Title

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

Journal

The Journal of Infectious Diseases, 212(1)

ISSN

0022-1899

Authors

Marchelletta, Ronald R
Gareau, Melanie G
Okamoto, Sharon
et al.

Publication Date

2015-07-01

DOI

10.1093/infdis/jiu829

Peer reviewed

MAJOR ARTICLE

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

Ronald R. Marchelletta,[1] Melanie G. Gareau,[1] Sharon Okamoto,[2] Donald G. Guiney,[2] Kim E. Barrett,[1] and Joshua Fierer[2,3]

Divisions of [1]Gastroenterology, [2]Infectious Diseases, University of California, San Diego, School of Medicine, La Jolla, and [3]VA San Diego Healthcare System

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

Received 28 August 2014; accepted 11 December 2014; electronically published 23 December 2014.

Correspondence: Joshua Fierer, MD, FIDSA, VA San Diego Healthcare System, 3350 La Jolla Village Dr, San Diego, CA 92161 (jfierer@ucsd.edu).

The Journal of Infectious Diseases® 2015;212:128–36

DOI: 10.1093/infdis/jiu829

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 $Nramp1^{D169}$ mutant mice die of overwhelming systemic infection 2–3 days after infection. Marchelletta et al recently described the course of infection in BALB/c.$D2^{Nramp1}$ mice that are congenic for a segment of chromosome 1 that carries the wild-type (WT) copy of $Nramp1^{G169}$ 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^-/HCO_3^- 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 $Nramp1^{G169}$, 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.$D2^{Nramp1}$ 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-$Alox5^{tm1Fun}$ (arachidonate 5–lipoxygenase–deficient) [36] mice from the Jackson Laboratory. $FPR1^{tmGao}$ 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 *Slc11ac* 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 5×10^3 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 5×10^3 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 chnges 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

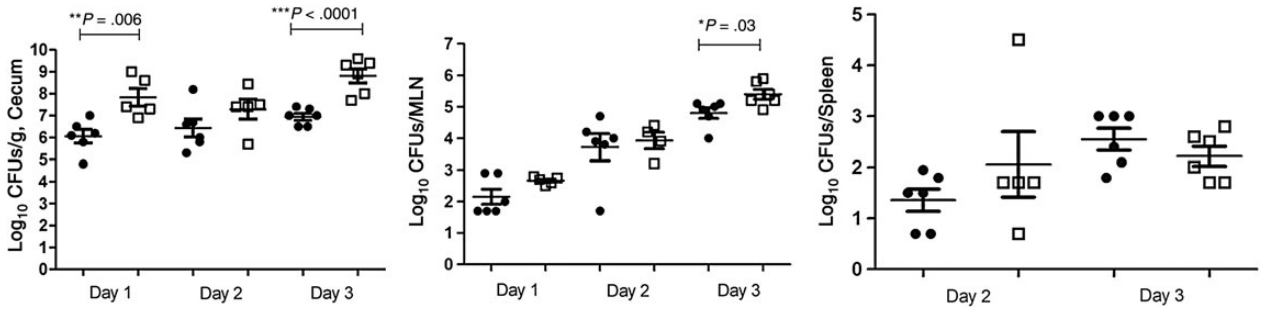

Figure 1. Interleukin 8R (IL-8R) knockout (KO) mice are more susceptible to *Salmonella enterica* serovar Typhimurium colitis. Groups of IL-8R KO and control mice were infected as described in "Methods" section and killed 1, 2, or 3 days after infection. Each symbol represents an individual mouse (closed 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.

