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

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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 Fc-independent effect of HLA I antibodies on endothelial cell activation, leading to monocyte recruitment. 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, but had little effect on antibody-induced endothelial injury. Our data suggest that antagonism of P-selectin may ameliorate accumulation of macrophages in the allograft during antibody-mediated rejection.

Keywords

Endothelial cells; HLA antibody; signal transduction; monocytes; P-selectin

Introduction

Donor specific antibodies to HLA class I and II molecules have a deleterious impact on graft survival in solid organ transplantation (1-4), and correlate with chronic rejection (5-8). HLA I antibody binding to vascular endothelial and smooth muscle cells activates cell signaling

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Disclosure

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pathways that are required for increased proliferative capacity, migration, stress fiber formation, and survival (9-17). 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).

Antibody-mediated rejection is a complex process, with characteristic features such as endothelial cell swelling and complement deposition in the vessels of the graft (19). In addition, macrophage infiltration frequently accompanies antibody-mediated rejection (AMR), and correlates with donor specific HLA antibodies and poor graft outcome (20-24). Macrophages comprise a significant proportion of graft infiltrating cells in acute rejection (25-29), and are found in lesions of chronic allograft vasculopathy (CAV) in human biopsies and experimental models (21, 29, 30). Macrophages promote deposition of extracellular matrix by SMC (31), release soluble mediators that cause endothelial cell proliferation (32), and may exhibit direct alloreactivity against the allograft (33, 34). Importantly, depletion of macrophages reduces rejection of murine cardiac allografts (35). Therefore, the understanding of the mechanism(s) that promote monocyte recruitment may lead to novel approaches to reduce allograft rejection.

It is incompletely understood how HLA I antibodies may promote monocyte recruitment into the allograft. In this study, we used an *in vitro* approach to measure endothelial cell activation and the Fc-independent recruitment of monocytes in response to HLA I antibody. Our results show that HLA I signaling in endothelial cells is sufficient to increase monocyte adhesion mediated by HLA I-induced P-selectin. Using an *in vivo* model of antibody-mediated rejection, we confirmed that donor specific MHC I antibodies elicit macrophage infiltration into the allograft. Importantly this influx can be blocked by the selectin antagonist rPSGL-1-Ig. This study demonstrates that selectin blockade prevents monocyte adherence *in vitro* and intragraft macrophage accumulation *in vivo* in response to MHC I antibodies.

Materials and Methods

Reagents

Pan-HLA I antibody (murine monoclonal W6/32, mIgG2a) was obtained from BioXCell. The $F(ab')_2$ fragment of W6/32 was generated as previously described (36), or using a kit from Thermo Scientific. Neutralizing goat (Santa Cruz) or sheep (R&D) antibody to P-selectin, or antibody to PSGL-1 (Biolegend, San Jose, CA) were used. Purified polyclonal human IgG was obtained from Fisher Scientific. Calcium inhibitor BAPTA-AM was from Calbiochem. Recombinant soluble PSGL-1 Fc chimera (rPSGL-1-Ig) was provided by Y's Therapeutics (San Bruno, CA). It contains mutations in the crystallizable fragment (Fc) region to eliminate interactions with complement and FcγRs, and inhibits the initial tethering of leukocytes to selectins (37, 38).

Cell Culture

The use of the human aortic tissue for the research described herein was approved by the OneLegacy Biomedical Review Board under the agreement #RS-02-10-2 and UCLA MTA2009-561. Primary human aortic endothelial cells (HAEC) were isolated from aortic rings and cultured as previously described (11). Endothelial cells were seeded in 24 or 48 well plates, and grown to confluence before use in experiments. The monocytic cell line Mono Mac 6 (39, 40) was cultured in RPMI-1640 supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, antibiotics, and insulin, and maintained at fewer than 10^6 cells per mL.

Primary Human Monocytes

Human peripheral blood mononuclear cells (PBMC) were collected under our institutional review board-approved study and isolated by Ficoll density centrifugation. Monocytes were enriched using MACS Monocyte Isolation Kit II (Miltenyi). Purity was confirmed by CD14 staining and to be greater than 85%.

Measurement of von Willebrand Factor

Confluent aortic endothelial cells were stimulated with HLA I antibody, thrombin, histamine or phorbol myristate acetate (PMA) in M199 supplemented with 5% fetal bovine serum (assay medium) for one hour. Supernatant was collected and von Willebrand Factor (vWF) was measured using an ELISA kit (Helena Laboratories) according to the manufacturer's protocol.

Flow Cytometric Analysis

Cell surface expression of HLA I, E-selectin or P-selectin was measured by flow cytometry. Basal expression of HLA I on endothelial cells is shown in Supplemental Figure 1. Endothelial cells were treated with stimuli for the indicated times in assay medium and detached using Accutase (Innovative Cell). To preserve epitopes, trypsin was not used (41). Cells stained with PE-conjugated anti-human P-selectin or anti-E-selectin (BD) were assayed on a FACSCalibur flow cytometer (BD). P-selectin results are expressed as proportion of cells staining positively, normalized to untreated endothelial cells.

Intracellular Calcium Measurement

HAEC were plated onto gelatin-coated glass coverslips, then labeled with 5μM Fura-2 AM for 20min at 37°C. Cells were washed and mounted in a standard cuvette filled with saline (at 37°C) (ANO-2100, Hitachi Instruments). The cuvette was placed in a fluorimeter (F-2000, Hitachi Instruments) with a heated jacket (37°C), and continuously stirred. Samples were taken every 0.5 secs (excitation 340nm, emission 380nm) using associated software (F-2000 Intracellular Cation Measurement System). The ratio of the signals at 340 nm/380 nm provided a monitor of Ca^{2+} concentration.

Monocyte Adhesion Assay

Preliminary studies determined the optimal concentration of blocking reagents. Adherence of the monocytic cell line Mono Mac 6 or freshly isolated primary monocytes to endothelial monolayers was adapted from previously described methods (42). Due to the established affinity of human FcγRs for murine IgG2a (43, 44), we sought to eliminate antibody-FcγR interactions. Briefly, HAEC were stimulated and carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen)-labeled Mono Mac 6 were added at a ratio of approximately 3 monocytes per endothelial cell for 20min. Nonadherent cells were removed by washing three times. Adherent monocytes were imaged by fluorescence microscopy (Nikon Eclipse Ti) and counted in 8-10 fields per sample using automated software (CellProfiler or Image J) (45). HAEC were pretreated with BAPTA-AM (20μM) for 30min, or rPSGL-1-Ig (20μg/mL) or P-selectin neutralizing antibody (10μg/mL) in the last 10min of stimulation. Monocytes were pretreated with a neutralizing antibody to PSGL-1 (10μg/mL), or with polyclonal human IgG (20μg/mL) to block FcγRs where indicated, for 20min (46).

