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Rational clinical trial design for antibody mediated renal allograft injury

Shaifali Sandal[1] and **Martin S. Zand**[1,2]

[1]Department of Medicine, Division of Nephrology, University of Rochester Medical Center, Rochester NY

[2]Center for Biodefense Immune Modeling, University of Rochester Medical Center, Rochester NY

Abstract

Antibody mediated renal allograft rejection is a significant cause of acute and chronic graft loss. Recent work has revealed that AMR is a complex processes, involving B and plasma cells, donor-specific antibodies, complement, vascular endothelial cells, NK cells, Fc receptors, cytokines and chemokines. These insights have led to the development of numerous new therapies, and adaptation of others originally developed for treatment of hemetologic malignancies, autoimmune and complement mediated conditions. Here we review emerging insights into the pathophysiology of AMR as well as current and emerging therapies for both acute and chronic AMR. Finally, we discuss rational clinical trial design in light of antibody and B cell immunobiology, as well as appropriate efficacy metrics to identify robust protocols and therapeutic agents.

Keywords

Kidney Transplant; Antibody Mediated Rejection; Alloantibody; Apheresis; B cells; Review

2. INTRODUCTION

Antibody mediated renal allograft rejection (AMR) has become one of the most pressing clinical issues in kidney transplantation, accounting for a large percentage of allograft losses (1). The majority of AMR is caused by the emergence of recipient anti-HLA antibodies (alloantibody) directed against donor HLA markers to which the recipient has developed B cell and complement binding IgG antibody immunity. The timing of AMR with respect to the time of transplant can be within hours or days (hyperacute and acute), weeks (acute) or months to years (chronic). Definitive diagnosis of AMR is also complex, requiring findings of complement activation (C4d) on renal biopsy, the presence of tissue damage, and the presence of donor specific alloantibodies. (2) Recent developments have revealed a complex and emerging pathogenesis of AMR, involving not just alloantibody, B and plasma cells, but complement, endothelial cells, NK cells, inflammatory cytokines and chemokines. In the first portion of this review, we review the evolving pathogenesis, new therapeutic targets, and rational clinical trial design of antibody mediated rejection in renal transplantation.

Send correspondence to: Martin S. Zand, Professor of Medicine, University of Rochester Medical Center, 601 Elmwood Ave – Box 675, Rochester, NY 14642, Tel: 585-275-4517, Fax: 585-846-1043, Martin_Zand@urmc.rochester.edu.

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Evolving pathogenesis includes the evolution in our knowledge of the biologic complexity of AMR, from which new therapeutic targets are identified.

The basic principles of AMR treatment have changed little in the last decade. Most protocols seek to (1) reduce or remove the deleterious antibody (2) kill the plasma cells secreting donor specific antibody (DSA), (3) prevent the differentiation of activated donor-specific B cells into antibody secreting plasma cells or memory B cells, (4) prevent complement mediated damage to graft vascular endothelium, and (5) prevent graft parenchymal damage. Given our increased mechanistic knowledge of the immunobiology of AMR, many new therapeutic agents and protocols are currently being developed. In the second section of this review, we discuss both conventional and emerging treatments.

Several features of B cell, plasma cell and IgG physiology make clinical trial design for treatment of AMR significantly more challenging than similar trials for cellular rejection. For example, the half-life of human IgG is 27–35 days, making it difficult to assess therapeutic efficacy if one must wait 5 half-lives to assess post-treatment steady state levels of alloantibody. Other challenges exist for assessing the success of plasma cell and B cell lytic therapies. In addition, it is not technically possible at present to measure the number and distribution (e.g. splenic, bone marrow, etc.) of donor-specific memory B cells, which will secrete DSA if activated. Current measures of efficacy are focused simply on the presence of DSA, which is generally alloantibody except in the cases of ABO incompatible transplants and the rarer AMR episodes linked to tissue-specific antigens.(3–5) Trial design becomes much more complicated when multiple therapies are employed in concert. This is especially true when many of the biologic therapies themselves are IgG antibodies (e.g. rituximab, belimumab, etc) and affected by interventions that alter IgG metabolism such as total plasma exchange and intravenous immunoglobulin. For example, what should be the timing and dosing intervals for a protocol that involves plasma exchange, proteasome plasma cell lytic therapy, IVIG, and B cell lytic therapy? In the last section of this review, we discuss rational design for clinical trials of therapies and protocols to treat AMR, and define a basic questions which robust trials should attempt to address.

3. EVOLVING PATHOGENESIS OF ANTIBODY MEDIATED RENAL ALLOGRAFT INJURY

The classical understanding of AMR emphasizes the importance of DSA and complement activation as the major modes of antibody-mediated renal allograft damage. However, mounting evidence suggests that AMR involves an extremely complex relationship between DSA, recipient endothelial cells, the complement system, innate immune cells, the coagulation system, platelets and the extracellular matrix (6–15). Here we highlight some aspects of this evolving understanding of AMR pathogenesis, which also suggests potential targets for therapeutic interventions.

3.1. Acute antibody mediated rejection

AMR occurs when a transplant recipient develops complement binding IgG antibodies directed against donor antigens accessible to the immune system within the renal transplant.

