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Antibodies in Transplantation: The Effects of HLA and Non-HLA Antibody Binding and Mechanisms of Injury

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Abstract

Until recently, allograft rejection was thought to be mediated primarily by alloreactive T cells. Consequently, immunosuppressive approaches focused on inhibition of T cell activation. While short-term graft survival has significantly improved and rejection rates have dropped, acute rejection has not been eliminated and chronic rejection remains the major threat to long-term graft survival. Increased attention to humoral immunity in experimental systems and in the clinic has revealed that donor specific antibodies (DSA) can mediate and promote acute and chronic rejection. Herein, we detail the effects of alloantibody, particularly HLA antibody, binding to graft vascular and other cells, and briefly summarize the experimental methods used to assess such outcomes.

Keywords

HLA antibodies; Non-HLA antibodies; Donor specific antibodies; Endothelial cell antibodies; Endothelial cell activation; Vascular injury

1 Clinical Frequency and Relevance of Donor Specific Antibodies

1.1 Frequency and Association with Outcome

Sensitization to polymorphic proteins, especially HLA class I and HLA class II molecules, occurs when a patient is exposed to cells from other individuals, via pregnancy, transfusion, or transplantation. Antibodies to donor antigens, called donor specific antibodies (DSA), can be directed against polymorphic HLA class I (HLA I), HLA class II (HLA II), or minor histocompatibility molecules such as MICA and MICB, or against non-HLA antigens expressed on endothelial cells, epithelial cells, or organ specific targets [1]. The frequency of donor specific HLA antibodies among transplant patients ranges greatly, from as low as 4 % to more than 50 % [2]. Based on data from the Organ Procurement and Transplantation Network as of March 2012, 18.7 % of renal transplant recipients had pretransplant antibodies with a calculated panel reactive antibody (cPRA) greater than 10 %, with 5 % of all patients being highly sensitized (>80 % C-PRA). Everly et al. found that 31 % of patients developed DSA de novo after transplant [3]. In one study of renal transplant patients, 30 % had HLA antibodies of any specificity, and 30 % of antibody-positive patients had HLA antibodies of donor specificity [4, 5]. Another study found that 35 % of renal patients had donor specific HLA IgG [6], while another report found DSA in as many as 57 % of renal recipients [7–12]. The rates of sensitization are higher in patients awaiting a second renal graft, where only 19.3 % of patients lacked antibody to HLA I (PRA <4 %), with 14.5 % of patients on the waitlist for a second transplant having very high PRA (>60 %). Here, 55 % were also positive for antibodies against HLA II [13].

Despite the variation in reported estimates of DSA prevalence, the literature clearly illustrates that DSA adversely affect the survival of a transplanted organ (Table 1). Numerous studies have linked the presence of preexisting or de novo antibodies to poor

graft outcome. For example, renal recipients with positive panel reactive antibodies (PRA recognizing >10 % of HLA alleles) or with DSA have lower graft survival at 3 and 5 years post-transplant [2, 14, 15]. Accordingly, patients diagnosed with antibody mediated rejection are likely to have donor specific HLA antibodies [16], and episodes of AMR predispose recipients to lower renal allograft survival [3], regardless of whether antibodies were preexisting or produced late after transplantation (from 83 % survival without HLA antibodies to 49 % with HLA antibodies) [4]. In a study with more than 1,000 patients, half of those with failed grafts had HLA antibodies, and 21 % had donor specific HLA antibodies [5]. If desensitization or other therapy effectively reduced DSA levels, long-term survival was significantly superior than if the DSA was persistent [17].

The effects of DSA on graft survival are not restricted to renal transplantation. Heart allograft patients with DSA also experience lower graft survival, especially if the antibodies appear after 1 year post-transplant [18]. Further, the presence of HLA specific DSA and the incidence of AMR correlate with chronic rejection in the heart [1, 19]. Even if patients are asymptomatic, HLA-DSA significantly lowers freedom from chronic allograft vasculopathy (CAV) compared to those without DSA [19]. In addition to HLA, DSA to MICA or to non-donor derived endothelial antigens correlate with chronic transplant rejection in the heart (CAV) [1]. Finally, DSA is a strong risk factor for rejection episodes in small bowel transplantation [20–22].

A recent study describes the clinical significance of and risk factors for development of *de novo* donor specific HLA antibody 6 months or later post-transplant. Wiebe et al. found that 15 % of low-risk renal transplant patients without presensitization developed DSA late after transplantation, which reduced graft survival at 10 years. Interestingly, the investigators found that a mismatch at HLA-DRB1 was an independent predictor of the production of *de novo* DSA, as was recipient nonadherence to immunosuppression [23]. These results and those of Smith et al. [24] point to a model of the “natural history of *de novo* DSA” describing the progressive nature of antibody-mediated rejection leading to graft failure. The authors propose that inflammatory cytokines expressed early after transplant increase HLA expression by the graft, which in turn promotes B cell allorecognition and production of donor specific HLA antibodies. Biopsies may reveal capillaritis with or without C4d staining, but graft function remains stable and any injury is subclinical. Over time in the presence of donor specific HLA antibodies, the graft progresses to clinical dysfunction and ultimately failure due to sustained microvascular injury and cellular infiltration.

While most studies uncovered a correlation of donor specific HLA antibodies with allograft outcome, only a few reports could not find an association. One study found that acute rejection in renal transplants could not be predicted by DSA [25], and in another CAV incidence did not correlate with DSA but rather with T cell alloreactivity [26]. Overall, however, it is well established that preexisting or *de novo* donor specific HLA antibodies have a deleterious effect on graft outcome across solid organ transplants.

1.2 Diagnosis of Antibody-Mediated Rejection

Antibody mediated rejection is a distinct entity from, but can occur concurrently with, T cell-mediated rejection. In kidney and heart transplantation, consensus criteria have been established for the histological characteristics and diagnosis of antibody-mediated rejection. Antibody-mediated rejection in renal transplantation is diagnosed by poor graft function, evidence of complement deposition (C4d) in the peritubules of the graft and/or DSA in the circulation [27]. Intravascular macrophages, endothelial cell swelling, C4d staining and donor specific HLA antibodies indicate antibody-mediated rejection in cardiac transplantation [28]. Similar criteria have been suggested for the diagnosis of antibody-mediated rejection in liver transplantation [29].

