Title

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Journal

Current Transplantation Reports, 2(3)

ISSN

2196-3029

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Publication Date

2015-09-01

DOI

10.1007/s40472-015-0065-6

Peer reviewed

Published in final edited form as:

Curr Transplant Rep. 2015 September ; 2(3): 222–232. doi:10.1007/s40472-015-0065-6.

Antibodies to HLA Molecules Mimic Agonistic Stimulation to Trigger Vascular Cell Changes and Induce Allograft Injury

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Abstract

Human leukocyte antigen (HLA)-induced signaling in endothelial and smooth muscle cells causes dramatic cytoskeletal rearrangement, increased survival, motility, proliferation, adhesion molecule and chemokine expression, and adhesion of leukocytes. These mechanisms are directly related to endothelial activation, neointimal proliferation, and intragraft accumulation of leukocytes during antibody-mediated rejection (AMR) and chronic rejection. Clustering of HLA by ligands in *trans*, such as in antigen-presenting cells at the immune synapse, triggers physiological functions analogous to HLA antibody-induced signaling in vascular cells. Emerging evidence has revealed previously unknown functions for HLA beyond antigen presentation, including association with coreceptors in *cis* to permit signal transduction, and modulation of intracellular signaling downstream of other receptors that may be relevant to HLA signaling in the graft vasculature. We discuss the literature regarding HLA-induced signaling in vascular endothelial and smooth muscle cells, as well as under endogenous biological conditions, and how such signaling relates to functional changes and pathological mechanisms during graft injury.

Keywords

HLA antibodies; Endothelial cells; Antibody-mediated rejection; Leukocyte recruitment; Signal transduction; Mammalian target of rapamycin (mTOR)

Introduction

Antibodies against donor human leukocyte antigen (HLA) molecules and subsequent rejection episodes are strongly associated with risk of chronic rejection and late graft failure [1–3]. The general mechanisms of graft injury by HLA antibodies during antibody-mediated rejection (AMR) include endothelial injury and dysfunction, infiltration of innate immune cells, and activation of the classical complement cascade. While canonical antibody Fc-mediated activation of complement- and FcγR-dependent functions are undoubtedly

Compliance with Ethics Guidelines

Conflict of Interest Nicole M. Valenzuela and Elaine F. Reed declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

This article is part of the Topical Collection on *Immunology*

important for the pathogenesis of antibody-mediated rejection, agonistic actions of HLA antibodies on donor vascular cells cause functional and phenotypic changes leading to endothelial and smooth muscle cell dysfunction that contribute to graft injury and rejection. This review will summarize and discuss the current knowledge about HLA-induced signaling in vascular cells and how it relates to graft injury during rejection. In addition, we will attempt to address *how* and *why* HLA molecules induce intracellular signaling upon cross-linking and what can be inferred from the known physiological roles of HLA in the immune system and beyond.

Although this review focuses on the mechanisms of HLA-mediated signaling within vascular cells, which contribute to graft pathology and injury, it is important to keep in mind that antibody-mediated rejection is a multifaceted process that involves other mechanisms of injury. We and others have proposed that the concomitant effects of antibodies, $F(ab')_2$-dependent endothelial activation, Fcγ R-dependent leukocyte functions [4, 5], and Fc-dependent activation of complement [6••], synergize during antibody-mediated rejection to culminate in graft injury. Thus, parallel mechanisms of complement activation and Fcγ R functions likely exacerbate endothelial dysfunction and graft injury initiated by HLA signaling.

HLA Signaling in Vascular Cells in Response to Antibodies: Mechanisms Related to Graft Injury

It has been well established that agonistic cross-linking of HLA I by alloantibodies activates a phosphorylation-dependent signaling cascade in vascular cells that leads to functional changes that may contribute to the histological manifestations of graft injury during acute and chronic rejection (Fig. 1a). In contrast, study of HLA II in cultured vascular cells has been technically challenging. Recently, two approaches to circumvent this complication have been described [7, 8•], which will likely permit much-needed studies of HLA II antibody effects on endothelium in the near future. For the purposes of this review, we will indicate, where possible, any existing knowledge regarding HLA II in vascular cells or any relevant literature from other cell types, but the reader should recognize that the evidence is currently limited.

