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The Perfect Storm: HLA Antibodies, Complement, FcγRs and Endothelium in Transplant Rejection

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Abstract

The pathophysiology of antibody-mediated rejection (AMR) in solid organ transplants is multi-faceted and predominantly caused by antibodies directed against polymorphic donor human leukocyte antigens (HLA). Despite the clearly detrimental impact of HLA antibodies (HLA-Ab) on graft function and survival, the prevention, diagnosis and treatment of AMR remain a challenge. Histological manifestations of AMR reflect 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 identify HLA-Ab more likely to cause rejection. We also highlight recent advancements that may pave the way for new, more effective therapeutics.

Keywords

Organ Transplantation; Antibody-mediated Rejection; HLA Antibodies; Classical Complement Pathway

Rejection of solid organ transplants challenges long term allograft survival

Organ failure is an immense human and economic burden, which can be successfully reversed with transplantation, substantially improving quality of life and life expectancy. In the United States, more than 100,000 patients currently await transplant of major solid organs. Significant advances in histocompatibility and immunosuppression have dramatically improved short-term graft and patient survival rates. Recipient recognition of donor human leukocyte antigen (HLA; see Glossary) present in the allograft induces an allogeneic immune response, resulting in the production of donor specific HLA antibodies (DSA). These antibodies, through many different effector functions, are responsible for the damage, and ultimately graft rejection, which occurs in antibody mediated rejection (AMR).

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AMR has emerged as a leading cause of graft dysfunction and reduced outcomes, yet it is often unresponsive to current therapies [1]. Histological markers of AMR are often unreliable, and it is controversial whether intervention is required for patients with DSA but no graft dysfunction. Clinical evidence suggests that DSA 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 the recent developments in the understanding of how antibodies against HLA (HLA-Ab) function to cause graft injury, emphasizing the multiple effector mechanisms of HLA-Ab, specifically IgG, and how they relate to risk and manifestations of AMR.

The alloimmune response

Immunity to alloantigens is surprisingly robust, mediated by the major histocompatibility complex (MHC), and based on exposure to allogeneic tissues. The MHC locus covers nearly 4000kb on human chromosome 6, and is polygenic, containing 3 loci of HLA class I (HLA-A, -B, and -C), 6 to 9 functional HLA class II loci (α and β chains of HLA-DR, -DP, and -DQ), as well as many non-classical MHC, minor histocompatibility antigens 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, either through pregnancy, transfusion, or transplantation. Twenty percent of healthy individuals [8, 9] and up to 30% of transplant candidates have HLA-Ab. Another 8–25% of recipients develop *de novo* DSA after receiving a graft [10–12]. Half of pre-sensitized patients and one third of patients with *de novo* DSA will experience AMR within the first year after transplant [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 one year post-transplant [13]. DSA 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 DSA (Figure 1) [17, 19, 20]. While HLA-Ab are indeed 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 antibodies and subclass biology

The fact that some patients with DSA 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 are reflective of the 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. Immunoglobulin G (IgG) is the most common isotype of circulating Ig, 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 IgG2 [27]. IgG4 has no detectable complement activity, and is often linked with IgG2 as “noncomplement fixing.” However, it should be noted that under unique conditions, such as high antigen/epitope density or increased concentrations of complement 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 lowly complement fixing IgG subclasses [30–32]. While not yet explored using human IgG and complement, this 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 vivo* mechanistic studies of IgG subclass effector functions in murine models of AMR to human disease [33].

After transplant, IgG1 antibodies are directed against approximately 90% of HLA specificities, whereas those of IgG2/3/4 recognize roughly 40% or less of HLA specificities [34–36]. These results are indicative of 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 DSA were associated with increased risk of allograft loss in liver [35] and renal transplantation [38]. In 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 DSA [39].

