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The Link Between MHC Antibodies and Cell Proliferation

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Abstract

Experimental evidence indicates that donor specific antibodies targeting MHC class I and class II molecules can elicit the key features of transplant vasculopathy by acting on the graft vasculature in three ways: directly activating proliferative, pro-survival, and migratory signaling in the target endothelial and smooth muscle cells; increasing expression of mitogenic factors in vascular endothelial cells, creating a potential proliferative autocrine loop; and promoting recruitment of inflammatory cells, which produce mitogenic factors and elicit chronic inflammation, proliferation, and fibrosis. Here we review the experimental literature showing the complement and Fc-independent effects of MHC class I and II antibodies on graft vascular cells which may directly contribute to the proliferative aspect of transplant vasculopathy.

Introduction

Advances in immunosuppression and patient management have greatly lowered the incidence of acute rejection in solid organ transplantation. However, long-term survival of solid organ allografts has not improved at the same rate due to chronic rejection. Based on Organ Procurement and Transplantation Network data as of August 2010, five year survival of primary cardiac and renal transplants in the United States is 70%, and by ten years post transplant, 50% of the grafts have failed. Chronic rejection is also a major limitation in lung and liver transplantation. Chronically rejected vascularized allografts develop a progressive and insidious vascular disease known as transplant or allograft vasculopathy (TV or AV). Histologically, vessels exhibit perivascular fibrosis, smooth muscle cell (SMC) proliferation and concentric neointimal thickening resulting in occlusion of the lumen. These vascular lesions are often accompanied by subendothelial lymphocytes and macrophages (1, 2).

TV manifests as bronchiolitis obliterans syndrome (BOS) in lung, cardiac allograft vasculopathy (CAV) in heart, and renal transplant arteriosclerosis in kidney transplantation. There are distinct features of disease in each organ undergoing chronic vascular rejection. In the heart, TV particularly affects the epicardial and intramyocardial arteries. The vascular

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lesions increase in area of the necrotic core, calcification, plaque area and burden with progression exhibit migration of SMC into the intima and neointimal thickening which reults in vessel occlusion (3). Renal arteriosclerosis results in inflammatory cell infiltration and a fibrous thickening of the vascular intima due to myofibroblast proliferation (4). This accompanies other facets of nephropathy, such as a duplication of the glomerular basement membrane and persistent capillaritis (5, 6). Chronic rejection in the lung is described as a "fibroproliferative disorder" with increased lymphocyte infiltration and disruption of the epithelium. Granulation tissue invades the small airway lumen and fibrous scarring blights the bronchioles (7–9).

Proliferation is a central feature of TV lesions. Expression of proliferating cell nuclear antigen (PCNA) is elevated in grafts with TV (10, 11). Further, increased expression of mitogenic factors, such as PDGF, TGFalpha (12), and TGFbeta (13), is observed. Chronically rejected allografts also have an increase in vascular endothelial growth factor (VEGF), an essential soluble factor which regulates angiogenesis and inflammation and is highly proliferative for vascular cells (14).

Clinical and Experimental Evidence Linking Donor Specific Antibodies to Transplant Vasculopathy

In addition to nonimmune factors, the development of TV is elicited by the alloimmune response to mismatched antigens expressed in the graft—in particular, the classical major histocompatibility molecules (MHC; also called human leukocyte antigen, HLA, in humans) on the endothelial cells (EC) lining the blood vessels of the allograft. Several *in vivo* animal studies have provided experimental evidence that T cell mediated alloimmunity is necessary and sufficient to cause transplant vasculopathy (15–20). In addition, donor specific antibodies (DSA) are an important clinical risk factor for development of TV (21, 22). Due to the advent of C4d staining that identifies antibody-mediated rejection, there has been a renewed interest in understanding the role of alloantibodies in TV. Indeed, animal models have shown that passive transfer of alloantibodies can mediate development of TV. When RAG-1 knockout or SCID recipient mice, immunodeficient mice which lack B and T cells, were transplanted with an MHC class I and II molecule mismatched organ and reconstituted with donor specific alloserum or MHC class I antibodies, the allografts had increased macrophage infiltration, exhibited the classic signs of antibody mediated rejection (AMR) and developed arteriosclerotic lesions and fibrosis (10, 16, 23–28). While DSA may not be necessary to cause TV, animal models of transplantation have established that MHC class I and/or II antibodies are sufficient to elicit vasculopathy in the absence of cellular immunity.

Clinical studies in cardiac, renal and lung transplantation have found significant correlations between the presence of DSA and decreased graft function, increased mortality and the incidence of chronic vascular rejection (14, 22, 29–34). The mechanisms by which antibodies may contribute to transplant arteriosclerosis are not yet fully clear. Antibody functions are mainly effected by the Fc region of the antibody, which can engage receptors on innate immune cells to provoke NK cell-mediated lysis or enhance recruitment into the tissues. Some classes of antibody can fix and activate complement through the Fc region, generating split products which are chemotactic for immune cells and which cause endothelial cell lysis, apoptosis or activation. The significance of complement during AMR has been thoroughly reviewed elsewhere (35, 36).

