

## **Antibody-mediated rejection across solid organ transplants: manifestations, mechanisms, and therapies**

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*J Clin Invest.* 2017;127(7):2492-2504. <https://doi.org/10.1172/JCI90597>.

### **Review Series**

Solid organ transplantation is a curative therapy for hundreds of thousands of patients with end-stage organ failure. However, long-term outcomes have not improved, and nearly half of transplant recipients will lose their allografts by 10 years after transplant. One of the major challenges facing clinical transplantation is antibody-mediated rejection (AMR) caused by anti-donor HLA antibodies. AMR is highly associated with graft loss, but unfortunately there are few efficacious therapies to prevent and reverse AMR. This Review describes the clinical and histological manifestations of AMR, and discusses the immunopathological mechanisms contributing to antibody-mediated allograft injury as well as current and emerging therapies.

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# Antibody-mediated rejection across solid organ transplants: manifestations, mechanisms, and therapies

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**Solid organ transplantation is a curative therapy for hundreds of thousands of patients with end-stage organ failure. However, long-term outcomes have not improved, and nearly half of transplant recipients will lose their allografts by 10 years after transplant. One of the major challenges facing clinical transplantation is antibody-mediated rejection (AMR) caused by anti-donor HLA antibodies. AMR is highly associated with graft loss, but unfortunately there are few efficacious therapies to prevent and reverse AMR. This Review describes the clinical and histological manifestations of AMR, and discusses the immunopathological mechanisms contributing to antibody-mediated allograft injury as well as current and emerging therapies.**

## Introduction

Solid organ transplantation is a lifesaving, curative therapy for patients with end-stage kidney, heart, liver, lung, pancreas, or bowel disease. More than 100,000 patients await a transplant from the deceased-donor pool in the United States. In 2015, 30,000 patients received an organ transplant (organdonor.gov; ref. 1). Based on Organ Procurement and Transplantation Network (OPTN) data (February 15, 2017), more than 90% of renal allografts in the United States are still functioning by 1 year after transplant. However, the lifetime of allografts does not match the life expectancy of patients. Twenty percent of recipients will lose their graft within 5 years, and 50% within 10 to 12 years. Many other organs fare worse; for example, lung allograft survival at 5 years is only 50% (<http://optn.transplant.hrsa.gov>). Moreover, although the number of patients who need a transplant has steadily grown in the last two decades, the number of organ donors per year is stagnant (organdonor.gov). Therefore, it is of high interest to extend the survival of transplanted solid organs and the patients who receive them.

A major driver of organ transplant injury is the alloimmune response, predominantly to polymorphic HLA. HLA allosensitization occurs after exposure to non-self tissue, through pregnancy, cardiac mechanical assist device placement, transfusion, solid organ transplantation, and tissue grafting (e.g., burn patients, homograft repair of congenital heart defects, or coronary artery bypass graft). The pathogenic role of alloantibodies in solid organ transplantation was recognized in the 1960s, when Patel and Terasaki (2) seminally showed that donor-specific antibodies, detected by cytotoxic crossmatch, were associated with immediate failure of renal allografts. Since this era, advances in the detection of antibodies against HLA, the diagnosis of antibody-mediated rejection (AMR), and our knowledge of the mechanisms of antibody-mediated allograft injury have expanded our understanding of AMR.

According to OPTN data, approximately 60% of the US renal transplant waitlist is sensitized to HLA, with an overrepresentation of women and retransplant candidates. In the post-transplant period, production of donor-specific antibodies against HLA (DSAs) among previously nonsensitized adult renal allograft recipients is 7% by 5 years (3) and nearly 20% after 10 years (4–6). The incidence tends to be higher (up to 40%) in pediatric (7, 8) and medication-nonadherent adult transplant patients, with the latter constituting nearly two-thirds of adult transplant patients by 12 years after transplantation (6). Post-transplant DSAs are also produced by a significant proportion of heart (9), lung (10), liver (11), and intestinal transplant recipients (12). Risk factors for de novo DSA (dnDSA) production also include recipients of African American ethnicity, episodes of acute cell-mediated rejection in the first year after transplant (3, 6), donor-recipient mismatching for HLA-DQ (3), and pretransplant sensitization to HLA (nondonor) (4).

Patients transplanted with preformed DSAs are at higher risk of acute AMR, chronic rejection, and allograft loss across all solid organs (8, 10, 12–22). AMR prevalence among presensitized patients is more than 20% (23). Production of dnDSAs after any solid organ transplantation is a major risk factor for decreased long-term graft survival. By 5 years after the appearance of dnDSAs, 40% of patients lose their renal allografts, compared with better than 80% survival of patients without DSAs (6). Half to all of late allograft failures can be attributed to HLA DSA-mediated chronic rejection (4, 24–28).

This Review will focus on the clinical impact and mechanisms of AMR mediated by antibodies against classical HLA molecules, and discuss the current and emerging therapies to prevent and combat AMR.

## Manifestations and mechanisms of acute AMR

Acute T cell-mediated rejection typically responds well to increased immunosuppression, while AMR is poorly responsive to standard therapy. Diagnostic criteria for AMR were first established for renal and heart allografts (29, 30), and subsequently for pancreas, liver, and lung allografts (31–33). Features of AMR in bowel and vascularized composite tissue allografts (CTAs) have been described

**Conflict of interest:** E.F. Reed received research grant funding from CSL Behring and Immucor Inc. E.F. Reed is an inventor on US Patent Publication No. 20170030928.

