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PECAM-1 dampens cytokine levels during LPS-induced endotoxemia by regulating leukocyte trafficking

Jamie R. Privratsky^{a,b}, Sarah B. Tilkens^a, Debra K. Newman^{a,b,c}, and Peter J. Newman^{a,b,d,e}

^aBlood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI 53201

^bDepartment of Pharmacology, Medical College of Wisconsin, Milwaukee, WI 53226

^cDepartment of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI 53226

^dDepartment of Cellular Biology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226

^eThe Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, WI 53226

Abstract

Aims—To investigate the mechanism by which platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31), an immunoglobulin (Ig)-superfamily cell adhesion and signaling receptor, regulates pro-inflammatory cytokine levels. The purpose of the present investigation was to test the hypothesis that PECAM-1 influences circulating cytokine levels by regulating the trafficking of activated, cytokine-producing leukocytes to sites of inflammation.

Main methods—PECAM-1^{+/+} and PECAM-1^{-/-} mice were subjected to lipopolysaccharide (LPS)-induced endotoxemia, and systemic cytokine levels were measured by Bioplex multiplex cytokine assays. Flow cytometry was employed to enumerate leukocytes at inflammatory sites and to measure cytokine synthesis in leukocyte sub-populations. Enzyme-linked immunosorbent assay (ELISA) was used to measure cytokine levels in tissue samples and in supernatants of in vitro-stimulated leukocytes.

Key findings—We confirmed earlier reports that mice deficient in PECAM-1 had greater systemic levels of pro-inflammatory cytokines following intraperitoneal (IP) LPS administration. Interestingly, expression of PECAM-1, in mice, had negligible effects on the level of cytokine synthesis by leukocytes stimulated in vitro with LPS and in peritoneal macrophages isolated from LPS-injected mice. There was, however, excessive accumulation of macrophages and neutrophils in the lungs of PECAM-1-deficient, compared with wild-type, mice - an event that correlated with a prolonged increase in lung pro-inflammatory cytokine levels.

Significance—Our results demonstrate that PECAM-1 normally functions to dampen systemic cytokine levels during LPS-induced endotoxemia by diminishing the accumulation of cytokine-

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Address correspondence to: Peter J. Newman, Blood Research Institute, BloodCenter of Wisconsin, P.O. Box 2178, 638 N. 18th Street, Milwaukee, WI 53201, Phone: (414) 937-6237, Fax: (414) 937-6284, peter.newman@bcw.edu.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

producing leukocytes at sites of inflammation, rather than by modulating cytokine synthesis by leukocytes.

Keywords

PECAM-1; leukocyte trafficking; cytokines; sepsis

Introduction

PECAM-1 is a 130 kDa cell adhesion and signaling receptor of the Ig-superfamily that is expressed on most cells of the hematopoietic lineage including platelets, monocytes/macrophages, neutrophils, and certain lymphocyte subsets (Newman 1997; Newman 1999; Newman and Newman 2003). PECAM-1 is also a major constituent of the endothelial cell intercellular junction in confluent vascular beds (Muller et al. 1989; Newman et al. 1990; Albelda et al. 1990; Newman 1997). A growing number of studies in C57BL/6 mice have revealed that a prominent function of PECAM-1 is to dampen inflammatory responses, one mechanism being by lowering levels of pro-inflammatory cytokines. For instance, PECAM-1 expression has been shown to protect mice from LPS-induced endotoxemia (Carrithers et al. 2005; Maas et al. 2005), an established animal model of sepsis (Cohen 2002), which is due, in part, to lower systemic levels of pro-inflammatory cytokines in PECAM-1-expressing, relative to PECAM-1-deficient, mice (Carrithers et al. 2005; Maas et al. 2005). The dampening of circulating pro-inflammatory cytokines by PECAM-1 has been confirmed in two other in vivo models of inflammation, collagen-induced arthritis (Tada et al. 2003) and atherogenic diet-induced steatohepatitis (Goel et al. 2007). Taken together, these studies provide compelling evidence that a prominent function of PECAM-1 is to prevent excessive levels of circulating pro-inflammatory cytokines.