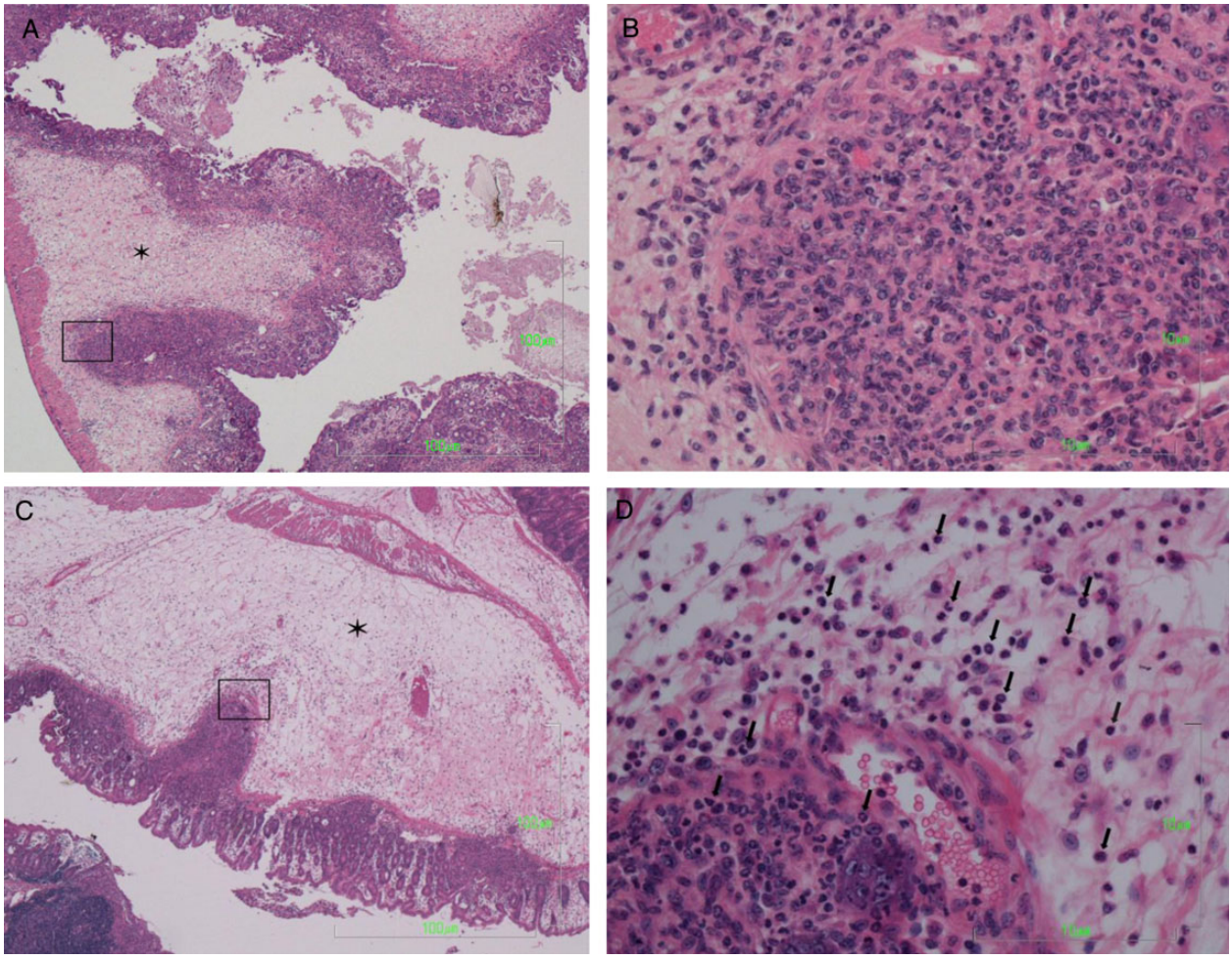

Figure 2. Cecal inflammation occurs independently of interleukin 8R (IL-8R). Hematoxylin–eosin-stained sections of the cecum are shown from representative mice, 3 days after infection. *A, B,* IL-8R knockout (KO) mice. Asterisk indicates area of submucosal edema; bar, 100 μm; area within box in *A* (×40) is shown at 10-fold higher magnification (×400) in *B.* The base of the crypts is heavily infiltrated with mononuclear cells, and few polymorphonuclear neutrophils (PMNs) are visible, if any. *C, D,* Sections from control BALB/c.D2 mice. Boxed area in *C* is shown at ×400 in *D,* with arrows pointing to some of the many PMNs that infiltrated the base of the crypts. PMNs can be seen exiting a venule.

