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Lack of Functional P-Selectin Ligand Exacerbates Salmonella Serovar Typhimurium Infection

Winnie W. S. Kum,* Sansan Lee,* Guntram A. Grassl,* Roza Bidshahri,* Kimberly Hsu,* Hermann J. Ziltener,† and B. Brett Finlay2*‡

The selectin family of adhesion molecules mediates the recruitment of immune cells to the site of inflammation, which is critical for host survival of infection. To characterize the role of selectins in host defense against Salmonella Typhimurium infection, wild-type (WT) mice and mice lacking P-selectin glycoprotein ligand-1 (PSGL-1), P-, E-, or L-selectin, or the glycosyltransferase C2GlcNAcT-1 (core 2) were infected using a Salmonella acute gastroenteritis model. Mice were monitored for survival and assessed for intestinal inflammation at 1 and 4 days postinfection. Infected mice lacking core 2, PSGL-1, or P-selectin showed a more pronounced morbidity and a significantly higher mortality rate associated with higher bacterial load and proinflammatory cytokine production, including that of TNF-α, MCP-1, and IL-6, from the colons at 4 days postinfection as compared with WT control. Surprisingly, at 1 day postinfection, more severe inflammation and higher neutrophil infiltration were observed in the ceca of mice lacking core 2, PSGL-1, or P-selectin compared with WT control. Enhanced levels of αiβ7+ and MAdCAM-1+ cells were observed in the ceca of infected mice lacking core 2, PSGL-1, or P-selectin. Neutrophil recruitment, cecal inflammation, and mortality rates were dramatically reduced in infected P-selectin knockout mice receiving blocking mAb to αiβ7 integrin, indicating that this alternative adhesion molecule may attempt to compensate for the loss of selectins in neutrophil recruitment. These results demonstrate a definitive phenotypic abnormality in mice lacking core 2, PSGL-1, or P-selectin, suggesting that the interaction of functional PSGL-1 with P-selectin is an important process in host defense against Salmonella infection. The Journal of Immunology, 2009, 182: 6550–6561.

Salmonella enterica is a Gram-negative, facultative, intracellular bacterial pathogen capable of infecting a number of hosts and causing significant morbidity and mortality globally (1). S. enterica serovar Typhimurium infection in humans is typically acquired by the ingestion of contaminated food or water leading to acute gastroenteritis with clinical manifestations of diarrhea, abdominal pain, nausea, and vomiting (1, 2). It has been shown that in the early phases of Salmonella infection, the bacteria induces cytoskeletal rearrangements of intestinal epithelial cells leading to uptake and subsequent transcytosis of the bacteria across the epithelium into the lamina propria (3, 4). Such bacterial-epithelial cell interactions result in the secretion of chemokine molecules such as cytokines and chemokines that recruit neutrophils, monocytes, dendritic cells and lymphocytes from the circulation to the site of infection (5). Although the recruited phagocytes engulf and destroy the invading bacteria, which helps to control the infection (6), neutrophil recruitment to the intestinal epithelium also gives rise to the histopathological hallmark of intestinal inflammation (1). Studies of the cellular and molecular mechanisms involved in Salmonella-induced intestinal inflammation have been greatly facilitated by the development of an acute gastroenteritis model in mice in response to oral S. Typhimurium infection (7). In this model, following oral administration of streptomycin, mice infected orally with S. Typhimurium develop acute colitis, showing signs of intestinal inflammation that manifests itself most prominently in the cecum and has many of the pathological features of Salmonella enterocolitis in humans (8, 9).

Recruitment of leukocytes to the sites of inflammation is, on the one hand, a requirement for ensuring host immune surveillance and defense against pathogens, but it also plays a key role in the pathogenesis of inflammatory diseases. Leukocyte recruitment into inflamed tissue is a multistep process comprising initial rolling along the microvascular endothelium followed by firm leukocyte adhesion and transmigration (10). Tethering and rolling of leukocytes along inflamed tissue are mediated by selectins (11–13) and α4 integrins (14). Selectins are a family of cell adhesion molecules consisting of three cell surface glycoproteins expressed by leukocytes (L-selectin), vascular endothelium (E-selectin), and platelets and endothelium (P-selectin). Whereas L-selectin is constitutively expressed on leukocytes, E- and P-selectins are inducible on endothelial cells in response to inflammatory stimuli such as TNF-α and IL-1 (15, 16). The best characterized ligand for selectins is P-selectin glycoprotein ligand (PSGL)-1 (11), a homodimeric mucin-like glycoprotein (17, 18) expressed on the surface of almost all circulating leukocytes (19, 20). Although PSGL-1 can bind to

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Abbreviations used in this paper: PSGL, P-Selectin glycoprotein ligand; CBA, cytometric bead array; KO, knockout; MAdCAM, mucosal addressin cell adhesion molecule; MPO, myeloperoxidase; WT, wild type.

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all three selectins under inflammatory conditions (21–23), it is the predominant ligand for P-selectin (24, 25).

