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## Monocyte recruitment by HLA IgG-activated endothelium: The relationship between IgG subclass and Fc $\gamma$ RIIa polymorphisms

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### Abstract

It is currently unclear which donor specific HLA antibodies confer the highest risk of antibody-mediated rejection (AMR) and allograft loss. In this study, we hypothesized that two distinct features (HLA IgG subclass and Fc $\gamma$  receptor (Fc $\gamma$ R) polymorphisms), which vary from patient to patient, influence the process of monocyte trafficking to and macrophage accumulation in the allograft during AMR in an interrelated fashion. Here, we investigated the contribution of human IgG subclass and Fc $\gamma$ R polymorphisms in monocyte recruitment *in vitro* by primary human aortic endothelium activated with chimeric anti-HLA I human IgG1 and IgG2. Both subclasses triggered monocyte adhesion to endothelial cells, via a two-step process. First, HLA I crosslinking by antibodies stimulated upregulation of P-selectin on endothelium irrespective of IgG subclass. P-selectin-induced monocyte adhesion was enhanced by secondary interactions of IgG with Fc $\gamma$ Rs, which was highly dependent upon subclass. IgG1 was more potent than IgG2 through differential engagement of Fc $\gamma$ Rs. Monocytes homozygous for Fc $\gamma$ RIIa-H131 adhered more readily to HLA antibody-activated endothelium compared with Fc $\gamma$ RIIa-R131 homozygous. Finally, direct modification of HLA I antibodies with immunomodulatory enzymes EndoS and IdeS dampened recruitment by eliminating antibody-Fc $\gamma$ R binding, an approach that may have clinical utility in reducing AMR and other forms of antibody-induced inflammation.

### Introduction

Twenty to 30% of transplant candidates are HLA sensitized prior to transplantation (1, 2), and 8–25% of recipients will develop donor specific HLA antibodies (DSA) in the post-transplant period (3–5). Up to 50% of these recipients will experience antibody-mediated rejection (AMR) within the first year post-transplant (3, 5–8). Despite substantial evidence

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#### Supporting Information

Additional Supporting Information may be found in the online version of this article.

that DSA and AMR significantly reduce graft outcome (5) and increase risk of chronic rejection (reviewed in (9)), it is yet unclear what antibody characteristics (titer, subclass, clonality and/or specificity) determine the highest risk for rejection and allograft loss. Further, there is no mechanistic evidence that demonstrates how the various IgG subclasses of HLA antibodies may differentially injure allografts. Finally, it has not been definitively determined whether other immunologic patient determinants, including polymorphisms in immunologic receptors, influence a sensitized transplant recipient's risk of AMR.

AMR is a complex process with multiple concurrent mechanisms of injury. HLA antibodies binding to endothelial cells of the allograft vasculature cause endothelial activation, trigger the classical complement cascade, and/or recruit effector cells (10–16), mechanisms which are paralleled by the diagnostic criteria for AMR (17–20). We have reported that intracellular signaling when antibodies agonistically crosslink targets on the endothelial cell surface does not require the Fc portion (10–13, 15, 21, 22); signaling may synergize with Fc-dependent effector functions, including complement activation and binding to Fc $\gamma$  receptors (Fc $\gamma$ Rs), to promote an enhanced state of inflammation in the allograft (16).

Human IgG3 and IgG1 are strong complement activators with high affinity for Fc $\gamma$ Rs, while IgG2 weakly activates the classical cascade and generally has the lowest affinity for all Fc $\gamma$ Rs. IgG4 has little to no recognized complement activity but binds Fc $\gamma$ RI with moderate affinity (reviewed in (23)). In transplant waitlist candidates and recipients, all subclasses of HLA antibodies have been reported, with IgG1 most frequently found (24–31). However, initial studies have not yielded a consensus regarding which are most detrimental to graft outcome (3, 24–27, 29, 32, 33). Further, complement is involved in but not absolutely required for clinical and experimental AMR (27, 32, 34–39), and it is likely that other Fc-dependent and Fc-independent mechanisms are at play.

Allografts undergoing AMR almost universally present with perivascular and microvascular macrophages (12, 15, 18, 40) and/or polymorphonuclear leukocytes (41), and intragraft macrophage burden correlates with worse outcome (42–44). The predominant subset of monocytes in circulation coexpresses the high affinity Fc $\gamma$ RI and intermediate affinity Fc $\gamma$ RIIa. While Fc $\gamma$ RI has no known functional polymorphisms, Fc $\gamma$ RIIa is dimorphic (45), with Fc $\gamma$ RIIa-H131 having a higher affinity for all subclasses of IgG compared with Fc $\gamma$ RIIa-R131 (46, 47). Consequently, Fc $\gamma$ RIIa-R131 is associated with decreased responsiveness to antibody-based anti-tumor regimens (45, 48, 49), and susceptibility to autoimmune diseases (50, 51) and infection (52, 53). It has not yet been determined whether Fc $\gamma$ R polymorphisms constitute a risk factor for AMR.

Although the affinity of Fc $\gamma$ R classes and alleles for different IgG subclasses has been characterized in detail (46, 47, 54), previous studies of immune cell trafficking to sites of injury triggered by autoantibodies or immune complexes have not compared the effect of human IgG subclasses nor accounted for polymorphisms in human Fc $\gamma$ Rs (47, 55–57). To our knowledge this is the first report evaluating Fc $\gamma$ R-dependent leukocyte trafficking in the context of different human IgG subclasses and human Fc $\gamma$ R alleles.

Immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* (IdeS) is a secreted cysteine proteinase that cleaves IgG into an intact Fc region and an F(ab')<sub>2</sub> fragment (58–60). An endoglycosidase (EndoS) expressed by *S. pyogenes* hydrolyzes the N-linked carbohydrate from the Fc region of IgG (61–65). Both enzymes have high specificity for IgG, abolish FcγR-dependent functions, and reduce autoimmune inflammation in animal models (66–71). Using an *in vitro* system with chimeric pan HLA I antibodies, primary human endothelial cells, and human monocytes, we compared the effect of the two most abundant subclasses in circulation, human IgG1 (hIgG1) and hIgG2. We assessed the influence of FcγRIIIa polymorphisms on monocyte recruitment and the therapeutic potential of IdeS and/or EndoS to reduce FcγR-dependent immune cell trafficking. Our findings provide insight into the subclass-dependent pathogenicity of antibodies in transplantation, and further elucidate the mechanism by which macrophages infiltrate allografts during AMR.

## Materials and Methods

### Ethics Statement

Use of all human samples and cells was approved by the UCLA Institutional Review Board (IRB#10-001689-CR-00003 and IRB#00-01-023) and all donors gave written informed consent.

### HLA Antibodies

To measure the effects of IgG subclasses using antibodies with the same variable region, we made chimeric mouse/human pan HLA I antibodies, termed HLA I hIgG1 and hIgG2, using the variable regions of the heavy and light chain of the murine monoclonal antibody W6/32, which binds all alleles and loci of HLA I (72). Variable regions were sequenced (Lake Pharma) (73), and assembled into expression vectors carrying human κ light chain, human γ1 or γ2 constant regions (Supplemental Figure 1a) as described (74, 75). Antibodies were expressed in Chinese hamster ovary cells (CHO-K1) cells and purified from supernatant using protein G (GE Healthcare), dialyzed to PBS, and sterile filtered (0.2μm). The subclasses of chimeric HLA I hIgG1 and hIgG2 were verified using an in-house subclass-specific IgG enzyme-linked immunosorbent assay (ELISA) (Supplemental Figure 1b). In preliminary experiments, glycan content of chimeric antibodies was evaluated using lectin LCA blotting (64, 65) and mass spectrometry. Both subclasses had glycosylated heavy chains with glycans similar in structure to those present on naturally occurring human serum IgG (data not shown), as has been reported (76).

