Salmonella-induced Diarrhea Occurs in the Absence of IL-8 Receptor (CXCR2)-Dependent Neutrophilic Inflammation

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Background. Gastroenteritis is the most common manifestation of nontyphoidal Salmonella enterica infections, but little is known about the pathogenesis of diarrhea in this infection.

Methods. To determine whether polymorphonuclear neutrophils (PMNs) are required for diarrhea for Salmonella colitis, we infected kanamycin-pretreated interleukin 8R (IL-8R) mutant mice and controls, both with nonmutant Slc11a1 (Nramp1, ItyR). We compared the 2 mouse strains for increases in fecal water content (diarrhea) 3 days after infection, changes in expression of ion transporters in colonic epithelial cells, proliferation of epithelial cells, and severity of infection as measured by colony-forming units (CFUs).

Results. The IL-8R knockout mice had fewer PMNs in the colon but the other variables we measured were unaffected except for an increase in CFUs in the colon. The pathologic changes in the cecum were similar in both groups except for the lack of PMNs in the IL-8R knockout mice. There was minimal damage to the colon more distally.

Conclusions. In the early stage of Salmonella colitis, PMNs are not required for diarrhea or for the decrease in expression of colonic epithelial cell apical ion transporters. They contribute to defense against infection in the cecum but not extracolonically at this stage of Salmonella colitis.

Keywords. diarrhea; Salmonella; colitis; neutrophil; epithelial cell; ion transporter.

Salmonella gastroenteritis is the most common manifestation of nontyphoidal Salmonella (NTS) infection [1]. In the United States it is estimated that there are 1.2 million cases annually of NTS gastroenteritis. Less than 1% of the >2100 serovars of Salmonella account for >80% of human gastroenteritis, and S. enterica serovar Typhimurium (S. Typhimurium) is one of the most commonly isolated serovars [1]. There have been many major international outbreaks of NTS gastroenteritis owing to the globalization of food production and distribution [2, 3]. The most common manifestations of NTS gastroenteritis are fever, watery diarrhea, and cramping abdominal pain, but more severe manifestations, including colitis, can occur [3–5]. Most episodes of NTS gastroenteritis are self-limited but they still cause substantial morbidity; NTS are notably responsible for nearly half of all deaths in the United States among persons with laboratory-confirmed infections by food-borne pathogens [6].

Despite its public health importance, relatively little is known about the pathogenesis of diarrhea in NTS infection. NTS are locally invasive in the intestine, infecting primarily the ileum and cecum [7], but, unlike Shigella sp., they do not directly destroy epithelial cells [8]. They cause diarrhea that is characterized by polymorphonuclear neutrophils (PMNs) in the stool after their transcytosis across the intestinal wall [9], but our understanding of the connection between intestinal inflammation and the pathogenesis of Salmonella diarrhea is still incomplete, in part owing to the absence of a small animal model to study the pathogenesis of...
diarrhea. Salmonella enteritis has been studied in calves, but this is an expensive model [10]. Blind loops of small intestine can also be infected, which has the advantage that fluid accumulation can easily and accurately be measured and different loops injected with isogenic mutants to study the roles of virulence factors in producing diarrhea [11]. In both of these models there is an association between infiltration of the intestine by PMNs and macrophages and fluid exudation [12].

