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The Complement System and Antibody Mediated Transplant Rejection

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Abstract

Complement activation is an important cause of tissue injury in patients with antibody mediated rejection (AMR) of transplanted organs. Complement activation triggers a strong inflammatory response, and also generates tissue-bound and soluble fragments that are clinically useful markers of inflammation. The detection of complement proteins deposited within transplanted tissues has become an indispensible biomarker of AMR, and several assays have recently been developed to measure complement activation by antibodies reactive to specific donor human leukocyte antigens (HLA) expressed within the transplant. Complement inhibitors have entered clinical use and have shown efficacy for the treatment of AMR. New methods of detecting complement activation within transplanted organs will improve our ability to diagnose and monitor AMR, and will also help guide the use of complement inhibitory drugs.

Transplantation is the best treatment for end stage disease of the kidneys, heart, liver, or lungs. Acute rejection events are associated with worse graft outcomes, and AMR imparts a poorer prognosis compared to cellular rejection (1-3). AMR is now the leading cause of late graft failure in kidney transplant recipients (4-7). Complement activation within allografts is caused by ischemia at the time of transplantation, and also by immunoglobulin bound to antigens within the allograft during acute and chronic AMR (8).

Antibody mediated rejection

AMR is a clinical and histopathologic diagnosis based on detection of allograft dysfunction with evidence of endothelial inflammation, and it is mediated by circulating antibodies directed against donor antigens in the allograft (9). Allelic variation in the genes for ABO blood group antigens and HLA cause these proteins to be recognized as foreign by the recipient's immune system (10). Recipients of organs that are mismatched at these loci can develop antibodies (referred to as donor specific antibodies, or DSA) that bind these antigens on the surface of endothelial cells in the microvasculature and activate the classical pathway of complement (Figure 1A). Although protocols for desensitizing patients to the ABO antigens have shown some promise, ABO-incompatible transplantation is not widely

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performed; thus, class I and class II HLA antigens are the most common targets of DSA. Preformed DSA can exist prior to transplant due to exposure to the antigens though pregnancy, blood transfusions, or previous transplants. *De novo* DSA can develop after transplantation. Recipients can also generate a humoral response to non-HLA antigens (11, 12).

Terasaki and Patel first observed the high incidence of immediate graft failure due to widespread capillary thrombosis and necrosis in sensitized recipients over 40 years ago (13). Terasaki assessed preformed DSA by mixing recipient sera with donor lymphocytes. This method became known as the complement-dependent cytotoxicity (CDC) crossmatch and was the gold standard for assessing donor/recipient compatibility for decades (14). The rapid occurrence of rejection and poor outcomes of transplantation across a positive CDC crossmatch highlighted the importance of preformed antibodies and complement activation in hyperacute rejection and graft loss.

Prior to the early 1990s, acute rejection events were widely thought to be primarily due to T-cell mediated immunity (15). However, several studies later showed that acute rejection in renal transplant recipients who developed DSA after transplant was clinically and pathologically distinct from rejection events in patients without DSA. Rejection events with DSA were associated with swollen and detached endothelial cells, glomerulitis (inflammation of glomerular capillaries), small-vessel vasculitis, neutrophil infiltration, and vascular occlusion. Acute rejection with DSA was also more severe and resulted in worse outcomes. Interestingly, deposited immunoglobulin was rarely seen in the allografts (16, 17). A similar pattern of endothelial inflammation is also seen in cardiac and lung transplants with DSA and antibody-mediated injury (18, 19).

The link between complement and rejection was solidified when Feucht demonstrated the complement split product C4d in transplant biopsies, implicating the classical complement pathway in acute and chronic rejection (20, 21). This landmark observation revealed that C4d is a durable biomarker of AMR. These and subsequent, corroborating studies led to the development of consensus diagnostic criteria in renal allografts for acute AMR at the 2001 Banff Conference on Allograft Pathology (22), and for chronic, active AMR at the 2007 Banff conference (9). These criteria included detection of DSA in serum and C4d in tissues as part of the diagnosis of renal AMR, and similar criteria have been proposed for monitoring cardiac AMR (23).