Sepsis is an overwhelming and aberrant systemic inflammatory response to infection (Cohen 2002). It is the leading cause of death in non-cardiac intensive care units (Cohen 2002; Levy et al. 2003; Martin et al. 2003). Sepsis arises when the initial, appropriate host immune response to infection becomes dysregulated and uncontrolled (Cohen 2002). During the development of sepsis, there is an initial systemic increase in pro-inflammatory cytokines that triggers a variety of events, including endothelial cell activation, leukocyte recruitment, and an increase in vascular permeability, all resulting in the local accumulation of leukocytes at sites of inflammation (Cohen 2002). Though leukocytes perform a protective function by killing invading organisms, they also damage tissue by releasing lysosomal enzymes, by generating free radicals, and by continuing to release pro-inflammatory cytokines (Cohen 2002). If excessive trafficking and/or accumulation of leukocytes at inflammatory sites is not kept in check, cytokines continue to be released, causing further tissue damage, uncorrectable hypotension, and vascular instability, which results in multi-organ dysfunction and failure, also known as septic shock (Cohen 2002). Consequently, controlling excessive accumulation of activated leukocytes at inflammatory sites has the potential to prevent multi-organ failure and the high rates of mortality associated with sepsis.

The mechanism by which PECAM-1 regulates systemic cytokine levels during inflammatory responses in vivo is still poorly understood. We reasoned that PECAM-1 might regulate systemic cytokine levels either by (1) modulating the level of cellular cytokine synthesis, as has been reported in lymphocytes and macrophages (Tada et al. 2003; Rui et al. 2007), or alternatively (2) affecting the trafficking and accumulation of cytokine-producing leukocytes at sites of inflammation. The purpose of the present investigation, therefore, was to determine the mechanism by which PECAM-1 regulates pro-inflammatory cytokine levels during LPS-induced endotoxemia.

Materials and Methods

Animals

C57BL/6J wildtype (PECAM-1^{+/+}) and PECAM-1-deficient (PECAM-1^{-/-}) mice were maintained in a pathogen-free facility under the supervision of the Biological Resource Center at the Medical College of Wisconsin (MCW). PECAM-1-deficient mice (Duncan et al. 1999) used in this study have been backcrossed for > 12 generations onto the C57BL/6J genetic background. Animal protocols were approved by the MCW Institutional Animal Care and Use Committee. Mice were maintained under alternating 12-hour light and dark cycles, and had free access to food and water.

Antibodies

The following anti-mouse antibodies were used in flow cytometric analysis: eFluor 450 anti-CD3, allophycocyanin (APC) anti-CD11b, phycoerythrin (PE) anti-F4/80, APC-eFluor780 anti-F4/80, PerCP-Cy 5.5 anti-CD11c, APC anti-CD45, PE and FITC anti-PECAM-1 (MEC 13.3, BD Biosciences), PE-Cy7 anti-CD19, FITC anti-interleukin (IL)-6. Respective isotypes were also used for each antibody. All antibodies were obtained from eBioscience (San Diego, CA) unless otherwise specified.

LPS-induced endotoxemia

LPS O55:B5 (Sigma-Aldrich, St. Louis, MO) was reconstituted in sterile PBS (Mediatech, Inc., Manassas, VA), sonicated to disperse particulates, and aliquotted and stored at -20°C until use. Age-matched wild-type and PECAM-1-deficient males from 8 – 12 weeks old were injected IP with 35 µg/g LPS (volume injected in µl of 7 µg/µl solution of LPS in PBS was 5X body weight in grams) or matching volume of sterile PBS (volume injected in µl of PBS was 5X body weight in grams). This dose had previously been determined for this lot of LPS to be lethal for ~50% of wild-type mice.

Luminex bead multiplex cytokine measurements

Blood samples were obtained from the retro-orbital plexus of anesthetized mice at 3 or 18 hours after PBS or LPS injection, and anti-coagulated with 3.8% sodium citrate (final dilution 1/10). Plasma was obtained by centrifugation at 1,000 g for 5 min. Plasma samples were stored at -20°C until further use. Plasma concentrations of interleukin (IL)-6, monocyte chemotactic protein (MCP)-1 (CCL2), KC (GROα), IL-1β, tumor necrosis factor (TNF)α, IL-12, interferon (IFN)γ, and macrophage inflammatory protein (MIP)-1β were measured by Bioplex luminex bead cytokine assays (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions.

Peritoneal lavage

Non-injected, or PBS-, or LPS-injected mice were anesthetized at 18 hours post-injection, and the skin and fur overlying the peritoneum was removed, leaving the peritoneum intact. Five mls of ice-cold sterile PBS was injected and removed three successive times using a 22 gauge × 1 inch safelet catheter (Exelint International, Co., Los Angeles, CA). Cells were spun down at 250 g for 5 minutes, resuspended, and counted using a hemacytometer.