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), housed under pathogen-free conditions in filter-top cages throughout the experiments and 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

BALB/c (H-2^d) hearts were heterotopically transplanted into abdominal great vessels of B6.RAG1 KO (H-2^b) recipients as previously described (16). Briefly, under phenobarbital 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 daily by abdominal palpation. On day 30 post-operatively, the donor and native hearts were recovered. All allografts included in the study were functioning at the time of sacrifice. The base of the heart was fixed in formalin and paraffin-embedded.

Adoptive transfer of monoclonal antibodies

All reagents to which mice were exposed were sterile and azide free. Allograft recipient mice were passively transferred with 30μg (approximately 1.5mg/kg) isotype control antibody (clone MOPC-173, Biolegend) or donor specific anti-MHC I antibodies (anti-K^d mIgG2a, clone SF1-1.1; anti-D^d mIgG2a, clone 34-2-12, Biolegend; diluted in sterile saline) by tail vein injection the beginning day 3 post-transplant, continuing weekly thereafter. 70μg of rPSGL-1-Ig (approximately 3.5mg/kg) was injected intravenously beginning on day 3 after transplant and continuing biweekly. The dose and administration regimen were based on previously published studies detailing the pharmacokinetics and efficacy of rPSGL-1-Ig in mouse (47, 48).

Histologic Analysis of Graft Infiltrating Cells

Histological features of antibody-mediated rejection and immunohistochemical staining in allografts were assessed by a pathologist blinded to the experimental groups. Microvascular changes were assessed, including endothelial cell swelling and increased number of intravascular cells, as previously described (16, 19). Cross sections of formalin-fixed tissue were stained for Mac-2 (Acris) or CD45 (Millipore). A Mac-2 or CD45 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, or biweekly administration of 30μg anti-K^d antibody, revealed no statistically significant difference between the three groups when compared by unpaired T test. Therefore, we combined animals from these groups into a single group, the anti-K^d antibody treated group. Groups treated with control antibody at 5μg and 30μg were merged into one control antibody treated group.

Flow Cytometric Crossmatch

To confirm the presence of circulating donor specific antibodies, blood was collected at the time of sacrifice, and serum was separated using a Capiject tube (Terumo T-MG). BALB/c splenocytes were incubated with 25μL of serum for 30min on ice, then stained with fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse-Fcγ and anti-mouse CD3-phycoerythrin (PE). MHC I antibody binding to T cells was measured by gating on CD3+ using flow cytometry (FACSCalibur, BD).

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. Statistical significance

between controls and samples was determined using one-way ANOVA followed by individual comparisons (student's T-test).

Results

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 preformed von Willebrand Factor (vWF) and P-selectin from Weibel-Palade bodies (WPb), we treated confluent monolayers of human aortic endothelial cells with a monoclonal murine pan-HLA I antibody (W6/32), or with classical agonists thrombin, PMA and histamine. Stimulation of endothelium with positive controls thrombin, PMA or histamine, or with HLA I antibody significantly increased vWF secretion by more than 1.5-fold over untreated control ($p=0.025$). Negative isotype control murine IgG (mIgG) had no effect (Figure 1a).

HLA I antibody (5μg/mL) significantly increased the proportion of cells staining positively in flow cytometry for P-selectin by 2.0+/−0.14 fold over control after 30min (Figure 1b). Untreated endothelial cells were negative for P-selectin staining. Dose response and kinetics studies revealed a maximal effect on P-selectin induced by 5μg/mL HLA I antibody at 30 minutes (Supplemental Figure Figure 2a, 2b and 2c). This response was specific to HLA I antibodies, as treatment of endothelial cells with nonbinding mIgG or non-HLA antibody against endoglin (CD105) did not significantly alter cell surface P-selectin (Fig. 1b). In contrast, P-selectin expression was elevated on positive control thrombin, PMA or histamine-treated endothelial cells. HLA class I mediated WPb exocytosis comparable to induction by classical agonists (49-54), which trigger rolling of leukocytes on blood vessels *in vivo* (55-58). Treatment of EC with HLA class I antibody had no effect on expression of E-selectin over a 48-hr time frame (Supplemental Fig. 3), whereas TNFα treatment increased cell surface E-selectin as previously reported (59, 60).

We postulated that HLA I-induced exocytosis might be dependent on Ca^{2+} signaling. Pretreatment of endothelial cells with BAPTA-AM to chelate intracellular Ca^{2+} significantly inhibited HLA I-induced P-selectin induction, from 1.70+/−0.11-fold without inhibitor to 1.15+/−0.37-fold over control (Figure 1c). Likewise, vWF secretion from endothelium in response to HLA I antibodies or thrombin was completely prevented by BAPTA-AM (data not shown). To further confirm that HLA I antibodies increase Ca^{2+}-dependent signaling, we measured $[Ca^{2+}]i$ in real time after HLA I crosslinking. Endothelial cells treated with HLA I antibody exhibited a rapid increase in $[Ca^{2+}]i$ levels. As a positive control, we verified that thrombin treatment rapidly increased free cytosolic Ca^{2+}. In contrast, isotype control mIgG had no effect on $[Ca^{2+}]i$ levels (Figure 1d).

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 and to eliminate interaction of FcγRs with the Fc fragment of antibody, 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 ($p<0.0001$) (Figure 2a). Representative micrographs and raw data from one representative experiment are given in Figure 2b. As an alternative approach, we treated endothelial cells with intact HLA I antibody and preincubated Mono Mac 6 with soluble IgG to block FcγRs. Treatment of

endothelial cells with HLA I antibody significantly increased monocyte adherence by 1.48+/−0.09 fold ($p<0.0001$). Thrombin also increased Mono Mac 6 binding, while exposure of endothelial cells to isotype control mIgG did not (Figure 2a).

