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Role of neutrophils and macrophages in the pathogenesis of necrotizing enterocolitis caused by *Cronobacter sakazakii*

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Abstract

Background—*Cronobacter sakazakii* (CS) is a highly virulent gram-negative opportunistic pathogen that has been implicated in clinical outbreaks of necrotizing enterocolitis (NEC). The role of mucosal immune cells in CS infection is not well understood. In this study, we sought to elucidate the role of neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages in the pathogenesis of NEC induced by CS using a novel newborn mouse model.

Materials and Methods—PMNs and macrophages were depleted in newborn mice using Gr-1 antibody and carrageenan respectively, and then infected with 10³ CFU of CS. The development of NEC in these mice was assessed by a pathologist based on the morphological changes in the intestine. Cytokine production was determined in the serum and intestinal homogenates of infected mice by ELISA. Inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production was determined by flow cytometry and Greiss method, respectively.

Results—Depletion of PMNs and macrophages in newborn mice led to increased recruitment of dendritic cells (DCs) in the intestine compared to wild-type mice upon infection with CS. PMN- and macrophage-depleted mice showed increased bacterial load, production of pro-inflammatory cytokines, iNOS expression and NO production in the intestines in comparison to wild-type mice fed with CS. In addition, depletion of PMNs and macrophages prior to infection in mice resulted in severe inflammation, villus destruction and enhanced enterocyte apoptosis in the intestines compared to CS-infected wild-type mice.

Conclusions—Our data suggest that depletion of PMNs and macrophages from the lamina propria (LP) exacerbates experimental NEC, indicating that both of these immunocytes play an important role in the clearance of CS during the initial stages of infection. The increased mucosal

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cytokine response and NO production in the absence of these immunocytes may be responsible for the observed increase in mucosal injury. Understanding how CS manipulates these cells employing novel mouse model of NEC reported in this study will provide significant insights for the development of novel therapeutic and preventive strategies to combat NEC.

Keywords

Cronobacter sakazakii; Macrophages; PMNs; Dendritic cells; Nitric Oxide; Necrotizing Enterocolitis

Introduction

Necrotizing enterocolitis (NEC) is the most common life-threatening gastrointestinal surgical emergency encountered in premature infants [1, 2]. Despite recent advances in neonatal medicine, the morbidity and mortality from NEC have remained largely unchanged [3, 4]. In fact, current trends suggest that NEC may soon become the leading cause of mortality for premature infants in the United States. Although multiple risk factors have been implicated in the pathogenesis of NEC, the exact etiology of the disease remains undefined [5, 6]. The only consistent epidemiologic precursors of NEC are prematurity, bacterial colonization and enteral alimentation [7, 8]. Bacterial colonization of the immature gut and the resultant bacterial-epithelial interactions appear to play a leading role in the activation of the mucosal immune system that is characteristic of NEC [9-12]. Understanding the role of bacterial infection and how it contributes to disease susceptibility is important for developing novel strategies for the prevention and treatment of NEC.

Cronobacter sakazakii (CS), formerly known as *Enterobacter sakazakii*, is a virulent gram-negative pathogen that has been implicated in clinical outbreaks of NEC in premature infants [13, 14]. Powdered infant formula (PIF) contaminated with CS has been implicated as a putative source of these outbreaks. CS-induced NEC results in a mortality rate of 40-100% [15]. CS has been designated by the International Commission for Microbiological Specifications for Foods (ICMSF) as a severe hazard for restricted populations, associated with life-threatening chronic sequelae. We have previously demonstrated that CS suppresses the maturation of dendritic cells (DCs) by down-regulating the expression CD40, CD86 and MHC class II antigen [16]. In addition, CS utilizes DCs as a vehicle for propagation and survival, hence evading potential immune surveillance [16].

Neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages play an important role in the clearance of infections [17-21]. Despite the fundamental function of PMNs and macrophages in innate immunity, the role of these immune cells in CS-induced NEC *in vivo* is not known. Here, we report that the oral feeding of CS results in acute intestinal inflammation and death in newborn mouse pups. We also demonstrate that the presence and recruitment of PMNs and macrophages to the lamina propria (LP) is important for clearance of the bacteria during the initial stages of infection. Furthermore, their absence exacerbates mucosal injury by increasing the levels of pro-inflammatory cytokines and iNOS expression.

Materials and Methods

Bacterial strain

C. sakazakii (strain 51329) was obtained from American Type Culture Collection. CS was transformed with a GFP containing plasmid as previously described [22]. The resulting colonies were selected for ampicillin resistance. For experiments, the bacteria were grown in Luria Broth containing ampicillin (50 µg/ml).

Animal experiments

The animal studies were approved by the institutional animal care and use committee (IACUC) of the Saban Research Institute at Children's Hospital Los Angeles and followed National Institutes of Health guidelines for the performance of animal experiments. Time pregnant C57BL/6 mice were obtained from Charles River at E18. After delivery, the pups were kept with the mothers and housed at CHLA. Three day old pups were fed 10^3 CFU of CS in 10 μ l of sterile PBS orally and left with the mother. The control animals received sterile PBS at day 3. Blood was collected from the tail or facial vein at different time points post-infection. Dilutions were made and plated on ampicillin LB agar plates to determine the bacteremia levels and success of infection. Mice were perfused with 0.9% saline intracardiacally at 24, 48 and 72 h post-infection to remove blood and contaminating intravascular leukocytes. Intestines were aseptically removed, weighed and homogenized in sterile PBS. Bacterial counts were determined by plating ten-fold serial dilutions of intestinal homogenates on ampicillin LB agar plates. The results were obtained from six independent experiments with 15 animals per group.

PMN and macrophage depletion

For PMN and macrophage depletion, mice received 6 injections of Gr-1 antibody (BD Biosciences, San Diego, CA) and carrageenan (Sigma, MO), respectively in sterile PBS (dose) (50 μ l) by intra-peritoneal injections as described previously [23-25]. Gr-1 antibody has been shown to selectively bind to and deplete PMNs [23, 24]. Carrageenan is a sulfated polysaccharide extracted from cell walls of certain algae of the Rhodophyta. Due to its unique structure, carrageenan is ingested by macrophages but not by other immunocytes that are not actively phagocytic and lack well developed lysosomal complex. Once ingested by macrophages, carrageenan causes the lysis of phagolysosomes and the release of hydrolytic enzymes leading to bursting of macrophages. Therefore carrageenan is widely used to deplete macrophages in animals to study their role during infection [25]. Mice received three injections of Gr-1 antibody or carrageenan 6 h apart on Day 1; two injections of Gr-1 antibody or carrageenan 6 h apart on Day 2 and final injection of Gr-1 antibody or carrageenan on Day 3 prior to infection with CS. Depletion was confirmed using flow cytometry for Gr-1 (specific to PMNs) and F4/80 (specific to macrophages) expression in liver, spleen and the intestinal homogenized tissue. The results were obtained from five independent experiments with 12 animals per group.

Histopathological examination

Intestine, spleen and liver specimens were collected at 24 h, 48 h and 72 h post-infection from at least 12 mice, fixed in formalin, 3-5 micron sections cut with microtome and stained with H&E [22]. At least 10 sections from each organ were examined for morphological changes by a pathologist. In addition, intestinal sections were graded microscopically by a pathologist blinded to groups, from grade 0 (normal) to grade 4 (severe), based on pathological manifestations including submucosal edema, villus core edema, epithelial sloughing/obliteration, neutrophil infiltration, intestinal perforation, and necrosis [22].