Cytoskeletal Reorganization: Migration, Permeability, and Signal Localization

One of the earliest functional changes occurring after exposure of endothelial cells to HLA I antibodies is the dynamic remodeling of the actin cytoskeleton, resulting in rapid and dramatic stress fiber formation [9–11, 12•]. Rho GTPase is activated after HLA I ligation by antibodies [10, 13]. Src protein kinase is rapidly activated and subsequently phosphorylates focal adhesion kinase (FAK) in endothelium [14] and smooth muscle cells [15]. FAK targets paxillin, an adaptor found in focal adhesion which transmits adhesion-dependent intracellular signaling. Although the role of HLA II in regulating endothelial cytoskeleton has not yet been reported, FAK was activated in fibroblasts after stimulation with HLA II antibodies [16]. Rho and Rho kinase were critically required for HLA I-induced FAK and paxillin phosphorylation as well as stress fiber formation [10, 11]. Other GTPases, Rac and Cdc42, which also regulate actin cytoskeletal organization, were unchanged in endothelium

after HLA I cross-linking [10]. This finding suggests that the type of stress fiber formed after HLA I cross-linking is distinct from Rac- and Cdc42-associated lamellipodia and filopodia and rather results in the formation of focal adhesion [17]. This is consistent with the observation that the pattern of stress fibers is different from those stimulated by thrombin [11]; thrombin acts on both Rho and Rac to control the actin cytoskeleton [18], which have opposing actions on the cytoskeleton in endothelium.

We also reported that ERK1/2 phosphorylation was dependent on mammalian target of rapamycin (mTOR) complex 2 (mTORC2) following HLA I ligation [19]. ERK proteins are vital for growth factor and integrin signaling and also regulate the cytoskeleton. Ultimately, Rho and ERK converge to activate myosin light chain (MLC) [11], which plays a central role in actin contractility and stress fiber assembly. Notably, activation of ERK through mTORC2 was unique to HLA I and integrin-induced signaling but was not observed with growth-factor-induced signaling [19], pointing to a distinct pathway of ERK activation that is common to MHC class I and integrins. Subsequent studies revealed that both mTORC2 and ERK were required for HLA I-mediated stress fiber formation in endothelium [11] and that pharmacological inhibition of mTOR with rapalogs could prevent stress fiber formation after exposure to HLA I antibodies [19].

What is the purpose or functional outcome of actin stress fiber formation? The cytoskeleton is integral to cell motility, cytokinesis, endocytic and exocytic events, cell-cell interactions, and invasion, as well as facilitating inside-out signaling of transmembrane receptors and integrins [20, 21]. The function of stress fibers is context dependent on their intracellular location, morphology, and associated proteins [22]. Stress fiber connection to the extracellular matrix through focal adhesion, such as those observed after HLA I antibody activation in endothelial cells, permits mechanotransduction. Stress fibers of this type are also likely important for endothelial rigidity during tethering of leukocytes [23] and during formation of cell-cell synapses.

It is increasingly appreciated that the cytoskeleton is also an active organizer of intracellular signaling cascades, controlling localization and compartmentalization of discrete processes and pathways. Indeed, disruption of the endothelial cytoskeleton abolishes all downstream signaling [14] and prevents molecular aggregation of HLA I by antibodies. Consistent with its central role in coordinating signaling molecules after HLA I cross-linking by antibodies in endothelial cells, the repertoire of phosphorylated signaling proteins associated with the actin cytoskeleton was dramatically altered [12•]. Using mass spectrometric analysis, Ziegler et al. verified that proteins involved in catalytic functions, protein biosynthesis, and actin cytoskeletal organization were dynamically associated with the cytoskeletal fraction in response to HLA ligation [12•]. mTORC2 and ERK were shown to translocate to the plasma membrane from the cytoplasm, where they may colocalize with transmembrane receptors and lipid rafts to facilitate signaling [11]. HLA I cross-linking by antibodies also resulted in increased endothelial and smooth muscle cell motility in in vitro wound-healing models of cell migration, a process which is important for angiogenic functions [24•].

