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Absence of Platelet Endothelial Cell Adhesion Molecule 1, PECAM-1/CD31, *In Vivo* Increases Resistance to *Salmonella enterica* Serovar Typhimurium in Mice

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PECAM-1/CD31 is known to regulate inflammatory responses and exhibit pro- and anti-inflammatory functions. This study was designed to determine the functional role of PECAM-1 in susceptibility to murine primary *in vivo* infection with *Salmonella enterica* serovar Typhimurium and in *in vitro* inflammatory responses of peritoneal macrophages. Lectin profiling showed that cellular PECAM-1 and recombinant human PECAM-1-Ig chimera contain high levels of mannose sugars and *N*-acetylglucosamine. Consistent with this carbohydrate pattern, both recombinant human and murine PECAM-1-Ig chimeras were shown to bind *S.* Typhimurium in a dose-dependent manner *in vitro*. Using oral and fecal-oral transmission models of *S.* Typhimurium SL1344 infection, PECAM-1$^{-/-}$ mice were found to be more resistant to *S.* Typhimurium infection than wild-type (WT) C57BL/6 mice. While fecal shedding of *S.* Typhimurium was comparable in wild-type and PECAM-1$^{-/-}$ mice, the PECAM-1-deficient mice had lower bacterial loads in systemic organs such as liver, spleen, and mesenteric lymph nodes than WT mice, suggesting that extraintestinal dissemination was reduced in the absence of PECAM-1. This reduced bacterial load correlated with reduced tumor necrosis factor (TNF), interleukin-6 (IL-6), and monocyte chemoattractant protein (MCP) levels in sera of PECAM-1$^{-/-}$ mice. Following *in vitro* stimulation of macrophages with either whole *S.* Typhimurium, lipopolysaccharide (LPS) (Toll-like receptor 4 [TLR4] ligand), or poly(I·C) (TLR3 ligand), production of TNF and IL-6 by PECAM-1$^{-/-}$ macrophages was reduced. Together, these results suggest that PECAM-1 may have multiple functions in resistance to infection with *S.* Typhimurium, including binding to host cells, extraintestinal spread to deeper tissues, and regulation of inflammatory cytokine production by infected macrophages.

Pathogen recognition by the immune system is crucial for the induction and maintenance of protective immunity (1). Rapid clearance of pathogens is essential for successful control of pyrogenic bacterial infections. Microbial pathogens have developed a variety of strategies to resist the innate and adaptive immune responses in order to survive and multiply in specific sites (2). The first step in bacterial recognition and pathogenesis is the interaction of bacterial structures expressed by the pathogen with host cell surface receptors, which, depending on the bacterial pathogen, may lead to bacterial intake by host cells. Bacteria may use intracellular niches as protection from the immune system and a safe site for replication and/or dissemination. *Salmonella enterica* serovar Typhimurium is a Gram-negative bacterium capable of evading the immune system by using macrophages as a protective niche for transport, replication, and survival (3).

The Ig-ITIM superfamily member platelet endothelial cell adhesion molecule 1 (PECAM-1) negatively regulates hematopoietic cell function, leukocyte transmigration, immune homeostasis, thrombosis, cutaneous anaphylaxis, apoptosis, and vascular permeability (4–12). Recent studies have suggested that PECAM-1 may also play a role in resistance to bacterial infections *in vivo*. It has been demonstrated that PECAM-1 knockout mice are more susceptible to lipopolysaccharide (LPS) endotoxic shock than their wild-type (WT) counterparts (13, 14). In addition, PECAM-1 is constitutively expressed on macrophages, and its expression is upregulated by LPS stimulation (15). Ligation of PECAM-1 with CD38 has been associated with negative regulation of Toll-like receptor 4 (TLR4) signaling in macrophages (16). While human CD38 has been proposed as a coreceptor for PECAM-1, these findings have not been reproduced by studies in mice, so this issue remains unresolved (17). In addition, while these earlier studies suggest a role for PECAM-1 as a negative regulator of LPS-induced inflammatory responses in macrophages and endotoxic shock, these studies did not examine virulence factors associated with *in vivo* infections with Gram-negative bacteria.

Recent studies suggest that a related Ig-ITIM superfamily member, carcinoembryonic cell adhesion molecule 1 (CEACAM-1), serves as a receptor for bacterial pathogens, including *S.* Typhimurium, *Escherichia coli*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis* in humans (18, 19). Based upon molecular modeling, crystallographic, and mutagenesis studies, a central paradigm has been proposed where the most distal N-terminal IgV-like domain 1 of CEACAM-1 is the target for binding to all currently identified bacterial ligands (20). However, the interactions between the

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closely related immunoreceptors such as PECAM-1 and bacterial ligands are less well defined.

In this study, we addressed whether PECAM-1 has the capacity to interact with the Gram-negative pathogen *S.* Typhimurium. We determined the carbohydrate moieties on PECAM-1 with lectin profiling and examined direct binding of *S.* Typhimurium to human and mouse recombinant PECAM-1 *in vitro*. In addition, to address whether PECAM-1 plays a role in the pathogenesis of *S.* Typhimurium infection *in vivo*, we examined the control of *S.* Typhimurium infection in PECAM-1$^{-/-}$ mice. Our results indicate that the absence of PECAM-1 resulted in reduced extraintestinal dissemination of *S.* Typhimurium to systemic sites, resulting in reduced proinflammatory responses. Further, macrophages from PECAM-1$^{-/-}$ mice are less responsive to TLR ligands in *in vitro* stimulations, suggesting that PECAM-1 may play a role in modulating the innate immune response following microbial exposure. Together, these results suggest that PECAM-1 may have multiple functions in resistance to infection with *S.* Typhimurium, including binding to host cells, extraintestinal spread to deeper tissues, and regulation of inflammatory cytokine production by infected macrophages.

MATERIALS AND METHODS

Reagents. *S.* Typhimurium virulent strain SL1344 was provided by P. Coloe and P. Smooker (RMIT University, Bundoora, Victoria). The mouse cytometric bead array (CBA) inflammation kit was obtained from BD Biosciences (Franklin Lakes, NJ). Cell culture reagents, including Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 200 mM glutamine, premixed penicillin (100 U/ml) and streptomycin (10,000 μg/ml), and 1 M HEPES buffer (pH 7.2). were from Gibco (Invitrogen, Life Technologies). SEW16 anti-human PECAM-1 antibody was obtained from Peter Newman (Blood Research Institute, Milwaukee, WI), anti-mouse PECAM-1 antibody–fluorescein isothiocyanate (FITC) was purchased from Santa Cruz (Santa Cruz, CA), 4D1C2 anti-CEACAM-1 antibody was obtained from Nicole Beauchemin (McGill University, Montreal, Quebec, Canada), and F4/80 antimacrophage antibody and streptavidin-phycoerythrin (PE) were obtained from BD Biosciences (Franklin Lakes, NJ). *Salmonella* lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (St. Louis, MO). CpG, poly(I·C), and Loxoribine (LXR) were obtained from Invivogen (San Diego, CA). Peptidoglycan (PGN) was purchased from Fluka (Ronkonkoma, NY).

Mice. The construction of PECAM-1$^{-/-}$ (PECAM-1 knockout) mice has been previously described (21). These PECAM-1$^{-/-}$ mice were backcrossed eight generations onto the C57BL/6 background. Mice were housed in a specific-pathogen-free facility at the Burnet Institute animal house, Heidelberg, Melbourne, Australia. For mouse genotyping, the primers for PECAM-1 forward (sense oligonucleotide) (5′-ATGGAACT GGCACCCATCACTTA-3′), PECAM-1 reverse (antisense oligonucleotide) (5′-GGTCACGTCTCGCCTATTAAGC-3′), and neomycin (antisense oligonucleotide) (5′-GTCTTCTTGAGCAGTTCTTCCGCTATC-3′) were obtained from Sigma Proligo (Castle Hill, New South Wales, Australia). Age- and sex-matched groups of 6- to 8-week-old wild-type C57BL/6 and PECAM-1$^{-/-}$ mice were used for *in vitro* and *in vivo* experiments. Wild-type C57BL/6 and PECAM-1$^{-/-}$ mice were genotyped using PCR-restriction fragment length polymorphism specific for the guanine-to-adenine point mutation associated with the susceptibility allele of the Slc11a1 (Nramp1) gene. Nramp1 primer sequences Ity3′ (ACA GCC CGG ACA GGT GGG), Ity5′S (ACG CAT CCC GCT GTG GGA) (susceptible or Nramp1$^{-/-}$ primer), and Ity5′R (ACG CAT CCC GCT GTG GGG) (resistant or NRamp1$^{+/+}$ primer) were obtained from Sigma Proligo. Both wild-type and PECAM-1$^{-/-}$ mice were confirmed to carry the homozygous susceptible allele of Slc11a1 (Nramp1) by PCR (22). All animal experiments were approved by the Austin Health Animal Ethics Committee and complied with the Prevention of Cruelty to Animals Act (1986) and the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

Lectin profiling and Western blotting. Platelet lysates were precleared twice with 50 μl of a 50% suspension of CNBr-activated Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) for 15 min at 4°C and then centrifuged at 4,000 rpm for 5 min, and the supernatant was retained. Precleared platelet lysates (1.5 mg) were incubated with 10 μg of biotin-labeled lectins (Vector Laboratories, Burlingame, CA) at 4°C for 2 h. The lectin-carbohydrate-protein complexes were then isolated with 50 μl of a 50% suspension of streptavidin-agarose beads (Sigma Chemical Company, St. Louis, MO) incubated for 1 h at 4°C. Beads were then washed five times with immunoprecipitation buffer (20 mM Tris [pH 7.4] containing 50 mM NaCl and 2% [vol/vol] Triton X-100). Bound proteins were eluted from the streptavidin-agarose beads by boiling for 10 min in 30 μl SDS reducing buffer and resolved on a 10% SDS-polyacrylamide gel, followed by Western blot analysis using either SEW16 anti-PECAM-1 antibody (20 μg/ml) or monoclonal 4D1C2 anti-CEACAM-1 antibody (5 μg/ml) and then appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1/20,000), followed by ECL detection.