While Fc and complement interactions are important contributors to inflammation, particularly during acute rejection, clinical observations have shown that CAV can occur in the absence of complement split product deposition (37). This is in line with other experimental demonstrations that complement fixation is not necessary for MHC class I antibodies to activate EC *in vitro* or elicit vasculopathy in animal models (10, 25, 28, 38–

40). These data suggest that complement fixation may not be a requisite for disease in chronic AMR (39). Therefore the complement-independent actions of antibodies on the graft will be the focus of this review.

MHC Class I Molecule-Mediated Signaling in Vascular Cells

The importance of antibodies targeting HLA molecules has been demonstrated *in vivo* when HLA class I antibodies but not anti-MICA antibodies stimulated SMC proliferation and consistently induced neointimal thickening in a murine recipient of a human arterial graft (10). These results agree with findings in a rat cardiac allograft model where only endothelial-reactive allosera directed against MHC molecules contributed to vasculopathy and rejection (41, 42). Accordingly, anti-endothelial cell antibodies from autoimmune sera do not induce endothelial proliferation *in vitro* (43). This evidence suggests that MHC antibodies function in unique manner in which the ability to induce TV may depend on the signaling capacity of the antibody target, similar to agonistic antibodies like anti-angiotensin type 1 receptor antibodies.

HLA Antibodies Directly Induce Proliferative and Survival Signaling

Antibodies which crosslink HLA class I or II molecules induce signaling in human lymphocytes, as well as EC and SMCs. The effects of alloantibodies on EC *in vitro* are dependent on antibody recognition and crosslinking of the target rather than interactions with target cell Fc receptors (28, 44). Although it is still unclear how the classical HLA molecule transmits its biological signal, it is well-established that crosslinking of HLA class I or II molecules with antibodies in B cells, T cells or antigen presenting cells results either in programmed cell death or activation and proliferation (45). While the mechanisms influencing the differential outcomes are incompletely understood, the pathways leading to death or proliferation in immune cells have common second messengers, including intracellular calcium, PLC, PKC and Src family kinases (46–49). By activating similar signaling pathways in the donor vasculature and inducing proliferation, HLA class I antibodies may directly promote neointimal thickening.

HLA Class I Antibodies Induce Proliferation *In Vitro*—While animal models using passive transfer of MHC class I antibodies or alloserum have shown repeatedly that antibodies are sufficient to elicit transplant vasculopathy, the mechanism is incompletely understood. Antibodies which crosslink HLA class I molecules consistently cause *in vitro* proliferation in vascular smooth muscle (10, 50) and EC (51–56). Our group was the first to report that HLA class I antibodies promoted proliferation of SMCs (50), which Galvani et al. recently confirmed using a SMC line (10). We also demonstrated that HLA class I antibodies induce proliferation in human aortic EC rendered quiescent by serum starvation (51, 52, 57), and these findings are supported by similar reports from other groups using endothelium from a variety of vascular beds (55, 56) and various assays to measure proliferation (55, 58). HLA class I antibodies also cause proliferation in airway epithelial cells, suggesting a process by which alloantibody may contribute to BOS (59, 60).

HLA Class I Antibodies Induce Phosphorylation of Signaling Proteins—Longstanding investigations by our group have sought to dissect the signaling pathways triggered by crosslinking of HLA class I molecules in endothelial and SMCs (61, 62). HLA class I molecule ligation induces signal transduction pathways which dictate mitogenesis and survival in the endothelial cell within minutes of antibody treatment, diagramed in Figure 1. One of the earliest events is the GTP loading and membrane localization of RhoA (54, 63). RhoA activation is likely key to HLA class I molecule-induced proliferation, as inhibition of Rho abrogates select downstream signals (63), simvastatin reduces HLA class I

molecule-mediated proliferation of EC *in vitro* (54), and Rho kinase inhibitors prevent rejection and transplant arteriosclerosis in animals (64, 65).

After HLA class I molecule crosslinking, the tyrosine kinase Src is phosphorylated at an autophosphorylation site in the catalytic domain (57, 58). Src is a crucial signal transducer which regulates such cell functions as cytoskeletal changes, migration, mitogenesis, cell cycle progression, and cell survival. Indeed, Src inhibition during HLA class I signaling prohibits activation of most downstream events (57). We reported that focal adhesion kinase (FAK) phosphorylation permits complex formation with Src family kinases (57, 58, 63), allowing for maximal kinase activity. These phosphorylation events are dependent on Rho/Rho kinase and Src activity in HLA class I molecule signaling (57, 63).

FAK then facilitates phosphorylation of paxillin (58), an adaptor protein involved in focal adhesion formation which can recruit a variety of other signaling molecules to specific compartments in the cell. One major functional consequence of FAK and paxillin activation is cytoskeletal rearrangement and formation or stabilization of focal adhesions. We have observed the reorganization of the actin cytoskeleton after HLA class I molecule ligation, with dramatic stress fiber formation (58, 63). Cytoskeletal tension and remodeling in adherent cells is required for proliferation, as many cytoskeletal regulators including Rho and FAK additionally control cell growth (66). Indeed, inhibition of FAK by siRNA during HLA class I molecule signaling reduces proliferative capacity (44, 58). The cytoskeleton is important in regulation of endothelial permeability as well as signal transduction, since Akt/PI3K complex formation is abrogated when the cytoskeleton is disrupted (44).