**Reference information:** *J Clin Invest.* 2017;127(7):2492–2504.

<https://doi.org/10.1172/JCI90597>

**Table 1. The overarching features of AMR for cardiac, renal, pancreas, liver, lung, bowel, and composite tissue allografts**

Organ/ Tissue	Consensus criteria	Evidence of HLA DSAs needed	Features of acute antibody-mediated rejection				Notes
			Concurrent allograft dysfunction needed	C4d	Microvascular inflammation	Immune cell infiltration	
Heart	Yes (29, 39)	May or may not be present; but consideration to return to criteria (40)	No; may be clinical or subclinical	May or may not be present; linear capillary staining i <sup>+</sup> <sup>A</sup> if positive	May or may not be present; “EC swelling,” dilation, CD31 h <sup>+</sup> <sup>A</sup> if positive	May or may not be present; intracapillary CD68 macrophages and other IAMCs	Not all are required for a definitive diagnosis
Kidney	Yes (30)	Yes; required (HLA or non-HLA)	No; may be clinical or subclinical	Yes; linear PTC staining	Yes; glomerulitis or peritubular capillaritis	Yes; predominant monocyte component (116)	All are required for a definitive diagnosis
Pancreas	Yes (32)	Yes; required (HLA); “suspicious” if missing	Unknown; in ref. 192, all were for-cause biopsies	Yes; only if in IACs	Acinar injury, cytoplasmic swelling, vacuolization, capillary dilatation	Interacinar capillaritis with mononuclear cells or PMNs; CD68 can be helpful	C4d alone is insufficient but with MVI
Lung	Yes (33)	Yes; required (HLA)	No; may be clinical or subclinical	May or may not be present; alveolar capillary staining only	Not explicitly required; but described in ref. 95, as capillaritis	Neutrophilic capillaritis and arteritis; CD68 not informative	All are required for a definitive diagnosis
Liver	Yes (31); others: refs. 100, 118	Yes; required (HLA)	Unknown; studies report only for-cause biopsies, since protocol biopsy of this organ is rare	Yes; diffuse, >50% portal tract staining microvasculature, with or without sinusoidal or central vein involvement (98, 100)	Yes; portal microvascular endothelial hypertrophy, portal capillary and inlet venule dilatation	Monocytic, eosinophilic, and/or neutrophilic portal microvasculitis; CD68 less informative in this organ	
Intestine	No (193)	Recommended (35)	Unknown; lack of consensus: yes (194), no (195)	Recommended (35)	Possibly; capillary dilatation and congestion (196)	Adherent inflammatory cells in vessels; more severe rejection with transmural inflammation (195, 196)	Not well defined because of nonspecific C4d staining and paucity of vessels in biopsies
Composite tissue	No; early mention in ref. 34, but AMR is not part of these Banff 2007 criteria	Unknown	Unknown; not enough clear evidence	May not be informative (99)	Possibly; vasculitis (34)	Neutrophil margination	

Where consensus criteria have been described, the most recent citation is given. Where consensus criteria have not yet been published (intestine and composite tissue), relevant references describing putative features of AMR in these organs are given. <sup>A</sup>For cardiac transplantation, “pathological” AMR (pAMR) may be diagnosed in the setting of histological evidence of allograft injury (h<sup>+</sup>), such as capillary endothelial changes, and/or immunopathological evidence of AMR (i<sup>+</sup>), such as positive C4d staining. EC, endothelial cell; IACs, interacinar capillaries; IAMCs, intracapillary activated mononuclear cells; MVI, microvascular inflammation; PMN, polymorphonuclear cells, such as neutrophils; PTC, proximal tubule cell.

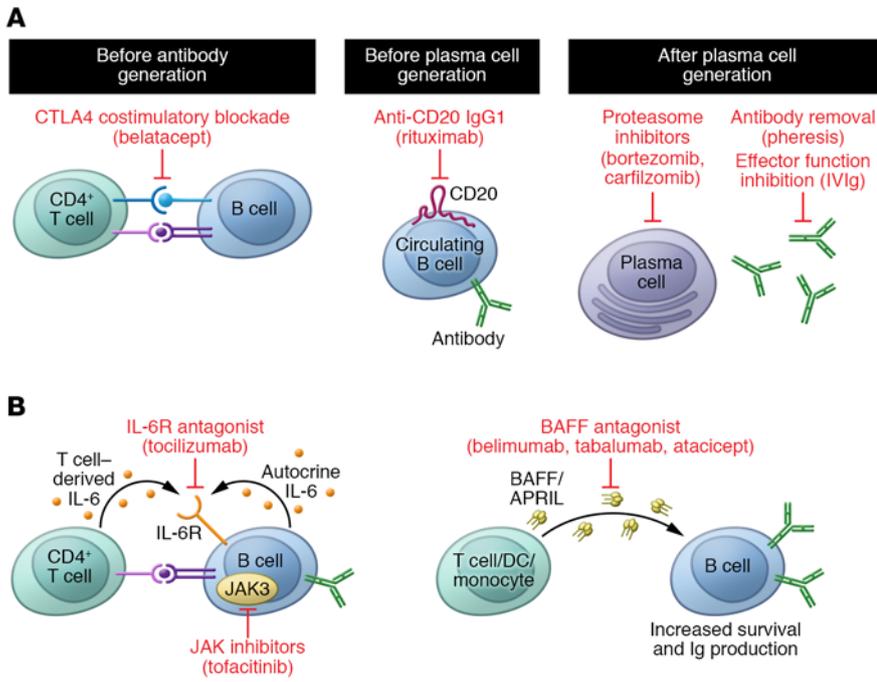
(34, 35), but consensus criteria for AMR in these tissues are still needed. Although growing evidence shows that antibodies to non-HLA antigens contribute to allograft dysfunction (36–38), no clear diagnostic criteria for non-HLA AMR have been established. Table 1 provides an overview of the current and proposed consensus criteria for the diagnosis of HLA AMR across solid organs and CTAs. Notably, four overarching features form the cornerstone of acute AMR diagnosis in transplanted solid organs (Table 1): serological evidence of antibodies, histological evidence of endothelial cell injury, complement activation, and infiltration of innate immune cells. These common features of AMR are discussed below in a framework to inform regarding their use for diagnosis, treatment, and understanding of the mechanisms of AMR.

### HLA donor-specific antibodies

**Diagnostic tools.** The presence of circulating DSAs is required for the diagnosis of AMR in renal (30), pancreas (32), liver (31), and

lung (33) transplantation. Currently, diagnosis of AMR in cardiac allografts does not require this “serological” component (39), but it may be reintroduced (40) with the acknowledgement that either HLA DSAs or non-HLA antibodies may contribute to AMR.