Cytokine measurements on supernatants of peritoneal cells

Peritoneal cells from above were plated at 1×10^5 /well in 96 well flat bottom tissue culture plates (Corning Inc., Corning, NY), maintained in 200 µl of leukocyte media – RPMI 1640, 10% FBS (Sigma-Aldrich), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 1X nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol (Sigma-Aldrich) – and incubated at 37°C with 5% CO₂. All cell culture

reagents were obtained from Mediatech, Inc. (Manassas, VA), unless otherwise specified. Cells were allowed to adhere to tissue culture dishes and then were stimulated with PBS or LPS at 0.1, 0.5, or 1 $\mu\text{g/ml}$ for 8 or 24 hours. Supernatants were removed and spun down at 500g for 5 minutes, after which 150 μl of supernatant was removed and stored at -20°C until further use. Levels of IL-6 and MCP-1 in supernatants were measured with ELISA Ready-SET-Go kits (eBioscience) according to manufacturer's instructions.

Intracellular IL-6 staining and flow cytometric analysis

For in vitro experiments, peritoneal leukocytes were isolated from mice as described above, plated at $1 \times 10^6/\text{well}$ in 6 well tissue culture dishes (Corning Inc.), allowed to adhere, and stimulated with PBS or LPS (1 $\mu\text{g/ml}$) in 1 ml of leukocyte media. Three hours following LPS stimulation, 1 μl of BD GolgiPlug (brefeldin A, BD Biosciences, San Jose, CA) was added, and cells were stimulated for an additional 5 hours (8 hours total). For in vivo experiments, peritoneal leukocytes were isolated from mice that had been injected 18 hours previously with PBS or LPS (35 $\mu\text{g/g}$), spun down, resuspended at $3 \times 10^6/\text{ml}$ in 1 ml of leukocyte media with 1 μl BD GolgiPlug, and incubated at 37°C with 5 % CO_2 for 3 hours. For both in vitro and in vivo experiments, 1×10^6 cells for each sample were incubated with anti-mouse CD16/CD32 (BD Biosciences) in staining buffer (PBS, 1% FBS, 0.09% sodium azide) for 15 minutes at 4°C to block Fc receptors. Cells were washed and then incubated in staining buffer containing antibodies to cell surface receptors for 30 minutes at 4°C . Cells were washed, fixed, permeabilized, and stained for intracellular IL-6 using the BD Cytotfix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. Samples were analyzed on an LSRII flow cytometer. Leukocytes were gated by forward scatter (FSC) and side scatter (SSC) to remove dead cells and cell debris from analysis, and subpopulations were then gated based on the following markers: total leukocytes-CD45, macrophages-CD11b⁺/F4/80⁺, dendritic cells-CD11b⁺/CD11c⁺, B cells-CD19⁺, and T cells-CD3⁺. Cells in each subpopulation were then analyzed for intracellular IL-6 levels. For graphic representation of IL-6 levels, mean fluorescence intensity of IL-6 staining was normalized to the mean fluorescence intensity of IL-6 in PBS-stimulated PECAM-1^{+/+} cells for each respective experiment.

Lung perfusion and isolation

PBS- or LPS-injected mice were anesthetized at 18 hours after LPS injection for leukocyte enumeration or 24 hours after injection for lung cytokine measurements, the abdomen and chest were surgically opened, and the inferior vena cava was cut. Mice were perfused through the right ventricle with 15 mls of PBS + 5 mM EDTA at 80 mmHg of pressure to clear the lungs of blood, and the entire right lung was removed and used in subsequent experiments.

Cytokine measurements in lung homogenates

Perfused lungs were homogenized in a lysis buffer containing 1% Nonidet P-40, 500 mM NaCl, 50 mM Hepes, and 1:100 Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA) using a tissue homogenizer. Homogenates were incubated on ice for 30 min and were then centrifuged at 20,000g for 20 min at 4°C . Supernatants were collected and stored at -20°C until further use. Levels of IL-6 and MCP-1 in homogenates were measured with ELISA Ready-SET-Go kits (eBioscience) according to manufacturer's instructions. The protein concentration of the homogenates was determined using the bicinchoninic assay (Thermo Scientific), and levels of IL-6 and MCP-1 were normalized to protein concentration of the homogenate.