HLA class I molecule ligation activates phosphatidylinositol 3 kinase (PI3K), via FAK-dependent phosphorylation of the p85 regulatory domain (44, 58, 67, 68). PI3K phosphorylates the membrane bound phospholipid phosphatidylinositol 4,5-bisphosphate (simply known as PIP_2) to yield PIP_3. PIP_3 can recruit 3-phosphoinositide dependent protein kinase (PDK1) and Akt, to the membrane (68), where phosphorylation of Akt by PDK1 permits a second modification by mammalian target of rapamycin (mTOR), resulting in full activation. The Akt pathway is an important regulator of cell growth, survival and migration. Akt contributes to cell proliferation by setting in motion the mTOR pathway and by regulating cell cycle progression.

Akt promotes mTOR activation through inhibition of mTOR antagonists. mTOR is present in two complexes in the cell. mTOR complex 1 (mTORC1) regulates ribosomal biogenesis and protein synthesis, and comprises mTOR, raptor and GbetaL. mTOR complex 2 (mTORC2) includes mTOR, Sin1, GbetaL and rictor, and participates in cytoskeletal regulation and feedback to Akt (69). We found that after HLA class I molecule ligation, mTOR is phosphorylated and forms signaling complexes (69).

Proliferation requires de novo protein synthesis, and the factors which regulate or execute translation of mRNA are targeted by the mTOR complex. Our group reported that 4EBP-1 (or PHAS-1), an inhibitor of the translation factor eIF-4E, is phosphorylated by mTOR after HLA class I molecule crosslinking (69). This modification of 4EBP1 is considered a translation initiation signal, as it triggers release of eIF-4E translation factor. p70 S6 kinase and ribosomal protein are also substrates for mTORC1 during HLA class I molecule signaling (69–71). Activation of ribosomal proteins complements the release of the translation factor eIF-4E to enhance protein synthesis. We found that none of the above events occur after HLA class I molecule ligation when mTOR signaling is impaired, emphasizing the central role of the mTOR complex in HLA class I molecule-mediated proliferative signaling. Further, both small interfering RNA knockdown (69) and

pharmacological inhibition (55) of mTOR prevent cell proliferation in response to HLA class I antibodies.

We have also demonstrated the relevance of these pathways *in vivo*. Histological examination of biopsies from cardiac transplant patients showed that phosphorylated S6 ribosomal protein was a more specific indicator of AMR than C4d staining, and correlated with circulating levels of HLA class II antibodies (71). Our group also used an animal model to confirm that activation of these pathways, including S6RP, S6K, ERK, mTOR, and Akt, was significantly increased during chronic AMR (69).

HLA Class I Antibodies Increase Cell Sensitivity to Growth Factor—As described, cells treated with HLA class I antibodies proliferate in the absence of exogenous growth signals. In addition to immediate activation of growth-promoting signaling, HLA class I antibody-stimulated vascular cells also acquire proliferative capacity by augmenting their sensitivity to growth factors. Our group reported that treatment of EC with HLA class I antibodies led to rapid translocation of the fibroblast growth factor receptor (FGFR) at the cell surface and redistribution within the cell (50, 57).

The increase in surface FGFR results in amplified sensitivity to bFGF. The upregulation of FGFR is expected to be central to the resulting proliferative response of the cells, since addition of bFGF to the culture medium significantly potentiates cell growth in response to HLA class I antibodies and because neutralizing antibody to soluble bFGF abrogates HLA class I-induced cell proliferation (50–52). FGFR signaling converges on ERK, a MAP kinase. Indeed, our group found that ERK is phosphorylated at two key sites after HLA class I molecule ligation (72), in an mTORC2 dependent manner, which allows dimerization and nuclear translocation. Since activation of ERK appears to be tied to FGFR signaling, while activation of Akt is not dependent on FGFR, we hypothesize that FGFR signaling must act in parallel with other HLA class I molecule-mediated signaling events (44).

HLA Class I Antibodies Promote Cell Cycle Progression—HLA class I molecule signaling also regulates cell cycle progression. One inhibitor of cell survival and proliferation, glycogen synthase kinase 3 beta (GSK3beta) is targeted by Akt to promote growth. Akt is known to inactivate GSK3beta (54), thereby relieving inhibition of cyclins, translation initiator eIF2B and other cell cycle regulators. It has also been reported that the activity of another cell cycle inhibitor is altered after HLA class I molecule crosslinking. Nath et al. showed that Rb was inactivated as a result of the action of cyclin dependent kinase 2 (Cdk2). Further, the inactivation of Rb was likely mediated by FGFR signaling, since inhibition of bFGF abolished the observed effect (73).

