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# The Mechanisms of Monocyte Recruitment by Graft Endothelium in Response to HLA I Antibody Binding

A dissertation submitted in partial satisfaction of

the requirements for the degree Doctor of Philosophy

in Cellular and Molecular Pathology

by

Nicole Marie Valenzuela

2012

ABSTRACT OF THE DISSERTATION

The Mechanisms of Monocyte Recruitment by Graft Endothelium

in Response to HLA I Antibody Binding

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Doctor of Philosophy in Cellular and Molecular Pathology

University of California, Los Angeles, 2012

Professor Elaine F. Reed, Chair

Successful organ transplantation is challenged by the recipient's immune responses against the donor's polymorphic HLA molecules. The humoral response produces antibodies against mismatched HLA class I and class II molecules, which have a significant negative impact on long-term graft survival. Antibody-mediated rejection is characterized by intragraft macrophages, which may be elicited by HLA I antibody-induced endothelial cell activation. Given that the presence of intragraft macrophages significantly correlates with poor graft outcome, we investigated the mechanisms of HLA I antibody leading to recruitment of monocytes by endothelial cells, to determine effective therapeutic strategies to reduce infiltration into the graft. First, we determined the effect of endothelial cell activation by crosslinking of HLA I. Using an in vitro system with human endothelial cells stimulated with murine monoclonal HLA I antibodies, we found that HLA I

ligation triggers endothelial exocytosis via calcium signaling, leading to increased cell surface P-selectin, which was required for increased monocyte adherence. We confirmed these results using an in vivo murine model of antibody-mediated rejection, where monocyte infiltration was significantly increased in allografts treated with donor specific MHC I antibodies. Macrophage accumulation in the graft could be prevented by therapy with the P-selectin antagonist rPSGL-1. Next, we explored the contribution of antibody-Fc receptor interactions to recruitment. Adhesion was stimulated by HLA I antibodies through P-selectin, irrespective of the isotype of HLA I antibody. Further, HLA I antibodies of an isotype with a high affinity for human Fc receptors elicited significantly more monocyte binding, and predominantly utilized FcγRI. Firm adherence to ICAM-1 on the endothelium required monocyte Mac-1 but not LFA-1. Finally, we investigated the activation of monocytes to a pro-adhesive state. Monocytes required intact calcium, Src and PI3 kinase signaling to adhere to HLA I antibody-treated endothelial cells, but not Rho kinase. Crosslinking of the P-selectin receptor PSGL-1, or of Fcγ receptors, triggered stable adhesion to ICAM-1. Deposition of low titer HLA I antibody on cytokine-activated endothelial cells enhanced monocyte binding, likely due to antibody-Fc receptor interactions. To conclude, we have elucidated the mechanisms of how HLA I antibody binding to endothelium promotes monocyte recruitment.

The dissertation of Nicole Marie Valenzuela is approved.

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2012

# DEDICATION

I would like to dedicate this work to my mother, who taught me that your only limitations are those you impose on yourself; to my father, who taught me that any problem can be solved, as long as you have the right tool for the job; and to my husband, who taught me to say "It is what it is," and who has been alternately my sounding board, cheerleader and support these last years. To all my friends and family, for your encouragement, thank you.

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# CHAPTER 1:

# HLA Antibodies and Cellular Proliferation

## 1.1 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.

## 1.2 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 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*).

**1.3 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.

### 1.4 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.

### 1.5 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.

## 1.6 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 (FcγR) 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*).

### 1.7 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.

**Figure 1-1**

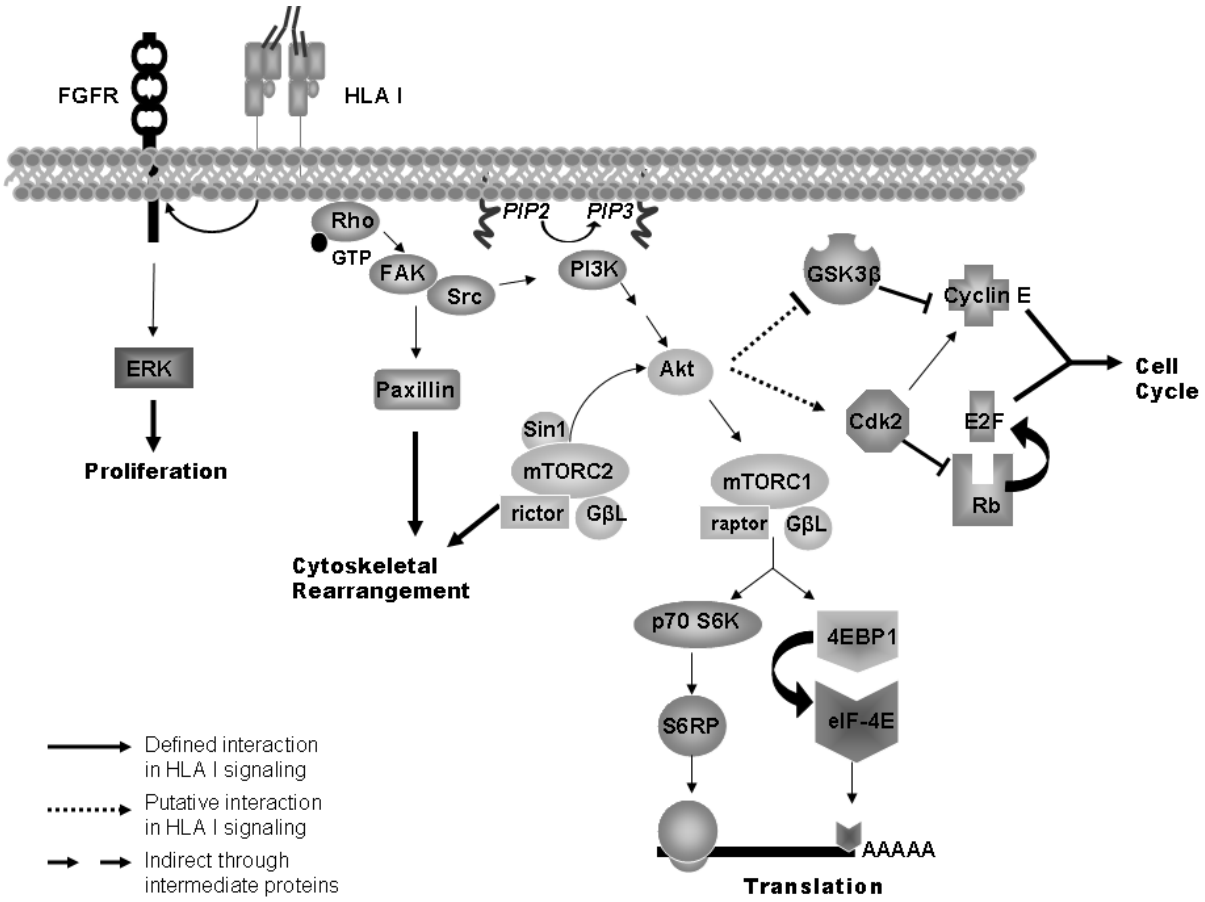


**Figure 1-1**. Crosslinking of HLA class I molecules by antibodies induces cell signaling pathways which promote cell growth. FAK activates paxillin, an important regulator of the cytoskeleton. FAK activation also permits complex formation with Src. PI3K-dependent activation of Akt through PDK1 is central 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 1-2**

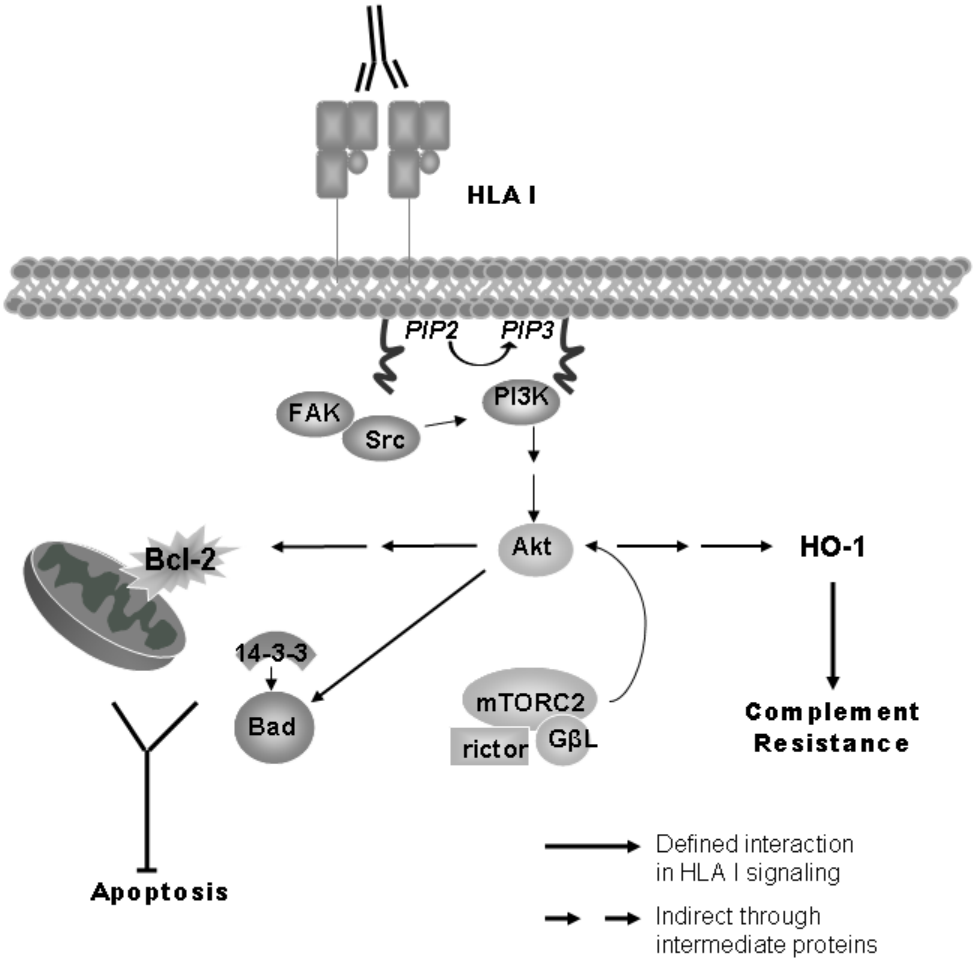


**Figure 1-2**. Crosslinking of HLA class I molecules with antibodies activates Akt, which promotes cell survival. PI3K catalyzes phosphorylation of $PIP_2$ to yield $PIP_3$ which can interact with a discrete region known as the pleckstrin homology domain. Akt and PDK1 localize to the membrane in response 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 1-3**

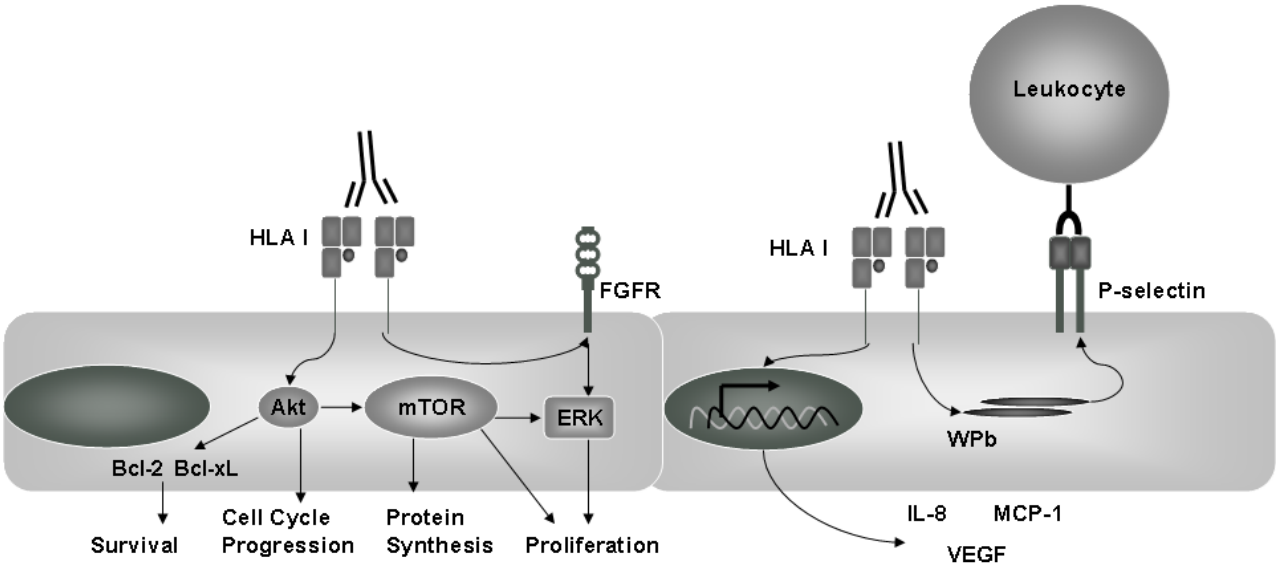


**Figure 1-3**. Three-step model of the Fc independent effects of HLA class I antibodies on vascular endothelium. HLA class I molecule ligation immediately activates cell signaling pathways which cause cell cycle progression, promote cell survival and protein synthesis, and ultimately induce cellular 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.

**Table 1-1.** 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*)<br>Regulates EC proliferation and apoptosis (*92*) | Initiator of coagulant cascade<br>Leukocyte diapedesis<br>Promotes fibrin deposition | Px with TV had higher expression in endomyocardial biopsy (*95*)<br>Early biopsy staining predictive of TV (*96*) | Upregulated in rat coronary intima and adventitia, of EC and mononuclear cell origin (*93*)<br>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<br>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*)<br>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<br>SMC chemoattractant (*125-127*) | Regulates vascular permeability (*128*)<br>Induces EC adhesion molecules (*129-131*)<br>Monocyte chemokine (*132*) | Upregulated in biopsies from patients with TV (*14, 98*) | VEGFR antagonism reduced TV and decreased mononuclear cell recruitment (*100*)<br>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*)<br>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*) |

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# CHAPTER 2:

# Antibodies in Transplantation: The effects of HLA antibody binding and mechanisms of injury.

**Abstract**

Until relatively recently, allograft rejection was thought to be mediated primarily by alloreactive T cells. Consequently, immunosuppressive approaches focused on inhibition of T cell mediated activity. While short-term graft survival has significantly improved and rejection rates have dropped, acute rejection has not been eliminated and chronic rejection threatens long-term graft survival (by ten years post-transplant, approximately half of all solid organ transplants will have failed). Increased attention to humoral immunity in both experimental systems and the clinic has revealed that donor specific antibodies can mediate and promote acute and chronic rejection. Here, we detail the effects of alloantibody, especially HLA antibody, binding to graft vascular and other cells, and briefly summarize the experimental methods used to assess such outcomes.

## 1. Clinical Frequency and Relevance of Donor Specific Antibodies

### 1.1 Frequency and Association with Outcome

Sensitization to polymorphic proteins, especially HLA class I and HLA class II molecules, occurs when a patient is exposed to cells from other individuals, due to pregnancy, transfusion or transplantation. Antibodies to donor antigens, called donor specific antibodies (DSA), can be directed against polymorphic HLA class I, HLA class II or minor histocompatibility molecules such as MICA and MICB, or against non-polymorphic endothelial, epithelial or cardiac targets. The estimated frequency of preexisting or de novo donor specific HLA antibodies among transplant patients ranges from as low as 4% to more than half (1). Based on OPTN data as of March 2012, 18.7% of renal transplant recipients had panel reactive antibodies (PRA) against greater than 10% of all HLA alleles, with 5% of all patients being highly sensitized (>80% PRA) (2). Everly et al. found that 31% of patients developed DSA *de novo* (3). In one study of renal transplant patients, 30% had HLA antibodies of any specificity, and 30% of antibody-positive patients had HLA antibodies of

donor specificity (4, 5). Another study found that 35% of renal patients had donor specific HLA IgG (6), while in yet another report DSA was found in as many as 57% of renal recipients (7). The rates of sensitization are obviously higher in patients awaiting a second renal graft, where only 19.3% of patients had negative (<4%) panel reactive antibodies (PRA) for HLA I, with 14.5% of patients on the waitlist for a second transplant having very high PRA (>60%). Here, 55% were also positive for antibodies against HLA II (8).

Despite the variation in reported estimates of donor specific antibody prevalence, the literature clearly illustrates that donor specific antibodies adversely affect the survival of a transplanted organ. Numerous studies have linked the presence of preexisting or *de novo* antibodies to poor graft outcome. For example, renal recipients with positive panel reactive antibodies (PRA recognizing >10% of HLA alleles) or with donor specific antibodies have lower graft survival at 3 and 5 years post-transplant (1, 2, 9). Accordingly, patients diagnosed with antibody mediated rejection are highly likely to have donor specific HLA antibodies (10), and episodes of AMR predispose recipients to lower renal allograft survival (3), regardless of whether antibodies were preexisting or produced late after transplantation (from 83% survival without HLA antibodies to 49% with HLA antibodies) (4). In a very large study with more than one thousand patients, half of those with failed grafts had HLA antibodies, and 21% had donor specific HLA antibodies (5). If desensitization or other therapy effectively reduced DSA levels, long-term survival was significantly superior than if the DSA was persistent (11).

The effects of donor specific antibody on graft survival are not restricted to renal transplantation. Heart allograft patients with DSA also experience lower graft survival, especially if the antibodies appear after 1yr post-transplant (12). Further, the presence of HLA specific DSA and the incidence of AMR correlate with chronic rejection in the heart (TCAD) (13, 14). Even if patients are asymptomatic, HLA-DSA significantly lowers freedom from CAV compared to those without

DSA (14). HLA or MICA DSA, or non-donor derived EC antigens correlated with chronic transplant rejection in the heart (TCAD) (13). Finally, DSA is a strong risk factor for rejection episodes in small bowel transplantation (15, 16).

Only a few reports could not find an association between outcome and HLA-DSA. A conflicting study found that acute rejection in renal could not be predicted by DSA (17), and in another CAV incidence did not correlate with DSA but rather with T alloreactivity (18).

1.2 Diagnosis of Antibody-Mediated Rejection

Antibody mediated rejection is distinct entity from, but can occur concurrently with, cell-mediated rejection. In kidney and heart transplantation, consensus criteria have been established for the histological characteristics and diagnosis of antibody-mediated rejection. In renal transplantation, a diagnosis of antibody-mediated rejection is called for if there is poor graft function, evidence of complement deposition (C4d) in the peritubules of the graft and donor specific antibodies in the circulation (19). Intravascular macrophages, endothelial cell swelling, C4d staining and donor specific HLA antibodies indicates antibody-mediated rejection in cardiac transplantation (20).

## 2. Experimental Techniques to Measure Effects of Antibodies

Given the strong association of HLA antibodies with inferior graft function and survival, it is crucial to understand the mechanisms of HLA antibody-mediated graft injury. A variety of experimental models are available in which to test the effects of HLA antibodies binding to cells of the graft. The first is a simplified system with cultured graft cells (endothelium, smooth muscle or airway epithelium), where cell signaling can be dissected in detail and specific functional changes can be analyzed. The more complicated but more physiological system utilizes *in vivo* transplantation of immunodeficient recipients that lack B and T cells, reconstituted with donor specific antibodies. Finally, mechanisms uncovered by experimental models can be confirmed in human biopsies. A

brief description of the common methods is given immediately below. A more detailed description of each *in vitro* assay is provided at the end of the chapter.

## 2.1 *In Vitro* Techniques

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

### 2.1.1 Measurement of HLA Antibody Binding Capacity

The actual amount of HLA I antibody on a donor cell can be measured using flow cytometric methods and normalized against fluorescent beads. In this way, concentration can be related to target expression and uniformly measured from machine to machine and across acquisition settings. This technique is also useful when comparing two monoclonal antibodies with differing affinity or two targets with differing expression on the cell surface.

### 2.1.2 Analysis of Intracellular Signaling

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

### 2.1.3 Determination of Cell Growth

Cellular proliferation can be measured by several techniques, which involve either radioactive substrates or flow cytometry. Our lab uses a flow cytometric assay exploiting the dilution of fluorescent vital dyes during cell division to measure proliferation.

2.1.4 Measurement of Leukocyte Adherence

Leukocyte adherence is measured by an adhesion assay in which endothelial monolayers are stimulated and fluorescently labeled lymphocytes or myelocytes are overlaid. Adherent cells are counted using fluorescence microscopy.

2.1.5 Determination of Cytoskeletal Changes and Cell Migration

Migration of cells is assessed by the scratch, or wound healing, assay. Cells are cultured, a wound is introduced by scratching the plate with a pipet tip, and closure of the wound in the absence or presence of stimulants is measured after 24 hours using microscopy. Cytoskeletal changes are monitored by immunofluorescent staining of the actin cytoskeleton using phalloidin and visualized by fluorescent microscopy.

2.1.6 siRNA and Pharmacological Inhibitors

Once a signaling molecule has been identified, its significance to downstream phosphorylation or functional events is determined by pharmacological inhibitors or siRNA against the protein of interest.

2.2 *In vivo* Models of Antibody-Mediated Rejection

Animals deficient in T cells are incapable of mounting a complete humoral response, due to the requirement of antibody-secreting B cells for T cell help during maturation and isotype switching. Therefore, an alternative system was required to assess antibody mediated rejection in the absence of cell-mediated alloimmunity. Murine models are particularly useful because they allow genetic manipulation of putative targets, using knockout or transgenic animals. Russell et al. first described the model in which alloserum alone is sufficient to elicit allograft rejection (21). In general,

a murine or rat immunodeficient recipient, such as severe combined immunodeficiency (SCID) or recombinase activating gene-1 (RAG1) knockout mice, is used, which lacks T cells and B cells, but retains an intact innate immune system, including monocytes, natural killer (NK) cells, and neutrophils. Recipients transplanted with an MHC mismatched organ are passively transferred with alloserum from sensitized animals or with monoclonal donor specific antibodies. This system has advanced knowledge about antibody-mediated rejection in the physiological setting. Such models have uncovered direct evidence that donor specific MHC antibodies are sufficient to trigger acute and chronic rejection, and yielded some insights into the mechanisms of antibody induced graft injury. Alternatively, human tissue can be transplanted onto an immunodeficient murine recipient, and anti-HLA antibodies or human immune cells are administered (22-24).

There are several important considerations to bear in mind when selecting an animal model of antibody-mediated rejection. The first is the availability of reagents, particularly of donor specific monoclonal antibodies. Secondly, chronic rejection in the mouse varies in location and severity of disease from model to model and from mouse to human. It is likely that monoclonal MHC antibodies are not potent enough to produce fulminant vascular lesions, whereas MHC alloserum can (25). Finally, it is obligatory to point out the caveat that the murine and human systems differ in important ways. For example DQ is not found in the mouse and mice do not have the same FcγR system. Finally, murine anatomical differences result in rejection models that are not always physiologically analogous to the clinical setting—i.e. the location of chronic vascular lesions.

2.3 Patient Biopsies

The final step in translational studies of the effects of HLA antibodies on the graft is to assess changes to transplanted organs during antibody mediated rejection in humans. *In vitro* findings are confirmed in patient biopsies using immunohistochemical techniques to detect total protein expression or phosphorylation status in the graft.

## 3. Mechanisms of Injury: Fc-dependent Effects of Antibodies

The effects of HLA antibody binding to graft vascular cells are manifold, and depend on both the canonical antibody functions mediated by the Fc portion of the molecule, and on crosslinking of the target molecule present on donor cells. Such Fc-dependent functions occur irrespective of the target protein or receptor.

### 3.1 Hyperacute and Acute Rejection

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

#### 3.1.1 Complement

The complement cascade is a complex system consisting of proteases that become sequentially activated upon cleavage. There are three pathways of complement, which mediate immunity against different immunologic threats and are activated by different signals. The classical pathway bridges the adaptive and innate immune responses by partnering with substrate bound antibodies. The lectin pathway mediates immunity against bacterial and fungal pathogens by recognizing mannan carbohydrate motifs. The alternative complement pathway is effective against cellular pathogens and facilitates opsonization. For the purposes of this review, we will restrict our discussion to the classical pathway. In humans, IgM, IgG1 and IgG3 are effective activators of complement. IgG or IgM on the cell surface binds to the low affinity, multivalent C1q molecule, which once "fixed" cleaves C2 and C4. The resultant cleavage products C2a and C4b complex to a protease capable of catalyzing activation of C3, which is a central mediator in all three complement pathways. This event triggers the production of C3b, which in the classical pathway activates C5, yielding the C5a and C5b fragments. C5a is an important inflammatory factor, which acts as a chemoattractant for monocytes.

Local synthesis of C5b at the C1q-antibody site causes complex formation of C5b with C6, C7, C8 and C9 into the macromolecule known as the membrane attack complex (MAC). MAC forms transmembrane pores which cause lysis of the target cell by disrupting the plasma membrane. Cells may resist complement-induced lysis by expressing CD59, which inhibits C5b binding to C9. In addition to the MAC, complement split products have a myriad of other functions, including chemoattraction, viral neutralization, opsonization, immune complex clearance, and activation of macrophages and other cells (reviewed (26)).

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

Complement is an important mediator of acute antibody-mediated rejection as well. Animal models have demonstrated that donor specific MHC antibodies are sufficient to mediate acute rejection of cardiac and corneal allografts (27, 28). Histologically, interaction of donor specific antibody with the graft is detected by deposition of the complement split product C3d or C4d, which is generally a fair indicator of antibody injury. Allograft bound complement split products are only found in murine models of rejection when DSA is also present (29). A majority of renal patients with donor specific HLA antibodies had peritubular deposition of C4d (1) and likewise all patients with C4d positive chronic renal rejection had donor specific HLA antibodies (30), which associated with inferior graft survival. However, C4d may not be an ideal marker, as several reports suggest that C4d deposition may not have sufficiently reliable predictive value for graft outcome (31, 32).

Antibody subclass and isotype define the effector functions of the molecule, including complement fixation. The literature on the pathogenicity of an antibody with respect to its isotype is conflicting. In murine models, IgKO allograft recipients have significantly longer graft survival than their wild-type counterparts. Acute rejection could be restored by passive transfer of donor MHC antibodies of the complement fixing isotype murine IgG2b but not the noncomplement fixing isotype mIgG1 (33). HLA I antibodies in patients reportedly are predominantly IgG1 (34), but can be of any of the four IgG isotypes (35). Recently, an assay has been developed which can identify both the specificity and the complement fixing ability of HLA antibodies by binding of C1q on single antigen beads. In several studies, C1q positive donor specific antibodies highly correlate with antibody mediated rejection in cardiac and renal transplantation when compared with antibodies identified only by IgG (36). Thus, C1q positive antibodies have a positive predictive value for early episodes of antibody-mediated rejection (37). It is postulated that noncomplement fixing antibodies will have a lower pathogenicity; indeed, three patients who were transplanted across high titer anti-donor HLA II antibodies had long-term AMR-free survival, possibly because the preexisting antibodies were of noncomplement fixing isotypes IgG2 and IgG4 (38). In contrast, another group reported that, while IgG-DSA positive patients had a significantly lower graft survival, there was no significant difference when groups with C1q-fixing and non-C1q fixing DSA were compared (6). Similarly, antibodies detected using flow PRA could predict early rejection episodes, while CDC-identified antibodies could not (39). Particularly when DSA was at lower titers, complement fixing ability could not predict early antibody-mediated rejection (35, 40).

Moreover, the clinical relevance of an HLA antibody's capacity to fix complement in chronic rejection is less defined. Murine models of AMR have demonstrated that chronic rejection lesions can develop in response to MHC antibodies that are not complement fixing or in complement deficient allograft recipients (41). Taken together, the literature suggests that complement fixation,

while important, may not be the only factor influencing the pathogenesis of a donor specific antibody.

3.1.2 ADCC

Antibodies bridge the innate and adaptive arms of the immune system by interacting with natural killer (NK) cells through the FcγRIII (CD16), which binds the constant Fc tail of the antibody. NK cells recognize antibody-coated cells and cause cell lysis. Although the general mechanism of antibody-dependent cell-mediated cytotoxicity is understood, there is limited evidence about ADCC in alloimmune responses. Antibodies from sensitized patients increased the lytic capacity of CD3-CD16+ (NK) peripheral blood mononuclear cells against renal epithelium (42). Long ago it was recognized that a positive ex vivo ADCC reaction using patient sera containing alloantibody bound to target endothelial cells was a risk factor for transplant rejection (43, 44). NK cell cytotoxicity against HUVEC could also be facilitated by IgG1 non-HLA anti-endothelial cell antibodies (45). Although this topic has cooled over the last decade, recent reports implicating NK cells in MHC antibody-mediated chronic rejection in the mouse (46, 47) and in human antibody-mediated rejection (48) will hopefully excite more interest in this area.

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

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

4.1 Survival and Accommodation

The presence of circulating donor specific HLA antibodies may not always indicate ongoing rejection. Some patients, as many as 24%, maintain good graft function despite detectable titers of HLA-DSA, in a poorly defined state known as transplant accommodation (5). Little is understood

about accommodation, but it is expected that antibody titer may be an important factor. For example, a high titer of antibody in renal patients (cutoff of 4487 MFI for class II) correlated with and accurately predicted AMR incidence (7), suggesting that symptomatic rejection has a threshold for amount of antibody to which the graft is exposed. However, the persistence of DSA on its own is a strong predictor of late graft loss independently of AMR episodes; thus accommodation does not indicate indefinite graft survival, and likely there are ongoing subclinical events in response to alloantibody that damage the graft and result in chronic rejection. For example, asymptomatic AMR cardiac recipients had lower 5 year freedom from chronic rejection than those without any AMR (14), suggesting that accommodation is better defined as silent antibody-mediated graft damage that slowly causes failure.

Several *in vitro* studies indicate a possible mechanism for HLA I antibody-mediated transplant accommodation, where the graft is resistant to acute antibody-mediated damage, including complement activation and apoptosis. One group reported that while HLA I antibodies from patient sera or monoclonal antibodies trigger endothelial cell death at high concentrations, at low concentrations HLA I antibodies confer resistance to complement induced cell death by inducing heme oxygenase (HO-1) and activating cAMP-dependent protein kinase A (PKA) (49, 50). HLA I antibodies also increase endothelial Nrf-2 mediated antioxidant responses, which provide protection of endothelial cells from complement induced death via HO-1 and ferritin H (51). Additionally, treatment of HLA I antibody at low doses increases PI3K/Akt signaling, which results in inactivation of the proapoptotic factor Bad and increased expression of apoptosis inhibitors Bcl-2 and Bcl-xL *in vitro* (52). These observations were confirmed in a murine transplant model, where Bcl-2 and HO-1 were upregulated in an HLA-A2 transgenic allograft by pretreatment with low titer of HLA I antibodies, which prevented subsequent rejection by high titers of HLA I antibodies (53).

Further, biopsies from patients with AMR have elevated Bcl-2 expression (52), demonstrating that donor specific HLA antibodies activate these pathways in the clinical setting.

4.2 Cell Proliferation

Chronic rejection, or transplant vasculopathy, is a proliferative disease, in which the vessels of the graft become occluded by a severely thickened intima invaded by smooth muscle cells. Donor specific HLA antibodies are strongly associated with vasculopathy (CAV) in heart and kidney transplantation (13).

*In vitro*

There is strong evidence that ligation of HLA I by antibodies triggers proliferation and cytoskeletal changes in vascular cells. Rapidly following HLA I stimulation, Rho-GTP, Src and focal adhesion kinase (FAK) become activated (54, 55). Src activation triggers the PI3K/Akt signaling axis, which is a key regulator of survival and cell cycle progression. Activation of Akt promotes the activity of mammalian target of rapamycin (mTOR), a serine/threonine kinase which exists in two complexes. mTORC1, composed of raptor, mTOR, and GβL, targets S6 kinase (S6K), S6 ribosomal protein and 4EBP1, molecules involved in protein synthesis which become phosphorylated after HLA I ligation (56). mTORC2, which contains rictor, mTOR, GβL, is a central regulator of the cytoskeleton as well as of extracellular regulated kinases (ERK1/2) (57, 58). HLA I crosslinking activates the mitogen-activated protein kinases ERK1/2, which promote cell proliferation through a parallel pathway. Consequently, stimulation of endothelial cells and smooth muscle cells with HLA I antibody causes increased cellular proliferation in the absence of other exogenous growth factors (59-61). Interestingly, HLA I antibody stimulation of smooth muscle cell growth was dependent on the activation of matrix metalloproteases/sphingolipid signaling, a stress-induced pathway (62).

Because HLA I molecule has no known signaling motif, we postulated that HLA I associated with a coreceptor to carry out its proximal signaling requirements. Using immunoprecipitation, we

discovered a molecular association between HLA I and integrin β4 which was required to activate downstream signaling and cell proliferation (61).

In addition to regulating signaling molecules central to cell cycle progression and proliferation, HLA I ligation causes dramatic reorganization of the actin cytoskeleton. FAK mediates cytoskeletal changes by acting on paxillin. We observe increased stress fiber formation and association of a variety of proteins with the cytoskeleton after treatment of endothelial cells and smooth muscle cells with HLA I antibody (59, 63, 64). Further, HLA I antibody increases cell migration and wound healing (59). These cytoskeletal rearrangements are relevant to cell migration and proliferation, as well as endothelial cell permeability.

These observations of the direct effects of HLA I antibody are not restricted to vascular cells. Stimulation of lung epithelial carcinoma with HLA sera from patients increases proliferation and tyrosine phosphorylation (65), pointing to a mechanism by which HLA antibodies may provoke BOS.

*In Vivo*

The experimental evidence linking MHC I antibodies and cellular proliferation *in vivo* is emerging. Initially the proliferative changes observed in chronic rejection were thought to be T cell mediated (25). However, when murine cardiac allograft recipients were depleted of T cells after generation of MHC alloantibodies, the grafts still developed arteriosclerosis to a greater extent than those without DSA (21). Further, in murine recipients deficient in B cells but with an intact T cell immune response, the allograft did not progress to vasculopathy (21), suggesting that the humoral response was required for full chronic rejection. In a seminal study, transfer of allosera to SCID recipients reproduced these obstructive lesions (21), results which have been confirmed more recently using donor specific MHC I monoclonal antibodies (41, 47). Further, vasculopathy is elicited by human antibodies in human grafts. SCID mice were transplanted with human mesenteric

arteries and passively transferred with HLA I antibody. The investigators observed increased neointimal thickening in response to HLA I antibody in this study (62). These data demonstrate that humoral immunity is sufficient to produce vasculopathy.

Importantly, MHC I antibodies elicited vascular lesions in RAG knockout recipients of cardiac allografts even when the antibodies were of noncomplement fixing isotypes or allograft recipients were deficient in complement (41, 46). These results strongly indicate that MHC I antibodies mediate chronic rejection via a complement-independent mechanism and are consistent with the *in vitro* work demonstrating that HLA I crosslinking directly triggers proliferation and cell signaling.

We confirmed HLA I-induced cell signaling *in vivo*. Allografts from RAG knockout mice reconstituted with anti-donor MHC I antibody had significantly increased phosphorylated Akt, mTOR and S6K compared with the control group (66). Further, our group investigated endothelial cell signaling downstream of HLA I crosslinking in patient cardiac biopsies. We found that phosphorylated S6 ribosomal protein (S6RP) was increased in AMR patients and was a more sensitive marker of AMR than C4d (67). Therefore, activation of signaling molecules which promote proliferation occurs in both animal models of antibody-mediated rejection and in the clinical setting.

4.3 Induction of Secondary Factors

In addition to direct activation of intracellular signaling cascades, *in vitro* experiments have revealed that HLA I antibodies increase sensitivity to and production of soluble mediators with the potential to promote autocrine proliferative signaling. HLA I crosslinking rapidly increases cell surface expression of fibroblast growth factor receptor (FGFR) on endothelial cells. The proliferative response to bFGF is significantly enhanced by sensitization with HLA I antibodies (68-70). Stimulation of HLA I also triggers endothelial cell production of cytokines which may have a secondary effect on cell growth. For example, HLA I antibodies increase endothelial production of vascular endothelial growth factor (VEGF), which activates cells in an autocrine manner via

VEGFR2 (71). Treatment of lung airway epithelial cells with HLA I antibodies increased PDGF, IGF-1, and bFGF production, which promoted paracrine proliferation of fibroblasts (72). Therefore, HLA I activation of endothelial cells increases sensitivity to bFGF and production of other soluble mediators which increase cell proliferation.

### 4.4 Leukocyte Recruitment

#### 4.4.1 Infiltration in Antibody-Mediated Rejection

In addition to antibody-induced complement deposition, antibody mediated rejection is frequently characterized by intravascular macrophages (73), which can promote both acute and chronic rejection (74, 75). Further, NK cells are required for CVR lesion development in the murine AMR model (46). Thus, recruitment of immune cells into the allograft facilitates innate cell-mediated injury.

#### 4.4.2 HLA I Antibody-Induced Leukocyte Recruitment

Endothelial cells contain intracellular rod-shaped vesicles called Weibel-Palade bodies, which contain preformed von Willebrand Factor, P-selectin, and other vascular mediators. Release of these vesicles, which fuse with the cell membrane and secrete contents into the extracellular space, is regulated by calcium or cAMP-dependent secretagogues, such as thrombin, histamine, or forskolin [reviewed in (76)]. Recently, it was reported that HLA I ligation by monoclonal murine antibody triggers exocytosis of these vesicles, externalizing vWF and increasing cell surface P-selectin. HLA I antibody-induced P-selectin caused increased adherence of the promyelocytic cell line HL60 *in vitro*. Further, treatment of SCID mice engrafted with human skin with the monoclonal HLA I antibody *in vivo* also caused vWF externalization and neutrophil influx (77). In a similar study, wild-type murine cardiac recipients had evidence of vWF release and P-selectin expression by allograft endothelium during acute rejection which was absent in IgKO recipients (33). In a murine lung transplant model, treatment with MHC antibodies increased neutrophil infiltration and inflammation

(78). Thus, MHC or HLA I antibody binding to endothelial cells causes rapid endothelial cell activation, promoting recruitment of leukocytes.

The clinical relevance of this pathway to AMR has not been definitively elucidated; however, in human cardiac biopsies, capillary endothelium displayed increased expression of vWF during rejection (79, 80). In a variety of murine allograft models, selectin deficiency in the donor graft prolongs graft survival and reduces cellular infiltration (81-83), suggesting that HLA I antibody-induced P-selectin and/or vWF may be a rational therapeutic target to decrease immune cell recruitment.

4.5 Therapies suggested from experimental evidence

The knowledge gained from the experimental systems described above reveals potential therapeutic targets to alleviate the proliferative and inflammatory effects of HLA I antibodies. For example, we and others found that HLA I crosslinking on endothelial cells activated RhoA, which was necessary for proliferation *in vitro* (54, 64). Inactivation of Rho and Rho Kinase by pharmacological inhibitors in animal models reduces neointimal thickening: in a rabbit model of vein graft, intimal thickening was inhibited by fasudil (ROCK inhibitor) (84); a murine model of chronic cardiac rejection, intimal thickening was suppressed by fasudil (85), while another Rho kinase inhibitor, Y-27632, prolonged survival, reduced infiltration and prevented intimal thickening (86).