We examined whether P-selectin was involved in mediating the increased adherence of monocytes. rPSGL-1-Ig was added to block P-selectin. Mono Mac 6 adherence to endothelium was increased by 1.61+/−0.06 fold over control in response to HLA I $F(ab')_2$, but was significantly reduced to 1.13+/−0.09 fold over control by rPSGL-1 (76.7+/−12.8% inhibition). Mono Mac 6 binding to thrombin-stimulated endothelium was also reduced by the presence of rPSGL-1 (Figure 2c). We substantiated the requirement for P-selectin with neutralizing antibody to P-selectin, or to monocytic PSGL-1, both of which significantly prevented Mono Mac 6 recruitment to HLA I $F(ab')_2$-treated endothelial cells (Figure 2d). Similar results were obtained using human monocytes enriched from peripheral blood (Figure 2e), where HLA I $F(ab')_2$ fragment or positive control PMA increased the number of monocytes bound to endothelium by more than 2-fold ($p<0.01$), while anti-CD105 antibody had no significant effect. Binding of peripheral blood monocytes was significantly inhibited by P-selectin antagonism (Figure 2e).

To determine whether endothelial cells required intact Ca^{2+} signaling to recruit monocytes after HLA I crosslinking, endothelial cells were pretreated with BAPTA-AM. Activation of BAPTA-AM treated endothelial cells with HLA I $F(ab')_2$ or thrombin failed to trigger Mono Mac 6 adherence ($p<0.001$ compared with no inhibitor) (Figure 2f).

Passive transfer of donor specific MHC I antibodies increases macrophage infiltration into the cardiac allograft

To elucidate the role of P-selectin under physiological conditions, we utilized an *in vivo* model of AMR (16, 61). C57BL/6 RAG1 KO (H-2*b*) recipients of BALB/c donor (H-2*d*) hearts were transferred with murine monoclonal IgG2a recognizing donor MHC I molecules (anti-K^d or anti-D^d) or control murine IgG2a (MOPC-173). The treatment regimen and dose of MHC I antibody were based on a previously described mouse model of antibody-mediated rejection (61, 62). Grafts were recovered on day 30 post-transplant and analyzed as outlined in Figure 3a.

We performed flow crossmatching to measure circulating antibodies. The flow cytometric cross-match revealed no binding of sera from control antibody-treated recipients to BALB/c CD3+ splenocytes. In contrast, sera from animals passively transferred with MHC I antibodies bound highly to donor $CD3^+$ splenocytes, and cells stained positively even when serum was diluted to 1:4. These results document the presence of anti-donor antibodies in the circulation of passively transferred cardiac allograft recipients (Figure 3b).

We then characterized the infiltration of macrophages in cardiac allografts in response to MHC I antibody treatment by immunohistochemistry. Less than half (42%) of grafts from animals which received control mIgG2a antibody were positive for macrophages, with a score of 2 or greater, and none (0%) were strongly positive with a score of 3 or 4 (Table 1). Strikingly, 84% of cardiac allografts from animals treated with anti-K^d antibody stained positively for the macrophage marker, with 40% having strong infiltration. Macrophage staining in anti-K^d antibody-treated allografts was significantly greater ($p<0.001$) when compared with allografts from control antibody-treated animals. We confirmed this phenomenon using a different monoclonal donor specific MHC I monoclonal antibody. Eighty percent of anti-D^d antibody treated allografts scored positively for macrophages, with 40% having strong macrophage infiltration. When allograft recipients were given both anti-K^d and D^d antibodies together, all transplanted hearts had a score of two or greater for

Mac-2 macrophage marker (Table 1 and Figure 3c). These results demonstrate that donor specific MHC I antibodies promote macrophage accumulation at the allograft site.

The selectin antagonist rPSGL-1 significantly reduces MHC I antibody-elicited macrophage and CD45+ accumulation in the allograft

We sought to determine whether antagonism of P-selectin could reduce immune cell recruitment in response to MHC I antibodies *in vivo*. While greater than 80% of anti-K^d antibody treated allografts were positive for Mac-2 staining, only 25% of allografts which received concurrent rPSGL-1 therapy had a Mac-2 score of 2 or higher. Remarkably, rPSGL-1 abolished the strong macrophage infiltration induced otherwise 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. Allografts from control antibody-treated animals were negative for CD45 staining. In contrast, 57% of allografts from animals which received anti-K^d antibody were positive for CD45 (score ≥2), and nearly one third (29%) stained strongly for CD45. Increased CD45 infiltration elicited by MHC I antibody (anti-K^d mIgG2a) was abolished by concurrent administration of rPSGL-1-Fc ($p<0.05$), as no allografts from animals which received both anti-K^d antibody and rPSGL-1 were positive for CD45 (Table 3 and Figure 4B). Therefore, rPSGL-1 therapy specifically reduced macrophage infiltration and prevented CD45+ immune cell infiltration.

The allografts were examined for histological features of antibody-mediated rejection. H&E sections of cardiac allografts showed characteristic histological features of AMR including microvascular changes and endothelial cell swelling (Figure 4c) (16, 19, 20, 27). No hemorrhage or intravascular neutrophils, as commonly seen in severe acute humoral rejection, were observed. An increase in overall cellularity in the interstitium, with cells in a linear arrangement indicative of intracapillary macrophages (19, 27), was found in allografts when compared with native hearts. However, allografts treated with donor specific MHC I antibody had notably greater cellularity and endothelial cell swelling due to intravascular macrophages when compared with control antibody-treated grafts (Figure 4c). While rPSGL-1-Ig therapy decreased cellularity, allografts receiving this treatment had no detectable change in the histological pattern of endothelial cell injury.

Discussion

In this study, we established that MHC I crosslinking on endothelial cells increases cell surface P-selectin, which is required for recruitment of monocytes independently of antibody-FcγR interactions. We also determined that $[Ca^{2+}]i$ signaling downstream of HLA I ligation is necessary for exocytosis, consistent with previous studies (18, 63). Importantly, we confirmed these results in an *in vivo* system, where donor specific MHC I antibodies increased macrophage infiltration into cardiac allograft, which could be abolished by P-selectin antagonism.

Given the Ca^{2+} dependence of exocytosis, we postulated that HLA I crosslinking elicited an increase in $[Ca^{2+}]i$ in the endothelial cell. HLA I antibodies (1μg/mL) stimulated a sustained increase in $[Ca^{2+}]i$, suggesting that Ca^{2+}-dependent pathways are activated shortly after HLA I signaling. Our group has previously demonstrated that HLA ligation rapidly stimulates several intracellular signaling pathways, necessary for proliferation and cytoskeletal changes (14, 64). Increased intracellular Ca^{2+} (reviewed in (65)) triggers a variety of signaling pathways, including protein kinase C (PKC), calmodulin and myosin light chain kinase (MLCK) (66) which are not yet explored in the context of HLA I signaling. It is interesting that the potent diacylglycerol (DAG) surrogate PMA stimulated P-

selectin and increased adherence of monocytic cells, suggesting that DAG/PKC acts in conjunction with Ca^{2+} signaling in mediating HLA I-triggered events.