Human monoclonal allele specific antibodies against HLA-A2 (clone SN230G6) or HLA-A3 (clone MUL2C6), were produced by human hybridomas generated as previously described (77–79), and were purified by protein A affinity chromatography. The UCLA Immunogenetics Center maintains a repository of polyclonal reference sera from multiparous women consented for research purposes (80) that are well defined for HLA antibodies. Sera were selected based on the presence of relevant antibodies against HLA I alleles expressed by endothelial cells, and heat-inactivated at 56°C for 30min.

## Cells and Cell Culture

Primary human aortic endothelial cells (HAEC) were isolated from the aortic rings of explanted donor hearts consented for research purposes as described previously (10) or were obtained from ATCC (donor 3F1153). HAEC were cultured as described (10, 16) and HLA typed at the UCLA Immunogenetics Center.

Human monocytic cell lines U937 (81) and THP-1 were from ATCC; Mono Mac 6 (MM6) were provided by Dr. Loems Ziegler-Heitbrock, Gauting, Germany. All monocytic cell lines were cultured as previously described (16, 82). Human monocytes were isolated from the peripheral blood of healthy volunteers, using MACS pan-monocyte negative selection kit (Miltenyi).

## Enzymatic Modification of Immunoglobulin

N-linked glycans were removed from IgG by incubating with EndoS (65) (New England Biolabs, Ipswich, MA). IgG was digested to F(ab')<sub>2</sub> fragments using IdeS/FABricator (Genovis/Bulldog Bio, Portsmouth, NH) according to vendor protocol. Cleaved Fc fragments were removed by protein G column. Western and LCA lectin blotting (65) were used to confirm IgG digestion and deglycosylation.

## Cell-Based ELISA

Because cell surface P-selectin is difficult to measure using flow cytometry (83), it was detected on adherent unpermeabilized endothelium using a colorimetric method (16, 84) that permits robust detection of P-selectin but not cell-by-cell analysis of P-selectin upregulation.

Binding of HLA antibodies to adherent endothelial cells was also determined by cell-based ELISA. Chimeric HLA I antibodies or 25µL serum were incubated with viable endothelial cells for 30min. Total IgG bound to the cell surface was probed with anti-human Fcγ-horseradish peroxidase (HRP) (Jackson Immuno, West Grove, PA), and detected with TMB substrate.

## Flow Cytometric Determination of FcγR Expression and Allotype

Cell surface expression of FcγRs on monocytes was determined by flow cytometry on an LSRII Fortessa flow cytometer (BD). FcγRIIa allotype was determined using a flow cytometric method as described (16, 85).

## Determination of Monocyte Binding to Human IgG

Monocytic cell FcγR-dependent interaction with human IgG1 and IgG2 was measured by soluble binding assay with flow cytometry and by immobilized IgG adhesion assay as published (46, 47).

## Static Monocyte Adhesion Assay

Adhesion of monocytes to endothelium was measured as we described (15, 16). HAEC were cultured to confluence, and stimulated with control or chimeric HLA I hIgG1 or hIgG2, or HLA serum diluted 1:2 for 15min. Stimulation medium was removed and carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled monocytes were added at

ratio of 2–3 monocytes per endothelial cell. After 20min at 37°C, nonadherent monocytes were decanted and wells were washed three times. Adherent monocytes were counted by fluorescence microscopy (Nikon Eclipse Ti) and automated software (CellProfiler) in 5 (48 well plate) or 10 (24 well plate) fields per well.

P-selectin/PSGL-1 interactions were inhibited by sheep anti-P-selectin (10µg/mL) (Millipore), rPSGL-1-Ig antagonist (25µg/mL) (Y's Therapeutics, Tokyo, Japan), or anti-PSGL-1 (10µg/mL) (Biolegend). Individual FcγRs were blocked by preincubation of monocytes with negative control mIgG1 (MOPC-21), or neutralizing antibodies to FcγRI (clone 10.1) or FcγRII (clone AT10) at 10µg/mL for 15min.

### Shear-Based Monocyte Adhesion Assay

Monocyte adhesion to endothelial monolayers was assessed under laminar flow conditions using a closed perfusion system (ibidi, Munich, Germany) (86). Endothelial cells ( $10^5$  cells/cm<sup>2</sup>) were seeded in gelatin-coated flow chamber slides (µ-slide I<sup>0.4</sup> Luer) and cultured for 2–24hr. EC were prestimulated with HLA-specific antibodies for 15min at 37°C. CFSE-labeled monocytic cells were resuspended in RPMI + 5% fetal bovine serum (FBS) at  $6.25 \times 10^5$ /mL, and added to the syringe of a white perfusion set. Monocytes were perfused under continuous unidirectional flow over the endothelial cell monolayer at 0.8dynes/cm<sup>2</sup> (comparable to small arterioles and capillaries (87, 88)) and recorded in real time on a live cell fluorescence microscope (Nikon Eclipse Ti). Supplemental Video 1 illustrates adhesion, slow rolling, and fast rolling behavior on endothelial monolayers. Data were analyzed using ImarisTrack software (BitPlane). Supplemental Video 2 shows a representative processed video.

### Statistical Analyses

Statistical differences between groups were determined using ANOVA followed by individual t tests with Bonferroni or Tukey's multiple comparisons test, or Fisher's LSD test, using GraphPad Prism software.

## Results

### Chimeric HLA I Antibodies Promote Monocyte Adhesion via Endothelial P-selectin Induction

Both HLA I hIgG1 and hIgG2 recognized all alleles and loci of HLA class I on Luminex Single Antigen Beads (One Lambda, data not shown), and bound to native antigen expressed on the surface of primary human aortic endothelial cells (HAEC) comparably to parental murine W6/32 (Figure 1a, Supplemental Figure 1c).

We assessed recruitment of human monocytic cell lines, U937 and MM6, to HAEC treated with chimeric HLA I hIgG1 and hIgG2 under static and shear conditions. Activation of endothelium with HLA I hIgG1 significantly increased the adherence of U937 ( $3.12 \pm 0.32$ -fold at 100ng/mL, n=6) and MM6 ( $5.33 \pm 1.32$ -fold at 100ng/mL, n=9) in a dose dependent manner (Figure 1b). HLA I hIgG2 also triggered significant adhesion of U937 ( $1.48 \pm 0.25$ -fold at 100ng/mL, n=6) and MM6 ( $2.08 \pm 0.25$ -fold at 100ng/mL, n=9) above background

(Figure 1b), although the magnitude was lower compared with hIgG1. Representative fields of fluorescently labeled monocytes bound to endothelial cells can be found in Figure 1b, bottom panel. HLA-specific antibodies also promoted adhesion of monocytes under shear conditions. When MM6 were perfused over endothelial monolayers, MM6 rolled more slowly on average in response to HLA I hIgG1 compared with HLA I hIgG2, with approximately 50% of monocytes travelling less than 50 $\mu$ m/sec on HLA I hIgG1-activated endothelium, compared with 25% on HLA I hIgG2 (Figure 1c and Supplemental Videos 3–5). Moreover, HLA I hIgG1 stimulated significantly more firmly adherent monocytes, defined by traveling <2 $\mu$ m/sec for >4sec (88), compared with HLA I hIgG2 (Figure 1c).

Endothelial exocytosis resulting in expression of P-selectin on HAEC was significantly and rapidly increased in response to both HLA I hIgG1 and hIgG2 (Figure 1d), consistent with previous reports using murine monoclonals (15, 22). U937, THP-1 and MM6 monocyte adhesion to endothelium activated with either HLA antibody subclass could be significantly reduced by more than 90% by inhibition of PSGL-1/P-selectin (Figure 1e; Supplemental Figures 2a–d), demonstrating that P-selectin is universally required for capture of monocytes by HLA I antibody-activated endothelium.