2 Experimental Techniques to Measure Effects of Antibodies

Given the strong association of HLA antibodies with inferior graft function and survival, it is crucial to understand the mechanisms of HLA antibody-mediated graft injury. A variety of experimental models are available to test the effects of HLA antibody binding to cells of the graft. The first is a simplified system with cultured graft cells (endothelium, smooth muscle, or airway epithelium), where intracellular signaling and cell–cell interactions can be dissected in detail and specific functional changes can be analyzed. The more complicated but more physiological system utilizes in vivo transplantation into immunodeficient recipients lacking B and T cells, which are passively transferred with DSA to recapitulate antibody-mediated rejection. Finally, the mechanisms uncovered by experimental models can be confirmed in human biopsies. A brief description of methods commonly utilized by our group and others groups follows.

2.1 In Vitro Techniques

Endothelial, smooth muscle, or epithelial cells are cultured and stimulated in vitro with HLA antibodies, and the direct effects can be analyzed in detail. Multiple clones and isotypes of murine or rat origin against human HLA molecules, which recognize monomorphic epitopes on all HLA I, are commercially available from several sources (our lab primarily uses the murine IgG2a clone W6/32). There are also murine anti-HLA antibodies with allele or locus specificity (for example against HLA-A2, A3, or B44) available from Abcam, BioLegend, and other commercial sources. For analysis using human antibodies, polyclonal HLA antibodies can be isolated from the IgG fraction of sensitized patient sera. More recently, human monoclonal antibodies of a single specificity have been developed [30], although these antibodies are only of complement fixing isotypes and are not yet commercially available.

2.1.1 Measurement of HLA Antibody Binding Capacity—Fluorescence intensity in flow cytometry is influenced by a variety of factors, including antibody concentration or titer, the antibody's affinity for its ligand, whether the epitope is monomorphic or polymorphic, and the expression level on the target cell. Further, fluorescence intensity may vary from machine to machine and with changes in machine settings. The actual amount of HLA I antibody on a donor cell can be measured using flow cytometric methods and normalized using fluorescence calibration Molecules of Soluble Fluorochrome (MESF) beads (Simply Cellular). In this way, concentration can be related to target expression and uniformly measured from machine to machine and across acquisition settings. This technique is also useful when comparing two monoclonal antibodies with differing affinity or two targets with differing expression on the cell surface.

2.1.2 Analysis of Intracellular Signaling—HLA I antibody binding to target cells induces a myriad of cell signaling and function events. Intracellular signaling cascades entail sequential phosphorylation of proteins and kinases. These phosphorylation events are monitored in the cells using Western Blotting with phosphorylation site-specific antibodies.

Briefly, endothelial cells are stimulated with HLA I antibody for defined time period (usually between 1 and 30 min depending on the signaling molecule of interest, as phosphorylation events are rapid). Cell lysates are separated by SDS-PAGE, then transferred onto a PVDF membrane, and probed with phosphorylation specific antibodies for Western Blot (Cell Signaling is an excellent source).

2.1.3 Determination of Cell Growth—Cellular proliferation can be measured by several techniques, which involve either radioactive substrates incorporated during division or using

flow cytometry. Our lab utilizes a flow cytometric assay exploiting the dilution of fluorescent vital dyes (such as carboxyfluorescein succinimidyl ester, CFSE) during cell division to measure proliferation. A shift of the histogram to the left (i.e., a decrease in fluorescence intensity) indicates increased cell division due to dilution of the dye, which is analyzed using FlowJo or ModFit (Verity) to calculate the proliferation index. As an alternative approach, cells can be labeled with the pyrimidine nucleotide analog 5-bromo-2'-deoxyuridine (BrdU), which is incorporated into DNA in lieu of thymidine upon cell replication, and which can be detected using antibodies by flow cytometry [31].

2.1.4 Measurement of Leukocyte Adherence—Leukocyte adherence is measured by an adhesion assay in which endothelial monolayers are stimulated and lymphocytes or myeloid cells which are fluorescently labeled with a vital dye such as CFSE are overlaid. Nonadherent cells are washed off and adherent cells are imaged in many fields per sample using fluorescence microscopy. Cells are counted using automated quantification software, such as Image J or CellProfiler (MIT). CellProfiler has excellent counting algorithms that can effectively distinguish single cells even in dense clusters.

2.1.5 Determination of Cytoskeletal Changes and Cell Migration—Migration of cells is assessed by the scratch, or wound healing, assay. Cells are cultured, a wound is introduced by scratching the plate with a pipet tip, and closure of the wound in the absence or presence of stimulants is measured after 24 h using microscopy. Cytoskeletal changes, including stress fiber formation, are monitored by immunofluorescent staining of the actin cytoskeleton using phalloidin, which selectively labels F-actin, and visualized by fluorescent microscopy.

2.1.6 siRNA and Pharmacological Inhibitors—In order to definitively identify a role for proteins in HLA I-mediated intracellular signaling leading to functional changes, it is important to utilize strategies to inhibit the protein's function to verify the upstream and downstream relationship to other proteins in the pathway. Two commonly used methods involve knockdown of expression of the protein of interest by small interfering RNA (siRNA), or pharmacological antagonism of the protein with commercial inhibitors. A variety of inhibitors are available commercially (especially from Tocris, Sigma, or Calbiochem) for the enzymes noted to be crucial to HLA I signaling.

2.2 In Vivo Models of Antibody-Mediated Rejection

Animals deficient in T cells are incapable of mounting a complete humoral response, due to the requirement of antibody-secreting B cells for T cell help during maturation and isotype switching. Therefore, an alternative system was required to assess antibody mediated rejection in the absence of cell-mediated alloimmunity. Murine models are particularly useful because they allow genetic manipulation of putative targets, using knockout or transgenic animals. Russell et al. first described the model in which alloserum alone is sufficient to elicit allograft rejection [32]. In general, a murine or rat immunodeficient recipient, such as severe combined immunodeficiency (SCID) or recombina-activating gene-1 (RAG1) knockout mice, is used, which lacks T cells and B cells, but retains an intact innate immune system, including complement components, monocytes, natural killer (NK) cells, and neutrophils. Recipients transplanted with an MHC mismatched organ are passively transferred with alloantiserum from sensitized animals or with monoclonal DSA. This system has advanced knowledge about antibody-mediated rejection in the physiological setting. Such models have uncovered direct evidence that donor specific MHC antibodies are sufficient to trigger acute and chronic rejection, and yielded some insights into the mechanisms of antibody induced graft injury. Alternatively, human tissue can be

transplanted onto an immunodeficient murine recipient, and anti-HLA antibodies or human immune cells are administered [33–35].

