HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging FcγRs

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HLA Class I Antibodies Trigger Increased Adherence of Monocytes to Endothelial Cells by Eliciting an Increase in Endothelial P-Selectin and, Depending on Subclass, by Engaging FcγRs

Nicole M. Valenzuela,* Arend Mulder,† and Elaine F. Reed*‡

Ab-mediated rejection (AMR) of solid organ transplants is characterized by intragraft macrophages. It is incompletely understood how donor-specific Ab binding to graft endothelium promotes monocyte adhesion, and what, if any, contribution is made by the Fc region of the Ab. We investigated the mechanisms underlying monocyte recruitment by HLA class I (HLA I) Ab–activated endothelium. We used a panel of murine mAbs of different subclasses to crosslink HLA I on human aortic, venous, and microvascular endothelial cells and measured the binding of human monocytic cell lines and peripheral blood monocytes. Both anti–HLA I murine (m)IgG1 and mIgG2a induced endothelial P-selectin, which was required for monocyte adhesion to endothelium irrespective of subclass. mIgG2a but not mIgG1 could bind human FcγRs. Accordingly, HLA I mIgG2a but not mIgG1 treatment of endothelial cells significantly augmented recruitment, predominantly through FcγRI, and, to a lesser extent, FcγRIIa. Moreover, HLA I mIgG2a promoted firm adhesion of monocytes to ICAM-1 through Mac-1, which may explain the prominence of monocytes during AMR. We confirmed these observations using human HLA allele-specific mAbs and IgG purified from transplant patient sera. HLA I Abs universally elicit endothelial exocytosis leading to monocyte adherence, implying that P-selectin is a putative therapeutic target to prevent macrophage infiltration during AMR. Importantly, the subclass of donor-specific Ab may influence its pathogenesis. These results imply that human IgG1 and human IgG3 should have a greater capacity to trigger monocyte infiltration into the graft than IgG2 or IgG4 due to enhancement by FcγR interactions. The Journal of Immunology, 2013, 190: 000–000.

Organ transplantation is a life-saving therapy for end-stage organ failure. Advances in histocompatibility testing, patient management, and immunosuppression have improved short-term graft survival, estimated at 75–90% for most solid organ transplants at 1 y after surgery (Organ Procurement and Transplantation Network data as of April 20, 2012 [http://optn.transplant.hrsa.gov/]). However, long-term graft survival has continued to be low; 50% or more of all solid organ grafts are lost at 10 y after transplant. The major challenge to achieving long-term graft survival is chronic rejection, or 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 Abs, primarily targeting the donor’s polymorphic HLA molecules. Many studies have correlated the presence of anti-donor HLA Abs with Ab-mediated rejection (AMR), poor graft outcome (1, 2), and chronic rejection (3, 4). A histological hallmark of 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 they are also found in the vascular lesions of transplant vasculopathy (12, 13). Depletion of macrophages ameliorates chronic rejection in experimental models (14), and recently Bruneau et al. (15) reiterated the significance of intragraft leukocytes, including monocytes, proposing that the process of “leukocyte-induced angiogenesis” drives chronic rejection.

Donor-specific HLA Abs binding to the endothelial and smooth muscle cells of the graft vasculature can trigger activation of the complement cascade. However, complement deposition is not always observed in acutely injured allografts, even when patients have histological evidence of AMR and donor-specific Abs (16). Our group has proposed that the pathogenesis of HLA class I (HLA I) Abs derives in part from their ability to directly activate the graft vascular cells via crosslinking of HLA I molecules by the F(ab′)2 portion. We and others have demonstrated in vitro, in experimental animals models, and in patient biopsies that HLA or MHC I Ab binding to endothelial and smooth muscle cells triggers intracellular signaling cascades mediating proliferation and cellular resistance to death (17–20), primarily via Src, PI3K/Akt, ERK, and mammalian target of rapamycin (mTOR). Additionally, HLA I
molecule ligation mobilizes endothelial vesicles called Weibel-Palade bodies (WPb), the exocytosis of which results in a rapid increase in von Willebrand Factor (vWF) secretion, cell surface P-selectin, and adherence of neutrophilic cells and monocytes (21, 22).

Activated endothelial cells express E- or P-selectin, 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 Ref. 23). Upon activation, leukocyte integrins are converted from a low-affinity to high ligand–binding state, permitting them to firmly adhere to their endothelial cell–expressed ligands, the cellular adhesion molecules VCAM-1 and ICAM-1 (reviewed in Refs. 24, 25); subsequently, they transmigrate into the tissue. Endothelial ICAM-1 is bound by two leukocyte integrins that contain the β2 subunit, whereas the α4 subunit pairs with β2 integrin to form LFA-1, expressed on most lymphoid and myeloid subsets, whereas the α4α2 subunit complexed with β2 integrin forms Mac-1, which is restricted to monocytes and activated neutrophils.

In addition to adhesion molecules, monocytes express receptors for the Fc region of IgG. Humans have three families of FcR. FcγRI (CD64) is the highest affinity receptor for IgG by several orders of magnitude and is expressed on monocytes as well as activated neutrophils. Activating FcγRIIA and FcγRIIBa multimeric or immune-complexed IgG (reviewed in Refs. 26, 27) and mediate adhesion to immune complexes deposited on endothelial cells or plastic (28–30). The Ab subclass controls its effector functions, including affinity for FcRys. In humans, IgG3 and IgG1 are the most effective activators of complement among the human allele-specific mAbs and IgG purified from transplant recipients. Results were confirmed using LABScreen single Ag beads (One Lambda) on the Luminex platform.

**Materials and Methods**

**Reagents and Abs**

Mouse monoclonal anti-human HLA I Abs (clone W6/32, mlgG2a, from BioXCell; clone 246-B8.67, mlgG2a, clones MEM-147 and MEM-81, mlgG1, from Abcam) were chosen as model Abs because they are well characterized, recognize monomorphic epitopes on all HLA I Ags (36), and are available in distinct IgG subclasses. Human allele-specific Abs (37) were derived from heterohybridomas established at the Leiden University Medical Center and purified by protein A chromatography. Specificity for HLA was confirmed using complement-dependent cytotoxicity assay (CDC) and flow cytometric cross-match against single HLA-transfected cells (38). Clones used were human (hlgG1: clone MULC26 (L) recognizes HLA-A3/A11, whereas clone SN2306G (L) recognizes HLA-A2/B17.

Irrelevant control hlgG1 was obtained from Sigma-Aldrich. These Abs were used to ligate HLA I on endothelial cells. Isootype-matched control Abs, nonspecific IgG, anti-endoglin (CD105; mlgG2a, clone P3D1, Millipore; mlgG1, Abcam), or anti-integrin β3 mlgG1 (CD61, Abcam) were used as negative controls.

**Cells and culture conditions**

The human microvascular endothelial cell line HMEC-1 (provided by Dr. Judith Berliner, Department of Pathology and Laboratory Medicine, University of California, Los Angeles) was grown on 0.1% gelatin in IMDM supplemented with 10% heat-inactivated FBS and antibiotics. Primary human aortic endothelial cells (HAEC) were isolated from aortic rings of explanted donor hearts as previously described (19). Primary HUVEC were obtained from the American Type Culture Collection. HAEC and HUVEC were grown to confluence on 0.1% gelatin in PBS and cultured as previously described (22). All experiments were repeated with endothelial cells from at least two different donors, with different HLA genotypes (CAR, A*02, A*68, B*09, B*06; CAS, A*02, A*30, B*15, 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). Primary endothelial cells were used at passages four through eight.

The human monocytic cell line Mono Mac 6 (39, 40) (a gift of Dr. Judith Berliner, Department of Pathology and Laboratory Medicine, University of California, Los Angeles) was cultured in RPMI 1640 supplemented with 10% FBS, 10 µg/ml insulin, sodium pyruvate, penicillin-streptomycin, and nonessential amino acids (Life Technologies). THP-1 mononuclear cells from the American Type Culture Collection were maintained in RPMI 1640 supplemented with 5% FBS and antibiotics. Monoytic cell lines were subcultured every 2–3 d and maintained at a density of <10^6 cells/ml.