Classical pathway activation by immune-complexes

Classical pathway activation is initiated when plasma C1q binds to the Fc segments of IgM and IgG (24). The relative ability of human immunoglobulin to activate the classical pathway is: IgM > IgG3 > IgG1 > IgG2 >> IgG4 (25). Although the C1q binding sites on free IgG are ordinarily exposed, the relative affinity of C1q for a single IgG is low (the Kd is approximately 100 μM) (26, 27). C1q is hexameric, however, and when IgG is aggregated on a target surface C1q can bind multiple IgGs, greatly increasing its affinity (~10 nM) (26). The affinity of C1q for IgG is even greater if the Fc regions of IgG are packed in a hexamer conformation, leading to effective classical pathway activation (27). Thus, antibody isotype,

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antigen density, and conformation of aggregated IgG all affect C1q binding affinity and classical pathway activation.

Complement regulatory proteins also affect the degree of complement activation (Figure 1A). C4 binding protein (C4bp) is a soluble regulatory protein that inactivates C4b and the classical pathway convertase (28). Membrane cofactor protein (MCP, or CD46) and decay accelerating factor (DAF, or CD55) control activity of the classical and alternative pathway convertases, and are expressed on endothelial cells. Antibody mediated complement activation on endothelial cells may upregulate the expression of MCP and CD59, increasing the resistance of the cells to complement-mediated injury (29). Complement receptor 1 (CR1, or CD35) is another membrane-bound regulator with both classical and alternative pathway regulatory activity. CR1 is not expressed on endothelial cells constitutively but has been shown to be inducible *in vitro* (30). The alternative pathway amplifies complement activation, even when activation is initially triggered by the classical pathway, and this process is limited by a soluble protein called Factor H and also by a cell surface regulator called CRIg (Figure 1A) (31). Efficient complement regulation limits downstream complement activation (32, 33), and it is possible to have abundant C4 fragment deposition on a surface in the absence of detectable C3 (34).

The extent of complement activation by immune-complexes is determined by multiple factors, including the isotype of the antibody, the abundance of the target antigen and density of immunoglobulin, and the local concentration of complement regulatory proteins. Prior exposure to the target antigens and treatment with immunosuppressive drugs affects antibody generation. *In vitro* studies have shown that expression of HLA antigens and complement regulator proteins on endothelial cells is altered by inflammatory stimuli (35). Thus, antibody-mediated allograft injury is not simply a function of the presence of DSA in serum, but is influenced by numerous local and systemic factors.

Mechanisms of complement-mediated endothelial injury

Complement activation produces several biologically active fragments (Figure 1B). C3b is covalently fixed to surfaces and acts as an opsonin. C5b-9 (the membrane attack complex or MAC) forms pores through the outer membrane of cells and can cause cellular activation, signaling, cell lysis, and possibly proliferation (36). MAC on the surface of endothelial cells rapidly induces activation of the non-canonical pathway of NF-κB, which then induces the expression of several pro-inflammatory proteins including IL-6, E-Selectin, and VCAM-1 (37). Although ion flux through the MAC can activate endothelial cells, clathrin mediated endocytosis of MAC from the cell surface was also shown to activate NF-κB via stabilization of NF-κB-inducing kinase on the surface of early endosomes (38).

C3a and C5a signal through G-protein coupled receptors expressed on a wide variety of cells, including hematopoietic cells, endothelial cells, and epithelial cells (39). These protein fragments, referred to as anaphylatoxins, are potent inducers of inflammation. Signaling through the C5a receptor on endothelial cells causes the release of heparan sulfate from the cell surface (40) and it amplifies production of inflammatory chemokines when the cells are

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also exposed to other cytokines (41). C5a also directly triggers the release of pre-formed von Willebrand Factor from endothelial cells (42).