There are several important considerations to bear in mind when selecting an animal model of antibody-mediated rejection (reviewed in [36, 37]). The first is the availability of reagents, particularly of donor specific monoclonal antibodies. Secondly, chronic rejection in the mouse varies in location and severity of disease from model to model and from mouse to human. It is likely that monoclonal MHC antibodies are not potent enough to produce fulminant, widespread vascular lesions, as observed with polyclonal MHC alloserum [38]. In designing in vivo experimental systems, one must keep in mind the crucial caveat that the murine and human immune systems differ considerably. For example, the murine system lacks the activating FcγRIIA (CD32A) and FcγRIIC (CD32C) molecules. Inhibitory NK cell receptors are highly divergent between mouse and human—for example, mice do not express KIRs—as are the ligands for the NKG2D molecule (MHC class I chain-related MICA in humans). Further, immunoglobulin isotypes differ between mouse and human, and cytokine regulation of P-selectin expression is distinctive to the mouse. Many chemokines identified in humans are not found in the murine system (extensively reviewed in [39]). Finally, murine anatomical differences result in rejection models that are not always physiologically analogous to the clinical setting—i.e., the location of chronic vascular lesions [37].

2.3 Patient Samples

The final step in translational studies of the effects of HLA antibodies on the graft is to assess changes in transplanted organs during antibody mediated rejection in humans. In vitro findings are confirmed in patient biopsies using immunohistochemical techniques to detect histological changes, complement deposition, cellular infiltration, total protein expression, or phosphorylation status of specific proteins in the graft. RNA from patient biopsies can also be evaluated by microarray and/or sequencing to determine differential expression of proteins of interest and other changes in the transcriptome.

3 Mechanisms of Injury: Fc-Dependent Effects of Antibodies

The effects of HLA antibody binding to graft vascular cells are manifold, and depend on both the canonical antibody functions mediated by the Fc portion of the molecule, and on binding via the Fab fragment, which cross-links the target molecule on donor cells. Fc-dependent functions occur irrespective of the target protein or receptor, but vary depending on the isotype and subclass of the DSA.

3.1 Hyperacute and Acute Rejection

Alloantibody binding to endothelial and smooth muscle cells promotes Fc-dependent functions such as complement cascade activation and antibody-dependent cell-mediated cytotoxicity (ADCC). These effects are particularly relevant in hyperacute and acute humoral rejection.

3.1.1 Complement Activation—The complement cascade is a complex system consisting of proteases that become sequentially activated upon cleavage. There are three pathways of complement, which mediate immunity against different immunologic threats and are activated by different signals. The classical pathway bridges the adaptive and innate immune responses by partnering with substrate bound antibodies. The lectin pathway mediates immunity against bacterial and fungal pathogens by recognizing mannan carbohydrate motifs. The alternative complement pathway is effective against cellular pathogens and facilitates opsonization by phagocytes. For the purposes of this review, we

will restrict our discussion to the classical antibody-mediated pathway. In humans, IgM, IgG1, and IgG3 are effective activators of complement. IgG or IgM on the cell surface binds to the low affinity, multivalent C1q molecule, which once “fixed” cleaves C2 and C4. The resultant cleavage products C2a and C4b complex into a protease capable of catalyzing activation of C3, which is a central mediator in all three complement pathways. This event triggers the production of C3b, which in the classical pathway activates C5, yielding the C5a and C5b fragments. C5a is an important inflammatory factor, which acts as a chemoattractant for neutrophils, monocytes and T cells. Local synthesis of C5b at the C1q-antibody site causes complex formation of C5b with C6, C7, C8, and C9 into the macromolecule known as the membrane attack complex (MAC). MAC forms transmembrane pores which cause lysis of the target cell by disrupting the plasma membrane. Cells may resist complement-induced lysis by expressing CD59, which inhibits C5b binding to C9. In addition to forming the MAC, complement split products have a myriad of other functions, including chemoattraction, viral neutralization, opsonization, immune complex clearance, and activation of macrophages and other cells (reviewed in [40]).

Complement fixing ability is particularly relevant to hyperacute and acute rejection. Hyperacute rejection is a predominantly complement-mediated severe injury to the allograft occurring within hours of transplantation. It is caused by high titer of preexisting HLA, or in rare cases non-HLA, antibodies in presensitized patients. The incidence of hyperacute rejection has dropped significantly due to improved detection of DSA and desensitization protocols.

Complement is also an important mediator of acute antibody-mediated rejection. Animal models have demonstrated that donor specific MHC antibodies are sufficient to mediate acute rejection of cardiac and corneal allografts [41, 42]. Histologically, interaction of DSA with the graft is detected by deposition of the complement split product C3d or C4d, which has been shown to associate with diagnosis of antibody-mediated rejection [43]. Allograft bound complement split products are only found in murine models of rejection when DSA is also present [44], and donor specific HLA antibodies correlate with C4d deposition [2, 45], which associated with inferior graft survival. However, C4d may not be a sensitive marker, as several reports suggest that C4d deposition may not have sufficiently reliable predictive value for diagnosis of antibody mediated rejection or graft outcome [46–51].

