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The Anti-Inflammatory Actions of Platelet Endothelial Cell Adhesion Molecule-1 Do Not Involve Regulation of Endothelial Cell NF-κB

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PECAM-1 is a cell adhesion and signaling receptor that is expressed on many hematopoietic cells and at endothelial cell–cell junctions. Accumulating evidence from a number of in vitro and in vivo model systems suggests that PECAM-1 suppresses cytokine production and vascular permeability induced by a wide range of inflammatory stimuli. In several of these models of inflammatory disease, endothelial, and not leukocyte or platelet, PECAM-1 conferred protection against inflammatory insult. However, the mechanism by which endothelial PECAM-1 functions as an anti-inflammatory protein is poorly understood. It was recently suggested that PECAM-1 exerts its anti-inflammatory effects in endothelial cells by inhibiting the activity of NF-κB, a proinflammatory transcription factor. To confirm and extend these observations, we examined the effect of engaging, cross-linking, or expressing PECAM-1 on NF-κB activation in a variety of human cells. PECAM-1 had no effect on the phosphorylation of the NF-κB inhibitory protein, IκBα; on the nuclear translocation of NF-κB; on the suppression of cytokine-induced transcriptional activation of an NF-κB luciferase reporter plasmid; or on the cytokine-stimulated upregulation of ICAM-1, an NF-κB target gene, in endothelial cells. Taken together, these studies strongly suggest that the anti-inflammatory actions of PECAM-1 in endothelial cells are not likely to involve its regulation of NF-κB. ***The Journal of Immunology*****, 2010, 184: 3157–3163.**

PECAM-1 (CD31) is a member of the Ig superfamily of cell adhesion molecules that is expressed on most cells of the hematopoietic lineage, including platelets, monocytes, neutrophils, and certain lymphocyte subsets (1–3). PECAM-1 is also a major constituent of endothelial cell intercellular junctions in confluent vascular beds where it was shown to play an important role in leukocyte diapedesis (4–6). However, in addition to its support of this proinflammatory event, a growing number of studies in C57BL/6 mice revealed that PECAM-1 can also function prominently in dampening the inflammatory response in a variety of clinically relevant acute and chronic inflammatory conditions, including collagen-induced arthritis (7, 8), late-stage autoimmunity (9), autoimmune encephalitis (10), LPS-induced endotoxic shock (11, 12), atherogenic diet-induced steatohepatitis (13), and atherosclerosis (14). In the case of LPS-induced endotoxic shock and autoimmune encephalitis, it was shown through the use of bone marrow chimeric animals that endothelial, but not leukocyte, PECAM-1 is largely responsible for protection against excessive inflammation (10, 11). However, the mechanism by which PECAM-1, and specifically endothelial PECAM-1, is protective against exaggerated inflammation is poorly understood.

The NF-κB/Rel family of transcription factors is made up of seven proteins (p105, p100, p50, p52, RelA (p65), c-Rel, and RelB) that are important in cell growth, differentiation, inflammation, and survival (15). The NF-κB molecule typically consists of a heterodimer (the prototype is RelA and p50) that is sequestered in the cytosol and inhibited by its cytosolic binding partner, IκBα. Upon cellular activation by various stimuli, the phosphorylation and subsequent degradation of IκBα releases the NF-κB dimer, allowing it to translocate to the nucleus where it acts as a transcription factor upregulating the expression of numerous proinflammatory genes (16). Proinflammatory genes turned on by NF-κB include acute phase proteins in the liver; endothelial cell adhesion molecules, such as ICAM-1 and VCAM-1; and various leukocyte and endothelial cell cytokines that exacerbate the inflammatory response (15).

A recent study reported that engagement of PECAM-1 results in downregulation of nuclear levels of NF-κB in cytokine-stimulated endothelial cells (17). These investigators put forward the attractive hypothesis that PECAM-1–PECAM-1 interactions between transmigrating leukocytes and endothelial cells initiate a negative feedback loop that prevents excessive leukocyte recruitment to sites of inflammation by dampening the expression of proinflammatory adhesion molecules on the endothelial cell surface (17). Based on these observations, we sought to confirm and extend our understanding of the mechanism by which PECAM-1 functions as an anti-inflammatory signaling receptor in the vascular endothelium. However, contrary to this previous report, we found by using a series of complementary experimental systems that neither PECAM-1 engagement nor cross-linking inhibits NF-κB activity, as determined by Western blot for phosphorylated and total IκBα, immunofluorescence for translocation of NF-κB subunits, or on binding to NF-κB target oligonucleotides by EMSA. We also found that PECAM-1 expression

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Abbreviations used in this paper: eGFP, enhanced GFP; dsRed, Discosoma red fluorescent protein; MFI, mean fluorescence intensity; NSC, nonsilencing control; PAF, platelet-activating factor; siRNA, small interfering RNA.

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does not inhibit NF-κB transcriptional activity in cytokine-stimulated HEK293 cells containing an NF-κB luciferase reporter plasmid or prevent the upregulation of ICAM-1, an NF-κB target gene, in cytokine-stimulated endothelial cells. Taken together, these studies demonstrate that the anti-inflammatory actions of PECAM-1 in endothelial cells are likely not due to regulation of NF-κB activity.

Materials and Methods

Endotoxin detection and removal

All primary and secondary Abs used in cross-linking experiments were tested for endotoxin contamination using the QCL-100 *Limulus* amebocyte lysate kit (Lonza, Walkersville, MD), according to the manufacturer's instructions. Reagents that tested positive for endotoxin contamination were subsequently decontaminated using Detoxi-Gel endotoxin removal columns (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. Detoxified reagents were below the limit of detection in subsequent assays using the QCL-100 kit.