Quantification of leukocyte subpopulations in the lung

Perfused lungs were finely minced and digested in 10 mls DMEM (Mediatech, Inc.) with 1 mg/ml collagenase/dispase (Roche Diagnostics, Indianapolis, IN) for 45 minutes at 37°C. Lungs were triturated with a 14 gauge cannula and strained through a 70 µm cell strainer (BD Biosciences). Cells were spun down, resuspended, and counted on a hemacytometer. One million cells from each lung were stained for cell surface markers, analyzed by flow cytometry, and gated as described above. The percentage of cells in each leukocyte subpopulation was then multiplied by the total number of cells isolated from its respective lung to obtain the final cell numbers.

Statistical analysis

Results, where applicable, are expressed as mean $\pm$ standard deviation (SD). Statistical analysis (Mantel-Cox log rank test for Fig. 1A; and two-way ANOVA for Figs. 1B, 2A, 2C, 3B, and 4A –D) was performed on GraphPad Prism 5 software and is indicated within figure legends.

Results

Mice deficient in PECAM-1 display a prolonged elevation in systemic pro-inflammatory cytokine levels following LPS-induced endotoxemia

Previous studies have demonstrated that expression of PECAM-1 is protective following LPS-induced endotoxemia in mice, due in part to lower systemic levels of pro-inflammatory cytokines (Maas et al. 2005; Carrithers et al. 2005). To confirm these findings and characterize the cytokine storm following LPS-induced endotoxemia, we subjected wild-type and PECAM-1-deficient mice to LPS challenge using a dose (35 µg/g) that is lethal in 50% of wild-type mice (Fig. 1A). As shown in Fig. 1B, multiplex cytokine assays on plasma samples of LPS-injected mice revealed that wild-type and PECAM-1-deficient mice produced similar systemic levels of IL-6, MCP-1, and KC initially at 3 hours following LPS administration. Whereas PECAM-1-expressing mice were able to dampen their elevated cytokine levels 24 hours following LPS administration, PECAM-1-deficient mice displayed a hyper-exaggerated elevation of these pro-inflammatory cytokines (Fig. 1B). Other cytokines measured, but that were not differentially regulated by PECAM-1, included IL-1 β , IL-12, IFN γ , MIP-1 β , and TNF α . These results demonstrate that mice deficient in PECAM-1 have decreased survival, at least in part, due to the inability to dampen systemic cytokine and chemokine levels following LPS injection.

Expression of PECAM-1 in leukocytes has negligible effects on the synthesis of pro-inflammatory cytokines in response to LPS

The sustained elevation of pro-inflammatory cytokines in PECAM-1-deficient mice following LPS administration could be due to either: (1) PECAM-1 dampening cytokine *synthesis* by leukocytes at the cellular level, or (2) PECAM-1 preventing the excessive *accumulation* of cytokine-producing cells at inflammatory sites. Since PECAM-1 has been shown to dampen activation of a variety of blood and vascular cells through ITIM-mediated inhibitory signaling (Newton-Nash and Newman 1999; Newman et al. 2001; Patil et al. 2001; Wilkinson et al. 2002; Wong et al. 2002; Falati et al. 2006; Rui et al. 2007), we first examined whether PECAM-1 regulated cellular cytokine *synthesis*.

Mononuclear cells, namely activated macrophages, release the vast majority of IL-6 in response to LPS (Kishimoto 1989), and additionally, are primary producers of MCP-1 and KC (Geiser et al. 1993; Feghali and Wright 1997). Since the peritoneum is a rich source of macrophages, and since this is the location where the innate immune response is initiated after IP LPS injection, peritoneal cells were isolated from PECAM-1^{+/+} and PECAM-1^{-/-}

mice and stimulated with LPS at various concentrations for 8 and 24 hours. PECAM-1 did not significantly affect the generation of either IL-6 (Fig. 2A) or MCP-1 (data not shown) in peritoneal leukocytes. Moreover, flow cytometric analysis revealed that the expression of PECAM-1, in mice, had no effect on upregulation of IL-6 in LPS-stimulated peritoneal macrophages (Fig. 2B – C) or in other leukocyte subsets including B lymphocytes and CD11b⁺/CD11c⁺ cells (data not shown). These results demonstrate that PECAM-1 does not modulate the rate or extent of IL-6 synthesis in macrophages or other leukocyte subsets when stimulated with LPS in vitro.

