Absence of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1/CD31) in vivo increases resistance to oral Salmonella enterica serovar Typhimurium infection in mice

By Michael D. Lovelace, May Lin Yap, Jana Yip, William Muller, Odilia Wijburg, and Denise E. Jackson

From Burnet Institute, Melbourne, Victoria, Australia, Northwestern University, Chicago, IL, USA, Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia, RMIT University, Bundoora, Victoria, Australia.

Footnotes - Current affiliations: Discipline of Anatomy and Histology and Bosch Institute, Sydney Medical School, The University of Sydney, Sydney, New South Wales and Centenary Institute of Cancer Medicine and Cell Biology, Sydney, New South Wales, Australia.

MY is a postgraduate student of the University of Melbourne.
DEJ is a recipient of an NHMRC Senior Research Fellowship.
OW is a recipient of an NHMRC Career Development Fellowship.

Running Title: PECAM-1 attenuates oral S. Typhimurium infection in vivo.


Address correspondence to:
Prof. Denise E. Jackson, FAIMS, PhD, FFSc, NHMRC Senior Research Fellow and Discipline Head of Laboratory Medicine, RMIT University, P. O. Box 71, Bundoora. Victoria 3083. Australia. Phone: 61 3 9925-7392; Fax: 61 3 9925-7063 E-mail: denise.jackson@rmit.edu.au
Abstract

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* var Typhimurium (S. Typhimurium) and in in vitro inflammatory responses of peritoneal macrophages. Lectin profiling showed that cellular and recombinant human PECAM-1-Ig contains 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 faecal-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 faecal shedding of S. Typhimurium was comparable between 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 compared to WT mice, suggesting that extraintestinal dissemination was reduced in the absence of PECAM-1. This reduced bacterial load correlated with reduced TNF, IL-6 and MCP levels in serum of PECAM-1−/− mice. Following in vitro stimulation of macrophages with either whole S. Typhimurium, LPS (TLR4 ligand) or polyI:C (TLR3 ligand), production of TNF and IL-6 was reduced by PECAM-1−/− macrophages. 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.
Introduction

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 response in order to survive and multiply in specific sites (2). The first step of 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. S. 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, PECAM-1 negatively regulates haematopoietic 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 LPS endotoxic shock than their wild-type 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 TLR4 signalling in macrophages (16). While human CD38 has been proposed as a co-receptor 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
Recent studies suggest that a related Ig-ITIM superfamily member, Carcinoembryonic Cell Adhesion Molecule-1 (CEACAM1) serves as a receptor for bacterial pathogens, including *S. Typhimurium*, *Escherichia coli* (*E. coli*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Neisseria meningitides* (*N. meningitides*) in humans (18,19). Based upon molecular modelling, 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 interaction between the closely related immunoreceptors like 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 pro-inflammatory responses. Further, macrophages from PECAM-1−/− mice are less responsive to TLR ligands *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 Prof. P. Coloe and Dr. P. Smooker (RMIT University, Bundoora, Victoria). 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), foetal bovine serum (FBS), 200 mM glutamine, premixed penicillin (100 U/mL) and streptomycin (10,000 μg/mL) and 1M HEPES buffer, pH 7.2 were from Gibco (Invitrogen, Life Technologies). SEW16 anti-human PECAM-1 antibody was obtained from Prof. Peter Newman (Blood Research Institute, Milwaukee, WI). Anti-mouse PECAM-1 antibody-FITC was purchased from Santa Cruz (Santa Cruz, CA), 4D1C2 anti-CEACAM1 antibody was obtained from Prof. Nicole Beauchemin (McGill University, Montreal, QC), F4/80 anti-macrophage antibody and streptavidin-PE was obtained from BD Biosciences (Franklin Lakes, NJ). *Salmonella* lipopolysaccharide (LPS) was purchased from Sigma Chemical Co., (St. Louis, MO). CpG, Poly I:C and Loxiridine (LXR) were obtained from Invivogen (San Diego, CA). Peptidoglycans (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'-
ATGGAACTGGCACCCATCACTTA-3', PECAM-1 reverse (antisense oligonucleotide), 5'-
GGTCACGTCTCGCCTATTAAGC-3' and Neomycin (antisense oligonucleotide; 5'-
GTCTTCTTGAGCAGTTCTTCCGCTATC-3' were obtained from Sigma Proligo (Castle
Hill, NSW). Age and sex-matched groups of 6-8 weeks 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 GGA (resistant or NRamp1 +/- primer) were obtained from Sigma Proligo
(Castle Hill, NSW). 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 pre-cleared twice with 50 μL
of 50% suspension of CNBr-activated Sepharose beads (Amersham Pharmacia Biotech AB,
Uppsala, Sweden) for 15 mins at 4°C, then centrifuged at 4,000 rpm for 5 mins and
supernatant retained. 1.5 mg pre-cleared platelet lysates were incubated with 10 μg of Biotin-
labelled lectins (Vector Laboratories, Burlingame, CA) at 4°C for 2 hours. The lectin-
carbohydrate-protein complexes were then isolated with 50 μL of 50% suspension of
streptavidin-agarose beads (Sigma Chemical Company, St. Louis, MO) incubated for 1 hour at 4°C. Beads were then washed five times with immunoprecipitation buffer (20 mM Tris pH 7.4 containing 50 mM NaCl and 2% (v/v) Triton X-100). Bound proteins were eluted from the streptavidin-agarose beads by boiling for 10 mins in 30 μL SDS reducing buffer and resolved on a 10% SDS-PAGE gel, followed by Western Blot analysis using either SEW16 anti-PECAM-1 antibody (20 μg/mL) or monoclonal 4D1C2 anti-CEACAM1 antibody (5 μg/mL), then appropriate HRP-conjugated secondary antibody (1/20,000) followed by ECL detection.