Orally infected mice do not develop intestinal inflammation unless they are germ free [13] or have been pretreated with an oral aminoglycoside [14, 15]. In streptomycin-pretreated mice lacking a functional natural resistance-associated macrophage protein 1 (Nramp1) S. enterica causes marked submucosal edema in the cecum, with infiltration of inflammatory cells into the submucosa and mucosa, a decrease in goblet cells, and disruption of the cecal epithelium [16]. This model has been very useful for confirming the biologic significance of bacterial genes required for invasion of cultured epithelial cells by NTS and for discovering host-resistance genes [14]. The model is not useful for studying diarrhea, however, because Nramp1G169 mutant mice die of overwhelming systemic infection 2–3 days after infection. Marchelletta et al recently described the course of infection in BALB/c.D2Nramp1 mice that are congenic for a segment of chromosome 1 that carries the wild-type (WT) copy of Nramp1G169 derived from DBA/2 mice [15, 17]. These mice survive for approximately 8 days after infection, dying of hemorrhagic colitis, not systemic infection. However, as early as day 3 after infection they have diarrhea [18]. At this time there is decreased expression of the electron-neutral Cl-/HCO3- exchanger DRA (down-regulated in adenoma) in the proximal colon and of the epithelial sodium channel (ENaC) in the distal colon, even though there is almost no inflammation in the colon distal to the cecum at this time [18]. We know that loss of DRA leads to diarrhea both in mice with mutations in Slc26a3 (DRA), and in humans who have severe congenital chloride diarrhea owing to mutations in the same gene [19].

S. enterica are macrophage-tropic bacteria, and macrophages are both the host cell for bacterial growth and ultimately the mediator of bactericidal activity; mutants that cannot survive in unactivated macrophages are not virulent in systemic infections [20]. PMNs also play a role in the host defense against systemic NTS infections [21–23], but their importance in the host defense against Salmonella gastroenteritis has not been established. However, invasion of the colon by Salmonella causes an influx of PMNs into the mucosa and submucosa of experimentally infected guinea pigs [24] calves [25], mice [14], and nonhuman primates [26].

Epithelial cells respond to bacterial invasion by basolateral secretion of proinflammatory cytokines and chemokines through multiple mechanisms [27]. Human intestinal epithelial cells secrete interleukin 8 (IL-8) [28] in response to S. enterica invasion, probably through the activation of several innate immune signaling pathways [29]. ELR-positive (glutamic acid, leucine, arginine) CXC chemokines are also synthesized by macrophages and dendritic cells, and even PMNs themselves [30]. The subsequent paracellular movement of PMNs into the intestinal lumen is mediated by apical secretion of a novel eicosanoid, hepoxilin A3 [31]. It has been suggested that the infiltrating PMNs are responsible for diarrhea in Salmonella gastroenteritis [32, 33]. In this study we tested the hypothesis that PMN infiltration of the colon was necessary for production of diarrhea by infecting mice that have a mutated CXCR2, which encodes the IL-8R in mice. We studied the effect of that mutation on severity of infection, intestinal pathology, expression of ion transporters in the colon, and diarrhea.

METHODS

Mice

We purchased IL-8R (CXCR2) KO mice on a BALB/c background from the Jackson Laboratory, which uses heterozygous female mice for breeding because homozygous female mice are not very fertile [34]. We were able to do homozygous breeding by keeping the mice free of infection with a combination of sterile food and sterile water containing trimethoprim-sulfamethoxazole (TMX) oral suspension containing 4 mg/20 mg per 5 mL. We determined the Nramp1 status of these mice using polymerase chain reaction, as described elsewhere [35], and we found that despite backcrossing to BALB/c they were Nramp1G169, because Cxcr2 is within 0.14 cM of Slc11a1 (encodes Nramp1) on chromosome 1 and the embryonic stem (ES) cells used to make the mutant Cxcr2 were 129, a strain that has a WT Slc11a1. Because TMX affects the composition of the intestinal flora, control BALB/c.D2Nramp1 congenic mice were also treated with TMX oral suspension, beginning with the pregnant mothers and continuing until the time of the experiment, so that any effects on the fecal flora would be comparable in both groups of mice.

We purchased C57BL/6J (B6), DBA/2, A/J, BALB/c, and B6. Cg-Alox5tm1Fun (arachidonate 5-lipoxygenase-deficient) [36] mice from the Jackson Laboratory. Fpr1−/−Gao in the fifth generation backcross to B6 mice were a gift from Ji-Lang Gao (National Institutes of Health). The mice were genotyped to identify those that still carried the wild-type Slc11a1 allele from the 129 ES cells and those mice were our breeders.