***Salmonella* enzyme-linked immunosorbent assay (ELISA).** Microtiter plates (Maxisorp; Nunc, Wiesbaden, Denmark) were coated with 100 μl of human recombinant PECAM-1-Ig chimera protein at various doses (0 to 5 μg/ml). PECAM-1-Ig chimera was diluted in 0.2 M carbonate buffer (pH 8.3), and plates were coated overnight at 4°C. Nonspecific binding sites were blocked with 200 μl of 1% (wt/vol) bovine serum albumin (BSA) (Bovogen Biologicals, Keilor East, Victoria, Australia) in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) for 1 h at 37°C. The wells were washed three times with 0.01 M PBS (pH 7.4) containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, MO). Bacteria were collected from an overnight culture by centrifugation at 2,400 rpm for 30 min and resuspended in blocking buffer, and 100 μl of bacterial suspension was added and incubated for 2 h at 37°C. The wells were washed six times with wash buffer, and then rabbit anti-*Salmonella* serum was added and incubated for 2 h at 37°C. Wells were washed six times with wash buffer, and alkaline phosphatase-conjugated sheep anti-rabbit antibody (Rockland Inc., Gilbertsville, PA) was added to each well and incubated for 1 h at 37°C. Bound antibodies were visualized using *para*-nitrophenyl phosphate (pNPP) as the substrate. The reaction was terminated with 50 μl of 2 N NaOH, and absorbance at 405 nm was measured using a Fluostar Optima plate reader (BMG Labtechnologies, Offenburg, Germany).

Bacterial culture and preparation. *S.* Typhimurium SL1344 was cultured overnight in 10 ml of Luria broth (LB) containing 25 μg/ml streptomycin at 37°C with agitation. For *in vitro* infections, *S.* Typhimurium SL1344 was collected from cultures by centrifugation and incubated at 37°C for 30 min in 300 μl of 10% (vol/vol) normal mouse serum in DMEM. Opsonized bacteria were collected by centrifugation at 13,000 rpm for 5 min, and serial dilutions were prepared in incomplete DMEM for infection of peritoneal macrophages. To heat kill the bacteria, after opsonization, *S.* Typhimurium SL1344 was heated at 65°C for 20 min. Killing of bacteria was confirmed by culture for 48 h on LB-streptomycin agar at 37°C. The viable count in overnight SL1344 cultures was determined by plating serial dilutions onto XLD plates and incubating at 37°C for 24 h.

Oral infection of mice with *S.* Typhimurium SL1344. Bacteria were cultured to mid-log growth phase and prepared as described previously (23). Mice were infected with 0.1 ml containing 10^3 to 10^5 CFU *S.* Typhimurium SL1344 by oral gavage under light inhalation anesthesia (22). Mice were observed for symptoms of salmonellosis and euthanized when humane endpoints were reached. The clinical symptoms that were monitored included fur condition, alertness, mobility, body weight loss, posture, and breathing rate.

Natural *S.* Typhimurium infection model. To mimic the natural fecal-oral route of *S.* Typhimurium transmission, a single C57BL/6 mouse

was infected with 1×10^6 CFU *S.* Typhimurium SL1344 by oral gavage and placed in a cage with naive animals (23). The acquisition and development of *S.* Typhimurium infection in the naive cohoused animals was monitored by detection of *S.* Typhimurium in fresh fecal samples plated on selected indicator medium (XLD). The absence of *S.* Typhimurium in feces of all mice was confirmed before commencement of the experiments (24).

Enumeration of bacterial organ load from infected mice. Mice were euthanized 5 days after oral infection, and livers, spleens, mesenteric lymph nodes (MLN), and brains were harvested into 5 ml of sterile cold PBS. Organs were homogenized using a Stomacher 80 Biomaster homogenizer (Seward, United Kingdom) at low speed for 5 min. Viable counts in serial dilutions of homogenates were determined following culture on LB agar containing 25 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) for 24 h at 37°C.

Cytokine analyses. Mouse sera collected at day 5 after infection with *S.* Typhimurium SL1344 and supernatants collected from *in vitro* macrophage invasion assay were aliquoted and stored at −80°C. For analysis of mouse serum cytokine levels, blood was obtained by cardiac puncture, incubated at 37°C for 1 h, and then centrifuged at 4,000 rpm for 10 min to separate the serum. Sera and supernatants were analyzed for cytokine levels using the mouse inflammation cytometric bead array (CBA) assay kit from BD Biosciences according to the manufacturer's instructions. The cytokines analyzed were interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein 1 (MCP-1), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-12p70 (IL-12p70). Data were acquired on a FACSCanto flow cytometer, and cytokine levels were analyzed using the CBA software.

Culturing of murine peritoneal macrophages for flow cytometry and cytokine analyses. Mouse resident peritoneal macrophages were harvested by intraperitoneal lavage with PBS from six wild-type C57BL/6 and PECAM-$1^{-/-}$ mice and cultured in a 1:1 ratio of complete DMEM (supplemented with 10% [vol/vol] heat-inactivated fetal calf serum [FCS], 2 mM glutamine, 50 μg/ml penicillin-streptomycin antibiotic, and 15 mM HEPES, pH 7.2) and 50% L-cell conditioned medium (as a source of macrophage colony-stimulating factor [M-CSF]). These cells were plated in 24-well plates at a density of 2×10^5 cells per well containing 1 ml of medium and grown at 37°C in a humidified 5% CO_2 incubator (Sanyo Fisher Company, Chatsworth, CA). The culture medium was changed after 1 day to remove nonadherent cells, and the cells were cultured for 6 days. For analysis of cytokines, the cells were grown for an additional 2 days in the above-described medium before the experiment. Cell viability was assessed by trypan blue exclusion.

Flow cytometry. Cultured peritoneal macrophages (2.5×10^5) from wild-type C57BL/6 and PECAM-$1^{-/-}$ mice were collected and suspended in fluorescence-activated cell sorter (FACS) buffer (PBS [pH 7.4] containing 1% [vol/vol] FCS). To prevent nonspecific antibody binding, macrophages were incubated with 2.4G2 anti-FcγRII antibody (provided by P. M. Hogarth, Burnet Institute, Heidelberg, Victoria, Australia) before staining with either anti-F4/80 (biotin conjugated [BD Pharmingen, San Diego, CA] or PE conjugated [eBioscience, San Diego, CA]), polyclonal rabbit anti-mouse TLR3/CD283 antibody (Imgenex, San Diego, CA), rat anti-mouse TLR4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or rat anti-mouse PECAM-1 IgG2a antibody (BD Pharmingen, San Diego, CA). Goat anti-rat IgG–FITC and PE-conjugated streptavidin were used as secondary antibodies (BD Pharmingen, San Diego, CA). Following staining, cells were resuspended in ice-cold FACS buffer, and data were acquired on a FACSCanto flow cytometer (BD Biosciences) and analyzed using DIVA software.

Statistical analysis. Results are expressed as means ± standard errors of the means (SEM). Group survival data were analyzed using a log rank *p* test. All other data were analyzed with Student's *t* test (two tailed) or the nonparametric Mann Whitney U test. All statistical analyses were performed using GraphPad Prism software, with a P value of <0.05 considered to represent statistical significance.