Antibody subclass and isotype define the effector functions of the molecule, including complement fixation. The literature on the pathogenicity of an antibody with respect to its isotype is conflicting. In murine models, immunoglobulin knockout (IgKO) allograft recipients have significantly longer graft survival than their wild-type counterparts. Acute rejection could be restored by passive transfer of donor MHC antibodies of the complement fixing isotype murine IgG2b but not the noncomplement fixing isotype murine IgG1 [52]. HLA I antibodies in patients reportedly are predominantly IgG1 [53], but can be of any of the four IgG iso-types [54]. Recently, it has been reported that skewing of DSA to complement fixing IgG3 predisposes the recipient to rejection [55]. An assay has been developed that can identify both the specificity and the complement fixing ability of HLA antibodies by binding of C1q on single antigen beads. The specificity of a patient’s HLA antibodies can be determined using single antigen luminex beads that consist of fluorescent microbeads conjugated to single recombinant HLA class I and class II molecules. Complement fixing ability is assessed by the binding of C1q to HLA antibodies present in the serum, which are bound to the HLA antigen coated microbeads. In several studies, C1q positive DSA associate with antibody mediated rejection in cardiac and renal transplantation when compared with antibodies identified only by IgG [56]. C1q positive antibodies had a positive predictive value for early episodes of antibody-mediated rejection [57]. It is

postulated that noncomplement fixing antibodies will have a lower pathogenicity; indeed, three patients who were transplanted across high titer anti-donor HLA II antibodies had long-term AMR-free survival, possibly because the preexisting antibodies were of non-complement fixing isotypes IgG2 and IgG4 [58]. In contrast, another group reported that, while IgG-DSA positive patients had a significantly lower graft survival, there was no significant difference when groups with C1q-fixing and non-C1q fixing DSA were compared [6]. Similarly, antibodies detected using solid phase flow cytometry detection of DSAA flow PRA could predict early rejection episodes, while CDC-identified antibodies could not [59]. Particularly when DSA was at lower titers, complement fixing ability could not predict early antibody-mediated rejection [54, 60]. Therefore, the clinical data regarding the relationship between antibody isotype and its pathogenicity is conflicting.

Moreover, the clinical relevance of an HLA antibody's capacity to fix complement in chronic rejection is less well defined. Murine models of AMR have demonstrated that chronic rejection lesions can develop in response to MHC antibodies that are not complement fixing, or in complement deficient allograft recipients [61]. Taken together, the literature suggests that complement fixation, while important, may not be the only factor influencing the pathogenesis of a donor specific HLA antibody.

3.1.2 Antibody-Dependent Cell-Mediated Cytotoxicity—Antibodies bridge the innate and adaptive arms of the immune system by interacting with natural killer (NK) cells through their FcγRIII (CD16), which binds the constant Fc region of the antibody. NK cells recognize antibody-coated cells and cause cell lysis. Although the general mechanism of antibody-dependent cell-mediated cytotoxicity is understood, there is limited evidence about ADCC in alloimmune responses. Antibodies from sensitized patients increased the lytic capacity of CD3-CD16+ (NK) peripheral blood mononuclear cells against renal epithelium [62]. Long ago it was recognized that a positive ex vivo ADCC reaction using patient sera containing alloantibody bound to target endothelial cells was a risk factor for transplant rejection [63, 64]. NK cell cytotoxicity against human umbilical vein endothelial cells (HUVEC) could also be facilitated by IgG1 non-HLA anti-endothelial cell antibodies [65]. Although this topic has received less priority over the last decade, recent reports implicating NK cells in MHC antibody-mediated chronic rejection in the mouse [66, 67] and in human antibody-mediated rejection [68] will likely excite more interest in this area.

4 Mechanisms of Injury: Target Cell Signaling Induced by HLA I Antibodies

HLA I ligation directly induces intracellular signaling cascades which have important implications for cell functional changes, especially cellular proliferation which is central to the pathogenesis of chronic rejection.

4.1 Survival and Accommodation

The presence of circulating donor specific HLA antibodies may not always indicate ongoing rejection. Some patients, as many as 24 %, maintain good graft function despite detectable titers of HLA-DSA, in a poorly defined state thought to represent transplant accommodation [5]. Little is understood about accommodation, but it is expected that antibody titer may be an important factor. For example, a high titer of antibody in renal patients (cutoff of 4,487 MFI for class II) correlated with and accurately predicted AMR incidence [7], suggesting that symptomatic rejection has a threshold for the amount of antibody to which the graft is exposed. However, the persistence of DSA on its own is a strong predictor of late graft loss independently of AMR episodes; thus, accommodation does not indicate indefinite graft survival, and likely there are ongoing subclinical events in response to alloantibody that damage the graft and result in chronic rejection. For example, cardiac transplant recipients with asymptomatic AMR had a lower 5 year freedom from chronic rejection than those

without any AMR [19], suggesting that accommodation is better defined as clinically silent antibody-mediated graft damage that slowly causes failure.

Several in vitro studies indicate a possible mechanism for HLA I antibody-mediated transplant accommodation, where the graft is resistant to acute antibody-induced damage, such as complement activation and apoptosis. One group reported that while HLA I antibodies from patient sera or monoclonal antibodies trigger endothelial cell death at high concentrations, at low concentrations HLA I antibodies confer resistance to complement induced cell death by inducing heme oxygenase (HO-1) and activating cyclic AMP-dependent protein kinase A (PKA) [69, 70]. HLA I antibodies also increase endothelial Nrf-2 mediated antioxidant responses, which provide protection of endothelial cells from complement induced death via HO-1 and ferritin H [71]. Additionally, treatment with HLA I antibody at low doses increases PI3K/Akt signaling, which results in inactivation of the proapoptotic factor Bad and increased expression of apoptosis inhibitors Bcl-2 and Bcl-xL in vitro [72]. These observations were confirmed in a murine transplant model, where Bcl-2 and HO-1 were upregulated in an HLA-A*02 transgenic allograft by pretreatment with low titer of HLA I antibodies, which prevented subsequent rejection by high titers of HLA I antibodies [73]. Further, biopsies from patients with AMR have elevated Bcl-2 expression [72], demonstrating that donor specific HLA antibodies activate these pathways in the clinical setting.

4.2 Cell Proliferation

Chronic rejection, or transplant vasculopathy, is a predominantly proliferative disease, in which the vessels of the graft become occluded by a severely thickened intima invaded by smooth muscle cells. Donor specific HLA antibodies are strongly associated with vasculopathy in heart and kidney transplantation [1]. There is strong evidence that ligation of HLA I by antibodies triggers proliferation and cytoskeletal changes in vascular cells.