Preparation of nuclear extracts

Passage 3 HUVECs were obtained from human volunteer donors by the Hybridoma and Endothelial Cell Core laboratory, Blood Research Institute, and maintained in RPMI 1640, 10% FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 500 μg/ml gentamicin, 6.45 U/ml heparin (Sigma-Aldrich; H3149), and 500 μg/ml endothelial cell growth supplement (BD Biosciences, San Jose, CA). All cell culture reagents were obtained from Mediatech (Manassas, VA), unless otherwise specified. Confluent monolayers of HUVECs were stimulated with PBS or IL-1β (1 ng/ml; PeproTech, Rocky Hill, NJ) + platelet-activating factor (PAF; 10^{-10} M, Sigma-Aldrich) for 4 or 5 h (see *Results*). Cell surface PECAM-1 was then cross-linked with 20 μg/ml Hec7 (a mouse mAb specific for PECAM-1 Ig domain 1 that mimics PECAM-1/PECAM-1 homophilic interactions; a kind gift of Dr. William Muller, Northwestern University), followed by the addition 30 min later of 5 μg/ml goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then lifted with trypsin/EDTA, washed, and incubated on ice for 15 min in Buffer A (200 μl 10 mM KCl, 10 mM HEPES [pH 7.9], 0.1 mM EDTA [pH 8.0], 0.1 mM EGTA, and 1 mM DTT) with 1:100 Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA) before the addition of Nonidet P-40 to a final concentration of 0.5%. After vortexing for 10 s, the mixture was centrifuged at 20,000 × *g* for 30 s, the resulting nuclear pellet was resuspended in 40 μl Buffer B (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA [pH 8.0], 1 mM EGTA, 1 mM DTT, 1:100 Protease Inhibitor Cocktail Set I), and incubated on ice for an additional 20 min. Nuclei were finally pelleted by centrifugation at >20,000 × *g* for 10 min, and the protein concentration of the resulting supernatant was determined using the bicinchoninic assay (Thermo Scientific). Glycerol was added to a final concentration of 10%, and aliquots were stored at −80°C until use.

EMSA

Forward (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and reverse (5′-GCCTGGGAAAGTCCCCTCAACT-3′) oligonucleotides containing the consensus NF-κB–binding sequence were obtained from Integrated DNA Technologies (San Diego, CA). Ten micrograms of each were annealed for 5 min at 90°C in 1× T4 kinase buffer (GE Healthcare Life Science, Piscataway, NJ) in a final volume of 100 μl, cooled to room temperature, and finally incubated at 37°C for 1 h to create the double-stranded probe. Double-stranded Oct1 oligonucleotide was obtained from Santa Cruz Biotechnology and used as a loading control. Both probes were labeled with (γ-[^{32}P]) ATP using a DNA 5′ End Labeling Kit (Promega, Madison, WI), according to the manufacturer's instructions, and had specific activities >2 × 10^9 cpm/μg. Nuclear extracts (15 μg) were incubated with 2 μg poly-deoxy-inosinic-deoxy-cytidylic acid and 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% weight to volume ratio glycerol, and labeled probe (>2 × 10^6 cpm/reaction). The reactions were electrophoresed on a 5% polyacrylamide gel containing 0.5× Tris/borate/EDTA and 2.5% glycerol, dried onto Whatman paper, and then exposed by autoradiography. For band intensity determination, the dried gel and film were aligned and bands were traced and cut out of the dried gel and put in scintillation fluid. β-particle emission counts of individual bands were measured on a Wallac 1410 liquid scintillation counter.

Determination of IκBα phosphorylation and degradation

Passage 3 HUVECs were grown to confluence and stimulated with 1 ng/ml IL-1β+PAF (10^{-10} M) in the presence of 20 μg/ml Hec7 or PECAM-1.3 mAbs. After 10, 30, or 60 min, cells were lysed in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 10 mM NaF) containing 1:100 Phosphatase Inhibitor Cocktail Set I (Calbiochem). Lysates were incubated at 4°C for 30 min and spun down at >20,000 × *g* for 20 min at 4°C. Supernatants were removed, and 30 μg each lysate were loaded onto a 10% SDS-PAGE gel, electrophoresed, and transferred to an Immobilon polyvinylidene difluoride membrane. Membranes were blocked with 5% milk and incubated with anti–phospho-IκBα (1:1000, no. 9246, Cell Signaling Technology, Danvers, MA) and HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). After image detection, membranes were washed and incubated with anti-IκBα (1:1000, no. 9842, Cell Signaling Technology) and HRP–anti-rabbit IgG, imaged, stripped, and reprobed with anti–α-tubulin (1:1000, no. 2125, Cell Signaling Technology) and HRP–anti-rabbit IgG. Images were obtained on the Kodak Image Station 2000R or an X-ray film developer. Band densitometry quantitation was measured using Kodak Molecular Imaging software.

Localization of NF-κB subunits by immunofluorescence

Passage 3 HUVECs were plated and grown to confluence on gelatin-coated eight-chamber glass slides (BD Biosciences). Confluent monolayers of HUVECs were stimulated with PBS or IL-1β (1 ng/ml) + PAF (10^{-10} M) for 1, 3, or 5 h. One hour prior to the end of stimulation for each time point, cell surface PECAM-1 was cross-linked with 20 μg/ml mAb Hec7, mAb PECAM-1.3, or PECAM-1/IgG chimeric fusion protein, followed 30 min later by the addition of 5 μg/ml goat anti-mouse IgG (for Hec7 and PECAM-1.3) or 5 μg/ml goat anti-human IgG (for PECAM-1/IgG). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were blocked with 3% BSA and then incubated sequentially with anti–NF-κB p65 (1:200, no. sc109, Santa Cruz Biotechnology), Texas Red goat anti-rabbit (1:100), and FITC–β-catenin (1:65, no. 2849, Cell Signaling Technology). Nuclei were counterstained with DAPI, and slides were mounted with ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA). Images were obtained using an ×40 oil objective and lasers at 405, 488, and 559 nm on an Olympus FluoView FV1000 MPE microscope (Center Valley, PA). Z-stack fluorescent images were acquired at 4 μs per

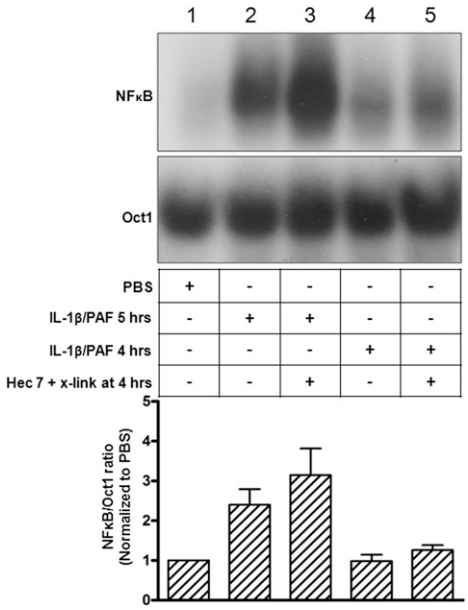

FIGURE 1. Engagement of PECAM-1 does not decrease binding ability of NF-κB to target oligonucleotides. HUVECs were stimulated with PBS (*column 1*), IL-1β (1 ng/ml) + PAF (10^{-10} M) for 5 h (*columns 2* and *3*), or IL-1β+PAF for the first 4 h and then replaced with normal media (*columns 4* and *5*). mAb Hec7 was added at 4 h, and anti-mouse IgG was added 30 min after primary Ab for cross-linking. Nuclear extracts were obtained and subjected to EMSA for binding to target NF-κB oligonucleotides. Oct1 served as a loading control. The photographs show images from a representative EMSA. The histograms depict the quantified ratio of NF-κB/Oct1 for each lane of β-particle emission counts of individual bands that were cut out of the gel. Results are mean ± SD of two independent experiments. Differences between *lanes 2* and *3* and *lanes 4* and *5* did not reach statistical significance using one-way ANOVA with Bonferroni multiple comparison post test.