Therefore, HLA I induction of cytoskeletal reorganization is a critical step in coordinating intracellular signaling pathways and downstream functional changes in vascular endothelial

and smooth muscle cells. In addition, dynamic regulation of the actin cytoskeleton following HLA antibody binding might account for the microvascular endothelial changes observed by electron microscopy in renal allograft AMR. However, it should be noted that antagonism of endothelial cytoskeletal functions may also have detrimental effects on wound healing, as has been observed in patients receiving mTOR inhibitors such as rapamycin.

Survival: Endothelial Resistance to Injury and Persistence

Survival of donor vascular cells despite ongoing graft injury, in particular complement-mediated inflammation, is thought to be an important contributor to chronic rejection. Low concentrations of HLA antibodies induce a cytoprotective program in endothelial cells that prevents lysis upon exposure to high doses of HLA antibody. HLA cross-linking activated the PI3K/Akt signaling axis at low antibody concentrations of antibody, causing upregulation of heme oxygenase-1 (HO-1) and ferritin [25, 26]. Additionally, PI3K/Akt upregulated the anti-apoptotic proteins Bcl-2 and Bcl-xL, as well as induced phosphorylation of Bcl-2-associated death promoter (Bad) [27]. These effects negatively regulate mitochondrial-controlled cell death, by attenuating apoptosis and antagonizing the cytotoxic effects of complement. HLA class II also appears to regulate endothelial cell signaling. Cross-linking of HLA II on IFN-activated endothelium activates intracellular signaling preventing apoptosis [28], which the authors noted was a distinct response of endothelium compared with B cells.

Recent work indicates that accommodation of allografts in the presence of HLA antibodies may not be an indefinite state, however. Several studies have uncovered “indolent,” “subclinical,” or “asymptomatic” inflammatory [29–32] markers in grafts of patients with digital subtraction angiography (DSA) but without current dysfunction. The “natural history of antibodies” proposed by Wiebe et al. [33••] suggests that ongoing inflammation in the graft occurs early after DSA appears, although histological and clinical presentation of graft rejection does not manifest until later. Therefore, the pro-survival signaling cascades initiated by low titers of HLA antibodies may protect endothelial cells from primary injury or death by complement-induced lysis but permit donor endothelial cell persistence that sets the stage for cell growth observed during chronic rejection, described in the next section.

Proliferation: Intimal Expansion and Angiogenesis

Antibodies are significantly associated with chronic rejection and late allograft loss in many solid organs [32, 34]. Chronic rejection manifests generally as perivascular fibrosis and expansion of the intimal layer of the donor vasculature. For example, in transplant vasculopathy of cardiac allografts, donor coronary arteries become occluded, with a hypertrophic endothelial layer invaded by smooth muscle cells, resulting in narrowing of the lumen [35]. Chronic rejection in kidney transplants, transplant glomerulopathy, appears as duplication of the basement membrane and interstitial fibrosis with obliteration of the glomerular and peritubular capillary lumens [36]. In lung allografts, bronchiolitis obliterans results in fibrosis affecting the small airways; pulmonary vascular rejection may also be present [37, 38]. Angiogenic processes appear to be central to formation of vascular lesions in chronic rejection, where vascular remodeling results in new smooth-muscle-lined microvessels [39–42].