4.2.1 In Vitro Evidence—We and others found that, rapidly following HLA I ligation by antibodies, Rho-GTP, Src and focal adhesion kinase (FAK) become activated [74, 75]. Src activation triggers the PI3K/Akt signaling axis, which is a key regulator of survival and cell cycle progression. Akt promotes the activity of mammalian target of rapamycin (mTOR), a serine/threonine kinase which exists in two complexes. mTOR complex 1, composed of raptor, mTOR, and GβL, targets S6 kinase (S6K), S6 ribosomal protein and 4EBP1, molecules involved in protein synthesis which become phosphorylated after HLA I ligation [76]. mTORC2, which contains rictor, mTOR, GβL, is a central regulator of the cytoskeleton as well as of extracellular regulated kinases (ERK1/2) [77, 78]. HLA I cross-linking activates the mitogen-activated protein kinases ERK1/2, which promote cell proliferation through a parallel pathway. Consequently, stimulation of endothelial cells and smooth muscle cells with HLA I antibody directly increases cellular proliferation in the absence of exogenous growth factors [31, 79, 80]. Interestingly, HLA I antibody stimulation of smooth muscle cell growth was dependent on the activation of matrix metalloproteases/sphingolipid signaling, a stress-induced pathway [81], illustrating the pleiotropic effects of HLA I ligation leading to cellular proliferation.

Because HLA I molecule has no known signaling motif, we postulated that HLA I associated with a coreceptor to carry out its proximal signaling requirements. Using immunoprecipitation, we discovered a molecular association between HLA I and integrin β4 which was required to activate downstream signaling and cell proliferation [31]. These results demonstrated for the first time the mechanism by which HLA I molecules transduce signals into the cell.

In addition to regulating signaling molecules central to cell cycle progression and proliferation, HLA I ligation causes dramatic reorganization of the actin cytoskeleton. Cytoskeletal rearrangements are relevant to cell migration and proliferation, as well as endothelial cell permeability. FAK mediates cytoskeletal changes by acting on paxillin. We observe increased stress fiber formation and association of several unique proteins with the cytoskeleton after treatment of endothelial cells and smooth muscle cells with HLA I antibody [79, 82, 83]. Further, HLA I antibody increases cell migration and wound healing [79].

These observations of the direct effects of HLA I antibody are not restricted to vascular cells. Stimulation of lung epithelial carcinoma with HLA sera from patients increases proliferation and tyrosine phosphorylation [84], pointing to a mechanism by which HLA antibodies may provoke bronchiolitis obliterans syndrome (BOS).

4.2.2 In Vivo Evidence—The experimental evidence linking MHC I antibodies and cellular proliferation in vivo is emerging. Initially the proliferative changes observed in chronic rejection were thought to be T cell mediated [38]. However, when murine cardiac allograft recipients were depleted of T cells after generation of MHC alloantibodies, the grafts still developed arteriosclerosis to a greater extent than those without DSA [32]. Further, in murine recipients deficient in B cells, but with an intact T cell immune response, the allograft did not progress to vasculopathy [32]. These data suggest that the humoral response is required for full chronic rejection. In a seminal study, transfer of allosera to SCID allograft recipients reproduced these obstructive lesions [32], results which have been confirmed more recently using donor specific MHC I monoclonal antibodies [61, 67]. Further, vasculopathy is elicited by human antibodies in human grafts. SCID mice were transplanted with human mesenteric arteries and passively transferred with HLA I antibody. The investigators observed increased neointimal thickening in response to HLA I antibody in this study [81]. These data demonstrate that humoral immunity is sufficient to produce vasculopathy.

Importantly, MHC I antibodies elicited vascular lesions in RAG1 knockout recipients of cardiac allografts even when the antibodies were of noncomplement fixing isotypes or allograft recipients were deficient in complement [61, 66]. These results strongly indicate that MHC I antibodies mediate chronic rejection via a complement-independent mechanism and are consistent with the in vitro work demonstrating that HLA I cross-linking directly triggers proliferation and cell signaling.

We confirmed HLA I-induced cell signaling in vivo. Allografts from RAG1 knockout mice reconstituted with anti-donor MHC I antibody had significantly increased phosphorylated Akt, mTOR and S6K compared with the control group [85]. Further, our group investigated endothelial cell signaling downstream of HLA I cross-linking in patient cardiac allograft biopsies. We found that phosphorylated S6 ribosomal protein (S6RP) was increased in AMR patients and was a more sensitive marker of AMR than C4d [86]. Therefore, activation of signaling molecules which promote proliferation occurs in both animal models of antibody-mediated rejection and in the clinical setting.

4.3 Induction of Secondary Factors

In addition to direct activation of intracellular signaling cascades, in vitro experiments have revealed that HLA I antibodies increase sensitivity to and production of soluble mediators which promote autocrine proliferative signaling. HLA I cross-linking rapidly increases cell surface expression of fibroblast growth factor receptor (FGFR) on endothelial cells. The proliferative response to bFGF is thus significantly enhanced by sensitization with HLA I antibodies [87–89]. Stimulation of HLA I also triggers endothelial cell production of

cytokines which may have a secondary effect on cell growth. For example, HLA I antibodies increase endothelial production of vascular endothelial growth factor (VEGF), which activates cells in an autocrine manner via its receptor VEGFR2 [90]. Treatment of lung airway epithelial cells with HLA I antibodies increased platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and fibroblast growth factor (bFGF) production, which promoted paracrine proliferation of fibroblasts [91]. Therefore, HLA I activation of endothelial cells increases sensitivity to bFGF and production of other soluble mediators which trigger autocrine and paracrine cell proliferation.

4.4 Leukocyte Recruitment

4.4.1 Infiltration in Antibody-Mediated Rejection—In addition to antibody-induced complement deposition, antibody mediated rejection is frequently characterized by intravascular macrophages [92], which can promote both acute and chronic rejection [93, 94]. Further, NK cells were required for neointimal thickening in a murine AMR model [66]. Thus, recruitment of immune cells into the allograft facilitates innate cell-mediated injury.