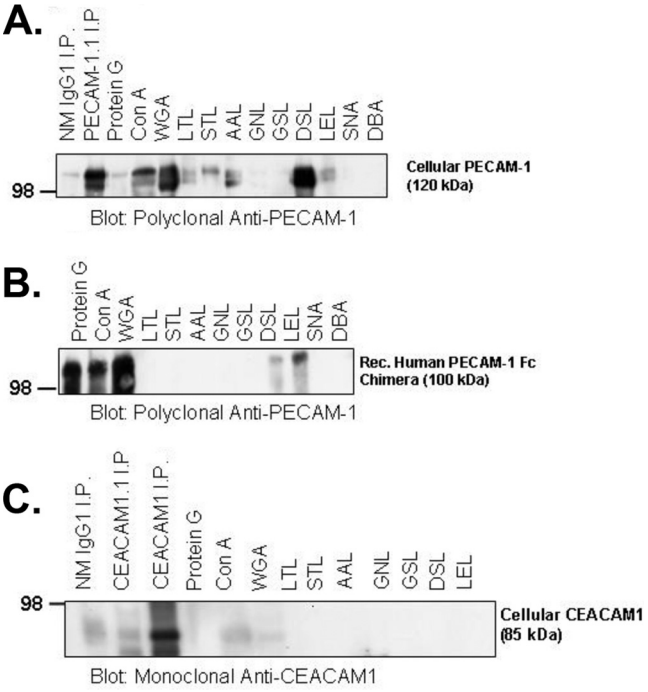

FIG 1 PECAM-1 contains α-linked mannose and *N*-acetylglucosamine sugars as shown by lectin profiling. Biotinylated lectins (10 μg) were added to precleared 1.5-mg 15K Triton-soluble human platelet lysates and incubated at 4°C for 2 h with mixing. A 50-μl suspension of 50% streptavidin-agarose beads was then added and incubated at 4°C for 1 h with constant mixing. Immunoprecipitation products were washed and resuspended in SDS reducing buffer, and proteins were separated by 10% SDS-PAGE and then transferred by Western blotting and probed with either a polyclonal anti-PECAM-1 antibody (A and B) or an anti-CEACAM-1 antibody (C) using ECL as the substrate to visualize bound antibody.

RESULTS

Lectin profiling of carbohydrates on PECAM-1. Previous studies have shown that high levels of mannose sugars on CEACAM-1 can be targeted by mannose-specific lectins such as type I fimbriae of *E. coli* and *S.* Typhimurium (19). Based upon these findings, we wanted to determine what carbohydrates are present on PECAM-1. In order to define the carbohydrate residues present on PECAM-1, a lectin profiling approach was used based upon carbohydrate-lectin recognition: a series of biotin-labeled lectins was used to capture immunoreceptor carbohydrate-lectin interaction using biotin-streptavidin and immunodetection of PECAM-1 with specific antibodies. The following biotin-labeled lectins were tested: *Aleuria aurantia* lectin (AAL) [fucose (α1,6)-*N*-acetylglucosamine], concanavalin A (ConA) (α-linked mannose), *Datura stramonium* lectin (DSL) (β-1,4-linked acetylglucosamine), *Galanthus nivalis* lectin (GNL) (α-1,3-mannose), *Griffonia simplicifolia* lectin (GSL) (α-*N*-acetylgalactosamine α-galactose), *Lycopersicon esculentum* lectin (LEL) (*N*-acetylglucosamine), *Lotus tetragonolobus* lectin (LTL) (α-linked fucose), *Solanum tuberosum* lectin (STL) (*N*-acetylglucosamine), *Sambucus nigra* lectin (SNA) (α-2,6-linked sialic acid), *Dolichos biflorus* agglutinin (DBA) (α-linked *N*-acetylgalactosamine), and wheat germ agglutinin (WGA) (*N*-acetylglucosamine). Figure 1 shows the lectin profiles of cellular PECAM-1 (Fig. 1A), cellular CEACAM-1 (Fig. 1C), and recombinant human PECAM-1-Ig chimera (Fig. 1B). As shown in

TABLE 1 Reactivities of cellular PECAM-1 and CEACAM-1 and PECAM-1-Ig chimera with biotinylated lectins of different sugar specificities

Biotinylated lectin[a]	Specificity	Activity[b]		
		Cellular PECAM-1	PECAM-1-Ig chimera	Cellular CEACAM1
AAL	Fucose(α1,6)-*N*-acetylglucosamine	++	−	−
DSL	β-1,4-Linked *N*-acetylglucosamine	++++	+	−
GNL	α-1,3-Mannose	−	−	−
GSL	α-*N*-Acetyl galactosamine α-galactose	−	−	−
LEL	*N*-Acetylglucosamine	+	++	−
LTL	α-Linked fucose	+	−	−
STL	*N*-Acetylglucosamine	+	−	−
SNA	α-2,6-Linked sialic acid	−	−	−
DBA	α-Linked *N*-acetylgalactosamine	−	−	−
ConA	α-Linked mannose	+++	++++	++
WGA	*N*-Acetylglucosamine	++++	+++++	+
Protein G	Binds via Ig portion	−	+++++	−

[a] AAL, *Aleuria aurantia* lectin; DSL, *Datura stramonium* lectin; GNL, *Galanthus nivalis* lectin; GSL, *Griffonia simplicifolia* lectin; LEL, *Lycopersicon esculentum* lectin; LTL, *Lotus tetragonolobus* lectin; STL, *Solanum tumerosum* lectin; SNA, *Sambucus nigra* agglutinin; DBA, *Dolichos biflorus* agglutinin; ConA, concanavalin A; WGA, wheat germ agglutinin.
[b] −, no reactivity; weak to strong reactivity is graded + to +++++.

Fig. 1A, cellular PECAM-1 showed different degrees of binding with the lectins ConA, WGA, LTL, STL, AAL, DSL, and LEL but had no or little binding to protein G, GNL, GSL, SNA, or DBA. In comparison, recombinant human PECAM-1-Ig chimera showed a similar pattern of reactivity with protein G (via the Ig portion), ConA, WGA, DSL, and LEL but did not bind LTL, STL, AAL, GNL, SNA, and DBA (Fig. 1B). This is in contrast to the case for cellular CEACAM-1, which showed a different carbohydrate profile with various degrees of weak binding to ConA and WGA but not LTL, STL, AAL, GNL, GSL, DSL, and LEL (Fig. 1C). A summary of the strengths of reactivity of various lectin interactions as determined by band intensity in Western blotting with either cellular PECAM-1, cellular CEACAM-1, or recombinant human PECAM-1-Ig chimera protein is shown in Table 1. These data indicate that PECAM-1 and CEACAM-1 both have α-linked mannose sugars and *N*-acetylglucosamine sugars, with no binding affinity to *N*-acetylgalactosamine or sialic acid sugars, indicating that neither has these carbohydrate modifications.

Human PECAM-1 and murine PECAM-1 bind *S.* Typhimurium *in vitro*. Previous studies have shown that high levels of mannose sugars on CEACAM-1 can be targeted by mannose-specific microbial lectins such as type 1 fimbriae of *E. coli* and *S.* Typhimurium (19). These original studies on CEACAM-1 and bacterial pathogens were restricted to humans, as mouse CEACAM-1 does not recognize many of the identified bacterial pathogens, including *S.* Typhimurium. Based upon these findings, it was important to determine whether human PECAM-1 and mouse PECAM-1 bind to Gram-negative bacteria, including *S.* Typhimurium, before embarking on mouse models of *S.* Typhimurium infection *in vivo*. In order to address this issue, a whole-cell *Salmonella* ELISA was performed to study the interaction of human and murine PECAM-1-Ig chimera and *S.* Typhimurium SL1344 *in vitro*. Bovine serum albumin (BSA)-coated plates were used as a negative control, as BSA lacks any high-mannose-type carbohydrate chains (Fig. 2).

As shown in Fig. 2A, bound bacteria were detected on plates coated with human PECAM-1-Ig chimera using rabbit anti-*Salmonella* antibody. The binding reached a plateau at an optical density (OD) at 405 nm of 1.25 when the protein concentration was 2.0 μg/ml. Little or no binding of *S.* Typhimurium was observed when BSA-coated plates were used as negative controls or when normal mouse serum was used instead of rabbit anti-*Salmonella* antibodies. There was a 3-fold increase in *S.* Typhimurium binding to PECAM-1-Ig chimera compared to binding to BSA. Similarly, *S.* Typhimurium bacteria bound to murine PECAM-1-Ig chimera, and this binding was quantified with a rabbit anti-*Salmonella* antibody. The binding reached a plateau at an OD at 405 nm of 1.05 when the protein concentration was 2.0 μg/ml, and there was an approximately 2-fold increase in binding compared to that with the negative-control antigen, BSA. No binding was observed when normal rabbit serum was used (Fig. 2B). Taken together, these results demonstrate that both human and murine PECAM-1-Ig chimera can bind *S.* Typhimurium *in vitro* in a dose-dependent, saturable manner.