In the absence of inflammation or injury, endothelial cells are quiescent and cell growth is static. Chronic inflammation and injury trigger endothelial cell turnover and repair mechanisms. Ample evidence has shown that cross-linking of MHC class I on endothelial and smooth muscle cells triggers increased proliferation in vitro [9, 15, 43, 44] and in vivo [45, 46]. Cell growth is dependent upon proximal activation of RhoA [13], mTOR, S6 kinase and S6 ribosomal protein (S6RP), and ERK [19, 24•]. Activation of these signaling molecules can be readily detected in murine cardiac allografts exposed to anti-donor MHC class I antibodies [43] as well as in cardiac biopsies from patients with donor-specific HLA antibodies [47, 48, 49•]. Indeed, phosphorylated S6RP is a useful marker of ongoing AMR. Pharmacological antagonism of mTOR using the rapalogs sirolimus and everolimus significantly attenuated HLA I antibody-induced endothelial proliferation in vitro [24•]. Interestingly, the capacity of everolimus but not sirolimus to inhibit mTORC2 and ERK1/2 was essential for full prevention of endothelial proliferation. Recently, a novel endogenous regulator of mTOR, called DEPTOR, was described in endothelial cells and may represent a therapeutic target to modulate HLA antibody-induced proliferation [50•]. These results suggest that mTOR inhibitors, particularly those that effectively block mTORC2, may have utility in preventing chronic AMR. Indeed, patients treated with everolimus experienced a slower progression of chronic cardiac allograft rejection (CAV) compared with standard MMF-based immunosuppression [51, 52].

A central stimulator of angiogenic responses is vascular endothelial cell growth factor (VEGF), which is upregulated in the circulation and in graft endothelial cells of patients with vascular lesions [53, 54]. VEGF was critical for neovascularization and neointimal formation in a rat model of chronic rejection [55]. Endothelial cells stimulated with HLA I antibodies upregulate VEGF [56], which may act in an autocrine fashion to enhance endothelial growth. Furthermore, inside-out signaling in endothelium after stimulation with VEGF causes increased vascular permeability through FAK and VE-cadherin, which may allow paracellular leakage of proteins, including immunoglobulin IgG. It is plausible that changes in endothelial permeability permit access of HLA antibodies to the subendothelial space, where they may bind to smooth muscle cells.

Smooth muscle cells contribute to transplant vasculopathy by invading and proliferating in the subendothelial and intimal layer [39]. Recent work demonstrated that HLA I signaling in smooth muscle cells promotes mitogenesis and migration through FAK, Akt, and ERK/12 [15], as well as increased activity of matrix metalloproteinase (MMP) and sphingomyelinases, leading to intimal expansion in a murine xenograft model of human arterial segments [57]. Pharmacological inhibitors of MMPs reduced HLA antibody-associated intimal expansion in vivo, implicating, but not conclusively defining, a role for autocrine signaling by the sphingolipid sphingosine-1-phosphate (S1P) in this process.

Inflammation and Alloimmunogenicity

Accumulation of myeloid cells within the allograft is an important histological marker of antibody-mediated rejection, and macrophages populate the vascular lesions in chronic rejection [39]. In lung allografts, neutrophilic capillaritis is associated with circulating donor-specific antibodies [3], while the macrophage marker CD68 is central to the diagnosis

of antibody-mediated rejection of cardiac allografts [58], and macrophage burden predicts poorer outcome in cardiac and renal transplantation [59, 60]. In addition, molecular diagnostic studies of antibody-mediated rejection of renal allografts have revealed NK cell- and macrophage-associated signatures [29]. NK cell and macrophage accumulation in allografts has been recapitulated in murine models of AMR [45, 61–63].

