The perfect storm: HLA antibodies, complement, FcγRs, and endothelium in transplant rejection

Kimberly A. Thomas*, Nicole M. Valenzuela*, and Elaine F. Reed

Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA

The pathophysiology of antibody-mediated rejection (AMR) in solid organ transplants is multifaceted and predominantly caused by antibodies directed against polymorphic donor human leukocyte antigens (HLAs). Despite the clearly detrimental impact of HLA antibodies (HLA-ABs) on graft function and survival, the prevention, diagnosis, and treatment of AMR remain a challenge. The histological manifestations of AMR reflect the signatures of HLA-Ab-triggered injury, specifically endothelial changes, recipient leukocytic infiltrate, and complement deposition. We review the interconnected mechanisms of HLA-Ab-mediated injury that might synergize in a ‘perfect storm’ of inflammation. Characterization of antibody features that are critical for effector functions may help to identify HLA-ABs that are more likely to cause rejection. We also highlight recent advances that may pave the way for new, more effective therapies.

Rejection of solid organ transplants challenges long-term allograft survival

Organ failure is an immense human and economic burden that can be successfully reversed with transplantation, substantially improving quality of life and life expectancy. In the USA, more than 100 000 patients currently await transplantation of major solid organs. Significant advances in histocompatibility and immunosuppression have dramatically improved short-term graft and patient survival rates. Recipient recognition of donor HLA (see Glossary) present in the allograft induces an allogeneic immune response resulting in the production of donor-specific HLA-ABs (DSAs). These antibodies, through many different effector functions, are responsible for the damage, and ultimately graft rejection, that occurs in AMR. AMR has emerged as a leading cause of graft dysfunction and reduced outcomes but is often unresponsive to current therapies [1]. Histological markers of AMR are frequently unreliable and it is controversial whether intervention is required for patients with DSAs but no graft dysfunction. Clinical evidence suggests that DSAs alone, in the absence of histological or molecular evidence of antibody-mediated injury, is not detrimental to renal allograft survival [2,3]. However, long-term follow-up studies of asymptomatic or subclinical AMR in cardiac [4,5] and renal [6] transplantation have demonstrated increased risk for chronic rejection. Consequently, AMR remains a diagnostic and therapeutic challenge. Here we highlight recent developments in our understanding of how HLA-ABs function to cause graft injury, emphasizing the multiple effector mechanisms of HLA-ABs, specifically IgG, and how they relate to the risk for and manifestations of AMR.

Glossary

Acute rejection: commonly refers to rejection that arises rapidly and causes graft dysfunction within days to weeks, often occurring in the early post-transplantation period (less than 1 year). May be mediated primarily by T cells [TCMR or acute cellular rejection (ACR)] or primarily by antibodies (humoral rejection or AMR) and can often be reversed with aggressive treatment.

Allograft: transplanted cells or solid organ from a genetically disparate member of the same species.

Alloimmunity: adaptive immune responses against non-self cells or tissue from members of the same species as a result of polymorphisms in proteins that are then recognized as foreign antigens.

Chronic rejection: also called transplant allograft vasculopathy (TAV), transplant arteriopathy, or transplant arteriosclerosis (TA) in cardiac allografts, transplant glomerulopathy (TG) in renal allografts, bronchiolitis obliterans syndrome (BOS) in lung allografts, and vanishing bile duct syndrome in liver allografts. Progressive and irreversible fibrosis and occlusion of the donor vasculature, distinct from native atherosclerosis in that it is concentric rather than focal and affects only the vessels of the allograft. Thought to result from repair mechanisms in response to successive insults or indolent, ongoing injury from antibodies and/or CD4 T cells; manifests as an expanded subendothelial layer comprising ECs and smooth muscle cells that have migrated and proliferated in the neointima, as well as CD4 T cells and macrophages.

Classical complement pathway: a system of proteases that consecutively cleave downstream components to generate catalytically active or inflammatory and cytolytic products. The classical pathway is activated by immunoglobulin and initiated by binding of C1 complex to the Fc region of IgM or IgG.

Donor-specific HLA-Ab (DSAs): antibodies directed against polymorphic HLA molecules expressed by donor tissue.

Fc receptors: receptors for the crystallizable fragment (Fc) of immunoglobulin, expressed by myeloid and some lymphoid cells. Link the innate immune system with adaptive immunity; binding to complexed or immobilized antibody triggers intracellular signaling leading to activation and inflammatory effector functions.

Human leukocyte antigen (HLA): genes encoded by the MHC. These proteins function in antigen presentation of peptides to T cells and are the most polymorphic loci in the human genome.

Transplant rejection: alloimmune response of the recipient against transplanted donor cells, tissues, or organs.

**Corresponding author:** Reed, E.F. (ereed@mednet.ucla.edu).

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*These authors contributed equally to this work.

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The alloimmune response

Immunity to alloantigens is surprisingly robust, mediated by MHC molecules, and based on exposure to allogeneic tissues. The MHC locus covers almost 4000 kb on human chromosome 6 and is polygenic, containing three loci of HLA class I (HLA-A, -B, and -C) and six to nine functional HLA class II loci (α and β chains of HLA-DR, -DP, and -DQ), as well as many nonclassical MHC, minor histocompatibility antigen, and immune-related genes. Balancing selection has resulted in extreme polymorphism within HLA class I and class II genes. To date, over 10,000 nucleotide sequences encoding more than 6000 class I and 2000 class II unique proteins have been reported [7]. The high allelic diversity of MHC genes is advantageous for protection of populations against pathogens but is highly unfavorable for cell and organ transplantation.