Increased resistance of PECAM-1$^{-/-}$ mice to oral infection with *S.* Typhimurium SL1344. Considering that PECAM-1 is expressed by epithelial cells and macrophages, which are known host cells for *S.* Typhimurium, and having demonstrated that PECAM-1 contains lectin-binding sites that specifically interact with bacterial structures, including *S.* Typhimurium, we next investigated the effect of absence of PECAM-1 in *S.* Typhimurium infection (25, 26). Mice were orally infected with various doses of virulent *S.* Typhimurium SL1344 (10^5 CFU, 10^4 CFU, and 10^3 CFU). Mice were observed for symptoms of salmonellosis for 7 to 10 days and were euthanized when humane endpoints were reached. Fig. 3A and B show that at higher infectious doses (10^5 and 10^4 CFU, respectively) there was little difference in the susceptibilities of PECAM-1$^{-/-}$ and wild-type C57BL/6 mice, but at a lower infectious dose of 10^3 CFU, PECAM-1$^{-/-}$ mice were clearly more resistant to infection (80% survival) than wild-type C57BL/6 mice (Fig. 3C). Subsequent experiments to further investigate the increased resistance to *S.* Typhimurium infection by PECAM-1$^{-/-}$ mice were therefore performed with an infectious dose of 10^3 CFU SL1344.

As *S.* Typhimurium infection is normally acquired through ingestion of contaminated water or food, we employed a natural oral-fecal transmission model as well as the oral infection model to further explore the susceptibility of PECAM-1$^{-/-}$ mice to infection with *S.* Typhimurium (24). To facilitate transmission of *S.* Typhimurium from infected to uninfected mice, naive mice were

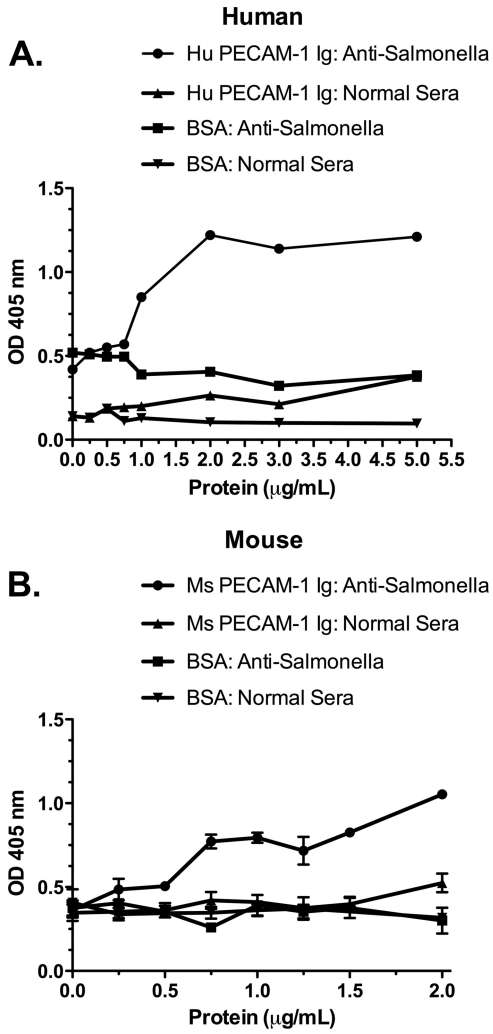

FIG 2 Dose response of binding of PECAM-1-Ig chimera to *S.* Typhimurium determined by ELISA. Binding of human PECAM-1-Ig chimera (0 to 2.0 μg/ml) (A) or murine PECAM-1-Ig chimera (B) and bovine serum albumin (0 to 2.0 μg/ml) to *S.* Typhimurium was visualized using an ELISA-based assay, in which microtiter plates coated with the indicated amounts of PECAM-1-Ig or BSA were used and binding of *S.* Typhimurium to the chimeric proteins was visualized using rabbit anti-*Salmonella* antibody and anti-rabbit alkaline phosphatase-conjugated antibody.

cohoused with orally infected (10^6 CFU) mice. Transmission was monitored by measuring the number of *Salmonella* bacteria in fecal pellets, and the onset of salmonellosis was monitored by observation for clinical signs. Figure 3D demonstrates that naive PECAM-$1^{-/-}$ mice showed 100% survival when cohoused with infected animals, while 75% of cohoused naive C57BL/6 mice became infected and moribund. To further demonstrate reduced susceptibility of PECAM-$1^{-/-}$ mice to oral infection with *S.* Typhimurium, the experiment was repeated with larger groups of mice ($n = 15$), which were all infected with 10^3 CFU *S.* Typhimurium SL1344 by oral gavage and monitored for onset of disease. Figure 3E shows that PECAM-$1^{-/-}$ mice were significantly more resistant to infection ($P < 0.005$, log rank p test) than wild-type C57BL/6 mice, confirming our observations with the natural transmission infection model.

Resistance of PECAM-$1^{-/-}$ mice to infection with *S.* Typhimurium was further characterized by enumeration of bacterial burdens in organs of infected mice. Mice were orally infected with 10^3 CFU SL1344, and at 5 days after infection, the bacterial loads in liver, spleen, mesenteric lymph nodes (MLN), and brain were determined by viable counts in tissue homogenates. In addition, the bacterial load in freshly collected fecal pellets was determined by viable count. Figure 4A shows that at early time points after infection, no difference in the number of SL1344 bacteria shed in feces was detected between C57BL/6 and PECAM-$1^{-/-}$ mice, suggesting that in the gastrointestinal tract, the bacterial load was comparable between mouse strains. At 5 days after oral infection, the bacterial load in PECAM-$1^{-/-}$ mice was significantly ($P < 0.05$, $n = 14$) lower in all systemic organs tested than that in C57BL/6 mice (Fig. 4B). Taken together, these data imply that dissemination of *S.* Typhimurium from the gastrointestinal tract into deeper tissues is delayed in mice lacking PECAM-1.

Proinflammatory cytokine responses are altered in PECAM-$1^{-/-}$ mice during *S.* Typhimurium SL1344 infection. Previous studies have shown that the production of cytokines and soluble factors by macrophages is important during the early phases of *S.* Typhimurium infection (25). These earlier studies showed that macrophage-derived cytokines such as IL-6, TNF-α, and IFN-γ are typically enhanced during *S.* Typhimurium infection (27). As PECAM-1 is expressed on macrophages and these cells serve as the major host cell, we next investigated whether the resistance to *S.* Typhimurium infection in PECAM-$1^{-/-}$ mice was associated with altered circulating levels of these cytokines during infection. Mice were infected orally with 10^3 CFU *S.* Typhimurium SL1344, and blood was taken from the mice at 5 days after oral infection for cytokine analyses. As shown in Fig. 5A to F, PECAM-$1^{-/-}$ mice produced significantly lower levels of proinflammatory cytokines IFN-γ ($P < 0.01$, $n = 11$), MCP-1 ($P < 0.001$, $n = 11$), IL-6 ($P < 0.05$, $n = 11$), and TNF-α ($P < 0.05$, $n = 11$) than did wild-type C57BL/6 mice, whereas no difference in the levels of IL-12p70 ($P > 0.05$, $n = 11$) and anti-inflammatory cytokine IL-10 ($P > 0.05$, $n = 11$) (using nonparametric Mann Whitney U test) was observed. Importantly, no significant differences were found in any of the cytokine levels between uninfected PECAM-$1^{-/-}$ and uninfected wild-type C57BL/6 mice (Fig. 5).

PECAM-$1^{-/-}$ peritoneal macrophages release reduced amounts of proinflammatory cytokines following stimulation with live or heat-killed *S.* Typhimurium or with TLR ligands *in vitro*. Next, we compared cytokine production by cultured resident peritoneal macrophages from PECAM-$1^{-/-}$ mice and wild-type C57BL/6 mice in response to *Salmonella* infection. Initially, we confirmed the expression of PECAM-1 and TLR4 on the surface of resident peritoneal macrophages and intracellular expression of TLR3 using flow cytometry. As shown in Fig. 6A, the purity of the peritoneal macrophages was confirmed using the F4/80 macrophage marker, and no difference between wild-type C57BL/6 and PECAM-$1^{-/-}$ macrophages was detected. The F4/80-positive macrophages from both strains of mice expressed equivalent levels of TLR4 (Fig. 6C) and TLR3 (Fig. 6D), while PECAM-1 was detected at modest levels on F4/80^+ cells from C57BL/6 mice and was absent on cells from PECAM-$1^{-/-}$ animals (Fig. 6B).