**Isolation of primary human monocytes**

Whole blood was obtained from healthy volunteers in accordance with the University of California, Los Angeles Institutional Review Board (no. 10-001689). Monocytes were enriched from peripheral blood using Ficoll-Paque and MACS. Briefly, PBMC were isolated using Ficoll-Paque density centrifugation (GE Healthcare). Monocytes were enriched from PBMC using a negative selection Pan Monocyte Enrichment Kit (Miltenyi Biotec) according to the manufacturer’s protocol. Purity of enriched monocytes was >85% as determined by flow cytometric staining for CD14.

**Purification of IgG from transplant recipient sera**

Patient sera from sensitized transplant recipients were obtained in accordance with the University of California, Los Angeles Institutional Review Board (no. 11-000577). Sera were selected for reactivity against HLA Ags expressed by the panel of endothelial cells, pooled (PS6), and subsequently used in experiments. The presence of polyclonal anti–HLA Ab was confirmed using LABScreen single Ag beads (One Lambda) on the Luminex
platform. Ab reactivity was to the following Abs expressed by aortic endo-
thelial cells: A3, A29, B35, B44 (donor H126); A2, A11, B44, B56 (donor 3F1153); A1, A2, B8, B60 (donor D121).

The IgG fraction from the pooled highly sensitized transplant recipient sera (PS6) or from negative serum lacking HLA Abs (NS) was purified using protein G column chromatography. Briefly, sera were diluted to 1:12 in 20 mM phosphate buffer (pH 7.0) (binding buffer). Diluted sera were mixed with beads at a ratio of 4 ml sera/1 ml beads and incubated in a centrifuge (Colworth) with end-over-end rotation overnight at 4°C. The column was washed with binding buffer until the A280 returned to baseline. IgG was eluted using 0.1 M citrate buffer (pH 2.16) and im-
nediately neutralized to physiological pH with 1 M Tris (pH 9.0). Purified IgG was concentrated and the buffer was exchanged to PBS using Amicon Ultra (0.5 ml) centrifugal filters for protein (molecular mass cutoff of 50 kDa, Millipore) according to the manufacturer’s recommended methods. IgG concentration was measured using a NanoDrop 8000 spectrophotometer assuming an extinction coefficient of 1.4 for absorbance at A280.

Binding of the IgG fraction of transplant recipient sera to unfixed HAEC was determined by cell-based ELISA method from Florey et al. (28). To evaluate the individual subclasses in hlgG bound to HAEC, biotinylated anti-hlgG1, anti-hlgG2, or anti-hlgG3 (Sigma-Aldrich) was used as detection Ab. Preliminary experiments confirmed that IgG from HLA-reactive donor sera bound to endothelial cells and contained hlgG1, hlgG2, and hlgG3 (N.M. Valenzuela and E.F. Reed, unpublished observations), as previously reported (41–43).

Ab binding to endothelial cells

Endothelial cells were detached by Accutase and stained with 1 μg HLA I Ab, anti-CD61 Ab, or anti-CD105 Ab in 0.1 ml PFA flow buffer (PBS with 2.5% FBS and 0.1% sodium azide) for 30 min on ice. Cells were washed twice and a secondary antibody-FITC conjugated Ab at 1:100 (Jackson ImmunoResearch) was used to detect Ab binding. Altern-
avatively, cells were stained with PE-conjugated ICAM-1 (BD Biosciences). Fluorescence was measured by flow cytometry on a FACS Calibur cytometer (Becton Dickinson).

Flow cytometric determination of monocyte receptor expression

Expression of FcyRs on unfixed monocytes was measured by flow cytometry. Abs to integrin β2, αε, αSM (BioLegend), FcγRI (anti-CD64, BioLegend), FcγRII (anti-CD32, StemCell Technologies), FcγRIII (anti-CD16, BioLegend), and Fcα/μR (anti-CD351, BioLegend)—all murine monoclonal IgG1—were used at 1 μg/0.1 ml PFA flow buffer on ice for 30–45 min, followed by secondary Ab anti-mouse Fcγ-FITC (Jackson ImmunoResearch). Results are shown as representative his-
tograms or average median channel fluorescence ± SEM from three experiments.

The FcγRIIa allototype of monocytes was determined by a flow cyto-
metric method as previously described (44–46). Briefly, monocytes were stained with pan-FcγRIIa Ab (clone AT10) or with clone 3D3, which recognizes only the R131 allele of FcγRIIa (BD Pharmingen). Cells hom-
ogenous for FcγRIIa-H131 stain negatively for 3D3, whereas cells hom-
ogenous for R131 will have approximately equal amounts of staining for pan-FcγRIIa and R131-specific Ab. Cells are considered heterozygous when the ratio of R131 staining to total FcγRIIa is ~0.5. As a control, the monocyte cell line U937 was stained in parallel, which is reportedly homzygous for R131 (46).

Determination of cell surface P-selectin

Cell surface expression of P-selectin was measured on adherent endothelial cells by cell-based ELISA (adapted from Ref. 47). Briefly, confluent endo-
thelial cells in a 96-well plate were treated with control or HLA I Abs in M199 with 5% FBS for 30 min. Cells were washed once with PBS and fixed in freshly prepared 2.5% paraformaldehyde in PBS for 5 min at room temperature. Unpermeabilized cells were washed and blocked with 5% BSA in PBS. Sheep anti-P-selectin (R&D Systems) was added at 1 μg/ml for 2 h. Wells were washed three times and donkey anti-sheep HRP (Millipore) was added at 1:1000 for 2 h at room temperature. After washing, tetramethylbenzidine substrate was added and OD (A405) was read on a SpectraMax plate reader (Molecular Devices).

Blockade of FcγRs

Because monocytes, Mono Mac 6 cells, and THP-1 cells express high levels of FcγRs, preliminary studies were undertaken to determine the ability of Mono Mac 6 cells to take up soluble monomeric mlgG and to assess the effectiveness of blocking strategies. According to these findings, in specified adhesion experiments monocytes, Mono Mac 6 cells, or THP-1 cells were incubated with 20 μg/ml purified hlgG in PBS (Fisher Scien-
tific) or 10 μg/ml monomonal mlgG2a (MOPC-173) for 15 min to block the FcγR from binding HLA I IgG on the surface of the endothelial cells.

Static adherence assay

Adhesion of monocytes to endothelial monolayers was measured as previously described (22). Briefly, confluent endothelial cells were treated with mlgG, anti–CD105 mlgG2a, HLA 1 Abs, or positive control thrombin in M199 with 2% FBS (assay medium) for the indicated times. Mono-
cytes or monocyte cells were fluorescently labeled with CFSE (Vybrant Cell Trace, Invitrogen) at 2 μM in HBSS with Ca2+ and Mg2+ for 10 min at 37°C, then washed. Monocytes were added at ~2 × 104 cells/well in a 24-well plate (roughly equivalent ratio of two to three monocytes/one endothelial cell) for 20 min at 37°C. The coculture was washed three times and fixed. Images of adherent monocytes were acquired by fluo-
rescence microscopy on a Nikon Eclipse Ti, in 8–10 ×4 objective fields for each condition. The number of adherent cells in each field was quan-
tified using automated software from the Massachusetts Institute of Technology called CellProfiler (19, 22, 48). 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 cells ± SEM.

To determine the effect of adhesion molecules in monocyte adherence to Ab-activated endothelium, receptors were blocked on either the monocytes or the endothelial cells prior to coculture. To block P-selectin and ICAM-1, endothelial cells were treated as above, and functional grade P-selectin or ICAM-1 blocking Abs were added at 10 μg/ml for at least 10 min before coculture with monocytes. To block monocyte receptors, cells were preincubated with neutralizing Abs to β2 integrin, αε (LFA-1), αSM (Mac-1), PSGL-1, FcγRI, FcγRII, FcγRIII, or Fcα/μR at 10 μg/ml for 20 min prior to the adhesion assay. All blocking Abs were mlgG1 isotype, except anti–P-selectin, which was goat Ig. Alterna-
tively, to block selectins, PSGL-1 was added at 20 μg/ml, a concentration that maximally inhibited Mono Mac 6 cell adherence to purified immo-
obilized P-selectin in preliminary experiments. Percent inhibition of adhesion by each inhibitor was calculated as follows: [(fold change without inhibitor – 1) – (fold change with inhibitor – 1)]/(fold change without inhibitor – 1) × 100%.