Immune sensitization to HLA occurs after exposure to allogeneic tissue through pregnancy, transfusion, or transplantation. Twenty percent of healthy individuals [8,9] and up to 30% of transplantation candidates have HLA-Abs. Another 8–25% of recipients develop de novo DSAs after receiving a graft [10–12]. Half of pre-sensitized patients and a third of patients with de novo DSAs will experience AMR within the first year after transplantation [10,12]. Antibody responses against donor HLA proteins are not well controlled by current immunosuppression regimens [1]. Therefore, AMR can occur at any time and is a common occurrence more than 1 year post-transplantation [13]. DSAs and subsequent rejection episodes are strongly associated with risk of chronic rejection and late graft failure [13–15].

Histological manifestations and diagnostic criteria of AMR

AMR is best defined in renal [16], cardiac [17], and pancreas [18] transplantation, although the diagnostic histological criteria for AMR differ somewhat from organ to organ. Central features include endothelial cell (EC) swelling, microvascular inflammation (subendothelial mononuclear cell infiltration), and intravascular CD68+ macrophages, with or without complement C4d deposition, often in the presence of circulating DSAs (Figure 1) [17,19,20]. While HLA-Abs are detrimental to liver [21], lung [22], and small bowel [23] allograft survival, clear pathological definitions of AMR remain contentious [17], as the utility of C4d and other histological markers remains unclear in these tissues.

The donor vasculature present at the interface between donor tissue and the recipient immune system is the primary target of the alloimmune response. AMR is increasingly viewed as predominant endothelial injury and vascular inflammation [24,25] and the principal involvement of the endothelium in AMR has been revealed by gene-profiling studies of renal biopsies undergoing AMR [2,3,26].

HLA-Abs and subclass biology

The fact that some patients with DSAs do not experience AMR suggests that other factors influence susceptibility or risk of rejection in the presence of antibodies that bind the graft. The histological manifestations of AMR reflect the

![Figure 1. Human leukocyte antigen (HLA) antibodies cause graft injury by inducing phenotypic changes in the donor vasculature. HLA crosslinking by antibodies of any subclass causes intracellular signaling leading to endothelial cell (EC) activation. Activated ECs express P-selectin, which promotes recruitment of leukocytes via interactions with P-selectin glycoprotein ligand-1 (PSGL-1). Recruited monocytes differentiate into CD68+ macrophages, which can be detected histologically in the capillaries and subendothelial space. Crosslinking of HLA molecules also enhances EC immunogenicity to recipient CD4 T cells, which proliferate and differentiate in response to alloantigen HLA class II. Complement-activating antibodies trigger the classical pathway through binding of C1q, resulting in production of the anaphylatoxins C3a and C5a, which have the potential to directly augment leukocyte recruitment and T cell alloresponses. Complement activation can be detected by immunohistochemical staining for C4d.

Monocytes, neutrophils, and natural killer (NK) cells also express Fc receptors (FcγRs), which can interact with the heavy chain of HLA antibodies bound to donor ECs. FcγR functions augment leukocyte recruitment and mediate phagocytosis and antibody-dependent cellular cytotoxicity. Taken together, the pleiotropic functions of HLA antibodies on the allograft ECs cause the microvascular inflammation characteristic of antibody-mediated rejection. Antibodies in the figure with the same coloration as the Fc region are of the same subclass, whereas the various colors within the F(ab′)2 denote unique antigenic specificities.

injurious functions of HLA-Ab binding to the vasculature, causing endothelial signaling and inflammation, activation of the classical complement cascade, and recruitment of effector cells. IgG is the most common isotype of circulating immunoglobulin and is divided into four subclasses with unique patterns of biological activity. IgG3 is the strongest activator of complement, followed closely by IgG1, and, to a far lesser extent, by IgG2 [27]. IgG4 has no detectable complement activity and is often linked with IgG2 as ‘non-complement fixing’. However, it should be noted that, under unique conditions such as high antigen/epitope density or increased concentrations of complement

Review
Table 1. Summary of the biological properties of human FcγRs and IgG subclasses

<table>
<thead>
<tr>
<th>Expression</th>
<th>FcγRI, CD64</th>
<th>FcγRIIα, CD32a</th>
<th>FcγRIIb, CD32b</th>
<th>All immune cells except T and NK</th>
<th>FcγRIIα, CD16a</th>
<th>FcγRIIb, CD16b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating or inhibitory</td>
<td>Activating</td>
<td>Activating</td>
<td>Inhibitory</td>
<td>Activating</td>
<td>Activating</td>
<td>Activating</td>
</tr>
<tr>
<td>Polymorphism</td>
<td>None known</td>
<td>R131</td>
<td>H131</td>
<td>I232T</td>
<td>F158</td>
<td>V158</td>
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<tr>
<td>Antigen for:</td>
<td></td>
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<tr>
<td>IgG1</td>
<td>++++</td>
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<td>IgG2</td>
<td>–</td>
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<tr>
<td>IgG3</td>
<td>+++</td>
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<tr>
<td>IgG4</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>Murine counterpart</td>
<td>FcγRI</td>
<td>FcγRIIα</td>
<td>FcγRIIb</td>
<td></td>
<td>FcγRIV</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from [33] and [75].

