THE ROLE OF NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 1 (Nramp1) IN SALMONELLOSIS

by

YANET VALDEZ

B.Sc. The National University of San Antonio Abad, Cusco, Peru 1994
M.Sc. Cayetano Heredia University, Lima, Peru 2001

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ABSTRACT

Salmonellosis poses a global threat to human health. Host resistance against *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) in the murine model is mediated by Natural resistance-associated macrophage protein 1 (Nramp1/Slc11a1). Nramp1 is critical for host defense, as mice lacking Nramp1 fail to control bacterial replication and succumb to low doses of *S*. Typhimurium. Despite this critical role, the mechanisms underlying Nramp1’s protective effects are unclear.

This thesis presents a detailed analysis of Nramp1 expression in the murine gastrointestinal tract and its impact on *S*. Typhimurium infection following oral infection. Dendritic cells (DCs) that sample the intestinal lumen are among the first cells encountered by *S*. Typhimurium and play an important role in *Salmonella* pathogenesis. Intestinal, splenic and bone marrow derived DCs (BMDCs) all expressed Nramp1 protein. In intestinal DCs, Nramp1 expression is restricted to a discrete subset of DCs (CD11c^+^ CD103^-^) that express elevated levels of pro-inflammatory cytokines in response to bacterial products. While Nramp1 expression did not affect *S*. Typhimurium replication in DCs, infected Nramp1^+/+^ DCs secreted more inflammatory cytokines (IL-6, IL-12 and TNF-α) than Nramp1^-/-^ DCs. This suggests that Nramp1 expression promotes accelerated inflammatory responses to *S*. Typhimurium. This hypothesis was tested using the *Salmonella*-induced colitis model, where pre-treatment of mice with antibiotics enhances colonization of the cecum/colon and induces massive inflammation. We found that Nramp1^+/+^ mice mounted a faster and more robust inflammatory response characterized by elevated pro-inflammatory cyto/chemokines (IFN-γ, TNF-α and MIP1-α) and recruitment of neutrophils and macrophages, thereby limiting spread of *S*. 
Typhimurium to systemic sites and ultimately protecting the host.

Nramp1+/+ mice also developed a chronic *Salmonella* infection of the gastrointestinal tract that led to severe tissue fibrosis. Intestinal fibrosis is a serious complication of Crohn’s disease, often requiring surgical intervention but the mechanisms underlying its development are poorly understood due to the lack of relevant animal models. A novel model of severe and persistent intestinal fibrosis caused by chronic bacterial induced colitis was developed. Since the pathology closely resembles human fibrosis, we present a valuable tool for investigating host and bacterial contributions to inflammatory bowel diseases, as well as infectious colitis.
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>BMDC</td>
<td>Bone marrow derived dendritic cells</td>
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<tr>
<td>BMMØ</td>
<td>Bone marrow derived macrophage</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<tr>
<td>CFU</td>
<td>colony forming units</td>
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<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FAE</td>
<td>follicle associated epithelium</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
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<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosine</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel diseases</td>
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<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
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<tr>
<td>IFN-γ</td>
<td>interferon-γ</td>
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<td>IgA</td>
<td>immunoglobulin A</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IL (e.g. IL-1)</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KC</td>
<td>keratinocyte chemoattractant</td>
</tr>
<tr>
<td>L</td>
<td>lumen</td>
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<tr>
<td>Lamp1</td>
<td>lysosome-associated membrane protein</td>
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<td>LM</td>
<td>lamina propria</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>M</td>
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<td>M6PR</td>
<td>mannose-6-phosphate receptor</td>
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<td>MAPK</td>
<td>MAP kinase</td>
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<tr>
<td>M cell</td>
<td>Microfold cell</td>
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<tr>
<td>MHC-II</td>
<td>major histocompatibility complex II</td>
</tr>
<tr>
<td>MLN</td>
<td>mesenteric lymph node</td>
</tr>
<tr>
<td>MP</td>
<td>muscularis propriae;</td>
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<td>MIP-1α</td>
<td>monocyte chemoattractant protein-1</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<td>SM</td>
<td>submucosa</td>
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<tr>
<td>MØ</td>
<td>macrophage</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<tr>
<td>Nramp</td>
<td>Natural resistance-associate macrophage protein</td>
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<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pi</td>
<td>post-infection</td>
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<tr>
<td>phox</td>
<td>phagocyte oxidase</td>
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<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
<td>PP</td>
<td>Peyer’s patches</td>
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<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>PTP</td>
<td>protein tyrosine phosphatases</td>
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<tr>
<td>q-PCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RNI</td>
<td>reactive nitrogen intermediate</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediate</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>SCV</td>
<td>Salmonella containing vacuole</td>
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<tr>
<td>S. enterica</td>
<td>Salmonella enterica</td>
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<tr>
<td>S. Enteritidis</td>
<td>Salmonella enterica serovar Enteritidis</td>
</tr>
<tr>
<td>SCL11A1</td>
<td>solute carrier family of multimembrane spanning protein 11</td>
</tr>
<tr>
<td>SI</td>
<td>small intestine</td>
</tr>
<tr>
<td>Sp</td>
<td>spleen</td>
</tr>
<tr>
<td>SPI</td>
<td>Salmonella pathogenicity island</td>
</tr>
<tr>
<td>Stat1</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-β1</td>
</tr>
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<td>TNBS</td>
<td>trinitrobenzene sulfonic acid</td>
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<tr>
<td>TNFα</td>
<td>tumour necrosis factor-α</td>
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<td>T3SS</td>
<td>type three (III) secretion system</td>
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<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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</table>
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CO-AUTHORSHIP STATEMENT

Some chapters of this thesis have been published in collaboration with other members of the Dr. Finlay’s and Dr. Vallance’s laboratories at UBC and in collaborations with the external laboratories of Dr. Philippe Gros at McGill University and Dr. Dan Littman at New York University School of Medicine. Formal contributions of the authors are as follow:

Chapter 2: I designed and performed all the experiments described in this chapter with the following exceptions: Gretchen E. Diehl, performed the experiment shown in the figure 2.6 B. Bruce Vallance guided and trained me in mouse infections and mouse ileal loop analyses. Guntram A. Grassl helped performing experiments in vivo and ELISA assays after in vitro infection of dendritic cells and macrophages with Salmonella. Julian A. Guttman offered guidance in immunofluorescence tissue staining and made micrographs of the ileal loops that I prepared (figure 2.3A). Nat F. Brown capture confocal images of dendritic cells that I prepared and stained (figure 2.4F), and he trained me in confocal microscopy. Carrie M. Rosenberger designed the PCR primers use for Nramp1 expression analysis. Mice were provided by the Philippe Gros laboratory and I received training in the isolation of intestinal dendritic cells in Dan Littman’s laboratory. I was primarily responsible for writing the manuscript with important input, suggestions and editing by the co-authors Bruce A. Vallance, Dan R. Littman, Philippe Gros and Brett Finlay.
Chapter 3: Guntram A. Grassl and I contributed equally to designing and performing the experiments as well as preparation of figures and tables for publication. Julian A. Guttman took pictures of infected tissues stained by me and he prepared figure 3.1A. Bryan Coburn performed the histopathology scoring. I was primarily responsible for writing the manuscript with guidance and intellectual input from co-authors Philippe Gros, Bruce A. Vallance and Brett Finlay.

Chapter 4: Guntram A. Grassl and I contributed equally to designing and performing the experiments as well as preparation of the figures for this publication. We received guidance and intellectual input from Bruce A. Vallance. Kirk S. B. Bergstrom performed the tissue staining and took the pictures shown in figures 4.3 F and 4.4A and B. Guntram Grassl was primarily responsible for writing the manuscript with important input from myself, Bruce A. Vallance and Brett Finlay.
Chapter 1

INTRODUCTION\(^1\)

Infectious diseases are a major cause of mortality in the world, particularly in developing countries where poverty and associated factors like malnutrition and poor sanitary conditions allow their development and persistence [1]. With the constant threat of antibiotic resistance, rapid dispersion via ever-increasing intercontinental travel, emergence of new pathogens, and re-emergence of old pathogens, there is an increasing necessity for a greater understanding of host-pathogen interactions [2]. The lifestyle of many of these pathogens requires them to establish infection in the face of a well-adapted host immune response. Upon entering a host, a series of interactions occur between host and pathogen and the constant interplay between these participants will determine the course and outcome of the infection [3]. Although there are many ongoing efforts to understand these complex interactions, the mechanisms by which disease initiates and progresses remain poorly understood [4]. Studying the host-pathogen relationship offers a way of understanding the process of disease progression, which in turn we hope will lead to the design of appropriate and effective strategies in order to control or eradicate diseases.

1.1 *Salmonella Typhimurium* (*S. Typhimurium*)

Salmonelloses are diseases caused by *Salmonella* species. *Salmonella* are Gram-negative, facultative intracellular bacteria. The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella bongori* resides

\(^1\) A version of this chapter has been submitted for publication as: Yanet Valdez, Rosana B. R. Ferreira and B. Brett Finlay (2009). Molecular mechanisms of *Salmonella* virulence and host resistance.
primarily in reptiles and infrequently causes disease in warm blooded animals. The species *Salmonella enterica* contains over 2000 serovars [5] a few of which are etiological agents of two major human diseases causing significant morbidity and mortality worldwide, particularly in the developing world. *Salmonella enterica* serovar Typhi (*S. Typhi*) and Paratyphi (*S. Paratyphi*), cause typhoid fever, a systemic disease characterized by fever, intestinal perforation and hemorrhage, enlargement of the mesenteric lymph nodes (MLN), spleen and liver [6]. *S. Typhi* is a host restricted, highly adapted human pathogen and does not cause disease in animals. Humans are the major reservoir for *S. Typhi*, which is spread through the fecal-oral route, usually by ingestion of food or drinking water contaminated with the bacteria. In the absence of complications, the disease usually is resolved after 4 months, although asymptomatic maintenance and shedding of the bacteria can continue in a minority of individuals (1-6%) for up to a year or longer [7]. The disease is endemic in Asia, Africa and South America with an estimated incidence of 17-21 million cases worldwide each year, leading to 600,000 deaths [8].

*Salmonella enterica* serovars Enteritidis (*S. Enteritidis*) and Typhimurium (*S. Typhimurium*) cause gastroenteritis or “food poisoning”, a self-limiting disease characterized by diarrhea with abdominal pain, nausea, vomiting and fever. Acute enteritis is characterized by mucosal edema and inflammation mostly of the large intestine with recruitment of polymorphonuclear leukocytes (PMN) [9]. Symptoms occur between 6 to 72 hours after consumption of contaminated animal products such as chicken or eggs, last for up to a week, and resolve spontaneously. Enteritis represents a major economic problem worldwide for both man and animals and is one of the most
frequent causes of bacterial food-borne disease in North America [10]. Immunocompromised and very young children are particularly vulnerable to severe forms of enteritis, and in isolated cases the infection can spread to systemic sites resulting in death [11].

1.2 Animal models and overview of the disease

Animal models are frequently used to develop an improved understanding of the complex mechanisms that lead to salmonellosis in humans [9, 10]. These models have been invaluable in defining both the bacteria’s key virulence factors and the host’s responses, thereby clarifying the process and outcome of these diseases. Many models are used to study the two major human diseases, typhoid fever and gastroenteritis, and they encompass human volunteers, monkeys, calf, cow ileal loops, rabbits, rodents and the nematode *Caenorhabditis elegans* [9, 12, 13]. The most widely used animal model is the mouse model, since it offers genetic homogeneity, along with well-defined genetic mutants that permit study of specific genes, cell types and pathways in host-pathogen interactions [14]. Below, a description of the mouse models related to this work is presented for the study of both typhoid fever and gastroenteritis.

1.2.1 The typhoid model

To study the pathogenesis of human typhoid fever a surrogate host (genetically susceptible inbred mice) and bacterium (*S. Typhimurium*) are used, providing a useful model for this disease (reviewed in [9]). *S. Typhi* does not naturally infect rodents while *S. Typhimurium* is a natural mouse pathogen. Importantly, the pathology associated with *S. Typhimurium* infection of mice closely resembles that of *S. Typhi* in humans. Hallmarks include elevated temperature 4-8 days after oral infection (indicated by ruffled
fur), enlarged Peyer’s patches and thickening of ileal mucosa. Other areas of the small intestine show a diffuse enteritis characterized by mononuclear cell infiltration, with no signs of intestinal epithelium destruction (reviewed in [6]). Like humans infected with S. Typhi, S. Typhimurium infection in mice leads to disseminated infection and multiplication of bacteria in the liver and spleen where large granulomatous lesions develop around bacteria-infected macrophages. For all these reasons the S. Typhimurium model has become the accepted model for human infections with S. Typhi.

It is important, to note a key shortcoming of this model, however, that being that S. Typhimurium, will infect humans but leads to enteritis rather than the typhoid fever like symptoms described above. This would suggest that in the context of different hosts, the same pathogen can cause widely different symptoms. Also, it is known that many of the bacterial pathogenicity genes are not shared between S. Typhi and S. Typhimurium. Therefore, although S. Typhimurium can be used to model the pathology associated with S. Typhi infections in human, it is not an ideal model to study the role of specific bacterial pathogenesis genes and extrapolate to their role in human disease. In fact, genes that are present in S. Typhi required to cause typhoid fever in humans, are absent in S. Typhimurium or vice versa. With this important caveat in mind, however, the S. Typhimurium model is useful in studying general host responses to typhoid fever type infections.

With regard to the specifics of infection in the murine typhoid model, orally ingested S. Typhimurium cross the intestinal barrier by 3 mechanisms: 1) invasion of specialized epithelial cells, termed M cells, that are situated in the Peyer’s patches and are known to sample antigens from the intestinal lumen, 2) active invasion of enterocytes, and 3)
uptake by intestinal dendritic cells (DCs) that express tight-junction proteins and extend dendrites between epithelial cells for direct bacterial uptake (Figure 1.1) [15-18].

Figure 1.1 Strategies that allow *Salmonella* to cross the intestinal barrier, survive in intestinal tissues and spread systemically. *Salmonella* cross M (microfold) cells of the follicle-associated epithelium mainly in the Peyer’s patches of the ileal portion of the small intestine. *Salmonella* virulence factors provide the bacteria with the capacity to both invade epithelial cells and to disseminate systemically. Alternatively, *Salmonella* can also directly enter intestinal cells by the apical pole of the cell or be captured by dendritic cells that emit pseudopods between epithelial cells. The latter process may promote systemic dissemination. Reproduced from Sansonetti et al. [18]
Once the bacteria cross the mucosal epithelia, they encounter cells of the gut-associated lymphoid tissue (GALT) that include DCs, macrophages (MØ), B, and T cells [19]. Contact of S. Typhimurium with these cells initiates a series of interactions between the bacteria and the host cells that initiates the disease [20]. *Salmonella* gain access to the host circulation within CD18+ cells [15]. Whether these represent MØ, DCs, or other myelomonocytic cells remains to be clarified. The bacteria then reach mesenteric lymph nodes (MLN), spleen and liver and replicate within phagocytic cells in these organs to levels that induce sepsis in susceptible mice.

*S. Typhimurium* can survive within phagocytic and non-phagocytic cells. During late stages of infection in vivo, *Salmonella* can be found within macrophages [21, 22], DCs [23], neutrophils [24, 25], B cells, T cells and hepatocytes [25, 26]. *S. Typhimurium* resides in granulomatous foci in the spleen and liver, predominantly within phagocytes. The dynamics of *S. Typhimurium* spread in the body at the level of individual infected cells is poorly understood. It has been suggested that, in the initial stages of the infection, each focus of infection consists of an individual phagocyte containing only one bacterium and that *Salmonella* growth in the tissues results in the continued passage of the bacteria to uninfected cells. Thus, *S. Typhimurium* growth in the tissues appears to result in an increased number of infected foci, with only a small increase in the number of bacteria per cell [27]. The increase in the number of infected foci triggers inflammatory responses, responsible for the recruitment and priming of phagocytes, cytokine release, hepatosplenomegaly, sepsis and ultimately leads to the death of susceptible animals [28, 29]. Chronic carriers have been reported in *S. Typhimurium* infections of resistant mice (Nramp1+/+, see below) [7, 22]. Although this model is just beginning to be explored, it
has enormous potential, since it may more accurately reflect the naturally occurring \textit{S. Typhi} carrier state in humans [7].

\subsection{1.2.2 The enterocolitis model}

\textit{S. Typhimurium} infection of calves and of cow ileal loops can induce gastroenteritis with clinical manifestations similar to those found in humans. Although the information obtained from these infections is valuable, the use of these animals presents serious limitations for extensive experimentation. Cattle are outbred, thus creating an inherent variability between subjects. In addition, large animal models are expensive, making the use of these animals costly and less than ideal. This issue was overcome with the development of a mouse model for enterocolitis [30]. This model relies on the premise that oral infection of mice with \textit{S. Typhimurium} results in modest colonization of the intestine and with little or no inflammation. This was referred to as “colonization resistance”. However, this resistance can be disrupted by treatment of mice with antibiotics prior to infection with \textit{S. Typhimurium}, resulting in a major increase in bacterial colonization. Although the molecular mechanisms responsible for colonization resistance are still under debate, it is believed that the antibiotics transiently alter the resident gut microflora, allowing the colonization of the cecum and colon by the incoming pathogen. This colonization is accompanied by significant inflammation that is characterized by the infiltration of PMNs [30] and intestinal pathology and pathophysiology that culminates in watery stools [31]. These changes closely resemble the features of enterocolitis in humans [32]. This model is now being used extensively to examine the course of the disease in a large number of genetically uniform inbred mice. This enables the study of specific cell signaling pathways and the contribution of specific
cells of the immune system in the development of colitis. It also provides a means of assessing S. Typhimurium mutant strains for their ability to induce colitis or modify the disease course [33-35]. However, a caveat of this model is the development of “typhoid–like” disease that occurs in parallel to colitis, rendering mice susceptible to systemic infection and death. Thus, this model generates a mixed symptomatology of both typhoid fever and colitis. More recently, the use of genetically-resistant mice has provided valuable information about the dynamics of S. Typhimurium and host interactions at later time points, thereby providing information on the resolution of the “colitis-like” component of the disease [31, 36, 37].

1.3 S. Typhimurium virulence determinants

S. Typhimurium, as a pathogen, has acquired an arsenal of genes that are required to establish a successful infection within the host. Many of the virulence traits of Salmonella are directly linked to genes encoded within large regions of the bacterial chromosome called Salmonella pathogenicity islands (SPIs). Pathogenicity islands are discrete chromosomal regions harboring virulence genes. These are common attributes of Gram-negative bacterial pathogens and encode virulence factors, together with machinery for their regulation and secretion. Such pathogenicity islands are absent from related, non-pathogenic species [38]. Pathogenicity islands have a DNA G+C contents that are noticeably different from the rest of the bacterial genome, likely indicating horizontal transmission from other bacteria at some point during evolution. S. Typhimurium contains at least 10 identified SPI, termed SPI-1, SPI-2, etc. SPI-1 and SPI-2 are of
particular relevance for pathogenesis, since mutations in these genes impair the bacteria’s ability to induce infection in their host [39]

The pathogenicity of S. Typhimurium is contingent on specialized machinery called a type III secretion system (T3SS). Effectively, these are molecular syringes used to deliver virulence proteins (effectors) directly into the host cells where they modify many aspects of host cell function [40, 41]. S. Typhimurium possesses two distinct virulence-associated T3SSs encoded within SPI-1 and SPI-2. Each of the T3SSs are used to translocate a unique set of effectors during different phases of the infection in order to manipulate various host pathways (reviewed in [39, 42]).

1.3.1 Salmonella pathogenicity island 1 (SPI-1)

SPI-1 is present in both S. enterica and S. bongori and it is has been hypothesized that its acquisition allowed Salmonella to become an enteric pathogen [42]. SPI-1 mutants are attenuated for oral but not intraperitoneal (systemic) infections in the murine typhoid model [43] and display attenuated enteropathogenicity in bovine ileal loops [10]. SPI-1 effectors influence a variety of host cell functions such as cytoskeletal rearrangements (e.g. ruffling) that mediate bacterial uptake by epithelial cells [39], tight junction disruption [44-46], macrophage apoptosis and neutrophil recruitment [47, 48]. SPI-1 induces inflammation in the murine and bovine enterocolitis models as well as in rabbit ileal loops [10]. Thus, SPI-1 appears to be important for the initial steps of active Salmonella invasion following oral infection of epithelial cells as well as the consequent inflammatory cascade characteristic of intestinal salmonellosis.
1.3.2 *Salmonella* pathogenicity island 2 (SPI-2)

SPI-2 is present in *S. enterica* but absent in *S. bongori* and its acquisition is thought to have been a key step in the evolution of *Salmonella* as a systemic and intracellular pathogen [42]. In contrast to SPI-1, the SPI-2 T3SS does not appear to play a role in the early phase of infection. Instead it is thought to play a role in the later, systemic phase of the disease. SPI-2 mutants are severely attenuated for virulence in the mouse typhoid model and fail to proliferate in internal organs [49]. Thus SPI-2 is essential for intracellular replication, which is necessary for systemic disease [50-52]. Specific defects attributed to SPI-2 mutants are a reduced ability to survive in macrophages [52] perhaps due to a failure to form the *Salmonella* containing vacuole (SCV). This vacuole is thought to be a unique intracellular niche in which *Salmonella* survives and replicates [53]. SPI-2-mediated secretion impairs trafficking of the oxidase-containing vesicles [54-56] and iNOS to the SCV [57]. These are important mediators of the oxidative and nitrosative burst, thereby enhancing the survival of *Salmonella* within macrophages. SPI-2 also inhibits antigen presentation and T cell activation by DCs [58-60]. Thus, SPI-2 allows *Salmonella* to avoid intracellular killing by both the innate and the adaptive immune system.