**Salmonella ELISA** - Microtiter plates (Maxisorp; Nunc, Wiesbaden, Denmark) were coated with 100 μL of human recombinant PECAM-1-Ig chimera protein at varying doses (0 – 5 μg/mL). PECAM-1-Ig chimera was diluted in 0.2 M carbonate buffer (pH 8.3) and coated overnight at 4°C. Non-specific binding sites were blocked with 200 μL of 1% (w/v) bovine serum albumin (BSA) (Bovogen Biologicals, Keilor East, Victoria) in 0.01M PBS (pH 7.4) for 1 hour at 37°C. The wells were washed three times with 0.01M PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (Sigma Chemical Co., St. Louis, MO). Bacteria were collected from an overnight culture by centrifugation at 2,400 rpm for 30 mins, resuspended in blocking buffer and 100 μL of bacterial suspension added and incubated for 2 hours at 37°C. The wells were washed six times with wash buffer, then Rabbit anti-Salmonella serum was added and incubated for 2 hours 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 hour at 37°C. Bound antibodies were visualised using paranitrophenyl phosphate (pNPP) as substrate. The reaction was terminated with 50 μL of
2N 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 mins in 300 μL of 10% (v/v) normal mouse serum in DMEM media. Opsonised bacteria were collected by centrifugation at 13,000 rpm for 5 mins and serial dilutions were prepared in incomplete DMEM for infection of peritoneal macrophages. To heat kill the bacteria, after opsonisation, *S.* Typhimurium SL1344 was heated at 65°C for 20 mins. Killing of bacteria was confirmed by culture for 48 hrs on LB-streptomycin agar at 37°C. The viable count in overnight SL1344 cultures was enumerated by plating serial dilutions onto XLD plates and incubation at 37°C for 24 hours.

**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 of 10³ – 10⁵ CFU *S.* Typhimurium SL1344 by oral gavage under light inhalation anaesthesia (22). Mice were observed for symptoms of salmonellosis and euthanased 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 faecal-oral route of *S.* Typhimurium transmission, a single C57BL/6 mouse was infected with 1 x 10⁶ CFU *S.* Typhimurium SL1344 by oral gavage and placed in a cage with naïve animals (23). The
acquisition and development of *S*. Typhimurium infection in the naïve co-housed animals was monitored by detection of *S*. Typhimurium in fresh faecal samples plated on selected indicator media (XLD). Absence of *S*. Typhimurium in faeces of all mice was confirmed before commencement of the experiments (24).

**Enumeration of bacterial organ load from infected mice** - Mice were euthanased 5 days after oral infection and livers, spleens, mesenteric lymph nodes and brains were harvested into 5 mL of sterile cold PBS. Organs were homogenised using a Stomacher 80 Biomaster homogeniser (Seward, UK) at low speed for 5 minutes. Viable count 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 hours at 37°C.