HLA antibodies and complement activation

The historical paradigm of AMR was one of complement-mediated damage caused by classical pathway activation by Fc regions of DSA bound to the allograft [30]. In recent years, complement fixing DSA have become a controversial topic. C4d-negative AMR is

becoming increasingly recognized, and the diagnostic schema for heart and renal AMR have been updated to reflect this entity [20]. Experimental mouse models of AMR suggest that acute rejection is dependent upon complement fixation [40]. In contrast, intimal thickening during antibody-induced chronic rejection occurred in complement-deficient murine recipients, suggesting there was no requirement for complement in this process [41]. Importantly, local production of complement by donor EC could not be ruled out [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 DSA 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 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 EC 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]. Indeed, early complement proteins, rather than terminal MAC formation, are likely to be the mediators of the majority of complement-associated damage to the graft (Figure 2A).

Factors which 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 and 2C). 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 affinity of DSA over time, resulting in HLA-Ab that are more inflammatory and induce 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 cues [54]. Many *in vitro* studies have shown that increased alloantibody bound to cells resulted 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 DSA, polyclonal antibodies should elicit more complement activation than monoclonal antibodies. Indeed, sera with >80% PRA (panel reactive antibody) 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 DSA bound to the graft (Figure 2D).

Lastly, variations in complement can determine the degree of activation. 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 indeed 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 extrahepatic complement production during episodes of rejection. As lowly 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 DSA may have a higher degree of damage during rejection episodes, when complement and antigen are more abundant.

Measuring DSA induced complement activation

Complement activation by DSA is a highly dynamic process responsible for mediating damage to the allograft, therefore clinical assays which discern the complement fixing potential of DSA are in high demand. The lymphocytotoxicity crossmatch (CDC-XM) assay developed by McClelland and Terasaki [60] was established for highly sensitive detection of DSA to recipient HLA. Although this assay utilizes complement fixation as a readout, it is not fully reflective of potential physiological capacity of DSA 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 at activation of rabbit complement [61]; consequently DSA subclass and CDC-XM results may not always correlate.

Development of high-throughput single antigen bead-based assays has been an important tool for risk stratifying patients with complement fixing DSA [45, 62, 63]. Specifically, the C1q assay measures HLA-Ab that bind C1q, and although informative, this assay only recognizes binding, not physiological complement activation [62]. Recently, a new assay measuring DSA-induced complement deposition (C3d) reported C3d+ DSA were significant predictors of allograft loss [64]. Collectively, these *in vitro* diagnostics attempt to measure the pathogenicity of HLA antibodies with regard to their complement fixing potential. However, results differ regarding the predictive value of detecting complement fixing HLA-Ab *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 antibodies and FcγRs

A nearly 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], results which 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 which 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 anti-tumor therapeutics, 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 reflective 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 quite dissimilar from the murine system, complicating study of FcγRs *in vivo* and confounding 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 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-Ab trigger NK cell degranulation and cytotoxicity against allogeneic target cells *in vitro* [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-Ab 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 capture of leukocytes by immune complexes 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 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 endothelial cells coated with monomeric IgG but not with immune complexes. It was recently demonstrated that monocyte recruitment to HLA-Ab activated endothelium was augmented by interaction of monocyte FcγRs with the Fc portion of HLA-Ab [82, 83]. This interaction was subclass-dependent, influenced by

monocyte FcγRIIa allelic variants, and was 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 by using HLA-Ab without preactivating endothelial cells with inflammatory cytokines, and it has been hypothesized that HLA-Ab 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 EC activated with HLA-Ab 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-Ab, predisposing them to AMR.

HLA antibodies 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 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 with agalactosylated Fc-glycans are more pro-inflammatory than those containing glycans with terminal galactosylation or sialic acid. As properties of glycosylation moieties modulate the inflammatory nature of IgG, the Fc-glycan may participate in determining the degree of pathogenicity of DSA in regards to AMR.