Detection of HLA antibodies uses a multiplexed assay with nearly 200 HLA antigens coupled to fluorescent beads to characterize an individual’s alloantibody repertoire. Extraordinarily high levels of pretransplant DSAs (greater than 10,000 median fluorescence intensity units [MFI] in our experience), especially against HLA class I antigens, may be cytotoxic and place patients at risk of hyperacute rejection. Patients are more likely to experience worse outcomes with stronger pretransplant DSA (21, 41). After transplant, DSAs of 2,500–3,000 MFI or above at the time of biopsy-proven rejection are associated with worse long-term outcome (42, 43) and increased incidence of AMR (3). Additionally, the persistence of DSAs is associated with worse outcome (19, 44).



**Figure 1. Current and emerging therapies to prevent HLA antibody production.** (A) Naive B cells rely on signals from CD4<sup>+</sup> T cells for full activation, Ig class switching, and Ig production. Blockade of costimulation using the CTLA4 fusion protein belatacept inhibits this important signal and hampers B cell activation. Naive as well as antigen-experienced B cells may be depleted from the circulation with antibodies against CD20, such as rituximab. Once HLA antibody-producing B cells have differentiated into plasma cells, they are more difficult to target. Because these antibody “factories” are metabolically active and reliant on the function of the proteasome, inhibitors such as bortezomib and carfilzomib, which block the 20S proteasome, may trigger apoptosis of these cells. Bortezomib is a reversible inhibitor, while carfilzomib is irreversible. Antibodies may also be physically removed from the circulation with plasmapheresis, and their effector functions inhibited by intravenous immunoglobulin (IVIg). (B) Emerging therapies to reduce alloantibody production include antagonism of the IL-6 receptor (tocilizumab), BAFF (belimumab, tabalumab, atacept), and inhibition of intracellular JAK (tofacitinib), which are needed for B cell activation and Ig production. BAFF/APRIL, B cell-activating factor/a proliferation-inducing ligand.

Solid-phase assays to detect HLA antibodies, although quite sensitive, may not predict the true pathogenic potential of DSAs. Several modifications of the HLA single-antigen test have been introduced that may provide useful information on the effector function of the DSA. In vitro assays measure binding of C1q (45) or deposition of C3d (46) or C4d (47, 48) to single-antigen beads, enabling identification of potentially complement-activating antibodies as well as their HLA specificity. The prognostic value of these assays remains controversial (5, 7, 20, 49–52), in part because in vitro complement activity appears to be tightly tied to antibody titer (20, 44, 53, 54).

Human IgG is composed of four subclasses. The subclass repertoire is potentially more informative for assessing the pathogenicity of DSAs than complement assays, as subclass predicts complement activation, Fcγ receptor-dependent (FcγR-dependent) functions, and the immunobiology of the alloantibody response. A growing number of reports suggest that characterization of DSA IgG subclass may have utility in identifying patients at risk of rejection or graft loss (5, 55–60). While enlightening, these studies have yet to capture the impact of mixtures of subclasses or multiple concurrent DSA specificities. It will be interesting to see how

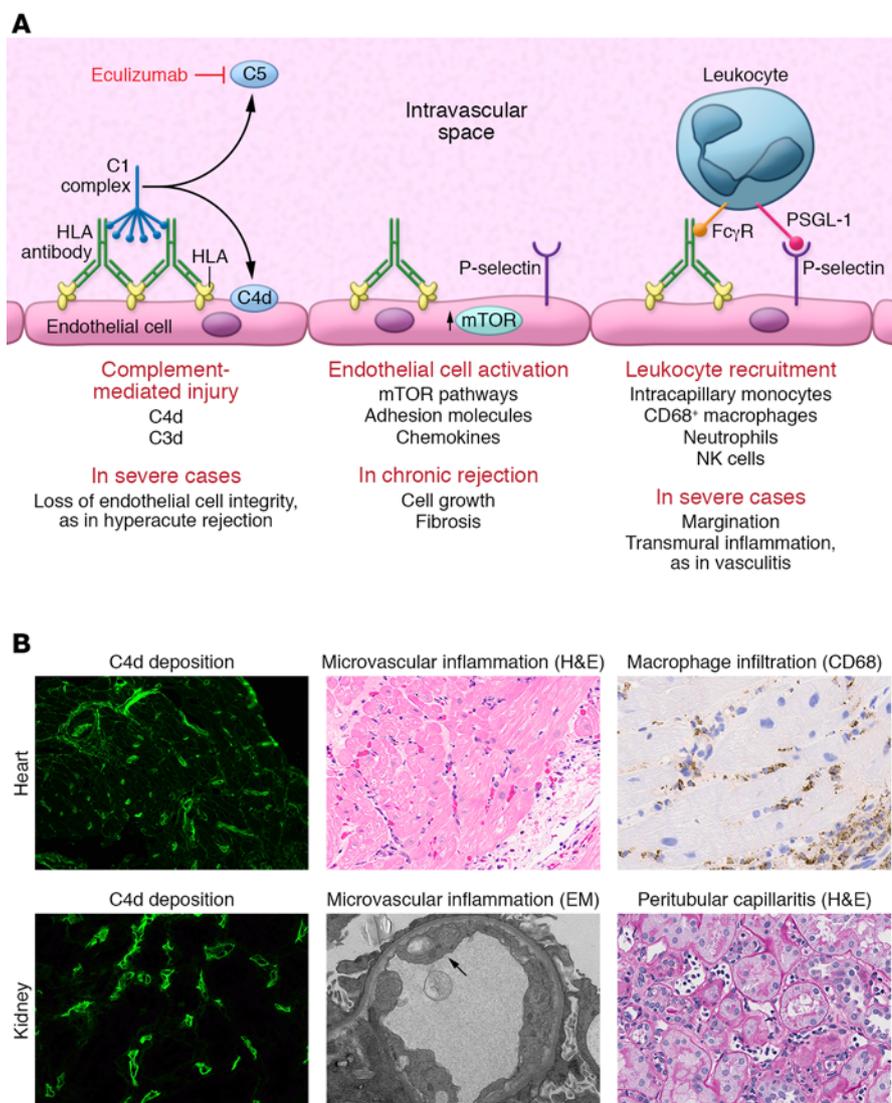
HLA IgG subclasses evolve longitudinally and correlate with graft pathology in transplant patients.