**Cytokine analyses** - Mouse serum 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 hour, and then centrifuged at 4000 rpm for 10 mins to separate the serum. Sera and supernatants were analysed for cytokine levels using the Mouse Inflammation Cytometric Bead Array (CBA) assay Kit from BD Biosciences according to manufacturer’s instructions. Cytokines analysed were interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor (TNF-α), and interleukin-12p70 (IL-12p70). Data was acquired on a FACSCanto flow cytometer and cytokine levels were analysed 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 1:1 ratio of DMEM complete media (supplemented with 10% (v/v) heat inactivated FCS, 2 mM Glutamine, 50 µg/mL Penicillin/Streptomycin antibiotic and 15 mM HEPES pH 7.2) and 50% L-cell conditioned media (as a source of M-CSF). These cells were plated in 24 well plates at a density of 2 x 10^5 cells per well containing 1 mL of media and grown at 37 °C in a humidified 5% CO₂ incubator (Sanyo Fisher Company, Chatsworth, CA). Culture medium was changed after 1 day to remove non-adherent cells, and the cells were cultured for 6 days. For analysis of cytokines, the cells were grown for an additional 2 days in the media described, before experiment. Cell viability was assessed by trypan blue exclusion.

Flow cytometry - Cultured peritoneal macrophages (2.5 x 10^5) from wild-type C57BL/6 and PECAM-1−/− mice were collected and suspended in FACS buffer (PBS, pH 7.4 containing 1% (v/v) FCS). To prevent non-specific antibody binding, macrophages were incubated with 2.4G2 anti-FcγRII antibody (provided by Prof. PM Hogarth, Burnet Institute, Heidelberg, Victoria) 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 was
acquired on a FACSCanto flow cytometer (BD Biosciences) and analysed using DIVA software.

Statistical analysis - Results are expressed as mean ± S.E.M. Group survival data was analysed using a Log-Rank p-test. All other data was analysed with Student’s $t$ test (two tailed) or non-parametric Mann Whitney U-test. All statistical analysis were performed using GraphPad Prism software, with $P<0.05$ considered to represent statistical significance.
Results

Lectin profiling of carbohydrates on PECAM-1

Previous studies have shown that high levels of mannose sugars on CEACAM1 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 performed based upon carbohydrate:lectin recognition, a series of biotin-labelled lectins were used to capture of immunoreceptor carbohydrate:lectin interaction using biotin:streptavidin and immunodetection of PECAM-1 using specific antibodies. The following biotin-labelled lectins were tested including AAL (Fucose (α1,6)-N-acetylglucosamine), Con A (α-linked mannose), DBA (N-acetylgalactosamine), DSL (β-1,4-linked acetylglucosamine), GNL (α-1,3-mannose), GSL (α-N-acetylgalactosamine α-galactose), LEL (N-acetylglucosamine), LTL (α-linked fucose), STL (N-acetylglucosamine), SNA (α-2,6-linked sialic acid), DBA (α-linked N-acetylglactosamine), and WGA (wheat germ agglutinin; N-acetylglucosamine). Figure 1 shows the lectin profiles of cellular PECAM-1 (Panel A), cellular CEACAM-1 (Panel C) and recombinant human PECAM-1-Ig chimera (Panel B). As shown in Figure 1A, cellular PECAM-1 showed different degrees of binding with Con A, WGA, LTL, STL, AAL, DSL and LEL lectins 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 reactivity with Protein G (via Ig-portion), Con A, WGA, DSL and LEL, but did not bind LTL, STL, AAL, GNL, SNA and DBA (Figure 1B). This is in contrast to cellular CEACAM-1 that showed a different carbohydrate profile with varying degrees of weak binding to Con A and WGA, but not LTL, STL, AAL, GNL, GSL, DSL and LEL (Figure 1C). A summary of the strength of reactivity of various
lectin interactions as determined by band intensity in western blot 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 neither have these carbohydrate modifications.

Human 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 have been restricted to humans as mouse CEACAM-1 does not recognise many of the identified bacterial pathogens including *S. Typhimurium*. Based upon these findings, it was important to determine whether human 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 assay 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. (Figure 2).

