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Cross-Talk between HLA Class I and TLR4 Mediates P-Selectin Surface Expression and Monocyte Capture to Human Endothelial Cells

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Abstract

Donor-specific HLA Abs contribute to Ab-mediated rejection (AMR) by binding to HLA molecules on endothelial cells (ECs) and triggering intracellular signaling, leading to EC activation and leukocyte recruitment. The molecular mechanisms involving donor-specific HLA Ab–mediated EC activation and leukocyte recruitment remain incompletely understood. In this study, we determined whether TLRs act as coreceptors for HLA class I (HLA I) in ECs. We found that human aortic ECs express TLR3, TLR4, TLR6, and TLR10, but only TLR4 was detected on the EC surface. Consequently, we performed coimmunoprecipitation experiments to examine complex formation between HLA I and TLR4. Stimulation of human ECs with HLA Ab increased the amount of complex formation between HLA I and TLR4. Reciprocal coimmunoprecipitation with a TLR4 Ab confirmed that the crosslinking of HLA I increased complex formation between TLR4 and HLA I. Knockdown of TLR4 or MyD88 with small interfering RNAs inhibited HLA I Ab–stimulated P-selectin expression, von Willebrand factor release, and monocyte recruitment on ECs. Our results show that TLR4 is a novel coreceptor for HLA I to stimulate monocyte recruitment on activated ECs. Taken together with our previous published results, we propose that HLA I molecules form two separate signaling complexes at the EC surface, that is, with TLR4 to upregulate P-selectin surface expression and capture of monocytes to human ECs and integrin β_4 to induce mTOR-dependent firm monocyte adhesion via ICAM-1 clustering on ECs, two processes implicated in Ab-mediated rejection.

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Ab-mediated rejection (AMR) is a major obstacle for long-term survival of allograft transplants and is estimated to affect 10–20% of allografts (1–3). The production of anti-donor HLA class I (HLA I) and class II (HLA II) Abs is a risk factor for development of chronic rejection, which manifests as transplant vasculopathy (4–6). Although the development of posttransplant anti-HLA Ab is linked to transplant vasculopathy, the physiologic and pathologic effects of their binding to the endothelium of the transplanted organ have only recently been explored. Donor-specific HLA Abs contribute to the process of AMR by binding to the HLA molecules on endothelial cells (ECs) and triggering intracellular signaling networks, leading to EC activation and leukocyte recruitment. In previous studies, we showed that Ab crosslinking of HLA I molecules on ECs stimulates intracellular signaling, including FAK/Src, ERK, PI3K, and mTORC1 (7–11). HLA I triggers these signaling pathways by physically associating with integrin β_4 in ECs, an interaction that is required for HLA I Ab–induced signaling, leading to cytoskeletal remodeling, proliferation, and migration (12). Thus, integrin β_4 functions as a coreceptor for HLA I in signal transduction in ECs.

ECs contain specialized secretory granules, termed Weibel–Palade bodies (WPBs), that store von Willebrand factor (vWF) and P-selectin (13, 14), which are the best characterized constituents of WPBs. The trafficking of vWF and P-selectin to the EC surface in response to a variety of stimuli plays a critical role in converting the nonadhesive surface of ECs into a highly adhesive surface that captures blood cells, including monocytes, which are crucial in the pathogenesis of vascular inflammation in AMR (15). Indeed, vWF knockout mice display defective leukocyte attachment, in line with the involvement of P-selectin externalization in monocyte adherence (16). Our studies demonstrated that HLA I engagement with Abs directed against monomorphic or polymorphic residues on HLA I promotes P-selectin expression on the EC surface and consequent monocyte recruitment (17–19). Collectively, these results imply that these changes in the adhesive properties of the EC surface initiated by HLA I Ab play a critical role in AMR. However, the specific mechanisms by which Ab binding to HLA I induces P-selectin expression and subsequent monocyte capture by ECs remain poorly understood, and the role of integrin β_4 in these processes has not been investigated.

In the current study, we demonstrate that HLA I Abs induce P-selectin externalization and monocyte binding to ECs through an integrin β_4–independent mechanism. Given that HLA molecules lack signal motifs and kinase activity in their short cytoplasmic tail, we postulated that HLA I triggers these changes in the properties of EC surface by forming a signaling complex with other molecules. TLR4 is known to regulate P-selectin expression and vWF release on ECs in response to its ligand, LPS (20, 21). We found that TLR4 is the only TLR expressed on the EC surface and accordingly hypothesized that HLA I molecules associate with TLR4 to transduce signals that lead to P-selectin externalization and monocyte recruitment.

Pursuing this hypothesis, we found that Ab ligation of HLA I molecules on ECs stimulated the formation of a molecular complex between HLA I and TLR4. These results support the notion that HLA I molecules physically associate with TLR4 together with the adaptor proteins MyD88 and TIRAP. Our data also show that HLA I Ab–stimulated P-selectin

expression on the surface of ECs is abrogated by knocking down TLR4 or MyD88 with small interfering RNAs (siRNAs). Reciprocally, our results also imply that HLA I functions as a coreceptor for TLR4-mediated P-selectin externalization and monocyte adhesion. We conclude that HLA I forms two separate molecular complexes at the cell surface, that is, with integrin β_4 or TLR4, each of which plays a critical role to elicit signals that regulate fundamental functions in ECs that are implicated in the pathogenesis of AMR.

Materials and Methods

Abs and chemicals

Cell culture reagents were from Invitrogen. Purified allele-specific human mAbs HLA-A2/A28 (clone SN607D8, IgG1), HLA-A3/A11/A24 (clone MUL2C6, IgG1), and HLA-B12 (clone DK7C11, IgG1) were gifts from Dr. Sebastiaan Heidt. Purified mouse mAbs against HLA-A2/A28 (clone H0037, IgG) were provided by Dr. Jar-how Lee (One Lambda, Canoga Park, CA). Anti–HLA I mAb W6/32 (mouse IgG2a), recognizing a conformational epitope on all HLA-A, HLA-B, and HLA-C H chains when they are in association with β_2-microglobulin (22), was purified from cultured supernatants of the hybridoma HB-95 (American Type Culture Collection, Manassas, VA). The $F(ab')_2$ fragments of mAbs were generated by using IdeZ protease (Promega, Madison, WI) according to the manufacturer's protocol. The mouse mAb EMR8–5, recognizing the denatured H chain of HLA-A, HLA-B, and HLA-C, and anti–HLA-A2 mAb for Western blot were obtained from Medical & Biological Laboratories. The mouse mAb TLR4 (HTA125, mouse IgG2a) for immunoprecipitation was from eBioscience. Ab against integrin β_4 was purchased from BD Biosciences (San Diego, CA). Sheep anti-human P-selectin Ab and donkey anti-sheep IgG HRP-conjugated Ab were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal Abs against TLR1, TLR2, TLR3, TLR4, TLR6, TLR7, TLR8, TLR9, MyD88, and β-actin were purchased from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal Ab against β-tubulin (H-235), mAbs against TLR4, TLR5, and TLR10, MD2 (J-12B), GAPDH, goat anti-rabbit HRP, goat anti-mouse HRP IgG, and Protein A/G Plus agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal Ab against TIRAP was from Novus (Centennial, CO). The monoclonal anti-vinculin (clone Hvin-1) and mouse isotype control were from Sigma-Aldrich (St. Louis, MO). PE-conjugated Abs against TLR2, TLR3, TLR4, TLR5, and TLR10, as well as mouse IgG2a, were purchased from BioLegend. PE-conjugated Ab against TLR1 and a Vybrant CFDA SE cell tracer kit (V-12883) were purchased from Molecular Probes (Eugene, OR). Mouse mAb against TLR4 (76B357.1) for immunohistochemistry and rabbit polyclonal against TRIF (Toll/IL-1R domain–containing adaptor inducing IFN-β) were purchased from Abcam (Cambridge, MA). FITC-conjugated donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell culture

Human aortic ECs were isolated from the aortic rings of explanted donor hearts, as described previously (9), or obtained from Lonza/Clonetics (Walkersville, MD). ECs were cultured in M199 medium (Mediatech, Manassas, VA) supplemented with 20% (v/v) FBS (HyClone), penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively; both from

Invitrogen), sodium pyruvate (1 mmol/l), heparin (90 μg/ml, Sigma-Aldrich), and EC growth supplement (20 μg/ml, BD Biosciences). Cells were used for experiments from passage 3 to 8. Prior to use in experiments, cells were grown for 6 h in medium M199 containing 0.2% FBS.

