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HLA class I antibody-mediated endothelial and smooth muscle cell activation

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Abstract

Purpose of review—Advances in immunosuppression and patient management have successfully improved 1-year transplant outcome. Unfortunately, antibody-mediated rejection is a major barrier to long-term graft survival. This study summarizes the effects of antibodies on endothelial cell and smooth muscle cell (SMC) migration, proliferation and leukocyte recruitment, emphasizing the intracellular signaling pathways that orchestrate these distinct functional outcomes.

Recent findings—Several studies have provided further insight into the effects of human leukocyte antigen (HLA) class I antibodies on vascular cells. We found that HLA I molecules partner with integrin $\beta 4$ to transduce proliferative signaling, and identified proteins that associate with the cytoskeleton after HLA class I crosslinking. Natural killer cells have been strongly implicated in a murine model of donor-specific major histocompatibility complex I antibody-triggered neointimal thickening. A recently developed human arterial graft model revealed the role of matrix metalloproteinases in SMC mitogenesis by HLA class I antibodies. Using a donor transgenic for HLA-A2, Fukami *et al.* investigated the mechanisms of accommodation induced by low titers of HLA class I antibodies.

Summary—Ligation of HLA class I molecules with antibodies leads to the activation of intracellular signals in endothelial cells and SMCs, which in turn promote actin cytoskeletal remodeling, survival, proliferation, and recruitment of leukocytes.

Keywords

antibody-mediated rejection; HLA class I; integrin $\beta 4$; natural killer cells

INTRODUCTION

Antibody-mediated rejection (AMR) remains a major obstacle to the long-term survival of solid organ transplants [1]. Numerous studies have shown that the production of donor-specific antibodies to human leukocyte antigen (HLA) is associated with increased chronic rejection and graft loss in patients with heart, lung, small bowel, or kidney transplants [2–4]. The hallmark of chronic rejection is transplant vasculopathy, which is characterized by increased intimal thickness of the vessels of the allograft. Histologically, the vessels of the graft display smooth muscle cell (SMC) proliferation accompanied by subendothelial

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Conflicts of interest

The authors have no conflicts of interest to disclose.

lymphocytes and macrophages [5,6]. The endothelium is located at the interface between the allograft and recipient blood and is directly targeted by HLA class I antibodies. Given the strong association between the development of donor-specific HLA antibodies with inferior graft survival and function, it is important to understand the mechanisms underlying HLA antibody-mediated injury of the endothelium in the vasculature of the solid organ. Historically, it was suggested that HLA antibodies caused endothelial injury mainly through complement activation [7]. Detection of the complement split product complement component 4d (C4d) in the graft by immunohistochemical staining is frequently used to diagnose AMR, and to demonstrate HLA antibody binding to the graft. However, C4d is not always an ideal marker, as several studies have shown that C4d deposition was not present in all grafts with AMR [8–10]. Murine models of AMR also demonstrated that transplant vasculopathy can develop in response to major histocompatibility complex (MHC) class I antibodies that are not complement fixing or in complement-deficient allograft recipients [11]. These findings suggest HLA class I antibodies may cause endothelial injury through other mechanisms.

LIGATION OF CLASS I MOLECULES ON ENDOTHELIAL CELLS STIMULATE ACTIN CYTOSKELETON REMODELING

The cytoskeleton consists of actin microfilaments, microtubules, and intermediate filaments. These structures provide the framework needed for cell motility, organelle transport, and cell division [12]. Crosslinking of HLA class I molecules by anti-HLA antibodies activates Rho signaling triggering reorganization of the cytoskeleton and formation of F-actin stress fibers [13]. Coupel *et al.* [14] showed upregulation of the guanosine-5'-triphosphate (GTP)-binding protein RhoA and its association with stress fibers following antibody ligation of class I molecules on endothelial cells. RhoA mediated phosphoinositide 3-kinase (PI3K) dependent endothelial cell proliferation [14]. Both Rho GTPase and Rho kinase are involved in class I-mediated stress fiber formation and phosphorylation of focal adhesion kinase (FAK) and paxillin [13].

FAK is a cytoplasmic protein kinase that discretely localizes to regions of the cell that attach to the extracellular matrix called focal adhesions. FAK regulates cell survival, proliferation, and migration, and therefore plays a critical role in wound repair, atherosclerosis, and cancer. Ligation of HLA class I on endothelial cells results in phosphorylation of FAK and Src and subsequent activation of paxillin [15,16]. The phosphorylation of Src and paxillin and the translocation of paxillin into focal adhesions following class I ligation were markedly decreased by small interfering RNA (siRNA) knockdown of FAK [15].