As shown in Figure 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 OD 405 nm of 1.25 when 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 OD 405 nm of 1.05 when the protein concentration was 2.0 μg/mL and there was an approximate 2-fold increase in binding compared to negative control antigen, BSA. No binding was observed when normal rabbit serum was used (Figure 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<sup>−/−</sup> 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<sup>5</sup> CFU, 10<sup>4</sup> CFU and 10<sup>3</sup> CFU). Mice were observed for symptoms of salmonellosis for 7 to 10 days and were euthanased when humane endpoints were reached. Figures 3A and B show that at higher infectious doses (10<sup>5</sup> and 10<sup>4</sup> CFU, respectively) there was little difference in susceptibility of PECAM-1<sup>−/−</sup> and wild-type C57BL/6 mice, but at lower infectious dose of 10<sup>3</sup> CFU PECAM-1<sup>−/−</sup> mice were clearly more resistant (80% survival) to infection compared with wild-type C57BL/6 mice (Figure 3C). Subsequent
experiments to further investigate the increased resistance to *S. Typhimurium* infection by PECAM-1<sup>−/−</sup> mice were therefore performed with infectious dose of 10<sup>3</sup> CFU SL1344.

As *S. Typhimurium* infection is normally acquired through ingestion of contaminated water or food, we employed a natural oral-faecal transmission model as well as the oral infection model to further explore susceptibility of PECAM-1<sup>−/−</sup> mice to infection with *S. Typhimurium* (24). To facilitate transmission of *S. Typhimurium* from infected to uninfected mice, naïve mice were co-housed with orally infected (10<sup>6</sup> CFU) mice. Transmission was monitored by measuring the number of *Salmonella* in faecal pellets and the onset of salmonellosis was monitored by observation for clinical signs. Figure 3D demonstrates that naïve PECAM-1<sup>−/−</sup> mice showed 100% survival when co-housed with infected animals, while 75% of co-housed naive C57BL/6 mice became infected and moribund. To further demonstrate reduced susceptibility of PECAM-1<sup>−/−</sup> mice to oral infection with *S. Typhimurium*, the experiment was repeated with larger groups of mice (n=15), which were all infected with 10<sup>3</sup> CFU *S. Typhimurium* SL1344 by oral gavage and monitored for onset of disease. Figure 3E shows that PECAM-1<sup>−/−</sup> mice were significantly more resistant to infection (**P<0.005, Log Rank p test) compared to wild-type C57BL/6 mice, confirming our observations with the natural transmission infection model.

Resistance of PECAM-1<sup>−/−</sup> mice to infection with *S. Typhimurium* was further characterised by enumeration of bacterial burden in organs of infected mice. Mice were orally infected with 10<sup>3</sup> CFU SL1344 and 5 days after infection the bacterial load in liver, spleen, mesenteric lymph nodes (MLN) and brain were determined by viable count of tissue homogenates. In addition, the bacterial load in freshly collected faecal pellets was determined.
by viable count. Figure 4A shows that at early time points after infection, no difference in number of SL1344 shed in faeces was detected between C57BL/6 and PECAM-1<sup>−/−</sup> mice, suggesting that in the gastrointestinal tract, the bacterial load was comparable between mouse strains. At 5 days after oral infection, the bacterial load of PECAM-1<sup>−/−</sup> mice was significantly (P<0.05, n=14) lower in all systemic organs tested compared with C57BL/6 mice (Figure 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.

Pro-inflammatory cytokine responses are altered in PECAM-1<sup>−/−</sup> mice during S. Typhimurium SL1344 infection

Previous studies have shown that the production of cytokines and soluble factors by macrophages are 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<sup>−/−</sup> mice was associated with altered circulating levels of these cytokines during infection. Mice were infected orally with 10<sup>3</sup> CFU S. Typhimurium SL1344, and blood was taken from the mice at 5 days after oral infection for cytokine analyses. As shown in Figure 5A-F, PECAM-1<sup>−/−</sup> mice produced significantly lower levels of pro-inflammatory 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 was observed in levels of IL-12p70 (P>0.05, n=11) and anti-inflammatory cytokine IL-10 (P>0.05, n=11 using non parametric Mann Whitney U test).
Importantly, no significant differences were found in any of the cytokine levels between uninfected PECAM-1−/− and uninfected wild-type C57BL/6 mice (Figure 5).