The human monocytic cell line Mono Mac 6 (23, 24) (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 mg/ml insulin, sodium pyruvate, penicillin-streptomycin, and nonessential amino acids (Life Technologies).

Human peripheral blood monocytes were isolated from healthy donors as previously reported (18). Briefly, PBMCs were isolated using Ficoll-Paque density centrifugation (Eppendorf centrifuge 5810; Eppendorf, Hauppauge, NY). Total monocytes were then enriched from PBMCs using a MACS negative selection Pan Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of monocytes was > 90% as determined by flow cytometric analysis with CD14 Ab.

Coimmunoprecipitation

ECs (4×10^6 to 5×10^6) were treated with W6/32 $F(ab')_2$ for 20 min, giving results comparable to those in cells treated with whole IgG of W6/32 (7); mouse IgG2a $F(ab')_2$ was used as a negative control. The W6/32 $F(ab')_2$ fragments were used instead of the whole IgG to prevent the Fc parts from interfering with immunoprecipitation with protein A/G agarose beads. Cells were lysed in ice-cold buffer containing 1% CHAPS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF, 1.5 mM Na_3VO_4, 0.5 mM PMSF, 0.2 μg/ml aprotinin, and 0.5 μg/ml leupeptin, and cell lysates were collected after spinning down at 10,000 × *g* for 10 min. Precleared cell lysates were immunoprecipitated with anti–HLA I mAb W6/32, murine anti–HLA-A2, or TLR4 mAb (HAT125) at 4°C on a rotator overnight. Then, samples were incubated with Protein A/G Plus agarose beads for 4 h. Immunoprecipitates were washed three times with immunoprecipitation wash buffer (40 mM HEPES [pH 7.5], 10 mM β-glycerophosphate, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1.5 mM Na_3VO_4, 0.3% CHAPS, 0.2 μg/ml aprotinin, 0.5 μg/ml leupeptin), and samples were resolved using SDS-PAGE. Five percent of the total amount of protein used in the immunoprecipitation was loaded onto the gel as an input control.

Western blot

Cell lysates for Western blot were prepared as previously described (9). Briefly, cells cultured in a low-serum concentration for 6 h, stimulated with human or murine anti–HLA I mAb or control IgG at 37°C, and lysed in buffer (containing 20 mM Tris [pH 7.9], 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 10 min on ice. The cell lysates were resolved by SDS-PAGE and proteins were transferred overnight onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked using 5% nonfat dry milk in TBS (pH 7.4) containing 0.05% Tween 20 (TBST) for 1 h at room temperature and incubated overnight with appropriate primary Abs. The blots were

incubated with HRP-conjugated secondary Abs and developed with ECL (Amersham). The phosphorylated protein bands were scanned using the Epson Perfection V700 photo scanner (Epson) and were quantified using the ImageJ program (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, MD).

siRNA transfection

The human integrin β_4 siRNA (target sequence, 5′-CAG AAG AUG UGG AUG AGU UUU-3′), the siRNAs against the HLA I H chain targeting the HLA-A and HLA-B alleles (5′-GCA GAG AUA CAC CUG CCA U-3′ and 5′-GAG CUC AGA UAG AAA AGG A-3′), TLR4, MyD88, TLR3, and TRIF smart pool siRNA, and control nontargeting siRNA duplexes (5′-UAG CGA CUA AAC ACA UCA AUU-3′ and 5′-AAU UGA UGU GUU UAG UCG CUA-3′) were synthesized by Dharmacon (Lafayette, CO). ECs were plated at a density of 70% confluence and transfected with siRNA using Mirus TransIT-TKO transfection reagents (Mirus Bio, Madison, WI) according to the manufacturer's protocol. Experiments were conducted 48 h after transfection (8).

Flow cytometry determination

ECs were grown in 2 ml of complete medium in 35-mm dishes coated with 0.1% gelatin until confluent and detached by trypsin-EDTA (Life Technologies) and incubated with HLA I Ab (1 μg/ml) in 0.1 ml of PBS with 2.5% FBS and 0.1% sodium azide for 30 min on ice. Cells were washed twice with PFA and incubated with FITC-conjugated anti-mouse secondary Ab at 1:100 (Jackson ImmunoResearch Laboratories) used to detect HLA I molecule expression. Alternatively, ECs or monocytes were incubated with PE-conjugated TLR1, TLR2, TLR3, TLR4, TLR5, or TLR10 Abs. Cell fluorescence was measured by flow cytometry on a LSRFortessa cytometer, calculated with the BD FACSDiva program (Becton Dickinson, Mountain View, CA), and analyzed with FlowJo v10 software.

Cell surface P-selectin expression

Cell surface P-selectin expression on ECs was detected by cell-based ELISA as described previously (18). Briefly, ECs were cultured in 35-mm dishes to 70% confluence and transfected with siRNA for 24 h. Transfected cells were detached with Accutase, seeded in a 96-well plate to confluence for 48 h, and treated with Ab against HLA I or mouse IgG2a as an isotype control for 30 min. Cells were fixed with freshly prepared 2.5% PFA in PBS for 5 min at room temperature. Unpermeabilized cells were blocked with 5% BSA in PBS and incubated with sheep anti–P-selectin Ab (1 μg/ml) for 2 h followed by incubation with anti-sheep HRP secondary Ab at 1:1000 for 2 h at room temperature. After washing three times, tetramethylbenzidine substrate was added into wells and OD was read at 650 nm on a SpectraMax plate reader (Molecular Devices).

Blockade of FcγRs

FcγRs were blocked as previously described (18). Briefly, in monocyte adhesion experiments, monocytes or Mono Mac 6 cells were incubated with 10 μg/ml purified human IgG in PBS for 15 min to block the FcγRs from binding HLA I IgG on the surface of ECs.

Monocyte adhesion assay

Monocyte adhesion on ECs was described previously (25, 26). Briefly, ECs were cultured in 24-well plates coated with 0.1% gelatin and were transfected with siRNA and treated with Abs against HLA I, or HLA-A2, HLA-A3, or HLA-B12. CFSE (Invitrogen)-labeled primary monocytes or Mono Mac 6 were added onto EC monolayers at a ratio of approximately three monocytes per EC for 20 min at 37°C. After washing three times, adherent monocytes were imaged by fluorescence microscopy (Nikon Eclipse Ti) with magnification ×4 and analyzed with software CellProfiler or ImageJ. Results are expressed for each condition as fold change in mean of adhesion monocytes per field normalized to control cells ± SEM.

Immunofluorescence staining

Indirect immunofluorescence analysis of cell surface molecules was previously described (12). For P-selectin expression and vWF release, ECs were transfected with TLR3, TRIF, TLR4, MyD88, or control siRNA (100 nM each) for 48 h, or pretreated with TLR4 inhibitor TAK242, and cells were stimulated with an anti–HLA I Ab $F(ab')_2$ fragment or a mouse IgG2a $F(ab')_2$ fragment (1 μg/ml) for 30 min. Cells were fixed with freshly prepared 4% PFA, and surface P-selectin was stained on unpermeabilized cells with mouse anti-human P-selectin mAb and rabbit anti-human vWF Ab followed by Alexa Fluor 488 rabbit anti-mouse IgG1, Alexa Fluor 594 goat anti-rabbit IgG (H1L), and DAPI. Images were obtained with a Zeiss LSM 880 fluorescence microscope using a ×40 objective lens.

Statistical analysis

Data are presented as mean ± SEM. Differences were calculated using a one-way ANOVA with Tukey's multiple comparison test, or a Student *t* test. A p value <0.05 was considered significant.

Results

Ligation of HLA I by Abs induces P-selectin externalization through an integrin β_4–independent pathway

We previously reported that HLA I molecules physically associate with integrin β_4 in ECs, an interaction that is required for HLA I Ab–induced signaling, leading to EC proliferation and migration (12). Given that HLA I Abs also trigger WPB exocytosis and adherence of monocytes (18, 25), we asked whether integrin β_4 was similarly required for eliciting this effect. To answer this question, we knocked down integrin β_4 with siRNA in primary ECs and tested whether P-selectin surface expression and monocyte adherence were reduced after HLA I Ab stimulation. Transfection with siRNA targeting integrin β_4 drastically diminished integrin β_4 protein levels without affecting Akt or ERK levels (Fig. 1A), or cell surface HLA I expression (Fig. 1B). In line with previous results, ECs treated with either anti–HLA-A2 Ab or anti–HLA-B12 Ab significantly upregulated cell surface P-selectin (Fig. 1C). The salient feature of the data shown in Fig. 1 is that ECs with significantly reduced integrin β_4 expression retained the capacity to exocytose P-selectin (Fig. 1C) as compared with cells transfected with nontargeting siRNA. Concordantly, knockdown of integrin β_4 had no effect on HLA I Ab-induced monocyte adherence (Fig. 1D), which was not significantly different

compared with control siRNA conditions (Fig. 1E). These experiments indicate that HLA I induces P-selectin externalization through an integrin β_4–independent pathway and raised the possibility that HLA molecules interact with other, as yet unidentified coreceptors to rapidly upregulate cell surface P-selectin expression and promote monocyte adherence.

