly in heart transplantation, IVIG has been used in conjunction with plasmapheresis (PP). Pisani et al. used PP and IVIG immediately prior to transplant and suggested that outcomes were similar to those of a contemporary unsensitized cohort (107). Similarly, Leech et al. found that plasmapheresis, with low dose IVIG reduced the PRA in many but not all heart transplant candidates (108). Underlying etiology and magnitude of the HLA antibody response may contribute to this heterogeneity as all non-responders in this study were women. Contemporary studies of perioperative plasmapheresis with IVIG also suggest that post-transplant rejection is common, likely because this approach does not address the underlying immune response (9, 109, 110).

Plasmapheresis has also been used as an adjunct to plasma cell therapies (discussed below) with the concept that antibody removal may decrease negative feedback inhibition on plasma cells enhancing protein production thereby sensitizing them to proteasome inhibition (PI). This effect remains to be formally evaluated but recent studies challenge this view. In an iterative trial of PI with and without intermittent plasmapheresis, both strategies led to a similar reduction in bone marrow plasma cells and circulating immunodominant HLA antibodies (10). Our preliminary work has also recently questioned the need for plasmapheresis during desensitization (12). Given i) the increased risk for infection in an already tenuous patient population, ii) challenges managing periprocedural anticoagulation, and hence iii) need for inpatient treatment, contemporary strategies may forgo plasmapheresis. Nonetheless, in very highly sensitized patients, when crossing DSA, its use in the peri-transplant setting may still be warranted.

Mechanistic Considerations

Multiple immunomodulatory effects have been proposed including the neutralization and enhanced elimination of pathogenic antibodies, inhibition of downstream complement activation, and direct inhibitory effects on the cellular immune response (reviewed in (97, 98, 111, 112)). From a desensitization perspective, early work proposed that IVIG effectively neutralized the pathogenic effects of anti-HLA antibodies through antiidiotypic antibodies and potentially complement inhibition (95, 113–116). The presence of anti-idiotypic antibodies to autoantibodies in IVIG preparations has been described (117). In a similar manner, Tyan et al, found that IVIG effectively inhibited cytotoxicity *in vitro*, which was attributed to the Fab2' portion of anti-idiotypic antibodies (114, 115). It was further proposed that IVIG may stimulate endogenous production of anti-idiotypic antibodies with potential protective effects (118). While this may explain the observed reduction in CDC PRA, it has also been attributed to complement inhibition. Either way, this could explain the reduction in CDC PRA seen in at least some patients but would have lesser effect on antibody titer.

IVIG may also enhance the elimination of pathogenic antibodies by saturating endogenous Fc neonatal receptors (FcRns) (119–121). Under physiological conditions, endogenous FcRns rescue endocytosed immunoglobulin, recycling it to the cell surface thereby preventing degradation and extending its half-life in the serum. When FcRns become saturated due to supraphysiological levels of IgG, immunoglobulin is targeted for lysosomal degradation thereby reducing its half-life in the serum. Since FcRn can mediate IgG recycling at a rate 42% higher than production (122), this pathway is critical for maintaining protective immunity but may also drive the persistence of pathogenic antibodies. FcRn-/- mice eliminate IgG markedly faster than wild type, an effect which has been capitalized on to study the use of exogenous IVIG in the treatment of autoimmune disease. In an experimental model of bullous pemphigus, FcRn-deficient mice were resistant to disease and had lower levels of pathogenic IgG, an effect that could be recapitulated in disease-prone wild-type mice by the administration of high-dose IVIG (123). In time course studies, Bleeker et al, used a monoclonal antibody (mAb) to trace the effect of single-dose IVIG on antibody elimination (124). In mice this resulted in a 40% reduction with maximal effect at 3 days. Extrapolating this model to humans, a 25% reduction in autoantibody was predicted with maximal effects at 3-4 weeks although differences between species make interpretation difficult (121). Importantly, it should be noted that enhanced Ig elimination has ramifications for treatment regimens that incorporate mAb therapies with high-dose IVIG. Total dose of mAbs may be reduced or augmented depending on their Fc properties. Thus, until strategic, rigorously tested multidrug regimens are designed to specifically capitalize on these properties, administration of IVIG should be temporally separated from that of mAbs by at least 2 weeks (125).

While IVIG and therapeutic modulation of its pathway may reduce pathological antibodies in the circulation, the underlying immune response is largely unaffected. In patients treated with IVIG and plasmapheresis followed by splenectomy at the time of transplant, histological analysis suggested that immune cell composition was undisturbed (110). Moreover, examination of paired bone marrow samples obtained before and after treatment with plasmapheresis, IVIG, and anti-thymocyte globulin found no significant effect of treatment on the number of alloantibody secreting cells (109).

New Antibody Targeted Therapies for Highly Sensitized Transplant Candidates

Taking advantage of a potent bacterial immune evasion strategy, the IgG-degrading enzyme derived from *Streptococcus pyogenes* (IdeS) has been used for perioperative desensitization in kidney transplant recipients (126). IdeS hydrolizes IgG into the Fab2' fragment and Fc portion thus effectively preventing antibody

dependent cellular cytotoxicity (ADCC) and complement activation. Twenty-five kidney transplant candidates received IdeS 4-6 hours before transplant. All received lymphodepletion (Atgam® or alemtuzumab). Some patients also received IVIG and rituximab. As expected, all circulating IgG was cleaved within 6 hours and remained inactivated for 1-2 weeks after which IgG levels increased. DSA rebound was especially notable in the absence of adjunctive B cell therapies. However, patients who received rituximab/IVIG as adjunctive therapy nonetheless had inflammation on 6-month protocol biopsies. Furthermore, infectious complications were common, and would be anticipated to be even higher/more serious amongst heart transplant recipients many of whom are supported on a LVAD as bridge to transplant. A more 'tempered' approach could be to enhance elimination of pathogenic antibodies by blocking FcRN, a strategy being actively studied in the setting of autoimmunity (127–130). IgG concentrations (but not IgA or IgM) are reduced by ~50% but interestingly, tetanus and influenza A reactive antibodies were preserved (127). The implications for HLA antibodies are currently being investigated. If proven, FcRN inhibition with adjunctive therapies to modulate the underlying immune response (discussed below) could reduce HLA antibodies, preserve protective immunity, and be used to suppress DSA until cellular donor reactivity is controlled.

An alternative approach is to block the downstream effects of pathogenic HLA antibodies. The complement inhibitor eculizumab has been studied in kidney transplant recipients and more recently in heart transplantation with favorable short-term outcomes (131, 132). The strength of this approach is that it permits transplantation across DSA and a positive flow-crossmatch without hyperacute rejection. However, long-term outcomes remain suboptimal. Amongst 30 sensitized kidney transplant recipients with a positive FCXM treated with eculizumab at the time of transplant, 56.7% had evidence of chronic AMR on 5-year protocol biopsy (133). Death-censored allograft survival was similar to FCXM+ controls both of which were reduced compared to those with a negative FCXM. Because this approach does not target the underlying immune response, >50% of patients in this cohort remained FCXM+ at 1-year post-transplant, albeit that the channel shift was reduced. Whether eculizumab can be used in conjunction with approaches that better target the underlying alloimmune response requires further study.

Plasma Cell Therapies

Clinical

Proteasome inhibitors (PIs) induce apoptosis in response to the accumulation of misfolded proteins. The exceptionally high rate of immunoglobulin synthesis by antibody secreting cells (ASCs) underlies their susceptibility to PI-based therapies and their efficacy in treating plasma cell dyscrasias (134, 135). Bortezomib is a first-generation PI that reversibly binds the 20S proteasome. Patel et al. were amongst the first to describe its use in 7 highly sensitized heart transplant candidates all except one of whom had undergone attempted desensitization with other therapies (7 to 177 days prior) (136). Four doses of Bortezomib with corticosteroids were administered, each immediately following 2 sessions of plasmapheresis which was used in an attempt to stimulate protein synthesis by PCs. Treatment led to a marked reduction in cPRA from 62% to 35% (p=0.01) although this was determined 1-2 weeks following treatment making it difficult to differentiate between the effect of plasmapheresis, bortezomib, and prior cycles of treatment. Five patients were transplanted, 4 without evidence of rejection. One died early post-transplant in the setting of graft dysfunction and sepsis. Notably however, only one patient was transplanted against a moderate level DSA. These preliminary results were to be further tested in a multicenter, randomized-controlled trial (CTOT-13). However, this was terminated due to inadequate enrollment highlighting the challenges of these nonetheless important clinical trials. Larger studies in kidney transplant candidates suggest a modest response to bortezomib. Woodle and colleagues used an iterative study design to investigate the use of a bortezomib based protocol (with rituximab and plasmapheresis) (137). In this cohort, 43.2% of patients were successfully desensitized and transplanted, all with a negative flow crossmatch and undetectable DSA. An encouraging reduction in immunodominant antibody was observed, especially when more frequent/higher density dosing was used (8 doses). Yet the response for very high MFI antibodies was less robust. Using a cPRA MFI cut off of 8000, only 50% of patients were defined as responders and this decreased to 38.3% when the CDC PRA was used. Thus, while promising, when extrapolating to the very highly sensitized cohort, the approach remains somewhat limited. In a separate cohort of kidney transplant candidates, 32 doses of bortezomib monotherapy did not significantly reduce cPRA despite a modest reduction in unacceptable antigens (138). Whether the addition of plasmapheresis and rituximab would have improved outcomes, as was used in the study by Woodle et al, is unknown. Either way, the regimen was poorly tolerated with only 50% of candidates receiving the full treatment course without dose reduction.

More recently carfilzomib, a second-generation proteasome inhibitor that binds irreversibly to the 20S proteasome has been used for desensitization. Its superior efficacy to bortezomib for the treatment of multiple myeloma and reduced incidence of peripheral neuropathy make it an attractive alternative (139, 140). In a recent report, 9 treatment naïve heart transplant candidates underwent desensitization with plasmapheresis and carfilzomib 20mg/m^2 followed by 2g/kg of IVIG (141). IgG-cPRA decreased from 76% to 40% (p=0.01) immediately after the last dose of carfilzomib (day 16). Six patients were transplanted, 5 across previously moderate MFI DSA which responded to desensitization (mean MFI pre/post desensitization 5360 and 2012 respectively). All patients received thymoglobulin induction. There was only 1 documented rejection during a median follow up of 35.1 months. This occurred in the patient with a C1q-PRA of 54% suggesting yet again a limitation for more highly sensitized candidates. Carfilzomib was studied in a cohort of kidney transplant candidates divided into two groups (10). A major strength of the study design was that it allowed for the effect of carfilzomib alone to be determined and directly compared to a protocol using pre-carfilzomib plasmapheresis. Both groups received 2 cycles with each cycle consisting of 6 doses of carfilzomib up to 36mg/m^2. Group A received 3

plasmapheresis sessions after the last dose of carfilzomib while Group B had an additional plasmapheresis session added each week prior to carfilzomib. Median maximal immunodominant antibody reduction was 72.8% (Group 1, 69.8%; Group B, 80.1%) with no significant difference between treatment groups (p=0.698). Notably, this included an assessment at day 45 (before both groups received post-carfilzomib plasmapheresis) therefore demonstrating the direct effect of PI on reducing circulating HLA antibodies in the absence of plasmapheresis (Group A). However, antibody rebound was observed within 30 days of completing treatment underscoring the need for adjunctive therapies to sustain the response.

Both bortezomib and carfilzomib carry distinct, but not insignificant side-effect profiles, the former being associated with peripheral neuropathy while the latter, based on the myeloma literature, carries a theoretical risk of cardiotoxicity (140, 142). The magnitude of risk in heart transplant candidates is unknown and must be balanced with the potential benefits given the ultimate goal. Other complications include reversible acute kidney injury, thrombocytopenia, gastrointestinal side effects, and infection (143).

Collectively, these studies highlight the potential of plasma cell directed therapies as part of desensitization regimens, but this remains limited by i) inadequate response, particularly in very highly sensitized candidates, ii) antibody rebound/PI resistance, and iii) their side-effect profile.