Cell Survival Pathways—While HLA class I and II antibodies can cause apoptosis in lymphocytes, stimulation of EC with a low dose of HLA class I antibodies or alloserum promotes cell survival and resistance to death (44, 67, 74). Akt lies at the heart of endothelial survival in the presence of HLA class I antibodies, as illustrated in Figure 2. We reported that after HLA class I molecule crosslinking, the pro-apoptotic protein Bad is phosphorylated at two Akt-responsive sites, which results in its sequestration from the mitochondria by the 14-3-3 proteins (44). Our group and others also showed that the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL are increased downstream of the PI3K/Akt/mTOR signaling axis (44, 68, 69). These Bcl family members suppress programmed cell death by regulating mitochondrial membrane permeability to prevent activation of caspases. Biopsies from patients with circulating DSA and mouse cardiac allografts undergoing AMR also had increased Bcl-2 expression, confirming these findings *in vivo* (28, 44, 74).

Other genes which protect from cell death during stress are upregulated after HLA class I molecule ligation. For example, expression of heme oxygenase 1 (HO-1), a factor which is cytoprotective against oxidative stress and other forms of cellular injury, is increased in endothelium after HLA class I molecule ligation and confers resistance to complement-mediated lysis (67, 75). Evidence also points to a protective effect of intragraft HO-1 against rejection and TV (76–78).

Cell survival and proliferative pathways are concurrently active and share key mediators, such as PI3K/Akt/mTOR pathway. Akt is the central signaling hub whose pleiotropic effects are accomplished by a broad array of targets. Akt activation imparts resistance to death signals (67) by modulating the balance between pro- and anti-apoptotic factors. The Akt signal also causes proliferation by regulating the activity of cyclins, thereby driving cell cycle progression, and diverges into the two mTOR complexes to influence protein synthesis. It is therefore difficult to dissect the signaling which increases cell viability from that which promotes active division because, while the outcomes are distinct, they are also strongly linked.

HLA Class I Molecule Signal Transduction—How classical HLA molecules signal is still a matter of investigation. While they do not have intrinsic kinase activity, it is postulated that HLA molecules associate with other proteins at the membrane which are capable transducing signals, an arrangement common to many receptors. For example, MHC class I molecules have been coimmunoprecipitated with growth factor receptors (IGFR and EGFR) (79, 80), whereas HLA class II molecules have been shown to interact with a tetraspanin network which links them to integrins such as integrin alpha4beta1 and alpha6beta1 (81). Indeed the signaling cascade induced by crosslinking of the HLA class I molecule is markedly similar to integrin and growth factor receptor signaling.

In order to explain the ability of HLA class I molecules to transduce signals in EC, our lab investigated the function of integrins in HLA class I molecule-mediated signaling. A molecular association between HLA class I molecules and the integrin subunit beta4 was found, which was increased following antibody ligation of HLA class I molecules. Deletion of the cytoplasmic domain of the HLA class I molecule, which is required for the association with integrin beta4, suppressed HLA class I molecule signaling. HLA class I molecules required integrin beta4 expression in order to cause phosphorylation of Src, Akt and ERK after crosslinking. Furthermore, knockdown of integrin beta4 abolished proliferation in response to HLA class I antibodies, demonstrating a dependency of HLA class I molecules on integrin beta4 for induction of cell growth. Importantly, we also uncovered a previously unknown role for the HLA class I molecule in integrin beta4 signaling, revealing for the first time that HLA class I molecule expression is required for integrin beta4-mediated functions such as migration and ERK phosphorylation (82). These studies give fresh and valuable insight into the molecular mechanisms by which HLA class I molecules stimulate cellular proliferation.

MHC Antibodies Activate Transcription and Synthesis of Mitogenic Mediators

MHC class I antibodies have also been observed to induce transcription factor activity and production of cytokines in EC. Cytokine production could initiate autocrine signaling that reiterates the endogenous proliferative signaling, and promotes recruitment of immune cells.

The Role of NF-kappaB

NF-kappaB is one of the most well-recognized transcription factors in inflammation. It controls cell growth and apoptosis, as well as production of inflammatory mediators in a variety of cells, and plays an important role in endothelial cell activation (83). Treatment of EC with HLA class I antibodies increases mRNA and protein expression of NF-kappaB dependent gene targets, such as cytokines and anti-apoptotic factors, suggesting a role in AMR. Traditional models of NF-kappaB signaling involve degradation of the inhibitory IkappaB proteins, which sequester the NF-kappaB transcription factor in the cytoplasm. Serine phosphorylation of IkappaBalpha causes release of NF-kappaB, ubiquitination and degradation of IkappaB, resulting in translocation of NFkappaB into the nucleus.

HLA class I antibodies activate NF-kappaB in immune cells (84). Ligation of HLA class I by antibody increased the DNA binding ability of NF-kappaB and tyrosine phosphorylation of IkappaBalpha in both human umbilical vein EC (HUVEC) and cardiac microvascular EC (53). However, the investigators were not able to consistently show degradation of IkappaBalpha with different HLA class I antibodies. Another group likewise failed to show a reduction in protein levels of IkappaBalpha which would correspond to its degradation (54). It may be that HLA class I ligation activates a less familiar NF-kappaB pathway, such as the tyrosine phosphorylation and nondegradory dissociation described in VEGF signaling (85).