*Monocyte.

*Macrophage.

Polymorphonuclear leukocyte.

*Dendritic cell.

*Antigen-presenting cell.

and IgG [28,29], all subclasses, including IgG2 and IgG4, effectively activate complement. In addition, work with murine MHC antibodies has demonstrated synergism between high- and low-complement-fixing IgG subclasses [30–32]. While this has not yet been explored using human IgG and complement, it is pertinent given that most antibody responses are polyclonal and HLA is often recognized by an admixture of subclasses.

IgG subclass interaction with Fc receptors (FcγRs) is more complex (Table 1). In general, IgG3 and IgG1 have the highest affinity for most FcγRs, while IgG2 and IgG4 are bound by a more restricted repertoire of FcγRs. Unfortunately the disparity between murine and human immunoglobulin systems limits the translation of in vitro mechanistic studies of IgG subclass effector functions in murine models of AMR to human disease [33].

After transplantation, IgG1 antibodies are directed against approximately 90% of HLA specificities whereas those of IgG2/3/4 recognize about 40% or less [34–36]. These results indicate a polyclonal response wherein each donor HLA antigen is recognized by multiple subclasses, most commonly including IgG1. It has been difficult to reconcile the apparently conflicting results regarding the association of DSA subclass and clinical outcome, despite reports of IgG1/3 dominating the alloantibody responses [37]. IgG3 DSAs were associated with increased risk of allograft loss in liver [35] and renal [38] transplantation. By contrast, others have reported no correlation between DSA subclass and risk of AMR or graft loss, although one study found a trend toward lower AMR in patients with only IgG2/4 DSAs [39].

Factors that dictate complement activation

The historical paradigm of AMR was one of complement-mediated damage caused by classical pathway activation by Fc regions of DSAs bound to the allograft [30]. In recent years, complement-fixing DSAs have become a controversial topic. C4d-negative AMR is becoming increasingly recognized and the diagnostic schemata for heart and renal AMR have been updated to reflect this entity [20]. Experimental mouse models of AMR suggest that acute rejection is dependent on complement fixation [40]. By contrast, intimal thickening during antibody-induced chronic rejection occurred in complement-deficient murine recipients, suggesting that there was no requirement for complement in this process [41]. Importantly, local production of complement by donor ECs could not be excluded [42]. These results from animal models are consistent with clinical observations that terminal complement inhibitors could not prevent chronic rejection [43,44]. Furthermore, studies using methods to define DSAs that are complement fixing, and determine whether complement fixation translates to graft damage, have had conflicting results [45–49].

Of the three complement pathways [50], the classical pathway is primarily responsible for DSA-mediated complement activation. Early activation results in the production of soluble mediators such as the anaphylatoxins C3a and C5a, which are potent chemoattractants for leukocytes and alter the microvasculature by increasing vascular permeability and inducing expression of adhesion molecules. The later stages are characterized by membrane attack complex (MAC) formation, which causes osmotic lysis of the target. Given the general resistance of ECs to complement-mediated lysis due to high expression of complement regulatory proteins, the physiological relevance of lytic terminal MAC formation during rejection is unclear [30]. Early complement proteins, rather than terminal MAC formation, are likely to be the mediators of most complement-associated damage to the graft (Figure 2A).

Factors that dictate complement activation

Many components modulate complement fixation by IgG. Of these, three are intrinsic to the antibody itself: IgG subclass, glycosylation, and affinity (Figure 2B,C). Multiple studies have defined the importance of antibody affinity in dictating the level of complement activation [51]. Repeated injury and consistent antigen exposure may increase the affinity of DSAs over time, resulting in HLA-Abs that are more inflammatory and cause robust complement induction.

Additionally, extrinsic factors such as antigen density/epitopes and complement concentration also regulate antibody-induced complement activation [52]. Despite constitutive allograft endothelium expression of HLA class I and II [53], these levels are altered in response to inflammatory
complement cues [54]. Many in vitro studies have shown that increased alloantibody bound to cells results in enhanced complement deposition, and this was augmented under inflammatory conditions [55,56]. Moreover, binding of multiple antibodies with distinct epitopes to a single HLA molecule synergistically enhanced complement activation [36]. If antibody subclass and antigen density/epitopes coordinate to determine complement activation by DSAs, polyclonal antibodies should elicit more complement activation than monoclonal antibodies. Sera with >80% panel reactive antibody (PRA) are strong inducers of complement activation [55,56], supporting the notion that differing levels of HLA antigen/epitopes determine both the quantity and quality of DSAs bound to the graft (Figure 2D).

Lastly, variations in complement can determine the degree of activation, as some complement proteins are located in the MHC locus (C2, C4) and are also polymorphic [57]. Genetic predisposition to specific polymorphisms may be useful for risk stratifying patients, and polymorphisms in complement C4 [58] but not C3 [59] have been shown to influence renal allograft outcome. In addition, complement concentration is potentiated in response to local inflammation. Renal epithelium, macrophages, cardiocytes, and vascular endothelium [42] are sources of extraperitoneal complement production during episodes of rejection. As low-lytic antibodies have enhanced activity when complement is elevated, and IgG4 activates complement when antigen density and complement levels are increased [28], patients with minimal complement-fixing DSAs may have a higher degree of damage during rejection episodes, when complement and antigens are more abundant.