Flow Cytometry

Animals were sacrificed at 24, 48 and 72 h post-infection, the intestines were dissected, Peyer's patches removed, the specimen placed in RPMI medium and prepared for percoll gradient. The epithelial and lamina propria (LP) layers were isolated on the percoll gradient as previously described [26]. Percentage of different immunocytes recruited to the lamina propria (LP) layer was determined by staining with fluorochrome coupled primary antibodies. DCs were identified by CD11c staining (Abcam, Cambridge, MA), PMNs by Gr-1 antibody and macrophages by F4/80 antibody. Mouse IgG isotype matched antibodies

was used as control. Cells were analyzed by four-color flow cytometry using FACS Calibur analyzer (Becton Dickinson, San Diego, CA) and cell Quest Pro software (BD Biosciences, San Diego, CA) and at least 10000 events were collected for analysis. Enterocytes were isolated from the intestines of mice by flow cytometry based on E-cadherin staining.

Measurement of NO Production as nitrite

To determine NO production in intestines of mice, equal weight of the intestine harvested from infected mice was homogenized in sterile PBS (1 ml); centrifuged; supernatants were collected, and analyzed for NO production by the modified Greiss method as described [27,28]. Briefly, nitrate was converted to nitrites with β -nicotinamide adenine dinucleotide phosphate (NADPH; 1.25 mg/ml) and nitrate reductase followed by addition of Greiss reagent. The reaction mixture was incubated at room temperature for 20 minutes followed by addition of trichloroacetic acid (TCA). Samples were centrifuged, clear supernatants were collected, and optical density was recorded at 550 nm. The amount of NO produced was determined by calibrating a standard curve using sodium nitrite.

Detection of iNOS expression

The expression of iNOS was examined in the intestines of mice by flow cytometry. Intestines were harvested from mice after 72 h post-infection, transferred to ice-cold Hanks buffer/ 3% fetal calf serum, homogenized using a glass potter, and passed through a stainless-steel sieve. The dissociated sample was collected by centrifugation and digested for 60 minutes at 37°C with 1.4 ml of 0.75% (w/v) with type II collagenase (0.95 unit/mg, Sigma) and 104 units of DNase I (Sigma) in dissociation buffer (42 mmol/L MgCl₂/23 mmol/L CaCl₂/50 mmol/L KCl/153 mmol/L NaCl). The digested sample was pelleted, resuspended in PBS and layered on percoll gradient to obtain the epithelial layer as described earlier. The epithelial cells from the epithelial layer were double stained for E-cadherin (Cell signaling) and iNOS (BD Biosciences). The cells were first pre-incubated for 30 minutes with IgG blocking buffer to mask nonspecific binding sites, fixed with 2% paraformaldehyde, and permeabilized using BD cytofix and cytoperm kit. Cells were then incubated with E-cadherin or iNOS, or an isotype matched antibody for 30 minutes at 4°C and then washed with BD perm wash buffer. Fluorochrome-conjugated secondary antibodies were then added, incubated for 30 minutes at 4°C, and washed with permwash buffer. The stained cells were then analyzed by flow cytometry using FACS Calibur analyzer (Becton Dickinson, San Diego, CA) and Cell Quest Pro software (BD Biosciences, San Diego, CA), and at least 10,000 events were collected for analysis. Results are expressed as mean fluorescence intensity subtracted from isotype matched control.

Determination of apoptosis

Apoptosis of enterocytes isolated from the intestines of WT, PMN-depleted and macrophage-depleted CS infected mice was assessed by TUNEL staining using ApoTag kit (Chemicon), Annexin-V and 7AAD staining (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Cytokine measurement

Biosource ELISA kits (Invitrogen, Carlsbad, CA) were used to measure cytokine production in the serum and intestinal homogenates of mice. The animal tissues were homogenized immediately post mortem or after euthanization in case of control animals. The intestinal weight was measured prior to homogenization. Equal weights of intestinal tissue were homogenized in PBS, centrifuged, clear supernatants were collected, and used for ELISA as per manufacturer's instructions.

Statistical analysis

Wilcoxon signed rank test, ANOVA and Fisher's exact test are used to determine the statistical significance. P values <0.05 was considered to be statistically significant.

