Nod1 and Nod2 regulation of inflammation in the *Salmonella* colitis model

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Abstract

The pattern recognition molecules Nod1 and Nod2 play important roles in intestinal homeostasis, however, how these proteins impact on the development of inflammation during bacterial colitis has not been examined. In the streptomycin-treated mouse model of *Salmonella* colitis, we found that mice deficient for both Nod1 and Nod2 had attenuated inflammatory pathology, reduced levels of inflammatory cytokines, and increased colonization of the mucosal tissue. Nod1 and Nod2 from both hematopoietic and non-hematopoietic sources contributed to the pathology and all phenotypes were recapitulated in mice deficient for the signaling adaptor protein Rip2. However, the influence of Rip2 was strictly dependent on infection conditions that favored expression of the *Salmonella* pathogenicity island (SPI)-2 type III secretion system (TTSS), as Rip2 was dispensable for inflammation when mice were infected with bacteria grown under conditions that promoted expression of the SPI-1 TTSS. Thus, Nod1 and Nod2 can modulate inflammation and mediate efficient clearance of bacteria from the mucosal tissue during *Salmonella* colitis, but their role is dependent on the expression of the SPI-2 TTSS.
Introduction

The first step in initiating an inflammatory response to a microbial pathogen is to recognize the presence of an infection. Pattern recognition molecules (PRMs) serve this role by monitoring for the presence of microbe associated molecular patterns (MAMPs) in extracellular or intracellular locations and triggering inflammation when MAMPs are detected by specific cell types or within normally microbe-free environments of the host (26). One family of extracellular PRMs is the Toll-like receptor (TLR) family whose members include TLR4 and TLR5 that recognize bacterial lippopolysaccharide and flagellin, respectively (22, 25). When a TLR interacts with its specific ligand, MyD88 dependent signaling cascades are initiated that result in activation of NF-κB leading to the transcription of genes encoding pro-inflammatory proteins. Another group of PRMs is the Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLR) family of intracellular receptors, which includes Nod1 and Nod2, that recognize unique MAMPs found in bacterial peptidoglycan (4, 10, 11, 14, 39, 42). Nod1 recognizes -D-glutamyl-meso-diaminopimelic acid a molecule found predominantly in peptidoglycan from Gram-negative bacteria (5, 16) while Nod2 recognizes muramyl dipeptide (MDP) a motif common to both Gram-negative and Gram-positive bacteria (17, 24). Upon recognition of their specific ligands, Nod1 and Nod2 associate with a common adaptor protein called Rip2 (or RICK) and initiate a signal transduction cascade that leads to expression of NF-κB-dependent pro-inflammatory mediators (23, 38).

A specific role for Nod1 and Nod2 in regulating intestinal inflammation is evidenced by
the association of mutations in Nod1 and Nod2 with inflammatory bowel disease (IBD) (4, 10, 11, 14). In particular, Nod2 has received much attention within the research community due to its association with Crohn’s disease. A Crohn’s disease-associated frame-shift mutation in Nod2 results in loss of NF-κB activation in response to MDP (2, 6, 17, 24, 29). However, the reasons why inactivation of Nod2 can result in chronic colitis remain largely speculative. IBD is thought to be a bacterial driven inflammatory response therefore it is important to understand the function of risk factors such as Nod1 and Nod2 in the context of bacterial colitis. Although several studies have shown that Nod1 and Nod2 modulate inflammatory responses to various bacterial pathogens in both in vitro and in vivo infection models (14), surprisingly little is known about their specific roles during infectious colitis. Therefore, the investigation of the role of Nod1 and Nod2 in a bacterial colitis model is likely to provide valuable insights into the etiology of IBD.