In addition to directing inflammatory responses, NF-kappaB regulates the decision between death and survival in a variety of cell types (86). Experimental evidence suggests that suppression of NF-kappaB in the allograft by anti-inflammatory proteins may reduce neointimal thickening and extend graft survival during chronic rejection (77, 87). Mechanistic studies of NF-kappaB are needed to understand its connection to the processes which promote vasculopathy during AMR. Further, it is almost certain that other transcription factors are active during HLA class I signaling and are waiting to be discovered.

Induction of Proliferative Factors

While complement split products can induce expression of inflammatory factors in EC (88, 89), MHC class I antibodies can elicit this response in the absence of complement. Rahimi et al. showed that high doses of both complement fixing and noncomplement fixing MHC class I antibodies can induce expression of KC in a murine endothelial cell line (SVEC-10) (38, 39). This finding is supported by the reported induction of interleukin-8 (IL-8, the human homolog of KC) in arterial EC after treatment with HLA class I antibodies (56). IL-8 is well-characterized as a chemokine which attracts neutrophils and other leukocytes to promote their recruitment to sites of inflammation. In addition to its function as a chemokine, IL-8 has an autocrine proliferative effect on EC (90).

Tissue factor (TF) plays a role as a procoagulant and has also been implicated in neointimal hyperplasia, fibrosis, EC and SMC proliferation (91, 92). In a rat cardiac transplant model of chronic rejection, TF was increased in the coronary intima (93), and inhibition of TF expression reduced neointimal thickening (94). In addition, patients exhibiting vasculopathy had nearly eight-fold higher expression of TF in endocardial biopsy than those without vasculopathy (95), which was determined to be predictive of TV (96). Naji et al. treated HUVEC with allele-specific HLA-A antibodies *in vitro* and showed that TF was increased (97), demonstrating that HLA class I antibodies can directly induce this important mediator.

VEGF is a key regulator of vascular function. In addition to its importance during vascular development, VEGF also induces proliferation and survival signaling in EC. Several reports have shown that VEGF is upregulated in human cardiac transplant biopsies with evidence of

TV, and localizes to EC (14, 98). Further, VEGF expression combined with macrophage infiltration increases the risk for development of TV by 2.5-fold (14, 98). A rat model of chronic rejection revealed the importance of VEGF signaling in chronic transplant rejection. It was found that overexpression of VEGF in the graft exacerbated intimal thickening while antagonism decreased vessel occlusion and mononuclear cell infiltration (99, 100). HUVEC treated with HLA class I antibodies *in vitro* increase production of VEGF mRNA. In addition, HLA class I-mediated cell migration was dependent on VEGFR2, implicating an autocrine signaling pathway in which secreted VEGF alters the endothelial cell phenotype (55). Therefore, VEGF plays a prominent role in antibody-mediated vasculopathy and may be the direct result of HLA antibody effects.

In addition to acting on EC to induce factors which promote vascular cell growth and migration, HLA class I antibodies may target other cells to elicit vascular disease. Rat SMCs produce PDGF, FGF-2 and IGF-1 upon exposure to donor specific antibodies (101). Additionally, airway epithelial cells treated with HLA class I antibodies elaborated growth factors which promote fibrosis, such as PDGF and bFGF, and stimulated proliferation of fibroblasts (102). This study highlights the effects of HLA class I antibodies during BOS in lung allografts. The authors postulated that expression of these growth factors promotes survival and proliferation of the SMCs in an autocrine fashion.

The mediators produced by EC upon MHC class I stimulation are summarized in Table 1. Importantly, while many studies reported that MHC class I antibodies cause both cytokine secretion and proliferation of EC, none have conclusively demonstrated the direct contribution of cytokine production to MHC class I molecule-induced proliferation. Further work should be correlative and focus on directly identifying the contribution of each cytokine to *in vitro* proliferation and the development of vasculopathy *in vivo*. The induction of the above factors by initial MHC class I molecule signaling may reinforce the proliferative signaling observed following ligation, by creating autocrine feedback from a highly proliferative cytokine milieu.

MHC Antibodies Promote Recruitment of Leukocytes

Macrophages in Vasculopathy

Mononuclear cell infiltration is a key feature of AMR, and macrophages are found in high frequency in the thickened intima of chronically rejected grafts from multiple species. Macrophages have been extensively studied in atherosclerosis, where they play an active pathogenic role. For example, macrophages can promote alteration of extracellular matrix production by SMC (103) and can directly cause endothelial cell proliferation by releasing a variety of growth factors and cytokines (104).

Mononuclear cells are not only a hallmark of chronic rejection, but they also are influential in the pathophysiology of rejection. Depletion of macrophages or transplant into a macrophage deficient allograft recipient significantly reduces intimal thickening (17, 105, 106). Further, antagonism of MCP-1, a chemokine signal important to monocyte recruitment from the blood into the tissue, increases graft survival and decreases transplant vasculopathy in an animal model of lung transplantation (107). Macrophage-derived factors in the graft neointima can contribute to local inflammation by causing tissue damage, fibrosis and SMC proliferation (108, 109). While the potential for macrophages to cause progression of arterial lesions is clear, evidence for a causative effect of macrophage function in TV is mostly indirect.