Endothelial cells are active modulators of the immune response. At the interface between the donor tissue and the recipient immune system, they coordinate recruitment of leukocytes from the blood into sites of inflammation. Endothelial cells store vasoactive mediators, chemokines, and adhesion molecules in intracellular vesicles called Weibel-Palade bodies (WPb) which are rapidly mobilized during type I endothelial activation [64]. Two notable constituents of WPb are von Willebrand factor (vWF), a regulator of clotting, and P-selectin, an initiator of leukocyte tethering to endothelium. Yamakuchi et al. first demonstrated the rapid increase in cell surface P-selectin in response to HLA I cross-linking, which supported increased neutrophil adherence in vitro and in vivo [65]. vWF was also rapidly released into the extracellular space after HLA antibody-induced exocytosis. Calcium-dependent exocytic pathways involving the ATPase *N*-ethylmaleimide sensitive factor (NSF) were critical for this response [65]. vWF staining could be observed in vessels of grafts treated with anti-donor MHC antibody but was lost 7 days after exposure to antibody, suggesting that this process is transient [66]. Monocytes were also recruited through endothelial P-selectin, and antagonism of P-selectin abrogated infiltration of macrophages into murine cardiac allografts [62]. Both vWF and P-selectin can promote platelet aggregation in the microvasculature, and anti-donor MHC class I antibodies induced rolling of platelets in the vessels of an allogeneic skin graft [67]. In addition to their central role in thrombosis, platelets can potently bridge leukocyte tethering to the vessel wall, and in vivo, this may be an important mechanism of efficient monocyte recruitment [68].

There is also evidence that HLA cross-linking causes late phase endothelial cell activation. Stimulation of microvascular endothelial cells with HLA antibodies caused activation of the transcription factors, with subsequent upregulation of cytokines and chemokines [6••, 69, 70]. Evidence from work with IFNγ-activated fibroblasts suggests that HLA II cross-linking also upregulates inflammatory factors [71]; but this has not been clearly defined in endothelial or smooth muscle cells. Abe et al. demonstrated in a murine model of AMR that allografts deficient in the chemokine monocyte chemoattractant protein-1 (MCP1/CCL2) had a reduction in intragraft macrophages [63], highlighting the importance of production of chemotactic factors by the graft during AMR. mTOR antagonism with rapamycin has been shown to inhibit endothelial activation, including adhesion molecule and chemokine production, by TNFα [72]. Therefore, it is possible that mTOR inhibition might prevent HLA antibody-induced transcriptional changes as well.

Emerging literature also supports the function of endothelial cells as semiprofessional antigen-presenting cells capable of stimulating T cell activation [73–75], a capacity that smooth muscle cells, epithelial cells, and fibroblasts lack [73, 76]. Activated endothelial cells (for example, with IFNγ) express, among many other factors, HLA II and are capable of activating T cells through CD58/CD2 interactions. Endothelium is sufficient to activate resting murine memory T cells but not naïve cells, due to a lack of B7.1 and B7.2 (CD80/

CD86) expression [74, 77]. Experimental evidence shows that HLA II-expressing endothelial cells trigger allogeneic CD4 T cell proliferation and promote generation of Th17 and Treg subsets [7, 8•, 78]. Interestingly, rapamycin treatment of endothelial cells resulted in selective expansion of Tregs via PD-L1 and PD-L2 [8•], suggesting that mTOR regulates endothelial alloimmunogenicity. These novel T cell interactions are relevant to antibody-mediated rejection, as the role of CD4 cells in so-called "mixed" AMR is increasingly reported in experimental models [79]. It has also been proposed that HLA antibodies may modulate endothelial immunogenicity [6••], a fascinating avenue of investigation that remains to be fully illuminated.

Why Does HLA Signal in Cells? Understanding the Role of Cross-linking by Trans Associations with Ligands

Although the literature clearly demonstrates that ligation of HLA molecules by antibodies causes intracellular signaling and cell functional changes in vitro and in vivo, it is not generally appreciated in the transplant community why this occurs. Is it an unfortunate phenomenon idiosyncratic to humoral alloimmunity or hijacking of a real biological function of HLA signaling?

Classical Roles of MHC in the Immune System

The physiological role of classical HLA is presentation of antigen and self/non-self-recognition through binding in *trans* (i.e., on the plasma membrane of a different cell) to the T cell receptor (TCR) on T cells or to MHC ligands (e.g., killer cell immunoglobulin-like receptors, KIRs) on NK cells. It is readily apparent from studies of the immunologic synapse that engagement of TCR by MHC presenting cognate peptides causes clustering of MHC, with enrichment at the T cell-antigen-presenting cell (APC) interface [80, 81]. Therefore, *trans* association of MHC molecules with cognate receptors on other cells, such as in the case of biological interactions with the TCR, causes crosslinking of MHC analogous to alloantibody-mediated cross-linking (Fig. 1b).