Proteomic studies were conducted to gain novel insights into signal pathway utilization during actin remodeling induced by class I antibodies and compared this with other agonists including thrombin and basic fibroblast growth factor [17^{''}]. Analysis by tandem mass spectrometry revealed unique cytoskeleton proteomes for each treatment group. Using annotation tools, a candidate list was created that revealed 12 proteins that were unique to the HLA class I stimulated group. Interestingly, 11 of 12 of the candidate proteins were phosphoproteins and exploration of their predicted kinases showed that 35 kinase families could be responsible for their activation. Among the kinase predictions, cyclin-dependent kinase 2 (CDK2) has the potential to phosphorylate three of the candidate proteins. CDK2 has already been established as a regulator of HLA class I signal transduction [18]. Another kinase family predicted to be involved in the phosphorylation of the candidate proteins is 70-kDa S6 protein kinase (p70S6k) family in which the specific kinase was ribosomal protein S6 kinase 1. HLA class I ligation leads to the phosphorylation of p70S6k and S6 ribosomal protein, which are downstream of mammalian target of rapamycin (mTOR) [19]. The importance of ribosomal protein S6 kinase 1 in class I signaling has not been

established, yet it has the potential to phosphorylate two of the 12 candidate proteins – ATP-dependent RNA helicase DDX3X and nuclear pore complex protein Nup153. Nup153 is crucial for nucleoskeleton and cytoskeleton architecture maintenance and is necessary for cell cycle progression and cell migration [20]. TPM4 was another protein identified in the HLA class I treated group and may regulate HLA class I-induced cytoskeleton remodeling downstream of extracellular regulated kinases (ERK). The eIF4A1 protein identified in this study functions downstream of mTOR complex 1 following class I ligation to promote translation and cell proliferation [17²²,21].

These in-vitro findings are supported by in-vivo studies. Mice treated with antidonor MHC I antibodies showed increased phosphorylation of the proteins involved in the HLA class I-mediated proliferation signaling pathway on the endothelium of the cardiac allograft [19]. Phosphorylation of S6 ribosomal protein (S6RP), a protein involved in the HLA class I proliferation signaling pathway was increased in biopsies from patients diagnosed with AMR, and was suggested to be a more sensitive marker of AMR than C4d [22]. These findings indicate that HLA class I antibodies have the capacity to activate proliferation signaling in cultured cells *in vitro*, in allografts of experimental models, and in patients with AMR.

HLA CLASS I ANTIBODIES INDUCE PROLIFERATION SIGNALING IN ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS

Investigation of the signaling networks involved in endothelial cell and SMC proliferation has identified FAK as a key regulator of this process. Antibody crosslinking of HLA I leads to rapid activation of Rho GTP and phosphorylation of Src and FAK in endothelial cells [13–15,23]. Src activation triggers the PI3K/AKT signaling axis, which is a key regulator of cell proliferation and survival. Phosphorylation of PI3K/AKT activates mTOR, a serine/threonine kinase. mTOR exists in two distinct protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is composed of raptor, mTOR, and mLST8, and stimulates protein synthesis and cell proliferation via activation of S6K, S6RP, and 4EBP1 [21]. mTORC2, which contains mTOR, rictor, mLST8 and Sin1, is a central regulator of cell survival and the cytoskeleton, as well as of ERK1/2 in the HLA class I-mediated signaling pathway [24]. Treatment of endothelial cells with HLA class I antibodies increases the phosphorylation of cyclic adenosine monophosphate (cAMP) response element-binding protein, a nuclear transcription factor that regulates cell proliferation, survival and differentiation [25].

FAK also plays an important role in SMC proliferation and migration [26²⁷]. Transplant vasculopathy is caused by proliferation of both endothelial cells and SMC, which is evident from proliferating cell nuclear antigen staining in chronic rejection lesions [27,28]. Recently, Li *et al.* [26²⁷] showed that, similar to endothelial cells, HLA class I antibodies stimulated cell proliferation and migration in SMC. Class I ligation increased phosphorylation of FAK, AKT, and ERK1/2 in SMC. Knockdown of FAK by siRNA attenuated class I-induced phosphorylation of AKT and ERK1/2, as well as cell proliferation and migration. These findings demonstrate the central role of FAK in HLA class I antibody-induced cellular proliferation.

Passive transfer of HLA class I antibodies in murine recipients of human mesenteric arteries led to neointimal thickening [27]. Matrix metalloproteinases (MMPs) are necessary for SMC migration and proliferation by regulating growth factor availability and by modulating cell–cell contacts [29]. Galvani *et al.* [30³¹] showed that HLA I antibody stimulation of SMC triggers proliferation by upregulation of the MMP2/neutral sphingomyelinase-2 pathway. MMP2 was required for SMC proliferation *in vitro* and in the human arterial grafts, as

therapy with MMP inhibitors prevented alloantibody-provoked neointimal thickening [30*]. These results suggest that MMP2 is involved in vascular remodeling leading to HLA I antibody-induced transplant vasculopathy.