HLA I physically associates with TLR4

Several previous studies suggest that HLA II is functionally linked to TLRs in innate immune cells (27–31), but it is not known whether HLA I interacts physically and functionally with TLRs in ECs. As a first step to explore this possibility, we determined the protein expression of different TLRs in ECs and used human monocytes or Ramos cells, which are known to express multiple TLRs, as positive controls. ECs expressed TLR3, TLR4, TLR5, and TLR10, as shown by Western blot analysis. In contrast, TLR1, TLR2, TLR6, TLR7, TLR8, or TLR9 was not detected (Supplemental Fig. 1A). Importantly, only TLR4 was detected on the surface of ECs by flow cytometry (Supplemental Fig. 1B). In addition, ECs express the Toll/IL-1R domain–containing adaptors TIRAP, MyD88, and TRIF, but not TRAM, the MyD88-independent component of TLR4 signaling (Supplemental Fig. 1C, 1D).

The preceding results prompted us to determine whether any of the endothelial-expressed TLRs physically associates with HLA I, as judged by coimmunoprecipitation. ECs were left untreated or stimulated with HLA I Ab. TLR4, which is the only TLR expressed on the surface of ECs, was also the only TLR detected when HLA I was pulled down (Fig. 2A). In addition, MD2, MyD88, and TIRAP were also found in the complex with HLA I, and their association was increased in cells treated with HLA I Ab (Fig. 2A). TIRAP is an adaptor protein that connects MyD88 with TLR4 (32). In contrast, TRIF was not detected in HLA I immunoprecipitates (Fig. 2A). Similar results were obtained when cells were challenged with an Ab that binds HLA-A2 (Fig. 2B). These results suggest that HLA I physically associates with TLR4 in ECs at the cell surface. Reciprocal experiments using TLR4 pulldown confirmed that HLA I associated with TLR4 (Fig. 2C, 2D). Because we demonstrated previously that HLA I interacts physically with integrin β_4, we asked whether this integrin was present in the HLA I/TLR4 complex. Integrin β_4 was not detected in TLR4 immunoprecipitates (Fig. 2C). Similarly, pulldown of integrin β_4 yielded an increased association with HLA I in response to HLA I stimulation, but TLR4 was not detected (Fig. 2E). Furthermore, knockdown of integrin β_4 did not alter the association of TLR4 with HLA I (Fig. 2F) despite extensive loss of integrin β_4 protein (Fig. 2G). These experiments used coimmunoprecipitation of endogenous rather than expressed tagged proteins because molecular complexes between expressed proteins of the TLR system might represent artifacts of protein overexpression (33). Our results indicate that HLA I molecules form two separate signaling complexes at the cell surface, that is, with either integrin β_4 or TLR4.

Knockdown of TLR4 impairs HLA I–induced P-selection expression at the surface of ECs

Because HLA I promotes P-selectin expression at the cell surface in an integrin β_4–independent manner, we tested whether the HLA I/TLR4 complex, identified in the current study, mediates P-selectin externalization. To determine whether TLR4 was required for

EC exocytosis of P-selectin in response to HLA I Abs, we tested P-selectin expression under TLR4 or MyD88 knockdown with siRNA. TLR4 siRNA specifically reduced TLR4 expression, but not HLA I or signaling adaptor molecules (Fig. 3A, 3C). Similarly, MyD88 siRNA reduced MyD88 protein but not TLR4 or HLA I (Fig. 3B, 3D). When either TLR4 or MyD88 was knocked down, HLA I Ab treatment failed to elicit P-selectin exocytosis (Fig. 3E). As a control, we verified that LPS-induced P-selectin was also abrogated under TLR4 or MyD88 deficiency (Fig. 3F). These results were substantiated using immunofluorescence imaging of cell surface P-selectin and externalized vWF, another component stored in the WPB. Neither VWF nor P-selectin was externalized by HLA I Ab in ECs that had been treated with TLR4 or MyD88 siRNAs (Fig. 3G). A pharmacological inhibitor of TLR4, TAK242 (34), also blocked HLA I Ab-induced P-selectin presentation (Fig. 3G). In contrast, siRNA targeting TLR3 or TRIF had no detectable effect (Fig. 3G).

TLR4 associates with HLA I to increase monocyte adherence to ECs

We and others previously showed that HLA Ab-induced P-selectin supported increased adherence of monocytes and platelets to endothelium (18, 25, 35). In this study, we demonstrate that HLA I induces P-selectin externalization through a novel interaction with TLR4. Consequently, we tested whether loss of TLR4 impairs P-selectin externalization and dampened monocyte adhesion to the endothelium. First, we corroborated that treatment of ECs with HLA I Ab markedly increased monocyte adhesion (2.7-fold). Similar results were obtained in ECs stimulated with either the TLR4 agonist LPS or thrombin, as positive controls, but not elicited by treatment with mouse IgG2a or non-HLA Ab CD105 (Fig. 4A, 4B). As observed with P-selectin surface expression, siRNA knockdown of TLR4 or MyD88, or pretreatment with the TLR4 inhibitor TAK242, markedly reduced monocyte adhesion to endothelium stimulated with HLA I Abs (Fig. 4C, 4D). In contrast, siRNA against TLR3 or TRIF had no effect (Fig. 4C, 4D). As controls, we found that TLR4 or MyD88 siRNA blocked monocyte adherence to LPS-stimulated endothelium, whereas siRNA-mediated knockdown of TLR3 or TRIF did not produce any appreciable inhibitory effect (Fig. 4C, 4E).

Given the results shown in Fig. 4, it was important to rule out that the effects of HLA I Ab were due to LPS contamination. To this end, we tested whether polymyxin B, a potent LPS neutralizer, has any effect on monocyte binding to ECs in response to Ab-mediated crosslinking of HLA I. Initially, we verified that pretreatment with polymyxin B completely blocked LPS-stimulated monocyte adhesion (Fig. 5A, 5B). Next, we excluded the presence of TLR4 ligand in our HLA Ab preparations by testing monocyte adherence induced by HLA Ab in the absence or presence of polymyxin B (Fig. 5C), at the concentration that neutralized LPS. Although purified LPS itself increased monocyte adherence to endothelium, polymyxin B did not significantly affect HLA I Ab-induced monocyte binding (Fig. 5C, 5D). In addition, ligation of HLA I with the mAb W6/32 stimulated an increase in monocyte adhesion on ECs transfected with nontargeting siRNA (Fig. 5C). Knockdown of TLR4 or MyD88 with siRNA or pretreated with TAK242 inhibited HLA Ab-induced monocyte adhesion to ECs (Fig. 5C). Similar to W6/32 treatment alone, W6/32 treated with polymyxin B also stimulated increased monocyte adhesion on ECs transfected with control siRNA, which eliminated the possibility of contamination by LPS. Knockdown of TLR4

or MyD88 with siRNA or pretreated with TAK242 inhibited PB + HLA I Ab-stimulated monocyte adhesion on ECs. Both W6/32 and W6/32 + polymyxin B–mediated monocyte adhesion was not affected by TLR3 siRNA and TRIF siRNA (Fig. 5C, 5D).

HLA I is required for TLR4-mediated P-selectin externalization and monocyte binding

Thus far, our results demonstrated that TLR4 is a coreceptor for HLA I mediating rapid Ab-induced P-selectin externalization and monocyte adhesion. We next interrogated whether HLA I was reciprocally required for TLR4-triggered endothelial responses elicited by LPS. Stimulation of ECs with LPS resulted in complex formation between HLA I, MyD88, and TLR4, but not TRIF, as shown by coimmunoprecipitation of HLA I (Fig. 6A) and reciprocal pulldown of TLR4 (Fig. 6B). Knockdown of HLA I in ECs prevented LPS-induced externalization of P-selectin and vWF release (Fig. 6C), as well as monocyte adherence to endothelium (Fig. 6D, 6E). In contrast, we found that knockdown of HLA I did not prevent expression of ICAM-1 or E-selectin in response to treatment with LPS for 18 h (data not shown). Collectively, our results demonstrate that HLA I functions as a coreceptor for TLR4-mediated rapid P-selectin externalization and monocyte adhesion.