To test the hypothesis that PECAM-$1^{-/-}$ peritoneal macrophages may have altered inflammatory responses to *S.* Typhimurium infection, we examined cytokine levels released by peritoneal macrophages upon exposure to live and heat-killed *S.* Typhimu-

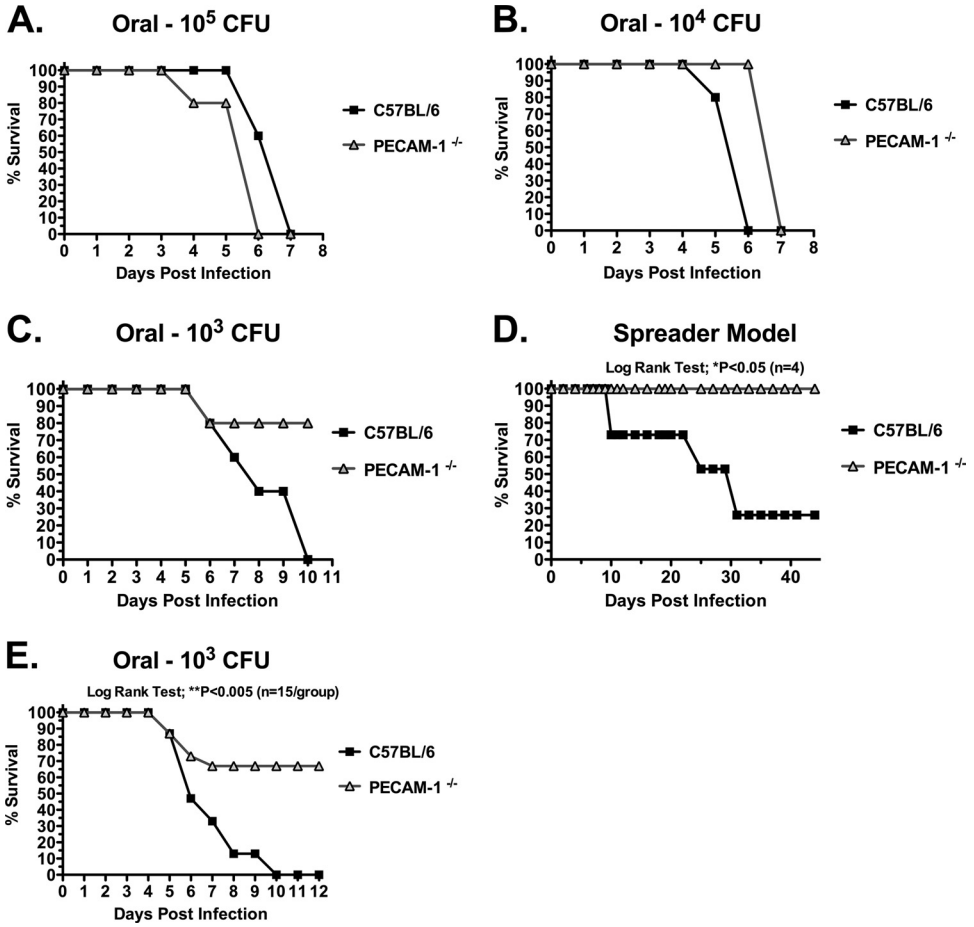

FIG 3 Absence of PECAM-1 increases resistance to *S.* Typhimurium SL1344 infection *in vivo*. (A to C) Groups of five naive C57BL/6 WT (■) or PECAM-$1^{-/-}$ (△) mice were infected with either 10^5 CFU (A), 10^4 CFU (B), or 10^3 CFU (C) virulent *S.* Typhimurium SL1344 by oral gavage. For ethical reasons, mice were culled when they became moribund; these mice were deemed "nonsurvivors." (D) One wild-type C57BL/6 mouse was orally inoculated with 10^6 CFU *S.* Typhimurium SL1344 and cohoused with four naive C57BL/6 mice or four PECAM-$1^{-/-}$ mice. The course of infection of all mice was followed by measuring survival over time. The experiment was performed with two cages for each mouse treatment, and the data are represented as means ± SEM. (E) Age- and sex-matched mice, C57BL/6 (■) and PECAM-$1^{-/-}$ (△) mice ($n = 15$) were infected orally with 10^3 CFU of *S.* Typhimurium SL1344, and their survival was monitored for 12 days. The *y* axis indicates survival, and the *x* axis indicates days after infection. For ethical reasons, mice were culled when moribund.

rium SL1344 *in vitro* (Fig. 7). Supernatants were collected 24 h after exposure to *Salmonella* and then analyzed using the CBA mouse inflammation kit. Stimulated macrophages produced both IL-6 and TNF-α, and the amount of cytokines produced increased with increasing multiplicity of infection (MOI) (Fig. 7). In comparison with C57BL/6 macrophages, PECAM-$1^{-/-}$ macrophages infected with live or heat-killed *S.* Typhimurium SL1344 produced reduced levels of IL-6 and TNF-α ($P < 0.05$, $n = 3$) (Fig. 7).

One of the major pathways leading to production of pro- and anti-inflammatory cytokines by macrophages is following activation of Toll-like receptors (TLRs) by their respective ligands. Consequently, it was important to investigate whether any specific TLR was affected by deficiency of PECAM-1 in macrophages. For these experiments, peritoneal macrophages were harvested from wild-type C57BL/6 and PECAM-$1^{-/-}$ mice, cultured for 2 days as described above, and then treated with either the TLR2 agonist peptidoglycan (PGN), the TLR4 agonist *Salmonella* lipopolysaccharide (LPS), the TLR7 agonist Loxoribine (LXR), the TLR9 agonist CpG oligonucleotide, or the TLR3 agonist poly(I·C) for 8 h before supernatants were removed for cytokine analysis.

The results in Fig. 8 demonstrate that a lack of PECAM-1 expression on macrophages affected cytokine production in a TLR ligand-dependent manner. Following stimulation with LPS, production of IL-6, TNF-α, and MCP-1 was significantly $P < 0.001$) reduced in PECAM-$1^{-/-}$ macrophages compared to C57BL/6 macrophages. Similarly, stimulation of PECAM-$1^{-/-}$ macrophages with poly(I·C) also showed significantly ($P \leq 0.05$) reduced TNF-α and MCP-1 levels compared to that of wild-type C57BL/6 macrophages (Fig. 8B). In contrast, stimulation of C57BL/6 and PECAM-$1^{-/-}$ macrophages with PGN showed high IL-6, TNF-α, and MCP-1 responses; however, there were no significant difference between the two genotypes. In addition, cytokine release following stimulation with CpG or LXR was no different between cultures of C57BL/6 and PECAM-$1^{-/-}$ macrophages ($P > 0.05$, $n = 3$) (Fig. 8). The differences in cytokine responses were not due to altered cell viability (Fig. 8D). These results suggest a possible

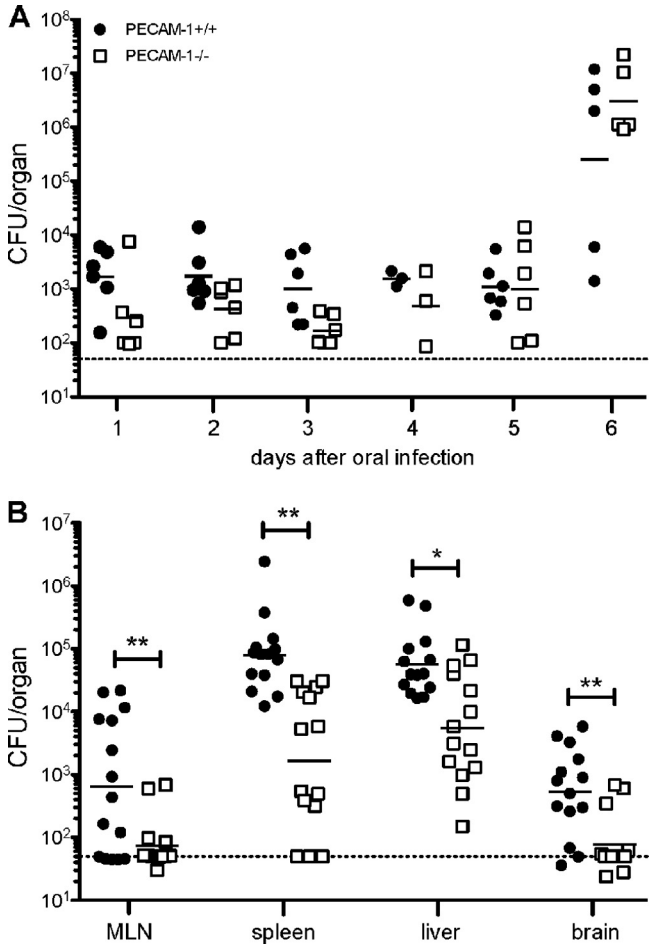

FIG 4 Reduced bacterial loads in PECAM-1$^{-/-}$ mice compared to C57BL/6 mice following oral *S.* Typhimurium SL1344 infection. C57BL/6 (PECAM-1$^{+/+}$) (black symbols) and PECAM-1$^{-/-}$ (white symbols) mice were inoculated orally with 10^3 CFU *S.* Typhimurium SL1344. (A) At the indicated time points after infection, fresh fecal pellets were collected and bacterial load was determined by viable count. Results were obtained in 2 independently performed experiments. Shown are results from individual mice (symbols); the horizontal bar indicates the geometric mean of each group. (B) At 5 days after infection, mice were euthanized, and bacterial loads in liver, spleen, MLN, and brain were determined by viable count. Results were obtained in 3 independently performed experiments. Shown are results from individual mice (symbols) ($n = 14$ for each organ); the horizontal bar indicates the geometric mean of each group. Data were analyzed using a nonparametric Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$.

