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Nramp1 Expression by Dendritic Cells Modulates Inflammatory Responses During *Salmonella* Typhimurium Infection

Yanet Valdez^{1,2}, Gretchen E. Diehl³, Bruce A. Vallance⁴, Guntram A. Grassl¹, Julian A. Guttman^{1,5}, Nat F. Brown¹, Carrie M. Rosenberger⁶, Dan R. Littman^{3,7}, Philippe Gros⁸, and B. Brett Finlay^{1,2,*}

¹ Michael Smith Laboratories, University of British Columbia, Vancouver, BC, V6T 1Z3

² Department of Microbiology and Immunology, University of British Columbia

³ The Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, New York, United States of America

⁴ Division of Gastroenterology, BC Children's Hospital, Vancouver, British Columbia, Canada

⁵ Simon Fraser University, Department of Biological Sciences, Burnaby, BC V5A 1S6

⁶ Institute for Systems Biology, Seattle, Washington

⁷ Howard Hughes Medical Institute, Departments of Microbiology and Pathology, New York University School of Medicine, New York, New York, United States of America

⁸ Department of Biochemistry, Center for the Study of Host Resistance, McGill University, Montreal, Quebec, Canada H3A 2T5

Abstract

Host resistance against *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is mediated by Natural resistance-associated macrophage protein 1 (Nramp1/Slc11a1). Nramp1 is critical to host defense, since mice lacking Nramp1 fail to control bacterial replication and succumb to low doses of *S. Typhimurium*. Despite this crucial role, the mechanisms underlying Nramp1's protective effects are unclear. Dendritic cells (DCs) that sample the intestinal lumen are among the first cells encountered by *S. Typhimurium* following oral infection and act as a conduit for *S. Typhimurium* to cross the intestinal epithelial barrier. We report that DCs, including intestinal, splenic and bone marrow derived DCs (BMDCs), express Nramp1 protein. In the small intestine, Nramp1 expression is greater in a subset of DCs (CD11c⁺ CD103⁻) characterized by the elevated expression of pro-inflammatory cytokines in response to bacterial products. While Nramp1 expression did not affect *S. Typhimurium* replication in BMDCs, infected Nramp1^{+/+} BMDCs and intestinal CD11c⁺ CD103⁻ DCs secreted more inflammatory cytokines (IL-6, IL-12 and TNF- α) than Nramp1^{-/-}, suggesting Nramp1 expression may promote a more rapid inflammatory response following infection. Collectively, these findings reveal a new role for DCs and Nramp1 in modulating the host inflammatory response to *S. Typhimurium*.

Keywords

Dendritic cells; Macrophages; Nramp1; *Salmonella*; Cytokine

*Corresponding author: Michael Smith Laboratories, 301-2185 East Mall, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada. Tel: (+1) 604-822-2210. Fax: (+1) 604-822-9830., bfinlay@interchange.ubc.ca.

INTRODUCTION

Salmonella enterica serovar Typhi is the causative agent of typhoid fever. Similarly, *S. Typhimurium* causes systemic salmonellosis in mice, thus offering a robust model of this disease (Finlay and Brumell, 2000). In the murine typhoid model, ingested *S. Typhimurium* penetrate the intestinal epithelial barrier, migrate to the lymph nodes, spleen and liver and proliferate to levels that induce sepsis in susceptible mice. The ability to replicate within host tissues is contingent on key *Salmonella* virulence factors (effectors) delivered into host cells by two type III secretion systems (T3SS), where they modify many aspects of host cell function (Marcus *et al.*, 2000). The T3SS encoded by *Salmonella* pathogenicity island 1 (SPI1) is required for invasion of non-phagocytic cells in the intestinal mucosa (Haraga *et al.*, 2008; Ellermeier and Slauch, 2007; Zhou and Galan, 2001), whereas in its absence, a recently published report (Niess *et al.*, 2005) suggests that orally delivered *S. Typhimurium* require uptake by local and recruited DCs to cross the intestinal epithelium.

Immature DCs reside in the intestinal mucosa where they i) induce tolerogenic responses to food and commensal organisms and ii) promote inflammatory responses against intestinal pathogens (Steinman and Banchereau, 2007). Recently, further characterization of intestinal DCs has shown that a subset of lamina propria and mesenteric lymph node (MLN) DCs, characterized by the expression of integrin α chain, CD103 (CD103⁺ DCs) promote the generation of T regulatory cells, thus inducing oral tolerance (Coombes *et al.*, 2007; Sun *et al.*, 2007). In contrast, the MLN CD103⁻ DC subset, exclusively produce pro-inflammatory cytokines in response to microbial products like LPS, suggesting they may play a role in protective immunity against intestinal pathogens (Coombes *et al.*, 2007). Based on these diverse actions, it is hypothesized that the immunoregulatory functions of DCs is crucial to maintain immune homeostasis in the gut.

In addition to their role in innate immunity to microbes and their products, DCs are recognized as the most potent antigen presenting cells (Steinman and Banchereau, 2007). They are able to phagocytose enteric microbes in the Peyer's patches and present antigenic peptides to T cells (Salazar-Gonzalez *et al.*, 2006). Also, lamina propria DCs (LPDCs) sample luminal *S. Typhimurium* by sending processes between epithelial cells into the gut lumen and this mechanism is dependent on the chemokine receptor CX₃CR1 (Niess *et al.*, 2005). Interestingly, mice lacking CX₃CR1 display increased susceptibility to *S. Typhimurium* compared with their wild type counterparts, suggesting that luminal sampling by LPDCs aids in defense against *S. Typhimurium*. Aside from the fact that DCs are among the first immune cells to encounter *S. Typhimurium* during oral infection, and act as a shuttle for this pathogen, their potential role in providing innate host defense against this and other enteric pathogens is not well understood.