pixel at optimal stepwise progression through the entire thickness of the monolayer. Images were reconstructed and analyzed with SlideBook 5.0 imaging software. All images were uniformly background subtracted, and a universal mask was generated to identify nuclei based on the DAPI channel. The mask excluded all objects <500 pixels and along the edge of the image. Nuclear levels of NF-κB were obtained by measuring the mean fluorescence intensity (MFI) of the Texas Red (NF-κB p65) channel within the mask throughout the whole z-stack image. Three images from each experimental group were analyzed, and graphs depict the MFI of nuclear NF-κB for all nuclei analyzed. For representative images, a projection image of combined z-stacks was created using SlideBook 5.0 software. Images were uniformly background subtracted, and brightness and contrast were adjusted in PowerPoint separately, but uniformly, for images at each time point.

NF-κB luciferase reporter assays

HEK293 cells were transfected with pcDNA3.1 vector and pNifty2-Luc NF-κB luciferase reporter plasmid (Invitrogen) using Superfect transfection reagent (Qiagen, Valencia, CA). Double stable clones were selected with G418 and Zeocin. Expressing clones were plated onto 96-well tissue culture plates and grown to 80% confluence. Cells were cotransfected with 0.2 μg pWPT-GFP (no.12255, Addgene, Cambridge, MA) and 0.2 μg plasmid containing Discosoma red fluorescent protein (dsRed) or human PECAM-1 cDNA at a 4:1 lipid/DNA ratio with Lipofectamine LTX transfection reagent (Invitrogen), according to the manufacturer's instructions. Three days later, cells were stimulated with IL-1β (1 ng/ml) + PAF (10^{-10} M) or TNF-α (1 ng/ml; R&D Systems, Minneapolis, MN) + PAF for 6 h. Enhanced GFP (eGFP) fluorescence was measured using a Victor2 multilabel photon counter. Luciferin salt (a kind gift of Dr. Mike Dwinnell, Medical College of Wisconsin) was subsequently added to the wells, and luciferase activity was measured on a Victor2 multilabel photon counter. After luciferase readings, cells were lifted, and eGFP fluorescence was analyzed on an LSRII flow cytometer to ensure similar transfection efficiency and eGFP fluorescence intensity (data not shown). Because transfection efficiency and eGFP fluorescence intensity were similar for all wells, we divided the relative light units from luciferase readings by the eGFP readings obtained on the Victor2 multilabel photon counter to normalize for cell number. For Western blots assessing PECAM-1 expression, lysates were made from transfected cells; 40 μg lysate was loaded onto an SDS-PAGE gel, electrophoresed, and transferred to membranes; and blots were imaged on the Kodak Image Station 2000R, as described above. Sew 32–34 (1 μg/ml, a rabbit polyclonal Ab directed against human PECAM-1) and anti–α-tubulin (1:1000) were used for blotting.

Flow cytometric analysis of ICAM-1 upregulation in cytokine-stimulated endothelial cells

Passage 3 HUVECs were grown to confluence and stimulated with IL-1β (1 ng/ml) + PAF (10^{-10} M). Four hours after the addition of cytokines, 20 μg/ml mAb Hec7, mAb PECAM-1.3, or PECAM-1/IgG, chimeric fusion protein was added, allowed to incubate for 30 min, and then cross-linked by the addition of 5 μg/ml species-specific secondary Ab. Two hours later (6 h total), cells were lifted with trypsin/EDTA and double-stained for ICAM-1 and PECAM-1 using FITC–ICAM-1 (1:100, no. sc-107, Santa Cruz Biotechnology) and Alexa647–PECAM-1.3 (50 μg/ml). In other experiments, HUVECs were stimulated with 10 ng/ml TNF-α; 10 min after the addition of TNF-α, 50 μg/ml mAb PECAM-1.3 was added, allowed to incubate for 10 min, and then cross-linked by the addition of 100 μg/ml goat anti-mouse IgG. Six hours later, cells were lifted with trypsin/EDTA and double-stained for ICAM-1 and PECAM-1 using FITC–ICAM-1 (1:100, no. sc-107, Santa Cruz Biotechnology) and Alexa680–PECAM-1.3 (50 μg/ml). In some experiments, PECAM-1 expression was knocked down using lentivirus constructs encoding PECAM-1 small interfering RNA (siRNA) (18) before stimulation with 1 ng/ml IL-1β + PAF (10^{-10} M) or TNF-α (20 ng/ml) for 6 or 24 h, after which cells were lifted with trypsin/EDTA and double-stained, as above. FACS was performed using an LSRII flow cytometer. Alexa647–PECAM-1.3 and Alexa680–PECAM-1.3 were created by labeling mAb PECAM-1.3 with the Alexa Fluor 647 and Alexa 680 mAb labeling kits (Molecular Probes, Eugene, OR), according to the manufacturer's instructions.