IL-8R KO mice than in controls until day 3 after infection. 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 4*A*). 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 4*B,* on day 3 after infection there was a 30% increase in fecal water in both IL-8 KO and control mice,

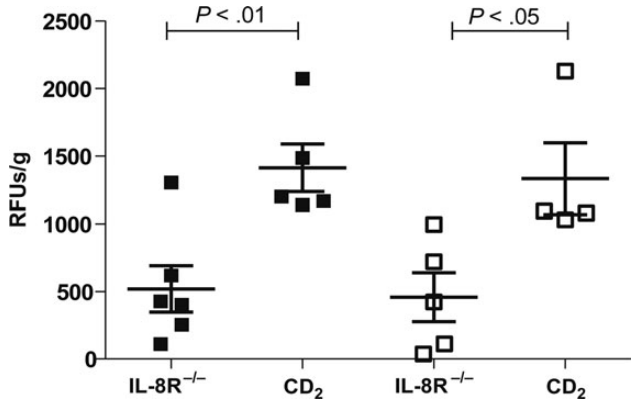

Figure 3. Myeloperoxidase activity in cecal tissue 3 and 5 days after infection is reduced in interleukin 8R (IL-8R) knockout (KO) mice. Enzymatic activity was measured as described in "Methods" section and expressed per gram of tissue. Each symbol represents the enzymatic activity in the tissue from 1 mouse. CD_2, BALB/c.D2^{Nramp1} congenic mice; IL-8R$^{-/-}$, IL-8 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.

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

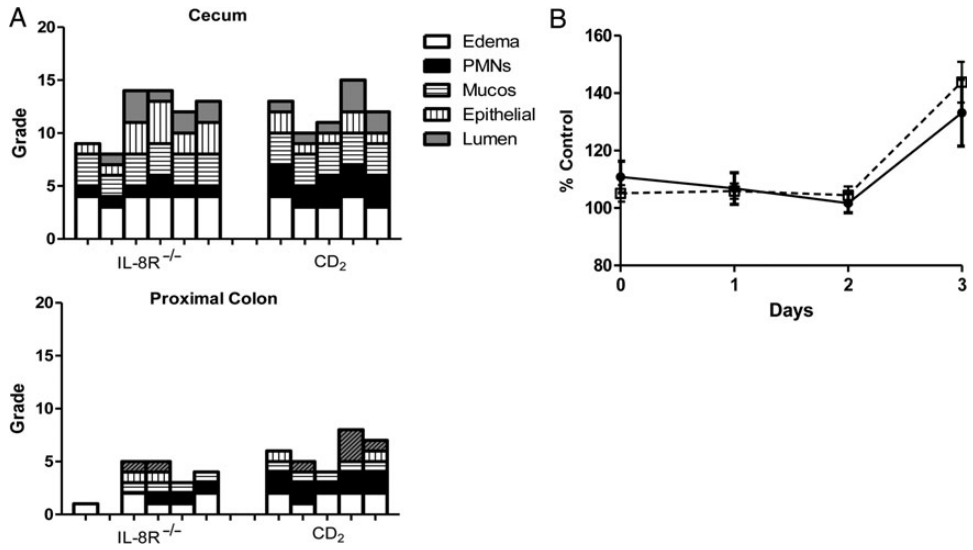

Figure 4. *A*, Pathologic severity scores for the cecum and proximal colon 3 days after infection. Hematoxylin–eosin-stained sections of cecal tissue were scored with respect to edema in the submucosa, polymorphonuclear neutrophil (PMN) infiltration, reduction in the number of goblet cells, erosion/ulceration 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. CD_2, BALB/c.D2^{Nramp1} congenic mice.

