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CXCR2-mediated immune cell recruitment to the intestinal mucosa confers protection against infection with *Salmonella* Typhimurium

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

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by

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7. **Klaus S**, Behnsen J, Nuccio SP, Raffatellu M. Molecular pathogenesis of non-typhoidal *Salmonella* diarrhea. (*In Preparation*), EcoSal Plus (2018).

8. **Klaus S**, Valeri M, Sharma P, Wang M, Lei V, Ransohoff R, Lane TE, Edwards RA, Raffatellu M. Leukocyte CXCR2 directs protective immunity against *Salmonella* in the intestinal mucosa. (*In Preparation*).

ABSTRACT OF THE DISSERTATION

CXCR2-mediated immune cell recruitment to the intestinal mucosa confers protection against infection with *Salmonella* Typhimurium

By

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Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2018

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Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a food-borne pathogen that causes severe diarrhea but can also cause fatal bacteremia if it reaches the bloodstream. During *S.* Typhimurium infection, a massive number of neutrophils migrate to the intestine; this response is important to prevent dissemination of *S.* Typhimurium into the bloodstream. Although some of the mechanisms by which neutrophils are protective during *S.* Typhimurium infection are known, the specific signals regulating their recruitment to the intestinal mucosa are not well elucidated. Most neutrophils express the chemokine receptor CXCR2, through which neutrophils sense CXC chemokines from inflamed or infected tissues. To better understand the role of CXCR2 during *S.* Typhimurium infection, we depleted CXCR2+ neutrophils in mice using antibody treatment, and we employed mice with a deletion of the *Cxcr2* gene. During *S.* Typhimurium infection, mice lacking CXCR2+ neutrophils showed significantly fewer neutrophils in intestinal tissue, indicating that CXCR2 is a major receptor for intestinal neutrophil recruitment during *S.* Typhimurium colitis. Consistent with a

protective role for neutrophils, *S.* Typhimurium numbers in peripheral organs were higher in mice lacking CXCR2+ neutrophils. Notably, and reported here for the first time, *Cxcr2*$^{-/-}$ mice exhibit fewer B cells in Peyer's patches, which correlated to a nearly 100-fold higher *S.* Typhimurium burden in this tissue. Furthermore, we demonstrated that Peyer's patch B cells migrate in response to CXCR2 chemokines, and that the role of Peyer's patch B cells in protection against *S.* Typhimurium is independent of intestinal IgA levels. To better understand the specific contribution of neutrophil recruitment in protection against *Salmonella,* we generated mice selectively lacking *Cxcr2* in granulocytes, which exhibited similar defects in neutrophil migration and *S.* Typhimurium dissemination as *Cxcr2*$^{-/-}$ mice. In contrast to *Cxcr2*$^{-/-}$ mice, granulocyte-specific deletion of *Cxcr2* was not associated with higher *S.* Typhimurium colonization in Peyer's patches, and Peyer's patch B cells were not reduced. Altogether, this work expanded our understanding of CXCR2-mediated neutrophil recruitment in influencing outcomes of *Salmonella* infection and uncovered an important, IgA-independent role for CXCR2 on B cells in Peyer's patches and in host defense against *Salmonella* intestinal infection.

Chapter 1: Molecular Pathogenesis of non-typhoidal *Salmonella* diarrhea

1.1 Non-typhoidal *Salmonella* and inflammatory diarrhea

Salmonella enterica is one of the leading causes of foodborne infection worldwide. Clinically, *S. enterica* infection results in two distinct diseases. A small number of serovars, the most notable of which is serovar Typhi, cause enteric fever, also known as typhoid fever. This disease is characterized by fever, bradycardia, constipation more often than diarrhea, and the absence of intestinal inflammation. In contrast, the vast majority of serovars are termed non-typhoidal serovars. They cause an infection localized to the intestine, known as inflammatory diarrhea, which is characterized by a severe intestinal inflammation (1). In this review, we summarize the molecular mechanisms by which non-typhoidal *S. enterica* serovars cause inflammatory diarrhea.

Diarrhea - loose, frequent bowel movements - is a relatively common disease manifestation associated with food poisoning. The greatest risk to health in patients with diarrhea is dehydration. In the most severe cases, diarrhea can lead to life-threatening ionic imbalances that interfere with nervous and cardiac system functions. Clinically, inflammatory diarrhea can be distinguished from secretory diarrhea, which is characterized by high-volume watery stools and the absence of fecal leukocytes and it is caused by non-invasive pathogens like *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. In contrast, small-volume stools with numerous fecal leukocytes usually indicate inflammatory diarrhea, which is caused by a small group of bacteria that

can invade the intestinal mucosa, including *Campylobacter jejuni*, *Shigella* spp., and non-typhoidal *S. enterica* serovars.

Among the more than 1,000 non-typhoidal *S. enterica* serovars that cause gastroenteritis and inflammatory diarrhea, the two most common isolates from patients are serovars Typhimurium (*S*. Typhimurium) and Enteritidis (Center for Disease Control and Prevention (CDC), Foodborne Outbreak Online Database (FOOD), 2015, http://wwwn.cdc.gov/foodborneoutbreaks). *S.* Typhimurium and other non-typhoidal serotypes of *Salmonella* are leading causes of bacterial food poisoning and account for over a million infections and 20,000 hospitalizations per year in the United States alone (2). Worldwide, especially in Africa, infections are even more prevalent. As symptoms usually resolve in four to seven days without medical intervention, approximately 29 out of 30 cases of non-typhoidal *Salmonella* diarrhea in the United States are undiagnosed (2). Reliable infection rates for non-typhoidal *Salmonella* are difficult to ascertain but are estimated to be in the range of 78 million (WHO estimates of the global burden of foodborne diseases, Foodborne disease burden epidemiology reference group 2007-2015) to >90 million cases per year, with mortality rates of 59,000 per year (3). Infection of healthy individuals with non-typhoidal *Salmonella* causes fever, chills, and inflammatory diarrhea characterized by massive numbers of neutrophils in the intestine, but the illness is usually self-limiting and does not spread beyond the intestinal tract (2). A clinically diagnosed *Salmonella* infection in an immunocompetent patient is treated with supportive hydration therapy, as antibiotic administration leads to protracted fecal shedding of *Salmonell*a (4). Children, elderly, and immunocompromised individuals (particularly those with HIV and AIDS), are more likely to be infected and have a higher

risk for bacteremia and focal infections, so antibiotic treatment may be necessary in these patients (5, 6). Potential complications of disseminated non-typhoidal *Salmonella* infection include focal infection, septicemia, shock, organ failure, and death.

In contrast to the typhoidal serovars, which infect only the human host, non-typhoidal *Salmonella* can grow in a wide range of hosts. This feature provides not only numerous reservoirs for infection, but also multiple animal models in which to study this host-pathogen interaction. To understand the molecular mechanisms of *Salmonella* diarrhea, it is important to know the characteristics of the animal models that have been developed to study this infection, which are summarized below.