Statistical analysis

Where applicable, results are expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

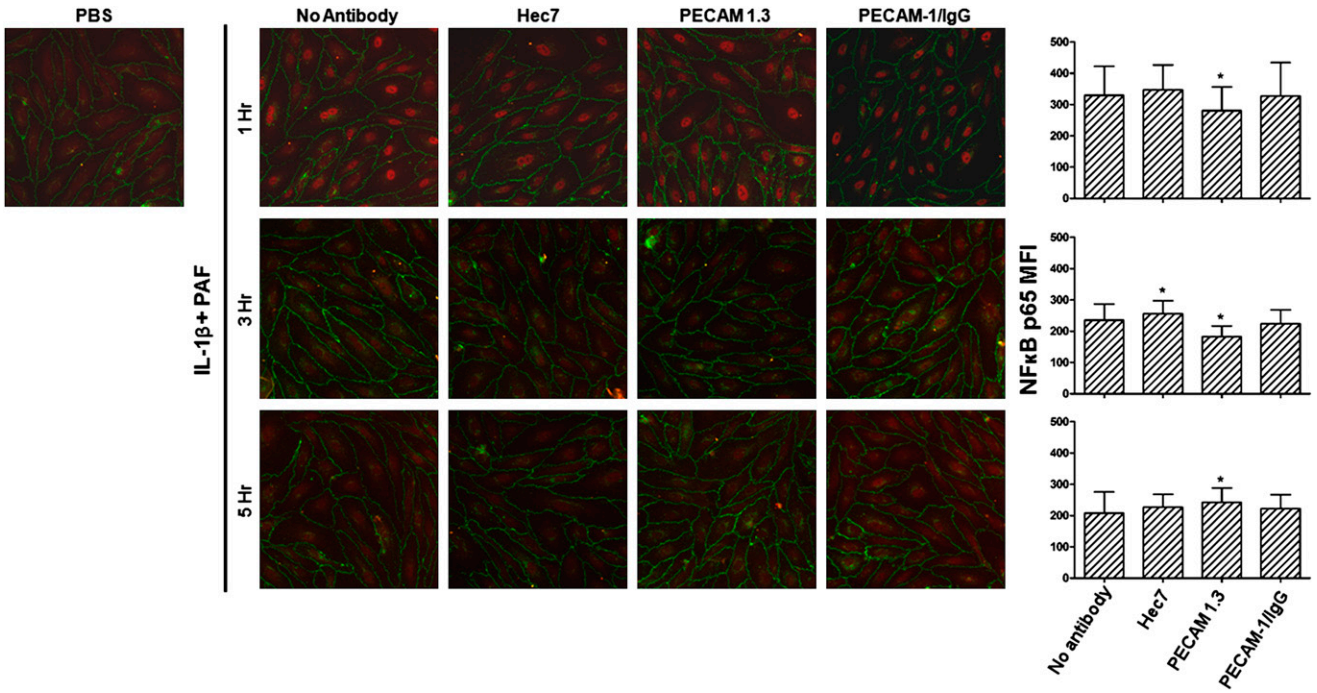

FIGURE 2. Engagement of PECAM-1 does not decrease nuclear translocation of NF-κB. HUVECs were stimulated with PBS or IL-1β (1 ng/ml) + PAF (10^{-10} M) for 1, 3, or 5 h. One hour prior to the end of stimulation for each time point, cell surface PECAM-1 was cross-linked with 20 μg/ml of mAb Hec7 or mAb PECAM-1.3 or PECAM-1/IgG, followed 30 min later by the addition of 5 μg/ml species-specific secondary Ab. Thirty minutes later, cells were fixed, permeabilized, and stained for NF-κB (red), β-catenin (green), and DAPI. Confocal images were obtained, and one representative image for each treatment condition is shown at ×40 magnification. Nuclear NF-κB was quantified (see *Materials and Methods*), and graphs depict the mean ± SD of the MFI of nuclear NF-κB from 57–107 nuclei from three separate images at each time point. Statistics were obtained by one-way ANOVA statistical analysis with the Tukey multiple comparison post test (*$p < 0.05$). Results are representative of three independent experiments.

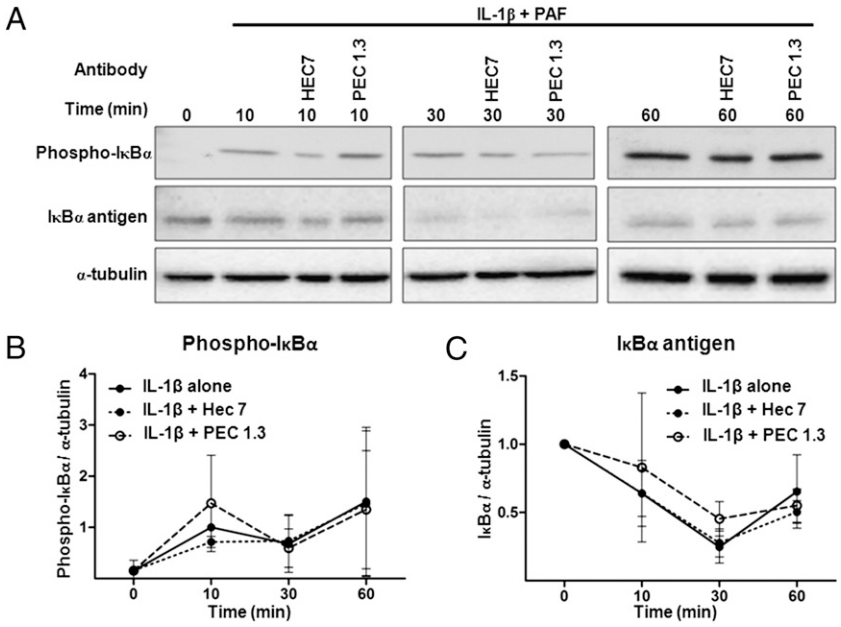

FIGURE 3. Engagement of PECAM-1 does not prevent the early activation of NF-κB. HUVECs were stimulated with IL-1β (1 ng/ml) + PAF (10^{-10} M) in the presence of 20 μg/ml of Hec7 or PECAM-1.3 mAbs for various time points. *A*, Representative Western blot of cell lysates showing phospho-IκBα, total IκBα Ag, and α-tubulin. Graphs below the blots show quantitation of phosphorylated (*B*) or total IκBα Ag (*C*) normalized to α-tubulin from three independent experiments. Results are mean ± SD. Means between groups at each time point were not statistically significant as determined by two-way ANOVA with Bonferroni post tests.

Results

Engaging PECAM-1 has no effect on nuclear levels of NF-κB

To confirm an earlier report (17) that engagement of PECAM-1 functions to suppress NF-κB activity in the vascular endothelium, we stimulated HUVECs with IL-1β+PAF for 4 h and then cross-linked PECAM-1 with mAb Hec7+anti-mouse IgG for an additional hour, either in the presence or absence of continuing exposure to the activating cytokines. As shown in Fig. 1, Ab-mediated engagement of PECAM-1 did not decrease cytokine-initiated translocation of NF-κB to the nucleus, as measured by gel-shift analysis, under the original conditions described by Cepinskas et al. (17), in which IL-1β+PAF was withdrawn prior to PECAM-1 engagement (compare *lane* 4 with *lane* 5), or under the more physiological conditions recently described by this group (19), in which these activating cytokines were allowed to remain present during the PECAM-1 cross-linking process (compare *lane* 2 with *lane* 3).