We also reported that mTOR is a central signaling molecule in HLA I-induced cellular proliferation (57). Pretreatment of endothelial cells with rapamycin, an mTOR inhibitor, reduced HLA I Ab-triggered endothelial proliferation, Akt phosphorylation and Bcl-2 expression (57). Recent reports suggest that rapamycin and its analogs (everolimus, sirolimus) may have clinical therapeutic potential. For example, everolimus therapy prevented remodeling after heart transplantation in human patients (87). The exact mechanism of protection from rejection conferred

by rapamycin is not understood, but mTOR inhibition in endothelial and smooth muscle cells may prevent HLA I antibody-induced proliferation.

Other therapies for antibody mediated rejection may include adhesion molecule antagonists to reduce leukocyte infiltration and complement inhibitors to prevent complement-mediated tissue injury. In a presensitized primate renal allograft model, complement inhibition reduced AMR and prolonged allograft survival (88). Use of eculizimab, a monoclonal antibody which blocks activation of C5, reduced antibody-mediated rejection incidence in patients with DSA (89). Therefore, the progress which has been made in understanding the mechanisms of HLA I antibody-mediated graft injury leads to rational clinical therapies in the treatment and prevention of AMR.

## 5. HLA II Antibodies

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

### 5.1 Association with outcome

Antibodies to HLA II frequently accompany chronic rejection in renal transplants (90) and correlate with AMR incidence in the absence of a positive T cell crossmatch (7, 91). Positive PRA for HLA II significantly correlates with worse graft outcome, in addition to PRA of HLA I (90).

### 5.2 Limitations to Studying HLA II in model systems

Investigation of the effects of HLA II antibodies on graft cells has been limited by its restricted expression pattern and by constraints in experimental systems. HLA II is not expressed on endothelial cells of most vascular beds, with the exception of renal microvasculature, but it can be upregulated after inflammatory insult (92-95). Indeed, several reports have demonstrated HLA II

expression on endothelia in grafts (80, 96), which suggests that transplantation or allogeneic attack itself may upregulate MHC II expression as reported in murine cardiac allografts (97-99).

*In vivo* studies on MHC II are further constrained by the different expression pattern of MHC II in mice, where normal cardiac tissue does not express MHC II molecules but which may upregulate these molecules after transplantation (98, 99). Similarly, in human heart, HLA-DR is increased during rejection (80). Human microvascular endothelia have been found to express HLA II, but cultured endothelial cells rapidly lose constitutive expression. HLA II can be reinduced by treatment of endothelium with cytokines such as TNFα or IFNγ. In our hands, however, cytokine treatment introduces a layer of complexity with respect to cell activation that masks many intracellular signals, upregulates a variety of other factors, and thus obscures analysis and conclusions. There is, therefore, a need for an *in vitro* system in which the effects of HLA II antibodies on vascular cells can be investigated without additional confounding factors.

5.3 Mechanisms

Due to the limitations discussed above, the consequences of crosslinking of HLA II on vascular cells are not well defined. Some insight may be inferred from data regarding HLA II engagement on other cell types. In B lymphocytes and antigen presenting cells, HLA II ligations triggers increase in intracellular calcium and tyrosine phosphorylation, with varying functional effects. Cells become activated and proliferate or undergo apoptosis depending on which intracellular pathway is predominantly activated (100-102). Fibroblasts stimulated through HLA II increase production of prostaglandin E (103), RANTES, IL6, MCP-1, and GRO (104, 105), through Janus kinase (JNK) and FAK (106). These HLA II-induced soluble factors produced by fibroblasts could promote proliferation of endothelial cells (104). However, little to no data is available to establish how HLA II crosslinking on endothelial or smooth muscle cells might impact rejection.

## 6. Non-HLA Antibodies

A new appreciation of non-HLA antibodies has arisen due to reports of antibody-mediated rejection or C4d deposition in the absence of circulating donor specific HLA antibodies. Non-HLA antibodies in transplantation can be directed against either polymorphic or nonallelic proteins. The development of antibodies against nonpolymorphic targets may be due to upregulation during inflammation, in response to transplantation, or when proteins are exposed to the immune system during injury. It is thought that the intragraft microenvironment or rejection may break humoral tolerance to autoantigens (107). Moreover, the indication for renal transplantation is often autoimmunity, where patients may be predisposed to humoral responses against self antigens.

### 6.1 Frequency and Outcome

Non-HLA antibodies are commonly found in transplant recipients. Le Bas-Bernadet reported that nearly half of renal patients who had donor specific antibodies to HLA also had non-HLA anti-endothelial cell antibodies (AECA) (108). Another study found anti-endothelial cell antibodies in 23 percent of renal patients, which associated with a greater rate of cellular rejection and lower graft function (109). Similarly, a significantly higher rate of AECA positivity was found in patients with failed renal transplants compared to those with functioning grafts (110). Non-HLA antibodies binding to airway epithelial cells were detected in lung transplant patients with chronic rejection, bronchiolitis obliterans syndrome (BOS), but not in patients without BOS (111). One group identified endothelial cell-binding IgM and IgG using flow cytometry in about half of cardiac transplant patients with TCAD and in patients with failed renal allografts. Interestingly, they did not find such antibodies in pretransplant patients or controls (112). Another group reported that non-HLA antibodies were found at a higher rate in cardiac and renal transplant recipients with or without rejection than in normal or waitlisted subjects (113), suggesting that these antigens become immunogenic during transplantation. Also remarkable is the observation that the array of non-HLA

antigens recognized by the alloimmune response varies from patient to patient, with only a few markers overlapping (114).

Several of the non-HLA ligands have been identified. Nonclassical histocompatibility antigens are frequent ligands for the alloimmune humoral response. The major histocompatibility class I related chain (MIC) genes are non-HLA proteins with some homology to HLA class I molecules. MICA and MICB do not associate with β2 microglobulin and function as ligands for the NK cell receptor NKG2D rather than the T cell receptor. Although not nearly as polymorphic as HLA molecules, MICA and MICB have more than 30 alleles each. Antibodies to MICA and MICB are found in renal, as well as cardiac transplant recipients, where they associate with TCAD/CAV (10, 13, 115).

Other targets of antibodies in transplantation include minor histocompatibility antigens, vascular receptors, adhesion molecules and intermediate filaments (reviewed in (13, 116)). Using immunoprecipitation and mass spectrometry, Qin et al. revealed the nucleolar protein nucleolin as a target of non-HLA antibodies in transplant patients (113). Neuropilin, a cell surface coreceptor for VEGFR; heterogenous nuclear ribonucleoprotein K; an intermediate filament protein; ribosomal protein L7; and CD36, a scavenger receptor for oxidized LDL and other lipoproteins, have all been identified as ligands for non-HLA endothelial cell antibodies in patients with chronic cardiac allograft rejection (TCAD) (114, 117-119). Antibodies to nondonor derived antigens, particularly vimentin and cardiac myosin (115, 120), are present in a majority (65%) of cardiac transplant patients with chronic rejection and correlate with CAV incidence (13, 121). In renal transplantation, antibodies recognizing glutathione-S-transferase (GST), a cytosolic enzyme which metabolizes toxins, were found in patients experiencing antibody-mediated rejection (122, 123).

6.2 Mechanistic Studies/Experimental Models

Not much is known about the mechanism of graft injury by non-HLA antibodies. The clinical relevance and pathogenic mechanism of antibodies to intracellular proteins, such as GST, vimentin and ribonucleoprotein, is chiefly unclear. Such antibodies may represent a marker for injury or humoral alloreactivity rather than having independent pathogenic potential. Non-HLA antibodies against cell surface markers may fix complement or mediate ADCC. Moreover, considering the agonistic function of an antibody when it crosslinks its target, the mechanism of action may depend on the signaling capacity and biological function of the ligand. For example, angiotensin II receptor AT1 autoantibodies are well-established agonists, which promote hypertension and may contribute to renal allograft rejection (124-126). Antibodies from autoimmune sera upregulate adhesion molecules and cytokines on endothelial cells (127-129), but when the target is unknown the precise mechanism remains elusive. To date, little has been studied on the actions of non-HLA antibodies during the humoral alloimmune response.

In some cases, non-HLA antibodies do not cause injury. For example, rat allosera lacking MHC antibodies caused only minor complement-mediated cytotoxicity against rat endothelial cells when compared with MHC specific allosera (130, 131). Further, C3 deposition was only observed in the cardiac allograft vasculature of an MHC-mismatched rat model but not in a rejection model with non-MHC alloserum production (131). A possible explanation may be that the isotype of non-HLA antibodies is different. One clinical study found non-HLA antibodies predominantly of the noncomplement fixing isotypes IgG2 and IgG4 (109). Further, as mentioned above, the signaling capacity of the target molecule may shape the effect of antibody binding. For example, anti-MICA antibodies did not increase neointimal thickening of human mesenteric arteries grafted in SCID mice, while HLA I antibody was sufficient to elicit lesions (60).

In contrast, other studies have suggested that non-HLA antibodies have pathogenic potential. Anti-airway epithelial cell antibodies isolated from patients induced calcium increase, proliferation

and tyrosine phosphorylation in airway epithelium, although the cell surface ligands remain unknown (111). Anti-donor antibodies produced by rat cardiac recipients could induce apoptosis of rat vascular endothelial cells (14). Antibodies to nucleolin or unidentified endothelial targets also caused apoptosis of human endothelial cells (108, 113). Although nucleolin is primary localized to the nucleus, this protein was detected on the cell surface in proliferating endothelial cells (113), suggesting that some intracellular targets of the humoral response may be exposed under certain conditions. Moreover, mice sensitized against vimentin experienced rejection, C3d deposition and P-selectin expression of the cardiac allograft, effects which were specifically mediated by antibodies to vimentin (132).

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

## 7. Conclusions

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

## 8. METHODS

Determination of Antibody Binding Capacity

Purpose: To measure the actual amount of antibody bound to the target cell surface. Fluorescence intensity in flow cytometry is influenced by antibody concentration/titer, antibody affinity (monomorphic or polymorphic epitopes), and expression level on the target cell. Further, fluorescence intensity may vary from machine to machine and with changes in machine settings, thus the goal is to normalize antibody binding across these variables.

General Method: A standard curve of fluorescence is generated using MESF or Simply Cellular beads incubated with the primary and secondary antibodies of interest. These beads enable correlation of mean fluorescence intensity (MFI) on stained cells to the actual number of antibodies bound to each cell, by generating an f/p ratio, which is the ratio of the fluorescence of a bead to the defined constant number of binding sites on that bead.

Endothelial cells are detached and incubated with HLA antibody in PFA flow buffer (PBS+ 0.1% sodium azide + 1% FBS) on ice. Cells are washed, and fluorescently conjugated secondary antibody is added in flow buffer on ice. Cells are washed and fluorescence is measured on a flow cytometer. The antibody binding capacity is determined by dividing the MESF value of the cell sample by the f/p ratio determined using the Simply Cellular beads.

Detection of Protein Phosphorylation by Western Blotting

Purpose: To measure cell signaling and phosphorylation cascades activated by HLA I ligation.

General Method:

Endothelial cells are grown and starved overnight with 0.2% FBS without endothelial cell growth serum. Cells are stimulated with HLA I antibody or positive control such as FBS for defined time period. Medium is removed and cells are washed once with ice cold sodium orthovanadate in PBS.

Cells are lysed in the plate on ice, with buffer containing phosphatase inhibitors, and scraped. The lysate is centrifuged to remove DNA and other debris. The lysate is separated by SDS-PAGE, then transferred onto a PVDF membrane overnight at 4°C. The membrane is blocked with 5% milk in TBST or other blocking buffer, and probed with phosphorylation specific antibodies for Western Blot (Cell Signaling is an excellent source). Phosphorylation is detected by an HRP conjugated secondary antibody and chemiluminescent substrate, measured on X-ray film or on a digital system. The membrane is stripped and blotted for total protein and a house-keeping protein such as actin for equal loading and normalization purposes.

Important Considerations: Starvation is necessary to reduce background levels of signaling. Lysis buffer may need to be optimized depending on the localization of the target protein (ex MLC is membrane-associated).

Measurement of Cell Division by Flow Cytometry

Purpose: To measure cellular proliferation induced by HLA I stimulation.

General Method:

Endothelial cells are labeled with the vital dye CFDA-SE (CFSE), plated and starved overnight. Cells are treated with HLA I antibody or positive control such as FBS in starvation medium. After two days, cells are detached and fluorescence is measured by flow cytometry. A shift of the histogram to the left indicates increased cell division due to dilution of the dye, which is analyzed using FlowJo or ModFit (Verity) to yield the proliferation index.

Important Considerations: Starvation is necessary throughout the assay to eliminate nonspecific stimulation by growth medium.

Cell Migration

Purpose: To measure migration of cells in the presence of HLA I antibodies.

In the in vitro wound healing assay, cell monolayers are scratched with a pipet tip and treated with HLA I antibodies or other stimulants for 24 hours, then stained with Giemsa or DAPI Stain to ease visualization. Wound closure is measured by calculating the number of migrating cells in the wound area or the percent closure of the area compared with control samples.

Detection of Cytoskeletal Changes

Purpose: To determine changes in the cytoskeleton in response to HLA I antibodies.

General Method:

Endothelial cells are cultured to subconfluence and starved for two hours, then treated with HLA I antibodies or positive control thrombin for 10min. EC are fixed, permeabilized, and directly stained with Texas-Red Phalloidin to visualize the actin cytoskeleton. Cells are analyzed using fluorescence microscopy.

Adhesion of Leukocytes to the Endothelial Monolayer

Purpose: To measure leukocyte recruitment by endothelium stimulated with HLA I antibodies.

General Method:

Endothelial cells are cultured to a confluent monolayer in a 24 or 48 well plate. Primary monocytes or monocytic cell lines (such as THP-1 or Mono Mac 6) are fluorescently labeled with a dye such as CFSE for 10min in HBSS with calcium and magnesium, then serum rescued and washed. Inhibitors can be added. Endothelial cells are stimulated with HLA I antibodies or positive control such as thrombin or TNF in M199 with 2% FBS. Medium is removed and monocytes are overlaid on the endothelial cell monolayer at a target ratio of 3 monocytes per 1 endothelial cell for 20min at 37C. Nonadherent cells are washed off three times by gently pipetting down the side of each well and

decanting. The coculture is fixed and at least 5 fields per well are collected by fluorescence microscopy. The number of adherent fluorescently labeled monocytes is counted using automated software, and averaged across fields for each condition.

Important Considerations: The washing technique is critical. If antibody-Fc receptor interacts are desired to be eliminated, we recommend generating an F(ab')2 fragment of the HLA I antibody, or alternatively blocking Fc receptors on the monocyte using a saturating concentration of human IgG. The isotype of any neutralizing antibodies should have low to no affinity for human Fc receptors, as it can otherwise result in nonspecific blockade of Fc receptors.

In general, we recommend using automated software such as ImageJ or CellProfiler (MIT) for analysis of microscopy data. While fields may be counted or scored by hand, automated quantification is critical to ensure objectivity. For example, CellProfiler has excellent counting algorithms that can effectively distinguish single cells even in dense clusters.

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# CHAPTER 3:

# HLA Class I Antibody Mediated Endothelial and Smooth Muscle Cell Activation

**3.1 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 report 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 HLA class I (HLA I) antibodies on vascular cells. We found that HLA class I molecules complex with integrin β4 to transduce proliferative signaling, and identified proteins that associate with the cytoskeleton after HLA I crosslinking. NK cells have been strongly implicated in a murine model of donor specific MHC I antibody-triggered neointimal thickening. A recently developed human arterial graft model revealed the role of MMPs in SMC mitogenesis by HLA I antibodies. Using a donor transgenic for HLA-A2, Fukami et al. investigated the mechanisms of accommodation induced by low titers of HLA I antibodies.

*Summary*

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

### 3.2 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 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 proliferation accompanied by subendothelial 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 C4d in the graft by immunohistochemical staining is frequently used to diagnose antibody mediated rejection, and to demonstrate HLA antibody binding to the graft. However, C4d is not always an ideal marker, as several reports 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 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.

### 3.3 Ligation of class I molecules on EC stimulates 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. showed upregulation of the GTP-binding protein RhoA and its association with stress fibers following antibody ligation of class I molecules on EC [14]. RhoA mediated PI3-kinase (PI3K) dependent EC proliferation [14]. Both Rho GTPase and Rho-kinase (ROK) 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 ECs 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 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 to other agonists including thrombin and bFGF [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, 11/12 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, where 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 ERK. The eIF4A1 protein identified in this study functions downstream of mTOR complex 1 to phosphorylate 4E-BP1 following class I ligation to promote translation and cell proliferation [17, 21].

These in vitro findings are supported by in vivo studies. Mice treated with anti-donor 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 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.

### 3.4 HLA class I antibodies induce proliferation signaling in endothelial cells and smooth muscle cells.

Investigation of the signaling networks involved in EC and smooth muscle cell proliferation have identified focal adhesion kinase as a key regulator of this process. Antibody crosslinking of HLA I leads to rapid activation of Rho-GTP and phosphorylation of Src and focal adhesion kinase (FAK) in EC [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 S6 kinase (S6K), S6 ribosomal protein (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 extracellular regulated kinases (ERK1/2) in the HLA class I mediated signaling pathway [24]. Treatment of EC with HLA class I antibodies increases the phosphorylation of cAMP response element binding protein (CREB), a nuclear transcription factor that regulates cell proliferation, survival and differentiation [25].

FAK also plays an important role in smooth muscle cell proliferation and migration [26] . Transplant vasculopathy is caused by proliferation of both EC and SMC, which is evident from PCNA staining in chronic rejection lesions [27, 28]. Recently, Li et al. showed that, similar to EC, HLA class I antibodies stimulated cell proliferation and migration in SMC [26]. 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 smooth muscle cell migration and proliferation by regulating growth factor availability and by modulating cell-cell contacts [29]. Galvani et al. showed that HLA I antibody stimulation of SMC triggers proliferation by upregulation of the MMP2/nSMase2 pathway [30]. MMP2 was required for smooth muscle cell 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.

### 3.5 HLA I partners with integrin β4 to elicit endothelial cell proliferation and migration.

The HLA I molecule has no known signaling motif, suggesting that HLA I molecules associate with other co-receptor(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 β4 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 β4 ligation and HLA class I antibodies, we postulated that HLA class I molecules partner with integrin β4 to transduce intracellular signals. We found that ligation of HLA class I with antibodies prompted complex formation between integrin β4 and HLA class I [33]. Furthermore, knockdown of integrin β4 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 β4 in transplant vasculopathy is corroborated by the association between the elevated expression of integrin β4 in the thoracic aortic endothelia and atherosclerosis [34].

### 3.6 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 while 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 up-regulation of cell protection genes such as heme oxygenase 1 (HO-1) and activation of cAMP-dependent PKA [36, 37]. Ligation of HLA class I with antibodies also activates the PI3K/Akt pathway and increases endothelial antioxidant responses, which protect EC 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 Bad and increased expression of anti-apoptotic proteins Bcl-2 and Bcl-xL *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.

### 3.7 HLA I Antibodies Induce Leukocyte Recruitment.

EC contain specialized storage vesicles called Weibel-Palade bodies, which harbor preformed von Willebrand Factor, P-selectin, and other vascular mediators. Exocytosis of Weibel-Palade bodies is regulatedby calcium or Toll like receptor signaling [41]. HLA class I ligation with antibodies also triggers Weibel-Palade bodies exocytosis, externalizing von Willebrand Factor and increasing P-selectin expression on the cell surface. Up-regulation of P-selectin on the cell surface causes

increased adherence of leukocytes to endothelial cell. Crosslinking 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, while monovalent Fab fragments cannot activate von Willebrand Factor 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 von Willebrand Factor release and neutrophil influx in the grafts [42]. In a similar study, the release of von Willebrand Factor in cardiac allografts transplanted into immunoglobulin knock-out 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 EC, 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. showed that administration of MHC I antibodies into the mouse native lung increased neutrophil infiltration [44]. Moreover, treatment of EC with HLA class I antibodies stimulated the production of monocyte chemattractant protein-1 (MCP-1) and neutrophil chemattractant growth-related oncogene□□ (KC), which attract macrophages to the endothelium [45].

Antibodies engage the innate immune system by interacting with the Fc receptor FcγRIIIa 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]. Colvin et al. demonstrated that MHC I antibodies cause transplant vasculopathy in RAG 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 □-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 EC with killer cell immunoglobulin-like receptors (KIR) 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 EC by relieving KIR mediated inhibition. Interestingly, depletion of NK 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].

### 3.8 Conclusion

HLA class I antibodies activate EC and SMC through a variety of signaling pathways (Figure 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.

**Key points**

1. HLA class I antibodies stimulate survival and proliferation signaling pathways in endothelial cells and smooth muscle cells.
2. HLA class I antibodies mediate actin cytoskeleton reorganization in endothelial cells which is functionally linked to cell proliferation.
3. HLA class I partners with integrin β4 to transduce signals into the cell.
4. HLA class I antibodies promote leukocyte recruitment by induction of endothelial cell exocytosis and upregulation of adhesion molecules at the cell surface.

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# CHAPTER 4:

# Blockade of P-selectin is Sufficient to Reduce MHC I Antibody-Elicited Monocyte Recruitment *In Vitro* and *In Vivo*.

**Abstract**

Donor specific HLA antibodies significantly lower allograft survival, but as yet there are no satisfactory therapies for prevention of antibody-mediated rejection. Intracapillary macrophage infiltration is a hallmark of antibody-mediated rejection, and macrophages are important in both acute and chronic rejection. The purpose of this study was to investigate the effect of HLA I antibodies on endothelial cell activation, leading to monocyte adherence. We used an *in vitro* model to assess monocyte binding to endothelial cells in response to HLA I antibodies. We confirmed our results in a mouse model of antibody-mediated rejection, in which B6.RAG1-/- recipients of BALB/c cardiac allografts were passively transferred with donor specific MHC I antibodies. Our findings demonstrate that HLA I antibodies rapidly increase intracellular calcium and endothelial presentation of P-selectin, which supports monocyte binding. In the experimental model, donor specific MHC I antibodies significantly increased macrophage accumulation in the allograft. Concurrent administration of rPSGL-1-Ig abolished antibody-induced monocyte infiltration in the allograft, demonstrating the dependence on selectins. Our data suggest that antagonism of P-selectin may ameliorate accumulation of macrophages in the allograft during antibody-mediated rejection.

## 4.1 Introduction

The presence of donor specific antibodies to HLA class I and II molecules has a significant negative impact on graft survival in heart (*1-3*) and renal transplantation (*4*), and correlates with chronic rejection (*5*). Donor specific HLA antibodies significantly increase allograft loss in the clinic (7, 8), but as yet there are no satisfactory therapies for prevention of antibody-mediated rejection (AMR). It is incompletely understood how HLA I antibodies may promote monocyte recruitment into the allograft. HLA I antibody binding to vascular endothelial and smooth muscle cells activates cell signaling pathways including PI3K/Akt, mTOR and ERK that are required for increased proliferative capacity and resistance to complement-mediated cell death (9-14). These signals are

activated in murine cardiac allografts exposed to DSA (15) and in AMR positive biopsies (11,16). Further, HLA I ligation causes stress fiber formation and increases cell migration (14,17). Finally, endothelial vesicles known as Weibel-Palade bodies (WPb) are mobilized after HLA I crosslinking, leading to adherence of the immature neutrophilic cell line HL60 (18). Macrophage infiltration frequently accompanies antibody-mediated rejection (19).

Macrophages mediate both acute and chronic rejection, and comprise a significant proportion of graft infiltrating cells in acute renal rejection, correlating with rejection severity (20, 21). In high grade renal allograft rejection, and in acute cellular rejection of cardiac allografts, macrophages outnumber T cells (22, 23). Many groups have described the correlation between high number of interstitial macrophages and poor graft outcome (24-26). In addition to acute rejection, macrophages are found during late/chronic rejection, and correlate with donor specific HLA antibodies (24, 27). There is a significant increase in intragraft macrophages in rejectors compared with nonrejectors in intestinal transplantation as well (28), demonstrating that macrophages universally promote rejection in a variety of tissues. Macrophages have a myriad of functions by which they may promote acute and chronic rejection. They present alloantigen to T lymphocytes, promote deposition of extracellular matrix by SMC (29) and release a variety of growth factors and cytokines that cause endothelial cell proliferation (30). Further, recent work has suggested that macrophages may have direct alloreactivity against the allograft (31, 32). Several experimental models have highlighted the central contribution of macrophages to allograft rejection. For example, depletion of macrophages ameliorates rejection of murine cardiac allografts (33). Therefore, it is desirable to reduce their entry into the allograft.

We investigated endothelial cell activation and recruitment of monocytes after HLA I crosslinking by antibody. Our results show that HLA I signaling in endothelial cells is sufficient to increase monocyte adhesion mediated by HLA I-induced P-selectin. In an *in vivo* model of antibody-mediated

rejection, we found that donor specific MHC I antibodies elicit macrophage infiltration into the allograft, that can be blocked by the selectin antagonist rPSGL-1-Ig. This study demonstrates that selectin blockade ameliorates monocyte adherence *in vitro* and intragraft macrophage accumulation *in vivo* in response to MHC I antibodies.

## 4.2 Materials and Methods

*Reagents*

Azide free sterile HLA I antibody (murine IgG2a, clone W6/32) was obtained from BioXCell. The $F(ab')_2$ fragment of W6/32 was generated as previously described (34), or using a kit from Thermo Scientific according to the manufacturer's recommendations. Neutralizing antibody to P-selectin was of goat (Santa Cruz) or sheep (R&D) origin; low endotoxin azide free neutralizing antibody to PSGL-1 was purchased from Biolegend (San Jose, CA). Purified polyclonal human IgG in sterile PBS was obtained from Fisher Scientific. Calcium inhibitor BAPTA-AM was purchased from Calbiochem. Recombinant soluble PSGL-1 Fc chimera (rPSGL-1-Ig) was provided by Y's Therapeutics (San Bruno, CA), and contains point mutations in the Fc region to eliminate interactions with complement and FcγRs.

*Cell Culture*

Primary human aortic cells were isolated from aortic rings from consenting donors as previously described (9). Endothelial cells were cultured on 0.1% gelatin coated tissue culture plates in growth medium composed of M199 supplemented with 20% heat inactivated fetal bovine serum (FBS), sodium pyruvate, heparin, endothelial cell growth serum, and penicillin/streptomycin. Endothelial cells were detached with trypsin EDTA and seeded in 24 or 48 well plates, and grown to confluence before use in experiments.

The monocytic cell line Mono Mac 6, which closely resembles primary blood monocytes in phenotype and adhesive properties (35, 36), was cultured in RPMI-1640 supplemented with 10% FBS, sodium pyruvate, non-essential amino acids, antibiotics, and insulin. The cell line was subcultured every 2 to 3 days and maintained at fewer than $10^6$ cells per mL.

*Measurement of von Willebrand Factor by ELISA*

Confluent aortic endothelial cells were stimulated with HLA I antibody at 5μg/mL, thrombin at 1U/mL, histamine at 10mM or PMA at 200nM in M199 supplemented with 5% FBS for one hour. Supernatant was collected and von Willebrand Factor (vWF) was measured in the medium using an ELISA kit (Helena Laboratories) according to the manufacturer's protocol.

*Flow Cytometric Measurement of P-selectin Expression*

Cell surface expression of P-selectin was measured by flow cytometry. Briefly, endothelial cells were treated with HLA I antibody at 5μg/mL, thrombin 1U/mL, histamine at 10mM, or PMA at 200nM for the indicated times in M199 with 5% FBS and detached using Accutase (Innovative Cell). Trypsin was not used to detach cells for flow cytometry because epitopes of P-selectin are sensitive to trypsin digestion (37). Cells were washed once with PFA flow buffer composed of PBS, 2.5% FBS and 0.1% sodium azide, then stained with anti-human P-selectin-PE conjugated antibody (BD) for 45min on ice. Cells were washed twice with PFA flow buffer and resuspended in 4% buffered formalin. Fluorescence was measured on FACSCalibur flow cytometer (BD). Results are expressed as proportion of cells staining positively for P-selectin, normalized to untreated control endothelial cells. In preliminary experiments, we determined the tempo of P-selectin induction and found that each stimulant triggered maximal P-selectin expression at a different time point. Based on these findings, we treated endothelial cells with each agonist according to its peak stimulation.

*Measurement of Intracellular Calcium*

***Cell Plating.*** Cells were plated onto 10 mm by 22 mm glass coverslips which were placed inside 35 mm plastic petri dishes filled with growth media and placed in the incubator.

***Fura-2 loading.*** Cells were removed from the incubator, then incubated in saline containing 5 μM Fura-2 AM for 20 minutes at 37°C. Cells were than washed with saline, and replaced in the incubator for at least 5 min. before the start of the experiment.

***Fluorimetry.*** Coverslips containing cells were mounted in a standard 1 cm path length cuvette filled with saline (37 oC ) using a special holder (ANO-2100, Hitachi Instruments). The cuvette was placed in a fluorimeter (F-2000, Hitachi Instruments) with a heated cuvette jacket (37°C), and solution was continuously stirred with the aid of a small magnetic stir bar. Small volumes of concentrated test solutions were introduced into the bottom 1/3 of the cuvette, with a Hamilton syringe. All concentrations reported are the final steady state mixed value. Injection was completed within 1 sec. Measurements of mixing kinetics showed that introduced test solutions were completely mixed (at the level of the detection window, about the middle 1/3 of the cuvette) within 2 secs, and with no sizable overshoot. The size of the detection window allowed measurement on the order of $10^5$ cells. Excitation was set to 340 nm and 380 nm, and emission signal collected at 380 nm, all with a 10 nm bandwidth. Samples were taken every 0.5 secs. using associated software ("F-2000 Intracellular Cation Measurement System", Hitachi Instruments). At each time point, the ratio of the signals at 340 nm / 380 nm provided a monitor of $Ca^{2+}$ concentration.

*Static Adhesion Assay*

Preliminary studies were undertaken to determine the optimal concentration of blocking reagents. Adherence of the monocytic cell line Mono Mac 6 to endothelial monolayers was adapted from previously described methods (38). Briefly, endothelial cells were stimulated with HLA I antibody at 5μg/mL, HLA I $F(ab')_2$ at 10μg/mL or thrombin at 1U/mL in M199 with 2% FBS for the indicated times. To inhibit calcium signaling, endothelial cells were pretreated with BAPTA-AM (Calbiochem) at 20μM for 30min prior to stimulation with HLA I antibody or thrombin. In specified experiments,

rPSGL-1-Ig at 20ug/mL or anti-P-selectin neutralizing antibody at 10μg/mL was added to the endothelial cells for the last 10min of stimulation. Mono Mac 6 were fluorescently labeled with CFDA-SE (Invitrogen) at 1μM for 10min in HBSS with calcium and magnesium, then washed and resuspended in M199 with 2% FBS. In indicated experiments, monocytes were incubated with a neutralizing antibody to PSGL-1 at 10μg/mL, or with soluble polyclonal human IgG at 20μg/mL to block FcγRs, for 20min prior to the assay (39). Medium was removed from the endothelial cells, and Mono Mac 6 were added at a ratio of approximately 3 monocytes per endothelial cell for 20min in the presence or absence of inhibitors. Nonadherent cells were washed off by gentle pipetting and decanting three times, then the coculture was fixed. Adherent monocytes were counted by collecting 8-10 fields per sample at a fluorescence microscope, and quantitated using automated software (CellProfiler and Image J) (40). Results are shown as fold increase in the mean number of adherent monocytes per field for each condition +/- standard error of the mean.

*Mice*

Male B6.129S7-Rag1$^{tm1Mom}$ (B6.RAG1$^{-/-}$, H-2$^{b}$) and male BALB/c (H-2$^{d}$) mice aged 7-10 weeks old were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed under pathogen-free conditions in filter-top cages throughout the experiments and were cared for according to The Guidelines for Animal Care. All reagents and experiments in this study were reviewed and approved by the UCLA Animal Research Committee.

*Murine heterotopic heart transplantation and histological techniques*

BALB/c (H-2$^{d}$) hearts were heterotopically transplanted into B6.RAG1 KO (H-2$^{b}$) recipients as previously described (15). Briefly, under isoflurane anesthesia, the donor aorta was anastomosed to the recipient abdominal aorta and the donor pulmonary artery was joined to the recipient inferior

vena cava. Graft function was monitored by abdominal palpation daily until recipient mice were sacrificed.

*Adoptive transfer of monoclonal antibodies*

All reagents to which mice were exposed were sterile filtered and azide free. Allograft recipient mice were passively transferred with isotype control antibody (mIgG2a, clone MOPC-173, Biolegend, San Jose, CA) or with donor specific anti-MHC I antibodies (anti-$K^d$ mIgG2a, clone SF1-1.1; anti-$D^d$ mIgG2a, clone 34-2-12, Biolegend) by intravenous injection into the tail vein beginning on day 3 post-transplant, continuing weekly thereafter and ending on day 28. Antibodies were diluted in sterile saline to 30ug per 0.2mL per animal (approximately 1.5mg/kg). rPSGL-1-Ig (Y's Therapeutics, San Bruno, CA) was diluted in sterile saline to 70ug per 0.2mL (approximately 3.5mg/kg) and injected intravenously via the tail vein beginning on day 3 after transplant and continuing twice weekly until the end of the experiment. The dose and administration regimen were based on previously published studies detailing the pharmacokinetics and effects of rPSGL-1-Ig in mouse, where 0.1mg/kg was detectable in the serum with a half life of 121 hours (41), and doses of 1-30mg/kg were sufficient to reduce inflammation and leukocyte rolling (42).

*Histologic Analysis of Graft Infiltrating Cells*

On day 30 post-operatively, animals were deeply anaesthetized (isoflurane) and the donor hearts were recovered from recipients. All allografts included in the study were still beating at the time of sacrifice. For some animals, the native heart was also removed and analyzed in parallel with donor allografts. Hearts were divided into two pieces along the transverse plane, into base and apex. The base was fixed in 10% phosphate buffered formalin for at least 24 hours at room temperature,

washed once in PBS, then transferred to 70% alcohol. The base was then embedded in paraffin and used for immunohistochemistry and H&E staining.

Cross sections were made on a microtome. Formalin-fixed tissue was stained for Mac-2 (Acris) or CD45 (Millipore). Immunohistochemical staining in allografts was scored by a blinded pathologist. A score of 0 indicates negative staining, 1 is rare, 2 is rare/focal, 3 is focal staining, and 4 is strongest, diffuse staining.

Initial experiments comparing macrophage infiltration into the allograft between animals receiving 5μg and 30μg of anti-$K^d$ antibody revealed no statistically significant difference between the two groups. We also tested a small group of animals which received biweekly administration of 30μg anti-$K^d$ antibody, and did not observe a significant difference in macrophage staining when compared by unpaired T test. Therefore, we combined animals from these groups into a single group, the anti-$K^d$ antibody treated group. Macrophage infiltration was also the same for allografts treated with control antibody at 5μg and 30μg, and these two groups were merged into one control antibody treated group.

*Flow Cytometric Crossmatch*

To confirm the presence of circulating donor specific antibodies, at the time of sacrifice, blood was collected directly from the inferior vena cava using an 18G needle, transferred into a Capiject tube containing a gel barrier and clot activator (Terumo T-MG), and allowed to clot at room temperature for at least 30min. Serum was separated by centrifuging for 10min at 1200RCF. The supernatant was centrifuged for a further 10min at 2500x*g* in a new tube to remove residual cell contaminate, then stored at -80°C until use.

BALB/c splenocytes were isolated from freshly procured donor spleens using a Cell Strainer (BD). Banked frozen splenocytes were thawed and washed with RPMI supplemented with 10% heat inactivated FBS and pen/strep. Sera were serially diluted in PFA flow buffer, and $2.5x10^5$–$1x10^6$

BALB/c splenocytes were incubated with 25μL of each serum dilution for 30min on ice. Cells were washed twice with PFA, then stained with goat-anti-mouse Fc-FITC (to minimize nonspecific staining of B cells) in 50μL for 30min on ice. After washing, rat anti-mouse CD3-PE was added for an additional 20min on ice. Cells were resuspended in 4% formalin and MHC I antibody binding to T cells was measured by gating on CD3+ using flow cytometry on FACSCalibur (BD). Results are expressed as median fluorescence intensity of CD3+ cells in the FITC/FL-1 channel.

*Statistical Analysis*

Groups were compared using the student's T test.

## 4.3 Results

### *4.3.1 HLA I antibodies trigger endothelial exocytosis of Weibel-Palade bodies via intracellular calcium.*

To examine whether HLA I crosslinking on endothelial cells could cause release of Weibel-Palade bodies (WPb), we treated confluent monolayers of human aortic endothelial cells with a monoclonal murine pan-HLA I antibody (W6/32), or positive controls thrombin, histamine and PMA. We collected the medium and measured secreted von Willebrand Factor (vWF) by ELISA. Stimulation of endothelium with HLA I antibodies significantly increased vWF in the supernatant by 1.62+/-0.01 fold over untreated control ($p=0.025$). Activation of endothelial cells with positive controls thrombin, PMA or histamine had a comparable effect and significantly increased vWF secretion by 1.97+/-0.31 fold ($p=0.048$), 1.89+/-0.04 fold ($p=0.006$), and 1.82+/-0.01 fold ($p=0.007$), respectively. Treatment of endothelial cells with negative isotype control murine IgG (mIgG) did not increase vWF in the supernatant (1.04+/-0.09 fold) (Figure 1a).

Because P-selectin, an adhesion molecule, is stored in WPb and presented on the cell surface upon secretion of these vesicles, we tested expression of P-selectin on endothelial cells stimulated with HLA I antibodies using flow cytometry. Untreated endothelial cells were negative for P-selectin staining. Stimulation of endothelium with a monoclonal HLA I antibody (5μg/mL) significantly increased the proportion of cells staining positively for P-selectin by 2.0+/-0.14 fold over control after 30min. Similarly, P-selectin expression was elevated on thrombin (1.67+/-0.08 fold), PMA (2.20+/-0.28 fold) or histamine (1.90+/-0.15 fold)-treated endothelial cells (Figure 1b).

Because HLA I crosslinking stimulates endothelial cell signaling, and because traditional WPb agonists cause an increase in intracellular calcium which leads to exocytosis, we postulated that HLA I-induced exocytosis might be dependent on calcium. Endothelial cells were pretreated with BAPTA-AM in order to chelate intracellular calcium, then stimulated with HLA I antibody. P-

selectin induction by HLA I antibodies was reduced in endothelium with impaired calcium signaling, from 1.70+/-0.11-fold over control to 1.15+/-0.37-fold (Figure 1C). Likewise, vWF secretion in response to HLA I antibodies or thrombin was completely prevented when endothelial cells were pretreated with the calcium inhibitor BAPTA-AM, but not EGTA, suggesting that the source of calcium is intracellular stores (data not shown).