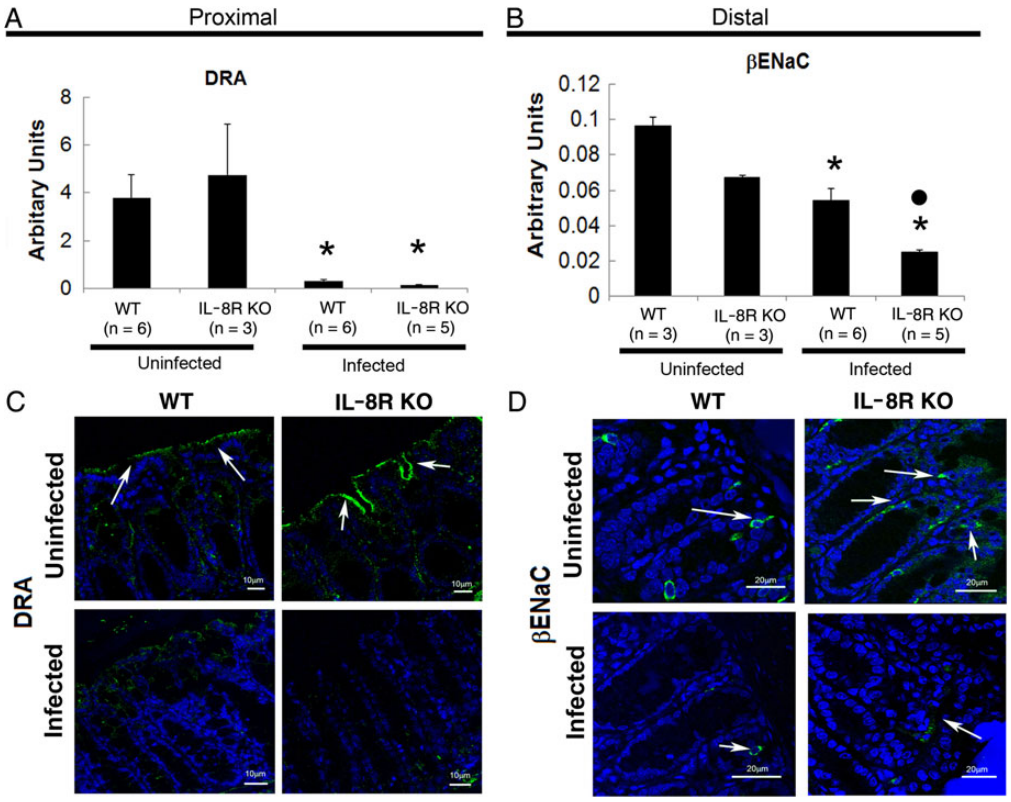

Figure 5. Infection reduced expression of DRA and βENaC. In panel panels A and B the changes in mRNA levels for DRA and βEnaC after *Salmonella* infection are shown for both C.D2 control (wild-type) and IL-8R KO mice. After Salmonella infection, wild-type and IL- 8R KO demonstrated a similar and significant 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 IL8rKO 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.