1.4 Host response to *Salmonella* Infection: Resistance/Susceptibility genes

Susceptibility to *S. Typhimurium* in mice is determined by virulence factors expressed by bacteria as well as by the host genetics [61]. Additional experimental factors can influence the severity of the disease. These include route of infection, dose, immunological status, and stress of the host.
The host response to systemic *S.* Typhimurium infection is complex and it is under the influence of many genes. Several of the genes conferring susceptibility have been identified and a table describing some of the most informative examples of mouse susceptibility is presented (See Table 1.1). These are grouped according to “phases” that correspond to immunological events described by Mastroeni [29]. Compiling studies using sublethal infections, Mastroeni divided the course of the infection into four distinct phases (see Figure 1.2). The **first phase** involves rapid clearance of the bacteria from the bloodstream (within 2 hours after oral infection). After clearance from the circulation, *Salmonella* reach intracellular locations within macrophages, polymorphonuclear cells, and DCs in the spleen and liver. Although phagocytes kill some of the bacteria in the next few hours, the surviving bacteria undergo exponential replication, initiating the **second phase**. The growth rate in this phase is determined by the size of the inoculum and the innate resistance of the host. The natural resistance-associated macrophage protein 1 (Nramp1), plays a critical role in controlling *Salmonella* replication in this phase [62]. Other mediators such as reactive oxygen intermediates are also crucial for *Salmonella* killing in this phase. The **third phase** is initiated by the activation of the innate immune system, and is characterized by the production of several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin-12 (IL-12). These arise as a consequence of signaling by pattern recognition receptors (PRR) on the immune cells that recognize pathogen-associated molecular patterns (PAMPS) such as LPS. The action of these cytokines is essential for activation of immune cells, which in turn produce antimicrobial mediators such as reactive nitrogen species, which suppress bacterial growth (plateau phase). This phase is
also important for activation of the adaptive immune response. The **fourth phase**, or resolution of the infection, is a function of the adaptive immune system. The key players in this phase are antigen presenting cells, B, and T cells and the activation of the antigen-specific humoral immune response (antibody production) as well as T-helper and cytotoxic T-lymphocyte responses. If these responses are not efficient, a relapse or carrier state can develop in the host [29].
Figure 1.2 The four phases of a primary *Salmonella* infection. Solid lines show the course of a sublethal infection in wild type (WT) immunocompetent mice. Dotted lines show the course of the infection when the immunological mechanisms required at points A-D are absent. The Nramp1 gene and reactive oxygen species (ROS) influence the net growth rate and their absence causes a shift of the curve from B-C to B-B1. Point C coincides with the onset of the cytokine response. The lack of any of the immunological factors in C and D determines failure to suppress bacterial growth in the tissues and the unrestrained progression of the infection process. Point D coincides with the intervention of antigen-specific immunity that is required to clear the infection and prevent relapse (D) or the establishment of a chronic carrier state (D2). Reactive nitrogen species (RNS), Toll-like receptors (TLR) and Antigen presenting cells (APC). Modified from Mastroeni [29].
Table 1.1 Key host genes conferring susceptibility in the murine typhoid model

<table>
<thead>
<tr>
<th>Phases of <em>Salmonella</em> infection</th>
<th>Gene targeted mice</th>
<th>Deficiency function/ Mechanism of action</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase 2</strong></td>
<td>- <em>gp91phox</em>&lt;sup&gt;−/−&lt;/sup&gt; mice (NADPH oxidase)</td>
<td>Dramatic exacerbation of <em>Salmonella</em> infection in the early phase of infection by inability to produce ROIs</td>
<td>[63]</td>
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<tr>
<td></td>
<td>- Natural mutation in Nramp1 mice: BALB/c, C57BL/6, DBA/1 &lt;br&gt; - <em>Nramp1</em>&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Failure to control bacterial growth in the exponential phase, although controversial, it is believed that Nramp1 alters the intravacuolar environment of the SCV</td>
<td>[61, 64, 65]</td>
</tr>
<tr>
<td></td>
<td>- Natural mutation in TLR4 mice: C3H/HeJ &lt;br&gt; - <em>lbp</em>&lt;sup&gt;−/−&lt;/sup&gt; mice (LPS protein binding) &lt;br&gt; - <em>Cd14</em>&lt;sup&gt;−/−&lt;/sup&gt; mice &lt;br&gt; - <em>Tlr4</em>&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Deficiency in induction of a rapid inflammatory response, decreased expression of TNF-α and IL-6</td>
<td>[66, 67]</td>
</tr>
<tr>
<td></td>
<td>- <em>Nos</em>&lt;sup&gt;−/−&lt;/sup&gt; mice (inducible nitric oxide synthase)</td>
<td>Mice can control early <em>Salmonella</em> replication, but are unable to suppress bacterial growth later and die earlier than control mice, deficient in generation of RNI</td>
<td>[29, 63, 68]</td>
</tr>
<tr>
<td></td>
<td>- <em>Tnfr55</em>&lt;sup&gt;−/−&lt;/sup&gt; (TNF deficient mice)</td>
<td>Fail to localize NADPH oxidase-containing vesicles to SCV leading to impaired bacterial killing</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>- <em>Ifngr</em>&lt;sup&gt;−/−&lt;/sup&gt; (IFNg deficient mice)</td>
<td>Failure to form focal granulomas during <em>Salmonella</em> infection. Inability to activate phagocytes and to influence antibody class switching.</td>
<td>[69]</td>
</tr>
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<td></td>
<td>- <em>Il12a</em>&lt;sup&gt;−/−&lt;/sup&gt; and <em>Il12b</em>&lt;sup&gt;−/−&lt;/sup&gt; (IL-12 deficient mice) &lt;br&gt; - neutralizing antibodies to IL-12</td>
<td>Deficiency in the production of IFNγ and increase production of IL-10 and IL-4</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>- <em>Il-6</em>&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Lower levels of IgA against <em>Salmonella</em> LPS</td>
<td>[71]</td>
</tr>
<tr>
<td><strong>Phase 3</strong></td>
<td>- <em>cd28</em>&lt;sup&gt;−/−&lt;/sup&gt; mice and &lt;br&gt; - <em>tcr-b</em>&lt;sup&gt;−/−&lt;/sup&gt; mice (lacking mature T a/b T cells)</td>
<td>Impaired T cells activation and reduced T-B cells activation, higher bacterial load and deficient in <em>Salmonella</em> clearance</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>- <em>xid mice</em> (impaired B cell activation and function) and &lt;br&gt; - <em>Igh-6</em>&lt;sup&gt;−/−&lt;/sup&gt; (B cell deficient mice)</td>
<td>Defect in antibody production and deficiency in establishment of protective long-lasting Th1 type T cell immunity to <em>Salmonella</em></td>
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</table>
Due to its specific relevance to this thesis, a detailed review of the Nramp1 gene product, and its role in salmonellosis, is presented below.

1.5 Nramp1

Natural resistance to S. Typhimurium, *Mycobacterium bovis* BCG and *Leishmania donovani* in mouse was described over 30 years ago. Three independent groups reported that inbred mouse strains could be segregated according to their susceptibility or resistance to these pathogens. This is phenotypically manifested by differential growth rates of the pathogen in the spleen and liver in the early phase of infection [72, 73]. This phenomenon led to the hypothesis that resistance to unrelated intracellular pathogens was conferred by a single locus or a group of tightly linked loci termed *Ity/Bcg/Lsh*. Experiments using F1 backcrosses and F2 generations of hybrids between resistant and susceptible inbred strains demonstrated later that a single gene, mapped to mouse chromosome 1, conferred resistance to the unrelated pathogens [74, 75] and (reviewed in [76]). Further positional cloning experiments isolated a candidate gene for *Ity/Bcg/Lsh*. The mRNA for this novel gene was described as being expressed exclusively in the spleen and liver and was enriched in the macrophages derived from these organs. The gene was named, Nramp1 (natural resistance-associated macrophage protein 1) [77, 78], and analysis of knock-out [64] or transgenic mice *in vivo* [65], as well as transfected macrophage cell lines *in vitro* [79], confirmed its function.
1.5.1 *Nramp1 Structure*

Identification of the mouse Nramp1 gene established that the Nramp1 belongs to an ancient family of proteins with homology to membrane-bound transporter proteins that are conserved in bacteria, plants, yeast, insects and mammals [80]. In mice, *Nramp1* maps to chromosome 1, the gene is composed of 15 exons and it spans 11.5Kb of genomic DNA [81]. It encodes a 90 to 100 kDa protein with: 10-12 transmembrane domains, a heavily glycosylated extracellular loop, several phosphorylated sites, a proline and serine rich putative Src homology (SH3) binding domain, and a consensus transport signature [82] (figure 1.3). Sequence analysis of Nramp1 cDNA clones from inbred mouse strains determined that susceptibility to infection was associated with a single nucleotide change resulting in a glycine to aspartic acid substitution at position 169 (G169D) within the predicted transmembrane 4 helix [83].
Figure 1.3 Predicted topology of Nramp1. The amino acid sequence of the Nramp1 protein is shown, using single color letter code. The number, position and polarity of the 12 predicted trans-membrane domains are shown with respect to the cytoplasmic face and the luminal face of the lysosome. Modified from Lam-Yuk-Tseung and Gros [84].
Initial experiments showed that *Nramp1* mRNA was expressed in phagocytes, displaying a striking pattern of tissue- and cell-specific expression in mice and humans [77, 85, 86]. *Nramp1* is highly expressed in the spleen and liver, particularly in the membrane fraction of cells of monocyte/macrophage and granulocyte lineages [86]. In addition, we recently characterized the expression of *Nramp1* in the lamina propria of the small intestine and found that *Nramp1* is expressed in a subset of DCs [87] (See chapter 2 and 3).

The subcellular localization of *Nramp1* was analyzed by double immunofluorescence studies [88], which showed that it is recruited to the late endocytic compartment after phagocytosis of latex beads in macrophages. Confocal microscopy in DCs revealed colocalization with Lamp1⁺ compartments [89]. Granule fractionation experiments showed that *Nramp1* is present in the membrane of gelatinase-positive tertiary granules in neutrophils [90]. Together, these experiments suggest that *Nramp1* is recruited to endocytic compartments placing it in close proximity to intracellular pathogens.

1.5.2 *Nramp1 Function*

Although *Nramp1* has been convincingly linked to resistance to intracellular microorganisms for over 20 years, how this resistance is achieved is still an area of controversy. Several models of how *Nramp1* confers resistance to *S. Typhimurium* are delineated below.
- **Nramp1 as a cation transporter modifying the intraphagosomal environment**

Insights about Nramp1’s function as a divalent cation transporter came from studies of homologous proteins, in particular from a second protein termed Nramp2 [91]. Nramp2 is an ion transport protein, expressed at the cell membrane and in recycling endosomes of many cells especially, enterocytes [92]. Studies of Nramp2-/- mutant mice (mk mouse) and the Belgrade rat showed that these animals have impaired intestinal iron uptake and deficient iron metabolism, leading to very severe microcytic anaemia. The function of Nramp2 was further determined by experiments in which the protein was expressed in Xenopus oocytes. These studies revealed a pH-dependent transport function for many divalent cations including Fe$^{2+}$, Zn$^{2+}$, Mn$^{2+}$ and Cd$^{2+}$ [93]. Nramp1 homologues have been identified in bacteria and yeast and are involved in acquiring divalent metals from the extracellular environoment (reviewed in [94]). Based on the documented ability of Nramp1 to transport divalent metal ions, and its sequence homology to member of the solute carrier family of multimembrane spanning proteins, Nramp1 was recently renamed SCL11A1 [95].

Recently, it was proposed that Nramp1 affects intraphagosomal microbial replication by modulating divalent cation content in this organelle. Two biochemical functions for Nramp1 affecting intracellular survival and replication were suggested. One is that Nramp1 functions to deprive intraphagosomal bacteria of the availability of Fe$^{2+}$ (critical for growth) and other divalent cations (Mn$^{2+}$ and Zn$^{2+}$), which are cofactors for superoxide dismutase, catalase and peroxidase, enzymes that are critical for intraphagosomal bacteria to mount an effective antioxidant defense [96-98]. An
alternative hypothesis is that Nramp1 increases the intraphagosomal Fe\(^{3+}\) in order to provide the catalyst for the Fenton/Haber-Weiss reaction, in which iron interacts with superoxide and hydrogen peroxide to generate highly reactive and extremely damaging hydroxyl radicals [99-101]. Thus the direction of substrate cation transport of remains controversial.
Figure 1.4 Metal transport by Nramp1 at the interface of host-pathogen interaction. Nramp1-mediated efflux of divalent metals from the lumenal space (according to one model). Nramp bacterial homologues compete with host Nramp1 by importing divalent metal from the phagosome space. Reproduced from [84].
• **Nramp1 altering the vesicular transport**

It has been proposed that Nramp1 can facilitate bacterial killing by modifying phagosomal maturation [102]. A few studies suggest that Nramp1 alters intracellular vesicular trafficking in macrophages. *Salmonella* are known to survive and replicate inside cells by creating a particular vesicle, known as the SCV. This vesicle doesn’t undergo the normal endocytic vesicle maturation and thereby *Salmonella* avoids lysosomal degradation [103]. In Macrophages the SCV transiently interacts with normal early endosomal compartments and matures into a modified late endosomal/lysosomal compartment expressing the Lysosomal marker Lamp1 and not the mannose-6-phosphate receptor (M6PR) [103]. Nramp1 appears to have an impact on SCV maturation: in Nramp1-deficient macrophages, SCVs fails to acquire M6PR, a protein known to regulate the delivery of a subset of lysosomal enzymes from the trans-Golgi network to the pre-lysosomal compartment, thereby facilitating bacterial killing [104]. Thus, Nramp1 appears to alter the trafficking patterns of bacteria-containing vacuoles and, as a result, the vacuoles are no longer sequestered from lysosomal trafficking and are subject to the full battery of bactericidal agents present in these vacuoles (figure 1.5).
Figure 1.5 Divalent-metal transport by Nramp1 proteins at the interface of host-pathogen interactions. a) Nramp1^{++} macrophages phagocyted bacteria are enclosed within a phagosome. Lamp1^{+} lysosomal/late-endosomal vesicles positive for Nramp1 fuse with the bacterial phagosome, eventually leading to a mature acidified and fully bactericidal/bacteriostatic phagolysosome thus restricting bacteria replication. b) In the absence of Nramp1, bacteria within the phagosomes are able to inhibit the maturation of endosomes. Reproduced from [102].
• **Nramp1 augmenting host defense mechanisms**

In addition to the direct role proposed for Nramp1 in restricting intracellular microbial replication (discussed above), an indirect function for Nramp1 in priming the immune system has been suggested. Many studies have shown that Nramp1 facilitates innate host defense mechanisms in macrophages such as the production of reactive oxygen and nitrogen species as well as pro-inflammatory cytokines [105]. In comparison to Nramp1-deficient macrophages, Nramp1\(^{+/+}\) macrophages make more cytokines in response to LPS and IFN-\(\gamma\) stimulation (reviewed in [106]). Nramp1-dependent upregulation of the chemokine KC (keratinocyte chemoattractant) was also reported in macrophages stimulated with mycobacterial lipoarabinomannans [107]. Similarly, high levels of TNF-\(\alpha\) expression by Nramp1\(^{+/+}\) macrophages after TLR7 activation has also been noted [108]. In conclusion, it has been suggested that Nramp1 may also enhance the ability of macrophages to orchestrate an immune response.

Nramp1 was proposed to influence adaptive immune responses, since it has been shown that Nramp1-deficient macrophages have an impaired ability to process and present antigen. This was attributed to the decreased upregulation of MHC class II molecules in Nramp1\(^{-/-}\) macrophages [109]. Similarly, a recent study suggested that Nramp1\(^{+/+}\) DCs are better antigen processing and presenting cells than Nramp1\(^{-/-}\) DCs, inducing greater activation of antigen-specific T cells and higher induction of IL-12 [89]. Overall, these data suggest that Nramp1 has an impact on the type and magnitude of the inflammatory response in the host [110].

Unfortunately, the influence of Nramp1 on cytokine production in response to *Salmonella* is poorly documented, and many of the studies performed are contradictory.
For example analysis of cytokine secretion in an *in vivo* study suggested that the kinetics and magnitude of the *Salmonella*-induced cytokine response, are similar between Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> mice following infection with *S. Dublin* [111]. In contrast, other studies have suggested that Nramp1 has a potent effect on cytokine responses. For instance, macrophages from Nramp1<sup>−/−</sup> mice have a diminished capacity to induce the secretion of IFN-γ by NK cells in response to infection with *S. Typhimurium*. Lower and slower expression of IFN-γ was observed in Nramp1<sup>−/−</sup> mice compared to congenic Nramp1<sup>+/+</sup> counterparts after *S. abortusovis* infection [112]. Similarly, IL-2 and IFN-γ production by T cells in response to a recombinant *Salmonella* vaccine strain in Nramp1<sup>+/+</sup> mice was associated with an enhanced resolution of *Leishmania* infection used as secondary challenge, whereas Nramp1<sup>−/−</sup> mice mounted a Th2 response with higher levels of IL-4 [113]. Nramp1 was also postulated to mediate a bias towards the development of Th1 responses in a model of chronic *Salmonella* (*S. enteritidis*) infection in mice. Infection led to elevated expression of Th1 cytokines such as IFNγ and IL-12 in Nramp1<sup>+/+</sup> mice, whereas Nramp1 deficient mice showed a Th2 biased response [114].

In summary, there are conflicting data on the role of Nramp1 in guiding cytokine dependent immune responses. This may be due, in part, to the failure of the previous studies to use a consistent model of infection. Each of the mentioned studies utilized different routes of infection, bacterial doses, and strains of bacteria. Therefore the true role of Nramp1 in cytokine-mediated responses to *Salmonella* pathogenesis and disease resistance remain unknown.

How Nramp1 enhances pro-inflammatory cytokine production is not known. However, recent studies are providing important insights. In a study by Gomez *et al.*
Nramp1 was shown to play a role in modulating the activity of protein tyrosine phosphatases (PTP). Expression of Nramp1 results in a transient inhibition of macrophage PTP activity via direct PTP-metal interaction and/or by reactive oxygen species-dependent PTP oxidation. Since these phosphatases dampen signals involved in cell activation and cytokine production, their inactivation via Nramp1-dependent iron transport could lead to amplified cytokine secretion. In another proteomic study in which signal transduction pathways were analyzed, Nramp1 expression was associated with increase phosphorylation of Stat1 (signal transducer and activator of transcription) in response to IFN-γ, as well as a rapid decline in the level of inhibitory kB-α induced by LPS. These are important macrophage proteins, which in turn, regulate the expression of many activation associated genes [115]. Thus, it would be valuable to examine at the molecular level, the Nramp1-mediated events that may influence cytokine release.

1.6 Dendritic cells and their role in S. Typhimurium infection

DCs are professional antigen presenting cells that play a crucial role in linking innate and adaptive immunity. Immature DCs are situated in peripheral and lymphoid tissues where they are highly phagocytic and able to recognize microbes via microbe recognition receptors on their surface. Following phagocytosis, DCs mature and migrate to the lymph nodes and spleen where they present antigenic peptides to other cells of the immune system [116, 117]. These properties of DCs are important in orchestrating an efficient immune response.

DC biology is very complex. DCs are versatile cells both in function and phenotype. They are often divided into conventional and plasmacytoid DC. In mice, conventional
DCs are defined by high expression of the integrin CD11c while plasmacytoid DCs express an intermediate level of CD11c. Conventional DCs are subsequently divided into two major groups based on the expression of the alpha chain of the CD8 molecule, defining CD8− (previously known as myeloid) and CD8+ DCs (lymphoid cells) [118]. A table summarizing the major subsets of conventional DCs that have been shown to play a role in salmonellosis and their distribution in anatomical sites is presented below.

### Table 1.2 Relative composition (%) of conventional DCs subsets in different organs

<table>
<thead>
<tr>
<th>Subset</th>
<th>CD8+CD4−CD11b−</th>
<th>CD8−CD4+CD11b+</th>
<th>CD8−CD4+CD11b+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP (SI)</td>
<td>NA</td>
<td>NA</td>
<td>~50</td>
</tr>
<tr>
<td>PP</td>
<td>40</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MLN</td>
<td>30</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Spleen</td>
<td>25</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Liver</td>
<td>10</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

Modified from: Shortman et al. [119] The values are based on references sources [118, 120] and Diehl G. personal communication.
LP (SI): Small intestinal lamina propria, PP: Peyer’s patches, MLN: Mesenteric Lymph nodes and NA: Non-applicable

Most of our current knowledge about Salmonella molecular pathogenesis comes from *in vitro* studies of infected epithelial cell lines or macrophages. However, in addition to their documented survival inside macrophages, *Salmonella* is also taken up by and survives within DCs [16, 121], which represent a static non-dividing reservoir [122]. Recent *in vivo* studies have shown that DCs are among the first cells that *Salmonella* encounter when infecting their murine hosts through the oral route [17]. DCs were shown to sample *Salmonella* in the gut by sending processes between intestinal epithelial cells.
(Figure 1.6). These processes maintain the integrity of the epithelial tight junctions and require the expression of the chemokine receptor CX3CR1. Mice lacking CX3CR1 displayed enhanced susceptibility to S. Typhimurium compared to their wild type counterparts, most likely as a consequence of impaired bacterial sampling by DCs. This highlights the importance of DCs in bacterial sampling in the lamina propria and their antibacterial defense during infections [17].

DCs harbor Salmonella in vivo after oral, intraperitoneal or intravenous infections [123]. Furthermore, all three of the major splenic DCs subsets, as well as DCs from Peyer’s patches can take up Salmonella. These in vivo studies reported an increase in the absolute number of splenic DCs after Salmonella infection. These DCs have the capacity to produce TNF-α, IL-12 and IFN-γ after Salmonella infection in vivo. The levels of these cytokines, however, are lower compared to the secretion by splenic DCs primed with Salmonella in vitro. These findings suggest a key involvement of DCs in Salmonella pathogenesis.