The conserved yet highly heterogenous N297 glycan present on Fc of all IgG [27, 86] contains a biantennary core heptasaccharide that is further modified by addition of fucose (over 90% of IgG), galactose, and sialic acid to further diversify the IgG glycoform pool. Various changes to this structure can completely alter the function of IgG in regards to both FcγR and complement dependent activities (thoroughly reviewed elsewhere [27, 87]). In brief, removal of fucose increases ADCC whereas removal of galactose residues reduces ADCC mediated by FcγRIIIa and complement-dependent cytotoxicity (CDC). Interestingly, sialic acid has been identified as the mediator of anti-inflammatory properties of intravenous immunoglobulin (IVIg) [84], a common modality used in treating 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 FcγR. Although there is minimal literature regarding differential glycosylation patterns of DSA, 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 antibodies in causing allograft injury, the Fc region of 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 an $F(ab')_2$ 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-Ab with either EndoS or IdeS dramatically reduced recruitment of monocytes to EC [83]. Clinical trials are currently underway 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 of agalactosylated IgG. Moreover, IgG from active immune responses have altered glycan profiles which differ from normal serum IgG [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 which 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, no matter the inflammatory surroundings, IgG were sialylated and immunosuppressive [93].

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

HLA antibodies and endothelial activation and regulation of immunogenicity

There has been resurgence in the appreciation of EC as important regulators of the immune response. EC can undergo acute (Type I) and 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 regulated kinase (ERK1/2) in endothelial and smooth muscle cells leading to dynamic cytoskeletal reorganization, 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-Ab requiring the bivalent $F(ab')_2$ region of IgG, and does not appear to depend upon subclass, complement or FcγRs. Alternatively, complement

activation, antigen expression, epitope density and antibody affinity will all significantly impact binding to and crosslinking of HLA on EC, in turn affecting intracellular signaling.

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

An expanding paradigm of vascular endothelium in directly stimulating adaptive immune responses has garnered attention [104, 105]. HLA class II-expressing ECs trigger allogeneic CD4 T cell proliferation and promote generation of Th17 and Treg subsets [106, 107]. Interestingly, rapamycin treatment of ECs resulted in selective expansion of Tregs via PD-L1 and PD-L2 [107], pointing to a role for mTOR in regulation of endothelial alloimmunogenicity through modulation of costimulatory molecule expression. mTOR inhibitors sirolimus and everolimus also prevent HLA I antibody-induced endothelial migration and proliferation [108], suggesting that rapalogues may be beneficial in preventing multiple manifestations of graft injury by HLA-Ab. A recent study showed HLA-Ab increased expression of proinflammatory cytokines and activation of noncanonical NFκB [56], indicating that HLA-Ab 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 immune complexes (IC); 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 IC, 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 DSA can activate macrophages, and increase FcγR expression, which may bind sequestered DSA-IC, thereby augmenting local C5a production (Figure 4A). In addition to direct effects of complement on macrophages, anaphylatoxins and MAC complex enhance EC activation. Endothelial NFκB signaling and inflammatory gene expression induced by DSA binding was augmented in the presence of sublytic MAC, and increased T cell stimulation [56]. These findings demonstrate an additional mechanism of synergy between complement and

HLA-Ab on endothelial activation (Figure 4B). As C5a is a potent mediator of leukocyte recruitment, as well as a novel modulator of T cell alloimmunity [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), both 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, likely synergize to cause damage to the allograft. Features such as antibody subclass, Fc glycosylation and FcγR polymorphisms may be key determinants of HLA-Ab pathogenicity and recipient risk of AMR. Therefore, characterization of both patient DSA and immune repertoire provides a foundation for individualized medicine, as well as possible guidelines for risk stratification of transplant patients. Highly tailored and specific immunotherapies could be used in the transplant field to modulate patient immune responses according to the details of the patient immune repertoire. Further experimental dissection of alloimmunity variables (Box 1) will guide future practice in allocation/antigen avoidance, management in sensitized patients, and development of new drugs to prevent and treat AMR.

Box 1

Outstanding Questions

1. Which mechanisms of HLA-Ab 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?

2. Can we reliably define the HLA-Ab repertoire, including specificity, glycosylation, complement fixing capacity and subclass distribution, of transplant patients?

   In particular, do current *in vitro* assays of complement detection reliably predict whether HLA-Ab will cause complement-mediated injury?

3. Are some patients predisposed to experience rejection in the presence of antibodies?

   Does the glycan profile of the DSA or recipient FcγR genotype influence transplant outcome?