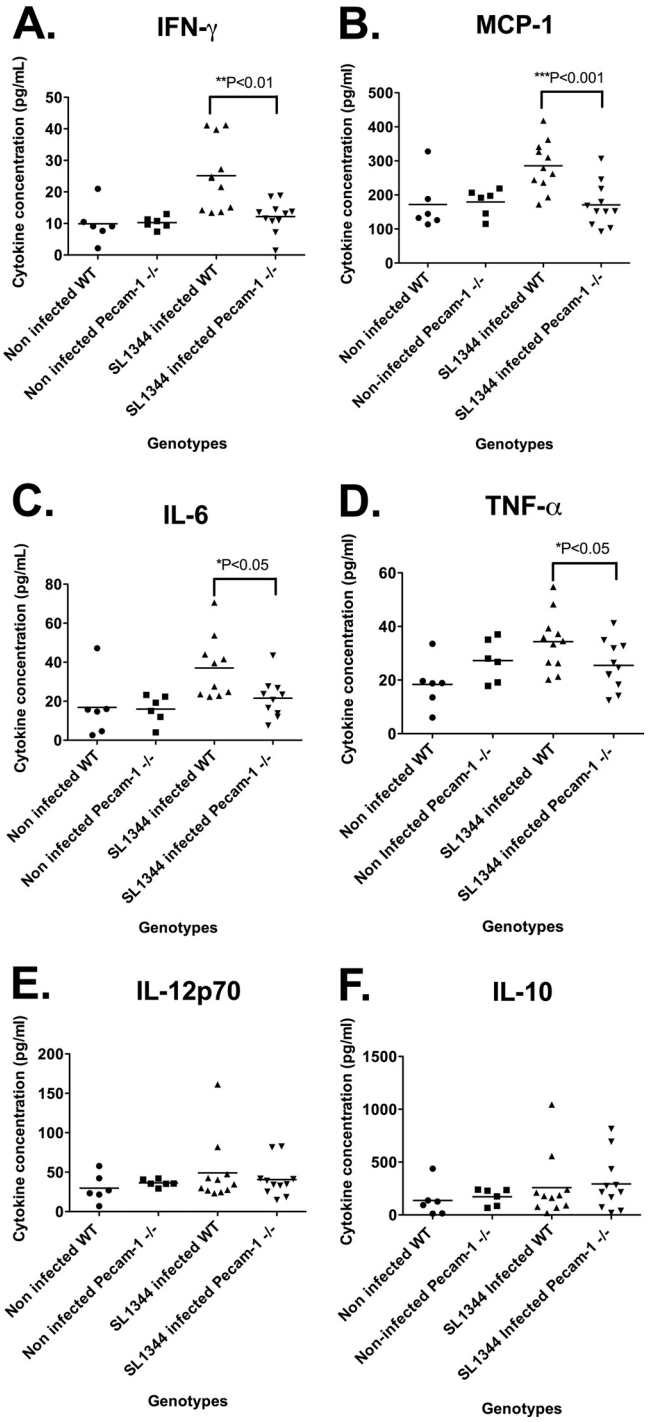

FIG 5 PECAM-1$^{-/-}$ mice have reduced serum proinflammatory cytokine responses following *S.* Typhimurium SL1344 infection *in vivo*. Wild-type C57BL/6 and PECAM-1$^{-/-}$ mice were infected orally with 1×10^3 CFU *S.* Typhimurium SL1344 or left uninfected. Both cohorts were sacrificed on day 5 after infection. Blood samples were collected via cardiac puncture, and serum was separated for cytokine analysis. The following cytokines were analyzed using a mouse CBA inflammation kit: IFN-γ (A), MCP-1 (B), IL-6 (C), TNF-α (D), IL-12-p70 (E), and IL-10 (F). Data were collected from a total of three independent experiments ($n = 11$ infected mice of each strain and $n = 6$ uninfected mice of each strain). Data were analyzed using a nonparametric Mann-Whitney U test. *, $P < 0.05$.

relationship between PECAM-1 and TLR3 and TLR4 signaling pathways involving cytokine release.

DISCUSSION

Recent evidence suggests that the Ig-ITIM-bearing receptor CEACAM-1 serves as a receptor for bacterial pathogens, including *S.* Typhimurium, *E. coli*, *N. gonorrhoeae*, and *N. meningitidis* in humans (18). CEACAM-specific adhesins allow bacterial or viral pathogens to attach to, invade, and/or transcytose polarized epithelia and endothelia to colonize host tissues. Whether this is the case for closely related immunoreceptors such as PECAM-1 is unknown. Studies on CEACAM-1 and bacterial pathogens have been restricted to humans, as mouse CEACAM-1 does not recognize many of the identified bacterial pathogens, including *S.* Ty-

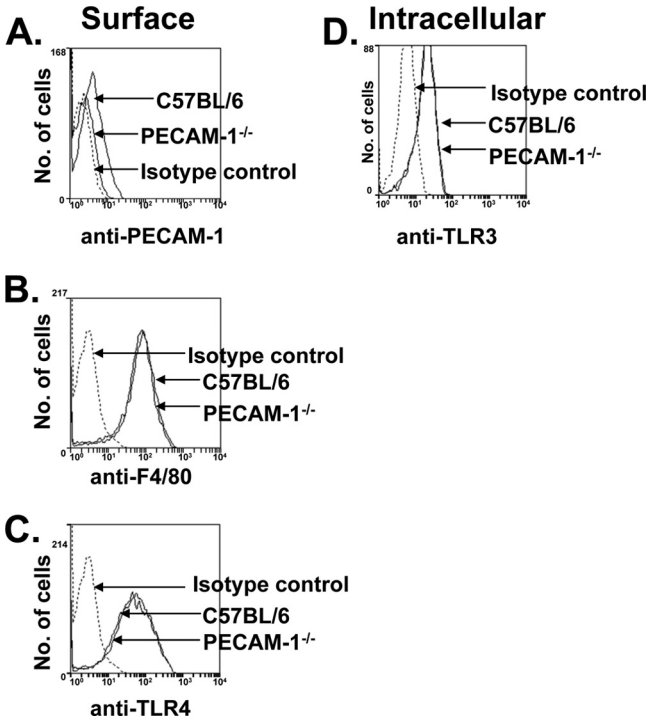

FIG 6 PECAM-1, F4/80, TLR3, and TLR4 expression by macrophages derived from wild-type C57BL/6 and PECAM-1$^{-/-}$ mice. (A) Flow cytometric analysis of F4/80 surface expression on murine macrophages. Macrophages were stained with PE-conjugated monoclonal antibody (MAb) F4/80. (B) Flow cytometric analysis of PECAM-1 surface expression on murine macrophages. Macrophages were stained with anti-PECAM-1 antibody followed by a secondary FITC-conjugated anti-rat antibody. (C) Flow cytometric analysis of TLR4 surface expression on murine macrophages. Macrophages were stained with PE-conjugated anti-TLR4 antibody. (D) Flow cytometric analysis of intracellular TLR3 expression on murine macrophages. Macrophages were stained with FITC-conjugated anti-TLR3 antibody. All results are representative of three independent experiments. In each panel, the dotted line represents the isotype control antibody.

phimurium. Therefore, it was important to define whether murine PECAM-1 binds to *S.* Typhimurium *in vitro* before embarking upon *in vivo* mouse models of systemic infection. Our results show that PECAM-1, like CEACAM-1, contains *N*-acetylglucosamine and α-linked mannose sugars as determined by lectin profiling (Fig. 1 and Table 1). We showed that cellular PECAM-1 derived from primary cells (platelets or leukocytes) and CHO-K1-derived recombinant human PECAM-1 Ig chimera bound specifically to *N*-acetylglucosamine and α-mannose sugars (ConA) (GlcNAc ≫ Man) but not other lectins (fucose, GalNAc, and mannan) (Table 1). As *N*-acetylglucosamine is a major component of peptidoglycan, it is likely that PECAM-1 binds Gram-positive bacteria (such as staphylococci, streptococci, *Listeria*, and *Bacillus*) that are known to be associated with infectious diseases in humans. In addition, the α-mannose sugars will target binding of type I fimbrial lectins of *E. coli* and *S.* Typhimurium, suggesting that PECAM-1, like CEACAM-1, may also bind some Gram-negative organisms. Indeed, we demonstrated that mouse and human PECAM-1 Ig chimeric proteins were able to bind *S.* Typhimurium *in vitro*.