Because these findings differed from those previously reported (17, 19), the ability of PECAM-1 engagement to suppress cytokine-induced nuclear translocation of NF-κB was also examined by immunofluorescence microscopy. As shown in Fig. 2, engagement of cell surface PECAM-1 with mAb Hec7 or a bivalent PECAM-1/IgG chimeric fusion protein, both of which bind to extracellular Ig domain 1 (20), had no effect on the translocation of NF-κB p65 to the nucleus after 1, 3, or 5 h of IL-1β+PAF stimulation. A second anti-PECAM-1 mAb, PECAM-1.3, had only a minor effect and only at earlier time points. These data, like those shown in Fig. 1, suggest that engagement of endothelial cell surface PECAM-1 following cytokine stimulation has little, if any, biologically significant ability to affect translocation of NF-κB to the nucleus.

To examine the possibility that engagement of PECAM-1 might be acting upstream of NF-κB to suppress its activation, we examined the effect of mAb-mediated engagement of PECAM-1 on IL-1β+PAF–induced phosphorylation and degradation of IκBα, a cytosolic suppressor of NF-κB activation. Binding of mAbs Hec7 and PECAM-1.3 did not inhibit the initial phosphorylation (Fig. 3*A*, 3*B*), degradation (Fig. 3*A*, 3*C*), or rate of Ag recovery (Fig. 3*A*, 3*C*, Supplemental Fig. 1) of IκBα in HUVECs. Together with the data shown in Figs. 1 and 2, we conclude that engagement of PECAM-1 has negligible effects on cytokine-stimulated activation of NF-κB.

PECAM-1 expression does not inhibit NF-κB–driven transcriptional activity in cytokine-stimulated HEK293 cells

Although PECAM-1 engagement per se has negligible effects on NF-κB activity (Figs. 1–3), it is possible that the expression of PECAM-1 alone might be sufficient to modulate NF-κB

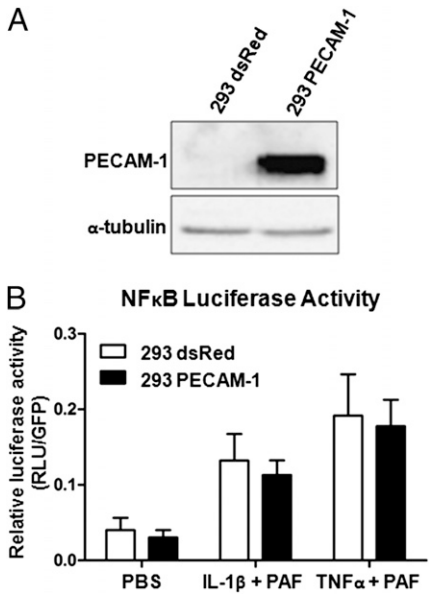

FIGURE 4. PECAM-1–expressing HEK293 cells do not display differences in NF-κB transcriptional activity when stimulated with cytokines. *A*, dsRed or human PECAM-1 were transfected into HEK293 cells that stably expressed an NF-κB–luciferase reporter plasmid. Expression of PECAM-1 was assessed by Western blot. *B*, 293 dsRed and 293 PECAM cells were stimulated for 6 h with IL-1β (1 ng/ml) + PAF (10^{-10} M) or TNF-α (1 ng/ml) + PAF. NF-κB transcriptional activity, as measured by luciferase reporter activity following the addition of luciferin salt, was assessed on a luminometer. eGFP fluorescence was read on a fluorometer to normalize for cell number and transfection efficiency (see *Materials and Methods*). Results are mean ± SD of 8–10 wells over three independent experiments in duplicate or triplicate. Means for each treatment group were not statistically different as determined by two-way ANOVA with Bonferroni post test.

responsiveness. To determine whether PECAM-1 expression affects the transcriptional activity of NF-κB, we transfected a dsRed control plasmid or a cDNA encoding human PECAM-1 into HEK293 cells that had been stably transfected with an NF-κB–responsive luciferase reporter plasmid (Fig. 4*A*). As shown in Fig. 4*B*, dsRed- and PECAM-1–transfected cells responded to exposure to IL-1β+PAF and TNF-α+PAF with an increase in NF-κB transcriptional activity. However, expression of PECAM-1 had no effect on the extent of NF-κB activation following stimulation with either inflammatory cytokine combination. As such, it seems that the expression of PECAM-1 is not able to inhibit transcriptional activation of NF-κB.

Neither expression nor cross-linking of PECAM-1 prevents upregulation of the NF-κB target gene, ICAM-1, in HUVECs

ICAM-1 serves as an important counterreceptor for leukocyte integrins during the leukocyte adhesion cascade (21), and it becomes markedly upregulated in an NF-κB–dependent manner on the surface of cytokine-activated endothelial cells (15). To determine whether PECAM-1 engagement might be sufficient to dampen the upregulation of ICAM-1 in cytokine-activated endothelial cells, we performed flow cytometric analysis of HUVECs, in which PECAM-1 was cross-linked with anti–PECAM-1 mAbs or a bivalent PECAM-1/IgG chimeric protein. As shown in Fig. 5*A*, engagement of PECAM-1 by mAbs Hec7 or PECAM-1.3 or with bivalent PECAM-1/IgG was not able to suppress IL-1β+PAF–induced upregulation of ICAM-1 after 6 h of cytokine stimulation. Similar results were seen after 24 h of IL-1β+PAF stimulation (data not shown). Likewise, engagement using mAb PECAM-1.3 also showed no effect on TNF-α–induced upregulation of ICAM-1 (Fig. 5*B*). siRNA-mediated silencing of PECAM-1 also failed to have an effect on cytokine-induced ICAM-1 expression in these cells (Fig. 5*C*, 5*D*). Taken together, these studies demonstrate that neither engagement nor expression of PECAM-1 is able to modulate NF-κB–dependent responses of the inflamed endothelium.

Discussion

PECAM-1 is a well-studied cellular adhesion and signaling receptor that plays an important role in supporting leukocyte diapedesis during the leukocyte adhesion cascade (4, 21). In contrast to this proinflammatory effect, PECAM-1 was shown in a number of situations to function as an ITIM-containing inhibitory receptor capable of dampening cellular activation events in lymphocytes (9, 22, 23), mast cells (24), and platelets (25–28). Numerous reports also demonstrated an anti-inflammatory role for PECAM-1 in well-established acute and chronic inflammatory disease models. For example, mice expressing PECAM-1 produce lower levels of inflammatory cytokines (7, 11–13), exhibit enhanced vascular barrier protection (10–12), and paradoxically, accumulate fewer leukocytes at sites of inflammation (10–13). However, the mechanisms by which PECAM-1 serves to confer protection in inflammation and how it regulates these aspects of the inflammatory response are still poorly understood.