Although the complement system is often regarded as a downstream effector system for antibody-mediated injury, complement activation also promotes the adaptive immune response. Several studies have shown that complement activation increases T cell alloreactivity against transplanted tissues (43, 44). Furthermore, antibody-induced MAC on endothelial cells enhances the response of alloreactive T cells and increases their production of IFN-γ (37). IFN-γ, in turn, increases the expression of HLA I and II by the endothelial cells, leading to further antibody-mediated complement activation on the cell surface. Complement receptor-2 (CR2) is expressed on B cells, and the ligation of CR2 by C3d amplifies the B cell response to target antigens (45). Complement activation within the ischemic kidney was found to amplify the humoral immune response to foreign antigens, possibly via this mechanism (46). Thus, the complement system potentially influences AMR at many different steps. It may promote the development of DSA, it mediates the downstream pathogenic effects of DSA, and it can increase the expression of HLA on endothelial cells.

Complement activation on endothelial cells is not always pathogenic, and it may be protective under some circumstances. Transient depletion of DSA in recipients of ABO incompatible allografts leads to long-term graft survival in some patients. Yet DSA titers rebound in these subjects and C4d can be detected within the allografts. This phenomenon is termed "accommodation", and it may require complement activation to occur (47). In a study of sensitized primates, depletion of C3 with Yunnan-cobra venom factor (Y-CVF) for two weeks prior to kidney transplant prevented accelerated AMR and resulted in prolonged graft survival (29). The animals had persistent DSA and C3 levels returned to normal after Y-CVF was discontinued, yet none of the animals subsequently developed AMR. The acquired resistance to complement mediated injury may involve increased expression of complement regulatory proteins and upregulated mechanisms for removing sC5b-9 from the plasma membrane (29, 48).

C4d deposition within allografts

A unique feature of the complement system is that circulating C3 and C4 proteins are cleaved and covalently fixed to nearby surfaces during activation, providing a durable marker of inflammation. The detection of C4d deposits in capillaries is a marker of acute and chronic AMR in transplanted kidneys (21, 49) and hearts (50) (Figure 2A). C4d is a more sensitive indicator of AMR than IgG deposition, probably because it is more abundant and it is covalently fixed to the cell surface. Isolated C4d deposition in kidney and heart allografts is not specific for AMR, however. In biopsies performed on patients who did not have evidence of renal allograft injury ("protocol biopsies"), only about half of the C4d-positive patients developed AMR (51). As mentioned above, C4d deposition in ABO incompatible allografts can actually be associated with accommodation and improved outcomes (52). Thus, detection of C4d without histologic or functional evidence of allograft injury is not sufficient to predict AMR or to guide treatment. The significance of C4d

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staining in lung and liver allografts is controversial (53-55). C3 and C4 are produced in the liver, and hepatocytes may be intrinsically resistant to complement-mediated injury (56).

The convertases deposit multiple C3b molecules on target surfaces, and the abundance of C3b can be many fold higher than that of C4b (57). C3d deposits can be seen in an identical pattern to C4d in some, but not all, allografts (58, 59). Cleavage of C3 occurs downstream of C4 in the classical pathway. Consequently, C3d is not seen in the absence of C4d in AMR (58, 60). On the other hand, the detection C4d in the absence of C3d may represent effective complement regulation (Figure 2B). Thus, C3d may be more specific than C4d as a marker of complete complement activation and AMR (58), although this requires further validation.

Although detection of C4d has long considered essential for the diagnosis of AMR, there is emerging evidence that DSA cause injury to allografts in the absence of C4d deposits. Ultrastructural changes and endothelial-specific gene expression profiles characteristic of AMR are observed in some C4d-negative biopsies (61, 62). Some reports now suggest that up to 20% of acute cases and up to 60% of cases of chronic AMR may be C4d-negative (9, 63). The absence of C4d in these biopsies may be due to technical reasons, but DSA may also cause injury through complement-independent mechanisms, such as signaling through FcR receptors on natural killer (NK) cells or other cell types (64-66). Based on these observations, a category of C4d-negative AMR was included in the most recent revision of the Banff criteria for the diagnosis of AMR (9). Diagnosis of C4d-negative AMR requires DSA, histologic evidence of injury, and detection of microvascular injury by histology or gene expression profile. Patients with C4d-negative AMR are at risk for long-term graft failure (62) and treatment of these patients improves outcomes, confirming the clinical importance this subgroup (67).