### **Involvement of Monocyte Fc $\gamma$ Rs in Adhesion is Dependent upon Antibody Subclass and Fc $\gamma$ RIIa Alleles**

We tested whether Fc $\gamma$ R interactions accounted for greater recruitment of monocytes by HLA I hIgG1. MM6, THP-1 and U937 all expressed Fc $\gamma$ RI (CD64) and Fc $\gamma$ RIIa (CD32a) (16, 89, 90), similar to the phenotype of the “classical” CD14<sup>hi</sup>Fc $\gamma$ RI<sup>+</sup>Fc $\gamma$ RIII<sup>neg</sup> monocyte in circulation (Supplemental Figure 3a). We previously described our panel of monocytic cell lines representing three genotypes of Fc $\gamma$ RIIa (16). U937 monocytic cells were homozygous for the low affinity allele Fc $\gamma$ RIIa-R131 (Figure 2a) and bound only human IgG1 (Supplemental Figures 3b, 3c). MM6 were homozygous and THP-1 were heterozygous for Fc $\gamma$ RIIa-H131 (Figure 2a), and both cell lines bound to human IgG1 and as well as IgG2 (Supplemental Figures 3b, 3c).

To define the respective roles of Fc $\gamma$ RI and Fc $\gamma$ RIIa, we inhibited Fc $\gamma$ Rs using neutralizing antibodies. U937 (Fc $\gamma$ RIIa-R131 homozygous) binding to HAEC stimulated with HLA I hIgG1 was significantly reduced by neutralizing antibody to Fc $\gamma$ RI (77.5 $\pm$  14.4% inhibition, averaged over 2 or more independent experiments, Figure 2b), but only weakly by blocking Fc $\gamma$ RIIa (19.4 $\pm$ 11.1% inhibition). Neither Fc $\gamma$ RI nor Fc $\gamma$ RIIa blocking antibodies could significantly decrease adhesion of U937 to HLA I hIgG2-stimulated endothelium (17.1 $\pm$ 11.5% and 0.7 $\pm$ 0.6% inhibition, respectively, Figure 2b). Binding of heterozygous THP-1 monocytes to endothelial cells activated by HLA I hIgG1 could be significantly reduced by antibody to Fc $\gamma$ RI or Fc $\gamma$ RIIa, but THP-1 adhesion to HLA I hIgG2-stimulated endothelium was not reduced by antagonism of any Fc $\gamma$ R (Figure 2c). Binding of Fc $\gamma$ RIIa-H131 homozygous MM6 to endothelium activated with HLA I hIgG1 was significantly inhibited by neutralizing Fc $\gamma$ RI and Fc $\gamma$ RIIa (47.4 $\pm$ 5.4% and 34.7 $\pm$ 4.8% inhibition, respectively). Notably, MM6 binding to endothelium in response to HLA I hIgG2 was dependent upon Fc $\gamma$ RIIa (76.0 $\pm$ 0.0% inhibition) but not Fc $\gamma$ RI (15.7 $\pm$ 2.5% inhibition) (Figure 2d), the only condition in which hIgG2 utilized Fc $\gamma$ Rs.

## Enzymatic Modulation of HLA Antibodies with IdeS and EndoS Reduces Fc $\gamma$ R-dependent Monocyte Adherence

Given that antibody antagonism of Fc $\gamma$ Rs reduced monocyte recruitment in most settings, we hypothesized that modulation of HLA I antibody by therapeutic enzymes would reduce the pathogenicity of these antibodies, as has been reported in autoimmunity (62, 65, 68, 71). Treatment of chimeric HLA I hIgG1 and hIgG2 with IdeS generated F(ab')<sub>2</sub> and Fc fragments of HLA I IgG (61). Neither IdeS treatment nor glycan truncation with EndoS altered antigen recognition (Supplemental Figure 4a) or capacity to trigger endothelial cell P-selectin (Supplemental Figure 4b) by HLA antibodies.

IdeS-digested HLA I hIgG1 stimulated a significant increase in U937 and MM6 adhesion to endothelium (1.74 $\pm$ 0.4-fold and 1.33 $\pm$ 0.0-fold, respectively) above background; however, this adhesion was lower compared with intact chimeric HLA I hIgG1 (3.49 $\pm$ 1.0-fold, 88.4 $\pm$ 3.6% reduction for U937; and 5.24 $\pm$ 1.4-fold, 71.9 $\pm$ 4.2% reduction for MM6; Figure 3a, 3b). Binding was comparable to when Fc $\gamma$ Rs were blocked on monocytes (Figure 3b). Similarly, HLA I hIgG1 deglycosylated by EndoS treatment stimulated lower adhesion of both monocytic cell lines compared with intact antibody (Figure 3c, 3d), although binding was still significantly greater than to untreated cells (2.16 $\pm$ 0.4-fold, 84.4 $\pm$ 5.4% reduction for U937; and 1.86 $\pm$ 0.5-fold, 71.9 $\pm$ 4.2% reduction for MM6). These enzymes were also effective against fully human monoclonal HLA allele specific antibodies (78). Endothelial cells expressing HLA-A3 were stimulated with intact or enzymatically modified anti-HLA-A3 hIgG1. Adhesion of U937 monocytic cells and of primary monocytes isolated from peripheral blood was significantly decreased by EndoS and IdeS treatment of antibodies (Figure 3e). Similar results were obtained using HLA-A2 expressing endothelial cells and anti-HLA-A2 hIgG1 (Supplemental Figure 4c). Importantly, residual monocyte adhesion to IdeS-HLA I hIgG1 treated endothelial cells could be completely abolished by the presence of a P-selectin inhibitor (Figure 3b), supporting the idea that adhesion molecules and Fc $\gamma$ Rs synergize to promote maximal adhesion of leukocytes, as we and others have previously proposed (16, 55).

We next assessed the effect of IdeS and EndoS treatments on HLA antibodies of the IgG2 subclass. Neither IdeS digestion nor EndoS deglycosylation of HLA I hIgG2 reduced recruitment of U937 compared with intact IgG (1.81 $\pm$ 0.6-fold for intact; 1.73 $\pm$ 0.4-fold, 9.5 $\pm$ 9.6% inhibition for IdeS; and 1.70 $\pm$ 0.3-fold, 15.5 $\pm$ 15.5% inhibition for EndoS; Figure 4a and 4b), consistent with our observations that recruitment of Fc $\gamma$ RIIa-R131 homozygous monocytes by hIgG2 is Fc $\gamma$ R-independent (Figure 2b). When P-selectin was blocked, U937 recruitment by IdeS-treated HLA I hIgG2 was completely abrogated (Figure 4b), suggesting that capture of Fc $\gamma$ RIIa-R131 monocytes by hIgG2 is due entirely to P-selectin. However, both IdeS and EndoS treatment of HLA I hIgG2 significantly reduced adherence of MM6 compared with intact IgG2 (2.22 $\pm$ 0.1-fold for intact; 1.61 $\pm$ 0.1-fold, 50.8 $\pm$ 2.6% inhibition for IdeS; 1.79 $\pm$ 0.1-fold, 27.0 $\pm$ 8.0% inhibition for EndoS; Figure 4c), pointing to a role for the Fc portion of IgG2, and in agreement with our results showing that Fc $\gamma$ RIIa is required for maximal binding of Fc $\gamma$ RIIa-H131 homozygous cells to HLA I hIgG2-stimulated endothelium.



As with monocytic cell lines, recruitment of peripheral blood monocytes was increased in response to HLA I chimeric antibodies. Adhesion of Fc $\gamma$ RIIa-R131 homozygous monocytes to endothelium treated with HLA I hIgG1 was significantly greater compared with hIgG2; however, Fc $\gamma$ RIIa-H131 homozygous monocytes bound hIgG1 and hIgG2 to a comparable extent (Figure 5b–f).