In humans S. typhimurium infection causes acute colitis, however, in mice the same organism causes a systemic disease with little or no intestinal inflammation. Interestingly, pre-treatment with the antibiotic streptomycin alters the infection in mice so that S. typhimurium causes acute colitis. This streptomycin pretreated mouse model has been useful for dissecting both bacterial and host factors that modulate inflammation (18). For example, the roles Salmonella pathogenicity island-1 (SPI-1) and Salmonella pathogenicity island-2 encoded type III secretion systems (TTSS) have been investigated in mice that are lacking MyD88, the adaptor protein that regulates NF-κB in response to TLR signaling (20). Through these studies, it was determined that MyD88 signaling is not essential for mediating inflammation when streptomycin pretreated mice were
infected with wild-type (WT) *S. typhimurium* (encoding both the SPI-1 and SPI-2 TTSS). However, MyD88 signaling mediates inflammation when mice were infected with a strain of *S. typhimurium* that lacks the SPI-1 TTSS.

Using the streptomycin pretreated mouse model we investigated the role of Nod1 and Nod2 in *S. typhimurium* colitis. Although mice deficient in either Nod1 or Nod2 did not display any significant changes in inflammatory pathology, mice deficient for both Nod1 and Nod2 (DKO mice) had significantly reduced inflammation and cytokine production in response to infection. The reduced inflammation resulted in increased bacterial burden in the inflamed tissue but did not affect translocation to systemic sites or shedding in feces. Furthermore, Nod1 and Nod2 signaling from both hematopoietic and non-hematopoietic cells were found to contribute to the pathology. Although another group reported that Rip2-deficient mice are not impaired for inflammation in this model (3), all phenotypes we observed in DKO mice were identical in Rip2-deficient mice. We therefore investigated whether bacterial growth conditions could affect the outcome of the infection as a possible explanation for this discrepancy. We found that Rip2 mediated inflammation when mice were infected with *Salmonella* cultures that were grown under conditions that favor SPI-2 TTSS expression whereas Rip2 was dispensable for inflammation when mice were infected with cultures that favor SPI-1 TTSS expression. Thus, Nod1 and Nod2 can play an important role in modulating the intensity of inflammation during *Salmonella* colitis but the influence of Nod1 and Nod2 on colitis depends upon the expression of the SPI-1 and SPI-2 TTSS.
Materials and methods

Mice. C57Bl/6, Nod1-/-, Nod2-/-, Nod1Nod2-/- (DKO), and Rip2-/- mice were bred and housed according to specific pathogen free conditions in the animal facility of the Center for Cellular and Biomolecular Research, Toronto, Canada. Nod1-/- mice were originally from Millennium Pharmaceuticals, Nod2-/- from Marco Giovaninni and Jean-Pierre Hugot and Rip2-/- from Richard Flavell. Mutant mice were back-crossed at least 10 generations. All animal experiments were approved by the Animal Ethics Review Committee of the University of Toronto. Rip2-/- mice and WT littermates were obtained by crossing heterozygous mice and using PCR for genotyping.

