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Antibody-Mediated Graft Injury: Complement-Dependent and Complement-Independent Mechanisms

Nicole M Valenzuela, **Jeffrey T McNamara**, and **Elaine F Reed**
Department of Pathology and Laboratory Medicine, UCLA Immunogenetics Center, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

Abstract

Purpose of Review—Antibody-mediated rejection (AMR) is emerging as the leading cause of chronic rejection and allograft failure. Traditionally, the mechanisms of graft injury mediated by donor specific antibodies beyond complement activation were not well appreciated. However, an evolving paradigm of Fc-independent antibody functions, along with clinical recognition of C4d negative AMR, has increased awareness of the action of antibodies leading to endothelial activation and dysfunction.

Recent Findings—Herein, we address current clinical trends, including the signature of microvascular inflammation in biopsies of grafts undergoing AMR, the prevalence of antibodies to HLA-DQ and non-HLA targets, and the functional characterization of HLA IgG subclasses and complement fixing capacity. We also discuss recent experimental evidence revealing new mechanisms of endothelial and smooth muscle cell activation by HLA antibodies, which may contribute to vascular inflammation and chronic rejection. Finally, we touch upon novel discoveries of the interplay between antibodies, the complement system and CD4 T cell-mediated alloimmunity.

Summary—The current literature suggests that, although complement fixing antibodies may have some prognostic value for graft outcome, complement-independent mechanisms of graft injury are increasingly relevant. Therapeutic strategies which target endothelial activation induced by antibodies may ameliorate vascular inflammation and mononuclear cell infiltration characteristic of AMR.

Keywords

Microvascular Inflammation; Endothelial Cell Activation; Complement; Antibody-mediated Rejection

Introduction

Antibody-mediated rejection (AMR) is an increasingly problematic entity in solid organ transplantation that leads to graft dysfunction and loss. AMR often occurs late (>1 year)

Corresponding author: Dr. Elaine F. Reed UCLA Immunogenetics Center Department of Pathology and Laboratory Medicine David Geffen School of Medicine, University of California at Los Angeles Los Angeles, CA 90095 Phone: 310-794-4943 Fax: 310-206-3216 ereed@mednet.ucla.edu.

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after transplantation with an incidence of approximately 30% (1), and was recently attributed to cause half of all renal allograft failures (2). An understanding of how antibodies cause graft injury and promote acute and chronic rejection is critical for the management of sensitized recipients and improvement of therapeutics. Studies from our group and others have previously demonstrated that HLA I antibodies act as agonists, inducing molecular aggregation of HLA I class I molecules to trigger intracellular signaling pathways that are critical in the regulation of cell survival, proliferation and migration in graft vascular cells (reviewed in (3)). Recent investigations have revealed additional functional responses of vascular cells to antibodies, including induction of inflammatory mediators by endothelial cells and recruitment of immune cells. Moreover, the relevance of complement fixing capacity of donor specific antibodies has received increasing attention. Herein, we discuss the implications of recent work in these areas.

Characteristics of AMR and Drawbacks of Current Histological Criteria

Consensus guidelines for the histological diagnosis of antibody-mediated rejection center around circulating DSA, vascular injury, microvascular changes and complement (C4d) deposition. However, AMR criteria have in some cases proved inadequate because of the reliance on complement staining (1, 2), and, while C4d appears to be a specific marker in cardiac and renal transplantation, it is estimated that half of AMR events are missed due to the lack of sensitivity of C4d staining (4). To date, no consensus AMR diagnostic criteria exist in lung and liver (5, 6), due to controversy over the clinical significance of DSA in liver transplantation and a lack of specificity of C4d staining in lung allografts (5-8).

Recent efforts have aimed at discovery of superior histological indicators that can discriminate antibody-mediated rejection from acute cellular rejection, infection, drug toxicity and stable graft function, and identify C4d-negative AMR. Extracellular matrix regulators matrix metalloproteinase-2 (MMP2) and TIMP1 were upregulated in renal biopsies during AMR (9), which may play a role in fibrosis during chronic rejection. Several groups have recently reported that glomerulitis and peritubular capillaritis (microcirculation or microvascular inflammation) correlate with DSA and graft failure in renal transplants (10-12). Moreover, activation of capillary endothelial cell signaling correlates closely with AMR severity, DSA and microcirculation inflammation, and is a more sensitive AMR marker than C4d deposition (13, 14) cardiac transplantation. A “molecular phenotyping” approach, predominantly composed of endothelial-associated transcripts expressed in the renal allograft, could also identify C4d negative AMR (15). A new sub-classification of AMR with vasculitis was recently proposed, which increased predictive value for renal allograft loss (16).