The human immune response is dynamic. Some patients exhibit “natural” or false-positive reactivity with recombinant HLA antigens (61), which do not detrimentally affect allograft outcome (62). On the other hand, patients with a known history of sensitization events may not exhibit detectable circulating anti-HLA antibodies at the time of transplant evaluation. This has prompted development of alternative methods to measure alloreactivity, including an HLA-specific B cell enzyme-linked immunospot (ELISPOT) (63, 64), ex vivo stimulation of B cells (65, 66), and staining of peripheral B cells with HLA tetramers (67). More work is needed to understand the clinical relevance of alloreactive memory B cells in the absence of circulating HLA DSAs.

**Therapies.** Nonadherence to maintenance immunosuppression is an important risk factor for dnDSA production in a number of studies (6, 68), suggesting that T cell inhibition is at least partly efficacious in delaying alloantibody formation. However, typical maintenance therapies (such as tacrolimus or mycophenolate) have little to no known effect on memory B cells or plasma cells.

Costimulatory blockade reduces the incidence of dnDSA production. Treatment of mice with cytotoxic T lymphocyte-associated protein-4-Ig (CTLA4-Ig) prevented alloantibody production when given in the first 2 weeks after sensitization (69, 70). Similarly, the CTLA4-Ig fusion protein belatacept reduced the risk of dnDSA formation in low-risk transplant patients (71). However, once patients present with preformed or de novo HLA antibodies, they become more challenging to manage. In combination with i.v. immunoglobulin, which has numerous proposed mechanisms of action (72), plasmapheresis can be effective at temporarily reducing circulating HLA antibodies. Peripheral B cells can be depleted with rituximab or other anti-CD20 immunotherapies (73). Eighty percent of highly sensitized transplant candidates responded to rituximab-based desensitization, enabling transplantation in patients who may otherwise have waited longer and possibly died before receiving an organ (74).

Importantly, CD20-directed biologics have little effect on the plasma cells responsible for the majority of serum antibodies. Peripheral concentrations of IgG are mostly unaltered in patients after rituximab treatment, despite depletion of more than 95% of circulating B cells (75). Thus, other therapies are needed to target the antibody-producing plasma cells within bone marrow niches. Proteasome inhibitors such as bortezomib (76) and carfilzomib (77) may more effectively target metabolically active plasma cells. On its own, a single dose of bortezomib is insufficient to reduce



**Figure 2. The known mechanisms of HLA antibody-mediated allograft injury and their therapeutic targets. (A)** HLA antibody binding to donor endothelial cells can trigger activation of the classical complement cascade. First, the complement C1 complex, which includes C1q, C1r, and C1s, binds to the IgG heavy chain Fc region. The C1 complex next sequentially activates serum complement proteins, catalyzing the generation of immunologically active split products. Activation of complement C5 protein occurs at the terminal stage of the signaling pathway, generating the highly potent anaphylatoxin C5a and initiating assembly of the membrane attack complex (MAC). In the process, the split product C4d becomes covalently bound to the endothelial cell surface and can be detected in biopsies of allografts undergoing rejection through immunofluorescent or immunohistochemical staining. Eculizumab is an mAb that prevents C5 cleavage. HLA binding also activates intracellular signaling within the donor endothelium, mainly via the mTOR signaling axis, and upregulation of the adhesion molecule P-selectin. P-selectin, in conjunction with the interaction between immune cell Fc $\gamma$  receptors (Fc $\gamma$ R), enhances leukocyte-endothelial adhesion. PSGL-1, P-selectin glycoprotein 1. **(B)** Representative micrographs illustrating the three main histological features of AMR in heart (top panels) and kidney (bottom panels) allografts. Deposition of C4d within the microvasculature is visualized by immunofluorescence in green. H&E staining demonstrates increased capillary endothelial cell size and numerous leukocytes in the intravascular space. Immunohistochemical staining for CD68 highlights intracapillary macrophages (original magnification,  $\times 400$ ). EM, electron micrograph.

DSA levels (78), but when bortezomib was combined with plasmapheresis and rituximab, 40% of renal transplant candidates with DSAs were successfully desensitized (76). Patients who undergo desensitization have higher rejection rates (79), but long-term outcomes are comparable to those of lower risk patients (80, 81). Figure 1A presents the stages at which B cell production of HLA antibodies may be targeted.

Tabalumab is an mAb that neutralizes B cell activating factor (BAFF/BlyS), a critical B cell factor. Disappointing results from clinical trials suggest that, on its own, BAFF antagonism is ineffective to durably reduce HLA antibody levels (82). However, given that higher BAFF levels are associated with greater sensitization to HLA (83, 84) and AMR (85), it remains an intriguing therapeutic target in the quest for suppressors of HLA antibody production. Another BAFF antagonist, atacicept, reduced dnDSA production in a nonhuman primate model of renal transplantation (86), and belimumab, which showed efficacy in systemic lupus erythematosus (87), is being investigated to reduce pretransplant HLA allosensitization (NCT02500251, ClinicalTrials.gov).

Tocilizumab is an mAb antagonist of the IL-6 receptor, which is critical in B cell activation and antibody production. Half of sen-

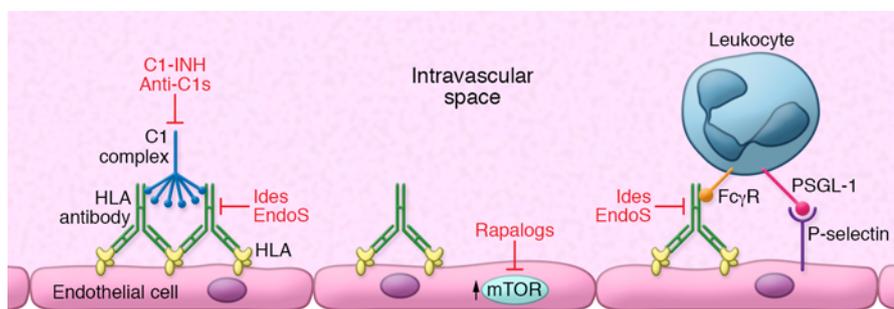
sitized kidney transplant patients who had failed desensitization with other methods experienced tocilizumab-induced reductions in HLA antibodies that facilitated transplant (88). Therefore, IL-6 antagonism appears useful in restraining HLA antibody production in sensitized individuals (Figure 1B), and may also be useful as a therapy for chronic AMR (89).