Discussion

Donor-specific HLA Abs can contribute to AMR by binding to the HLA molecules on the surface of ECs and triggering intracellular signaling, leading to EC activation and leukocyte recruitment. Despite the importance of understanding these processes to identify novel targets to prevent or treat AMR, the molecular mechanisms involved remain incompletely understood. Our previous work demonstrated that HLA I forms a molecular complex with integrin β_4 in response to Ab binding (12). Integrin β_4, acting as a coreceptor, mediates HLA I–induced signaling pathways, including Src/FAK, ERK, and PI3K/AKT/mTORC1, leading to EC proliferation and migration. In addition to these signaling pathways and cellular responses, HLA I crosslinking also elicits rapid trafficking of P-selectin to the surface of ECs, which plays a pivotal role in the capture of monocytes that are critical in the pathogenesis of vascular inflammation in AMR (15). Surprisingly, we show in the present study that integrin β_4 is not required for surface expression of P-selectin and monocyte capture to ECs in response to HLA I crosslinking. These new findings prompted us to hypothesize that in addition to integrin β_4, HLA I coopts other surface molecules that have the capacity to transduce intracellular signals that elicit P-selectin externalization and monocyte recruitment.

A recent study demonstrated that TLR4 mediates P-selectin externalization in ECs in response to intravascular hemolysis (21) and previous studies indicated that HLA II interacts functionally with TLRs (27, 28), but it was not known whether HLA I interacts physically and functionally with these receptors in ECs. We therefore focused on TLRs as possible mediators of HLA I–induced P-selectin expression and monocyte adhesion to ECs. As a first step to investigate this possibility, we found that ECs express TLR3, TLR4, TLR5, and TLR10. Of significance, TLR4 was the only TLR detected on the EC surface. Consequently, we performed coimmunoprecipitation experiments to examine whether HLA I and TLR4 form a physical complex in these cells. Stimulation of ECs with anti-HLA Ab increased the

amount of complex formation between HLA I and TLR4, as shown by Western blot analysis of either HLA I or TLR4 immunoprecipitates. These findings prompted us to determine whether HLA I couples with TLR4 to induce P-selectin externalization, vWF release, and monocyte adhesion to ECs.

We found that siRNA-mediated knockdown of TLR4 inhibited HLA I Ab-stimulated P-selectin expression, vWF release, and monocyte recruitment on ECs, strongly implicating TLR4 in HLA I signal transduction. TLR4 is the only TLR that signals both through MyD88 to induce proinflammatory cytokines and via TRIF to induce type I IFN (36). Of significance, TLR4/MyD88 signals from the cell surface, whereas signaling from TLR4/TRIF occurs from endosomes (37). Accordingly, the new signaling complex identified in this study between HLA I and TLR4 signals from the EC surface and contains MyD88. Furthermore, siRNA-mediated knockdown of MyD88 prevented P-selectin surface expression induced by Ab binding to HLA I as effectively as knockdown of TLR4 or treatment with the TLR4 inhibitor TAK242. Our results imply that TLR4 is a novel coreceptor for HLA I to stimulate P-selectin externalization and monocyte capture on activated ECs, although the interaction between anti–class I Ab, HLA-I, and TLR4 may activate additional mechanisms that increase monocyte adhesion that remain to be identified. Taken together with our previous results (12), we propose that in response to Ab binding, HLA I molecules form two separate signaling complexes at the surface of ECs, that is, one with integrin β_4 and another with TLR4/MyD88 to regulate different EC functions of great significance in the pathogenesis of AMR. A corollary of this conclusion is that the TLR4/MyD88 emerges as a new target for therapeutic interventions in the context of AMR.

Our data also reveal that TLR4 and HLA I form a molecular signaling complex in response to bacterial LPS promoting endothelial P-selectin exocytosis and leukocyte recruitment. These results implicate HLA I molecules in the generation of innate immune responses. Consistent with this possibility, associations between MHC molecules and TLRs have been reported to enhance TLR-mediated cellular activation and antimicrobial effector mechanisms. Ab ligation of HLA II on human monocytes induced MyD88 expression followed by NF-kB activation and proinflammatory cytokine production (29). Deficiency in MHC class II expression by macrophages and dendritic cells attenuated the production of proinflammatory cytokines and type I IFN triggered by TLR3, TLR4, or TLR9 signaling (30). Furthermore, coexpression of HLA II molecules and TLR4 enhanced macrophage production of the antimicrobial peptide human β-defensin 2 after treatment with LPS (27). Moreover, bacterial superantigen exotoxins, signaling through HLA II, upregulate the transcription and membrane expression of TLR4 in human monocytes (31). Further elucidation of molecular interactions between HLA I and TLR4 may well provide new insights into the regulation of inflammatory responses to infectious pathogens in the vasculature.

One of the most intriguing questions raised by the identification of two separate signaling complexes induced by Ab-induced HLA I activation is their functional interaction in the context of AMR. Substantial evidence indicates that surface-expressed P-selectin and ICAM-1 cooperate in promoting monocyte adhesion to the endothelium during their recruitment. Previously, we demonstrated that treatment of ECs with HLA I Ab not only

induces P-selectin surface expression but also firm adhesion of monocytes via ICAM-1 (17). These studies revealed that mTOR plays a critical role in promoting ICAM-1–mediated firm monocyte adhesion whereas Ca^{2+} flux stimulates P-selectin externalization. Given the results presented in the current study, we speculate that the two signaling complexes formed by HLA I play a key role in orchestrating the cooperative interaction between P-selectin surface expression and ICAM-1 in monocyte recruitment. Specifically, the HLA I/integrin β_4 complex mediates mTOR-dependent firm monocyte adhesion via ICAM-1 while the HLA I/TLR4 complex identified in this study mediates the P-selection externalization, the first step in monocyte interaction with ECs. Thus, we postulate that the concomitant functions of both HLA I signaling complexes cooperate in inducing monocyte recruitment, a critical event in the inflammatory cascade implicated in AMR.

In conclusion, donor-specific HLA Abs are increasingly recognized to contribute to AMR by binding to HLA molecules on the surface of ECs and triggering intracellular signaling pathways leading to EC activation and leukocyte recruitment, but the molecular mechanisms involved remain incompletely understood. The mechanistic experiments presented in the present study identify, to our knowledge for the first time, TLR4 as novel coreceptor for HLA I to upregulate rapid P-selectin surface expression and stimulate monocyte recruitment on activated ECs. Taken together with our previous studies, we propose that HLA I molecules form two separate physical complexes at the EC surface, that is, with either integrin β_4 or TLR4, thereby mediating an array of signaling pathways leading to EC activation. We postulate that the concomitant functions of both signaling complexes formed by HLA I in ECs cooperate in inducing monocyte recruitment, a critical event in the inflammatory cascade implicated in AMR. A corollary of this conclusion is that the novel HLA I/TLR4/MyD88 signaling axis in ECs emerges as a new target for therapeutic intervention to prevent monocyte recruitment to the graft and prevent AMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article:

AMR	Ab-mediated rejection

EC	endothelial cell
HLA I	HLA class I
HLA II	HLA class II
PFA	paraformaldehyde
siRNA	small interfering RNA
TRIF	Toll/IL-1R domain–containing adaptor inducing IFN-β
WPB	Weibel–Palade body
vWF	von Willebrand factor