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Such antigens include disparate ABO carbohydrate moieties, HLA-antigens, and tissue specific antigens, all generally found on the surface of the vascular endothelium in peritubular and glomerular endothelial capillaries (1, 6). These antibodies are high affinity, produced by activated, class switched B cells and plasma cells and of the complement binding IgG isotypes. Even low levels of DSA in patients with a negative crossmatch has been shown to precipitate AMR (16). Both acute and chronic AMR share this fundamental physiology, but differ in the time over which the damage accumulates. Diagnostic criteria for AMR require the presence of at least 3 of the following features: the presence of serum donor specific antibody, histologic features of antibody mediated graft injury (e.g. C4d staining in peritubular cappilaries), tissue injury, and evidence of clinical graft dysfunction. (2, 17, 18)

Binding of DSA to vascular endothelial cells triggers a cascade of events that results in endothelial cell destruction and graft micro-ischemia. DSA activates complement through the classical pathway by binding C1, or by the lectin activated alternative pathway (9, 19). Once activated, C3 splits into C3a and C3b (9). C3b amplifies the alternative pathway, while the chemoattractant C3a and C5a recruit macrophages and neutrophils, causing additional endothelial injury(9, 19). Covalent binding of C4d to the plasma membrane is one diagnostic hallmark of AMR, although C4d negative AMR was recently recognized.(20, 21) Damaged endothelial cells release numerous proteins that play different roles in the pathogenesis of AMR. These include von Willebrand factor and P-selectin that promote platelet aggregation, IL-1α, IL-8, and chemokine ligand 2 (CCL2) that induces leukocyte endovascular adherence within the glomeruli, causing glomerulitis or to dilated peritubular capillaries (1, 6). C5b triggers the assembly of the membrane-attack complex (C5b–C9) leading to endothelial necrosis and apoptosis and detachment of endothelial cells from the basement membrane(1, 9). In severe cases, microthrombi can occur with hemorrhage and arterial-wall necrosis and infarction, causing a clinical syndrome of thrombotic microangiopathy (TMA) in the transplant recipient. (1, 6). Acute AMR has a wide spectrum and can easily be missed by histologic criteria alone as renal biopsies may show acute cellular rejection, acute tubular injury, or thrombotic microangiopathy (6, 22).

3.2. Chronic antibody mediated rejection

CAMR is strongly associated with circulating antibodies against donor-specific HLA antigens. Patients with a positive pre-transplant cross-match have a higher incidence of CAMR, and previous AMR is a risk factor for CAMR (6). However, a substantial number of cases of CAMR have no detectable circulating HLA antibody or C4d in the graft at the time of diagnosis (6). Possible mechanisms of CAMR include complement mediated injury as in AMR followed by maintenance of cellular injury, complement independent pathway, non-HLA antibody induced damage(3–5) direct activation induced proliferation or apoptosis of endothelial cells by DSA (9, 23). Histological features of CAMR include glomerular and peritubular injury, cellular hypertrophy, subendothelial deposition of fibrillary material, expansion and duplication of the glomerular basement membrane, and, mesangial-cell interposition (1, 6). Transplant glomerulopathy is a morphologic lesion associated with CAMR, and characterized by duplication and multilayering of the glomerular basement membrane (24). Considered a criteria for CAMR, transplant glomerulopathy may also be

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seen in Hepatitis C and thrombotic microangiopathy (24). A similar chronic injury pattern is peritubular capillaritis, best seen on electron microscopy (9).

A substantial number of cases of CAMR have no detectable circulating HLA antibody or C4d graft deposition. Some have suggested that the graft itself acts as a “sink” for DSA, that low or absent DSA levels simply reflect ongoing graft deposition. This hypothesis is supported by the finding that anti-HLA and anti-non-HLA DSA can be recovered from failed renal allografts even in the absence of similar circulating antibodies in the blood.(25–27) This has led some to characterize four stages of CAMR: stage I alloantibody production, stage II antibody interaction with alloantigens resulting in the deposition of C4d in PTC and possibly glomeruli, stage III pathologic changes and stage IV graft dysfunction (6). Thus, stages III and IV may persist after antibody and C4d disappear (6, 9).

3.3. Subclinical antibody mediated rejection

Subclinical AMR (SAMR) that is diagnosed in surveillance (not for-cause) biopsies, and is seen more frequently in highly sensitized recipients with a positive crossmatch (10). It is associated with the development of transplant glomerulopathy and reduced graft survival(10). In one study, SAMR occurred in approximately 8% of surveillance biopsies, and 30% of patients progressed to clinically evident AMR between 14–20 months post-transplant (13). This observation prompted the hypothesis that AMR is a continuous spectrum of antibody related injury starting with microvascular endothelial injury, progressing to injury of larger vessels and the interstitium, and finally to clinically evident CAMR (13). Microvascular injury is an essential component of AMR that can be reliably used to increase the sensitivity of the histologic diagnosis (13). Recognizing this, many have recommended that the Banff criteria for diagnosis of AMR be expanded to encompass a wider spectrum of lesions identified in protocol biopsies (10, 13). The challenge remains of creating a working diagnostic scheme with sufficient sensitivity to minimize under treatment and high specificity to avoid giving unwarranted immunosuppression (10).

3.4. AMR without stigmata of complement activation

Until recently, complement activation was considered essential for diagnosis of AMR. However, C4d negative AMR is being increasingly recognized, where DSA may cause graft injury similar to AMR in the absence of C4d deposition (10, 28). The mechanisms behind such damage is unclear. One hypothesis is that DSA of IgG isotypes unable to fix complement may activated endothelial signaling pathways associated with surface HLA, resulting in endothelial apoptosis or a pro-fibrotic state leading to basement membrane secretion (6). Alternatively, NK calls may participate in the pathogeneisis AMR by inducing antibody dependent cellular cytotoxicity (ADCC) (29). Elevated NK cell number and related transcripts encoded by six unique genes that were selective for NK cells were present in PTC from biopsies of patients with AMR (11). NK cells, via the FcγRIIIa (CD16), recognize the Fc portion of antibodies bound to allo-antigens on target cells resulting in an ADCC-like release of the T cell trophic cytokine gamma-interferon (29). NK released chemokines also attract macrophages which are also mportant mediators of allograft injury in AMR (11).