Finally, cytokine signaling in immune cells is dependent on intracellular actions of JAKs (Figure 1B). One example is JAK3, which is important for activation and Ig production in naive B cells (90). Although trials of the JAK inhibitor tofacitinib were halted because of adverse events (91), recent reviews have advocated reconsideration of the suitability of this and other JAK inhibitors in maintenance immunosuppression for transplant (92).

To summarize, there is a need for additional approaches to prevent alloantibody generation and more effective therapies to specifically deplete DSA-producing B and plasma cells.

## Complement deposition

**Diagnosis.** Figure 2 illustrates the mechanisms of allograft injury by HLA donor-specific antibodies, and provides exemplary histological images of AMR features in cardiac and renal allografts. His-



**Figure 3. Emerging therapies to inhibit HLA antibody-induced allograft injury.** Emerging therapies for AMR include upstream complement inhibition with C1 esterase (C1-INH) or antibody against C1s. DSAs are capable of cross-linking HLA molecules on the donor endothelial and smooth muscle cells, resulting in activation of the mTOR signaling axis. Rapamycin (sirolimus) and other rapalogs (everolimus) inhibit mTOR and may dampen vascular cell growth and fibrosis, which contributes to chronic rejection. Finally, abolishing Fc-dependent antibody effector functions through cleavage of the Fc region with IdeS, or removal of the N-linked glycan with EndoS, is likely to hamper activation of the classical complement cascade as well as prevent interactions with FcγRs on monocytes, neutrophils, and NK cells.

tological detection of endothelial membrane-associated complement split product C4d is widely used to provide evidence of DSA interaction with the allograft vasculature, first described in refs. 93 and 94. C4d is a reliable and specific, but not sensitive, marker of AMR in heart, kidney, and pancreas allografts when deposited in the capillaries. C4d may be nonspecifically positive in larger vessels or the interstitium. In contrast, there is a lack of consensus regarding the utility of C4d staining in lung (95–97), visceral, liver, and composite tissue transplantation, although it has been recommended in conjunction with other indicators of antibody-mediated graft injury (31, 35, 98–100). C4d positivity is considered but not required for a diagnosis of AMR in cardiac and renal allografts, and C4d detection often misses cases of AMR (101–103).

**Mechanisms and therapies.** Antibodies interacting with their targets may activate the classical complement cascade, the initiation of which is regulated by the C1 complex that binds to the Fc portion of IgG. The complement pathway is a sequential series of amplified catalytic events leading to activation of successively more potent serum complement factors. The end result of terminal complement activation is generation of the potent anaphylatoxin C5a and formation of the membrane attack complex (MAC). Even during acute AMR, MAC-mediated cell lysis is uncommon. Endothelial cells, which are constantly exposed to serum complement factors, express a repertoire of cell surface complement regulatory proteins (CD59, DAF, Cr1) that actively antagonize and limit complement activation. Complement activation is also negatively regulated by serum factors, such as the serpin C1 esterase (C1-INH).

At sublytic levels, MAC has been shown to activate noncanonical NF-κB signaling in endothelial cells, leading to cytokine and adhesion molecule expression (104). Upstream complement components such as C3a, C3b, and C5a are physiologically active and act on both endothelial cells and leukocytes to enhance inflammation (105). Eculizumab is a recombinant antibody against complement C5. Eculizumab may facilitate transplantation across high levels of donor-specific antibodies (106) and be effective as salvage therapy for aggressive AMR (107). However, patients treated with eculizumab remain at risk of acute AMR and chronic rejection

(108, 109). These findings suggest that upstream complement activation and/or non-complement-mediated effector functions mediate allograft damage.

Emerging therapies to suppress activation of complement include those that act at earlier points in the pathway (Figure 3). One example is the serum-derived endogenous factor C1-INH (Berinert, Cinryze, NCT02547220; ref. 110). Alternatively, an anti-C1s antibody is in clinical trials for the treatment of complement-mediated diseases (NCT02502903). Since the capacity of IgG to activate complement depends on interaction with C1q via the glycosylated CH2 domain in the Fc region, other possible therapies to attenuate complement-mediated injury include the *Staphylococcus aureus* enzymes IdeS and EndoS (111–113).

These enzymes cause cleavage of the Fc tail (IdeS) or of the N-linked glycan (EndoS) and abolish complement activation in animal models of antibody-mediated inflammation (111–114). Clinical trials for desensitization with IdeS are currently under way in Europe and the United States (NCT02426684); however, such approaches are likely to be limited because of (a) risk of allergic reaction due to antibody responses against these bacterial enzymes, and (b) reconstitution of circulating IgG, which occurs in 2–3 weeks. Fc inactivation could be a salvage therapy, but likely needs to be combined with effective B cell depletion for durable protection of the allograft from DSAs.

In summary, complement activation in the allograft can be detected by immunohistochemistry for C4d, which is an important but not universal feature of AMR. Several established and emerging therapeutics exist for antagonism of complement activation in transplant recipients, but they do not represent a “silver bullet,” since there are concurrent, non-complement-mediated mechanisms of allograft injury triggered by HLA antibodies.