We confirmed our findings using sera from HLA sensitized individuals paired to HLA-typed endothelial cells expressing relevant HLA I alleles (Table 1). All sera contained IgG that bound to surrogate endothelial cells (Figure 5a, 5d). When HAEC H126 (A3, A29) were stimulated with serum 152-SF containing antibodies against HLA-A3 and HLA-A29, primary monocytes from donors homozygous for Fc $\gamma$ RIIa-R131 (Figure 5b) and for Fc $\gamma$ RIIa-H131 (Figure 5c) adhered. Similar results were observed using HAEC 3F1153 (A2, A11, B44) stimulated with sera M4277 and Q2045 which contained relevant HLA antibodies (Figure 5e, 5f). Interestingly, we observed varying levels of adhesion triggered among sera, despite similar antibody strength and amount bound to endothelium. For example, monocytes from an Fc $\gamma$ RIIa-H131 homozygous individual (Figure 5e) bound to endothelium activated with serum Q2045 at a higher magnitude than Fc $\gamma$ RIIa-R131 homozygous monocytes (Figure 5f). Finally, sera digested with IdeS and EndoS triggered significantly lower recruitment of peripheral blood monocytes from both Fc $\gamma$ RIIa-H131 homozygous and Fc $\gamma$ RIIa-R131 homozygous individuals to endothelial cells compared with untreated HLA serum (Figure 6a–e), demonstrating that polyclonal HLA I IgG can be modulated in serum to reduce Fc $\gamma$ R-dependent monocyte recruitment.

## Discussion

We investigated the mechanisms of monocyte recruitment by HLA antibody-activated endothelial cells, using chimeric antibodies with broadly specific murine HLA I variable regions and human IgG1 or IgG2 constant regions. This approach enabled us to study the effect of IgG subclass of HLA antibodies recognizing the same antigen epitope. Our data indicate that monocyte recruitment by HLA antibodies is a multistep process. HLA I antibodies universally stimulate the initiating event of endothelial P-selectin presentation (Figure 7a), although this appears to be a unique function of HLA antibodies, and has not been reported for autoimmune and endothelial binding antibodies (55, 56). The second step is strongly influenced by several independent variables that differ among individuals, including antibody subclass and Fc $\gamma$ R polymorphisms (Figure 7b–d).

With the increasingly recognized importance of AMR in solid organ transplant outcome, it has become essential to define antibody and recipient determinants of risk for AMR and understand the mechanisms of HLA antibody-mediated vascular injury. Features that differ among transplant recipients include antibody titer, subclass and glycosylation (all of which affect complement fixation as well as Fc $\gamma$ R binding), and polymorphisms in innate immunity genes. It is not unequivocally settled whether certain subclasses are more pathogenic than others. There is experimental evidence to support both that subclass is important for injury in murine allograft models (91, 92), and conversely that all HLA antibodies have potentially detrimental effects (10, 13, 15, 93). Clinical studies focusing on

*in vitro* complement fixing capacity suggest but do not definitively demonstrate that complement activation is important for outcome (24, 27, 29, 32, 36, 94).

Our results highlight the importance of antibody subclass in monocyte trafficking, and indicate distinct roles for Fc $\gamma$ R classes and alleles in HLA antibody-induced monocyte recruitment. Auto-antibodies and anti-endothelial cell antibodies are often IgG2 and IgG4 (95, 96), while HLA-specific IgG in serum is polyclonal and may be a mix of all four subclasses (4, 25, 26, 33, 97, 98). The current study focused on the effect of IgG1 and IgG2, which are most prevalent in serum, but the functions of IgG3 and IgG4 in this system have yet to be determined. Despite lower frequency in circulation, IgG3 has a high affinity for Fc $\gamma$ Rs (54) and IgG3 autoantibodies are the most potent stimulators of neutrophil recruitment (99). Moreover, IgG4 binds to Fc $\gamma$ RI with moderate affinity (46) and can mediate monocyte phagocytosis (100). Therefore, we expect that IgG3 and IgG4 might also promote efficient Fc $\gamma$ R-dependent recruitment of leukocytes

Circulating monocytes are a heterogenous population with distinct functional responses after activation. The monocytic cell lines used here are phenotypically similar to the classical CD14<sup>+</sup> Fc $\gamma$ RI(CD64)<sup>+</sup> Fc $\gamma$ RIIa(CD32a)<sup>+</sup> Fc $\gamma$ RIIIb(CD16b)<sup>neg</sup> subset, which composes over 80% of monocytes in circulation. Intermediate (CD14<sup>+</sup>Fc $\gamma$ RIIIb<sup>+</sup>) and nonclassical (CD14<sup>dim</sup>Fc $\gamma$ RIIIb<sup>+</sup>) monocytes carry low levels of Fc $\gamma$ RI but express Fc $\gamma$ RIIIb (reviewed in (101)). In our experiments, the peripheral blood monocytes tested were a mixture of all subsets in circulation, and we did not examine each subset individually. IgG4 preferentially binds Fc $\gamma$ RI expressed on classical monocytes (46), and IgG3 has high affinity for the Fc $\gamma$ RIII family expressed by NK cells, neutrophils, and intermediate and nonclassical monocytes. Therefore, it is tempting to speculate that allo- or auto-antibody subclasses may differentially recruit adaptive and innate immune cell subsets (102), particularly since recent clinical and experimental evidence indicate a role for NK cells in AMR (37, 103, 104).

We report that monocytes from individuals with high affinity Fc $\gamma$ RIIa-H131 have greater adhesive behavior to HLA I antibody-activated endothelium. In two early clinical reports, there was no association between Fc $\gamma$ RIIa genotypes and kidney allograft rejection (105, 106). However, other investigators uncovered a higher rate of acute cellular rejection in transplant recipients homozygous for Fc $\gamma$ RIIa-R131 (48, 49), which may reflect the lower activity of Fc $\gamma$ RIIa-R131 in antibody-based lymphocyte depletion during induction. No studies have specifically evaluated the impact of Fc $\gamma$ R polymorphisms with AMR. However, the combination of IgG2 anti-cardiolipin antibodies and Fc $\gamma$ RIIa-H131 correlated with disease state in anti-phospholipid antibody syndrome (107). Similarly, we hypothesize that transplant recipients with Fc $\gamma$ RIIa-H131 might have an increased susceptibility to AMR with exacerbated cellular infiltration in the presence of IgG1 or IgG2 DSA. Unfortunately, antibody/Fc $\gamma$ R systems are sufficiently disparate between mouse and human that *in vivo* study of Fc $\gamma$ R-subclass interactions and of Fc $\gamma$ R polymorphisms was not feasible at this time (reviewed in (108)). Recently a novel transgenic mouse was described which expresses the full complement of human Fc $\gamma$ Rs (109). This constitutes an important new tool for research on humoral immune responses.

The emergence of AMR as a leading risk factor for graft loss has highlighted the need for better preventative approaches and treatments. The effects of IdeS and EndoS *in vivo* are rapid, within 1–4hr of injection into rabbits (65), but transient, declining as new IgG replaces modulated IgG (68). In animal models, IdeS was immunogenic (58), although immune responses against IdeS did not reduce its *in vivo* activity (65). The HLA chimeric antibodies used in this study were produced in CHO cells in order to generate Fc glycosylation that is similar to that seen on human serum IgGs (76, 110). Antibody glycosylation patterns impact autoimmune disease activity and severity (reviewed in (111)), and may impact transplant rejection as well. In our study, cleavage of this N-linked glycan by EndoS significantly impaired Fc $\gamma$ R-dependent monocyte recruitment by HLA antibodies. The fine structure of the heavy chain glycan is critical for Fc domain conformation and interaction with effector systems, including activating and inhibitory Fc $\gamma$ Rs and complement C1q (112–116). Preclinical models have indicated utility for EndoS and IdeS in the treatment of autoimmune disorders (60, 63, 66–68, 71, 117), and currently clinical trials for IdeS are underway in the United States (NCT01802697), and Sweden. We hypothesize that EndoS and/or IdeS treatment of patients undergoing AMR will reduce graft damage by eliminating both complement activation and Fc $\gamma$ R-dependent cellular recruitment by HLA antibodies.