Mechanistic Considerations

Bone marrow CD138+ plasma cells (PCs) secrete antibodies with specificities that mirror those in the peripheral blood underscoring their contribution to HLA sensitization (23). Bortezomib and carfilzomib reduce CD138+ bone marrow plasma cells (BMPCs) by ≥ 50%, including the HLA reactive repertoire in sensitized patients (23, 144, 145). PIs disrupt the balance between protein synthesis and destruction leading to accumulation of misfolded proteins, activation of the unfolded protein response (UPR), and apoptosis. In a mouse model of lupus, bortezomib reduces both short and long-lived BMPCs which correlated with the activation of the unfolded protein response (UPR) (146). Similar activation of the UPR has been observed in BMPCs from patients undergoing desensitization with carfilzomib (23). Nonetheless, the response is incomplete and accompanied by brisk rebound following treatment completion (10). Several factors may contribute. Firstly, some PCs may be particularly resistant to PIs owing to their tightly regulated and highly protective bone marrow niche. Indeed, protective immunity appears to be only partially affected with persistence of adequate, albeit somewhat reduced titers of tetanus and measles antibodies (145, 147, 148). Whether CD19-CD38hiCD138+ long-lived plasma cells (LLPCs), which harbor specificities for protective viral immunity also contribute to HLA specificities requires further study (149). Secondly, some PCs may develop PI resistance, potentially through β5 subunit mutations and/or upregulation of components of the immunoproteasome (23, 150). Thirdly, whether PI-induced apoptosis enhances the secretory rate of remaining PCs to maintain equilibrium remain to be established. Importantly, homeostatic proliferation in germinal centers (GCs) is thought to drive repopulation and has been elegantly demonstrated in a non-human primate model of AMR (151). This highlights the critical contribution of memory B cells, which can enter the GC leading to robust recall responses. Taken together, these observations argue for coordinated combinatorial therapies targeting not only the PC compartment but also GCs and memory B cells.

New Plasma Cell Therapies With Potential for Desensitization

Daratumumab is a mAb targeting CD38 which is highly expressed on the surface of plasma cells. Its success in treating myeloma (152) and more recently AL amyloidosis (153), has raised interest in its use for desensitization/AMR (154, 155). Notably, CD38 is also expressed on NK cells, which may provide additional benefit, particularly in the setting of AMR and chronic rejection (35, 36). Doberer et al. described a case of combined smoldering myeloma and DSA+ chronic active AMR treated with daratumumab (156). This report is notable for the extensive analysis of the peripheral blood, bone marrow, and allograft tissue. Consistent with the established mechanism of daratumumab, blood and bone marrow PCs were effectively depleted. This included DSA secreting BMPCs and was paralleled by the elimination of circulating DSA. NK cells were also reduced in the peripheral blood and allograft. Importantly, this was accompanied by a reduction in the molecular AMR score and microcirculatory inflammation. Similar resolution of AMR was described in another case (157). However, in contrast, class II DSA did not appear to be reduced by daratumumab. The authors suggested NK cell depletion as the mechanism underlying this apparently divergent response. While cautious enthusiasm is merited, a concern is that CD38 is also expressed on multiple suppressor cell lineages including IL-10 secreting Bregs, a subset of CD4+CD25+CD127lo Tregs with particularly potent suppressive capabilities, and myeloid-derived suppressor cells (158). In myeloma patients, this was accompanied by augmented CD8+ T effector memory responses with enhanced IFNγ secretion in response to viral antigens (158). Given the potential role of heterologous immunity and/or bystander T-cells in rejection, this latter finding is also noteworthy (28, 159). Interestingly, both of the aforementioned cases showed signs of TCMR on follow up biopsies consistent with the findings in a non-human primate model (155–157). Finally, as with PIs, daratumumab is unlikely to abolish upstream GC reactions and thus, antibody rebound may occur. Nonetheless, daratumumab, potentially in combination with additional immunomodulatory therapies may provide benefit in the pre-transplant setting. Proof-of-concept has been described with partial response in a heart transplant candidate allowing for transplantation across two previously unacceptable antigens (155). A pilot study in highly sensitized heart transplant candidates is awaited (NCT04088903).

The inability to adequately deplete the bone marrow compartment of HLA secreting PCs suggests that their tightly regulated microenvironments (i.e. 'bone marrow niche') may be a barrier to direct PC targeted therapies. Plerixafor inhibits

CXCR4-CXCL12 interactions between PCs and bone marrow stromal cells, with the potential to release them from their niche thus raising the possibility that it could enhance PI mediated effects (160). This question is being addressed in kidney transplant desensitization with preliminary results showing promise (161, 162). Other possible approaches of interest include cytokine modulation and metabolic regulation. These have been reviewed elsewhere (160).

B-Cell Therapies

Clinical

The brisk rebound after plasma cell directed therapies emphasizes the need to target B cell memory. To date, the most commonly used B cell therapy in heart transplantation is rituximab, a chimeric murine/human monoclonal IgG1 antibody directed against CD20, expressed on mature B cells. Near complete depletion of peripheral blood B cells is achieved through a combination of antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and apoptosis (163).

While there are no randomized controlled trials in heart transplant desensitization, evidence from the kidney literature suggests that rituximab 1) has a modest, albeit heterogenous effect on the CDC PRA and T-cell flow crossmatch, 2) may be more effective than IVIG alone at preventing severe AMR/graft loss, and 3) has some effect on rebound but this is often incomplete (164). In an early clinical trial, Vo et al. investigated the benefit of adding rituximab (1g given on days 7 and 22) to an IVIG based desensitization protocol. This approach reduced the mean CDC PRA by 33%. However, like IG02, the PRA remained above 40% and confidence intervals were wide, consistent with a heterogeneous response (mean CDC T-cell PRA 77 ± 18% before desensitization, 44 ± 30% after desensitization) (165). Furthermore, 50% of the patients transplanted had a rejection episode, 31% of which had evidence of C4d staining suggesting that constraint of the amnestic response is incomplete. Nonetheless, the addition of rituximab may have some benefit over IVIG at tempering rebound. A trial comparing IVIG alone to IVIG + rituximab was stopped early due to AMR and 2 graft losses, both of which occurred in the placebo arm (166).

In heart transplantation, Patel et al. described the successful desensitization of 4 sensitized heart transplant candidates with rituximab and IVIG (167). The same group later reported long-term outcomes in 21 heart transplant recipients treated with IVIG and plasmapheresis, 5 of whom also received rituximab (168). Desensitization led to a reduction in antibody, albeit with a heterogeneous response, and patients were transplanted with a negative CDC crossmatch. Five-year survival and freedom from CAV were comparable to non-sensitized recipients. However, this study only considered patients who were successfully transplanted and therefore does evaluate treatment effect *per se*. Further small single-center experience in pediatric heart transplantation has also been cautiously favourable (169, 170). Schumacher et al, described a cohort of 14 heart transplant candidates of whom 8 were classified as responders and 5 were transplanted (170). Treatment with IVIG and rituximab increased the donor pool from 10% to 85% (range 2-100%) amongst responders. Rituximab was continued post-transplant with good short-term outcomes. However, despite cautious optimism based on these select, non-randomized reports, clinical experience parallels the heterogeneous and often inadequate response observed in the kidney RCTs.

Several other clinical observations are noteworthy. Firstly, as seen in CTOT-11, a trial of rituximab in non-sensitized heart transplant recipients, rituximab does not appear to prevent *de novo* DSA, a finding which has also been described in kidney transplantation (171, 172). Secondly, non-selective B cell depletion also affects B cells with regulatory/tolerogenic properties. This has been raised as a potential explanation for the increase in percent atheromatous volume (PAV) in CTOT-11, as well as the higher rate of cellular rejection in at least one rituximab study in kidney transplantation (171, 172). More recently, the ratio of IL-10/TNFα expressed by transitional B cells (T1B, CD24+++ CD38+++) was found to predict T-cell mediated rejection in kidney transplant recipients (173). Perhaps most importantly, as further discussed below, B cell depletion in lymph nodes, spleen, and bone marrow is incomplete. Therefore, there is persistent potential for ongoing GC and extrafollicular antibody responses, as well as antigen presentation to T cells.

Mechanistic Considerations

While the CD20 antigen is widely expressed on most B cell subsets, it is absent on cells at both extremes of the B cell lineage, namely B cell precursors and antibody secreting plasmablasts/plasma cells. With respect to the former, this implies that B cell repopulation will follow depletion, as is typically seen within 12 months post-infusion (174). In terms of the latter, because bone marrow plasma cells can survive decades in protective bone marrow microenvironments, removing precursor B cells will not address HLA secreting PCs persisting in these reservoirs.

However, beyond this, rituximab incompletely eliminates B cells, and in particular, CD27+ memory B cells (Bmem), from the bone marrow, spleen, and lymph nodes (110, 175–178). Ramos and colleagues determined the effect of desensitization on splenic histology and cellular composition in five splenectomized patients who had undergone recent desensitization with rituximab/IVIG/plasmapheresis (110). Despite depletion of CD20+ and CD79+ B cells, CD27+ Bmem persisted. Similarly, CD27+IgD- switched Bmem persisted in lymph nodes from patients with rheumatoid arthritis (176). In contrast, naïve and unswitched Bmem were reduced. Clinically this is consistent with the robust recall response to influenza vaccination following rituximab monotherapy despite the near absence of circulating influenza specific Bmem in peripheral blood (179). Similarly, amongst sensitized kidney transplant recipients treated with rituximab, 34 of 39 HLA antibodies that increased after transplant were associated with epitopes shared with previous allografts or pregnancy (164).

An important implication is the persistence of lymphoid germinal center (GC)-like structures in patients treated with rituximab (180). Histological assessment is further supported by the failure to suppress LN IL-21 mRNA transcription, a

surrogate for Tfh activity (74). Consistent with this, in a humanized CD20+ transgenic mouse model, GC B cells were most resistant to rituximab (181). Together, the persistence of Bmem, GCs, and LLPCs underly the limited ability of rituximab to adequately suppress established HLA antibody responses.

New B cell Targeting Strategies

Inebilizumab is a CD19-directed antibody recently approved for use in patients with neuromyelitis optica spectrum disorder (NMOSD) (182). Because CD19 is expressed on a subset of plasma cells, inebilizumab is an attractive target for desensitization which is being investigated in sensitized kidney transplant candidates (NCT04174677). An alternative approach is to modulate the factors required for B cell survival. To this end, BAFF (anti-B cell activating factor of the tumor necrosis alpha family) modulation has been attempted for desensitization without significant benefit. More recently, the BAFF inhibitor belimumab had modest benefit in preventing proteasome inhibitor induced rebound (183). Targeting survival factors and cytokines may be of benefit when used as part of a multidrug regimen but this requires further study.

Costimulation Blockade Disrupts the Germinal Center and Suppresses the DSA Response

Thus far, the therapeutic strategies discussed focus on eliminating antibodies or the cells responsible for their production. The T-dependent nature of the anti-HLA antibody response suggests T cell- B cell interactions as a rationale therapeutic target. The critical players include CD28:CD80/86, CD40:CD40L, and ICOS : ICOSL and the ability to block these interactions is collectively referred to as costimulation blockade (184). The major clinical target to date has been the CD28:CD80/86 pathway. This has been achieved using the fusion protein CTLA4-Ig (abatacept) and its high affinity variant belatacept (185). CTLA4 binds to CD80/86 thereby preventing CD28 mediated signaling critical for T cell activation (signal 2). This underlies its rationale as maintenance immunosuppression in kidney transplantation with the theoretical advantage of inducing anergy in an alloantigen specific manner when T-cell receptor signaling is left undisturbed (signal 1 in the absence of signal 2). Importantly, in pre-clinical non-human primate studies, belatacept potently suppressed the primary antibody response following sheep red blood cell immunization (185) suggesting a potential advantage over contemporary T-cell focused immunosuppression. Clinically this translated into a strikingly low incidence of *de novo* DSA in phase 3 clinical trials (186).