Bacterial infections. For infections, mice were fasted for 3 hours then orally administered 20 mg of streptomycin. 21 hours after streptomycin treatment, mice were again fasted for 3 hours then orally infected with either 5 X10^7 or 1 X 10^3 colony forming units (CFU) of SL1344 a streptomycin resistant strain of Salmonella enterica serovar Typhimurium. Except where indicated, overnight cultures were used for infections and were washed with PBS then diluted to the desired CFU level based on optical density readings at 600 nm. For SPI-1 inducing conditions, overnight cultures were diluted 1:33 and grown at 37°C with aeration for 3 hours prior to dilution to the desired CFU concentration. For SPI-2 inducing conditions overnight culture were used. These growth conditions have previously been shown to allow for specific activity of the SPI-1 and SPI-2 TTSS (13, 15).
Pathological scoring. Cecum samples were collected after mice were sacrificed, and the distal half (containing the cecal patch) was fixed in 10% formalin, then stained with hematoxylin and eosin (H/E) at the Toronto Center of Phenogenomics using standard histological staining procedures. Some samples were also stained with alcian blue to visualize mucous. H/E stained cecum samples were then analyzed by a pathologist specializing in intestinal inflammation who was blinded to the experimental conditions. The scoring system was based on one that was previously published that was slightly modified due to differences in microscopy instrumentation and also to improve the dynamic range and the empirical nature of the scoring system. Edema and epithelial erosion scores were performed as previously described (1). Neutrophil recruitment was scored by calculating the average number of neutrophils present from 10 microscopic fields of a given sample. The average neutrophil count was then divided by 120 (the maximum average) then multiplied by 4 (see supplemental table 1 for raw PMN counts and corresponding scores). The goblet cell depletion score was calculated by counting the average number of goblet cells present in 10 microscopic fields of a sample, this average was divided by 65 (the average number of goblet cells present in uninfected samples) and the log of the resulting fraction was multiplied by -1.5, and the maximum value for the goblet score was capped at 4 (if the average number of goblet cells was 0 then a score of 4 was given) (see supplemental table 2 for raw goblet cell counts and corresponding scores). The resulting neutrophil and goblet scores were similar to those already published with the additional benefit that the score is continuous.
Cytokine ELISAs. Serum samples were collected from mice and allowed to coagulate at room temperature for 2 hours then centrifuged to remove red blood cells and coagulated material. The half of the cecum proximal to the ileum was collected, the contents removed, then the tissue was placed in PBS, weighed, and homogenized using a rotor homogenizer. A small sample (10µl) was removed to count CFUs then the samples were centrifuged to remove non-soluble material and the supernatants were collected to use in ELISAs. Serum samples and cecum samples were diluted (beginning with a 1:5 dilution) and ELISAs (R and D systems) were used to measure KC and IL-1β levels and was normalized to tissue weight for cecal tissue samples (detection threshold for serum = ~80 pg/ml, detection threshold for cecal tissue = ~500 pg/g).

Bacterial load quantification. The liver, spleen, mesenteric lymph node and fecal pellets were collected from infected mice and placed in PBS containing 1% triton X-100 then homogenized using a rotor homogenizer. Cecal tissue samples were collected as described above and a small (10µl) sample was diluted in PBS containing 1% triton X-100. Samples were serially diluted in PBS and plated on MacConkey agar containing 50µg/ml streptomycin.

Mouse chimeras. WT or DKO mice were lethally irradiated with 1000 centi-Gray of ionizing radiation and 24 hours later these mice were reconstituted with bone marrow from the tibia and femurs of either WT or DKO mice and allowed to reconstitute for 6-8 weeks prior to any experimental manipulation.
Results

**Nod1 and Nod2 have redundant roles in Salmonella colitis.** To investigate the role of Nod1 and Nod2 in *Salmonella* colitis, wild-type (WT), Nod1-deficient, and Nod2-deficient mice as well as mice deficient for both Nod1 and Nod2 (DKO) were treated with streptomycin and infected with $5 \times 10^7$ CFU of the streptomycin resistant *S. typhimurium* strain SL1344. Mice were sacrificed at 24-hour intervals following infection (see Table 1 for numbers of mice used for each time point and group) then their cecums were removed and stained for histological analysis. Samples were analyzed for neutrophil accumulation, goblet cell depletion, edema, and epithelial erosion and were scored using a previously established scoring system (1) (see materials and methods). Although neither Nod1 nor Nod2 deficient mice had any significant changes in pathology (Supplemental Fig. 1), DKO mice had reduced inflammation from 24 to 72 hours after infection. This was reflected by decreased neutrophil recruitment, and goblet cell depletion as well as overall pathological score in infected DKO mice when compared to WT mice (Fig. 1). Thus, Nod1 and Nod2 modulate the intensity of inflammation during *Salmonella* colitis and either protein is sufficient to complement the lack of the other.