PSGL-1 binding to the three selectins requires appropriate glycosylation and sulfation modifications (26–28). A minimal recognition motif that enables adhesion under flow for all selectins is the sialyl-Lewisx tetrasaccharide (29–31). Targeted gene deletion studies have demonstrated that several glycosyltransferases are important for the formation of the sialyl-Lewisx carbohydrate structure (32). Particularly, the core 2 β1,6-N-acetylgalactosaminyltransferase-I (or C2GlcNAcT-I, henceforth referred to as core 2) has been implicated in the biosynthesis of functional PSGL-1. Studies in mice with gene deletion of core 2 have shown a partial or complete lack of PSGL-1 expression, leading to increased susceptibility to infections and inflammation.

**FIGURE 1.** Interaction of functional PSGL-1 with P-selectin controls host survival of S. Typhimurium infection. WT control mice and mice lacking core 2, PSGL-1, P-selectin, E-selectin, and L-selectin were treated with streptomycin before infection with S. Typhimurium and monitored for signs of morbidity and mortality. Significantly higher mortality rates were observed in core 2 KO (p < 0.0001) (A), PSGL-1 KO (p = 0.0081) (B), and P-selectin KO (p = 0.0009) (C) mice when compared with those in WT controls, whereas no difference was noted in E-selectin KO (D) and L-selectin KO (E). p values for survival curves of KO mice compared with those of WT controls were determined using a log-rank test. Data are representative of three independent experiments with a total of at least 15 animals from each genotype and WT control.

**FIGURE 2.** S. Typhimurium colonization in the colon is enhanced in core 2-, PSGL-1-, and P-selectin-deficient mice at 4 days postinfection. Mice deficient in core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with S. Typhimurium and sacrificed at 4 days postinfection. Bacterial loads harvested from the spleens (A) were similar among all groups of mice, but significantly increased in the colons (B) of core 2 KO (p < 0.05), PSGL-1 KO (p < 0.01), and P-selectin KO (p < 0.05) mice as compared with those of WT controls. p values for the differences in bacterial loads between the KO mice and the WT controls were determined by one-way ANOVA with Dunnett’s post-test. Bars indicate geometric means from each genotype of at least four mice. Data are representative of three independent experiments.

**FIGURE 3.** S. Typhimurium induced severe inflammation in the ceca of core 2-, PSGL-1-, and P-selectin-deficient mice at 1 day postinfection. Mice defective in core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with S. Typhimurium and sacrificed at 1 day postinfection. Representative photographs of ceca of one mouse from each group of at least four mice were shown. More severe inflammation was in the ceca of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice as demonstrated by the observation that the ceca from these mice were all filled with pus and had shrunk (A), and there was a significant reduction in cecal weights (p < 0.01) in these mice as compared with the WT controls (B). p values for the differences in cecal weights between the KO mice and the WT controls were determined by one-way ANOVA with Dunnett’s post-test. Data are representative of three independent experiments.
complete loss of PSGL-1 binding to each of the three selectins. Binding of PSGL-1 to P-selectin and L-selectin is entirely dependent on the activity of core 2 (33, 34) whereas interaction of PSGL-1 with E-selectin can occur in the absence of core 2 (35).

The firm leukocyte adhesion and transmigration steps (10, 36) that follow the selectin-mediated leukocyte tethering and rolling are mediated primarily by integrins, a large family of heterodimeric transmembrane glycoproteins expressed on a leukocyte cell surface that mediate cell adhesion (37, 38). The \( \alpha_4\beta_7 \) integrins interact with members of the Ig superfamily of cell adhesion molecules. The \( \alpha_\beta_1 \) integrin binds to vascular cell adhesion molecule-1, which is induced on the endothelium in nonmucosal sites during inflammation (39). The \( \alpha_\beta_2 \) integrin is critical for the homing of lymphocytes to mucosal addressin cell adhesion molecule (MAdCAM) 1 (39, 40). MAdCAM-1 can be induced on endothelial cells by bacterial LPS and inflammatory cytokines including TNF-\( \alpha \) and IL-1 (41). The interaction between \( \alpha_\beta_1 \) integrin and MAdCAM-1 has been shown to be the key step in lymphocyte homing to gut mucosa (42), playing an active role in both mucosal immune homeostasis and intestinal inflammation.

Whether selectins that are important mediators of leukocyte recruitment play a role in infectious colitis has not yet been explored. In the present study, we used Salmonella-induced intestinal inflammation in mice deficient in PSGL-1, P-, E-, L-selectin, or core 2 to delineate the role of selectins and their ligand in the recruitment of neutrophils and host survival to Salmonella infection. We found that the interaction of functional PSGL-1 with P-selectin plays a critical role in host defense against Salmonella infection.

Materials and Methods

Bacterial culture

Salmonella enterica serovar Typhimurium SL1344 (43) was grown overnight with shaking (200 rpm) in Luria-Bertani broth supplemented with 50 \( \mu \)g/ml streptomycin at 37°C for 18 h.