Figure 2. Complement activation by antibody/antigen determinants. (A) Activation of the classical complement pathway by human leukocyte antigen (HLA) antibodies (HLA-Abs) is mediated by C1q recognition of the Fc region of IgG. Through a subsequent series of enzymatic cleavages, the complement pathway yields the soluble anaphylatoxins C3a and C5a, which are potent chemoattractants and stimulators of immune responses. C4d is covalently linked to the cell surface and is a defining marker of antibody-mediated rejection (AMR) in renal and cardiac transplants. Additionally, sublytic membrane attack complex (sMAC), the terminal complex bound to cells but unable to induce lysis, is proving to be an important mediator of endothelial cell (EC) activation. Differences in antibody clonality, as demonstrated by the antibodies of varying specificity (red or blue F(ab')2 side region), allow increased ratios of IgG:HLA, allowing more C1q binding. (B) Antibody subclass determines the propensity for C1q binding, as IgG3, a prominent complement fixer, is recognized by C1q whereas the structure of IgG4 makes it a poor C1q binding partner. (C) Differential patterning of the N297 glycan (blue square) of IgG also modulates the level of C1q interaction. Terminal galactose residues confer maximal C1q binding to antibodies. (D) The density of HLA antigen on the surface of the cell, as well as the number of epitopes, strongly dictates the level of complement activation. The proximity of antibody Fc regions is increased when multiple antibodies can bind the same molecule of HLA. Patients with high-titer polyclonal donor-specific antibodies (DSAs) may be predisposed to exacerbated complement activation during times of heightened inflammation, such as infection, when HLA expression is increased on the surface of endothelium.

Measuring DSA-induced complement activation

Complement activation by DSAs is a highly dynamic process responsible for mediating damage to the allograft; therefore, clinical assays that discern the complement-fixing potential of DSAs are in high demand. The lymphocytotoxicity cross-match (CDC-XM) assay developed by McClelland and Terasaki [60] was established for highly sensitive detection of DSAs to recipient HLAs. Although this assay utilizes complement fixation as a read-out, it is not fully reflective of the potential physiological capacity of DSAs to activate human complement due to the use of rabbit serum as a source of complement. It should also be noted that human IgG2 is highly effective in the activation of rabbit complement [61]; consequently, DSA subclass and CDC-XM results may not always correlate.

The development of high-throughput, single-antigen, bead-based assays has been an important tool for risk stratifying patients with complement-fixing DSAs [45,62,63]. Specifically, the C1q assay measures HLA-Abs that bind C1q and, although informative, recognizes binding only, not physiological complement activation [62]. Recently, a new assay measuring DSA-induced complement deposition (C3d) reported that C3d+ DSAs were significant predictors of allograft loss [64]. Collectively, these in vitro diagnostics attempt to measure the pathogenicity of HLA-Abs with regard to their complement-fixing potential. However, results differ regarding the predictive value of detecting complement-fixing HLA-Abs in vitro with respect to clinical outcomes [39,45,49,62,65–67], and new diagnostic criteria for AMR include rejection without histological evidence of complement activation (C4d deposition) [17,20,68].

HLA-Abs and FeYRs

An almost universal histological feature of AMR is the infiltration of CD68+ macrophages in the microvascular and perivascular spaces of heart and renal allografts [17,19,69,70] and neutrophils in lung transplants [22], which is predictive of worse outcome [69]. In addition, gene-expression profiling studies have uncovered a natural killer (NK) cell signature during AMR [2,71,72], findings

![Figure 2](https://example.com/f2.png)
that were paralleled by experimental animal models of AMR implicating NK cells in chronic antibody-mediated rejection [73]. Monocytes, macrophages, neutrophils, and NK cells express receptors for the Fc region of antibodies (Table 1) [74] and FcγRs mediate innate immune cell functions such as leukocyte recruitment, cytotoxicity, and phagocytosis that are highly relevant to the etiology of AMR (Figure 3).

**FcγR families and alleles**

FcγR families and alleles have distinct subclass specificities and divergent activities (Table 1) [33,75]. Moreover, functional polymorphic variants of FcγRIIa (H131R), FcγRIIIa (F158V), and FcγRIIIb (NA1/NA2 alleles) are associated with differential phenotypes in response to antibody-based antitumor therapies, susceptibility to infection, and risk of autoimmune disease [76]. In the context of transplantation, the low-affinity FcγRIIa-R131 allele was associated with increased risk of acute T cell-mediated rejection (TCMR) [77], but this is likely a reflection of reduced responsiveness to antibody-based leukocyte depletion induction regimens rather than predisposition to rejection per se. However, the effect of transplant recipient FcγR polymorphism on risk of AMR has not yet been studied and warrants investigation.

As with IgG subclasses, the human FcγR system is dissimilar from the murine system, complicating the study of FcγRs in vivo and confounding the translation of experimental results in murine models of AMR to the human setting. A recently described novel transgenic mouse carrying the full repertoire of human FcγRs [78] may enable future studies. Several important caveats, however, including cross-reactivity of human FcγRs with endogenous murine IgG and the representation of only one FcγR genotype, may limit findings [33].