One intriguing possibility might be that PECAM-1 inhibits signaling of a canonical proinflammatory signaling pathway,

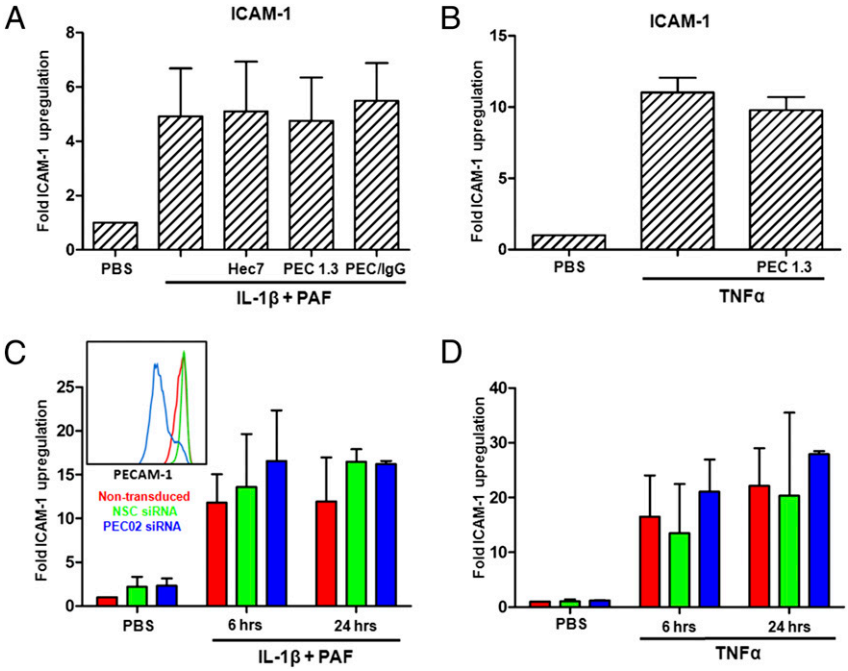

FIGURE 5. Neither PECAM-1 engagement nor expression is able to prevent the upregulation of ICAM-1 in HUVECs following cytokine stimulation. *A*, HUVECs were stimulated with IL-1β (1 ng/ml) + PAF (10^{-10} M). PECAM-1 was engaged with mAb Hec7, mAb PECAM-1.3, or PECAM-1/IgG 4 h after the start of stimulation and subsequently cross-linked 30 min later with species-specific secondary Ab. ICAM-1 expression was measured by flow cytometry after 6 h of stimulation and is displayed as the fold ICAM-1 upregulation compared with PBS-stimulated cells. Results are mean ± SD of two or three independent experiments. Means for cytokine-stimulated groups were not statistically significant as assessed by one-way ANOVA. *B*, HUVECs were stimulated with TNF-α (10 ng/ml). PECAM-1 was engaged with mAb PECAM-1.3 and subsequently cross-linked with anti-mouse IgG. ICAM-1 expression was measured by flow cytometry after 6 h of stimulation and is displayed as fold ICAM-1 upregulation compared with PBS-stimulated cells. Results are mean ± SD of two independent experiments. Means for cytokine-stimulated groups were not statistically significant as assessed by one-way ANOVA. *C* and *D*, HUVECs were nontransduced or transduced with lentiviruses expressing nonsilencing control (NSC) or PECAM-1–specific (PEC02) siRNA. Expression of PECAM-1 was measured by flow cytometry for each experiment, and a representative graph is displayed as an inset in *C*. Cells were stimulated with IL-1β (1 ng/ml) + PAF (10^{-10} M) (*C*) or TNF-α (20 ng/ml) (*D*) for 6 or 24 h, and ICAM-1 expression was measured by flow cytometry. Results depict mean ± SD of fold ICAM-1 upregulation compared with nontransduced, nonstimulated cells from two independent experiments. Means for groups at each time point were not statistically different as assessed by two-way ANOVA with Bonferroni post test.

thereby dampening cellular activation and inflammation. Cepinskas et al. (17) reported that PECAM-1 engagement on the endothelial cell surface was able to decrease nuclear levels of NF-κB, which they hypothesized might be responsible for initiating a negative feedback loop to dampen further cellular activation, namely prolonged expression of endothelial adhesion molecules. Because endothelial PECAM-1 likely confers much of the protection against excessive inflammation, at least in models of LPS-induced endotoxic shock (11, 12) and murine autoimmune encephalitis (10), we hypothesized, like Cepinskas et al., that endothelial PECAM-1 might function to dampen inflammatory activation of endothelial cells through inhibition of NF-κB.

To test this hypothesis, we engaged and cross-linked PECAM-1 on the surface of IL-1β+PAF–stimulated HUVECs using two anti–PECAM-1 mAbs, as well as a homophilically binding PECAM-1/IgG chimeric protein construct. In preliminary experiments, we found no detectable decrease in nuclear translocation of NF-κB, as detected by EMSA, when we engaged PECAM-1 with mAbs PECAM-1.3 and PECAM-1.2 or with PECAM-1/IgG (data not shown). It was subsequently revealed (19) that the observation that PECAM-1 modulated NF-κB activity had been made under conditions in which the stimulatory cytokines IL-1β+PAF were removed prior to Ab addition. Therefore, we performed a side-by-side comparison of the effects of PECAM-1 engagement in the continuous presence (our protocol) or following removal (17, 19) of IL-1β+PAF. Although the amount of NF-κB detected in the nucleus decreased following cytokine removal (Fig. 1), engaging PECAM-1 had no discernable additional effect, even when using the same anti–PECAM-1 mAb (Hec7) that had been used in the previously published experiments (17). Similarly, the addition of anti–PECAM-1 mAbs or PECAM-1/IgG had no effect on the rate or extent of phosphorylation and degradation of IκBα (Fig. 3) or on the upregulation of the NF-κB–inducible leukocyte adhesion molecule, ICAM-1, in IL-1β+PAF– or TNF-α–stimulated HUVECs (Fig. 5*A*, 5*B*). Cross-linking of cell surface PECAM-1 with mAb PECAM-1.3 caused a slight decrease in the amount of translocated NF-κB p65 at 1 and 3 h of IL-1β+PAF stimulation (Fig. 2), which supports the findings by Cepinskas et al. (17). However, the small increment of this decrease, coupled with the fact that two other PECAM-1–specific cross-linking reagents had no effect on the translocation of NF-κB (Fig. 2) and that PECAM-1.3-mediated cross-linking of PECAM-1 had no effect on cytokine-induced upregulation of ICAM-1 (Fig. 5*A*, 5*B*), strongly suggests that engaging and cross-linking PECAM-1 homophilically is unable to dampen cytokine-mediated activation of the NF-κB pathway in the vascular endothelium. However, the possibility remains that during the process of neutrophil transmigration, PECAM-1 becomes engaged and activated in a manner that has not been adequately mimicked by the reagents used in this study and that such interactions might initiate anti-inflammatory signaling pathways mediated by PECAM-1. Given the recent identification of CD177 as a high-affinity heterophilic ligand for PECAM-1 (29), the possibility that engagement of PECAM-1 in this manner might be able to send inhibitory signals into the cell would be an interesting line of future investigation.