4. Should patients be treated when they have donor specific antibodies, yet no evidence of graft dysfunction?

5. 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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Glossary

Acute rejection	commonly refers to rejection that arises rapidly and causes graft function within days to weeks, often occurring in the early post-transplant period (less than one year); may be mediated primarily by T cells (called T cell-mediated rejection, TCMR, or acute cellular rejection, ACR) or primarily by antibodies (called humoral or antibody-mediated rejection, AMR); 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 arteriosclerosis (TA) in cardiac allograft, transplant glomerulopathy (TG) in renal allograft, bronchiolitis obliterans syndrome (BOS) in lung allograft, and vanishing bile duct syndrome in liver allograft; 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, consisting of endothelial cells and smooth muscle cells which have migrated and proliferated in the neointima, as well as CD4 T cells and macrophages
Classical complement pathway	a system of proteases which consecutively cleave downstream components to generate catalytically active or inflammatory and cytolytic products; the classical pathway is activated by immunoglobulin (Ig), and initiated by binding of C1 complex to the Fc region of IgM or IgG
Donor specific HLA antibodies (DSA)	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 major histocompatibility complex; 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

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Highlights

- Antibody-mediated rejection is a major challenge to solid organ transplantation.
- Complement, endothelial and FcγR mechanisms synergize to exacerbate inflammation.
- A variety of antibody characteristics influence Fc-dependent effector functions.

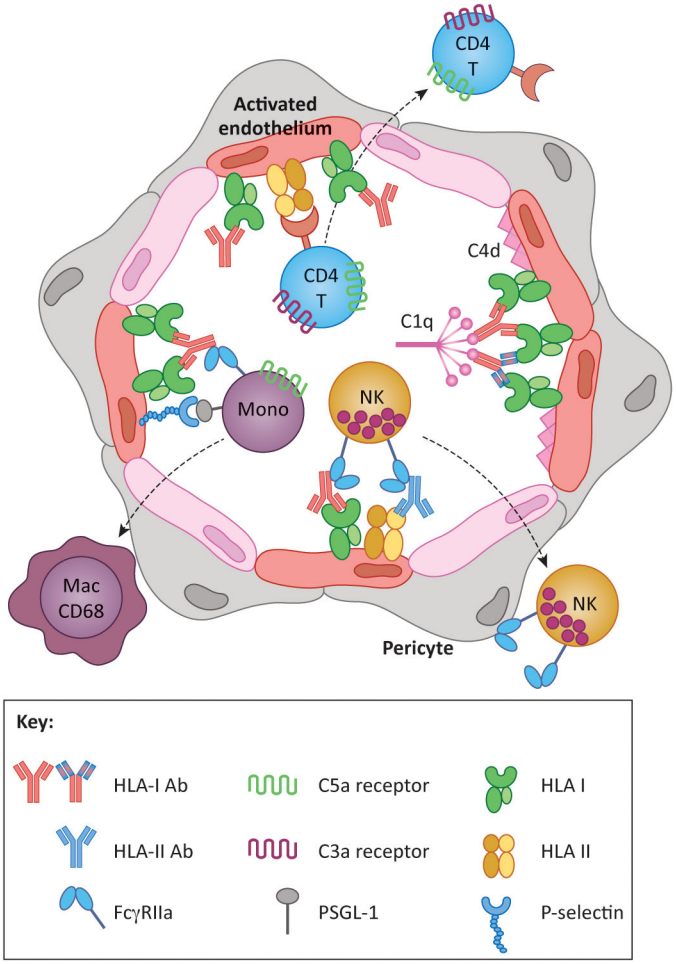

Figure 1. HLA antibodies cause graft injury by inducing phenotypic changes in the donor vasculature

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 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 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 NK cells also express 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 microvascular inflammation characteristic of antibody-mediated rejection. Antibodies in the figure with the same coloration of the Fc region are of

the same subclass, whereas the varied colors within the $F(ab')_2$ denote unique antigenic specificities.