Mice

C57BL/6 wild-type (WT), PSGL-1-deficient C57BL/6 (PSGL-1 knockout (KO)), and P-selectin-deficient C57BL/6 (P-selectin KO) mice, originally from The Jackson Laboratory, were bred at the Biomedical Research Centre (University of British Columbia, Vancouver, Canada). The generation of C2GlcNAcT-I deficient C57BL/6 (core 2 KO) mice (34). E-selectin-deficient C57BL/6 (E-selectin KO) mice (44), and L-selectin-deficient C57BL/6 (L-selectin KO) mice (45) has been described. All mice were backcrossed at least eight generations on a C57BL/6 background. Mice

![Image](https://via.placeholder.com/150)

**FIGURE 4.** Histology of ceca from S. Typhimurium-infected mice showing severe inflammation in core 2-, PSGL-1-, and P-selectin-deficient mice at 1 day postinfection. Mice defective in core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with S. Typhimurium and sacrificed at 1 day postinfection. A–E, Representative H&E staining of the cecum of one mouse from each group of at least four mice showing severe inflammation as demonstrated by submucosal edema and polymorphonuclear leukocyte infiltration, the formation of crypt abscesses in the mucosa, desquamation in the surface epithelium, and neutrophil infiltration in the lumen of the ceca of infected core 2 KO (A), PSGL-1 KO (B), and P-selectin KO (C) mice, whereas only mild inflammation was observed in the WT controls (D). These pathological features of inflamed cecum were absent from uninfected mice among all groups (E). Images are shown at original magnifications of \( \times 50 \) and \( \times 200 \). F, Pathological scoring revealed significantly more severe cecal inflammation in the ceca of core 2 KO (\( p < 0.01 \)), PSGL-1 KO (\( p < 0.01 \)), and P-selectin KO (\( p < 0.01 \)) in comparison with the WT controls at 1 day postinfection. \( p \) values for the differences in pathological scoring of the ceca between the KO mice and the WT controls were determined by one-way ANOVA with Dunnett’s post-test. Data are representative of three independent experiments.
were maintained in specific pathogen-free conditions and all animal experiments were done according to institutional guidelines and were approved by the Animal Care Committee of the University of British Columbia.

**Mouse model of Salmonella-induced intestinal inflammation**

The protocol for *S.* Typhimurium-induced enterocolitis was used as described (7, 46). Briefly, mice aged 8–10 wk were given 20 mg of streptomycin by oral gavage with a 21-gauge feeding needle. Twenty-four hours later, 3 × 10^8 S. Typhimurium were administered by oral gavage. Mice were assigned randomly to a survival group to monitor for survival and an organ harvest group to assess for intestinal inflammation. To allow a humane end point, mice in the survival group were monitored twice daily for 10 days after bacterial inoculation for signs of morbidity (reduced level of motion, piloerection, labored breathing, and weight loss). Mice that showed distress of these signs or became moribund were euthanized with CO2 and considered as nonsurvival mice. For other experiments, mice were euthanized at designated time points. Spleens and colons were harvested aseptically for bacterial enumeration and ceca were collected for histopathology.

**Bacterial enumeration**

Spleens and colons were harvested at 1 and 4 days postinfection with *S.* Typhimurium and weighed, and each sample was collected in 1 ml of sterile PBS and homogenized with an MM 301 mixer mill (Retsch). Serial dilutions of the resulting homogenates were plated on Luria-Bertani agar plates containing 100 μg/ml streptomycin. Plates were incubated at 37°C for 24 h. Colony counts were expressed as CFU per milliliter.

**Histopathology**

Ceca of experimental animals were fixed in 10% formalin for 18 h followed by 18 h in 70% ethanol before being embedded in paraffin, sectioned, and stained with H&E by Wax-it Histology Services. Stained sections were examined in a blinded fashion for signs of inflammation, polymorphonuclear leukocyte infiltration, edema, crypt abscess formation, regenerative changes, and necrosis. Pathological scores were determined by grading the histopathologic findings from 0 (none) to 1+ (mild), 2+ (moderate), and 3+ (severe) and averaging six fields per sample according to a scoring system previously described (46).

**Colonic cytokine measurements**

Colon samples collected at 1 and 4 days postinfection were assayed for the presence of cytokines. Colon homogenates collected as described above were spun twice at 13,200 rpm in an Eppendorf bench top centrifuge (5415R) for 30 min each at 4°C to remove insoluble matter. Colon supernatants were frozen at −80°C until assayed for cytokines using the mouse inflammation cytometric bead array (CBA) assay kit (BD Biosciences) according to the manufacturer’s instructions.

**Myeloperoxidase staining**

For myeloperoxidase (MPO) immunofluorescent staining for the detection of neutrophils in the cecum of infected mice, paraffin-embedded tissues were
postinfection. Cytokines present in the colon extracts were determined by CBA assay. Significantly elevated levels of TNF-α, MCP-1, and IL-6 in the colons of core 2-, PSGL-1-, and P-selectin-deficient mice. Mice lacking core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with S. Typhimurium and sacrificed at 4 days postinfection. Cytokines present in the colon extracts were determined by CBA assay. Significantly elevated levels of TNF-α (p < 0.005) (B), MCP-1 (p < 0.05) (D), and IL-6 (p < 0.05) (F) were detected in the colons of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice as compared with infected WT controls. The levels of IL-12 p70 (A), IFN-γ (C), and IL-10 (E) present in the colons were similar among all groups of infected mice. p values for the differences in colonic cytokines between the infected KO mice and the infected WT controls were determined by two-tailed Student’s t test. U. Colon cytokines from uninfected mice: I, colon cytokines from infected mice. Results (mean ± SEM) were from five mice per genotype. Data are representative of three independent experiments.