Host resistance genes play a critical role in controlling the severity of *S. Typhimurium* infections. Nrp1 (also called S1c11a1) is an important innate host resistance factor to *S. Typhimurium* (Vidal *et al.*, 1993), *Mycobacterium* and *Leishmania* (Wyllie *et al.*, 2002). Many commonly studied inbred mouse strains, including C57Bl/6 and Balb/C, possess a point mutation in the *Nrp1* gene, resulting in a non-functional protein. As a result, these mice are susceptible to low doses of *S. Typhimurium* and die due to uncontrolled bacterial replication, whereas Nrp1 expressing mice are resistant and survive the infection (Roy and Malo, 2002) although, approximately 5% of these survivors became chronic carriers (Monack *et al.*, 2004). Nrp1 is expressed by professional phagocytes, including macrophages and granulocytes in the spleen and liver of mice and humans (Cannon-Hergaux *et al.*, 2002; Govoni *et al.*, 1997). However, the mechanism by which Nrp1 controls replication of intracellular pathogens is poorly understood. Structurally, Nrp1 is a 12 pass membrane spanning protein and has been proposed to function as a cationic

transporter (Vidal *et al.*, 1996). While it is recruited to the late endocytic compartment of macrophages after phagocytosis of latex beads (Gruenheid *et al.*, 1997) and may affect intraphagosomal microbial replication by modulating the divalent cation content, both the direction of transport of substrate cations, and Nramp1's role in this process, remain controversial (Wyllie *et al.*, 2002).

Here, we analyzed Nramp1 expression in the small intestine and evaluated its impact on *S. Typhimurium* survival following oral infection. Furthermore, since intestinal DCs clearly play a role in *Salmonella* pathogenesis and they are also derived from myeloid precursors, we hypothesized that they may comprise an important, but as yet unrecognized Nramp1-expressing cell population contributing to host defense against *S. Typhimurium*. We find that, indeed, intestinal DCs do express Nramp1 protein and that the expression of Nramp1 in the small intestine is greater in a subset of DCs (CD11c⁺ CD103⁻). This subset expressed higher levels of pro-inflammatory cytokines after *Salmonella* infection in the gut compared to CD11c⁺ CD103⁺ DCs. Similarly, *in vitro*, Nramp1 expressing BMDCs secrete significantly more IL-6, IL-12 and TNF- α after *S. Typhimurium* infection than Nramp1^{-/-} DCs. These findings suggest that DCs from resistant mice promote host defense by generating a stronger and more rapid inflammatory response to *S. Typhimurium* infection.

RESULTS

Nramp1 is expressed in cells of the small intestinal lamina propria

Many groups have studied the role of Nramp1 in host defense against *S. Typhimurium*, yet its function is still poorly understood. Most studies have focused on the actions of this protein in macrophages *in vitro*, or in systemic infections, whereas the natural route of *Salmonella* infection is through oral ingestion. Here we focused on oral infection and the small intestinal lamina propria that underlies the gut epithelium. This region is densely populated with phagocytes and the function of these cells is believed to be crucial in the host defense against *S. Typhimurium*, (reviewed in Wick 2007 (Wick, 2007)). Since Nramp1 is expressed in cells derived from the myeloid lineage, we hypothesized that phagocytes in the lamina propria express Nramp1. We analyzed Nramp1 expression by immunofluorescence and by RT-PCR in mice. As shown in the Figure 1A, Nramp1 is strongly expressed in the lamina propria of Nramp1^{+/+} mice but not in Nramp1^{-/-} mice. Its expression appears restricted to discrete populations of cells in the lamina propria and not to the epithelium. As expected, using RT-PCR, a specific band for Nramp1 was amplified only in the small intestines of Nramp1^{+/+} and not in Nramp1^{-/-} mice (Fig. 1B). Our results are in contrast to observations by Vidal *et al.*, (Vidal *et al.*, 1993) who were unable to detect Nramp1 expression in the murine small intestine, using Northern blots. Thus, we show here the expression of Nramp1 in the lamina propria of the small intestine of mice both at RNA and protein level.

Nramp1 expression is protective at earliest stages of *S. Typhimurium* infection

Previous studies have suggested that following oral infection, luminal *S. Typhimurium* can use two major routes to translocate across the intestinal epithelium and reach systemic sites: 1) invasive *S. Typhimurium* can penetrate intestinal Peyer's patches by invading M cells whereas 2) it has been suggested that non-invasive *S. Typhimurium* mutants can only cross the epithelial barrier after being phagocytosed by DCs (Niess *et al.*, 2005). Since no studies have yet addressed the actions of Nramp1 on *S. Typhimurium* infection through its natural, oral, route (Vidal *et al.*, 1995), we tested the impact of Nramp1 expression by the lamina propria using these two main pathways of *Salmonella* entry through the gut.

First, we assessed survival of Nrp1^{+/+} and ^{-/-} mice following infection with invasive *S. Typhimurium* (M cell pathway). In the representative infection shown in Fig 2A, 86% of Nrp1^{-/-} mice died between days 6 to 8 post-infection, whereas all Nrp1^{+/+} mice survived until the end of the experiments (day 29 post infection). Assessment of pathogen load within the small intestine (SI), Peyer's patches (PP), mesenteric lymph nodes (MLN) and spleen (SP), at one and three days post-infection revealed a significantly higher bacterial load in most organs of Nrp1^{-/-} mice (Fig 2B), confirming that Nrp1 functions as a resistance gene in this oral infection model.

We next assessed survival of Nrp1^{+/+} and ^{-/-} mice after infection with the non-invasive *invA* *S. Typhimurium* mutant (DC pathway). While 70% of Nrp1^{-/-} mice died, all Nrp1^{+/+} mice survived (Fig 2A). Colony counts revealed more *S. Typhimurium* in the small intestine and Peyer's patches of Nrp1^{-/-} mice at one day post-infection compared to Nrp1^{+/+}, and colony counts were also higher in the spleens of Nrp1^{-/-} three days post-infection (Fig 2B). Thus, we find an early effect of Nrp1 on *S. Typhimurium* infection in the gut-associated tissue, independent of the route by which the bacteria cross the intestinal epithelium. Interestingly, we observed differential bacterial replication using WT or *InvA* mutant *S. Typhimurium* in distinct anatomical sites. Higher bacterial counts in Nrp1 deficient mice were evident in the PP and MLN using WT *S. Typhimurium* for infections, whereas using *InvA* mutant, this effect was observed mainly in the small intestine and PP and later in the spleen. These differences could be a consequence of the route of entry of *Salmonella*. Since WT *S. Typhimurium* penetrate the intestinal epithelium and enter the PP, these bacteria may encounter many cells that lack Nrp1 expression including epithelial cells, therefore the effects of Nrp1 may not be immediate. Conversely, since the *InvA* mutant is directly taken up by DCs in the PP or small intestine lamina propria, the expression of Nrp1 by these cells may rapidly act to control *S. Typhimurium* replication.