4.4.2 HLA I Antibody-Induced Leukocyte Recruitment—Endothelial cells contain intracellular rod-shaped vesicles called Weibel–Palade bodies, which contain preformed von Willebrand Factor (vWF), P-selectin, and other vascular mediators. Release of these vesicles, which fuse with the cell membrane and secrete contents into the extracellular space, is regulated by calcium or cAMP-dependent secretagogues, such as thrombin, histamine, or forskolin (reviewed in [95]). Recently, it has been reported that HLA I ligation by monoclonal murine antibody triggers exocytosis of these vesicles, externalizing vWF and increasing cell surface P-selectin. HLA I antibody-induced P-selectin mediated increased adherence of the promyelocytic cell line HL60 in vitro. Further, treatment of SCID mice engrafted with human skin with the monoclonal HLA I antibody in vivo also triggered vWF externalization and neutrophil influx [96]. In a similar study, wild-type murine cardiac recipients had evidence of vWF release and P-selectin expression by allograft endothelium during acute rejection, which was absent in IgKO recipients [52]. In a murine lung transplant model, treatment with MHC antibodies increased neutrophil infiltration and inflammation [97]. Thus, MHC or HLA I antibody binding to endothelial cells causes rapid endothelial cell activation, promoting recruitment of leukocytes.

The clinical relevance of this pathway to AMR has not been definitively elucidated; however, in human cardiac biopsies, capillary endothelium displayed increased expression of vWF during rejection [98, 99]. In a variety of murine allograft models, selectin deficiency in the donor graft prolongs graft survival and reduces cellular infiltration [100–102], suggesting that HLA I antibody-induced P-selectin and/or vWF may be a rational therapeutic target to decrease immune cell recruitment.

4.5 Therapies Suggested from Experimental Evidence

The knowledge gained from the experimental systems described above reveals potential therapeutic targets to alleviate the proliferative and inflammatory effects of HLA I antibodies. For example, we and others found that HLA I cross-linking on endothelial cells activated RhoA, which was necessary for proliferation in vitro [74, 83]. Inactivation of Rho and Rho kinase (ROCK) by pharmacological inhibitors in animal models reduces neointimal thickening: in a rabbit vein graft model, intimal thickening was inhibited by fasudil (ROCK inhibitor) [103]; in a murine model of chronic cardiac rejection, intimal thickening was suppressed by fasudil [104], while another Rho kinase inhibitor, Y-27632, prolonged survival, reduced immune cell infiltration, and prevented intimal thickening [105].

We also reported that mTOR is a central signaling molecule in HLA I-induced cellular proliferation [77]. Pretreatment of endothelial cells with rapamycin, an mTOR inhibitor, reduced HLA I Ab-triggered endothelial proliferation, Akt phosphorylation, and Bcl-2 expression [77]. Recent reports suggest that rapamycin and its analogs (everolimus, sirolimus) may have clinical therapeutic potential. For example, everolimus therapy prevented remodeling after heart transplantation in human patients [106]. The exact mechanism of protection from rejection conferred by rapamycin is not understood, but mTOR inhibition in endothelial and smooth muscle cells may prevent HLA I antibody-induced proliferation.

Other potential therapies for antibody mediated rejection include adhesion molecule antagonists to reduce leukocyte infiltration and complement inhibitors to prevent complement-mediated tissue injury. In a presensitized primate renal allograft model, complement inhibition reduced AMR and prolonged allograft survival [107]. Use of eculizimab, a monoclonal antibody which blocks activation of C5, reduced antibody-mediated rejection incidence in patients with DSA [108]. Therefore, the progress which has been made in understanding the mechanisms of HLA I antibody-mediated graft injury has suggested rational clinical therapies in the treatment and prevention of AMR.

5 HLA II Antibodies

Although the mechanism by which HLA I antibodies promote inflammation and proliferation has been revealed by experimental models, the pathogenesis of HLA II antibodies is less defined.

5.1 Association with Outcome

Antibodies to HLA II frequently accompany chronic rejection in renal transplants [43] and correlate with AMR incidence in the absence of a positive T cell crossmatch [7, 109]. The presence of antibody to HLA II significantly correlates with worse graft outcome, in addition to antibody to HLA I [90].

5.2 Limitations to Studying HLA II in Model Systems

Investigation of the effects of HLA II antibodies on graft cells has been limited by its restricted expression pattern and by constraints in experimental systems. HLA II is not expressed on endothelial cells of most vascular beds, with the possible exception of renal microvasculature [110, 111], but it can be upregulated after inflammatory insult [112–115]. Indeed, several reports have demonstrated HLA II expression on endothelia in grafts [99, 116], which suggests that allogeneic attack or transplantation itself may upregulate MHC II expression as reported in murine cardiac allografts [117–119].

In vivo studies on MHC II are constrained by the different expression pattern of MHC II in mice, where normal cardiac tissue does not express MHC II molecules. In the mouse, MHC II may be upregulated after transplantation [118, 119], similar to reports in human cardiac allografts, where HLA-DR was increased during rejection [99]. Human microvascular endothelia have been found to express HLA II, but cultured endothelial cells rapidly lose constitutive expression. HLA II can be reinduced by treatment of endothelium with cytokines such as $\text{TNF}\alpha$ or $\text{IFN}\gamma$. However, cytokine treatment introduces a layer of complexity with respect to cell activation that masks many intracellular signals, upregulates a variety of other factors, and thus obscures analysis and conclusions. There is, therefore, a need for an in vitro system in which the effects of HLA II antibodies on vascular cells can be investigated without additional confounding factors.

5.3 Mechanisms

Due to the limitations discussed above, the consequences of cross-linking of HLA II on vascular cells are not well defined. Some insights may be inferred from data regarding HLA II engagement on other cell types. In B lymphocytes and antigen presenting cells, HLA II ligation triggers an increase in intracellular calcium and tyrosine phosphorylation, with varying functional effects. Cells become activated and proliferate or undergo apoptosis depending on which intracellular pathway is predominantly activated [120–122]. Fibroblasts stimulated through HLA II increase production of prostaglandin E [123], RANTES, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and GRO α [124, 125], through Janus kinase (JNK) and FAK signaling [126]. These HLA II-induced soluble factors produced by fibroblasts could promote proliferation of endothelial cells [124]. However, little to no data is available to establish how HLA II cross-linking on endothelial or smooth muscle cells might trigger functional changes leading to rejection.

6 Non-HLA Antibodies

A new appreciation of non-HLA antibodies has arisen due to reports of antibody-mediated rejection or C4d deposition in the absence of circulating donor specific HLA antibodies. Non-HLA antibodies in transplantation can be directed against either polymorphic or nonallelic proteins. The development of antibodies against nonpolymorphic targets may be due to upregulation during inflammation, in response to transplantation, or when proteins are exposed to the immune system during injury. It is thought that the intraorgan microenvironment or rejection may break humoral tolerance to autoantigens [127]. Moreover, the indication for renal transplantation is often autoimmunity, where patients may be predisposed to humoral responses against self antigens.