Results

CS infection results in acute intestinal inflammation and enterocolitis in a novel newborn mouse model

To study the pathogenesis of a disease, availability of an acceptable animal model which closely mimics human clinical conditions is of utmost importance. Therefore we set out to establish a mouse model of NEC by feeding 3 day old pups with 10^3 CFU of CS orally which is a natural route of entry of this bacteria in humans. In order to investigate the natural progression of CS infection in this mouse model, we followed clinical signs as well as histological manifestation of NEC in a time-dependent manner. Mortality was also used as a measure of disease severity. The animals began showing clinical signs of disease such as lethargy and decreased activity within 24 h post-infection. Oral infection of mice with CS resulted in 100% mortality by 96 h post-infection (Fig. 1A). The bacterial load was examined in the blood of mice at different post-infection time periods. The bacterial load in the blood increased from log 1.0 CFU ml⁻¹ at 24 h to log 5.0 CFU ml⁻¹ by 72 h post-infection (Fig. 1 B). There was an increase in the bacterial load in the intestine from log 3.0 CFU ml⁻¹ at 24 h to log 6.0 CFU ml⁻¹ by 72 h post-infection (Fig. 1C). Influx of immune cells (DCs, PMNs and macrophages) was significantly higher in the intestine of infected mice starting at 24 h post-infection compared to control saline fed uninfected mice (Fig 1D; *p<0.001). However, CS infected mice recruited significantly greater number of DCs compared to both PMNs and macrophages (p<0.001, DCs vs PMNs influx or DCs vs macrophage recruitment, by ANOVA and Fisher's exact test). To evaluate disease progression, histopathological examination of intestinal tissue from infected mice was conducted at various time points post-infection (Fig. 2A). Intestinal tissue from CS-infected pups showed dilated and necrotic intestine with perforation and destruction of villi by 72 h post-infection. The average pathological scores increased from 1.71 at 24 h post-infection to 2.8 by 72 h post-infection (Fig 2A-B). Morphological examination of other organs such as liver and spleen showed an acute inflammatory response characterized by the presence of many PMNs (and fewer monocytes) in response to CS infection, but in contrast to the intestine, tissue damage was less frequent (Fig. 3).

Depletion of PMNs or macrophages exacerbates CS infection

To elucidate the role of PMNs and macrophages in CS-induced NEC, we used Gr-1 antibody and carrageenan to deplete PMNs and macrophages, respectively as described previously [23-25]. The depletion of PMNs and macrophages was confirmed by flow cytometry prior to infection with CS which revealed >95% depletion of PMNs or macrophages (Fig. 4A and B). PMN-depleted mice demonstrated clinical signs of infection by 12-18 h following CS challenge and succumbed to the infection within 48 h (Fig. 4C). A similar trend was observed in macrophage-depleted mice upon CS infection (Fig. 4C). The intestinal bacterial load was significantly higher in PMN- and macrophage-depleted mice challenged with CS compared to WT animals (Fig. 4D, p<0.05 WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test). Histopathological examination of intestines from PMN- and macrophage-depleted mice revealed severe inflammation, extensive villus destruction and hemorrhage compared to WT mice (Fig. 5A). Consistent with these findings pathological scores for the intestine were significantly higher in PMN- and macrophage-depleted mice compared to WT animals; PMN- and macrophage-depleted mice had an average score of 3.8 and 3.9 respectively compared to 2.9 for WT mice (Fig. 5B; p<0.05 WT vs PMN-depleted or WT vs macrophage-depleted by Wilcoxon signed rank

test). Taken together these results suggest that PMNs and macrophages play an important role in the initial clearance of bacteria.