Infection

We discontinued TMX 3 days before administering a 40-mg dose of oral kanamycin. Two days after the kanamycin, we infected the mice by gavage with 5 × 105 of kanamycin-resistant S. Typhimurium 14028s [38] in 0.1 mL of 0.1 mol/L sodium bicarbonate. We collected feces from mice the next morning for culture to ensure that the mice were infected and then collected
feces daily to measure water content, as described elsewhere [15].
For controls, we treated mice with TMX and kanamycin but did not infect them. At necropsy we performed quantitative bacteriology of cecum, mesenteric lymph nodes, and spleens [15]. To study PMN chemotaxis into the peritoneal cavity, we injected mice with $5 \times 10^7$ colony-forming units (CFUs) of S. Typhimurium and killed them 3 hours later before performing peritoneal lavage. All studies were approved by the Institutional Animal Care and Use Committee of the VA San Diego Healthcare System.

Pathology
Three days after infection (5 days after oral kanamycin) mice were killed by cervical dislocation. Their colons were removed, luminal material was rinsed out with sterile saline, and the colons were perfused with and then immersed in methacarn solution [39] and curled into a “Swiss roll,” with the rectum in the center of the spiral. Paraffin-embedded fixed tissue was then sectioned and stained with hematoxylin-eosin. Immunostaining of the tissue for DRA, beta subunit of ENaC (βENaC), and the epithelial marker villin was done as described elsewhere [18]. To quantify the severity of the pathologic changes, we used a slight modification of the scoring system devised by Coburn et al [40], adding 1 point for blood in the lumen. We graded the pathologic changes in the cecum and the proximal colon. Both segments were scored separately. The maximum number of points in this scoring system is 26. For consistency, the slides were coded and read by a single investigator (J. F.) blinded to the experimental conditions.

Real-Time Polymerase Chain Reaction
Some mice were used to obtain fresh full-thickness samples of proximal and distal colonic tissues that were placed in Trizol (Sigma) per the manufacturer’s instructions. The expression of DRA and βENaC messenger RNAs in the proximal colon and distal colons, respectively, were normalized to the expression of villin and compared in the infected and control tissues, as described elsewhere [18].

Myeloperoxidase
The proximal half the cecum was resected and weighed and then processed for measurement of myeloperoxidase according to manufacturer’s instruction (Cell Technology). The amount of fluorescence (in relative fluorescent units) was calculated per milligram of tissue.

Statistical Analysis
We log-transformed CFU and PMN counts in the peritoneal exudates and then determined the geometric means. We used GraphPad Prism software (version 5.04) to compare group means, using a 2-tailed $t$ test to compare 2 groups and analysis of variance to compare >2 groups.

RESULTS
We compared the severity of S. Typhimurium colitis in male IL-8R KO mice and C.D2 controls. All the IL-8R KO mice died before day 6, the planned day of euthanasia, whereas all the C.D2 controls survived, strongly suggesting that the IL-8R, and by extension ELR-positive CXC chemokines, were necessary for resistance to this or infection. We then infected female mice, which are more resistant than male mice to Salmonella infection. By 24 hours after infection, the ceca were shrunken in all infected mice in both groups, and this abnormality grew progressively more severe with time, without any apparent difference between the 2 groups of mice. However, IL-8R KO mice had approximately 1000-fold more bacteria per gram of feces on the day after infection (not shown), and we recovered significantly more Salmonella from cecal tissue from the IL-8R KO mice than from control mice (Figure 1). The CFU counts from mesenteric lymph nodes were not significantly higher in

![Figure 1](https://academic.oup.com/jid/article-abstract/212/1/128/1844564)