### Intravascular mononuclear cells

**Diagnosis.** Another key characteristic of AMR across solid organs is the presence of intracapillary mononuclear cells (leukocytes) in the allograft (Figure 2). While larger vessels may be affected in more severe cases of AMR (“arteritis” or “vasculitis”; ref. 42), capillary dilatation with inflammatory cell margination with characteristic linear staining is a key feature of AMR. Staining for the macrophage marker CD68 has been shown to be informative for diagnosis of AMR in cardiac allografts (115). Although not included in the diagnostic criteria, increased glomerular macrophage burden correlates with AMR in renal allografts and is a histological feature of acute tissue injury in AMR with or without C4d deposition (30, 116, 117). The utility of CD68 staining in lung and liver is questionable. Rather AMR is characterized by a monocytic, neutrophilic, or eosinophilic infiltrate in these organs (31, 33, 95, 118), again with inflammatory cells adherent to microvascular endothelial cells.

Growing evidence in human and murine transplantation shows that other leukocyte subsets play an important role in AMR. T lymphocytes may also be found in allografts undergoing AMR

(119). Infiltrating B cells form ectopic lymphoid structures within the allograft and locally secrete HLA antibodies (120, 121). NK cells and their associated transcripts are increased in human renal, cardiac, and lung allografts during AMR (122–126) and mediate acute and chronic AMR in mouse transplant models (127–130).

**Mechanisms and therapies.** In vitro, HLA I antibodies mobilize endothelial vesicles called Weibel-Palade bodies, externalizing the adhesion molecule P-selectin (131, 132). Cell surface P-selectin expression by endothelial cells in turn supports adhesion of platelets (133), neutrophils (132), and monocytes (131) in vitro and in vivo, which likely contributes to the infiltration of recipient immune cells. Interactions between endothelial-bound IgG and myeloid cell Fc $\gamma$ Rs can facilitate tethering and adhesion of leukocytes in autoimmune inflammation (134) and in response to HLA antibodies (refs. 135, 136, and Figure 2). Active complement-mediated inflammation also promotes recruitment of T cells, monocytes, and neutrophils via direct chemotactic effects on immune cells, and by amplifying endothelial cell activation (104). Intra-graft macrophages have a proinflammatory phenotype during acute rejection, and a repair/profibrotic phenotype during chronic rejection (137), suggesting that macrophage effector functions differentially contribute to acute and chronic AMR.

NK cells express one receptor for IgG, Fc $\gamma$ RIIIA (CD16a), which facilitates antibody-dependent cell-mediated cytotoxicity against target cells. Stimulation of NK cells through Fc $\gamma$ RIIIA increased expression of a number of proinflammatory genes in vitro that were also validated in biopsies from human renal (138) and heart allografts (124) undergoing AMR. IFN- $\gamma$  production by NK cells can be triggered by allogeneic target cells coated with alloantibody (139), and contributes to chronic rejection in murine models (129). Current immunosuppressive drugs incompletely inhibit NK cell-mediated alloreactivity (140).

There are currently no therapies directly aimed at preventing recruitment or function of allograft infiltrating leukocytes. It is possible that treatment of DSA-positive patients with IdeS and/or EndoS to cleave the Fc region of circulating IgG might be efficacious in reducing Fc $\gamma$ R-mediated monocyte recruitment into the allograft (ref. 136 and Figure 3), but this application has yet to be tested in the clinic. Although we can conjecture from findings in other disease models, it is not clear exactly what intravascular macrophages, NK cells, and neutrophils are doing in the allograft that contributes to injury. Moreover, the interrelated mechanisms, including additive effects, mutual regulation, and feedback, of these distinct leukocyte subsets are a complex and fascinating issue that remains to be elucidated. Consequently, the functions of such innate immune cells, and how they interweave, may represent new targets of immunosuppressive therapy to treat AMR.

## Microvascular inflammation and endothelial injury

**Diagnosis.** Donor endothelial cells are in direct contact with the recipient immune system and are the predominant target of the alloimmune response. AMR typically manifests in the microvasculature of the transplanted organ. Microvascular inflammation appears as endothelial cell or cytoplasmic swelling or enlargement and vacuolization. In cardiac allografts, staining of endothelial cells with CD31 highlights capillaries with endothelial dilation,

nuclear enlargement, or “swelling” (29, 115). In the lung, capillary inflammation was associated with HLA DSAs (96), and in liver, endothelial cell hypertrophy and dilatation are noted in the peribiliary capillaries and portal microvasculature (31).

HLA antibodies promote activation of intracellular signaling in vitro and in animal models (141–147). Several studies have demonstrated that detection of phosphorylated signaling molecules that are activated downstream of HLA cross-linking, including S6 ribosomal protein and S6 kinase, aids in detection of AMR in cardiac allografts (101, 102, 148). Sis and colleagues first reported that rejecting renal transplants exhibit increased expression of endothelial-associated transcripts (149, 150), a finding that has been further substantiated by the same group in cardiac allografts (124, 151). Thus, the “molecular microscope” may enable pathologists to further distinguish AMR from other forms of allograft injury (152), and facilitate a better understanding of intra-graft changes during AMR, but the field awaits confirmation of these studies by other laboratories.

**Mechanisms and therapies.** Endothelial cell injury and activation lie at the heart of antibody-mediated allograft damage. Extensive in vitro evidence points to an intrinsic role for HLA signaling within the graft vasculature that promotes angiogenic and inflammatory responses (Figure 2). For example, cross-linking of HLA class I with bivalent antibodies mimics *trans* interactions with T cell receptors, triggering phosphorylation of Src and focal adhesion kinases (FAKs), mTOR, ERK, and S6 ribosomal protein in endothelial and smooth muscle cells (143, 145, 146). These signaling pathways promote cytoskeletal rearrangements, as well as increased wound healing and cell growth (143, 145, 146). Activation of these signaling molecules during AMR has been substantiated in vivo in a murine model (147) as well as in human cardiac allografts (101, 102). Much less is known about the signaling events in endothelium after HLA II cross-linking. This is a significant gap in our knowledge, since numerous studies have found a strong relationship between DSAs against HLA-DQ and chronic rejection. Exposure of HLA-DR-expressing endothelial cells increased ERK and Akt phosphorylation (153), as well as activation of proinflammatory Th17 cells (154). More studies are needed to elucidate the effect of antibodies against HLA II on vascular endothelial cell signaling, and how they contribute to acute and chronic AMR.