Table 1: Cytokine levels in the colons of infected mice

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Untreated</th>
<th>Infected</th>
</tr>
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<tbody>
<tr>
<td>IL-12 p70</td>
<td>50 pg/ml</td>
<td>100 pg/ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>200 pg/ml</td>
<td>700 pg/ml</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>120 pg/ml</td>
<td>240 pg/ml</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2500 pg/ml</td>
<td>5000 pg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>50 pg/ml</td>
<td>100 pg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>200 pg/ml</td>
<td>500 pg/ml</td>
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Flow cytometry analysis

Single cell suspensions of cecal lamina propria cells were prepared following a standard protocol (47). Briefly, ceca from naive or infected mice were rinsed in HBSS to remove fecal contents. Epithelial cells were removed by shaking for 30 min at 37°C in HBSS containing 5 mM EDTA (Sigma-Aldrich), 5% FCS (HyClone), and 1 mM DTT (Sigma-Aldrich). The digested fragments were passed through a 70-mm nylon mesh and digested with a mixture of collagenase/Dispase (Roche) for 1 h at 37°C. The digested fragments were passed through a 70-mm nylon mesh and further purified by centrifugation over a discontinuous Percoll gradient (40/70%) for 20 min at 2000 rpm. Purified cells from the cecal lamina propria were surface stained with fluorescein-conjugated Abs against CD11b (clone M1/70; BD Biosciences), Gr-1 (clone RB6-8C5; BD Biosciences), αβ integrin (clone DATK32; BD Biosciences), and MD-2 (clone M2-A5; BD Biosciences) before being subjected to flow cytometry analysis with a FACSCalibur (BD Biosciences) using CellQuest software.

Ab blocking of αβ integrin

The purified no azide/low endotoxin monoclonal rat anti-mouse αβ integrin (clone DATK32; rat IgG2a, κ) and rat IgG2a κ isotype control (clone R35-95) were obtained from BD Biosciences. To determine the role of the adhesion molecule αβ integrin in the recruitment of neutrophils and the mortality in mice deficient for functional P-selectin, 50 μg of blocking anti-αβ integrin mAb or isotype control in 100 μl of PBS was administered i.p. to streptomycin-pretreated mice following the Salmonella-induced intestinal inflammation protocol as described above.

Statistical analysis

The survival curves of infected mice were compared using Kaplan-Meier analysis followed by log-rank test. Bacterial loads, cecal weights, and total pathological scores of infected ceca were compared using one-way ANOVA with Dunnett’s post-test. CBA assays for cytokines in the colons of infected mice were compared using two-tailed, unpaired t test. All analyses were performed with a 95% confidence interval using GraphPad Prism software, version 4.0.

Results

Interaction of functional PSGL-1 with P-selectin is critical for host survival in S. Typhimurium infections

During Salmonella-induced intestinal inflammation, phagocytic cells are recruited to the site of inflammation (5, 6). Because selectins are the critical mediators of such leukocyte recruitment, we hypothesized that deficiency of these mediators would impair the host’s defense mechanisms against Salmonella infection, leading to increased morbidity and mortality. To address this, we orally infected mice deficient in the selectin ligand PSGL-1 and P-, E-, or L-selectin or the glycosyltransferase core 2 with S. Typhimurium. Significantly higher mortality rates were noted throughout the period of observation in core 2 KO (p < 0.005) (Fig. 1A), PSGL-1 KO (p = 0.0081, Fig. 1B), and P-selectin KO (p = 0.0009, Fig. 1C), whereas the mortality rates for E-selectin KO (Fig. 1D) and L-selectin KO mice (Fig. 1E) were not different from those of WT controls. These data indicate that the interaction of functional
PSGL-1 with P-selectin may play an important role in host survival during Salmonella infections. Because significant differences in survival were observed in core 2 KO, PSGL-1 KO, and P-selectin KO mice, our subsequent studies were focused on using mice with these deficiencies.

S. Typhimurium colonization in the colon is enhanced in mice with core 2, PSGL-1, and P-selectin deficiencies at 4 days postinfection

Selectins play a major role in the recruitment of leukocytes to the site of infection, which helps in either killing the bacteria and eliminating the infection or in preventing the spreading of the bacteria to systemic organs. Thus, we determined the ability of S. Typhimurium to translocate and colonize the colons and spleens of mice with deficiencies in selectins. Bacterial loads harvested from the colons and spleens of core 2 KO, PSGL-1 KO, P-selectin KO, and WT control mice at 1 day postinfection were similar (data not shown). At 4 days postinfection, bacterial loads harvested from the spleens were also similar among all groups of infected mice (Fig. 2A). However, significantly higher bacterial loads were harvested from the colons of core 2 KO (p < 0.05), PSGL-1 KO (p < 0.01), and P-selectin KO (p < 0.05) mice at 4 days postinfection when compared with WT controls (Fig. 2B), indicating that the loss of P-selectin-PSGL-1 interaction is associated with enhanced Salmonella colonization of the colon.