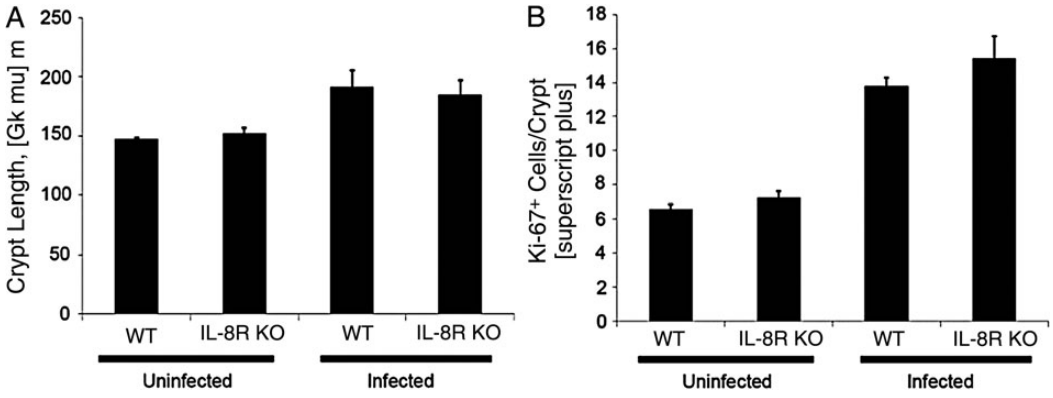

Figure 6. Measures of epithelial proliferation in infected wild-type (WT) and interleukin 8R (IL-8R) knockout (KO) mice 3 days after infection. *A*, Crypt length in the proximal colons of both control (WT) and IL-8R KO mice increases after *Salmonella* infection compared with uninfected mice, (n = 3–4, 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 hyperproliferation of epithelial cells.

Table 1. Influx of PMNs Into the Peritoneal Cavity of Different Strains of Inbred Mice 3 Hours After Injection of *Salmonella enterica* serovar Typhimurium

Mouse Strain	Mice, No.	PMNs, Mean (SEM), Log_{10} No.	Proportion of Control, %	*P* Value
C57BL/6	16	5.64 (0.08)	. . .	Control
ALOX 5 KO	7	5.53 (0.12)	78.0	>.05
FPR1 KO	3	5.48 (0.29)	78.0	>.05
DBA2[a]	5	6.08 (0.07)	270[b]	NA
A/J[a]	5	5.84 (0.12)	150[b]	NA
IL-8R$^{-/+}$	4	6.48 (0.13)	. . .	Control
IL-8R KO	4	5.33 (0.30)	7.0	<.01

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

[a] C5-deficient mice.

[b] Compared to C57BL/6.

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^{Nramp1}; IL-8R KO and control mice had comparably severe diarrhea 3 days after infection, as assessed by fecal water content (Figure 4*B*). As we expected, mice lacking IL-8R had a marked decrease in PMNs in the hematoxylin-eosin–stained sections of their infected colons (Figure 2). This was confirmed by measuring myeloperoxidase in the cecum

It has been shown elsewhere that diarrhea occurs in BALB/c.D2^{Nramp1} mice on the third day after infection, when there is only minimal mucosal damage distal to the cecum [15, 18], and this observation was confirmed in our current experiments. At this time point, we also detected major changes in the expression (transcription and translation) of 2 important colonic ion transport proteins, DRA and βENaC [18]. Here we showed that these changes in transporter expression also occur in IL-8R KO mice. The changes in DRA and βENaC expression, and increased epithelial cells proliferation, were detected in parts of the colon with only minimal evidence of inflammation, further evidence that these changes were not a response to damage caused by the PMNs. The signals responsible for altered gene expression may be due to the release of host mediators made by other cells. Interestingly, the dramatic pathologic changes in the cecum, such as massive submucosal edema and epithelial disruption, were observed to a comparable extent in IL-8R KO and control mice, even in the absence of PMN infiltration in the former case (Figure 2). This was true even though the IL-8R KO mice had increased CFU counts in the colon and mesenteric nodes after infection (Figure 1).

The role of PMNs in contributing to or defending against infectious diarrhea has been postulated but not been extensively studied. Giannella [32] used a rabbit ligated loop assay to show that rabbits made neutropenic with nitrogen mustard had less fluid accumulation after *Salmonella* infection. Spehlmann et al [41] reported that IL-8R KO mice have more severe diarrhea than WT mice after *Citrobacter rodentium* infection, that infection is more severe in IL-8R KO mice, and that they have more decrements in expression of normal electrolyte transporters, including cystic fibrosis transmembrane conductance regulator, and DRA. *Citrobacter rodentium* causes attaching and effacing lesions in the colon, so it is logical that more organisms would cause greater disturbances in epithelial function. In our experiments *S.* Typhimurium infection was more severe in IL-8R KO mice, as judged by the higher numbers of bacteria in the stool (not shown) and in the cecal wall after infection. Nevertheless, the increased bacterial load did not affect the severity of the diarrhea. We therefore conclude that PMNs play different roles in *C. rodentium* versus *Salmonella* colitis and that the pathophysiologic changes underlying these related disease states are accordingly of interest.