In summary, we have shown that interaction of certain subclasses of human anti-HLA IgG with Fc $\gamma$ Rs on the monocyte significantly enhances their adhesion to endothelium. Expression of the high affinity Fc $\gamma$ RIIa-H131 by monocytes lead to greater adhesion to endothelial cells stimulated with HLA I antibodies, suggesting that transplant recipients carrying this allele may experience severe macrophage accumulation during AMR. Notably, HLA I hIgG2, a subclass not generally considered clinically relevant in transplantation due to its low complement fixing capacity (27, 33), potentially activated endothelial exocytosis and supported significant monocyte adherence, which was enhanced in combination with Fc $\gamma$ RIIa-H131. Finally, *in situ* modulation of antibody Fc effector functions with therapeutic enzymes may dampen graft damage or other antibody-mediated vascular injury through parallel inhibition of complement and Fc $\gamma$ R-mediated inflammation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

**AMR**                      antibody-mediated rejection

<b>CFSE</b>	carboxyfluorescein diacetate succinimidyl ester
<b>CHO-K1</b>	Chinese Hamster Ovary cells
<b>DSA</b>	donor specific HLA antibodies
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>EndoS</b>	endoglycosidase S
<b>FBS</b>	fetal bovine serum
<b>FcγR</b>	Fc gamma receptor
<b>FITC</b>	fluorescein
<b>HAEC</b>	human aortic endothelial cells
<b>HRP</b>	horseradish peroxidase
<b>IdeS</b>	Immunoglobulin G-degrading enzyme of <i>Streptococcus pyogenes</i>
<b>MFI</b>	mean fluorescence intensity
<b>MM6</b>	Mono Mac 6

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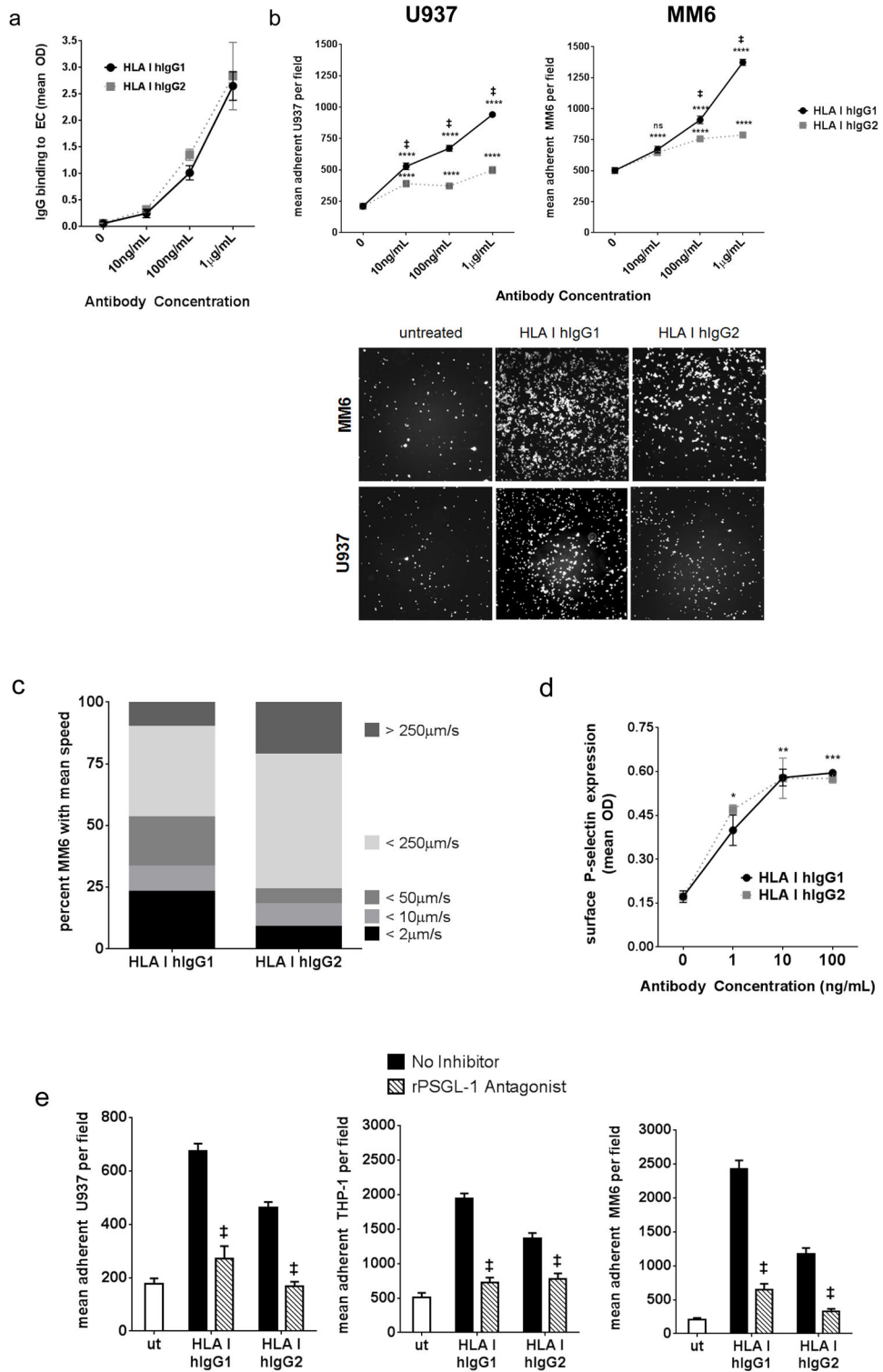
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**Figure 1. Comparison of the ability of different subclasses of anti-HLA I antibody to activate endothelial cells and promote adhesion of monocytes**  
**(a)** Human aortic endothelial cells (EC) were exposed to varying concentrations of chimeric human HLA I hlgG1 and IgG2 for 30min, and hlgG bound to the cell surface was detected

by cell-based ELISA probing with anti-hIgG-HRP. The average of two independent experiments using endothelial cells from different donors (3F1153 and H126) are shown as mean optical density at 650nm  $\pm$  SEM read on a microplate reader (Molecular Devices). No statistically significant difference was found between the two subclasses comparing each dose by Two way ANOVA.

**(b)** Confluent HAEC were stimulated for 15min with indicated concentrations of chimeric human HLA I IgG1 and IgG2. Fluorescent CFSE-labeled U937 (left panel) and MM6 (right panel) were added at a ratio of 2–3 monocytes to 1 endothelial cell and allowed to adhere for 20min. Nonadherent cells were removed by washing, and adherent cells were visualized by fluorescence microscopy and counted using automated software. Results in top panel are presented as mean number of adherent monocytes per 4x field averaged from 8–10 fields per condition,  $\pm$  SEM. Adherent monocytes were quantitated using CellProfiler software (MIT) with the following pipeline: LoadImages, ApplyThreshold with 0.5 and 1.0 lower and upper bounds on threshold, respectively, and IdentifyPrimaryObject (diameter 3–30 pixel units, 0.6 to 1.0 bounds) modules.