S. Typhimurium-induced cecal inflammation is more severe in mice deficient in P-selectin-PSGL-1 interaction

To assess the role of selectins in S. Typhimurium-induced cecal inflammation, we compared the cecal pathology of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice with that of the WT control mice. The ceca of uninfected mice appeared similar among all groups of mice (data not shown). At 1 day postinfection, ceca of S. Typhimurium-infected core 2 KO, PSGL-1 KO, and P-selectin KO mice were filled with pus and appeared shrunken (Fig. 3A) and had a significant reduction in cecal weights (p < 0.01) compared with the infected WT control mice (Fig. 3B), indicating enhanced inflammation of the ceca in the KO mice. Histopathological analysis of H&E-stained tissue sections revealed extensive pathological changes of the ceca from infected core 2 KO (Fig. 4A), PSGL-1 KO (Fig. 4B), and P-selectin KO (Fig. 4C) mice at 1 day postinfection. In the ceca of these mice we observed marked edema and infiltration of PMNs in the submucosa, the formation of crypt abscesses in the mucosa, marked regenerative changes, and desquamation in the surface epithelium layer, as well as neutrophil infiltration into the lumen. Only mild inflammation was observed in the cecum of infected WT control mice at 1 day postinfection (Fig. 4D). These pathological features of inflamed cecum were absent from uninfected mice among all groups (Fig. 4E). Using the pathological scoring scheme, we compared the severity of inflammation between the different groups of KO mice with that of the WT control mice. We found that at 1 day postinfection with S. Typhimurium, cecal inflammation was significantly more severe in core 2 KO (p < 0.01), PSGL-1 KO (p < 0.01), and P-selectin KO (p < 0.01) mice than in WT control mice (Fig. 4F).

At 4 days postinfection, more severe cecal inflammatory features including edema, ulcerations, crypt destruction, and desquamation as well as the presence of necrotic epithelial cells and the infiltration of neutrophils into the lumen were observed in core 2 KO (Fig. 5A), PSGL-1 KO (Fig. 5B), and P-selectin KO (Fig. 5C) mice. Cecal inflammation in infected WT control was also observed, although to a lesser extent (Fig. 5D). Pathological scoring also indicated that cecal inflammation was significantly more severe in core 2 KO (p < 0.05), PSGL-1 KO (p < 0.05), and P-selectin KO (p < 0.05) mice in comparison with the WT control mice (Fig. 5E) at 4 days postinfection.

Consistent with Salmonella colonization of the colon, we found that at 4 days but not at 1 day postinfection there was a significant increase in proinflammatory cytokines such as TNF-α (p < 0.005; Fig. 6A), MCP-1 (p < 0.05; Fig. 6D), and IL-6 (p < 0.05; Fig. 6F) in the colons of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice in comparison with infected WT controls, whereas levels of IL-12 p70 (Fig. 6A), IFN-γ (Fig. 6C), and IL-10 (Fig. 6E) present in the colons were similar among all groups of infected mice. Enhanced levels of these proinflammatory cytokines and chemokines may contribute to increased cecal inflammation in mice with selectin function deficiencies.

S. Typhimurium-induced neutrophil recruitment is more pronounced in ceca of core 2-, PSGL-1-, and P-selectin-deficient mice

Because selectins mediate the recruitment of immune cells to the site of inflammation, we would expect reduced neutrophil recruitment in the ceca of mice with core 2, PSGL-1, and P-selectin

deficiencies. However, histopathological analysis of the H&E sections revealed more pronounced neutrophil infiltration in the ceca of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice in comparison with the WT control mice at 1 day postinfection. To confirm this, we stained tissue sections of infected ceca harvested at 1 day postinfection with Abs against MPO. As shown in Fig. 7, infiltration of MPO positively stained neutrophils was observed in the lamina propria and predominantly in the submucosa of the ceca of infected core 2 KO (Fig. 7A), PSGL-1 KO (Fig. 7B), and P-selectin KO (Fig. 7C) mice but was absent in the cecum of infected WT control (Fig. 7D), comparable to the uninfected mice among all groups (Fig. 7E).

To further characterize the phenotype of the neutrophil populations induced in the ceca of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice, lamina propria leukocytes were harvested from the ceca of naive and infected mice and analyzed for Gr-1 and CD11b expression using flow cytometry. Fig. 8 illustrates representative FACS plots comparing expressions of CD11b and Gr-1 on neutrophils harvested from the ceca of core 2 KO, PSGL-1 KO, P-selectin KO, and WT control mice. Only 1% of cells from the ceca of uninfected mice from the various groups were mature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>+</sup> (Fig. 8A–D, upper right quadrants). At 1 day postinfection with S. Typhimurium, the percentage of immature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>−</sup> (upper left quadrants) from the ceca of infected core 2 KO (Fig. 8F), PSGL-1 KO (Fig. 8G), and P-selectin KO (Fig. 8H) mice increased to 6, 10, and 15% respectively, whereas CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from infected WT control (Fig. 8E) remained unchanged. The percentage of mature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>+</sup> (upper right quadrants) from the ceca of infected core 2 KO (Fig. 8F), PSGL-1 KO (Fig. 8G), and P-selectin KO (Fig. 8H) mice increased to 15, 69, and 68%, respectively, whereas CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from infected WT control mice (Fig. 8E) remained unchanged. FACS plots are representative of two independent experiments with a total of four mice from each genotype and WT control.