**FcγR functions relevant to graft injury**

FcγRs on monocytes, macrophages, and NK cells facilitate antibody-dependent cell-mediated cytotoxicity (ADCC). While HLA-Abs trigger NK cell degranulation and cytotoxicity against allogeneic target cells in vivo [79], and macrophages also perform ADCC, currently there is no direct evidence that these cells cause cytotoxicity in the graft. However, murine models of chronic AMR have revealed a novel role for NK cells in MHC antibody-induced transplant vasculopathy [73], through undefined FcγR-dependent mechanisms. An elegant study imaging the trafficking of recipient immune cells into murine cardiac allografts revealed elevated phagocytic activity during rejection, mediated by recipient macrophages [80]. HLA-Abs may provoke antibody-dependent cellular phagocytosis (ADCP) by macrophages and neutrophils, contributing to enhanced presentation of alloantigen to T cells, but the pathophysiological relevance of phagocytosis during rejection remains to be explored.

Finally, FcγRs are involved in the capture of leukocytes by immune complexes (ICs) and monomeric anti-endothelial cell antibodies, and enhanced trafficking of neutrophils to inflamed endothelium in autoimmune settings [81]. It is notable that there was a prerequisite for tumor necrosis factor alpha (TNFα) activation of endothelium, as deposition of antibody on resting cells did not cause efficient neutrophil adhesion. Moreover, concurrent expression of chemokines was required for efficient neutrophil adhesion to ECs coated with monomeric IgG but not with ICs. It was recently demonstrated that monocyte recruitment to HLA-Ab-activated endothelium was augmented by the interaction of monocyte FcγRs with the Fc portion of the HLA-Abs [82,83]. This interaction was subclass dependent, influenced by monocyte FcγRIIa allelic variants, and abrogated by enzymatic modulation of antibody Fc regions using EndoS or IdeS [83]. In contrast to reports using murine anti-endothelial cell antibodies [81], efficient recruitment was observed using HLA-Abs without pre-activating ECs with inflammatory cytokines, and it has been hypothesized that HLA-Abs are unique in their capacity to trigger direct endothelial activation and expression of selectins as well as stimulate FcγRs [82]. Interestingly, monocytes from
donors who expressed the high-affinity FcγRIIa-H131 allele exhibited significantly greater FcγR-dependent adhesion to ECs activated with HLA-Abs of both IgG1 and IgG2 subclasses compared with monocytes expressing only FcγRIIa-R131. These results suggest that transplant recipients carrying high-affinity FcγR alleles may experience exacerbated leukocyte infiltration in response to HLA-Abs, predisposing them to AMR.

**HLA-Abs and glycosylation**

Patterns of antibody glycosylation strongly influence affinity of FcγRs [84]. The bulk of evidence comes from the fields of tumor immunology and recombinant therapeutic antibodies, through glycoengineering of antibodies to alter ADCC and complement-dependent cytotoxicity (CDC) properties. In addition, several studies have correlated the degree of IgG-Fc glycosylation with the severity of antibody-mediated disease [85]. A common theme appears: antibodies withagalactosylated Fc-glycans are more proinflammatory than those containing glycans with terminal galactosylation or sialic acid. As the properties of glycosylation moieties modulate the inflammatory nature of IgG, the Fc-glycan may participate in determining the degree of pathogenicity of DSAs with respect to AMR. The conserved yet highly heterogeneous N297 glycan present on the Fc of all IgGs [27,86] contains a biantennary core heptasaccharide that is further modified by the addition of fucose (over 90% of IgGs), galactose, and sialic acid to greatly diversify the IgG glycoform pool. Various changes to this structure can completely alter the function of IgG with respect to both FcγR and complement-dependent activities (reviewed elsewhere [27,87]). In brief, removal of fucose increases ADCC whereas removal of galactose residues reduces ADCC (mediated by FcγRIIIa) and CDC. Interestingly, sialic acid has been identified as the mediator of the anti-inflammatory properties of intravenous immunoglobulin (IVIg) [84], a common modality in the treatment of AMR. Whereas all sialic acid linkages contribute to decreased ADCC, the alpha-2,6 version is responsible for the anti-inflammatory effects of sialylated IgG, through direct binding of SIGN-R1/DC-SIGN, causing upregulation of inhibitory FcγRs. Although there is minimal literature regarding differential glycosylation patterns of DSAs, one would be remiss to disregard the potential role of DSA glycan heterogeneity during the course of AMR.

**Regulation of IgG glycosylation**

Given that both complement activation and FcγR engagement are key effector functions of HLA-Abs in causing allograft injury, the Fc region of the antibody is a potential therapeutic target. The Gram-positive bacterium *Streptococcus pyogenes* expresses a battery of immunomodulatory enzymes that aid in its pathogenicity, two of which have shown promise in preclinical autoimmune models through specific actions on IgG [88]. The peptidase IdeS cleaves off the Fc fragment of human IgG, generating a Fab′ fragment, while the endoglycosidase EndoS hydrolyzes the N297-linked Fc glycan. Both ameliorate inflammation, complement activation, and FcγR-dependent leukocyte recruitment in several experimental models and treatment of HLA-Abs with either EndoS or IdeS dramatically reduced the recruitment of monocytes to ECs [83]. Clinical trials are currently under way testing the efficacy of IdeS in sensitized kidney transplant recipients (NCT02224820).