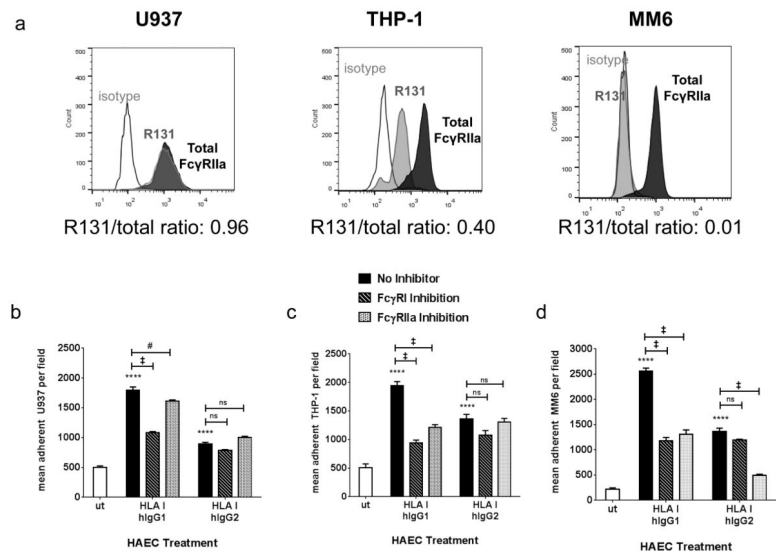
\*\*\*\*  $p < 0.0001$  versus untreated (0); ns  $p > 0.05$ , ‡  $p < 0.0001$  comparing hIgG1 to hIgG2 at the same concentration using One-way ANOVA followed by individual t tests. One representative experiment out of 6 (U937) and 9 (MM6) is given. Representative 4x fluorescence microscopy fields of adherent CFSE-labeled monocytes acquired with NIS Elements using the fluorescein (FITC) filter of a Nikon Eclipse Ti are shown in the bottom panel. Results show that stimulation of HAEC with IgG1 or IgG2 HLA I antibodies causes dose-dependent adhesion of monocytes. However, IgG1 triggers significantly more monocyte adhesion than IgG2.

**(c)** Monocytes were perfused at 0.8dynes/cm<sup>2</sup> over an endothelial monolayer stimulated with HLA I hIgG1 or hIgG2 at 500ng/mL. Three five second videos were collected in real-time for each condition. The mean velocity ( $\mu$ m/sec) of each monocyte over 5 seconds was determined using ImarisTrack software (Bitplane). Results are presented as the percent of total monocytes observed in the 5sec video with each indicated speed (i.e. 50–10 $\mu$ m/sec). Adherent monocytes were defined as those with a speed of  $\geq 2\mu$ m/sec over 5 seconds. Results from one representative experiment show the distribution of monocytes with each mean velocity. Slow rolling monocytes have a mean velocity of  $\approx 50\mu$ m/sec, while fast rolling monocytes moved at  $> 50\mu$ m/sec. Firmly adherent monocytes were identified as those which traveled  $< 2\mu$ m/sec for more than 4 seconds.

**(d)** HAEC were cultured to confluence in a 96-well tissue culture plate, then stimulated with HLA I antibody for 15min. Cells were fixed for 5min in freshly prepared 4% paraformaldehyde (without permeabilization), then stained with sheep-anti-human P-selectin (R&D), followed by anti-sheep-HRP (Millipore). TMB Substrate was added to each well and cell surface P-selectin was determined by measuring optical density at 650nm using a microplate reader. Results are presented as mean optical density (OD) at 650nm in duplicate wells  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  versus untreated (0) for both hIgG1 and hIgG2 by Two way ANOVA. There was no statistically significant difference between hIgG1 and hIgG2 at the same dose. Each condition was assayed in duplicate, and each experiment was independently repeated using endothelial cells from at least three different donors. Results from one representative experiment are shown demonstrating that IgG1 and

IgG2 antibodies to HLA I both stimulate endothelial exocytosis resulting in P-selectin presentation in a dose dependent manner

(e) Confluent HAEC were stimulated for 15min with 100ng/mL chimeric human HLA I IgG1 and IgG2 in the presence (striped bars) or absence (solid bars) of rPSGL-1-Ig at 25µg/mL. Fluorescent CFSE-labeled U937, THP-1 and MM6 were allowed to adhere for 20min. Adherent cells were visualized and counted as in (b). Results from one representative experiment out of two are presented as mean number of adherent monocytes per 4x field averaged from 8–10 fields per condition, ± SEM. ‡ p<0.0001 comparing no inhibitor to P-selectin antagonist in each condition by One way ANOVA followed by individual t tests. Results are shown from one experiment representative of two or three independent experiments on endothelial cells from different donors with each monocytic cell line.



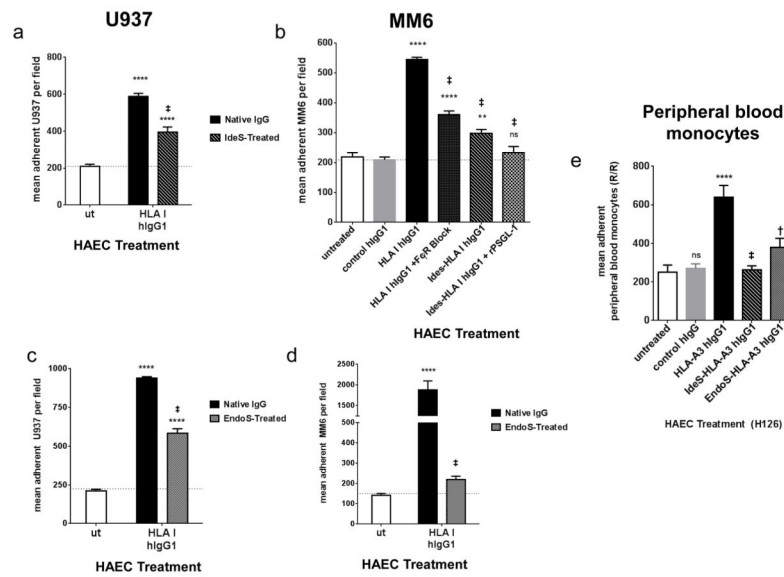
**Figure 2. Monocyte Fc $\gamma$ R utilization during adhesion to HLA antibody-activated endothelial cells, and the role of monocyte Fc $\gamma$ RIIa variants**

(a) Cell surface expression on U937, THP-1 and MM6 human monocytic cells of Fc $\gamma$ RIIa was measured by flow cytometry on an LSRII Fortessa (BD). Monocytic cells were stained with unconjugated isotype control mIgG1 (MOPC-21), mouse anti-human total Fc $\gamma$ RII (clone AT10) or mouse anti-Fc $\gamma$ RIIa-R131 allele-specific clone 3D3 (BD), followed by anti-mouse Fc $\gamma$ -FITC. Results are presented as an overlay of total Fc $\gamma$ RIIa expression (black fill), Fc $\gamma$ RIIa-R131 expression (grey fill), and isotype control background staining (grey line). R131 homozygous U937 exhibit approximately equal staining for total and R131 antibodies, while H131 homozygous MM6 are negative for the anti-Fc $\gamma$ RIIa-R131 antibody. The ratio of mean fluorescence intensity (MFI) of R131 staining to total Fc $\gamma$ RIIa normalized to background isotype control staining ( $MFI_{R131} - MFI_{isotype} / (MFI_{total} - MFI_{isotype})$ ) is shown below each histogram. Monocytes were judged to be homozygous for Fc $\gamma$ RIIa-R131 if the ratio of fluorescence of anti-Fc $\gamma$ RIIa-R131 to anti-total Fc $\gamma$ RII was approximately 1, heterozygous if the ratio was near 0.5, and homozygous for Fc $\gamma$ RIIa-H131 if the ratio was near 0.

Confluent HAEC were stimulated with 100ng/mL HLA I hlgG1 or hlgG2. CFSE-labeled U937 (b), THP-1 (c), or MM6 (d) were left untreated (solid dark bar) or preincubated with 10 $\mu$ g/mL neutralizing antibody to Fc $\gamma$ RI (CD64, striped bar) or Fc $\gamma$ RIIa (CD32, hatched bar) for 15min prior to addition to activated endothelial cells. Adherent monocytes were quantitated as above.

\*\*\*\*  $p < 0.0001$  versus untreated. ns  $p > 0.05$ , #  $p < 0.05$ , ‡  $p < 0.0001$  comparing no inhibitor to neutralizing antibody condition.

Data are representative of two to three independent experiments for each monocytic cell line using endothelium from different donors.