The ability to control established immune responses with costimulation blockade has been addressed in mouse and non-human primate models which we have recently translated to the clinical setting amongst some of the most highly sensitized heart transplant candidates (cPRA>99%). Early work in mouse models from Anita Chong's lab demonstrated that CTLA4-Ig could inhibit the memory B cell response, collapse ongoing GC B cell reactions, and halt the rise in alloantibody following heart transplantation (24, 187). While post-GC plasma cells were less well controlled, the addition of bortezomib to a continuous CTLA4-Ig regimen, but not bortezomib alone, led to sustained alloantibody suppression which mirrored the clinical response in a small cohort of kidney transplant recipients (188). Parallel studies, in non-human primates performed by Kwun/Knechtle and colleagues demonstrated that belatacept could abrogate the potent GC homeostatic proliferation associated with PI administration (25). This 'dual targeting' approach, tested in a rigorous model of allosensitization, decreased bone marrow plasma cells, lymph node isotype switched B cells and T follicular helper cells resulting in a reduction in DSA before transplant and improved post-transplant survival (148, 189). Moreover, a direct effect on BMPCs has been proposed given the expression of CD28 on a subset of long-lived plasma cells (190). Building on this work, we recently reported on the combination of belatacept with sequential cycles of PI therapy in four extremely highly sensitized heart transplant candidates (12). Our studies revealed a marked reduction in both class I and II HLA antibodies, including those with high MFI that were C1q positive and successful transplantation with a negative CDC crossmatch in three cases. Preliminary findings suggest that the reduction in many HLA antibodies may be sustained with ongoing monthly belatacept infusions. This is bolstered by observations in kidney transplant recipients where, compared to calcineurin inhibitors, belatacept more effectively constrained pre-existing DSA and led to a modest reduction in non-DSA (191, 192). Taken together, this highlights the relevance of rationally designed strategies that integrate clinical observations, with hypothesis driven findings, that have been specifically tested in model systems.

Using Il-6 Inhibition to Modulate the Immune Response in Highly Sensitized Transplant Candidates

A conceivable alternative to direct immune cell targeting is to modulate the cytokine milieu. The theoretical advantage is that multiple levels of the immune response can be simultaneously targeted. Given its pleiotropic nature, IL-6 is an attractive cytokine that is important for both the innate and adaptive immune response. IL-6 supports Th1 and Th2 proliferation, promotes proinflammatory Th17 lineage commitment at the expense of Tregs, contributes to Tfh development, sustains B cell survival, and favors plasmablast differentiation (193). Inhibition of IL-6 can be achieved either by blocking the IL-6 receptor (tocilizumab) or direct neutralization (clazakizumab). Both have been used in kidney transplantation for desensitization and AMR. Tocilizumab is also being studied in non-highly sensitized heart transplant recipients early post-transplant to establish whether it can reduce the incidence of rejection, *de novo* DSA, re-transplantation and death at 1-year post transplant (NCT03644667).

Tocilizumab has been used for desensitization in two distinct cohorts of kidney transplant recipients. Vo et al. reported their experience with tocilizumab + IVIG in a phase I/II

desensitization study (194). All patients had failed treatment with IVIG and rituximab. Five of 10 patients were transplanted with a reduction in their immunodominant DSA. However, the pre-treatment MFIs were <10,000 suggesting a potential limitation for patients with high titer antibodies. In a separate study of 13 highly sensitized (mean cPRA>97%) kidney transplant candidates, a more muted response was observed (195). Twelve patients had at least 1 prior transplant and in 92% the prior allograft remained *in situ* (off immunosuppression). Efficacy assessment was evaluated based on the immunodominant MFI, which in most cases was >10,000. In this cohort, tocilizumab monotherapy had only a marginal effect on HLA antibodies as assessed by MFI with dilutions used when prozone was detected. While an increase in naïve B cells and decrease in plasmablasts was observed, tocilizumab did not significantly change the percentage of pre/post-germinal center B cells, Tfh, and Tfh subsets between baseline and 6 months and there was no significant augmentation of CD3+CD4+CD127-FoxP3+ Tregs. Nonetheless, Treg augmentation with tocilizumab has been reported by others (196, 197). Subsequently, Vo et al, described the use of clazakizumab in combination with plasma exchange and IVIG for desensitization (198). A reduction in class I and II HLA antibody MFI was observed although there was substantial variability in response between patients. Alemtuzumab was used for induction along with standard immunosuppression and clazakizumab was continued post-transplant. Notably, only 1 patient had detectable DSA at 12 months raising the possibility that adjunctive post-transplant IL-6 blockade may be of benefit.

A potential role for IL-6 inhibition has also been described in the setting of AMR. Choi et al, reported on a cohort of 36 kidney transplant recipients with cAMR treated with tocilizumab after failing standard therapies (199). A significant reduction in DSA, C4d deposition, glomerulitis and peritubular capillaritis scores was observed. Graft survival was 80% at 6-years which is higher than that reported in the literature without treatment although a control arm was not included. A subsequent study randomized 20 patients with DSA positive late AMR to clazakizumab *vs.* placebo followed by an open label extension where all participants received study drug (200). Clazakizumab reduced DSA with improvements in intragraft AMR associated findings in some patients during the extended follow up period. Early attenuation of eGFR decline was observed in the clazakizumab arm compared with placebo which was no longer apparent when the placebo arm was transitioned to clazakizumab. Serious infection (n=5) and diverticular disease with complications (n=2) were noted suggesting the need for careful pre-treatment evaluation and close monitoring.

Collectively, these studies suggest that IL-6 inhibition may be of benefit to the allosensitized transplant patient both before and after transplant. The full potential of IL-6 inhibition together with adjunctive strategies for the highly sensitized candidate remains to be determined. Conceptually, synergism between IL-6 inhibition and costimulation blockade could augment GC inhibition while counterbalancing the potential reduction in Tregs. Other combinations are also possible and future studies, perhaps driven by a translational approach, should be considered.

Considering Cell-Based Therapies for the Allosensitized Heart Transplant Candidate

A potential alternative or complementary strategy to the ones described thus far is to modulate the immune response using cell-based therapies such as mesenchymal stromal cells (MSCs) or regulatory immune cells (e.g. Tregs, dendritic cells, macrophages). This approach remains largely speculative for desensitization and a full discussion of the topic is beyond the scope of this review. However, several pre-clinical and conceptual considerations are noteworthy. MSCs have been shown to inhibit alloantibody production *in vitro (*201), reduce DSA in a rat model of transfusion associated allosensitization (202), and increase rejection-free survival in a high-risk corneal transplant model (203). Clinical translation was reported in three HLA sensitized patients (204) although cautious interpretation is warranted given the need to address potential risks of MSC-based therapy. Firstly, the use of allogeneic MSCs has been associated with the development of low-level DSA to the MSC and/or shared kidney-HLA (205). This was prevented when repeat mismatches (MSC and allograft) were avoided (206). Secondly, MSCs have been associated with transient kidney graft dysfunction when given in the immediate post-transplant period (207). This was attributed to early post-transplant inflammation resulting in MSC recruitment to the allograft where they potentiated the proinflammatory response. MSC administration prior to transplant may prevent this. Nonetheless, the possible implications for the heart transplant recipient are noteworthy given that hemodynamic compromise can be fatal. Thirdly, the risk of opportunistic infection and malignancy requires ongoing evaluation (208). Nonetheless, in addition to the aforementioned humoral alloimmune effects, MSCs may modulate multiple layers of the immune response enhancing T-cell suppression, augmenting transitional/regulatory B cells, modulating dendritic cells and macrophage activity (reviewed in (208, 209)). The potential to use 3rd party MSCs in the pre-transplant setting would significantly enhance feasibility in heart transplantation where the timing of transplant is not *known a priori.*

An alternative strategy is to use Tregs to suppress the anti-donor response, ideally in an antigen specific manner. This could theoretically target alloreactive effector T cells while leaving infectious immunity intact. Safety has been preliminarily established in phase I/II trials including those in the multicenter ONE Study (210, 211). However, these trials were done in low-to-intermediate risk subjects with the goal of demonstrating safety and/or permitting immunosuppression minimization. Less is known about the effect on humoral alloimmunity and more saliently, the relevance to pre-transplant allosensitization. In a mouse model of AMR, induced Tregs reduced DSA and allograft infiltration/damage in some, but not all animals (212). More recently, Sicard et al, investigated the effect of donor specific CAR-Tregs (dsCAR-Tregs) on the humoral response in a mouse allogeneic skin transplant model (213). Compared to controls, dsCAR-Tregs reduced the number of DSA secreting B cells, decreased the amount of *de novo* DSA, and prolonged survival. In contrast,

when mice were sensitized prior to transplant, dsCAR-Tregs were neither able to constrain the humoral alloimmune response nor prolong survival suggesting that this approach may not be as effective for sensitized transplant candidates. Whether combining Tregs with adjunctive strategies such as B cell depletion would be of benefit, requires further study. However, practical issues (such as the unpredictability of timing in heart transplantation) and safety implications (including the stability of Treg phenotype) are important to consider in this setting.

DISCUSSION: CHALLENGES AND FUTURE DIRECTIONS

In this review the literature relevant to heart transplant desensitization has been summarized, supported by finding at the cellular level in human studies, and objective rationale derived from model systems. Interpretation is limited by 1) a paucity of standardized clinical trials in heart transplantation, 2) ethical limitations in assigning a control arm, 3) immense variability in the etiology and 'degree' of sensitization, 4) lack of standardization across HLA labs, and 5) deficits in our understanding of the factors driving memory B cell and plasma cell differentiation, persistence, and resistance to desensitization. Given that humoral sensitization rates have doubled over the past 2 decades (4) and that highly sensitized candidates are more likely to die waiting for transplant or become too sick (3), developing safe and effective desensitization strategies is an urgent priority.

One aspect that has as yet not been addressed in this review is the sensitization 'cut-off' meriting desensitization. This remains a challenging issue. Using cPRA based on MFI can be problematic. Not only is MFI an inadequate surrogate for antibody titer, but the choice of MFI cut-off will determine whether the candidate is desensitized thus introducing heterogeneity. Attesting to the variability in practice, in a recent international survey, 21% of centers used cPRA>80% and 21% used cPRA>50% as their cut off. The remainder described using a range of cut-offs from >10% to >90% (4). The next important consideration is whether the desensitization strategy will alter long-term outcomes. Approaches that have the potential to modulate the underlying donor reactive immune phenotype and/or repertoire, as discussed in this review, may be of benefit even when sensitization is more modest. This has yet to be formally evaluated. However, at present, the most commonly used approaches for desensitization are plasmapheresis, IVIG, and rituximab (4). Neither alone nor in combination are these likely to suffice, particularly in the very highly sensitized candidate and the potential for durable effects is modest.