Glycan analysis of antibodies produced during inflammation in response to pathogens or autoimmune disease have shown an increased proportion ofagalactosylated IgG. Moreover, IgGs from active immune responses have altered glycan profiles that differ from normal serum IgGs [89,90]. The mechanism by which antibodies are glycosylated during immune responses is not well understood, although distinct glycan profiles from individual patients suggest differential glycosylation by unique B cell subsets [91,92]. This indicates that the ability to regulate levels of glycosylation relies on B cell-intrinsic factors and would be subject to the immune milieu. In this regard, B cells presented with T-dependent antigens under proinflammatory conditions produced antibody that lacked galactose (proinflammatory) [89,93], whereas antibodies produced in response to T-dependent antigens but under tolerogenic settings were heavily sialylated (anti-inflammatory) [89]. Finally, in the context of T-independent antigens, regardless of the inflammatory surroundings IgGs were sialylated and immunosuppressive [93].

This comprehensive understanding of the contribution of Fc-glycan to the immune function of IgG and the circumstances modulating the production of these glycosylated antibodies allows conjecture regarding the pathogenic potential of DSAs. One could surmise that acute rejection episodes increase levels ofagalactosylated DSAs, which would incur damage to the graft through both complement and FcγR pathways, whereas DSAs present in accommodated grafts may be heavily sialylated and somewhat tolerogenic. Future work detailing the glycan profiles of DSAs would determine whether antibody glycosylation status correlates with the severity of AMR. Additionally, a new methodology described to simultaneously measure both the subclass and glycosylation of antigen-specific IgG [94] may be adapted to transplantation.

**HLA-Abs and endothelial activation and regulation of immunogenicity**

There has been resurgence in the appreciation of ECs as important regulators of the immune response. ECs can undergo acute (Type I) or chronic (Type II) activation, leading to expression of chemokines and adhesion molecules and recruitment of leukocytes to sites of inflammation [95]. Past work showed that crosslinking of HLA by antibodies triggers intracellular signaling through focal adhesion kinase (FAK), Akt, mammalian target of rapamycin (mTOR), S6 kinase (S6K), S6 ribosomal protein (S6RP), and extracellular signal-regulated kinase (ERK1/2) in endothelial and smooth muscle cells leading to dynamic cytoskeletal reorganization and increased cell proliferation, migration, and survival [96]. Multiple groups recently confirmed the activation of these signaling pathways in biopsies from cardiac allografts undergoing AMR [97,98]. Importantly, the agonistic signaling capacity is an observed property of
all HLA-Abs requiring the bivalent F(ab')2 region of IgG and does not appear to depend on subclass, complement, or FcγRs. Alternatively, complement activation, antigen expression, epitope density, and antibody affinity will all significantly impact the binding to and crosslinking of HLAs on ECs, in turn affecting intracellular signaling.

Recent studies demonstrated that HLA class I signaling triggers Type I EC activation, resulting in a rapid increase of cell surface P-selectin and the adhesion of neutrophils and monocytes to endothelium [99,100]. Exocytosed von Willebrand factor (vWF) and P-selectin also facilitated the capture and activation of platelets, which aggregate in the microvasculature and support the tethering of monocytes [101]. Platelets express FcγRIIa [102]; therefore, additional mechanisms of FcγR-dependent platelet adhesion cannot be excluded. HLA crosslinking also activated the transcription factors cAMP response element-binding protein (CREB) and noncanonical nuclear factor kappa B (NF-κB), resulting in increased expression of late phase adhesion molecules, cytokines, and chemokines [56,103], consistent with Type II EC activation.

An expanding paradigm of the role of vascular endothelium in directly stimulating adaptive immune responses has gained attention [104,105]. HLA class II-expressing ECs trigger allogeneic CD4 T cell proliferation and promote generation of T helper 17 (Th17) and regulatory T cell (Treg) subsets [106,107]. Interestingly, rapamycin treatment of ECs resulted in selective expansion of Tregs via programmed death ligand (PD-L) 1 and 2 [107], indicating a role for mTOR in the regulation of endothelial alloimmunogenicity through modulation of costimulatory molecule expression. The mTOR inhibitors sirolimus and everolimus also prevent HLA I antibody-induced endothelial migration and proliferation [108], suggesting that rapalogs may be beneficial in preventing multiple manifestations of graft injury by HLA-Abs. A recent study showed HLA-Abs increased expression of proinflammatory cytokines and the activation of noncanonical NF-κB [56],