CD103⁻ DCs from the lamina propria express Nrp1 and expression levels increase after exposure to *S. Typhimurium*

The above experiments suggest that Nrp1 is protective against *S. Typhimurium* and, moreover, that DCs are important in both scenarios of *Salmonella* entry through the gut. While preliminary studies by Stober et al. suggest bone marrow derived DCs can express Nrp1 (Stober *et al.*, 2007), its expression *in vivo* has, thus far, only been described in macrophages (MØ) and neutrophils. We therefore assessed Nrp1 expression by intestinal DCs. First, we used immunofluorescence to examine Nrp1 expression in CD11c⁺ cells, both before and after *S. Typhimurium* infection. Figure 3A shows the presence of Nrp1 associated with a subset of CD11c⁺ cells in the small intestinal lamina propria of both uninfected and *S. Typhimurium* infected Nrp1^{+/+} mice. Since DCs are a heterogeneous population and it has been suggested that discrete subsets may have different functions during an immune response (Niess and Reinecker, 2006; Wick, 2002), we examined Nrp1 expression in these subsets. LPDCs from 129sv mice were separated by FACS sorting based on the expression of surface markers. CD11c^{high} MHC-II^{high} cells were defined as DCs and these cells were further divided based on expression of CD103: CD11c⁺CD103⁺ have been defined as tolerogenic DCs, or CD11c⁺CD103⁻ which defines pro-inflammatory DCs (Coombes *et al.*, 2007). We used quantitative PCR (q-PCR) to evaluate Nrp1 expression at the RNA level. As shown in figure 3B, Nrp1 mRNA levels in the CD11c⁺CD103⁻ subpopulation were approximately 7 fold higher than in CD11c⁺CD103⁺ cells. We also analyzed the expression of Nrp1 in other cell populations. Nrp1 was not detected in CD11c⁻MHC-II^{high} cells (likely B cells) and very low expression was detected in the CD11c⁺CD103⁺ subset, whereas moderate expression was detected in the CD11c^{med} MHC-II⁻ cells (likely neutrophils) (Fig 3B).

Next, we tested for expression of the Nramp1 protein using intracellular FACS analysis in intestinal DCs isolated from Nramp1^{-/-} and ^{+/+} mice. Figure 3C shows Nramp1 expression in the MHC-II^{high}CD11c⁺CD103⁻ population while the MHC-II^{high}CD11c⁺CD103⁺ cells show little expression. Only background (no expression), levels were detected in both subsets of DCs from Nramp1^{-/-} mice. A small but consistent upregulation of Nramp1 was observed in the CD11c⁺CD103⁻ subset very early (30, 60, 90 and 120 minutes) after *S. Typhimurium* infection, (Fig. 3D), whereas no changes in the Nramp1 expression was observed in the CD11c⁺CD103⁺ at the above times points analyzed (data not shown), indicating that bacteria or their byproducts increase Nramp1 expression in the CD11c⁺CD103⁻ DCs. Our results demonstrate the expression of Nramp1 in a previously unrecognized cell population. Interestingly, this expression appears to be significantly higher in the CD11c⁺CD103⁻ subset of DCs which have been previously characterized as high secretors of pro-inflammatory cytokines (Coombes *et al.*, 2007), suggesting a potential role in host defense against *S. Typhimurium*.

Nramp1 expression is not restricted to gut DCs

We next examined whether Nramp1 expression by DCs is exclusive to the intestinal lamina propria by analyzing Nramp1 expression in splenic DC populations. First, we purified splenic mononuclear cells from Nramp1^{+/+} mice and analyzed the expression of Nramp1 by RT-PCR. DCs as well as MØs expressed Nramp1, while B cells were Nramp1 negative (Fig. 4A). It has been shown previously that CD11c⁺CD103⁻ DC subset is absent in this organ (Sun *et al.*, 2007; Johansson-Lindbom *et al.*, 2005), so in order to define the DC populations expressing this protein, we divided splenic DCs into major subsets based on the surface expression of discriminating molecules such as CD11c⁺, CD4⁻, CD8⁺ and CD11c⁺, CD4⁺ CD8⁻. As shown in Figure 4B, Nramp1 was expressed in both subsets of DCs in the spleen.

We next asked whether DCs derived from bone marrow also express Nramp1. To test this, we derived DCs from the bone marrow of Nramp1^{+/+} and ^{-/-} mice (BMDC). Figure 4C shows that Nramp1 is expressed in Nramp1^{+/+} BMDCs but not in Nramp1^{-/-} BMDCs and this expression increased 24 hrs after *S. Typhimurium* infection. Bone marrow derived macrophages (BMMØ) were also analyzed and, as expected, expressed Nramp1 mRNA (data not shown).

Nramp1 expression at the protein level was verified by intracellular FACS analysis. Forty one percent of BMDCs from Nramp1^{+/+} mice expressed high levels of Nramp1 protein, with a mean fluorescent intensity (MFI) = 120, suggesting that BMDCs constitutively express Nramp1 (Fig. 4D). The level of Nramp1 expression increased significantly in these cells after *S. Typhimurium* infection MFI = 277, whereas Nramp1 expression in BMDC derived from Nramp1^{-/-} mice was not detectable before or after infection with *S. Typhimurium* MFI = 21.9 and MFI = 11.9 respectively (Fig 4E). Levels of MHC-II expression also increased in the Nramp1^{+/+} and ^{-/-} BMDCs following *S. Typhimurium* infection. However, levels of MHC-II were slightly higher in the Nramp1^{+/+} BMDC compared to Nramp1^{-/-} BMDC (data not shown).

To explore the subcellular compartmentalization of Nramp1, confocal microscopy was used to analyze its expression in BMDC. We observed a punctate distribution of Nramp1 in intracellular membranes throughout the cytoplasm as has been reported previously in MØs (Fig. 4F) (Gruenheid *et al.*, 1997). Based on these findings, we conclude that, like intestinal DCs, splenic and BMDCs express Nramp1 protein and its expression increases with *S. Typhimurium* infection.