In addition to their role in innate immunity to microbes and their products, DCs are the most potent antigen presenting cells [124]. This, together with the observation that DCs internalize Salmonella, has prompted researchers to study the role of DCs as antigen presenting cells during Salmonella infection. Indeed, it has been demonstrated that DCs can process and present Salmonella antigens to specific CD4+ and CD8+ T cells, in vitro [125, 126]. In vivo, DCs expressing CCR6+ in the Peyer’s patches are rapidly recruited and activate Salmonella specific T cells following oral infection (figure 1.6). These data suggest that DCs likely initiate the adaptive immune response by stimulating Salmonella-specific T cells during infection. At the same time, these properties of DCs make them
attractive targets for intracellular pathogens like *Salmonella*, since successful colonization of their hosts may require inhibition of DC function. In fact, recent reports have suggested that *Salmonella* inhibits antigen presentation and expression of MHCII by DCs [127, 128]. This effect was dependent on the induction of inducible NO synthase by DCs and on the function of SPI-2 by *Salmonella* [58]. However, the molecular mechanisms by which intracellular *Salmonella* interfere with DC functions remain to be elucidated. Thus future work to clarify these mechanisms and their implications in *Salmonella* pathogenesis is needed.
Figure 1.6 Dendritic cell subsets are responsible for antigen recognition in the intestine. CX3CR1+ DCs populate the entire intestinal lamina propria, as well as dome regions of Peyer’s patches. These DCs are responsible for continuous antigen acquisition from the intestinal lumen, and transport this antigen for presentation from the lamina propria to mesenteric lymph nodes. CCR6+ DCs are associated with Peyer’s patches and are recruited from the interfollicular to dome region upon pathogen challenge for pathogen recognition induction of defense.

MLN: mesenteric lymph node, FAE: follicle associated epithelium, IEC: intestinal epithelial cell. Reproduced from [129].
1.7 Hypothesis and research objectives

Nramp1 is a critical host resistance factor for *S. Typhimurium* infection. Previously, it was proposed that Nramp1 plays an important role in macrophage activation, subsequently affecting many functions including oxidative burst, antigen presentation and cytokine release. This led to the suggestion that Nramp1 might have a function as a modulator of the host immune response. However, there has been a paucity of studies addressing this potential role. The objective of this study is to characterize the expression of Nramp1 in cells of the gastrointestinal tract, since no other study has addressed this expression, and to analyze in detail the immunological events following *S. Typhimurium* oral infection. The overall hypothesis of this thesis is that the severity and outcome of *S. Typhimurium* infection is determined by the influence of Nramp1 on the quality of the immune response mounted. Dissecting this response will clarify and identify new mechanisms of host response to *Salmonella* infection.
1.8 Literature Cited


Chapter 2

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

2.1 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 [1]. 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 [2]. The T3SS encoded by Salmonella pathogenicity island 1 (SPI1) is required for invasion of non-phagocytic cells in the intestinal mucosa [3-5], whereas in its absence, a recently published report [6] 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 [7]. Recently, further characterization of intestinal DCs has shown that a subset of lamina propria and MLN DCs, characterized by the expression of integrin a chain, CD103 (CD103+ DCs) promote the generation of T regulatory cells, thus

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inducing oral tolerance [8, 9]. In contrast, the 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 [8]. 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 [7]. They are able to phagocytose enteric microbes in the Peyer’s patches and present antigenic peptides to T cells [10]. 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 [6]. 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, 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. Nramp1 (also called S1c11a1) is an important innate host resistance factor to S. Typhimurium [11], Mycobacterium and Leishmania [12]. Many commonly studied inbred mouse strains, including C57Bl/6 and Balb/C, possess a point mutation in the Nramp1 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 Nramp1 expressing mice are resistant and survive the
infection [13] although, approximately 5% of these survivors became chronic carriers [14]. Nramp1 is expressed by professional phagocytes, including macrophages and granulocytes in the spleen and liver of mice and humans [15, 16]. However, the mechanism by which Nramp1 controls replication of intracellular pathogens is poorly understood. Structurally, Nramp1 is a hydrophobic protein with 12 membrane-spanning domains and has been proposed to function as a cationic transporter [17]. While it is recruited to the late endocytic compartment of macrophages after phagocytosis of latex beads [18] 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 [12].

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 expresses 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.
2.2 Results

2.2.1 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 [19]). 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 2.1 A, 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. 2.1 B). Our results are in contrast to observations by Vidal et al, [11] 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.
**Figure 2.1 Nramp1 is expressed in the small intestine.**

**A.** Immunofluorescence analysis of small intestine from Nramp1<sup>+/+</sup> and <sup>-/-</sup> mice. Uninfected tissues were stained for Nramp1 and DAPI and analyzed by microscopy. Single color images are shown in black and white to maximize contrast. For merge images, Nramp1=red, DAPI=blue. **B.** Nramp1 gene expression in small intestine of uninfected and *Salmonella* infected mice. q-PCR was used to analyze the expression of Nramp1 from small intestines of uninfected Nramp1<sup>-/-</sup> and Nramp1<sup>+/+</sup> mice or orally *Salmonella* infected Nramp1<sup>+/+</sup> mice (24 hours post-infection). The Nramp1 expression was normalized relative to expression of GAPD. Amplified bands were resolved in a 2% agarose gel.
2.2.2 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 [6]. Since no studies have yet addressed the actions of Nramp1 on *S. Typhimurium* infection through its natural, oral, route [20], 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 Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> mice following infection with invasive *S. Typhimurium* (M cell pathway) [21]. In the representative infection shown in Fig 2.2 A, 86% of Nramp1<sup>−/−</sup> mice died between days 6 to 8 post-infection, whereas all Nramp1<sup>+/+</sup> 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 Nramp1<sup>−/−</sup> mice (Fig 2.2 B), confirming that Nramp1 functions as a resistance gene in this oral infection model.

We next assessed survival of Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> mice after infection with the non-invasive *invA S. Typhimurium* mutant (DC pathway) [6, 22-24]. While 70% of Nramp1<sup>−/−</sup> mice died, all Nramp1<sup>+/+</sup> mice survived (Fig 2.2 A). Colony counts revealed more *S. Typhimurium* in the small intestine and Peyer’s patches of Nramp1<sup>−/−</sup> mice at one
day post-infection compared to Nramp1+/+, and colony counts were also higher in the spleens of Nramp1−/− three days post-infection (Fig 2.2 B). Thus, we find an early effect of Nramp1 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 Nramp1 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 Nramp1 expression including epithelial cells, therefore the effects of Nramp1 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 Nramp1 by these cells may rapidly act to control S. Typhimurium replication.
Figure 2.2 Nramp1\(^{+ +}\) mice succumb to S. Typhimurium and show higher bacterial load.

A. Nramp1\(^{+ +}\) and Nramp1\(^{- -}\) mice were orally infected with 3\(\times10^6\) CFU WT or with 1\(\times10^8\) \(\Delta\text{invA}\) 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 Nramp1\(^{+ +}\) and Nramp1\(^{- -}\) 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 \(\Delta\text{invA}\)) or 3 independent experiments with > 4 mice/time point. SI: small intestine; PP: Peyer’s patches; MLN: Mesenteric Lymph Nodes; SP: Spleen. Data for day 1 was analyzed using Mann-Whitney test. Data for day 3 was analyzed with one-way ANOVA with Kruskal-Wallis and Dunn’s post-test. See Figure 2.7 in appendix 1 (addendum for chapter 2) for more detail.
2.2.3 CD103’ DCs from the lamina propria express Nramp1 and expression levels increase after exposure to S. Typhimurium

The above experiments suggest that Nramp1 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 Nramp1 [25], its expression in vivo has, thus far, only been described in macrophages (MØ) and neutrophils. We therefore assessed Nramp1 expression by intestinal DCs. First, we used immunofluorescence to examine Nramp1 expression in CD11c+ cells, both before and after S. Typhimurium infection. Figure 2.3 A shows the presence of Nramp1 associated with a subset of CD11c+ cells in the small intestinal lamina propria of both uninfected and S. Typhimurium infected Nramp1+/+ mice. Since DCs are a heterogeneous population and it has been suggested that discrete subsets may have different functions during an immune response [26, 27], we examined Nramp1 expression in these subsets. LPDCs from 129sv mice were separated by FACS sorting based on the expression of surface markers. CD11c<sup>high</sup> MHC-II<sup>high</sup> cells were defined as DCs and these cells were further divided based on expression of CD103: CD11c<sup>+</sup>CD103+ have been defined as tolerogenic DCs, or CD11c<sup>+</sup>CD103- which defines pro-inflammatory DCs [8]. We used quantitative PCR (q-PCR) to evaluate Nramp1 expression at the RNA level. As shown in figure 2.3 B, Nramp1 mRNA levels in the CD11c<sup>+</sup>CD103- subpopulation were approximately 7 fold higher than in CD11c<sup>+</sup>CD103+ cells. We also analyzed the expression of Nramp1 in other cell populations. Nramp1 was not detected in CD11c<sup>-</sup>MHC-II<sup>high</sup> 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 2.3 B).
Figure 2.3  A subset of DCs express Nramp1 in the lamina propria of the small intestine.

A. Tissue sections of ileal loops of uninfected or S. Typhimurium infected Nramp1+/+ and Nramp1−/− mice, were stained for Nramp1, CD11c and Salmonella and analyzed by microscopy. Single color images are shown in black and white to maximize contrast. For merge images, Nramp1=red, CD11c=green and Salmonella=blue. Data are representative of two independent experiments. Note: Because CD11c is a cell surface molecule and Nramp1 is intracellular molecule co-stained cells do not show co-localization for these antigens. For more detail see Figure 2.8 in appendix 1 (addendum for chapter 2)
Figure 2.3 Nramp1 is expressed at highest level by CD103− DCs in the small intestine.

B. Lamina propria DCs were isolated and different populations of cells were separated by FACS sorting as: MHC II^{high}CD11c^{+}CD103^{+} cells (green), MHC II^{high}CD11c^{+}CD103^{−} cells (red), CD11c^{−}MHC-II^{high} cells (light blue) and CD11c^{med} MHC-II^{−} cells (yellow). Nramp1 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.
Next, we tested for expression of the Nramp1 protein using intracellular FACS analysis in intestinal DCs isolated from Nramp1−/− and Nramp1+/+ mice. Figure 2.3 C shows Nramp1 expression in the MHC-II\textsuperscript{high}CD11c\textsuperscript{+}CD103\textsuperscript{−} population while the MHC-II\textsuperscript{high}CD11c\textsuperscript{+}CD103\textsuperscript{+} 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\textsuperscript{−}CD103\textsuperscript{−} subset very early (30, 60, 90 and 120 minutes) after S. Typhimurium infection, (Fig. 2.3 D), whereas no changes in the Nramp1 expression was observed in the CD11c\textsuperscript{+}CD103\textsuperscript{+} at the above times points analyzed (data not shown), indicating that bacteria or their byproducts increase Nramp1 expression in the CD11c\textsuperscript{−}CD103\textsuperscript{−} 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\textsuperscript{−}CD103\textsuperscript{−} subset of DCs which have been previously characterized as high secretors of pro-inflammatory cytokines [8], suggesting a potential role in host defense against S. Typhimurium.
Figure 2.3 Nramp1 is expressed at highest level by CD103− DCs in the small intestine.

C Nramp1 protein levels in CD103+ and CD103− DCs (MHC II\textsuperscript{high} CD11c+) from the small intestinal lamina propria of uninfected Nramp1+/− and Nramp1+/+ 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.
2.2.4  *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. 2.4 A). It has been shown previously that CD11c\(^{-}\)CD103\(^{-}\) DC subset is absent in this organ [9, 28] and (Valdez, personal observation). 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 2.4 B, *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 *Nramp1*\(^{-/-}\) mice (BMDC). Figure 2.4 C 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).
Figure 2.4 Nramp1 is expressed in all the Major Subsets of DCs in the spleen

A. Splenocytes from Nramp1+/+ mice were stained with antibodies against B cells (B220⁺CD11c⁻), MØ (F4/80⁺CD11c⁻), DC (CD11c⁺F4/80⁻) and CD8α⁺ DC (CD11c⁺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. The numbers in the plots show the percentage of cells falling within the indicated gates. The numbers in the histograms indicate the percentage of cells positive for Nramp1. Data are representative of three independent experiments.
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. 2.4 D). 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 2.4 E). Levels of MHC-II expression also increased in the Nramp1$^{+/+}$ and Nramp1$^{-/-}$ 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. 2.4 F) [18]. Based on these findings, we conclude that, like intestinal DCs, splenic and BMDCs express Nramp1 protein and its expression increases with $S$. Typhimurium infection.
Figure 2.4 Nramp1 is expressed in all the Major Subsets of DCs and is localized to Intracellular Membranes

C. Nramp1+/+ 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 Nramp1+/+ was assessed by intracellular FACS staining.

F. BMDC from uninfected and S. Typhimurium infected Nramp1+/+ and Nramp1-/- were stained with CD11c (red) and Nramp1 (green) and analyzed by confocal microscopy. The figure shows Nramp1+/+ BMDC.
2.2.5  *S. Typhimurium do not replicate in Nramp1**/+ or Nramp1**/−** BMDCs.

In previously published studies utilizing the Raw264.7 MØ-like cell line as well as peritoneal and splenic MØ, loss of Nramp1 protein was found to facilitate intracellular replication of *S. Typhimurium* [29-31]. The inability of Nramp1**/−** DCs to control *S. Typhimurium* replication could add to the differences in pathogen loads we detected in Nramp1**/+** and Nramp1**/−** mice following oral infection (Figure 2.2 B). Since our experiments showed high levels of Nramp1 expression in BMDCs and these cells are easily obtained in vitro, we used BMDCs from Nramp1**/+** and Nramp1**/−** mice to test whether Nramp1 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 Nramp1**/+** and **/−** BMDCs phagocytosed similar numbers of *S. Typhimurium*, (Nramp1**/+** = 461666.7 ± 72341.8 and Nramp1**/−** = 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 Nramp1**/+** and Nramp1**/−** BMDCs. These data suggest that *S. Typhimurium* do not replicate in DCs (Figure 2.5 A), a finding supported by other studies [32]. Interestingly, we also observed a similar decline in *S. Typhimurium* numbers in infected BMMØ with only slight differences observed between Nramp1**/−** and **/+** BMMØ (Figure 2.5 B). The lack of an effect of Nramp1 on bacterial replication in BMDC and BMMØ in vitro is in contrast to previous studies using Raw264.7 cells [29, 33] and peritoneal and splenic MØ [30, 31]. Our data are, however, supported by studies by Ables et al. who found that Nramp1 expression by peritoneal MØ influence bacterial replication only during the first hour of infection, with no differences found in CFU at later time points [34]. Similarly, other studies have found
no significant differences in the survival of WT S. Typhimurium following infection of
Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> congeneric peritoneal MØ [35]. These divergent results may
reflect the use of MØ from different sources, or differences in experimental protocols.
Our results however, suggest that Nramp1 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 Nramp1, perhaps on the
inflammatory milieu encountered by S. Typhimurium.
Figure 2.5  *S. Typhimurium* do not replicate in BMDCs and BMMØ. BMDC and BMMØ were infected with WT *S. Typhimurium* and analyzed by gentamicin protection assays. 2, 8 and 24 hours after infection, colonies were enumerated and expressed as CFU per $2 \times 10^5$ cells. The data is representative of 3 experiments. See Figure 2.9 in appendix 1 (addendum for chapter 2) for more information.
2.2.6 Nramp1⁺⁺ 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 inducing 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 2.6 A). Two hours following S. Typhimurium infection, IL-6, IL-12p40 and TNF-α gene expression were all upregulated in both subsets of DCs (Fig 2.6 A). 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 Nramp1 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 Nramp1⁻⁻ 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 2.6 B). Similarly, higher levels of TNF-α was observed in Nramp1+/+ CD103- DCs at both time points analyzed. We did not observe any differences in the cytokine secretion in CD103+ DCs from Nramp1+/+ or Nramp1−/− 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 Nramp1−/− BMDCs before and after infection with S. Typhimurium over the course of 24 hours. We found that both Nramp1+/+ and Nramp1−/− 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. 2.6 C). We also analyzed the cytokine secretion by BMMØ and found higher secretion of IL-6 and TNF-α by Nramp1 producing BMMØs, but unlike BMDCs we did not find differential secretion of IL-12p70 in BMMØ from Nramp1+/+ and Nramp1−/− (Figure 2.6 D). These results demonstrate that Nramp1 augments DC secretion of several important inflammatory cytokines in response to S. Typhimurium infection.
Figure 2.6 Nramp1<sup>++</sup> DCs and macrophages secrete more inflammatory cytokines than Nramp1<sup>−/−</sup> cells.
Figure 2.6 Nramp1<sup>+/+</sup> DCs and macrophages secrete more inflammatory cytokines than Nramp1<sup>−/−</sup> cells.

A. CD103<sup>+</sup> and CD103<sup>−</sup> 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 show as an average of triplicate samples, with the exception of IL-6 secretion by CD103<sup>+</sup> cells in which only duplicate samples were analyzed.

B. CD103<sup>+</sup> and CD103<sup>−</sup> DCs were FACS sorted from Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> mice. IL-6 and TNF-α secretion was assessed using luminex beads. Triplicates samples of CD103<sup>+</sup> 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<sup>+</sup> DCs, only duplicates or single wells were assessed and treated as for CD103<sup>−</sup> DCs.

C. BMDC from Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> were infected with S. Typhimurium MOI=10 or mock infected and supernatants were taken at indicated times 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.

D. BMMØ from Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> were infected with S. Typhimurium or mock infected and supernatants were taken at indicated times 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.
2.3 Discussion

Nramp1 is known to be a key mediator of host resistance to intracellular pathogens, including *Mycobacterium bovis* BCG and *Leishmania*, yet the mechanisms underlying Nramp1’s role in host resistance remain unclear and controversial [36-38]. 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* [25], 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 [39]. Although the involvement of DCs in tolerance is well documented [40], 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 [8]. 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 [36] 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 [41]. TNF-α is also essential for this response, since deficient mice are highly susceptible to S. Typhimurium infection [36]. 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 [42]. This appears to be a conserved host defense mechanism since IL-12 receptor deficient humans are also immunocompromised and highly susceptible to Salmonella infections [43]. Interestingly, our data show that Nramp1 positive 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<sup>+/+</sup> mice demonstrate significantly more severe inflammation early in the Salmonella-induced colitis model compared to Nramp1<sup>-/-</sup> mice (Y. Valdez, personal observation). These findings are consistent with previous reports suggesting that Nramp1 modulates acute inflammatory reactions [44] [38]. Nramp1 was previously shown to promote higher induction of pro-inflammatory cytokines in MØ. For example, elevated levels of IL-1β and TNF-α were reported in Nramp1<sup>+/+</sup> MØ after LPS and IFN-γ stimulation [45]. Similarly, Nramp1 dependent upregulation of the chemokine KC (keratinocyte chemoattractant) was reported in MØ stimulated with Mycobacterial lipoarabinomannans [46]. High levels of TNF-α expression by Nramp1<sup>+/+</sup> MØ after TLR7 activation has also been noted [47]. 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. [38] 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 [48] and that SPI2 gene expression is substantially greater within an Nramp1 expressing environment [49].

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.
2.4 Experimental procedures

**Bacteria.** Wild-type (WT) S. Typhimurium (SL1344) [50] and isogenic ΔinvA mutant (invA::kan SB103; SPI1) [51] were grown overnight at 37°C with shaking (200 rpm) in 10 ml of LB broth (stationary phase), supplemented with 100 µg/ml streptomycin (to select for WT S. Typhimurium) and/or 50 µg/ml kanamycin (to select for ΔinvA mutant). 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 [20]. 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 ΔinvA 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 [48]. 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 $10^7$ 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 [48]. The cells were harvested at day 7 of culture, on 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-Aβ+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 Nramp1−/− 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 (a source of macrophage-colony stimulating factor) 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.

**Gentamicin protection assays.** BMDC and BMMØ were infected with opsonized bacteria with a MOI = 10 as described previously [48]. 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 + steptomycin (50 μg/ml) agar.

**Isolation of small intestine Lamina propria DCs.** Cells were prepared as previously described [6] 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 cm 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 150μg/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 [52]. In brief, each spleen was collected in 1 ml of RPMI, 10mM HEPES and 0.01mg/ml of Liberase Blenzyme 3 (Roche Diagnostics), 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) and FloJo (Tree Start, Inc.) cell sorting was performed using FACS Vantage and MoFlo cytometer (DAKO Cytomation). Antibodies to CD11c, CD11b, I-A^b^, B220, CD8a, CD4, TCRab, CD103 and CD16/32, were from BD PharMingen or from e-Bioscience. Affinity purified rabbit anti-mouse Nramp1 antibody was from P. Gros [18]. 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. 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^, and 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, TCRab 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 x 10⁴/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 (Upstate). Cytokine beads analytes were purchased from Upstate and data analysis was performed using BiedView Software (Upstate).

**ELISA assays.** BMDC (2. 5 x 10⁵) 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 Pharmigen). The signals 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 RNAlater (Qiagen). 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) and reverse transcription was performed with the Quantitect RT kit (Qiagen) using 1µg RNA. For PCR reactions, the cDNA was diluted 1:10 and 1µl 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**: TCATAGCCGAAGGTCAAAAGC
- **GAPD fw**: ATTTGTCAGCAATGCATCTG
- **GAPD re**: ATGGACTGTGGTCATGAGCC
- **IL-12b fw**: GGAAGCACGGCAGCAGAATA
- **IL-12b re**: AACTTGAGGGAGAAGTAGGAATGG
- **IL-6 fw**: GAGGATACCACTCCCCAAGAGCC
- **IL-6 re**: AAGTGCTCGATCGTTGTTCCATA
- **TNFα fw**: CCACCACGCTTCTCTGTCTAC
- **TNFα 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 Liquid nitrogen 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 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 (goat anti-rabbit Alexa-488 or Alexa-594). 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) and analyzed by confocal microscopy using a 60× 1.4 numerical aperture objective. Images were captured and analyzed using Nikon C1 and Slidebook software (Intelligent Imaging Innovations).

**Statistical analysis.** Data was analyzed using the following statistic test:

Logrank test was used to compare survival curves between Nramp1+/+ and Nramp1-/- mice infected with *Salmonella*.

Student T test (parametric test) was used to compare two groups assuming that the data in these groups follow approximately a Gaussian bell-shaped distribution. Unpaired T test was used to analyze experiments performed *in vitro* such as cytokine secretion measured by ELISA.