1.2 Animal models of non-typhoidal *Salmonella* infection

Animals that develop diarrhea when infected with non-typhoidal *Salmonella* include calves and primates like rhesus macaques. In these animals, non-typhoidal *Salmonella* induces the classic hallmarks of inflammatory diarrhea, i.e. neutrophil infiltration and fluid accumulation in the intestine, within hours of infection (reviewed (7, 8)). Studies in non-human primates have the potential to answer questions about co-infections or underlying disease. For example, rhesus macaques infected with the Simian Immunodeficiency Virus develop a similar disease to humans infected with HIV (9, 10) and can be co-infected with *S. enterica* to understand the mechanism by which patients with HIV are highly susceptible to non-typhoidal *Salmonella* infection (11). In cattle, the rare but natural occurrence of leukocyte adhesion deficiency has allowed researchers to probe the role of neutrophils in the intestinal mucosa during non-typhoidal *Salmonella* infection (12). Experimental infections in calves and macaques

largely use ligated ileal loops, which allow testing of multiple *S. enterica* strains within a single animal, but require direct injection of *S. enterica* into the intestinal lumen. One issue with this approach is that in human infections, *S. enterica* must first survive passage through the acidic stomach before gaining access to the small intestinal sites of invasion. Moreover, these studies are limited by the short time course of infection and by the lack of possibilities for genetic manipulation of the host. Nevertheless, they have been essential to understand the mechanisms by which non-typhoidal *S. enterica* causes gastroenteritis.

Mice are also natural hosts for *Salmonella*, but the serovars that cause inflammatory diarrhea in humans cause mouse typhoid, a disease that shares some features with typhoid fever, including the absence of intestinal inflammation and neutrophil influx (13). Many studies have employed the typhoid model of infection to investigate *Salmonella* pathogenicity and have yielded important insights on the molecular factors and mechanisms underlying bacterial pathogenesis. Nevertheless, earlier studies in the typhoid model overlooked some virulence factors and mechanisms of host defense that are important players in the pathogenesis of inflammatory diarrhea rather than in typhoid fever. More recently, many investigators have used a model in which mice develop cecal and colonic inflammation upon infection, namely the streptomycin-treated mouse model (14, 15). Streptomycin treatment prior to *S. enterica* infection diminishes the natural intestinal microbiota and induces a low-level of inflammation (16), two conditions that enable *S. enterica* to thrive in the intestinal lumen. Although mice experience a less dramatic change in stool consistency than humans, macaques, and cattle, their immune response and tissue inflammation in cecal and

colonic tissue recapitulate several aspects of infection in larger mammals, including neutrophil infiltration, antimicrobial protein expression, and cytokine production (17-19). Moreover, genetic manipulation of mice has enabled researchers to probe the specific roles of host immune proteins during *Salmonella* infection. Complementary genetic manipulation of *S. enterica* strains has further clarified the specific nature of the host-pathogen interactions that lead to inflammatory diarrhea and gastroenteritis.

In this chapter, we focus on the mechanisms by which non-typhoidal *Salmonella* causes inflammatory diarrhea. We discuss how non-typhoidal *Salmonella* actively induces its own uptake into a variety of different host cells and initiates a cascade of immune cell and cytokine communication, culminating in inflammatory diarrhea. Remarkably, although some immune mechanisms play a role in preventing *Salmonella* dissemination to extraintestinal organs, other aspects of the host mucosal response enhance *Salmonella* growth in the intestinal lumen. We also elaborate on the fitness factors and virulence genes that enable *Salmonella* to effectively compete with commensal intestinal microbes for nutritional resources and evade killing by host antimicrobial proteins, thereby increasing likelihood of host transmission. Additionally, we describe host immune dysfunctions that predispose patients to *Salmonella* bacteremia.

Figure 1.1 Non-typhoidal *Salmonella* intestinal infection, immune activation, and proliferation