Having confirmed that mouse PECAM-1 binds to *S.* Typhimurium SL1344, we used virulent *S.* Typhimurium SL1344 in a mouse model of salmonellosis to determine the role of PECAM-1 in resistance to *Salmonella* infection. We show that PECAM-1$^{-/-}$ mice are more resistant to oral *S.* Typhimurium infection than wild-type (WT) C57BL/6 mice, as evidenced by reduced morbidity and mortality (Fig. 3) and lower bacterial loads in systemic organs, including spleen, liver, MLN, and brain (Fig. 4), following oral infection with low doses. Interestingly, our data showed that at early time points after infection, the bacterial loads in the gastrointestinal tissues, determined by viable count in fecal pellets, are comparable in C57BL/6 and PECAM-1-deficient mice, suggesting that the reduced susceptibility of PECAM-1-deficient mice to oral infection with *S.* Typhimurium is due to reduced extraintestinal dissemination of the infection from the gut to the systemic organs. Additional experiments using systemic infection routes are warranted to further address whether this diminished spread explains why PECAM-1-deficient mice are less susceptible to *S.* Typhimurium infection. However, others have shown that following intraperitoneal infection with an attenuated strain of *S.* Typhimurium, PECAM-1 deficiency results in delayed clearance of *Salmonella* infection as a result of impaired T-cell responses (28). However, the initial establishment of infection and bacterial loads during the early-phase response, which is known to be T-cell independent, were not altered (29). Thus, these data would suggest that following systemic infection, initial establishment of infection and spread within the reticulo-endothelial system (RES) would be comparable in PECAM-1-deficient and wild-type mice. Therefore, these data imply that PECAM-1 deficiency might affect susceptibility to *Salmonella* infection due to reduced extraintestinal dissemination but that once the bacteria reach systemic organs, the lack of PECAM-1 does not affect further spread of the infection.

S. Typhimurium is an invasive enteric pathogen that preferentially breaches the epithelial barrier by targeting M cells in Peyer's patches but can also invade epithelial cells or be directly taken up from the lumen by CD18^{+} phagocytic dendritic cells (30–33). PECAM-1 is unlikely to play a role in *S.* Typhimurium entry into M cells, as previous studies have shown an absence of PECAM-1 expression on *in vitro* cultured M-cell-like cells (25). However, PECAM-1 is expressed on the surfaces of macrophages and dendritic cells and thus may be important in interaction with these host cells (15, 34). Uptake of *S.* Typhimurium is enhanced by opsonization with antibodies through Fc receptor-mediated phagocytosis, but it is thought that other receptors on host cells also play a role in internalization. As we have shown that PECAM-1 can bind to *Salmonella* adhesins, it would be interesting to further explore the role of PECAM-1 in *Salmonella* uptake by macrophages and dendritic cells. In this context, it is noteworthy that Ross et al. found that at least in *in vitro* experiments, adherent splenic cells from wild-type and PECAM-1$^{-/-}$ mice infected with *S.* Typhimurium contain comparable numbers of intracellular bacteria (28). Indeed, while PECAM-1 may play a role in uptake of *Salmonella* by phagocytic cells, there are likely other receptors involved.

While macrophages are used as an intracellular niche for replication, *S.* Typhimurium does not replicate in dendritic cells. Instead, the *Salmonella* bacteria interfere with the induction of adaptive immune responses and with the trafficking of dendritic cells to draining lymph nodes and the bloodstream. Residing sheltered from immune attack inside dendritic cells, *S.* Typhimurium exploits the migrating properties of dendritic cells to reach into

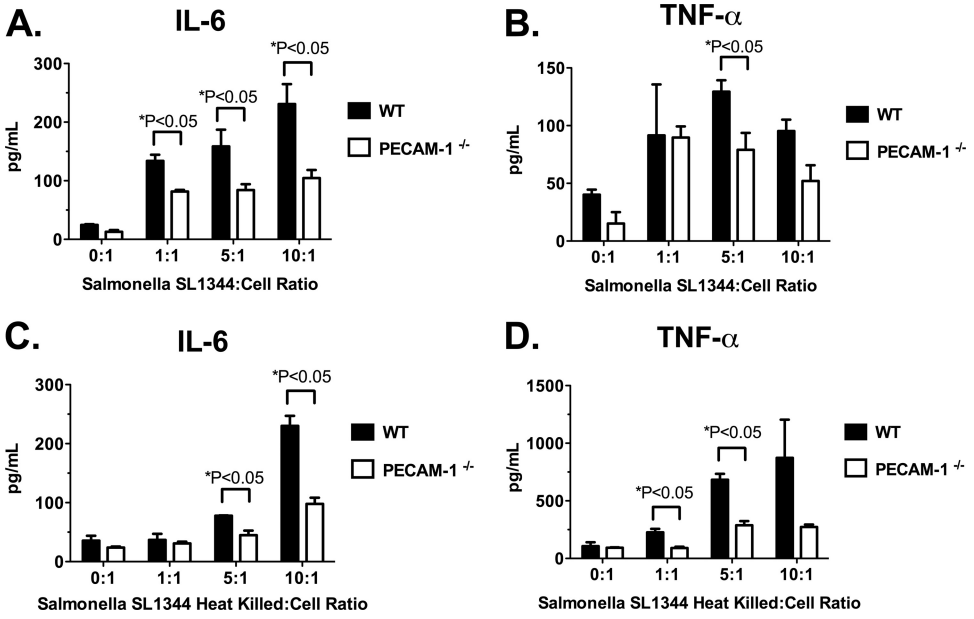

FIG 7 Live and heat-killed *S.* Typhimurium SL1344-infected cultured PECAM-1$^{-/-}$ macrophages display reduced IL-6 (A and C) and TNF-α (B and D) cytokine levels at all bacterium/cell ratios tested (1:1, 5:1, and 10:1) compared to wild-type C57BL/6 macrophages. Peritoneal macrophages were obtained from wild-type C57BL/6 and PECAM-1$^{-/-}$ mice and cultured in the presence of 10% (vol/vol) FCS and L-cell conditioned medium for 2 days. Cells were seeded and either left untreated or infected with *S.* Typhimurium SL1344 (live or heat killed) at different bacterium/cell ratios. Following 1 h of infection, bacteria were removed by washing and treatment with 300 μl of 50 μg/ml gentamicin for 40 min at 37°C and then 24 h of incubation, and supernatants were collected. Cytokine analysis was performed using the mouse CBA inflammation kit, with detection on a FACSCanto flow cytometer. Three independent experiments were performed, with each treatment being performed in triplicate. Data are presented as means ± SEM.

deeper tissues and organs (35). Interestingly, our data showed reduced dissemination of *Salmonella* from the gastrointestinal tract into systemic organs in the absence of PECAM-1. PECAM-1 was shown to be essential for human dendritic cell migration through the lymphatics, and it is thus likely that PECAM-1 is involved in dendritic cell trafficking (34). No differences in the number of CD11c^{+} cells in the spleens of naive and infected PECAM-1$^{-/-}$ mice in comparison with wild-type mice have been reported, and the antigen-presenting capacities of cultured dendritic cells of PECAM-1$^{-/-}$ and wild-type mice are comparable (28). However, the functional abilities of dendritic cells in PECAM-1-deficient mice and particular dendritic cell populations in the gastrointestinal lymphoid tissue have not been studied. Thus, while outside the scope of this study, investigations into the migratory capacity of dendritic cells in *Salmonella*-infected PECAM-1$^{-/-}$ mice and thorough analysis of uptake of *S.* Typhimurium by gastrointestinal dendritic cells are warranted to clarify whether the reduced extraintestinal dissemination of *Salmonella* in PECAM-1-deficient mice could be due to reduced uptake by dendritic cells and/or diminished migration of dendritic cells.

Various biological roles for PECAM-1 in thrombosis, inflammation, and the immune system have been identified (35). The role of PECAM-1 in the inflammatory process seems to depend on the stimulus and tissues involved and the genetic background when murine studies are concerned (reviewed in references 35, 36 and 37). The role of PECAM-1 in leukocyte extravasation, an essential process in inflammation, is well established. However, C57BL/6 mice (which were used in this study) have normal leukocyte migration responses when interactions with PECAM-1 are blocked (38), and thus any effects of the absence of PECAM-1 are likely due to role of PECAM-1 in maintaining vascular integrity or in regulation of inflammatory cytokine production. The role of PECAM-1 protein in inflammation has been explored in several models of inflammatory disease, including collagen-induced arthritis, experimental autoimmune encephalitis, croton oil-induced dermatitis, and endotoxic shock (35, 36), and these studies have alluded to complex pro- and anti-inflammatory roles for PECAM-1. In the C57BL/6 background, many of these studies suggest an anti-inflammatory role for PECAM-1, attributed mostly to modulation of cytokine production and/or changes in vasculature. However, the role of PECAM-1 in mediating aspects of the inflammatory process may be stimulus dependent and influenced by the cytokine milieu. For instance, leukocyte emigration is dependent on PECAM-1 expression following exposure to IL-1β but not in the presence of TNF-α. Thus, further investigations are needed to completely understand the complexity of these interactions.