Mann-Whitney test (non-parametric test) was used to compare CFU from internal organs of *Salmonella* infected mice at day 1 post-infection. Due to the asynchrony inherent to oral infections and to the variability between individuals, we analyzed the data assuming that they do not follow a Gaussian distribution.
One-way ANOVA with Krukal-Wallis (non-parametric) with Dunn’s post-test (to compare pairs) was performed using a 95% confidence interval. This test was used to analyze *in vivo* data comparing CFU from internal organs of *Salmonella* infected mice at day 3 post-infection, since at this time the bacteria was recovered from many organs and more animals were used at this time point.

All analyses were performed using GraphPad Prism version 4.0.
2.5 Literature cited


Chapter 3

Nramp1 drives an accelerated inflammatory response during

Salmonella-induced colitis in mice³

3. 1 Introduction

Salmonella enterica serovar Enteritidis (S. Enteritidis) and serovar Typhimurium (S. Typhimurium) cause gastroenteritis in human or “food poisoning”, a self-limiting disease characterized by diarrhea. A recently developed model for enterocolitis in mice involves pre-treatment with the antibiotic streptomycin prior to infection with S. Typhimurium. This pre-treatment, dramatically enhances S. Typhimurium colonization of the cecum and colon leading to significant inflammation [1]. This host response is accompanied by both intestinal pathology and pathophysiology, culminating in watery stools [2]. These changes closely resemble the features of enterocolitis in humans and this model has been used to study both bacteria and host components important for human enterocolitis [3].

Susceptibility to S. Typhimurium in mice is determined by virulence factors expressed by bacteria as well as by the host genes conferring resistance. Although many host resistance factors to virulent S. Typhimurium have been identified [4], arguably, the most important innate resistance protein is Nramp1 (Natural resistance-associate macrophage protein 1), also called Slc11A1 [5]. The susceptibility of several common inbred mouse strains to S. Typhimurium is the result of a single mutation of amino acid 169 of the Nramp1 protein from Gly to Asp (G169D), leading to impaired folding and

* Authors contributed equally to this work
loss of the mature protein in these mice [6]. Susceptible mouse strains succumb to a low
dose of *S*. Typhimurium due to uncontrolled bacterial replication, while resistant mice
control the infection and are thus able to survive the infection. Interestingly, even
resistant mouse strains are often fail to completely clear *Salmonella*, and these mice, like
some human patients, become chronic carriers of *Salmonella* [7].

The *in vivo* role of Nramp1 in response to *S*. Typhimurium and other microbial
pathogens has been studied primarily in systemic infections [8, 9], whereas very few
studies have addressed the role of Nramp1 in mucosal immunity. Recently, we
demonstrated that Nramp1 is expressed in the lamina propria of the small intestine [10].
Although one previous study examined colitis in a variety of Nramp1 positive and
negative inbred mouse lines (C57Bl/6 versus 129SvJ and DBA/2) [11] these mice were
not on an isogenic background and thus the importance of Nramp1 in this disease could
easily have been obscured by the plethora of other allogenic differences that exist
between these strains. Indeed, these mice are known to harbour many genetic differences
with known impacts on inflammatory responses that would confound the role of Nramp1
[12-15].

Here we used isogenic Nramp1+/+ and Nramp1−/− mice to analyze the actions of
Nramp1 in the large intestine using the *Salmonella*-induced enterocolitis model. We
demonstrate that Nramp1 is expressed in the lamina propria of the large intestine (cecum
and colon) and increases rapidly following *S*. Typhimurium infection. Moreover, we
show that functional Nramp1 is associated with a faster pro-inflammatory response, with
more severe inflammation identified in the colonic mucosa one day after *S*. Typhimurium
infection when compared to Nramp1+/− mice. This response was characterized by elevated
expression of the pro-inflammatory cytokines IFN-γ and TNF-α and chemokines such as MIP-1α as well as the strong and rapid recruitment of neutrophils and macrophages to the cecal mucosa. This heightened inflammatory response correlates with better control of S. Typhimurium infection and, correspondingly, Nramp1+/+ mice showed lower bacterial burdens than Nramp1−/− mice. Our findings highlight the role of Nramp1 in controlling the host response to enteric Salmonella infections and suggest that an early and rapid inflammatory response contributes significantly to protection of the host from these infections.

3.2 Results

3.2.1 Cecal Nramp1 expression rapidly increases following S. Typhimurium infection

Recently, we have shown that Nramp1 is expressed in the small intestinal lamina propria of mice and that in the typhoid model [10], this expression influences S. Typhimurium replication, since mice lacking Nramp1 showed higher bacterial counts in the small intestine and Peyer’s patches early following oral infection. Using this typhoid model, colonization of S. Typhimurium of the cecum and colon is very low. However, pre-treatment with streptomycin prior to Salmonella infection facilitates bacterial colonization of the lower bowel and induces severe inflammation in these organs [1] thus enabling the study of Nramp1 in the S. Typhimurium-induced enterocolitis model [3]. Since Nramp1 is expressed in phagocytic cells recruited to sites of inflammation [16], we assessed the changes in Nramp1 expression in the cecum and colon, before and after S. Typhimurium infection using the colitis model. Nramp1+/+ mice (129SvJ) were treated
with streptomycin 24 h prior to infection with *S. Typhimurium* and Nramp1 expression was evaluated by immunofluorescence (IF) and quantitative PCR (q-PCR). Figure 3.1 A shows an increased number of cells expressing Nramp1 in the cecum of Nramp1^{+/+} mice one day after infection with *S. Typhimurium*. Similarly, analysis of the Nramp1 transcripts revealed a ~10 fold increase in Nramp1 mRNA expression following *S. Typhimurium* infection compared with the uninfected controls (Fig. 3.1 B). Nramp1^{-/-} mice, which lack the exons containing these primers showed no PCR products (data not shown). These results demonstrate that Nramp1 expression in the lamina propria of the cecum is rapidly increased early after *Salmonella* infection, probably as a result of the recruitment of phagocytic cells expressing Nramp1 to the site of infection and/or upregulation of Nramp1 by resident cells after *S. Typhimurium* exposure similar to our previously reported observations in the small intestine [17].
Figure 3.1 Nramp1 expression is upregulated in the cecum of *S. Typhimurium* infected mice. 129SvJ mice (Nramp1<sup>+/+</sup>) and isogenic controls (Nramp1<sup>-/-</sup>) were treated with 20 mg streptomycin (Sm) 24 hours prior the infection with 3x10<sup>6</sup> *S. Typhimurium* or sham infected. Mice were sacrificed 1 day after infection. **A.** Tissue sections were stained for Nramp1 and DAPI and analyzed by microscopy. Scale bar = 50µm. **B.** Cecum tissues of uninfected controls (*n* = 5) or *S. Typhimurium* infected (*n* = 10) Nramp1<sup>+/+</sup> and Nramp1<sup>-/-</sup> mice were analyzed for the expression of Nramp1 by quantitative PCR (qPCR). Data were normalized to GAPD levels. No PCR products were detected in Nramp1<sup>-/-</sup> mice. *p* value was calculated using Student T test. The data are compiled from 2 independent experiments and mean ± SEM is shown.
3.2.2 Kinetics of S. Typhimurium replication in Nramp1\textsuperscript{+/+} and Nramp1\textsuperscript{-/-} mice

Following pre-treatment with streptomycin and subsequent infection with S. Typhimurium, we recently showed that Nramp1\textsuperscript{+/+} mice become chronically infected, displaying high numbers of bacteria in their ceca and colons which leads to a severe transmural inflammation and ultimately to fibrosis in the infected organs [18]. However it is unknown how Nramp1 affects bacterial replication in the early phase of the infection. It has been suggested that Nramp1’s action takes place early after bacterial infection [19, 20]. To address the consequence of Nramp1 expression in the ceca and colon of \textit{Salmonella}-infected mice, we analyzed S. Typhimurium colonization in these organs. Nramp1\textsuperscript{+/+} and isogenic Nramp1\textsuperscript{-/-} control mice were treated with streptomycin 24 hours prior to infection with $3 \times 10^6$ S. Typhimurium. Mice were sacrificed at one and four days post-infection. As shown in Figure 3.2 A, bacteria replicated to high levels in the ceca of both Nramp1\textsuperscript{+/+} and Nramp1\textsuperscript{-/-} mice at early time points (day 1). However, this replication was influenced by the Nramp1 status of the host, since we found five fold more bacteria in the ceca of Nramp1\textsuperscript{-/-} mice. No differences in bacterial counts were observed in the colon and mesenteric lymph nodes (MLN) at this time point. The number of bacteria in the spleens at day 1 was below the threshold of detection. The impact of Nramp1 as a resistance factor against S. Typhimurium was more obvious at day 4 post-infection (Figure 3.2 B). At this point we found between 10 to 50 times more bacteria in the Nramp1\textsuperscript{-/-} mice compared to Nramp1\textsuperscript{+/+} mice in all organs analyzed. At later time points, levels of bacterial replication reached very high numbers in the Nramp1\textsuperscript{-/-} mice, leading to sepsis and death of these mice starting at day 5 post infection (see appendix 2 for survival curves), whereas Nramp1\textsuperscript{+/+} mice were able to control the infection. These
data suggest that Nramp1 mediates early control of *S. Typhimurium* replication and limits the spread of bacteria to systemic sites.
Figure 3.2 Bacterial colonization of mouse organs by *S. Typhimurium*. Nramp1<sup>+/+</sup> mice and isogenic control Nramp1<sup>-/-</sup> mice were pre-treated with Sm prior to infection with *S. Typhimurium*. Serial dilutions of homogenized tissues were plated on LB+Sm plates. A. Bacterial counts one day post infection of spleen, mesenteric lymph nodes (MLN), cecum and colon. Dashed line at 100 indicates limit of detection; cfu, colony forming units. *P* values were calculated using Mann-Whitney test. B. Bacterial counts 4 days post infection.
3.2.3 Nramp1<sup>+/+</sup> mice show acute inflammation early after S. Typhimurium infection

A hallmark of S. Typhimurium-induced enterocolitis is the acute inflammation of the cecum of infected mice [3]. This inflammation starts early (approximately 8 hours after S. Typhimurium infection) and triggers many pathological changes including epithelial damage and even ulceration, submucosal edema and substantial infiltration of PMN cells into the lamina propria [1]. Many of the phagocytic leukocytes recruited to the site of infection express Nramp1 and we hypothesized that this molecule would influence the onset of the inflammatory response. Therefore, we induced colitis in Nramp1<sup>+/+</sup> and isogenic Nramp1<sup>−/−</sup> controls and evaluated both the macro- and microscopic pathological changes in these mice at different time points after S. Typhimurium infection. One day post infection, macroscopic analysis of the ceca of Nramp1<sup>+/+</sup> mice revealed classic characteristics of cecal inflammation including a reduction in cecum size, and the replacement of fecal material in this organ with purulent exudates (Fig. 3.3 A). Strikingly, these changes were absent in the ceca of Nramp1<sup>−/−</sup> mice at this time point (Fig 3.3 A). Another quantitative measure of intestinal inflammation is reduced cecum weights, which have been shown to decrease as a reflection of a higher degree of increased inflammation (due to loss of cecal contents as a consequence of enhanced mucus production) [1]. We found that Nramp1<sup>+/+</sup> mice had markedly decreased cecum weights at 1 day post-infection compared to Nramp1<sup>−/−</sup> mice (Fig. 3.3 B). In contrast, macroscopic examination at day 4 post-infection showed increased signs of inflammation in Nramp1<sup>−/−</sup> mice and this was associated with reduced cecal weight (Fig. 3.3 B). Histological analysis of the ceca of these mice confirmed our macroscopic observations.
As shown in Figure 3.3 C, at day 1 post-infection, Nramp1\(^{+/+}\) mice displayed clear pathological changes in their infected ceca such as submucosal edema, thickened mucosa and increased numbers of PMN infiltrating into the lamina propria. In contrast, pathological changes were mild or absent in Nramp1\(^{-/-}\) mice (Fig. 3.3 C). However, at day 4 post-infection we observed profound inflammation in the ceca of both Nramp1\(^{+/+}\) and Nramp1\(^{-/-}\) mice. At this time point Nramp1\(^{-/-}\) mice showed greater pathological changes compared to Nramp1\(^{+/+}\) mice (Fig. 3.3 D-E). Overall these data demonstrate a profound influence of Nramp1 on intestinal inflammation very early after S. Typhimurium infection, whereas at later time points, the inflammation is influenced by mechanisms unrelated to Nramp1.
Figure 3.3 S. Typhimurium induced inflammation is delayed in Nramp1<sup>-/-</sup> mice.

A. Photographs of ceca with colon removed from Nramp1<sup>+/+</sup> and Nramp1<sup>-/-</sup> uninfected and infected mice at day 1 and 4 post infection. Red circle indicates cecum B. Cecum weights of Nramp1<sup>+/+</sup> and Nramp1<sup>-/-</sup> uninfected and infected mice at day 1 and 4 post infection. Data is representative of three independent experiments. P values were calculated using one-way analysis of variance with Bonferroni’s multiple comparison post test, *, p < 0.05; ***, p < 0.001.
Figure 3.3 S. Typhimurium induced inflammation is delayed in Nramp1⁻/⁻ mice.

C. H&E staining of cecum sections revealing submucosal edema, mucosal and muscle hyperplasia and granulocyte infiltration in Nramp1⁺/+ mice at day 1 and in Nramp1⁺/+ and Nramp1⁻/⁻ mice at day 4 (see also Figure 3.8 in the appendix 2). D. Pathology score of infected ceca, each bar represents a mouse. E. Quantification of pathology score of uninfected and infected mice. Data are compiled from three independent experiments. P values were calculated using one-way analysis of variance with Bonferroni’s multiple comparison post test, *, p < 0.05; ***, p < 0.001.
3.2.4 Nramp1\(^{+/+}\) mice exhibit higher expression of chemokines/cytokines in the cecum early after S. Typhimurium infection

Godinez et al. [21] have recently shown that numerous cytokines and chemokines are upregulated in the ceca of mice following S. Typhimurium infection using the enterocolitis model. To assess if the early inflammation observed in Nramp1\(^{+/+}\) correlated with an increased production of cytokines, we analyzed the production of cytokines and chemokines in the cecum one day after S. Typhimurium infection by q-PCR. mRNA was extracted from the ceca of streptomycin pre-treated Nramp1\(^{+/+}\) and Nramp1\(^{-/-}\) mice uninfected or infected with S. Typhimurium. As shown in Figure 3.4, production of TNF-\(\alpha\), MIP-1\(\alpha\), IFN-\(\gamma\), IL-6 and KC were readily detected in both Nramp1\(^{+/+}\) and Nramp1\(^{-/-}\) mice day 1 post-infection with S. Typhimurium (Fig. 3.4). However, significantly higher levels of TNF-\(\alpha\), MIP-1\(\alpha\) and IFN-\(\gamma\) were observed in the ceca of Nramp1\(^{+/+}\) mice compared to Nramp1\(^{-/-}\) mice. These data demonstrate that Nramp1 is associated with enhanced production of several inflammatory cytokines in response to S. Typhimurium. This increased inflammatory response likely results in the activation of resident immune cells and/or recruitment of other inflammatory cells to the site of infection, thus controlling the progression of the infection.
Figure 3.4 Induction of TNF-α, MIP-1α, IFN-γ, IL-6 and KC. Expression of cytokine and chemokine transcripts in uninfected (n = 5) and infected (n = 10) ceca were determined by q-PCR; data were normalized to GAPD levels and are relative to uninfected controls. Protein levels for these factors are too low to be accurately measured necessitating assessment of transcript levels. Data shown are compiled of 2 independent experiments and analyzed using Student T test, mean ± SEM is shown.
3.2.5 Neutrophils and macrophages are the main cells recruited to the site of infection and limit spread of S. Typhimurium to systemic sites

Infiltration of PMNs was observed previously in the ceca of S. Typhimurium infected mice undergoing acute colitis [1]. However, the analysis of infiltrating cells under the influence of Nramp1 early after the infection has not been characterized. Therefore, we performed an immunohistochemical analysis of sections of the ceca of Nramp1+/+ and Nramp1−/− mice one day after infection. Large numbers of myeloid cells (CD11b+), including Gr-1+ neutrophils and macrophages (F4/80+) (Fig. 5A), were recruited to the site of inflammation (ceca) of Nramp1+/+ mice. In contrast, far fewer infiltrating cells were observed in the ceca of Nramp1−/− mice at this time point (Fig. 5A). This difference is likely a consequence of elevated cytokine/chemokine expression in the ceca of Nramp1+/+ versus Nramp1−/− mice.

Myeloperoxidase (MPO) is an enzyme and an important component of the innate immune defense by virtue of its ability to generate reactive oxidants and diffusible radical species mediating significant microbicidal activity. MPO is highly expressed in neutrophils and to a lesser extent in monocytes and certain types of macrophages [22]. Interestingly, many MPO positive cells were found in the ceca of Nramp1+/+ infected mice compared to very few to no MPO positive cells in Nramp1−/− mice at day 1 post-infection. At the same time we found that most S. Typhimurium bacteria were localized to the lumen and/or associated with the luminal surface of the cecal epithelium of Nramp1+/+ mice and no bacteria were detected in deeper tissue layers such as the mucosa or submucosa. While we also found S. Typhimurium in the cecal and colonic lumen of Nramp1−/− mice, substantial numbers of bacteria were also identified in the mucosa,
submucosa and even muscle layers (Table 1 and Fig. 5B). Overall our data suggest that Nramp1 activity results in rapid recruitment of cells with antibacterial capacity – such as neutrophils and thus restricts the spread of *Salmonella* to internal organs.
Figure 3.5 S. Typhimurium-induced recruitment of inflammatory cells to the cecum is Nramp1 dependent. Nramp1+/+ and isogenic controls Nramp1−/− were pre-treated with streptomycin (Sm) prior to infection with S. Typhimurium. (A) One day post-infection ceca were collected in OCT as described in experimental procedures and sections were stained with antibodies against CD11b, Gr-1 and F4/80. Scale bar = 50µm. (B) Ceca were collected at day 1 and 4 days post-infection and fixed in formalin. Sections were stained with antibodies against MPO and S. Typhimurium LPS, original magnification: 100x. Arrowheads indicate bacteria in the lumen or attached to the surface epithelium. Arrows indicate bacteria that have penetrated into deeper tissue layers such as mucosa and...
submucosa. See Figure 3.9 in appendix 2 (addendum for chapter 3) for higher magnification.
Table 3.1 Localization of *S*. *Typhimurium* in the cecum

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 4</th>
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<tbody>
<tr>
<td></td>
<td>N ramps1+/+</td>
<td>N ramps1-/-</td>
</tr>
<tr>
<td>tissue</td>
<td>4.0 ±1.7</td>
<td>66.53 ±10.5</td>
</tr>
<tr>
<td>adherent to</td>
<td>73.3 ±11.2</td>
<td>50.27 ±9.4</td>
</tr>
<tr>
<td>epithelium</td>
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Table 3.1 summarizes quantification of *S*. *Typhimurium* in different localizations of the cecum layers, as described in experimental procedures. Due to the high number of bacteria in the cecal lumen in both mouse strains, we could not accurately enumerate the bacteria in this compartment. Thus data are only given for bacteria within the tissue or adherent to the epithelium. Data were analyzed using Student T test and are shown as mean ±SEM.
Figure 3.6 Schematic diagram summarizing temporal response by Nramp1<sup>+/+</sup> and Nramp1<sup>-/-</sup> cells following S. Typhimurium infection. Within hours (day1) following S. Typhimurium infection, Nramp1-expressing resident cells respond by secreting cytokines and chemokines thus activating and attracting new phagocytic cells to the site of infection creating an antimicrobial environment. Nramp1-deficient cells exhibit a delayed response, allowing bacterial replication and penetration to internal layers of the cecum. By day 4, both Nramp1<sup>+/+</sup> and Nramp1<sup>-/-</sup> cells have responded to the bacteria eliminating them from the deeper layers of the mucosa and sequestered them in the lumen of the cecum (although the bacteria have now colonized peripheral tissues). In both luminal cecum and peripheral sites, bacterial numbers are substantially lower, likely due to the more rapid, Nramp1-mediated cytokine response).
3. 3 Discussion

There have been a paucity of studies that have attempted to address the potential role played by Nramp1 in the development of colitis [2, 11]. Here we analyzed the impact of Nramp1 using the S. Typhimurium-induced colitis model. We tested the hypothesis that bacterial numbers are limited in an Nramp1 dependent manner in the gut mucosa through an accelerated early inflammatory response. We show here that Nramp1+/+ mice respond faster to S. Typhimurium infection with elevated production of cytokines/chemokines and the rapid recruitment of phagocytic cells to the site of infection. Our data suggest that an early response (one day after S. Typhimurium) results in rapid priming of the immune system and restriction of bacterial replication, thus limiting the spread of Salmonella to systemic sites.

Previously, the role of Nramp1 in conferring resistance to S. Typhimurium has been widely studied using systemic models of infection (intravenous or intraperitoneal) [8, 9, 23, 24]. Surprisingly, only recently has the natural route of S. Typhimurium infection, the oral route, been analyzed [10, 25]. We recently showed an early effect of Nramp1 in S. Typhimurium replication in the small intestine and gut-associated lymphoid tissues such as Peyer’s patches and MLN [10]. However, we were unable to address the role of Nramp1 during colitis, since the large intestine in these mice is poorly colonized with S. Typhimurium during oral infection with no signs of inflammation. However, treatment of mice with streptomycin prior to infection with S. Typhimurium leads to markedly enhanced colonization and severe inflammation in the cecum and colon [3]. Here we demonstrate that Nramp1+/+ mice exhibit much more intense inflammation at day 1 in comparison to Nramp1−/− mice. In contrast, another group reported that inflammation
in Nramp1-sufficient and -deficient mouse strains is very similar [11]. However in that study the authors compared mice of distinctly different genetic backgrounds including C57Bl6, DBA/2 and 129Sv mice. Such studies are inherently problematic since the susceptibility of these mouse strains to S. Typhimurium often varies because of differences in background genetics, independent of their Nramp1 status. Since these mice have differential expression of a plethora of other genes - the lack of an obvious effect by Nramp1 in this study could have been the result of a polygenic suppression of an early Nramp1-associated phenotype. Here, using isogenic mouse strains for comparison, we show an unequivocal role for Nramp1 in stimulating a faster inflammatory response in S. Typhimurium induced colitis.