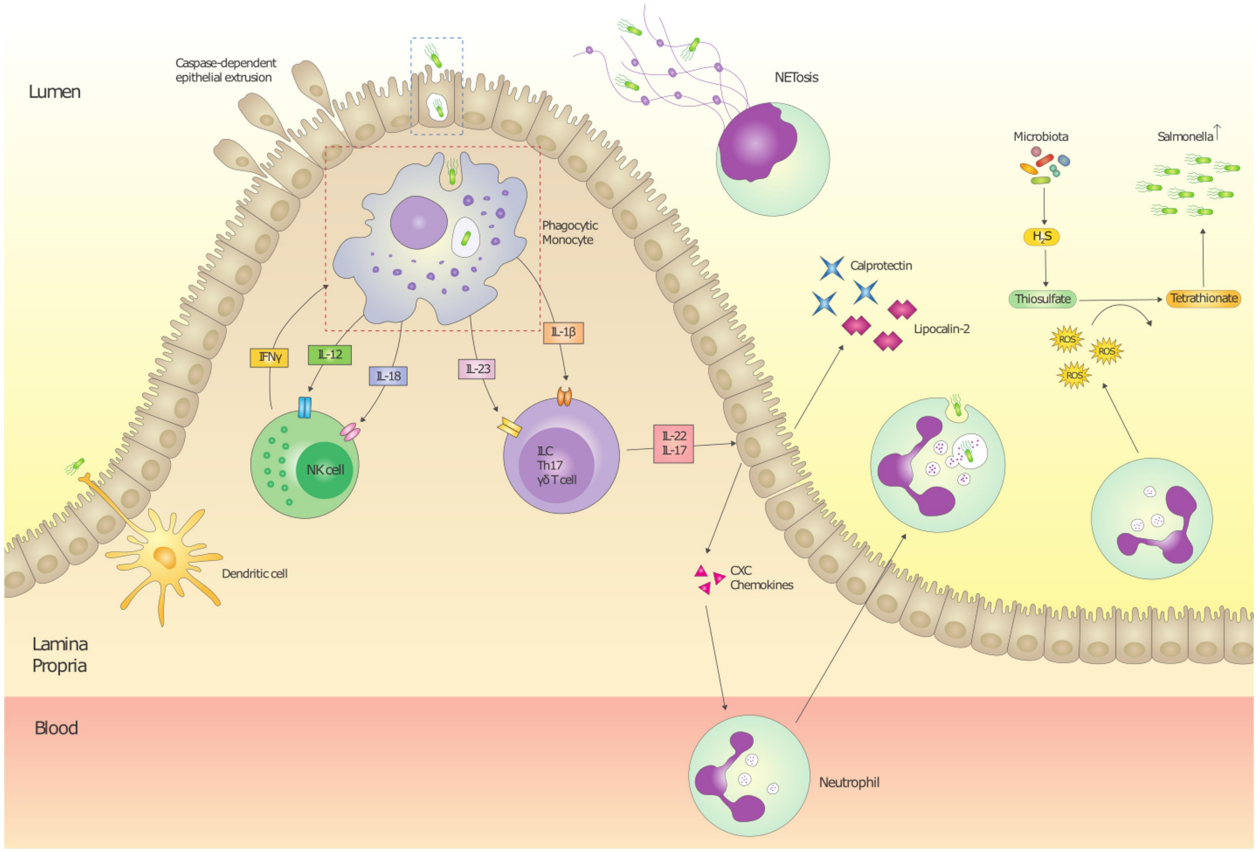

Figure 1.1 After entering the lumen of the gastrointestinal tract, *Salmonella* invades epithelial cells and transmigrates into the lamina propria. For further detail on this process, see Figure 1.2. In the lamina propria, *Salmonella* invades or is phagocytosed by macrophages. Figure 1.3 describes the processes triggered by *Salmonella* inside macrophages. *Salmonella* is also sampled from the lumen of the gastrointestinal tract by dendrites of dendritic cells and responses are similar as will be described for macrophages. Macrophages respond to *Salmonella* infection by production of cytokines IL-1β, IL-23, IL-18, and IL-12. Amongst other cells, NK cells respond to IL-18 and IL-12 by production of IFNγ, which acts on monocytes and amplifies the signal in a paracrine loop. Cytokines IL-1β and IL-23 trigger innate lymphoid cells (ILCs), Th17 cells and γδ T cells to produce IL-17 and IL-22. Receptor engagement of these cytokines on epithelial cells prompts the production of CXC chemokines. Neutrophils follow the chemokine gradient and extravasate from the blood through the epithelial layer into the lumen of the gut. Some neutrophils die in a process called NETosis and entrap *Salmonella* in neutrophil extracellular traps. Furthermore, via NADPH oxidase, neutrophils produce reactive oxygen species (ROS), which, in an oxidative environment, are crucial for *Salmonella* to gain access to otherwise unavailable nutrient sources. The microbiota produces H_2S, which enterocytes convert to thiosulfate, which in turn is oxidized to tetrathionate. *Salmonella* can use tetrathionate as a terminal electron acceptor for respiration of e.g. ethanolamine in the gut. Finally, neutrophils, but also enterocytes, are a source of antimicrobial proteins calprotectin and lipocalin-2 in the gut. Calprotectin binds zinc and manganese, and lipocalin-2 binds enterochelin, a siderophore secreted by *Salmonella* and some members of the microbiota to acquire iron. Calprotectin and lipocalin-2 are released in response to *Salmonella* infection, but inhibit the growth of the microbiota instead of *Salmonella*, as *Salmonella* is resistant to metal withholding via these antimicrobial proteins. *Salmonella* expresses high affinity metal transporters for zinc and manganese and a stealth siderophore that cannot be bound by lipocalin-2. In summary, the neutrophil response to *Salmonella* is eventually effective, but initial inflammatory responses promote *Salmonella* growth instead of restricting it.

1.3 *Salmonella* invasion of the intestinal mucosa

The intestinal epithelium is a single layer of columnar cells with apical microvilli, which provide a large luminal surface area to accommodate nutrient uptake, ion exchange, and water absorption. The trillions of commensal bacteria that reside in the intestinal lumen, known as the intestinal microbiota, are separated from host mucosal tissues by this thin barrier of enterocytes and by a thick mucus layer. When *Salmonella* encounters the nutrient-rich, neutral pH, and low oxygen environment in the distal ileum, a cluster of genes named *Salmonella* Pathogenicity Island-1 (SPI-1) is expressed (20). The proteins encoded by SPI-1 form a type-three secretion system (TTSS), a needle-like structure through which bacterial proteins, called effectors, are injected into the host epithelial cell (21). Some effector proteins are encoded on SPI-1, while others are encoded on other pathogenity islands scattered throughout the chromosome (22). These effector proteins induce actin remodeling through numerous effectors that nucleate (SipC) (23), assemble (SipA) (24), and energetically drive actin polymerization through GTP exchange (SopE, SopE2, and SopB), leading to the activation of Rho GTPases Rac1 and CDC42 (25, 26). As a consequence of actin polymerization, membrane ruffles of intestinal epithelial cells surround and engulf *Salmonella*, taking it up from the intestinal lumen via induced pinocytosis to form a *Salmonella* Containing Vacuole (SCV) (27). Actin organization returns to normal partly through the actions of another effector, SptP, which promotes Rho GTPase-mediated cleavage of GTP to GDP (28) and dephosphorylates host proteins (29). The phosphatase activity of SptP promotes actin ruffle resolution by regulating the phosphorylation state of villin, a host cytoskeletal protein (30), and stimulates development of *Salmonella*-induced filaments

(Sifs) around the SCV by dephosphorylating ATPase *N*-ethylmaleimide-sensitive factor (NSF) (31).

A subset of SPI-1-secreted effectors (SipA, SopA, SopB, SopD, SopE, and SopE2) has been shown to promote *Salmonella* invasion of a variety of epithelial cell lines (22, 32-34) and to contribute to the development of inflammatory diarrhea both in calves and mice (35, 36). From within the SCV, *Salmonella* expresses a cluster of genes on a second pathogenicity island, termed SPI-2, which encodes another type-three secretion system and effectors that promote intracellular survival, localization near the nucleus, and eventual release from the basal side of epithelial cell (37). SPI-2 effectors with identified functions are discussed in the context of SCVs formed in macrophages.

1.4 Enterocyte inflammatory signals

The gut is exposed to commensal and pathogenic microbes, which express conserved patterned molecules termed pathogen-associated molecular patterns (PAMPs). Gut cells detect these PAMPs through specialized receptors, termed pattern-recognition receptors (PRRs), that are located either in the cell membrane or in the cytosol (reviewed in (38)). Membrane-associated PRRs include Toll-like receptors (TLRs), whereas cytosolic PRRs include nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs). Once TLRs and NLRs bind their ligands, several signaling cascades are initiated; the cellular responses induced by TLR activation promotes the upregulation of genes encoding for pro-inflammatory cytokines, whereas the activation

of NLRs induces the cleavage of pro-inflammatory caspases, and the consequent maturation and release of the cytokines IL-1β and IL-18 (38).

In intestinal epithelial cells (IECs), PRRs are dominantly distributed intracellularly and on the basolateral surfaces. Thus, IECs are primarily stimulated to trigger an inflammatory response when the epithelial barrier has been breached, e.g. by an invasive pathogen like *Salmonella*. Extracellular *Salmonella* is recognized through TLR2, TLR4, and TLR5 (35, 39-44). TLR2, which binds many bacterial ligands including lipoprotein, and TLR4, which binds to lipopolysaccharide, are expressed at very low levels on IECs in non-inflamed tissue (45). TLR5, which binds bacterial flagellin, is present on the basolateral side of IECs *in vivo* (46) and in cell culture (47). Therefore, *Salmonella* flagellin in the intestinal lumen does not activate enterocyte inflammatory signals, but flagellin present on the basal side of the epithelial layer after invasion and transcytosis initiates an inflammatory response. Infection of calves with *Salmonella* mutants lacking flagellin results in fewer neutrophils migrating to the calf ileal mucosa than during infection with wild-type *Salmonella*, indicating that flagellin is important for initiating the cytokine signaling cascade that leads to neutrophil recruitment (48). However, as *Salmonella* strains lacking flagellin are also less invasive due to reduced motility, impaired invasion of the host epithelium may also contribute to the observed phenotype.

Activation of NLRs by *Salmonella* is mostly studied in macrophages (discussed in detail in the section “Macrophage inflammatory responses”), but it was also recently described in IECs. In macrophages, *Salmonella* activates two NLRs, NLRC4 and NLRP3 (reviewed in (49)). Activation of these receptors leads to the assembly of a

multiprotein complex called the inflammasome, which in turn activates caspase-1 (canonical pathway). Caspase-1 activation has two main consequences: 1) the maturation and secretion of IL-1β and IL-18 and 2) the induction of pyroptosis, which is defined as an inflammatory process of caspase 1-dependent programmed cell death (49). A non-canonical inflammasome has also more recently being described and involves the participation of caspase-11 in mice (caspase 4-5 in humans) in response to LPS (50, 51). Caspase-11 is a member of the caspase-1 subfamily of proteases and shares 46% identity with caspase-1. During *Salmonella* infection *in vivo*, inflammasome activity in IECs leads to detachment from the basal lamina and to the extrusion of infected cells into the lumen, which reduces the number of *Salmonella* in close proximity to the mucosa (52, 53). The extrusion process is dependent on caspase activity, as depletion of caspases leads to more intracellular *Salmonella* in enterocytes and decreased detachment (52-54).