6.1 Frequency and Outcome

Non-HLA antibodies are commonly found in transplant recipients. Le Bas-Bernadet et al. reported that nearly half of renal patients who had DSA to HLA also had non-HLA anti-endothelial cell antibodies (AECA) [128]. Another study found anti-endothelial cell antibodies in 23 % of renal patients, which associated with a greater rate of cellular rejection and lower graft function [129]. Similarly, a significantly higher rate of AECA positivity was found in patients with failed renal transplants compared to those with functioning grafts [130]. Non-HLA antibodies binding to airway epithelial cells were detected in lung transplant patients with chronic rejection, bronchiolitis obliterans syndrome (BOS), but not in patients without BOS [131]. One group identified endothelial cell-binding IgM and IgG using flow cytometry in about half of cardiac transplant patients with transplant coronary artery disease (TCAD) and in patients with failed renal allografts. Interestingly, they did not find such antibodies in pretransplant patients or controls [132]. Another group reported that non-HLA antibodies were found at a higher rate in cardiac and renal transplant recipients with or without rejection than in normal or wait-listed subjects [133], suggesting that these antigens become immunogenic during transplantation. Also remarkable is the observation that the array of non-HLA antigens recognized by the alloimmune response varies from patient to patient, with only a few markers overlapping [134].

Several of the ligands for non-HLA antibodies have been identified. Nonclassical histocompatibility antigens are frequent targets of the alloimmune humoral response. The major histocompatibility class I related chain (MIC) genes are non-HLA proteins with some homology to HLA class I molecules. MICA and MICB do not associate with β 2 microglobulin and function as ligands for the NK cell receptor NKG2D rather than the T cell receptor. Although not nearly as polymorphic as HLA molecules, MICA and MICB have more than 30 alleles each. Antibodies to MICA and MICB are found in renal [135, 136], as

well as cardiac transplant recipients, where they associate with chronic rejection [1, 16, 137].

Other targets of antibodies in transplantation include vascular receptors, adhesion molecules, and intermediate filaments (reviewed in [1, 138]). Using immunoprecipitation and mass spectrometry, Qin et al. revealed the nucleolar protein nucleolin as a target of non-HLA antibodies in transplant patients [133]. Further, neuropilin, a cell surface coreceptor for VEGFR; heterogenous nuclear ribonucleoprotein K; an intermediate filament protein; ribosomal protein L7; and CD36, a scavenger receptor for oxidized LDL and other lipoproteins, have all been identified as ligands for non-HLA endothelial cell antibodies in patients with chronic cardiac allograft rejection [134, 139–141]. Antibodies to nondonor derived antigens, particularly vimentin and cardiac myosin [137, 142], are present in a majority (65 %) of cardiac transplant patients with chronic rejection and correlate with CAV incidence [1, 143]. In renal transplantation, antibodies recognizing glutathione-S-transferase (GST), a cytosolic enzyme which metabolizes toxins, were found in patients experiencing antibody-mediated rejection [144, 145].

6.2 Experimental Models

Not much is known about the mechanism of graft injury by non-HLA antibodies. The clinical relevance and pathogenic mechanism of antibodies to intracellular proteins, such as glutathione-S-transferase (GST), vimentin, and ribonucleoprotein, is unclear. Such antibodies may represent a marker for injury or bystander humoral activation rather than having independent pathogenic potential. Non-HLA antibodies against cell surface markers may fix complement or mediate ADCC. Considering the agonistic function of an antibody when it cross-links its target, the mechanism of action may depend on the signaling capacity and biological function of the ligand as with HLA I molecules. For example, angiotensin II receptor AT1 autoantibodies are well-established agonists, which promote hypertension and may contribute to renal allograft rejection [146–148]. Antibodies from autoimmune sera upregulate adhesion molecules and cytokines on endothelial cells [149–151], but when the target is unknown the precise mechanism remains elusive. To date, little has been studied on the actions of non-HLA antibodies during the humoral alloimmune response.

In some experimental studies, non-HLA antibodies do not cause injury. For example, rat allosera lacking MHC antibodies caused only minor complement-mediated cytotoxicity against rat endothelial cells when compared with MHC specific allosera [152, 153]. Further, C3 deposition was only observed in the cardiac allograft vasculature of an MHC-mismatched rat model but not in a rejection model with non-MHC alloserum production [153]. A possible explanation may be that the isotype of non-HLA antibodies is different from that of HLA antibodies. One clinical study found non-HLA antibodies predominantly of the noncomplement fixing isotypes IgG2 and IgG4 [129]. Further, as mentioned above, the signaling capacity of the target molecule may shape the effect of antibody binding. For example, anti-MICA antibodies did not increase neointimal thickening of human mesenteric arteries grafted in SCID mice, while HLA I antibody was sufficient to elicit lesions [80].

In contrast, other studies have suggested that non-HLA antibodies have pathogenic potential. Anti-airway epithelial cell antibodies isolated from patients induced intracellular calcium increase, proliferation, and tyrosine phosphorylation in airway epithelium, although the cell surface ligands remain unknown [131]. Anti-donor antibodies produced by rat cardiac recipients could induce apoptosis of rat vascular endothelial cells [19]. Antibodies to nucleolin or unidentified endothelial targets also caused apoptosis of human endothelial cells [128, 133]. Although nucleolin is primarily localized to the nucleus, this protein was detected on the cell surface in proliferating tumor and endothelial cells [133], suggesting that intracellular targets of the humoral response may be exposed under certain conditions.

Moreover, mice sensitized against vimentin experienced rejection, C3d deposition and P-selectin expression in cardiac allografts, effects which were specifically mediated by antibodies to vimentin [154].

Therefore, antibodies to donor antigens correlate with rejection and may promote graft injury. More work is needed to understand whether non-HLA antibodies are relevant only as a marker of humoral activation and graft injury, or whether they also function as independent actors of alloreactivity.