Additionally, intravascular macrophages, identified by CD68 staining, appear to be a common feature of AMR (17), and provide a more sensitive histological marker compared with C4d in cardiac allografts (18). Intragraft macrophages also increase the risk of renal allograft dysfunction (11), although macrophage staining is not currently included in the Banff criteria. Natural killer (NK) cells have also been implicated in antibody-mediated graft injury, particularly in late AMR (19). These clinical results are paralleled by observations in experimental models, where depletion of NK cells ameliorated MHC I antibody-induced

chronic rejection in murine cardiac allografts (20). Taken together, these clinical observations highlight the central role of endothelial activation and innate immune infiltration in graft injury by donor specific antibodies.

New Trends

There is a wealth of literature regarding the incidence and deleterious impact of donor specific HLA antibodies, and this topic has been thoroughly reviewed elsewhere (21, 22).

HLA-DQ Antibodies

HLA I antibodies appear early, while HLA II DSA tends to prevail late after transplantation and associates with chronic graft dysfunction (23, 24). An emerging trend is the intriguing but little understood phenomenon of predominant DSA to HLA-DQ in lung (25), cardiac (26), renal (27) and liver transplant recipients (28), particularly during chronic rejection (28). The possible pathogenic mechanisms of antibodies to HLA-DQ are not yet clear, although recipients tend to have worse outcome than when anti-DQ antibodies are absent (29). Interestingly, DQ mismatch increases the risk of *de novo* DSA development (27). It is yet unknown whether antibodies to HLA-DQ are a marker of end-stage graft injury and/or nonadherence; whether they are increased in circulation due to low HLA-DQ expression in the graft; or whether they represent an independent pathogenic actor.

Non-HLA Antibodies

There is growing evidence to suggest that antibodies against non-HLA antigens may contribute to AMR in solid organ transplantation. Reports show that 10-23% of recipients are pre-sensitized to non-HLA antigens (30, 31), while 22% form non-HLA antibodies after transplant (32). Endothelial reactive antibodies have been used clinically to identify cases of AMR (33) and the nature of these targets is starting to be elucidated. Non-HLA antibodies are found in renal, lung and cardiac transplant recipients, but the targets appear to be unique to each solid organ. A recent study revealed that endothelial-reactive antibodies may recognize nonpolymorphic and polymorphic antigens on the endothelial surface (34). However, conflicting reports suggest either that non-HLA antibodies independently reduce graft outcome (35-37) or that they have no significant impact (38). The issue is complicated by observations that autoantibodies can occur independently of or concurrently with donor specific HLA and MICA antibodies. In lung transplants, non-HLA antibodies against K-α tubulin and collagen are associated with rejection, but these antibodies were preceded by HLA DSA and persisted after elevated HLA antibody levels subsided (39), suggesting that non-HLA antibodies are markers of alloimmune damage. Anti-endothelial cell antibody subclasses are reportedly IgG2 and IgG4 predominant, which are non-complement fixing, suggesting a complement-independent mechanism of action (30). However, antibodies against vimentin, K-α tubulin, and collagen have been associated with C4d deposition on erythrocytes, suggesting they could contribute to classical complement activation (40). It is also likely that the pathogenic potential of non-HLA antibodies depends upon the target. For example, anti-angiotensin II type 1 receptor (AT1R) antibodies contribute to hypertension and vasculopathy in renal and cardiac transplantation (41) and increase the risk of graft failure (42). In summary, the targets of antibodies to non-HLA endothelial proteins are only

beginning to be identified, and, for the most part, their mechanism of action beyond Fc-mediated functions remain to be further elucidated.

The Mechanisms of Antibody-Mediated Graft Injury

Antibody-mediated acute rejection is primarily driven by the effector functions of the Fc fragment of HLA antibodies, while experimental evidence indicates that the Fc-independent effects of antibodies promote chronic inflammation and proliferation.