**Nod1 and Nod2 regulate the levels of key inflammatory cytokines.** In mice, Nod1 and Nod2 lead to NF-κB-dependent expression of chemokines such as the keratinocyte-derived chemokine (KC) (similar to human IL-8) and play a critical role in neutrophil recruitment in different models of inflammation (14). Activation of NF-κB also leads to expression of the pro-form of IL-1β, another cytokine that plays a central role in
mediating inflammation. However, release of mature IL-1β requires cleavage of pro-IL-1β by caspase-1, an enzyme whose activity is regulated via a complex termed the “inflammasome”. Caspase-1 activation and IL-1β release have been shown to be regulated by another NLR family member, IPAF, in the context of Salmonella infection (9, 27, 34). Thus, although Nod1 and Nod2 activation lead to increased expression of pro-IL-1β, release of IL-1β is likely independent of Nod1 and Nod2 during Salmonella colitis. Hence, it is not clear what influence disruption of Nod1 and Nod2 would have on IL-1β production. Therefore, we assessed the impact of Nod1 and Nod2 on the expression of the cytokines KC and IL-1β during infection.

To this end, portions of the same cecum samples that were used for histological analysis as well as serum samples from these same mice were used to measure cytokine levels by ELISA. Infected Nod1- and Nod2-deficient mice had no change in cecal KC when compared to infected WT mice (Supplemental Fig. 1). However at 72 hours post infection there was a significant decrease in cecal IL-1β in Nod2 deficient mice but this did not match the pathology observed in the same mice. In infected DKO mice, there was significantly reduced levels of both KC and IL-1β in the cecal tissue at all time points compared to WT mice, however there was no difference in the levels of KC in the serum (Fig. 2). The greatest differences were detected at 72 hours post infection, where the levels of KC were quite consistent between samples and closely matched the pathology. In contrast, the levels of IL-1β were generally more variable and did not necessarily correlate with the pathology, perhaps reflecting an indirect influence of Nod1/2 on IL-1β production. Overall, these findings indicate that Nod1 and Nod2 regulate local tissue KC
and IL-1β levels and the levels of KC closely parallel the pathology.

**DKO mice have increased bacterial load in cecal tissue at 48 and 72 hours post infection.** WT, Nod1-deficient, Nod2-deficient and DKO mice were also examined for differences in colonization by SL1344. Pellets, spleens and cecal tissue samples were collected from the same infected mice used for histological analysis and the CFUs in the samples were determined. No differences in CFUs were observed in any samples in either Nod1- or Nod2-deficient mice (Supplemental Fig. 1). DKO mice had similar levels of colonization to WT in both spleen and pellet samples, however there were significantly less bacteria isolated from cecal tissue at 24 hours post infection and significantly more bacteria in cecal tissues at 48 and 72 hours (approximately 4-fold increase at both time points) (Fig. 3A-D). Thus time dependent differences in colonization were observed between WT and DKO mice.

It has previously been reported that *Salmonella* utilizes Caspase-1 dependent inflammatory pathways to enhance host colonization (35). Although these results are somewhat controversial (27) it is possible that the increased cecal colonization of DKO mice at 24 hours reflects a requirement for Nod1/2 driven inflammation to efficiently colonize the host. Most of the analysis was performed following infection with a relatively high infectious dose of 5 X 10^7 CFU because this dose produces the most consistent results for pathology. We repeated some of the analyses in a smaller group of mice using a lower dose of 10^3 CFU. Using this low dose we found that the pathology was similar to that seen using a high dose with similar trends for differences in KC and
IL-1β production (Supplemental Fig. 2). At this lower dose, the reduced colonization of DKO mice persisted from 24 to 72 hours post infection in the cecal tissue as well as systemic sites (the spleen, liver and mesenteric lymph nodes) and this translated to decreased shedding of bacteria in fecal pellets (supplemental figure 2, note that statistics could not be performed on most 24 hour samples because most DKO samples had colonization or cytokine levels below the detection threshold). By 120 hours post infection, the level of colonization in cecal tissue and systemic sites was similar in both WT and DKO mice and appears to be trending towards increased colonization of DKO cecums similar to what was seen using the higher infectious dose. However, experiments at low dose were not carried out beyond 120 hours because the mice began to die after this time point. Notably, there were no differences in mortality observed between WT and DKO mice at either low or high dose (data not shown). Overall, we observed that differences in colonization of WT and DKO mice were dependent on timing and the size of the inoculum.