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3.5. Emerging role of endothelial cells

Endothelial cells (ECs) as illustrated above are the prime targets in AMR however they might be key players as illustrated by a review by Yamaguci of 12 studies conducted by his group (12). For instance in one study, they studied c-Jun, a transcription factor that is activated in glomerular and tubular cells and plays a major role in renal pathophysiology (12). An increase in c-Jun, a transcription factor that is activated in glomerular and tubular cells and plays a major role in renal pathophysiology, was observed in injured ECs, as well as infiltrating mononuclear cells, in the glomerular and PTCs and correlated with changes in creatinine in CAMR (30). The investigators hypothesized a role for endoplasmic reticulum stress-induced c-Jun activation in EC in AMR and postulated that blockade of the JNK/c-Jun pathway may prevent endothelial injury in AMR (12, 30). While endothelial cell activation is gaining increasing importance as an underappreciated aspect of CAMR, this has not yet translated into new therapies.

3.6. Antibody-mediated vascular rejection and arteriosclerosis

There is evidence that both active and chronic lesions in arteries, beyond the recognized lesions of transmural necrosis and intimal fibrosis, play a role in the pathogensis of AMR (6, 10, 31). In a study of 302 patients with acute rejection, four distinct forms were identified: 9% were T cell-mediated vascular rejection, 46% were T cell-mediated rejection without vasculitis, 24% were antibody mediated rejection without vasculitis and 21% antibody-mediated vascular rejection (AMVR)(32). The latter were associated with the highest risk for graft loss. Of patients with AMVR, 52% had endarteritis graded as v1 with Banff criteria, 30% had v2, and 19% had v3. Patients with v1 or v2 lesions are judged to have T cell-mediated rejection based on the current Banff criteria. 42/64 patients diagnosed with AMVR were misclassified at time of biopsy as having T cell-mediated rejection and received inappropriate treatment perhaps explaining the high risk of graft loss. Authors conclude that lesions of endarteritis form part of the range of general endothelial inflammatory changes mediated by antibodies and advocated for the division of all cases of endarteritis into two separate profiles of rejection based on the presence of absence of antibodies.

Another important emerging pathology is arteriosclerosis which is used to diagnose CAMR and in AMR it can occur in the absence of evident concurrent or prior intimal vascular inflammation (6, 31). One study noted that arteriosclerosis is accelerated after transplant in both DSA+ and DSA− patients but progresses three times faster in the former while de novo DSA positivity and had an intermediate rate of progression (31). Authors postulate that accelerated arteriosclerosis is a muted, largely subleukocytic inflammatory and proliferative response forming a continuum with more overt vasculytic lesions and DSA seem to further enhance this acceleration in patients manifesting SAMR (31).

3.7. Emerging role of Fc receptors

Fc receptors (FcRs) for IgG play an important role in innate and acquired immunity, including that of allograft rejection(33). Their roles include antigen presentation and immune-complex-mediated maturation of dendritic cells (DCs), and in the regulation of B-cell activation and plasma-cell survival and each step is a potential therapeutic target (33).

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The inhibitory FcRIIB is the most broadly expressed FcR, and is present on virtually all leukocytes with the exception of NK cells and T cells and are involved in endocytosis or phagocytosis of immune complexes (33). A recent study on FcRIIb signaling in cardiac allografts in mice showed that it regulates chronic but not acute rejection (34). Authors postulated that helper CD4 T cell response in acute rejection overcomes FcRIIb-mediated inhibition of the effector B cell population. Similar studies in renal allografts are lacking (34). In another study in cardiac allografts in mice, monoclonal alloantibodies to immunoglobulin to MHC I antigens can augment graft injury by stimulating EC to produce MCP-1 and by activating mononuclear cells through their Fc receptors.

4. EMERGING TARGETS FOR CLINICAL INTERVENTION

The basic principles of treating antibody-mediated rejection have not changed significantly in the last decade. These include (1) removing the injurious donor-specific antibodies from the circulation, (2) preventing B cell activation and differentiation into DSA secreting plasma cells, (3) removal of donor-specific memory B cells, short- and long-lived plasma cells, and (4) blocking antibody dependent graft damage. In general, current treatment protocols for acute AMR combine several agents and therapies to achieve these goals. Here we briefly review each class of therapy, with an emphasis on emerging biological targets and newer modalities.

4.1. Removal of antibodies

Removal of antibodies is a mainstay of therapy for AMR, and is currently accomplished by three interventions: therapeutic plasma exchange (TPE) or double-filtration plasmapheresis (DFPP), immunoadsorption, and blockade of the neonatal Fc receptor by high dose intravenous immunoglobulin (IVIG). Experimental therapies for antibody removal include genetically engineered agents that block the FcRn and decrease circulating IgG half-life, thereby increasing clearance of the deleterious IgG species. Despite their extensive use, there are a paucity of well controlled, randomized, prospective trials of TPE and DFPP in the treatment of AMR. Several significant questions remain unresolved included the duration and intensity of therapy, how to monitor efficacy, optimal replacement intervals for clotting factors. Currently, most protocols administer several daily treatments initially, followed by every-other-day therapy until clinical improvement occurs, or doubts about the utility of continued therapy ensue. The emerging challenges for this class of therapies includes minimizing side effects of the therapies, and determining treatment intervals, duration, and effective combinations with other therapeutic modalities.