HLA Class I Antibodies Cause Leukocyte Recruitment by Endothelial Cells

Leukocyte recruitment from the blood by EC is a complex cascade with discrete stages directed by an array of adhesion molecules and chemokines. The first step, known as "rolling," is a low affinity interaction between the leukocyte and endothelial cell mediated by selectins. Subsequently, high affinity integrin receptors on the leukocyte convert rolling to firm adhesion and arrest. Finally, remodeling of the endothelial barrier and polarization of leukocyte receptors permits extravasation into the subendothelial space.

There is mounting proof that antibodies can trigger endothelial cell recruitment of immune cells to the graft. Passive transfer of MHC class I antibody into an immunodeficient recipient of a mismatched allograft elicits strong macrophage and perivascular cellular infiltration (23, 28, 38, 110). When EC are coated with antibody, leukocyte receptors for antibody Fc regions (FcR) can enhance leukocyte recruitment by initiating inside-out signaling which supports integrin-mediated firm adhesion and arrest (111). While Fc functions can be a significant factor in leukocyte-endothelial interactions during rejection, as demonstrated by Lee et al. (38), other work suggests that the Fc is not necessarily required for antibodies to elicit leukocyte recruitment. Treatment of the immunodeficient allograft recipient with an $F(ab')_2$ fragment of the MHC class I antibody does not preclude recruitment of leukocytes into the graft (28, 38). This observation is strongly indicative of functions mediated by the antibody independent of Fc interactions or complement, in which alloantibody-induced signaling in EC increase endothelial adhesivity.

HLA class I antibodies may cause leukocyte recruitment through induction of adhesion molecules and chemokines in the endothelium. While previous work by our group ruled out the upregulation of the endothelial adhesion molecules as ICAM-1, VCAM-1 and E-selectin (112), ligation of HLA class I molecules causes release of endothelial vesicles known as Weibel-Palade bodies and rapid presentation of P-selectin, which facilitated leukocytic cell adherence (113). P-selectin participates in neutrophil and macrophage recruitment during inflammation, and study of chronic rejection in rat cardiac allografts uncovered a correlation between intimal thickening and increased P-selectin expression in the intima (114, 115).

Cytokine production by MHC class I-activated EC could initiate an autocrine inflammatory loop to promote leukocyte recruitment. MHC class I antibodies cause cultured EC to produce a variety of inflammatory cytokines, including IL-6 and IL-1beta, and chemokines, such as MCP-1, VEGF and IL-8 (38, 55, 56). These factors could contribute to the mobilization of leukocytes into the graft, either by directly guiding leukocyte migration or by enhancing and extending endothelial cell activation (summarized in Table 1). For example, intragraft expression of VEGF facilitates recruitment of mononuclear cells directly and augments endothelial expression of other chemokines, thereby amplifying the rejection response (116).

Certainly macrophage functions and the leukocyte recruitment cascade represent important targets in chronic allograft vasculopathy. Antagonism of mononuclear cell recruitment or the endothelial signaling which promotes recruitment may reduce lesion burden (17).

Conclusions and Future Directions

In summary, HLA class I antibodies can activate a variety of signaling pathways in vascular endothelial and SMCs which may contribute to transplant vasculopathy. As shown in Figure 3, HLA class I antibodies cause immediate activation of proliferative and survival pathways. They also induce production of mitogenic factors which may reinforce the proliferative milieu, and inflammatory mediators which can recruit leukocytes to the graft. We propose

that these effects compound during chronic AMR to provoke proliferation of the graft vasculature, culminating in TV.

While extensive work has yielded insight into the effects of DSA on the graft vasculature, much more investigation is needed to fully understand how HLA antibodies contribute to transplant arteriosclerosis. In particular, the intracellular signaling pathways should be further explored in order to ascertain the potential therapeutic benefits of modulating this system and to identify other possible biomarkers of AMR which may be early indicators of vasculopathy. Additionally, the specific contribution of cytokines and other mitogenic factors should be clarified with correlative studies, as cytokine milieu and microenvironments are known to influence disease outcomes. Finally, it will be important to more specifically investigate the role of macrophages in vasculopathy, as has been done in atherosclerosis, to fully reveal their contribution to the pathogenesis of chronic rejection.