It appears that the reduced inflammation in the DKO mice initially resulted in less efficient colonization, however the colonization of cecal tissue increased at later time points. This may be due to inefficient clearance of bacteria at the mucosal surface as a direct consequence of reduced fluid and mucous secretion. This is supported by the observation that in some infected DKO samples there was intense alcian blue staining, a dye that stains mucous, that persisted in the cecal lumen at 72 hours post infection that was never observed in WT samples (Fig. 3E). Thus, we postulate that Nod1- and Nod2-dependent inflammation is important for physically removing Salmonella from the
Both hematopoietic and non-hematopoietic Nod1 and Nod2 are involved in *Salmonella* colitis. Chimeras were generated to determine the contribution of Nod1 and Nod2 signaling from different cellular compartments. Lethally irradiated WT and DKO mice were reconstituted with either WT or DKO bone marrow generating mice with WT radio-resistant (or non-hematopoietic) cells with DKO radio-sensitive (or hematopoietic) cells \((\text{DKO} \rightarrow \text{WT})\), mice with DKO radio-resistant cells with WT radio-sensitive cells \((\text{WT} \rightarrow \text{DKO})\), as well as control mice with both compartments derived from WT \((\text{WT} \rightarrow \text{WT})\) or DKO \((\text{DKO} \rightarrow \text{DKO})\) mice. These chimeric mice were treated with streptomycin and infected with SL1344, then the mice were sacrificed after 72 hours of infection and the cecums analyzed for histology and cytokine levels. DKO→DKO mice had significantly reduced overall inflammatory pathology when compared to WT→WT mice while DKO→WT and WT→DKO had intermediate phenotypes (Fig. 4A and B). Similar results were seen for KC levels measured in cecal tissue with both DKO→WT and WT→DKO having intermediate levels of KC (Fig. 4C). Again, the levels of KC closely matched the observed pathology. Interestingly, the levels of IL-1β were uncoupled from Nod1 and Nod2 in the chimeric mice perhaps reflecting an influence of irradiation on the IL-1β responses (Fig. 4D). All groups of chimeras had similar levels of IL-1β in the cecal tissue and the IL-1β levels did not correlate with observed differences in pathology. From these experiments we conclude that both hematopoietic and non-hematopoietic Nod1/2 are involved in modulating *Salmonella* colitis.
The role of Rip2 in *Salmonella* colitis is dependent on SPI-1 or SPI-2 growth conditions for infection. While the studies on Nod1/2 deficient mice were in progress, Bruno et al published a report indirectly suggesting that Nod1/2 do not play a role in *Salmonella* colitis (3). In this report, Rip2, the adaptor protein for both Nod1 and Nod2, was found to be dispensable for inflammation in the *Salmonella* colitis model. These findings were surprising since reports had already shown that Nod1 and Nod2 can influence responses to *Salmonella* (21, 28). Moreover, we found that all the phenotypes observed in DKO mice during *Salmonella* colitis were recapitulated in Rip2-deficient mice (data not shown, Fig. 3D and Fig. 5). We investigated the possibility that different pre-growth conditions could affect the influence of Rip2 in *Salmonella* colitis since we used growth conditions that favor SPI-2 TTSS expression in our experiments while Bruno et al used growth conditions that favor SPI-1 TTSS expression in their report. Therefore, streptomycin treated Rip2 deficient mice or WT littermates, generated from breeding mice heterozygous for the Rip2 null allele, were infected using SL1344 grown under SPI-1 or SPI-2 favoring conditions. As expected, when we infected with SPI-2 expressing SL1344, Rip2 deficient mice had reduced inflammation and cytokine production when compared to their WT littermates (Fig. 5). Surprisingly, when mice were infected with SPI-1 expressing SL1344 cultures, Rip2 deficient mice actually had a trend towards an increase in overall inflammation and cytokine production. Of note, SPI-1 and SPI-2 conditions did not significantly change colonization levels in the spleen of cecum (supplemental figure 3). Overall these results reproduce the previous conclusion by Bruno et al that Rip2 is dispensable for inflammation when mice are infected with bacteria expressing the SPI-1 TTSS. However, we found that this is strictly dependent on
growth conditions since Rip2 plays a significant role in inflammation when mice are infected with bacteria expressing the SPI-2 TTSS.