**PECAM-1+ peritoneal macrophages release reduced amounts of pro-inflammatory cytokines following stimulation with live or heat-killed S. Typhimurium or with TLR ligands in vitro**

Next, cytokine production by cultured resident peritoneal macrophages from PECAM-1−/− mice and wild-type C57BL/6 mice in response to *Salmonella* infection was compared. 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 Figure 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 (Figure 6C) and TLR3 (Figure 6D), while PECAM-1 was detected in modest levels on F4/80+ cells from C57BL/6 mice and absent on cells from PECAM-1−/− animals (Figure 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. Typhimurium* SL1344 *in vitro* (Figure 7). Supernatants collected were collected 24hrs after exposure to *Salmonella*, then analysed using the CBA mouse inflammation kit. Stimulated macrophages produced both IL-6 and TNF-α and the amount of cytokines produced increased with increasing MOI (Figure 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; Figure 7).

One of the major pathways leading to production of pro- and anti-inflammatory cytokines by macrophages is following activation of Toll-like receptors (TLR) by their respective ligands. Consequently, it was important to investigate if 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, and then treated with either TLR2 agonist peptidoglycan (PGN), TLR4 agonist *Salmonella* lipopolysaccharide (LPS), TLR7 agonist Loxoribine (LXR), TLR9 agonist CpG oligonucleotide or TLR3 agonist PolyI:C (TLR3) for 8 hours before supernatants were removed for cytokine analysis.

The results in Figure 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<0.05) reduced TNF-α and MCP-1 levels when compared to wild-type C57BL/6 macrophages (Figure 8B). In contrast, stimulation of C57BL/6 and PECAM-1−/− macrophages with PGN showed a high IL-6, TNF-α and MCP-1 response, 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; Figure 8). The differences in cytokine responses were not due to
altered cell viability (Figure 8D). These results suggest a possible relationship between PECAM-1 and TLR3 and TLR4 signalling pathways involving cytokine release.

Discussion

Recent evidence suggests that the Ig-ITIM bearing receptor, CEACAM1 serves as a receptor for bacterial pathogens, including *S. Typhimurium*, *E. coli*, *N. gonorrhoea* and *N. meningitidis* in humans (18). CEACAM-specific adhesins allow bacterial or viral pathogens to attach to, invade and/or transcytose polarised epithelial and endothelial cells to colonise host tissues. Whether this is the case for closely related immunoreceptors like PECAM-1 is unknown. Studies on CEACAM-1 and bacterial pathogens have been restricted to humans, as mouse CEACAM-1 does not recognise many of the identified bacterial pathogens including *S. Typhimurium*. Therefore, it was important to define if murine PECAM-1 binds to *S. Typhimurium* 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 by lectin profiling (Figure 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-acetyl-glucosamine 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 fimbriae lectins of *E. coli* and *S. Typhimurium* suggesting that PECAM-1, like CEACAM1, 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 C57BL/6 (WT) mice, as evidenced by reduced morbidity and mortality (Figure 3) and lower bacterial loads in systemic organs including spleen, liver, MLN and brain (Figure 4), following oral infection with low doses. Interestingly, our data showed that at early time points after infection, the bacterial load in the gastrointestinal tissues, determined by viable count in faecal pellets, is comparable between 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 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 RES would be comparable between 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 once the bacteria reach systemic organs, 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 absence of PECAM-1 expression on in vitro cultured M cell-like cells (25).

However, PECAM-1 is expressed on the surface 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 opsonisation 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 number 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 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 blood stream. Residing sheltered from immune attack inside dendritic cells, S. Typhimurium exploits the migrating properties of dendritic cells to reach into 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 spleen of naïve and infected PECAM-1−/− in comparison with wild type mice have been reported, and the antigen presenting capacity of cultured dendritic cells of PECAM-1−/− and wild type mice is 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 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 if 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 genetic background when murine studies are concerned (reviewed in 35-37). The role of PECAM-1 in leucocyte extravasation, an essential process in inflammation, is well established. However, C57BL/6 mice (which were used in this study) have normal leucocyte migration responses when interactions with PECAM-1 are blocked (38) and thus any effects of 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 eluted to complex pro- and anti-inflammatory roles for PECAM-1. In 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, leucocyte 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 compared to 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 intracellular during infection, preferably in macrophages of the RES (39). In addition, in our 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, as 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 are 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 load) compared with the spleen or liver (increased bacterial load in PECAM-1 deficient animals) to better understand the complexity of local tissue specific pro-inflammatory 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 load or TLR ligands. The outcome of these experiments highlighted an interesting difference between responses from PECAM-1 deficient macrophages compared with 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, IL-6) by PECAM-1 deficient macrophages was reduced (43).