**Figure 3. Enzymatic modulation of HLA I hIgG1 with IdeS and EndoS reduces recruitment of monocytes to endothelium**

(a, b) HLA I IgG1 was digested with IdeS for 1hr at 37°C. Confluent HAEC were activated with intact (solid bar) or IdeS-treated (striped bar) HLA I hIgG1 (500ng/mL) for 15min in the absence or presence of rPSGL-1 to block P-selectin (crossed bar). Where indicated, MM6 were preincubated with polyclonal hIgG at 10μg/mL to block FcγRs.

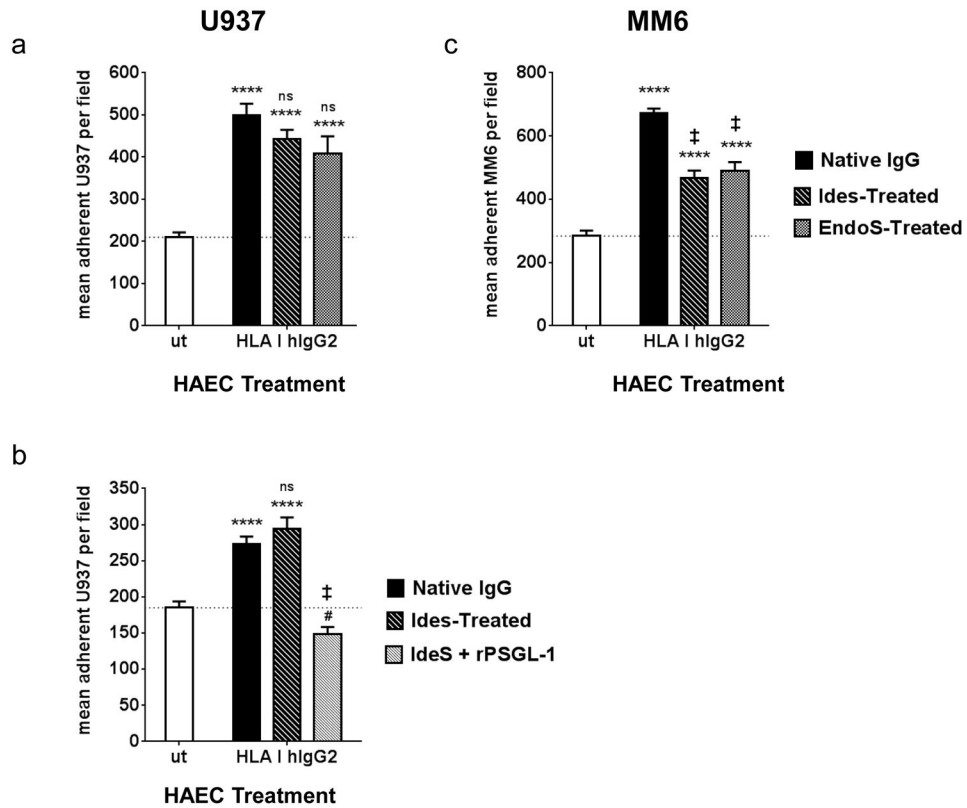
(c, d) Purified human IgG1 and IgG2 were treated with EndoS for 1hr at 37°C. Confluent HAEC were activated with intact (solid bar) or EndoS-treated (dotted bar) HLA I hIgG1 (500ng/mL) for 15min.

Adherence of U937 (a, c) or MM6 (b, d) monocytic cells was measured as above.

Results are representative of three independent experiments with endothelial cells from different donors. ns  $p > 0.05$ , \*\*\*\*  $p < 0.0001$  versus untreated; ‡  $p < 0.0001$  comparing enzyme-treated IgG to intact IgG.

(e) HLA-A3 expressing endothelial cells (donor H126) were stimulated with intact or enzymatically treated fully human monoclonal anti-HLA-A3 IgG1 at 100ng/mL for 15min. Adhesion of primary monocytes isolated from the peripheral blood of healthy volunteers was measured as above.

ns  $p > 0.05$ , \*\*\*\*  $p < 0.0001$  versus untreated; †  $p < 0.001$ , ‡  $p < 0.0001$  comparing enzyme-treated IgG to intact IgG.

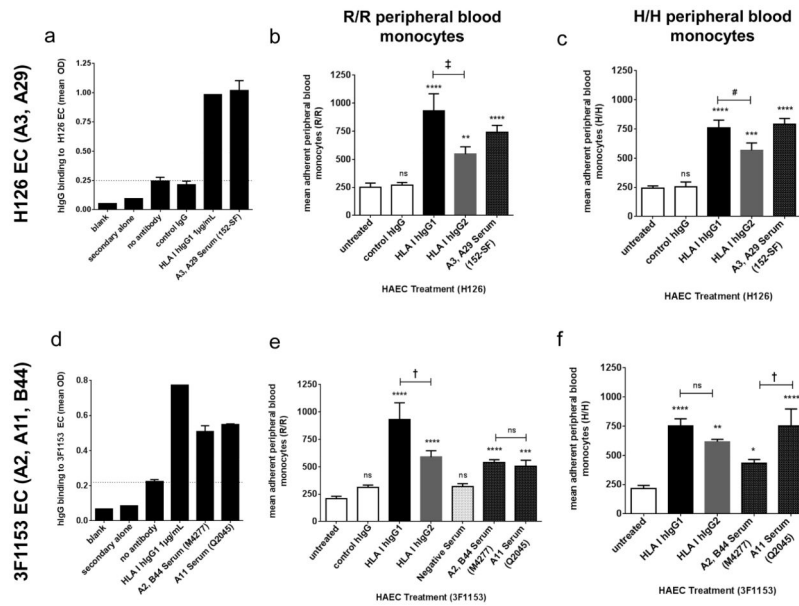


**Figure 4. Enzymatic modulation of HLA I hIgG2 with IdeS and EndoS reduces recruitment of monocytes to endothelium**

Confluent HAEC were activated with intact (solid bar), IdeS-treated (striped bar) or EndoS (dotted bar) HLA I hIgG2 and adherence of U937 (**a**, **b**) or MM6 (**c**) monocytic cells was measured as above. Where indicated, rPSGL-1-Ig was added to endothelial cells at 10 $\mu$ g/mL to block P-selectin (**b**). #  $p > 0.05$ , \*\*\*\*  $p < 0.0001$  versus untreated; ns  $p > 0.05$ , ‡  $p < 0.0001$  comparing inhibitor group to native intact IgG.

Results are representative of three independent experiments with endothelial cells from different donors.