**Discussion**

Although Nod1 and Nod2 are key regulators of intestinal homeostasis, this is the first in-depth analysis of their role in an animal model of infectious colitis. We found that in the *Salmonella* colitis model, mice lacking both proteins have reduced overall pathology and cytokine production, coinciding with an increased bacterial burden in the mucosal tissue. Furthermore, Nod1/2 signaling from both hematopoietic and non-hematopoietic cells contribute to the pathology. Finally, the role of Rip2, the adaptor protein for both Nod1 and Nod2 signaling, was dependent on bacterial growth conditions that favored expression of SPI-2 TTSS.

An important paradox in the field of Nod2 research is how mutations that disrupt its function and impair its ability to induce inflammatory responses could lead to the increased inflammation observed in Crohn’s disease (4, 10, 11, 14). It has been postulated that defects in Nod2 result in a constitutively weak inflammatory response that could lead to increases in intestinal bacterial load and over time lead to chronic states of inflammation (33). We found that the weaker inflammatory response of Nod1/2 deficient mice allowed for increased association of bacteria with the mucosal tissue. It is possible that in the long term this could lead to chronic states inflammation. Unfortunately, this could not be investigated in our mice since they are deficient for the nramp1 resistance
gene and die within 5-7 days post infection, making it impossible to perform long-term infection studies. In addition to increased bacterial colonization, other work has shown that Nod1/2 deficiency can affect T-cell polarization skewing responses from a Th2 to Th1 bias (12, 32). Therefore it is also possible that such influences on adaptive responses could further contribute towards the establishment of chronic colitis in the *Salmonella* colitis model.

It has previously been shown that host responses to *Salmonella* infection are influenced by Nod1 and Nod2 (21, 28), however none of these previous reports provided in-depth analysis of the role of Nod1/2 in mediating inflammation during *Salmonella* colitis. Bruno et al were the only other group to investigate the role of Nod1/2 in the *Salmonella* colitis model and contradictory to our results, came to the conclusion that NOD signaling is not involved in the inflammatory response in this model (3). In the 1990s, there was a similar controversy regarding the mechanism of cell death that various groups observed in cultured macrophages during *Salmonella* infection (7, 30, 31, 36). This controversy was resolved when it was reported that infection using pre-growth conditions that favors SPI-1 expression (log phase cultures) resulted in rapid macrophage death whereas infection using pre-growth conditions that favors SPI-2 expression (stationery phase cultures) results in delayed cell death (40). We therefore considered the possibility that different pre-growth conditions could influence the role of Nod1/2 in the *Salmonella* colitis model. This was an attractive hypothesis since it had already been reported that the pathology in this model is differentially influenced by *Salmonella* mutants that are deficient for either SPI-1 or SPI-2 (8, 20). This is precisely what we observed; indeed, the
influence of Nod1 and Nod2 on inflammation in the *Salmonella* colitis model was entirely dependent on growth conditions.