Inflammatory cell recruitment is often reported in the S. Typhimurium induced enterocolitis model and is likely a consequence of the localized expression of chemokines and cytokines at the site of infection, leading to an inflammatory response [3]. Surprisingly, in most studies the expression of these mediators has not been analyzed in detail using this model [2, 26-28]. Although Godinez et al. [21] recently showed upregulation of numerous cytokines and chemokines 48 hours after S. Typhimurium infection in the ceca of C57/Bl6 mice, this was not correlated to Nramp1 expression. Here we have addressed this issue and found that the extent of cytokine production is dependent on Nramp1 expression as Nramp1 mutant mice had elevated expression of TNF-α, MIP-1α and IFN-γ, early after infection (day 1). Analogous chemokines have also been reported in human enterocolitis and in a bovine cecal ligation model of colitis [3]. Overall, the data suggest that expression of cytokines and chemokines probably precedes PMN infiltration and the stronger inflammation observed in Nramp1 mutant mice is most
likely a consequence of higher cytokine expression. We present here the first analysis of the effect of Nramp1 on cytokine and chemokine expression after infection with S. Typhimurium using the mouse enterocolitis model.

Recent studies have provided insights into the molecular mechanisms governing Nramp1’s role in enhanced cytokine production. In a study by Gomez et al. [29] Nramp1 was shown to play a role in modulating the activity of protein tyrosine phosphatases (PTP). Expression of Nramp1 results in lower macrophage PTP activity [29]. Since these phosphatases are well known to dampen signals involved in cell activation and cytokine production, their inactivation via Nramp1-dependent iron transport leads to amplified cytokine secretion. Although this study clarified Nramp1’s role in mediating enhanced cytokine production, to our knowledge the present work represents the first detailed in vivo evaluation of how this enhanced cytokine production leads to a more rapid and protective immune response to a bacterial pathogen.

Chemokines such as KC and MIP-1α are important chemoattractants of neutrophils and monocytes respectively [30, 31], and we observed significantly higher numbers of these cell types in the ceca of Nramp1+/+ mice following S. Typhimurium infection. Cytokines such as TNF-α and IFN-γ are master regulators of the immune system with the ability to activate various cell types [32, 33]. That these cytokines are crucial for S. Typhimurium clearance is highlighted by the fact that TNF-α deficient mice are highly susceptible to Salmonella infection [34-36]. Similarly, IFN-γ receptor knockdown mice (in a Nramp1+/+ background) succumb to systemic S. Typhimurium infection (Vallance et al. unpublished observations), probably due to a deficient activation of phagocytic cells. Indeed, IFN-γ primed macrophages [10, 37] and DCs
(Valdez, unpublished data), have more potent bactericidal activity killing S. Typhimurium faster than non-primed cells in in vitro assays. We suggest that the accelerated cytokine/chemokine expression observed in Nramp1+/+ animals results in activation and recruitment of other cells of the immune system to the site of inflammation, creating an anti-microbial environment in the gut and limiting spread of Salmonella to systemic sites. Consistent with this hypothesis, we found most S. Typhimurium bacteria in the lumen and adherent to epithelial cells in the mucosa of Nramp1+/+ mice but not within the mucosa which contained numerous MPO positive cells. In contrast, in Nramp1−/− mice we found very few MPO positive cells but high numbers of S. Typhimurium in the mucosa and submucosa. Therefore, the early inflammation influences the location of bacteria rather than total numbers. At day 4, inflammation was stronger in Nramp1−/− mice and at this time point bacteria were almost exclusively found in the lumen in both mouse strains. This late inflammation is independent of Nramp1 and likely a compensatory mechanism subsequent to the high proliferation of S. Typhimurium in Nramp1−/− mice.

We have previously identified a specific subset of DCs (CD11c+ CD103−) as the major cell type secreting proinflammatory cytokines following S. Typhimurium infection in the lamina propria of the small intestine. Interestingly, this subset was characterized by a higher expression of Nramp1 [17]. It is plausible that the same subset of DCs could be a major source of cytokine secretion in the Nramp1+/+ mice in S. Typhimurium induced colitis. Recently, numerous reports point to NK and NKT cells as major cell types secreting IFN-γ early after S. Typhimurium infection, both in the spleen and liver, following intravenous infection [38] and in the Peyer’s patches and MLN after oral
infection [39]. It is likely that NK and NKT cells could be the source of IFN-γ in early colitis. It is tempting to speculate that cytokines such as IL-12, which is known to be a key activator of NK and NKT cells, are secreted at high levels by CD103+ DCs (Nramp1 expressing DCs) early following S. Typhimurium infection in Nramp1+/+ mice. This would permit a faster stimulation of NK and NKT cells in these mice, leading to higher production of IFN-γ by these cells. Thus, paracrine activation of IFN-γ on different cell types could explain the faster inflammation observed in Nramp1+/+ mice.

A model for how Nramp1-dependent cytokine secretion leads to protection is presented in Figure 3.6. Following pathogen detection, chemokines and cytokines are rapidly released in Nramp1 expressing mice but accumulate only after a substantial lag in Nramp1 negative mice. This results in a more rapid phagocyte (neutrophil/macrophage) activation and influx into the lamina propria, epithelia and lumen of the gut. Nramp1 negative mice fail to induce this rapid response thus providing the pathogen with sufficient time to replicate and breach the epithelial layer and compromise the ability to contain the infection.

In conclusion our data show that Nramp1 plays a critical role in orchestrating the inflammatory response in the gut mucosa. The early expression of Nramp1 during the intestinal inflammatory response to *Salmonella* results in a stronger inflammatory response and correlates highly with decreased bacterial loads in all organs, thereby controlling the infection.
3. 4 Experimental procedures

Mice

129SvJ mice and isogenic controls were described previously [24]. Nramp1\(^{-/-}\) mice have a null allele at the Nramp1 locus (the Nramp1 gene was interrupted by an inserted Neomycin cassette), thus mRNA transcripts are absent. Sex and age matched mice (8-12 weeks old) were pretreated with 20 mg of streptomycin (Sm) by oral gavage, 24 hours prior to infection [18]. Mice were infected orally with 3x10\(^6\) in 100 µl HEPES buffer (100 mM, pH 8.0). Control mice that were also pretreated with Sm, were given 100 µl HEPES buffer. Mice were euthanized at designated time points and tissues were harvested aseptically for bacterial enumeration and histopathology. All animal experiments were conducted following the exact ethical requirements of the Animal Care Committee at the University of British Columbia.

Bacterial strains

*S. enterica* serovar Typhimurium strain SL1344 was grown at 37°C with shaking (200 rpm) in Luria-Bertani (LB) broth supplemented with 100 µg/ml streptomycin.

Tissue collection and bacterial enumeration

Tissues were collected at various time points into 1 ml of sterile PBS and homogenized with a MixerMill 301 (Retsch, Newtown, PA, USA). For cecum counts, the proximal half including all luminal contents was used for determination of bacterial colonization. For colon colonization, the whole organ including luminal contents was used for
homogenization. Serial dilutions of the homogenate were plated on LB agar plates containing 100 µg/ml streptomycin.

**Histology**

Tissues were fixed in 10% neutral buffered formalin overnight and then placed into 70% ethanol. Fixed tissues were embedded in paraffin and cut into five µm sections. Tissues were stained with H&E using standard techniques by Wax-it Histology Services (Vancouver, BC, Canada).

**Tissue Pathology Scoring**

Tissue pathology in the infected cecum was scored blind using number coded H&E stained sections as previously described [26]. Briefly, the scoring system was as follows: for lumen, sum of empty (score 0), necrotic epithelial cells (scant 1, moderate 2, dense 3), and polymorphonuclear cells (PMNs) (scant 2, moderate 3, dense 4); for surface epithelium, sum of no pathological change (score 0), regenerative change (mild 1, moderate 2, severe 3), desquamation (patchy 1, diffuse 2), and PMNs in epithelium (score 1), ulceration (score 1); for mucosa, sum of no pathological change (score 0), crypt abscesses (rare [15%] 1, moderate [15% to 50%] 2, abundant [50%] 3), presence of mucinous plugs (1), and presence of granulation tissue (1); for submucosa, sum of no pathological change (0), mononuclear cell infiltrate (1 small aggregate 0, more than one aggregate 1, large aggregates plus increased single cells  2), PMN infiltrate (none 0, single 1, aggregates 2), and edema (mild 0, moderate 1, severe  2).
**Immunofluorescence, frozen sections**

Cecal tissues were removed from mice, transferred into blocks containing OCT (optimum cutting temperature) compound and then frozen in liquid nitrogen, followed by storage at -80°C. Five µm sections were cut by Wax-it Histology Services, Inc. (Vancouver, Canada). Sections were then plunged into –20°C acetone for 5 minutes and 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 the primary antibodies. After incubation, the slides were washed 3 times for 10 min each with TPBS/BSA buffer then incubated for 1-2 hours at 37°C with secondary antibodies conjugated to Alexa fluorochromes. The slides were washed 3 times for 10 min each with TPBS/BSA buffer, mounted with Prolong Gold Antifade reagent (Invitrogen) and analyzed using a Zeiss Axiophot epifluorescence microscope.

**Immunofluorescence, paraffin sections**

5 µm sections were deparaffinized and rehydrated. After heat-induced antigen retrieval in citrate buffer, immunostaining was carried out using antibodies against myeloperoxidase (MPO, Thermo Scientific, Fremont, CA, USA) to visualize neutrophils and F4/80 (Serotec) for macrophages and anti-*Salmonella* LPS to visualize *S. Typhimurium*, followed by fluorescently labelled secondary antibodies. Images were obtained using a Zeiss Axiohot microscope equipped with an AxioCam HRm camera. To evaluate localization of *S. Typhimurium*, pictures of three random fields of view of 5 ceca for each condition were taken at 200x and bacteria were counted according to their localization as
a) within the tissue (mucosa, submucosa and muscle layers), b) adherent or in proximity to the epithelium or c) in the cecal lumen.

**RNA isolation and cDNA synthesis**

The terminal 2 - 3 mm of the ceca were submerged in RNAlater (Qiagen, Mississauga, ON, Canada) and stored at -70°C. RNA was extracted using RNeasy Mini kit (Qiagen) according to manufacturer’s instructions. RNA concentration was determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and reverse transcription was performed with the Quantitetc RT kit (Qiagen) using 1 µg RNA as starting material. Final cDNA was diluted 1:10 and 1 µl of diluted cDNA was used for PCR reactions.

**Quantitative polymerase chain reaction**

Quantitative PCR (q-PCR) was performed using Quantitetc SYBR-Green Mastermix (Qiagen) and following forward (fw) and reverse (re) primers:

- **NRAMP1 fw**: GCCATCTCTACTACCCCAAGG
- **NRAMP1 re**: TCATAGGGGAAGGTCAAAAGC
- **GAPD fw**: ATGTCAGCAATGCATCCTG
- **GAPD re**: ATGGACTGTGGTCATGAGCC
- **IL-6 fw**: GAGGATAACCACCCTCCCAACAGACC
- **IL-6 re**: AAGTGTCATCATCGTTGTTCATACA
- **TNFa fw**: CCACCACGCTCTTCTCTGCTAC
- **TNFa re**: AGGGTCCTGGGCCATAGAACT
- **KC re**: TCTCCGTACTGGGGACAC
- **KC fw**: ACCCAAACCGAAGTCATAGC
- **IFN-γ fw**: TCAAGTGGCATAGATGTGGAAAGAA
- **IFN-γ re**: TGGCTCTGAGATTTCATAG
- **MIP-1α fw**: ACCATGACACTCTGCAACCA
- **MIP-1α re**: GTGGATACCTCTCCGGCTGCTAG
PCR was performed on 7500 Fast Real Time System (Applied Biosystems, Foster City, CA, USA) and cycles consisted of 50°C, 2 min, 95°C, 10 min and 39 cycles of 95°C, 15 s, 60°C, 1 min.

**Statistical analysis** Data was analyzed using the following statistic test:

Unpaired Student T test (parametric test) was used to compare two groups (control vs treatment) for experiments performed *in vitro* such as evaluation of genes expression by q-PCR.

Mann-Whitney test (non-parametric test) was used to compare CFU from internal organs of *Salmonella* infected mice at day 1 and day 4 post-infection.

One-way ANOVA with Bonferroni’s multiple comparison post-test (to compare pairs) was performed using a 95% confidence interval, this test was used to analyze *in vivo* data comparing cecal weight and pathology scores at different times post-infection with *Salmonella*.

All analyses were performed using GraphPad Prism version 4.0.
3. 5 Literature cited


Chapter 4

Chronic enteric *Salmonella* infection in mice leads to severe and persistent intestinal fibrosis

4.1 Introduction

The inflammatory bowel diseases (IBD) comprising ulcerative colitis (UC) and Crohn’s disease (CD) are characterized by chronic inflammation, severe intestinal pathology and changes in intestinal physiology that cause the characteristic symptoms of pain, nausea and diarrhea. In recent years, significant progress has been made in our understanding of the pathogenesis as well as the treatment of IBD. Several groundbreaking studies have identified genetic factors that predispose to the development of CD and UC, while research has also helped define specific immune mediators that play a key role in their pathogenesis. Moreover, studies have clarified that bacterial stimulation of the mucosal immune system is a key factor in the etiology of IBD. While these findings have not led to a cure, they have helped improve the available treatment options, including the targeting of TNF-α as well as the use of immunomodulatory therapies, antibiotics and probiotics. Notably, many of these advances have originated through discoveries made using experimental mouse models of IBD [1-5].

Despite these findings, significant morbidity still arises from the fibrosis that frequently occurs in the inflamed bowel segments of CD patients. For reasons that are currently unclear, the repair processes that develop in the inflamed intestines of CD patients contribute to ongoing inflammation and scarring. Aversion of this chapter has been published as: Guntram A. Grassl*, Yanet Valdez*, Kirk S. B. Bergstrom, Bruce A. Vallance and B. Brett Finlay (2008). Chronic enteric *Salmonella* infection in mice leads to severe and persistent intestinal fibrosis. Gastroenterology, 134 (3): 768-80

* Authors contributed equally to this work
patients can progress uncontrollably, leading to the proliferation of mesenchymal cells and the extensive deposition of extracellular matrix (ECM). These processes thicken the gut wall, often resulting in obstructive strictures.

Whereas new therapies are urgently needed to prevent and/or treat intestinal stricturing [6], to date our understanding of the mechanisms that lead to intestinal fibrosis and stricture formation are limited, in large part due to the lack of simple, effective and reproducible mouse models of intestinal fibrosis. While rats have been shown to develop fibrosis following injection of peptidoglycan-polysaccharide into their gut walls [7] and following repeated bouts of trinitrobenzene sulfonic acid (TNBS) colitis [8], mice represent a preferred model because of the genetic and immunologic tools available for their study. Unfortunately, mice are relatively resistant to developing fibrotic disease, both in the intestine and at other mucosal sites [9]. Despite these difficulties, intestinal fibrosis in mice has been shown to occur following repeated induction of TNBS colitis [10], and following adenoviral gene transfer and over-expression of transforming growth factor-b1 (TGF-β1) [11], and monocyte chemoattractant protein-1 (MCP-1) in the colon [12]. Unfortunately these models are not ideal, since they can be labour intensive, lead to frequent mortality, and the resulting fibrosis can vary in intensity and position within the GI tract. Moreover, while the over-expression of pro-fibrotic cytokines has proven useful in determining their potential role in fibrosis, these models are limited in their ability to address how fibrotic processes develop naturally following inflammatory events in the intestine.

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a clinically important intracellular bacterial pathogen that causes food poisoning and gastroenteritis
in millions of people each year. Following oral inoculation of mice, only small numbers of these bacteria infect the intestine, causing modest inflammation. However when mice are pre-treated with the antibiotic streptomycin prior to infection with *S. Typhimurium*, the resulting enteric phase of the infection is far more successful, leading to heavy *S. Typhimurium* colonization of the cecum and colon and significant colitis [13]. It has also been shown that the duration of *S. Typhimurium* infection and the resulting colitis differs amongst mouse strains [14] so we hypothesized that prolonged infections might also lead to intestinal fibrosis.

Here, we show that *S. Typhimurium* infection in several mouse strains leads to chronic infection and colitis in association with extensive transmural ECM deposition within the cecum and colon. This fibrotic response was accompanied by elevated expression of Th1 cytokines as well as several pro-fibrotic cytokines including TGF-β1, connective tissue growth factor (CTGF), insulin-like growth factor (IGF)-1 and MCP-1. These data indicate that oral *S. Typhimurium* infection in mice offers a robust model of bacterial induced intestinal fibrosis that will aid in elucidating the contributions of both host and bacterial factors to the development of intestinal fibrosis and stricture formation.

4.2 Results

4.2.1 Chronic infection with *S. Typhimurium* induces inflammation and tissue pathology

Following oral inoculation, *S. Typhimurium* infects the small and large intestines of mice, although in relatively small numbers. In susceptible mouse strains, carrying the null mutation in the Nramp1 gene (*nramp*<sup>D169</sup>), this pathogen subsequently spreads from
the intestines to the mesenteric lymph nodes, spleen and liver where \textit{S. Typhimurium} proliferate to large numbers, causing lethal sepsis. Interestingly, some mouse strains including 129Sv/J mice (\textit{nramp}\textsuperscript{G169}) exhibit innate resistance to \textit{S. Typhimurium} such that they limit the spread and virulence of these bacteria, resulting in chronic infections of the spleen and mesenteric lymph nodes [15]. To address whether \textit{S. Typhimurium} could also chronically infect the intestines, 129Sv/J mice were infected with $3 \times 10^6$ cfu of \textit{S. Typhimurium} and host survival, as well as bacterial counts, inflammation and tissue pathology were assessed. As expected, the majority (95\%) of mice survived the infection and when assessed at day 29 post-infection (pi), \textit{S. Typhimurium} were detected at low levels ($10^2$-$10^3$ bacteria) in the cecum and colon while similar numbers were recovered from the spleen ($10^4$ bacteria) and mesenteric lymph nodes ($10^3$ bacteria). Despite the chronic infection few signs of inflammation were detected in these tissues (not shown).

Pre-treatment with the antibiotic streptomycin facilitates \textit{S. Typhimurium} infection of the murine GI tract [13]. 129Sv/J mice were treated with streptomycin twenty-four hours prior to infection with $3 \times 10^6$ \textit{S. Typhimurium} and sacrificed at specific time points thereafter. By day 7 pi the ceca and colons of infected mice were heavily colonized with the pathogen burden ranging from $10^5$ to $10^9$ \textit{Salmonella} in these tissues (Figure 4.1 A), which is in line with previous studies [14]. Colonization of all organs was monitored for 40 days; \textit{Salmonella} numbers remained at these high levels during the entire time course (Figure 4.1 A).

Chronic infection of 129Sv/J mice was accompanied by watery stool and a pronounced reduction in the size of the cecum (Figure 4.1 B, C). Moreover, the colons and ceca of infected mice developed a thickened appearance and “rubbery” consistency.
Histochemical analysis of H&E stained tissue sections showed that infected tissues displayed extensive submucosal edema, as well as a dramatically thickened mucosa indicative of crypt hyperplasia as well as goblet cell depletion. Extensive transmural inflammation was evident primarily in the cecum but also in the colon throughout the time course, from day 7 to day 40 pi (Figure 4.2 A-B). H&E and naphthol chloroacetate-esterase (stain for neutrophils) staining revealed a massive influx of mononuclear cells and neutrophils predominantly in the submucosa by day 7 pi, in concert with focal mucosal ulcerations and widespread sloughing of epithelial cells into the lumen of the gut. By day 14 pi, neutrophil infiltration was even greater, within both the cecal mucosa and lumen, while epithelial destruction was maximal, with the entire crypt epithelium lost in many regions and replaced by infiltrating cells. Submucosal edema followed a similar time course peaking at day 14 pi, while the muscularis mucosa was almost entirely destroyed (day 14, arrowheads). By day 21 and at day 40 pi, the makeup of the inflammatory cell infiltrate had changed, such that only a few neutrophils were observed. Instead, aggregates of mononuclear cells including lymphocytes were seen at later time points. Evidence of epithelial regeneration and restitution was also seen at day 21 pi and further repair of the epithelia and crypts was evident at days 29 and 40 pi, although the crypt epithelium was still not fully restored by day 40 pi, with significant pathology still evident (Figure 4.2 A-B).
Figure 4.1 Chronic colonization of mouse organs by *S. Typhimurium*. A. Bacterial counts of colon, cecum, and spleen; dashed line: limit of detection; cfu, colony forming units. B. Photographs of ceca with colon removed from mice over the time course of infection; C. Cecal weights were assessed over a similar time course. *p* values for infected ceca vs. uninfected control were calculated using one-way ANOVA with Bonferroni’s multiple comparison post test. *= p < 0.05; **= p < 0.01; ***= p < 0.001
Figure 4.2 A Chronic S. Typhimurium infection-induced inflammation and fibrosis in mouse cecum. H&E and trichrome staining of mouse ceca. H&E staining revealing mucosal hyperplasia, edema and crypt abscesses at day 7, 14, 21, 40. Trichrome staining showing transmural collagen deposition (blue) including mucosa, submucosa, muscularis mucosa and muscularis propria; bar: 500 µm. Trichrome at higher magnification; bar: 100 µm. L, lumen; M, mucosa; MP, muscularis propria; SM, submucosa; arrowheads, muscularis mucosa.
Figure 4.2 B Pathology score of infected ceca. Pathological changes were severe at day 7, peaked at day 14 when especially lumen and surface epithelium scores reached maximum values but stayed high throughout the time course. Each bar represents one animal.
4.2.2  *S. Typhimurium* infection leads to transmural fibrosis in the cecum and colon

Next, we investigated whether the hardened and rubber-like texture of the infected ceca and colons reflected the development of fibrosis in these tissues. The presence of ECM can be visualized by Masson’s trichrome (trichrome) staining and as shown in Figure 4.2 A, Masson’s staining of uninfected ceca showed only a thin layer of ECM within the submucosa. In contrast, by day 7 pi, extensive ECM deposition was detected surrounding many of the cells infiltrating the mucosa, while numerous fibrils of collagen could be seen within the muscularis mucosa as well as interspersed between cells scattered throughout the edematous submucosa. By day 14, the ECM staining was increased, such that extensive ECM deposition was found spanning the entire cecal wall. Heavy fibrosis was observed within and surrounding the muscularis propria and the muscularis mucosa. Moreover the previously edematous submucosa was filled with ECM, as was much of the mucosa, with the fibrosis reaching almost to the cecal lumen. Interestingly, by day 21 and day 40 pi, the extent of trichrome staining within the mucosa had lessened, in keeping with the partial restoration of the mucosal architecture. In contrast, ECM staining in the muscularis mucosa, muscularis propria and submucosa remained similar, or was even increased compared to that seen at day 14 pi. Trichrome staining of fibrotic colons was similar to that found in the ceca (Figure 4.7 in appendix 3). The fibrosis, particularly in the deeper layers of the gut was persistent, since significant tissue fibrosis was detected even 70 days pi (not shown).