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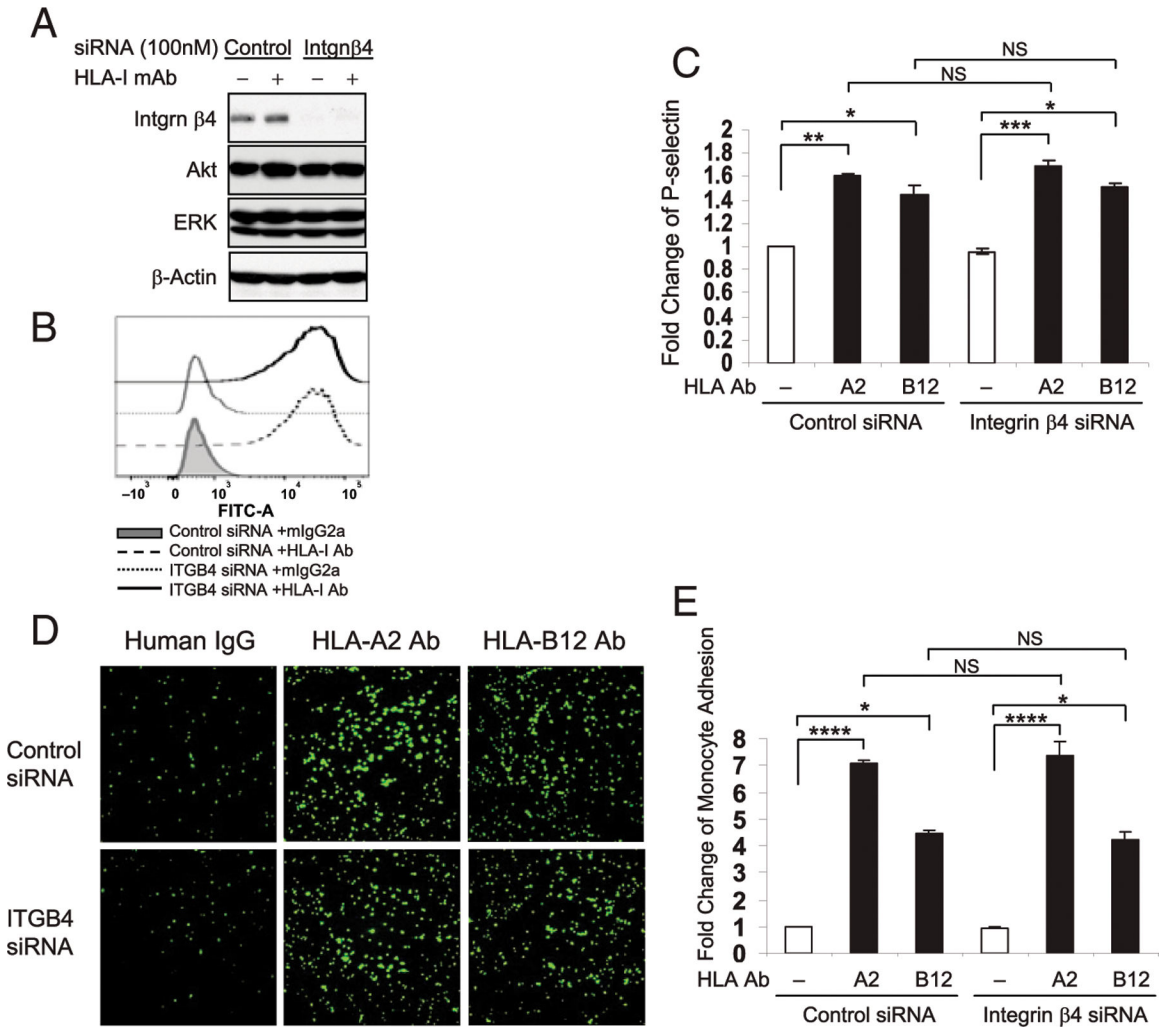

FIGURE 1.

HLA I Ab induces P-selectin expression and monocyte adhesion through an integrin β_4–independent pathway on human ECs. (**A**) ECs were transfected with 100 nM single duplex siRNA against the integrin β_4 subunit (sequence, 59-GAGAGCAGCUUCCAAAUCA-39) or with nontargeting control siRNA and were incubated for 48 h. Cells were lysed and subjected to Western blotting analysis with Abs against integrin β_4 (205 kDa), Akt, and ERK and with β-actin as a loading control. (**B**) Expression of HLA I on the surface of ECs transfected with siRNAs was analyzed by flow cytometry with the HLA I–specific Ab W6/32 or isotype control mouse IgG2a. Negative control cells transfected with nontargeting control siRNA + mouse IgG2a are shown as a filled peak in half-offset histography, whereas the dashed line represents cells transfected with control siRNA + HLA I Ab, the dotted line represents cells transfected with integrin β_4 siRNA + mouse IgG2a, and the thick solid line represents cells transfected with integrin β_4 siRNA + HLA I Ab. (**C**) ECs were transfected with integrin β_4–specific siRNA or control siRNA (100 nM each). After 48 h, cells were stimulated with anti–HLA-A2 or anti–HLA-B12 human mAb (1 μg/ml) for 30 min. Cell surface P-selectin was stained on unpermeabilized cells with sheep anti-human P-selectin Ab followed by donkey anti-sheep HRP-conjugated secondary Ab, detected by

adding tetramethylbenzidine substrate and measuring the OD at 650 nm. The bar graph shows the fold change of the mean ± SEM of the OD value from triplicate wells for each condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ analyzed by one-way ANOVA with Tukey's multiple comparison test. Results represent at least three independent experiments. (**D** and **E**) ECs were transfected with integrin β_4–specific siRNA or control siRNA (100 nM each). ECs were stimulated with HLA-A2 or HLA-B12 human mAb (1 μg/ml) for 30 min. CFSE-labeled Mono Mac 6 (MM6) with an FcγR blockade was added to HLA I Ab-stimulated EC monolayers. Adherence of MM6 cells was measured by a Nikon Eclipse Ti live cell inverted microscope with a ×4 objective lens and (E) analyzed with CellProfiler. Bar graph represents fold change of adherent MM6 mean ± SEM from at least three independent experiments. *$p < 0.05$, ****$p < 0.0001$ analyzed by one-way ANOVA with a Tukey's multiple comparison test.

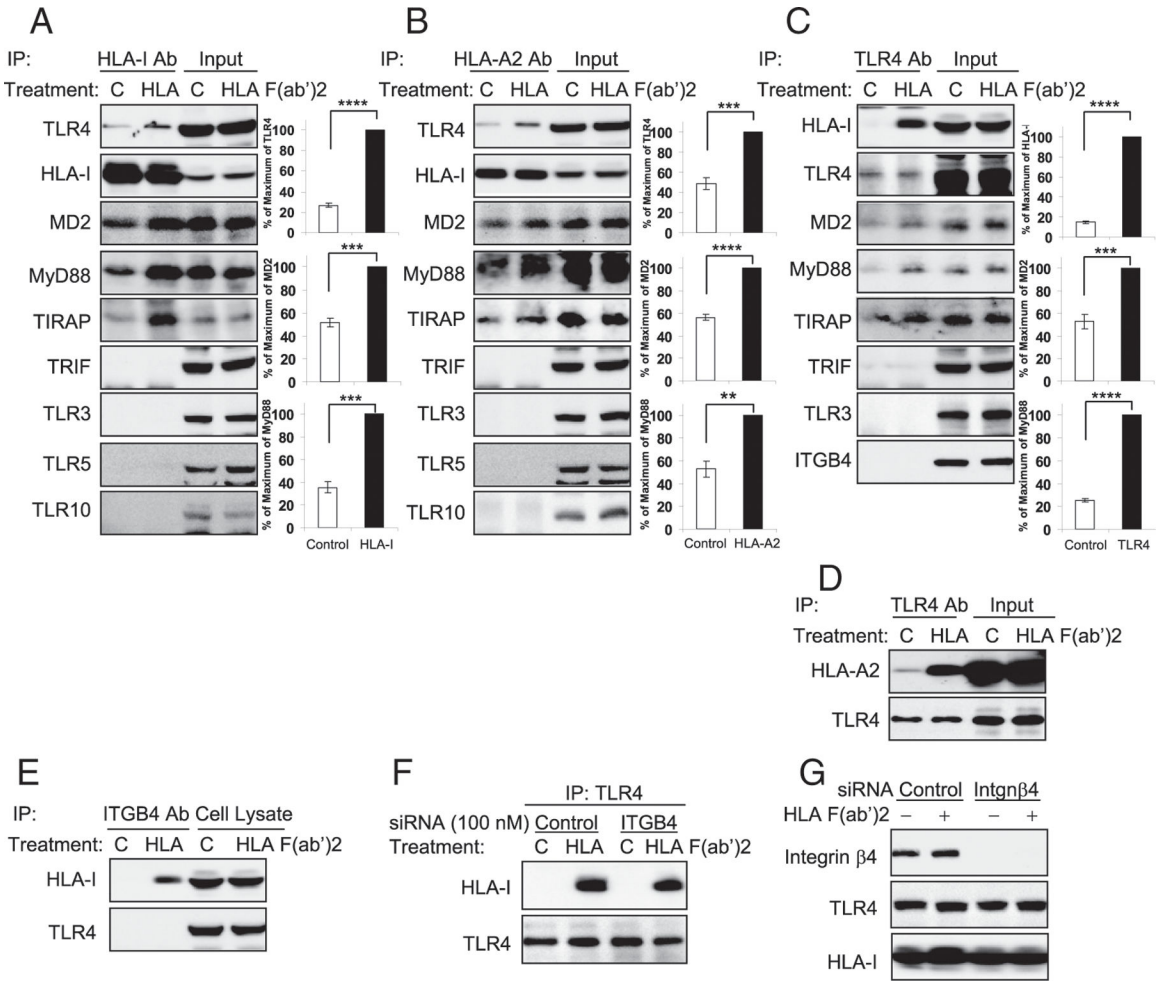

FIGURE 2.