Imobilized Ig adherence assay

Purified polyclonal hlgG (Fisher Scientific), mlgG2a (MOPC-173), or mlgG1 (AK4) was diluted in carbonate/bicarbonate buffer and coated onto high protein binding (Nunc MaxiSorp) 96-well plates at 0.1 ml/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 2 h at room tem-
perature. Mono Mac 6 cells were labeled with 2 μM CFSE in HBSS for 10 min, then serum was rescued with 5% FBS in HBSS, spun down, and resuspended at 105 cells/ml. Cells were added to the plate at 105 cells in 0.1 ml/well and incubated at 37°C for 30 min. Wells were washed three times with HBSS using the flock method and then fixed. Adherent cells were visualized by fluorescence microscopy using a ×4 objective, and five fields per well were counted using ImageJ or CellProfiler.

Dynamic adherence assay

Confluent HAEC in a 12-well plate were treated with mlgG, anti–CD105 mlgG2a, or HLA I Abs in M199 with 2% FBS (assay medium). Mono Mac 6 cells were fluorescently labeled with CFSE as above and then washed. Mono Mac 6 cells were added at ~5 × 104 cells/well and allowed to adhere for 20 min at 37°C on an orbital shaker (New Brunswick Scientific) at 40 rpm. At this speed, the τmax was calculated at 1.5 dynes/cm2, similar to shear stress in capillaries (49). Dynes per square centimeter were esti-
mated using the following formula: \( \tau_{\text{max}} = \frac{p}{d}(\frac{4}{3}) \), where \( p \) is the viscosity of the medium, \( d \) is the density of the medium, and \( j \) is the frequency of rotation in revolutions per second. The coculture was washed three times and fixed. Adherent monocytes were counted as above. The center of the well was avoided, as shear stress becomes negligible here on under orbital conditions (50).

Results are expressed for each condition as the mean number of adherent monocytes per field ± SEM.

Statistical analysis

One-way ANOVA with a Tukey multiple comparisons test, a protected Fisher least significant difference test, or a Dunnett multiple comparisons test was used for statistical comparison of means, with \( p < 0.05 \) considered significant. Data are presented as means ± SEM.
Results
The magnitude of HLA I Ab–induced monocyte adhesion to endothelial cells is dependent on HLA I Ab subclass
As a surrogate for donor-specific HLA I Ab, we tested a panel of monoclonal murine Abs, with each recognizing a monomorphic epitope on human HLA I molecules, and we assessed recruitment of human monocytes to human endothelial cells stimulated with these pan–HLA I Abs. The monocytic cell line Mono Mac 6 bound to untreated HAEC (from donor 3F1153) at 297.7 ± 24 cells per ×4 field. Treatment of HAEC with HLA I Ab led to a large increase in the number of Mono Mac 6 cells adherent on the endothelial monolayer (Fig. 1A). HLA I mIgG1 (clone MEM-147) increased the number of bound Mono Mac 6 cells, as did endothelial stimulation with HLA I mIgG1 (clone MEM-81), to a mean of 508 ± 25 and 445 ± 7 adherent monocytes per field, respectively (p ≤ 0.001). Both clones of mlgG2a pan–HLA I Abs (clones W6/32 and 246.B8-E7) dramatically increased adherence of Mono Mac 6 cells to endothelial cells to an average of 777 ± 21 and 999 ± 60 per field, respectively. Similar observations were made when the panel was used to stimulate endothelial cells from a donor with a different HLA genotype (H126). Representative fluorescence microscopy fields demonstrating Mono Mac 6 cells adherent to the endothelial monolayer (3F1153) are given in Fig. 1A.

We observed a notable difference in the magnitude of Mono Mac 6 cell adherence stimulated by mIgG1 compared with mIgG2a HLA I Abs and confirmed these findings with a second monocytic cell line, THP-1 (Fig. 1B). Adhesion of both Mono Mac 6 and THP-1 monocytes was significantly increased to endothelial cells stimulated with HLA I mlgG1 (MEM-147) by 2.16 ± 0.21-fold and 2.17 ± 0.16-fold over untreated cells, respectively. Stimulation of endothelial cells with HLA I mlgG2a (W6/32) markedly increased adherence of Mono Mac 6 cells by 4.50 ± 0.49-fold over control and THP-1 cells by 4.64 ± 0.7-fold over control. When the two isotypes were compared in parallel, the degree of monocytic cell adherence to HLA I mlgG2a (W6/32)–treated endothelial cells was 2.5-fold higher than to HLA I mlgG1 (MEM-147)–treated endothelial cells in more than four experiments (p < 0.01) (Fig. 1B). We further validated these observations with human CD14+ monocytes enriched from peripheral blood (PBMC–monocytes). Stimulation of HAEC with HLA I mlgG2a (W6/32) significantly increased PBMC-monocyte adherence from 73.4 ± 8.4 to 898.2 ± 38.5 per field. HLA I mlgG1 (MEM-147) treatment also significantly increased monocyte binding, although to a lesser extent than did mlgG2a, at 501.9 ± 62.8 monocytes per field (Fig. 1C). This observed difference in recruitment capacity between isotypes could not be attributed to unequal amounts of HLA I Ab bound to the endothelial cell surface, as the binding of each HLA I monoclonal was comparable on endothelia from several different donors (Supplemental Fig. 1A).

Monocyte recruitment by endothelial cells is a specific response to HLA I Abs
To determine whether endothelial cell Abs could promote monocyte adherence as observed with HLA I Abs, HAEC were treated with non–HLA or HLA I Abs at the same concentration (1 μg/ml). All Abs bound to endothelial cells (Supplemental Fig. 1B). Coating of aortic endothelial cells with murine Ab to integrin β3 (anti–CD61 mlgG1) was not sufficient to cause adherence of Mono Mac 6 cells (1.03 ± 0.025-fold over untreated cells) to HAEC. In contrast, treatment of endothelium with HLA I–specific mlgG1 (MEM-147) significantly increased adherence of Mono Mac 6 cells by 2.71 ± 0.46-fold (Fig. 1D). Similarly, whereas Ab recognizing endoglin (anti–CD105 mlgG2a) did not stimulate monocyte cell binding (1.17 ± 0.11-fold over untreated cells), HLA I mlgG2a (W6/32) provoked extensive Mono Mac 6 cell recruitment (4.39 ± 0.32-fold). Similar results were obtained when THP-1 monocytes were used (Fig. 1D).

Additionally, control anti–CD105 mlgG2a had no significant effect on Mono Mac 6 cell adherence to HUVEC or on the human dermal microvascular cell line HMEC-1. In contrast, treatment with HLA I mlgG1 (MEM-147) or HLA I mlgG2a (W6/32) at 1 μg/ml significantly increased Mono Mac 6 cell binding to both HUVEC and HMEC-1 (Fig. 1E). Therefore, monocyte recruitment is specific to Abs recognizing HLA I molecules and is not induced by isotype-matched non–HLA I Ab bound to endothelium.

HLA I Abs differentially recruit monocytes depending on their capacity to engage FcyRs
Mono Mac 6 and THP-1 cells express FcyRI (CD64) and FcyRII (CD32), with little detectable FcyRIII (CD16) (Supplemental Fig. 2A), which is similar to the predominant monocyte subset in peripheral blood (CD64+CD14+CD16−) (reviewed in Ref. 51). The ability of human FcyRs to interact with mlg, particularly with mlgG2a, has been previously characterized (52, 53). Therefore, we postulated that the difference in the capacity of HLA I Ab IgG1 and IgG2a subclasses to stimulate monocyte adherence was due to unequal affinities of the Fc portion for human FcyRs on monocytes. To test the ability of each mlg isoform to engage FcyRs on the monocyte, we used a solid phase assay in which irrelevant Ig was immobilized and Mono Mac 6 cell adherence was measured (Supplemental Fig. 2B). Mono Mac 6 cells did not adhere to wells coated with mlgG1 or BSA alone. In contrast, Mono Mac 6 cells adhered to immobilized positive control polyclonal mlgG, or to mlgG2a, to a significantly greater extent than to BSA alone. Thus, Mono Mac 6 monocytes have minimal ability to bind mlgG1, whereas binding to mlgG2a through FcyRs was high, consistent with previous studies (54, 55).