In Vitro Assays for Donor Specific Antibodies

The original CDC assay provides a functional readout of complement activation by DSA (i.e. lymphocyte lysis) (13). A positive crossmatch with this assay is highly predictive of acute rejection and is a contraindication to transplantation. The assay is not sensitive and is cumbersome to perform, however, and several other assays for DSA have subsequently been developed [the technical aspects of these assays have recently been reviewed (68)]. In 1983, flow cytometric crossmatching using recipient serum and donor lymphocytes was introduced as an alternative method (69), and flow cytometry has largely replaced the CDC crossmatch. In addition, methods to detect DSA reactive to specific donor antigens were developed in the mid-1990s (70). In these assays, recipient serum is mixed with beads coated with either HLA class I or class II antigens, and IgG bound to specific antigens is detected by flow cytometry (71, 72).

Hyperacute rejection has become rare since the adoption of crossmatching using a combination of flow cytometry crossmatch and the solid phase single antigen bead assay. Unfortunately these assays do not predict or detect AMR as well as originally hoped. Most studies agree that pre-transplant DSA is associated with worse graft outcomes and a higher incidence of acute and chronic AMR in kidney, heart, lung, liver, and pancreas transplantation (73-77). In a study by Amico *et al*, for example, pre-transplant DSA was

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associated with an increased incidence of AMR (55% vs 6%) and a reduced 5-year death-censored graft survival compared with patients without detectable DSA. However, 45% of the patients with detectable DSA did not develop AMR and did not have reduced 5-year graft survival (78, 79). The poor specificity of the standard DSA assays for predicting AMR may be due to the fact that they do not capture information about antibody isotype or its ability to activate complement.

DSA assays that measure complement binding/activation

To improve the ability of solid-phase assays to predict allograft injury, the single antigen bead assay has been modified to measure complement activation by DSA against specific antigens. Assays have been developed that measure C1q or C3d binding by the DSA, or that measure C4d deposition onto the bead surface (80-83). The C4d-deposition DSA assay correlates with C4d deposition in renal allografts (84), and it predicts worse allograft survival in cardiac (85) and renal transplants (86). In a recent study of 1016 renal transplant patients, those with C1q-binding DSA had worse graft survival than those who did not: 18% of the patients developed T cell mediated rejection and 48% of patients developed AMR (87). However, detection of C1q-binding DSA has not correlated with AMR or allograft failure in all studies (88). Patients with C3d-binding DSA were found to have worse renal allograft survival than those who do not, even in patients with low overall DSA titers (81). The evaluation of C3d-binding DSA was performed at the time of biopsy and was not evaluated over time, so the significance of these findings is limited to acute AMR.

A recent study reported that detection of IgG3 DSA in the single antigen bead assay is associated with a higher risk of allograft loss than other DSA isotypes (89). This is probably due to the effects of isotype on the ability of immunoglobulin to activate complement. Complement activation is also a function of DSA titer, however, since bound IgG must achieve a certain degree of density to bind C1q. Indeed, positivity in the C1q-binding assay is more strongly correlated with the overall level of DSA than it is with the isotype (90, 91). Thus, further study will be needed to determine whether the complement binding assays yield clinically useful information beyond that provided by the standard single antigen bead assay and antibody titer, and whether the results of these assays will improve patient care.

Complement-targeted therapies

Currently, the treatment of AMR is based upon strategies to remove pre-formed antibodies (plasmapheresis), or to prevent production of new antibodies (IVIg, rituximab, bortezomib). Unfortunately, the treatment of acute and chronic AMR is often unsuccessful. Inhibition of the complement cascade is an attractive therapeutic option, particularly in patients with acute disease and complement-activating DSA detected by the solid-phase assays (81, 87).

Eculizumab is a monoclonal antibody to C5 that blocks C5 cleavage and activation. The FDA has approved eculizumab for treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. The first case report of eculizumab used for AMR involved a highly sensitized kidney transplant recipient who developed severe AMR within days of transplantation (92). The patient was resistant to standard antibody-depleting therapies but recovered renal function after treatment with two doses of eculizumab.