Currently, no treatments exist to directly modulate graft vascular function. However, given what is known from experimental models and clinical studies, the mTOR signaling axis is activated by HLA antibodies and represents a viable therapeutic target to dampen endothelial cell activation during AMR (Figure 3).

## Allograft dysfunction

AMR manifests as a broad spectrum both histologically and symptomatically. Certainly, AMR identified on for-cause biopsies concomitant with overt allograft dysfunction warrants treatment. Patients who experience clinically symptomatic AMR and severe AMR have significantly worse long-term outcomes compared with stable patients or those experiencing other forms of rejection, such as acute cell-mediated rejection (155). However, patients may also have detectable circulating DSAs but no histological rejection or allograft dysfunction. In other cases, DSAs may be present with subtle changes on biopsy, but no impairment of function.

**Table 2. The features of transplant vasculopathy across solid organs and composite tissue allografts**

Organ/ Tissue	Primary reference	See also	Organ-specific name	Features of transplant vasculopathy			Notes
				Vascular lesions	Fibrosis	Inflammatory component	
Heart	29, 39	25, 40	Cardiac allograft vasculopathy or arteriosclerosis	Arteriosclerosis with concentric and diffuse proliferative lesions; epicardial and myocardial arteries; loss of capillaries/microvascular density; apparent with light microscopy/H&E	May have medial atrophy	May or may not be present with arteritis, endothelialitis, or transmural inflammation; may have concurrent acute microcirculation inflammation	Usually missed by endomyocardial biopsy; distinct from atherosclerotic plaques
Kidney	30	152, 173	Transplant glomerulopathy in glomerular capillaries; transplant arteriopathy in arteries, arterioles and other vessels	Glomerular hypertrophy; may also be a component of chronic arteriolar change (intimal fibrosis, transplant arteriopathy), luminal narrowing of arteries	Duplication of glomerular BM, multilamination of the peritubular capillary BM; neomedial formation and fibrosis in arteries	Often intracapillary mononuclear cells are present; may have concurrent acute glomerulitis	Visualized by light microscopy (severe) and ultrastructural (EM) for BM lamination
Pancreas	32	32	Arteriopathy; graft sclerosis	Luminal narrowing	Arterial intimal fibrosis	Accompanied by mononuclear cell infiltration	Larger vessels often missed on biopsy; accompanied by acinar loss
Lung	33	96	Chronic vascular rejection	Fibrointimal thickening of pulmonary arteries and veins	Intimal fibrosis of larger pulmonary vessels, may have medial atrophy	Mononuclear cell infiltration	Linked with but distinct from obliterative bronchiolitis, which affects the airways; often missed by transbronchial biopsy
Liver	31	28, 100, 118	Chronic rejection	Obliterative arteriopathy	Noninflammatory fibrosis	Mononuclear periportal and/or perivenular inflammation; accumulation of foam cell macrophages in the intima	Accompanied by bile duct loss
Intestine	Not well defined (193)	197	Obliterative arteriopathy	Arteriopathy affecting the mesenteric and other submucosal arteries, concentric intimal thickening; intimal hyperplasia and luminal narrowing	Fibrosis of submucosa		Often missed by surveillance biopsy
Composite tissue	34	166	Chronic vasculopathy	Possibly vascular narrowing, myointimal proliferation; seen in major vessels (e.g., donor radial and ulnar arteries in hand)	Duplication of elastic lamina		Often missed by punch biopsy

Note that chronic AMR is a distinct diagnosis that is often concurrent with transplant vasculopathy. BM, basement membrane; EM, electron microscopy.

Rush et al. first noted subclinical AMR in protocol renal allograft biopsies (156), and subsequently there has been increased appreciation that patients without overt graft dysfunction may exhibit abnormal histology on biopsy of renal (6, 43, 157), cardiac (155, 158), and liver (159) allografts. For example, among patients who developed dnDSAs but had stable allograft function, 25% exhibited acute subclinical AMR and 7.5% had chronic AMR on surveillance biopsy (3). One year after dnDSA appearance, these patients had further increased incidence of AMR (3). Importantly, patients with subclinical AMR experience faster rates of allograft function decline, higher incidence of transplant vasculopathy (TV), and greater risk of graft loss (6, 21, 43, 160), falling at intermediate risk for graft failure between those without DSAs or dysfunction and those with clinically symptomatic AMR.

Patients treated with plasmapheresis for subclinical AMR had no significant difference in their risk of graft loss at 5 years com-

pared with when subclinical AMR was left untreated (21). Taken together, these studies provide compelling evidence that patients with DSAs but without overt clinical dysfunction are at higher risk for graft loss compared with their DSA-negative counterparts. Intervention is therefore warranted, particularly when AMR is severe (161), but current therapies do not appear efficacious to improve long-term outcomes.

## Manifestations and mechanisms of chronic AMR and TV

*Incidence and clinical impact.* Repeated injury of the graft vasculature and uncontrolled repair responses contribute to chronic rejection (25, 162) that, in many cases, progresses to TV. Thirty percent of heart transplant recipients are diagnosed with TV by 5 years after transplant, and 50% by 10 years after transplant (163). TV is a leading cause of retransplantation. While nonim-

mune factors also contribute to TV, the majority of renal allograft loss due to chronic rejection was associated with HLA DSAs (24, 164). Chronic rejection is also an important cause of late failure of vascularized CTA (165, 166). Similarly, chronic damage and TV in heart (25), lung (95, 167, 168), pancreas (169), and liver (11, 28, 170) allografts are more common among patients with a history of biopsy-proven AMR and HLA DSAs.

Only half of renal allografts were functioning within 18 months of diagnosis of chronic AMR (164). Median survival time of lung allografts after diagnosis of bronchiolitis obliterans syndrome (BOS) is less than 3 years (171). TV accounts for one-third of cardiac allograft recipient deaths late after transplant (163). Therefore, chronic rejection culminating in TV is currently an irreversible process with significant impact on patient morbidity and mortality.