In light of these findings, we determined whether HLA I mlgG2a–triggered monocyte recruitment had an Fc-mediated component. Mono Mac 6 or THP-1 cells were pretreated with a blocking Ab to FcyRI, FcyRII, or FcyRIII, or with soluble IgG to inhibit all FcyRs, and adherence to HLA I mlgG2a (W6/32)–treated HAEC was measured. HLA I mlgG2a–stimulated endothelial cells demonstrated a >5-fold increase in THP-1 monocyte binding compared with untreated endothelium (Fig. 2A). Inhibition of FcyRI significantly reduced the number of THP-1 cells adherent to HLA I mlgG2a (W6/32)–treated endothelial cells by 89.3 ± 4.4%, whereas a blocking Ab against FcyRII had a minor inhibitory effect at 24.4 ± 4.9%. In contrast, blocking Abs to FcyRIII had no significant effect (Fig. 2A, 2B), consistent with the absence of this receptor on the monocytic cell lines (Supplemental Fig. 2A). Similar results were obtained when Mono Mac 6 cell binding was arrested. Inhibition of FcyRI reduced Mono Mac 6 cell binding to HLA I mlgG2a (W6/32)–stimulated endothelium by 82.6 ± 8.1%, whereas a neutralizing Ab to FcyRII resulted in lesser inhibition of adherence (59.2 ± 8.8%). FcyRII antagonism did not significantly affect the ability of Mono Mac 6 monocytes to adhere to HLA I mlgG2a (W6/32)–treated endothelial cells, nor did a control Ab against Fcα/γR (N.M. Valenzuela and E.F. Reed, unpublished observations). Pretreatment of monocytes with a total FcγRII blockade inhibited THP-1 cell adherence by 72.0 ± 8.8% and Mono Mac 6 cell adherence by 85.6 ± 4.8% (Fig. 2B). Mono Mac 6 cell recruitment by HAEC treated with the second clone of HLA I mlgG2a (246-B8-E7) was also FcyRII-dependent (N.M. Valenzuela and E.F. Reed, unpublished observations). These results demonstrate that FcyRI is predominantly involved in
FIGURE 1. HLA I Abs trigger adherence of monocytes to endothelium differentially depending on subclass. (A) Confluent HAEC (donor 3F1153, filled bars; or donor H126, open bars) were stimulated with murine monoclonal HLA I Abs of isotype IgG1 (clones MEM-147 or MEM-81) or IgG2a (clones W6/32 or 246.B8-E7) at 1 μg/ml for 30 min. CFSE-labeled Mono Mac 6 (MM6) cells were overlaid and allowed to bind for 20 min. Nonadherent cells were washed off and adherent cells were counted in 10 fields per condition. Results are displayed as the mean number of adherent MM6 cells ± SEM. Groups were compared using two-way ANOVA and a Holm–Sidak multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 versus untreated cells. Representative fluorescence microscopy fields for CFSE-labeled MM6 cells binding to 3F1153 HAEC are given in the bottom panel. Original magnification ×4. (B) HAEC were treated as in (A), and adhesion of MM6 (filled bars) or THP-1 cells (open bars) was measured. Results are expressed as the fold increase in the number of adherent monocytes from three or more independent experiments. Groups were compared using one-way ANOVA followed by a Tukey multiple comparisons test. **p ≤ 0.01, ***p ≤ 0.001 comparing HLA I mIgG1 to mIgG2a. (C) HAEC were treated as in (A), and adhesion of CFSE-labeled human CD14+ monocytes enriched from peripheral blood was measured. Results are displayed as (Figure legend continues)
monocytic cell adherence to endothelial cells activated with HLA I mlgG2a.

We observed a difference in FcyR utilization between Mono Mac 6 and THP-1 cells; that is, blockade of FcyRIIa largely impaired Mono Mac 6 but not THP-1 cell recruitment (Fig. 2A, 2B). We posited that this discrepancy was due to allelic differences in FcyRIIa, which is dimorphic in humans. The R131 and H131 alleles have divergent affinities for the various subclasses of mlgG and hlgG, and H131 has higher overall affinity for IgG than does R131. We determined the alleotype of FcyRIIa on each monocytic cell line using flow cytometry method as previously described (45, 56), where the binding of an R131 allele–specific Ab (clone 3D3) can be compared with staining for total FcyRIIa to determine homozygosity or heterozygosity. This method revealed that Mono Mac 6 cells are homozygous for FcyRIIa-H131, whereas THP-1 cells are heterozygous (H/R). As a control, the U937 monocytic cell line carried only the R131 allele as previously documented (Supplemental Fig. 2C) (45, 46, 56, 57).

Although our data indicated that FcyRs participated in monocytic recruitment in response to HLA I Abs with a high affinity for FcyRs, previous studies (57, 58) and our preliminary data implied that mlgG1 would have a weaker capacity to engage human FcyRs, which may explain the lower capacity of HLA I mlgG1 to trigger adherence of monocytes. Therefore, to determine whether HLA I mlgG1–stimulated adherence to endothelial cells was also dependent on FcyRs, we treated HAEC with HLA I mlgG1 (MEM-147), and Mono Mac 6 cells were left untreated or incubated with soluble IgG to block FcyRs prior to the adherence assay. HLA I mlgG1 (MEM-147) significantly increased the adherence of Mono Mac 6 cells to endothelium, by 2.34 ± 0.24-fold over untreated cells. In contrast to HLA I mlgG2a, HLA I mlgG1–triggered Mono Mac 6 cell binding was not significantly different when FcyRs were blocked, at 2.11 ± 0.25-fold over untreated cells (mean, 18.16 ± 5.19% inhibition for eight experiments) (Fig. 2C). Similar results were obtained when endothelial cells were stimulated with HLA I mlgG1 (MEM-81) (data not shown). Therefore, inhibition of FcyRs by preincubation with soluble IgG did not impair the ability of monocytes to bind to endothelial cells stimulated with HLA I mlgG1, in agreement with our observation that mlgG1 did not bind human FcyRs on Mono Mac 6 cells.

Peripheral blood CD14+ monocytes highly express FcyRI and FcyRIII, but only a small subset (<10%) express FcyRIII (N.M. Valenzuela and E.F. Reed, unpublished observations, and Ref. 51). Whereas treatment with anti–CD105 mlgG2a did not increase recruitment of monocytes, HLA I mlgG2a (W6/32) stimulated a 5.27-fold increase in peripheral blood monocytes bound to HAEC. Adherence of monocytes was partially dependent on interaction between FcyRs and Ab, as FcyR blockade significantly reduced adherence to HAEC to 2.08-fold over untreated cells (74.7% inhibition, Fig. 2D). In contrast, FcyR blockade had no inhibitory effect on peripheral blood–monocyte recruitment to HLA I mlgG1 (MEM-147)–activated endothelial cells (data not shown). To validate the contribution of the Fc fragment of HLA I Ab to monocytic recruitment, we stimulated HAEC with an F(ab’2) fragment of W6/32, which lacks the Fc portion of the Ab but retains Ag recognition sites. HLA I crosslinking with W6/32 F(ab’2) resulted in significantly lower numbers of adherent monocytes compared with intact Ab, identical to results with an FcyR blockade (Fig. 2D). Notably, however, monocyte binding in response to HLA I F(ab’2) was 1.94-fold higher than to unstimulated endothelial cells (p < 0.001), consistent with our previous observation (22). Additionally, whereas FcyR antagonism significantly impaired Mono Mac 6 cell adherence to HUVEC (Fig. 2E) and HMEC-1 (Fig. 2F) stimulated with HLA I mlgG2a (W6/32), FcyR blockade had no effect on Mono Mac 6 cell recruitment in response to endothelial activation with HLA I mlgG1 (MEM-147).