Despite the inability of PECAM-1 homophilic engagement to suppress NF-κB activation, we used two complementary approaches to examine the possibility that PECAM-1 expression per se might be sufficient to dampen NF-κB activation in response to inflammatory mediators. In the first, we compared NF-κB–mediated luciferase activity in cytokine-stimulated PECAM-1–positive versus PECAM-1–negative HEK293 cells that harbored an NF-κB–responsive luciferase reporter gene (Fig. 4). In the second, we examined the effect of siRNA-induced PECAM-1 silencing on the upregulation of ICAM-1 expression in cytokine-stimulated HUVECs (Fig. 5*C*, 5*D*). In neither case did PECAM-1 expression have an inhibitory effect in the presence of IL-1β+PAF or TNF-α. As such, we conclude that PECAM-1 expression, like PECAM-1 engagement, is not a regulator of cytokine-induced activation of NF-κB in endothelial cells.

In summary, PECAM-1 seems to play a number of prominent, although sometimes opposing, roles in regulating specific components of the inflammatory response. On the positive, proinflammatory side, PECAM-1 supports leukocyte transendothelial migration and functions as a positive regulator of NF-κB in atheroprone areas of the vasculature where endothelial cells are subjected to oscillatory, disturbed fluid shear (30, 31). On the anti-inflammatory side, PECAM-1 plays a role in maintaining the barrier function of endothelial cell–cell junctions (10–12) and suppresses cytokine production (7, 11–13). In this regard, Rui et al. (32) reported that heterophilic engagement of macrophage PECAM-1 with a CD38-Fc fusion protein depressed LPS-induced proinflammatory cytokine and type I IFN production by inhibiting JNK, IFN regulatory factor-3, and NF-κB. Therefore, it seems that the ability of PECAM-1 to influence inflammation, in general, and NF-κB, in particular, may depend on how PECAM-1 is engaged, the cell type in which it resides, and the environmental conditions to which that cell is subjected. Evidence that cells use PECAM-1 in a situation- and site-specific manner to fine-tune their response to cellular stress can be found in three recent reports, each of which examined the role of PECAM-1 in atherosclerosis, a complex disorder whose development and progression is influenced by rheological factors, the presence of inflammatory stimuli, and the ease with which leukocytes migrate into the vessel wall. Thus, the proinflammatory properties of endothelial cell PECAM-1, likely acting as a sensor of shear stress that activates NF-κB, were found to dominate and promote atherosclerosis in the aortic arch (14, 31, 33), whereas the ability of PECAM-1 to suppression inflammation, namely the production of proinflammatory cytokines (7, 11–13) and reactive oxygen species (13, 34), seems to play a more prominent role within the descending aorta (14). The molecular details of how PECAM-1 supports anti-inflammatory signaling in platelets, leukocytes, and endothelial cells to attenuate inflammation are not known. We are in the process of analyzing real-time PCR-based microarrays to generate PECAM-1–regulated candidate genes that might confer such protection.

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Disclosures

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References

1. Newman, P. J. 1997. The biology of PECAM-1. *J. Clin. Invest.* 100: (11), Suppl.) S25–S29.
2. Newman, P. J. 1999. Switched at birth: a new family for PECAM-1. *J. Clin. Invest.* 103: 5–9.
3. Newman, P. J., and D. K. Newman. 2003. Signal transduction pathways mediated by PECAM-1: new roles for an old molecule in platelet and vascular cell biology. *Arterioscler. Thromb. Vasc. Biol.* 23: 953–964.
4. Muller, W. A., S. A. Weigl, X. Deng, and D. M. Phillips. 1993. PECAM-1 is required for transendothelial migration of leukocytes. *J. Exp. Med.* 178: 449–460.