The mechanism leading to differential role of Nod proteins during infection with SPI-1 versus SPI-2 expressing *Salmonella* remains unknown. One possible explanation stems from the fact that SPI-1 deficient *S. typhimurium* enter the host exclusively through a monocyte and dendritic cell dependent uptake mechanism that differs from the M-cell dependent entry mechanism employed by WT *S. typhimurium* (41, 43). This difference in uptake mechanism could lead to differential means of NF-κB activation in different cell types. Thus, SPI-1 or SPI-2 dependent infections could affect the interactions with different cell types at very early stages of infection and lead to distinct mechanisms of activating the inflammatory response. In support of this idea, it has been shown that when streptomycin treated mice are infected with *Salmonella* using SPI-1 conditions or using SPI-1 deficient bacteria, the bacteria reside within unique subsets of monocytic cells (20). Interestingly SPI-1 deficient *Salmonella* specifically associate with CX3CR1+ dendritic cells (19) and these cells have been implicated in mediating inflammation in the CD45RB transfer model colitis (37). Another possibility is that effectors delivered into host cells via the SPI-1 TTSS subvert the need for host PRM recognitions pathways. Indeed, SPI-1 effectors have been implicated in PRM independent NF-κB activation (3). Therefore using SPI-1 expression conditions for infection could be masking the influence of Nod-driven inflammatory pathways.

NOD receptors are key regulators of inflammatory responses that serve a critical function
in controlling acute stages of bacterial infections by coordinating events such as the recruitment of phagocytic cells, mucous release and programmed cell death. These aggressive measures, designed to physically remove or contain the pathogen, often come at the expense of causing localized tissue damage. Thus it is essential to carefully regulate inflammation in order to prevent excessive damage to the host organism. This is evidenced by the fact that improper activation of these inflammatory pathways leads to various autoimmune and inflammatory disorders and is therefore an intense area of research. This report expands our understanding of how NOD receptors contribute to infectious colitis, however much work remains to fully understand how the pleitropic affects of Nod1/2 deficiency can result in chronic inflammation.
Acknowledgements

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References


Table 1. Mice used in experiments for figures 1-4. The numbers of mice of different genotypes that were either uninfected or infected with SL1344 for 24-72 hours is shown.

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Figure Legends

Figure 1. DKO mice have reduced inflammation following SL1344 infection. Streptomycin treated WT or DKO mice were either left uninfected or infected with 5 X 10^7 CFU of SL1344 for 24-72 hours prior to sacrifice then their cecums were examined for histological changes. A pathological score was assigned to each sample and the average score for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion and epithelial erosion) for all mice from each group was calculated (A) as well as the average total sum of the pathological score (B). The line graphs show the average number of neutrophils (C) and goblet cells (D) observed per microscopic field from infected samples over the period of infection. Error bars represent one standard error of the mean. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001)

Figure 2. Reduced cytokine production in cecal tissue from DKO mice. ELISAs were used to measure the level of KC and IL-1β in samples from streptomycin treated WT and DKO mice infected with 5 X 10^7 CFU of SL1344 for 24-72 hours. Line graphs depict the average serum levels of KC (A) and the average cecal tissue levels of KC (B) and IL-1β (C) in infected samples over time. Error bars represent one standard error of the mean. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001)

Figure 3. Increased colonization of cecal tissue from DKO mice at 72 hours post infection. Streptomycin treated WT and DKO mice were infected with 5 X 10^7 CFU of SL1344 for 24-72 hours then CFU counts were performed on spleen (A), pellet (B), and
cecals tissue (C) samples. In addition, the colonization of the cecum of streptomycin treated Rip2 deficient mice infected with SL1344 for 72 hours was determined (D). Hematoxylin and eosin stained cecum samples from 72 hour infected WT (E, top row) and DKO (E, bottom row) mice were also stained with alcian blue to visualize mucous. Arrowheads indicate mucinous material in the cecal lumen that is staining with alcian blue. The left hand panels show 2.5X magnification while the right panels show 20X magnification of the inset black rectangles. All graphs show the median CFU levels and error bars depict the interquartile range. (* = p < 0.05, ** = p < 0.01)