4.1.1. Plasmapheresis and Plasma Exchange—Therapeutic plasma exchange (TPE) or double-filtration plasmapheresis (DFPP) non-specifically immune complexes, protein bound toxins, circulating antibodies, complement components, and coagulation factors (35, 36). In TPE, the plasma fraction of whole blood is removed by either filtration or differential centrifugation (36). Plasma colloid is replaced by either albumin or fresh-frozen plasma during the treatment (36). In DFPP, plasma is removed as in TPE and then passed through a second filter, whose smaller pore size traps larger molecules, especially immunoglobulins (37). A study comparing TPE and DFPP showed that while the maximal plasma volume

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treated with DFPP was almost twice that of TPE and DFPP removed total IgG effectively, there was no overall advantage in reduction in DSA levels (37). In renal transplantation these modalities have also been used to for removal of isohemaggluinins (anti-ABO antibodies) in ABO incompatible transplantation, removal of donor-specific allo- antibodies in HLA sensitized patients pre-transplant and during AMR (35, 36).

Early studies using TPE in treatment of AMR were disappointing, likely because concurrent therapies to suppress antibody production were not used (38–40). Later studies showed graft salvage with TPE when used in combination with IVIG, rituximab, eculizumab, and bortezomib (41–48). In 39 AMR patients, who received TPE or DFPP in addition to other concomitant treatments, 24/39 (61.5.%) subjects continued to have functioning grafts after therapy (49). In another study, 8/9 grafts with AMR were salvaged with TPE and IVIG and a 30% decline in serum creatinine was noted after an average of 10 days of therapy (50). In CAMR, results have been less encouraging (49). A single-center, observational study showed 1-year graft survival of 86% in patients with AMR who responded to TPE and a 3 and 5 year graft survival of 86% and 78% respectively (51). Complications of TPE or TPE are related to complications of vascular access, allergic and infections reactions to transfusion products, hypotension, increased risk of hemorrhage, and hypocalcemia (35, 38). The cause of the hypocalcemia is likely two-fold: removal of plasma calcium bound to albumin and free calcium by binding to citrate. Compared with PE, DFPP is associated with less loss of albumin and clotting factors (49) . Arrhythmias and pulmonary edema have also been reported, albeit rarely (49).

4.1.2. Immunoadsorption—Immunoadsorption (IA) is an emerging and potent tool for rapid and targeted removal of donor-specific antibodies. It has been used for pre-transplant immune desensitization for allo- and anti-ABO antibodies, AMR treatment, and cessation of complement activation (52–57). IA involves passing plasma through single or double columns coated with ligands that bind IgG antibodies and remove them from the circulation. The ligands may be non-specific, binding all immunoglobulins or all IgG isotypes, such as with protein A, the peptide-GAM and sepharose-immobilized polyclonal sheep anti-human Ig antibodies (53, 58). Columns coated with blood group carbohydrates can specifically remove isohaemagglutinin IgG and IgM antibodies, and are useful in treating AMR in patients who have undergone ABO incompatible kidney transplants.(59) Similar columns for removal of donor-specific alloantibody would require large amounts of donor HLA antigens, which is technically and financially unfeasible at the current time. In general, IA can remove up to 87% of plasma IgG and over 50% of the IgA and IgM per treatment, as well as Ig-bound complement proteins (54, 57, 58, 60). IA has several advantages over TPE: it does not remove clotting factors, requires no substitution of plasma, antibodies against previously encountered antigens are somewhat preserved, and has the potential of reusable adsorption systems (58, 60, 61). Similar to TPE, however, IA does not alter IgG synthesis or redistribution from tissue compartments, and additional B and plasma cell depleting therapies are needed for sustained DSA reduction (58).

As with TPE, there are no robust trials to assess efficacy or to guide therapy. Several uncontrolled studies have demonstrated IA is effective in the treatment of AMR (52, 56, 62–64). A randomized, controlled, open-label trial involving 10 patients with C4d-positive graft

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dysfunction showed that 5 patient who had 9 –14 IA sessions with tacrolimus conversion had a 100% response rate, while the control group had a high graft loss rate (55). In a case series, 2 patients with evidence of AMR, DSA could be eliminated after treatment with IA and rituximab (52). Reported adverse effects of AI include high cost, citrate toxicity, reduced antibodies against pneumococcus and haemophilous polysaccharide antigens, and anaphylaxis with concurrent ACEI use (57, 58, 60). Additional studies directly comparing TPE and IA are needed.

4.1.3. Neonatal Fc receptor blockade—As noted above, the half-live of circulating IgG is greatly increased by a recycling pathway that depends on the neonatal Fc receptor. FcRn deficient mice have lower serum IgG levels and the half-life of IgG is reduced from 21 days to 3 (65). Thus, one strategy for non-specifically clearing circulating IgG is to block the FcRn. IVIG infusion causes supra-physiologic levels of IgG to saturate the FcRn. This leads to a rapid clearance of all IgG, decreasing the circulating half-life from 27 days to 3.5. days, and increased clearance of DSA. In general, a 1–2 g/kg dose is needed to achieve this effect for 5–8 days, after which the circulating levels of IgG fall below that needed to saturate the FcRn. Other experimental approaches to FcRn blockade include therapeutic anti-FcRn monoclonal antibodies, genetically engineered high-affinity mutants of the IgG1 Fc region, and FcRn agonist peptides. (66, 67). Recent work in mice showed efficacy of an anti-human FcRn monoclonal antibody, which increased clearance of human IgG in transgenic mice expressing the human FcRn gene, although the effects were transient and required repeated dosing (68). No clinical trials have been performed, and current data is limited to rodents and nonhuman primates (66, 67).