The signalling 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 signalling pathways in PECAM-1−/− macrophages following exposure to *S. Typhimurium*, LPS or polyI: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, NFκB 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 signalling.
Acknowledgements

This work was supported by grants from the National Health and Medical Research Council of Australia #603812 (to DEJ). MDL is supported by a Rebecca Cooper Foundation grant.
References

1. Lanoue, A., Clatworthy, M. R., Smith, P., Green, S., Townsend, M. J., Jolin, H. E.,
Smith, K. G., Fallon, P. G., and McKenzie, A. N. SIGN-R1 contributes to protection

2. Cossart, P., and Sansonetti, P. J. Bacterial invasion: The paradigms of enteroinvasion

macrophages in vivo in pathogenesis and control of murine Salmonella enterica var.

E. 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

5. Jones, K. L., Hughan, S. C., Dopheide, S. M., Farndale, R. W., Jackson, S. P., and
Jackson, D. E. Platelet endothelial cell adhesion molecule-1 is a negative regulator of

1 serves as an inhibitory receptor that modulates platelet responses to collagen. Blood
2001;97:1727-1732.

7. Graesser, D., Solowiej, A., Bruckner, M., Osterweil, E., Juedes, A., Davis, S., Ruddle,
N. H., Engelhardt, B., and Madri, J. A. Altered vascular permeability and early onset of


**Figure Legends**

**Figure 1.** PECAM-1 contains α-linked mannose and N-acetylglucosamine sugars 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 hours with mixing. 50 μL suspension of 50% streptavidin-agarose beads were then added and incubated at 4°C for 1 hour with constant mixing. Immunoprecipitations were washed, resuspended in SDS reducing buffer and proteins separated on a 10% SDS-PAGE and then transferred by western blot and probed with either a polyclonal anti-PECAM-1 antibody (A, B) or anti-CEACAM1 antibody (C) using ECL as substrate to visualise bound antibody.

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

**Figure 3.** Absence of PECAM-1 increases resistance to *S.* Typhimurium SL1344 infection *in vivo.* Groups of five naïve C57BL/6 WT (■) or PECAM-1−/− (Δ) mice were infected with either 10⁵ CFU (A), 10⁴ CFU (B) or 10⁳ CFU (C) virulent *S.* Typhimurium SL1344 by oral gavage. For ethical reasons, mice were culled when they became moribund; these mice were deemed “non-survivors”. (D) One wild-type C57BL/6 mouse was orally inoculated with 10⁶ CFU *S.* Typhimurium SL1344 and co-housed with four naïve 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 represented as mean ± S.E.M. (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.

Figure 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−/− mice (white symbols) were inoculated orally with 10^3 CFU S. Typhimurium SL1344. (A) at indicated time points after infection, fresh faecal 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 geometric mean of each group. (B) At 5 days after infection, mice were euthanased and bacterial load 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 geometric mean of each group. Data was analysed using a non-parametric Mann-Whitney U test, * denotes P<0.05 and ** denotes P<0.01.

Figure 5. PECAM-1−/− mice have reduced serum pro-inflammatory cytokine responses following S. Typhimurium SL1344 infection in vivo. Wild-type C57BL/6 and PECAM-1−/− mice were infected orally with 1x10^3 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. Cytokines were analysed using
mouse CBA inflammation kit, and were (A) IFN-γ; (B) MCP-1; (C) IL-6; (D) TNF-α; (E)
IL12-p70; (F) IL-10. Data was collected from a total of three independent experiments
(n=11 infected mice of each strain, n=6 uninfected mice of each strain). (Data was analysed
using a non-parametric Mann-Whitney U test, * denotes P<0.05).

Figure 6. PECAM-1, F4/80, TLR3 and TLR4 expression of 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 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 (A-D) are representative of three independent experiments. In each panel, the dotted
line represents the isotype control antibody.