The Significance of Complement

IgG1 and IgG3 are efficient activators of the classical complement cascade, as well as high affinity ligands for Fc gamma receptors expressed on myeloid cells including macrophages and NK cells (reviewed in (43)). In recent years, new assays have been developed to characterize the functional deposition of complement components, including C1q, C3d and C4d, by HLA antibody in solid phase assays or cell-based protocols (1, 44-46). However, contradictory conclusions have been reached regarding the predictive significance of complement fixing DSA detected by these assays (47, 48), and the results often do not correlate with lymphocytotoxicity (CDC-XM) results (46). Moreover, despite the predominance of complement-fixing IgG1 DSA in recipients (28, 49), only certain specificities exhibit cytotoxicity or C1q deposition. Antigen density or synergism of multiple low-level allele-specific antibodies may explain the differences (reviewed in (50)). Additionally, complement fixation appears to be closely dependent upon antibody titer (51) and confounded by the presence of IgM (52). Indeed, poorer outcomes associated with complement fixing antibodies in some studies may be in fact reflective of high donor specific antibody levels (48). Duquesnoy et al. proposed the theory that complement fixation is a function of the epitope itself, which induces conformational changes in the antibody to confer binding to the C1 complex (53). Additionally, it is possible that differences in Fc glycan composition may lead to increased cytotoxicity of certain HLA DSA (reviewed in (54)). While interesting, experimental evidence addressing these putative mechanisms remains to be reported.

The utility of C4d staining has recently been questioned (16), particularly late in transplantation (23). Antibody-mediated chronic injury does not always correlate with or require complement activation in clinical studies (55) or experimental models (20). Whereas HLA antibodies trigger complement-dependent cytotoxicity of target lymphocytes, endothelial cells—the primary target of antibody-mediated alloimmunity—are generally resistant to complement-mediated cell lysis in response to HLA antibodies. Despite evidence of terminal complement activation at the endothelial cell surface (44, 56), endothelial cells may upregulate cytoprotective factors and survival signaling to prevent HLA antibody-induced cytotoxicity (57, 58). Therefore, the physiological relevance of complement activating antibodies detected by C1q or C3d-fixing assays remains unclear, and the mechanisms of antibody-mediated graft injury in C4d negative AMR have yet to be fully elucidated.

Complement-Independent Antibody-Mediated Endothelial and Smooth Muscle Cell Activation

HLA antibodies also act as agonists to induce intracellular signaling in endothelial and smooth muscle cells. Notably, these effects are independent of the Fc fragment, and may therefore be elicited by any IgG subclass. HLA I antibodies promote focal adhesion kinase (FAK)-dependent proliferation and migration of smooth muscle cells (59), which may contribute to the neointimal changes seen in chronic rejection. Recent evidence has also implicated matrix metalloproteinase-2 (MMP2) and neutral sphingomyelinase-2 (nSMase2) in HLA I antibody-induced smooth muscle cell proliferation *in vitro* and *in vivo* (60), which is interesting in light of clinical observations of increased intragraft expression of MMP2 during AMR (9). Active MMP2 acts on nSMase2 to stimulate, among other effects, production of ceramide. Ceramide is itself a proinflammatory stimulus, and can be converted to sphingosine-1-phosphate (S1P), which is mitogenic through MAPK/ERK pathways (61). In this study, the authors speculated that nSMase2 activation generates S1P, but as yet no evidence has linked HLA I antibodies with ceramide and/or S1P production. Further studies are needed in this novel area to dissect the proximal signaling leading to MMP2 and nSMase2 activation, to confirm the role of S1P in smooth muscle cell proliferation. If S1P is indeed produced in response to HLA I crosslinking, it would be of interest to examine other phenotypic changes triggered by this mediator, such as regulation of cell motility, control of barrier function, and modulation of endothelial inflammatory activation.

Cell proliferation and migration are dependent upon reorganization of the cytoskeleton. Recently, our group characterized HLA I-induced cytoskeletal rearrangement in greater detail, demonstrating that HLA I crosslinking activated Rho kinase, myosin light chain kinase and ERK1/2 in a calcium-independent manner to stimulate stress fiber formation. This study also revealed a novel molecular association of ERK1/2 with mTORC2, and showed that Rictor and ERK relocalize to the plasma membrane after HLA I antibody stimulation. These results suggest that ERK1/2 is a critical regulator of endothelial cytoskeletal changes, migration and motility (62).