4.2. Modulating b cell activation and plasma cell differentiation

Blocking B cell activation, division, and differentiation into antibody secreting plasma cells is a key but underappreciated part of AMR treatment. B cell activation requires the help of CD4 T follicular helper cells within germinal centers, and differentiation requires several cytokines and factors which support the differentiation of activated B cells into antibody secreting short and long-lived plasma cells. Suffice to say, adequate T cell immunosuppression is absolutely necessary during treatment of AMR. On the B cell front, several new agents have emerged which are able to directly modulate B cell activation and prevent plasma cell differentiation. These include agents which interfere with the action of BLyS and Baff, IL-6, and TNF-alpha/Lymphotoxin-beta. Most entered the therapeutic arena for treatment of autoimmune B cell disease, and show varying degrees of promise for treatment of AMR.

4.2.1. Soluble BLyS receptors—Therapies which block the action of the B cell trophic factors BLyS and BAFF are powerful immune modulating therapies which interfere with B cell activation, differentiation, and plasma cell development. B- Lymphocyte stimulator (BLyS) and B-cell activating factor (BAFF) are a family of TNF receptors and ligands that are critical for normal B cell homeostasis, including preventing apoptosis of activated antibody secreting B cells. This family also includes ligand APRIL and the B cell receptors BR3, transmembrane activator and CAML interactor (TACI), and B cell maturation protein A (BCMA) (69). BLyS binds all three receptors, whereas APRIL binds only TACI and

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BCMA (69). Anti-BLyS agents cause partial B cell depletion, especially in activated B cells, while anti- agents disrupt germinal centers and prevent plasma cell development. Several agents currently approved for treatment of lupus, or are in clinical trails, may also have promise for the treatment of AMR (70). Five anti-BLyS agents are currently in clinical development for treatment of systemic lupus erythematosus: belimumab is a monoclonal antibody that binds and neutralizes soluble BLyS and is currently FDA approved, atacicept is a receptor fusion that binds and neutralizes both BLyS and APRIL, blisibimod is a fusion between the Fc portion of IgG and a peptide sequence with high affinity to BLyS, tabalumab is an anti-BLyS mAb that binds to both soluble and membrane BLyS and lastly a monoclonal antibody specific for APRIL (71).

In the context of AMR, therapies targeting these ligands may be more useful for abrogating de novo antibody production rather than in desensitization. In addition, therapeutically depleting patients of specific activated B cell and newly differentiated plasma cell subsets, while leaving their immunological history intact, might be advantageous as the memory cells will persist and allow recall responses against previously encountered pathogens (69, 72). Supra-physiologic BLyS levels may potentiate alloreactive B cell immunity and may promote allograft rejection (70). In young adult renal transplant recipients, rituximab treatment caused a significant elevation of BLyS levels at 3 months and this was positively correlated with DSA specific for HLA-class I and negatively correlating with creatinine clearance (73).

4.2.2. Anti-IL-6—Interleukin (IL)-6 is a pleiotropic cytokine, primarily involved in the regulation of immune and inflammatory responses and generated by both B and T cells (74). It is an essential growth factor of B cells and plasma cells, and blockade of IL-6R signaling induces B cell apoptosis and abrogates plasma cell differentiation. In addition, IL-6 signaling blockade may protect against renal ischemia. In IL-6 knockout mice, ischemia induced renal injury, dysfunction, and inflammation was significantly less than that of wild-type controls (74). Similarly, elevated IL-6 levels in a cohort of renal transplant recipients were associated with graft loss and inflammation, suggesting a potential therapeutic role for tocilizumab, a monoclonal antibody against IL-6-receptor (75). Tocilizumab impairs B cell differentiation and plasma cell development in human memory B cells, which could prevent or treat early AMR.(76, 77) In addition, tocilizumab reduced alloantibody levels in a sensitized mouse model. (78) A clinical trial of tocilizumab to improve transplant rates in highly sensitized patients awaiting kidney transplantation is currently underway (http://clinicaltrials.gov/show/NCT01594424).

4.3. Removal of donor-specific B cell and plasma cells

Removal of donor-specific B and plasma cells is the “holy grail” of AMR treatment and prevention. Unfortunately, all current therapies non-specifically remove the B cells or plasma cells that they target, not just the donor-specific subset. Many of these agents are monoclonal antibodies directed against B cell surface markers, but expression of these markers changes during B cell differentiation into plasma cells. For example CD20, the target of rituximab, is rapidly down-regulated on activated B cells, and absent on plasma cells. Thus, rituximab is only effective during the early AMR period, when activated B cells

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still express CD20. To make matters worse, any therapy that removes immunoglobulin or increases Ig clearance, such as therapeutic plasma exchange and IVIG, has the potential to interfere with these other agents.

4.3.1. Anti-CD20 Monoclonal Antibodies—The development of anti-CD20 monoclonal antibodies provided a non-surgical method of reducing B cell mass, and was sometimes referred to as a "chemical splenectomy". Rituximab is a chimeric monoclonal anti-CD20 antibody directed against CD20, which is expressed on immature and mature B cells, but not on plasma cells. Rituximab binding to target cells causes apoptosis and antibody mediated cell lysis (79). Rituximab has a well established role as induction agent in ABO blood group incompatible kidney transplantation (80). However, its role in AMR is still evolving with only case series and controlled studies. In a retrospective analysis, 26 patients with AMR who received 375 mg/m^2 of rituximab after each TPE, 2 year graft survival was 92% versus 60% in 28 patients treated with TPE alone (81). Overall, a recent meta-analysis suggested that rituximab is a clinically effective treatment for AMR.(82) Treatment with rituximab is currently an expensive approach, and some recommend use only in AMR patients with low initial DSA titers (<1:128 dilution), creatinine of <3 mg/dL at the time of rejection, and mild tubular atrophy/interstitial fibrosis on renal biopsy (83). Reported adverse effects of rituximab therapy include lung toxicity, greater need for IVIg supplementation, infectious and infusion complications including fatal Jacob-Kreutzflet encephalitis (79, 84).