To quantify collagen deposition in this model, pieces of cecum were excised and digested with pepsin for 24 h. Collagen determination identified a striking 8.4 (± 1.9) fold
increase in collagen content in these tissues at day 21 compared to uninfected tissues, confirming the results of the histochemical staining.

4.2.3 **S. Typhimurium infection upregulates Th1 cytokines, fibrotic growth factors and procollagen type I.**

CD is associated with strong Th1 immune responses, including elevations in the expression of the pro-inflammatory cytokine TNF-α. Moreover, we and others have already identified specific pro-fibrotic mediators including TGF-β1 and MCP-1 that contribute to bowel fibrosis, putatively by inducing the expression of the fibrosis inducing CTGF. To address whether inflammatory mediators and pro-fibrotic growth factors previously implicated in bowel fibrosis were expressed in this model, we assayed these responses using cytometric bead array (CBA) on supernatants from cecal homogenates.

We found a significant increase in TNF-α, IFN-γ and MCP-1 expression in the ceca of infected mice when compared to control mice (Figure 4.3 A). Conversely, we could not detect any increase in IL-10, confirming a strong Th1 immune response in this model. Recently, it has also been shown that levels of IL-17, a key cytokine produced by Th17 cells are also increased in Crohn’s patients [16]. Quantitative real time PCR (qPCR) showed that IL-17 was also highly induced in the ceca of S. Typhimurium infected mice (Figure 4.3 B) compared to controls.

CTGF, TGF-β1 and IGF-1 have been shown to be upregulated in fibrotic tissues of CD patients. We performed q-PCR in order to assess if these cytokines were upregulated in the fibrotic ceca of S. Typhimurium-infected mice. CTGF expression was
significantly upregulated at day 7 pi and TGF-β1 and IGF-1 was significantly upregulated from day 14 onwards (Figure 4.3 C). We also analyzed latent and active TGF-β1 protein levels from tissue homogenates by ELISA and identified a significant increase in both forms of TGF-β1 in infected ceca (Figure 4.3 D). Overall, the observed pattern of cytokine expression in this model resembles the cytokine profile found in Crohn’s patients [16], strengthening its relevance in studying intestinal fibrosis.

Collagen types I and III have been shown to be increased in fibrotic tissues from CD patients. Using q-PCR analysis of samples from fibrotic ceca we found upregulation of procollagen-1 (Col1a2) but no significant induction of procollagen-3 (Col3a1). Immunofluorescence staining of ceca from uninfected mice showed collagen I expression in the submucosa while collagen III was seen in the serosa. At day 21 we found a strongly increased signal for collagen I in the submucosa (SM; Figure 4.3 F) and within the muscularis propria (MP; arrowheads). In contrast, staining against collagen III was found to be patchy in the submucosa and muscularis, and the strongest signal was seen in proximity to the muscularis mucosa and around blood vessels (Figure 4.3 F, arrows).
Figure 4.3 Induction of Th1 cytokines and pro-fibrotic cytokines in chronically infected ceca. A. Cytokine levels of in tissue homogenate determined by CBA. Gene expression of IL-17 (B), TGF-β1, CTGF and IGF-1 (C) in ceca of chronic S. Typhimurium infected mice as determined by q-PCR. Data were normalized to GAPD levels and are relative to uninfected control. D. Protein levels (pg/ml) of latent and active TGF-b1 in cecum homogenate measured by ELISA. E. Quantification of procollagen I (col1a2) and procollagen III (col3a1) by q-PCR. F. IF staining of collagen I and III; magnification: 200x. M, mucosa; MP, muscularis propriae; SM, submucosa. p values were calculated using students t test (A, D, E), Mann-Whitney (B) or one-way ANOVA with Bonferroni’s multiple comparison post test (C).
4.2.4 Fibroblasts and myofibroblasts are found in large numbers in fibrotic areas

Mesenchymal cells are thought to be responsible for ECM deposition in many fibrotic diseases. While fibroblasts, myofibroblasts and smooth muscle cells have all been implicated in the widespread deposition of ECM found in the strictured tissues of CD patients, it remains unclear if all these cell types contribute equally, and if there are regional and tissue specific differences in their actions or involvement. Answering these questions is of considerable importance since these different cell types constitute potential targets for therapeutic intervention. To identify the mesenchymal cell types involved in development of *S. Typhimurium* induced fibrosis, tissue sections were stained with antibodies to vimentin (V), α-smooth muscle actin (A) and desmin (D). The staining pattern allows for distinction between fibroblasts (V+, A−, D−), myofibroblasts (V+, A+, D+/−) and smooth muscle cells (V−, A+, D+).

In uninfected mice, the smooth muscle cells within the muscularis externa and muscularis mucosa stained strongly for both actin and desmin (A+, D+). In addition, muscle cells in the lamina propria (LP) stained A+ and D+, as did the vascular smooth muscle cells within blood vessel walls in the submucosa (Figure 4.4 A). In contrast, few V+ cells (fibroblasts or myofibroblasts) were found in the LP and they were virtually absent in the submucosa of uninfected mice. During *S. Typhimurium* infection, few overt changes were seen in the number or location of the smooth muscle cells, although the staining was patchier, suggesting the influx of other cell types into the muscle layers. Infection was however associated with a marked increase in the number of V+ cells present within fibrotic tissues, with many V+ cells found in the submucosa, and mucosa of infected ceca, as well as within selected regions of the muscle layers. While an
increase in A+ cells was also seen during infection, it was limited to the mucosa. Interestingly, in the mucosa, most of the A+ cells were also V+ and weakly D+ and these V+ A+ D+ cells showed fibroblast-type morphology suggestive of myofibroblasts (white arrows). In contrast, all the V+ cells in the submucosa were A- and D-, while a majority of the V+ cells in the mucosa also presented with this phenotype, suggesting they were fibroblasts. These staining patterns suggest that different populations of mesenchymal cells are recruited to the inflamed cecum, with fibroblasts found throughout the gut wall, whereas myofibroblasts are only found in the mucosa. The distribution of mesenchymal cell types in the fibrotic colons was similar to that found in the ceca (Figure 4.8 in appendix 3). These data thus suggest that fibroblasts are likely the key cell types contributing to ECM production in this model. However the proximity of ECM to myofibroblasts and smooth muscle cells in the mucosa and intermixed with and surrounding the muscle layers indicates that these cells also likely contribute to the fibrosis.
Figure 4.4 Distribution of mesenchymal cell types in ceca of uninfected control mice or the fibrotic ceca of mice chronically infected with *S. Typhimurium* for 21 days. Mesenchymal cells were differentiated by staining for vimentin (V), $\alpha$-smooth muscle actin (A) and desmin (D). V+ A+ D- and V+ A+ D+ (white arrows) cells were only found in the mucosa. Original magnification of the first and second column of images: 200x; third column: higher magnification of the area indicated by white rectangle in the images of second column. Merge: vimentin (red); a-smooth muscle actin (green); desmin (blue).
4.2.5 The severity of intestinal fibrosis induction depends on host genetics

While S. Typhimurium infection can cause severe intestinal fibrosis in 129Sv/J mice, it is unclear if this is a strain-dependent pathology or if S. Typhimurium can induce similar fibrosis in mice of different genetic backgrounds. To address this issue, three other mouse strains (129Sv/ImJ, DBA/2 and C3H/HeOuJ) were orally infected with 3 x $10^6$ S. Typhimurium for 28 days and monitored for infection, inflammation and the development of fibrosis. S. Typhimurium colonized all tested mouse strains to a similar level (Figure 4.5 A), however, inflammation and fibrosis were considerably less pronounced in C3H/HeOuJ mice compared to 129Sv/ImJ and DBA/2 mouse strains (Figure 4.5 C). Fibrosis was primarily restricted to the submucosa of the C3H/HeOuJ mice and almost no collagen staining was visible within the external muscle layers or in the mucosa. In contrast, in 129Sv/ImJ and DBA/2 mice, collagen staining was observed throughout the muscle layers, submucosa and in 129Sv/ImJ mice also in the mucosa. These data demonstrate that chronic S. Typhimurium infection can lead to cecal and colonic fibrosis in a variety of mouse strains; however the resulting fibrosis was qualitatively and quantitatively different in these strains, emphasizing the importance of host genetics in modulating the development of fibrosis.

4.2.6 S. Typhimurium ΔaroA induces fibrosis in C57/Bl6 mice

Further assessment of the host contribution to the fibrosis in this model would be facilitated by infection of gene knockout mice. However, the majority of available knockout mice are maintained on a C57/Bl6 background. These mice carry alleles encoding an amino acid substitution in Nramp1 ($nramp^{D169}$) resulting in a non-functional
protein. As a result, C57/Bl6 mice are extremely susceptible to infection with wild-type S. Typhimurium and die between day 6 and day 9 after oral infection. To test if we could chronically infect and induce fibrosis in nrampD169 mice, we infected streptomycin-treated C57/Bl6 mice for 21 days with the attenuated S. Typhimurium ΔaroA mutant. This is an auxotrophic Salmonella mutant strain carrying non-reverting deletion in the aroA gene, making it dependent for growth on certain aromatic compounds not found in mammalian hosts [17]. As shown in Figure 4.5 B, S. Typhimurium ΔaroA mutant bacteria colonized the spleen, colon and cecum. Colonization of the intestine was between 10^4 – 10^5 cfu/organ and thus 1 - 2 orders of magnitude lower than colonization of colon and cecum of 129SvJ mice with wild-type S. Typhimurium. However, S. Typhimurium ΔaroA induced pronounced inflammation and fibrosis in the cecum of infected mice. Collagen deposition was strongest in the submucosa and muscle layers (Figure 4.5 C). Quantification of collagen showed a marked increase of collagen in the ceca of infected mice at day 21 and confirmed the observations made by trichrome staining (Figure 4.5 D). These data show that infection with S. Typhimurium ΔaroA mutant bacteria permits the analysis of intestinal fibrosis in mice of the nrampD169 background such as C57/Bl6.
Figure 4.5: Chronic colonization and induction of fibrosis by S. Typhimurium in various mouse strains. A - B. Bacterial counts in spleen, colon and cecum. Dashed lines: limit of detection C. H&E staining of the ceca showing inflammation including edema, hyperplasia and crypt abscesses in 129Sv/ImJ and DBA/2 mice. Trichrome staining of 129Sv/ImJ, DBA/2 and C3H/HeOuJ mice; bar: 500 µm. D. Quantification of pepsin-soluble collagen in caecal tissues by Sircol assay of caecum from uninfected C57/Bl6 mice (cntrl.), compared to mice infected with S. Typhimurium ΔaroA. Data were normalized for tissue weight. p values were calculated using students t test.
4.2.7 *Salmonella* is found primarily in the cecal lumen of chronically infected mice and requires specific virulence factors to induce fibrosis

To chronically infect the intestine, bacteria must colonize a niche where they are protected from eradication by the host’s immune system, either because of defects in the host response or in the case of pathogens, because they can circumvent these defenses through the actions of specific virulence factors. To address the location and strategy by which *S*. Typhimurium chronically infects the intestine, we stained infected cecal tissues for *Salmonella* LPS by immunofluorescence. As shown in Figure 4.6 A, *S*. Typhimurium were found predominantly in the lumen of the cecum, while a small number were detected in the mucosa (white arrows). This localization indicates that the chronic inflammation and fibrosis seen in this model may be a response to luminal *Salmonella*.

It is well established that *S*. Typhimurium uses several pathogenicity islands, including SPI-1 and SPI-2 to elaborate virulence factors into host cells, allowing these bacteria to infect their hosts and evade both extracellular and intracellular defenses. SPI-1 is important for translocation of *S*. Typhimurium through the gut wall and SPI-2 is crucial for intracellular survival within phagocytes. To examine if *Salmonella* effectors secreted by SPI-1 or SPI-2 are required for the development of fibrosis, we orally infected streptomycin-pretreated mice with *S*. Typhimurium ΔinvA (SPI-1 mutant) or *S*. Typhimurium ΔssaR (SPI-2 mutant) for 21 days and analyzed bacterial colonization, cecal weight, inflammatory changes in the cecum and ECM deposition. Both mutants heavily colonized the GI tract to a similar extent as wild-type bacteria but surprisingly; neither mutant was capable of inducing significant fibrosis (Figure 4.6 B-C). Thus, although SPI-1 and SPI-2 are dispensable for chronic colonization of the murine GI tract,
both pathogenicity islands are essential for the induction of fibrosis. These data suggest that in the absence of the genetic susceptibility exhibited by CD patients these microbes can only trigger chronic inflammation and fibrosis when they express specific virulence factors.

In contrast, commensal bacteria do not possess pathogenicity islands or translocated effectors, therefore the fibrosis seen in CD patients is rather thought to be the result of genetic susceptibility combined with an aberrant immune response to microbial products. Flagellin is a major antigenic determinant and it is highly conserved between pathogenic and commensal bacteria. To assess whether flagellin expression by *S. Typhimurium* contributed to fibrosis, we infected mice with a *S. Typhimurium* Δ*fljB/ΔfliC* double mutant, which is missing both major flagellar subunits. Our results show that *S. Typhimurium* Δ*fljB/ΔfliC* colonized 129SvJ mice as well as wild-type bacteria and caused pronounced inflammation and collagen deposition at day 21 pi (Figure 4.6 B-C). However, the amount of collagen induced by the *S. Typhimurium* Δ*fljB/ΔfliC* mutant was approximately 50% of the level induced by wild-type bacteria (Figure 4.6 D). Thus flagellin expression contributed significantly to the fibrotic response seen in our model.
Figure 4.6 *Salmonella* pathogenicity islands (SPI)-1 and SPI-2 are required for the induction of fibrosis. Mice were treated with streptomycin 24 h prior to oral infection with *S.* Typhimurium wild-type, ΔssaR, ΔinvA or ΔfljB/ΔfliC.

**A.** Immunostaining of *S.* Typhimurium (red) in the cecum showing that the majority of bacteria are found in the cecal lumen. Only a few bacteria were found in the mucosa (white arrows). Left panel: uninfected control, centre and right panels: infected with wild-type *S.* Typhimurium day 7 pi. L, lumen; M, mucosa; SM, submucosa; magnification left and middle panel: 100x, right panel: higher magnification of the area indicated by white rectangle.

**B.** Bacterial counts in colon, cecum and spleen.

**C.** H&E staining shows massive inflammation in ΔfljB/ΔfliC infected ceca and some inflammatory infiltrates in ΔinvA infected ceca whereas there is no overt evidence of inflammation in the ceca of ΔssaR infected mice. Trichrome staining shows no significant or abnormal collagen deposition within the ceca of mice infected with either *S.* Typhimurium ΔinvA or *S.* Typhimurium ΔssaR and pronounced collagen staining in ceca of ΔfljB/ΔfliC infected mice; bar: 500 µm.

**D.** Quantification of pepsin-soluble collagen in cecal tissues by Sircol assay of cecum from uninfected control mice, compared to mice infected with WT, ΔssaR, ΔinvA or ΔfljB/ΔfliC *S.* Typhimurium. Data were normalized for tissue weight. *p* values were calculated using one-way ANOVA with Bonferroni’s multiple comparison post test.
4.3 Discussion

Intestinal fibrosis and stricture formation are among the most problematic clinical features of CD, in large part because they are strikingly resistant to anti-inflammatory treatments. As a result, therapeutic options are limited, with endoscopic balloon dilatation usually only delaying intervention with stricturoplasty or surgical resection of affected segments. While the etiology underlying the development of intestinal fibrosis remains unknown, commensal bacteria and/or bacterial products are key factors in triggering IBD [18] and experimental colitis [19, 20]. As highlighted in several recent reviews, the testing of potential therapies would be greatly advanced by the development of a robust model of intestinal fibrosis. In addition, the relevance of such a model would be heightened if the resulting fibrosis was associated with bacterial induced inflammation [9, 21, 22]. Unfortunately it has proven difficult to model intestinal fibrosis in laboratory animals since they generally do not develop intestinal fibrosis in response to commensal microbes, even in the context of chronic bowel inflammation.

By chronically infecting the large bowel and cecum of mice with the clinically important and genetically well defined enteric bacterial pathogen S. Typhimurium, we overcame these difficulties, generating a novel model of persistent bowel inflammation and transmural fibrosis that mimics the pathology seen in Crohn’s patients [23-27]. At early time points the fibrosis spanned the entire cecal wall whereas from day 21 onwards the fibrosis was mainly localized to the muscle layers and the submucosa similar to observations made in CD patients [28].
Since many well-defined genetic mutants are available for *S. Typhimurium*, we tested mutants lacking either SPI1 (*ΔinvA*)- or SPI2 (*ΔssaR*)-encoded factors and a mutant lacking flagellin expression. Both ΔSPI1 and ΔSPI2 mutants have been previously shown to cause considerable gut inflammation in an acute infection model [29, 30]. While all three mutants colonized the gut to a similar degree as wild-type *S. Typhimurium*, only the ΔfljB/ΔfliC mutants caused chronic inflammation and fibrosis. Interestingly, infection with the ΔfljB/ΔfliC mutant triggered only 50% of the amount of collagen deposition induced by wild-type bacteria. These findings suggest that colonization on its own is insufficient to cause these pathologies, in keeping with the absence of fibrosis following chronic infections with most other enteric bacterial pathogens such as *Helicobacter pylori*. While *S. Typhimurium* infections in humans are not associated with intestinal fibrosis, the requirement for specific virulence factors suggests that *S. Typhimurium*’s pathogenic strategy and accompanying inflammation rather than its colonization is what mimics the maladaptive host-bacterial interactions that cause fibrosis and stricturing in a subset of CD patients. We hypothesize that determining which bacterial structures and virulence factors are involved in fibrosis can serve to unlock the molecular mechanisms underlying the induction and maintenance of intestinal fibrosis.

While *S. Typhimurium* infection caused fibrosis along the entire length of the colon, the most severe and extensive fibrosis was focused in the cecum. This consistent localization should facilitate future investigation of the mechanisms underlying the fibrotic response as well as the testing of potential therapies.

Our studies showed that fibrosis could be induced in other *S. Typhimurium*-resistant mouse strains, but with variations in regard to the amount and localization of fibrotic
Furthermore, we demonstrated that infection with the attenuated strain *S. Typhimurium* ΔaroA triggers pronounced inflammation and fibrosis in C57/Bl6 mice. These results indicate that *S. Typhimurium*-triggered fibrosis is strongly dependent on host genetics, a feature that is currently being used to identify additional host factors involved in fibrosis development.

While *S. Typhimurium* and its virulence factors are necessary for fibrosis induction, it is the host response that ultimately mediates the fibrosis in this model. During active clinical disease, the involved tissues of CD patients display evidence of severe inflammation, with transmural infiltration of neutrophils, macrophages and other mononuclear cells. Moreover, affected gut tissues often exhibit extensive damage to the epithelium and underlying tissues, along with the expression of a characteristic cytokine profile dominated by Th1 and Th17 cytokines such as TNF-α, IFN-γ and IL-17 [16, 31]. In keeping with this profile, we found upregulated expression of TNF-α, IFN-γ, MCP-1, and IL-17 in the *S. Typhimurium* infected ceca in concert with transmural infiltration of inflammatory and immune cells. No differences were found in IL-10 levels in infected versus uninfected ceca, which further resembles the situation found in CD patients [16].

Along with the increased expression of Th1 and Th17 cytokines, we found upregulated expression of the pro-fibrotic growth factors TGF-β1, CTGF and IGF-1. While the GI tract is a site of substantial TGF-β1 expression, it is typically released in its latent form, and requires proteolytic cleavage to become active. Recent studies suggest that active TGF-β1 causes fibrosis through the induction of CTGF. Collagen production is positively regulated by TGF-β1, IGF-1 and CTGF whereas its degradation is achieved by matrix metalloproteinases (MMPs). It is thought that interactions between these
factors, along with MCP-1 are key effector determinants for intestinal fibrosis, since TGF-β1 is strongly induced in patients with CD [32] and similarly CTGF expression is upregulated in fibroblasts of CD patients [33]. This is in marked contrast to UC patients, since although they also have upregulated levels of TGF-β1, they express normal levels of CTGF [33], and do not suffer fibrostenotic disease. While the basis for the differential implication of TGF-β1 is not clear, one possibility is that TGF-β1 is more frequently activated in CD, than in UC and thus exerts its effect on downstream mediators of fibrosis, like CTGF, which is also upregulated in CD but not UC. Interestingly, TGF-β1 and IGF-1 are both potent inducers of collagen I [34], which we found strongly upregulated in the submucosa and external muscle layers in infected ceca.

The immunofluorescence studies identified a massive accumulation of V+ cells in the submucosa and mucosa of infected ceca. Most of these cells were also A- D-, identifying them as fibroblasts. Fibroblasts appear to be the predominant mesenchymal cell type within fibrotic tissues in CD [9, 27] and since these cells were also found in the midst of the extensive collagen deposition seen in the infected ceca, it appears that fibroblasts are the main collagen producers in our model. Aside from fibroblasts, strong collagen staining was also found adjacent to V- A+ D+ (smooth muscle) and V+ A+ cells (putative myofibroblasts) indicating that these cells may also contribute to collagen production similar to what has been observed in other murine models of fibrosis [10-12] and to findings in CD patients [28].