To further confirm that HLA I antibodies increase calcium-dependent signaling, we measured intracellular calcium in real time after HLA I crosslinking. Endothelial cells treated with HLA I antibody at 1μg/mL exhibited a rapid increase in intracellular calcium levels (Figure 1d). A slight increase in intracellular calcium was also observed with HLA I antibody at 5μg/mL. As a positive control, endothelium was treated with thrombin at 0.01U/mL and 1U/mL, which rapidly increase free cytosolic calcium (Figure 1d).

### *4.3.2 HLA I antibody-induced endothelial P-selectin is necessary and sufficient to promote monocyte adherence* in vitro.

In order to focus on monocyte recruitment due exclusively to HLA I antibody-provoked endothelial cell signaling, we used two strategies to eliminate the potential confounding effects by the Fc region of the antibody, and measured monocyte adherence to endothelial monolayers. Endothelial cells were treated with an $F(ab')_2$ fragment of HLA I antibody (W6/32) and fluorescently labeled Mono Mac 6 were added. Crosslinking of HLA I on endothelium by HLA I $F(ab')_2$ significantly increased the number of adherent monocytes by 1.66+/-0.07 fold compared with untreated endothelial cells ($p<0.0001$). As an alternative approach to measure monocyte adherence in the absence of Fc-mediated effects, we treated endothelial cells with an intact HLA I antibody (W6/32) and preincubated Mono Mac 6 with soluble IgG to block FcγRs. Mono Mac 6 adhesion to HLA I antibody-stimulated endothelial monolayers was significantly increased compared with untreated

endothelial cells, by 1.48+/-0.09 fold ($p<0.0001$). Monocyte adherence was not significantly different between the two conditions ($p=0.121$). Exposure of endothelial cells to isotype control mIgG did not stimulate Mono Mac 6 adherence (0.99+/-0.07 fold over untreated, $p>0.05$). Thrombin, which increased P-selectin expression, was used as a positive control; endothelial cells activated with thrombin recruited 1.64+/-0.07 fold more monocytes than untreated EC ($p<0.0001$) (Figure 2a).

Leukocyte capture is mediated by endothelial selectins. Since we observed that thrombin and HLA I antibody stimulation of endothelial cells increased cell surface P-selectin, we determined whether P-selectin was involved in the increased adherence of monocytes. EC were treated with HLA I $F(ab')_2$ (W6/32) or thrombin, and rPSGL-1-Ig was added at 20μg/mL to block P-selectin. Mono Mac 6 were allowed to adhere in the presence of the antagonist, and bound monocytes were counted. The number of adherent Mono Mac 6 was increased by 1.61+/-0.06 fold over control in response to HLA I $F(ab')_2$. When rPSGL-1 was present, Mono Mac 6 binding to HLA I antibody-treated endothelial cells was reduced to background levels of binding, at 1.13+/-0.09 fold over control ($p=0.003$ versus no inhibitor). The presence of rPSGL-1 during adherence diminished Mono Mac 6 binding to HLA I antibody stimulated endothelial monolayers by 76.7+/-12.8%. As a control, we measured the effect of the selectin antagonist on thrombin-induced monocyte adherence. Mono Mac 6 binding to thrombin-stimulated endothelium was reduced by the presence of rPSGL-1 (Figure 2b). We confirmed the contribution of P-selectin to HLA I antibody-mediated monocyte recruitment under the Fc blockade. Endothelial cells were treated with intact HLA I antibody (W6/32), and Mono Mac 6 were pretreated with soluble IgG to block their FcγRs. rPSGL-1 inhibited Mono Mac 6 recruitment by 81.3+/-18.7% when compared with no antagonist (data not shown).

We substantiated the requirement for P-selectin by incubating the stimulated endothelial cells with neutralizing antibody to P-selectin, or the monocytes with a neutralizing antibody to PSGL-1. These complementary strategies of antagonizing P-selectin/PSGL-1 interactions also significantly diminished Mono Mac 6 recruitment to HLA I $F(ab')_2$-treated endothelial cells, with 94.2+/-5.8% inhibition by anti-P-selectin antibody, and 100+/-0.0% inhibition by anti-PSGL-1 antibody (Figure 2c).

HLA I crosslinking triggered an intracellular calcium increase which was required for P-selectin presentation. Therefore, we ascertained whether endothelial cells required intact calcium signaling to recruit monocytes after HLA I crosslinking. Endothelial cells were pretreated with BAPTA-AM to chelate calcium, then stimulated with HLA I $F(ab')_2$ or thrombin. Mono Mac 6 were incubated with stimulated EC. HLA I $F(ab')_2$ treatment increased the number of bound monocytes by 1.65+/-0.06 fold over control. In contrast, stimulation of BAPTA-AM treated endothelial cells with HLA I $F(ab')_2$ failed to trigger Mono Mac 6 adherence, which was not significantly different than background binding (0.90+/-0.05 fold, $p<0.0001$ versus no BAPTA). Therefore, inhibition of endothelial calcium signaling prevented monocyte adhesion by 90.0+/-10.0%. Pretreatment of endothelial cells with the calcium chelator also decreased monocyte adherence in response to thrombin stimulation, from 1.63+/-0.18 fold to 0.77+/-0.17 fold, (100.0+/-0.0% inhibition, $p=0.007$). Thus, endothelial cells which were pretreated with BAPTA were significantly impaired in their ability to support binding of Mono Mac 6 (Figure 2d). BAPTA-AM pretreatment did not alter expression of HLA I on the endothelial surface [data not shown].

### *4.3.3 Passive transfer of donor specific MHC I antibodies increases macrophage infiltration into the cardiac allograft.*

In order to extend our *in vitro* observation that HLA I antibodies provoked monocyte adherence to endothelial cells, we utilized an *in vivo* model of antibody-mediated rejection. C57BL/6 recombinase activating gene (RAG1) knockout (H-$2^b$) recipients of a heterotopic cardiac allograft from BALB/c donors (H-$2^d$) were passively transferred with control murine IgG2a (MOPC-173) or with a monoclonal murine IgG2a recognizing donor MHC I molecules (anti-$K^d$ or anti-$D^d$). Grafts were recovered on day 30 post-transplant and examined for cellular infiltration (diagrammed in Figure 3a). To measure the levels of circulating anti-donor antibodies in allograft recipients, we performed flow crossmatching using recipient sera and splenocytes of donor BALB/c origin. The flow cytometric cross-match revealed no binding of sera from control antibody-treated recipients to donor T cells; sera from control antibody-treated animals did not stain BALB/c T cells (MFI: 8.5+/-2.3). In contrast, sera from animals treated with anti-$K^d$ or anti-$D^d$ antibody bound highly to donor $CD3^+$ splenocytes (neat serum MFI: 57.1+/-3.5 and 59.0+/-3.3), and cells stained positively even when serum was diluted to 1:4. Sera from animals that were passively transferred with a both anti-$K^d$ and anti-$D^d$ antibodies together strongly stained donor $CD3^+$ splenocytes (MFI 126+/-14). These results demonstrate the presence of anti-donor antibodies in the circulation of passively transferred cardiac allograft recipients (Figure 3b).

We characterized the infiltration of macrophages in cardiac allografts in response to MHC I antibody treatment. By immunohistochemistry, grafts from animals that received control mIgG2a antibody had low Mac-2 staining. Six out of 14, less than half (42%) of, control allografts were positive for macrophages with a score of two or greater, and none (0%) were strongly positive with a score of 3 or 4. The mean score for the control antibody-treated group was 1.43+/-0.14. Strikingly, 21 out of 25, or 84%, of cardiac allografts from animals treated with anti-$K^d$ antibody were positive for macrophage infiltration, with 40% of those grafts having strong Mac-2 staining. The degree of macrophage staining in anti-$K^d$ antibody-treated allografts was significantly greater ($p=0.0002$) when

compared with allografts from control antibody-treated animals, with a mean score of 2.30+/-0.18 for the group. We confirmed this phenomenon using a different monoclonal donor specific MHC I monoclonal antibody. Eighty percent, four out of five, of anti-$D^d$ antibody treated allografts scored positively for macrophages, with 40% of anti-$D^d$ antibody treated grafts having strong macrophage infiltration. The mean score was 2.20+/-0.37 ($p=0.025$ versus control). When allograft recipients were given both anti-$K^d$ and $D^d$ antibodies together, all transplanted hearts from this group had a score of two or greater for Mac-2 macrophage marker ($p=0.012$ versus control) (Table 1 and Figure 3c). These results demonstrate that donor specific MHC I antibodies are sufficient *in vivo* to increase macrophage accumulation in the allograft.

### *4.3.4 Treatment with the selectin antagonist rPSGL-1 significantly reduces MHC I antibody-elicited macrophage and CD45+ accumulation in the allograft.*

Since our *in vitro* data showed that HLA I antibody treatment of endothelial cells increases surface P-selectin, and that blocking of P-selectin was sufficient to abolish resultant monocyte recruitment, we sought to determine whether antagonism of PSGL-1/P-selectin interactions *in vivo* could also reduce immune cell recruitment in response to MHC I antibodies. We postulated that, since P-selectin initiates the recruitment cascade and licenses the leukocyte to firmly adhere, blockade of P-selectin would preclude ICAM-1, chemokine, FcγR or other interactions that may occur *in vivo*. Administration of rPSGL-1 prevented macrophage infiltration into the allografts of anti-$K^d$ antibody-treated animals ($p=0.004$). While greater than 80% of anti-$K^d$ antibody treated allografts were positive for Mac-2 staining, only 25% of allografts that received concurrent rPSGL-1 therapy had a Mac-2 score of 2 or more. Remarkably, rPSGL-1 abolished the incidence of strong macrophage infiltration induced by donor specific MHC I antibodies, from 40% to 0% (Table 2 and Figure 4a and 4c).

We also examined the allografts for total immune cell infiltration by immunohistochemistry. Native or transplanted hearts were stained for CD45, a marker of hematopoietic cells. No native hearts were positive for CD45 staining. Allografts from control antibody-treated animals were also negative for CD45 staining. In contrast, 4 out of 7 allografts (57%) from animals which received anti-$K^d$ antibody were positive for CD45, with a score of 2 or greater. Nearly one third (29%) of those grafts stained strongly for CD45, having a score of 3 or 4. The mean score for the group was 2.0+/-0.52. Increased CD45 infiltration elicited by MHC I antibody (anti-$K^d$ mIgG2a) was wholly inhibited by concurrent administration of rPSGL-1-Fc ($p=0.037$), and no allografts from animals that received both anti-$K^d$ antibody and rPSGL-1 were positive for CD45 staining (Table 3 and Figure 4b). Therefore, rPSGL-1 therapy specifically reduces macrophage infiltration and also prevents total CD45+ immune cell infiltration.

## 4.4 Discussion

We found that HLA I crosslinking on human aortic endothelial cells triggers an upregulation of P-selectin at the cell surface and increased adherence of monocytic cells. Monocyte binding to HLA I antibody-stimulated endothelial cells was inhibited completely by blockade of P-selectin. Further, we confirmed these results using an *in vivo* system, where donor specific MHC I antibodies increased macrophage infiltration into the allograft, that could be abolished by antagonism of P-selectin.

We report that HLA I antibodies trigger exocytosis of Weibel-Palade bodies (WPb), specialized endothelial vesicles which contain von Willebrand Factor (vWF), P-selectin, and other inflammatory and vascular mediators. We also determined that intracellular calcium signaling downstream of HLA I ligation is necessary for exocytosis, dependence which has been reported for other secretagogues, such as thrombin and histamine. Our results are consistent with the study by Yamakuchi et al., who also observed increased vWF secretion and P-selectin expression from endothelial cells shortly after treatment with HLA I antibodies (18).

Given this calcium dependence, we postulated that HLA I crosslinking elicited an increase in intracellular calcium in the endothelial cell. HLA I antibodies at a high dose (1μg/mL) stimulated a modest but sustained increase in cytosolic calcium, suggesting that calcium-dependent pathways are activated shortly after HLA I signaling. Interestingly, the tempo of calcium increase in endothelial cells induced by HLA I antibodies was slower than that observed with thrombin. This observation parallels our preliminary studies on the kinetics of P-selectin expression, in which HLA I antibody-induced P-selectin peaked much later (30-60min) than when thrombin (10-20min) or histamine (5-10min) was the stimulus. Our group has previously demonstrated that HLA ligation rapidly stimulates several intracellular signaling pathways, leading to functional changes. For example, Rho-GTP, Src and FAK become activated and are necessary for cytoskeletal changes and stress fiber formation (12, 43). Further, the PI3K/Akt signaling axis is turned on, which targets cell cycle

regulators and mTOR to promote proliferation (12). Calcium agonists act through phospholipase C β1, which catalyzes the production of the intracellular second messengers $IP_3$ and diacyl glycerol (DAG). $IP_3$ binds to a receptor (IP3R) on the membrane of the endoplasmic reticulum and causes opening of calcium release channels, increasing cytosolic calcium (44). Increased intracellular calcium is required for a variety of signaling pathways not yet explored in the context of HLA I signaling. For example, classical forms of PKC (α) are dependent upon calcium, as are calmodulin and myosin light chain kinase (MLCK), a cytoskeletal regulator. Other physiological agonists of WPb release, such as thrombin and histamine, increase intracellular calcium and modulate the cytoskeleton and endothelial cell permeability. Calcium signaling is important for modulation of the endothelial cell barrier and permeability (45).

Herein, we describe HLA I antibody-elicited recruitment of monocytic cells with an adhesive phenotype analogous to that of peripheral blood monocytes (36). This binding was dependent upon P-selectin, as it could be blocked by the antagonist rPSGL-1, neutralizing antibody to P-selectin, or neutralizing antibody to monocyte PSGL-1. P-selectin is an important early mediator of the leukocyte recruitment cascade, and facilitates capture of immune cells from the blood and rolling (low-affinity interactions) on the endothelium of the vessel wall. P-selectin is particularly important for the influx of neutrophils and granulocytes during ischemia/reperfusion and other acute injury. However, its role in allograft rejection is not well defined. P-selectin was found to be upregulated on microvascular endothelium in acutely rejecting cardiac allografts in rat, and on thickened intima of arteries during chronic rejection (46). Upregulation of P-selectin during rejection was also observed in a murine model (47). Allografts from murine donors deficient in both ICAM-1 and P-selectin or in all three selectins (ELP-/-) had significantly longer graft survival than their wild-type counterparts (48, 49). Rat lung allograft recipients treated with SLX (an oligosaccharide antagonist of selectin) reduced early rejection and interstitial accumulation of leukocytes (50).

Ours is the first report showing that inhibition of selectins *in vivo* ameliorates cellular infiltration during antibody-mediated rejection. Our group previously reported increased macrophage infiltration into allografts treated with a single high dose of MHC I $F(ab')_2$ (15), which we substantiated using a long-term administration of two different intact donor specific MHC I monoclonal antibodies. In a murine cardiac allograft model of acute rejection, IgKO recipients that could not produce donor specific antibodies had significantly longer graft survival than their wild-type counterparts. Increased P-selectin and vWF were observed in allografts from wild-type but not IgKO recipients, suggesting that these mediators are specifically induced in the presence of MHC antibodies (51, 52). Moreover, *in vitro* stimulation of endothelial cells with HLA I antibody triggered WPb release and increased adherence of HL60 cells through P-selectin (18). The HL60 cell line is an immature promyelocytic cell line with a primarily neutrophilic phenotype (53). The same group used an *in vivo* model to demonstrate that HLA I $F(ab')_2$ caused rapid neutrophil recruitment and acute rejection of human skin grafts on immunodeficient mice (18). We demonstrate macrophage accumulation *in vivo* in a long-term vascularized model of antibody-mediated rejection, which can be completely prevented by concurrent rPSGL-1 therapy.

The effects of the soluble antagonist rPSGL-1-Fc are well-documented in ischemia/reperfusion injury, where early administration reduces neutrophil, macrophage, and CD45+ cell infiltration after balloon artery occlusion or other injury, and decreases resultant neointimal hyperplasia in multiple species (54-57). Currently, clinical trials are underway to investigate the efficacy of rPSGL-1-Ig as a treatment to reduce ischemia/reperfusion injury. Initial results indicate that rPSGL-1-Ig is safe, and may reduce early graft dysfunction and expression of rejection markers (58-60). However, the effect of rPSGL-1-Ig on long-term survival and leukocyte recruitment in human transplantation has yet to be revealed.

Selectin antagonism has been used to reduce leukocyte infiltration in a variety of experimental models. rPSGL-1-Ig reduced surgery-induced rolling of leukocytes on P-selectin in cremaster muscle (42), ischemia/reperfusion injury in feline and rat models (55, 61), and neointimal hyperplasia in rat diabetic and porcine coronary artery balloon injury models (56, 57). Other immune cells that mediate rejection utilize selectins to transmigrate from the blood into tissue. The selectin ligand PSGL-1 is expressed on NK cells, neutrophils, monocytes and T cells (62), while P-selectin is expressed by endothelial cells and platelets. In cell-mediated rejection, CD8+ T cells required P-selectin for entry into the allograft (63), and activated T cells utilized P-selectin during recruitment to skin allograft (64). Recent data points to a central role for NK cells in antibody-mediated rejection. NK cells were present in allografts from animals passively transferred with donor specific MHC I antibody, and were required for antibody-induced chronic vascular lesions (65, 66). Moreover, the clinical signature of antibody mediated rejection in renal transplantation was significantly enriched for NK cell-derived transcripts (67). Given that NK cells adhere to platelets and P-selectin during recruitment to atherosclerotic lesions (68), it is possible that the recombinant PSGL-1 antagonist may reduce infiltration of other immune cells as well as monocytes in an immunocompetent host.

While P-selectin is the preferred ligand of PSGL-1, it can interact with other adhesion factors. Injection of rPSGL-1-Ig polyplexes showed localization to inflamed venules of wild-type but not P-selectin deficient mice, demonstrating *in vivo* interactions of this antagonist with P-selectin (69). PSGL-1 also mediates adherence to E- and L-selectin, as well as von Willebrand Factor. Therefore, rPSGL-1-Ig may inhibit other targets *in vivo*, promiscuity which may be advantageous from the therapeutic standpoint, given the redundancy of the adhesion molecule system.

MHC I antibodies also increase recruitment of platelets, which may facilitate monocyte infiltration. Injection of skin allograft recipients with donor specific MHC I antibodies causes platelet activation (likely via endothelial-derived vWF) and interaction with graft endothelium (51), which mediated

neutrophil trafficking to the graft. Platelet attachment to endothelium or extracellular matrix can support monocyte tethering through platelet-derived P-selectin, acting as a "bridge" between the monocyte and endothelium which is particularly important under shear stress (70). In our *in vivo* model, it is possible that administration of rPSGL-1-Ig antagonized the function of platelet P-selectin as well as MHC I antibody-induced endothelial P-selectin, both of which would have the consequence of reducing monocyte infiltration.

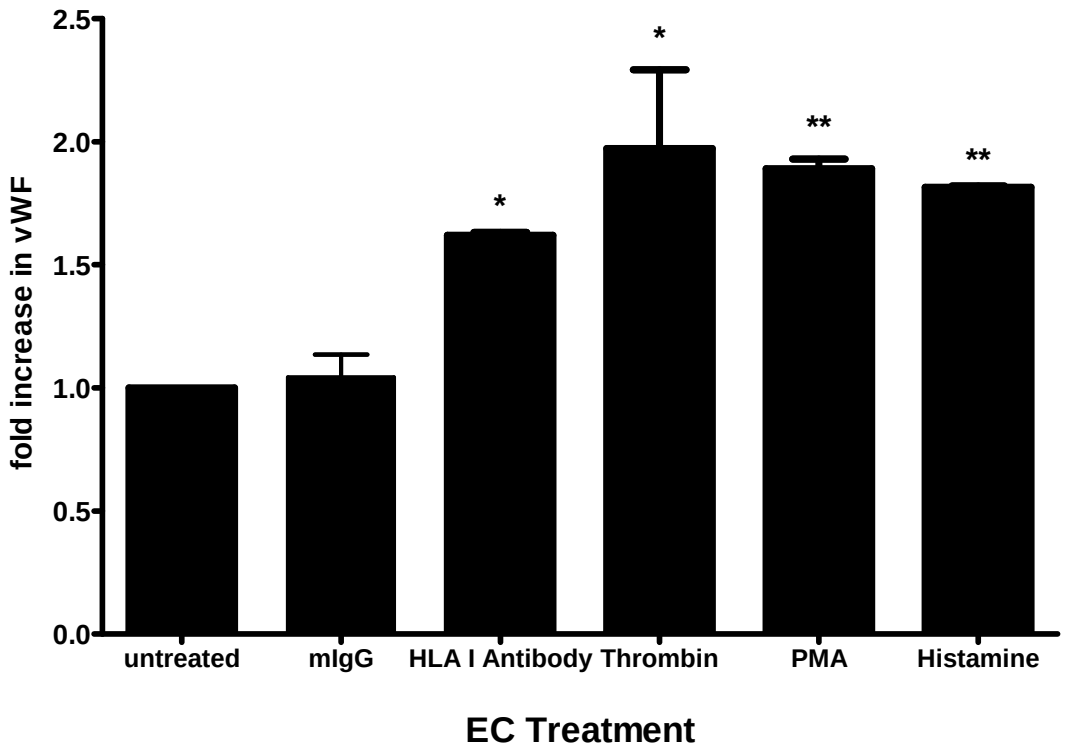
Unlike several reports from the Colvin group using a similar mouse model, we did not observe chronic vascular lesions in allografts from animals transferred with MHC I antibody (65, 66). This disagreement may be due to the restricted localization of the disease in this model, which is reported to occur only in the proximal coronary arteries. Alternatively, there may be differences in donor animals. Our donors were of the BALB/c (H-2*d*) strain, while Uehara et al. utilized B10.BR (H-2*k*) donors. There may be disparities between these genetic backgrounds, or between the donor specific monoclonal MHC I antibodies used.

The contribution of antibody-FcγR interactions has not yet been established in HLA I antibody-mediated cellular recruitment. We found *in vitro* that HLA I antibodies could promote monocyte adherence in the absence of Fc interactions. We attempted to confirm our findings with an MHC I $F(ab')_2$ fragment, but were unable to achieve consistently detectable levels of circulating antibody with a biweekly dosing. $F(ab')_2$ fragments have well-established instability and a short half-life *in vivo*, which makes such studies difficult. Nevertheless, the *in vivo* data reported here suggest that P-selectin is an important initiator which is both necessary and sufficient to initiate recruitment. In our system, MHC I antibodies directly activate the endothelium to induce selectin expression at the cell surface, independently of Fc interactions. Therefore, P-selectin is a priming or licensing step, inhibition of which precludes other Fc or chemokine effects. This idea is consistent with results from investigations of leukocyte recruitment in response to anti-endothelial cell antibodies. Florey et al.

demonstrated that anti-endothelial cell antibodies were not sufficient to increase neutrophil adherence to otherwise unstimulated endothelial cells, but that they augmented selectin-mediated recruitment by TNFα-activated endothelium (71).

In conclusion, we have demonstrated that monocyte infiltration is directly triggered by HLA I antibody activation of endothelial cell exocytosis and P-selectin expression. These results strongly suggest that selectin antagonism may reduce the accumulation of macrophages and other recipient immune cells into the allograft during antibody-mediated rejection.

**Figure 4-1a**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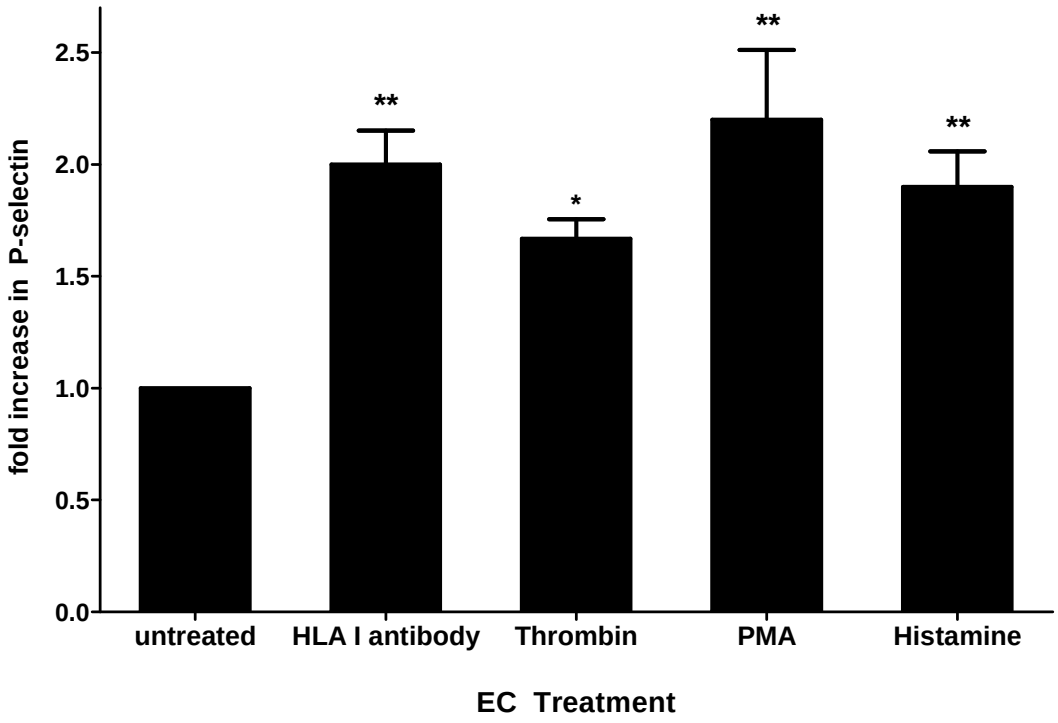
PMA
Histamine
EC Treatment

**Figure 4-1b**


fold increase in P-selectin
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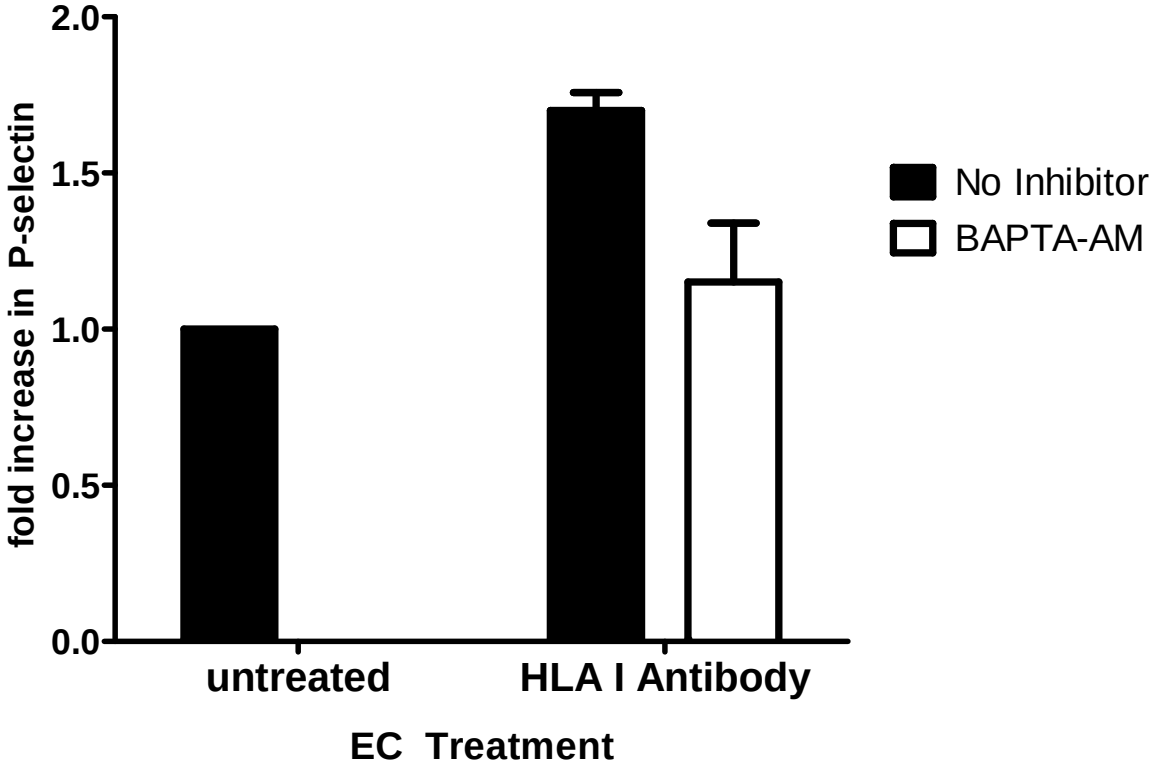
Histamine
EC Treatment

**Figure 4-1c**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HLA I Antibody
EC Treatment

**Figure 4-1d**

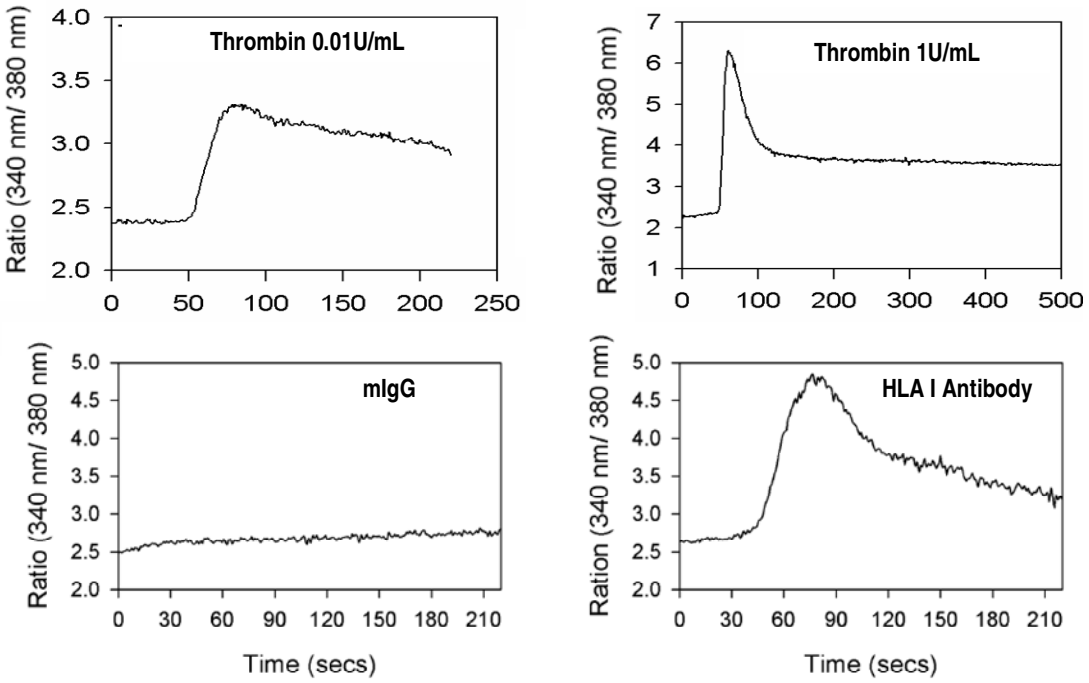
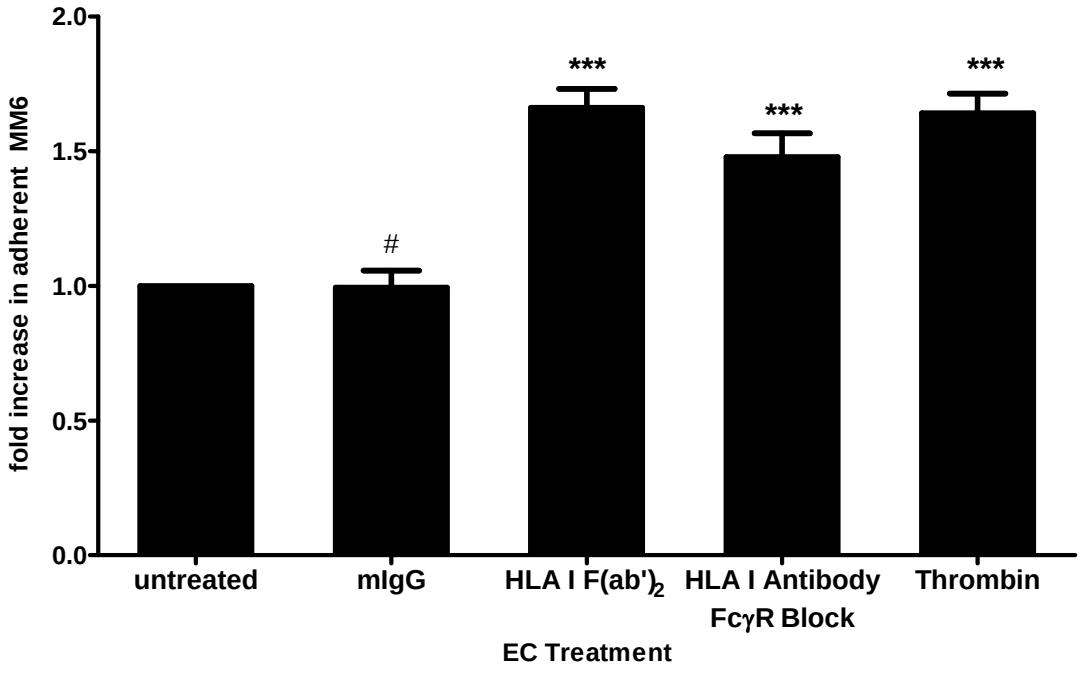



Figure 4-1. HLA I antibodies trigger Weibel-Palade body exocytosis via intracellular calcium. (a) Human aortic endothelial cells (HAEC) were treated with isotype control mIgG at 5μg/mL, HLA I antibody (clone W6/32, murine IgG2a) at 5μg/mL, thrombin at 10U/mL, PMA at 200nM, or histamine at 10mM for one hour. Supernatant was collected and vWF was measured by ELISA. Bar graph shows mean fold increase in optical density (OD) from two (PMA and histamine) or three (mIgG, HLA I antibody and thrombin) independent experiments for each condition. * $p<0.05$, ** $p<0.01$ versus mIgG. (b) HAEC were treated with HLA I antibody at 5μg/mL for 30min, thrombin at 1U/mL for 15min, PMA at 200nM for 20min, or histamine at 10mM for 10min and detached using Accutase. Cell surface P-selectin was stained with conjugated anti-P-selectin-PE and analyzed by flow cytometry. Bar graph shows mean fold increase in P-selectin positive cells from multiple independent measurements for each condition: HLA I antibody (n=10), thrombin (n=3), PMA (n=6), (Histamine n=23). * $p<0.05$, ** $p<0.001$ versus untreated. (c) HAEC were pretreated with BAPTA-AM at 20uM for 30min, then

stimulated with HLA I antibody at 5ug/mL for 1hr, or histamine at 10mM for 10min. P-selectin was measured. Bar graph shows summary of data from six experiments. Black bars show the fold increase in P-selectin expression without inhibitor, and white bars show P-selectin induction with inhibitor. (d) In order to monitor intracellular Ca2+ concentration, HAEC were loaded with Fura-2 and then treated with HLA I antibody at 1μg/mL, thrombin at 0.01U/mL and 1U/mL, or isotype control antibody (mIgG). Intracellular calcium was monitored in real-time using a fluorimeter. Data are presented as the ratio of emission at 340nm/380nm, and the time of introduction of reagents is marked with an arrow.