During the inflammatory response, cytokines such as IL-18 are released to promote epithelial cell proliferation, so the extrusion of IECs does not leave basolateral surfaces exposed for *Salmonella* invasion. Further research regarding inflammasomes in intestinal epithelial cells is reviewed by Sellin *et al*. (55). Recent evidence suggests an additional role for the NLRs NOD1 and NOD2, as they are also able to detect bacterial effector-activated GTPases (56) and thus differentiate when the cell is infected with a pathogenic organism and not a member of the commensal microbiota (which would not have effectors to activate GTPases). The interactions of NLRs and Rho GTPases in host defense against enteric pathogens are reviewed in (57).

Independent of SPI-1-induced epithelial invasion, *Salmonella* can gain access to the *lamina propria* via endocytosis by specialized epithelial Microfold cells (M cells), which regularly sample the intestinal lumen. M cells can also be induced to uptake *Salmonella* in a SPI-1 dependent manner (58). Situated on the luminal surface of Peyer's Patches, M cells transfer endocytosed antigen to underlying dendritic cells for processing (59). Alternatively, dendritic cells can extend a sampling dendrite directly into the intestinal lumen and phagocytose a sample of the luminal content without disrupting the tight junctions of the epithelial layer (60, 61). Although these alternative routes of accessing the *lamina propria* can be observed in *Salmonella* mutants deficient for invasion, invasion genes are of the uttermost importance for infection, as SPI-1 mutants do not cause diarrhea in calves (36, 62, 63) and induce lower levels of intestinal inflammation in the colon of streptomycin-treated mice (35). Only mice deficient in important inflammatory proteins, such as caspase-1 and NADPH oxidase, are susceptible to *Salmonella* disease from strains lacking SPI-1 (64, 65).

Figure 1.2 Non-typhoidal *Salmonella* SPI-1 mediated epithelial cell invasion

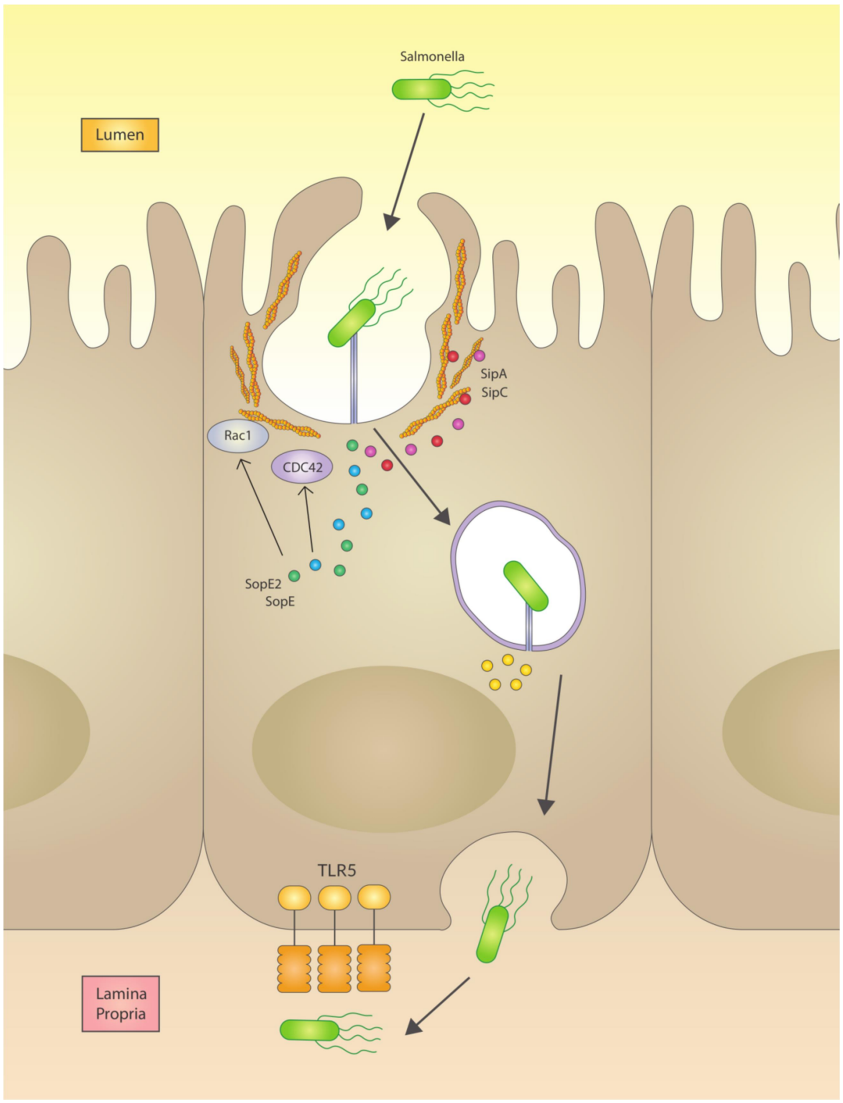

Figure 1.2 When *Salmonella* enters the distal ileum, the nutrient-rich, neutral pH, and low oxygen environment promotes expression of *Salmonella* Pathogenicity Island 1 (SPI-1) genes. The encoded proteins assemble into a needle like structure, a type three secretion system (T3SS), which injects effector molecules into epithelial cells. These effectors include SipA, SipC, SopE and SopE2, which induce the activation of Rac1 and CDC42. These small RhoGTPases can activate NFκB-dependent gene expression via the nodosome but they are also involved in actin remodeling. The membrane ruffles this process produces surround *Salmonella* and result in the uptake of *Salmonella* into a *Salmonella* containing vacuole (SCV). Inside the SCV, *Salmonella* expresses genes from a second pathogenicity island, SPI-2. These effectors were long thought to play a role in intracellular survival, but might be more important for *Salmonella* exit from the cell into the lamina propria. On the basolateral side of the epithelium, TLR-5 receptors recognize *Salmonella* flagellin.

1.5 *Salmonella* & Antigen Presenting Cell interactions

The intestinal *lamina propria* is home to mononuclear phagocytes like macrophages and dendritic cells, which express bacterial recognition receptors (TLRs and NLRs) as well as receptors for immunoglobulins and host complement proteins. *Salmonella* that passes through the intestinal epithelial layer is rapidly phagocytosed by these cells, leading to cytokine production and pyroptosis. However, as in enterocytes, intracellular *Salmonella* secretes effectors that counteract host defenses and promote intracellular survival and escape, which can lead to complications such as bacteremia and/or dissemination to other tissues. In the paragraph below, we discuss some of the signaling events and the resulting production of cytokines that occur in both macrophages and dendritic cells. The usage of the terms “macrophage” or “dendritic cell” is based on the specific cells used in the described studies and is not to be considered a hard differentiation of distinct functions in these related cell types.