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Additional cases have been reported of successful treatment of acute AMR in highly sensitized recipients of kidney (93, 94), kidney/pancreas (95), heart (96), lung (97), and intestine (98) transplants. In the largest published trial to date, prophylactic treatment of sensitized renal transplant patients with eculizumab in addition to plasmapheresis reduced the incidence of acute AMR at one year compared to historical controls (7.7% versus 42.2%), but it did not significantly reduce the incidence of chronic AMR at two years (99, 100). Of patients with high DSA levels post-transplant, positive C4d staining on biopsy was common in both eculizumab treated and control patients (100% and 91%, respectively), consistent with the downstream position of C5 in the complement cascade (Figure 1B). In patients with high DSA levels and positive C4d staining, however, only 15% of patients in the treatment group had inflammation consistent with AMR compared to 100% of the historical controls.

Further analysis of the two patients in the eculizumab treatment group that developed AMR revealed an association of IgM DSA with eculizumab failure (101). Burbach *et al* reported two other cases of eculizumab failure, one in prevention of acute AMR and one in treatment of acute AMR (102). Both cases met Banff criteria for acute AMR but were C4d-negative on biopsy. These cases may represent a subset of patients who develop injury through complement-independent mechanisms. It is also unknown how long treatment with eculizumab should continue, and it is possible that prolonged treatment will impede the development of accommodation. These are particularly important issues given the expense of the drug.

The early successes of eculizumab highlight the potential of complement modulating therapies for preventing and treating AMR, while its failures underscore the heterogeneity of acute and chronic AMR. Clinical trials of eculizumab in transplant patients are ongoing and should provide further insights into the optimal approach to complement inhibition in AMR (NCT01895127, NCT02113891, and NCT02013037). Several novel complement inhibitors are in development, some of which are being investigated as possible therapies for AMR (103). C1 blockade prevented acute AMR of kidney allografts in allosensitized baboons (104), for example, and a recent phase I/II trial of C1 inhibitor infusions showed promise in preventing acute AMR in highly sensitized renal transplant patients (105). Inhibition at the level of C3 may also be an effective approach given the importance of C3 fragments in the innate and adaptive immune responses (29). An agent that prevents C3 activation within the transplanted organs is currently being evaluated in a phase II study (106).

Conclusions

AMR is an important cause of acute and chronic allograft injury. Treatment is not always effective, and chronic AMR is now the most common cause of long-term allograft failure. The degree of tissue injury caused by the humoral immune system is a function of the titer, affinity, and isotype of the DSA, as well as the expression of antigens and complement regulatory proteins within the target tissue. These elements are influenced by tissue injury, illness, and by immunosuppressive medications (or non-compliance). Consequently, the overall tendency of DSA to cause injury varies over time, and the diagnosis of AMR

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requires the detection of DSA in serum and microvascular injury and/or C4d deposition within the allograft.

In recent years there have been important advances in our understanding of the role of complement in AMR. One innovation has been the development of assays to examine the complement-activating potential of DSA. These assays provide further evidence of the importance of the complement cascade in the pathogenesis of AMR. Eculizumab has shown efficacy for the prevention and treatment of acute AMR, and new therapeutic complement inhibitors are in development. Additional clinical experience with these drugs will provide important information about the benefits and limitations of complement inhibition in this disease. For example, there may be a subset of AMR patients in whom antibody-mediated injury of the allograft does not involve complement activation. Furthermore, C3a and/or C3b may contribute to allograft injury, and complement inhibitors that block the complement cascade at the level of C3 may have advantages over agents that block the cascade at the level of C5.

One of the primary remaining challenges in caring for transplant recipients is detecting and monitoring AMR. An ideal biomarker of AMR would specifically indicate active complement activation and tissue injury, not just future risk of injury. Furthermore, biomarkers that indicate ongoing complement activation could be used to stratify patients to treatment with complement inhibitory drugs. Molecular imaging methods have been developed to non-invasively detect deposition of complement fragments within tissues (107), and these techniques may be useful for monitoring AMR. These diagnostic tools and other future discoveries in the field of complement biology will undoubtedly have an important impact on prevention and treatment of AMR.