In summary, we describe a novel mouse model of chronic colitis and intestinal fibrosis that mimics many of the features of CD-induced fibrosis. Our studies suggest that chronic S. Typhimurium infection of the cecum on its own is insufficient to cause
fibrosis, but that particular bacterial virulence strategies and factors are necessary to initiate both the chronic inflammation and the fibrosis. Since current attempts at prevention or treatment of bowel strictures often prove ineffective, this model can be used to elucidate how bacteria can stimulate pro-fibrotic host responses, as well as define the likely complex pathways involved. Ultimately, this model may identify new therapeutic targets for the treatment of intestinal fibrosis.

4.4 Experimental procedures

Mice

129SvJ mice [35] were bred in the Animal Unit at the University of British Columbia (UBC). 129Sv/ImJ, DBA/2 and C3H/HeOuJ mice (Jackson Laboratories) were infected at an age of 8-12 weeks. Mice were given 20 mg of streptomycin orally twenty-four hours prior to infection with $3 \times 10^6$ or $3 \times 10^8$ bacteria in 100 µl HEPES buffer (100 mM, pH 8.0) by oral gavage. All animal experiments were conducted consistent with the ethical requirements of the Animal Care Committee at UBC.

Bacterial strains

*S. enterica* serovar Typhimurium strain SL1344 [36], $\Delta invA$ [37], $\Delta saaR$ [38], $\Delta aroA$ and $\Delta fljB/\Delta fljC$ [39] mutant strains were grown at 37°C with shaking (200 rpm) in Luria-Bertani broth containing 100 µg/ml streptomycin and/or 50 µg/ml kanamycin.

Tissue collection and bacterial enumeration

Tissues were collected at various time points in 1 ml of sterile PBS and homogenized
with a MixerMill 301 (Retsch). Serial dilutions of the homogenate were plated on LB agar plates containing 100 µg/ml streptomycin.

**Histology**

Formalin-fixed, paraffin embedded tissues were stained with H&E and Masson’s trichrome by Wax-it Histology Services (Vancouver, Canada).

**Immunohistochemistry**

5µm sections were deparaffinized and rehydrated. After antigen retrieval immunostaining was carried out using following antibodies: rabbit polyclonal anti-Vimentin (MBL; 1:50), mouse monoclonal anti-Desmin (Sigma; 1:200), FITC-conjugated mouse anti-α smooth muscle actin (Sigma; 1:100). Secondary antibodies used were AlexaFluor 568-conjugated goat anti-rabbit IgG (Molecular Probes; 1:500) for vimentin and biotinylated goat anti-mouse IgG F\textsubscript{ab}\textsuperscript{'} (Jackson ImmunoResearch; 1:400), followed by AlexaFluor 680-conjugated streptavidin (Molecular Probes; 1:200) for desmin. Tissues were mounted using ProLong Gold Antifade (Molecular Probes) that contains 4’,6’-diamidino-2-phenylindole (DAPI) for DNA staining. Images were obtained using a Zeiss AxioImager microscope equipped with an AxioCam HRm camera operating through AxioVision software.

**Tissue Pathology Scoring**

Tissue pathology in the infected cecum was scored using H&E stained sections as previously described [30].
**ELISA and cytometric bead array (CBA)**

Tissue homogenates were centrifuged for 10 min at 13000g and supernatants were collected. TGF-β1 concentration was determined by ELISA (R&D Systems) and levels of TNF-α, IFN-γ, MCP-1 and IL-10 were determined by CBA (BD Biosciences) according to manufacturer’s instructions.

**Real time polymerase chain reaction (q-PCR)**

RNA was extracted from the terminal 2 - 3 mm of the ceca using a RNeasy Mini kit (Qiagen, Mississauga, ON). Reverse transcription was performed with the Quantitect RT kit (Qiagen) and used for PCR reactions. qPCR was performed using Quantitect SYBR-Green Mastermix (Qiagen) and forward (fw) and reverse (re) primers as listed below. PCR was performed on an Opticon 2 (Bio-Rad) and cycles consisted of 95°C, 10 min and 39 cycles of 94°C, 15 s, 60°C, 30 s.

The following primer forward (fw) and reverse (re) were used:

- TGF-β1fw: 5’-TGACGTCACTGGAGTTGTACGG-3’;
- TGF-β1re: 5’-GGTTCATGTCATGGATGGTGC-3’;
- CTGFfw: 5’-CAGACTGGAGAAGCAGAGCC-3’;
- CTGFre: 5’-GCTTGGCGATTTTAGGTGTC-3’;
- IL-17fw: 5’-CTCTCCACCGCAATGCATCCTG-3’;
- GAPDfw: 5’-AGCTTTCCCTCCGCTATTGA-3’;
- GAPDre: 5’-ATGGACTGTTGTCATGAGCC-3’

**Collagen assay**

Pepsin-soluble collagen from 0.5 cm sections of caecum was quantified with the Sircol collagen assay according to manufacturer’s instructions. Briefly, 1 cm and 0.5 cm
sections of colon and cecum, respectively, were weighed, cut into small pieces and
digested overnight in 500 µl of pepsin solution (10 mg/ml in 0.5 M acetic acid) with
vigorous shaking at room temperature. Collagen was quantified using a standard curve
made with collagen supplied by the manufacturer.

Statistical analysis

Student T test was used to compare control vs day 21 for in vitro assays.

One-way ANOVA with Bonferroni’s multiple comparison post-test was performed using
a 95% confidence interval. This test was used to analyze data when more than two time
points were analyzed. Except in the figure 4.3B in which Mann-Whitney test was used
due to high levels of upregulation of IL-17 and variability of expression between
samples.

* = p < 0.05; ** = p < 0.01; *** = p < 0.001; ns, not significant (p > 0.05).

All analyses were performed using GraphPad Prism version 4.0.
4.5 Literature cited


Chapter 5
Conclusion and perspectives

5.1 Nramp1 expression in the gastrointestinal tract: moving beyond the classical role proposed for Nramp1 in macrophage and phagolysosomes

Research on *Salmonella* over the past 20 years has revealed Nramp1 to be one of the most important innate resistance factors to *S. Typhimurium* infection of mice [1]. *In vivo* and *in vitro* experiments have shown convincingly that a single amino acid substitution in this protein can determine the difference between life and death of the host during infection [2, 3]. However, unraveling the mechanisms of how this single molecule influences molecular and cellular responses has been, and still is, a matter of heated debate and controversy. Two decades of intense research are now bearing fruit by providing insights into the function of this protein in host defense. Nevertheless, many of the prevailing hypotheses on Nramp1 function have been heavily skewed towards a role in bacterial killing by macrophages, likely because this was one of the first cell types identified as expressing Nramp1 [4-6], as well as the important role played by macrophages in regulating *Salmonella* pathogenesis. Focus on Nramp1 as a cationic transporter led to the hypothesis that it played a bacteriostatic role [2, 7-9]. A subsequent focus on the complex interactions between host and pathogen at the subcellular level (cation transport and vacuole maturation) has proven a fruitful area of research [2, 10, 11]. However, this “trend” led to the neglect of other, possible functions for Nramp1 as a modulator of the host immune response [12, 13]. In this thesis, this older hypothesis has been revisited with the new knowledge that Nramp1 is prominently expressed by dendritic cells. I provide compelling evidence that Namp1 influences the speed and
intensity of the host inflammatory response, facilitating a rapid activation of host defense and thereby impacting on the severity and outcome of *S. Typhimurium* infection.

Historically, the study of murine typhoid fever has relied on the systemic administration of bacteria either by intraperitoneal or intravenous injection in naturally susceptible mice [14]. This was likely due to the fact that most studies were measuring the outcome of using different bacterial mutants or were focused on understanding the pathogenic events in systemic organs where *Salmonella* replicate such as the spleen and the liver [15-17]. Additionally, factors like the synchronicity of the infections were also important for comparing host parameters between mice in these studies and this is much more easily achieved via systemic injection, rather than via the natural oral route of infection. An unintended shortcoming of these studies was the lack of attention to oral infection, which most faithfully recreates the naturally occurring infections in humans. This has limited our knowledge of the initial stages of *Salmonella* infection and their interactions with the host. Consequently, very little attention was paid to the dynamics of oral *Salmonella* infection in the context of host resistance factors such as Nramp1. It was previously published that Nramp1 is not expressed in the gastrointestinal tract (GI). However, in these earlier studies the expression was analyzed only at the mRNA level using Northern blot assays. Chapter 2 and 3 of this thesis demonstrate that Nramp1 is indeed expressed in the lamina propria of the small and large intestine and this expression modulates the host immune response with important consequences for *Salmonella* replication.

Because we were studying immune responses of the host early after infection, our studies have put greater emphasis on the role of DCs, since they are among the first cells
to encounter *S.* Typhimurium following oral infection and are recognized to be the most potent initiators and orchestrators of the immune response [18-20]. Not only do we show for the first time that Nramp1 is expressed by this cell population in vivo, but we show that Nramp1 is differentially expressed in distinct subsets of DCs in the small intestine. Interestingly, the Nramp1 positive subsets (CD103⁺ DCs) show elevated expression of pro-inflammatory cytokines in response to *Salmonella* compared to the Nramp1 negative DCs (CD103⁺ DCs) [21]. Overall, these findings allow us to propose that Nramp1 affects the inflammatory status of the host and 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. We tested this hypothesis using the *Salmonella* colitis model (chapter 3), and found that Nramp1 acts rapidly by inducing an accelerated inflammatory response in the gut mucosa creating an anti-bacterial environment and limiting spread of *S.* Typhimurium to systemic sites. Collectively, these findings reveal a new role for DCs and Nramp1 in modulating the host inflammatory response to *S.* Typhimurium and potentially other intracellular pathogens.

### 5.1.1 Other functions of DCs expressing Nramp1

Although our studies on *Salmonella* infection were directed toward the initial stages of infection in the GI tract, we found that expression of Nramp1 is not restricted to DCs in the gut mucosa, but that Nramp1 is widely expressed by different subsets of DCs in the secondary lymphoid organs, including the spleen. It will be important in future studies to analyze the impact of Nramp1 expression at different stages of *Salmonella*
infection. It is tempting to speculate that Nramp1 may have an effect on the adaptive immune response at later stages of *S. Typhimurium* infection, in addition to its early role in promoting inflammation. A very recent paper suggests that Nramp1-expressing DCs show enhanced antigen presentation compared to Nramp1 deficient DCs [22]. Another report has suggested that Nramp1 can bias a T cell response to a Th1 type [23]. A provocative hypothesis is that Nramp1 expression in DCs, directs Th1/Th2 differentiation. In this regard, it is intriguing that *Salmonella* have been proposed to inhibit antigen presentation in DCs [24-27], by an unidentified mechanism. A partial explanation for these data may be that *Salmonella* avoids lysosomal degradation and survives within a modified late endosomal/lysosomal compartment (SCV) [28] characterized by delayed acidification thereby preventing antigen loading. It is noteworthy, however, that the experiments described above were performed exclusively using DCs derived from Nramp1 deficient mice. It will now be important to compare the antigen presentation capacity of *Salmonella* infected DCs from Nramp1+/+ and Nramp1−/− mice and dissect the role of Nramp1 in antigen presentation and helper T cell differentiation in vivo.

### 5.2 What are the molecular mechanisms involved in Nramp1 mediated resistance to colitis?

Our data using the colitis model demonstrated that Nramp1 promotes an accelerated inflammatory response characterized by: 1) a higher induction of pro-inflammatory cytokines and chemokines 2) greater recruitment of PMN cells and 3) containment of *Salmonella* to the mucosal sites early after the infection. However, many questions have
yet to be addressed. As with the typhoid model described above, most of the previous studies on inflammatory responses to *Salmonella* in the murine colitis model have relied on the use of Nramp1 deficient mouse strains [29-32]. It is important to note that inflammation is an extremely complex and intricate process involving the coordinated interactions of a wide variety of specialized cell types and soluble mediators. Given 1) our demonstration of a key role for Nramp1 in generating a more effective immune response in colitis, and 2) the fact that there is no evidence of frequent deficiency of Nramp1 in human, we would argue that evaluation of colitis in Nramp1\(^{+/+}\) mouse strains is more likely to give insights into human gastroenteritis and associated diarrhea than the use of Nramp1-deficient mouse models [33]. Further studies evaluating the major cell types that produce pro-inflammatory mediators such as IFN\(\gamma\) and the Nramp1-mediated molecular mechanism leading to control of the infection in our model would be needed to better understand enteric *Salmonella* infections.

### 5.3 Implications of the accelerated response driven by Nramp1 in pathogenesis

Our findings suggest that Nramp1 promotes an accelerated immune response irrespective of *Salmonella* virulence factors, including those proteins delivered via SPI-1 and SPI-2. We find that in all cases, Nramp1-expressing BMDC infected with different *Salmonella* mutants secreted higher levels of pro-inflammatory cytokines (personal observation). To test whether this response was specific to bacteria adapted to an intracellular life cycle, we infected Nramp1 expressing and deficient BMDC with *Campylobacter jejuni* a bacteria that, unlike *Salmonella*, is unable to survive for extended periods in phagocytic cells. We found that in fact, DCs derived from Nramp1\(^{+/+}\) mice
also produce higher levels of pro-inflammatory cytokines in this model of infection (See appendix 1), although the type and amount of cytokines secreted was distinct from those cytokines secreted from *Salmonella* infected cells. This suggests that Nramp1-expressing cells are rapidly activated in response to a variety of bacterial products and that this response is not restricted to a specific pathogen but reflects a more general effect of Nramp1 on the DC response. Thus, Nramp1-dependent responses are likely to be important in a variety of infection scenarios.

5.4 What is the specific contribution of subsets of Nramp1+/− host phagocytic cells in resistance to *Salmonella* infection in vivo?

Identifying the function of different cell populations involved in bacterial infection is of critical importance to understanding both innate and adaptive immunity induced in response to infection. Unfortunately, deciphering the role of distinct cell populations in vivo is complicated. This is particularly true for phagocytes, since they share expression of many of the same surface molecules and play overlapping roles in directing immune responses [34]. Neutrophils, macrophages and DCs are recruited early after *Salmonella* infection [35]. In addition, it has been demonstrated that these cell populations undergo a rapid expansion/recruitment to the Peyer’s patches and spleens of orally infected mice [36]. The protective role of neutrophils and macrophages during *Salmonella* infection is controversial. While some studies suggest that these cell types are important in defense against *Salmonella*, others propose that *Salmonella* uses both cell types for survival and replication [37-39] [40]. Moreover all of these studies, again, were performed in susceptible mice, where the bacteria replicate to high levels and rapidly
induce sepsis and death. With the exception of one study analyzing the degree of inflammation in mice depleted of DCs using the colitis model [29, 41, 42], no other studies have defined the specific contribution of this cell type in Salmonellosis. Thus, it is will now be important to identify the specific role of these cell types and their contribution during infection. One possibility would be the use of a conditional mutagenesis (Cre/loxP) system in mice [43], to study the role of macrophages/granulocytes and DCs lacking expression of Nramp1 in vivo. In this model, Nramp1 could be depleted exclusively in macrophages/granulocytes by using the currently available LysM-Cre mice [44], and crossing them with a deletable Nramp1 allele (loxP flanked). To analyze the role of Nramp1 expressing DCs, the same model could be used except that CD11c-ER-Cre T mice [45] would replace the LysM-Cre mice. The above strategy would allow the deletion of the functional Nramp1 gene in a cell type-specific manner (ie. lineage specific loss-of-function). Infection of mice lacking Nramp1 in either the macrophage/granulocyte lineage or the DC lineage could clarify which of these cell types is playing the predominant role in promoting Nramp1-dependent Salmonella immunity. An alternative to these “loss-of-function experiments” would be to do a “gain-of-function experiment” by inducing expression of Nramp1 in macrophages or DCs of Nramp1 deficient mice, using a similar strategy to that mentioned above. This system would offer a unique opportunity to analyze the contribution of each cell type in the pathogenesis of Salmonella and would provide a powerful research tool for the study of Nramp1 in immunity to Salmonella and other intracellular pathogens.
5.5 Models to study host-pathogen interaction

5.5.1 Mouse models to study Salmonella and data interpretation

Animal models are commonly used to reproduce a given disease and to identify the specific bacterial or host factors important in the disease pathogenesis. The choice of the most robust animal model to study this disease is thus critical, since the knowledge gained from these models will, ideally, be extrapolated later to humans. The most widely used animal model to study typhoid fever in humans is the mouse model, and the strains of choice are C57Bl/6 and Balb/C mice [41]. As mentioned before, these are susceptible mice and succumb to even low doses of Salmonella. This model has proven very useful in characterizing the function of individual bacterial effectors in the pathogenesis of Salmonella and to understand the immunological events in an acute infection [46]. However one caveat with using susceptible mice is that all of the animals die within the first week of the infection. This is not what happens in the human population infected with S. Typhi. The highest percentage of mortality in humans without antibiotic treatment is roughly 10%, whereas fewer than 1% of individuals treated with antibiotics die due to severe complications [47]. Thus, if the goal of a study is to understand the immunological events that allow the host to survive the infection, the host resistance factors should not be ignored. Therefore a reasonable and more informative model for typhoid fever would be to study the disease in the presence of a functional Nramp1 protein, as humans normally express this molecule. Otherwise the conclusions derived from susceptible mice do not accurately reflect naturally occurring Salmonella infections that occur in the presence of this protein in humans. Although few in number, more and more studies, including those outlined in this thesis, are addressing the interplay between
pathogen and host in the context of functional resistance mechanisms [21, 33, 48, 49]. One avenue for future studies in Nramp1\(^{+/+}\) and isogeneic Nramp1\(^{-/-}\) mice would be to assess the dynamics of S. Typhimurium spread and distribution \textit{in vivo}. These should be assessed at both the level of organs and of individual cells, in order to further clarify how this protein alters the pathogenesis of \textit{Salmonella} \textit{in vivo}. Experiments using bioluminescence imaging of \textit{Salmonella} during infection \textit{in vivo} could aid in determining if \textit{S}. Typhimurium colonize the same organs in these mice with and without Nramp1 and to determine if Nramp1 has an effect on cell trafficking.

Although, we have provided the first evidence of how Nramp1 modulates early inflammatory responses in the gut, a more exhaustive investigation is needed to rule out the influence of Nramp1 in systemic sites at later stages of \textit{S}. Typhimurium infection. Analysis of cytokine secretion in MLN, spleen and liver will provide further clarification of the effect of Nramp1 in the immunological status of the host. Evaluation of pathological lesions, and activation of the adaptive immunity, using Nramp1\(^{+/+}\) and Nramp1\(^{-/-}\) mice would clarify how Nramp1 contributes to the containment and suppression of \textit{S}. Typhimurium growth \textit{in vivo}. This ultimately could give us clues to the important immunological events that make Nramp1\(^{+/+}\) mice survive the infection and clarify the significance of previous reports that have overlooked the importance of this molecule.

5.5.2 \textbf{From colitis to the development of a new mouse model for the study of human intestinal fibrosis and beyond}

One of our motivations for studying colitis in resistant mice (Nramp1\(^{+/+}\)) was to decipher how the resolution of the inflammatory response and elimination of the
pathogen is achieved. Instead we obtained a very unexpected result. We found that the intestines of Nramp1++ mice became **chronically infected** with S. Typhimurium for long periods of time, up to 100 days. Interestingly, this chronic *Salmonella*-induced colitis and inflammation led to the development of fibrotic tissue within the infected organs (Chapter 4), probably as a consequence of an excessive healing and repair process in response to injury. Analysis of the pathology, cytokine profile, cell types involved in the production of extracellular matrix (ECM) and the type of ECM, revealed that this chronic inflammation mimics features of the fibrosis observed in Crohn’s disease patients. Thus, a byproduct of these studies was the development of a unique and extremely useful mouse model for the study of human intestinal fibrosis [50]. Although preliminary, this model has enormous potential for follow-up studies of microbial products, cellular interactions and the complex molecular pathways that contribute to the development of fibrosis. Likewise, it could provide insights into the mechanistic events leading tissue remodeling and healing. With the advent of new cutting-edge technology such as conditional mutagenesis and the development of new transgenic and KO mouse strains, it may be possible to determine the specific contribution provided by different immune cell populations to the development of chronic inflammation. It may also permit the identification of specific mesenchymal cells subsets (fibroblast, myofibroblast, smooth muscle cells) and their involvement in the production of ECM. All would be extremely helpful in the design of treatment options.
5.6 Concluding remarks

Some components of the immune defense to infectious agents can manifest themselves as genetic determinants conferring innate resistance or susceptibility. Characterizing the function of these genes and proteins could provide insights into the constant interplay between host and pathogen. The focus of this study was to dissect the cellular and immunological host response to *S. Typhimurium* in the context of host resistance mechanisms, an area that is currently neglected. We characterized the function of Nramp1 expressing DCs *in vivo* and proposed that Nramp1 promotes an accelerated immune response to *Salmonella*. We tested this hypothesis in the development of colitis in mice and found that in fact Nramp1 triggers a faster response that ultimately limits the dissemination of *Salmonella* from the gut to internal organs early after the infection. Nevertheless, pathogenic bacteria such as *Salmonella* are able to withstand the host resistance mechanisms and survive for long periods in the host tissues. The host response then, in its unsuccessful attempt to kill the bacteria, induces chronic inflammation leading to tissue damage. As a form of protection, the host then mobilizes a potent healing arsenal to repair this tissue insult resulting in scar formation and fibrosis. Finally, after a long-lasting, dynamic interaction between the pathogen and the host, the host prevails by eliminating the pathogen and restoring the equilibrium that existed before the assault. The studies performed in this thesis illustrate that disease is a very dynamic process, where sometimes the host or the pathogen dominate the disease outcome. In other cases, no winner is decided and both rivals rest, waiting for a new battle to arise. Such is the case of chronic infections.
5.7 Literature cited

27. Bueno, S.M., et al., The capacity of Salmonella to survive inside dendritic cells and prevent antigen presentation to T cells is host specific. Immunology, 2008.


Figure 2.7 Nramp1<sup>−/−</sup> mice show higher bacterial load.