1.6 *Salmonella* survival inside macrophages

Macrophages recognize *Salmonella* antigens through TLR2, TLR4, TLR5, and TLR9. Signaling through any of these TLRs is necessary and sufficient for phagosome acidification (66). Phagocytosed *Salmonella* detects vacuole acidification and the lack of inorganic phosphate, conditions that trigger expression of the SPI-2 genes (67-69). SsrA, a sensor kinase of the SsrA/SsrB two-component regulatory system, auto-phosphorylates in the acidic environment of the phagosome and induces expression of SPI-2 genes (70, 71). The SPI-2 T3SS complex is projected through the SCV membrane, and effectors are transferred to the macrophage cytosol. The SCV develops

features of an endosomal compartment but avoids fusing with lysosomal vesicles that contain degradative enzymes or NADPH oxidase, which produces reactive oxygen species (ROS) (72-77). In cultured macrophages, the *Salmonella* effector SpiC was found to prevent lysosome fusion with the SCV (78). Several SPI-2 effectors associate with the SCV membrane, stimulating Sif formation that support the SCV during its maturation (SifA, PipB2, SseJ, and SopD2) (79-85) and position the SCV near the nucleus (SseF) (86) and the Golgi apparatus (SseG) (87). Maintenance of Sifs and proximity to key organelles are associated with the ability of *Salmonella* to replicate in the SCV (88, 89) (see Figure 1.3).

Much of the understanding of individual effectors has been gained from *in vitro* infection of primary macrophages and macrophages cell lines, and the overall role of SPI-2 effectors was thought to be the maintenance of *Salmonella* intracellular survival. However, recent evidence from *in vivo* mouse infections suggests that SPI-2 effectors are not necessary to support intracellular survival of *Salmonella*. Intravenous infection of mice with *Salmonella* lacking the translocon component (SseB) of the SPI-2 type-three secretion system, and therefore lacking the ability to inject effectors into the host cytosol, was associated with higher numbers of *Salmonella* per macrophage in the liver and spleen than infection by wild-type *Salmonella* (90). However, contrary to wild-type *Salmonella*, *sseB* mutant *Salmonella* were less able to escape from their host cells and form new infection foci. These data show that *Salmonella* can survive intracellularly without SPI-2 effectors, and support a shift in thinking about the overall purpose of SPI-2 effectors. Instead of promoting intracellular growth and persistence, the SPI-2 effector

proteins help *Salmonella* to escape host cells and resist NADPH-oxidase mediated killing, allowing further spread of the infection (90).

1.7 Macrophage inflammatory responses to *Salmonella*

Macrophages defend against *Salmonella* survival and spread by releasing pro-inflammatory cytokines and undergoing pyroptotic cell death. In the following section, we discuss activation of the inflammasome and the resulting release of pro-inflammatory cytokines by macrophages (partially illustrated in Figure 1.4).

1.7.1 Inflammasomes

Intracellular *Salmonella* sets off a chain of activating events when NLRs, specifically NLRC4 and NLRP3, recognize cytoplasmic bacterial proteins or cellular changes caused by infection and initiate assembly of the multi-protein inflammasome complex (reviewed in (91)). The NLRC4 inflammasome is activated by flagellin and the *Salmonella* T3SS needle/rod proteins ((92), reviewed in (93)). The ability to identify these different ligands is mediated through multiple NAIPs (neuronal apoptosis inhibitory proteins), which each directly bind a bacterial antigen and then bind the NLRC4 protein (92, 94, 95). A *Salmonella*-specific ligand has not been identified for the NLRP3 inflammasome, but indirect signals, such as reactive oxygen species (ROS), can trigger potassium efflux and activate NLRP3 (96).

The inflammasome-induced host immune response is detrimental to *Salmonella* survival, so *Salmonella* evades NLRC4 inflammasome activation by downregulating expression of flagellin. It also reduces NLRP3 inflammasome activation via the oxidative metabolism enzymes aconitase and isocitrate dehydrogenase (97). Disruption of these

evasive mechanisms leads to higher cytokine secretion and clearance of *Salmonella* (97-99). In C57BL/6 mice, the NLRC4 and NLRP3 inflammasomes appear functionally redundant, as both NLRs must be deleted to impair host immunity, but BALB/c mice lacking NLRC4 alone have a higher *Salmonella* burden (100-102). Regardless of whether NLRC4 or NLRP3 signaling initiates inflammasome assembly, each cell forms a single inflammasome complex, which can contain both NLRC4 and NLRP3 (102).

1.7.2 Caspase-1 and Caspase-11

The first evidence of a role for caspases in the inflammation process was demonstrated by studies showing that caspase-1 is involved in the processing and maturation of IL-1 cytokine family members IL-1*β* and IL-18 through the inflammasome canonical pathway (103-105). Mice deficient for caspase-1 exhibit defective maturation of pro-IL-1*β* and pro-IL-18 (104-107) and the absence of this caspase confers susceptibility to *Salmonella* (108). In addition, caspase-1 in macrophages can be directly activated by the *Salmonella* effector SopE (109).

The role of caspase-11 in activation of noncanonical inflammasome was identified as a result of the finding that all *Casp1*$^{-/-}$ mouse strains generated from embryonic stem cells also lack caspase-11 (50). As caspase-11 and caspase-1 loci are located in close proximity, the mutations do not segregate during backcrossing, leading to double mutant (*Casp1*$^{-/-}$ *Casp11*$^{-/-}$) mice (50). Caspase-11 is able to induce macrophage death (50), but caspase-1-deficient mice are significantly more susceptible to *Salmonella* infection than mice deficient in both caspase 1 and 11 (110), indicating that caspase-1 primarily confers protective immunity to *Salmonella* (111). Caspase-1 contributes to host defense against *Salmonella* by producing IL-1β and IL-18, but also

by inducing pyroptotic cell death (98). When inflammatory caspases are activated, they cleave gasdermin D, whose N-terminal cleavage product oligomerizes in cell membrane and form pores, resulting in cytokine release and pyroptosis (112-114). When a cell dies of pyroptosis, the cytosolic contents are released into the extracellular space, triggering an inflammatory response (115). Macrophage pyroptosis during *Salmonella* infection releases live *Salmonella*, ejecting it from the SCV before it can multiply to high numbers. Extracellular *Salmonella* may then invade or be phagocytosed by another host cell.

1.7.3 Pro-inflammatory cytokines IL-1β and IL-18

As alluded to previously, active caspase-1 is necessary for secretion of inflammatory cytokines IL-1β and IL-18 by macrophages. However, another signal, independent of the inflammasome, is needed to produce sufficient amounts of inactive pro-IL-1β and pro-IL-18. For example, TLR signaling induces the production of pro-IL-1β and pro-IL-18 (116, 117), which are subsequently cleaved by active caspase-1 (105, 118). Once cleaved into their active forms, IL-1β and IL-18 are rapidly released from the cell by multiple mechanisms (reviewed in (119)).

IL-1β is a broadly-acting pro-inflammatory cytokine that activates immune cell proliferation and differentiation. Despite its high production during *Salmonella* infection in wild-type mouse cells (120, 121), deficiency of IL-1β is associated with only a modest increase in *Salmonella* burden, and it is primarily important during the intestinal phase of the infection (105). Recent work showed that the monocytic phagocytes residing in the intestinal mucosa are uniquely primed by their proximity to the microbiota to produce IL-1β in response to infection with pathogens like *Salmonella* (101). This is in contrast to

non-intestinal macrophages exposed to *Salmonella,* which instead produce TNFα and IL-6, a distinction which highlights the importance of IL-1β at intestinal sites of infection.

Released in concert with IL-1β, IL-18 is also a pro-inflammatory cytokine whose primary role is to cooperate with IL-12 to induce interferon gamma (IFNγ) production by NK cells and T cells (122) and to increase intestinal inflammation (123). This response is protective during *Salmonella* infection, as mice lacking IL-18, whether from antibody neutralization or genetic deletion, have lower IFNγ levels and higher *Salmonella* burden in the spleen and liver (65, 124).