S. Typhimurium do not replicate in Nrp1^{+/+} or ^{-/-} BMDCs

In previously published studies utilizing the Raw264.7 MØ-like cell line as well as peritoneal MØ, loss of Nrp1 protein was found to facilitate intracellular replication of *S. Typhimurium* (Govoni *et al.*, 1999; Lissner *et al.*, 1985). The inability of Nrp1^{-/-} DCs to control *S. Typhimurium* replication could add to differences in pathogen loads we detected in Nrp1^{+/+} and ^{-/-} mice following oral infection (Figure 2B). Since our experiments showed high levels of Nrp1 expression in BMDCs and these cells are easily obtained *in vitro*, we used BMDCs from Nrp1^{+/+} and ^{-/-} mice to test whether Nrp1 expression has a bacteriocidal/static effect on *S. Typhimurium* within DCs. BMDCs were infected with *S. Typhimurium* and colony counts were assessed over a 24-hour period. While Nrp1^{+/+} and ^{-/-} BMDCs phagocytosed similar numbers of *S. Typhimurium*, (Nrp1^{+/+} = 461666.7 ± 72341.8 and Nrp1^{-/-} = 403000.0 ± 63379.8) the numbers of viable bacteria subsequently decreased over the course of infection, with no significant difference in *S. Typhimurium* survival between Nrp1^{+/+} and ^{-/-} BMDCs. These data suggest that *S. Typhimurium* do not replicate in DCs (data not shown), a finding supported by other studies (Jantsch *et al.*, 2003). Interestingly, we also observed a similar decline in *S. Typhimurium* numbers in infected BMMØ with only slight differences observed between Nrp1^{-/-} and ^{+/+} BMMØ (data not shown). The lack of an effect of Nrp1 on bacterial replication in BMDC and BMMØ *in vitro* is in contrast to previous studies using Raw264.7 cells (Boyer *et al.*, 2002; Govoni *et al.*, 1999) and peritoneal MØ (Lissner *et al.*, 1985). Our data are, however, supported by studies by Ables *et al.* who found that Nrp1 expression by peritoneal MØ influence bacterial replication only during the first hour of infection, with no differences found in CFU at later time points (Ables *et al.*, 2001). Similarly, other studies have found no significant differences in the survival of WT *S. Typhimurium* following infection of Nrp1^{+/+} and ^{-/-} congenic peritoneal MØ (Zaharik *et al.*, 2004). These divergent results may reflect the use of MØ from different sources, or differences in experimental protocols. Our results however, suggest that Nrp1 does not alter the bacterial survival or proliferation in BMDCs or BMMØ *in vitro* and suggests that the control of *S. Typhimurium* infection *in vivo* may reflect other actions of Nrp1, perhaps on the inflammatory milieu encountered by *S. Typhimurium*.

Nrp1^{+/+} DCs exhibit a greater and more rapid pro-inflammatory cytokine response to S. Typhimurium infection

As mentioned previously, two functionally distinct mucosal DC subsets have recently been described, with the CD103⁺ DC subset inducing tolerogenic regulatory T cells, and the CD103⁻ subset pro-inflammatory cytokines in response LPS. While these findings suggest the CD103⁻ subset may play an important role in responding to pathogens such as *Salmonella* through the production of pro-inflammatory cytokines, this has not yet been investigated. We therefore tested the ability of these cells to rapidly produce pro-inflammatory cytokines in response to *S. Typhimurium* infection. CD103⁺ and CD103⁻ DCs were purified from the small intestines of 129svJ mice, before and 2 hrs after *in vivo* *S. Typhimurium* infection. The levels of cytokine mRNA were determined by q-PCR. We found that CD103⁻ DCs produced higher levels of TNF-α and lower levels of IL-12p40, before the infection with *S. Typhimurium* compared to CD103⁺ DCs (Figure 5A). Two hours following *S. Typhimurium* infection, IL-6, IL-12p40 and TNF-α gene expression were all upregulated in both subsets of DCs (Fig 5A). However, the fold induction of IL-12 mRNA was much higher (1.6-fold versus 5.9-fold) in the CD103⁻ DCs. Thus, we show that both mucosal DC subsets can be stimulated *in vivo* by *S. Typhimurium*, to rapidly produce pro-inflammatory cytokines.

Our studies confirmed that Nrp1 expressing CD103⁻ DCs produce higher pro-inflammatory cytokines mRNA than CD103⁺ DCs. We then further assessed the impact of

Nramp1 on pro-inflammatory cytokine responses to *S. Typhimurium*. We purified intestinal CD103⁺ and CD103⁻ DCs from Nramp1^{+/+} and ^{-/-} mice and assessed the cytokine secretion in uninfected or *S. Typhimurium* infected DCs. Levels of IL-6 secretion were significantly higher in CD103⁻ DCs isolated from Nramp1^{+/+} mice in comparison to CD103⁻ DCs purified from Nramp1^{-/-} mice, at 12 or 24 hours after the infection (figure 5B). Similarly, higher levels of TNF- α was observed in Nramp1^{+/+} CD103⁻ DCs at both time points analyzed. We did not observed any differences in the cytokine secretion in CD103⁺ DCs from Nramp1^{+/+} or ^{-/-} mice, however, due to the paucity of these cells attainable from primary tissue, only one or two samples were analyzed for each treatment.

Since the yield of the mucosal DC subsets from the intestines of mice is extremely low and in order to confirm that this enhanced cytokine response is not restricted to gut derived DCs, we examined cytokine secretion from Nramp1^{+/+} and ^{-/-} BMDCs before and after infection with *S. Typhimurium* over the course of 24 hours. We found that both Nramp1^{+/+} and ^{-/-} BMDCs secrete IL-6, IL-12p70 and TNF- α after *S. Typhimurium* infection. More importantly, Nramp1^{+/+} BMDC expressed significantly more IL-6 and IL-12p70 than Nramp1^{-/-} DCs at 6 or 7 and 24 hours after *S. Typhimurium* infection. Small, yet significant differences in TNF- α were also found 2 hours after infection (Fig. 5C). We also analyzed the cytokine secretion by BMM ϕ and found higher secretion of IL-6 and TNF- α by Nramp1 producing BMM ϕ s, but unlike BMDCs we did not find differential secretion of IL-12p70 in BMM ϕ from Nramp1^{+/+} and ^{-/-} (Figure 5D). These results demonstrate that Nramp1 augments DC secretion of several important inflammatory cytokines in response to *S. Typhimurium* infection and the resulting inflammatory response may aid in the control and progression of the infection.