Observations in PECAM-1-deficient mice in a model of endotoxic shock are seemingly in contrast to our observations during *Salmonella* infection. Inducing endotoxic shock by injecting lipopolysaccharide (LPS) (13), Maas et al. demonstrated that PECAM-1$^{-/-}$ mice had a higher mortality rate following LPS exposure than wild-type mice, due to lack of PECAM-1 expression in the vasculature, so that loss of PECAM-1 expression at endothelial cell-cell junctions resulted in increased vascular permeability, increased fluid loss, and failure to recover from hypotension. The discrepancy between the study by Maas et al. and the study presented here could be explained by the different disease models used, as oral infection with *S.* Typhimurium is not likely to result in endotoxic shock from high levels of soluble LPS. Rather, *S.* Typhimurium is known to reside intracellularly during infection, preferably in macrophages of the RES (39). In addition, in our

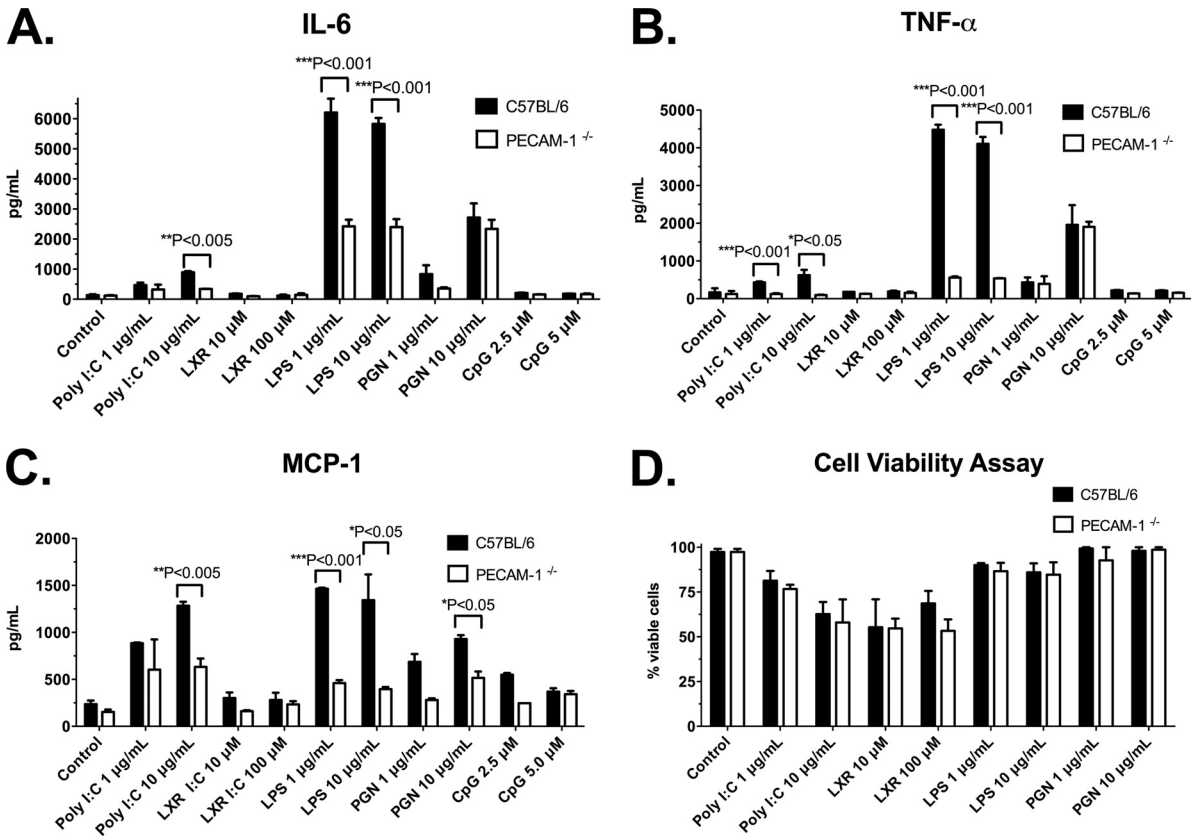

FIG 8 TLR ligand stimulation of cultured PECAM-1$^{-/-}$ macrophages revealed reduced inflammatory cytokine release compared to that with wild-type C57BL/6 macrophages. Resident peritoneal macrophages were obtained from wild-type C57BL/6 and PECAM-1$^{-/-}$ mice and cultured in the presence of 10% (vol/vol) FCS and 50% L-cell conditioned medium for 2 days. For experiments, the medium was changed and cells were seeded in 0.5% (vol/vol) FCS and either left untreated or stimulated with various TLR ligands [LPS, poly(I·C), PGN, CpG, or LXR) at the indicated concentrations. Following 1 h of exposure to the various ligands followed by a 24-h incubation, supernatants were collected. Cytokine analysis was performed using the mouse CBA inflammation kit, and detection was with a FACSCanto flow cytometer. Data are presented for the IL-6 (A), TNF-α (B), and MCP-1 (C) cytokines. A trypan blue cell viability assay was also performed (D). Three independent experiments were performed with each treatment in triplicate cultures, and data are presented as means ± SEM.

study the increased resistance of PECAM-1$^{-/-}$ mice to infection was especially evident with low doses of *S.* Typhimurium, which are more reflective of naturally acquired infections (24) and should not result in high levels of circulating, soluble LPS.

The results of this study demonstrated that increased resistance to *Salmonella* infection correlated with reduced levels of circulating cytokines. However, it is well known that early control of *Salmonella* infections is dependent on innate immune responses and that the production of inflammatory cytokines such as TNF-α is essential for control of infection (27, 40–42). The reduced cytokine levels in PECAM-1$^{-/-}$ mice can therefore not explain the relative increase in resistance. Instead, we suggest that the reduced cytokine levels are reflective of the lower bacterial load detected in these animals. It would be interesting to further explore the cytokine production on a tissue-specific level, i.e., in the gastrointestinal tract (comparable bacterial loads) compared with the spleen or liver (increased bacterial load in PECAM-1-deficient animals) to better understand the complexity of local, tissue-specific proinflammatory cytokine production in response to bacterial infection. However, we further explored the production of inflammatory cytokines by peritoneal macrophages in *in vitro* experiments in which the macrophages from each mouse strain were exposed to similar bacterial loads or TLR ligands. The outcome of these experiments highlighted an interesting difference between responses from PECAM-1-deficient macrophages and wild-type macrophages, namely, that macrophages from PECAM-1-deficient mice produced reduced levels of TNF-α, IL-6, IFN-γ, and MCP in response to culture with *S.* Typhimurium. Further, we demonstrate that following stimulation with LPS (TLR4 ligand) or to a lesser extent poly(I·C) (TLR3 ligand), but not other TLR ligands, production of inflammatory cytokines (TNF-α and IL-6) by PECAM-1-deficient macrophages was reduced (43).

The signaling pathways leading to pro- or anti-inflammatory pathways may be regulated differently in various cell types, and therefore ligation of PECAM-1 on different cells may result in different outcomes. In this study, we did not further investigate the activation of signaling pathways in PECAM-1$^{-/-}$ macrophages following exposure to *S.* Typhimurium, LPS, or poly(I·C). Other studies have shown that PECAM-1 plays an anti-inflammatory role by dampening cytokine production through ITIM/SHP-2 interactions. Studies by Rui et al. have shown that in T lymphocytes, ligation of PECAM-1 can result in interference with IRF-3, NF-κB, and JNK pathways (16). Other studies have demonstrated that in endothelial cells, ligation of PECAM-1 may prolong STAT3 activation, as the scavenging of SHP-2 by PECAM-1 ITIM prevents the dephosphorylation of STAT3. The molecular

mechanism by which *S.* Typhimurium-mediated ligation of PECAM-1 or LPS-mediated stimulation of TLR4 results in reduced production of inflammatory cytokines remains unknown and is worthy of further investigation.

In summary, our results indicate that PECAM-1 plays a role in modulating the innate immune response during *S.* Typhimurium pathogenesis both *in vivo* and *in vitro*. This study demonstrates that PECAM-1 plays a role in recognition and binding of *S.* Typhimurium, in resistance to extraintestinal dissemination of *S.* Typhimurium following oral infection *in vivo*, and in macrophage inflammatory responses *in vitro*. Further understanding of the mechanisms by which PECAM-1 regulates/interferes with *Salmonella* infections may open up opportunities to develop therapeutic approaches based on blockade of PECAM-1 ligation and signaling.

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