Chronic AMR occurs late (more than 1 year) after transplant but exhibits many of the features characteristic of acute AMR in each organ, including endothelial cell changes, microvascular inflammation, C4d, and circulating donor-specific antibodies. In addition, fibrosis is an important characteristic of chronic AMR (31, 172). For example, transplant glomerulopathy is a feature of chronic AMR in renal allografts, with duplication of the basement membrane in the glomerulus and peritubular capillaries (173).

Allografts with TV suffer from neointimal expansion and fibrosis in the larger vessels, which is largely considered a manifestation or consequence of chronic AMR. TV may be missed on routine biopsy owing to its predominant involvement of larger vessels that are not often observed except on deep tissue samples. For example, in the heart, cardiac allograft vasculopathy (CAV) is detected by imaging such as intravascular ultrasound or histologically only upon explant. CAV appears as arteriosclerotic lesions with inflammatory neointimal hyperplasia that can be clearly distinguished from eccentric atherosclerotic plaques (25). Intimal thickening may be so severe as to fully occlude the affected vasculature. Similarly, arteriopathy of kidney transplants is a consequence of intimal fibrosis and expansion in the renal arteries. An overview of the features of TV across organs and CTA is provided in Table 2.

*Mechanisms and therapies.* Although clinical evidence strongly supports the association between chronic AMR, TV, and HLA DSAs in numerous solid organs, the precise mechanisms by which neointimal proliferation and fibrosis occur are unclear. In part, this is because it is difficult to model this process *in vitro*, and there has been a historical lack of an appropriate animal model. Multiple other mechanisms contribute to TV, including chronic T cell-mediated injury and viral and/or metabolic risk factors, but HLA antibodies are both a major risk factor and a probable causative agent for chronic vascular changes. Our group and others have demonstrated in *in vitro* models that HLA antibodies trigger pro-survival and proliferative intracellular signaling via ERK and mTOR in vascular endothelial and smooth muscle cells, promoting growth, cytoskeletal changes, and migration (131, 141–147). Phosphorylation of these signaling molecules has been substantiated in AMR in both mouse and human allografts (102, 147, 148). HLA I antibodies also elicit matrix metalloproteinase- and sphingolipid-mediated signaling in vascular smooth muscle cells, which are thought to contribute to the intimal hyperplasia seen in TV (174,

175). Antibodies, NK cells, and complement likely promote endothelial apoptosis, enhancing leukocyte adhesion, matrix remodeling and fibrosis, and thrombosis (176). Indeed, in early TV, coronary artery endothelia exhibited apoptotic phenotypes (177). New evidence shows that apoptotic endothelial microparticles are increased in renal transplant recipients with AMR (178), in cardiac allograft recipients with CAV (179), and in lung recipients with BOS (180). Such graft-derived exosomes and microvesicles might represent an important marker of graft damage, as well as a potential source of alloantigen.

Endothelial cells exposed to HLA antibodies also elaborate IL-8, TNF- $\alpha$  (181), and VEGF (182), which can signal in an auto-crine fashion or act on smooth muscle cells in a paracrine manner. Complement activation at the endothelial surface can enhance these processes leading to intimal expansion by acting directly on endothelial cells and augmenting adhesion of leukocytes (104), although in murine models complement deficiency did not impact TV (127).

*In vivo* studies using T cell- and B cell-deficient murine recipients passively transferred with anti-donor MHC I antibodies demonstrated that DSAs triggered vascular lesions in cardiac allografts reminiscent of TV in humans (127, 129). This model has identified a key role for NK cells, IFN- $\gamma$ , and Fas/FasL-mediated injury in the process of DSA-induced TV (127, 183). An alternative model using passive transfer of anti-HLA antibodies and human T cells into murine recipients of human arterial grafts demonstrated that CD4<sup>+</sup> T cells also infiltrate the neointima and produce IFN- $\gamma$  (104).

No treatment options reverse TV once established. Use of statins is encouraged because of a substantial survival benefit and significantly lower incidence of CAV (184, 185). In addition, mTOR inhibitors reduce neointimal thickening in animal models and in human transplantation (186–188). This is likely partly attributable to general antiproliferative properties, since mTOR inhibitors also reduce neointimal hyperplasia in other vascular injury models (189). We have proposed that mTOR inhibition may also ameliorate progrowth signaling in the vasculature triggered by HLA antibodies (143). Recently, antibody-mediated TV in murine recipients capable of mounting a *de novo* antibody response against renal allografts was reported (190). This breakthrough holds promise to elucidate the physiological processes driving chronic allograft injury by alloantibodies, including the independent and interrelated contributions of complement, endothelial signaling, and different leukocyte subsets.

## Conclusions

The mechanisms of HLA antibody-mediated allograft injury are manifold, and manifest as universal histological features across all transplanted solid organs. AMR is characterized by circulating DSAs or non-HLA antibodies, evidence of complement deposition, microvascular inflammation with leukocyte infiltration, and endothelial injury. Repeated injury of the allograft culminates in TV, with fibrosis and neointimal expansion in larger vessels. The arsenal of therapies to combat AMR and chronic rejection is growing, but today's treatments do not address all of the components of antibody-mediated injury. In particular, the intracellular signaling pathways leading to endo-

thelial dysfunction and recruitment of leukocytes have yet to be effectively targeted. AMR also presents as a continuum of histological and clinical features. Advancing toward precision medicine in transplantation will require an understanding that each patient may need a different, combinatorial approach to prevent and alleviate AMR. Along these lines, improving the organ allocation system to favor HLA or HLA epitope matching or avoiding specific epitope mismatches that are highly immunogenic holds promise to reduce allosensitization and improve graft and patient outcomes (191). This strategy would be particularly useful in children, who will likely require two or more transplants during their lifetime.

## Acknowledgments

This work was supported by NIH grant RO1AI42819 (to EFR). The authors gratefully extend their thanks to W. Dean Wallace (UCLA Department of Pathology and Laboratory Medicine) for critical review of the manuscript and for providing the micrographs of allograft histology in Figure 2.

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