Because leukocyte recruitment occurs in the blood vessels under shear stress, we recapitulated more physiological endothelial cell–monocyte interactions by performing the adhesion assay under dynamic conditions. HAEC were treated with anti–CD105 mlgG2a, HLA I mlgG1 (MEM-147), or HLA I mlgG2a (W6/32). Mono Mac 6 cells with or without an FcyR blockade were overlaid and incubated with stimulated endothelial cells on an orbital shaker. Whereas inhibition of FcyRs had no effect on HLA I mlgG1–induced recruitment, HLA I mlgG2a–induced recruitment was significantly inhibited when FcyRs were blocked (Supplemental Fig. 2D).

**HLA I Ab–induced endothelial P-selectin is required for monocyte adherence irrespective of Ab isotype**

We observed that HLA I mlgG1–stimulated monocyte adherence to endothelial cells was not affected by an FcyR blockade. Moreover, inhibition of FcyRs did not completely eliminate monocyte binding in response to HLA I mlgG2a, and F(ab’2) HLA I Ab stimulated a significant increase in monocyte binding over untreated cells (22). Taken together, these results pointed to an additional mechanism of recruitment unrelated to the Fc fragment and dependent on HLA I recognition and dimerization. Therefore, we proposed that HLA I Abs activate endothelia, leading to increased adhesion molecule expression, which could independently support monocyte adhesion.

Given the rapidity of the recruitment response (<1 h), we first determined the effect of stimulation with monoclonal HLA I Abs on P-selectin, which is stored in WPb and rapidly translocated to the cell surface upon endothelial activation. Confluent endothelial cells were left untreated or exposed to isotype control mlgG2a, non–HLA endoglin Ab (anti–CD105 mlgG2a), Ab recognizing integrin β3 (anti–CD61 mlgG1), or HLA I Ab (clones W6/32, MEM-147, and MEM-81) for various times. Cell surface P-selectin was measured by cell-based ELISA. Crosslinking of HLA molecules with HLA I mlgG2a (W6/32), HLA I mlgG1 (MEM-81), or HLA I mlgG1 (MEM-147) significantly elevated P-selectin on the endothelial cell surface by >2-fold over baseline (Fig. 3A, 3B), with expression detected as early as 5 min, peaking at 30 min, and sustained up to 45 min after stimulation (Supplemental Fig. 3A). P-selectin induction by HLA I mlgG2a was dose dependent, with a maximal effect at 5 μg/ml (N.M. Valenzuela and E.F. Reed, unpublished observations), confirming previous findings (21, 22). Activation with classical WPb agonists histamine, PMA, and thrombin significantly increased endothelial P-selectin (Fig. 3A,
FIGURE 2. HLA I mlgG2a–mediated monocyte recruitment is dependent on FcγRs. (A) HAEC were stimulated with HLA I mlgG2a at 1 μg/ml for 30 min. Mono Mac 6 (MM6; left panel) or THP-1 cells (right panel) were pretreated with neutralizing Abs to FcγRI, FcγRII, or FcγRIII at 10 μg/ml for 15 min, and adherence to endothelium was measured. Results are from one representative experiment out of three. (B) HAEC were stimulated and MM6 (filled bars) or THP-1 cell (open bars) adherence was measured with or without FcγR inhibition as in (A). Total FcγRs were blocked by incubating monocytic cells with soluble hIgG at 10 μg/ml. Results are expressed as the average ± SEM percentage inhibition of monocyte binding from three or more independent experiments. (C) HAEC were stimulated with HLA I mlgG1 at 1 μg/ml for 30 min. Adherent MM6 cells with or without an FcγR blockade were counted. Results are shown as fold increase in the number of adherent MM6 cells normalized to untreated cells from three independent measurements (top panel). Comparison no inhibitor to FcγR blockade was not significant. Average percentage inhibition of adherence in eight independent experiments is given in the bottom panel. (D) HAEC were stimulated with intact HLA I Ab at 1 μg/ml or with an F(ab′)2 fragment of HLA I (Figure legend continues)
3B), as previously reported (59). In contrast, non-HLA anti–CD105 or anti–CD61 Abs did not stimulate cell surface P-selectin on HAEC (Fig. 3B). Similarly, P-selectin was not detected on unstimulated HUVEC or HMEC-1, and it was unchanged by isotype control mIgG or anti–CD105 mIgG2a. HLA I crosslinking by Abs significantly increased P-selectin detected on the surface of HUVEC and HMEC-1 by 1.6- and 3-fold, respectively (Fig. 3C). Thus, several clones of HLA I Abs elicited ectopic expression and increased P-selectin on aortic, venous, and microvascular human endothelial cells at a similar magnitude as traditional WPb agonists.

We hypothesized that endothelial P-selectin elicited by HLA I ligation would engage its ligand PSGL-1, expressed on monocyte cell lines and monocytes (Supplemental Fig. 2E). To test this hypothesis, endothelial cells were treated with HLA I mIgG2a (W6/32) or HLA I mIgG1 (MEM-147) and exposed to the soluble antagonist of P-selectin, rPSGL-1. Adherence of Mono Mac 6 cells was measured with and without the inhibitor. The presence of rPSGL-1 significantly inhibited Mono Mac 6 cell binding to endothelial cells stimulated with either HLA I mIgG2a (W6/32) or HLA I mIgG1 (MEM-147) by 69.4 ± 4.3 and 70.4 ± 8.4%, respectively (Fig. 3D, 3E), demonstrating the effectiveness of a P-selectin blockade against recruitment induced by both isoforms of HLA I Ab. As a control, rPSGL-1 antagonist also reduced monocyte adherence to thrombin-stimulated endothelial cells by 88.6 ± 6.9% (N.M. Valenzuela and E.F. Reed, unpublished observations). Representative fields illustrating Mono Mac 6 cell adherence to HLA I Ab–activated endothelial cells with and without the antagonist are shown in Fig. 3D.

As an alternative strategy, neutralizing Ab to P-selectin or to its receptor PSGL-1 reduced Mono Mac 6 cell adherence to HLA I Ab–activated endothelial cells by 40–65% (Supplemental Fig. 3B). rPSGL-1 or neutralizing Ab to PSGL-1 also significantly attenuated HLA I Ab–mediated Mono Mac 6 cell adherence to HMEC-1 (Fig. 3E). Similar results were observed with THP-1 cells (N.M. Valenzuela and E.F. Reed, unpublished observations). Because selectin-mediated leukocyte capture is most prominent under shear stress (25), we confirmed our observations under dynamic conditions. P-selectin antagonism with rPSGL1 significantly impaired Mono Mac 6 cell adherence to HLA I Ab–treated endothelium under flow conditions (Supplemental Fig. 3C). These results demonstrate that P-selectin is a critical mediator of HLA I Ab–triggered monocyte binding to endothelial cells irrespective of HLA I Ab subclass and under both static and flow conditions.

**HLA I Abs promote firm adherence to endothelial cells preferentially through Mac-1/ICAM-1 interactions**

Whereas P-selectin initiates capture of leukocytes from the blood and mediates rolling on endothelial cells, integrin binding to ICAM-1 or VCAM-1 is required for firm adherence and ultimately transmigration into the tissue. HAEC constitutively express ICAM-1 as previously documented (60, 61); however, we did not observe an alteration in ICAM-1 surface expression 1, 2, or 4 h after exposure to HLA I Abs (Fig. 4A), consistent with previous reports (62). Because Mono Mac 6 and THP-1 cells express the ICAM-1 binding integrins LFA-1 (αLβ2) and Mac-1 (αMβ2) (Supplemental Fig. 2E), we reasoned that ICAM-1/β2 integrins may be involved in stable adhesion of monocytes to HLA I Ab–stimulated endothelial cells after initial capture and activation by P-selectin.