1.8 Effector T cell activation and response to *Salmonella* infection

The dendritic cell response to *Salmonella* can be similar to that described for macrophages, (e.g., with inflammasome activation and production of IL-1β and IL-18) (122), but dendritic cells during *Salmonella* infection are also a major source of the cytokines IL-12 and IL-23 (125, 126), heterodimeric proteins that share the common subunit IL-12p40 (127). Despite their similarities, the downstream effects of IL-12 and IL-23 on the immune systems are distinct. IL-12 promotes the majority of IFNγ production (Th1 responses), and IL-23 induces the production of IL-17 and IL-22 (Th17 responses) (128, 129). Th1 responses are mechanisms of defense primarily during infection with intracellular pathogens, whereas Th17 responses play a major role in host defense against extracellular pathogens. Because *Salmonella* exhibits both an intracellular and an extracellular phase, both Th1 and Th17 responses play a role in the defense against this pathogen. In particular, Th1-primed immune cells control *Salmonella* replication at systemic sites (130, 131), whereas Th17 responses are of great importance in the intestinal mucosa (11, 132).

Whether dendritic cells produce IL-12 or IL-23 depends on the environment. Lipopolysaccharide (LPS) stimulation of TLR4 and flagellin stimulation of TLR5 induce the production of IL-23 by dendritic cells (127, 128, 133). The same ligands, in the presence of IFNγ, induce IL-12 production (133). *In vitro* studies show that IL-23 alone can induce IFNγ production by T cells (127), and that IL-23, in combination with IL-1β or IL-18, induces IFNγ production by NK cells (134, 135). Moreover, phagocytic monocytes infected with *Salmonella* secrete IL-23 but not IL-12, and IL-12 is instead induced indirectly, through IFNγ-dependent stimulation of dendritic cells (134).

1.8.1 Th1 responses

The importance of Th1 responses during *Salmonella* infection can be inferred by the observation that human genetic mutations in IL-12 or the IL-12 receptor are associated with increased susceptibility to severe *Salmonella* infections (136-141). Laboratory studies have shown that IL-12 produced by dendritic cells in response to *Salmonella* infection induces a potent IFNγ response from T cells and NK cells, especially in combination with IL-18 (131). Neutrophils are also an important source of IFNγ, especially early in infection (142), but neutrophil production of IFNγ is not dependent on TLR stimulation (143). Patients with mutations causing an IFNγ deficiency are also susceptible to complicated *Salmonella* infections, although to a lesser degree (144). Moreover, even though IFNγ is beneficial during *Salmonella* infection, a recent study showed that early IFNγ production during infection hinders host recovery after antibiotic treatment (145), providing one potential explanation as to why antibiotic treatment has limited or no benefit during *Salmonella* gastroenteritis.

Figure 1.3 Non-typhoidal *Salmonella* SPI-2 mediated survival inside phagocytes

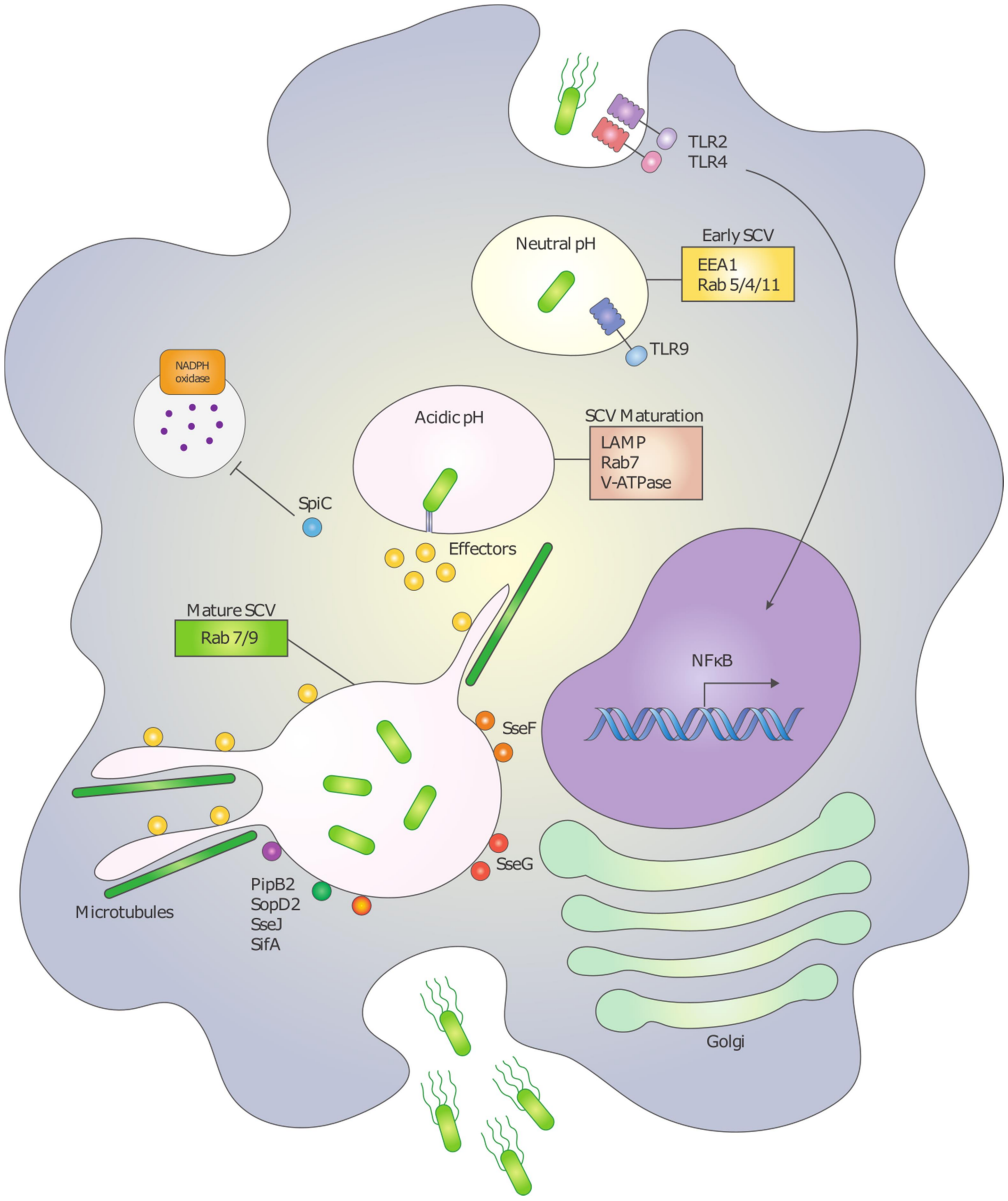

Figure 1.3 Macrophages recognize *Salmonella* antigens through TLR2, TLR4, TLR5, and TLR9. Signaling through any of these TLRs triggers phagosome acidification. Phagocytosed *Salmonella* detects vacuole acidification and turns on expression of SPI-2 genes. The SPI-2 T3SS complex releases effectors into the macrophage cytosol. The SCV develops features of an endosomal compartment but the effector SpiC helps prevent fusion with lysosomal vesicles that contain degradative enzymes or NADPH oxidase, which produces reactive oxygen species (ROS). Several SPI-2 effectors associate with the SCV membrane, stimulating Sif formation that support the SCV during its maturation (SifA, PipB2, SseJ, and SopD2) and position the SCV near the nucleus (SseF) and the Golgi apparatus (SseG). Maintenance of Sifs and proximity to key organelles are associated with the ability of *Salmonella* to replicate in the SCV. Ultimately, the SPI-2 effector proteins help *Salmonella* to escape host cells and resist NADPH-oxidase mediated killing, allowing further spread of the infection.