DISCUSSION

Nramp1 is known to be a key mediator of host resistance to intracellular pathogens, including *Mycobacterium* and *Leishmania*, yet the mechanisms underlying Nramp1's role in host resistance remain unclear and controversial (Gomez *et al.*, 2007; Lalmanach and Lantier, 1999; Eckmann *et al.*, 1996). In an oral infection model with *S. Typhimurium* we specifically examined whether Nramp1 was expressed by DCs, one of the first cell types to interact with pathogens following infection. Confirming recent studies *in vitro* (Stober *et al.*, 2007), we show that DCs do express Nramp1 *in vivo*. Moreover, we demonstrate that this expression endows DCs with the ability to secrete significantly more pro-inflammatory cytokines than Nramp1^{-/-} DCs in response to *S. Typhimurium* infection.

Intestinal DCs are thought to be key players in the establishment of gut homeostasis through sensing and sampling luminal intestinal contents, including commensal bacteria, and the subsequent modulation of host immunity (Bilsborough and Viney, 2004). Although the involvement of DCs in tolerance is well documented (von Boehmer, 2007), the shift to protection against pathogens is less well defined. Interestingly, recent studies suggest that LPDCs expressing CX₃CR1 sample intestinal bacteria and are important in providing immuno-protection against *Salmonella*. Recently, it has also been shown that CD103⁻ DCs from MLN produce TNF- α and IL-6 in response to LPS, potentially due to their elevated expression of TLR2 and TLR4 (Coombes *et al.*, 2007). Here we show that, in addition to these pattern recognition receptors, CD103⁻ DCs also express Nramp1 and that the expression of Nramp1 is upregulated after contact with *S. Typhimurium*. Moreover, we show that intestinal DCs express pro-inflammatory cytokines upon *Salmonella* infection. Thus, our data suggest an important role for these cells in immuno-protection against *S. Typhimurium* and other enteric pathogens.

The aforementioned cytokines (IL-6 and TNF- α) are crucial for host defense against *S. Typhimurium* (Lalmanach and Lantier, 1999) as mice deficient in these cytokines are extremely susceptible to *S. Typhimurium* infection. For example, IL-6 KO mice have impaired immune and acute-phase responses and deletion of nuclear factor-IL-6 (NF-IL-6), a key transcription factor required for IL-6 expression, renders mice highly susceptible to *S. Typhimurium* infection (Tanaka *et al.*, 1995). TNF- α is also essential for this response, since deficient mice are highly susceptible to *S. Typhimurium* infection (Lalmanach and Lantier, 1999). Finally, other cytokines like IL-12 play a crucial role in host defense against *S. Typhimurium* since deletion of the IL-12 gene or neutralization of IL-12 using a specific antibody leads to increased bacterial counts and decreases host survival. Correspondingly, IL-12 treatment increases host survival (Kincy-Cain and Bost, 1996). This appears to be a conserved host defense mechanism since IL-12 receptor deficient humans are also immunocompromised and highly susceptible to *Salmonella* infections (Sanal *et al.*, 2006). Interestingly, our data show that Nramp1 producing DCs (CD103⁺) produce higher levels of IL-6 and TNF- α after *Salmonella* contact. These data suggest that DCs from resistant mice can induce a faster and more potent inflammatory response following contact with *S. typhimurium*. The resulting inflammatory response may help to control the progression of the disease through the host.

Based on these observations, we hypothesize that the expression of Nramp1 by DCs facilitates a more rapid and effective immune response to *S. Typhimurium*. Our data suggest that once DCs detect the presence of *S. Typhimurium*, they rapidly respond with pro-inflammatory signals including IL-6, IL-12 and TNF- α . Such a response likely results in the activation of resident immune cells and/or the recruitment of other inflammatory cells to the site of infection. While the intestinal inflammatory response to orally delivered *S. Typhimurium* is relatively modest, our recent studies have found that Nramp1^{+/+} mice demonstrate significantly more severe inflammation early in the *Salmonella*-induced colitis model compared to Nramp1^{-/-} mice (Y. Valdez, in preparation). These findings are consistent with previous reports suggesting that Nramp1 modulates acute inflammatory reactions (Peters *et al.*, 2007) (Gomez *et al.*, 2007). Nramp1 was previously shown to promote higher induction of pro-inflammatory cytokines in M ϕ . For example, elevated levels of IL-1 β and TNF- α were reported in Nramp1^{+/+} M ϕ after LPS and IFN- γ stimulation (Blackwell, 1996). Similarly, Nramp1 dependent upregulation of the chemokine KC (keratinocyte chemoattractant) was reported in M ϕ stimulated with Mycobacterial lipoarabinomannans (Roach *et al.*, 1994). High levels of TNF- α expression by Nramp1^{+/+} M ϕ after TLR7 activation has also been noted (Moisan *et al.*, 2006). Overall these data suggest that Nramp1 affects the inflammatory status of the host. We propose that much of the impact of Nramp1 on the severity and outcome of *S. Typhimurium* infection is determined by its influence on the speed and intensity of the host inflammatory response, facilitating the rapid activation of host defense. It will be important to now explore the molecular mechanisms by which cytokine responses are augmented by Nramp1 in DCs and examine the precise cytokine-mediated events that may influence resistance or susceptibility to *S. Typhimurium in vivo*. Intriguingly, a new study suggests that this could be mediated by Nramp1's ability to transport metal ions. Gomez *et al.* (Gomez *et al.*, 2007) recently showed that transfection of Nramp1 into the Raw 264.7 macrophage cell line, leads to inactivation of cytosolic protein tyrosine phosphatases (PTPs). This inactivation is likely through Nramp1-dependent iron transport into the cytosol and the subsequent iron-dependent inhibition of PTP enzyme activity. PTPs are well-known for their ability to dampen cell activation and cytokine production. Thus, inhibition of PTPs via Nramp1-dependent iron transport provides a provocative new mechanistic explanation for the enhanced cytokine secretion we have observed in the present studies.

The discovery that DCs express Nramp1 provides a new concept for the role of DCs in the innate immune response to enteric pathogens such as *S. Typhimurium*. The fact that elevated responses by Nramp1 expressing cells was seen even 2 hours post-infection suggests that Nramp1's actions commence as soon as *S. Typhimurium* crosses the intestinal epithelial barrier and encounters DCs. In keeping with this hypothesis, we recently demonstrated that *Salmonella* pathogenicity island 2 (SPI2) genes are expressed while *S. Typhimurium* still reside on the luminal face of gastrointestinal tract (Brown *et al.*, 2005) and that SPI2 gene expression is substantially greater within an Nramp1 expressing environment (Zaharik *et al.*, 2002).