**S. Typhimurium induces enhanced levels of α<sub>4</sub>β<sub>7</sub>-Gr-1<sup>−</sup> and MadCAM-1<sup>+</sup> cells in ceca of core 2−, PSGL-1−, and P-selectin-deficient mice**

Interaction of α<sub>4</sub>β<sub>7</sub> integrin with its receptor MadCAM-1 is known to play a central role in the homing of leukocytes to gut-associated sites (42, 48). We thus determined whether these other intestinal mucosal adhesion molecules might compensate for the loss of Core2, PSGL-1, and P-selectin in recruiting neutrophils to the ceca during Salmonella infection. Lamina propria leukocytes were isolated from the ceca of naive and infected mice and analyzed for α<sub>4</sub>β<sub>7</sub> integrin and MadCAM-1 expression using flow cytometry. Fig. 9 illustrates representative FACS plots comparing expression of α<sub>4</sub>β<sub>7</sub> integrin on Gr-1<sup>−</sup> neutrophils harvested from naive and infected ceca of core 2 KO, PSGL-1 KO, P-selectin KO, and WT control mice. Only 1% of cells from the ceca of uninfected mice from the various groups were α<sub>4</sub>β<sub>7</sub>-Gr-1<sup>−</sup> (Fig. 9, A–D, upper right quadrants). At 1 day following infection with S. Typhimurium, the percentage of immature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>−</sup> (upper left quadrants) from the ceca of infected core 2 KO (Fig. 8F), PSGL-1 KO (Fig. 8G), and P-selectin KO (Fig. 8H) mice increased to 15, 69, and 68% respectively, whereas CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from infected WT control (Fig. 8E) remained unchanged. FACS plots are representative of two independent experiments with a total of four mice from each genotype and WT control.

**FIGURE 8.** S. Typhimurium infection is associated with an elevated influx of Gr-1<sup>+</sup>CD11b<sup>+</sup> and Gr-1<sup>−</sup>CD11b<sup>−</sup> cells in mice lacking functional P-selectin-PSGL-1 interaction. Mice deficient in core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with S. Typhimurium and sacrificed at 1 day postinfection. Single cell suspensions from ceca of infected and uninfected mice were subjected to FACS staining. Representative FACS plots comparing expression of CD11b and Gr-1 neutrophils harvested from the ceca of the various groups were shown. Only 1% of cells from uninfected mice of the various genotypes and WT mice were mature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>−</sup> cells (A–D, upper right quadrants). At 1 day postinfection, the percentage of CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from the ceca of infected core 2 KO (F), PSGL-1 KO (G), and P-selectin KO (H) mice increased to 6, 10, and 15% respectively, whereas CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from infected WT control (E) remained unchanged. The percentage of immature neutrophils expressing CD11b<sup>+</sup>Gr-1<sup>−</sup> (upper left quadrants) from the ceca of infected core 2 KO (F), PSGL-1 KO (G), and P-selectin KO (H) mice increased to 15, 69, and 68%, respectively, whereas CD11b<sup>+</sup>Gr-1<sup>−</sup> cells from infected WT control mice (E) remained unchanged. FACS plots are representative of two independent experiments with a total of four mice from each genotype and WT control.
infected core 2 KO (Fig. 9F), PSGL-1 KO (Fig. 9G), and P-selectin KO (Fig. 9H) mice increased to 6, 10, and 12% respectively, whereas $\alpha_4\beta_7$-Gr-1$^+$ cells from the ceca of infected core 2 KO (Fig. 9F), PSGL-1 KO (Fig. 9G), and P-selectin KO (Fig. 9H) mice increased to 9.4, 10.9, and 16.1% respectively, whereas $\alpha_4\beta_7$-Gr-1$^+$ cells from the ceca of infected WT mice (Fig. 9E) were similar to those of the uninfected controls.

Fig. 10 illustrates representative FACS plots comparing the expressions of MadCAM-1 on cells harvested from naive and infected ceca of core 2 KO, PSGL-1 KO, P-selectin KO, and WT control mice. Less than 2% of cells from the ceca of uninfected controls increased to 6, 10, and 12%, respectively, whereas $\alpha_4\beta_7$-Gr-1$^+$ cells from the ceca of infected core 2 KO (Fig. 10F), PSGL-1 KO (Fig. 10G) and P-selectin KO (Fig. 10H) mice increased to 9.4, 10.9, and 16.1% respectively, whereas MadCAM-1$^+$ cells from the ceca of infected WT mice (Fig. 10E) were similar to those of the uninfected controls.

Collectively, these data suggest that in the absence of functional P-selectin, other adhesion molecules including $\alpha_4\beta_7$ integrin and MadCAM-1 were up-regulated, thus enabling the recruitment of neutrophils to the site of inflammation during S. Typhimurium infection.