To more closely mimic the environment that macrophages and other leukocytes are exposed to in vivo, PECAM-1^{+/+} and PECAM-1^{-/-} mice were injected with LPS, peritoneal cells were isolated 18 hours later, and intracellular levels of IL-6 were measured by flow cytometry. IL-6 was upregulated to a similar extent in macrophages from PECAM-1^{+/+} and PECAM-1^{-/-} mice (Fig. 3) and other leukocyte subsets including B lymphocytes and CD11b⁺/CD11c⁺ cells (data not shown). Taken together, these results demonstrate that expression of PECAM-1, in mice, plays little to no role in regulating synthesis of pro-inflammatory cytokines in response to LPS in macrophages, and in other leukocyte subsets, either in vitro or in vivo.

Mice deficient in PECAM-1 have increased numbers of leukocytes, macrophages, and IL-6 in the lungs in response to LPS

The lungs are particularly susceptible to excessive leukocyte recruitment and subsequent tissue damage during sepsis since they receive and sample the entire blood supply, and thus are exposed to large amounts of cytokine- and pathogen-associated molecular pattern (PAMP)-containing blood. Consequently, the lungs are the most commonly reported organs to have dysfunction during severe sepsis (Angus et al. 2001). Since PECAM-1 was not found to modulate pro-inflammatory cytokine synthesis by leukocytes either in vitro or in vivo (Figs. 2 and 3), we sought to determine whether there might be increased leukocyte accumulation in the lungs of PECAM-1-deficient mice after LPS administration, and whether this correlated with elevated lung cytokine levels. As shown in Fig. 4A – C and Supp. Fig. 1, PECAM-1^{-/-} mice had significantly higher total numbers of leukocytes, macrophages, and neutrophils in the lungs compared with PECAM-1^{+/+} mice eighteen hours after LPS injection. Similar numbers of dendritic cells, B cells, and T cells were found in PECAM-1^{+/+} and PECAM-1^{-/-} mice following both PBS and LPS administration (data not shown). In addition, PECAM-1-deficient mice displayed a prolonged increase in lung IL-6 levels 24 hours after LPS administration (Fig. 4D), much like that which had been observed in plasma (Fig. 1A). These results suggest that the prolonged increase in cytokine levels systemically in the plasma and locally in the lungs of PECAM-1^{-/-} mice is due to increased accumulation of activated, cytokine-producing leukocytes, and they implicate PECAM-1 in the regulation of cytokine levels in vivo by modulating leukocyte trafficking, rather than by regulating cellular cytokine synthesis.

Discussion

PECAM-1 is a well-studied adhesion and signaling receptor that has been demonstrated to play both pro- and anti-inflammatory roles during inflammatory responses (Privratsky et al. 2010). For pro-inflammatory functions, it is well-established that PECAM-1 facilitates the transendothelial migration of leukocytes (Muller et al. 1993; Vaporciyan et al. 1993; Wakelin et al. 1996), and additionally, it helps to initiate pro-inflammatory signaling pathways in endothelial cells in response to fluid shear stress (Tzima et al. 2005). In contrast to its pro-inflammatory functions, PECAM-1 is capable of dampening the cellular activation of a variety of hematopoietically-derived cells (Newton-Nash and Newman 1999; Patil et al. 2001; Newman et al. 2001; Wilkinson et al. 2002; Wong et al. 2002; Falati et al. 2006; Rui

et al. 2007), and it also helps to lessen the severity of disease in a variety of acute and chronic inflammatory disease models in C57BL/6J mice. Previous studies have demonstrated that PECAM-1 dampens circulating levels of pro-inflammatory cytokines (Tada et al. 2003; Carrithers et al. 2005; Maas et al. 2005; Goel et al. 2007), however, the mechanisms by which PECAM-1 lowers circulating pro-inflammatory cytokine levels are still poorly understood. Consequently, the purpose of our study was to use LPS-induced endotoxemia as a model whereby we could determine the mechanism of PECAM-1 in the regulation of pro-inflammatory cytokine levels.