In conclusion, our results demonstrate that pro-inflammatory DCs express Nramp1 and that the expression of this host resistance factor by DCs may play a key role in promoting resistance to *Salmonella* via enhanced inflammatory cytokine release. Since DCs likely represent the first vanguard of effector cells to come in contact with *Salmonella*, and are the key orchestrators of both innate and adaptive immune responses, our data suggest that Nramp1 expression provides an important advantage for these cells in guiding the immune response during the earliest phase of infection. Future studies will be needed to more clearly delineate the relative role of Nramp1 expressing DCs, versus MØ and neutrophils, in mediating successful resistance to infection.

EXPERIMENTAL PROCEDURES

Bacteria

Wild-type (WT) *S. Typhimurium* (SL1344) (Hoiseth and Stocker, 1985) and isogenic $\Delta invA$ mutant (*invA::kan* SB103; SPI1) (Galan and Curtiss, 1991) were grown overnight at 37°C with shaking (200 rpm) in 10 mls of LB broth (stationary phase), supplemented with 100 µg/ml streptomycin and/or 50 µg/ml kanamycin or overnight cultures were diluted in LB 1:33 and grown for additional 3 hours (logarithmic phase).

Mice and *S. Typhimurium* infection

Female 129Sv/J mice (Nramp1^{+/+}) and isogenic Nramp1-deficient (Nramp1^{-/-}) mice have been previously described (Vidal *et al.*, 1995). Nramp1^{-/-} mice have a null allele at the Nramp1 locus, thus mRNA transcripts are absent. Groups of mice were infected by oral gavage with 100 µl of bacteria as indicated below. Mice were euthanized 1 or 3 days after infection, organs were homogenized, diluted in PBS and plated on LB + streptomycin (50 µg/ml) agar. Plates were incubated at 37°C for 24 hours, colony counts were expressed as colony forming units (CFU) per ml. Mice were infected orally with 3×10⁶ WT (logarithmic phase) or 1×10⁸ $\Delta invA$ CFU (stationary phase) in 100 µl HEPES buffer (100 mM, pH 8.0), control mice were given 100 µl HEPES buffer. Mice were monitored daily. Moribund mice were euthanized.

Ileal loops

Ileal loop experiments were performed as previously described (Brown *et al.*). Briefly, mice were anaesthetized by intraperitoneal injection of ketamine (70 ng/g) and xylazine (6 ng/g)/body weight, diluted in a volume of 300 µl of PBS. Following a midline abdominal incision, the small intestine was exposed and the terminal ileum was ligated twice, to create a loop of approximately 3–4 cm in length into which the inoculum was injected, bacterial inoculum was of approximately 10⁷ CFU in 100 µl. The intestine was then returned to the abdominal cavity and the incision closed. The mice were euthanized at different time points (30 minutes to 2 hours) and tissues were collected for Immunofluorescence.

Bone marrow derived dendritic cells (BMDCs)

BMDC were derived in GM-CSF and IL-4 as previously described (Brown *et al.*, 2005), the cells were harvested at day 7 of culture, in average this population contains between 60–70% CD11c⁺ DCs. The BMDC were purified using anti-CD11c-conjugated MACS microbeads and magnetic separation columns (Miltenyi). The purity, assessed by FACS, was over 95% CD11c⁺. The purified BMDCs were reseeded in 96-well plates in media containing 10% fetal calf serum, lacking any cytokines or antibiotics, the cells were rested for 24 hours prior to infection with *S. Typhimurium*. BMDC were infected as described below using the gentamicin protection assay with a multiplicity of infection (MOI) of 10. Alternatively, for RT-PCR experiments, CD11c⁺, I-A^b⁺, 7AAD[−] BMDCs were sorted for a purity of between 90–98%.

Bone marrow derived macrophages (BMMØ)

Bone marrow was extracted from the femurs and tibias of Nramp^{+/+} and ^{−/−} mice and cultured for 7 days in DMEM (HyClone), supplemented with 20% fetal bovine serum (Invitrogen), 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate and penicillin/streptomycin (Invitrogen) and 30% of L-cell conditioned media at 37°C in 5% CO₂. Twenty-four hours prior to the infection BMMØ were seeded in 24-well plate and were cultured in the above media under conditions lacking L-cell conditioned media and penicillin/streptomycin. The cells were infected using the gentamicin protection assay with a MOI = 10.

Gentamicin protection assays

BMDC and BMMØ were infected with opsonized bacteria as described previously (Brown *et al.*, 2005). At 2, 7 or 8 and 24 hours after infection, cells were washed with PBS and then lysed in 250 µl of 1% Triton X-100, 0.1% SDS in PBS. Lysates were diluted in PBS and plated on LB + streptomycin (50 µg/ml) agar.

Isolation of small intestine Lamina propria DCs

Cells were prepared as previously described (Niess *et al.*, 2005) with the following modification. Peyer's patches, fat and mesenterium were removed from the small intestine before the tissue was cut longitudinally. The tissue was then cut into 2 pieces and washed with calcium and magnesium free Phosphate Buffered Saline (PBS; Gibco) and the mucus removed with a 10 minute wash with 1 mM dithiothreitol (DTT; Sigma) in PBS. The intestinal epithelium was eluted with two 10 minute washes with 30 mM EDTA, 10mM HEPES in PBS, at 37 degrees. The tissue was washed with complete RPMI and then cut in to 2 cms pieces, digested for 1 hour at 37 degrees in 5% CO₂, humidified atmosphere, in complete RPMI containing 100 U/ml type VIII collagenase (Sigma) and 150ug/ml DNaseI (Sigma). After digestion, cells were eluted from the tissue by shaking. Live cells were isolated using a Percoll (Pharmacia) gradient.

Splenocyte preparation

Splenocytes were prepared as previously described (Valdez *et al.*, 2002), in brief, each spleen was collected in 1 ml of RPMI, 10mM HEPES and 0.01mg/ml of Liberase Blenzyme 3 (Roche Diagnostics, Indianapolis, IN), minced with razor blades and then incubated for 1 hour at 37°C. Fragments were dissociated by passing 5 times through a 21g needle in order to dissociate undigested material and then washed twice with complete RPMI medium.