Herein, we describe HLA I antibody-elicited recruitment of monocytic cells *in vitro*, which could be blocked by several strategies of P-selectin antagonism. P-selectin is an important early mediator of the leukocyte recruitment cascade, which initiates the capture of immune cells from circulation and precedes firm adhesion and extravasation events. P-selectin is upregulated on endothelium in acutely rejecting cardiac allografts, and on thickened arterial intima during chronic rejection in rodent models (67, 68). Allografts from murine donors deficient in both intercellular adhesion molecule (ICAM-1) and P-selectin or in all three selectins (ELP-/-) had significantly longer graft survival than their wild-type counterparts (69, 70). rPSGL-1 or SLX (antagonists of selectin) reduce immune cell infiltration, neointimal hyperplasia and rejection in many models (48, 71-76). Currently, clinical trials are underway to investigate the use of rPSGL-1-Ig in humans as a treatment to reduce ischemia/reperfusion injury (77-79). However, the effect of rPSGL-1-Ig on long-term survival and leukocyte recruitment in human transplantation has yet to be revealed.

Our group previously reported increased macrophage infiltration into allografts treated with a single high dose of MHC I intact antibody or $F(ab')_2$ (16). P-selectin and vWF were increased in acutely rejecting murine allografts from wild-type but not IgKO recipients, suggesting that these mediators are specifically induced in presence of donor specific antibodies (80, 81). Another group demonstrated that HLA I $F(ab')_2$ caused rapid neutrophil recruitment and acute rejection of human skin grafts on immunodeficient mice (18). We attempted confirm our findings *in vivo* with an MHC I $F(ab')_2$ fragment, but were unable to achieve consistently detectable levels of circulating antibody, likely due to their short half-life *in vivo* (82). Nevertheless, the data reported here show that P-selectin is a critical initiator which facilitates capture of monocytes from the circulation by graft endothelium in a long-term vascularized model of AMR.

We found that overall CD45+ infiltration was reduced by rPSGL-1 therapy. NK cells, neutrophils, monocytes and T cells utilize selectins to adhere to endothelium and transmigrate from the blood into tissue (83-86). Donor specific MHC I antibodies cause platelet activation in skin allograft recipients (80), and attachment of platelets to endothelium can support monocyte tethering through P-selectin (87). Moreover, recent data point to a novel role for NK cells in AMR (61, 62, 88). NK cells adhere to P-selectin during recruitment to atherosclerotic lesions (89). In our *in vivo* model, it is possible that administration of rPSGL-1-Ig also antagonized platelet or NK cell adhesion. While P-selectin is the preferred ligand of PSGL-1, PSGL-1 can interact with other adhesion molecules, such as E- and L-selectin and von Willebrand Factor (90). We did not observe an increase in E-selectin expression induced by HLA I crosslinking *in vitro*, but E-selectin may be involved *in vivo*. In light of the myriad parallel mechanisms which may promote leukocyte recruitment during antibody-mediated rejection, the promiscuity of rPSGL-1-Ig may be viewed as advantageous from the therapeutic standpoint since it can disrupt many of the interactions between leukocytes and endothelium.

Complement and endothelial activation are parallel pathways of AMR. rPSGL-1 therapy did not change the histological pattern with respect to endothelial cell injury (19, 27), emphasizing the complexity of antibody-mediated rejection. Our results suggest that concurrent therapies may be required to inhibit the multiple mechanisms of HLA antibody-mediated graft injury. We did not observe graft failure in this model, consistent with several studies (16, 61, 62), nor did we observe chronic vascular lesions in allografts from animals transferred with MHC I antibody, in disagreement with previous reports (61, 62). This

inconsistency may be due to the restricted localization of transplant arteriosclerosis in this model, which is reported to occur only in the proximal coronary arteries.

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 AMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMR	antibody mediated rejection
CAV	chronic allograft vasculopathy
CFSE	carboxyfluorescein diacetate succinimidyl
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
Fc	crystallizable fragment of immunoglobulin
FITC	fluorescein isothiocyanate
HAEC	human aortic endothelial cell
ICAM	intercellular adhesion molecule
PKC	protein kinase C
PMA	phorbol myristate acetate
vWF	von Willebrand Factor

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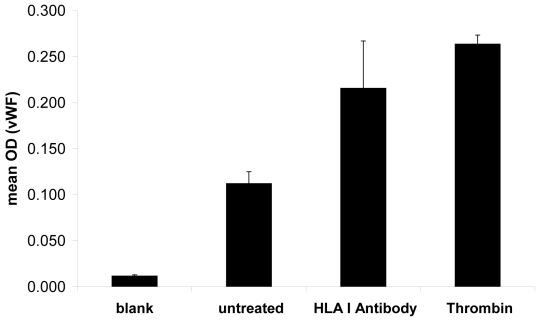
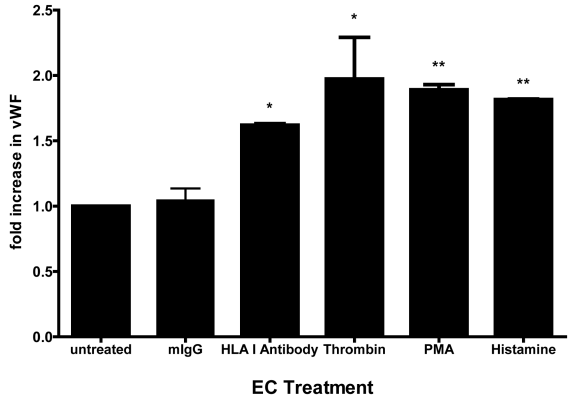
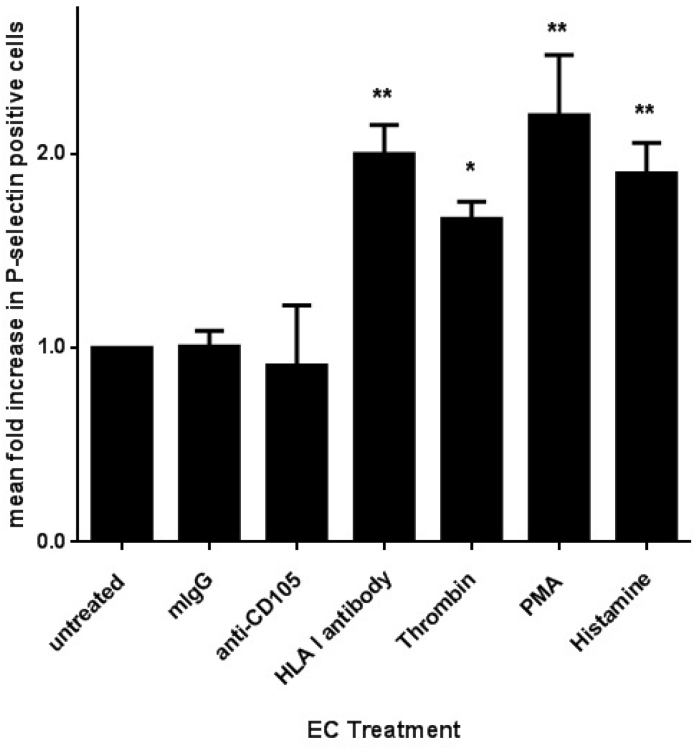