Developing a prioritization policy, as has been implemented in Canada (9), may be of value but is limited by the lack of standardization across HLA labs in the US, differences in what constitutes an unacceptable antigen, and difficulties defining an optimal cut-off. Furthermore, this approach may foster hesitancy to pursue robust immunomodulatory desensitization strategies with the potential to improve long-term outcomes. Whether some patients, perhaps those who are more advanced in age, may be better served by destination LVAD is another consideration. However, this is not an acceptable option in many cases where heart transplantation remains the gold-standard. The focus should therefore be on designing multidrug regimens, driven by drug repurposing, and developed in conjunction with findings from pre-clinical models. While the present review has put forth several strategies with potential to target each layer contributing to the immune response (**Figure 2**), other approaches are forthcoming. Improvements in our understanding of HLA reactive memory B cells and plasma cell characteristics is critical to defining who will derive the greatest benefit from targeted therapies. Post-transplant immunosuppression with better control of B cell memory, ideally in an antigen specific manner, will also be required. Long-term benefit will likely be derived from strategies that modulate multiple layers of the immune response. Despite inroads being made, much remains to be done to ensure equal access to heart transplantation for the highly sensitized

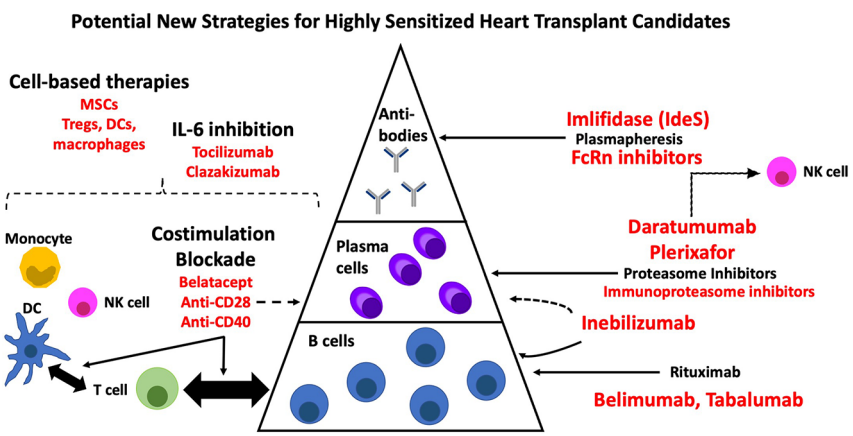


FIGURE 2 | New therapies with potential for desensitization in heart transplantation. DC, dendritic cell; MSC, mesenchymal stromal cell; NK, natural killer cell.

patient with end-stage heart disease. While desensitization continues to be a 'niche' field, the growing incidence in an already high-risk population along with the poor prognosis for those denied transplant underscores the urgent need for attention.

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REVIEW
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Cellular Therapies in Solid Organ Allotransplantation: Promise and Pitfalls

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Donor specific transfusions have been the basis of tolerance inducing protocols since Peter Medawar showed that it was experimentally feasible in the 1950s. Though trials of cellular therapies have become increasingly common in solid organ transplantation, they have not become standard practice. Additionally, whereas some protocols have focused on cellular therapies as a method for donor antigen delivery—thought to promote tolerance in and of itself in the correct immunologic context—other approaches have alternatively focused on the intrinsic immunosuppressive properties of the certain cell types with less emphasis on their origin, including mesenchymal stem cells, regulatory T cells, and regulatory dendritic cells. Regardless of intent, all cellular therapies must contend with the potential that introducing donor antigen in a new context will lead to sensitization. In this review, we focus on the variety of cellular therapies that have been applied in human trials and non-human primate models, describe their efficacy, highlight data regarding their potential for sensitization, and discuss opportunities for cellular therapies within our current understanding of the immune landscape.

Keywords: allotransplantation, donor specific transfusion (DST), donor specific antibodies, mesenchymal stem cell, sensitization, allosensitization, tolerance

INTRODUCTION

Presently, solid organ allotransplantation is hampered by poor outcomes due to the nonspecific effects of immunosuppressive medications, especially calcineurin inhibtors (1, 2). Indeed, improvements in long term graft outcomes in both liver and kidney transplant have been minimal and disappointing in the past 20 years (3–5). In spite of this, there has been a consolidation of immunosuppressive management, with the vast majority of patients now managed on a calcineurin-based regimen (6).

In a distinct vein, there has long been an interest in the ability to specifically modify the immune response to donor antigens. This research has focused on utilizing donor derived biological products across a number of cell types and preparations to condition the immune system to accept the allograft and is based, conceptually, on the work of Sir Peter Medawar (7). In spite of Medawar's early experiments, however, the ability to not simply blunt the total immune response but rather

specifically inhibit the response to the donor has been elusive. Indeed, it has been haunted by the fact that specific inhibition requires that an immune system be exposed to the antigens which it may respond to, giving rise to a risk of sensitization. The use of regulatory cell therapies may be a way to avoid this, but they remain incompletely explored. An overview of cellular therapies currently under investigation may be found in **Figure 1**.

In the present manuscript, we will review the progress made in the application of donor specific cellular therapies starting with an examination of early evidence. We will further describe the multiple cellular strategies currently being investigated in clinical trials including the combination of donor specific transfusion (DST) with novel immunosuppressive regimens, modified cellular therapies, and specific cell type based therapies. Finally, we will examine the future landscape of donor directed therapies and the unmet research need.

THE ORIGINS OF CELLULAR THERAPY IN TRANSPLANTATION

Few experiments in transplantation are as well-known as those of Sir Peter Medawar. In his seminal set of studies, Dr. Medawar built on the work of Dr. Ray Owen (8) and demonstrated that intra-embryonic injection of cells from future skin donors led to tolerance among a subset of mice (7). Ultimately, Medawar was awarded the Nobel Prize for this work and it remains the theoretical basis for many tolerance inducing protocols today. A few specific aspects of the experiment are worth noting. First, the injections occurred early in the development of the mice; therefore, the thymus was robust and actively educating T cells. Thymic development continues to adolescence when involution begins (9). Therefore, these injections in the fetal period had the advantage of potentially utilizing the positive and negative selection programs of the thymus to abrogate responses to the alloantigens.

Additionally, they utilized a mix of donor cells from the "testis, kidney, and splenic tissue" (7). Here, the splenic tissue used suggests a high proportion of B cells as well as professional antigen presenting cells (APCs) which are known to express higher levels of major histocompatibility complex (MHC) class II as well as class I, which is constitutively expressed. That is, there were a variety of antigens expressed by these tissues and therefore available to the recipients. Taken together, these details suggest that both the character of donor derived infusions and the overall immunologic context (in this case most modified by the pre-natal environment) may determine the fate of the immune response.

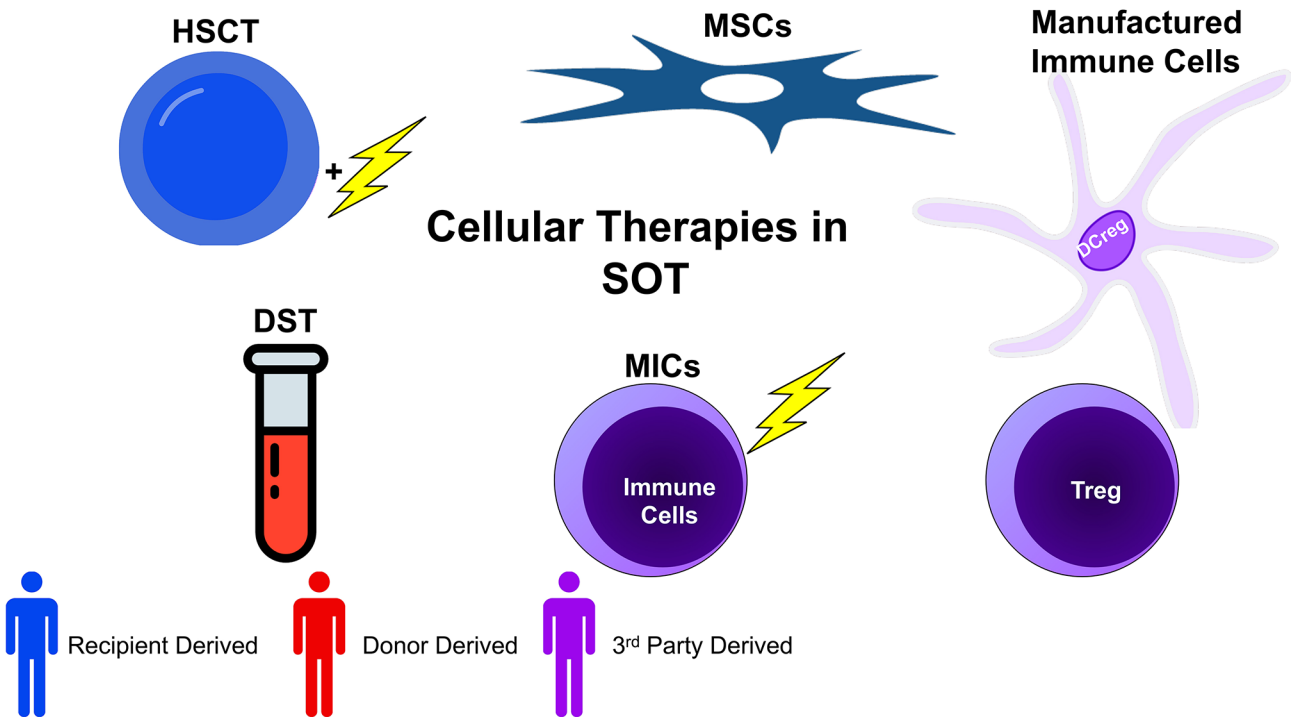

FIGURE 1 | Cellular therapies in transplantation can be broadly categorized into five groups: donor specific transfusions (DSTs), hematopoietic stem cell transplant (HSCT), mesenchymal stem cell based therapy (MSC), manufactured immune cell therapy (using various regulatory cells), and modified immune cell (MIC) therapy. These broad categories can be further subdivided by the source of the cells. Whereas recipient derived cells carry no risk of sensitization (or very little), donor derived cells inherently may sensitize the recipient. Additionally, 3rd party cells, depending on their genetic similarity with donor and recipient, may or may not be sensitizing. When evaluating cellular therapies, both the function and origin of cells are of import.

Though his experiments were conceptually very important, the clinical reality of early transplantation was more complex and the application of cellular products of as treatment remains elusive (10). As adults were the first recipients of transplanted organs, the selective programs of the thymus were less available. Early experience with DST showed the now predictable result of allosensitization leading to the inability to transplant and a divisive discussion within the transplant community regarding the benefit of transfusion pre-operatively (11). Still, results were heterogenous: some early animal models hinted at the benefit of DST (12) and a number of early studies indeed showed that non-leukoreduced transfusions in general (not donor specific) appeared to lengthen graft survival, though the mechanism was unknown (13, 14).

Salvatierra and colleagues were some of the first to specifically evaluate the role of DST on graft survival in a systematic way (15). In this seminal work, they showed that while 1/3 of patients became sensitized (as measured by persistently high levels of alloantibody), 2/3 of patients were able to be transplanted. These patients had improved graft survival compared to similarly mismatched controls (94% *vs*. 56%). Despite a high degree of donor responsiveness as measured by mixed-lymphocyte reaction (MLR), patients who received DST had lower rates of acute rejection in the first 3 months using similar immunosuppression (44% *vs*. 82%). From these experiments, it was difficult to determine the exact differentiating factor which caused sensitization in some and tolerogenic effects in others. However, the authors did show that all patients who became sensitized with DST had also received multiple other blood transfusions. In this instance, the treatment was standardized (non-frozen, non-leukoreduced DST) but the context, potentially measured by the amount of 3rd party (i.e., neither donor nor recipient) blood transfusions, may have modified the ultimate immune response to donor antigen. Concomitantly, experimentation in mouse models showed similar results with improved skin graft survival with DST but not with 3rd party transfusion (16).

Over the next decade, several studies continued to illustrate the principles by which DST may be beneficial. First, evidence showed that the salutary effect of DST was most apparent in human leukocyte antigen (HLA) mismatched recipients, whereas it had little effect in fully haploidentical recipients (17). This was predictable as HLA matched recipients may have fewer antigens to tolerize to and therefore the effect would be of a lower magnitude. Additionally, it is worth noting that even in the Salvatierra study, the graft survival of the high reactivity MLR group was worse than that of the low reactivity MLR group (which did not receive DST), even with the addition of DST (15). Other studies suggested that at least some degree of HLA similarity was needed for improved graft survival with DST (18). Further mechanistic studies were also undertaken around this time which showed that it was possible that APC-depleted DST were more effective at inducing tolerance (19), suggesting that the type of cell was of import. However, due to the continued risk of sensitization, DST fell out of favor throughout the 1990s and early 2000s, culminating in a meta-analysis in 2010 which concluded that the risks of DST outweighed the benefits in the modern immunosuppressive era (20).