To test this hypothesis, HAEC were treated with HLA I Ab, and a neutralizing Ab to ICAM-1 was added. HLA I mIgG2a (W6/32) increased adherence of Mono Mac 6 cells to endothelial cells to an average of 995 ± 31 per field, compared with adherence to untreated endothelial cells at 384 ± 27 per field. Mono Mac 6 cell adherence was significantly inhibited when ICAM-1 was blocked, by 75.7 ± 5.3% for HLA I mIgG2a and by 75.3 ± 14.9% for HLA I mIgG1 (Fig. 4B, 4C). We blocked ICAM-1 binding integrins by pretreating the Mono Mac 6 cells with a neutralizing Ab against β2 integrin prior to the adhesion assay. β2 integrin inhibition reduced Mono Mac 6 cell 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 cell ability to bind to HLA I mIgG1 (MEM-147)–treated endothelium, reducing the number of adherent cells by 84.1 ± 15.9% (Fig. 4C).

Because monocytes express two integrins that 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), whereas αM complexes with β2 to form the Mac-1 integrin. Mono Mac 6 cells were incubated with a neutralizing Ab to the αL subunit of LFA-1 or to the αM subunit of Mac-1 (Fig. 4B, 4D). Pretreatment of Mono Mac 6 cells with a blocking Ab targeting αL (LFA-1) did not significantly alter recruitment in response to HLA I mIgG2a, at 903 ± 17 per field. In contrast, inhibition of αM (Mac-1) on Mono Mac 6 cells significantly impaired the monocytes’ ability to adhere to HLA I mIgG2a–activated endothelial cells, reducing the number of adherent monocytes to a mean of 509 ± 21 per field (Fig. 4B). Neutralizing Ab to αM (LFA-1) had limited inhibitory effect, reducing adherence by 25.0 ± 8.4% (p > 0.01), whereas blockade of αM (Mac-1) abolished the adhesive capacity of Mono Mac 6 cells, by 78.1 ± 19.7% compared with monocytes without inhibitor (Fig. 4B, 4D). This suggests that Mac-1 integrin is the dominant molecule mediating firm adhesion of monocytes to endothelium activated with HLA I Abs, despite much higher expression of LFA-1. Similar results were obtained when THP-1 monocyte adherence was assessed (N.M. Valenzuela and E.F. Reed, unpublished observations).

**Human HLA I allele–specific IgG1 triggers recruitment of peripheral blood monocytes through P-selectin, ICAM-1, and FcγRs**

We next tested the effect of human HLA I Abs on recruitment of peripheral blood–monocytes. We predicted that human monoclonal HLA I allele–specific Abs derived from sensitized volunteers would trigger endothelial cell–monocyte interactions by similar mechanisms as above. Only human IgG1 HLA I Abs were available (37). We tested the reactivity of monoclonal hIgG1 recognizing HLA-A2 (clone SN230G6) and anti–HLA-A3 (clone MUL2C6) against HAEC with known HLA I genotypes (3F1153, A*02, A*68; H1256, A*03, A*29) and confirmed that each HLA I Ab bound to endothelial cells expressing its Ag (data not shown).

HLA-typed HAEC were stimulated with relevant allele-specific human HLA-A Abs. Binding of human HLA-A3–specific IgG1 on HLA-A*03+ endothelial cells significantly increased adherence of PBMC-monocytes in a dose-dependent manner (0.01–1 μg/ml), and stimulated adhesion of monocytes to HLA I Ab–stimulated endothelial cells after initial capture and activation by P-selectin. Therefore, P-selectin and ICAM-1 play a critical role in monocyte recruitment to HLA I Ab–stimulated endothelial cells.
FIGURE 3. HLA I crosslinking by Abs stimulates WPb exocytosis and increased cell surface P-selectin on endothelial cells. (A) HAEC were treated for 30 min with HLA I Ab at 1 μg/ml, 10 min with 5 U/ml thrombin, or 20 min with 200 nM PMA. Cell surface P-selectin was stained on unpermeabilized cells with sheep anti-P-selectin followed by anti-sheep HRP, and detected by adding tetramethylbenzidine substrate. Results are expressed as the mean OD of duplicate wells for each condition. Results from one representative experiment out of three total are shown. (B) HAEC were stimulated with anti-CD105, anti–CD61 Ab, or HLA I Ab at 1 μg/ml for 30 min, histamine for 5 min, or PMA at 200 nM for 15 min. P-selectin was measured as in (A). (C) HUVEC (dark gray bars) or HMEC-1 (light gray bars) were stimulated with control or HLA I Ab as in (B) and cell surface P-selectin was measured. (D and E) HAEC (D) or HMEC-1 (E) were stimulated with HLA I Ab at 1 μg/ml for 30 min. Adherence of Mono Mac 6 (MM6) cells was measured with or without rPSGL-1 at 20 μg/ml to block P-selectin or anti–PSGL-1 at 10 μg/ml. Results from one representative experiment of three are shown as the mean number of adherent MM6 SEM in the top panel. Bottom panel (D) shows representative fluorescence microscopy fields (original magnification ×4). Groups were compared by one-way ANOVA followed by a Dunnett multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 versus untreated cells; ‡p < 0.01 versus no inhibitor.
with a maximal effect at 1 \( \mu \text{g/ml} \) (Fig. 5A). Similar results were observed when HLA-A*02+ HAEC were treated with anti–HLA-A2 hIgG1. In contrast, stimulation of HLA-A*03+ HAEC with irrelevant anti–HLA-A2 hIgG1 did not significantly increase adhesion of peripheral blood monocytes.

Given that the Fc region of hIgG1 isotype has a high affinity for human Fc\(\gamma\)Rs, we postulated that hIgG1 may engage monocyte Fc\(\gamma\)Rs during the adhesion cascade. To determine whether stimulation of endothelial cells with HLA I–specific hIgG1 resulted in Fc\(\gamma\)R-dependent monocyte recruitment, individual Fc\(\gamma\)Rs were blocked with neutralizing Abs. HLA-A3 hIgG1–mediated adhesion of monocytes to HLA-A*03+ HAEC (H126) was significantly inhibited by antagonism of Fc\(\gamma\)RI, decreasing adherence from 3.1-fold over untreated cells to 1.8-fold (52% inhibition). Total Fc\(\gamma\)R blockade reduced monocyte adherence to 2.1-fold, a 50% inhibition. A minor effect was observed with neutralization of Fc\(\gamma\)RII (2.6-fold, 23% inhibition) and Fc\(\gamma\)RIII (2.9-fold, 10% inhibition) (Fig. 5B).

Similar results were obtained with HLA-A2 hIgG1 (Supplemental Fig. 4A, 4B). Taken together, these results suggest that donor-specific IgG1 predominantly engages Fc\(\gamma\)RI to promote monocyte–endothelial cell interactions, and that this interaction contributes to maximal HLA I Ab–triggered adhesion. Thus, antagonism of Fc\(\gamma\)R interactions with the Fc fragment of donor-specific Ab can significantly attenuate the recruitment of monocytes to endothelium by human HLA I IgG1.

To define the adhesion molecules participating in human HLA I Ab–induced recruitment of PBMC-monocytes, we blocked ICAM-1/integrin \(\beta_2\) and PSGL-1/P-selectin during monocyte adherence. HLA-A3 hIgG1 significantly increased the number of monocytes bound to HLA-A*03+ endothelial cells by >3-fold.