Dynamic crosstalk at the immune synapse provides bidirectional signaling into both the T cell and the APC regulating APC function as well as lymphocyte activation and differentiation [82, 83]. Many studies have elucidated the "reverse signaling" of MHC (analogous to outside-in signaling) into the APC after engagement by TCRs or antibodies. Engagement of MHC class II causes actin polymerization to facilitate its enrichment at the immunologic synapse, relying on Rho/Rac activation [84, 85]. MHC class II ligation also modulates survival of immature T cells during thymic development and of APCs and B cells [28, 86]. Cross-linking of MHC class I and class II on T cells, B cells, monocytes, and dendritic cells causes activation of protein kinase C (PKC) and increased tyrosine phosphorylation and intracellular calcium. Cell outcomes are cell lineage- and context-dependent, including activation, proliferation, or apoptosis (reviewed elsewhere [86–93]). Thus, in its normal guise, reverse signaling by MHC is thought to be a mechanism of homeostatic regulation of the immune response, which acts on the cytoskeleton and protein tyrosine kinases, to regulate cell survival and growth.

MHC Nonclassical Roles in the Immune System

In addition to their role in antigen presentation of peptides and self to T cells and NK cells, HLA molecules carry out a variety of “nonclassical” functions within and outside of the immune system [93]. Recent work has shown that MHC class II-associated invariant chain (CD74) negatively regulates macrophage and dendritic cell motility through actions on myosin II [94], demonstrating a coupling of MHC-dependent antigen presentation processes with cytoskeletal regulation. MHC class I reverse signaling also negatively regulates the function of NK cells after engagement by as yet unidentified ligands on target cells [95], and cross-linking of MHC class I inhibited cytotoxicity against tumor cells in response to CD16/FcγRIII but not NKG2D stimulation [96•]. Therefore, HLA signaling in immune cells modulates cell function beyond the immunologic synapse.

How Does HLATransduce Intracellular Signals? *Cis* Associations

The cytoplasmic tails of both HLA I and II are relatively short (30–40 amino acids) and lack any known signaling motif. However, the robust intracellular signaling and functional changes induced by engagement of HLA I and II strongly imply a *cis* association with other receptors capable of signal transduction (i.e., lateral interactions of HLA with other transmembrane receptors on the same cell) (Fig. 1b).

Cis-Interacting Partners of MHC

Many lines of evidence have demonstrated that the cytoplasmic domain of MHC is indispensable for transduction of signaling and association with the cytoskeleton after cross-linking [90, 97], including in endothelial cells [98]. Several serine residues and a tyrosine residue in the cytoplasmic domain of HLA I are known substrates for phosphorylation by Src family kinases and PKC [99–101], thought to be important for transport during peptide loading and endocytic recycling [102]. Phosphorylation of the HLA I intracellular domain leads to association with intracellular tyrosine kinases and subsequent HLA I-mediated signaling cascades in APCs [101]. It is possible that HLA also becomes phosphorylated in endothelial and smooth muscle cells after cross-linking, which would permit association with intracellular signaling adaptors, although this has not yet been demonstrated.

Given the similarities between HLA I-induced intracellular signaling and the signaling cascades downstream of integrins and growth factors, Zhang et al. hypothesized that HLA partnered with these receptors and discovered that HLA I associates with integrin β4 at the cell surface of endothelial cells after cross-linking of HLA. This molecular association was dependent upon the cytoplasmic domain of HLA I and was required for antibody-induced phosphorylation of Src, Akt, and ERK, as well as cell migration and proliferation [98]. Whether HLA II associates with integrins or other receptors in endothelial cells has yet to be determined but may be inferred from observations in other cell types.