Figure 4. Proposed models of donor-specific antibody (DSA)-induced inflammatory loops. (A) Macrophages perpetuate activation and recruitment via complement and Fc receptor (FcγR) pathways. DSA crosslinking of human leukocyte antigens (HLAs) on endothelium results in P-selectin mobilization to the cell surface and provides a binding platform for C1q (1). Classical complement activation produces C5a (2), which has two functions: (i) it recruits monocytes to activated endothelial cells (ECs), which tether to P-selectin via P-selectin glycoprotein ligand-1 (PSGL-1), promoting graft infiltration and differentiation into macrophages (3); and (ii) it may act on intragraft CD8+ macrophages and induce FcγR expression (4). These cells can recognize immune complexes (ICs) via FcγRs, which can upregulate C5a production (5). Newly synthesized C5a may signal in either an autocrine (6a) or a paracrine (6b) fashion, mediating further activation of intragraft macrophages or recruitment and activation of monocytes from the periphery, respectively. (B) Recent studies have identified a novel role for ECs and complement in antigen presentation and stimulation of allogeneic T cells. Under inflammatory conditions [such as interferon gamma (IFNγ) activation], ECs express HLA class II as well as ICAM-1, VCAM-1, and IL-6 – molecules that are critical for promoting allo-CD4 T cell proliferation (4) and differentiation into T helper 17 (Th17) and regulatory T cell (Treg) subsets. Preliminary work has shown that HLA antibodies modulate endothelial alloimmunogenicity through activation of the classical complement pathway (1) resulting in deposition of sublytic membrane attack complex (MAC) (2). MAC triggers noncanonical nuclear factor kappa B (NF-κB) signaling leading to inflammatory gene expression (3) and stimulation of allogeic CD4 T cells (4). T cells also express receptors for the complement split products C3a and C5a, which provide costimulatory signals and augment T cell proliferation. Therefore, it is likely that the presence of these anaphylatoxins at the endothelial–T cell interface might enhance T cell alloimmunity (5).
indicating that HLA-Abs modulate endothelial immunogenicity and antigen presentation to T cells.

**Inflammatory loops and interplay between antibody functions**

Concurrent processes of EC activation, classical complement activation, and FcγR-dependent immune cell functions are likely to independently and cumulatively promote graft inflammation during AMR. Crosstalk between FcγR and complement adds another level of complexity to IgG modulation of the immune response [109]. Abrogation of either Fc/FcγR or C5a/C5aR signaling abolished inflammation induced by ICs and it is known that both are necessary for robust immune responses. C5a acts directly on macrophages, simultaneously upregulating activating FcγR and downregulating inhibitory FcγR [110,111]. Additionally, IC binding to macrophages through FcγRIII induced C5a synthesis [112]. Furthermore, binding of C5a to Kupffer cells triggered increased expression of activating FcγR, which bound ICs, thereby stimulating C5a production and creating a proinflammatory loop [113]. This cycle could potentially translate to exacerbated AMR-associated pathophysiology. Local activation of complement in the graft by DSAs can activate macrophages and increase expression of FcγR, which may bind sequestered DSA ICs, thereby augmenting local C5a production (Figure 4A). In addition to the direct effects of complement on macrophages, anaphylatoxins and sublytic MAC enhance EC activation. Endothelial NF-κB signaling and inflammatory gene expression induced by DSA binding was augmented in the presence of sublytic MAC and led to increased T cell stimulation [56]. These findings demonstrate an additional mechanism of synergy between complement and HLA-Abs in endothelial activation (Figure 4B). As C5a is a potent mediator of leukocyte recruitment as well as a novel modulator of T cell allore cognition [114], this DSA-induced inflammatory loop could exacerbate damage during episodes of AMR.

**Concluding remarks and future perspectives**

In summary, graft injury results from the pleiotropic function of antibodies (Figure 5), through canonical Fc-mediated effector functions as well as novel agonistic actions on HLA molecules. The collective action of antibodies on donor vascular cells, including complement activation, FcγR-dependent macrophage and NK cell functions, and EC activation, are likely to synergize, causing damage to the allograft. Features such as antibody subclass, Fc glycosylation, and FcyR polymorphisms may be key determinants of HLA-Ab pathogenicity and recipient risk of AMR. Therefore, characterization of the patient’s DSAs and immune repertoire provides a foundation for individualized medicine as well as possible guidelines for the risk stratification of transplantation patients. Highly tailored and specific immunotherapies could be used in the transplantation field to modulate patient immune responses according to the details of an individual’s immune repertoire. Further experimental dissection of alloimmunity variables (Box 1) will guide future practice in allocation/antigen avoidance, management in sensitized patients, and the development of new drugs to prevent and treat AMR.

**Box 1. Outstanding questions**

- Which mechanisms of HLA-Abs are critical for rejection and graft injury and how do these mechanisms vary depending on antibody characteristics?
- What are the effector functions of NK cells during AMR? Do ADCC and ADCP play a mechanistic role in AMR?
- Can we reliably define the HLA-Ab repertoire, including specificity, glycosylation, complement-fixing capacity and subclass distribution, of transplantation patients? In particular, do current in vitro assays of complement detection reliably predict whether HLA-Abs will cause complement-mediated injury?
- Are some patients predisposed to experience rejection in the presence of antibodies? Does the glycan profile of DSAs or the receptor FcγR genotype influence transplant outcome?
- Should patients be treated when they have DSAs but no evidence of graft dysfunction?
- What is the significance of C4d-negative AMR? Does it represent complement-independent graft injury by non-complement-fixing antibodies or is it capturing AMR after complement is no longer active?

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**References**


antibody-mediated transplantation.


allografts.

antibodies. transplants.