1.8.2 Th17 responses

The cytokine interleukin-23 initiates Th17 responses by stimulating innate lymphoid cells, γδ T cells, and Th17 cells to release IL-17 and IL-22 (146-148). These IL-23-mediated T cell responses are protective during infection and orchestrate the mucosal barrier to *Salmonella* (17, 148). Mice lacking IL-23 (*Il23p19*$^{-/-}$) express lower levels of IL-17 and IL-22, as well as lower levels of antimicrobial proteins such as Reg3γ (148). IL-17 also induces epithelial cell secretion of CXC chemokines, which are neutrophil chemoattractants (reviewed in (149)). Although CXC chemokines are produced by direct contact of *Salmonella* with epithelial cells and macrophages (150), the response is greatly amplified by the IL-23-Th17 axis (148). Indeed, IL-17-mediated induction of CXC chemokines is the most effective mechanism to induce neutrophil recruitment to mucosal surfaces. Furthermore, Th17 cytokines enhance granulopoiesis through the induction of G-CSF, thereby contributing to the replenishment of neutrophils during infection (151, 152). Interference with this cascade through deletion of either IL-23 (*Il23p19*$^{-/-}$) or the IL-17 receptor (*Il17ra*$^{-/-}$) causes decreased neutrophil recruitment to the intestine and blunted expression of antimicrobial proteins during *Salmonella* infection (11, 148). Lower IL-17 levels also result in an increase of *Salmonella* dissemination to the reticuloendothelial system (mesenteric lymph nodes and spleen), indicating that IL-17 promotes antimicrobial responses that confine *Salmonella* to the intestine (11, 132).

Like IL-17, IL-22 enhances mucosal barrier defenses against intestinal pathogens, primarily by inducing the secretion of antimicrobial proteins by intestinal epithelial cells (153, 154). Two of these antimicrobial proteins, lipocalin-2 and

calprotectin, function by sequestering essential metal ions from microbes. Lipocalin-2 binds to the siderophore enterobactin and limits bacterial iron uptake (155), whereas calprotectin (a heterodimer/tetramer of S100A8 and S100A9), sequesters manganese, zinc, and, in some settings, ferrous iron (156, 157). However, these antimicrobial proteins are not very effective in sequestering metal ions from *Salmonella*. This pathogen overcomes lipocalin-2-mediated enterobactin sequestration by expressing a stealth siderophore, salmochelin (158), which cannot be bound by lipocalin-2 (159). In addition, *Salmonella* expresses high affinity zinc and manganese transporters (ZnuABC, ZupT, MntH, SitABCD) to overcome metal limitation in the host (160-164). These mechanisms of metal ion acquisition enable *Salmonella* to thrive in the inflammatory intestinal environment (165-168). Therefore, evasion of IL-22–mediated antimicrobial responses enables *Salmonella* to thrive, also by reducing the growth of closely related competing microbes such as *Escherichia* in the inflamed intestine (165, 166).

1.9 The hallmark of *Salmonella*-elicited diarrhea: Neutrophils

In contrast to other cells (intestinal epithelial cells and phagocytic monocytes) that are permissive to *Salmonella* replication, *Salmonella* is susceptible to killing by neutrophils. Neutrophils effectively control *Salmonella* with their prominent production of reactive oxygen and nitrogen species (ROS/RNS), and antimicrobial proteins. In the vast majority of cases, *Salmonella* gastroenteritis remains localized to the gut mucosa, where the pathogen is eventually cleared by the large number of neutrophils that migrate to the intestine. However, if the host neutrophil antimicrobial response is insufficient, for example because of deficiency in neutrophil numbers or functions,

Salmonella may persist in the intestine and potentially disseminate to and replicate in extraintestinal tissues.

1.9.1 CXC chemokines attract neutrophils to the site of infection

Salmonella invasion of intestinal epithelial cells induces production of CXC chemokines (150), and IL-17 stimulation significantly amplifies production of these chemokines (11). Circulating neutrophils detect CXC chemokines via the receptor CXCR2 (169) and extravasate to the subepithelium, following the chemokine gradient. Migration through the epithelial layer and into the lumen is further driven by apical release of hepoxilin A3, a derivative of arachidonic acid (170-172). Abundant accumulation of neutrophils in the intestine is apparent within 5 hours of *Salmonella* infection in calf ligated ileal loops, and by 20 hours after oral infection in streptomycin-treated mice (173, 174). Although the mechanism by which neutrophils control *Salmonella* infections is not completely understood, production of reactive oxygen and nitrogen species and the release of antimicrobial proteins during infection were shown to play major roles. We explain these key neutrophil defense mechanisms in the following paragraphs.

1.9.2 Antimicrobial mechanisms: RNS, ROS, and antimicrobial proteins

When neutrophils migrate to the intestinal mucosa during *Salmonella* infection, they are stimulated by IL-1β, TNFα, and IFNγ to produce inducible nitric oxide synthase (iNOS). This enzyme uses L-arginine as a substrate for the production of nitric oxide, a highly reactive compound that contains a radical electron that is toxic on its own and can form additional toxic molecules by reacting with other reactive molecules like superoxide.

The majority of reactive oxygen produced in response to infection is generated by NADPH oxidase, a transmembrane, multi-protein enzymatic complex that assembles after TLR stimulation (175, 176). The active NADPH oxidase complex produces superoxide, which can spontaneously interact with hydrogen to form hydrogen peroxide. Superoxide and hydrogen peroxide can react with other molecules, such as nitric oxide (see above), to produce a variety of toxic ROS/RNS. Reactive electrons in ROS kill bacteria by damaging proteins and nucleotides, and thus interfere with key metabolic and reproductive processes. ROS and RNS produced by NADPH oxidase and iNOS are needed for the most effective killing of *Salmonella* (77). Mice lacking a functional NADPH oxidase complex are highly susceptible to infection with wild-type *Salmonella* and even develop colitis and bacteremia upon infection with *Salmonella* lacking both SPI-1 and SPI-2 T3SSs, which is avirulent in wild-type mice (64). These studies highlight the importance of reactive oxygen and reactive nitrogen species in host defense against *Salmonella*.

In addition to the production of ROS/RNS, neutrophils harbor numerous digestive enzymes with antimicrobial function, as well as antimicrobial proteins, in their granules and cytosol. An important neutrophil enzyme is myeloperoxidase, which catalyzes the reaction of hydrogen peroxide and chloride ions into the toxic hypochlorous acid. Other enzymes include elastase and lysozyme, which damage bacteria by cleaving proteins and sugar linkages like those found in peptidoglycan. Together with epithelial cells, neutrophils are sources of antimicrobial proteins that normally keep the microbiota at bay, but that are highly upregulated upon infection (11, 168). Neutrophil antimicrobial molecules include defensins (177), antimicrobial peptides which primarily act by

creating pores in bacterial membranes, and proteins that sequester essential metals like iron, zinc, and manganese (lactoferrin, calprotectin, lipocalin-2) (178).