7 Conclusions

Alloantibody binding to donor cells injures the graft by a myriad of mechanisms (Fig. 1). Complement cascade activation by alloanti-body results in inflammation and destruction of the graft vasculature. Agonistic stimulation of HLA I, HLA II, endothelial or epithelial cell surface markers may induce intracellular signaling leading to recruitment of immune cells, apoptosis, survival or proliferation. Thus, alloantibody can elicit complement deposition and neutrophil infiltration, features of acute rejection, as well as cellular proliferation and vascular lesion formation, characteristic of chronic rejection.

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Fig. 1.

The pleiotropic effects of HLA I antibody binding to graft vascular cells. Donor specific HLA I antibodies bind to HLA I molecules expressed on the surface of endothelial cells lining the vasculature of the transplanted organ. In addition to triggering complement activation through their Fc γ fragment, antibodies recognizing HLA I molecules cross-link these receptors at the cell surface, inducing intracellular signaling. HLA I-mediated cell signaling results in increased FGFR at the endothelial cell surface, migration, proliferation, and resistance to apoptosis and complement-induced death. Cellular proliferation requires a molecular association between HLA I molecules and integrin $\beta 4$. HLA I cross-linking also increases cytosolic calcium (Ca^{2+}), which triggers mobilization of Weibel–Palade bodies and increased cell surface P-selectin, leading to adherence of selectin-ligand bearing immune cells

Table 1

Reports of correlation between HLA antibodies and graft outcome

Organ	Time after Tx	Frequency	Correlation with outcome	Number of patients	Assay to Detect DSA	Reference
Heart	Post	75 % of AMR + Px had HLA-DSA (14 % of total patients); 11 % of total had MICA-DSA	HLA or MICA-DSA associated with CAV	43	Luminex	Nath et al. [16]
Heart	Post	35 % developed de novo anti-HLA	Correlated with HLA-A mismatch; CAV correlated with HLA II Ab	71	Flow-PRA	Tambur et al. [155]
Heart	Post	60 % of AMR + Px had HLA-DSA, the remainder had MICA Ab	HLA±MICA Ab correlates with chronic rejection	168	Luminex	Zhang et al. [11]
Heart	Pre and post	16 % had preexisting anti-HLA; 48 % had post-Tx anti-HLA	Patients with persistent HLA Ab had significantly reduced survival (post±pre)	950	CDC and solid phase	Ho et al. [18]
Heart	Pre and post	32 % had anti-HLA preTx; 63 % had anti-HLA postTx	preTx anti-HLA correlated with rejection in the first year	219	FC (more sensitive than CDC)	Tambur et al. [59]
Intestine	Pre	12 % had positive PRA, 7 % had HLA-DSA	Correlated with reduced survival	88	CDC and Luminex	Farner et al. [22]
Intestine	Pre		PRA positivity was risk factor for acute rejection	324	PRA	Gonzalez-Pinto et al. [21]
Intestine	Pre	21 % had positive CM	Correlated with early poor function	28	CDC	Wu et al. [12]
Islet	Pre		Positive PRA associated with reduced survival	81	CDC	Campbell et al. [111]
Liver	Pre	70 % had anti-HLA I	Lower outcome 1 year, higher rejection rate	80	ELISA	Bishara et al. [8]
Lung	Pre	9 % had positive PRA	PRA positivity increased post-Tx complications	200	CDC	Lau et al. [156]
Lung	Post	HLA±MICA Ab correlated with BOS incidence		80	Luminex and Flow PRA	Angaswamy et al. [157]
Renal	Post	27 % had anti-HLA		22	ECXM	Canet et al. [53]
Renal	Post	5 % had HLA-DSA, 11 % had non-DSA HLA Ab	Both reduced survival	1,229	CDC and FC	Hourmant et al. [15]
Renal	Post	30 % of Px had anti-HLA; 31 % of those had HLA-DSA	DSA reduced survival	1,014	SAB	Lachmann et al. [5]
Renal	Post	15 % of nonsensitized Px developed de novo HLA-DSA	HLA-DSA reduced 10 year survival	315	Solid phase	Wiebe et al. [23]
Renal	Post (>6 months)	11 % had anti-HLA; 4 % had HLA-DSA	Correlated with reduced function	251	ELISA	Cardarelli et al. [2]
Renal	Post	21 % had anti-HLA I, 10 % had anti-HLA II, 10 % had anti-MICA		191	Luminex	Alvarez-Marquez et al. [144]

Organ	Time after Tx	Frequency	Correlation with outcome	Number of patients	Assay to Detect DSA	Reference
Renal	Pre	48 % HLA antibodies, 11 % had HLA-DSA		113	CDC	Aubert et al. [25]
Renal	Pre	80.6 % had PRA positive for HLA I		62	CDC	Barocci et al. [13]
Renal	Pre	17 % had C1q fixing HLA DSA	C4d fixing ability of DSA was not predictive of early rejection	64	SAB with C4d fixation	Hönger et al. [54, 60]
Renal	Pre	22 % were FCXM+, 66 % of those had HLA I-DSA, 34 % had HLA II-DSA	Persistent DSA reduced survival	308	FCXM	Kimball et al. [17]
Renal	Pre		Positive PRA reduced 2 year outcome	154	ELISA	Meng et al. [109]
Renal	Pre	Of those with HLA Ab, 57 % had HLA-DSA	HLA-DSA increased acute rejection incidence, especially HLA II DSA	28 sensitized Px	Luminex	Song et al. [7]
Renal	Pre	11 % had MICA-DSA	MICA Ab correlated with rejection, especially when HLA matching was good	1,910	MICA antigen beads	Zou et al. [136]
Renal	Pre and post	16 % had MICA Ab; 13 % had preTx PRA; 16 % had HLA Ab postTx	HLA±MICA Ab increased rejection episodes and decreased survival	185	Luminex and ELISA	Panigrahi et al. [135, 158, 159]

anti-HLA HLA Ab of any specificity, *BOS* bronchiolitis obliterans syndrome, *CAV/TCAD* chronic cardiac rejection, *CDC* complement dependent cytotoxicity, *CM* crossmatch, *DSA* donor specific antibodies, *ECXM* endothelial cell crossmatch, *FC* flow cytometric assay, *FCXM* flow crossmatch, *HLA-DSA* donor specific HLA Ab, *Px* patient, *SAB* single antigen beads