Figure 1. Interleukin 8R (IL-8R) knockout (KO) mice are more susceptible to Salmonella enterica serovar Typhimurium colitis. Groups of IL-8R KO and control mice were infected as described in “Methods” section and killed 1, 2, or 3 days after infection. Each symbol represents an individual mouse (closed circles represent control mice; open squares, IL-8R KO mice), and horizontal lines indicate geometric means with standard errors of the mean. The mean number of colony-forming units (CFUs) in the cecal walls of IL-8 KO mice was greater than in the controls, with statistically significant differences on days 1 and 3 after infection. The same was true for mesenteric lymph node (MLN) CFU counts, but that difference reached statistical significance only on day 3 after infection. There were <10 CFUs/spleen on day 1 after infection (not shown), with no significant differences between spleen CFU counts thereafter.
There was no difference in CFU counts in the spleens on days 2 and 3 after infection but, with a single exception, no spleen had >1000 CFUs, possibly because all the mice expressed WT Nramp1.

On day 3 after infection, pathologic changes in infected mice were limited to the cecum and proximal colon. As shown in Figure 2, there was severe submucosal edema and mucosal damage in the ceca in both IL-8 KO and control mice, and there was marked infiltration of inflammatory cells into the submucosa and mucosa. However, the inflammatory infiltrate in IL-8R KO mice was composed almost entirely of macrophages, whereas the infiltrate in the control mice was a mixture of PMNs and macrophages. The marked reduction in PMNs in the cecum of IL-8R KO mice was confirmed by measuring myeloperoxidase activity in ceca taken at necropsy 3 and 5 days after infection (Figure 3). The severity scores for pathologic changes in the ceca of the 2 groups of mice were similar except for PMNs, which were scored only 0 to 1+ in the IL-8R KO mice and usually 3+ in the control mice (Figure 4A). In the proximal colon, there was only a minimal inflammatory infiltrate and submucosal edema in both groups, but again there were essentially no PMNs in the mucosa or submucosa of IL-8R KO mice. There were almost no remaining goblet cells in the ceca of both groups of mice, and, surprisingly, in the proximal colon the number of Periodic acid–Schiff–positive goblet cells was decreased by approximately 25%, even in the absence of overt inflammation.

Next, we asked whether the lack of IL-8R had any influence on the development of diarrhea after Salmonella infection. As shown in Figure 4B, on day 3 after infection there was a 30% increase in fecal water in both IL-8 KO and control mice.
compared with the respective uninfected controls that received only TMX and kanamycin. There was no significant difference between the 2 groups of mice in this regard. Marchelletta et al [18] have reported that in this model of S. Typhimurium colitis the increase in fecal water coincides with decreased expression of DRA by epithelial cells in the proximal colon and of βENaC in epithelial cells in the distal colon. We therefore studied the expression of these 2 ion transporters in infected IL-8R KO mice by measuring messenger RNA levels in the colon and protein expression by immunostaining of tissue. DRA and βENaC expression were equally suppressed by infection in IL-8R KO and control mice indicating that neutrophils or their products were not responsible for the down-regulation of these genes (Figure 5). Marchelletta et al [18] proposed elsewhere that these changes in expression and localization of epithelial cell transporters reflected increased proliferation of epithelial cells, even in parts of the colon that showed little or no histologic evidence of inflammation. In the current study, we again found that crypt length was increased in the proximal colon of infected mice, and that there was increased expression of the proliferation-related protein Ki-67 in the epithelium of the proximal colon (Figure 6), and these changes did not require IL-8R.

We then asked whether the IL-8R was necessary for PMN chemotaxis beyond the colon in response to Salmonella infection. We injected live S. Typhimurium intraperitoneally into mice and killed them 3 or 6 hours later. The percentage of PMNs in the peritoneal cavity of normal mice rose from <5% to 35% and then 65% after 3 and 6 hours, respectively. We then compared the number of PMNs obtained from various

Figure 3. Myeloperoxidase activity in cecal tissue 3 and 5 days after infection is reduced in interleukin 8R (IL-8R) knockout (KO) mice. Enzymatic activity was measured as described in “Methods” section and expressed per gram of tissue. Each symbol represents the enzymatic activity in the tissue from 1 mouse. CD2, BALB/c.D2Nramp1 congenic mice; IL-8R−/−, IL8 R KO; RFUs, relative fluorescent units; closed squares, data points from day 3 after infection; open squares, data points 5 days after infection. Results for the IL-8R KO mice were at the lower limit of detection for this assay.