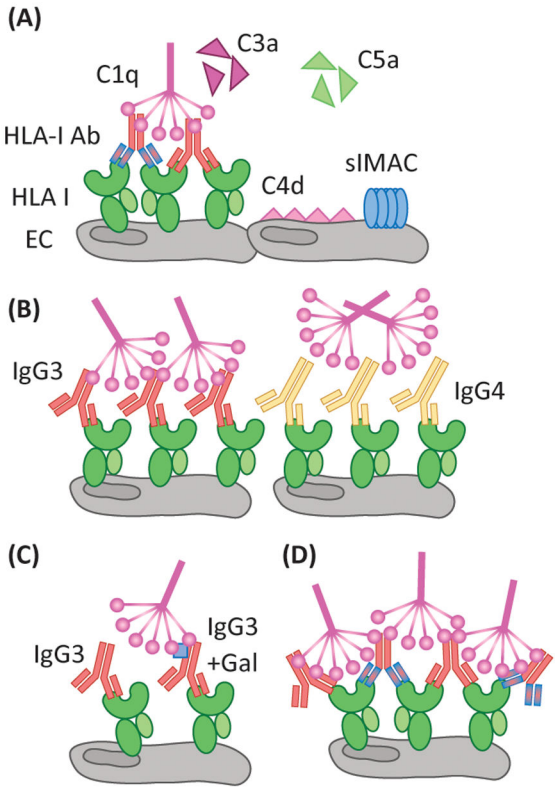

Figure 2. Complement activation by antibody/antigen determinants

(A) Activation of the classical complement pathway by HLA-Ab is mediated by C1q recognition of the Fc region of IgG. Through a series of subsequent 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 AMR in renal and cardiac transplants. Additionally, sublytic MAC (slMAC), 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 $F(ab')_2$ region), allow for increased ratios of IgG:HLA, allowing for more C1q binding. (B) Antibody subclass determines the propensity of 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, heavily 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 DSA 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.

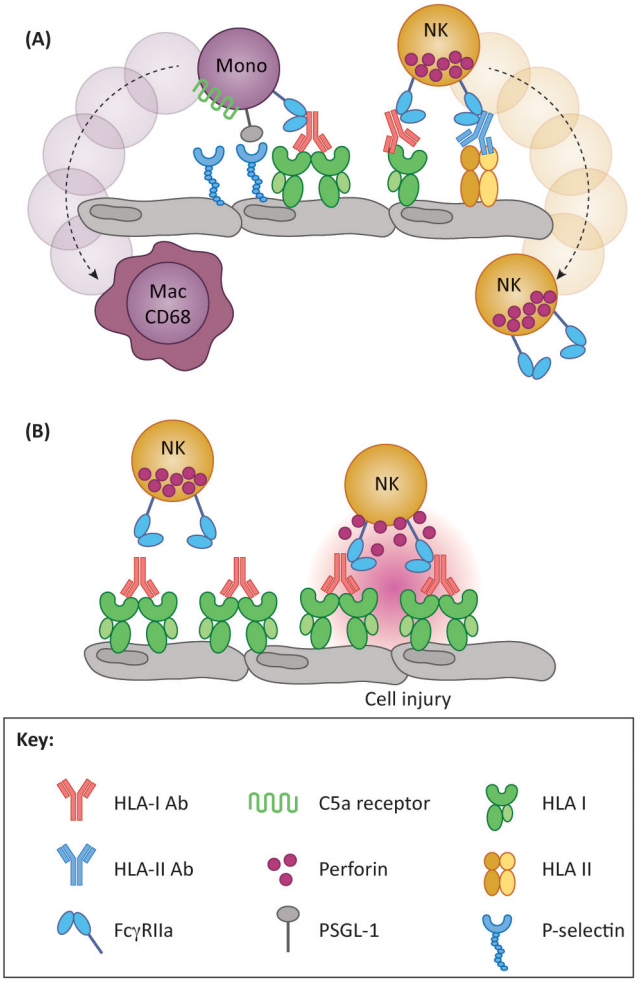

Figure 3. Fc-mediated functions which contribute to graft injury

(A) Increased leukocytic infiltrate is a hallmark feature of AMR, and this occurrence is mediated by the Fc region of donor specific antibodies (DSA). Upon DSA binding to HLA, DSA-Fc are recognized by FcγR expressed on myeloid and NK cells. Additionally, monocytes are also able to interact with the endothelium through HLA-induced P-selectin to enhance tethering and extravasation. (B) An important feature of FcγR is their role in antibody-dependent cell-mediated cytotoxicity (ADCC). DSA bind to HLA on the surface of the endothelium, facilitating Fc interaction with FcγR expressed by myeloid and NK cells. This can lead to perforin-mediated lysis of target cells, in this case, endothelium, resulting in damage to the allograft.