Figure 4-2a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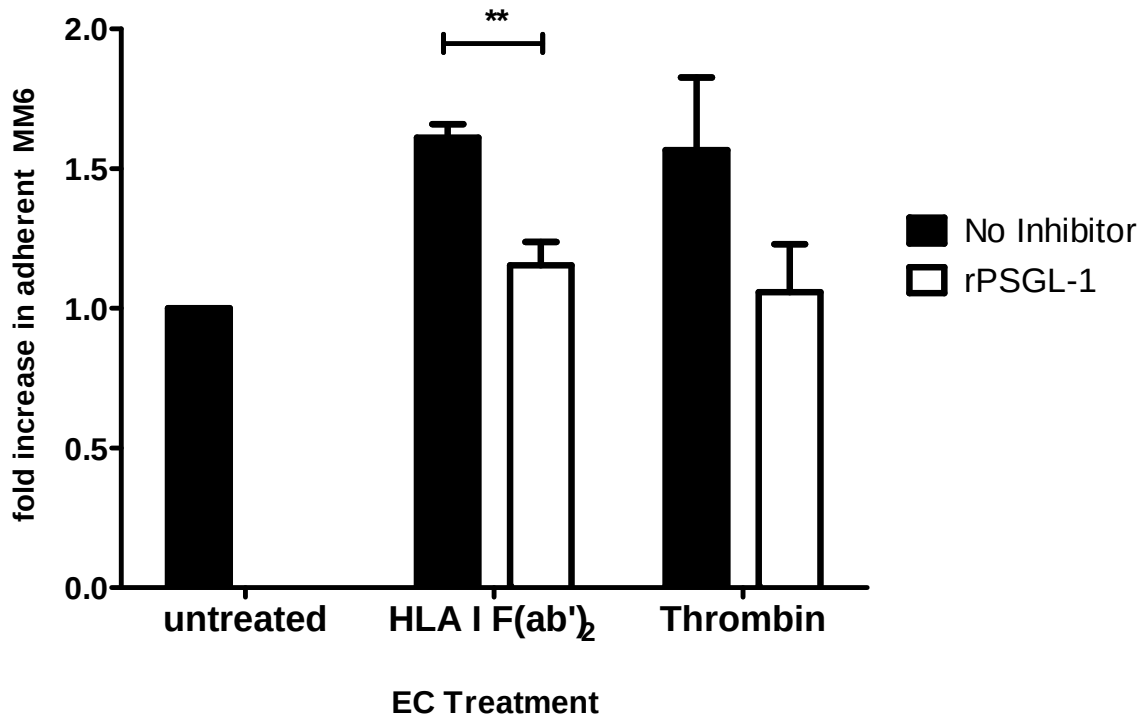
HLA I Antibody FcγR Block
Thrombin
EC Treatment

Figure 4-2b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fold increase in adherent MM6
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HLA I F(ab')2
Thrombin
EC Treatment
No Inhibitor
rPSGL-1

**Figure** 4-2c

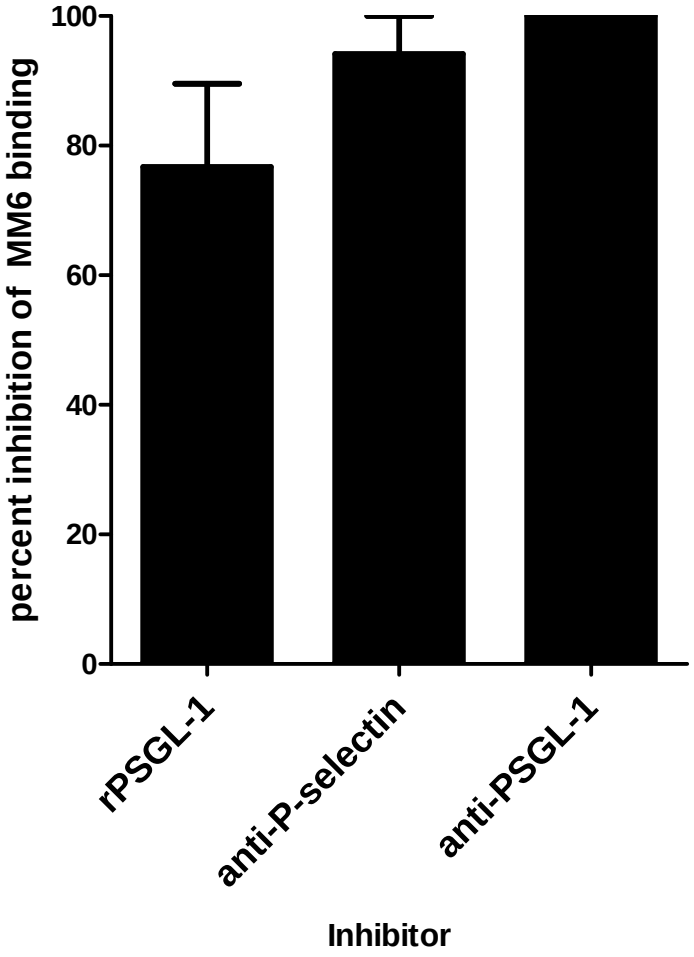

100
80
60
40
20
0
percent inhibition of MM6 binding
rPSGL-1
anti-P-selectin
anti-PSGL-1
Inhibitor

**Figure** 4-**2d**

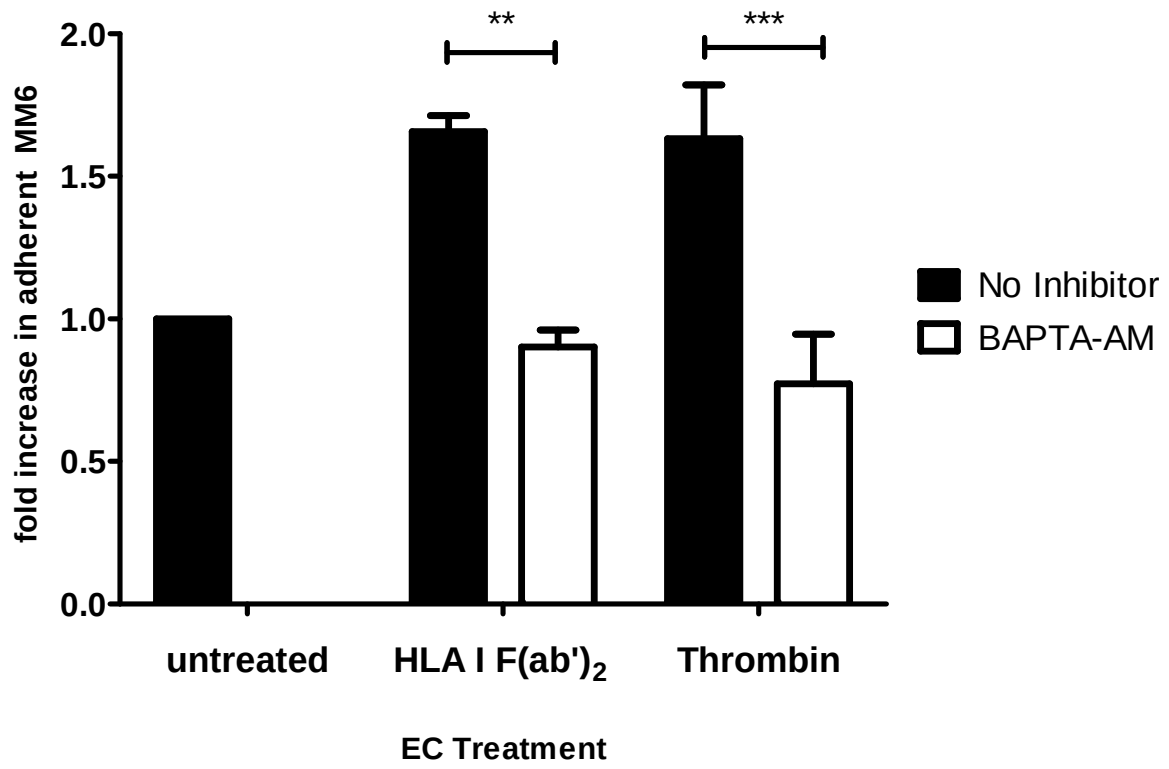



Figure 2. HLA I antibody-induced endothelial P-selectin is necessary and sufficient to support increased monocyte adherence *in vitro*. (a) HAEC were treated with isotype control mIgG (n=17), HLA I intact antibody at 5μg/mL (n=9) or F(ab')$_2$ fragment at 10μg/mL for 60min (n=18); or thrombin at 1U/mL for 20min (n=16). Fluorescently labeled Mono Mac 6 were left untreated or incubated with soluble human IgG to block FcγRs. Mono Mac 6 with or without an FcγR blockade were added to stimulated endothelial monolayers, and adherent cells were counted after 20min. Bar graph represents mean fold increase in the number of adherent Mono Mac 6 from multiple independent experiments +/-SEM, normalized to untreated. #, $p>0.05$; *** $p<0.0001$ versus untreated. (b) rPSGL-1 was added to block endothelial P-selectin, and Mono Mac 6 adherence was assessed with or without inhibitor as in (a). Graph shows mean fold increase in the average number of adherent monocytes per field over 5 independent experiments +/-SEM. Black bars show Mono Mac 6 binding without inhibitor, white bars with inhibitor. **

$p<0.01$ comparing no inhibitor to inhibitor. (c) Mono Mac 6 were incubated with anti-PSGL-1 blocking antibody (n=2), or stimulated HAEC were incubated with rPSGL-1 (n=5) or with neutralizing anti-P-selectin antibody (n=3) prior to adherence assay. Graph shows the mean percent inhibition +/- SEM of binding to HLA I $F(ab')_2$ treated endothelium by each inhibitor. (d) HAEC were pretreated with BAPTA-AM at 20μM for 30min, then stimulated with HLA I $F(ab')_2$ at 10μg/mL for 45min or thrombin at 1U/mL (n=6). Adherence of Mono Mac 6 was assessed. Data are presented as mean fold increase in adherent cells +/-SEM. Black bars are binding without inhibitor, white bars with inhibitor. ** $p<0.01$, *** $p<0.001$ versus comparing no inhibitor to inhibitor.

Figure 4-3a

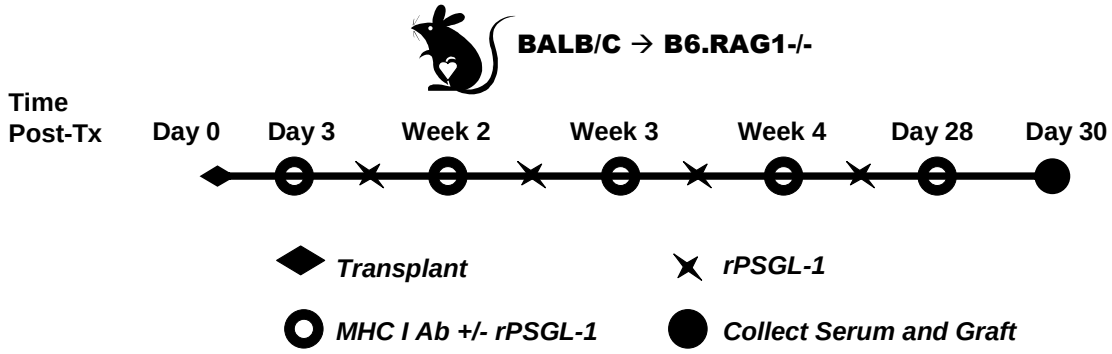
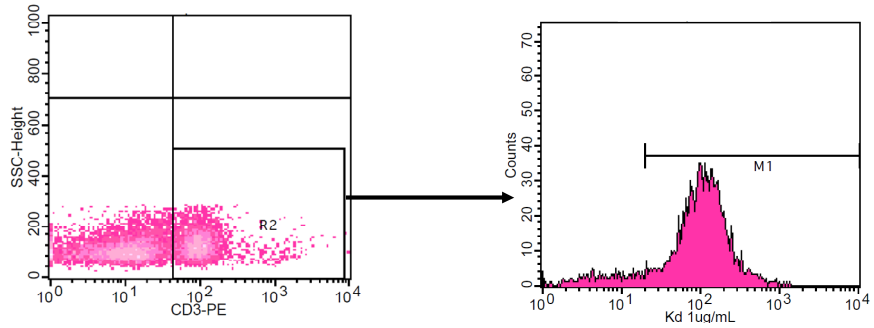

BALB/C → B6.RAG1-/-
Time
Post-Tx
Day 0
Day 3
Week 2
Week 3
Week 4
Day 28
Day 30
Transplant
rPSGL-1
MHC I Ab +/- rPSGL-1
Collect Serum and Graft

Figure 4-3b


SSC-Height
CD3-PE
R2
Counts
M1
Kd 1ug/mL

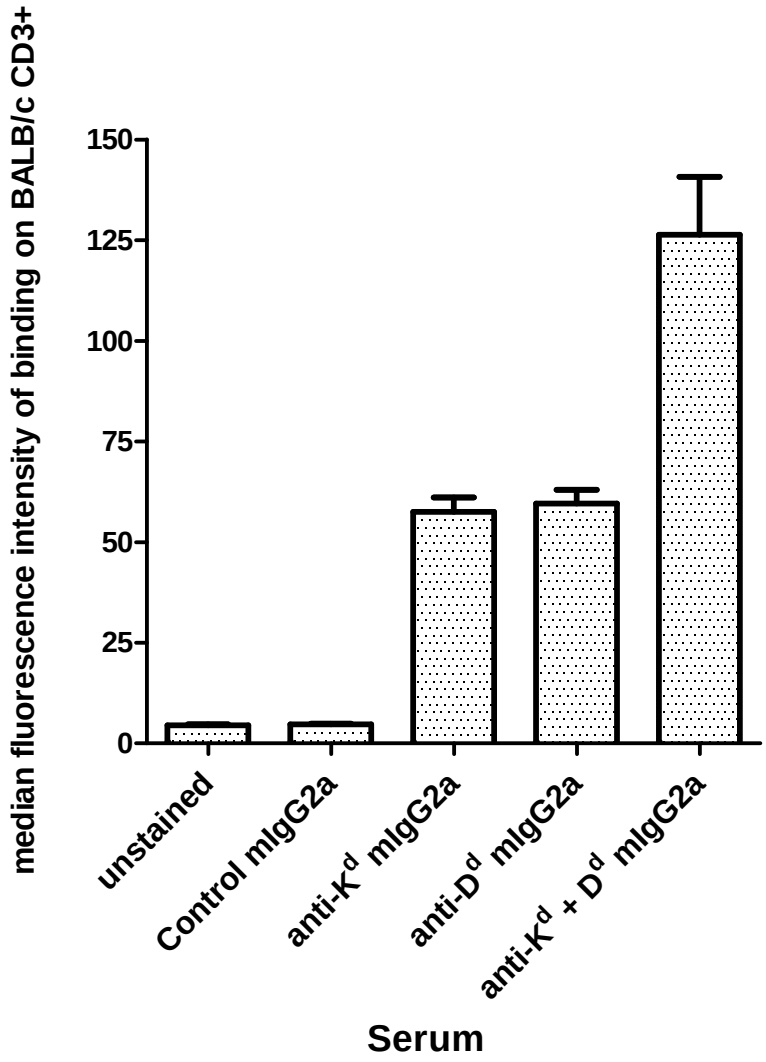

median fluorescence intensity of binding on BALB/c CD3+
150
125
100
75
50
25
0
unstained
Control mIgG2a
anti-K^d mIgG2a
anti-D^d mIgG2a
anti-K^d + D^d mIgG2a
Serum

**Figure** 4-3c

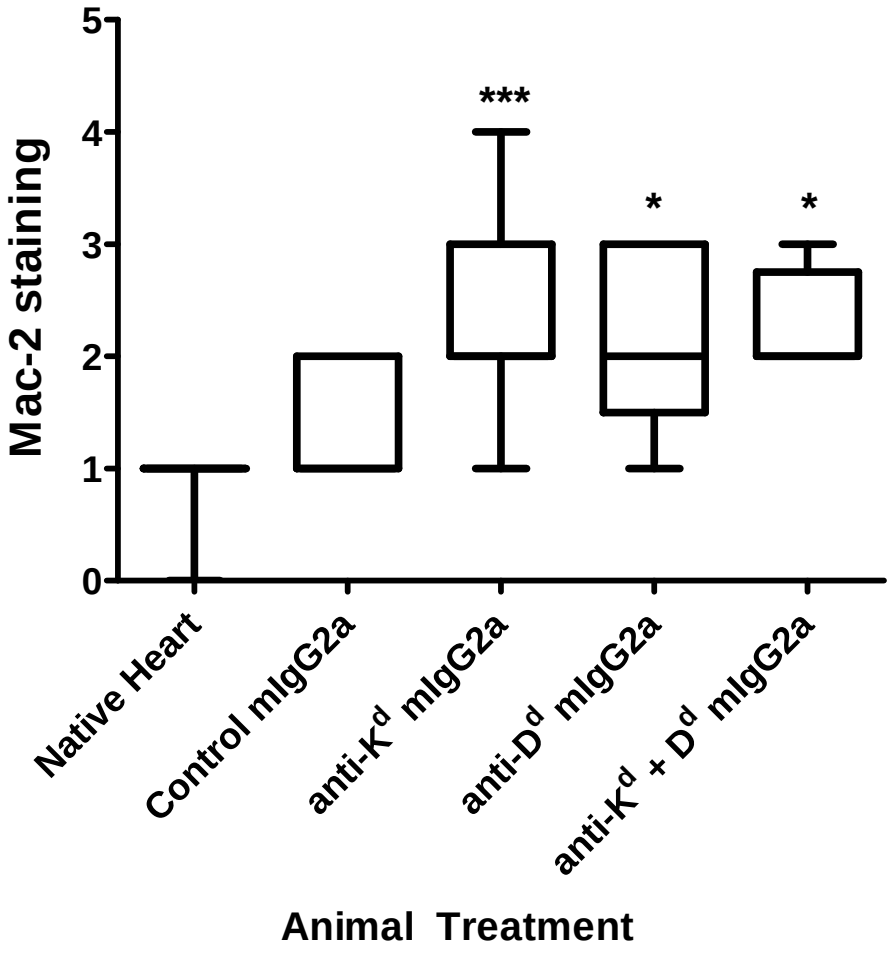



Figure 3. Donor specific MHC I antibodies increase monocyte recruitment into a murine cardiac allograft. (a) Diagram of antibody administration protocol. BALB/c (H-$2^d$) donor hearts were heterotopically grafted into RAG1 knockout recipients on a C57BL/6 background (H-$2^b$). Animals were passively transferred with a murine isotype control monoclonal IgG2a or monoclonal directed against the donor MHC I locus beginning on day 3 after transplant and continuing weekly thereafter until day 28. Grafts were recovered from recipients while still beating on day 30 post-transplant. Cellular infiltration in the base was assessed by immunohistochemical staining and scored by a blinded pathologist. The fusion protein rPSGL-1-Ig was administered to a group of animals biweekly beginning on day 3 post-transplant at 70μg

per animal. (b) The presence of circulating donor specific MHC I antibodies in recipient serum was confirmed using flow cytometric crossmatch on BALB/c splenocytes. BALB/c splenocytes were incubated with 25μL of neat serum collected from allograft recipients, and stained with anti-mouse Fcγ-FITC. Cells were stained with anti-CD3-PE and fluorescence was measured by flow cytometry. CD3-positive cells were gated as shown in the representative dot plot, and the median fluorescence in the FL-1 channel was determined. Results are shown as median fluorescence intensity of serum on CD3 positive cells +/-SEM, using sera from multiple animals for each condition: unstained (n=5), control mIgG2a (n=3), anti-$K^d$ mIgG2a (n=7), anti-$D^d$ mIgG2a (n=2), anti-$K^d$ + $D^d$ mIgG2a (n=2). (c) Native or transplanted hearts were assessed immunohistochemically for Mac-2 and staining was scored by a blinded pathologist. The box and whiskers graph shows the distribution of scores for each group. Infiltration near the suture site or in the endocardium was disregarded. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control mIgG2a group.

**Table 4-1.**

| Group | Donor | Treatment | frequency Mac-2 ≥2 | % positive | frequency Mac-2 >2 | % positive | p value |
|---|---|---|---|---|---|---|---|
| **1** | **n/a** | C57BL/6 Native Heart | 0/13 | ***0*** | 0/13 | ***0*** | |
| **2** | **BALB/c H-2*d*** | Control mIgG2a | 6/14 | ***42*** | 0/14 | ***0*** | |
| **3** | **BALB/c H-2*d*** | anti-$K^d$ mIgG2a | 21/25 | ***84*** | 10/25 | ***40*** | *0.0002 versus group 2* |
| **4** | **BALB/c H-2*d*** | anti-$D^d$ mIgG2a | 4/5 | ***80*** | 2/5 | ***40*** | *0.025 versus group 2* |
| **5** | **BALB/c H-2*d*** | anti-$K^d$ + anti-$D^d$ mIgG2a | 4/4 | ***100*** | 1/4 | ***25*** | *0.012 versus group 2* |

Figure 4-4a

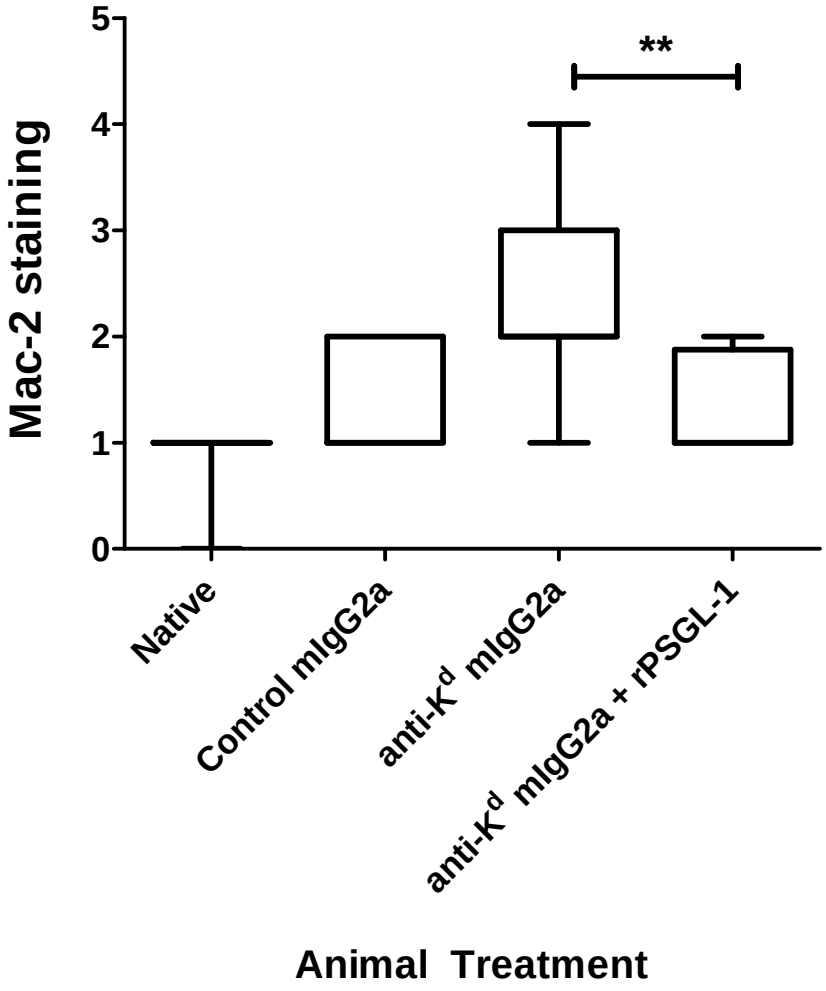

Mac-2 staining
5
4
3
2
1
0
**
Native
Control mIgG2a
anti-K$^d$ mIgG2a
anti-K$^d$ mIgG2a + rPSGL-1
Animal Treatment

**Table 4-2.**

| Group | Donor | Treatment | frequency Mac-2 ≥2 | % positive | frequency Mac-2 >2 | % positive | p value |
|---|---|---|---|---|---|---|---|
| **1** | **BALB/c H-2*d*** | Control mIgG2a | 6/14 | ***42.8%*** | 0/14 | ***0%*** | |
| **2** | **BALB/c H-2*d*** | Control mIgG2a + rPSGL-1 | 2/5 | ***40.0*** | 2/5 | ***40.0*** | |
| **3** | **BALB/c H-2*d*** | anti-$K^d$ mIgG2a | 21/25 | ***84.6*** | 10/25 | ***40.0*** | |
| **4** | **BALB/c H-2*d*** | anti-$K^d$ mIgG2a + rPSGL-1 | 2/8 | ***25.0*** | 0/8 | ***0*** | *0.004 versus group 3* |

Figure 4-4b

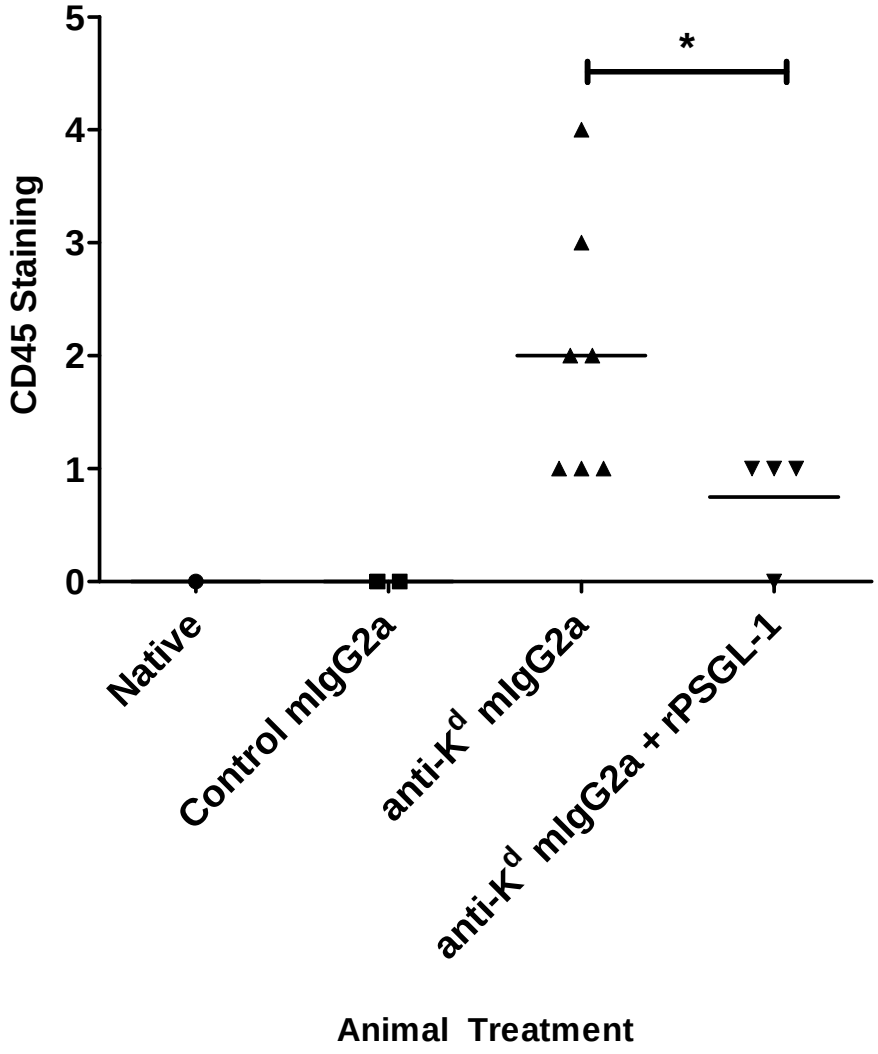

5
4
3
2
1
0
CD45 Staining
*
Native
Control mIgG2a
anti-K$^d$ mIgG2a
anti-K$^d$ mIgG2a + rPSGL-1
Animal Treatment

**Figure 4-4c**

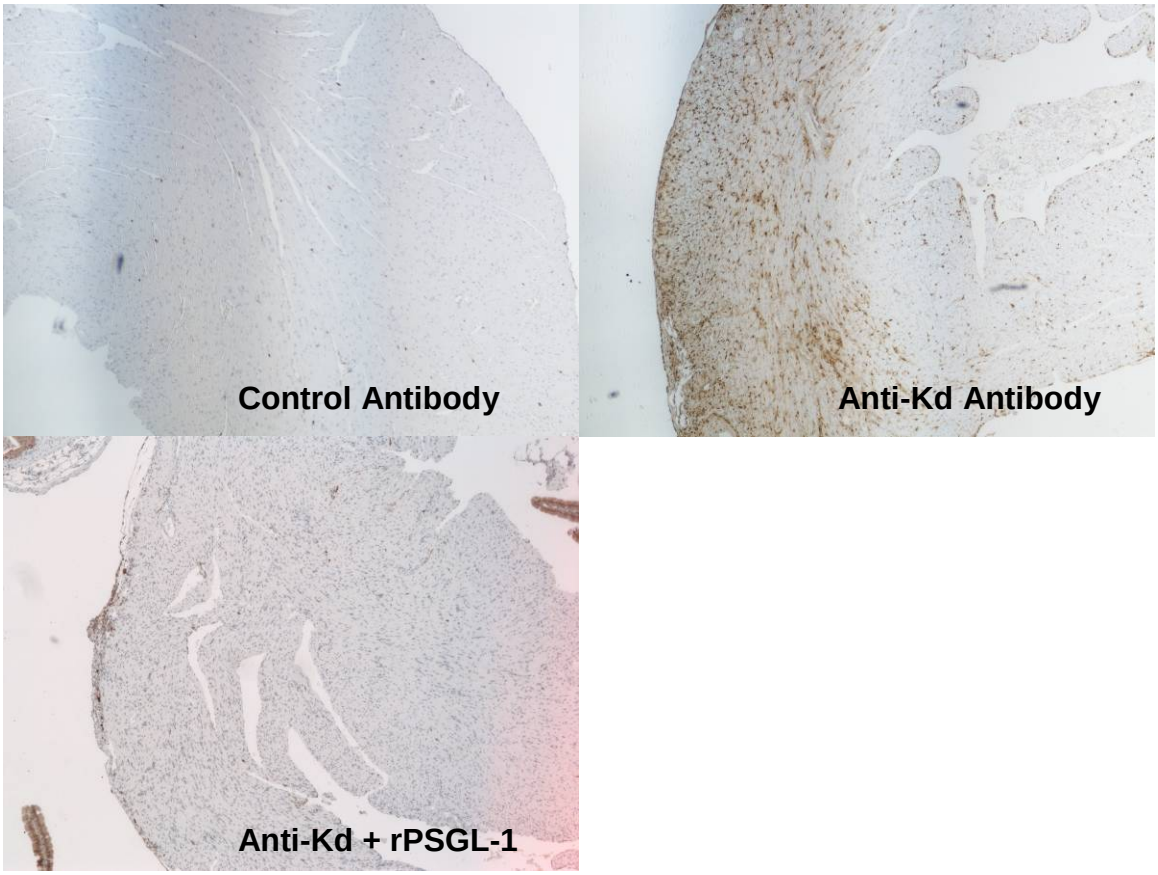



Figure 4. rPSGL-1-Ig therapy reduces macrophage and CD45+ accumulation in the allograft in response to MHC I antibody. Native or transplanted hearts were assessed immunohistochemically for Mac-2 (a) or CD45 (b) and staining was scored by a blinded pathologist. * $p<0.05$. *** $p<0.001$. Graphs show the distribution of scores for each group. Representative micrographs of Mac-2 staining are shown in (c).

Table 4-3.

| Group | Donor | Treatment | frequency CD45 ≥2 | % positive | frequency CD45 >2 | % positive | p value |
|---|---|---|---|---|---|---|---|
| **1** | **BALB/c H-2*d*** | Control mIgG2a | 0/2 | ***0%*** | 0/2 | ***0%*** | |
| **2** | **BALB/c H-2*d*** | anti-$K^d$ mIgG2a | 4/7 | ***57*** | 2/7 | ***29*** | *0.048 versus group 1* |
| **3** | **BALB/c H-2*d*** | anti-$K^d$ mIgG2a + rPSGL-1 | 0/4 | ***0*** | 0/4 | ***0*** | *0.037 versus group 2* |

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# CHAPTER 5:

# Antibody/FcγR Interactions Enhance Monocyte Recruitment in Response to HLA I Antibody-Mediated Endothelial Cell Activation by Augmenting Monocyte Activation

### *5.1 Abstract*

Antibody-mediated rejection of solid organ transplants is characterized by intragraft macrophages. HLA I crosslinking triggers endothelial release of Weibel-Palade bodies, leading to increased adhesion of neutrophils. However, it is incompletely understood how HLA I antibody binding to the graft endothelium promotes monocyte recruitment, and, what, if any, contribution is made by the Fc region of the antibody. We investigated the mechanisms underlying monocyte recruitment by HLA I antibody-activated endothelial cells, with a specific emphasis on endothelial activation by HLA I crosslinking, and on Fc fragment-mediated functions. We used a panel of murine monoclonal antibodies directed against human HLA I to crosslink these molecules on human aortic endothelial cells, and measured the binding of human monocytic cell lines. We compared the effects of antibody isotypes with a high affinity (murine IgG2a) or low affinity (murine IgG1) for human Fcγ receptors to determine the role of Fc-interactions. Treatment of endothelial cells with anti-HLA I mIgG1 significantly increased monocyte adherence in an Fcγ receptor-independent, P-selectin-dependent manner. Endothelial cells activated by anti-HLA I mIgG2a recruited monocytic cells utilizing both Fc/FcγR interactions and P-selectin. Our results demonstrate that HLA I antibodies universally elicit endothelial cell exocytosis leading to monocyte adherence, and that isotypes which can also engage FcγRs have a greater capacity to trigger recruitment.


### *5.2 Introduction*

Organ transplantation is a life-saving therapy for end-stage organ failure. Advances in histocompatiblity testing, patient management and immunosuppression have improved short-term graft survival, which is estimated between 75-90% for the majority of solid organ transplants at one year after surgery, based on OPTN data as of April 20, 2012. However, long-term graft survival has continued to be low; 50% or more of all solid organ grafts were lost at 10 years post-transplant. The

major challenge to achieving long-term graft survival is chronic rejection, which manifests as transplant vasculopathy, in which the blood vessels of the graft develop concentric neointimal thickening with ultimate lumen occlusion, necessitating retransplantation. Rejection of organ transplants is caused by alloimmune responses mediated by T cells and/or antibodies. The recipient's humoral response primarily targets the donor's polymorphic HLA molecules, and many studies have correlated the presence of anti-donor HLA antibodies with antibody-mediated rejection and poor graft (1)(2) and chronic rejection (3)(4). A histological hallmark of antibody-mediated rejection (AMR) is the presence of intragraft macrophages (5), and macrophages rather than T cells associate with decreased renal allograft function and poor survival (6-10). Macrophages can comprise up to 60% of the cellular infiltrate in acute rejection, including acute cellular rejection (11), and are also found in the vascular lesions of transplant vasculopathy (12, 13).

Donor specific HLA antibodies bind to the endothelial and smooth muscle layers of the graft vasculature which can trigger activation of the complement cascade. However, complement deposition is not always observed in acutely injured allografts, even when patients have diagnosed antibody-mediated rejection and donor specific antibodies (DSA) (14). Our group has proposed that the pathogenesis of HLA I antibodies derives in part from their ability to directly activate the graft vasculature. We and others have demonstrated *in vitro*, in experimental animals models and in patient biopsies that HLA or MHC I antibody binding to endothelial and smooth muscle cells activates intracellular signaling cascades mediating proliferation and cellular resistance to death (15-18), primarily via Src, PI3K/Akt, ERK and mTOR. Additionally, HLA I molecule ligation on endothelium mobilizes vesicles called Weibel-Palade bodies (WPb), the exocytosis of which results in a rapid increase in vWF secretion, cell surface P-selectin and adherence of HL60 leukocytic cells (19).

Activated endothelial cells express E- or P-selectin, molecules which capture leukocytes from the blood by binding to PSGL-1, and support low affinity adhesion known as rolling or tethering. This interaction exposes the leukocyte to endothelial-bound factors such as chemokines or complement split products, which activate the leukocyte through its G protein coupled receptors or complement receptors. The initial binding to P-selectin primes the leukocyte for a proadhesive state and is required for efficient recruitment (reviewed in (20)). Upon activation, leukocyte integrins are converted from a low affinity to high ligand binding state, permitting them to firmly adhere to their ligands, the cellular adhesion molecules VCAM and ICAM (reviewed in (21, 22)); subsequently they transmigrate into the tissue. Endothelial intracellular adhesion molecule 1 (ICAM-1) is bound by two leukocyte integrins which contain the β2 subunit. The αL subunit pairs with β2 integrin to form LFA-1, while the αM subunit complexed with β2 integrin forms Mac-1.

In addition to adhesion molecules, monocytes express receptors for the Fc region of IgG. Humans have three families of Fc gamma receptors (FcγRs), which are expressed on myeloid and lymphoid cells. FcγRI (CD64) is the highest affinity receptor for IgG, by several orders of magnitude. FcγRII and FcγRIII are thought to bind multimeric or immune complexed IgG (reviewed in (23, 24). The subclass and isotype of an antibody controls its effector functions, such as affinity for FcγRs. In humans, isotypes IgG3 and IgG1 are the most effective activators of complement among gamma globulins and have the highest affinity for FcγRs, while IgG2 and IgG4 have limited ability to activate the complement cascade. Murine IgG2a has the highest affinity for human FcγRs and can bind all three family members; murine IgG3 can only interact with human FcγRI; and murine IgG1 is, in general, the lowest affinity isotype (24, 25). These attributes are reflected in the different capacities of antibody isotypes to trigger FcγR-mediated functions in neutrophils, NK cells and

monocytes, such as antibody-dependent cellular cytotoxicity (cell depletion) *in vivo* (26, 27), phagocytosis (28), and FcγR-dependent cytokine (29) production.

Macrophages play a critical role in both acute and chronic allograft rejection. We therefore focused on the recruitment of monocytes in an *in vitro* model of HLA I antibody-mediated rejection to gain a better understanding of how HLA I antibodies promote accumulation of intragraft macrophages. We used an *in vitro* system of primary human endothelial cells and a panel of HLA I murine monoclonal antibodies of different isotypes to investigate monocyte recruitment in response to HLA I antibody binding to endothelial cells. We hypothesized that HLA I antibodies have a unique capacity compared with other endothelial cell antibodies to promote monocyte recruitment into the allograft because of their dual actions on the endothelium and on the monocyte. First, HLA I crosslinking by antibodies rapidly increases endothelial cell surface P-selectin, which is sufficient to initiate the recruitment of monocytes. Secondly, the engagement of monocyte FcγRs with endothelial-bound antibody can enhance adherence. An upshot of the involvement of FcγRs is that the isotype of the HLA I antibody may influence the antibody's ability to augment P-selectin-mediated recruitment.

### *5.3 Materials and Methods*

*Reagents and Antibodies*

Mouse anti-human HLA-ABC antibodies (clone W6/32, murine IgG2a, from BioXCell; clone 246-B8.E7, murine IgG2a, clones MEM-147 and MEM-81, murine IgG1, from Abcam) were used to ligate HLA I on endothelial cells. The monoclonal antibodies W6/32 and MEM-147 recognize monomorphic native epitopes common to all HLA I molecules (30). Isotype matched control antibodies, nonspecific mIgG or anti-endoglin (CD105, murine IgG2a, clone P3D1, Millipore) were used as negative controls. Isotype control murine IgG2a (MOPC-173) and murine IgG1 (MOPC-21), low endotoxin, azide free format, were purchased from BioLegend. Neutralizing antibodies to β2 (murine IgG1 clone TS1/18, BioLegend), αL (BioLegend), αM (BioLegend), ICAM-1 (sheep IgG, R&D; murine IgG1 clone HCD54, BioLegend), PSGL-1 (murine IgG1 clone KPL-1, BioLegend), P-selectin (clone AK4, murine IgG1, Biolegend; sheep or goat IgG, R&D), FcγRI (clone 10.1, BioLegend), FcγRII (clone AT10, Abcam), FcγRIII (clone 3G8, BioLegend), and Fcα/μR (BioLegend) were used and were of isotypes shown to have low affinity for human FcγRs (25) to minimize nonspecific blockade of FcγR. Recombinant soluble P-selectin-Fc chimera homodimer was obtained from R&D. Recombinant soluble PSGL-1 Fc chimera (YSPSL) was obtained from Y's Therapeutics (San Bruno, CA), which has an Fc region from human IgG1 containing point mutations to eliminate interactions with complement and FcγRs.

*Cells and Culture Conditions*

Primary human aortic endothelial cells (HAEC) were isolated from aortic rings in accordance with IRB as previously described (17). HAEC were grown to confluence on 0.1% gelatin in PBS. Cells were cultured in Medium 199 (Gibco) supplemented with 10% heat-inactivated FBS, 1X sodium-pyruvate, 4mg/mL heparin, penicillin-streptomycin, and endothelial cell growth supplement (BD),

and stimulated in low-serum medium (M199 with 2% FBS). All experiments were repeated with endothelial cells from at least two different donors, with different HLA haplotypes (CAR: A*02, A*68; B*60, B*65; CAS: A*02, A*30; B*35; B*35 ;3F1153: A*02, A*11; B*44, B*56; D121: A*01, A*02, B*08, B*60; H126: A*03, A*29; B*35, B*44). Endothelial cells were used at passages 4 through 8.

The human monocytic cell line Mono Mac 6 (kind gift of Dr. Judith Berliner, Department of Pathology and Laboratory Medicine, UCLA) was cultured in RPMI-1640 supplemented with 10% FBS, 10μg/mL bovine insulin, sodium-pyruvate, penicillin-streptomycin, and nonessential amino acids (Gibco). Mono Mac 6 adherence to endothelial cells has been reported to be more similar to that of primary monocytes than other monocytic cell lines (31, 32). THP-1 monocytic cells were maintained in RPMI-1640 supplemented with 5% FBS and antibiotics. Monocytic cell lines were subcultured every 2-3 days and maintained at a density of less than $10^6$ cells per ml.

*HLA I Antibody Binding*

Endothelial cells were detached by Accutase, and stained with 1μg of HLA I antibody in 0.1mL of PFA flow buffer for 30min on ice. Cells were washed twice and a secondary anti-mouse Fcγ FITC-conjugated antibody at 1:100 (Jackson Immuno) was used to detect antibody binding. Fluorescence was measured by flow cytometry.