Neutrophils play a complex role in *Salmonella* pathogenesis. One of the earliest responses to *S. enterica* infection is an influx of inflammatory cells, predominantly PMNs and macrophages [42], and PMNs are present in the submucosa and crypts of humans with NTS colitis [8]. Unlike tissue macrophages, PMNs are bactericidal for complement-opsonized *S. enterica* [22]; they are prominent in the cecum after *Salmonella* infection. Recently Winter et al [43] showed that *S. enterica* benefit from inflammation because the reactive oxygen species made by PMNs in the inflamed intestine converts thiosulfate to tetrathionate that *Salmonella* can use as a terminal electron receptor, allowing them to respire under anaerobic conditions in the gut. This group also showed that PMNs are a major source of interferon γ in the intestinal wall in the first few days after infection, implying an indirect role for PMNs in controlling that infection [44]. This could be part of the explanation for our finding of enhanced growth of *Salmonella* in IL-8R KO mice.

This study shows that the IL-8R, and presumably CXC chemokines that are ligands for the receptor (KC, macrophage inflammatory protein 2, and LIX in mice), are required for

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 the bronchial lumens and this exit is due to apical epithelial cell secretion of another PMN chemoattractant, hepoxilin A_3 [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

Financial support. This work was supported by NIAID (grant 1 R01 AI077661-01A1 to D. G. G., K. E. B, and J. F.), the University of California, San Diego, Digestive Diseases Research and Development Center, the National Institutes of Health (grant DK080506 to K. E. B. and grant T32 DK07202 to R. R. M.) the Crohn's and Colitis Foundation of America (career development award to R. R. M.), and the US Department of Veterans Affairs (Merit Review grant to J. F.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Jackson BR, Griffin PM, Cole D, Walsh KA, Chai SJ. Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998–2008. Emerg Infect Dis **2013**; 19:1239–44.
2. Le Hello S, Hendriksen RS, Doublet B, et al. International spread of an epidemic population of *Salmonella* enterica serotype Kentucky ST198 resistant to ciprofloxacin. J Infect Dis **2011**; 204:675–84.
3. Barbut F, Beaugerie L, Delas N, et al. Comparative value of colonic biopsy and intraluminal fluid culture for diagnosis of bacterial acute colitis in immunocompetent patients. Clin Infect Dis **1999**; 29:356–60.
4. Chaudhuri A, Bekdash BK. Toxic megacolon due to *Salmonella*: a case report and review of the literature. Int J Colorectal Dis **2002**; 17:275–9.
5. Mandal BK, Mani V. Colonic Involvement in salmonellosis. Lancet **1976**; 307:887–8.
6. Behravesh CB, Jones TF, Vugia DJ, et al. Deaths associated with bacterial pathogens transmitted commonly through food: foodborne diseases active surveillance network (FoodNet), 1996–2005. J Infect Dis **2011**; 204:263–7.
7. Goldberg MB, Rubin RH. The spectrum of *Salmonella* infection. Infect Dis Clin North Am **1988**; 2:571–98.
8. Day DW, Mandal BK, Morson BC. The rectal biopsy appearances in *Salmonella colitis*. Histopathology **1978**; 2:117–31.
9. Galyov EE, Wood MW, Rosqvist R, et al. A secreted protein of *Salmonella dublin* is translocated into eukaryotic cells and mediates inflammation and fluid secretion in infected ileal mucosa. Mol Microbiol **1997**; 25:903–12.
10. Tsolis RM, Adams LG, Ficht TA, Bäumler AJ. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect Immun **1999**; 67:4879–85.
11. Santos RL, Tsolis RM, Zhang S, Ficht TA, Bäumler AJ, Adams LG. *Salmonella*-induced cell death is not required for enteritis in calves. Infect Immun **2001**; 69:4610–7.
12. Santos RL, Tsolis RM, Baumler AJ, Adams LG. Pathogenesis of *Salmonella*-induced enteritis. Braz J Med Biol Res **2003**; 36:3–12.
13. Stecher B, Macpherson AJ, Hapfelmeier S, Kremer M, Stallmach T, Hardt W-D. Comparison of *Salmonella enterica* serovar Typhimurium colitis in germfree mice and mice pretreated with streptomycin. Infect Immun **2005**; 73:3228–41.
14. Barthel M, Hapfelmeier S, Quintanilla-Martínez L, et al. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun **2003**; 71:2839–58.
15. Woo H, Okamoto S, Guiney DG, Gunn JS, Fierer J. A model of *Salmonella* colitis with features of diarrhea in *SLC11A1* wild-type mice. PLOS One **2008**; 3:e1603.
16. Hapfelmeier S, Hardt W-D. A mouse model for *S.* Typhimurium-induced enterocolitis. Trends Microbiol **2005**; 13:497–503.
17. Fierer J, Okamoto S, Banerjee A, Guiney DG. Diarrhea and colitis in mice require the Salmonella pathogenicity island 2-encoded secretion function but not SifA or Spv effectors. Infect Immun **2012**; 80: 3360–70.
18. Marchelletta RR, Gareau MG, McCole DF, et al. Altered expression and localization of ion transporters contribute to diarrhea in mice with *Salmonella*-induced enteritis. Gastroenterology **2013**; 145:1358–68.
19. Schweinfest CW, Spyropoulos DD, Henderson KW, et al. *slc26a3 (dra)*-Deficient mice display chloride-losing diarrhea, enhanced colonic proliferation, and distinct up-regulation of ion transporters in the colon. J Biol Chem **2006**; 281:37962–71.