In addition to cell motility, the intact cytoskeleton is required for activation of critical kinases downstream of HLA I crosslinking. Proteomic analysis demonstrated that HLA I crosslinking induced the association of many phosphorylated proteins, including eukaryotic initiation factor 4A1 (eIF4A1), with the actin cytoskeletal fraction (63). eIF4A1 is a target of mTORC1 and is required for protein translation and cell proliferation. The cytoskeleton is actively involved in and required for translation, through spatial organization of the protein synthesis machinery, delivery of tRNAs to polysomes, and support of compartment-restricted localized translation of specific RNA (64). Taken together, these studies highlight the central role of cytoskeleton in mediating the cell signaling process and raise fascinating questions regarding the functional link between the cytoskeleton and protein synthesis machinery in HLA I signaling.

Intracapillary macrophages are an important hallmark of AMR in cardiac allografts (17), and macrophages are key immune mediators of graft injury. Endothelial cells actively regulate

infiltration of leukocytes from the bloodstream into the tissue. Type 1 endothelial cell activation involves rapid and transient induction of adhesion molecules and chemoattractants, while type 2 activation requires protein synthesis resulting in chemokine and adhesion molecule expression over the course of hours or days. Emerging evidence indicates that HLA crosslinking can trigger both type 1 and type 2 endothelial cell activation, providing some insight into the mechanisms of macrophage recruitment to the allograft during HLA antibody-mediated injury. We and others reported that HLA I crosslinking by antibodies triggers rapid endothelial exocytosis (<30min, type 1 activation) leading to cell surface presentation of the adhesion molecule P-selectin and release of vWF (65, 66) from aortic, venous and microvascular endothelial cells (67). This induction did not require the Fc portion of the antibody, suggesting that all IgG subclasses can elicit pathogenic effects by direct endothelial cell activation. P-selectin was indispensable for increased adhesion of monocytes *in vitro* and for accumulation of macrophages in murine cardiac allografts *in vivo* in response to MHC I antibodies (65). Interestingly, we observed that complement fixing HLA I antibody dramatically augmented basal P-selectin-mediated monocyte adhesion through engagement of monocyte Fc gamma receptors (FcγRs) (67), indicating that DSA subclasses that interact efficiently with FcγRs elicit enhanced recruitment of monocytes through dual activation of endothelium and monocytes.

In addition to early endothelial activation, HLA I antibodies induce late phase adhesion molecules and synthesis of chemoattractants and inflammatory cytokines that promote inflammation. For example, HLA I crosslinking activated the transcription factor cAMP response element binding protein (CREB), which lead to increased adhesion molecule and chemokine expression by microvascular endothelium, and monocyte adhesion (68). Similar studies found increased synthesis of cytokines and chemokines by endothelial cells from other vascular beds (69) upon exposure to HLA antibodies (Table 1).

The Relationship between Donor Specific Antibodies and T Cell-Mediated Alloimmunity

Most recently, a novel paradigm has emerged in which antibody-mediated graft injury may potentiate cell-mediated alloimmune responses. Jordan Pober's group demonstrated that complement activation can augment HLA I and II antibody activation of endothelium, which increased endothelial immunogenicity to T cells. Sublytic MAC deposition on endothelial cells in response to HLA antibodies exacerbated proinflammatory signaling through non-canonical NFκB, which in turn triggered CD4 T cell capture under shear stress. Moreover, allogeneic CD4 T cell activation was increased upon coculture with HLA antibody-activated endothelium (56). In another study, complement split products augmented costimulation of alloreactive CD4 T cell by dendritic cells (70). Taken together, these findings provoke interesting questions about the interplay between antibodies and alloreactive T cells, and highlight a role for complement in graft injury beyond lytic vascular damage. Indeed, a complete appreciation of the multifaceted mechanisms of antibody-mediated graft injury should consider the relationships between the humoral, cell-mediated and innate immune systems, which do not act in isolation under physiological conditions.