*Flow Cytometric Determination of FcγR Expression*

Expression of FcγRs on monocytes was measured by flow cytometry. FcγRI (anti-CD64, Biolegend), FcγRII (anti-CD32, StemCell), FcγRIII (anti-CD16, Biolegend), and Fcα/μR (anti-CD351, Biolegend)—all murine monoclonal IgG1—were used at 1μg per 0.1mL PFA (PBS with 2.5% FBS and 0.1% sodium azide) flow buffer on ice for 30-45min. Secondary antibody anti-mouse

Fcγ-FITC (Jackson Immuno) was used to stain the cells. Results are shown as average median channel fluorescence +/- SEM from three experiments.

*Determination of Cell Surface P-selectin*

Cell surface expression of P-selectin was measured on adherent endothelial cells by cell-based ELISA (adapted from (33)). Briefly, confluent endothelial cells in a 96-well plate were treated with control or HLA I antibodies in M199 with 5% FBS for 30min. Medium was removed. Cells were washed once with PBS and fixed in freshly prepared 2.5% paraformaldehyde in PBS for 10min at room temperature. Buffered formalin was not used because it is stabilized with methanol, which can permeabilize the cells. Cells were washed with PBS and blocked with 5% BSA in PBS for 30min. Sheep anti-P-selectin (R&D) was added at 1μg/mL for 2hr. Wells were washed three times and donkey anti-sheep-HRP (Millipore) was added at 1:1000 for 2hr at room temperature. After washing, TMB substrate was added and the reaction was stopped with sulfuric acid. Optical density was read at 450nm on a SpectraMax plate reader (Molecular Devices).

*Blockade of FcγRs*

Since monocytes and the Mono Mac 6 cell line express high levels of Fc gamma receptors (FcγR), preliminary studies were undertaken to determine the ability of Mono Mac 6 to take up soluble monomeric murine IgG, and to assess the effectiveness of blocking strategies. According to these findings, in specified adhesion experiments Mono Mac 6 or THP-1 were incubated with 20μg/mL of purified human IgG in PBS (Fisher Scientific) or 10μg/mL of monoclonal murine IgG2a (MOPC-173) for 15min to block the FcγR from binding murine IgG on the surface of the endothelial cells.

*Static Adherence Assay*

Confluent HAEC were treated with mIgG, anti-CD105 mIgG2a, HLA I antibodies, or positive controls thrombin or PMA in M199 with 2% FBS (assay medium) for the indicated times. Monocytic cells were fluorescently labeled with CFDA-SE (Vybrant Cell Tracer, Invitrogen) at 2μM in HBSS with $Ca^{2+}$ and $Mg^{2+}$ for 10min at 37°C, then washed. After treatment, medium was aspirated from HAEC, and Mono Mac 6 or THP-1 cells were added at approximately $5x10^5$ cells in M199 with 2% FBS per 35mm dish or $2x10^5$ cells per well in a 24 well plate (roughly equivalent ratio of 2-3monocytes:1EC). We used a low concentration of FBS to reduce stimulation by growth factors, and heparin was excluded from the medium because it can inhibit basal binding of Mono Mac 6 to endothelial cells (31). Monocytes and endothelial cells were cocultured for 20min at 37°C, then nonadherent cells were decanted. The coculture was washed three times by decanting and gentle pipetting down the wall of the well, and fixed in freshly made 4% paraformaldehyde in PBS for 20min. Images of adherent monocytes were acquired by fluorescence microscopy on a Nikon Eclipse Ti, in 8-10 4x objective fields for each condition. The number of adherent cells in each field was quantified using automated software: Image J Cell Counter or CellProfiler (17, 34). Results are expressed for each condition as the mean number of adherent monocytes per field or as fold change in mean adherent monocytes per field normalized to untreated, +/- standard error of the mean (SEM).

To determine the effect of adhesion molecules in monocyte adherence to antibody-activated endothelium, receptors were blocked on either the monocytes or the endothelial cells prior to coculture. To block P-selectin and ICAM-1, HAEC were treated as above, and functional grade P-selectin or ICAM-1 blocking antibodies were added at a concentration of 10μg/mL for 10min before coculture with monocytes. To block monocytic receptors, cells were preincubated with neutralizing antibodies to β2 integrin, αL (LFA-1), αM (Mac-1), PSGL-1, FcγRI, FcγRII, FcγRIII, or

Fcα/μR at 2μg/mL for 20min prior to the adhesion assay. All blocking antibodies were murine IgG1 isotype, except anti-P-selectin, which was goat immunoglobulin. Alternatively, to block selectins, rPSGL-1 was added at 20μg/mL, a concentration which maximally inhibited Mono Mac 6 adherence to purified immobilized P-selectin in preliminary experiments. Percent inhibition of adherence by each inhibitor was calculated as follows: ((fold change without inhibitor - 1) - (fold change with inhibitor – 1)) / (fold change without inhibitor – 1) x 100%.

*Immobilized Protein Adherence Assay*

Purified polyclonal human IgG (Fisher), murine IgG2a (MOPC-173), or murine IgG1 (AK4) were diluted in carbonate/bicarbonate buffer and coated onto high protein binding (Nunc Maxisorp) 96 well plates at 0.1mL per well, then incubated overnight at 4°C. The next day, the protein solution was removed, and the wells were blocked with 5% BSA in PBS for 2hr at room temperature. Mono Mac 6 cells were labeled with 2μM CFSE in HBSS for 10min, then serum rescued with 5% FBS in HBSS, spun down and resuspended at $10^6$ cells per mL. Cells were added to the plate at $10^5$ cells in 0.1mL per well and incubated at 37°C for 30min. Wells were washed three times with HBSS using the flick method, then fixed. Adherent cells were visualized by fluorescence microscopy using a 4x objective, and 5 fields per well were counted using Image J or CellProfiler.

*Dynamic Adherence Assay*

Confluent HAEC in a 12 well plate were treated with mIgG, anti-CD105 mIgG2a, or HLA I antibodies in M199 with 2% FBS (assay medium) for 45min. Mono Mac 6 were fluorescently labeled with CFDA-SE as above, then washed. Mono Mac 6 were added at approximately $5x10^5$ cells per well and allowed to adhere for 20min at 37°C on an orbital shaker (New Brunswick) at 40RPM. At this speed, the $\tau_{max}$ was calculated at 1.5 dynes/cm$^2$, similar to shear stress in capillaries (35). Dynes/cm$^2$ were estimated using the following formula: $\tau_{max} = a\sqrt{(\eta\rho(2\prod f)^3)}$ (35, 36), where a is the

radius of rotation for the shaker, $\eta$ is the viscosity of the medium, $\rho$ is the density of the medium, and $f$ is the frequency of rotation in revolutions per second. The coculture was washed three times by decanting and gentle pipetting, and fixed in freshly made 4% paraformaldehyde in PBS for 20min. Adherent monocytes were counted by fluorescence microscopy on a Nikon Eclipse Ti, in 8-10 4x objective fields for each condition using CellProfiler (34). The center of the well was avoided, as shear stress becomes negligible here on an orbital shaker (36). Results are expressed for each condition as the mean number of adherent monocytes per field +/- standard error of the mean (SEM).

*Statistical Analysis*

In the leukocyte adherence assay, the number of adherent cells was counted in 8-10 random fields for each sample, and the mean number of cells was determined. Error bars represent standard error of the mean (standard deviation/square root of field number). Statistical significance between controls and samples was determined using the student's T-test, using one-tailed distribution and two sample equal or unequal variance based on the results of an F test.

### *5.4 Results*

*5.4.1 The magnitude of HLA I antibody-induced monocyte adhesion to endothelial cells is dependent upon HLA I antibody isotype.*

To determine if HLA I antibody treatment of endothelial cells increased monocytic cell adherence, we tested a panel of monoclonal murine antibodies each recognizing all HLA I molecules. Treatment of endothelial cells with HLA I antibody significantly increased adherence of Mono Mac 6 (Figure 1A). HLA I mIgG1 (clone MEM-147) increased the number of bound Mono Mac 6 to 508.0+/-25.7 per field, as did endothelial stimulation with HLA I murine IgG1 (MEM-81), at 445.6+/-7.4 adherent monocytes per field. Both clones of mIgG2a pan-HLA I antibodies (W6/32 and 246-B8.E7) dramatically increased adherence of Mono Mac 6 to endothelial cells, to 777.2+/-21.0 and 999.4+/-60.3 per field, respectively. Mono Mac 6 bound to untreated endothelial cells from donor 3F1152 at a rate of 297.7+/-24.3 cells per 4x field. Similar results were observed when the panel was used to stimulate endothelial cells from a different donor (H126). Representative fields illustrating Mono Mac 6 adherent to the endothelial monolayer are given in Figure 1B.

We observed that there was a difference in the magnitude of Mono Mac 6 adherence stimulated by mIgG1 compared with mIgG2a HLA I antibodies. We confirmed this observation using a second monocytic cell line, THP-1. Adhesion of Mono Mac 6 and THP-1 monocytes was significantly increased to endothelial cells stimulated with HLA I mIgG1 (MEM-147), by 2.16+/-0.21 fold and 2.34+/-0.2 fold, respectively. Stimulation of endothelial cells with HLA I mIgG2a (W6/32) increased adherence of Mono Mac 6 by 5.17+/-0.72 fold over control, and THP-1 by 4.45+/-0.9 fold over control. When the two isotypes were compared in parallel, the degree of monocyte adherence to HLA I mIgG1 (MEM-147)-treated endothelial cells was significantly lower than to HLA I mIgG2a (W6/32) treated endothelial cells (Figure 1C). This observed difference between

isotypes could not be attributed to unequal amounts of antibody bound to the endothelial cell surface, as we used flow cytometry to assess the binding capacity of each HLA I monoclonal, and found staining to be comparable (Figure 1D).

We postulated that the difference in the capacity of HLA I antibody isotypes to stimulate monocyte adherence was due to unequal affinities of the Fc portion for human FcγRs. To test the ability of each isotype to engage FcγRs on the monocyte, we used a solid phase assay in which immunoglobulin was immobilized and Mono Mac 6 adherence was measured (Figure 2A and 2B). Mono Mac 6 did not adhere to wells coated with nonspecific murine IgG1 (clone AK4). In contrast, Mono Mac 6 adhered to plastic coated with positive control polyclonal human IgG, or to nonspecific murine IgG2a (clone MOPC-173), to a significantly greater extent than to BSA alone. Thus, murine IgG1 has a lower ability to bind Mono Mac 6 than murine IgG2a, consistent with previous studies (37, 38).

We next asked whether HLA I antibody-stimulated recruitment was dependent on FcγRs. We treated endothelial cells with HLA I mIgG1 (MEM-147). Mono Mac 6 were left untreated or incubated with soluble IgG to block FcγRs prior to the adherence assay. HLA I mIgG1 (MEM-147) significantly increased the adherence of Mono Mac 6 to endothelium, by 2.34+/-0.24 fold over untreated. Mono Mac 6 binding was not significantly different when FcγRs were blocked, at 2.11+/-0.25 fold over untreated (Figure 2C). Therefore, inhibition of FcγRs by preincubation with soluble IgG did not impair the ability of Mono Mac 6 to bind to endothelial cells stimulated with HLA I mIgG1 (MEM-147).

To determine whether HLA I mIgG2a-triggered monocyte recruitment had an Fc-mediated component, endothelial cells were treated with HLA I mIgG2a (W6/32). Since both monocytic cell lines express high levels of FcγRI, moderate levels of FcγRII, and no detectable FcγRIII or Fcα/μR (Supplemental Figure 1), we questioned which FcγR participated during adherence. Mono Mac 6 were pretreated with a blocking antibody to FcγRI, FcγRII, FcγRIII or Fcα/μR, or with soluble IgG to inhibit all FcγRs. Adherence to HLA I mIgG2a (W6/32)-treated endothelial cells was measured. HLA I mIgG2a-stimulated endothelial cells supported significantly more monocyte binding than untreated endothelium. Inhibition of FcγRI significantly reduced the number of monocytes adherent to HLA I mIgG2a (W6/32)-treated endothelial cells, whereas FcγRII or FcγRIII inhibition had little to no effect (Figure 2D). The neutralizing antibody to FcγRI significantly reduced Mono Mac 6 binding by 82.6+/-8.1%, while a blocking antibody against FcγRII had a lesser inhibitory effect at 59.2+/-8.8%. In contrast, blocking antibodies to FcγRIII or Fcα/μR had no significant effect, consistent with the lack of expression of these receptors on the monocytic cell lines (Figure 2E). Total FcγR blockade inhibited Mono Mac 6 and THP-1 adherence by 92.4+/-3.5%.

Similar results were obtained when THP-1 monocyte binding was assessed. FcγR blockade inhibited THP-1 adherence by 72.0+/-8.8%. Inhibition of FcγRI reduced THP-1 binding to HLA I mIgG2a (W6/32)-stimulated endothelium by 89.3+/-4.4%. Pretreatment of THP-1 with a neutralizing antibody to FcγRII resulted in minor inhibition of adherence (24.4+/-4.9%), whereas FcγRIII antagonism did not significantly affect the ability of THP-1 monocytes to adhere to HLA I mIgG2a (W6/32)-treated endothelial cells (Figure 2E). These results demonstrate that FcγRI is predominantly involved in monocyte adherence to endothelial cells activated with HLA I mIgG2a.

*5.4.2 HLA I antibodies increase endothelial P-selectin, which is required for subsequent monocyte adherence.*

We observed that HLA I mIgG1-stimulated monocyte adherence to endothelial cells was not affected by an FcγR blockade, pointing to an Fc-independent mechanism of HLA I antibody-elicited monocyte binding. Therefore, we hypothesized that HLA I antibody-induced endothelial adhesion molecules could be responsible. Previously, Yamakuchi et al. reported that HLA I mIgG2a (W6/32) induced endothelial P-selectin (19). Therefore, we determined the effect of stimulation with our additional monoclonal HLA I antibodies on early endothelial cell activation, and tested cell surface P-selectin by cell-based ELISA. Confluent endothelial cells were left untreated or exposed to isotype control mIgG, a non-HLA anti-endothelial cell antibody (anti-CD105 mIgG2a), or HLA I antibody (MEM-147 and MEM-81), and cell surface P-selectin was measured. We did not detect P-selectin on the cell surface of untreated endothelial cells. Neither mIgG nor anti-CD105 mIgG2a increased P-selectin above background. In contrast, HLA I antibodies elevated P-selectin on the endothelial cell surface, by more than 3-fold over untreated (Figure 3A).

We next determined whether antibody bound to endothelium was sufficient to increase monocyte adherence without P-selectin, or whether this effect was specific to antibodies recognizing HLA I. Endothelial cells were treated with HLA I mIgG1 (MEM-147) or a non-HLA antibody of the same isotype recognizing ICAM-1 (anti-ICAM-1 mIgG1). Endothelial cells were also treated with HLA I mIgG2a (W6/32) or an isotype matched anti-CD105 mIgG2a, or positive control PMA, and monocyte adherence was measured. Coating of endothelial cells with either mIgG1 against ICAM-1, or mIgG2a against CD105, did not significantly increase adherence of Mono Mac 6, despite FcγR expression. Treatment of endothelium with HLA I mIgG1 (MEM-147) or HLA I mIgG2a (W6/32), which increased endothelial P-selectin, significantly increased adherence of Mono Mac 6. Identical

results were obtained when THP-1 monocytes were used (Figure 3B). Therefore, non-HLA I antibodies bound to endothelial cells do not increase adherence of monocytes.

We found that the monocytic cell lines Mono Mac 6 and THP-1 expressed the ligand for P-selectin, PSGL-1 (Supplemental Figure 1). We hypothesized that endothelial P-selectin elicited by HLA I ligation may be responsible for monocyte adherence. To test the role of P-selectin, endothelial cells were treated with HLA I mIgG2a (W6/32), HLA I mIgG1 (MEM-147), or positive control thrombin, and exposed to the rPSGL-1 soluble antagonist of P-selectin. Adherence of Mono Mac 6 was measured with and without the antagonist. The presence of rPSGL-1 significantly inhibited Mono Mac 6 binding to endothelial cells stimulated with either HLA I mIgG2a (W6/32), HLA I mIgG1 (MEM-147) or with thrombin, by 69.4+/-4.3%, 70.4+/-8.4%, and 88.6+/-6.9%, respectively (Figure 3C), demonstrating the effectiveness of a P-selectin blockade against recruitment induced by both isotypes of HLA I antibody. Representative fields illustrating Mono Mac 6 adherence to HLA I antibody-activated endothelial cells with and without the antagonist are shown in Figure 3D. Similar results were observed using THP-1 monocytes (data not shown).

To verify the effects of rPSGL-1, we used two alternative strategies to block the interaction of P-selectin with monocyte PSGL-1. HLA I mIgG2a (W6/32)-stimulated endothelial cells were incubated with a neutralizing antibody to P-selectin, or Mono Mac 6 were treated with a blocking antibody to PSGL-1. Inhibition of P-selectin reduced Mono Mac 6 adherence by 40.7+/-3.5%, and antibody to PSGL-1 inhibited recruitment by 59.8+/-20.5% (Supplemental Figure 2). These results demonstrate that P-selectin is required for HLA I antibody-triggered monocyte binding.

*5.4.3 Mac-1/ICAM-1 interactions are required for HLA I antibody-dependent monocytic cell binding.*

While P-selectin initiates capture of leukocytes from the blood by endothelial cells, integrin-CAM interactions are required for firm adherence. Because endothelial cells constitutively express ICAM-1, and Mono Mac 6 and THP-1 express the ICAM-1 receptors LFA-1 (αLβ2) and Mac-1 (αMβ2) (Supplemental Figure 1), we reasoned that ICAM-1/β2 integrins were involved in stable adhesion of monocytes to HLA I antibody-stimulated endothelial cells. Endothelial cells were treated with HLA I mIgG2a (W6/32) or HLA I mIgG1 (MEM-147), and a neutralizing antibody to ICAM-1 was added. Mono Mac 6 adherence was significantly inhibited when ICAM-1 was blocked, by 75.7+/-5.3% for HLA I mIgG2a and 75.3+/-14.9% for HLA I mIgG1 (Figure 4A).

As a reverse experiment, we blocked the ICAM-1 binding integrins LFA-1 and Mac-1, by pretreating the Mono Mac 6 with a neutralizing antibody against β2 integrin prior to the adhesion assay. β2 integrin inhibition reduced Mono Mac 6 binding to HLA I mIgG2a (W6/32)-stimulated endothelium by 70.3+/-4.4% compared with no inhibitor. Blockade of β2 integrin also abolished Mono Mac 6 ability to bind to HLA I mIgG1 (MEM-147)-treated endothelium, reducing the number of adherent cells by 84.1+/-15.9% (Figure 4A). Similar results were observed with THP-1 (data not shown).

Because monocytes express two integrins which bind to ICAM-1, we explored the relative contribution of each to HLA I mIgG2a-triggered adherence. The β2 subunit pairs with the αL subunit to form LFA-1 (αLβ2), while αM complexes with β2 to form the Mac-1 integrin. Endothelial cells were treated with HLA I mIgG2a (W6/32). Mono Mac 6 were incubated with a neutralizing antibody to the αL subunit of LFA-1, to the αM subunit of Mac-1, or to the β2 subunit, which blocks both LFA-1 and Mac-1. The number of adherent Mono Mac 6 to untreated endothelial cells was measured at 384.6+/-27.6 per field. HLA I mIgG2a (W6/32) increased adherence of Mono

Mac 6 to endothelial cells to 995.8+/-31.19 per field. Pretreatment of Mono Mac 6 with a blocking antibody targeting αL (LFA-1) did not significantly alter recruitment in response to HLA I mIgG2a, with 903.6+/-17.9 per field. In contrast, inhibition of αM (Mac-1) on Mono Mac 6 significantly impaired the monocytes' ability to adhere to HLA I mIgG2a-activated endothelial cells, reducing the number of adherent monocytes to 509.4+/-21.6 per field. As in Figure 4A, blocking of endothelial ICAM-1 or total β2 integrins also significantly inhibited adhesion of Mono Mac 6 to endothelium in response to HLA I mIgG2a (Figure 4B). The neutralizing antibody to αL (LFA-1) inhibited adherence by 25.0+/-8.4% ($p>0.01$), while blockade of αM (Mac-1) abolished the adhesive capacity of Mono Mac 6, by 78.1+/-19.7% compared to monocytes without inhibitor (Figure 4C), demonstrating the dominance of this integrin in monocyte recruitment by HLA I antibodies. These results were similar when THP-1 monocyte adherence was assessed (data not shown).

*5.4.4 HLA I antibody stimulation of endothelial cells increases monocyte recruitment under dynamic conditions.* Finally, because a static adhesion assay is not always optimal, and findings may not translate to the physiological setting, we sought to confirm our observations using a dynamic adhesion assay. Endothelial cells were treated with anti-CD105 mIgG2a, HLA I mIgG1 (MEM-147) or HLA I mIgG2a (W6/32), Mono Mac 6 were allowed to adhere under dynamic conditions, on an orbital shaker which simulated shear stress equivalent to the dynamics in capillaries (39). Antibody to endoglin (anti-CD105) did not significantly increase adherence of Mono Mac 6 (1.14+/-0.2-fold over control). HLA I mIgG1 (MEM-147) treatment of endothelial cells significantly increased their capacity to recruit Mono Mac 6, by 3.46+/-0.64-fold compared with untreated. HLA I mIgG2a (W6/32) activation of endothelial cells also significantly increased the number of adherent Mono Mac 6 by 5.42+/-0.16 fold. To determine whether FcγR functions were relevant under simulated shear stress, Mono Mac 6 were pretreated with soluble IgG to block FcγRs. Inhibition of FcγRs did

not significantly alter adherence of Mono Mac 6 to endothelium treated with HLA I mIgG1 (MEM-147), at 2.77+/-0.05 fold. In contrast, blockade of FcγRs impaired monocyte binding in response to HLA I mIgG2a (W6/32), reducing adherence to 1.355+/-0.01 fold over control (Figure 5A).

To confirm the role of adhesion molecules during monocyte recruitment, we added rPSGL-1 to antagonize selectins, or anti-ICAM-1 blocking antibody to block ICAM-1. The presence of rPSGL-1 inhibited Mono Mac 6 adherence to endothelium in response to both HLA I mIgG1 (MEM-147) by 56.8% and HLA I mIgG2a (W6/32) by 86.7%. Antagonism of endothelial ICAM-1 also decreased Mono Mac 6 binding to background levels under flow conditions, by 60.5% for HLA I mIgG1 (MEM-147) and by 94.8% for HLA I mIgG2a (W6/32) (Figure 5B). Therefore Mono Mac 6 adherence under dynamic flow to HLA I antibody treated endothelial cells requires P-selectin and ICAM-1.

### *5.5 Discussion*

The mechanism(s) by which HLA I antibodies promote mononuclear cell infiltration are not fully understood. We report that HLA I antibodies are unique among endothelial-binding antibodies in that they activate endothelial cells to increase cell surface P-selectin, which supports increased monocyte binding *in vitro*. We propose a model in which selectin-mediated adherence is augmented by secondary engagement of FcγRs via the Fc tail of the antibody. A key finding of this study is that the capacity of an HLA I antibody to enhance selectin-triggered recruitment is determined by its ability to interact with FcγRs, which is dictated by its isotype. Together, P-selectin and the Fc region of the HLA I antibody promote maximal monocyte adherence by activating Mac-1 (illustrated in Figure 6).

How HLA antibodies modulate immune cell recruitment remains to be fully elucidated. In many models of inflammation, monomeric antibody or immune complexes deposited on endothelial cells can cooperate with chemokines or selectins present on the cell surface to augment recruitment of neutrophils (40, 41). Our results from both the static and dynamic adhesion assays were consistent with the findings of Florey et al. (42) demonstrating that anti-endothelial cell antibodies could enhance neutrophil recruitment to TNFα-stimulated endothelium

In this study, endothelial cells were immune activated by TNFα or other cytokines that induce expression of E-selectin. Then, IgG antibodies bound to the endothelium enhanced binding of neutrophils to endothelial monolayers via Fcγ receptor (FcγR)-mediated interactions. Therefore, antibody/FcγR-mediated neutrophil adherence has a prerequisite of selectin expression, and, while they can enhance binding, endothelial bound antibodies alone are not sufficient to increase adherence (42) or facilitate capture of immune cells (43). Other investigations have revealed that monomeric anti-endothelial cell antibodies from autoimmune sera activate endothelial cells and increase expression of late phase mediators such as E-selectin and ICAM-1, but the leukocyte

adherence may not be FcγR-dependent (44). It has not yet been explored which paradigm occurs in the context of HLA antibodies and recruitment of leukocytes into the transplanted organ.

Multiple changes occur to increase integrin ligand binding: conformational (inside-out signaling), expression changes, or clustering (avidity). P-selectin functions as a paracrine signaling molecule, mediating Mac-1 clustering (and therefore increased avidity) (45), and PSGL-1 engagement is sufficient to increase adherence to ICAM-1 or fibrinogen via Mac-1 by stimulating Src and PI3K signaling *in vitro* and *in vivo* (46-50). PSGL-1 ligation by P-selectin does not, however, induce activation epitopes (conformation change) or increase total αM and β2 subunit expression, and it triggers the "extended" but not the highest affinity conformation of LFA-1 (51). Thus P-selectin/PSGL-1 is thought to need an additional signal, typically chemokine/GPCR signaling, to contribute to maximal arrest (52). We hypothesize that FcγR engagement filled this gap.

We also discovered a role for FcγRs in HLA I mIgG2a-elicited monocyte recruitment. FcγRI or FcγRII crosslinking increases PI3K, Src family kinases, paxillin and FAK phosphorylation (53), signaling which is required for sustained binding and spreading of PMN to immune complex-coated plastic and adherence *in vivo* (52, 54). Immune complexes engage FcγRII and FcγRIII to increase Mac-1 activation epitope and total expression (54, 55), but cannot support leukocyte capture on endothelial cells without concurrent expression of selectins *in vitro* (42, 56) or *in vivo* (41, 57). Our results are consistent with the idea that endothelial bound antibody has a corequisite for selectins, since coating endothelial cells with anti-CD105 or anti-ICAM-1 antibody did not significantly increase monocyte adherence when P-selectin was absent. Therefore, selectin ligation of PSGL-1 may synergize with FcγR engagement to produce a highly adhesive state in the monocyte.

Despite higher expression of LFA-1 compared to Mac-1 on the monocytic cell lines, we uncovered a prominent role for Mac-1 in adhesion to HLA I antibody-treated endothelial cells. We propose two reasons for this dominance. First, Mac-1 can bind other ligands in addition to ICAM-1, including

von Willebrand Factor which is externalized after HLA I ligation on endothelium ((19), unpublished observations). Secondly, the crosstalk between integrins (particularly Mac-1) and FcγRs has been well described in a myriad of systems. FcγRs and Mac-1, but not LFA-1, synergize to accomplish FcγR effector functions such as phagocytosis (reviewed in (58)). Mac-1 in neutrophils physically associates with FcγRIIA (59), and facilitates sustained adherence and spreading on immune complexes (60). FcγR ligation with aggregated or immobilized IgG can increase Mac-1 avidity (43, 61). These data, and a vast number of additional studies, support the idea that FcγR stimulation directly targets Mac-1 to prime the integrin for ligand binding.

Although we identified FcγRI as the predominant antibody receptor in HLA I mIgG2a-stimulated monocyte adherence, FcγRII participated to a minor but significant extent as well. There are distinct roles for each FcγR depending on the type of antibody-mediated inflammation (41). We expected that FcγRI would be the principal and probably the only Fc receptor involved in HLA I antibody-mediated recruitment, given that it has the highest affinity for IgG, including murine IgG2a. As a result, FcγRI is the only FcγR which can bind to monomeric antibody. Our finding of FcγRII involvement is in agreement with evidence from Florey et al., who demonstrated that FcγRIIA mediated anti-endothelial cell antibody-enhanced recruitment of neutrophils. These cells lack FcγRI, but express FcγRIIIB, which facilitated adherence only in response to immune complexes (42). The interplay between activating and inhibitory FcγRs during antibody-mediated monocyte recruitment has not been investigated, but likely has important influence over the ultimate outcome of FcγR stimulation. The antibody we used to detect FcγRII expression and to inhibit FcγRII-mediated adherence recognizes both the activating FcγRIIa and inhibitory FcγRIIb forms (clone AT10). We did not determine the expression of the inhibitory FcγRIIb on our monocytic cells, so it is unknown whether this molecule was present in our system. The potential interactions between the

down-modulating FcγRIIb and the activating FcγRIIa could obscure our findings, as FcγRIIb dampens the function of FcγRIIa when the two are coligated (62).

We report that, while an HLA I antibody with low to no affinity for human FcγRs—mIgG1—promotes monocyte binding only through P-selectin, an HLA I antibody with a high affinity for human FcγRs—mIgG2a—can potentiate monocyte recruitment through both P-selectin and Fc-mediated interactions. Therefore, our data indicates that the isotype of a donor specific HLA I antibody and the FcγR(s) it engages shape the degree of infiltration of FcγR-bearing immune cells by endothelial cells. Donor specific HLA antibodies found in transplant patients can be of any of the four IgG isotypes, with IgG1 being the most prominent (63). Investigators examining the effect of antibody isotype on the pathogenesis of donor specific antibodies in transplant rejection have particularly focused on complement fixing ability, with mixed clinical results. Griffiths et al. reported that, in a small cohort, patients who had only IgG1 donor specific HLA antibodies had lower short term renal graft survival than those with other subclasses (64). On the other hand, one study found that when renal transplant patients with only non-complement fixing HLA antibodies (IgG2 or IgG4) and those with complement fixing antibodies (IgG1 and IgG3) were compared, no significant differences were found in the incidence of AMR (65). Further, the long-term prognosis of the transplant could not be predicted by antibody isotype when DSA was at low titers (66). Similarly, the Colvin group reported in a murine model of pure antibody-mediated rejection that both complement fixing and non-complement fixing antibodies could cause chronic rejection lesions in transplanted allografts (67, 68). These discrepancies suggest that not enough is understood about the mechanism of pathogenesis of HLA antibodies, and furthermore that complement fixation may not be the only effector function of antibodies which is important to clinical outcome. One explanation is that antibody isotype controls the ability to interact with FcγRs, necessary for a diverse array of neutrophil, NK cell, and macrophage effector functions.

We found a requirement for ICAM-1 in addition to P-selectin in HLA I antibody-stimulated monocyte recruitment, which is inconsistent with the findings of Yamakuchi et al., who reported that HLA I antibody-mediated binding of HL60 cells required P-selectin but not ICAM-1 (19). Our cell lines are monocytic, whereas HL60 are less differentiated and more closely resemble neutrophils (69, 70). Mono Mac 6, THP-1 and primary blood monocytes expressed higher levels of CD14 and β2 integrins, including LFA-1 and Mac-1, than HL60 (42). In addition, HL60 cells had lower expression of FcγRs, and their capacity to adhere to soluble mIgG2a was much less than that of THP-1 cells (unpublished observations). Therefore the effects of FcγRs and β2 integrins may be less evident in these cells. Secondly, we allowed the monocytes to adhere to stimulated endothelium for longer than the adherence time used by Yamakuchi et al.; the second phase of integrin-mediated arrest is more dominant at later time points than selectin-mediated binding, and is required under shear stress. Further, while this group did not explicitly examine the role of antibody-FcγR interactions, their *in vivo* findings demonstrate that HLA I $F(ab')_2$ is sufficient to increase vWF and accumulation of PMN in human skin grafts, supporting the line of reasoning that P-selectin itself is enough to elicit some leukocyte recruitment without FcγR involvement. Our results add to this knowledge by demonstrating that basal recruitment can be enhanced by concurrent engagement of PSGL-1 and FcγRI on the monocyte.

An obvious limitation of this study is the primary use of murine monoclonal HLA I antibodies rather than HLA I specific antibodies of human origin. Unfortunately, the availability of human monoclonal antibodies is generally limited. Those human HLA I antibodies that are available are the complement fixing isotypes IgG1 and IgG3, which also efficiently bind FcγRs. However, human HLA I monoclonal antibodies of the IgG2 and IgG4 isotypes, which have low-to-no interaction with FcγRs, are, to our knowledge, nonexistent. Therefore there is a need to generate and study

human or humanized HLA I monoclonals of distinct isotypes to assess their influence on adherence in a system that more closely mimics physiological conditions.

In the transplant setting, surgery, infection or alloimmune injury may increase the inflammatory milieu. Complement factors activated by antibody deposition are implicated in leukocyte recruitment. We observed that HLA I antibodies triggered WPb exocytosis from endothelial cells and monocyte recruitment in an otherwise noninflammatory setting, but recruitment may be augmented depending on the inflammatory state of the endothelium. The presence of complement factors at the endothelial surface augments endothelial and monocyte activation and recruitment (19, 71-74). Further, the content of Weibel-Palade bodies may be altered in endothelial cells of different vascular beds, or after previous inflammatory insult (75, 76), when IL-8 can be stored in WPb and released along with vWF, further increasing monocyte recruitment potential/synergizing with HLA I antibody-stimulated P-selectin.

Recruitment of immune cells, particularly monocytes, into the allograft represents an important therapeutic target to alleviate acute and chronic transplant rejection. Monocytes are a consistent histological indicator of rejection, especially of antibody-mediated rejection (5). In several experimental models, depletion of macrophages or inhibition of macrophage factors increased survival and conferred protection from allograft rejection (77-79), demonstrating their central contribution to alloimmune injury. Because of their pleiotropic functions, macrophages regulate both innate immune injury, such as tissue damage or vascular remodeling, as well as adaptive immune responses, through indirect presentation of alloantigen to T cells (11). Recent work suggests that macrophages may independently exhibit alloreactivity and actively mediate rejection even in the absence of T cells (80, 81). A fuller understanding of the mechanism of mononuclear cell recruitment during antibody-mediated rejection, including the contribution of adhesion molecules

and antibody-FcγR interactions, will reveal appropriate and effective therapies to reduce its occurrence in the clinic.

We found that inhibition of P-selectin reduced monocyte recruitment in response to both isotypes of HLA I antibody, suggesting that this response is a universal phenomenon following ligation of HLA I. Based on these data, and those of other investigators, we propose that adhesion molecules are potential targets to reduce macrophage infiltration into the graft. Selectin/PSGL-1 interactions have been antagonized in a variety of experimental models to reduce immune cell accumulation and improve graft function in renal, liver, lung, and intestinal murine models of ischemia/reperfusion (82-87). The preliminary results of Phase II clinical trials indicate that, although rPSGL-1 has little impact on early graft function in renal transplant patients (88), this therapy reduces the expression of rejection biomarkers shortly after transplantation (89). Further, high risk liver transplant recipients experienced less delayed graft function when they received YSPSL compared with placebo-treated controls (90). Long-term efficacy in humans or its effect on macrophage accumulation in the graft has yet to be revealed, but experimental models point to promising results beyond use in ischemia/reperfusion injury. Koskinen et al. reported high expression of P-selectin in the rat cardiac allograft microvasculature during acute renal transplant rejection, and on arterial endothelia in sclerotic lesions during chronic vascular rejection (91), suggesting that P-selectin may be a target in long-term allogeneic transplantation in addition to ischemia/reperfusion.

In this study, both PSGL-1 and Mac-1 were required for HLA I mediated monocyte recruitment. One important consideration is the well-established redundancy of the adhesion molecule system, and particularly relevant to this study is the promiscuity of the PSGL-1 and Mac-1 molecules. The primary ligand of PSGL-1 is endothelial and platelet P-selectin, but it can also tether to E- and L-selectin. von Willebrand Factor, which is present in Weibel-Palade bodies and released during HLA I antibody-triggered exocytosis (19), has been identified as an adhesive substrate for neutrophils,

through both PSGL-1 and the β2 integrin Mac-1 (60, 92). This may explain the greater inhibitory effect we observed with rPSGL-1-Fc (about 70%) when compared with a neutralizing antibody to P-selectin (about 40%); we cannot rule out the possibility that the increased external vWF is participating in recruitment via PSGL-1 and/or Mac-1 on the monocytes. These factors highlight the potential strength of using rPSGL-1-Fc chimera as a therapeutic agent, since its ability to interact with multiple ligands can be exploited to target multiple adhesion molecule interactions.

We also found that an ICAM-1 blockade abolished HLA I-mediated endothelial adhesivity. The role of ICAM-1 in allograft rejection has to some extent been explored *in vivo*. In murine models of cardiac or arterial transplantation, ICAM-1 deficiency in the donor or Mac-1 deficiency in the recipient resulted in significantly increased graft survival and lowered macrophage infiltration compared with wild-type controls (93, 94). Similar results were observed in a rat model using aortic valve allografts with β2 integrin antagonism therapy (95). Moreover, a cumulative effect on graft survival was observed when murine donor ICAM-1 deficiency was combined with impaired recipient selectin ligands (96), demonstrating the cooperative effect of ICAM-1 and selectins in leukocyte recruitment. Our data provide some insight into the role of ICAM-1 during HLA I antibody-mediated rejection.

The interaction of endothelial bound HLA I antibody with monocyte FcγRs occurred in addition to the activation of endothelial cells by HLA I antibodies, which was sufficient to increase monocyte adherence. Our group previously reported that administration of an $F(ab')_2$ fragment of donor specific MHC I antibody increased macrophage infiltration *in vivo* (97), as did Yamakuchi et al (19). The findings in this work reiterate the fact that donor specific antibodies have pathogenic potential due to their direct effects on the graft vasculature, which are unrelated to their complement fixing or FcγR engaging capacity. Moreover, HLA I antibodies did not require cytokine activation of the endothelium to enhance leukocyte recruitment by FcγR mechanisms, due to their unique capacity to

immediately activate the endothelium. In conclusion, we have shown that endothelial cell exocytosis downstream of HLA I crosslinking independently promotes recruitment of monocytes, and that concurrent FcγR engagement potentiates selectin-dependent monocyte adherence when the HLA I antibody isotype permits binding to the FcγR. Selectin inhibition may therefore alleviate monocyte trafficking into the allograft in response to donor specific HLA I antibodies, irrespective of isotype.