HLA I PARTNERS WITH INTEGRIN β 4 TO ELICIT ENDOTHELIAL CELL PROLIFERATION AND MIGRATION

The HLA I molecule has no known signaling motif, suggesting that HLA I molecules associate with other coreceptor(s) to transduce signals into the cell. Integrins are cell adhesion molecules that have the capacity to transduce signals regulating cell migration, proliferation, and survival. Integrin β 4 features a long cytoplasmic tail that interacts with FAK and Src to transduce cell proliferation and survival signals [31,32]. Given the similarity between the signals stimulated by integrin β 4 ligation and HLA class I antibodies, we postulated that HLA class I molecules partner with integrin β 4 to transduce intracellular signals. We found that ligation of HLA class I with antibodies prompted complex formation between integrin β 4 and HLA class I [33]. Furthermore, knockdown of integrin β 4 with siRNA prevented cell proliferation and protein phosphorylation of Src, ERK, and AKT induced by HLA I antibodies. These findings suggest HLA class I antibodies cause transplant vasculopathy by stimulating endothelial cell proliferation and migration via integrin β 4 signaling. The role of integrin β 4 in transplant vasculopathy is corroborated by the association between the elevated expression of integrin β 4 in the thoracic aortic endothelia and atherosclerosis [34].

HLA CLASS I ANTIBODIES STIMULATE CELL SURVIVAL AND PROMOTE ACCOMMODATION

The presence of circulating donor-specific HLA antibodies may not always indicate ongoing rejection or graft dysfunction. Some patients, as many as 24%, maintain good graft function in spite of detectable titers of donor-specific HLA antibodies [35]. Several in-vitro studies indicate possible mechanisms whereby HLA class I antibodies promote transplant accommodation in which the graft acquires resistance to AMR. It was shown that whereas HLA class I antibodies cause endothelial cell death at high concentrations, at low concentrations HLA class I antibodies confer resistance to complement induced cell death by upregulation of cell protection genes such as heme oxygenase 1 (HO-1) and activation of cAMP-dependent protein kinase A [36,37]. Ligation of HLA class I with antibodies also activates the PI3K/AKT pathway and increases endothelial anti-oxidant responses, which protect endothelial cells from complement-induced death via HO-1 and ferritin H [38,39]. Additionally, the PI3K/AKT signaling pathway triggered by HLA class I antibodies at low doses results in inactivation of the proapoptotic factor B-cell lymphoma 2 (Bcl-2) antagonist of cell death and increased expression of antiapoptotic proteins Bcl-2 and B-cell lymphoma-extra large *in vitro* [23,37]. Furthermore, biopsies from patients with AMR showed elevated Bcl-2 expression, indicating that donor-specific HLA antibodies activate these pathways in patients [23]. The importance of Bcl-2 and HO-1 in transplant accommodation is demonstrated by a HLA-mismatched murine model in which the recipient was heterotopically transplanted with a heart from a human HLA-A2 transgenic mouse [40*]. The expression of Bcl-2 and HO-1 was upregulated in the HLA-A2 transgenic allograft after pretreatment with low titer of HLA class I antibody (W6/32). Exposure of the graft to low titer of HLA class I antibodies prolonged graft survival in the recipient sensitized against HLA-A2 antigens. Moreover, knockdown of Bcl-2 with siRNA led to loss of protection induced by pretreatment with low titer of HLA class I antibodies [40*], demonstrating the critical role of Bcl-2 in cell survival.