Groups of mice Nramp1<sup>+/+</sup> and Nramp1<sup>−/−</sup> were infected orally with WT S. Typhimurium. Mice were sacrificed 1 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 ΔinvA) 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.
Figure 2.8 A subset of DCs express Nramp1 in the lamina propria of the small intestine. Tissue sections of ileal loops of uninfected (A) or S. Typhimurium infected Nramp1+/+ mice (B), were stained for Nramp1 (red), CD11c (green) and DAPI (blue) or Salmonella (blue) and analyzed by microscopy. Single color images are shown in black and white to maximize contrast.
Figure 2.9 Lack of an effect of Nramp1 on S. Typhimurium replication in BMDCs
BMDC were stimulated with IFNγ or left untreated. The cell were infected with WT S. Typhimurium and analyzed by gentamicin protection assays for 1, 2, 22 and 46 hours after infection, as indicated in experimental procedures. Colonies were enumerated and expressed as CFU per 2x10^5 cells.
Figure 3.7 Survival curves using the murine colitis model. Nramp1<sup>+/+</sup> mice and Nramp1<sup>-/-</sup> mice were pre-treated with Sm prior to infection with 3 x 10<sup>8</sup> CFU S. Typhimurium. Mice were monitored daily for development of disease and mortality. Data was analyzed using log-rank test $p=0.03$
Figure 3.8 S. Typhimurium induced inflammation is delayed in Nramp1\textsuperscript{−/−} mice. H&E staining of cecum sections revealing submucosal edema, mucosal and muscle hyperplasia and granulocyte infiltration in Nramp1\textsuperscript{+/−} mice at day 1 post infection. Higher magnification of the area indicated by black rectangle in the images of second column. Arrowheads indicate representative granulocytes.
Figure 3.9 Localization of *S. Typhimurium* in the cecum at day 1 post-infection. Higher magnification of the figure 3.5 B. Arrowheads indicate bacteria in the lumen or attached to the surface epithelium in Nramp1<sup>+/+</sup> or bacteria that have penetrated into deeper tissue layers such as mucosa and submucosa in Nramp1<sup>−/−</sup>.
Appendix 3: Addendum for Chapter 4

Figure 4.7 *S. Typhimurium* infection-induced inflammation and fibrosis in mouse colon at day 40 post infection. H&E and Masson’s trichrome staining of mouse colon. H&E staining revealing mucosal and muscle hyperplasia and crypt abscesses. Masson’s trichrome staining showing collagen deposition primarily in the submucosa, but also in the mucosa and muscularis propria; bar in left panels of each staining: 500 µm, right panels of each staining: 250 µm.
Figure 4.8 Distribution of mesenchymal cell types in the colon of uninfected control mice or in the fibrotic colons of mice chronically infected with *S. Typhimurium* for 21 days. Mesenchymal cells were differentiated by staining for vimentin (V), α-smooth muscle actin (A) and desmin (D) of formalin-fixed, paraffin embedded sections. Note that in the fibrotic colons, many V+ A- D- cells (fibroblast) were found in the lamina propria and the submucosa. V- A+ D- and V- A+ D+ smooth muscle cells were located in the muscle layers and V- A+ D- cells were also found around vessels in the submucosa. Note: there was some non-specific interaction of vimentin and desmin antibodies with luminal contents of control colons. Magnification: 200x. Merge: vimentin (red); α-smooth muscle actin (green); desmin (blue).
Appendix 4: A murine intraperitoneal infection model reveals that host resistance to *Campylobacter jejuni* is Nramp1 dependent

* Authors contributed equally to this work

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* Authors contributed equally to this work
Short communication

A murine intraperitoneal infection model reveals that host resistance to Campylobacter jejuni is Nrampl dependent

Olivia L. Champion a,b,1,3, Yanet Valdez a,b,3, Lisa Thorson b, Julian A. Gutman b,2, Alfredo Menendez b, Erin C. Gaynor a, B. Brett Finlay b,2

a University of British Columbia, Department of Microbiology and Immunology, Life Sciences Centre, 2558-2350 Health Sciences Mall, Vancouver, British Columbia V6T 1Z2, Canada
b University of British Columbia, Michael Smith Laboratories, 2315 East Mall, Vancouver, British Columbia V6T 1Z4, Canada

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Abstract

We tested the hypothesis that host resistance to Campylobacter jejuni is Nrampl dependent. Following intraperitoneal (IP) inoculation of Nrampl+/− and isogenic Nrampl-deficient (Nrampl−/−) mice C. jejuni primarily associated with muc-positive cells in liver tissue. A significant reduction of C. jejuni was observed in Nrampl+/+ mice 4 days post-infection (PI) (liver) and 8 days PI cecum–colon. In contrast, Nrampl−/− mice showed no significant reduction of C. jejuni and instead had a chronic inflammatory response and significant histopathological lesions 30 days PI. Differential cytokine profiles were observed in C. jejuni infected Nrampl+/+ and Nrampl−/− primary dendritic cells. Taken together these data indicate that Nrampl is critical for host resistance to C. jejuni.

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Keywords: Pathogenesis; Innate immunity; Intracellular

1. Introduction

Campylobacter jejuni is the leading bacterial cause of gastroenteritis in the UK and US [1]. C. jejuni manifests clinically in a variety of forms including asymptomatic carriage, diarrhoea, septicaemia and can lead to the peripheral neuropathy Guillain-Barré syndrome. Despite the morbidity and mortality caused by C. jejuni, little is known about its life cycle and mechanism of pathogenesis.

Natural resistance-associated macrophage protein 1 (Nrampl) is an integral membrane protein with the structural characteristics of an ion channel/transporter expressed exclusively by professional phagocytic cells. After phagocytosis, Nrampl colocalises to the phagosome membrane, remaining associated with this structure through its maturation to the phagolysosome [2–4]. Nrampl expression affects the capacity of the host to control survival of intracellular pathogens. It has been suggested that Nrampl functions by restricting the availability of luminal divalent cations to the intracellular bacteria, limiting replication [5,6]; however, the exact mechanism for this is not fully characterized. Nrampl-deficient mice are susceptible to infection by a number of unrelated intracellular pathogens including Salmonella, Leishmania and Mycobacterium spp. [7].

A role for Nrampl in the control of C. jejuni infection has been suggested through comparison of C. jejuni survival in Nrampl-deficient mice in a myeloid differentiation factor 88 (MyD88) deficient background [8]. MyD88 is a key factor in TLR signalling, and lack of myD88 results in a decreased innate response. As such, the specific effects of Nrampl

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8 Corresponding author. Tel.: +1 604 822 2210; fax: +1 604 822 9830.
E-mail address: bfinlay@interchange.ubc.ca (B.B. Finlay).
1 Present address: Exeter University, Biosciences, Geoffrey Pope Building, St Barh Road, Exeter, UK, EX4 4QD.
2 Present address: Simon Fraser University, University Drive, Burnaby Science Centre, Room B8276, Burnaby, British Columbia V5A 1S6, Canada.
3 These authors contributed equally to this work.

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deletion on C. jejuni infection are likely to be masked in the mdx88−/− murine model. We tested the hypothesis that host resistance to C. jejuni is Nnramp1 dependent using a murine IP infection model in an Nnramp1−/− otherwise wild-type mouse background.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Wild-type C. jejuni 81–176 (9) was cultured on Columbia agar supplemented with 5% (W/V) horse blood at 37 °C under 6% O₂/12% CO₂ conditions (Campypak, Oxoid).

2.2. Mice and C. jejuni infection

Female 129SvJ mice (Nnramp1+/+) and isogenic Nnramp1-deficient (Nnramp1−/−) mice have been previously described [10]. Groups of mice were IP injected with C. jejuni using a dose of 10⁶ CFU/mouse in 300 μl. Mice were euthanized 1, 4 or 8 days after infection, organs were homogenized, diluted in PBS and plated on Mueller Hinton agar supplemented with vancomycin and trimethoprim (50 μg/ml) agar. Plates were incubated at 37 °C under microaerobic conditions for 48 h, and recovered culturable bacteria were expressed as colony forming units (CFU) per milliliter.

2.3. Cytokine profiles of C. jejuni infected bone marrow derived dendritic cells

Bone marrow dendritic cells (BMDCs) were derived in GM-CSF and IL-4 as previously described [11]. The cells were harvested at day 7 of culture, in average this population contains between 60 and 70% CD11c+ DCs. BMDCs were derived in GM-CSF and IL-4 as previously described [19]. The cells were harvested at day 7 of culture, in average this population contains between 60 and 70% CD11c+ DCs. BMDCs were reseeded in 24 well plates in media containing 10% fetal calf serum, lacking any cytokines or antibiotics, the cells were rested for 24 h before infection with C. jejuni. BMDCs were infected with opsonized C. jejuni with a multiplicity of infection (MOI) of 10 as described previously [11]. At 4, 8, 12 and 24 h after infection supernatants were analyzed for secretion of cytokines IFN-γ, TNF-α, IL-6, IL-10, MCP-1 or IL-12p70 using commercial cytometric bead array kits (BD Biosciences, San Diego, CA). Flow cytometry was conducted on a BD FACS Calibur flow cytometer and data was analyzed using BD CellQuest and BD CBA software (Becton Dickinson, San Jose, CA).

2.4. Immunostaining of liver sections

Murine livers were dissected from mice and diced into 4–5 μm cubes that were fixed, washed and cut by wax-It Histology Services Inc. Immunostaining of tissues was carried out using the method published by Gunnetta et al. [12] Briefly, the tissue was blocked and treated with primary antibodies consisting of a rabbit anti-campylobacter antibody and a mouse anti-mac1 antibody followed by a goat anti-rabbit and anti-mouse secondary antibodies conjugated to Alexa flor 568 and 488, respectively. The tissue was visualized using a Zeiss Axioskop microscope.

2.5. Histopathology of liver sections

Livers were dissected from mice and diced into 4–5 μm cubes that were fixed, washed, cut and H&E stained by Wax-It Histology Services Inc. The tissue was visualized using a Zeiss Axioskop microscope.

3. Results

3.1. Nnramp1 is critical for rapid clearance of C. jejuni from the liver, cecum, colon and spleen

To determine the role of Nnramp1 in survival of C. jejuni in vivo isogenic Nnramp1+/+ and Nnramp1−/− mice were infected IP. After 1 day, the liver, cecum, colon, and mesenteric lymph node (MLN) tissues of both mouse strains were well colonised by C. jejuni. Nnramp1−/− mice subsequently exhibited no significant decrease of viable C. jejuni from the livers and cecum–colon up to 8 days post-infection, with a mean bacterial load of 1.29 × 10⁶ CFU/liver and 2.61 × 10⁵ CFU/cecum–colon. However, Nnramp1+/+ mice showed a significant reduction (P = 0.0148) of C. jejuni from the liver by day 4 and the cecum–colon by day 8. By day 4, no viable C. jejuni was recovered from the livers of 67% (8/12) of the Nnramp1−/− mice, whereas only 9% (1/11) of Nnramp1−/− mice harboured no culturable C. jejuni in the liver tissue. By day 8, clearance of C. jejuni from the liver was observed in 81% (9/11) of Nnramp1+/+ mice compared with 8% (1/13) in Nnramp1−/− mice. Similarly, 81% (9/11) Nnramp1+/+ had cleared C. jejuni from the cecum–colon by day 8 compared to 0% (0/13) Nnramp1−/− mice. On day 1, PL C. jejuni was isolated from the spleen of 100% (14/14) Nnramp1−/− mice with a mean bacterial load of 5.61 × 10⁶ CFU/spleen. However, C. jejuni was never observed to colonise the spleens of Nnramp1+/+ mice (0/15) with a significant difference of <0.0001. C. jejuni initially colonised the mesenteric lymph nodes (MLNs) of both Nnramp1−/− and Nnramp1−/− mice and was cleared from both by day 8 PI (Fig. 1). In summary, C. jejuni was rapidly cleared from Nnramp1−/− mice, and a significantly reduced rate of clearance was observed in Nnramp1−/− mice. In Nnramp1−/− mice the liver was heavily colonised with C. jejuni, and clearance was not observed up to 8 days PI.

3.2. Immunostaining of Nnramp1+/+ and Nnramp1−/− liver sections shows co-localization of C. jejuni with mac1

Due to high levels of colonisation in the liver, we aimed to identify the cells with which C. jejuni associated through immunostaining of liver sections. Nnramp1 is expressed exclusively by professional phagocytic cells. To determine whether
Fig. 1. C. jejuni bacterial loads in livers of Nparm1−/− and Nparm1−/− 129 mice. Three experimental replicates, 38 129s/cf mice (Nparm1−/−) and 38 isogenic sex and birth date matched Nparm1−/− mice were IP inoculated with approx. 10⁷ CFU/mouse C. jejuni. Liver, cecum–colon, spleen and MLN were collected on day 1, 4, 8 post-infection, homogenized, and CFUs organ were enumerated. Uninfected Nparm1−/− and Nparm1−/− mice were sacrificed and tissue homogenates plated out as negative controls. P-values were calculated using Welch corrected two-tailed t-tests from pooled data from three separate experiments. *Liver day 4 PI P = 0.0148, *Liver day 8 PI P = 0.0393, *cecum–colon day 8 PI P = 0.0393, *spleen day 1 PI P < 0.0001.

C. jejuni was associated with cells expressing Nparm1, mac-1 a marker of professional phagocytic cells, was stained for Nparm1+/+ and Nparm1−/− mice euthanized on day 1 PI revealed C. jejuni associated with mac-1-positive cells in the liver sinusoids (Fig. 2A). C. jejuni was not observed in liver sections taken from Nparm1−/− mice on day 8 PI. However, C. jejuni was clearly associated with mac-1-positive cells in liver sections from Nparm1−/− mice euthanized on day 8 PI (Fig. 2B). C. jejuni was unable to survive associated with mac-1 cells in Nparm1+/+ mice but survived up to 8 days associated with mac-1 cells in Nparm1−/− mice. Thus, C. jejuni clearance from mac-1-positive cells in the liver was Nparm1 dependent.

3.3. Nparm1+/+ BMDCs produce increased levels of proinflammatory cytokine IFN following infection with C. jejuni

Following infection with C. jejuni, Nparm1+/+ and Nparm1−/− differential cytokine profiles were measured and pooled from experimental replicates. In Nparm1+/+ BMDCs a significant increase of the proinflammatory cytokine interferon gamma was observed from as early as 8 h post-infection (P = 0.0394). Mean IFN-γ levels of 10.17 pg/ml (SE 3.296) were detected in culture supernatants of Nparm1+/+ and 0.9333 pg/ml (SE 0.5004) in Nparm1−/− BMDCs. This trend continued with the levels of IFN-γ increasing further in Nparm1+/+ infected cells (mean IFN 59.97 pg/ml SE 6.219) compared to Nparm1−/− cells (mean IFN-γ 23.93 pg/ml SE 5.146) by 12 h post-infection (P = 0.016). By 24 h post-infection mean IFN-γ levels of 86.25 pg/ml (SE 6.264) were recorded for Nparm1+/+ BMDCs compared to mean IFN-γ levels of 52.57 pg/ml (SE 3.142) in Nparm1−/− BMDCs (P = 0.002).

3.4. Histopathological examination demonstrates an inflammatory response and liver damage in Nparm1−/− liver sections

Histological analyses of livers from infected mice showed no gross pathological differences between Nparm1+/+ and Nparm1−/− mice at 4 or 8 days PI with only mild mononuclear cell infiltration and small areas of focal necrosis and hemorrhage. At day 30 PI the inflammatory lesions on the
livers of Nrpmp1+/+ mice had been resolved and the tissue appeared normal (Fig. 3A and B), which is in keeping with the lack of detectable bacteria at this time point. However, Nrpmp1−/− mice still shedding C. jejuni were euthanised on day 30 and liver sections demonstrated signs of a strong inflammatory response evidenced by large lesions with widespread lymphocytic infiltration. Extensive necrosis could be observed in perportal and lobular areas (Fig. 3C and D), often accompanied by local hemorrhage and zones of parenchymal degeneration characterized by disappearance of hepatocytes.

Fig. 2. Immunostaining of liver sections. C. jejuni, mac1, DAPI, Nrpmp1+/+ and Nrpmp1−/− day 1 PL. (A) Liver sections from nine Nrpmp1+/+ and nine Nrpmp1−/− mice infected with approx. 10⁶ CFU/mouse C. jejuni were collected day 1 PL. (B) Liver sections from nine Nrpmp1+/+ and nine Nrpmp1−/− mice infected with approx. 10⁹ CFU/mouse C. jejuni were collected day 8 PL. The cell type with which C. jejuni was associated was determined by immunostaining for C. jejuni and mac1-positive cells. Uninfected liver sections were stained as a negative control.

Fig. 3. Liver injury in mice infected with C. jejuni. Representative micrographs of livers from Nrpmp1+/+(A and B) and Nrpmp1−/−(C and D) mice infected for 30 days. Several large lesions are indicated by arrows in C. Arrowsheads in D point to zones of hepatocytes disappearance and hemorrhage. Scale bars on A and C are 200 μm and in B and D are 50 μm.
4. Discussion

Due to historical difficulties in genetic manipulation of C. jejuni and the lack of a small, reproducible animal model for studying pathogenesis in vivo, remarkably little is known about the mechanism of pathogenesis of this significant pathogen. A central role for Nbrnpl in the fate of intracellular pathogens residing within the macrophage phagosome has been demonstrated [7].

Here we used IP inoculation of 129SvJ9 mice (Nbrnpl−/−) and isogenic Nbrnpl−/− mice as a model to investigate the role of the host protein Nbrnpl in the survival of C. jejuni in vivo. Previously, Nbrnpl has been implicated in C. jejuni survival using a myd88−/− mouse model [8]. However, as the myd88−/− murine model may have decreased innate response due to loss of TLR signalling we aimed to demonstrate an unequivocal role for Nbrnpl in C. jejuni survival using an isogenic Nbrnpl−/− model. Nbrnpl-deficient mice were significantly impaired in their ability to clear C. jejuni from liver and intestinal organs, with no significant reduction observed 8 days PI. Furthermore, Nbrnpl−/− mice were impaired in clearing C. jejuni from the spleen, although no significant defect was observed for clearance from MLN. Conversely, Nbrnpl−/− mice showed a significant reduction of C. jejuni from liver, cecum—colon and spleen, indicating that C. jejuni clearance in vivo is Nbrnpl dependent. Moreover, our study demonstrates that in the absence of Nbrnpl, long-term colonization of C. jejuni occurs resulting in chronic inflammation and tissue damage. A differential cytokine mechanism for the Nbrnpl effect has also been demonstrated.

Mac1 is expressed on circulating monocytes, granulocytes (including neutrophils), subsets of dendritic cells and certain NK cells. Nbrnpl is expressed in macrophages [4,13], neutrophils [14], and in bone marrow derived dendritic cells [15]. Liver sections from infected Nbrnpl−/− and Nbrnpl−/− mice were immunostained to determine the cell type with which C. jejuni was associated during colonization. C. jejuni was associated with macrophages in both Nbrnpl−/− and Nbrnpl−/− 1 day PI. By day 8 post-infection C. jejuni was still associated with macrophages in Nbrnpl−/− mice.

Significantly, Nbrnpl−/− mice were able to clear colonising C. jejuni from the liver by 8 days post-infection, and gross pathology or histopathology was not observed in liver sections from Nbrnpl−/− mice 30 days post-infection. However, in contrast Nbrnpl−/− mice were still shedding C. jejuni 30 days post-infection. This indicates that Nbrnpl is critical for host clearance of C. jejuni. Although there was no obvious indication of gross liver pathology, signs of inflammation, including lymphocytic infiltration and liver injury, were readily observed in liver sections from Nbrnpl−/− mice 30 days post-infection. These results show that the absence of Nbrnpl is unable to eradicate C. jejuni, resulting in chronic inflammation and subsequent tissue injury.

Multiple Nbrnpl intracellular pathogen regulation mechanisms have been demonstrated, including restriction of microbial access to essential micro-nutrients such as iron and manganese [16], differential production of antimicrobial nitric oxide within the phagosome [17], a bias toward Th1 cell formation which aids host resistance to intracellular pathogens [15], enhanced macrophage apoptosis [18] and differential cytokine expression [19]. We have demonstrated a rapid and significant increase in levels of the proinflammatory cytokine interferon gamma in Nbrnpl−/− BMDCs infected with C. jejuni. These findings strengthen the hypothesis that expression of Nbrnpl promotes a more rapid inflammatory response following infection [19].

The aim of this research was to determine whether host resistance to C. jejuni was Nbrnpl dependent and to determine the cells with which C. jejuni associates in vivo. We have clearly demonstrated that C. jejuni survives in vivo associated with macrophages in Nbrnpl−/− mice resulting in chronic inflammation and liver damage. However, C. jejuni is unable to survive associated with macrophages in Nbrnpl−/− mice. Taken together these data indicate that Nbrnpl is critical for host resistance to C. jejuni.

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References


Appendix 5: Publications arising from graduate work


Valdez Y*, Grassl GA*, Guttmann JA, Coburn BA, Vallance BA, and Finlay BB. *Nramp1 promotes a faster inflammatory response in the Salmonella-induced murine colitis model*. Cell Microbiol 11(2)


Brown NF, Vallance BA, Coombes BK, Valdez Y, Coburn BA and Finlay BB. *Salmonella Pathogenicity Island-2 is expressed prior to penetrating the intestine*. PloS Pathog. 1(3): 0252-0258, 2005


Kujat Choy SL, Boyle EC, Gal-Mor O, Goode DL, Valdez Y, Vallance BA and Finlay BB. 
*SseK1 and SseK2 are novel translocated proteins of Salmonella enterica serovar typhimurium.* Infect Immun. 72(9): 5115-25, 2004

* Authors contributed equally to this work
Appendix 6: Animal Ethical Approvals


   THE UNIVERSITY OF BRITISH COLUMBIA

   Yanet Valdez

   has successfully completed the online training requirements of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program

   Chair, Animal Care Committee                                             Veterinarian

   Certificate #: 1075                                            Date Issued: October 05, 2005

2. Animal care ethical approval certificates:

   a) Number: A04-0058  
      Principal Investigator: Brett Finlay  
      Title: Bacterial infections and innate immunity

   b) Number: A04-0280  
      Principal Investigator: Brett Finlay  
      Title: Genomics of infection: Salmonella and pathogenic E. coli and Identification & Functional Characterization of Actin-Related Proteins Associated with Salmonella-containing Vacuoles

   c) Number: A05-1082  
      Principal Investigator: Brett Finlay  
      Title: Gates & Genome