COMBINING DST AND COSTIMULATION BLOCKADE

One area where DST continues to be investigated is in conjunction with costimulation blockade. Early murine experiments showed indefinite islet graft survival when DST with small lymphocytes (with or without T-cell depletion) was combined with anti-CD154 (21) or when DST with splenocytes was combined with CTLA4Ig (22) or anti-CD154 (23). Elegant mechanistic experiments showed that these effects may be mediated by deletion of alloreactive cells (24), the production of allospecific suppressor cells (25), or the induction of anergy (26) and appeared to depend at least in part on indirect presentation of donor antigens by recipient MHCs, whereas direct presentation was dispensible (27). Indeed, improved outcomes were achieved using DST in conjunction with rapamycin and anti-CD154 in a rhesus macaque model of skin transplantation, compared to rapamycin and anti-CD154 alone (28). This prompted further trials which showed operational tolerance in kidney transplant in both rhesus macaque (29) and cynomologous (30) models. Trials using belatacept based costimulation blockade, rapamycin, and DST in the form of donor bone marrow have been completed with many patients able to be maintained on belatacept monotherapy with excellent renal function outcomes (31, 32). In these studies, alemtuzumab—an anti-CD52 monoclonal antibody which is a potent depletional induction agent which causes near immediate (33) and long lasting lymphopenia, up to 6 months (34)—was used. This potentially allowed for remodeling of the immune system during its reconstitution (35). Of note, a total of n = 5 (12%) of patients on this protocol developed *de novo* DSA after transplant, though it is unclear what clinical significance these antibodies have.

DST USING MODIFIED CELLULAR PRODUCTS

Another distinct line of investigations has attempted to deliver donor antigen itself in a more tolerogenic manner. Beginning with multiple early studies in mice, Luo and colleagues have shown that pre-treating cellular infusions (in this case splenocytes) with ethylene carbodiimide (ECDI) leads to long term tolerance in a high proportion of mice undergoing islet transplantation into the kidney capsule (36). Of note, they showed that this effect was donor specific, with no prolongation of 3rd party grafts. Multiple findings from this first study are mechanistically interesting. First, they found that the ablation of Treg *via* an anti-CD25 antibody prior to ECDI-treated cell infusion abrogated tolerance induction but late (120 days after transplantation) depletion of Treg did not.

Additionally, they showed that the PD-1 axis was necessary for tolerance induction.

Following on from these studies, it was demonstrated that ECDI treated splenocytes could condition tolerance in murine cardiac allografts (37). Additionally this tolerance could be achieved not only with splenocytes but also culture-expanded B cells or cryopreserved cellular products and could be combined with clinically relevant immunosuppressive regimens (38). Mechanistically, these infusions cause an expansion of myeloid derived suppressor cells (MDSCs) both at the site of the allograft (37) and systemically (39) which appear to be important in abrogating deleterious T-cell responses. ECDI treatment leads to apoptosis of the infused cells and therefore presentation of donor antigen in an apoptotic context. This should attenuate any inflammatory immune response and is generally termed efferocytosis—the clearing of apoptotic cells in a non-inflammatory manner (40). Specifically, it has been demonstrated that this anti-inflammatory program is mediated by the receptor tyrosine kinase MerTK which participates in the phagocytosis of apoptotic cells in an allotransplantation context *via* suppression of IFN-α signaling and subsequent expansion of MDSCs (41).

Recently, this strategy has also expanded into a non-human primate (NHP) model of islet cell transplantation, again, using splenocytes as the donor cell source supplemented by culture-expanded donor B cells from the peripheral blood. This lead to long term tolerance in a high proportion of recipients (42). Multiple experiments confirmed that there was a decrease in donor-specific T-cell response and an increase in the proportion of MDSCs. Of note, this protocol was not successful when primates had previous sensitization to donor antigens. However, in mice, ECDI treated splenocyte infusion when combined with rapamycin and anti-CD154 (MR1) led to prolonged survival in sensitized recipients relative to rapamycin and anti-CD 154 alone (43). Overall, ECDI treated cells represent one cellular therapy that is donor specific, that does not appear to cause sensitization, and may allow for the reduction or withdrawal of immunosuppression in select patients. Further work is needed to translate this therapy into vascularized grafts in NHP models in anticipation of human trials.

Another line of investigation that parallels the treatment of splenocytes with ECDI is the use of modified immune cells (MICs). Terness and colleagues initially observed that DCs treated with mitomycin C become tolerogenic in an *in vitro* context, potentially *via* downregulation of costimulatory molecules CD80/CD86 and ICAM-1. Intriguingly, T cells exposed to these DCs became stably tolerized to the antigens presented as these cells could not be restimulated after co-culture with these MICs (44). Further experiments showed that MICs prolonged graft survival in rat heart transplantation and again showed a phenotype of downregulated costimulatory molecules. Indeed, they achieved the same clinical effect using antibodies that coated DC costimulatory molecules and blocked the productive interaction of other cells with these DCs (45). They further extended these results from sorted DCs to whole PBMC in both rat and porcine contexts with improved graft survival with mitomycin C treated PBMC infusion (46). They also showed improved survival in a vascular composite allograft context in rats (47). Recently, they completed a trial in humans where they showed both safety of MICs derived from whole PBMC as well as specific inhibition of donor responses in patients treated with MICs (48). However, as this was a phase 1 study, patients were maintained on CNI for immunosuppression and no comparator group was examined. Of note, they also showed an increase in regulatory B cells and did not observe any sensitization in the study. In sum, modified cellular therapies are a promising but incompletely studied form of cellular therapy that may specifically inhibit the recipient response to donor.

COMBINED HEMATOPOIETIC STEM CELL TRANSPLANT AND SOLID ORGAN TRANSPLANTATION

Another area where a form of DST continues to be investigated is in chimerism protocols. Early murine models showed tolerance could be achieved by the depletion of the recipient immune system followed by concomitant hematopoietic stem cell transplant (HSCT) and solid organ transplant from the same donor (49). Within a few years, this result had also been replicated in NHP (50). Mechanistically, this tolerance is thought to be due to chimerism (51). Though the outcomes were exciting, there was some hesitation as macrochimerism can lead to graft *vs.* host disease (GVHD), especially with the use of HLA-mismatched donors (52). Studies using combined HSCT in conjunction with solid organ transplant in humans were first pursued among patients with multiple myeloma who also qualified for kidney transplant. In these patients, they were conditioned using cyclophosphamide, anti-thymocyte globulin (ATG), and thymic irradiation. Though initial results with one (53), and then six subsequent patients (54) were encouraging, long term follow-up demonstrated GVHD (either acute or chronic) in 4/7 patients and the need for at least some immunosuppression in 3/7 patients (55).

In a more targeted way, the same group as above also performed combined HSCT and kidney transplant in five patients using a non-myeloablative regimen consisting of cyclophosphamide, thymic irradiation, and anti-CD2 monoclonal antibody, and a short course of cyclosporine. Four out of five patients were able to be weaned off all immunosuppression with stable graft function. Interestingly, the second patient on this protocol developed acute humoral rejection with early graft loss (day 10) in spite of a negative crossmatch, though none of the patients developed GVHD (56). Other groups have also attempted to achieve tolerance using combined HSCT and kidney transplant with variable success. One group has utilized total lymphoid irradiation and ATG for conditioning with a T-cell depleted HSCT infusion which has led to at least transient chimerism and the ability to withdraw immunosuppression in 16/22 (72% of

patients) who were HLA-matched (57–59). However, none of the patients on this protocol who were HLA-mismatched have yet been able to undergo immunosuppression withdrawal. A final group has attempted HSCT with fludrabine, cyclophosphamide, and whole-body irradiation condition in conjunction with kidney transplant and shown the ability to withdraw immunosuppression in most patients that have stable chimerism, with some now up to 9.5 years off all immunosuppression (60–62). However, only 6/20 total patients and 6/15 who underwent weaning are currently still off immunosuppression. Though these protocols have been very successful in achieving tolerance in a small number of patients, the high morbidity and continued concern regarding GVHD has limited their widespread adoption.

A SHIFT TO CELL TYPE BASED THERAPIES—THE RISE OF MESENCHYMAL STEM CELLS

As the field of transplantation shifted away from DST based strategies and early enthusiasm for non-CNI based therapies waned due to concerns about increased rates of acute rejeciton (63–66), there was a concomitant increase in interest in cellular therapies of specific cell types, especially ones that may be derived from the recipient themselves. One early stream of investigation targeted the use of mesenchymal stem cells (MSC). MSCs are heterogenous progenitor cells which can differentiate into mesodermal tissues, are adherent to plastic, and express certain cell surface markers. Of note, MSCs may be derived from many tissues but in practice are mostly isolated from bone marrow *via* culture methods that take advantage of the specific propensity of MSCs to adhere to plastic (67). Regardless, expansion of cells is required as the dose used is approximately 1.5 x 10^6 cells/kg body weight (68). It should be mentioned that the preparations of MSCs do vary widely, and some groups have shown that both cryopreservation itself and the way in which MSCs are prepared after cryopreservation, for example, influences their immunosuppressive effect (69).

The first description of MSCs as immunosuppressive was in 2002 when investigators utilized MSCs in a skin transplantation model among baboons (70). They noted that MSCs inhibited MLRs to alloantigens and that MSC administration to baboons prolonged skin graft survival. Importantly, they also noted that the effect of MSCs was independent of their origin. That is, MSCs prolonged skin grafts regardless of whether the grafts were from the same donor as the MSCs or 3rd party, suggesting a general immunosuppressive effect, not an allospecific one.

Mechanisms of MSC's immunosuppressive effect are various and have been reviewed extensively in the literature (71). For the purposes of this review, the most salient mechanisms are the generation of tolerogenic APC. Early experiments showed that MSCs decreased ability of co-cultured dendritic cells (DCs) to stimulate T cells, including in an allostimulatory context (72). Further experimentation showed that this effect was in part due to the inhibition of DCs from entering the cell cycle (73) and potentially mediated by the soluble factors IL-6, IL-10, and hepatocyte growth factor (74). Additionally, MSC conditioned DCs have been shown to preference the generation of Treg *via* a CCL18 dependent mechanism (75). Indeed, taken together, these data together suggest that MSCs may ultimately change the context in which antigen is presented and therefore promote a tolerogenic phenotype. However, it is sobering to think that while MSCs may modify APCs to prevent a productive response to certain antigens, other cellular therapies may instead potentiate responses.

Consistent with the above mechanisms, further animal studies after Bartholemew's initial description showed prolongation of graft survival regardless of origin of MSC. An early study in rat liver transplantation showed graft survival prolongation with MSC derived from donor, third-party, or syngeneic animals (76). Further studies in rat corneal transplant also showed prolongation of graft survival with third-party derived MSCs (77). Experiments in mouse models of heart (78) or kidney (79) transplantation showed prolongation of graft survival with the infusion of syngeneic MSCs when administered pre-transplant.

The first human trials of MSCs in transplantation were spurred on by safety in other fields (80, 81) and culminated in early clinical studies which showed the safety of MSC infusion among kidney transplant recipients (82, 83). Due to the nonspecific nature of the immunosuppressive effects of MSCs, early trials generally used autologously derived MSCs—thought to be the safest product—combined with kidney transplantation (84–86). In a randomized controlled trial setting, autologous MSC infusion with kidney transplantation and tacrolimus showed a lower incidence of acute rejection and opportunistic infection (83). However, the incidence of rejection in the standard therapy group was high (approximately 20%) and longer follow-up remains to be reported. Later trials in kidney (87, 88), liver (89, 90), and lung (91) transplantation utilized 3rd party derived MSCs and demonstrated a similarly good safety profile.