In the initial characterization of the LPS-induced cytokine storm in PECAM-1^{+/+} and PECAM-1^{-/-} mice, we found that PECAM-1 expression dampens systemic levels of the pro-inflammatory cytokine IL-6 and the pro-inflammatory chemokines MCP-1 and KC at later, but not earlier, timepoints following LPS administration (Fig. 1), providing circumstantial evidence that PECAM-1 does not inhibit the ability of cells to *initiate* cytokine synthesis. We also demonstrated that peritoneal leukocytes from PECAM-1-expressing mice produced similar levels of pro-inflammatory cytokines when stimulated with LPS in vitro (Fig. 2), and following LPS administration in vivo (Fig. 3), compared to leukocytes from PECAM-1-deficient mice. Based on these findings, we conclude that PECAM-1 does not play a significant role in regulating pro-inflammatory cytokine *synthesis* by leukocytes in response to LPS. A previous study, however, has observed differential regulation of cytokine synthesis by PECAM-1 in splenic and lymph node cells isolated from collagen-immunized mice that were further stimulated in vitro with collagen (Tada et al. 2003). The reasons for these divergent results are likely due to the use of different model systems, as Tada et al. analyzed the adaptive immune response in response to antigen encounter in lymphocytes (Tada et al. 2003), which is reliant on cellular activation mediated by ITAM-containing lymphocyte receptors. PECAM-1 has been demonstrated to regulate ITAM-mediated signaling through its cytoplasmic ITIMs (Newton-Nash and Newman 1999; Newman et al. 2001; Wilkinson et al. 2002), so it is possible that PECAM-1 is able to dampen cytokine production in response to stimuli that activate ITAM-containing receptors, whereas it appears not to play a role regulating cytokine synthesis downstream of PAMP receptors such as TLRs. On the other hand, in a model of CD38 (a postulated heterophilic binding partner on lymphocytes) (Deaglio et al. 1998)-mediated engagement of PECAM-1 on macrophages and siRNA-mediated acute silencing of PECAM-1 in macrophages, Rui et al. reported that PECAM-1 could regulate TLR4 signaling and cytokine production in macrophages following LPS-stimulation in vitro (Rui et al. 2007). Our results argue against CD38-mediated engagement of PECAM being able to dampen cytokine production in vivo, as we did not detect differences in intracellular levels of IL-6 in peritoneal macrophages isolated from LPS-injected PECAM-1^{+/+} and PECAM-1^{-/-} mice (Fig. 3). Additionally, unlike Rui, et al., we did not observe differential modulation of cytokine production based on expression of PECAM-1 in our in vitro studies utilizing peritoneal leukocytes from mice in which PECAM-1 had been chronically silenced through genetic deletion (Fig. 2). The differences between our results and theirs might be explained by compensatory pathways that are upregulated in macrophages in which PECAM-1 is chronically silenced. At any rate, it does not appear that PECAM-1 modulates the rate or extent of cytokine synthesis by leukocytes in vivo in our system, which mimics an innate immune response (i.e., LPS stimulation of TLR receptors); however, whether PECAM-1 might modulate cellular production of cytokines during the adaptive immune response to recognized antigens remains to be further characterized.

Since PECAM-1 did not inhibit cellular cytokine *synthesis* (Figs. 2 and 3), we next examined whether PECAM-1 regulates cytokine levels through leukocyte *trafficking and accumulation*. Previous studies have demonstrated that mice expressing PECAM-1 have decreased accumulation of leukocytes at inflammatory sites during EAE (Graesser et al.

2002), collagen-induced arthritis (Tada et al. 2003), atherogenic diet-induced steatohepatitis (Goel et al. 2007), and LPS-induced endotoxemia (Maas et al. 2005), with the latter three studies also reporting that PECAM-1 lowered systemic levels of pro-inflammatory cytokines (Tada et al. 2003; Maas et al. 2005; Goel et al. 2007). In the current study, we found that the lungs of PECAM-1-deficient mice, compared to wild-type mice, contained significantly more leukocytes, mainly macrophages and neutrophils, with a concomitant increase in IL-6 levels following LPS-injection (Fig. 4). Interestingly, once again, levels of IL-6 in the lung were only elevated at later, but not earlier, timepoints following LPS administration (Fig. 4D). Taken together, our data are consistent with a mechanism in which leukocytes synthesize and release pro-inflammatory cytokines during the early stages of LPS-induced inflammation in a PECAM-1-independent manner, followed by PECAM-1-dependent trafficking and accumulation of leukocytes in the lungs and other organs. If leukocytes are allowed to accumulate in the lungs and/or other organs, as they do in PECAM-1-deficient mice during LPS-induced endotoxemia (Fig. 4C) (Carrithers et al. 2005; Maas et al. 2005), they then continue to release pro-inflammatory cytokines, resulting in prolonged inflammation both locally and systemically.