Rituximab may also have a role in treatment of CAMR, although the evidence is limited to case series. In four patients with CAMR who received rituximab and IVIG, graft function improved at 6 months and in two patients DSA levels significantly dropped (84). In another case series, six pediatric patients with CAMR received four weekly doses of IVIG followed by a single dose of rituximab one week after the last IVIG infusion and four patients had improvement or stabilization of their GFR (85). A recent study compared outcomes of ABO incompatible patient treated with splenectomy (ABO-I-SPX) or rituximab (ABO-I-RIT) with ABO compatible patients (ABO-C)(86). CAMR rates 2 years after the operation were 8.8., 3.5. and 28.9.%, and de novo donor-specific anti-HLA antibody (DSHA) positive rates were 2.2., 1.7. and 18.1.% in the ABO-I-SPX, ABO-I-RIT and ABO-C groups, respectively. The authors proposed the that the primary role of B-cell depletion protocols may be for the prevention of de novo antibody formation rather than treatment of AMR. A clinical trial is currently underway to determine whether rituximab can stabilize or improve renal function and/or proteinuria in patients with CAMR in whom standard therapeutic approaches have failed. (http://clinicaltrials.gov/ct2/show/NCT00476164)

4.3.2. Anti-CD19 monoclonal antibodies—In B cells, expression of CD19 starts prior to the expression of CD20 and is maintained throughout differentiation and activation stages until terminal plasma cell differentiation when CD19 expression decreases (87). T cells have a receptor that recognize CD19 (87). Hence, depleting and modulating monoclonal antibodies targeting CD19 are currently being studied in various hematologic and rheumatologic disorders and have a potential role in transplant medicine as well (87). In mice with RT, while graft survival was similar in control and those treated with anti-CD20

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antibody with only 20–22% of mice surviving, in those treated with anti-CD19 antibody 67% of mice survived (88). Additionally, biopsies showed reduced renal pathology and lower C4d, IgG and IgM deposition in the anti-CD19 group (88).

4.3.2. Proteasome inhibitors—Bortezomib is a proteasome inhibitor that is thought to inhibit the clearance of unfolded proteins in cells, leading to death of mature plasma cells (89). It has been used as a salvage therapy for AMR refractory to standard protocols, and in allo-desensitization protocols (46, 89). A review of all case reports on bortezomib showed that over 95% of all patients treated for AMR with a bortezomib-based regimen and approximately 50% of patients with CAMR achieved allograft stabilization and/or rejection reversal and 50% of the patients achieved a reduction in DSA (89). These findings were higher than that reported in a randomized trial of rituximab based therapy (89).

More importantly, bortezomib may have a potential role in CAMR (89). A study prospectively comparing patients treated for CAMR with a rituximab versus a bortezomib based regimen demonstrated that renal function and graft survival was superior in bortezomib treated group (79). Some investigators have even suggested combining rituximab and bortezomib to effect the combined deletion of mature, antibody-secreting plasma cells and their precursors (46). Bortezomib-related toxicities include gastrointestinal toxicity, thrombocytopenia, paresthesias and sensory neuropathy, although these appear occur less frequently in renal transplant recipients than myeloma patients (90). A second-generation proteasome inhibitor carfilzomib with an improved side effect profile is currently under study for its potential role in multiple myeloma although its potential role in AMR is yet to be explored (91).

4.3.3. Cyclophosphamide—Cyclophosphamide is a DNA alkylating agent widely used in the treatment of many malignancies and kidney related disorders. Its role in AMR is largely unexplored. In a 1969 study on rat models, cyclophosphamide was noted to depress both cell mediated and AMR in transplanted allogeneic rat kidney (92). The authors proposed the cytotoxic effect on antibody producing cells as the likely etiology. Recently, there has been a resurgence of interest in its use to treatment AMR. A phase II pilot study of short-term, low dose, cyclophosphamide therapy in patients with late phase AMR refractory to current standard of care treatment is currently underway at the University of Manitoba (http://clinicaltrials.gov/ct2/show/NCT01630538).

4.3.4. Other anti-B cell therapies—Emerging therapies targeting B cell surface markers are on the horizon, including epratuzumab (anti-CD22), mederax and SGN-30 (anti-CD30). However neither agent has been used in renal transplantation, and clinical trials are not yet planned for this indication (93). The second-generation anti-CD20 monoclonal antibodies include ofatumumab, veltuzumab, and ocrelizumab that are humanized to reduce immunogenicity and the third generation such as obinutuzumab are currently in development for autoimmune disease and B cell malignancies (93). Identification of agents directed against other therapeutic targets, such as microRNAs (miRNA) and STAT signaling proteins, or the use of therapeutic regulatory T cells, are all nascent therapies which may eventually augment both acute and chronic AMR treatment protocols (70).

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4.3.5. Splenectomy—Splenectomy has been hypothesized to "debulk" the antibody-secreting cell mass during AMR, dramatically diminishing antibody production (94–96), and was one of the first therapies used for treatment. The major concern with splenectomy is sepsis, however recent developments in immunosuppression and antibiotics are believed to have decreased this (94). The spleen contains a very small proportion of direct antibody secreting cells that are CD138+ plasma cells (97). It has been postulated that in AMR the spleen either captures or sequesters CD138 cells or causes differentiation of B cells into antibody-secreting CD138+ cells (97). In one case series, splenectomy combined with TPE and IVIG was an effective rescue therapy in five highly sensitized patients who developed post-desensitization AMR, leading to improved creatinine, decrease DSA and renal allograft survival at 18 months in all patients (94). Similarly, another case series showed splenectomy was an effective rescue therapy for AMR refractory to IVIG and TPE for 9 of 11 patients, while 3 others required additional bortezomib administration and one patient died of sepsis (95).