Ligation of HLA I on ECs with Ab triggers formation of a signal complex containing TLR4 and HLA I. (**A**–**C**) ECs were stimulated with the $F(ab')_2$ fragments of W6/32 (a pan Ab against HLA I) or with a control mouse IgG2a $F(ab')_2$ fragment for 20 min. Cell lysates were subjected to immunoprecipitation (IP) with (A) W6/32, (B) anti–HLA-A2 human mAb, (C) TLR4 Ab (HTA125) and analyzed by Western blotting with Abs against the H chain of HLA I (EMR8–5), TLR4 (120 kDa), MyD88 (33 kDa), MD2 (20–25 kDa), TIRAP (30 kDa), TRIF (66 kDa), TLR3 (115 kDa), TLR5 (110–120 kDa), TLR10 (90 kDa), or (C) integrin β_4 (205 kDa) as indicated to the left of the blots. For each sample, 5% of the total cell lysate that was used in the immunoprecipitation was loaded as an input control. Data are representative of three independent experiments. Protein bands (A and B) TLR4, MD2, and MyD88 and (C) HLA I, MD2, and MyD88 were quantified by densitometry scan and the results are expressed as the mean ± SEM percentage of the maximal extent of protein. The data presented in (A)–(C) are representative of at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ analyzed by Student *t* test. (**D**) ECs were stimulated as described in (A), cell lysates were immunoprecipitated with an Ab against TLR4 (HTA125), and samples were analyzed by Western blotting with the HLA-A2 Ab (BB7.2) or TLR4 Ab. For each sample, 5% of the total cell lysate that was used in

the immunoprecipitation reaction was loaded as an input control. Data are representative of three independent experiments. (**E**) ECs were stimulated as described in (A), cell lysates were immunoprecipitated with an integrin β_4 Ab, and samples were analyzed by Western blotting with the HLA I H chain (EMR8–5) or TLR4 Ab. For each sample, 5% of the total cell lysate that was used in the immunoprecipitation reaction was loaded as an input control. Data are representative of three independent experiments. (**F** and **G**) ECs were transfected with 100 nM integrin β_4 siRNA or control siRNA. After 48 h, ECs were stimulated with the $F(ab')_2$ fragments of W6/32 or with control IgG2a $F(ab')_2$ for 20 min. (F) Cell lysates were immunoprecipitated with an Ab against TLR4 (HTA125), and samples were analyzed by Western blotting with Ab against HLA I H chain EMR8–5 or TLR4 Ab. Data are representative of two independent experiments. (G) ECs were subjected to Western blotting analysis with Abs against integrin β_4, TLR4, and HLA I (EMR8–5). Data are representative of three independent experiments.

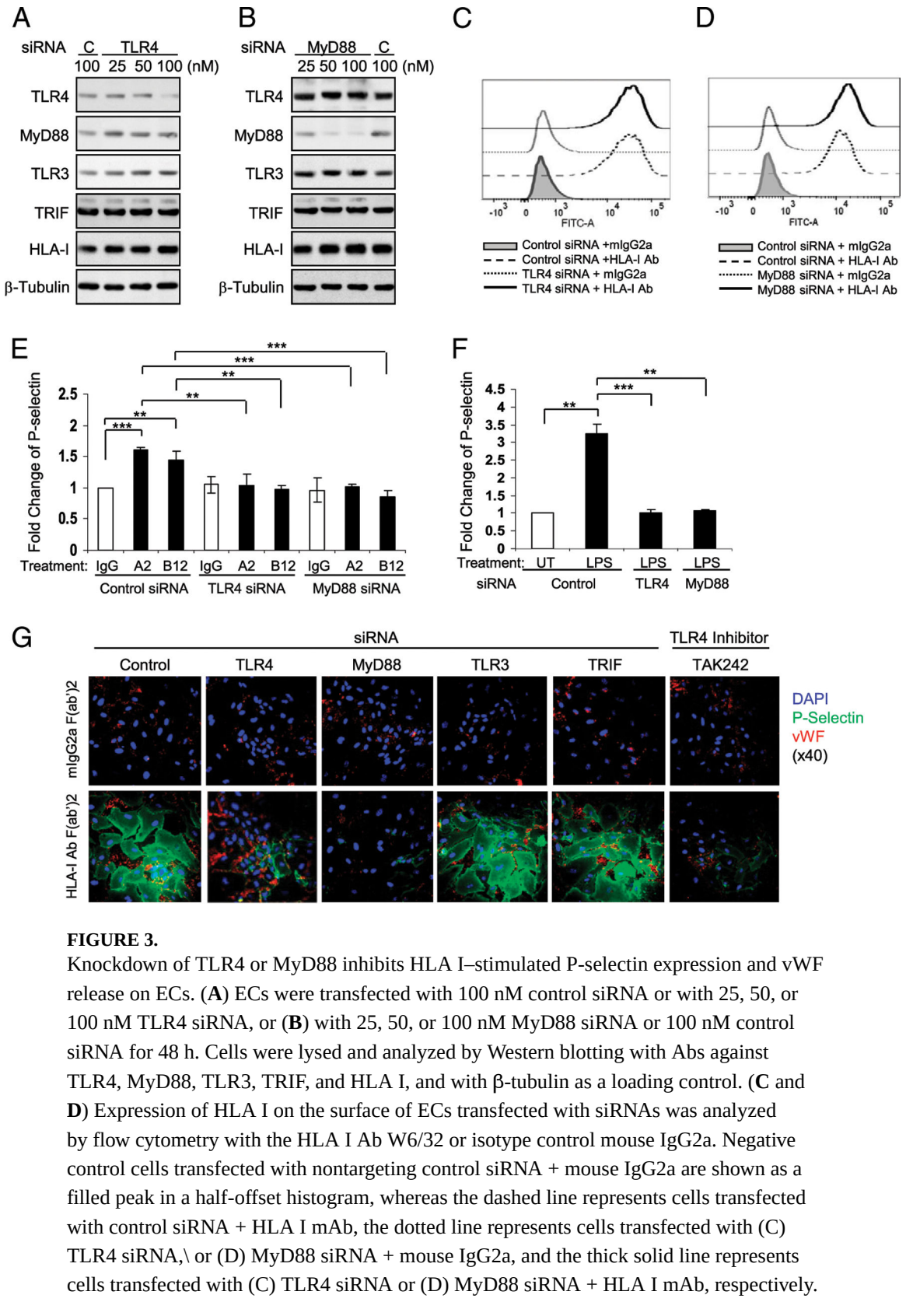

FIGURE 3.

Knockdown of TLR4 or MyD88 inhibits HLA I–stimulated P-selectin expression and vWF release on ECs. (**A**) ECs were transfected with 100 nM control siRNA or with 25, 50, or 100 nM TLR4 siRNA, or (**B**) with 25, 50, or 100 nM MyD88 siRNA or 100 nM control siRNA for 48 h. Cells were lysed and analyzed by Western blotting with Abs against TLR4, MyD88, TLR3, TRIF, and HLA I, and with β-tubulin as a loading control. (**C** and **D**) Expression of HLA I on the surface of ECs transfected with siRNAs was analyzed by flow cytometry with the HLA I Ab W6/32 or isotype control mouse IgG2a. Negative control cells transfected with nontargeting control siRNA + mouse IgG2a are shown as a filled peak in a half-offset histogram, whereas the dashed line represents cells transfected with control siRNA + HLA I mAb, the dotted line represents cells transfected with (C) TLR4 siRNA,\ or (D) MyD88 siRNA + mouse IgG2a, and the thick solid line represents cells transfected with (C) TLR4 siRNA or (D) MyD88 siRNA + HLA I mAb, respectively.