MHC molecules associate with a variety of tetraspanins in the endosomal compartment and at the plasma membrane in immune cells [103–107]. In addition, it has recently been elucidated that MHC class I ligands called leukocyte immunoglobulin-like receptors (LILRs/ILTs/CD85) interact with MHC molecules laterally, i.e., in the plane of the plasma

membrane on the same cell as well as in *trans* (i.e., as ligands on opposing cells) [108]. Unlike TCRs and KIRs, LILRB proteins appear to have broad specificity for HLA I alleles, as the recognition site lies at the relatively lowly polymorphic α3 domain and the invariant β2 microglobulin [109, 110]. Notably, though, there was a range of affinities of LILRB1 for different alleles of HLA [111], and it is possible that interactions with LILRs vary among individuals with different HLA genotypes.

MHC Role in *Cis* Regulation of Tonic Signaling in the Immune System and Beyond

MHC molecules modulate intracellular signaling downstream of other receptors. For example, HLA I-LILR complexes forming in *cis* dampen activation of mast cells through Fc receptors [112]. Loss of MHC I and II in mice causes unexpected impairment of a variety of biological responses not previously thought to involve these molecules. For example, constitutive MHC class I played an integral role in dampening intracellular toll-like receptor (TLR) signaling in response to endotoxin, preventing sepsis [101]. In contrast, MHC class II was required for TLR signaling in macrophages [113], suggesting that MHC class I and MHC class II might differ in their intracellular signaling functions.

Recent work has elucidated the critical role for MHC in neuronal synapse elimination and pruning during development [114•], at least in the mouse. MHC associates in *cis* with insulin receptors on neurons, hepatic cells, and B cells, forming a molecular complex which modulates insulin receptor-dependent signaling and glucose uptake [115, 116]. Complex formation of MHC class I with insulin receptor is thought to tonically inhibit insulin receptor signaling in neuronal pruning [117] and to downmodulate myeloma cell survival in the presence of low levels of insulin.

We also uncovered an unexpected requirement for HLA I in integrin β4-mediated signaling. Surprisingly, endothelial cells lacking HLA I exhibited impaired integrin β4-dependent angiogenic functions, including intracellular signaling, proliferation, and migration [98]. Given the role of integrin β4 in angiogenesis [118], HLA I molecules may act in concert with integrin β4 to promote tumor progression [119]. These results add to the list of unforeseen functions of HLA in modulating intracellular signaling by other receptors and suggest that the association between HLA I and integrin β 4 is a physiological norm.

Conclusions

HLA molecules display diverse functions both within and without the immune system. Alloantibodies to HLA in the context of transplantation mimic the physiological clustering of HLA molecules, as at the immunologic synapse, to induce “reverse signaling” in donor vascular cells that is reminiscent of modulation of APC function and survival. Cross-linking of HLA by alloantibodies stimulates tyrosine kinase cascades leading to cytoskeletal rearrangement, endothelial resistance to complement-mediated lysis, and increased survival proteins, proliferation, and leukocyte recruitment and may modulate alloimmunogenicity to T cells. In addition, recent evidence points to novel roles for HLA molecules in the regulation of intracellular signaling downstream of many other cell surface receptors, including CD16/FcγRIII in NK cells, integrin β4 during angiogenic responses of endothelial cells, TLR-dependent responses in monocytes, and insulin receptor during synaptic pruning

and hepatocyte glucose regulation. Taken together, the literature demonstrates the important and active role that HLA molecules play in transduction of intra-cellular signaling, mechanisms which are misappropriated in donor vascular cells upon engagement by donor-specific HLA antibodies, leading to graft injury.

Acknowledgments

The authors would like to thank KAT and AR for critical review of this manuscript. This work was supported by the National Institute of Allergy and Infectious Diseases Grant RO1 AI 042819 and NIH U018I077821.