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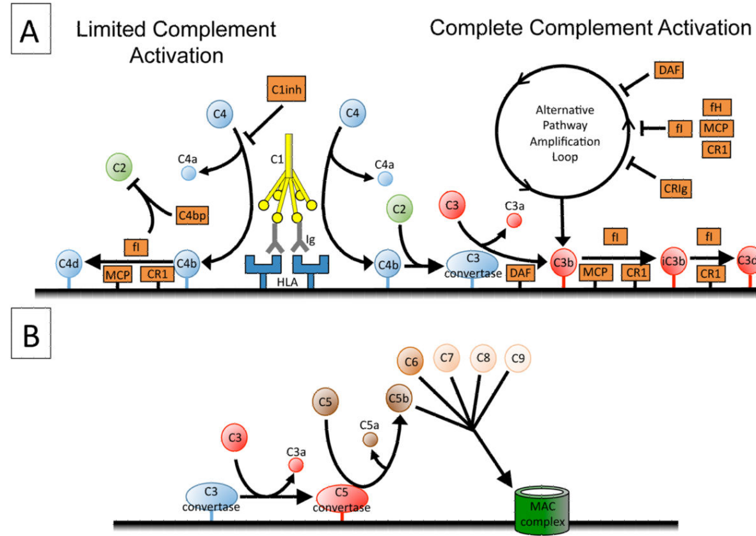

Figure 1. Complement activation in antibody mediated rejection

A) The classical pathway of complement is activated when C1q binds to IgG clustered on endothelial HLA. Each C1q deposits multiple C4 molecules on target surfaces. Efficient complement regulation stops the reaction before C3 molecules are deposited. In contrast, complete complement activation results in covalent fixation of multiple C3 fragments to the target tissue. This process is augmented by the alternative pathway amplification loop, although several regulatory proteins control the alternative pathway. Complement regulatory proteins metabolize the C3b to iC3b and then to C3dg. B) Complete complement activation generates several biologically active products, including C3a, C3b, C5a, and C5b-9. Abbreviations: C4bp, C4 binding protein; CR1, complement receptor 1; fI, Factor I; fH, Factor H; MCP, membrane cofactor protein; DAF, decay accelerating factor.

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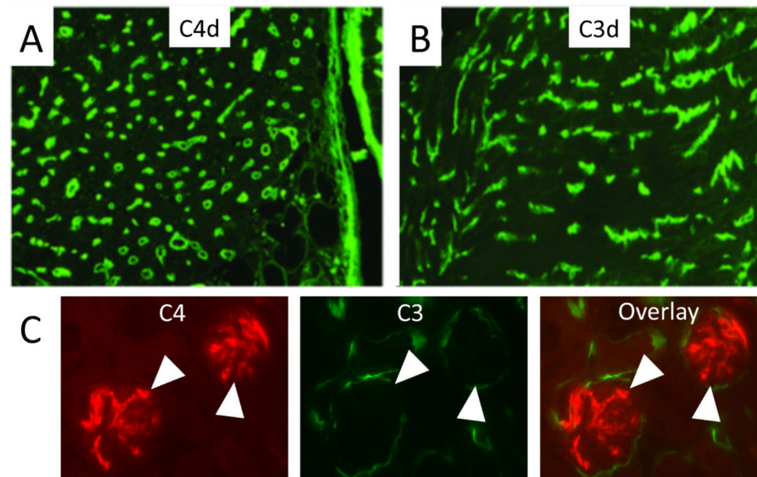

Figure 2. C4 and C3 deposition in tissues by the classical pathway of complement

A) Linear staining of capillary endothelium for A) C4d and B) C3d in a cardiac biopsy from a patient with AMR. The staining pattern for C4d and C3d was identical in this biopsy. C) C3 and C4 deposition in glomerulus of a mouse kidney. IgM deposition in the mesangium causes C4 deposition (red). Efficient complement regulation prevents C3 deposition (green) at the same location. Some alternative pathway-mediated C3 deposition is seen on Bowman's capsule and in the tubulointerstitium. Glomeruli are indicated with arrowheads. Modified with permission from references (34) and (59).