One study did use donor derived MSCs which have the theoretical benefit of both nonspecific immunosuppression combined with antigen specificity. Again, the authors demonstrated a good safety profile and were able to use a lower dose of tacrolimus in the MSC group compared to the standard immunosuppression group (92). Of note, there was no assessment of the development of alloantibody in this trial. In the opposite manner, a study used MSCs which were mismatched to the recipient HLA *and* the donor HLA (i.e., they were no "repeated mismatches") and showed good safety (93). This study did assess for sensitization to the specific 3rd party alloantigen and did not observe it. Though one recipient had pre-existing allo-antibody to an MSC donor antigen, the antibody titer did not change with MSC infusion.

An interesting result from an early autologous MSC safety trial was the detection of an MSC infiltrate in a kidney graft when MSCs were administered 7 days after transplant (82). Though this did not lead to any production of antibody (the MSCs, again, were autologous) or lasting graft damage, there was a transient increase in creatinine. This may have been due in part to the

proinflammatory environment of the recently implanted kidney. Indeed, a body of research exists that shows that innate signaling *via* molecules such as damage associated molecular patterns (DAMPs) (94) may modify the alloresponse. Similarly, these factors may be important when considering the infusion of dynamic cellular therapies.

One other important consideration of cellular therapies generally is their safety, which has been extensively reviewed previously for MSCs (95, 96). Important to transplant, prior studies have found that MSCs may promote a pro-coagulable state *via* the instant blood mediated inflammatory reaction (97), though administration directly into the bone marrow may mitigate this (98). Regardless, the clinical context of MSC therapy must be taken into consideration as this hypercoagulability may be deleterious to certain clinical conditions, for examples, in COVID-19 infeciton (99). In vascularized organ transplant, the potential for thrombosis of newly anastomosed vessels should lead to the tracking of these types of events when cellular therapies are utilized.

Overall, the data show that, while safe and slightly immunosuppressive in humans *in vivo*, MSCs do not themselves appear to condition for tolerance or cause drastic shifts in the recipient immune system. Indeed, a recent meta-analysis came to much the same conclusion (100). Of special note, present data do not suggest that MSCs are sensitizing, potentially due to their concomitant immunosuppressive effects, which raises the possibility of utilizing them as a delivery vector for alloantigen.

OTHER CELL TYPE BASED THERAPIES—TREG AND BEYOND

Besides MSCs, many other cell types have been investigated. The largest study of regulatory cell based immunosuppressive products, the ONE study (101), was recently published. In this study, the safety of six different autologously derived cellular products was assessed using seven single arm studies that were harmonized for comparison. A single control arm was standard of care therapy for living donor renal transplant recipients using basiliximab induction, tapered steroid, mycophenolate mofetil, and tacrolimus. The six phase 1/2a interventional single arm studies consisted of two polyclonal Treg studies, two donor-antigen specific Treg studies, a tolerogenic DC study, and a regulatory macrophage study. As these were safety studies, no minimal graft outcomes were reported but all infusions were well tolerated and rejection events were similar between the control and interventional studies. Additionally, there were similar rates alloantibody production between the control and interventional studies. As all products were derived from recipients, the risk of sensitization was low, though the donor specific Treg were incubated with donor cells in order to achieve their specificity. A recent subset analysis of the natural Treg infusion data from this study has shown that these patients were more likely to be weaned to a monotherapy immunosuppression regimen (102). Still, the preparation of these cells is laborious and the appropriate timing of infusions remains unknown.

CONCLUSIONS

Cellular therapies, especially those derived from donors, carry great potential but also great risk. Early studies showed that DST may have a salutary effect in certain patients, but an inability to determine who may benefit from these transfusions and the high degree of sensitization (up to 1/3 of patients) led clinicians to abandon these therapies. A shift towards specific cell type based therapies has been productive in improving the field's ability to manufacture and deliver consistent therapies but has yet to revolutionize the way in which we approach immunosuppression. Modified cellular therapies are on the horizon and represent an

TABLE 1 | Ideal properties of a cellular infusion and whether current therapies meet those criteria.

Characteristic	Explanation	DST	HSCT	Modified immune cells	MSC	Manufactured regulatory cells
Readily available	The ideal infusion would be readily available for administration at a reasonable timeframe with relation to transplant. E.g., Autologous therapies that could be derived over the course of a workup, donor derived therapies that are amenable to generation even in a time sensitive deceased donor context, or 3rd party infusions that could be used "off-the-shelf."	+/-	+/-	+/-	+	–
Specific to donor	Through either modification of the recipient immune system or delivery of donor antigens in a tolerogenic manner, the cellular infusion would specifically inhibit the recipient immune system from responding to donor antigens.	+	+	+	+/-	–
Lack of sensitization	Infusions should not cause sensitization (i.e., the generation of productive anti-HLA antibody) to either the donor or any other individual.	–	–	+	+ (autologous)	+
Effective regardless of sensitization status	The ideal infusion would be able to not only prevent the generation of responses to donor in recipients that were naïve to their donor but also delete pre-existing responses in order to expand the donor pool for highly sensitized individuals.	+/-	+/-	+/-	+	Unknown
Does not interfere with standard immunosuppression	Infusions should be compatible with a wide variety of immunosuppressions such that patients may be placed on the most appropriate therapy for their clinical condition	+/-	–	+	+	Unknown

(+), does meet the requirement; (-), does not meet the requirement; DST, donor specific transfusion; HSCT, hematopoietic stem cell transplant; MSC, mesenchymal stem cells.

exciting development that is grounded in long standing understanding of mechanisms of the immune response. Combinations of DST with costimulation blockade have emerged as a promising approach. However, questions remain regarding sensitization, long-term outcomes, and the pool of patients appropriate for costimulation blockade-based therapy. Hematopoietic stem cell transplant in conjunction with solid organ transplant is less clinically applicable due to the morbidity involved with conditioning, even in the absence of high rates of GVHD. Regardless of the cellular therapies pursued, there are key characteristics that these therapies must possess to be most useful (summarized in **Table 1**).

Further phase 1 studies should continue to follow the example of the ONE study and others who attempt to gain as much information as possible with the most parsimonious control group. Additionally, further investigations into modifications of donor cells may be one way to overcome the significant hurdle that is sensitization. Transplantation is the original precision medicine, with HLA matching and crossmatch testing ensuring that organs implanted function appropriately and for the longest possible time. Cellular therapies ought to consider this precision in their development given the cost and potential for adverse events. In the future, the extent to which a therapy can inhibit a donor specific response relative to its overall immunosuppressive effect may become the most important metric, rather than a simplistic view which preferences only the inhibition of rejection episodes.

AUTHOR CONTRIBUTIONS

BS drafted the manuscript. JO performed critical review and revision of the manuscript. CN performed critical review and revision of the manuscript. XL performed critical review and revision of the manuscript and oversaw the drafting process. All authors contributed to the article and approved the submitted version.

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Histopathologic Features of Antibody Mediated Rejection: The Banff Classification and Beyond

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Antibody mediated rejection (ABMR) in the kidney can show a wide range of clinical presentations and histopathologic patterns. The Banff 2019 classification currently recognizes four diagnostic categories: 1. Active ABMR, 2. Chronic active ABMR, 3. Chronic (inactive) ABMR, and 4. C4d staining without evidence of rejection. This categorization is limited in that it does not adequately represent the spectrum of antibody associated injury in allograft, it is based on biopsy findings without incorporating clinical features (e.g., time post-transplant, *de novo* versus preformed DSA, protocol versus indication biopsy, complement inhibitor drugs), the scoring is not adequately reproducible, and the terminology is confusing. These limitations are particularly relevant in patients undergoing desensitization or positive crossmatch kidney transplantation. In this article, I discuss Banff criteria for these ABMR categories, with a focus on patients with pre-transplant DSA, and offer a framework for considering the continuum of allograft injury associated with donor specific antibody in these patients.

Keywords: antibody mediated allograft rejection, pathology and clinical outcomes, diagnostic criteria, kidney, transplant, sensitized, alloantibody

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INTRODUCTION

Since its initial meeting in 1991, the Banff classification for allograft pathology has become the most commonly used classification system for renal allograft pathology. Pathologists, HLA laboratory directors, nephrologists, surgeons, and researchers gather every other year to discuss recent advances in the field and to revise the classification system, and a meeting report with consensus opinion is published. The Banff schema first recognized antibody mediated rejection (ABMR) as a diagnostic entity in 2001 (published in 2003), making use of the complement split product C4d as an immunophenotypic marker of ABMR on biopsy (1). The ABMR criteria have been revised over the years, and continue to be revised, as knowledge progresses.

OVERVIEW OF CLINICAL CATEGORIES OF ABMR AND BANFF ABMR CATEGORIES

ABMR can show a wide variety of clinicopathologic features, from hyperacute rejection with primary graft nonfunction, to early acute ABMR in positive crossmatch (+XM) kidney transplants, to progressive graft dysfunction or proteinuria years post-transplant. ABMR features are also seen commonly on protocol biopsies from patients who have preformed (pre-transplant) DSA; these patients have stable graft function and no proteinuria (2). There are additional clinicopathologic features in kidney transplant patients with *de novo* DSA, immunosuppressive medication nonadherence, or combined cellular rejection and ABMR (3–5). The challenge with any classification system is to fit a wide variety of potential clinicopathologic features into a reasonable number of distinct diagnostic categories, which ideally would have specific corresponding treatment protocols.

As of 2019, the Banff schema recognizes four diagnostic categories: 1. Active ABMR, 2. Chronic active ABMR, 3. Chronic (inactive) ABMR, and 4. C4d staining without evidence of rejection, The first category, "active ABMR", requires 3 diagnostic criteria: histologic evidence of acute tissue injury, evidence of current or recent antibody interaction with the endothelium (usually C4d), and serologic evidence of DSA (although C4d staining or validated transcripts may substitute for DSA). Histologically, microvascular inflammation (MVI), also known as capillaritis, qualifying for this category is a glomerulitis (g) score + peritubular capillaritis (ptc) score of 2 or greater. Other acute tissue injury patterns are acute tubular injury, thrombotic microangiopathy, and less commonly arterial lesions of endothelialitis, fibrinoid necrosis, or transmural inflammation. Chronic active ABMR has a similar three criteria, but with histologic evidence of chronic tissue injury, such as transplant glomerulopathy (TG) attributable to ABMR. Chronic (inactive) ABMR shows histologic evidence of chronic tissue injury, but without capillaritis and without C4d deposition in peritubular capillaries.

The final category is peritubular capillary C4d staining without evidence of rejection, previously referred to as "accommodation" (6). This category primarily applies to ABO blood group incompatible transplants, which show positive C4d staining in even 80% of protocol biopsies and the staining does not correlate with peritubular capillaritis (7, 8). Despite positive C4d staining, ABO blood group incompatible kidney transplants show the same rate of capillaritis as conventional kidney transplants (8). Although some protocol biopsies in patients with +XM (anti-HLA DSA) transplants show positive C4d staining and no histologic evidence of tissue injury, and thus qualify for the category of accommodation, the state of accommodation in patients with anti-HLA DSA is likely temporary and unstable (8).

EARLY ACUTE ABMR IN +XM KIDNEY TRANSPLANTS

The Banff 2019 category of "active" ABMR (previously known as "acute" or "acute/active" ABMR) by itself encompasses a wide variety of clinicopathologic features. Patients undergoing +XM kidney transplants are at increased risk of early acute ABMR within the first month post-transplant (9, 10). This type of ABMR is associated with very high serum DSA levels (measured at the time of biopsy), C4d deposition in peritubular capillaries, and graft acute tissue injury with thrombi and acute tubular injury (11). Clinically, although uncommon, this type of acute ABMR has been difficult to treat (11, 12). There is a high rate of graft loss if not recognized and treated (13, 14). More recently, early acute ABMR has been treated with the terminal complement inhibitor eculizumab (15). The current standard of care for treatment of this particular type of acute ABMR involves plasmapheresis, intravenous immunoglobulin, and corticosteroids, and adjunctive therapies of complement inhibitors, rituximab and splenectomy may be considered (16).