**Figure 5-1A**

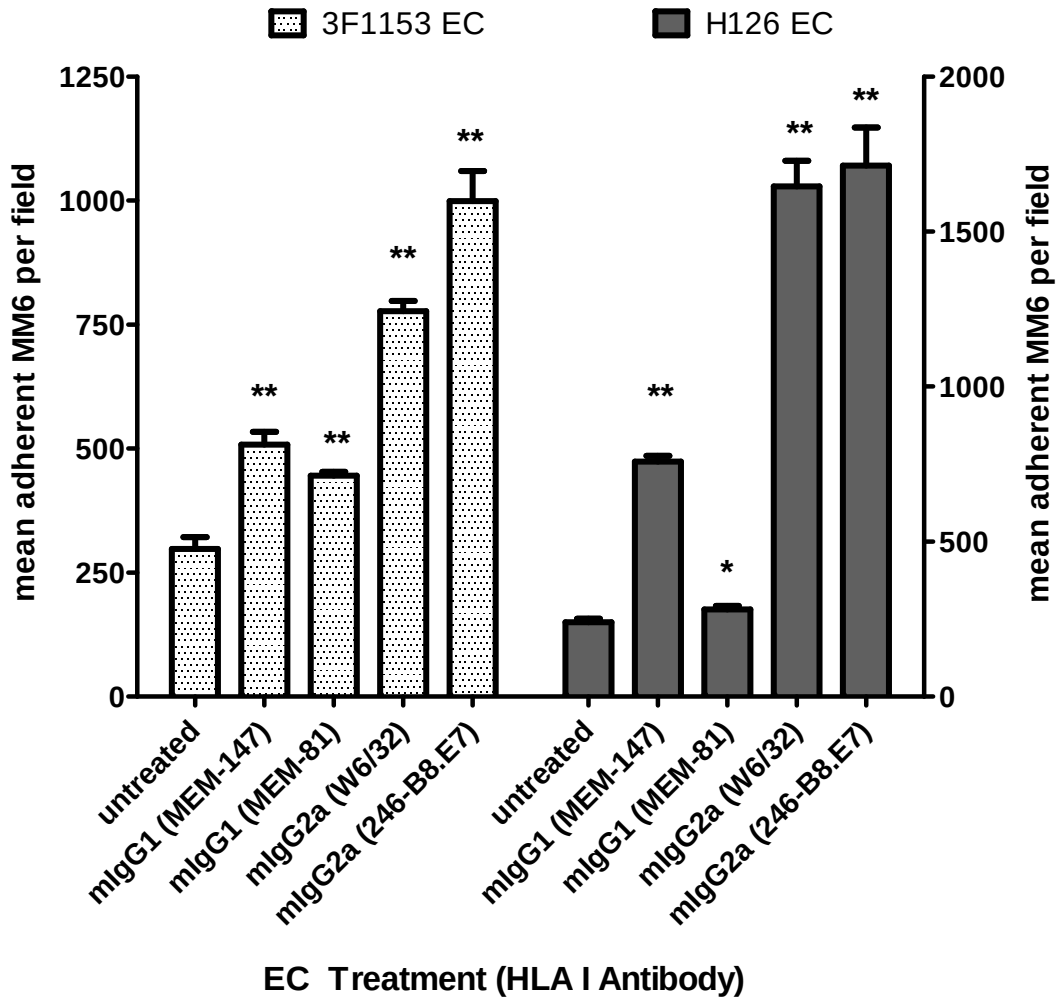

3F1153 EC
H126 EC
mean adherent MM6 per field
1250
1000
750
500
250
0
2000
1500
1000
500
0
**
**
**
**
**
**
*
untreated
mIgG1 (MEM-147)
mIgG1 (MEM-81)
mIgG2a (W6/32)
mIgG2a (246-B8.E7)
EC Treatment (HLA I Antibody)

**Figure 5-1B**

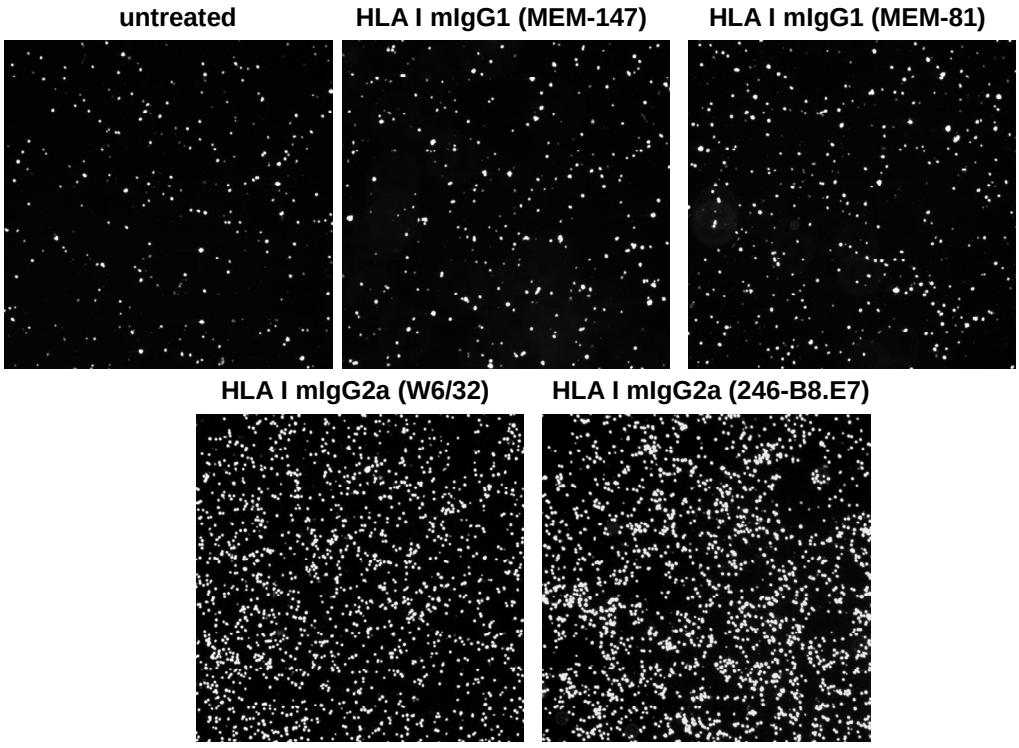

untreated
HLA I mIgG1 (MEM-147)
HLA I mIgG1 (MEM-81)
HLA I mIgG2a (W6/32)
HLA I mIgG2a (246-B8.E7)

Figure 5-1C

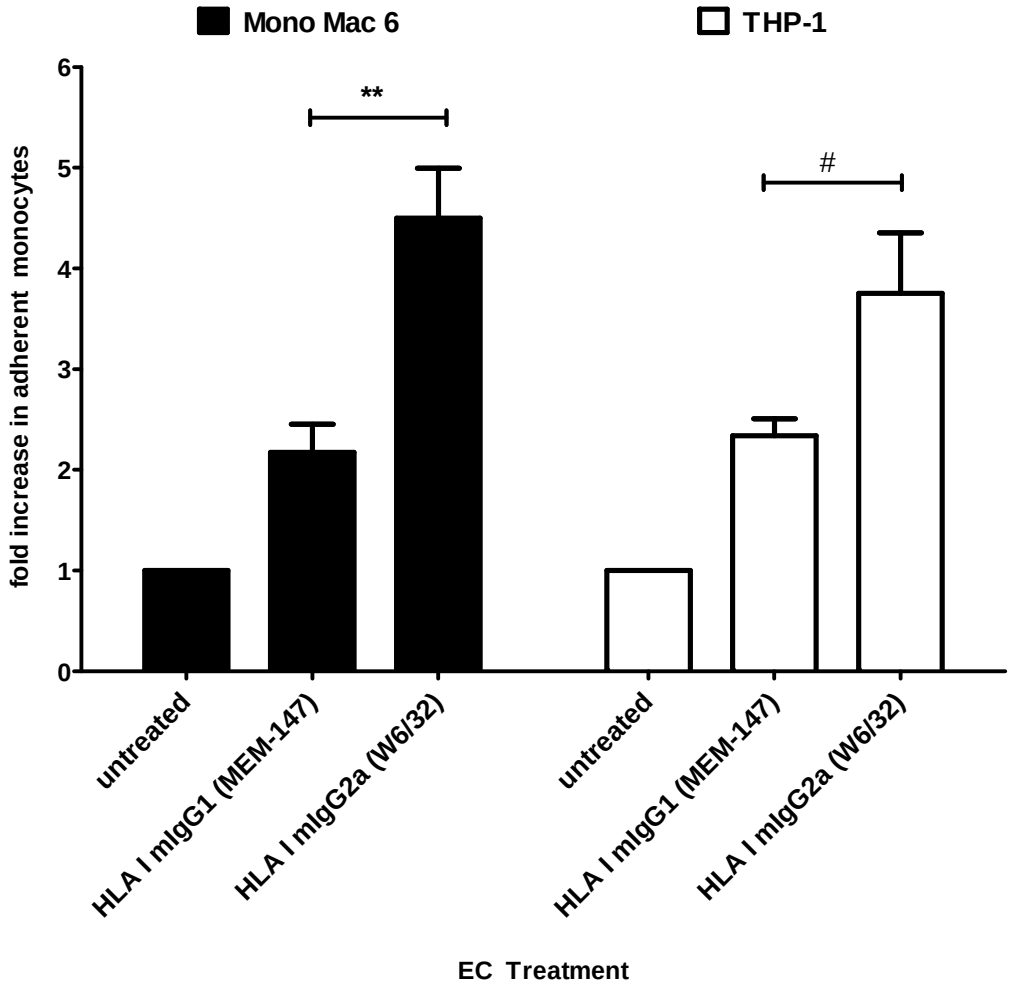

Mono Mac 6
THP-1
fold increase in adherent monocytes
0
1
2
3
4
5
6
**
#
untreated
HLA I mIgG1 (MEM-147)
HLA I mIgG2a (W6/32)
untreated
HLA I mIgG1 (MEM-147)
HLA I mIgG2a (W6/32)
EC Treatment

**Figure 5-1D**

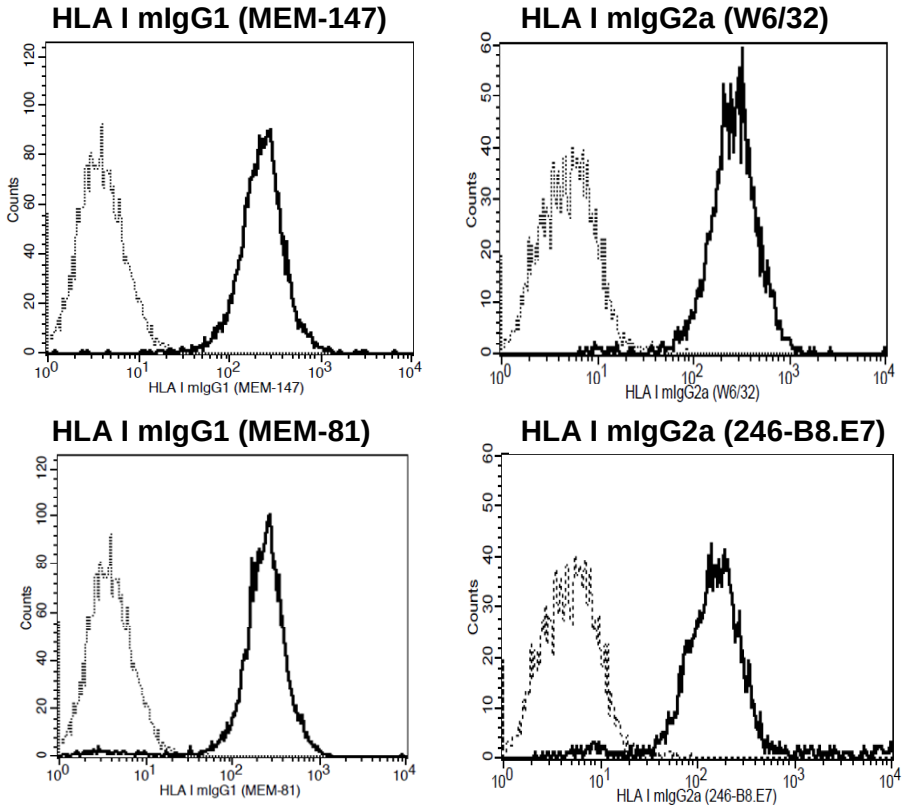



Figure 5-1. HLA I antibody treatment of endothelial cells increases adherence of monocytic cells.

A Endothelial cells were stimulated with HLA I mIgG1 (clone MEM-147 or MEM-81) or HLA I mIgG2a (clone W6/32 or 246-B8.E7) at 1μg/mL for 45min. Medium was aspirated and CFSE-labeled Mono Mac 6 or THP-1 cells were added to stimulated endothelial cells at a ratio of 3 monocytes per 1 endothelial cell for 20min at 37°C. Nonadherent cells were washed off, and adherent cells were counted by fluorescence microscopy. Fields were analyzed using automated software. Bar graphs show average number of adherent monocytes +/- SEM per field for each condition on EC from the donor H126 (black) and EC from the donor 3F1153 (white). ** $p<0.001$ versus untreated.

B Endothelial cells were stimulated and Mono Mac 6 adherence was measured as in (A). For each condition, one representative 4x fluorescence microscopy field showing monocyte binding to the endothelial monolayer is given.

C The endothelial monolayer was treated with murine monoclonal anti-HLA I mIgG1 (MEM-147) or HLA I mIgG2a (W6/32) at 1μg/mL for 45min in M199 supplemented with 2% FBS, and Mono Mac 6 adherence was measured as in (A). Bar graph shows mean fold increase in number of cells per field +/- SEM, from three or more independent experiments, using endothelial cells from at least two different donors. Black bars are binding of Mono Mac 6, white bars are binding of THP-1. # $p<0.05$; ** $p<0.001$ comparing HLA I mIgG2a to HLA I mIgG1.

D Endothelial cells were detached with Accutase and incubated with 1μg of each HLA I antibody in 100μL of PFA flow buffer. Antibody binding was detected by staining with anti-mouse-Fcγ-FITC and measured by flow cytometry. Histograms show fluorescence of each HLA I antibody on endothelial cells, and are representative of independent measurements on endothelial cells from at least two different donors.

Figure 5-2A

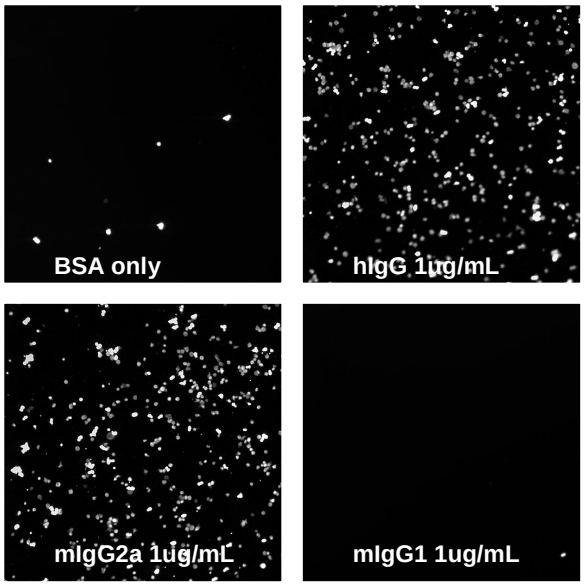



Figure 5-2B

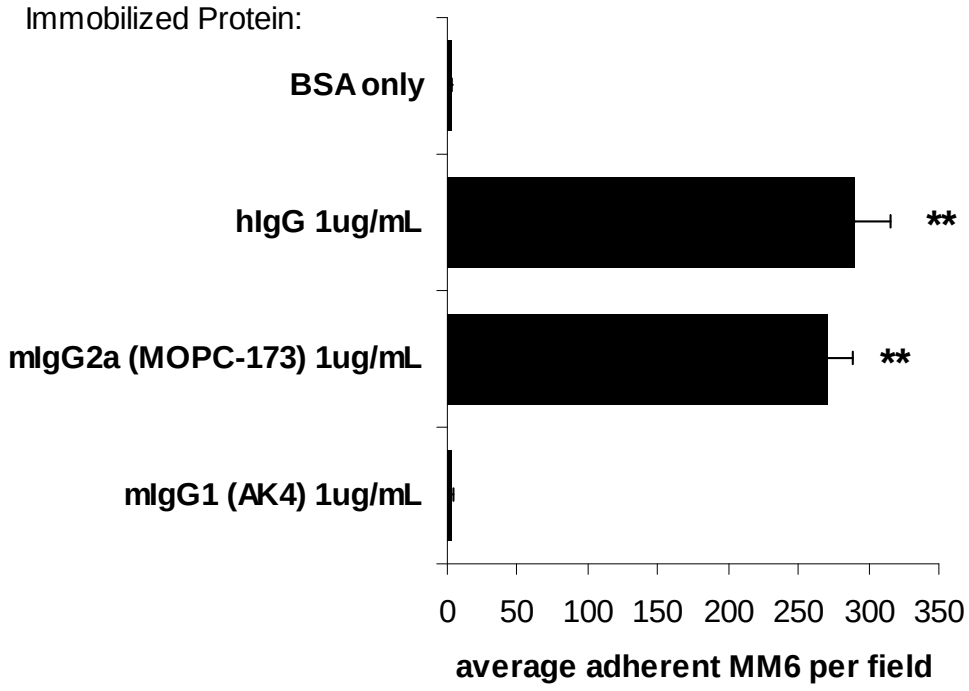


Figure 5-2C

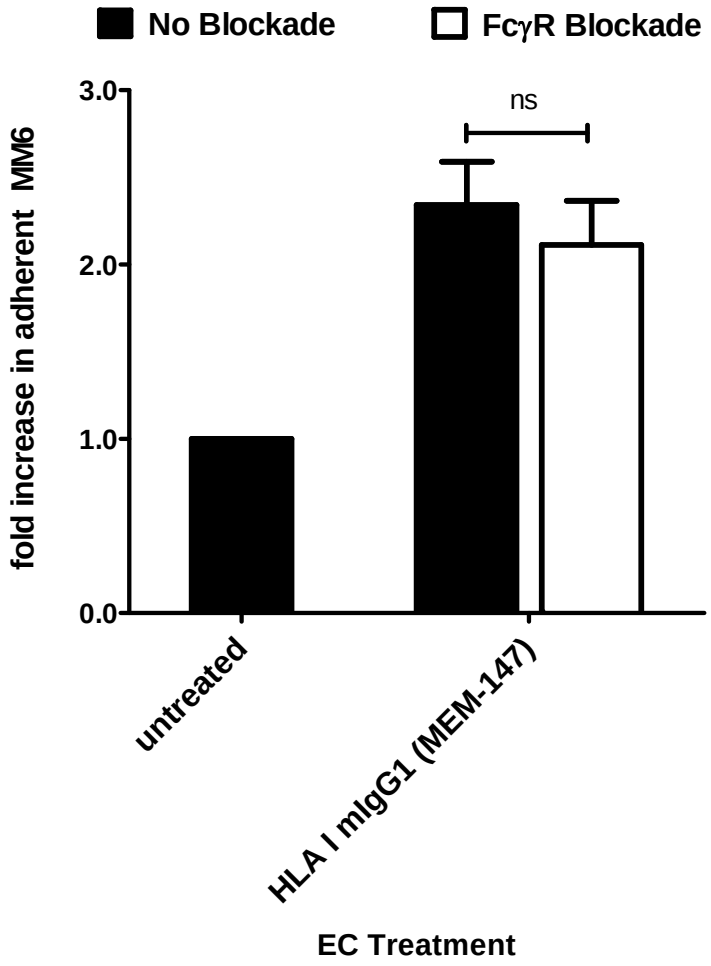

No Blockade
FcγR Blockade
ns
3.0
2.0
1.0
0.0
fold increase in adherent MM6
untreated
HLA I mIgG1 (MEM-147)
EC Treatment

**Figure 5-2D**

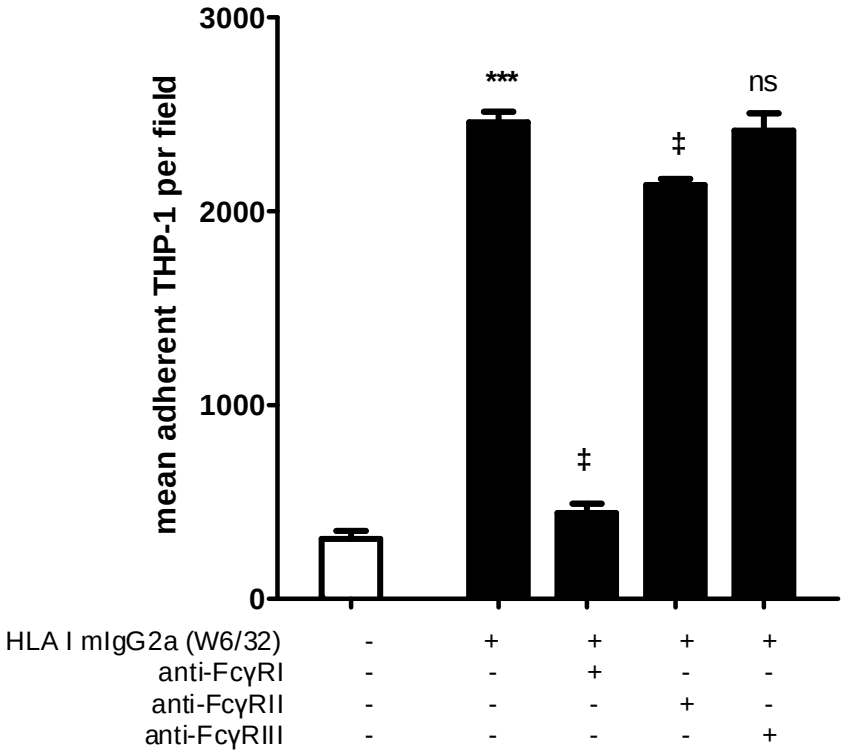

3000
2000
1000
0
mean adherent THP-1 per field
***
ns
‡
‡
HLA I mIgG2a (W6/32) - + + + +
anti-FcγRI - - + - -
anti-FcγRII - - - + -
anti-FcγRIII - - - - +

**Figure 5-2E**

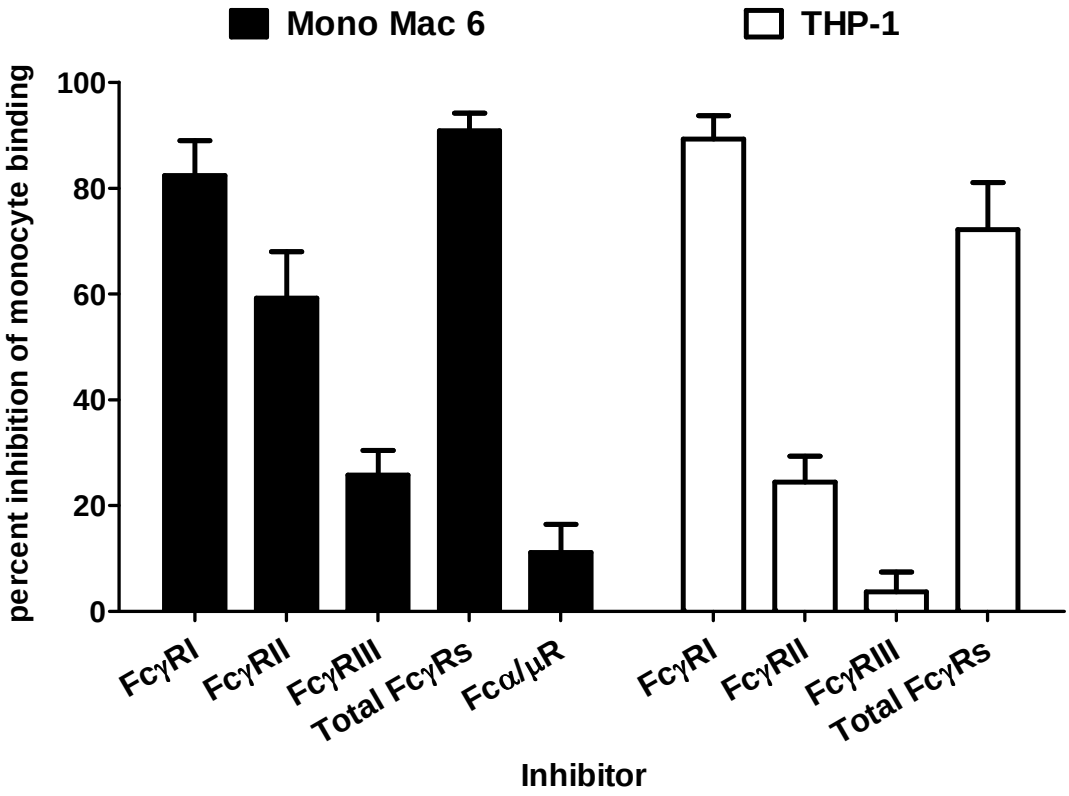



Figure 5-2. FcγRs are involved in HLA I antibody-mediated monocyte recruitment by endothelial cells.

A Nunc Maxisorp plastic wells were coated with 5% BSA in PBS alone, or nonspecific polyclonal human IgG, murine IgG1 (clone AK4), or murine IgG2a (clone MOPC-173) at 1μg/mL with BSA. CFSE-labeled Mono Mac 6 were added at $10^5$ cells/well in RPMI supplemented with FBS for 20min. Nonadherent cells were washed off. Representative 4x fluorescence microscopy fields are shown of fluorescently labeled Mono Mac 6 adherent to wells.

B Mono Mac 6 adherence to immobilized protein was performed as in (A), and the number of adherent cells was counted in 5 fields per well. The bar graph displays the mean number of cells per well +/- SEM. ** $p<0.01$ versus BSA alone.

C The endothelial monolayer was treated with HLA I mIgG1 (MEM-147) at 1μg/mL for 45 min. CFSE-labeled Mono Mac 6 were left untreated or preincubated with soluble hIgG at 10μg/mL for 20min, then added to treated endothelial cells in the presence of blocking reagents for 20min. Nonadherent cells were washed off, and adherent cells were counted. Bar graphs show average fold change in the number of adherent monocytes +/- SEM over four independent experiments. Black bars are binding without inhibitor, white bar is binding with FcγR blockade. n.s. $p>0.01$, comparing HLA I antibody with no inhibitor versus with inhibitor.

D The endothelial monolayer was treated with HLA I mIgG2a (W6/32) at 1μg/mL for 45 min. CFSE-labeled THP-1 were preincubated with soluble hIgG at 10μg/mL, anti-FcγRI, anti-FcγRII, anti-or FcγRIII at 5μg/mL for 30min, then added to treated endothelial cells in the presence of blocking reagents for 20min. Nonadherent cells were washed off, and adherent cells were counted by fluorescence microscopy. Panel shows results from one representative experiment. Data represent the mean number of THP-1 bound per field +/- SEM. ***$p<0.001$ versus untreated; n.s. $p>0.01$ versus no inhibitor, ‡ $p<0.001$ versus no inhibitor.

E The endothelial monolayer was treated with HLA I mIgG2a (W6/32) at 1μg/mL for 45 min. CFSE-labeled Mono Mac 6 or THP-1 were preincubated with soluble hIgG at 10μg/mL, anti-FcγRI, anti-FcγRII, anti-FcγRIII, or anti-Fcα/μR at 5μg/mL for 30min, then added to treated endothelial cells in the presence of blocking reagents for 20min. Nonadherent cells were washed off, and adherent cells were counted by fluorescence microscopy. Bar graph shows mean percent inhibition +/- SEM by each inhibitor of Mono Mac 6 (black bars) or THP-1 (white bars) adherence to HLA I antibody-treated

endothelium, from three or more experiments with at least two different endothelial cells.

**Figure 5-3A**

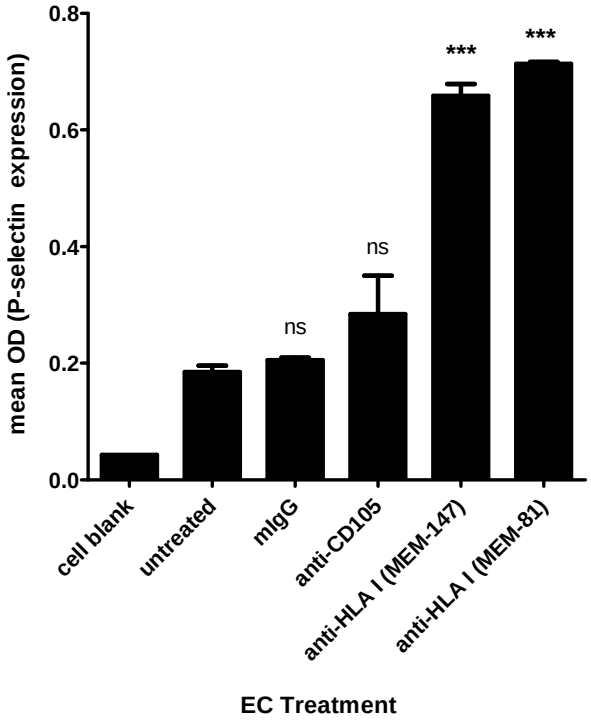

mean OD (P-selectin expression)
0.8
0.6
0.4
0.2
0.0
***
***
ns
ns
cell blank
untreated
mIgG
anti-CD105
anti-HLA I (MEM-147)
anti-HLA I (MEM-81)
EC Treatment

Figure 5-3B

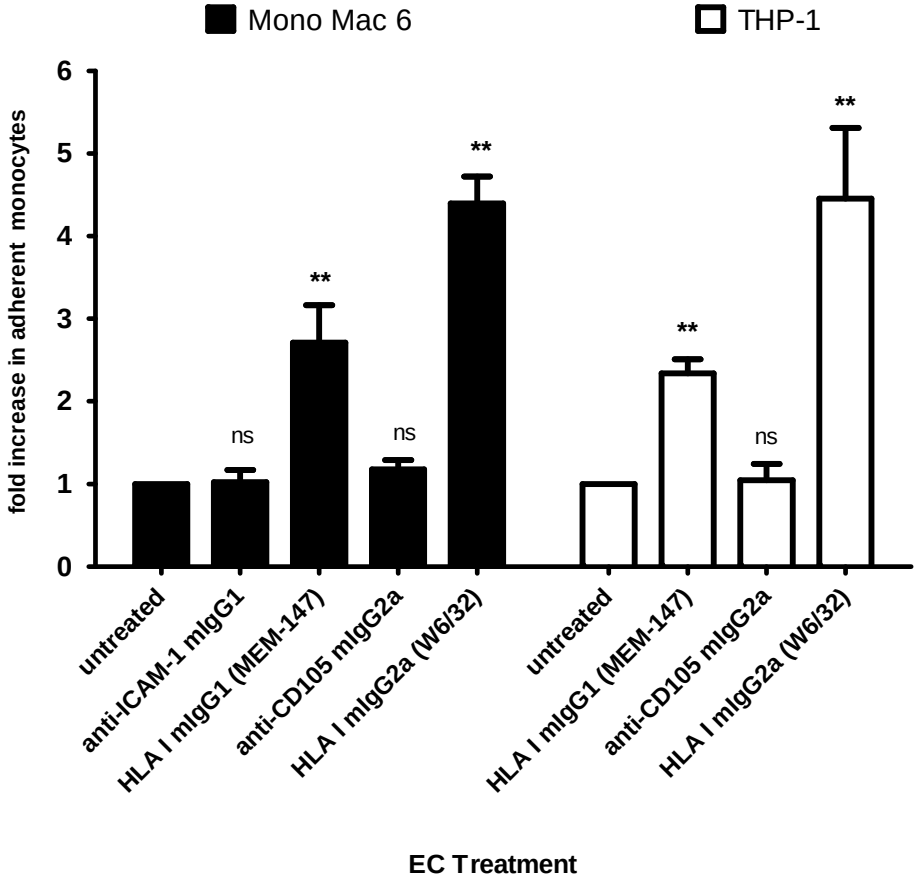



Figure 5-3C

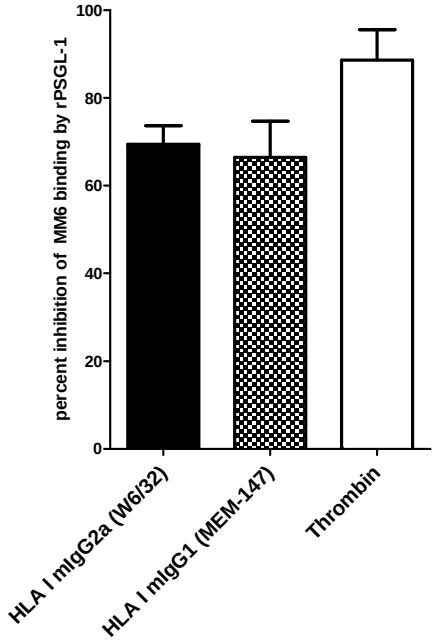



Figure 3D

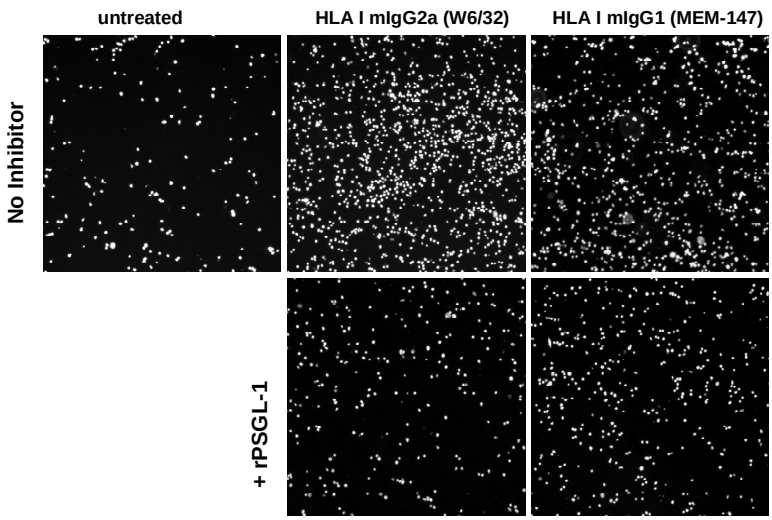


Figure 5-3. HLA I antibody-induced endothelial P-selectin expression is required for monocyte adherence.

A Endothelial monolayers were left untreated or were treated with control mIgG, anti-CD105 mIgG2a, HLA I mIgG1 (MEM-147 or MEM-81) at 1μg/mL for 30min, or with histamine at 10mM for 10min, in M199 with 5% FBS. Cells were fixed and left unpermeabilized, then stained with sheep anti-P-selectin, followed by anti-sheep-HRP secondary. Cell blank was left unstained. To detect P-selectin, TMB substrate was added, and optical density was measured. Data represent the mean optical density (OD) +/- SEM at 450nm of duplicate wells from one representative experiment out of two total. ns, $p>0.05$, * $p<0.05$, *** $p<0.001$ versus untreated.

B Confluent endothelial cells were exposed to anti-ICAM-1 mIgG1, anti-endoglin (CD105) mIgG2a, HLA I mIgG1 (MEM-147), or HLA I mIgG2a (W6/32) at 1μg/mL for 45min. CFSE-labeled Mono Mac 6 or THP-1 were added to treated endothelial cells for 20min, and nonadherent cells were washed off. Results are shown as mean fold increase in number of adherent Mono Mac 6 (black bars, n=9) or THP-1 (white bars, n=3) per field +/- SEM. n.s. $p>0.05$, * $p<0.01$, ** $p<0.001$ versus untreated.

C Endothelial monolayers were treated with HLA I mIgG2a (W6/32), HLA I mIgG1 (MEM-147) at 1μg/mL for 45min, or thrombin 1U/mL for 20min. rPSGL-1 was added at 10μg/mL for the last 10min of endothelial cell stimulation. CFSE-labeled Mono Mac 6 were allowed to adhere in the presence of rPSGL-1 for 20min, then nonadherent cells were washed off. Data are presented as mean percent inhibition of Mono Mac 6 binding +/- SEM from seven (HLA I mIgG2a and mIgG1) or six (thrombin) independent experiments using at least two different endothelial cells.

D Representative 4x microscopy fields from an experiment performed as in (C) demonstrate adherence of Mono Mac 6 to endothelium.

Figure 5-4A

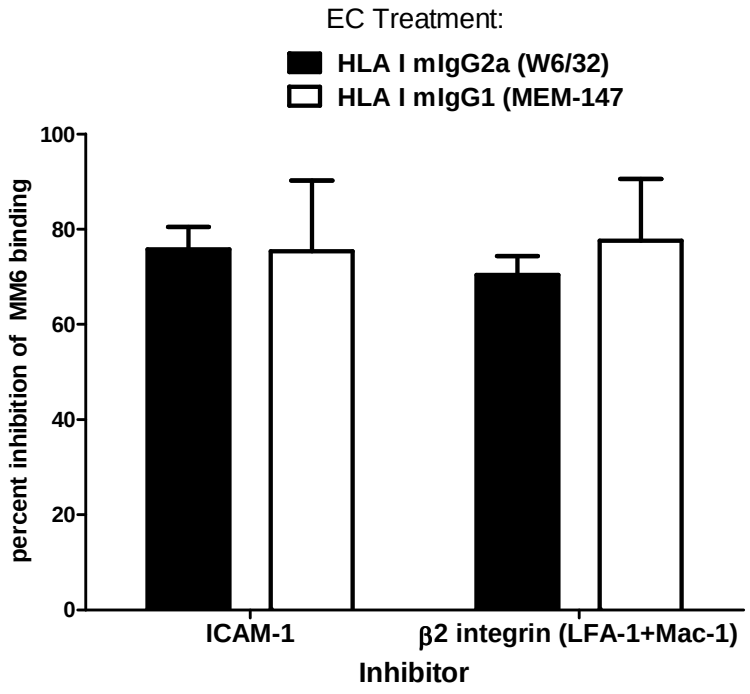



Figure 5-4B

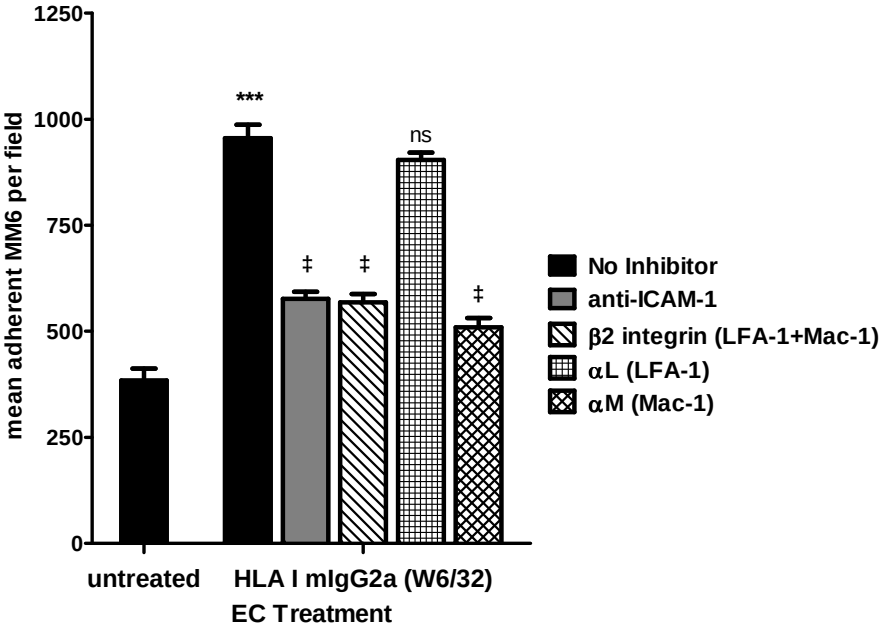


**Figure 5-4C**

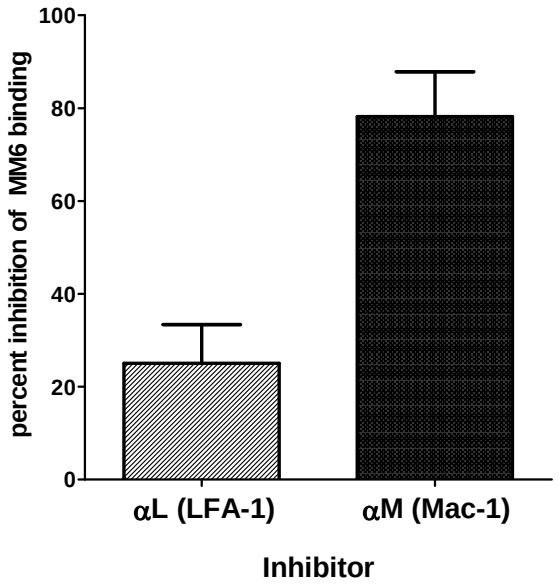



Figure 5-4. HLA I antibody-induced monocyte adherence to endothelial cells requires ICAM-1 and Mac-1.