**FIGURE 4.** Endothelium activated with HLA I Abs support firm adhesion of monocytes through ICAM-1 and Mac-1 interactions. (A) HAEC were treated with HLA I mIgG2a (W6/32) for 1, 2, or 4 h and then detached with Accutase. ICAM-1 was stained with a PE-conjugated Ab and expression was measured by flow cytometry. Histogram shows ICAM-1 basal expression on unstimulated endothelial cells (gray fill) and after exposure to HLA I Abs for 1 h (dark line). Mean fluorescence intensity (MFI) of ICAM-1 at 2 and 4 h after exposure to HLA I Ab is graphed. Results are representative of two independent measurements. (B) HAEC were treated for 30 min with HLA I Ab at 1 \( \mu \text{g/ml} \). Adhesion of Mono Mac 6 cells was measured as above, with or without neutralizing Ab at 10 \( \mu \text{g/ml} \) to the indicated adhesion molecules. Results are from one representative experiment out of three. ***\( p \leq 0.001 \); ****\( p \leq 0.0001 \) versus untreated cells; \( ^* p \leq 0.001 \) versus no inhibitor. (C) Mono Mac 6 cell adhesion to HAEC stimulated with HLA I mIgG2a (filled bars) or mIgG1 (open bars) was measured as in (B). Results are expressed as the average percentage inhibition \( \pm \) SEM of adherence by each inhibitor for each condition. (D) Mono Mac 6 cell adhesion to HAEC stimulated with HLA I mIgG2a was measured as in (B). Results are expressed as the average percentage inhibition \( \pm \) SEM of adherence by neutralizing Ab to LFA-1 or Mac-1 for each condition.
Antagonism of PSGL-1 reduced monocyte binding by 50.1% (Fig. 5C). Firm adhesion molecules were also required, as blockade of monocyte β2 integrins significantly prevented maximal recruitment 57.9% (Fig. 5C). Similar results were obtained using HLA-A2 hIgG1 on A*02-expressing HAEC (Supplemental Fig. 4C). Combinatorial blockade of P-selectin, FcγRs, and integrin β2 fully

**FIGURE 5.** Activation of endothelial cells with human anti–HLA I IgG1 triggers adherence of peripheral blood monocytes via FcγRI, P-selectin, and ICAM-1. (A) HAEC were stimulated with human mAb HLA I IgG1 at varying doses for 30 min. Top panel, HLA-A*03–expressing endothelial cells (HLA-A*03–expressing endothelial cells (H126) were treated with anti–HLA-A3 hIgG1 (black bars) or irrelevant anti–HLA-A2 hIgG1 (gray bar). Bottom panel, HLA-A*02–expressing HLA-A2 hIgG1 (gray bar). Bottom panel, HLA-A*02–expressing HAEC (3F1153) were treated with anti–HLA-A3 hIgG1. Measurement of peripheral blood CD14+ monocyte adherence was performed as above. (B) HLA-A*03+ HAEC were treated with anti–HLA-A3 hIgG1 at 1 µg/ml as in (A). Peripheral blood monocytes were left untreated or preincubated with neutralizing Ab to FcγRs. Results are shown as mean number of adherent monocytes per field ± SEM from one representative experiment out of three. (C) HLA-A*03+ HAEC were treated as in (A), with or without indicated neutralizing Abs at 10 µg/ml. Results are from one representative experiment out of three. (D) Peripheral blood monocyte adherence was measured as above with or without neutralizing Ab to integrin β2 and rPSGL-1 antagonist. *p ≤ 0.01, ****p ≤ 0.0001 versus untreated cells; #p ≤ 0.05, ‡p ≤ 0.001 versus no inhibitor.
abolished adhesion of monocytes to HLA-A2 hIgG1–treated HAEC, demonstrating the cooperative effect of these signals (Fig. 5D). In conclusion, these experiments demonstrate that human HLA I Abs can trigger FcγR-, P-selectin–, and ICAM-1–dependent recruitment of peripheral blood monocytes by endothelial cells.

HLA I Abs from sensitized transplant recipients stimulate binding of monocytes

Finally, we purified the IgG fraction of sera from highly sensitized transplant recipients and tested the effect of polyclonal HLA-specific hIgG on endothelial cell recruitment of monocytes. Binding of IgG from HLA I Ab⁺ sera to HAEC was confirmed using cell-based ELISA (data not shown). For subsequent experiments, we selected a concentration of HLA-reactive IgG with similar binding capacity as the unfractionated serum diluted 1:4 on endothelial cells from two different donors.

Adhesion of peripheral blood monocytes to HAECE exposed to polyclonal HLA I–reactive IgG or unfractionated sera at varying concentrations was measured. Stimulation of HAEC with unfractionated serum diluted 1:4 significantly increased adhesion of monocytes, as did HLA-specific IgG (0.2, 0.4, or 1.7 mg/ml) in a dose-dependent manner (Fig. 6A) compared with IgG from a negative control serum. Antagonism of FcγRs or FcγRI alone significantly attenuated monocyte binding by 52.3 and 46.6%, respectively (Fig. 6B). Moreover, inhibition of P-selectin, ICAM-1, or β2 integrin reduced monocyte binding by 46.6, 69.5, and 82.6%, respectively (Fig. 6B). Finally, combined blockade of both β2 integrin and P-selectin fully abolished monocyte binding to background levels (Fig. 6C).

Discussion

Taken together, our findings point to a complex model of monocyte–endothelial cell interactions during AMR. We propose that crosslinking of HLA I is a universal function of HLA I Abs irrespective of subclass (including hIgG2 and IgG4) and is sufficient to increase P-selectin and support an intermediate level of monocyte tethering through PSGL-1 (Fig. 7A). Complement-fixing subclasses of donor-specific Ab such as hIgG1 or hIgG3 can concurrently engage FcγRI (and to a lesser extent FcγRIIa) to amplify P-selectin–mediated adhesion of monocytes, intensifying infiltration during AMR (Fig. 7B). We speculate that both P-selectin–PSGL-1 and Ab–FcγR interactions promote firm adhesion to ICAM-1 by activating monocyte Mac-1 integrin.

Although macrophage infiltration frequently accompanies AMR and intragraft macrophages are associated with poor outcome, the mechanisms by which HLA I Abs promote mononuclear cell re-

![FIGURE 6.](http://www.jimmunol.org/)

Anti-HLA Abs from sensitized transplant recipients stimulate endothelial recruitment of peripheral blood monocytes. (A) HAEC (H126) were stimulated with unfractionated anti–HLA I-containing serum (diluted 1:4), the IgG fraction from anti–HLA I serum, or with the IgG fraction of a negative serum at the indicated concentrations for 30 min. Adherence of peripheral blood monocytes was measured. Results are representative of two independent experiments with monocytes from two different donors. (B and C) HAEC (CAR) were stimulated with the IgG fraction of anti–HLA I⁺ serum at 1.7 mg/ml. Inhibitors were added individually (B) or in combination (C) as above and adherence of peripheral blood monocytes was measured. ****p ≤ 0.0001 versus untreated cells; *p ≤ 0.05, †p ≤ 0.01, ‡p ≤ 0.001 versus no inhibitor.
recruitment to the graft are not fully understood. We report that HLA I–specific Abs can trigger monocyte adherence through two parallel mechanisms. The Ag recognition function is mediated by the V region, and, given its bivalent structure, IgG can dimerize (crosslink) its target. Our laboratory has demonstrated that intracellular signaling induced by HLA I Abs is dependent on this crosslinking ability (60). HLA I crosslinking by Abs of different subclasses increased P-selectin on the cell surface of endothelium, a response that was not observed with non–HLA Abs or third-party human HLA I Abs. Second, the degree to which HLA I Ab stimulated monocyte adherence varied depending on the Ig subclass owing to the capacity to interact with FcγRs on the monocyte. Abs against HLA I appear to have a unique capacity to trigger endothelial cell activation in addition to the canonical Fc fragment–mediated effector functions, derived from the signaling capacity of the target HLA I, which we have previously reported to transduce signals controlling proliferation, survival, and cytoskeletal remodeling (19, 20, 63, 64).

We demonstrated that P-selectin induction in response to HLA I crosslinking was a common feature of endothelial cells derived from aorta, umbilical vein, and dermal microvasculature, although responsiveness varied among cell types. P-selectin is stored in WPb as a rapidly releasable pool, which appears at the membrane intraluminally. Since the first description of vWF localization to WPb (65), endothelial cell biologists have determined that the density and content of WPb vary among the vascular beds and tissues (66, 67), suggesting that endothelia have unequal propensity to recruit leukocytes. The various vascular beds are also differentially susceptible to AMR and transplant vasculopathy. Alloantibody deposition occurs most prominently in the cardiac microvasculature, where intravascular macrophages are also commonly observed (68). In renal AMR, complement deposition affects the arteries or the peritubular capillaries. Veins are rarely affected in chronic allograft rejection, whereas the arterial vasculature and microvasculature undergo proliferative and fibrotic changes in renal and cardiac allografts. Our results point to a central role for HLA I–induced P-selectin across vascular beds, and they suggest that a therapeutic P-selectin antagonist, such as rPSGL-1, may be clinically useful in ameliorating macrophage accumulation in the allograft during AMR.