**Monoclonal Ab blockade of $\alpha_4\beta_7$ integrin abolishes neutrophil recruitment and reduces cecal inflammation and mortality rate in S. Typhimurium-infected, P-selectin-deficient mice**

We next evaluated the role of the gut-homing adhesion molecule $\alpha_4\beta_7$ integrin in the recruitment of neutrophils to the ceca of infected mice deficient for functional P-selectin. Blocking anti-$\alpha_4\beta_7$ integrin mAb or isotype control was administered i.p. to streptomycin-pretreated mice immediately before infection with S. Typhimurium. Consistent with our earlier findings, at 1 day postinfection, severe histopathology of the ceca was observed from infected P-selectin KO mice (Fig. 11B). Intriguingly, histopathological changes of the ceca in infected P-selectin KO mice receiving anti-$\alpha_4\beta_7$ integrin mAb were drastically reduced (Fig. 11C). In fact the only histopathological changes we observed in these mice were diffuse desquamation of the surface epithelium and regenerative changes in the surface epithelium (Fig. 11C). Severe histopathological changes were found in the ceca of infected P-selectin KO mice receiving isotype control Ab (Fig. 11D). Histopathological scoring also revealed that at 1 day postinfection, cecal inflammation was reduced remarkably in P-selectin KO mice receiving anti-$\alpha_4\beta_7$ integrin mAb but remained severely inflamed in P-selectin KO mice receiving isotype control Ab when compared with P-selectin KO mice (Fig. 11E).

Consistent with the histopathological scoring, we found that neutrophil recruitment was blocked in the ceca of infected P-selectin KO mice receiving anti-$\alpha_4\beta_7$ integrin mAb (Fig. 12C) comparable to that in uninfected control (Fig. 12A), whereas infiltration of MPO positively stained neutrophils was observed in the ceca of infected P-selectin KO mice receiving isotype control Ab (Fig. 12D), similar to that in infected P-selectin KO mice (Fig. 12B).

We further assessed the role of $\alpha_4\beta_7$ integrin in host survival in infected mice deficient of functional P-selectin. As shown in Fig. 13, compared with Salmonella-infected P-selectin KO mice, the mortality rate in P-selectin KO mice receiving anti-$\alpha_4\beta_7$ Ab ($p = 0.0090$) was significantly reduced to levels comparable to those of C57BL/6 WT mice, whereas the isotype control had no effect on survival ($p = 0.7959$).

Together, these results suggest that compensatory mechanisms that involved up-regulation of the gut-homing adhesion molecule $\alpha_4\beta_7$ integrin exist to allow the homing of neutrophils into the...
cecal tissues following infection with *S. Typhimurium* in the absence of functional P-selectin. However, such mechanisms are not sufficient to control *Salmonella*, indicating a defect in particular cell recruitment or some other imbalance in the process. This emphasizes the critical role of P-selectin and its ligand in controlling this enteric pathogen.

**Discussion**

Selectins and their ligands play a key role in leukocyte recruitment into sites of infection, which is essential for bacterial clearance and is a fundamental host defense against invading pathogens. In *S. Typhimurium*-induced colitis, the role of selectins has not been explored. To address this, we analyzed *S. Typhimurium*-induced intestinal inflammation in selectin- and selectin ligand-deficient mice. Increase in mortality rate in mice defective in core 2, PSGL-1, and P-selectin, but not in E-selectin- and L-selectin-deficient mice, following infection with *S. Typhimurium* points to the functional involvement of P-selectin in this disease model.

Leukocyte capture and rolling, mediated by selectins, is a generally accepted prerequisite for leukocyte recruitment to sites of inflammation. Thus, earlier studies using a chemical-induced acute peritonitis model by i.p. thioglycollate injection in mice with targeted gene deletion of core 2 (34), PSGL-1 (24), or P-selectin (49) demonstrated a drastic reduction in leukocyte rolling and in neutrophil recruitment to inflamed peritoneum in mice lacking core 2, PSGL-1, or P-selectin. In the present study, we used a bacteria-induced acute colitis model to investigate the role of selectins in mice with core 2, PSGL-1, or P-selectin deficiencies. Should P-selectin be functionally involved, we would thus expect a reduction in neutrophil recruitment in mice deficient in P-selectin function. Surprisingly, our data revealed a more pronounced neutrophil infiltration in the ceca of mice lacking core 2, PSGL-1, and P-selectin. The reason for such a discrepancy may have resulted in the difference in the infection/inflammation models studied. In the current study using the *S. Typhimurium*-induced colitis model in selectin- and selectin ligand-deficient mice, it is possible that the loss of functional P-selectin interaction may be compensated by up-regulation of other adhesion molecules. Indeed, our data show a strong up-regulation of the intestinal mucosal adhesion molecule αβ integrin and its ligand, the vascular mucosal addressin MadCAM-1. Both of these adhesion molecules are known to be central in the homing of leukocytes to gut-associated sites (42, 48), indicating that the up-regulation of these adhesion molecules is responsible for the increased influx of leukocytes to the ceca of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice. This is further confirmed by our finding that blockade of αβ integrin interaction with its ligand by using an anti-αβ integrin mAb has abolished neutrophil recruitment and reduced dramatically cecal inflammation and the mortality rate in mice lacking functional P-selectin when infected with *S. Typhimurium*.