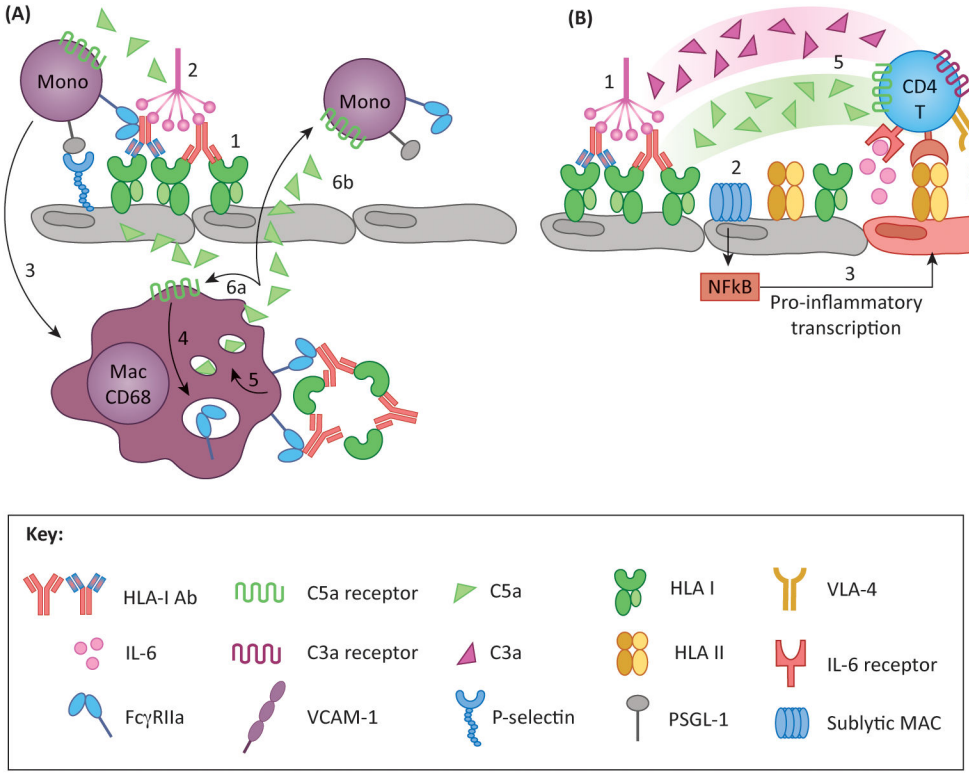

Figure 4. Proposed models of DSA-induced inflammatory loops

(A) Macrophages perpetuate activation and recruitment via complement and FcγR pathways. DSA crosslinking of HLA 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) C5a recruits monocytes to activated EC, which tether to P-selectin via PSGL-1, promoting graft infiltration and differentiation into macrophages (3); and (ii) C5a may act on intragraft CD68+ macrophages and induce FcγR expression (4). These cells can recognize immune complexes (IC) via FcγR, which can upregulate C5a production (5). Newly synthesized C5a may signal in either an autocrine (6a) or paracrine (6b) fashion, mediating further activation of intragraft macrophages and recruitment and activation of monocytes from the periphery, respectively. (B) Recent studies have identified a novel role for endothelial cells and complement in antigen presentation and stimulation of allogeneic T cells. Under inflammatory conditions (such as IFNγ activation) endothelial cells 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 Th17 and 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 MAC (2). MAC triggers non-canonical NFκB signaling leading to inflammatory gene expression (3) and stimulation of allogeneic CD4 T cells (4). T cells also express receptors for 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).