Flow Cytometry

Flow cytometry was conducted on FACS Calibur or LSRII flow cytometer and data analyzed using CellQuest, Diva software (Becton Dickinson, San Jose, CA) and FloJo (Tree

Start, Inc, CA) cell sorting was performed using FACS Vantage and MoFlo cytometer (DAKO Cytomation). Antibodies to CD11c, CD11b, I-A^b, B220, CD8 α , CD4, TCR $\alpha\beta$, CD103 and CD16/32, were from BD PharMingen (San Diego, CA) or from e-Bioscience (San Diego, CA). Affinity purified rabbit anti-mouse Nramp1 antibody was from P. Gros (Gruenheid *et al.*, 1997). Preliminary experiments were performed using this antibody for FACS and immunostaining, later the experiments were confirmed using the affinity purified rabbit anti-mouse Nramp1 antibody from Alpha Diagnostic, (San Antonio TX). Both antibodies have the same specificity. For intracellular FACS staining of Nramp1, BMDC were infected with *S. Typhimurium* for 24 hours, incubated with anti-CD16/32 for 30 minutes on ice, stained for CD11c and I-A^b, the cells were then permeabilized using FACS buffer with 0.2% saponin and incubated with primary antibody against Nramp1 following with a secondary antibody Goat anti rabbit-Alexa-488.

Cytokine analysis by CD103⁺ and CD103⁻ DCs

Small intestine LPDCs DCs were isolated as described above. Cells were stained with antibodies against CD11c, MHC-II, CD103, CD8, B220, TCR $\alpha\beta$ and FACS sorted as TCR/B220/CD8 negative, CD11c⁺, MHC-II⁺ and CD103⁺ or CD11c⁺, MHC-II⁺ and CD103⁻. Sorted DCs were cultured in round bottom TC plates at 2×10^4 /well and left untreated or infected with WT *S. Typhimurium* at MOI=5 or MOI=1 or treated with heat killed *S. Typhimurium* at 0.5ug/ml. Supernatants were collected at 12 and 24 hours. Cytokine level were assayed using luminex technology. Cytokine beads were purchased from Upstate and data analysis was performed using BiedView Software (Upstate).

ELISA assays

BMDC (2.5×10^5) were infected as previously described. At 2, 6 or 7 and 24 hours after infection supernatants were analyzed for secretion of cytokines, using commercial kits to detect IL-6, IL-12p70 and TNF- α (BD Pharmingen). The assays were detected using a Spectrafluor Plus (TECAN, Austria).

RT-PCR and q-PCR

To analyze the expression of Nramp1 in small intestine, between 2 – 3 mm of terminal ileum tissues were excised and immediately submerged in RNeasy lysis buffer (Qiagen, Mississauga, ON, Canada). RNA was extracted using RNeasy Mini kit (Qiagen) according to manufacturer's instructions. The concentration of RNA was determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and reverse transcription was performed with the Quantitect RT kit (Qiagen) using 1 μ g RNA. For PCR reactions, the cDNA was diluted 1:10 and 1 μ l of diluted cDNA was used. For Nramp1 expression in intestinal DCs, different populations of intestinal cells were FACS sorted and kept at -70 °C in Trizol until RNA isolation and cDNA preparation. For BMDC and spleen cells, similar numbers of DCs or splenocytes were FACS sorted and mRNA was isolated using magnetic beads, (Dynabeads, Dynal, Norway), as instructed by the supplier, cDNA was obtained using SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. q-PCR was performed using Quantitect SYBR-Green Mastermix (Qiagen) on an Opticon 2 (Bio-Rad) and cycles consisted of 95°C, 15 min and 39 cycles of 94°C, 1 min 15 s, 60°C, 30 s, 72°C, 30 s.

The Following forward (fw) and reverse (re) primers were used:

NRAMP1 fw	GCCATCTCTACTACCCCAAGG
NRAMP1 re	TCATAGCCGAAGGTCAAAGC
GAPD fw	ATTGTCAGCAATGCATCCTG

GAPD re	ATGGACTGTGGTCATGAGCC
IL-12b fw	GGAAGCACGGCAGCAGAATA
IL-12b re	AACTTGAGGGAGAAGTAGGAATGG
IL-6 fw	GAGGATACCACTCCCAACAGACC
IL-6 re	AAGTGCATCATCGTTGTCATACA
TNFA fw	CCACCACGCTCTTCTGTCTAC
TNFA re	AGGGTCTGGGCCATAGAACT

Immunofluorescence (IF) and Confocal Microscopy

The distal 1cm of terminal ileum or ileal loops were removed from mice and transferred into PBS ++ (containing 1mM MgCl₂ + 1mM CaCl₂). Tissue samples remained unfixed, but were frozen in LN2 and 5 µm sections cut by Wax-it Histology Services, Inc. (Vancouver, Canada). Sections were then plunged into -20°C acetone for 5 minutes then air dried. The slides were re-hydrated with 5% normal goat serum (NGS) in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% Bovine Serum Albumin) buffer for 20 minutes at room temperature. Slides were then incubated overnight at 4°C with of primary antibodies. After incubation, the slides were washed 3 times 10 min each with TPBS/BSA buffer then incubated for 1–2 hours at 37°C with secondary antibodies conjugated to Alexa Fluorochroms. The slides were washed 3 times 10 min each with TPBS/BSA buffer, mounted with Vectashield Hard Set with DAPI (Vector Labs) or with in Prolong Gold Antifade reagent (Invitrogen) and analyzed using a Zeiss Axiophot epifluorescent microscope.

For confocal microscopy cells were first stained for CD11c and Nramp1 as described above for FACS analysis and then fixed in 2.5% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. Samples were washed extensively with PBS, mounted on glass slides using Prolong Gold Antifade reagent (Molecular Probes, Eugene, Oregon, USA) and analyzed by confocal microscopy using a 60× 1.4 N.A. objective. Images were captured and analyzed using Nikon C1 and Slidebook software (Intelligent Imaging Innovations, Denver, Colorado, USA).

Statistical analysis

Logrank test, unpaired T test, Mann-Whitney and one-way ANOVA with Kruskal-Wallis with and Dunn's post-test were performed using a 95% confidence interval. All analyses were performed using GraphPad Prism version 4.0.

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Figure 1 A

Figure 1B

Fig. 1. Nrampl is expressed in the small intestine

A. Immunofluorescence analysis of small intestine from Nrampl^{+/+} and ^{-/-} mice. Uninfected tissues were stained for Nrampl and DAPI and analyzed by microscopy. **B.** Nrampl gene expression in small intestine of uninfected and *Salmonella* infected mice. Nrampl^{+/+} mice were infected orally with *S. Typhimurium* and tissues were collected 24 hours after the infection, q-PCR was used to analyze the expression and normalized relative to expression of GAPD. Amplified bands were resolved in a 2% agarose gel.