Concluding Remarks

In summary, vasculitis and microvascular inflammation have emerged as promising histological markers, underscoring the central role of endothelial injury in AMR. Experimental evidence suggests that HLA antibody-induced endothelial cell inflammatory activation may promote the microcirculation inflammation and innate immune infiltration characteristic of AMR. Moreover, Fc-mediated functions such as complement activation and engagement of Fc receptors act synergistically with endothelial activation to enhance inflammation and alloimmune damage. Many questions remain, including the physiological relevance of prevalent anti-DQ antibodies, non-HLA antibodies, and antibodies that fix complement *in vitro*; and the effects of HLA II antibodies on vascular cells.

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Abbreviations

AMR	antibody-mediated rejection
AT1R	angiotensin II type 1 receptor
CDC-XM	complement-dependent cytotoxicity crossmatch
DSA	donor specific (HLA) antibodies
FcγR	Fc gamma receptor
HLA	human leukocyte antigen
MICA	major histocompatibility complex class I-related chain A

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Key Points

- HLA antibodies may be pre-existing or develop at any time after transplant; however, most *de novo* antibodies form 6 months or later post-transplant. HLA I antibodies appear earlier, while HLA II antibodies (particularly anti-HLA-DQ antibodies) develop in the late post-transplant period.
- Donor specific HLA antibodies stimulate endothelial cell signaling, including rapid exocytosis of P-selectin and production of chemokines, leading to inflammatory activation and leukocyte recruitment.
- Engagement of Fc receptors on monocytes by certain subclasses of HLA I antibodies enhances monocyte recruitment by activated endothelial cells.
- It is yet unclear whether antibodies that fix complement in *in vitro* deposition assays have definitive clinical relevance. However, activation of complement by HLA antibodies in experimental models triggers production of complement split products that boost T cell-mediated alloimmune responses.

Table 1

Production of inflammatory mediators by endothelial cell antibodies stimulated with HLA antibodies.

Cell Type	HLA Antibody Source	Protein Induced by HLA antibody	Function	Target Cell	Reference
HAEC HMEC-1 HUVEC	Murine monoclonal W6/32 and broadly reactive HLA I anti-serum	P-selectin	rapidly induced initiator of leukocyte tethering	all leukocytes	(65-67)
HMEC-1	Murine monoclonal W6/32	ICAM-1	endothelial adhesion molecule	all leukocytes	(68)
		VCAM-1	endothelial adhesion molecule	lymphocytes and monocytes	
		IL-6	acute phase inflammatory cytokine	T cells (activation and proliferation) monocytes (differentiation)	
		IL-8 (CXCL8)	chemoattractant	neutrophils and monocytes	
Glomerular MVEC	Broadly reactive HLA I and II anti-serum	ICAM-1	endothelial adhesion molecule	all leukocytes	(69)
		VCAM-1	endothelial adhesion molecule	lymphocytes and monocytes	
		GM-CSF	growth factor and chemoattractant	granulocyte and monocyte	
		TNFα	inflammatory cytokine	all leukocytes and EC	
		MCP-1 (CCL2)	chemoattractant	monocyte and basophil	
		RANTES (CCL5)	chemoattractant	T cell and monocyte	
		IL-11	acute phase inflammatory cytokine extracellular matrix regulator	endothelial cells	
		IL-1β	inflammatory cytokine	endothelial cells, monocytes	
		Fractalkine	chemoattractant	NK cells, T cells and monocytes	
HUVEC	Broadly reactive HLA I and II anti-serum (with complement)	E-selectin	late phase initiator of leukocyte tethering	all leukocytes	(56)
		VCAM-1	endothelial adhesion molecule		
		RANTES	chemoattractant	T cells and monocytes	
		IL-6	acute phase inflammatory cytokine	T cells (activation and proliferation) monocytes (differentiation)	
		MIP3α (CCL20)	chemoattractant	immature dendritic cells	

Cell Type	HLA Antibody Source	Protein Induced by HLA antibody	Function	Target Cell	Reference
				memory CD4 T cells	

HAEC: human aortic endothelial cells, primary

HMEC-1: human microvascular endothelial cell line

MVEC: microvascular endothelial cells, primary

HUVEC: human umbilical vein endothelial cells