20. Fields PI, Swanson RV, Haidaris CG, Heffron F. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A **1986**; 83:5189–93.
21. Conlan JW. Critical roles of neutrophils in host defense against experimental systemic infections of mice by *Listeria monocytogenes*, *Salmonella typhimurium*, and *Yersinia enterocolitica*. Infect Immun **1997**; 65:630–5.
22. Fierer J. Polymorphonuclear leukocytes and innate immunity to *Salmonella* infections in mice. Microbes Infect **2001**; 3:1233–7.
23. Fierer J, Swancutt MA, Heumann D, Golenbock D. The role of lipopolysaccharide binding protein in resistance to *Salmonella* infections in mice. J Immunol **2002**; 168:6396–403.
24. Takeuchi A, Sprinz H. Electron-microscope studies of experimental *Salmonella* infection in the preconditioned guinea pig. Am J Pathol **1967**; 51:137–61.
25. Santos RL, Zhang S, Tsolis RM, Bäumler AJ, Adams LG. Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. Vet Pathol **2002**; 39:200–15.
26. Santos RL, Almeida AP, Xavier MN, et al. Enteric pathology and *Salmonella*-induced cell death in healthy and SIV-infected rhesus macaques. Vet Pathol **2011**; 48:933–41.
27. Eckmann L, Kagnoff MF, Fierer J. Intestinal epithelial cells as watchdogs for the natural immune system. Trends Microbiol **1995**; 3: 118–20.
28. Eckmann L, Kagnoff M, Fierer J. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun **1993**; 61:4569–74.
29. Tükel C, Raffatellu M, Chessa D, Wilson RP, Akçelik M, Bäumler AJ. Neutrophil influx during non-typhoidal salmonellosis: who is in the driver's seat? FEMS Immunol Med Microbiol **2006**; 46:320–9.
30. Bazzoni F, Cassatella MA, Rossi F, Ceska M, Dewald B, Baggiolini M. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. J Exp Med **1991**; 173: 771–4.
31. Mrsny RJ, Gewirtz AT, Siccardi D, et al. Identification of hepoxilin A3 in inflammatory events: a required role in neutrophil migration across intestinal epithelia. Proc Natl Acad Sci U S A **2004**; 101:7421–6.
32. Giannella RA. Importance of the intestinal inflammatory reaction in *Salmonella*-mediated intestinal secretion. Infect Immun **1979**; 23: 140–5.
33. Patel S, McCormick BA. Mucosal inflammatory response to *Salmonella typhimurium* infection. Front Immunol **2014**; 5:1–10.
34. Cacalano G, Lee J, Kikly K, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. Science **1994**; 265:682–4.
35. Weintraub BC, Eckmann L, Okamoto S, Hense M, Hedrick SM, Fierer J. Role of alphabeta and gamma delta T cells in the host response to *Salmonella* infection as demonstrated in T-cell-receptor-deficient mice of defined *Ity* genotypes. Infect Immun **1997**; 65:2306–12.