Our earlier studies demonstrated that CS suppresses the maturation of dendritic cells and exploits these immune cells as a replication-permissive niche to multiply in order to cause infection (16). Therefore, we hypothesized that depletion of PMNs and macrophages may increase the recruitment of DCs in the intestines leading to higher bacterial binding to the gut. To test this hypothesis, we examined the cellular infiltrate in the intestines of PMN- and macrophage-depleted mice by flow cytometry. Interestingly, 55% of the cells from PMN-depleted and 58% of the cells from macrophage-depleted mice were CD11c+ DCs, compared to only 29% of the cells in WT mice ($p < 0.01$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test) (Fig. 5C). These results suggest that decreased numbers of PMNs and macrophages may predispose to CS-induced NEC by recruiting greater numbers of DCs, which permit bacterial multiplication and hence, promote infection.

Depletion of PMNs and macrophages enhances inflammatory cytokine production in mice fed with CS

High levels of proinflammatory cytokines have been implicated in the pathogenesis of intestinal damage in NEC. Therefore, we investigated the cytokine profile in the plasma and intestinal homogenates of infected mice. Plasma levels of TNF- α , IL-1 β , IL-12 and IL-6 in PMN- and macrophage-depleted animals were increased in a time-dependent manner post-infection (Fig 6A-D). Although a similar pattern of cytokine production was observed in WT mice, the levels of these cytokines were significantly lower compared to PMN- and macrophage-depleted mice ($p < 0.05$ WT vs PMN-depleted or $p < 0.01$ WT vs macrophage-depleted by ANOVA and Fisher's exact test). Similarly, a significant increase in the levels of these cytokines was detected in the intestines of CS-infected PMN- and macrophage-depleted mice compared with WT animals at 48 h post-infection ($p < 0.05$ or $p < 0.01$ WT vs PMN-depleted or $p < 0.001$ WT vs macrophage-depleted by ANOVA and Fisher's exact test) (Fig. 7).

Increased expression of iNOS in the intestines of PMN- and macrophage-depleted mice upon CS infection

We have previously demonstrated that the expression of iNOS in the intestinal mucosa plays an important role in the pathogenesis of NEC (29). Therefore, we measured the levels of epithelial iNOS expression in response to CS infection using double staining for the epithelial marker E-cadherin, and iNOS. As expected, mucosal iNOS expression rises with progression of infection and peaks by 48 h post-infection (Fig. 8A). In both PMN- and macrophage-depleted mice, the levels of intestinal iNOS were significantly higher than WT animals ($p < 0.001$ WT vs PMN- depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test). On par with these findings, PMN- or macrophage-depleted mice showed greater levels of NO in the intestines compared to WT animals ($p < 0.001$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test) (Fig. 8B). Taken together, these results suggest that depletion of PMNs and macrophages leads to higher iNOS expression and hence, increased NO production, by intestinal epithelial cells in response to CS infection.

CS infected PMN- and macrophage-depleted mice exhibit increased enterocyte apoptosis

Enterocyte apoptosis is an important hallmark of NEC. Our previous studies have shown that iNOS plays an important role in apoptosis of intestinal epithelial cells. Knocking down iNOS by siRNA abrogates CS-induced apoptosis in IEC-6 cells (29). Therefore, we isolated enterocytes from the intestines of PMN- and macrophage-depleted as well as WT infected

mice and examined apoptosis using Apotag and AnnexinV/7AAD staining. There was a greater number of apoptotic enterocytes in the intestines of PMN- and macrophage-depleted mice compared to WT infected mice ($p < 0.01$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test) (Fig. 9A and B). AnnexinV/ 7AAD staining revealed 72% and 80% enterocyte apoptosis in PMN- and macrophage-depleted mice respectively, compared to 40% in WT infected animals ($p < 0.001$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test) (Fig. 9C). These results suggest that lack of PMNs and/or macrophages increases the apoptosis of enterocytes in the intestine upon infection with CS.