Data are representative of three independent experiments. (**E**) ECs were transfected with TLR4 siRNA, MyD88 siRNA, or control siRNA (100 nM each). After 48 h, cells were stimulated with anti–HLA-A2 or anti–HLA-B12 human mAb (1 μg/ml) for 30 min. The cells were fixed with freshly prepared 4% PFA, and cell surface P-selectin was stained on unpermeabilized cells with sheep anti-human P-selectin Ab followed by donkey anti-sheep HRP secondary Ab, detected by adding tetramethylbenzidine substrate and measuring the OD at 650 nm. Bar graph shows fold change of the mean ± SEM of OD from triplicate wells for each condition. $^{**}p < 0.01$, $^{***}p < 0.001$ analyzed by one-way ANOVA with a Tukey's multiple comparison test. Data are representative of at least three independent experiments. (**F**) ECs were transfected with TLR4 siRNA, MyD88 siRNA, or control siRNA (100 nM each). After 48 h, cells were stimulated with LPS (0.1 μg/ml) for 30 min. Cell surface P-selectin was stained on unpermeabilized cells with sheep anti-human P-selectin Ab followed by anti-sheep HRP secondary Ab, detected by adding tetramethylbenzidine substrate and measuring OD at 650 nm. Bar graph shows fold change of the mean ± SEM of OD from triplicate wells for each condition. $^{**}p < 0.01$, $^{***}p < 0.001$ analyzed by one-way ANOVA with a Tukey's multiple comparison test. Data are representative of at least three independent experiments. (G) ECs were transfected with TLR3, TRIF, TLR4, MyD88, or control siRNA (100 nM each) for 48 h, or pretreated with TLR4 inhibitor TAK242 (1.0 μM) for 30 min, and cells were stimulated with anti–HLA I Ab $F(ab')_2$ fragment or mouse IgG2a $F(ab')_2$ fragment (1 μg/ml) for 30 min. Cells were fixed with freshly prepared 4% PFA, and surface P-selectin was stained on unpermeabilized cells with mouse anti-human P-selectin mAb (mouse IgG1) and rabbit anti-human vWF Ab followed by Alexa Fluor 488 rabbit anti-mouse IgG1, Alexa Fluor 594 goat anti-rabbit IgG (H1L), and DAPI. Six middle fields per dish were imaged with a Zeiss LSM 880 fluorescence microscope using a ×40 objective lens. Data are representative of at least three independent experiments.

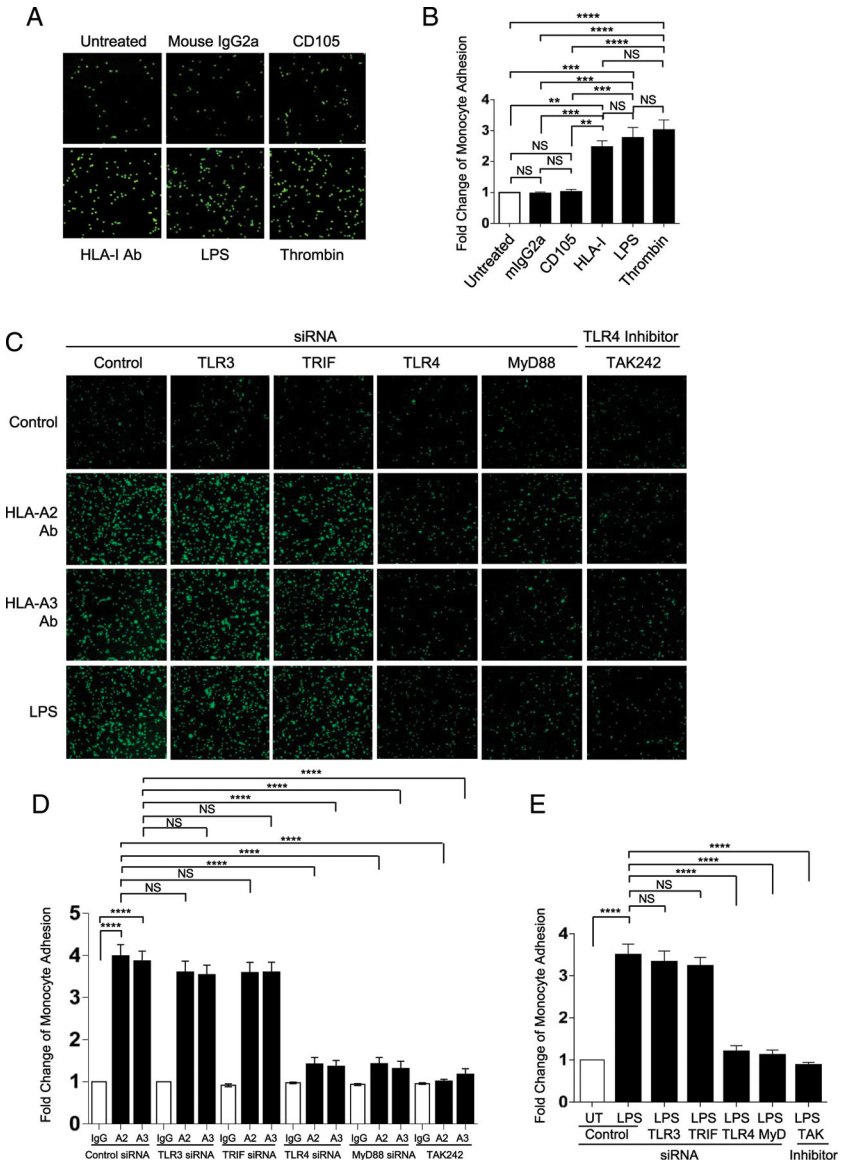

FIGURE 4.

Knockdown TLR4 or MyD88 inhibits HLA I–mediated monocyte adhesion on ECs. (**A**) ECs were untreated or treated with HLA I Ab, LPS, thrombin, anti-CD105 Ab, or mouse IgG2a for 30 min. CFSE-labeled fresh isolated monocytes from healthy donors with an Fc**γ**R blockade were added to stimulated endothelial monolayers. (**C**) ECs were transfe-cted with TLR3, TRIF, TLR4, MyD88, or control siRNA (100 nM each) for 48 h or treated with TLR4 inhibitor TAK242 (1.0 μM) for 30 min. Cells were untreated or stimulated with anti–HLA-A2 or anti–HLA-A3 human mAb (1 μg/ml) or LPS (0.1 μg/ml) for 30 min. CFSE-labeled fresh isolated monocytes from healthy donors with an Fc**γ**R blockade were added to stimulated endothelial monolayers. Adherence of monocytes was measured by a fluorescence microscope with a ×4 objective lens and analyzed with ImageJ. (**B**, **D**, and **E**) Bar graphs represent fold change of adherent monocyte mean ± SEM from (A) and (C). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ analyzed by one-way ANOVA with a Tukey's

multiple comparison test. Data represent at least four independent experiments using three different ECs from three different donors and fresh isolated monocytes from three different healthy donors.