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List of Abbreviations

HLA	human leukocyte antigen
MHC	major histocompatibility complex
TV	Transplant vasculopathy
BOS	bronchiolitis obliterans syndrome
CAV	cardiac allograft vasculopathy
VEGF	vascular endothelial growth factor
PCNA	proliferating cell nuclear antigen
RAG	recombinase activating gene
DSA	donor specific antibodies
AMR	antibody mediated rejection
FAK	focal adhesion kinase
PI3K	phospho inositol 3 kinase
PDK1	3 phosphoinositide dependent protein kinase
mTOR	mammalian target of rapamycin
FGFR	fibroblast growth factor receptor
EC	endothelial cell
SMC	smooth muscle cell

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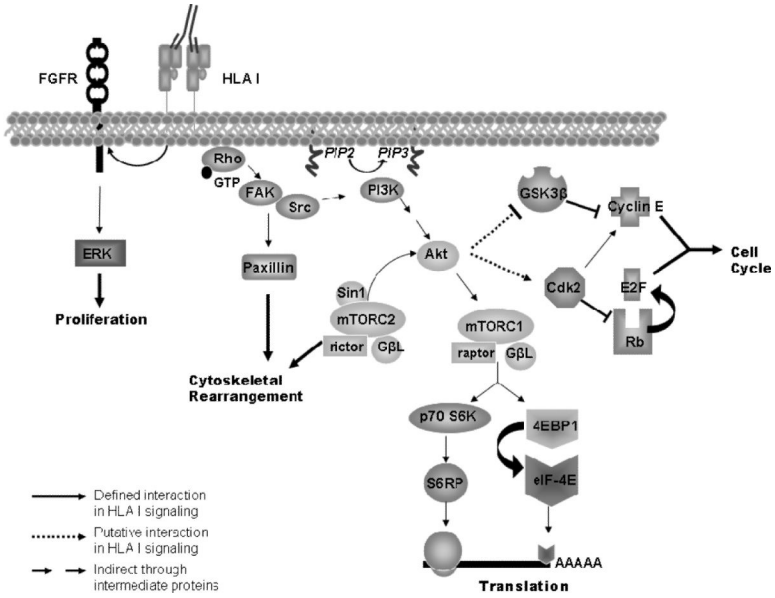

Figure 1.

Crosslinking of HLA class I molecules by antibodies induces cell signaling pathways which promote cell growth. FAK activates paxillin, an important regulator of the cytoskeleton. FAK activation also permits complex formation with Src. PI3K-dependent activation of Akt through PDK1 is central to cell growth. Akt inhibits GSK3beta, an antagonizer of cyclins such as cyclin E, which is vital to cell cycle progression. Akt also stimulates cyclin-dependent kinase 2, which causes the tumor suppressor Rb to release the transcription factor E2F, facilitating production of genes which promote G1/S transition.

Akt targets mTOR. mTOR complex 1 is responsible for S6K phosphorylation, which in turn activates S6 ribosomal protein. 4EBP-1, an inhibitor of the translation factor eIF-4E, is phosphorylated in an mTOR dependent manner after HLA class I molecule crosslinking. Phosphorylation ultimately triggers release of eIF-4E and allows recruitment of 40S ribosomal subunits to mRNA. Cumulatively, these events result in increased protein synthesis and are central to cell proliferation.

Finally, crosslinking of HLA class I molecules results in rapid translocation of FGFR to the cell surface and increased sensitivity to growth factor. The ERK MAP kinase is phosphorylated in an mTORC2 and FGF dependent manner, and actively promotes proliferation.

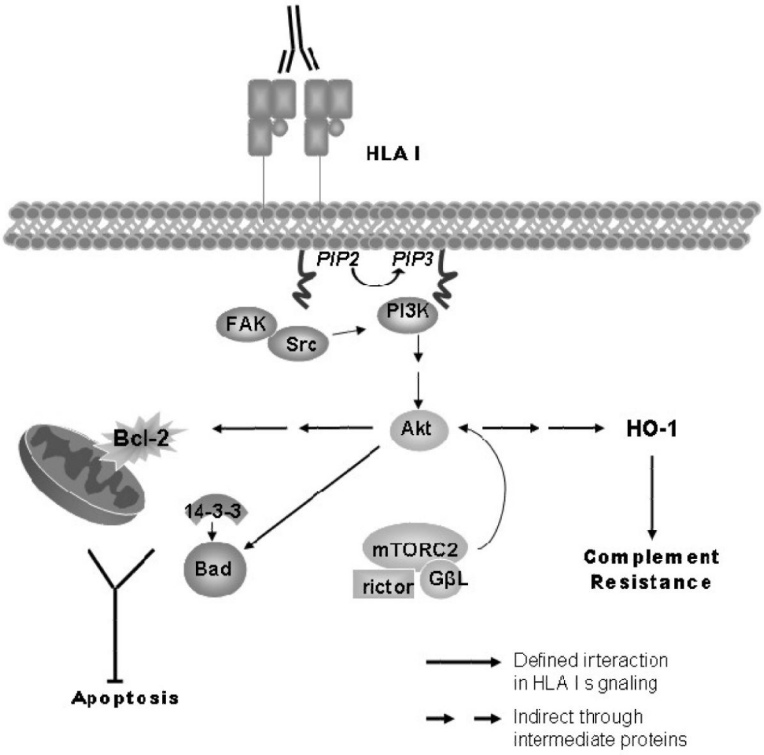

Figure 2.