Fig. 2. Nrp1^{-/-} mice succumb to *S. Typhimurium* and show higher bacterial load

A. Nrp1^{+/+} and ^{-/-} mice were orally infected with 3×10^6 CFU WT or with 1×10^8 $\Delta invA$ *S. Typhimurium*. Mice were monitored daily for development of disease and mortality and data analyzed using log-rank test. $P < 0.0001$ for WT and $P = 0.0041$ for *InvA*. Data are representative of 2 experiments.

B. Groups of mice Nrp1^{+/+} and ^{-/-} were infected orally with WT *S. Typhimurium*. Mice were sacrificed one or 3 days after infection, organs removed and homogenates were plated on LB agar. The data are compiled from 2 (for day 1 infected with WT and for day 3 infected with *invA*) or 3 independent experiments with > 4 mice/time point. SI: small intestine; PP: Peyer's patches; MLN: Mesenteric Lymph Nodes; SP: Spleen. Data was analyzed using Mann-Whitney test or one-way ANOVA with Kruskal-Wallis and Dunn's post-test.

Figure 3 A

Fig. 3. Nrp1 is expressed at highest level by CD103⁺ DCs in the small intestine

A. Tissue sections of ileal loops of uninfected or *S. Typhimurium* infected Nrp1^{+/+} and ^{-/-} mice, were stained for Nrp1 (red), CD11c (green) and *Salmonella* (blue) and analyzed by microscopy. Data are representative of two independent experiments.

B. Lamina propria DCs were isolated and different populations of cells were separated by FACS sorting as MHC II^{high}CD11c⁺CD103⁺, MHC II^{high}CD11c⁺CD103⁻, CD11c^{med}MHC-II^{high} and CD11c^{med} MHC-II⁻ cells, Nrp1 gene expression was assayed by q-PCR and normalized relative to expression of GAPD. Data is presented as the average of duplicate samples and the figure is representative of two independent experiments.

C. Nrp1 protein levels in CD103⁺ and CD103⁻ DCs (MHC II^{high}CD11c⁺) from the small intestinal lamina propria of uninfected Nrp1^{-/-} and Nrp1^{+/+} mice were measured by intracellular FACS analysis. Data are representative of two independent experiments.

D. 129sv Mice (Nramp1^{+/+}) were sham or infected orally with *S. Typhimurium* for 30, 60, 90 and 120 minutes. Nramp1 expression by intestinal DCs was measured by intracellular FACS analysis. The figure shows Nramp1 expression in CD103⁺ DCs uninfected or infected orally with *S. Typhimurium* for 90 minutes. This figure is representative of two independent experiments.

Figure 4 C, D, E and F

Fig. 4. Nramp1 is expressed in all the Major Subsets of DCs and is localized to Intracellular Membranes

A. Splenocytes from Nramp1 ^{+/+} mice were stained with antibodies against B cells (B220), MØ (F4/80), DC (CD11c) and CD8α⁺ DC (CD11c and CD8⁺). Cells were sorted and Nramp1 expression was evaluated by RT-PCR. Data are representative of two independent experiments.

B. Single cell suspensions were obtained from spleens of Nramp1^{+/+} mice. The cells were stained with antibodies against CD11c, CD4, CD8 and Nramp1 and analyzed by intracellular FACS analysis. Data are representative of three independent experiments.

C. Nramp^{+/+} and ^{-/-} BMDCs were sorted for the high expression of CD11c and MHC-II and low 7AAD. One fraction of cells was infected with *S. Typhimurium* and RT-PCR of the

sorted cells was performed for Nramp1 and GAPD. Data are representative of 5 independent experiments.

D. BMDCs from Nramp1^{+/+} and ^{-/-} mice analyzed for Nramp1 via intracellular FACS staining. Numbers indicate the percentage of cells in each quadrant. The data is representative of 5 experiments. **E.** The expression of Nramp1 by non-infected or 24 hours *S. Typhimurium* infected BMDC from Nramp1^{-/-} and ^{+/+} was assessed by intracellular FACS staining **F.** BMDC from uninfected and *S. Typhimurium* infected Nramp1^{+/+} and ^{-/-} were stained with CD11c (red) and Nramp1 (green) and analyzed by confocal microscopy. The figure shows Nramp^{+/+} BMDC.

Figure 5 A and B

Figure 5C and D

Fig. 5. Nrp1^{+/+} DCs and macrophages secrete more inflammatory cytokines than Nrp1^{-/-} cells

A. CD103⁺ and CD103⁻ DCs were FACS sorted from small intestine of uninfected mice or infected orally for 2 hours with *S. Typhimurium*. IL-6, IL-12 and TNF-α gene expression was analyzed by q-PCR and normalized to the expression of GAPD. Data is shown as an average of triplicate samples, with the exception of IL-6 secretion by CD103⁺ cells in which only duplicate samples were analyzed.

B. CD103⁺ and CD103⁻ DCs were FACS sorted from Nrp1^{+/+} and ^{-/-} mice. IL-6 and TNF-α secretion was assessed using luminex beads. Triplicate samples of CD103⁻ DCs were treated with *S. Typhimurium* at MOI=1 or MOI=5, duplicate samples were assessed with heat killed (HK) or left unstimulated. Data was analyzed using unpaired T test. For CD103⁺ DCs, only duplicates or single wells were assessed and treated as for CD103⁻ DCs.

C. BMDC from Nrp1^{+/+} and ^{-/-} were infected with *S. Typhimurium* MOI=10 or mock infected and supernatants were taken at indicated time points IL-6, IL-12 and TNF-α secretion was measured by ELISA. CFU at time of cytokine assessment are for Nrp1^{-/-} at 2 hours = 81000 ± 6557.4 at 6 hours = 99333.3 ± 2565.8 and 24 hours = 68000.0 ± 5766.3. For Nrp1^{+/+} at 2 hours = 83166.7 ± 2081.7 at 6 hours = 79333.3 ± 10396.3 and 24 hours = 48000.0 ± 14756.4. Data was analyzed using unpaired T test. Data is representative of 3 experiments.

D. BMMφ from Nrp1^{+/+} and ^{-/-} were infected with *S. Typhimurium* or mock infected and supernatants were taken at indicated time points IL-6, IL-12 and TNF-α secretion was measured by ELISA. Data was analyzed using unpaired T test. Data is representative of 3 experiments.