The mechanisms by which PECAM-1 expression prevents the accumulation of leukocytes at inflammatory sites are still poorly understood. One contributing factor could be through the regulation of cytokine and chemokine synthesis in endothelial cells. Alternatively, PECAM-1 could regulate leukocyte trafficking through modulation of vascular barrier function during leukocyte transendothelial migration. PECAM-1 has been demonstrated to play an important role in the regulation of vascular barrier function in vitro (Ferrero et al. 1995; Graesser et al. 2002; Biswas et al. 2006; Privratsky et al. 2011) and during LPS-induced endotoxemia and EAE in vivo (Graesser et al. 2002; Carrithers et al. 2005; Maas et al. 2005). In fact, decreased barrier function has previously been implicated in the observation that leukocytes accumulate to a higher degree in PECAM-1^{-/-} versus PECAM-1^{+/+} mice subjected to EAE (Graesser et al. 2002). Consequently, although expression of PECAM-1 promotes leukocyte transendothelial migration (Muller et al. 1993; Vaporciyan et al. 1993; Wakelin et al. 1996), its role in maintaining vascular barrier function appears to dominate in certain inflammatory conditions. Whether any or all of these mechanisms account for the increased accumulation of leukocytes seen in PECAM-1-deficient mice during inflammation remains an active area of investigation.

Conclusion

In conclusion, the major finding of the present work is that, rather than regulating cytokine synthesis per se, PECAM-1 appears to regulate systemic cytokine levels by controlling the localization of cytokine-producing leukocytes. Further insights underlying the mechanism by which PECAM-1 regulates cytokine production and leukocyte trafficking in a wider range of inflammatory conditions will be important for advancing the treatment of acute and chronic inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PECAM-1-deficient mice display a prolonged elevation of pro-inflammatory cytokines following LPS administration

PECAM-1^{+/+} and PECAM-1^{-/-} mice were either not injected (0 hrs) or injected IP with LPS (35 μ g/g). (A) Mortality curves were generated based on survival proportions of PECAM-1^{+/+} (solid line) and PECAM-1^{-/-} (dashed line) mice following 35 μ g/g LPS injection. Median survival was 62 and 28 hours for PECAM-1^{+/+} and PECAM-1^{-/-} mice, respectively. PECAM-1^{+/+} mice displayed improved survival compared to PECAM-1^{-/-} mice following LPS injection as determined by Mantel-Cox log rank test. (B) At 3 and 24 hours following LPS administration, plasma samples were collected and levels of pro-inflammatory cytokines were measured by luminex bead multiplex cytokine assays. Levels

of IL-6, MCP-1, and KC are shown in the figure. Other cytokines measured, but that were not differentially regulated by PECAM-1, included IL-1 β , IL-12, IFN γ , MIP-1 β , and TNF α . Results are displayed as mean $\pm$ SD of cytokines in pg/ml from 3–4 plasma samples for 0 and 3 hr groups and 7–10 plasma samples for the 24 hr group. PECAM-1^{-/-} mice produced similar levels of pro-inflammatory cytokines initially at 3 hrs, but displayed prolonged elevation of pro-inflammatory cytokines at 24 hrs compared to PECAM-1^{+/-} mice as determined by two-way ANOVA statistical analysis (*p<0.05, **p<0.01).

Figure 2. PECAM-1 expression has negligible effects on IL-6 synthesis in peritoneal leukocytes stimulated with LPS *in vitro*

Peritoneal leukocytes from PECAM-1^{+/+} and PECAM-1^{-/-} mice were plated and allowed to adhere to tissue culture dishes. (A) Leukocytes were stimulated with various concentrations of LPS for either 8 or 24 hours after which supernatants were collected and assayed for IL-6 levels by ELISA. Results are expressed as mean $\pm$ SD of IL-6 in pg/ml. Expression of PECAM-1 did not significantly modulate the production of IL-6 in either a time- or dose-dependent manner as determined by two-way ANOVA statistical analysis (n=4–5 separate measurements for each dose and timepoint). (B & C) Peritoneal leukocytes were stimulated with PBS or LPS (1 μ g/ml) for 8 hrs. Cells were collected, stained for cell surface markers

and intracellular levels of IL-6, and analyzed by flow cytometry. In (B), density plots demonstrate the macrophage population in peritoneal leukocytes from (i) PECAM-1^{+/+} and (ii) PECAM-1^{-/-} mice based on CD11b and F4/80 expression. In (C), CD11b / F4/80 double positive macrophages were analyzed for intracellular levels of IL-6. (i) Representative histogram demonstrating IL-6 expression in PBS-stimulated (dashed line), LPS-stimulated PECAM-1^{+/+} (gray fill), and LPS-stimulated PECAM-1^{-/-} macrophages (black line). (ii) Histogram displaying the fold upregulation of IL-6 in response to LPS for macrophages from PECAM-1^{+/+} (black) and PECAM-1^{-/-} (white) mice from two independent experiments. For each experiment, the mean fluorescence intensity of IL-6 staining for each sample was normalized to the mean fluorescence intensity of IL-6 staining in PBS-stimulated PECAM-1^{+/+} macrophages to obtain the fold upregulation. Macrophages from PECAM-1^{+/+} mice produced IL-6 in response to LPS stimulation to the same extent as did macrophages from PECAM-1^{-/-} mice as assessed by two-way ANOVA statistical analysis (n.s.-not significant)..