Neutrophils have been shown to produce enhanced levels of proinflammatory cytokines during infectious colitis (50). In the present study, significant increases in TNF-α, IL-6, and MCP-1 production were detected in the colons of infected mice with P-selectin function deficiencies in comparison with WT control at 4 days postinfection (Fig. 6). The enhanced levels of TNF-α, IL-6, and MCP-1 production may be attributable to the large influx of neutrophils. Intriguingly, MCP-1 is a pivotal chemokine in the recruitment of monocytes (51) and neutrophils (52); the expression of MCP-1 can be induced by a variety of factors, including inflammatory cytokines such as TNF-α (53, 54) and IL-6 (55). Thus, the increased levels of TNF-α, IL-6, and MCP-1 in mice lacking

**FIGURE 10.** *S. Typhimurium* induced elevated levels of MaDCAM-1$^+$ cells in the ceca of mice lacking functional P-selectin-PSGL-1 interaction. Mice deficient in core 2, PSGL-1, and P-selectin and WT controls were treated with streptomycin before infection with *S. Typhimurium* and sacrificed at 1 day postinfection. Representative FACS plots comparing expression of MaDCAM-1 on cells harvested from the ceca of the various groups were shown. Less than 2% of cells from the ceca of uninfected mice of the various genotypes and WT mice were MaDCAM-1$^+$ (A–D). The percentage of MaDCAM-1$^+$ cells from the ceca of infected core 2 KO (F), PSGL-1 KO (G), and P-selectin KO (H) mice increased to 9.4, 10.9, and 16.1%, respectively, whereas MaDCAM-1$^+$ from infected WT control mice (E) remained unchanged. FACS plots are representative of two independent experiments with a total of four mice from each genotype and WT control.
core 2, PSGL-1, or P-selectin would contribute to the additional neutrophil infiltration.

Several mechanisms have been shown that allow the bacteria to breach the intestinal epithelium in S. Typhimurium-induced systemic disease. Preferentially, the bacteria penetrate microfold (M)
cells, which subsequently transport them to lymphoid cells in the underlying Peyer’s patches (56, 57) where the bacteria multiply and disseminate throughout the body. Alternatively, they can be translocated across the intestinal epithelium after uptake by CD18-expressing phagocytes (58). However, in the streptomycin pretreatment, S. Typhimurium-induced intestinal inflammation model, colonization of Peyer’s patches is not required for the initiation of colitis or the spread of the bacteria to internal organs (7), suggesting that alternative pathways may be involved. Previous studies have shown that neutrophils were recruited to the ceca of mice infected with S. Typhimurium following pretreatment with streptomycin (46). Transmigration of neutrophils may play a role in promoting the bacteria to breach the intestinal barrier. Neutrophil infiltration through the intestinal epithelium results in the release of toxic products such as proteases and reactive oxygen intermediates (59), leading to endothelial damage and thus allowing the bacteria to invade the epithelium and cause intestinal inflammation.

In response to bacterial invasion, neutrophils are the first cells that migrate from the circulating blood to infected tissues where they efficiently bind, engulf, and inactivate the bacteria, thus playing a critical role in bacterial clearance and resolution of infection. It has been shown that neutrophils have the capacity to specifically overcome virulence factors of S. Typhimurium, hence enabling these cells to effectively kill the bacteria and protect the host from infection (60). In the current study, we found a more pronounced neutrophil infiltration in the ceca of infected core 2 KO, PSGL-1 KO, and P-selectin KO mice in comparison with the WT control mice at 1 day postinfection. Thus, we would expect that the rapid influx of neutrophils in these genetically deficient mice would help to protect them from S. Typhimurium infection. However, we found increased bacterial loads in the colons of core 2 KO, PSGL-1 KO, and P-selectin KO mice, and they were more susceptible to S. Typhimurium infection compared with the WT control mice (Fig. 1). Although neutrophil recruitment to sites of acute inflammation is beneficial for the host to fight infection, an overexuberant influx of neutrophils may enhance the translocation of bacteria to other internal organs and may also exacerbate proinflammatory cytokine production and excessive release of proteases and reactive oxygen intermediates, resulting in endothelial damage, capillary leakage, multiorgan failure, and consequently mortality. In the present study, the massive influx of neutrophils observed in the ceca of S. Typhimurium-infected mice lacking core 2, PSGL-1, and P-selectin may lead to more tissue damage and induction of the proinflammatory cytokines TNF-α and IL-6, which have been shown to contribute to the development of intestinal inflammation (61–64) due to their proinflammatory activity. The profound influx of neutrophils in core 2-, PSGL-1-, and P-selectin-deficient mice may also enhance the spreading of viable bacteria to the colons and, as a result, cause increased inflammation in mice with these deficiencies.

Taken together, using the murine S. Typhimurium-induced intestinal inflammation model, our data have revealed that the interaction of functional PSGL-1 with P-selectin has an important role in host survival to S. Typhimurium infection. In the absence of core 2, PSGL-1, and P-selectin there is an up-regulation of the intestinal mucosal adhesion molecule αIβ2 integrin on neutrophils and of the endothelial receptor MadCAM-1 on endothelial cells, leading to a massive influx of neutrophils associated with enhanced bacterial colonization, tissue damage, proinflammatory cytokines secretion, and fatality. Future studies investigating the dual roles of neutrophils that affect inflammation to stimulate host defenses against infection and limit the detrimental effects of inflammation because of the additional damage these cells cause will help further elucidate these mechanisms.

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Disclosures

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References