Discussion

Our central hypothesis regarding the pathogenesis of NEC focuses on the integrity of epithelial barrier and the role of bacterial colonization in the initiation and progression of the disease. Immaturity of the intestinal epithelial barrier and neonatal mucosal immune system predisposes the premature infant to intestinal mucosal injury [1-3]. Indigenous microorganisms or pathogenic bacteria from contaminated formula, for instance, then breach the compromised gut barrier and incite the production of pro-inflammatory cytokines by enterocytes or immunocytes in the epithelium or lamina propria [4-6]. These pro-inflammatory mediators, in turn, upregulate iNOS expression leading to local overproduction of NO and epithelial injury [30-31]. We have previously shown that iNOS upregulation induces enterocyte apoptosis or necrosis, while simultaneously inhibiting intrinsic repair mechanisms, namely enterocyte migration and proliferation [30]. The resulting imbalance between epithelial cell injury and repair leads to a persistent mucosal defect that promotes continued bacterial invasion, uncontrolled immune activation and an exuberant pro-inflammatory response that further exacerbates gut barrier failure, the hallmark of NEC. In this study, we examined the role of PMNs and macrophages in an acute CS-induced mouse model of NEC. CS is an opportunistic food-borne pathogen that has been implicated in multiple outbreaks of NEC in neonatal ICUs around the world [13-15]. These pathogenic bacteria can withstand extremes of temperature and therefore survive pasteurization and the desiccation process that is involved in producing infant formula. We have already demonstrated that in our formula feeding, hypoxia and hypothermia model of NEC, administration of formula contaminated with CS induces NEC-like pathology in mice [31]. In our current model, we use the lowest inoculum of CS to infect mice and cause NEC like pathology. Therefore, this model closely mimics human clinical conditions and can be used to study the pathogenesis of NEC. Our data showed that the oral feeding of CS leads to the recruitment of inflammatory cells to the intestinal mucosa. Consistent with previous observations, this phenomenon is followed by a progressive rise in the expression of pro-inflammatory cytokines and mucosal iNOS expression, which in turn leads to mucosal injury.

Following administration of CS, PMNs and macrophages appear to play a crucial role in the clearance of bacteria during the initial stages of the infection. Interestingly, depletion of PMNs and macrophages led to increased recruitment of DCs to the intestine of CS infected mice. We have previously demonstrated that CS survives and multiplies inside DCs *in vitro* and utilizes these immunocytes as safe haven to evade the host defense mechanisms and induce bacteremia [16]. Depletion of PMNs and macrophages resulted in increased DC recruitment, increased production of proinflammatory cytokines and enterocyte apoptosis. Enhanced enterocyte apoptosis in PMN- and macrophage-depleted mice may be attributed to the increase in pro-inflammatory cytokine production, which leads to intestinal iNOS expression and consequently greater NO production following CS infection. Our study demonstrates the importance of PMNs and macrophages for the first time in the pathogenesis of CS-induced NEC in newborn mice. Our data suggest that PMNs and

macrophages may help attenuate the inflammatory response since their absence leads to increased DC recruitment, which results in an increased production of pro-inflammatory cytokines. Further studies are warranted to elucidate the specific role of the innate immune system in the pathogenesis of NEC. Better understanding of the role of the innate immune response may lead to the development of novel therapeutic strategies to prevent disease progression in NEC.

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Figure 1. Oral feeding of CS to newborn mice causes bacteremia and intestinal colonization

3 day-old mouse pups were fed with 10^3 CFU of CS and various parameters of disease were examined. Survival curves of newborn mice infected with CS (A). Blood was collected at different times post-infection and analyzed for bacterial titers (B). Intestines from infected mice were homogenized and cultured on ampicillin-containing plates (C). The influx of DCs, PMNs and macrophages was determined in the intestines of CS infected and saline fed control uninfected mice by flow cytometry (D). Data represent mean $\pm$ SEM. * $p < 0.001$ DCs vs PMNs or DCs vs macrophage influx by ANOVA and Fisher's exact test.

Fig 2. Histopathological examination of intestinal tissues from CS infected and uninfected mice Intestines were harvested from infected and control mice and subjected to pathological examination (A). Intestinal sections were graded microscopically by a pathologist blinded to the groups from grade 0 (normal) to grade 4 (severe) based on morphological changes (B). Scale bars 10 μ m.