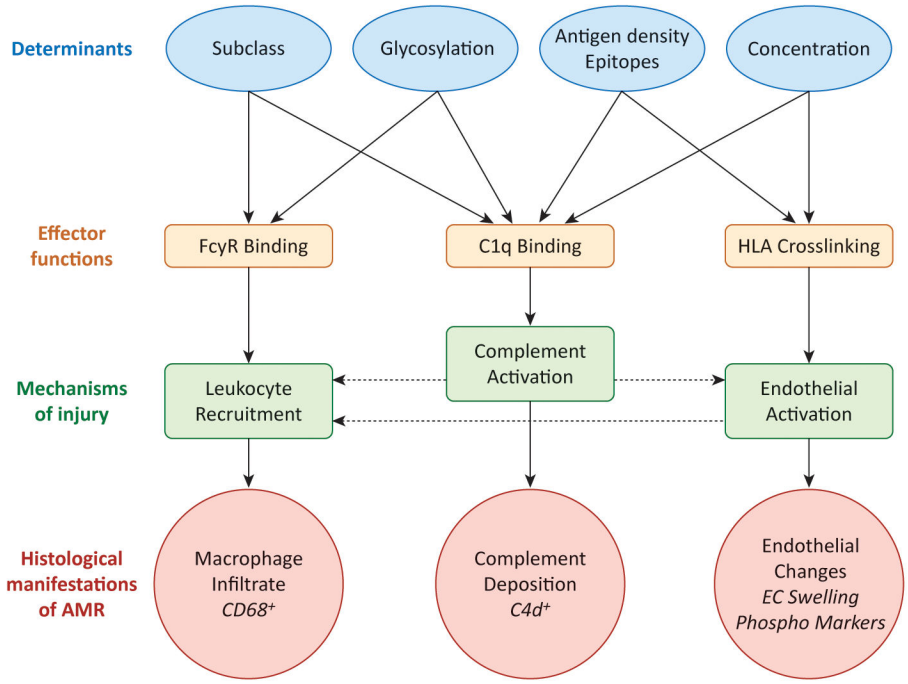

Figure 5. Mechanisms of DSA in graft pathogenesis

Features of antibody-antigen and antibody-effector system interactions that influence pathogenic functions and mechanisms of injury are shown. Variable factors regulating antibody-antigen interactions (blue ovals) directly influence the capacity of an antibody to trigger effector functions (green boxes), and mechanisms causing graft injury (purple boxes), which ultimately manifest in the graft as common histological features (red bursts). Linear effects are indicated by solid arrows.

The functional endpoints of antibody-mediated injury are interrelated (with potential inflammatory loops indicated by dashed arrows), and likely synergize to cause maximal inflammation during AMR. For example, direct endothelial cell activation by HLA antibodies triggers adhesion of leukocytes, which can be enhanced when those leukocytes bind antibody through FcγRs. Activation of complement at the endothelial cell surface may cause production of anaphylatoxins C3a and C5a, which can act on leukocytes as chemoattractants, or enhance endothelial activation.

Table 1

Summary of the biological properties of human FcγRs and IgG subclasses[a].

Name	**FcγRI**, CD64	**FcγRIIa**, CD32a	**FcγRIIb**, CD32b		**FcγRIIIa**, CD16a		**FcγRIIIb**, CD16b
Expression	Mono, Mac, Activated PMN	Mono, Mac, PMN, DC, platelets	All immune cells except T and NK		APCs (mono, DC, B), NK cells		PMN, some mono
Activating or Inhibitory	Act	Act	Inh		Act		Act
Polymorphism	None Known	R131	H131	I232T	F158	V158	NA1/NA2
Affinity for:							
IgG1	++++	+	++	±	+	+	±
IgG2	−	+	+	−	−	−	−
IgG3	++++	+	+	±	++	+++	+
IgG4	+++	+	+	±	±	±	−
Murine Counterpart	FcγRI	FcγRIII	FcγRIIb				FcγRIV

Adapted from [33] and [75].

[a]Abbreviations: APC, antigen presenting cell; DC, dendritic cell; Mac, macrophage; Mono, monocyte; NK, natural killer cell; PMN, polymorphonuclear leukocyte.