Histologically, these cases of early acute ABMR with high serum DSA levels often show an "ATN-like" (acute tubular necrosis) phenotype (Banff "grade 1") with few marginated inflammatory cells (particularly neutrophils) in glomerular and peritubular capillaries, along with glomerular thrombi (see **Figure 1**, left panel). Some cases do show moderate microvascular inflammation (g + ptc >/=2), particularly when the pathologist is aware of the clinical consideration of acute ABMR as a diagnostic possibility with therapeutic implications (Banff "grade 2") (11, 17, 18). These early acute ABMR biopsies are essentially always C4d positive, and C4d positivity correlates with the serum DSA level (11). It should be noted that the capillary inflammation does not mirror the clinical severity of this ABMR manifestation: a higher Banff capillaritis score does not indicate a more severe acute rejection phenotype. In this way, ABMR is different from acute cellular rejection, tubulointerstitial type (Banff grade IA or IB or borderline rejection), where more extensive inflammation with a higher Banff i or t score reflects a more severe rejection (19).

As these biopsies show evidence of complement activation, colleagues at Mayo Clinic conducted a pilot study of +XM kidney transplant recipients using a terminal complement inhibitor, eculizumab, to prevent early acute ABMR (20). Compared to historical +XM control patients, there was a marked reduction in the rate of early acute ABMR: overall, 41% (21/51) of controls developed early acute ABMR, compared to 7.7% of patients (2/26) in the eculizumab group (p=0.0031). Moreover, of those who developed high DSA levels within the first month post-transplant, and thus were at risk for early acute ABMR, 100% (20/20) of the control patients developed early acute ABMR, compared to 2/13 (15%) of the eculizumab group (p<0.0001). Both eculizumab treated and control patients in this group with high DSA (at the time of biopsy) showed C4d deposition in peritubular capillaries. Eculizumab is a terminal complement inhibitor, inhibiting the complement cascade downstream of C4d, and so even patients with effective terminal complement inhibition would be expected to show C4d deposition on the kidney biopsy. [These two patients who did develop early acute ABMR while receiving eculizumab responded well to treatment with plasmapheresis alone; further investigation of these patients' serum suggested an IgM anti-HLA DSA, which likely responds differently to plasmapheresis treatment (21)] The striking results of this study suggests that this early acute ABMR in +XM patients is complement-mediated, rather than

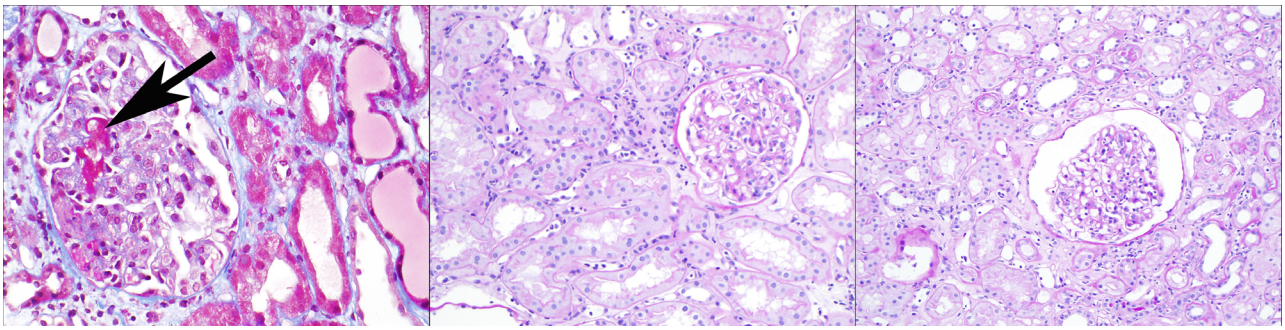

FIGURE 1 | Three clinicopathologic phenotypes of ABMR, one Banff diagnosis: In the left panel (Masson trichrome stain), a patient 2 weeks after positive-crossmatch kidney transplant has acute kidney injury; the biopsy shows glomerular thrombi, acute tubular injury, and minimal capillaritis, and diffuse C4d deposition in peritubular capillaries. In the middle panel (periodic acid Schiff stain), a patient 4 months after positive-crossmatch kidney transplant has stable graft function and undergoes protocol biopsy; the biopsy shows glomerulitis and peritubular capillaritis; C4d staining is negative. In the right panel (periodic acid Schiff stain), a patient 2 years after kidney transplant has stable graft function and undergoes protocol biopsy; the biopsy shows glomerulitis and peritubular capillaritis; C4d staining is negative. All of these biopsies pictured would be assigned the Banff diagnosis of "active ABMR", although clearly the clinical settings are different.

simply "antibody-mediated" as the name implies. I believe we should recognize this distinct clinicopathologic entity with its own name, such as early acute antibody and complement-mediated rejection in patients with preformed DSA.

ACTIVE (SMOLDERING) ABMR

After the first few months post-transplant, patients with pre-transplant DSA may develop a more indolent form of ABMR, characterized histologically by mild to moderate capillaritis involving the glomeruli, peritubular capillaries, or both ("microvascular inflammation", or MVI). These findings are commonly observed on protocol biopsies from patients with normal graft function and no proteinuria, ranging from 3 months to 5 years post-transplant (8, 22–24). This pattern of injury, in the absence of graft dysfunction, is sometimes referred to as "subclinical ABMR". C4d may be focally positive (more common) or diffusely positive, and ranges from approximately 50% to <20% (less commonly positive on 5 year post transplant protocol biopsies) (8, 24). These changes are associated with later development of TG, and the risk of TG is greater in patients with C4d positivity on biopsy. MVI also commonly occurs in conjunction with chronic ABMR lesions, such as TG, once they have developed.

It is unclear how patients with MVI on biopsy, with or without early TG, should be treated. Since C4d is generally not diffusely positive, we can hypothesize that: (1) the corresponding serum DSA levels are low, and thus likely not responsive to plasmapheresis; and (2) the mechanism of injury may not be complement-mediated, at least not in the way early acute ABMR is complement-mediated. Supporting (2), a subset of patients showed endothelial injury changes and developed MVI, and even TG, even while receiving the terminal complement inhibitor eculizumab (22, 25). Treatment of this lesion is under investigation in clinical trials. Also, prevention, including through kidney donor paired exchange programs and "low positive" crossmatch transplantation, will help to reduce graft loss due to chronic ABMR (12).

According to the Banff classification, MVI (with the threshold of g + ptc >/= 2) is an "active" ABMR lesion. The current Banff schema thus may place some cases of early acute ABMR in the same category as a 2 year post-transplant protocol biopsy with mild glomerulitis and peritubular capillaritis and is C4d negative. (See **Figure 2**) Early acute ABMR is an aggressive form of acute ABMR with specific treatment protocols; while the latter, which we could term "smoldering ABMR", appears to be more indolent and would not respond to the same treatment (14). It is important to recognize that the degree of capillaritis does not necessarily correspond to the severity or "activity" of the rejection. Incorporating time post-transplant, preformed versus *de novo* DSA status, and other clinical information such as acute kidney injury or protocol biopsy would inform pathologists and nephrologists as to a better classifying diagnosis along this spectrum of antibody associated disease.

CHRONIC ABMR

Chronic active ABMR occurs when one or more of the "chronic" features are present on a biopsy: TG, peritubular capillary basement membrane multilayering (PTCBMML, also known as peritubular capillaropathy), or transplant arteriopathy. Of these, TG is the best characterized lesion of chronic ABMR. By definition, TG is a pattern of injury with glomerular basement membrane duplication in the absence of subendothelial immune complex deposits. TG is most commonly due to chronic ABMR, but the pattern may also be related to chronic thrombotic microangiopathy and/or hepatitis C infection (26). Biopsies with TG due to ABMR may show negative, minimal, focal or multifocal, or diffuse C4d deposition in peritubular capillaries. Approximately 20-40% of biopsies with TG due to ABMR show positive C4d staining, but this percentage will vary widely and depend on many factors –

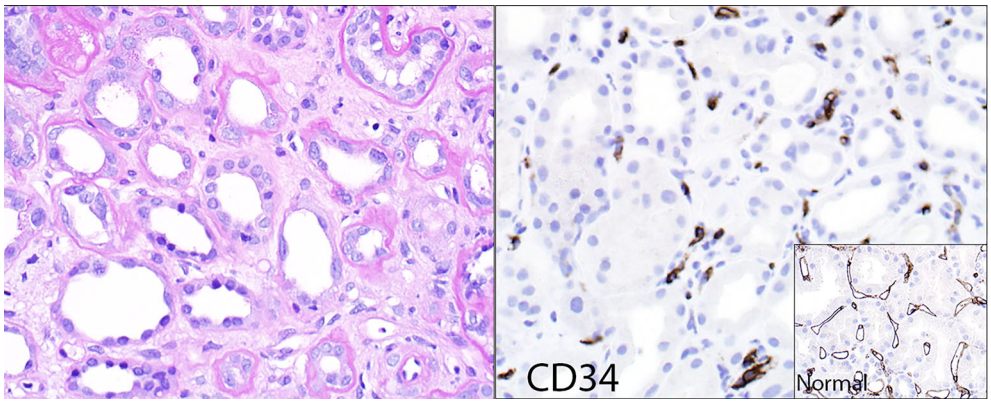

FIGURE 2 | Loss of peritubular capillaries. Some transplants with chronic ABMR show such significant loss of peritubular capillaries that the capillaries simply no longer exist to show capillaritis. The left panel (hematoxylin & eosin stain) shows minimal peritubular capillaritis; the right panel shows the endothelial marker CD34 of the same case, revealing remnants of peritubular capillaries that were presumably destroyed by rejection. The insert shows a CD34 stain of a reference kidney transplant case without peritubular capillary loss.

protocol versus indication biopsy, +XM kidney transplant status, preformed versus *de novo* DSA timing of the biopsy post-transplant, presence of concurrent cellular rejection, and immunosuppressive medication non-adherence (27–29). While TG overall has a bad prognosis, the prognosis is variable. TG does have a worse prognosis when peritubular capillaries are C4d positive – likely reflecting a more "active" rejection phenotype with higher DSA levels and complement activation (27, 28). Other clinical, laboratory, and biopsy features besides C4d may be helpful in determining the "activity" and prognosis of TG for a particular patient, and potential treatment or enrollment in clinical trials. These may include time post-transplant, DSA antibody level and type (e.g., anti-HLA class I or II), proteinuria, degree of capillaritis, peritubular capillaropathy (PTCBMML), concurrent cellular rejection, and medication non-adherence. Future directions in ABMR categorization would include validation of activity and chronicity scores such as for these features.

The current Banff classification lumps TG cases together in one diagnostic category, but TG has a variable prognosis. An outcomes-based approach may be used: a study looked at an "archetype" classification, incorporating clinical, histologic, and immunologic features, which placed patients with TG into different prognostic or outcomes groups (30). These clinicopathologic archetypes may be useful to incorporate into the current biopsy-centric diagnosis, for TG now and at some point for other manifestations of ABMR.

The Banff schema also has a category of chronic (inactive) ABMR. This category may be theoretical, since DSA and its resultant effects do not seem to disappear at any point post-transplant, at least with current therapies (or rather, lack of effective therapies). Biopsies from patients with advanced chronic kidney disease due to ABMR generally show capillaritis as long as capillaries exist. Occasional transplants show such significant loss of peritubular capillaries that the capillaries simply no longer exist to show capillaritis (**Figure 2**).

APPLICATION OF THE BANFF ABMR CRITERIA IN CLINICAL PRACTICE

At the 2013 Banff conference in Comandatuba, Bahia, Brazil, a new Banff Working Group was formed, the "Clinical and Laboratory Assessment of Highly Sensitized Patients Working Group", later referred to as the "Antibody-Mediated Injury Working Group" (31). This Working Group, in which the author (LDC) participates, conducted a survey of clinical practices related to antibody-mediated injury (32). This survey included six clinicopathologic ABMR scenarios and corresponding kidney biopsies, meant to reflect a broad spectrum of injury, including acute/active ABMR and chronic ABMR, and mixed cellular and antibody-mediated rejection. Both pathologists and clinicians (nephrologists/transplant surgeons) responded. There was a discrepancy between the reference (Banff assigned) diagnosis and the pathologist or clinician diagnosis overall approximately 30% of the time. Moreover, this discrepancy influenced treatment decisions. We concluded that the term "acute" in "acute/active ABMR" is confusing, and consequently it was removed from the Banff 2017 schema. We also found that clinicians often failed to recognize the "chronic" elements of ABMR, such as transplant glomerulopathy (TG). They were more likely to consider a diagnosis of chronic active ABMR if the C4d stain was negative, even if there was no TG, PTCBMML, or interstitial fibrosis/tubular atrophy.