The fusion protein rPSGL-1 effectively attenuated adhesion of monocytes to aortic, venous, and microvascular endothelial cells activated by murine or human HLA I Abs. In vivo, P-selectin is bound by and expressed by platelets, which bridge the monocyte and endothelium to support shear-resistant adherence (69, 70). HLA I Ab directly activates platelet exocytosis, promoting platelet–monocyte aggregates (71). Under physiological conditions, activated platelets are likely to play an important role in Ab-mediated

**FIGURE 7.** Proposed model of monocyte recruitment during AMR mediated by donor-specific HLA I Abs. (A) Human HLA I Abs of subclasses with low affinity for human FcγRs include IgG2 and IgG4. Crosslinking of HLA I on endothelial cells by IgG2 and IgG4 triggers endothelial cell exocytosis, leading to increased P-selectin at the cell surface. Monocytes are recruited through tethering via P-selectin/PSGL-1, which promotes firm adherence through ICAM-1/Mac-1. (B) Human HLA I Abs with high affinity for human FcγRs include IgG1 and IgG3. Abs that interact with FcγRs promote monocyte recruitment both through P-selectin as in (A) and through FcγRI- and FcγRIIa-dependent pathways. Engagement of PSGL-1 and FcγRs cooperatively augments firm adherence to ICAM-1.
leukocyte recruitment to the graft. vWF, which is present in WPb and released during HLA I Ab–triggered exocytosis (21), has been identified as an adhesive substrate for neutrophils, through both PSGL-1 and the β2 integrin Mac-1 (72, 73). These considerations highlight the potential strength of using rPSGL-1-Fc chimera as a therapeutic agent, because P-selectin is a critical initiator of the leukocyte recruitment cascade and PSGL-1 responds to a variety of ligands.

HLA I Abs were capable of both promoting P-selectin–induced recruitment and enhancing this recruitment via FcγR engagement, pointing to a unique model of HLA I Ab pathogenesis. In other models of inflammation, anti–endothelial cell Ab or immune complexes can cooperate with chemokines or selectins to augment recruitment of neutrophils by endothelial cells (74–76). Notably, Abs alone cannot support leukocyte capture on endothelial cells without concurrent expression of selectins in vitro (28, 29) or in vivo (75, 77), consistent with our finding that non–HLA Abs did not increase adherence of monocytes. Florey et al. (28) proposed that the amplification of recruitment by endothelial-bound Abs through FcγR-dependent mechanisms may cause inappropriately extensive leukocyte influx relative to the actual level of endothelial activation. In transplantation, the intragraft milieu may be altered by ischemia/reperfusion, infection, or allosreactivity, and endothelial cells may have a high background level of inflammation. Our data suggest that FcγR engagement by HLA class I Abs can augment signaling events that increase firm adhesion of monocytes to ICAM-1, as has been shown for immune complexes (30, 78–80). Owing to FcγR-mediated enhancement, anti–donor HLA I Abs or MICA Abs are likely to magnify monocyte recruitment above what would otherwise occur in response to cytokine activation of graft endothelium. Therefore, selectin tethering to PSGL-1 may synergize with FcγR engagement to produce a highly adhesive conformation of monocyte integrins.

We found that FcγRII prominently participated in recruitment by certain subclasses of HLA I Abs. Whereas FcγRIIa is extensively distributed on lymphoid and myeloid cells, FcγRII expression is generally restricted to monocytes and activated neutrophils. Moreover, despite several fold higher expression of LFA-1 compared with Mac-1 on monocytes, Mono Mac 6 and THP-1 cells almost exclusively used Mac-1 to adhere to HLA I Ab–stimulated endothelial cells. Finally, there is a well-studied signaling crosstalk between Mac-1 and FcγRs, particularly with FcγRII (72, 81, 82). For these reasons, monocytes may be more responsive to HLA Ab bound on the endothelium compared with other myeloid or lymphoid cells.

Recipient polymorphisms in FcγR may influence the extent to which FcγR-bearing cells are recruited. Although there are no known functional mutations in FcγRI, FcγRIIa (activating) exists in two major allotypes in the human population. FcγRIIa-H131 has very low affinity for the Fc portion of mlgG1 but is the only FcγR that can bind hIgG2. In contrast, FcγRIIa-R131 has increased affinity for mlgG1 but a lower overall affinity for hlgG (57, 83), and it is associated with increased susceptibility to autoimmune diseases and bacterial infection (84, 85). THP-1 cells carry both H131 and R131 alleles (86), whereas Mono Mac 6 cells are homozygous for H131. We noted a difference between THP-1 and Mono Mac 6 cells in FcγR-dependent recruitment, as well as some variation among healthy volunteer donors of the peripheral blood monocytes, possibly attributed to FcγRIIa polymorphism, suggesting that FcγRIIa polymorphism may influence cellular infiltration during AMR.

Our data indicate that the subclass of a donor-specific HLA I Ab and the FcγR(s) it engages shape the degree of infiltration of FcγR-bearing immune cells by endothelial cells. Donor-specific HLA Abs found in transplant patients can be of any of the four IgG subclasses, although IgG1 dominates (87). Recent advances in Ab detection technologies can reveal the subclass or complement-fixing capacity of donor-specific Abs (88, 89), but they raise the question of whether subclass is clinically significant to rejection risk and graft outcome. For example, complement-fixing donor-specific Abs predicted acute rejection episodes but not long-term outcome (42, 43, 90). In a murine model of AMR, both complement fixing and non–complement fixing Abs could cause chronic rejection lesions in transplanted allografts, indicating that MHC I Abs can promote proliferative changes in a complement-independent manner (91, 92), as observed in vitro (19). Our group and others reported that administration of an F(ab′)2 fragment of donor-specific MHC I Ab increased neutrophil and macrophage infiltration in vivo (21, 64) and in vitro (22). Thus, donor-specific HLA Abs may have a dual capacity to cause graft injury through both Fc-dependent and Fc-independent mechanisms.

We used monoclonal hlgG1 against two common HLA-A alleles and demonstrated increased P-selectin- and FcγR-dependent monocyte recruitment. However, we were unable to compare human subclasses, as HLA I monoclonal IgG2 and IgG4 isotypes, which have low-to-no interaction with FcγRs, are not available. Hence, there is a need to generate and study human or humanized HLA I monoclonals of multiple subclasses to assess their influence on adherence in a system that more closely mimics physiological conditions.

Our data differ from the findings of Yamakuchi et al. (21), who reported that HLA I Ab–mediated binding of HL60 cells required P-selectin but not ICAM-1. HL60 cells are less differentiated and more closely resemble neutrophils (93, 94). Mono Mac 6 cells, THP-1 cells, and primary blood monocytes express higher levels of FcγRs, CD14, and β2 integrins, including LFA-1 and Mac-1, than do HL60 cells (Ref. 28 and N.M. Valenzuela and E.F. Reed, unpublished observations). Thus, the effects of FcγRs and β2 integrins may be less evident with neutrophilic cells. Our results thus add to this work, demonstrating that basal recruitment can be enhanced by concurrent engagement of PSGL-1 and FcγRI on monocytes.

In conclusion, we have shown that endothelial cell exocytosis of P-selectin downstream of HLA I crosslinking independently promotes recruitment of monocytes. The findings reiterate the fact that donor-specific Abs have pathogenic potential due to their direct effects on the graft vasculature, which are in part unrelated to their complement-fixing or FcγR-engaging capacity. Therefore, selectin inhibition may alleviate monocyte trafficking into the allograft in response to donor-specific HLA I Abs, irrespective of subclass. Moreover, concurrent FcγR engagement by complement-fixing donor-specific Abs potentiates selectin-dependent monocyte adherence. This evidence points to an increased pathogenic potential of donor specific Abs of subclasses with high affinity for human FcγRs.

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Disclosures

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immune complex binding to the high-affinity IgG receptor, FcγRI, in the presence of monomeric IgG. *Blood* 116: 5327–5333.