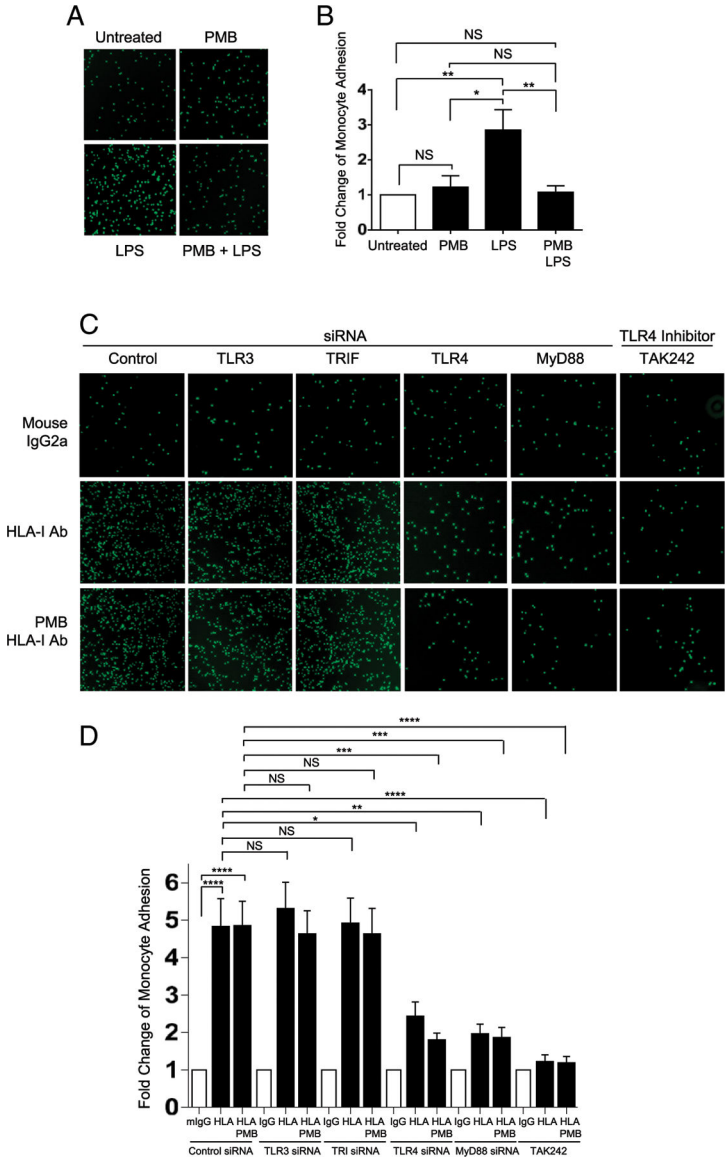

FIGURE 5.

Knockdown TLR4 or MyD88 inhibits HLA I Ab-mediated human peripheral blood monocyte adhesion on ECs. (**A**) ECs were untreated or treated with polymyxin B (PMB), or LPS, or PMB+LPS. (**C**) ECs were transfected with 100 nM TLR3, TRIF, TLR4, MyD88, or control siRNA for 48 h, or pretreated with TAK242 for 30 min. Cells were stimulated anti anti–HLA I mAb (1 mg/ml) or PMB preincubated HLA I Ab for 30 min. (A and C) CFSE-labeled primary human monocytes with an Fcγ**R** blockade were added to Ab-activated endothelial monolayer. Adherence of monocytes was measured by fluorescence microscopy using a ×4 objective lens and analyzed with ImageJ. (**B** and **D**) Bar graph represents fold change of monocyte adherent mean ± SEM from three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ analyzed by one-way ANOVA with a Tukey's multiple comparison test. Data are representative of a minimum of three independent experiments.

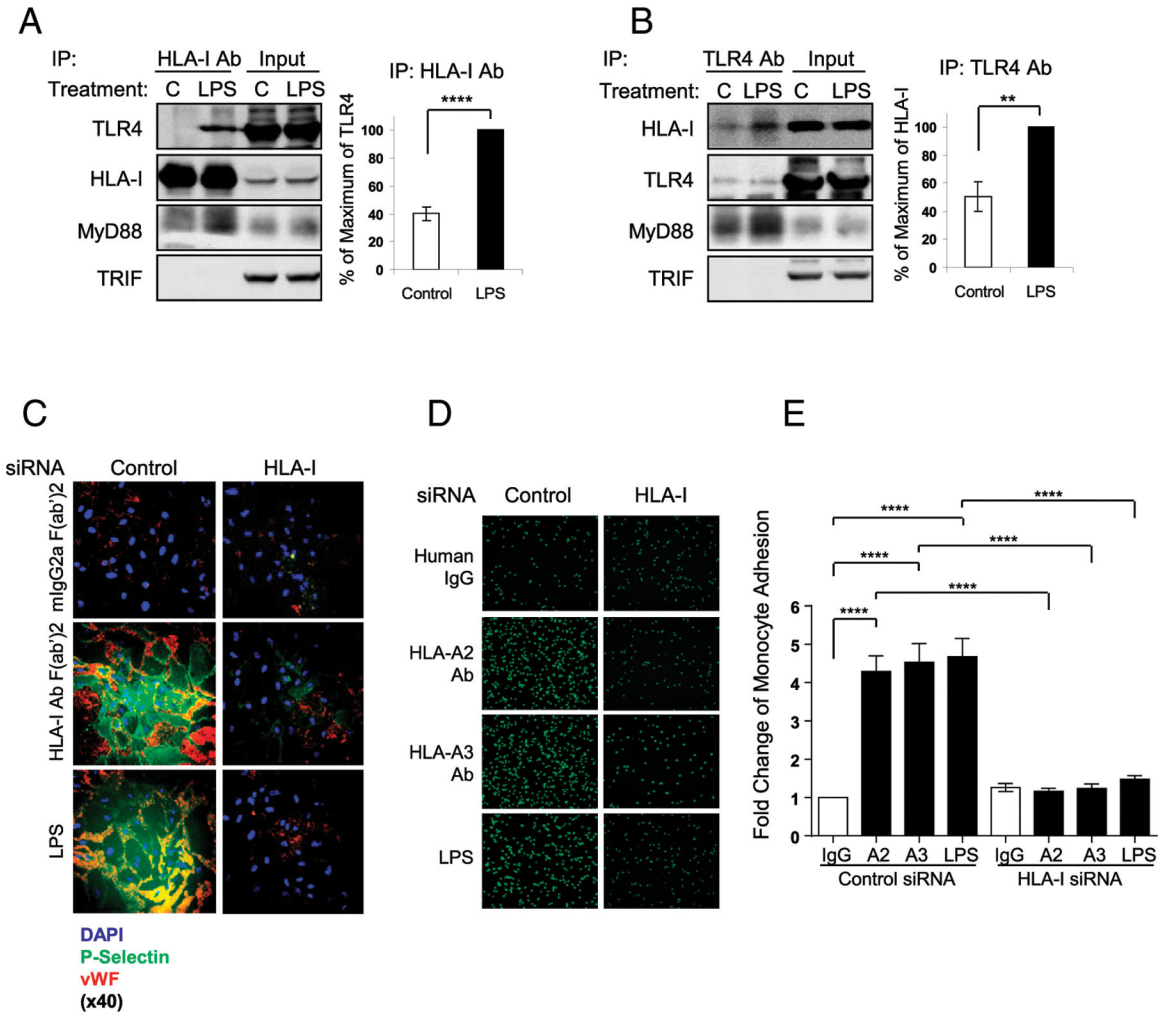

FIGURE 6.

An HLA I/TLR4 signaling complex is required to induce LPS-mediated P-selectin. (**A** and **B**) ECs were stimulated with LPS (0.1 μg/ml) for 15 min, and cell lysates were subjected to immunoprecipitation (A) with HLA I pan Ab W6/32 or (B) with TLR4 Ab and analyzed by Western blotting with Abs against the H chain of HLA I (EMR8–5), TLR4, MyD88, or TRIF, as indicated to the left of the blots. For each sample, 5% of the total cell lysate that was used in the immunoprecipitation was loaded as an input control. Protein bands (A) TLR4 and (B) HLA I were quantified by a densitometry scan, and the results are expressed as the mean ± SEM percentage of the maximal extent of protein. $^{**}p < 0.01$, $^{****}p < 0.0001$ analyzed by a Student *t* test. Data are representative of three independent experiments. (**C** and **D**) ECs were transfected with 100 nM HLA I or control siRNA for 48 h. (C) Cells were stimulated with anti–HLA I Ab $F(ab')_2$ fragment, mouse IgG2a $F(ab')_2$ fragment (1 μg/ml), or LPS (0.1 μg/ml) for 30 min. Cells were fixed with freshly prepared 4% PFA, and surface P-selectin was stained on unpermeabilized cells with mouse anti-human P-selectin mAb (mouse IgG1) and rabbit anti-human vWF Ab followed by Alexa Fluor 488 rabbit anti-mouse IgG1, Alexa Fluor 594 goat anti-rabbit IgG (H1L), and DAPI. Six middle fields per dish were image with a Zeiss LSM 880 fluorescence microscope using a

×40 objective lens. Data are representative of three independent experiments. (D) ECs were stimulated anti–HLA-A2, anti–HLA-A3 human mAb (1 μg/ml), or human IgG1 or LPS (0.1 μg/ml) for 30 min. CFSE-labeled primary human monocytes with an FcγR blockade were added to stimulated endothelial monolayers. Adherence of monocytes was measured by a fluorescence microscope using a ×4 objective lens and analyzed with ImageJ software. (**E**) Bar graph represents fold change of monocyte adherent mean ± SEM from at least three independent experiments. $^{**}p < 0.01$, $^{****}p < 0.0001$ analyzed by (A and B) Student *t* test or (E) one-way ANOVA with a Tukey's multiple comparison test. Data are representative of three independent experiments.