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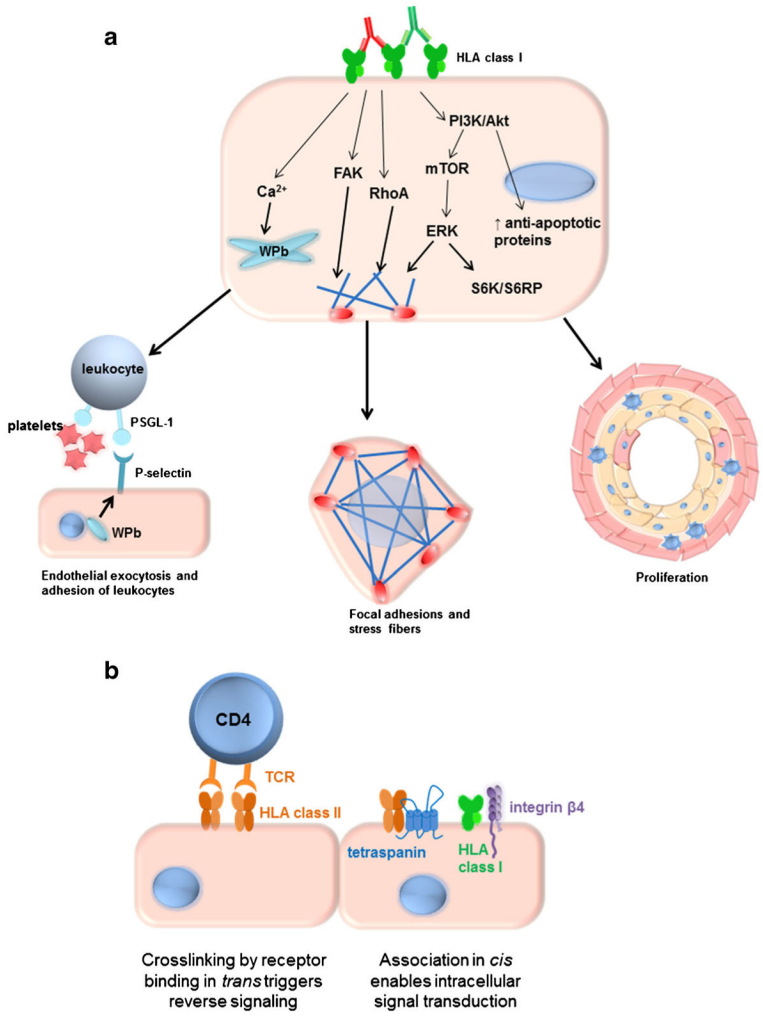

Fig. 1.

a Cross-linking of HLA I on the surface of endothelial and smooth muscle cells activates intracellular signaling programs that promote the mechanisms of graft injury. Intracellular calcium is released in endothelium, which triggers exocytosis of Weibel-Palade body (WPb) vesicles containing P-selectin. Rapid upregulation of P-selectin at the cell surface is sufficient to support adhesion of neutrophils, monocytes, and platelets, which likely contributes to the predominance of intragraft myeloid cells, such as CD68+ macrophages and neutrophils, during antibody-mediated rejection. HLA signaling also promotes activation of FAK, which facilitates formation of focal adhesions, and activation of RhoA, mTOR, and ERK, which act on the actin cytoskeleton to stimulate stress fibers. Stress fiber formation is dramatic and extends across the cell. mTOR and ERK are also key regulators of proteins involved in translation, S6 kinase (S6K) and S6 ribosomal protein (S6RP), and of cell growth. Activation of the PI3K/Akt axis causes upregulation of pro-survival proteins. Together, proliferative, survival, and migratory signaling likely contributes to chronic vascular rejection and intimal hyperplasia. **b** Schematic diagram of HLA binding in *trans* to receptors on opposing cells, such as the TCR on T cells, and of HLA association in *cis* with tetraspanins and integrins on the same cell. *Trans* interactions are likely the physiologically

relevant mechanisms of HLA cross-linking that leads to reverse signaling into the cell, pathways which become hijacked and inappropriately activated by bivalent alloantibody during antibody-mediated rejection. Associations in *cis* are required for HLA, which has no known signaling motifs, to transduce signaling through coreceptors, such as integrin β4 in endothelial cells