A Endothelial cells were treated with HLA I mIgG2a (W6/32) or HLA I mIgG1 (MEM-147) at 1μg/mL for 45min. Anti-ICAM-1 blocking antibody was added at 10μg/mL for the last 10min of endothelial cell stimulation. CFSE-labeled Mono Mac 6 were left untreated or incubated for 15min with blocking antibody to β2 integrin (a component of LFA-1 and Mac-1) at 5μg/mL, then allowed to adhere to endothelial cells in the presence of inhibitors for 20min. Adherent cells were counted. Bar graph shows mean percent inhibition +/- SEM of Mono Mac 6 adherence to HLA I antibody-treated endothelial cells, from three or more independent measurements with at least two different endothelial cell donors.

B The endothelial monolayer was treated with intact HLA I mIgG2a (W6/32) at 1μg/mL for 45min. Anti-ICAM-1 blocking antibody was added at 10μg/mL for the last 10min of endothelial cell stimulation. CFSE-labeled Mono Mac 6 were left untreated or incubated

for 15min with blocking antibody to β2 integrin (LFA-1+Mac-1), αL (LFA-1) or αM (Mac-1) integrin at 5μg/mL, then allowed to adhere to treated endothelial cells in the presence of blocking reagents for 20min. Nonadherent cells were washed off, and adherent cells were counted. One representative experiment illustrates adherence of Mono Mac 6 with and without inhibitors; average number of adherent cells per field +/- SEM. ** $p<0.01$ versus untreated; n.s. $p>0.05$, ‡ $p<0.01$ versus no inhibitor.

C Bar graph shows mean percent inhibition +/- SEM of Mono Mac 6 adherence to HLA I mIgG2a-treated endothelium, from three or more independent experiments with at least two different endothelial cells.

Figure 5-5A

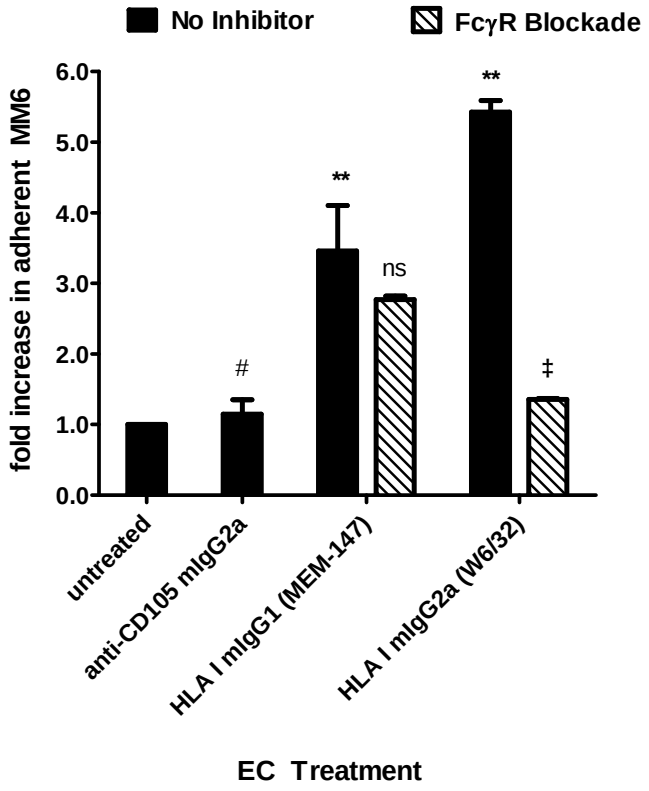



Figure 5-5B

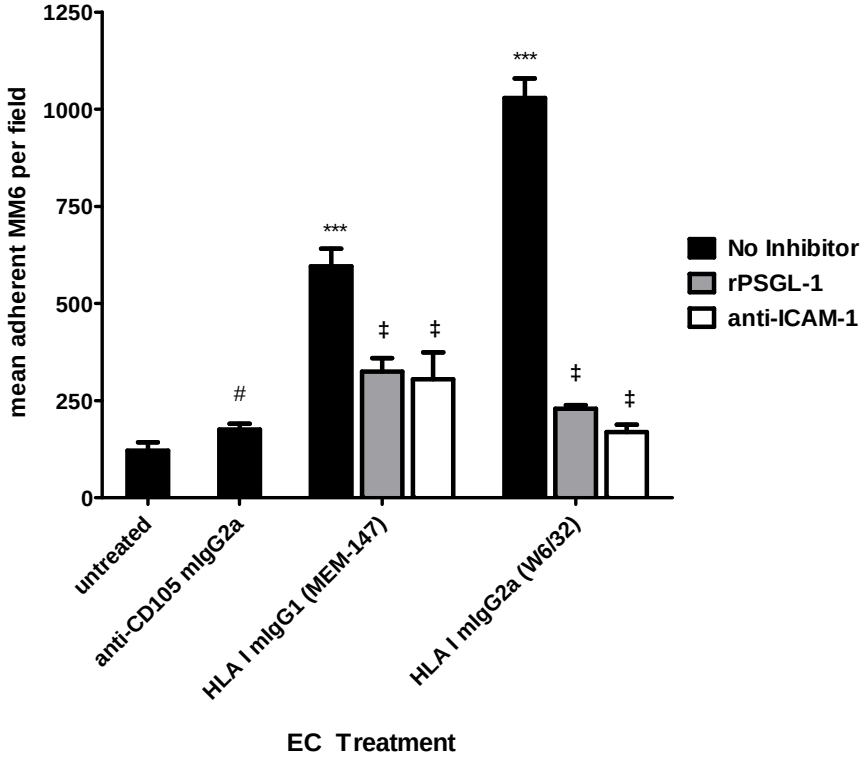


Figure 5-5. Monocyte recruitment to HLA I antibody-treated endothelial cells under dynamic conditions is significantly increased.

A Endothelial cells were stimulated with anti-CD105 mIgG2a, HLA I mIgG1 (MEM-147) or HLA I mIgG2a (W6/32) at 1μg/mL for 45min. CFSE-labeled Mono Mac 6 were left untreated or preincubated with soluble human IgG to block FcγRs, and overlaid on stimulated endothelial monolayers. Adherence was allowed for 20min on an orbital shaker at 40RPM. Nonadherent cells were removed by washing, and adherent cells were counted. Bar graph shows average fold increase in the number of adherent cells per field +/- SEM, n=2. # $p>0.01$, ** $p<0.001$ versus untreated; n.s. $p>0.01$, ‡ $p<0.001$ versus no inhibitor.

B Endothelial cells were stimulated with anti-CD105 mIgG2a, HLA I mIgG1 (MEM-147) or HLA I mIgG2a (W6/32) at 1μg/mL for 45min. rPSGL-1 at 20μg/mL or anti-ICAM-1 neutralizing antibody at 10μg/mL were added to endothelial cells. CFSE-labeled Mono Mac 6 were overlaid on stimulated endothelial monolayers. Adherence was allowed for 20min on an orbital shaker at 40RPM. Nonadherent cells were removed by washing, and adherent cells were counted. Bar graph shows average number of adherent cells per field +/- SEM, for one representative experiment out of two. # $p>0.01$, ** $p<0.001$ versus untreated; n.s. $p>0.01$, ‡ $p<0.001$ versus no inhibitor.

**Figure 5-6**

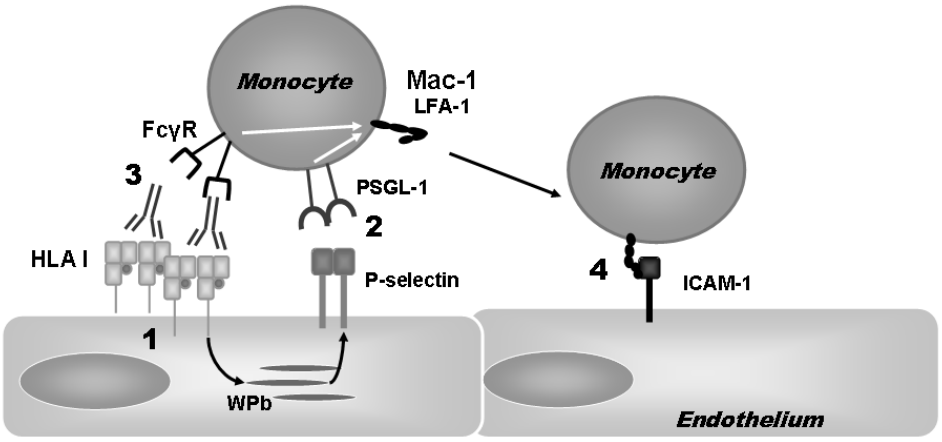
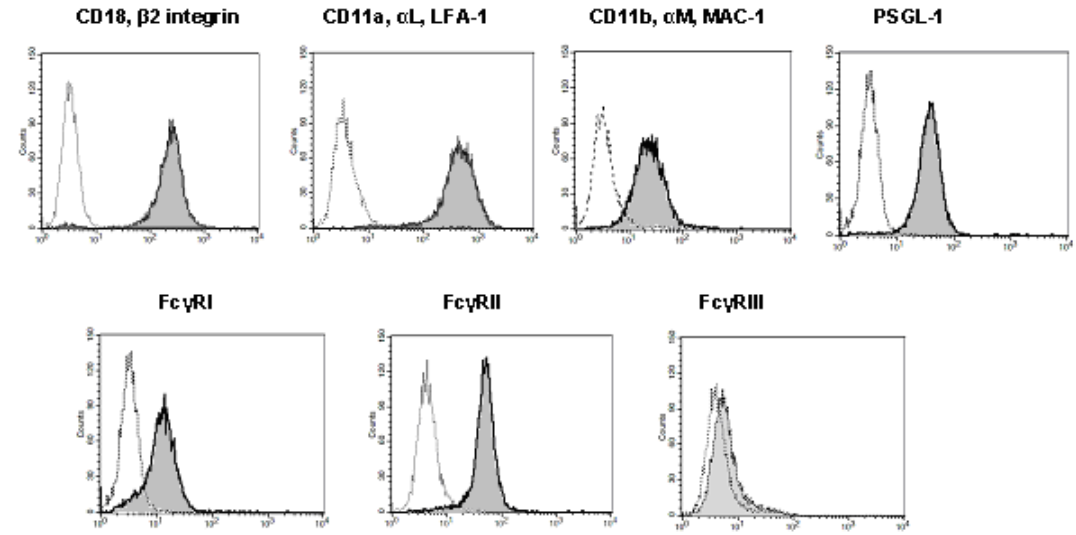


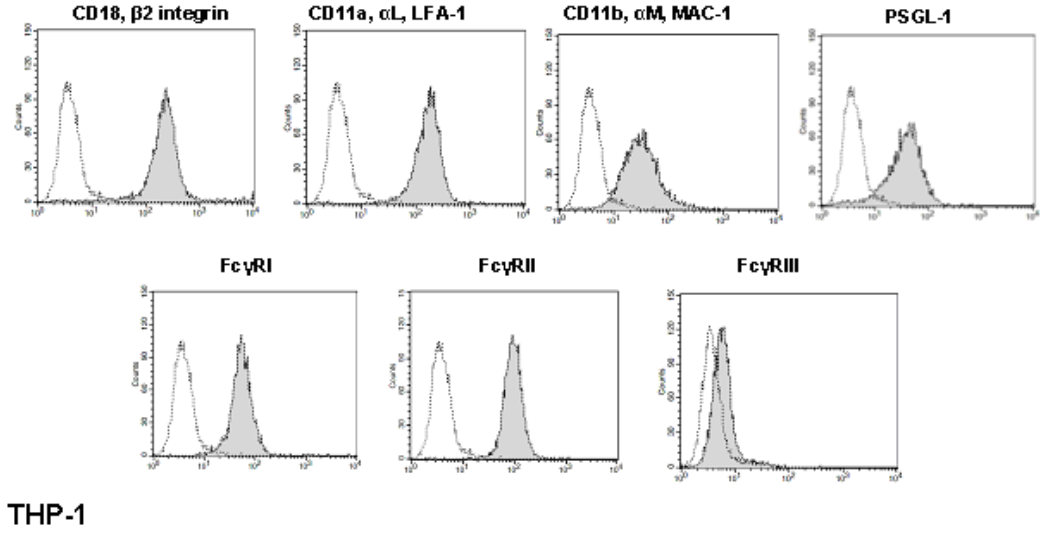

Figure 5-6. Proposed model of monocyte recruitment by HLA I antibodies.

(1) HLA I antibody binding to endothelial cells triggers an increase in cell surface P-selectin. Initial tethering of monocytes to endothelium is mediated through PSGL-1/P-selectin, as antagonism of this interaction reduces adherence. 2) Engagement of PSGL-1 activates monocytic β2 integrins, primarily Mac-1, to increase ligand affinity and promote firm adherence via ICAM-1 on the endothelium. 3) HLA I antibodies which are of an isotype with high affinity for human FcγRs can potentiate binding by augmenting the inside-out signaling to β2 integrins. 4) Stable adherence to the endothelial cell requires β2 integrin/ICAM-1 interactions, since antagonism of these molecules reduces adherence. Thus, maximal adhesion is promoted by concurrent engagement of PSGL-1 and FcγRs.

**Supplemental Figure 5-1A**



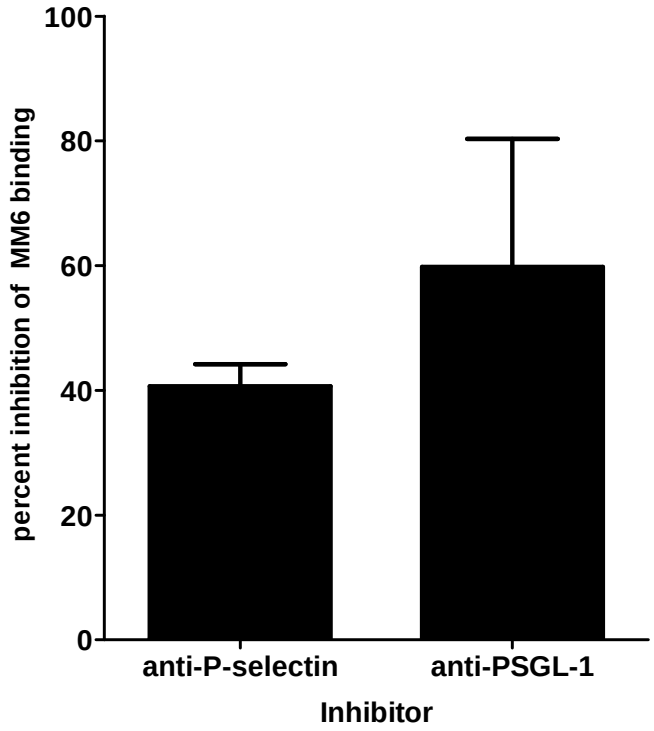

**Supplemental Figure 5-1B**




Supplemental Figure 5-1. Mono Mac 6 and THP-1 express FcγRs, PSGL-1, LFA-1 and Mac-1. Expression of FcγRs and cognate receptors for endothelial adhesion molecules on the Mono Mac 6 was assessed by flow cytometry. Mono Mac 6 (A) or THP-1 (B) cells were incubated with primary antibody to FcγRI, FcγRII, FcγRIII, Fcα/μR, β2 (LFA-1 and Mac-1), αL (LFA-1), αM (Mac-1) or

PSGL-1 at 1μg/0.1mL for 30min on ice in PFA buffer. Cells were washed twice and incubated with anti-mouse secondary antibody at 1:100 in PFA buffer for 30min on ice. Expression was assessed by flow cytometry. Representative histograms show expression, with the dotted line representing staining of secondary antibody alone, and the bold line showing staining for the marker of interest, from three or more experiments.

**Supplemental Figure 5-2**




Supplemental Figure 5-2. P-selectin or PSGL-1 antagonism reduces HLA I antibody-mediated monocyte recruitment.

Endothelial monolayer was treated with intact HLA I mIgG2a (W6/32) at 1μg/mL for 45min. Anti-P-selectin blocking antibody was added at 10μg/mL for the last 10min of endothelial cell stimulation. CFSE-labeled Mono Mac 6 were incubated for 15min with blocking antibody to PSGL-1 at 5μg/mL, then allowed to adhere to treated endothelial cells in the presence of blocking reagents for 20min. Nonadherent cells were washed off, and adherent cells were counted by fluorescence microscopy. Bar graph shows mean percent inhibition +/- SEM of Mono Mac 6 adherence to HLA I mIgG2a-treated endothelium, from three (anti-PSGL-1) or four (anti-P-selectin) independent experiments with at least two different endothelial cells.

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# CHAPTER 6:

# Monocyte Activation by Interaction with HLA I antibody-stimulated endothelial cells

## 6.1 Abstract

Macrophage infiltration is an important feature of antibody-mediated rejection. HLA I antibodies stimulate endothelial exocytosis, leading to increased P-selectin on the cell surface. Further, HLA I antibodies bound to endothelial cells interact with Fc gamma receptors on monocytes. We investigated the effect of monocyte interaction with HLA I antibody-treated endothelium, presenting P-selectin and clustered Fc regions, using an in vitro system. Our results demonstrate that monocytes require intracellular calcium, Src, PKC and PI3 kinase to be able to adhere to HLA I antibody-activated endothelium. Further, crosslinking of PSGL-1 or of Fc gamma receptors triggers inside-out signaling leading to increased ligand binding capacity for ICAM-1. Finally, deposition of HLA I antibody at low doses on cytokine-activated endothelial cells exacerbates the recruitment of monocytes above and beyond cytokine stimulation, likely through concurrent Fc receptor stimulation. These results demonstrate the cooperative effects of HLA I antibody-induced endothelial P-selectin and the Fc region of the antibody in promoting the stable adhesion of monocytes during antibody-mediated rejection.

## 6.2 Introduction

Macrophage accumulation in the allograft is a histological hallmark of antibody-mediated rejection. Macrophages are pathogenic in rejection, due to their antigen presentation, cytokine and growth factor production and generation of damaging reactive oxygen species. Recruitment of immune cells from the blood into the tissue is facilitated by endothelium, which is the located at the interface between the transplanted organ and the recipient immune system. Activated endothelial cells present P- or E-selectin, which captures leukocytes from the blood, a low affinity reversible interaction known as rolling. The proximity of the endothelium and leukocyte exposes the leukocyte to endothelial bound cytokines and/or complement, resulting in activation of the leukocyte leading to

"firm," shear force-resistant adhesion (also called "arrest") via ICAM or VCAM. Ultimately the leukocyte extravasates into the tissue [reviewed in (1)]. There is therefore a dual requirement for P-selectin and ICAM-1, as each molecule is involved in discrete stages of recruitment. Monocytes constitutively express receptors for ICAM-1 (αLβ2, LFA-1; and αMβ2, Mac-1), and yet they do not interact with either ICAM-1 protein or resting endothelial cells expressing ICAM-1. This is because the β2 integrins exist in an "inactive state" and must change conformation or increase in avidity downstream of outside-in signaling to permit adherence to their ligands (from such exogenous signals as chemokines, PSGL-1 ligation). Engagement of PSGL-1 has been demonstrated to promote activation of β2 integrins (2-5). Further, crosslinking of FcγRs promotes Mac-1 activation during phagocytosis and adhesion to immune complexes (6, 7).

In a variety of autoimmune models, antibody or immune complex binding to endothelial cells can promote recruitment of neutrophils (8). However, anti-endothelial cell antibodies or immune complexes cannot do so independently. Antibody/FcγR-mediated neutrophil adherence has a pre/corequisite of selectins, which means that endothelial bound antibodies alone are not sufficient to increase adherence (9). Given that ICAM-1 but not selectin/PSGL-1 or antibody/FcγRs interactions are required for firm adherence, we propose that interaction of the monocyte with the endothelial P-selectin and with the Fc region of the HLA I antibody licenses the monocytes for firm adherence via activation of the β2 integrins.

We previously found that HLA I antibodies triggered monocyte adherence via P-selectin and, depending on antibody isotype, FcγRs. Further, P-selectin and ICAM-1 cooperated to support monocyte binding. We hypothesized that, since monocytes must be activated to be able to firmly adhere to ICAM-1 via integrins, that the interaction of the monocyte with the HLA I antibody-

treated endothelium generates two activation signals: FcγR and PSGL-1 engagement. We sought to explore the regulation of the monocyte adhesive state by these two ligands.

## 6.3 Materials and Methods

*Reagents and Antibodies*

Mouse anti-human HLA-ABC antibody (clone W6/32, murine IgG2a, from BioXCell) which recognizes a monomorphic epitope common to all HLA I molecules was used to ligate HLA I. Isotype matched nonspecific mIgG was used as negative control. Neutralizing antibody to ICAM-1 (sheep IgG, R&D; murine IgG1 clone HCD54) was obtained from BioLegend. BAPTA-AM was from Alexis Biochemicals; Y26723 was from Calbiochem; wortmannin, LY294002 and PP2 from Sigma; and GF109203X was from Tocris. IL-1β was from R&D Systems.

*Cells and Culture Conditions*

Primary human aortic endothelial cells (HAEC) were grown to confluence on 0.1% gelatin in PBS. Cells were cultured in Medium 199 (Gibco) supplemented with 10% heat-inactivated FBS, 1X sodium-pyruvate, 4mg/mL heparin, penicillin-streptomycin, and endothelial cell growth supplement (BD), and stimulated in low-serum medium (M199 with 2% FBS). Endothelial cells were used at passages 4 through 8.

The human monocytic cell line Mono Mac 6 (kind gift of Dr. Judith Berliner, Department of Pathology and Laboratory Medicine, UCLA) was cultured in RPMI-1640 supplemented with 10% FBS, 10μg/mL bovine insulin, sodium-pyruvate, penicillin-streptomycin, and nonessential amino acids (Gibco). Mono Mac 6 adherence to endothelial cells has been reported to be more similar to that of primary monocytes than other monocytic cell lines, such as U937 (10). Monocytic cell lines were subcultured every 2-3 days and maintained at a density of less than $10^6$ cells per mL.

*Flow Cytometric Determination of Adhesion Molecule Expression*

Membrane localization of E-selectin was assessed by flow cytometry. Endothelial cells were cultured to a confluent monolayer, and treated with negative control mIgG at 5μg/mL or TNFα at 10pg/mL for 1, 2 or 4hr. The cells were washed with PBS without $Ca^{2+}$ or $Mg^{2+}$, then detached with Accutase (Innovative Cell Technologies) or scraped. Trypsin was not used to detach the cells because epitopes of cell surface markers are sensitive to trypsin digestion (11). Cells were stained with an anti-E-selectin antibody (PE-conjugated, BD Pharmingen) on ice at the manufacturer's recommended concentration, washed twice with PFA flow reagent (1% FBS, 0.1% sodium azide in PBS), fixed and analyzed by flow cytometry on a Becton Dickinson FACS Calibur or FACS Scan.

*Endothelial Cell Adherence Assay*

Confluent HAEC were treated with HLA I antibodies, or positive controls TNFα and IL-1β in M199 with 2% FBS (assay medium) for the indicated times. Leukocytes were fluorescently labeled with CFDA-SE (Vybrant Cell Tracer, Invitrogen) at 2μM in HBSS with $Ca^{2+}$ and $Mg^{2+}$ for 10min at 37°C, then washed. After treatment, medium was aspirated from HAEC, and Mono Mac 6 or THP-1 cells were added at approximately $5x10^5$ cells in M199 with 2% FBS per 35mm dish or $2x10^5$ cells per well in a 24 well plate (roughly equivalent ratio of 2-3monocytes:1EC). We used a low concentration of FBS to reduce stimulation by growth factors, and heparin was excluded from the medium at the time of stimulation because it can inhibit basal binding of Mono Mac 6 to endothelial cells (12). Monocytes and endothelial cells were cocultured for 20min at 37°C, then nonadherent cells were decanted. The coculture was washed three times by decanting and gentle pipetting, and fixed in freshly made 4% paraformaldehyde in PBS for 20min. Adherent monocytes were counted by fluorescence microscopy on a Nikon Eclipse Ti in 8-10 4x objective fields for each condition using automated software (CellProfiler) (13). Results are expressed for each condition as the mean

number of adherent monocytes per field or fold change in mean adherent monocytes per field normalized to untreated, +/- standard error of the mean (SEM).

*Immobilized Protein Adherence Assay*

hIgG, murine IgG2a, murine IgG1, or recombinant human ICAM-1 (R&D) were diluted in carbonate/bicarbonate buffer and coated onto high protein binding (Nunc Maxisorp) 96 well plates at 0.1mL per well, then incubated overnight at 4°C. The next day, the protein solution was removed, and the wells were blocked with 5% BSA in PBS for 2hr at room temperature. Mono Mac 6 cells were labeled with 2μM CFSE in HBSS for 10min, then serum rescued with 2.5-5% FBS in HBSS, spun down and resuspended at $10^6$ cells per mL. Cells were added to the plate at $10^5$ cells in 0.1mL per well and incubated at 37°C for 30min. Wells were washed three times with HBSS using the flick method, then fixed. Adherent cells were visualized by fluorescence microscopy using a 4x objective, and 5 fields per well were counted using Image J or CellProfiler.

For crosslinking experiments, CFSE-labeled Mono Mac 6 were treated with soluble ligands as surrogate stimuli to mimic interaction with ligands present on endothelia. To crosslink PSGL-1, monocytes were incubated with rP-selectin-Fc dimeric chimera at 20μg/mL, with or without an anti-human Fcγ secondary antibody. To crosslink FcγRs, Mono Mac 6 were incubated with purified human IgG at 5μg/mL, followed by anti-human $F(ab')_2$ in RPMI-1640 with 5% FBS, for 5min prior to addition to protein-coated wells. Adherent cells were counted 5 fields per well as described above.

*Statistical Analysis*

In the leukocyte adherence assay, the number of adherent cells was counted in 8-10 random fields for each sample, and the mean number of cells was determined. Error bars represent standard error of the mean (standard deviation/square root of field number). Statistical significance between

controls and samples was determined using the student's T-test, using one-tailed distribution and two sample equal or unequal variance based on the results of an F test.

## 6.4 Results

*6.4.1 Monocytes require intracellular signaling for efficient adherence to HLA I antibody-stimulated endothelium.*

Inside-out signaling regulates the avidity and conformation of β2 integrins (LFA-1 and Mac-1), thereby controlling ligand binding ability. PSGL-1 crosslinking upon engagement of E-selectin or P-selectin, or FcγR engagement with immune complexes, causes activation of intracellular signaling in neutrophils and monocytes (6, 14). Given the requirement for ICAM-1/Mac-1 interactions uncovered in Chapter 5, we postulated that the interaction of monocytes with endothelium promoted inside out signaling necessary for firm adherence. To this aim, we pretreated Mono Mac 6 with pharmacological inhibitors of key intracellular signaling molecules. We treated endothelial cells with HLA I antibodies and measured the ability of Mono Mac 6 to adhere with and without inhibition. As we previously showed, Mono Mac 6 binding to unactivated endothelium is low. Crosslinking of HLA I on endothelial cells with antibody significantly increases the number of adherent Mono Mac 6. When monocytes were pretreated with PP2 to inhibit Src, their ability to bind to HLA I antibody-activated endothelium was significantly impaired (Figure 1a). In contrast, inhibition of Rho kinase by pretreating Mono Mac 6 with Y27623 had no significant impact on their ability to adhere (Figure 2a). Cytoskeletal integrity was required for maximal monocyte adherence to HLA I antibody-treated endothelium, as pretreatment with cytochalasin D significantly reduced binding (Figure 2c). Other signaling molecules were necessary in addition to Src. We found that pretreatment of the endothelium with GF109203X, a pan inhibitor of the protein kinase C (PKC) family, wortmannin or LY294002, inhibitors of PI3 kinase (PI3K), or with BAPTA-AM, a chelator of intracellular calcium, all nearly or totally abolished monocyte adherence in response to HLA I crosslinking on endothelial cells (Figure 1d-f). Thirty minute exposure of Mono Mac 6 to these pharmacological inhibitors did not affect viability (data not shown). Taken together, these results

demonstrate that Mono Mac 6 utilize Src, PKC, PI3K and calcium, but not Rho kinase, to adhere to HLA I antibody-treated endothelial cells, consistent with our hypothesis of inside-out signaling.

*6.4.2 Activation of intracellular calcium signaling triggers monocyte adherence to ICAM-1.*

We next wanted to determine whether inside-out signaling regulated binding to endothelial ICAM-1, which is constitutively expressed and is a ligand for the monocyte β2 integrins LFA-1 and Mac-1. To that end, we stimulated monocytes with surrogate exogenous stimuli and assessed the capacity to adhere to unstimulated endothelial cells which express ICAM-1 but not selectins. Mono Mac 6 were treated with thapsigargin for 5 minutes, which causes a rapid and irreversible increase in cytosolic free calcium by emptying intracellular stores. We then measured binding of calcium agonist-activated monocytes to unstimulated endothelial cells, which lack P- or E-selectin expression but have high levels of ICAM-1. Unactivated monocytes adhered at a very low level to unstimulated EC, despite high expression of β2 integrin receptors for ICAM-1. Calcium activation in monocytes nearly doubled the number of adherent Mono Mac 6 per field (Figure 2a). To determine whether the proadhesive state induced in the monocyte by increased free calcium used ICAM-1 as a ligand, we pretreated endothelial cells with a neutralizing antibody to ICAM-1. When ICAM-1 was blocked, thapsigargin-treated Mono Mac 6 were no longer able to bind, with a significantly lower number of adherent cells per field than when ICAM-1 was available (Figure 2a). Therefore, triggering of an increase in intracellular calcium, which was required for Mono Mac 6 binding to HLA I antibody-treated endothelial cells, provokes increased adhesivity via ICAM-1.

To confirm ICAM-1 was a ligand for calcium-activated but not resting monocytes (i.e. that adhesion to ICAM-1 is regulated by calcium signaling), we devised an experimental system to measure the adhesive state of the monocyte. Purified ICAM-1 protein was immobilized on plastic with BSA, and

the ability of monocytes to adhere was measured with and without stimulation. Unstimulated Mono Mac 6, despite high expression of LFA-1 and Mac-1, did not bind to ICAM-1 at a greater rate than to BSA-blocked wells without ICAM-1 ($p>0.05$). Triggering of an increase in intracellular calcium in Mono Mac 6 by short-term treatment with thapsigargin significantly increased the capacity of Mono Mac 6 to adhere to purified ICAM-1, in a dose dependent manner (Figure 2b). Thus, expression of ICAM-1 receptors is not sufficient for monocyte adherence to ICAM-1; these results confirmed that monocytic cells required an exogenous signal such as a calcium agonist to be able to adhere to ICAM-1.

*6.4.3 Crosslinking of FcγRs or PSGL-1 activates monocyte adherence to ICAM-1.*

Since we demonstrated that monocyte binding to endothelial cells to immobilized ICAM-1 is regulated, we asked whether either of the two signals present on HLA I antibody-stimulated endothelial cells could activate the pro-adhesive state in the monocyte. HLA I antibody has an Fc tail which can engage and cluster FcγRs on the monocyte when the antibody is substrate bound. Therefore, monocytes were incubated with polyclonal soluble hIgG to occupy FcγRs, with or without an anti-human $F(ab')_2$ secondary antibody to crosslink the receptors. hIgG treatment alone did not increase Mono Mac 6 binding to immobilized ICAM-1; however, crosslinking of FcγRs induced a modest but significant increase in the monocytes' capacity to adhere to ICAM-1 (Figure 3a). Therefore, engagement of FcγRs on the monocyte by substrate-bound antibody can also activate the monocytes' integrins to promote binding to ICAM-1.

In order to validate the observation that FcγR engagement promotes adhesion to ICAM-1, we used a different approach to measure firm adherence. Here, we measured binding to immobilized hIgG, ICAM-1, or hIgG with ICAM-1 coated plates on an orbital shaker, with the expectation that hIgG

alone would not significantly facilitate shear-resistant binding. Mono Mac 6 did not adhere to BSA, ICAM-1 alone or hIgG at low concentrations (0.1μg/mL). When both hIgG and ICAM-1 were present as substrates, Mono Mac 6 binding was significantly promoted under dynamic conditions (Figure 3b).

We asked whether engagement of PSGL-1 by P-selectin could induce monocyte binding to ICAM-1. As a proxy for endothelial-presented P-selectin, Mono Mac 6 were Mono Mac 6 treated with soluble rP-selectin-Fc. Mono Mac 6 were incubated with rP-selectin-Fc with or without a secondary crosslinking antibody and assessed for their adhesion to purified immobilized ICAM-1. Mono Mac 6 treated with the crosslinking antibody alone did not bind to immobilized ICAM-1. Dimerization or clustering of PSGL-1 dramatically increased Mono Mac 6 binding to purified ICAM-1 (Figure 3c). Therefore, engagement of PSGL-1 on the monocyte by P-selectin can deliver a stimulating signal, increasing the monocytes' β2 integrin activation to promote binding via ICAM-1. These results support the premise that P-selectin and Fc receptors can cooperatively activate monocytic integrins to prime the monocyte for binding to ICAM-1.

We determined the ability of PSGL-1 engagement to promote firm adherence to ICAM-1 under dynamic conditions. Here, we measured binding to immobilized P-selectin, ICAM-1, or P-selectin with ICAM-1 coated plates on an orbital shaker. Again, P-selectin alone should not significantly facilitiate shear-resistant binding without ICAM-1, and unactivated monocytes should not be able to bind ICAM-1. There were very few Mono Mac 6 adherent to BSA, ICAM-1 alone or P-selectin at either 0.1μg/mL or 1μg/mL. When both P-selectin and ICAM-1 were present as substrates, Mono Mac 6 binding was significantly promoted under dynamic conditions, which was dependent on the density of P-selectin (Figure 3d). These results are consistent with the paradigm that P-selectin

captures leukocytes but cannot support stable, flow resistant binding, while binding to ICAM-1 is a secondary step that mediates firm adhesion but requires P-selectin.

We sought to validate our observations that crosslinking of FcγR or PSGL-1 on the monocyte could promote binding to ICAM-1, using endothelial cells as a substrate. Mono Mac 6 were left unactivated, or PSGL-1 or FcγRs were crosslinked as above. Their ability to adhere to unstimulated endothelial cells was measured. Engagement of PSGL-1 on the monocyte increased the number adherent to endothelial cells expressing ICAM-1 by 3-fold. Similarly, crosslinking of FcγRs with soluble IgG and a secondary antibody increased Mono Mac 6 binding to endothelium by more than 3-fold over untreated (data not shown).

*6.4.4 HLA I antibody binding to cytokine stimulated endothelial cells augments cytokine-mediated monocyte recruitment.*

Finally, we hypothesized that low levels of HLA I antibody, which stimulate little or no monocyte adherence, could enhance recruitment to cytokine-activated endothelial cells. TNFα and IL-1β treatment of endothelial cells increases expression of adhesion molecules such as E-selectin and ICAM-1. We treated endothelial cells with low concentrations of TNFα for 4hr to stimulate expression of E-selectin (Figure 4a). To determine whether HLA I antibody deposition could augment cytokine-mediated recruitment, endothelial cells were left cytokine stimulated, or coated with HLA I antibody at 0.1μg/mL. As expected, monocyte binding was significantly stimulated by preactivation of endothelial cells with TNFα or IL-1β. Deposition of HLA I antibody at 0.1μg/mL on unstimulated endothelial cells had no detectable effect on monocyte-endothelial cell interactions. However, deposition of low levels of HLA I antibody on TNFα and IL-1β activated endothelial cells significantly potentiated recruitment above background (Figure 4b). Thus, coating of endothelial

cells with HLA I antibodies enhanced cytokine-stimulated monocyte adherence, suggesting that HLA I antibody binding to endothelium exacerbates basal inflammation by engaging FcγRs.

## 6.5 Discussion

In this paper, we show that monocyte interaction with HLA I antibody-stimulated endothelium triggers inside-out signaling that is essential for firm adherence via ICAM-1. Further, engagement of FcγRs by deposition of low titers of HLA I antibody on endothelium, or by stimulation with immune complexes, augments recruitment by promoting adhesion to ICAM-1.

We found that monocytes required Src, PI3K, calcium and PKC signaling to adhere to HLA I antibody-stimulated endothelial cells. None of the pharmacological inhibitors we used are useful clinically, but this finding yields insight into the mechanisms of monocyte activation by endothelial cells. The inside-out signaling pathways required for β2 integrin activation are fairly well-characterized. An increase in calcium and activation of Src family kinases are necessary, as are PI3K and probably PKC. In neutrophils, immune complex stimulation of FcγRs promotes Mac-1 activation to support a variety of FcγR-mediated functions, such as phagocytosis and adhesion to immune complexes. FcγRIIa and FcγRIIIb both activate PI3k, Syk (a Src family kinase), PAK and PKA to regulate β2 integrins (15). FcγRI or FcγRII crosslinking increases PI3K, Src family kinases, paxillin and FAK phosphorylation (16), signaling which is required for sustained binding and spreading of PMN to immune complex-coated plastic and adherence *in vivo* (17, 18).

Multiple changes occur to increase integrin ligand binding: conformational (inside-out signaling), expression changes, or clustering (avidity). Immune complexes engage FcγRII and FcγRIII to increase Mac-1 activation epitope and total expression (7, 18), but cannot support leukocyte capture on endothelial cells without concurrent expression of selectins *in vitro* (9, 19) or *in vivo* (8, 20). P-selectin functions as a paracrine signaling molecule, mediating Mac-1 clustering (and therefore increased avidity) (21), and PSGL-1 engagement is sufficient to increase adherence to ICAM-1 or

fibrinogen via Mac-1 by stimulating Src and PI3K signaling *in vitro* and *in vivo* (5, 14, 22, 23). Neutrophil rolling on P-selectin or E-selectin causes PSGL-1 crosslinking, which also activates PI3K and Src family kinases (Fgr, Hck, and Lyn) to target Mac-1. The literature suggests that there is a role for calcium and PKC in PSGL-1-mediated Mac-1 activation as well (24). PSGL-1 ligation by P-selectin triggers the "extended" but not the highest affinity conformation of LFA-1 (25). Therefore, selectin ligation of PSGL-1 may synergize with FcγR engagement to produce a highly adhesive state in the monocyte, through stimulation of Src family kinases, PI3K and PKC.