Fig 3. Histopathological examination of spleen and liver tissues

Liver and spleen tissues were collected from mice at 72 h post-infection, fixed in formalin, 3-5 micron sections cut with microtome and stained with H&E. The specimens were examined by a pathologist blinded to the groups. The slides revealed acute inflammation (black arrow and inset) but the architecture of these tissues was well preserved in response to CS infection. Scale bars 10 μ m.

Fig 4. PMN- and macrophage-depleted mice develop severe CS induced NEC

The depletion of PMNs and macrophages in newborn mice upon administration of Gr-1 antibody and carrageenan, respectively was confirmed by flow cytometry using Gr-1 and F4/80 antibody respectively which revealed >95% depletion (A and B). PMN- and macrophage-depleted mice were challenged with CS and mortality rates were assessed (C). Intestines from infected mice were cultured on antibiotic containing plates to determine intestinal bacterial load (D). Data represent mean $\pm$ SEM. * $p < 0.05$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test.

Fig. 5. PMN- and macrophage-depleted mice infected with CS show increased intestinal damage Paraffin-embedded sections of intestinal segments from CS infected mice were stained with H&E and examined for morphological changes in response to bacterial infection. Severe inflammation, extensive villus destruction and hemorrhage were observed in the intestines of PMN- and macrophage-depleted mice compared to WT mice (A). The pathology scores by a pathologist blinded to the samples revealed significant damage in the intestinal sections obtained from PMN- and macrophage-depleted CS infected mice compared with WT mice (B). In addition recruitment of DCs, macrophages and PMNs in the intestines of infected mice was determined by flow cytometry (C). Data represent mean $\pm$ SEM. # $p < 0.05$ WT vs PMN-depleted or WT vs macrophage-depleted by Wilcoxon signed rank test. * $p < 0.01$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test. Scale bars 10 μ m.

Fig. 6. PMN- and macrophage-depleted mice exhibit increased serum cytokine production
 Blood was collected from infected PMN-or macrophage-depleted or WT mice and the production of TNF-α (A), IL-6 (B), IL-12 (C), and IL-1β (D) in serum was determined by ELISA. Data represent mean ± SEM. #p<0.05 WT vs PMN-depleted or *p<0.01 WT vs macrophage-depleted by ANOVA and Fisher's exact test.

Fig 7. Cytokine production in the intestines of PMN- and macrophage-depleted CS infected mice Intestines were harvested from infected mice at 48 h post-infection, weighed and homogenized in sterile PBS. The homogenized samples were centrifuged and the clear supernatants were collected. The levels of TNF- α , IL-1 β , IL-6 and IL-12 were determined by ELISA (A). Data represent mean $\pm$ SEM. # $p < 0.05$ or ** $p < 0.01$ WT vs PMN-depleted or * $p < 0.001$ WT vs macrophage-depleted by ANOVA and Fisher's exact test.

Fig. 8. iNOS expression and NO production in the intestines of PMN- and macrophage-depleted mice infected with CS

iNOS expression was determined in the intestines of infected animals 48 h post-infection by flow cytometry (A). NO production was determined in the intestinal homogenates of infected mice by Greiss reagent (B). Data represent mean $\pm$ SEM. * $p < 0.001$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test.

Fig. 9. PMN- and macrophage-depleted mice show enhanced apoptosis of enterocytes

Enterocytes were isolated from the intestines of mice by flow cytometry based on E-cadherin staining. Enterocytes were stained with ApopTag Red *in situ* Apoptosis Detection kit and DAPI (blue), and viewed under fluorescence microscope for apoptotic cells (red) (A). The number of apoptotic nuclei was measured per high power field (HPF) (B). Enterocyte apoptosis was also examined by AnnexinV/7AAD staining (C). Data represent mean $\pm$ SEM. * $p < 0.001$ WT vs PMN-depleted or WT vs macrophage-depleted by ANOVA and Fisher's exact test. Scale bars 10 μ m.