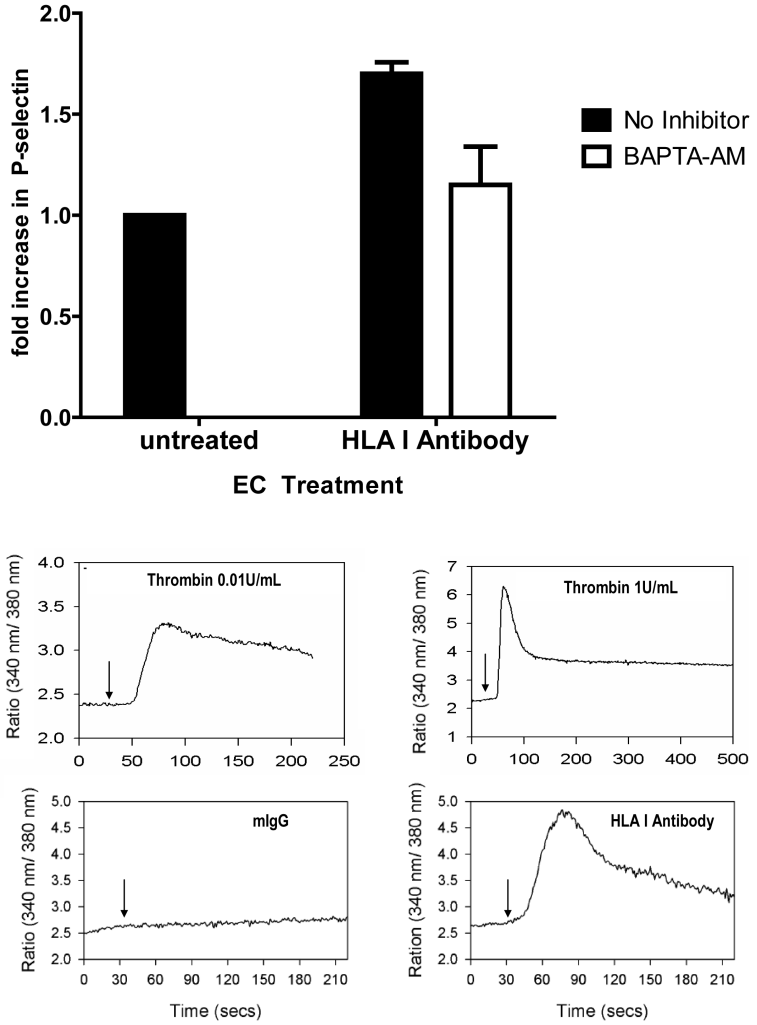


Figure 1.

HLA I antibodies trigger Weibel-Palade body exocytosis via intracellular calcium.

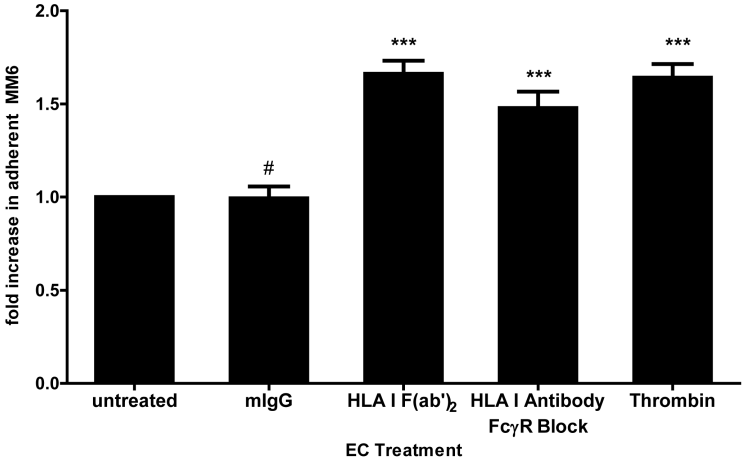
(a) Human aortic endothelial cells (HAEC) were treated with isotype control mIgG at 5μg/mL, HLA I antibody (clone W6/32, murine IgG2a) at 5μg/mL, thrombin at 10U/mL, PMA at 200nM, or histamine at 10mM for 1 hr. Supernatant was collected and vWF was measured by ELISA. An experiment showing the average optical density from duplicate measurements +/− SEM (upper panel). Bar graph in lower panel shows mean fold increase in optical density (OD) from 2 (PMA and histamine) or 3 (mIgG, HLA I antibody and thrombin) independent experiments for each condition. * $p<0.05$, ** $p<0.01$ versus mIgG.

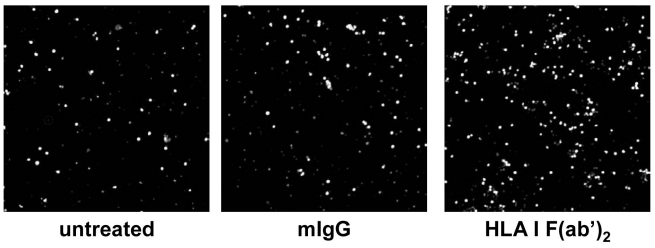
(b) HAEC were treated with mIgG, anti-CD105 antibody, or HLA I antibody at 5μg/mL for 30min, thrombin at 1U/mL for 15min, PMA at 200nM for 20min, or histamine at 10mM for 10min and detached using Accutase. Cell surface P-selectin was stained with conjugated anti-P-selectin-PE and analyzed by flow cytometry. Bar graph shows mean fold increase in P-selectin positive cells from multiple independent measurements for each condition: mIgG (n=5), anti-CD105 antibody (n=2), HLA I antibody (n=10), thrombin (n=3), PMA (n=6), histamine (n=23). * $p<0.05$, ** $p<0.001$ versus untreated.