Crosslinking of HLA class I molecules with antibodies activates Akt, which promotes cell survival. PI3K catalyzes phosphorylation of PIP_2 to yield PIP_3 which can interact with a discrete region known as the pleckstrin homology domain. Akt and PDK1 localize to the membrane in response to PIP3, where PDK1 phosphorylates Akt at Thr308. Akt promotes mTORC1 activation, while mTORC2 phosphorylates Akt at Ser473. Akt increases Bcl-2 and Bcl-xL levels in the cell and phosphorylates Bad, a pro-apoptotic mediator, which allows 14-3-3 proteins to bind. This association with 14-3-3 prevents Bad antagonism of Bcl-2 and Bcl-xL proteins and promotes cell survival. Finally, cells treated with HLA class I antibody upregulate the antioxidant HO-1 in a PI3K dependent manner, which contributes to the cells' resistance to complement mediated lysis.

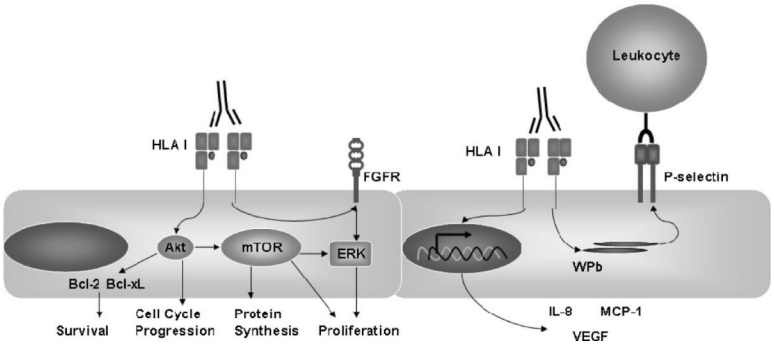

Figure 3.

Three-step model of the Fc independent effects of HLA class I antibodies on vascular endothelium. HLA class I molecule ligation immediately activates cell signaling pathways which cause cell cycle progression, promote cell survival and protein synthesis, and ultimately induce cellular proliferation. HLA class I molecule crosslinking also activates transcription, and endothelial cells begin producing inflammatory and proliferative factors such as IL-8, VEGF and MCP-1 after a lag period. Finally, HLA class I molecule signaling mobilizes endothelial vesicles known as Weibel-Palade bodies (WPb), resulting in presentation of the adhesion molecule P-selectin on the cell surface and increasing the binding of leukocytes. Immune cells such as T cells and macrophages can release mediators in the neointima which cause endothelial and smooth muscle cell proliferation and vascular remodeling.

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Table 1

Summary of cytokines and growth factors induced by MHC I antibodies in endothelial cells, their functions and role in allograft rejection.

Cytokine	Effect on Vascular Cells	Inflammatory Effect	Observations in Clinical Rejection	*In vivo* work showing its role in TV	Primary *In Vitro* Reference
IL-8	Causes autocrine proliferation of EC (90)	Promotes neutrophil and monocyte recruitment and activation (117)	Strong expression in high grade rejection heart biopsies (118)	None reported	MHC I Ab induce KC (38, 39) and IL-8 (56) production from ECs
Tissue factor	Induces mitogenic factors (91) Regulates EC proliferation and apoptosis (92)	Initiator of coagulant cascade Leukocyte diapedesis Promotes fibrin deposition	Px with TV had higher expression in endomyocardial biopsy (95) Early biopsy staining predictive of TV (96)	Upregulated in rat coronary intima and adventitia, of EC and mononuclear cell origin (93) Inhibition of TF expression reduces intimal thickening (94)	Increased TF mRNA and protein in HUVEC after HLA I Ab treatment (97)
IL-6	Induces production of VEGF (119) and PDGF Promotes SMC and EC migration (120) and proliferation (121)	Regulates EC adhesion molecule expression and permeability (122)	Increased in plasma of patients with TV (123) Strong correlation between intragraft mRNA and histological rejection (124)	None reported	Human EC produce IL-6 after HLA I Ab treatment (56)
VEGF	Causes EC proliferation and migration SMC chemoattractant (125–127)	Regulates vascular permeability (128) Induces EC adhesion molecules (129–131) Monocyte chemokine (132)	Upregulated in biopsies from patients with TV (14, 98)	VEGFR antagonism reduced TV and decreased mononuclear cell recruitment (100) Overexpression of VEGF increased intimal thickening and macrophage infiltration (99)	Treatment of HUVEC with HLA I Ab increased VEGF mRNA (55)
MCP-1 (CCL2)	None reported (133)	Monocyte chemokine (117)	Increased in plasma of patients with TV (123)	Increased expression in graft when recipient was treated with anti-donor Ab (38) Increased intragraft MCP-1 preceded intimal thickening (134)	Murine EC line produces MCP-1 after treatment with MHC I Ab (38, 56)
RANTES (CCL5)	None reported	Chemotactic for T cells and monocytes	Increased in graft vessels in patients with graft atherosclerosis (135) and rejection (136)	Expression correlated with mononuclear infiltration and intimal thickening (134, 136)	Murine EC line produces RANTES after treatment with MHC I Ab (38)