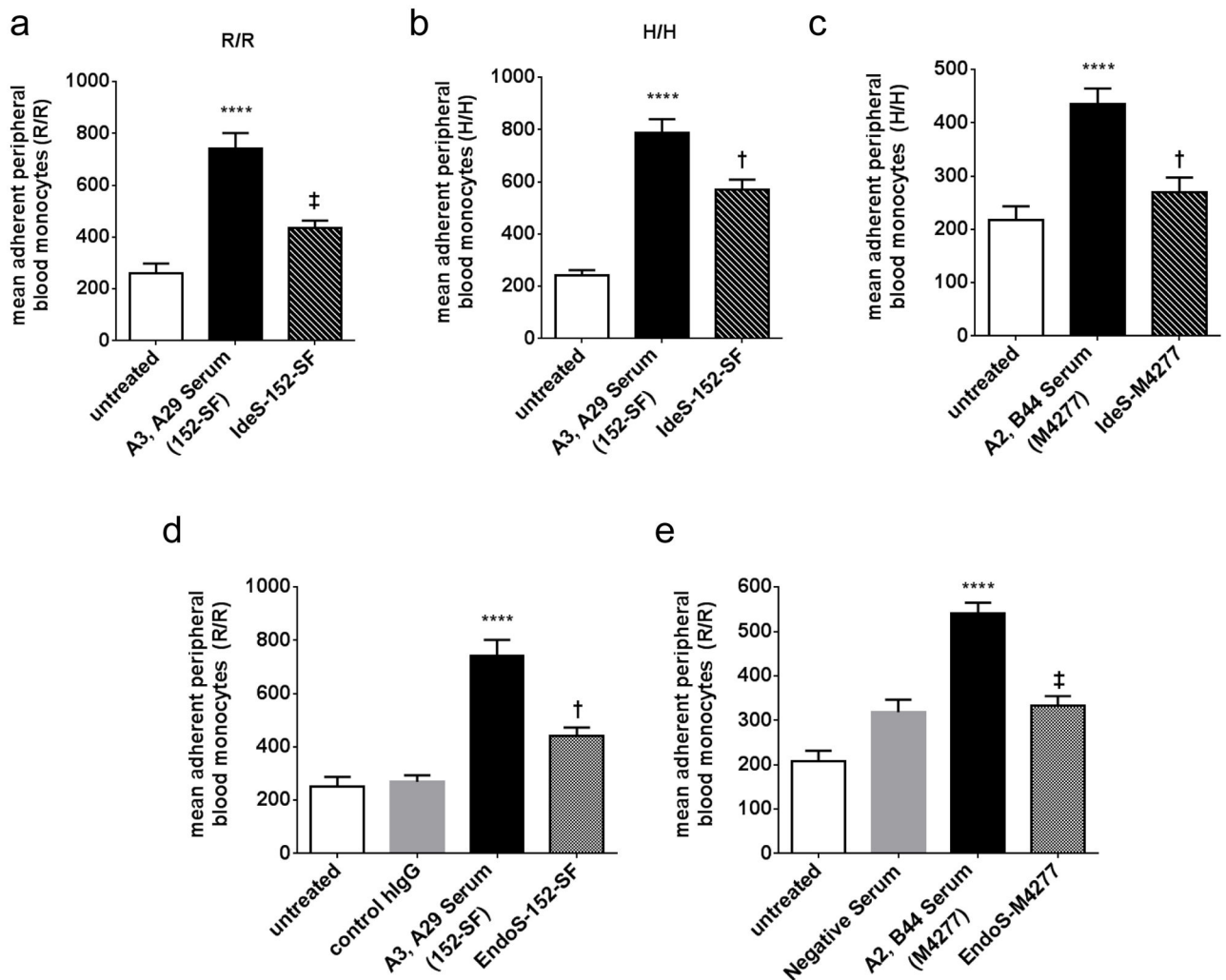




**Figure 5. Stimulation of endothelial cells with polyclonal anti-HLA alloserum from sensitized individuals triggers adhesion of monocytes**

HLA-typed H126 (a) or 3F1153 (d) endothelial cells were seeded at confluence in a 96 well tissue culture plate and incubated with 25 $\mu$ L of sera containing relevant HLA antibodies or with control or chimeric HLA I hIgG1 at 1 $\mu$ g/mL for 30min on ice. Wells were washed and total hIgG bound to the cell surface was measured by cell-based ELISA with anti-human Fc $\gamma$ -HRP followed by colorimetric detection with TMB substrate. Results are expressed as mean OD of hIgG bound to the endothelial cell surface in duplicate wells  $\pm$  SEM.

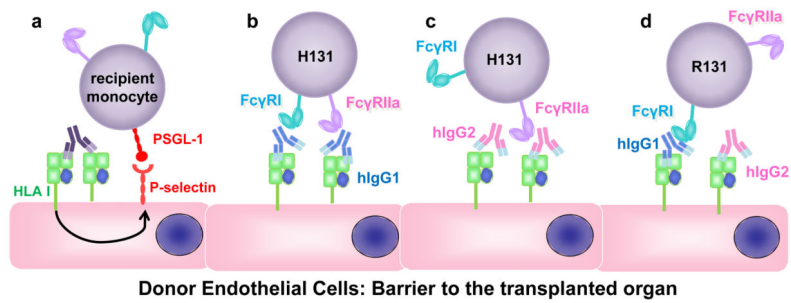
(b–f) Monocytes were isolated from the peripheral blood of healthy volunteers using Ficoll-Paque density centrifugation and MACS negative selection to obtain both Fc $\gamma$ RI+ (CD64) and Fc $\gamma$ RIIIa+ (CD16) monocyte populations. HAEC seeded at confluence in a 48-well plate were activated with control or chimeric HLA I hIgG, negative serum containing no HLA antibodies, or HLA-sensitized sera (3F1153: M4277, Q2045; H126: 152-SF) containing relevant HLA antibodies diluted 1:2 in M199 + 5% FBS for 15min at 37°C. Adhesion of CFSE-labeled peripheral blood monocytes from donors homozygous for Fc $\gamma$ RIIa-R131 (b, e) or H131 (c, f) to H126 (b, c) or 3F1153 (e, f) endothelium was measured as above. One Way ANOVA followed by individual t tests was used to find statistical differences between groups. ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  versus untreated. #  $p < 0.05$ , †  $p < 0.001$ , ‡  $p < 0.0001$  for indicated comparison.



**Figure 6. Modification of IgG in whole serum reduces adhesion of monocytes by HLA antibodies** (a) Whole sera were treated with IdeS or EndoS as described in the methods, and adhesion of peripheral blood monocytes to endothelial cells activated with negative control IgG, negative serum containing no HLA antibodies, native (solid bar), (a, b) IdeS-treated (striped bar) or (d, e) EndoS-treated (hatched bar) HLA-sensitized sera was measured. H126 endothelial cells (a, b, d) were stimulated with or 152-SF (A3, A29); 3F1153 endothelium (c, e) was stimulated with M4277 (A2, B44) serum.

Results are representative of multiple experiments using several other combinations of sera and endothelial cells.

\*\*\*\*  $p < 0.0001$  versus untreated; †  $p < 0.01$ , ‡  $p < 0.0001$  comparing IdeS- or EndoS-treated to intact serum.



**Figure 7. Proposed model of recruitment of recipient Fc $\gamma$ R-bearing immune cells by donor allograft endothelium during HLA antibody-mediated rejection: dependence on antibody subclass and recipient Fc $\gamma$ RIIa polymorphisms**

**(a)** Stimulation of endothelial cells with HLA I antibodies of any subclass stimulates exocytosis of Weibel-Palade bodies, leading to increased cell-surface P-selectin. P-selectin is sufficient to promote a basal level of recruitment of monocytes via interaction with PSGL-1. **(b)** Monocyte recruitment to endothelial cells stimulated with HLA antibodies of hlgG1 subclasses is enhanced by interaction with Fc $\gamma$ Rs. When monocytes express Fc $\gamma$ RIIa-H131 allele, both Fc $\gamma$ RI and Fc $\gamma$ RIIa bind to the Fc portion of hlgG1. **(c)** Additionally, Fc $\gamma$ RIIa-H131 can interact with the Fc region of hlgG2, augmenting recruitment of monocytes. **(d)** Monocytes homozygous for the low affinity Fc $\gamma$ RIIa-R131 bind only hlgG1 through Fc $\gamma$ RI, but do not exhibit Fc $\gamma$ RIIa-dependent enhancement in response to hlgG1 or hlgG2.

**Table 1**

HLA antigen specificity of serum-endothelial cell pairs.

Endothelium	Serum	HLA Specificity (Single Antigen MFI)
<b>3F1153</b> HLA typing: A2, A11 B44, B56	Q2045	A11 (22,203)
	M4277	A2 (22,300), B44 (1,200)
	M0546	A2 (23,000)
<b>H126</b> HLA typing: A3, A29 B35, B44	152-SF	A3 (10,500), A29 (7,200)
	Q2045	A3 (13,626), B35 (14,476)

The strength of relevant HLA antibodies present in the serum as defined by median fluorescence intensity (MFI) of IgG binding on Luminex single antigen LabScreen Beads (One Lambda) are presented in parentheses for each specificity. A cutoff of 1,000 was used to determine positive binding. MFI >10,000 is considered strong reactivity.

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