5. Vaporciyan, A. A., H. M. DeLisser, H. C. Yan, I. I. Mendiguren, S. R. Thom, M. L. Jones, P. A. Ward, and S. M. Albelda. 1993. Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. *Science* 262: 1580–1582.
6. Wakelin, M. W., M. J. Sanz, A. Dewar, S. M. Albelda, S. W. Larkin, N. Boughton-Smith, T. J. Williams, and S. Nourshargh. 1996. An anti-platelet-endothelial cell adhesion molecule-1 antibody inhibits leukocyte extravasation from mesenteric microvessels in vivo by blocking the passage through the basement membrane. *J. Exp. Med.* 184: 229–239.
7. Tada, Y., S. Koarada, F. Morito, O. Ushiyama, Y. Haruta, F. Kanegae, A. Ohta, A. Ho, T. W. Mak, and K. Nagasawa. 2003. Acceleration of the onset of collagen-induced arthritis by a deficiency of platelet endothelial cell adhesion molecule 1. *Arthritis Rheum.* 48: 3280–3290.
8. Wong, M. X., J. D. Hayball, P. M. Hogarth, and D. E. Jackson. 2005. The inhibitory co-receptor, PECAM-1 provides a protective effect in suppression of collagen-induced arthritis. *J. Clin. Immunol.* 25: 19–28.
9. Wilkinson, R., A. B. Lyons, D. Roberts, M. X. Wong, P. A. Bartley, and D. E. Jackson. 2002. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. *Blood* 100: 184–193.
10. Graesser, D., A. Solowiej, M. Bruckner, E. Osterweil, A. Juedes, S. Davis, N. H. Ruddle, B. Engelhardt, and J. A. Madri. 2002. Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. *J. Clin. Invest.* 109: 383–392.
11. Maas, M., M. Stapleton, C. Bergom, D. L. Mattson, D. K. Newman, and P. J. Newman. 2005. Endothelial cell PECAM-1 confers protection against endotoxic shock. *Am. J. Physiol. Heart Circ. Physiol.* 288: H159–H164.
12. Carrithers, M., S. Tandon, S. Canosa, M. Michaud, D. Graesser, and J. A. Madri. 2005. Enhanced susceptibility to endotoxic shock and impaired STAT3 signaling in CD31-deficient mice. *Am. J. Pathol.* 166: 185–196.
13. Goel, R., B. Boylan, L. Gruman, P. J. Newman, P. E. North, and D. K. Newman. 2007. The proinflammatory phenotype of PECAM-1-deficient mice results in atherogenic diet-induced steatohepatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293: G1205–G1214.
14. Goel, R., B. R. Schrank, S. Arora, B. Boylan, B. Fleming, H. Miura, P. J. Newman, R. C. Molthen, and D. K. Newman. 2008. Site-specific effects of PECAM-1 on atherosclerosis in LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 28: 1996–2002.
15. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16: 225–260.
16. Sun, S. C., P. A. Ganchi, D. W. Ballard, and W. C. Greene. 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science* 259: 1912–1915.
17. Cepinskas, G., J. Savickiene, C. V. Ionescu, and P. R. Kvietys. 2003. PMN transendothelial migration decreases nuclear NFκB in IL-1β-activated endothelial cells: role of PECAM-1. *J. Cell Biol.* 161: 641–651.
18. Bergom, C., R. Goel, C. Paddock, C. Gao, D. K. Newman, S. Matsuyama, and P. J. Newman. 2006. The cell-adhesion and signaling molecule PECAM-1 is a molecular mediator of resistance to genotoxic chemotherapy. *Cancer Biol. Ther.* 5: 1699–1707.
19. Stephen, J., K. Katada, M. Cukiernik, A. Bihari, R. F. Potter, and G. Cepinskas. 2008. Polymorphonuclear leukocyte (PMN) migration across vascular endothelial cells (EC) in the absence or presence of IL-1β differentially regulate expression of EC adhesion molecules ICAM-1 and E-selectin. *FASEB J.* 22: 166.
20. Sun, J., J. Williams, H. C. Yan, K. M. Amin, S. M. Albelda, and H. M. DeLisser. 1996. Platelet endothelial cell adhesion molecule-1 (PECAM-1) homophilic adhesion is mediated by immunoglobulin-like domains 1 and 2 and depends on the cytoplasmic domain and the level of surface expression. *J. Biol. Chem.* 271: 18561–18570.
21. Muller, W. A. 2002. Leukocyte-endothelial cell interactions in the inflammatory response. *Lab. Invest.* 82: 521–533.
22. Newman, D. K., C. Hamilton, and P. J. Newman. 2001. Inhibition of antigen-receptor signaling by Platelet Endothelial Cell Adhesion Molecule-1 (CD31) requires functional ITIMs, SHP-2, and p56(lck). *Blood* 97: 2351–2357.
23. Newton-Nash, D. K., and P. J. Newman. 1999. A new role for platelet-endothelial cell adhesion molecule-1 (CD31): inhibition of TCR-mediated signal transduction. *J. Immunol.* 163: 682–688.
24. Wong, M. X., D. Roberts, P. A. Bartley, and D. E. Jackson. 2002. Absence of platelet endothelial cell adhesion molecule-1 (CD31) leads to increased severity of local and systemic IgE-mediated anaphylaxis and modulation of mast cell activation. *J. Immunol.* 168: 6455–6462.
25. Patil, S., D. K. Newman, and P. J. Newman. 2001. Platelet endothelial cell adhesion molecule-1 serves as an inhibitory receptor that modulates platelet responses to collagen. *Blood* 97: 1727–1732.
26. Jones, K. L., S. C. Hughan, S. M. Dopheide, R. W. Farndale, S. P. Jackson, and D. E. Jackson. 2001. Platelet endothelial cell adhesion molecule-1 is a negative regulator of platelet-collagen interactions. *Blood* 98: 1456–1463.
27. Cicmil, M., J. M. Thomas, M. Leduc, C. Bon, and J. M. Gibbins. 2002. Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets. *Blood* 99: 137–144.
28. Falati, S., S. Patil, P. L. Gross, M. Stapleton, G. Merrill-Skoloff, N. E. Barrett, K. L. Pixton, H. Weiler, B. Cooley, D. K. Newman, et al. 2006. Platelet PECAM-1 inhibits thrombus formation in vivo. *Blood* 107: 535–541.
29. Sachs, U. J., C. L. Andrei-Selmer, A. Maniar, T. Weiss, C. Paddock, V. V. Orlova, E. Y. Choi, P. J. Newman, K. T. Preissner, T. Chavakis, and S. Santoso. 2007. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). *J. Biol. Chem.* 282: 23603–23612.
30. Tzima, E., M. Irani-Tehrani, W. B. Kiosses, E. Dejana, D. A. Schultz, B. Engelhardt, G. Cao, H. DeLisser, and M. A. Schwartz. 2005. A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* 437: 426–431.
31. Harry, B. L., J. M. Sanders, R. E. Feaver, M. Lansey, T. L. Deem, A. Zarbock, A. C. Bruce, A. W. Pryor, B. D. Gelfand, B. R. Blackman, et al. 2008. Endothelial cell PECAM-1 promotes atherosclerotic lesions in areas of disturbed flow in ApoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 28: 2003–2008.
32. Rui, Y., X. Liu, N. Li, Y. Jiang, G. Chen, X. Cao, and J. Wang. 2007. PECAM-1 ligation negatively regulates TLR4 signaling in macrophages. *J. Immunol.* 179: 7344–7351.
33. Stevens, H. Y., B. Melchior, K. S. Bell, S. Yun, J. C. Yeh, and J. A. Frangos. 2008. PECAM-1 is a critical mediator of atherosclerosis. *Dis. Model Mech.* 1: 175–181, discussion 179.
34. Liu, Y., A. H. Bubolz, Y. Shi, P. J. Newman, D. K. Newman, and D. D. Gutterman. 2006. Peroxynitrite reduces the endothelium-derived hyperpolarizing factor component of coronary flow-mediated dilation in PECAM-1-knockout mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 290: R57–R65.