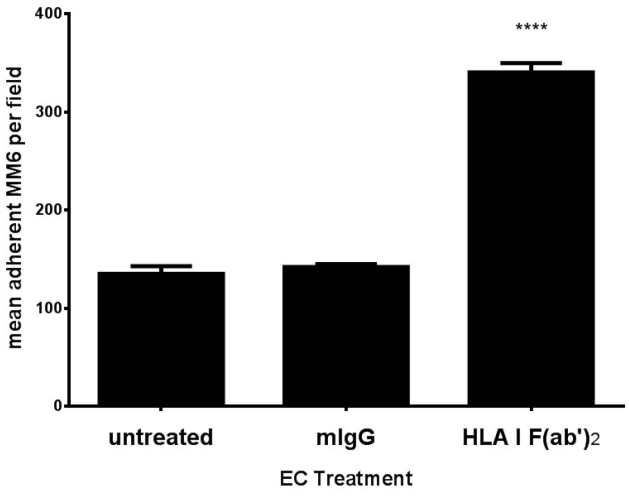
(c) HAEC were pretreated with BAPTA-AM at 20μM for 30min, then stimulated with HLA I antibody at 5ug/mL for 1 hour (h) and P-selectin was measured as in (b). Bar graph shows summary of data from 6 independent experiments. Black bars show the fold increase in P-

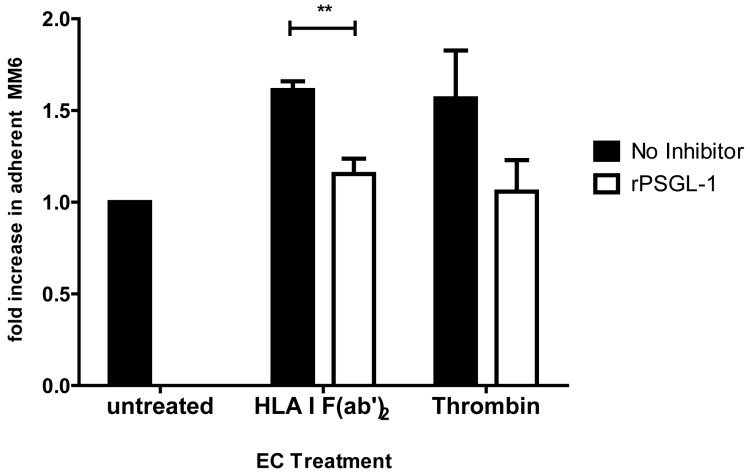
selectin expression without inhibitor, and white bars show P-selectin induction with inhibitor.

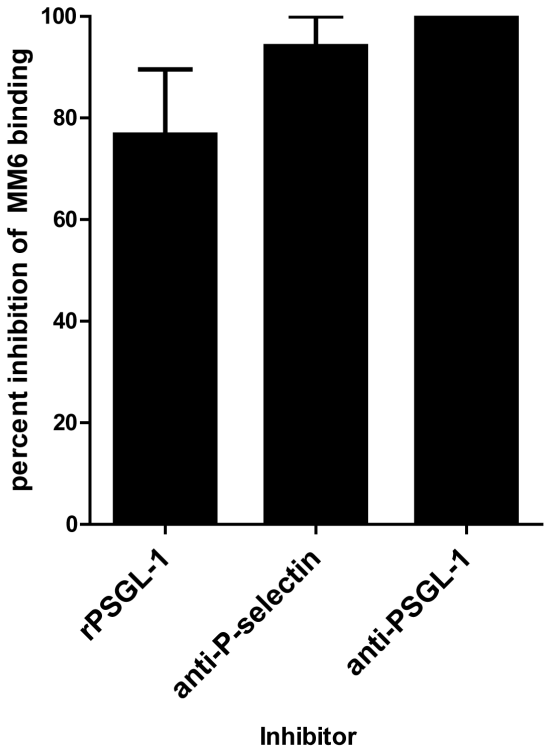
(d) In order to monitor intracellular Ca^{2+} concentration, HAEC were loaded with Fura 2-AM and then treated with HLA I antibody at 1μg/mL, thrombin at 0.01U/mL and 1U/mL, or isotype control antibody (mIgG) at 1μg/mL. Intracellular calcium was monitored in real-time using a fluorimeter. Data are presented as the ratio of emission at 340nm/380nm, and are representative of 2 (mIgG and thrombin 0.01U/mL) or 3 (HLA I antibody and thrombin 1U/mL) independent measurements. The time of introduction of reagents is marked with an arrow.