Figure 4. A, Pathologic severity scores for the cecum and proximal colon 3 days after infection. Hematoxylin–eosin-stained sections of cecal tissue were scored with respect to edema in the submucosa, polymorphonuclear neutrophil (PMN) infiltration, reduction in the number of goblet cells, erosion/ulceration of the epithelial layer, desquamation of cells in the lumen, and blood in the lumen (scored as 1 or 0). Scores were plotted as stacked vertical bars with a maximum possible score of 26. Each bar represents 1 mouse, and the same mice were scored separately for cecal and proximal colon pathologic changes. B, Fecal water content after infection with Salmonella enterica serovar Typhimurium. Control values were determined by measuring fecal water in mice treated with TMX and kanamycin but not infected. Graph shows fecal water in infected mice starting on the day of infection (day 0) and daily for the next 72 hours after infection. Dotted line represents interleukin 8R knockout (IL-8R−/−) mice. On day 3 after infection there was a marked increase in fecal water in both groups of infected mice, with no significant difference between groups. CD2, BALB/c.D2Nramp1 congenic mice.
Figure 5. Infection reduced expression of DRA and βENaC. In panel panels A and B the changes in mRNA levels for DRA and βENaC after Salmonella infection are shown for both C.D2 control (wild-type) and IL-8R KO mice. After Salmonella infection, wild-type and IL-8R KO demonstrated a similar and significant decrease in both messages when compared to their respective uninfected controls (*, P < .05, ANOVA, Newman–Keuls post-hoc test). Arbitrary Units were assessed by the 2-DCT method. In panels C and D. we show representative immunofluorescent stains for DRA (C) and βENaC (D) in colonic tissue using confocal microscopy (both transporters are shown in green). Nuclei are shown in blue (20 ×, Bar = 10 mm). The proximal colon from uninfected wild-type and IL-8R KO show extensive apical DRA localization (arrow). DRA is substantially diminished and internalized in infected mice of both genotypes. βENaC is enriched in a subpopulation of cells (arrow) within the distal colon of uninfected wild-type and IL-8R KO mice. These numbers of cells expressing βENaC are diminished or absent in infected wild-type and IL8R KO distal colon. (20 ×, Bar = 20 mm). Abbreviations: βENaC, beta subunit of ENaC; DRA, down-regulated in adenoma; IL-8R KO, interleukin 8R knockout; WT, wild type.

Figure 6. Measures of epithelial proliferation in infected wild-type (WT) and interleukin 8R (IL-8R) knockout (KO) mice 3 days after infection. A, Crypt length in the proximal colons of both control (WT) and IL-8R KO mice increases after Salmonella infection compared with uninfected mice, (n = 3–4, mean from 5–6 fields/replicate; P < .05 [1-way analysis of variance]). B, Increase Ki-67-positive nuclei in both infected WT and IL-8R KO colon compared with uninfected controls (n = 2–3, mean of 5–6 fields/replicate; P < .01 [1-way analysis of variance]). Together, these results suggest that infection causes hyper-proliferation of epithelial cells.
mutant and WT mice 3 hours after infection, because we were interested in the earliest responses to infection. To determine which chemokine receptors played a role in PMN chemotaxis into the peritoneum, we infected FPR1 KO, Alox 5 KO mutants, and 2 C5-deficient inbred strains (A/J and DBA/2), and they all responded normally. In contrast, the numbers of PMNs in the peritoneal cavity of IL-8R KO mice was reduced by approximately 90% (Table 1). Thus, chemotaxis of PMNs in this model of *Salmonella* peritonitis is dependent on the IL-8R but not on other chemokine receptors.