4.4. Blocking antibody dependent, complement mediated, graft damage

The humanized monoclonal antibody, eculizumab, binds to C5 with high affinity and prevents the formation of C5a and the membrane attack complex(9). A single-center, open-label study showed that treating allograft recipients with eculizumab could reduce the incidence of AMR in the first 3 months after positive crossmatch RT (98). The incidence of AMR was 7.7.% in the eculizumab-treated group compared with 41.2.% in the control group (98). Complications in this study were one episode of subclinical CMR, one wound infection and one patient with Burkitt's lymphoma 2.5. years after transplantation. A patient treated with eculizumab for severe AMR after ABOI kidney transplantation refractory to standard treatment showed normal biopsy at 6 months and stable creatinine at 1 year (99). An editorial pointed out the cost associated with this therapy, questioned the efficacy in the setting of higher levels of anti-donor HLA antibodies and asked for experience in larger populations to assess infectious risk (100). A phase 2 clinical trial examining the safety and efficacy of Eculizumab in the preventing AMR LDKT recipients requiring desensitization therapy is currently underway. (http://clinicaltrials.gov/show/NCT01399593)

Other C5 inhibitors in clinical trials include pexelizumab, a recombinant derivative of eculizumab, and Mubodina, a neutralizing antibody against C5 that recognizes a different C5 epitope to the one targeted by eculizumab. Neither have been studied in the field of RT (9). The Yunnan-cobra venom factor (Y-CVF) is an extremely potent anti-complement protein with excellent ability to deplete circulating C3(101). Y-CVF caused suppression complement activation in the first 2 weeks following RT in monkeys, AMR was successfully prevented and long-term renal allograft survival was achieved in most pre-sensitized recipients (101).

4.5. Intravenous immunoglobulin (IVIG)

IVIG has emerged as an important component of virtually all protocols for treatment of AMR, as well as for desensitization of patients with alloantibody prior to renal transplantation. (43, 45, 102, 103). Multiple mechanisms of immune modulating activity have been attributed to IVIG, including regulation of both innate and cellular immunity,

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inhibition of cytokine gene activation and/or anti-cytokine activity, inhibition of B-cell and T-cell activation, Fc receptor-mediated interactions and inhibition of complement activity and complement mediated inflammation (102, 104–106). A major but underappreciated mechanism for the effect of IVIG on endogenous alloantibody levels is saturation of the FcRn, leading to greatly increased clearance of endogenous IgG alloantibody.(107, 108) However, robust evidence for the clinical efficacy of IVIG in the form of prospective, randomized clinical trials is scarce. In one case series of renal transplant recipients with AMR, serum creatinine levels improved and cross-matches turned negative within 1–2.5. weeks of IVIG therapy (104). In a second report, a protocol combining TPE, IVIG and anti-CD20 lead to a graft survival of 91.7.% over 36 months and significantly greater diminution of DSA levels, compared to 50% graft survival in patients treated with IVIG alone (109). When given after TPE, IVIG can replenish gamma globulins decreasing the risk of infection (41). In addition, IVIG perhaps has a role in the treatment of chronic AMR (84). Possible adverse effects of IVIG are infrequent, and include volume overload and infusion-related complications such as aseptic meningitis, thrombotic events and bronchospasm (103, 104).

5. RATIONAL CLINICAL TRIAL DESIGN

Comprehensive clinical trails of therapies and treatment protocols for acute and chronic antibody mediated rejection have been rare. A recent review of antibody mediated rejection and desensitization protocols found only 5 robust prospective, randomized clinical studies that were appropriately statistically powered and analyzed. Several factors have contributed to the scarcity of AMR clinical trials. First, the incidence of acute AMR is modest, and there is currently no general consensus on what constitutes standard of care. The best evidence suggests that IVIG and rituximab are likely effective, TPE and bortezomib possibly effective, and the remainder of therapies lack good evidence for efficacy. Second, many of the agents now being used were first approved by regulatory agencies for other indications, such as treatment of hematologic malignancies or autoimmune disease. Given this reality, there may be little financial incentive for pharmaceutical companies to sponsor expensive clinical trials. Partnership with government funding agencies for funding well-designed clinical trials would serve many parties, advancing appropriate clinical rigor and science, and ensuring that data to justify standard-of-care guidelines is generated from each trial. Finally, the underlying immunobiology of human B cell activation, differentiation and antibody kinetics is highly complex, making it difficult to predict the effects of any B cell modulating agent. It is important to note, however, that although the paucity of robust clinical trials in AMR has made it difficult to judge protocol efficacy, many agents are widely used for treatment of AMR. With this in mind, we briefly discuss rational clinical trial design for antibody mediated rejection protocols.

5.1. There should be moderate pre-clinical evidence for efficacy in animal prior to clinical trials

Several recent agents used for AMR, notably bortezomib, eculizumab and rituximab, were developed and approved for use in other disease states prior to their use in case series for treatment of AMR in renal transplantation. The use of these therapies was based more on extrapolation from cancer and autoimmune disease trials than any specific pre-clinical

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studies related to AMR. As such, these agents generally lacked pre-clinical evidence for efficacy in treatment of AMR. For example, B and plasma cell depleting therapies could be studied in murine and non-human primate models of vaccination. Such experiments would involve vaccination against MHC antigens, experimental treatment, and measurements of control and treatment arms for allo-specific plasma and memory B cell frequency in spleen, lymph nodes and bone marrow by ELISPOT, with parallel serum alloantibody levels by ELISA or multiplex assay. Timing of administration and combination effects with other agents could be investigated to aid in rational protocol construction. Such work would frame realistic expectations for treatment efficacy, and help design clinical trials outcomes measures. When animal models are not available, at minimum human *in vitro* studies with B and plasma cells could be undertaken.