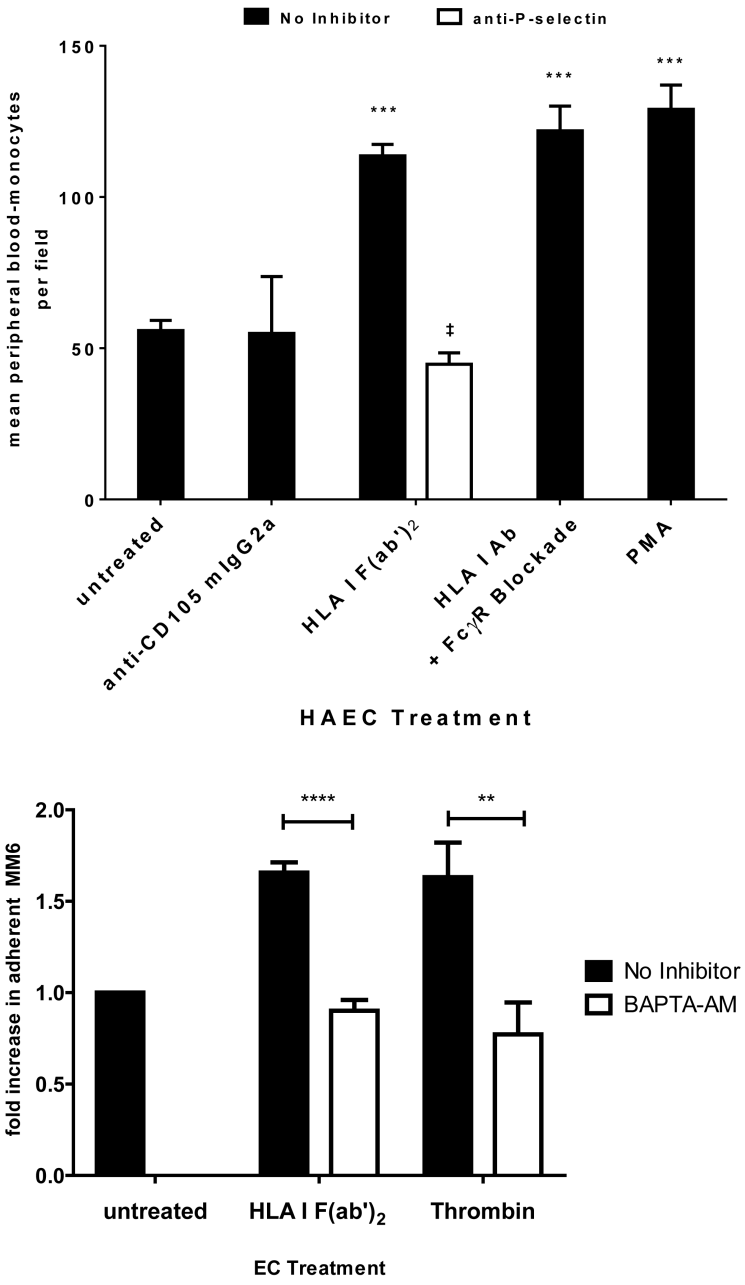


Figure 2.

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.

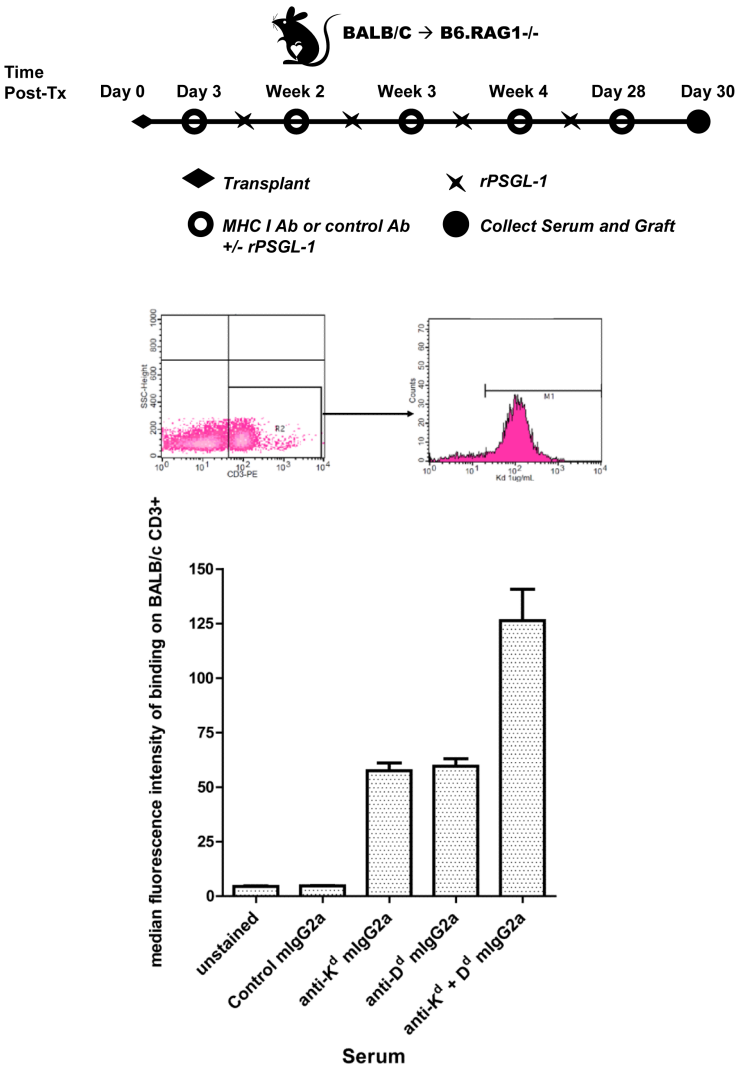
(b) Representative experiment performed as in (a), with data expressed as average number of adherent cells per field in 10 fields +/− SEM. Representative micrographs illustrate fluorescently labeled monocytic cells adherent to the endothelial monolayer. **** $p<0.0001$ versus untreated.

(c) 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 +/−SEM over 5 independent experiments. Black bars show Mono Mac 6 binding without inhibitor, white bars with inhibitor. ** $p<0.01$ comparing no inhibitor to inhibitor.

(d) 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.

(e) Experiments were performed as in (a) using human monocytes enriched from peripheral blood and rPSGL-1 was added to block endothelial P-selectin. Data is expressed as average number of adherent monocytes per field in 10 fields +/− SEM, and is representative of three independent experiments using monocytes isolated from three different donors. *** $p<0.001$ versus untreated. ‡ $p<0.0001$ versus no inhibitor.

(f) 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.