HLA Prevalence Incidence donor.

volunteer donors.

of C4d staining. Am. J. Transplant. 9, 2312–2323

Asymptomatic antibody-mediated rejection after heart transplantation predicts poor outcomes. J. Heart Lung Transplant. 28, 417–422

A longitudinal study of the course of asymptomatic antibody-mediated rejection in heart transplantation. J. Heart Lung Transplant. 31, 46–51

Outcome of subclinical antibody-mediated rejection in kidney transplant recipients with preformed donor-specific antibodies. Am. J. Transplant. 9, 2561–2570

The IMG7/HLA database. Nucleic Acids Res. 39, D1171–D1176

Screening for HLA antibodies in plateletpheresis donors with a history of transfusion or pregnancy. Transfusion 54, 3036–3042

Prevalence of HLA antibodies in remotely trans fused or alloexposed volunteer blood donors. Transfusion 50, 1329–1334

Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. Transplantation 95, 410–417


Disappearance of T cell-mediated rejection despite continued antibody-mediated rejection in late kidney transplant recipients. J. Am. Soc. Nephrol. Published online November 6, 2014. (http://dx.doi.org/10.1681/ASN.2014060588)

Circulating donor-specific anti-human leukocyte antigen antibodies and complement C4d deposition are associated with the development of cardiac allograft vasculopathy. Am. J. Clin. Pathol. 140, 809–815


The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of pathological terminology of heart allograft biopsies: diagnosis of antibody-mediated rejection in heart transplantation. J. Heart Lung Transplant. 32, 1147–1162


Morphologic and immunohistochemical findings in antibody-mediated rejection of the cardiac allograft. Hum. Immunol. 73, 1213–1217


Acute liver allograft antibody-mediated rejection: an inter-institutional study of significant histopathological features. Liver Transplant. 20, 1244–1255

Pathologic findings in lung allografts with anti-HLA antibodies. J. Heart Lung Transplant. 32, 326–332

Pretransplant predictors of survival after intestinal transplantation: analysis of a single-center experience of more than 100 transplants. Transplantation 90, 1574–1580


Endothelitis in cardiac allograft biopsy specimens: possible relationship to antibody-mediated rejection. J. Heart Lung Transplant. 30, 435–444


IgG subclasses and allotypes: from structure to effector functions. Front. Immunol. 5, 320


Mechanisms of complement activation, C4d deposition, and their contribution to the pathogenesis of antibody-mediated rejection. Transplant. Rev. (Orlando) 23, 139–150

Synergistic deposition of C4d by complement-activating and non-activating antibodies in cardiac transplants. Am. J. Transplant. 7, 2605–2614

Non-complement- and complement-activating antibodies synergize to cause rejection of cardiac allografts. Am. J. Transplant. 4, 326–334

Properties of mouse and human IgG receptors and their contribution to disease models. Blood 119, 5640–5649

Comparing antibody-mediated renal allografts. J. Am. Soc. Nephrol. 24, 168–175

Impact of IgM and IgG3 anti-HLA alloantibodies in primary renal allograft recipients. Transplantation 97, 504–501

CD4-fixing capability of low-level donor-specific HLA antibodies is not predictive for early antibody-mediated rejection. Transplantation 89, 1471–1475


Human leukocyte antigen-specific antibodies and gamma-interferon stimulate human microvascular and glomerular endothelial cells to produce complement factor C4. Transplantation 93, 867–873

Eculizumab and splenectomy as salvage therapy for severe antibody-mediated rejection after HLA-incompatible kidney transplantation. Transplantation 98, 857–863


Clinical usefulness of a novel Clq assay to detect immunoglobulin G antibodies capable of fixing complement in sensitized pediatric heart transplant patients. J. Heart Lung Transplant. 30, 158–163


Complement-fixing donor-specific antibodies identified by a novel Clq assay are associated with allograft loss. Pediatr. Transplant. 16, 12–17


Pretransplant IgG subclasses of donor-specific human leukocyte antigen antibodies and development of antibody-mediated rejection. Transplantation 92, 41–47

Crespo, Chen, Terasaki, Varagunam, Bay, Atkinson, and in enhancement of loss. 19, human subset variants kidney. superior rejection: cytotoxicity. genetically 315–322 L.

transcripts M.

IgG M.

al.

et al.

Soc.

12, 313–321

Nature

1989–1994


Mellor, J.D. et al. (2013) A critical review of the role of Fc gamma receptor polymorphisms in the response to monocolonal antibodies in cancer. J. Hematol. Oncol. 6, 1


Florey, O.J. et al. (2007) Antiendothelial cell antibodies mediate enhanced leukocyte adhesion to cytokine-activated endothelial cells through a novel mechanism requiring cooperation between FcγRIIa and CXCR1/2. Blood 109, 3881–3889

Valenzuela, N.M. et al. (2013) HLA class I antibodies trigger increased adherence of monocytes to endothelial cells by eliciting an increase in endothelial P-selectin and, depending on subclass, by engaging FcγRs. J. Immunol. 190, 6635–6650


Lundström, S.L. et al. (2014) IgG antibodies to cyclic citrullinated peptides exhibit profiles specific in terms of IgG subclasses, Fc-glycans and a Fab-peptide sequence. PLoS ONE 9, e113924


Li, F. et al. (2011) Antibody ligation of human leukocyte antigen class I molecules stimulates migration and proliferation of smooth muscle cells in a focal adhesion kinase-dependent manner. Hum. Immunol. 72, 1150–1159


103 Naemi, F.M. et al. (2013) Anti-donor HLA class I antibodies: pathways to endothelial cell activation and cell-mediated allograft rejection. Transplantation 96, 258–266