Figure 4. Nod1/2 from both hematopoietic and non-hematopoietic compartments contribute to *Salmonella* colitis. Lethally irradiated WT and DKO mice were reconstituted with WT or DKO bone marrow thus generating chimeric mice with DKO non-hematopoietic cells with WT hematopoietic cells (WT→DKO), mice with WT non-hematopoietic cells with DKO hematopoietic cells (DKO→WT), or control mice with both compartments derived from either WT (WT→WT) or DKO (DKO→DKO). These chimeric mice were streptomycin treated, infected with $5 \times 10^7$ CFU of SL1344 for 72 hours, then sacrificed and their cecums were analyzed for pathological changes and cytokine production. A pathological score was assigned to each sample and the average score for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion and epithelial erosion) for all mice from each group groups was calculated (A) as well as the average total sum of the pathological score (B). ELISAs were used also used to measure and calculate the average level of KC (C) and IL-1β (D) in the cecum samples. Error bars represent one standard error of the mean. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001)
Figure 5. Infection using SL1344 grown under SPI-1 or SPI-2 inducing growth conditions influences the role of RIP-2 in *Salmonella* colitis. Streptomycin treated Rip2 deficient mice or their WT littermates were infected with 5 \times 10^7 CFU of SL1344 grown under either SPI-1 or SPI-2 inducing conditions. After 72 hours of infection the mice were sacrificed and their cecums were analyzed for pathological changes and cytokine production. A pathological score was assigned to each sample and the average score for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion and epithelial erosion) for all mice from each group groups was calculated (A) as well as the average total sum of the pathological score (B) (error bars represent one standard error of the mean). ELISAs were used to measure KC (C) and IL-1β (D) in the cecum samples, the circles in the scatter plots show the data from individual mice and the horizontal bars represent the mean. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001)

Supplemental figure 1. SL1344 infected streptomycin treated Nod1 or Nod2 deficient mice have no significant differences in pathology, cytokine production or colonization when compared to WT mice. Streptomycin treated Nod1 deficient, Nod2 deficient and WT mice were infected with 5 \times 10^7 CFU of SL1344 for 24 or 72 hours. After sacrifice, the cecums from infected mice were analyzed to histological changes and a pathological score was assigned to each sample and the average score for each analyzed feature (edema, neutrophil recruitment, goblet cell depletion and epithelial erosion) for all mice from each group groups was calculated (A) as well as the average total sum of the pathological score (B). The average number of neutrophils (C) and goblet cells (D)
observed per microscopic field from infected samples at 24 and 72 hours was also calculated. In addition, ELISAs were used to measure the level of KC (D) and IL-1β (E) in cecal tissue. Finally, the median number of CFUs isolated from spleen samples (F), fecal pellets (G) and cecal tissue (H) was calculated. For graphs A-E error bars represent one standard error of the mean and for graphs F-H the error bars represent the interquartile range. (* = p < 0.05)

Supplemental figure 2. Nod1 and Nod2 play a role in mediating inflammation when streptomycin treated mice are infected with a low dose of SL1344. Streptomycin treated DKO mice were infected with 1 X 10^3 CFU of SL1344 for 24 – 120 hours. Groups of 3 mice were sacrificed at 24, 72 and 120 hours post infection and their cecums were removed and analyzed for cytokine production and CFU content, and their spleens, mesenteric lymph nodes (MLN), livers and pellets were also analyzed for colonization. The average level of KC (A) and IL-1β (B) detected in the cecal tissue is shown (error bars depict one standard error of the mean). The median number of CFUs isolated from the spleen (C), liver (D), MLN (E), cecal tissue (F) and fecal pellet (G) was also calculated (error bars depict the interquartile range). (* = p < 0.05, ** = p < 0.01, *** = p < 0.001)

Supplemental figure 3. Infection using SL1344 grown under SPI-1 or SPI-2 inducing growth conditions does not change spleen or cecum colonization. Streptomycin treated Rip2 deficient mice or their WT littermates were infected with 5 X 10^7 CFU of SL1344 grown under either SPI-1 or SPI-2 inducing conditions. After 72 hours of infection the
mice were sacrificed and samples from their cecums and spleens were analyzed for CFU content. Bar graphs show the median number of CFUs isolated and error bars depict the interquartile range.
Figure 1
Figure 2
Figure 4
Figure 5