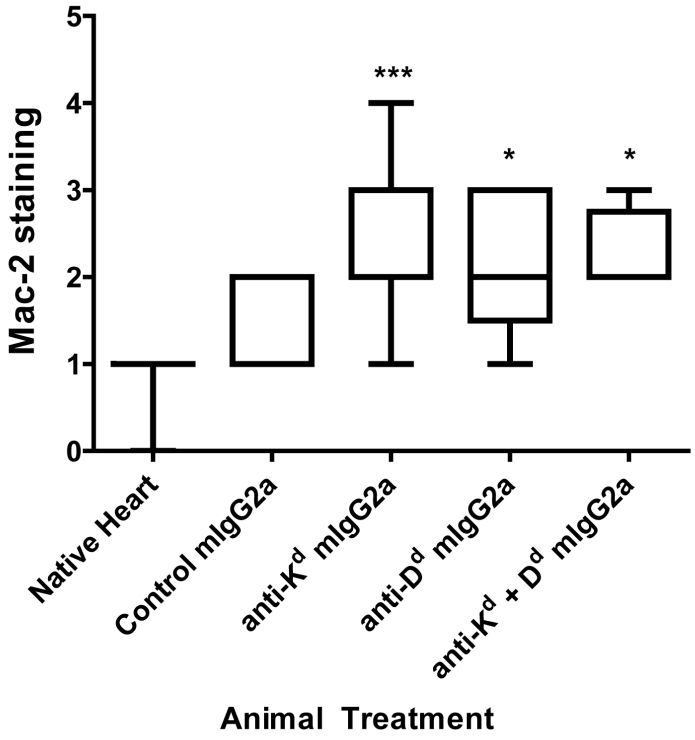


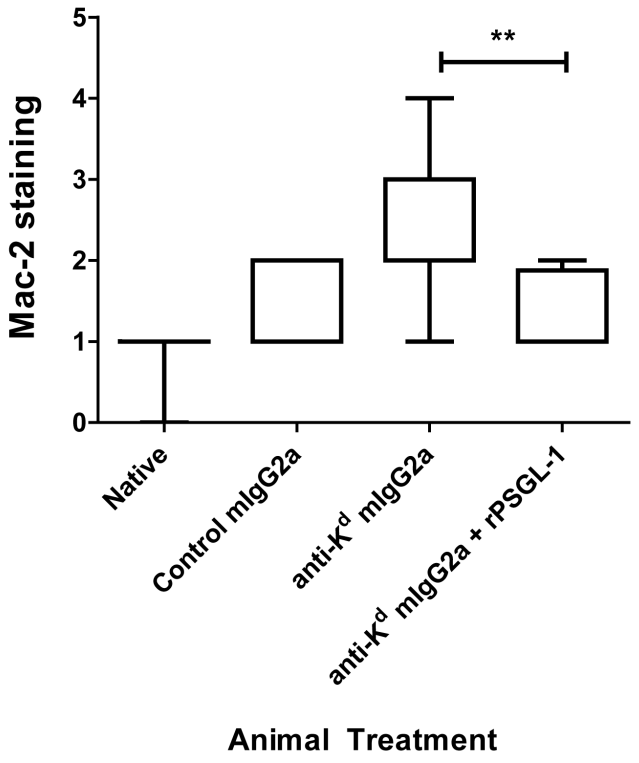
Figure 3.

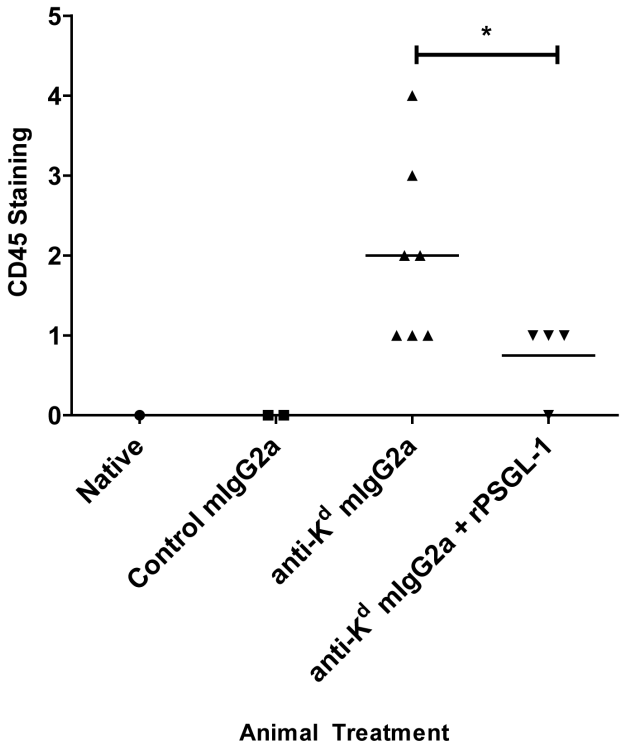
Donor specific MHC I antibodies increase monocyte recruitment into a murine cardiac allograft.

(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 pathologist blinded to the experimental groups. 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 pathologist blinded to the experimental groups. The box and whiskers graph shows the distribution of scores for each group. Infiltration near the suture site or in the endocardium was disregarded. Global ANOVA, then individual groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control mIgG2a group.





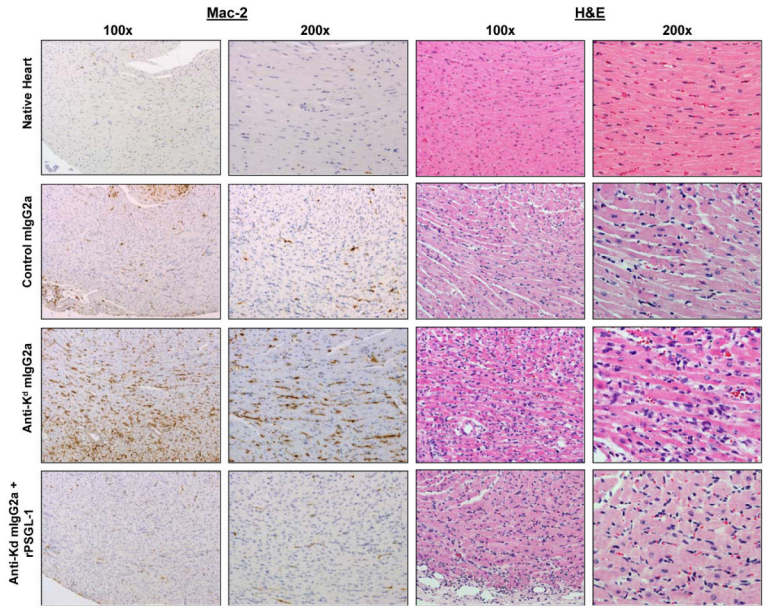


Figure 4.

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 pathologist blinded to the experimental groups. Global ANOVA and individual comparisons * $p<0.05$. *** $p<0.001$. Graphs show the distribution of scores for each group. Representative micrographs at 100x and 200x magnification of Mac-2 staining and H&E are shown in (c)

Table 1

MHC I antibody-induced macrophage accumulation in the allograft.

Group	Donor	Treatment	Hearts with Mac-2 ≥2	% positive	Hearts with Mac-2 ≥3	% 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*

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Table 2

rPSGL-1 therapy reduces MHC I antibody-induced macrophage infiltration into the allograft.

Group	Treatment	Hearts with Mac-2 score ≥2	% positive	Hearts with Mac-2 score ≥3	% positive	p value
1	Control mIgG2a	6/14	***42.8%***	0/14	***0%***	
2	Control mIgG2a + rPSGL-1	2/5	***40.0***	2/5	***40.0***	
3	anti-K^d mIgG2a	21/25	***84.6***	10/25	***40.0***	
4	anti-K^d mIgG2a + rPSGL-1	2/8	***25.0***	0/8	***0***	*0.004 versus group 3*

Table 3

rPSGL-1 therapy reduces CD45+ cell infiltration in donor specific MHC I antibody-treated allografts.

Group	Treatment	Hearts with CD45 score ≥2	% positive	Hearts with CD45 score ≥ 3	% positive	p value
1	Control mIgG2a	0/2	***0%***	0/2	***0%***	
2	anti-K^d mIgG2a	4/7	***57***	2/7	***29***	*0.048 versus group 1*
3	anti-K^d mIgG2a + rPSGL-1	0/4	***0***	0/4	***0***	*0.037 versus group 2*