Figure 7. Live and heat-killed S. Typhimurium SL1344 infected cultured PECAM-1−/−
macrophages display reduced IL-6 and TNF-alpha cytokine levels at all bacteria: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% (v/v) FCS and L-cell conditioned media for 2 days. Cells
were seeded and either left untreated or infected with S. Typhimurium SL1344 (live or heat-killed) at different bacteria:cell ratios. Following 1 hour infection, bacteria were removed by washing and treatment with 300 µL of 50 µg/mL gentamycin for 40 mins at 37°C then 24 hour incubation, supernatants were collected. Cytokine analysis was performed using the mouse CBA inflammation kit and detected on a FACSCanto flow cytometer. Three independent experiments were performed with each treatment being performed in triplicate. Data are presented as mean +/- S.E.M.

Figure 8. TLR ligand stimulation of cultured PECAM-1−/− macrophages revealed reduced inflammatory cytokine release compared to 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% (v/v) FCS and 50% L-cell conditioned media for 2 days. For experiment, the media was changed and cells were seeded in 0.5% (v/v) FCS and either left untreated or stimulated with various TLR ligands (LPS, Poly I:C, PGN, CpG, LXR) at indicated concentrations. Following 1 hour exposure to the various ligands, followed by a 24 hour incubation, supernatants were collected. Cytokine analysis was performed using the mouse CBA inflammation kit and detected on a FACSCanto flow cytometer. Data are presented are for (A) IL-6, (B) TNF-α and (C) MCP-1 cytokines. A trypan blue cell viability assay was performed and the results shown in (D). Three independent experiments were performed with each treatment in triplicate cultures, data are presented as mean +/- S.E.M.
Table 1: Reactivity of cellular PECAM-1 and CEACAM1, and PECAM-1-ig chimera with biotinylated lectins of different sugar specificity.

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Cellular PECAM-1</th>
<th>PECAM-1-ig Chimera</th>
<th>Cellular CEACAM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAL</td>
<td>Galactosyl(1,6)-N-acetylglucosamine</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DTL</td>
<td>(1,4-linked N-acetylglucosamine</td>
<td>++++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>OHL</td>
<td>α-1,3-mannose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GSL</td>
<td>α-N-acetyl galactosamine α-galactose</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LSL</td>
<td>β-N-acetylglucosamine</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>LTL</td>
<td>α-linked fucose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>STL</td>
<td>β-N-acetylglucosamine</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SNA</td>
<td>α-2,6-linked sialic acid</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DBA</td>
<td>α-linked β-N-acetylglucosamine</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Con A</td>
<td>α-linked mannose</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>WGA</td>
<td>β-N-acetylglucosamine</td>
<td>++++</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Protein G</td>
<td>Bind via Ig portion</td>
<td>–</td>
<td>+++++</td>
<td>–</td>
</tr>
</tbody>
</table>

- No reactivity; weak to strong reactivity is graded + to ++++

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Source</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAL</td>
<td>Acanthamoeba lectin</td>
<td>Galactosyl(1,6)-N-acetylglucosamine</td>
</tr>
<tr>
<td>DTL</td>
<td>Datura Stramonium lectin</td>
<td>SLT</td>
</tr>
<tr>
<td>OHL</td>
<td>Oenothera Lasiocarpa lectin</td>
<td>SNA</td>
</tr>
<tr>
<td>GSL</td>
<td>Ophiopogon Japonicus lectin</td>
<td>DBA</td>
</tr>
<tr>
<td>LSL</td>
<td>Lycopersicum Esculentum lectin</td>
<td>Con A</td>
</tr>
<tr>
<td>LTL</td>
<td>Lotus Tetragonolobus lectin</td>
<td>WGA</td>
</tr>
</tbody>
</table>

IAI Accepts published online ahead of print.
Human

A.  
- Hu PECAM-1 Ig: Anti-Salmonella
- Hu PECAM-1 Ig: Normal Sera
- BSA: Anti-Salmonella
- BSA: Normal Sera

Mouse

B.  
- Ms PECAM-1 Ig: Anti-Salmonella
- Ms PECAM-1 Ig: Normal Sera
- BSA: Anti-Salmonella
- BSA: Normal Sera