Our Working Group then conducted a follow up survey in 2019 of pathologists and transplant clinicians to address additional questions raised by the first clinical practices survey (33). We found that most (97%) respondents said they used the Banff ABMR classification at least sometimes; however, only 19% of pathologists and 41% of nephrologists/surgeons always had DSA results when the kidney biopsy was interpreted. In fact, 18% of 99 pathologists surveyed responded that they *never* had DSA

testing results when interpreting the kidney transplant biopsy. Recall that DSA information is central to Banff ABMR diagnosis, although not necessarily required for all kidney transplant biopsy diagnoses. 40% of pathologists agreed that the time post-transplant influenced their ABMR diagnosis and 58% agreed that they consider allograft dysfunction in considering a diagnosis. One of the main concerns identified was the dichotomous nature (active and chronic active ABMR) of the Banff ABMR categories. These results reflect the reality of renal pathology practice, and the recognition that the Banff schema does not reflect the full range of ABMR.

Another concern that some survey respondents raised was that the Banff ABMR classification changed too frequently for practical use (33). A recent paper from J Callemeyn and colleagues evaluated kidney transplant biopsies and classified their histologic features, C4d staining status, and DSA status into different ABMR categories by the Banff 2001, Banff 2013, and Banff 2017 classifications (34). The authors found – from the same set of biopsies – that there was significant reclassification of the biopsies, between no ABMR, "suspicious for ABMR", and ABMR. The Banff 2013 classification resulted in a marked increase in the number of biopsies classified as ABMR or suspicious for ABMR, and the 2017 classification adjusted this downward. Of course, it was by design that reclassification occurred, as the Banff schema was changed in an attempt to reflect updated knowledge of the ABMR process. But with potential future iterations of the Banff ABMR criteria, this kind of analysis could be used to gauge clinical impact, including incorporating clinical features such as graft dysfunction and time post-transplant, and molecular markers.

REPRODUCIBILITY OF HISTOLOGIC SCORES, ABMR GRADING, AND INCORPORATION OF CLINICAL INFORMATION

Besides determining the "ideal" histologic criteria and cut-off points for different categories of ABMR, the Banff schema should also take into account problems with reproducibility of the Banff scores. Recognizing problems with the Banff "antibody" scores reflecting microvascular inflammation and transplant glomerulopathy as currently defined, a number of studies have looked at reproducibility and methods to improve the scoring and the relation between antibody scores and graft outcome (35–38).

In a study we conducted at Mayo Clinic, colleagues and I reviewed a set of biopsies from patients with preformed DSA, and evaluated them for the "antibody" scores of g, ptc, and cg (transplant glomerulopathy) (39). This study included 6 renal pathologists from 3 Mayo Clinic sites. We found poor agreement for a specific score by any 2 pathologists, ranging from 45-66% agreement for g, 45-67% for ptc, and 54-81% for cg. Furthermore, in 22% of cases, review of the same slide by a different pathologist would result in a different Banff ABMR diagnosis based on histology alone (no ABMR, active ABMR, or chronic ABMR). An improvement analyzed in this paper could be made by a "majority rules" approach with review by three pathologists.

A randomized, multi-center clinical trial was conducted to determine the safety and efficacy of the C5 complement inhibitor drug, eculizumab, to prevent early acute ABMR in positive-crossmatch kidney transplant patients, following an initial pilot study at Mayo Clinic with positive results (18, 20). The primary endpoint of the multi-center study was a composite of biopsy-proven acute ABMR and graft loss or death. The initial results, surprisingly, showed no significant difference in the rate of acute ABMR among patients who received eculizumab versus the standard of care. How could this be? First, "positive crossmatch" was defined differently at different participating institutions, and so patients likely had different degrees of risk for early acute ABMR (early acute humoral rejection). Second, in the initial analysis, only Banff 2007 ABMR types/"grades" II and III were included (presumably as an indicator of rejection severity), as assessed histologically by a central pathologist. As discussed above, early acute ABMR often shows an "ATN-like" histologic pattern, which is ABMR type or "grade" I and would not qualify for the rejection endpoint in this study. The data were re-analyzed to include ABMR grades I, II, and III, and then there was a significant difference between the eculizumab and standard of care groups, the latter showing a higher rate of rejection. Third, the biopsies were reviewed initially by the local pathologist, who had knowledge of the clinical setting (e.g., graft dysfunction, DSA levels, communication with a clinician about the possibility of acute ABMR); the local pathologist rendered a diagnosis of acute ABMR (particularly grades I and II) more often than the central pathologist. The central pathologist was removed from the clinical setting, and rendered a diagnosis based on histology only, at least in the initial analysis. Conclusions we can draw from this study with regards to the Banff ABMR classification are: 1) Banff grading and categorization of the biopsies affected the design and outcome of this trial; 2) Histologic "grade" does not necessarily imply severity or treatment responsiveness in acute ABMR; and 3) Access to clinical information and laboratory results leads to a higher rate of rejection diagnosis in sensitized kidney transplant recipients. As more drugs are being developed and tested for kidney transplant patients with ABMR, there is an urgent need to re-evaluate biopsy-based clinical trial entry and endpoint criteria independent of the current Banff classification.

Like all of medical renal pathology, the renal transplant biopsy diagnosis is a clinicopathologic diagnosis. The renal pathologist identifies the histologic pattern, and then the disease, and then ideally the cause of that disease. For example, a biopsy shows a pattern of acute tubular injury with calcium oxalate crystals, the disease is acute oxalate nephropathy, and the cause is enteric hyperoxaluria due to Roux-en-Y gastric bypass. Just as renal pathologists use a clinical history of diabetes to diagnose diabetic glomerulosclerosis, a history of a vasculitic rash to diagnose Henoch-Schoenlein purpura, a history of antibiotic exposure to diagnose acute allergic interstitial nephritis, or a history of autoimmune pancreatitis and sialadenitis to diagnose IgG4-related tubulointerstitial nephritis, we similarly use the

clinical history and laboratory results to make a diagnosis on transplant biopsies. While there is value in reproducible histologic scoring systems with outcomes measures (such as the Oxford classification for IgA nephropathy), we should not expect any classification system to assign an accurate diagnosis based on histology alone, and particularly in the transplant, where most "native" kidney diseases may occur, and in addition, those diseases specific to the transplant may also occur.

ABMR CONTINUUM

Figure 3 shows a framework for considering the spectrum of ABMR, including some existing Banff categories, ranging from the most "acute" or active rejection on the left to the more chronic and progressive changes on the right. Clinically, positive crossmatch patients with early acute humoral rejection within the first month post-transplant present with acute kidney injury, show more "acute" features histologically, show diffuse C4d deposition in peritubular capillaries associated with high serum DSA levels. Acute ABMR shows similar features but may not have as severe a phenotype, and may occur later post-transplant. Active (smoldering) ABMR is subclinical, shows capillaritis only, and is seen on protocol biopsies from patients with DSA. Chronic active ABMR is subclinical and seen on protocol biopsy, or may be clinically apparent, and shows "chronic" features on biopsy. C4d staining and serum DSA levels may be variable depending on disease activity at the time of biopsy. This schematic shows only "pure" ABMR, as is seen in positive crossmatch or sensitized patients, and does not take into account combined ABMR and cellular rejection, which adds an additional level of complexity. While it is most convenient if patients have a known history of DSA at some point, detectable circulating DSA is not always present, or this information is not available at the time of biopsy. I believe the previous Banff designation of "suspicious" for ABMR would be useful in these situations when the allograft biopsy shows histologic features otherwise typical of ABMR.

FUTURE DIRECTIONS IN ABMR DIAGNOSIS

What are potential ways to address many of these concerns? First, the Banff diagnostic categories should recognize and incorporate the broad range of clinicopathologic phenotypes of ABMR. Different patterns of rejection occur in different settings (e.g., transplantation in sensitized patients) and at different times post-transplant, and so the clinical setting is useful for a more accurate histopathologic diagnosis. In addition to the ABMR spectrum in **Figure 3**, a range of markers of "activity", including C4d positivity and ultrastructural endothelial activation, for example, and "chronicity", including TG, could be incorporated into activity and chronicity scores, akin to the modified NIH lupus activity and chronicity indices (40). Such an ABMR index would need to be tested and validated, such as with the Callemeyn biopsy set, ideally including outcomes measures. Testing and validation of histologic and immunophenotypic markers, including capillaritis, individually and in combination could eventually clarify the meaning of confusing terms such as "acute", "active", and "chronic".

An "archetypes" approach, as has been evaluated in TG (30), could be taken with other antibody-associated patterns of injury. At some point, molecular markers may be incorporated into diagnosis (41, 42), although these would need to be validated and also widely available for clinical use. Clinical trials may be a

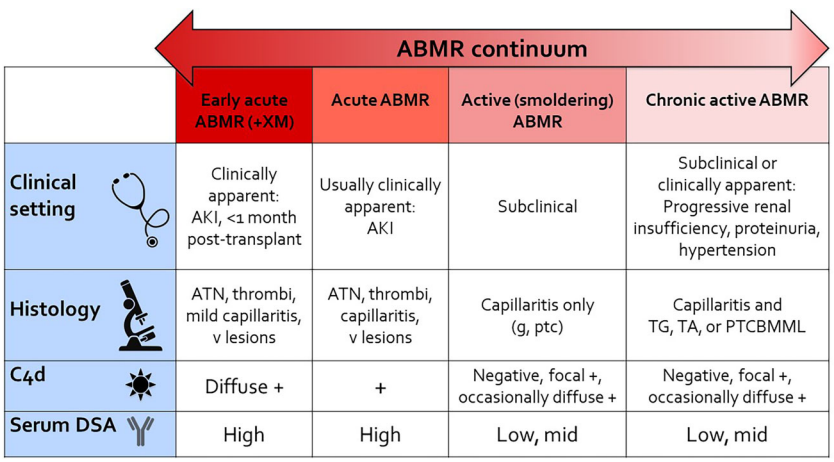

FIGURE 3 | ABMR continuum. This schematic provides a reference for thinking about the continuum of "pure ABMR" in kidney transplant recipients with preformed DSA, as detailed in this article. Not included in the figure is combined ABMR and T cell mediated rejection in patients with *de novo* DSA and under-immunosuppression (iatrogenic or due to nonadherence). AKI, acute kidney injury; ATN, acute necrosis/injury; g, glomerulitis; ptc, peritubular capillaritis; v lesions, Banff vascular lesions (endothelialitis, fibrinoid necrosis of vessels); TG, transplant glomerulopathy; TA, transplant arteriopathy; PTCBMML, peritubular capillary basement membrane multilayering (by electron microscopy); +XM, positive crossmatch.

starting point for incorporation of new molecular markers as classifiers of ABMR categories. Another approach may be to incorporate digital pathology and machine learning into the diagnostic schema, perhaps including immunohistochemical stains to quantify the capillary inflammation and endothelial reactive changes. Again, such tests would need to be validated. Availability of resources is a challenge for widespread incorporation of these techniques, although clinical trials could serve as a starting point.

CONCLUSION

ABMR in the kidney shows a wide range of clinicopathologic features that are not adequately represented in the current Banff diagnostic classification. For current clinical practice and clinical trials, we need to re-assess the usefulness of the current ABMR categories, and more accurately reflect the spectrum of antibody-associated injury and their prognostic categories.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

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

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