We did not find that Rho kinase was required for monocyte adherence to HLA I antibody-treated endothelium. This is consistent with the literature, which has demonstrated that Rho kinase regulates cytoskeletal changes important for leukocyte motility and migration, but is not required for the conversion event activating β2 integrins from rolling to stable adhesion (4). We did uncover a requirement for intact cytoskeleton in the monocyte during binding to endothelium. Again, this observation is in line with those of other groups, who found that disruption of cytoskeleton using jasplakinolide or cytochalasin D inhibited Mac-1-mediated adherence of neutrophils. The mechanisms may be that integrins need to attach to the cytoskeleton during activation or adherence (for clustering), or that the cytoskeleton is required for rapid cell surface upregulation of Mac-1 (for increased expression) (4, 26).

Lastly, we demonstrated that monocyte recruitment could be augmented by low levels of HLA I antibody bound to the endothelium. This low concentration, 0.1μg/mL, of HLA I antibody did not trigger endothelial exocytosis, nor did it stimulate Mono Mac 6 recruitment. However, when HLA I antibody was deposited on cytokine-activated endothelial cells, the antibody augmented recruitment. A similar phenomenon was reported in the context of autoimmune anti-endothelial cell antibodies

(9). The authors postulate that the amplification of recruitment by endothelial-bound antibodies may cause an inappropriate amount of leukocyte influx relative to the actual level of endothelial activation. Certainly in transplantation, the cytokine milieu may be altered by ischemia/reperfusion, infection or alloresponses, and endothelial cells may have a higher background level of inflammation. HLA I antibodies are likely to magnify monocyte recruitment above what would otherwise occur in response to cytokine activation of graft endothelium.

We conclude that HLA I antibodies have dual effects on the monocyte recruitment system. First, activation of endothelial P-selectin causes crosslinking of PSGL-1 on the monocyte, which promotes firm adherence to ICAM-1. Secondly, engagement of FcγRs with the Fc tail of the antibody also triggers inside-out signaling increasing the capacity to bind to ICAM-1. The HLA I antibody-activated endothelium provokes intracellular Src, calcium/PKC, PI3K and cytoskeletal activation that are required for stable adhesion and recruitment.

Figure 6-1

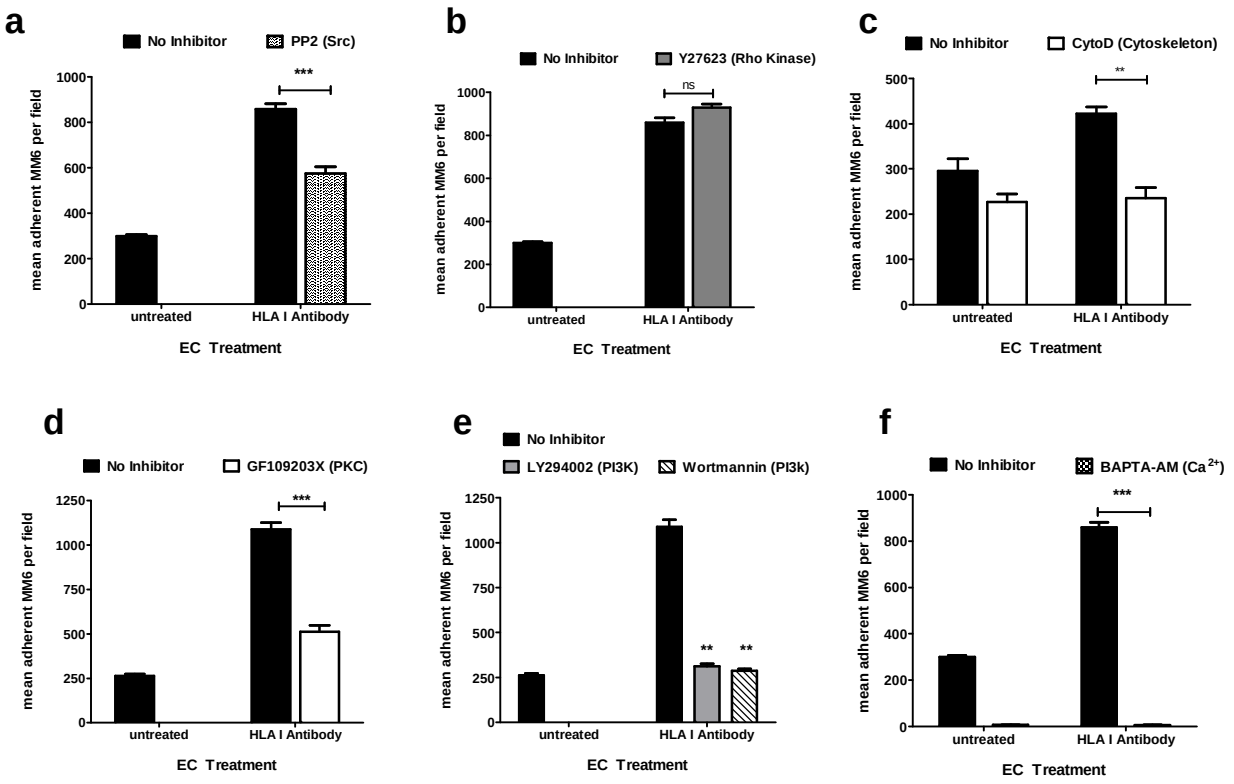



**Figure 6-1**. Monocytes require intracellular signaling for adherence to HLA I antibody-stimulated endothelium. Confluent human aortic endothelial cells (HAEC) were left untreated or treated with HLA I antibody at 1μg/mL for 45min. Mono Mac 6 were fluorescently labeled with CFSE, left untreated or pretreated with (a) PP2 at 10μM for 15min, (b) Y27632 at 10μM for 15min, (c) cytochalasin D (CytoD) at 1μM for 20min, (d) GF109203X at 10μM for 30min, (e) LY294002 or wortmannin at 25μM for 30min, or (f) BAPTA-AM at 10μM for 30min. Mono Mac 6 were added to endothelial monolayers for 20min at 37°C, nonadherent cells were removed by washing, and adherent cells were counted in 5-8 fields per condition. Data are presented as the average number of adherent Mono Mac 6 per field for each condition, +/- SEM. Each panel shows the results from one representative experiment out of two or three total measurements for each inhibitor. ns $p>0.05$, ** $p<0.001$, *** $p<0.0001$ versus no inhibitor.

Figure 6-2

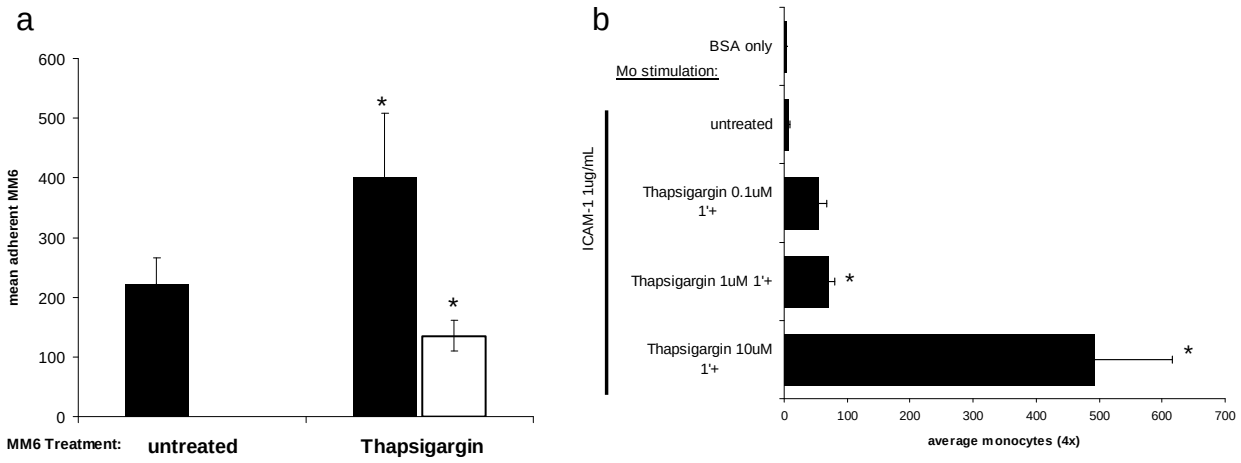
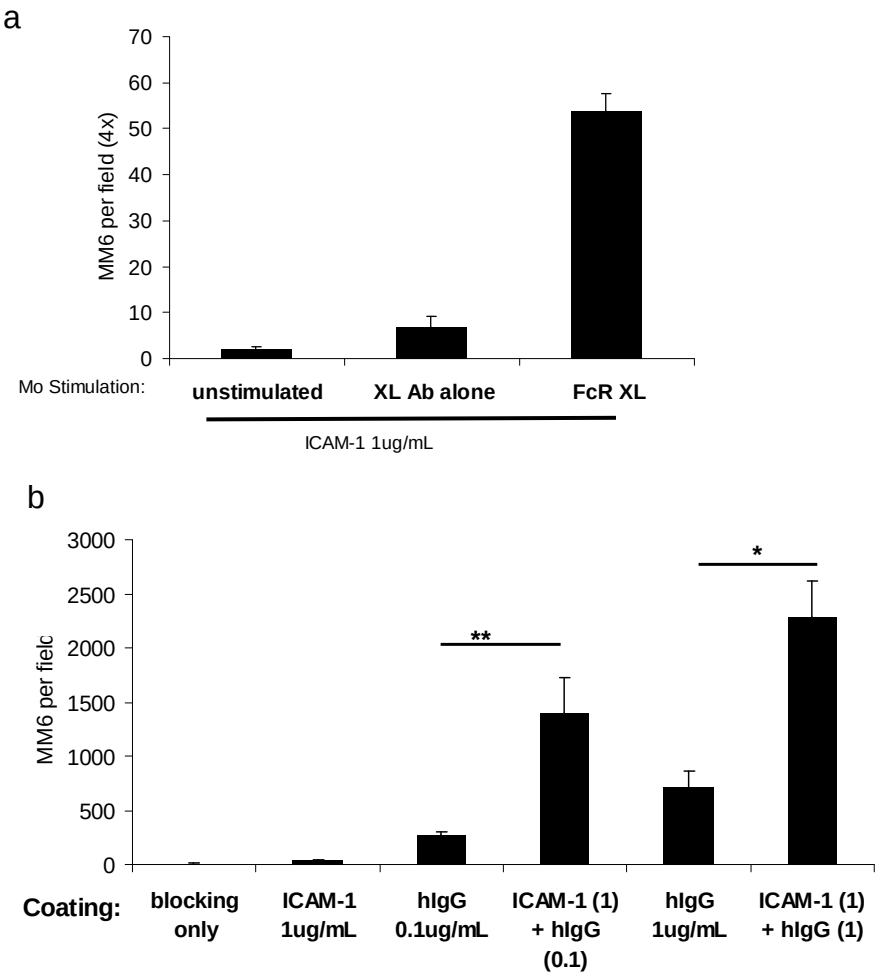



Figure 6-2. Treatment with a calcium agonist increases monocyte adhesion to ICAM-1.

(a) Mono Mac 6 were fluorescently labeled and left unstimulated or treated with thapsigargin at 3μM for 5min. Monocytes were overlaid on unstimulated endothelium in the presence or absence of a neutralizing antibody to ICAM-1 at 10μg/mL. Adherent cells were counted as in Figure 1.

(b) CFSE-labeled Mono Mac 6 were left unstimulated or treated with thapsigargin at 0.1μM, 1μM, or 10μM for 1min, then seeded into wells coated with 5% BSA or purified ICAM-1 protein at 1μg/mL. The number of adherent monocytes was counted, and data are presented as the mean number of monocytes per field for each condition +/- SEM. Graph shows one representative experiment out of three total. *$p<0.01$ versus untreated.

Figure 6-3


a
70
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MM6 per field (4x)
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MM6 per fielc
**
*
Coating:
blocking only
ICAM-1 1ug/mL
hIgG 0.1ug/mL
ICAM-1 (1) + hIgG (0.1)
hIgG 1ug/mL
ICAM-1 (1) + hIgG (1)

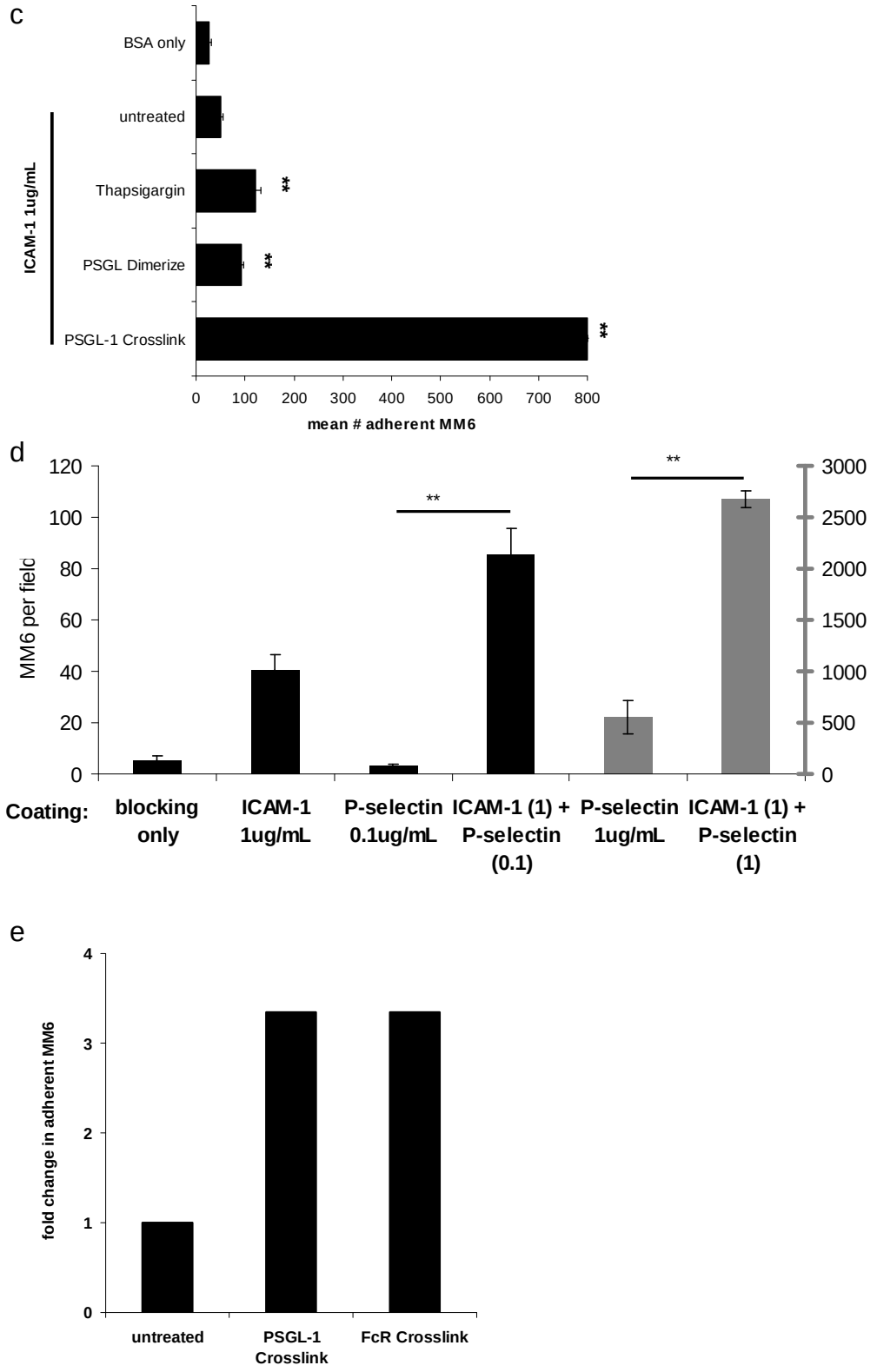


Figure 6-3. Crosslinking of PSGL-1 or FcγRs triggers adhesion to ICAM-1.

(a) Purified ICAM-1 protein was immobilized on Nunc Maxisorp wells, and the wells were blocked with BSA. Mono Mac 6 were incubated with hIgG with or without an anti-human $F(ab')_2$ crosslinking antibody to cluster the FcγRs occupied with IgG. CFSE-labeled Mono Mac 6 were aliquoted into protein-coated wells at $10^5$ cells per well and allowed to bind for 20min. Nonadherent cells were washed off and adherent cells were counted. Representative experiment out of two total. * $p<0.01$ binding to ICAM-1 versus untreated.

(b) Purified ICAM-1 protein at 1μg/mL, or hIgG at 0.1μg/mL or 1μg/mL alone or with ICAM-1 was immobilized on Nunc Maxisorp wells, and the wells were blocked with BSA. CFSE-labeled Mono Mac 6 were aliquoted into protein-coated wells at $10^5$ cells per well and allowed to bind for 20min on an orbital shaker at 45RPM. Nonadherent cells were washed off and adherent cells were counted. Representative experiment out of two total. ** $p<0.01$ binding to hIgG alone versus hIgG with ICAM-1.

(c) Purified ICAM-1 protein at 1μg/mL in 0.1mL was immobilized on Nunc Maxisorp wells, and the wells were blocked with 5% BSA. CFSE-labeled Mono Mac 6 were incubated with positive control thapsigargin at 10μM, or dimeric P-selectin-Fc chimera, with or without an anti-human Fcγ crosslinking antibody to cluster PSGL-1 at the cell surface. The Mono Mac 6 were aliquoted into protein-coated wells at $10^5$ cells per well and allowed to bind for 20min. Nonadherent cells were washed off and adherent cells were counted. Graphs show the average number +/- SEM of adherent Mono Mac 6 in five fields per sample. ** $p<0.01$ compared with untreated. Representative experiment out of two total.

(d) Purified ICAM-1 protein at 1μg/mL alone, or rP-selectin at 0.1μg/mL or 1μg/mL alone or with ICAM-1 was immobilized on Nunc Maxisorp wells, and the wells were blocked with BSA. CFSE-labeled Mono Mac 6 were aliquoted into protein-coated wells at $10^5$ cells per well and allowed to bind for 20min on an orbital shaker at 45RPM. Nonadherent cells were

washed off and adherent cells were counted. Representative experiment out of two total. ** $p<0.01$ binding to rP-selectin alone versus rP-selectin with ICAM-1.

(e) HAEC were cultured to a confluent monolayer and left untreated. Mono Mac 6 were stimulated with thapsigargin at 3μM for 2min, or hIgG followed by anti-human $F(ab')_2$ secondary antibody, then aliquoted at $2x10^5$ cells per well and allowed to bind for 20min in the presence or absence of a neutralizing antibody to ICAM-1 at 10μg/mL. Or, Mono Mac 6 were stimulated with anti-PSGL-1, then aliquoted at $2x10^5$ cells per well and allowed to bind for 20min. Nonadherent cells were washed off and adherent cells were counted. Representative experiment out of two total. Nonadherent cells were washed off and adherent cells were counted. Graphs show the fold change in the average number +/- SEM of adherent Mono Mac 6 in five fields per sample.

Figure 6-4

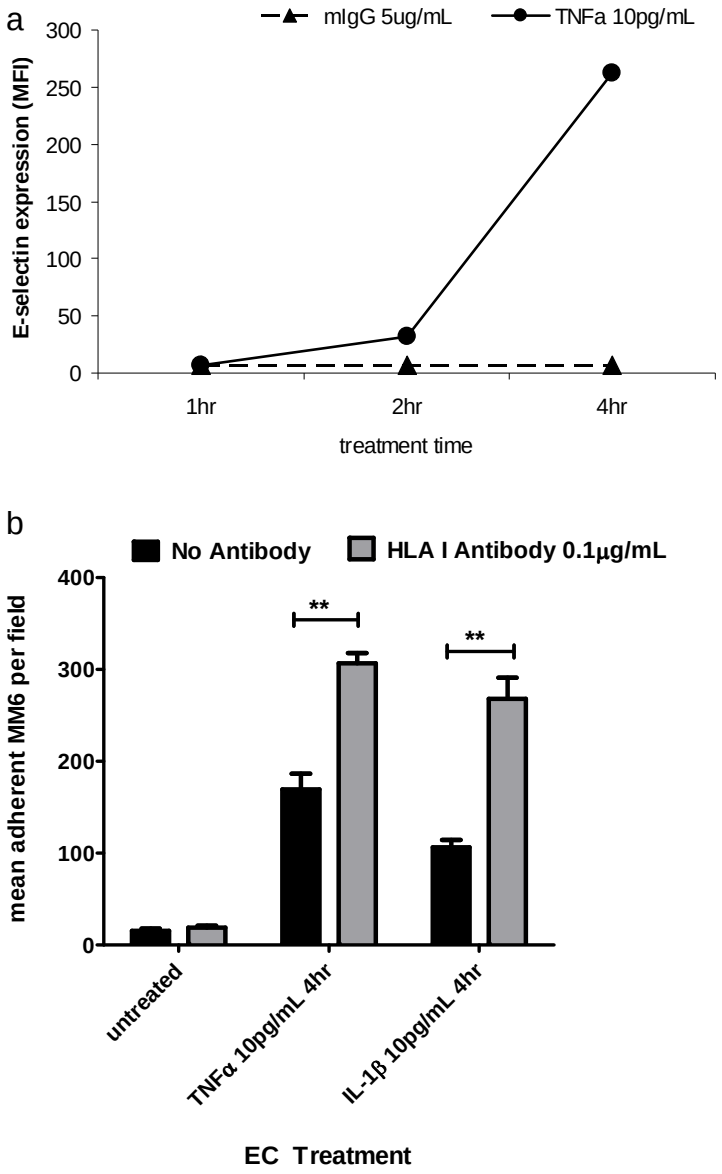


Figure 6-4. Low titers of HLA I antibody enhance monocyte adhesion to cytokine-activated endothelium.

(a) Endothelial cells were treated with negative control mIgG at 5µg/mL or TNFα at 10pg/mL for 1, 2, and 4hr. Cells were detached and stained with anti-E-selectin-PE. Fluorescence was

measured by flow cytometry. Data are presented as median channel fluorescence of E-selectin expression over time. Graph shows one representative experiment out of three total.

(b) Confluent HAEC were pretreated with TNFα or IL-1β at 10pg/mL for 4hr. Cells were then incubated with HLA I antibody at 0.1μg/mL for 30min, and CFSE-labeled Mono Mac 6 were allowed to adhere for 20min. Adherent cells were counted, and data are presented as the average number of bound monocytes per field for each condition +/- SEM. ** $p<0.001$ comparing cytokine alone to cytokine with HLA I antibody.

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# CHAPTER 7:

# Summary and Implications

## CHAPTER 7: Discussion and Future Directions

### 7.1 Introduction

Despite advances in immunosuppression, which have reduced the incidence of T cell-mediated and acute rejection, long-term graft survival is still threatened by the development of donor specific HLA antibodies and chronic rejection. Transplant arteriosclerosis or vasculopathy (TV) is a chronic disease unique to the transplanted vascularized organ, and is a leading cause of graft loss in cardiac, lung, and renal transplantation. TV is characterized by diffuse thickening of the intimal layer of allograft blood vessels which results in occlusion of the lumen and failure of the organ due to ischemia. TV is caused by proliferation of the vascular cells and recruitment of mononuclear cells. Although somewhat similar to traditional atherosclerosis, TV is primarily the result of the recipient's immune response to polymorphic proteins in the graft, particularly against HLA antigens. Infiltration of mononuclear cells, most notably macrophages, is a hallmark of TV and is critical to the pathogenesis of TV, as depletion of macrophages ameliorates TV in animal models (1). Current immunosuppressive regimens cannot mitigate antibody production against alloantigens such as HLA, and anti-donor HLA antibodies are highly associated with chronic graft rejection and poor outcome (2).

Antibodies to donor HLA molecules which are produced before or after transplantation cause graft injury and acute rejection, and promote allograft vasculopathy. Sensitization to polymorphic proteins, especially HLA class I and HLA class II molecules, occurs when a patient is exposed to cells from other individuals, due to pregnancy, transfusion or transplantation. In children, the primary cause of sensitization is transfusion during exposure to "bridge to transplant" mechanical support. For example, use of the ventricular assist device (VAD) in pediatric patients increased the risk for positive HLA I and HLA II PRA (3, 4).

Antibodies to HLA I bind and crosslink these molecules on the surface of graft endothelial and vascular cells, and transduce signaling which promotes proliferation and inflammation, suggesting a mechanism by which donor specific antibodies can elicit TV. In addition, antibody-mediated rejection is characterized by the accumulation of macrophages in the allograft (5), which have pathogenic potential due to their myriad functions. We postulated that HLA I antibodies can promote recruitment of monocytes to the graft in two ways; first, by activating endothelial cells to express inflammatory molecules which increase endothelial adhesivity, and secondly by potentiating the adherence of monocytes through FcγR ligation. These two signals additively (cooperatively) increase monocyte binding to ICAM-1, which mediates firm adherence. This work has demonstrated that a) HLA I antibody treatment of endothelial cells increases P-selectin expression and increases binding of MM6 b) inhibition of P-selectin inhibits (Fc-independent) or reduces (Fc dependent) HLA I Ab-induced MM6 binding; and c) the isotype of the HLA I antibody determines the degree to which it promotes adherence of monocytes.

**7.2 Key Findings**

The goal of this project was to elucidate the mechanisms by which HLA I antibody binding to the graft endothelium promotes recruitment of monocytes. The knowledge gained from these studies has important implications for the understanding of immunology and for the management of patients with antibody-mediated rejection. There are three key findings. First, we showed that HLA I ligation by antibodies increases free calcium in the endothelial cell, identifying a new signaling pathway. It is well-established that crosslinking of HLA I and II on professional antigen presenting cells increases intracellular calcium (6), but the effect of ligation of HLA I on endothelial calcium was unknown until now. Increased intracellular calcium is a catalyst for a variety of signaling cascades that regulate important cell functions. For example, myosin light chain kinase (MLCK) is

activated by calcium and regulates cytoskeletal changes (7). Further, activation of kinases such as calmodulin and classical protein kinase C (PKC) is calcium dependent. Future studies should expand on the area of HLA I antibody-induced calcium-mediated signaling in endothelial cells.

Secondly, we found that calcium-dependent Weibel-Palade body (WPb) exocytosis increased endothelial cell surface P-selectin, an early endothelial activation event that promotes rapid adhesion of leukocytes. P-selectin was an essential mediator of HLA I antibody-induced monocyte-endothelial interactions. Importantly, we validated its significance *in vivo*, where the selectin antagonist rPSGL-1 (already in use clinically) abolished donor specific MHC I antibody-elicited macrophage accumulation in the allograft. Other leukocytes, including T cells, neutrophils and NK cells, utilize P-selectin to egress into the tissue, and rPSGL-1 may have a therapeutic effect to block infiltration of these immune cells as well. Clinical trials of rPSGL-1 in transplantation currently focus on amelioration of ischemia/reperfusion injury and early graft function. Future studies should examine its effect on the influx of recipient immune cells into the graft.

Finally, our results suggest that the isotype of the HLA I antibody shapes the degree to which it promotes monocyte recruitment. Both HLA I antibodies with low affinity and high affinity for human FcγRs triggered monocyte adhesion to endothelial cells via P-selectin. However, the HLA I antibody of an isotype with high capacity to engage FcγRs on the monocyte was able to augment P-selectin-mediated binding.

### 7.3 Other Effects of HLA I Antibodies on Endothelium

Endothelial cells have been recently given more credit for their role in inflammation and regulation of immune responses. Over a decade ago, Hunt et al. proposed a model of "endothelial cell

activation," existing in two stages. Endothelial activation type 1 is rapid, does not require protein synthesis, and results in P-selectin and vWF release, as well as endothelial retraction. Type 2 activation, generally triggered by cytokines, requires protein synthesis, and upregulates chemokines and adhesion molecules at the level of gene transcription (8, 9). HLA I crosslinking in endothelial cells results in signaling which fit the criteria for type I endothelial cell activation. P-selectin and vWF are rapidly released and actin cytoskeleton rearrangement occurs, together with stress fiber formation. While our group reported that HLA I ligation did not upregulate late phase adhesion molecules, other groups have demonstrated that HLA I crosslinking may cause type II endothelial cell activation as well. For example, it has been shown that tissue factor, KC (the murine homolog of IL-8) and MCP-1 are produced and secreted (10, 11).

Given that we reported that calcium is increased, phospholipase C is implicated in HLA I signaling. PLC catalyzes the conversion of $PIP_2$ to $IP_3$ and diacyl glycerol. IP3 is an intracellular second messenger which binds a receptor on the endoplasmic reticulum and stimulates release of calcium into the cytosol. The increase in free calcium also implies that protein kinase C (PKC) will be activated following HLA I ligation. The classical family members of PKC are activated by DAG and by calcium, so it is possible that PKC may be activated upon HLA I crosslinking. Whether RalA is involved in HLA I signaling is unknown. RalA is a central mediator that can be activated by PDK1, or calcium/calmodulin. It is required for WPb release in response to thrombin and regulates exocytosis. Further, RalA is involved in proliferation along with mTOR, while RalB suppresses apoptosis. RalGDS functions as a scaffold for PDK1, localizing it near Akt to facilitates PDK1-dependent and independent Akt phosphorylation therefore the Rals are involved in proliferative and survival signaling that are central to HLA I antibody-mediated effects (reviewed in (12, 13)).

Our group has reported that fibroblast growth factor receptor (FGFR) is rapidly increased at the cell surface following HLA I ligation, which increases the cell's sensitivity to its growth factor ligand (14). However, the mechanism by which FGFR is mobilized is totally unknown. Given that P-selectin is similarly rapidly externalized by exocytosis, perhaps the same machinery, such as calcium-mediated signaling, directs the changes in FGFR localization.

### 7.4 The Inflammatory Milieu

The *in vitro* system we used enabled us to dissect the details of monocyte-endothelial interactions downstream of HLA I crosslinking. However, the physiological setting of transplantation is more complex. For example, ischemia/reperfusion injury elicits innate activation of the graft and introduces a background of inflammation even before alloresponses occur. The contents of WPb are altered by inflammatory insult (15) and may store chemokines such as IL-8. Further, inflammatory cytokines upregulated during injury, such as TNFα and IL-1β, increase expression of late activation adhesion molecules in endothelial cells, which support leukocyte recruitment. The effect of HLA I antibody binding to cytokine-activated endothelium is likely to enhance recruitment and magnify inflammation relative to the background inflammatory state of the endothelium (16).

In addition to rapidly triggering cell surface P-selectin on endothelial cells, anti-MHC I antibodies can also induce endothelial expression of chemokines, including KC (IL-8), MCP-1, VEGF, and RANTES (11). While we did not look at the effects of long-term treatment of endothelium with HLA I antibodies, under conditions of chronic stimulation, endothelial cell-derived chemokines may also contribute to monocyte recruitment and augment the system we have described. Certainly in our animal model, in which the graft was exposed to donor specific MHC I antibodies for 30 days, chemokine upregulation may be pertinent. Future studies could address whether MCP-1, VEGF and

KC are induced in the allograft during antibody-mediated rejection. Further, complement fixing isotypes of HLA I antibody cause local activation of the complement cascade, which generates inflammatory and chemotactic complement split products like C5a (17). In our animal model, the donor specific MHC I antibody used was of the complement fixing isotype mIgG2a, and these isotypes are common in the DSA of transplant patients. There is likely interplay in vivo between HLA I antibody-induced selectins, chemokines and complement. Therefore, complement activation and HLA I antibody-induced chemokine production by endothelial cells likely cooperates with HLA I-induced exocytosis to promote monocyte infiltration. Future experiments could compare the effect of complement fixing MHC I antibodies to noncomplement fixing MHC I antibodies on macrophage infiltration.

We also showed that a constituent of WPb, von Willebrand Factor (vWF), was externalized upon HLA I ligation. vWF is an important regulator of thrombosis and supports platelet accumulation at the endothelial surface (18). Activated platelets express P-selectin at high densities and act as a bridge to facilitate monocyte adherence, particularly necessary under shear stress. This function in the context of AMR has previously been described by the Baldwin group (19, 20). Moreover, vWF is itself a substrate for monocyte adherence, acting as a ligand for both PSGL-1 and Mac-1 (21). There is thus a likely role for vWF in monocyte recruitment by HLA I antibodies which has yet to be explored. The promiscuity of PSGL-1 is both a detriment in our studies and an advantage for therapeutic applications. The rPSGL-1 molecule may have bound and antagonized vWF as well as P-selectin, which may explain our observation of its greater inhibitory effect when compared with anti-P-selectin neutralizing antibody. In patient management, this feature is an advantage because rPSGL-1 should antagonize many adhesion molecule interactions, addressing the issue of redundancy in the leukocyte recruitment system.

### 7.5 Preferential Recruitment of Monocytes during Antibody-Mediated Rejection

Our data may provide some insight into why antibody-mediated rejection is characterized by intragraft macrophages. We hypothesize that HLA I antibody activation of endothelial cells preferentially recruits monocytes for two reasons. First, we found that Mac-1 rather than LFA-1 was the predominant integrin mediating firm adhesion, and we postulate that the reason is its well-documented cooperation with FcγRs (22). Monocytes express higher levels of Mac-1 than neutrophils. Further, FcγRI was the dominant Fc receptor involved in recruitment in response to HLA I antibodies. Neutrophils express only FcγRII and FcγRIII, and have lower overall expression of all FcγRs than monocytes. While neutrophils are recruited in response to immune complexes (an FcγR-mediated function), they do not utilize FcγRI (16, 23). This may mean that monocytes will be more responsive to antibody bound on the endothelium.

### 7.6 The Significance of FcγRs

A potential implication of the involvement of FcγRs is that recipient polymorphisms which increase or decrease the affinity for the Fc region of antibody may influence the extent to which FcγR-bearing cells are recruited. While there are no known functional mutations in FcγRI, FcγRIIa exists in two major allotypes in the human population. FcγRIIa-H131 has very low affinity for murine IgG1, but has the capacity to interact with human IgG2. In contrast, FcγRIIa-R131 has increased affinity for mIgG1, which cannot bind any other human FcγR. Based on typing using an allotype-specific antibody and flow cytometry, we determined that the Mono Mac 6 cell line was homozygous for the H131 allele, suggesting that will interact efficiently with murine IgG2a but not with murine IgG1. The THP-1 monocytic cell line was heterozygous, carrying both the R131 and H131 alleles (24), so the FcγRIIa of THP-1 may have decreased affinity for murine IgG2a but

increased affinity for murine IgG1. This phenotypic difference may explain the discrepancy in our date, where Mono Mac 6 had a moderate requirement for FcγRII to bind to HLA I mIgG2a-activated endothelial cells, while THP-1 did not. Future studies should more extensively investigate this difference in experimental models, and in clinical outcomes of patients carrying these allotypes.

One drawback to our studies is that we used murine monoclonal HLA I antibodies to stimulate endothelial cells. While the interaction of murine antibody isotypes with human Fc gamma receptors is well described, an ideal experimental system would assess monocyte recruitment in response to HLA I antibodies of human origin. To that end, it is necessary to develop chimeric antibodies containing the variable region of murine monoclonal HLA I antibodies, because they are well characterized and because they have the advantage pan-recognition (i.e. antibodies developed in the mouse recognize all alleles and loci of HLA class I molecules). These variable regions should be fused to human Fc regions from different gamma globulin isotypes, in order to more accurately assess the influence of antibody isotype on monocyte recruitment, with results that are more readily applicable to the clinical setting.

Other questions are raised by the results presented herein. For example, several studies have highlighted the role of NK cells in antibody-mediated rejection. NK cells were recruited to murine cardiac allografts in response to donor specific MHC I antibodies, and were necessary for antibody-induced chronic rejection (25). Further, biopsies from patients with antibody-mediated rejection were enriched for NK cell transcripts, suggesting that NK cells are recruited in the clinical setting (26). NK cells express FcγRIII, which have been shown to support adhesion to immune complexes, but not anti-endothelial cell antibodies (16). It remains to be seen whether NK cell recruitment may have an Fc-mediated component. Further, monocytes express both activating FcγRIIa and the

inhibitory FcγRIIb, which down-modulates the function of FcγRIIa. The effect of the interplay between these receptors should be determined in the context of Fc-dependent recruitment.

Intravenous IgG (IVIG), a polyclonal mixture of human IgG, is currently used as a therapy for antibody-mediated rejection (27, 28), although its precise mechanism of action is unclear. It is thought that, in addition to containing anti-idiotypic antibodies (which may neutralize donor specific antibodies) and cytokine neutralizing antibodies, IVIG may occupy and block FcγRs to prevent their function (29, 30). As we demonstrated, this may be useful to reduce HLA I antibody-induced recruitment, although it will not abolish it.

**7.7 Monocyte Subsets and Macrophage Polarization**

The phenotype of the monocytic cell lines used was similar to the predominant blood monocyte subset CD14+CD64++CD16-. It is worthwhile to note that both monocytic cell lines closely resemble the blood monocyte subset expressing CD14hi CD64+ CD32hi CD16-. The two major subsets of monocytes differ with respect to expression of FcγRs, chemokine receptors, and molecules involved in antigen presentation [reviewed in (31)]. Because these phenotypes are characterized by FcγR expression, there may be subtle or not-so-subtle differences with respect to recruitment to antibody-coated endothelium between the subsets expressing FcγRI and the minor subset expressing FcγRIII.

Herein we investigated the effects of FcγR and PSGL-1 engagement on rapid activation of β2 integrins and induction of a proadhesive phenotype. The downstream implications of such a stimulus are currently unknown. During initial stimulation, the monocyte differentiates and there are two pathways, termed classical and alternative, of activation. Polarization of macrophages influences

the phenotype and function of the tissue macrophage. There are two major classes of macrophages, termed M1 and M2, with subdivisions. In general, M1 macrophages are considered "proinflammatory," and produce reactive oxygen species, and TNFα, IL-1β and other inflammatory cytokines (32, 33). M2 macrophages, such as tumor infiltrating macrophages, have an "anti-inflammatory," profibrotic phenotype. The M2 macrophage marker is found in the arteriosclerotic lesions in cardiac transplant recipients (34), and may promote proliferation and fibrosis seen in chronic rejection. Given that immune complex stimulation of THP-1 or primary monocytes seemingly polarizes monocytes to the M2 phenotype (35-38), it is tempting to speculate that antibody stimulation of FcγRs during monocyte recruitment polarizes the macrophage to an M2 phenotype as it extravasates into the allograft, which may skew the macrophage to promote chronic rejection changes.

### 7.8 HLA II Antibodies

In the clinic, donor specific antibodies recognizing HLA class II molecules are pathologically important, and the presence of HLA II antibodies is detrimental to graft outcome. However, the effects of HLA II antibodies on endothelial cells are almost wholly unknown, due to the experimental limitations discussed in Chapter 1. More investigation is required in this area to understand the effect of HLA II antibodies on endothelial cell immune activation leading to leukocyte recruitment.

### 7.9 Conclusion

The data herein describe 1) that P-selectin is a central mediator of monocyte recruitment by HLA I antibodies and 2) that the isotype of an HLA I antibody shapes the degree of macrophage

infiltration. These results have important implications for patient management, and provide greater knowledge regarding the mechanisms of monocyte recruitment during antibody-mediated rejection.

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