36. Chen X-S, Sheller JR, Johnson EN, Funk CD. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature **1994**; 372:179–82.
37. Gao J-L, Lee EJ, Murphy PM. Impaired antibacterial host defense in mice lacking the *N*-formylpeptide receptor. J Exp Med **1999**; 189: 657–62.
38. Lesnick ML, Reiner NE, Fierer J, Guiney DG. The *Salmonella spvB* virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. Mol Microbiol **2001**; 39:1464–70.
39. Uneyama C, Shibutani M, Masutomi N, Takagi H, Hirose M. Methacarn fixation for genomic DNA analysis in microdissected, paraffin-embedded tissue specimens. J Histochem Cytochem **2002**; 50: 1237–54.
40. Coburn B, Li Y, Owen D, Vallance BA, Finlay BB. *Salmonella enterica* serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. Infect Immun **2005**; 73:3219–27.
41. Spehlmann ME, Dann SM, Hruz P, Hanson E, McCole DF, Eckmann L. CXCR2-dependent mucosal neutrophil influx protects against colitis-associated diarrhea caused by an attaching/effacing lesion-forming bacterial pathogen. J Immunol **2009**; 183:3332–43.
42. Wick MJ. Living in the danger zone: innate immunity to *Salmonella*. Curr Opin Microbiol **2004**; 7:51–7.
43. Winter SE, Thiennimitr P, Winter MG, et al. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. Nature **2010**; 467:426–9.
44. Spees AM, Kingsbury DD, Wangdi T, Xavier MN, Tsolis RM, Baumler AJ. Neutrophils are a source of IFN-χ during acute *Salmonella enterica* serovar Typhimurium colitis. Infect Immun **2014**; 82:1692–8.
45. Rivera-Chavez F, Winter SE, Lopez CA, et al. *Salmonella* uses energy taxis to benefit from intestinal inflammation. PLOS Pathog **2012**; 9: e1003267.
46. Szabady RL, McCormick BA. Control of neutrophil inflammation at mucosal surfaces by secreted epithelial products. Front Immunol **2013**; 4:1–6.
47. Loetscher Y, Wieser A, Lengfeld J, et al. *Salmonella* transiently reside in luminal neutrophils in the inflamed gut. PLOS One **2012**; 7:e34812.
48. Southgate EL, He RL, Gao J-L, Murphy PM, Nanamori M, Ye RD. Identification of formyl peptides from *Listeria monocytogenes* and *Staphylococcus aureus* as potent chemoattractants for mouse neutrophils. J Immunol **2008**; 181:1429–537.
49. McCormick BA, Miller SI, Carnes D, Madara JL. Transepithelial signaling to neutrophils by salmonellae: a novel virulence mechanism for gastroenteritis. Infect Immun **1995**; 63:2302–9.