5.2. The study design must account for the half-life of IgG

A critical but often overlooked issue is the effect of the long half-life of circulating IgG, 27–35 days, which is a function of FcRn binding saturation (108). For example, if the production rate of DSA changes after a plasma cell depletion therapy, it will take approximately five half-lives to reach new steady state DSA levels before measurements could be used to accurately judge long-term protocol efficacy. To adjust for this issue, we recommend two features should be added to any AMR study design. To more rapidly assess DSA levels accurately, the treatment regimen should include a single TPE treatment to lower DSA levels below steady state. Antibody redistribution and synthesis will occur over 5–7 days following the TPE resulting in a new steady state, after which DSA levels can be accurately measured. Second, we recommend frequent serum measurements of both total IgG and DSA levels at regular intervals during the protocol. This will provide some measure of how a therapy affects total IgG versus DSA levels.

5.3. AMR clinical trials should be designed to clearly answer questions regarding efficacy and mechanism of action

In order to evaluate the efficacy of a treatment protocol or new agent in AMR, rational trial design should include collection of data that answer the following clinical questions:

What is the clinical, serologic, and histologic evidence for AMR at enrollment? Patients enrolled in AMR protocol trials should meet accepted clinical criteria, such as Banff classification criteria for AMR. The current classification schema is flexible enough to accommodate C4d negative and non-HLA donor-specific antibody mediated rejection episodes. This will ensure that clinical practitioners seeking to apply the study protocol to their own patient populations will have an accepted standard for enrollment, and a more robust ability to advise patients on the chances of protocol success, side effects, and failure.

What are the 1, 3, 6, 12, and 24 month post-AMR treatment graft survival rates, glomerular filtration rates, and spot urine protein / creatinine ratios? While early post-treatment graft survival is a clean, hard endpoint, we know that most AMR can be treated to avert early graft loss, but substantial parenchymal and vascular damage may substantially increase the risks of early graft failure. Thus, patients should be followed for a minimum of two years post-treatment, and other non-invasive measures of graft

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damage and function, such as estimated glomerular filtration rate and degree of proteinuria, should be collected.

What are the pre- and post-treatment specificities of DSA and non-DSA? This seems an obvious metric that should be included, it has been omitted in favor of simple graft survival or panel reactive antibody levels. Given that the presence of DSA at almost any level is a substantial risk factor for early graft loss and CAMR, trials of protocols or newer agents for AMR should assay for the presence and specificity of DSA at relevant intervals. Successful treatments and protocols should eliminate or markedly reduce DSA.

How much has the DSA-secreting plasma cell mass been reduced? Reduction in memory B cell and bone marrow resident plasma cell mass by B cell modulating or lytic agents is a major mechanism for treating AMR, and preventing further CAMR. The ideal B and plasma cell agent would reduce the frequency of short and long lived DSA secreting plasma cells in the bone marrow and spleen. Such measurements, however, require bone marrow aspiration.

What are the pre- and post frequencies of memory B cells capable of secreting DSA after activation? Memory B cells are the iceberg beneath the surface: silent yet capable of rapidly expanding and secreting destructive DSA upon reactivation. Measurement of donor-specific memory B cells requires isolation of peripheral blood memory B cells, *in vitro* stimulation, and assay of secreted DSA, either by supernatant sampling and standard multiplex assay, or by ELISPOT assay. One goal of timely AMR treatment may be to prevent the long-term establishment of B cell memory. Protocols or agents that can demonstrate such an outcome in a trial would have a clear advantage in clinical use.

What long-term renal parenchymal damage (e.g. proteinuria, reduced eGFR, renal fibrosis and tubular dropout) is present pre- and post-treatment? Superior agents or protocols would not only abrogate an acute episode of AMR, but also prevent long-term microvascular, glomerular and tubular damage to the allograft. For example, complement inhibiting agents which reduce the post-AMR incidence of glomerular basement membrane duplication would be superior agents or protocols that abrogated AMR but did not prevent long-term allograft damage.

Are there post-treatment indicators of ongoing, low-level CAMR (e.g. continued presence of DSA, complement activation, biopsy evidence)? While related to the previous question, this is a distinct line of inquiry. Post-AMR damage may result from a cascade of inflammatory and fibrotic pathways which persist even in the eventual absence of DSA. In contrast, the presence of ongoing CAMR portends a worse long-term prognosis in terms of graft function and survival. Because study subjects may start with different initial levels of allograft damage, and sustain different levels of injury during the AMR episode, study designs that can demonstrate absence of CAMR measures after an AMR episode hold the promise of identifying successful interventions even in the presence of moderate but quiescent organ damage.

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Clearly this is an inclusive list, and measuring all indicators is probably not be feasible for every study. However, a study design which cannot answer a reasonable number of these questions is likely to decrease the value of the clinical trail or case series. Many of the questions can be answered with standard clinical tests, while some may require laboratory measures (ELISPOT assay for DSA, re-stimulation assays for donor-specific memory B cell frequencies). We also recognize that some of these questions are difficult to answer without invasive procedures, for example a bone marrow aspiration to assess donor-specific plasma cell mass reduction. Nevertheless, bone marrow aspiration to obtain plasma cells has been performed in recent studies, and has been highly informative assessing donor-specific long-lived plasma cell frequency (110).

In conclusion, the current practice in treatment of AMR is based on a moderate number of uncontrolled case series, single center studies, and sub-optimal clinical trials. Although a number of promising new therapies are emerging, we need more large, multicenter trials with adequate metrics to advance the field. Rational AMR clinical trial design can be achieved with current knowledge and analytic methods, and will likely improve the chances of identifying better therapies and protocols.

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