Figure 3. PECAM-1 expression has negligible effects on IL-6 production in peritoneal macrophages isolated from LPS-injected mice
 PECAM-1^{+/+} and PECAM-1^{-/-} mice were injected IP with either PBS or LPS (35 μ g/g). At 18 hours after injection, the peritoneum was lavaged, and the collected cells were first incubated with Golgi-Plug (see Methods), then stained for cell surface markers and intracellular levels of IL-6, and analyzed by flow cytometry. (A) Dot plots demonstrate the macrophage population in peritoneal cells from (i) PECAM-1^{+/+} and (ii) PECAM-1^{-/-} mice based on CD11b and F4/80 expression. (B) CD11b / F4/80 double positive macrophages from (A) were analyzed for intracellular levels of IL-6. (i) Representative histogram demonstrating IL-6 expression in macrophages from PBS-injected (dashed line), LPS-

injected PECAM-1^{+/+} (gray fill), and LPS-injected PECAM-1^{-/-} mice (black line). (ii) Histogram displaying the fold upregulation of IL-6 in response to LPS for macrophages from PECAM-1^{+/+} (black) and PECAM-1^{-/-} (white) mice from two independent experiments. For each experiment, the mean fluorescence intensity of IL-6 staining for each sample was normalized to the mean fluorescence intensity of IL-6 staining from macrophages isolated from PBS-injected PECAM-1^{+/+} mice to obtain the fold upregulation. Macrophages from LPS-injected PECAM-1^{+/+} mice (black) produced IL-6 to the same extent in response to LPS injection as did macrophages from PECAM-1^{-/-} mice (white) as assessed by two-way ANOVA statistical analysis (n.s.-not significant).

Figure 4. PECAM—1-deficient mice have increased numbers of total leukocytes and macrophages, and increased levels of pro-inflammatory cytokines, in the lung at later timepoints following LPS administration

PECAM-1^{+/+} (black dots) and PECAM-1^{-/-} (white dots) mice were injected IP with either PBS or LPS (35 µg/g). (A) At 18 hours after injection, the lungs were perfused and digested to obtain a single-cell suspension. Similar numbers of cells were stained for various cell surface markers and analyzed by flow cytometry. Live cells were gated into subpopulations of leukocytes based on the following markers: total leukocytes-CD45⁺, macrophages-CD11b⁺/F4/80⁺, dendritic cells-CD11b⁺/CD11c⁺, and B cells-CD19⁺. Panels display density plots showing the macrophage population based on expression of CD11b and F4/80. Lettering below the gate signifies the mean ± SD of the percentage of macrophages that were isolated from each lung. Differences in percentages of macrophages and other leukocyte subsets (not shown) did not reach statistical significance as determined by two-way ANOVA statistical analysis. (B & C) Percentages of cells from each subpopulation were multiplied by the total number of cells from its respective lung to obtain the total cell number, as displayed in the graphs for (B) total lung WBC - and (C) lung macrophages. Dots in graphs display cell numbers (× 10⁶) for each lung analyzed, and the solid horizontal line depicts the mean. Note that PECAM-1^{-/-} mice had increased numbers of total leukocytes and macrophages in the lungs following LPS-injection compared to PECAM-1^{+/+} mice as determined by two-way ANOVA statistical analysis. (D) At 3 or 24 hours after injection, the lungs were perfused to remove blood, homogenized, and lysed. Levels of IL-6 in whole lung homogenates were measured by ELISA and normalized to protein content. Dots in graphs display cytokine levels (pg/mg protein in homogenate) from each lung measured, and the solid line depicts the mean. Note that PECAM-1^{-/-} mice displayed a prolonged increase in IL-6 in the lungs at 24 hours following LPS injection as

compared to PECAM-1^{+/+} mice as determined by two-way ANOVA statistical analysis.
(*p<0.05)