**DISCUSSION**

In this article we show that in the first few days after *Salmonella* infection in kanamycin-pretreated mice diarrhea is induced independent of PMN infiltration into the colon. We compared the development of diarrhea in IL-8R KO mice and control BALB/c D2 N ramp1 mice, and 2 C5-deficient inbred strains (A/J and DBA/2), and they all responded normally. In contrast, the numbers of PMNs in the peritoneal cavity of IL-8R KO mice was reduced by approximately 90% (Table 1). Thus, chemotaxis of PMNs in this model of *Salmonella* peritonitis is dependent on the IL-8R but not on other chemokine receptors.

<table>
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<th>Mouse Strain</th>
<th>Mice, No.</th>
<th>PMNs, Mean (SEM), Log10 No.</th>
<th>Proportion of Control, %</th>
<th>P Value</th>
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<td>5.64 (0.08)</td>
<td>. . .</td>
<td>Control</td>
</tr>
<tr>
<td>ALOX 5 KO</td>
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<tr>
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<td>5.33 (0.30)</td>
<td>7.0</td>
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</tbody>
</table>

Abbreviations: IL-8R, interleukin 8R; KO, knockout; NA, not applicable; PMNs, polymorphonuclear neutrophils.

* C5-deficient mice.

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efficient chemotaxis of PMNs in the *Salmonella*-infected colon. KC and macrophage inflammatory protein 2 are synthesized in the cecum soon after infection in mice [45], but it is not known whether the chemokines are produced by epithelial cells, endothelial cells, or tissue macrophages, and the relative importance of each source could change during the course of infection. The fate of PMNs at epithelial surfaces is a special case, in that PMNs exit the body via the epithelium into the intestinal or bronchial lumens and this exit is due to apical epithelial cell secretion of another PMN chemoattractant, hepoxilin A3 [46]. Within the lumen, viable PMNs can ingest *Salmonella*, though it is unknown whether they reduce the bacteria burden [47]. It has been claimed that the movement of PMNs through and between epithelial cells is responsible for much of the pathologic changes induced in NTS colitis, but in our experiments the changes in the ceca of IL-8R KO mice were very similar to those in controls, except for the absence of PMNs in the former group.

We used a *Salmonella* peritonitis model to determine whether the IL-8R was also required for PMN chemotaxis outside the colon, and there was approximately 90% decrease in the influx of PMNs into the peritoneal cavity in IL-8R KO mice, whereas C5-deficient and leukotriene-deficient (Alox5 KO) mice responded normally. This was also the case for mice lacking the receptor for formylated peptides (FPR1 KO). This may be unique to mice, because human FPR1 recognizes formylated peptides made by both gram-negative and gram-positive bacteria, whereas the mouse receptor recognizes only peptides from gram-positive bacteria [48]. Thus, chemokines acting through CXCR2 are required for the PMN response to *Salmonella* infection. It was shown elsewhere that IL-8 was required for chemotaxis of PMNs across a monolayer of epithelial cell [49]. In conclusion, PMNs are important for host resistance to *S. Typhimurium* colitis, but they are not primarily responsible for the damage to the mucosa and submucosa that results from this infection, nor are they important for the alteration in DRA and ENaC expression that underlies the pathogenesis of diarrhea early in the course of infection while the epithelium of the colon is still intact. These findings challenge long-held assumptions about *Salmonella*-associated diarrhea and underscore the diverse mechanisms that result in diarrheal illness in response to gastrointestinal pathogens. Our results seem relevant to human infection, because *Salmonella* enteritis is usually a self-limited disease with early onset of watery diarrhea, so that knowledge of the mechanisms of abnormal ion transport physiology may provide new and effective therapeutic targets.

**Notes**

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