HLA I ANTIBODIES INDUCE LEUKOCYTE RECRUITMENT

Endothelial cells contain specialized storage vesicles called Weibel–Palade bodies, which harbor pre-formed von Willebrand factor (vWF), P-selectin, and other vascular mediators. Exocytosis of Weibel–Palade bodies is regulated by calcium or Toll-like receptor signaling [41]. HLA class I ligation with antibodies also triggers Weibel–Palade body exocytosis, externalizing vWF and increasing P-selectin expression on the cell surface. Upregulation of P-selectin on the cell surface causes increased adherence of leukocytes to endothelial cells. Cross-linking of HLA class I appears to be critical in the ability of HLA class I antibody to activate exocytosis, because only bivalent F(ab')₂ fragments of HLA class I antibodies are effective in triggering exocytosis, whereas monovalent Fab fragments cannot activate vWF release. Furthermore, injection of F(ab')₂ fragments of HLA class I antibodies into severe combined immune deficiency/beige mice engrafted with human skin causes vWF release and neutrophil influx in the grafts [42]. In a similar study, the release of vWF in cardiac allografts transplanted into immunoglobulin knockout mice was observed only after treatment with donor-specific MHC I antibodies [43]. The recruitment of neutrophils induced by HLA class I antibodies does not appear to be limited to endothelial cells, as ligation of HLA class I on epithelial cells also leads to the recruitment of neutrophils. Using a mouse model of obliterative airway disease, Saini *et al.* [44] showed that administration of MHC I antibodies into the mouse native lung increased neutrophil infiltration. Moreover, treatment of endothelial cells with HLA class I antibodies stimulated the production of monocyte chemoattractant protein-1 and neutrophil chemo-attractant growth-related oncogene α (KC), which attract macrophages to the endothelium [45].

Antibodies engage the innate immune system by interacting with the Fc receptor Fc γ RIIIa on natural killer (NK) cells through the Fc fragment. NK cells recognize antibody-coated cells and induce rapid apoptosis in target cells via the granzyme exocytosis pathway [46]. Hirohashi *et al.* demonstrated that MHC I antibodies cause transplant vasculopathy in recombination activating gene knockout recipients of cardiac allografts even when the antibodies were of noncomplement fixing isotypes or allograft recipients were deficient in complement [11,47^{***}]. In contrast, transplant vasculopathy failed to develop when the recipient mouse was depleted of NK cells or carried the γ -chain knockout mutation, thus, lacking mature NK cells. The importance of NK cells to AMR was substantiated clinically by the finding of enriched NK cell transcripts in kidney biopsies with AMR [48]. HLA class I antibodies may activate NK cells through other pathways besides the Fc receptor as well. For example, recruitment of human NK cells from the blood by endothelium is dependent on the adhesion molecule P-selectin [49,50]. Thus, increased P-selection expression stimulated by HLA class I antibodies may enhance the adhesion of NK cells to endothelium. Further, HLA class I antibodies may cause the activation of NK cells by blocking the interaction of HLA class I on endothelial cells with killer cell immunoglobulin-like receptors (KIRs) on NK cells. NK cells are not activated if the inhibitory KIR is engaged with the specific HLA class I ligand [51]. Blockade of interaction between HLA class I and KIR by class I antibodies might increase the NK cell cytotoxicity to endothelial cells by relieving KIR-mediated inhibition. Interestingly, depletion of natural killer cells alone did not prevent rejection in a mouse cardiac allograft model, suggesting that either antibodies or T cells in the adaptive immune system are needed for NK cells to mediate allograft rejection [52].

CONCLUSION

HLA class I antibodies activate endothelial cells and SMC through a variety of signaling pathways (Fig. 1), which may contribute to AMR. Elucidation of these pathways will help identify potential therapeutic targets to treat AMR. For example, inhibition of mTOR signaling may prevent HLA class I antibody-induced proliferation in endothelial cells and

SMC. It has been shown mTOR inhibitor rapamycin prevents endomyocardial remodeling after heart transplantation in human patients [53]. Recently, several studies demonstrated that HLA class I antibodies can modify the activity of leukocytes, such as macrophages, neutrophils, and NK cells. These findings will hopefully stimulate more interest in the role of crosstalk between HLA antibodies and the innate immune system in rejection.

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- of special interest
- of outstanding interest

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KEY POINTS

- HLA class I antibodies stimulate survival and proliferation of signaling pathways in endothelial cells and SMCs.
- HLA class I antibodies mediate actin cytoskeleton reorganization in endothelial cells that is functionally linked to cell proliferation.
- HLA class I partners with integrin $\beta 4$ to transduce signals into the cell.
- HLA class I antibodies promote leukocyte recruitment by induction of endothelial cell exocytosis and upregulation of adhesion molecules at the cell surface.

FIGURE 1.

HLA class I antibodies activate endothelial cells through several mechanisms. Ligation of HLA class I with antibodies increases its association with integrin $\beta 4$, which in turn triggers signaling cascades regulating cell proliferation, survival, and migration. Treatment of endothelial cells with HLA class I antibodies also leads to the release of von Willebrand factor and upregulation of P-selectin that increase the recruitment of leukocytes, such as neutrophils. In addition, HLA class I antibodies can activate NK cells through Fc receptor-mediated signals. ERK, extracellular regulated kinases; FAK, focal adhesion kinase; HO-1, heme oxygenase 1; KIR, killer cell immunoglobulin-like receptor; mTORC1, mammalian target of rapamycin complex 1; NK, natural killer; S6RP, S6 ribosomal protein; vWf, von Willebrand factor.