When bacteria are phagocytosed by neutrophils, granules containing NADPH oxidase fuse with the phagosomal membrane and expose the bacteria to digestive enzymes and concentrated levels of ROS. Neutrophil granules can also fuse with the cell membrane and release enzymes and ROS into the extracellular space. Alternatively, neutrophils can undergo a complete release of antimicrobial molecules through a specialized form of apoptosis termed netosis, characterized by the formation of Neutrophil Extracellular Traps (NETs) (179, 180). These structures are caused by the extrusion of nuclear, cytosolic, and granule contents, ultimately resulting in a DNA web that engulfs histones, calprotectin, myeloperoxidase, and numerous other antimicrobial proteins (181). *Salmonella* has been shown to be trapped by NETs *in vitro* (179), but a specific role for NET formation in defense against *Salmonella* enterocolitis has yet to be defined.

In addition to their direct antimicrobial activity, neutrophils are also important sources of cytokines during *Salmonella* infection. For example, neutrophils produce IFNγ in the intestinal mucosa during *Salmonella* enterocolitis (142, 182), and produce IL-1β in a model of *Salmonella* peritonitis (183). Studies with other pathogens have shown that neutrophils can also secrete IL-17 (184, 185), and IL-10 (186). However, these neutrophil populations have not yet been described during *Salmonella* acute colitis.

1.9.4 Role of neutrophils in promoting Salmonella persistence

Although the host immune response protects from dissemination, and the infection is usually resolved within a week, in some patients *Salmonella* shedding can occur for up to a month after infection. A similar phenomenon has been observed in mice, where about 30% of the population shed significantly more *Salmonella* (187). Depletion of immune cells prior to infection is not sufficient to induce "super-shedder" status (i.e. increased shedding of *Salmonella*), but antibiotic treatment that depletes the resident microbiota can induce at least transient super-shedding (188). Super-shedder status is associated with elevated splenic neutrophils and reduced Th1 cells (187), which suggests that splenic neutrophils are not activated in the same way for bacterial killing as neutrophils that are recruited to intestinal tissue. Whether or not this phenomenon also occurs in humans with prolonged fecal shedding of *Salmonella* remains to be determined.

1.10 A side effect of neutrophil recruitment: Inflammatory diarrhea

Although neutrophils play a major role in keeping *Salmonella* infection localized to the gut, recruitment of massive numbers of neutrophils also has some detrimental effects for the host. To access the intestinal lumen, neutrophils have to pass through the mucosal extracellular matrix and tight junctions between epithelial cells. Neutrophils can secrete proteinases to facilitate this process, and inflammatory cytokines like IL-1β and IL-18 have been shown to induce changes in epithelial cell tight junction integrity (189-192). Neutrophils extravasating into intestinal tissue and migrating past the epithelial barrier into the gut lumen damage the integrity of the epithelial barrier. The

epithelial barrier is further damaged when neutrophils generate extracellular ROS and RNS and release degradative enzymes when encountering *Salmonella*. This process causes the leakage of serum proteins and fluid from capillaries into the intestinal lumen, which ultimately results in the protein and fluid loss that are characteristics of inflammatory diarrhea. For example, calves with *Salmonella* diarrhea show epithelial disruption and fecal protein loss, which can be measured by decreased serum albumin (193, 194).

In addition to the damage inflicted by extravasating neutrophils, the epithelial lining is directly damaged and altered by *Salmonella*. The pathogen induces epithelial cell death and shedding, and alters epithelial cell ion transporter expression and localization (195, 196), leading to increased water content in the feces and host dehydration. Loss of chloride and sodium absorptive transporters such as DRA ("downregulated in adenoma"), CFTR ("cystic fibrosis transmembrane conductance regulator"), ENaC beta ("epithelial Na^+ channel beta"), and the chloride-bicarbonate exchanger (sodium channel B) in the apical surface of enterocytes have been detected in mice infected with *Salmonella* (196). Changes in ion transporter expression and localization are not exclusive to infected epithelial cells, suggesting that host soluble factors promote epithelial cell changes. At least one *Salmonella* effector (SopB) promotes chloride secretion by dephosphorylating inositol species to generate inositol 1,4,5,6 tetrakisphosphate, which interferes with EGF (epidermal growth factor) and PI3K (phosphoinositide-3 kinase) signaling in the epithelial cell (197). Deletion of the SopB gene in *Salmonella* also led to lower fluid accumulation in a model of calf ileal loop infection (36). Although neutrophil-mediated destruction of epithelial integrity is the

major contributor to diarrhea, the fluid accumulation in calf ileal loops and the lower fecal density in mice with defective neutrophil migration (198, 199) indicate at least a minor role for ion flux in the pathogenesis of diarrhea.

1.11 What enables *Salmonella* to thrive in the inflamed gut?

The inflammatory response and the recruitment of neutrophils contribute to the eventual clearance of *Salmonella* infection in immunocompetent patients. However, during early stages of infection, *Salmonella* has unique virulence factors that allow the pathogen to thrive in the inflamed intestine. One of these factors is *Salmonella* resistance to antimicrobial proteins that sequester metal ions, which we explained earlier. Metal starvation does not only slow bacterial growth due the lack of a nutrient, but also enhances oxidative damage due to chelation of metals that are essential cofactor for bacterial enzymes that detoxify ROS (200, 201). The majority of bacteria that comprise a healthy intestinal microbiota are strict anaerobes of the order Clostridiales and Bacteroidetes and cannot survive the oxidative environment created by host inflammation. On the contrary, *Salmonella* is able to grow in both aerobic and anaerobic conditions, can acquire metals under severely limiting conditions, and can neutralize ROS. To accomplish the latter, *Salmonella* expresses metal ion dependent enzymes. Multiple superoxide dismutase (SOD) and catalase proteins neutralize superoxide and hydrogen peroxide, into molecular oxygen, hydrogen, and water, respectively. *Salmonella* lacking SOD is more susceptible to neutrophil-mediated killing, but is rescued when the host NADPH oxidase complex is nonfunctional (202). Furthermore, acquisition of manganese, an essential cofactor for the *Salmonella*

enzyme SodA, helps *Salmonella* to thrive in the inflamed gut by detoxifying ROS in this environment (166).

Another major determinant of *Salmonella's* success in the inflamed intestine is its ability to use unique carbon sources for energy, an ability that is made possible by the electron acceptor tetrathionate, which is induced in sufficient amount only during inflammation (203). The presence of tetrathionate allows for anaerobic respiration of compounds like ethanolamine, made from host cell membranes; fucose, from dietary intake or breakdown of the mucus layer; 1,2-propanediol, a product of fucose fermentation; and fructose-asparagine, an Amadori product of glucose and asparagine. These molecules are not readily used by other microbes in the intestine, so their utilization provides *Salmonella* with a growth advantage (204-206). In addition to *Salmonella*-induced inflammation, antibiotic treatment can create conditions that favor *Salmonella* growth, both through killing competitors for nutrients and space, by increasing available fucose and sialic acid for *Salmonella* metabolism (207), and by inducing the production of novel acidic sugars that can be utilized by *Salmonella* (208). During early stages of infection, *Salmonella* growth is also enhanced by its ability to cleave hydrogen to produce protons that can be used for ATP production or transport of carbon-containing molecules. Hydrogen in the intestine comes from commensal bacteria, so killing the hydrogen-producing bacteria through antibiotic treatment eliminates the advantage that hydrogen provides for *Salmonella* colonization (209).

Figure 1.4 Non-typhoidal *Salmonella* activation of NLRP3 and NLRC4 Inflammasomes

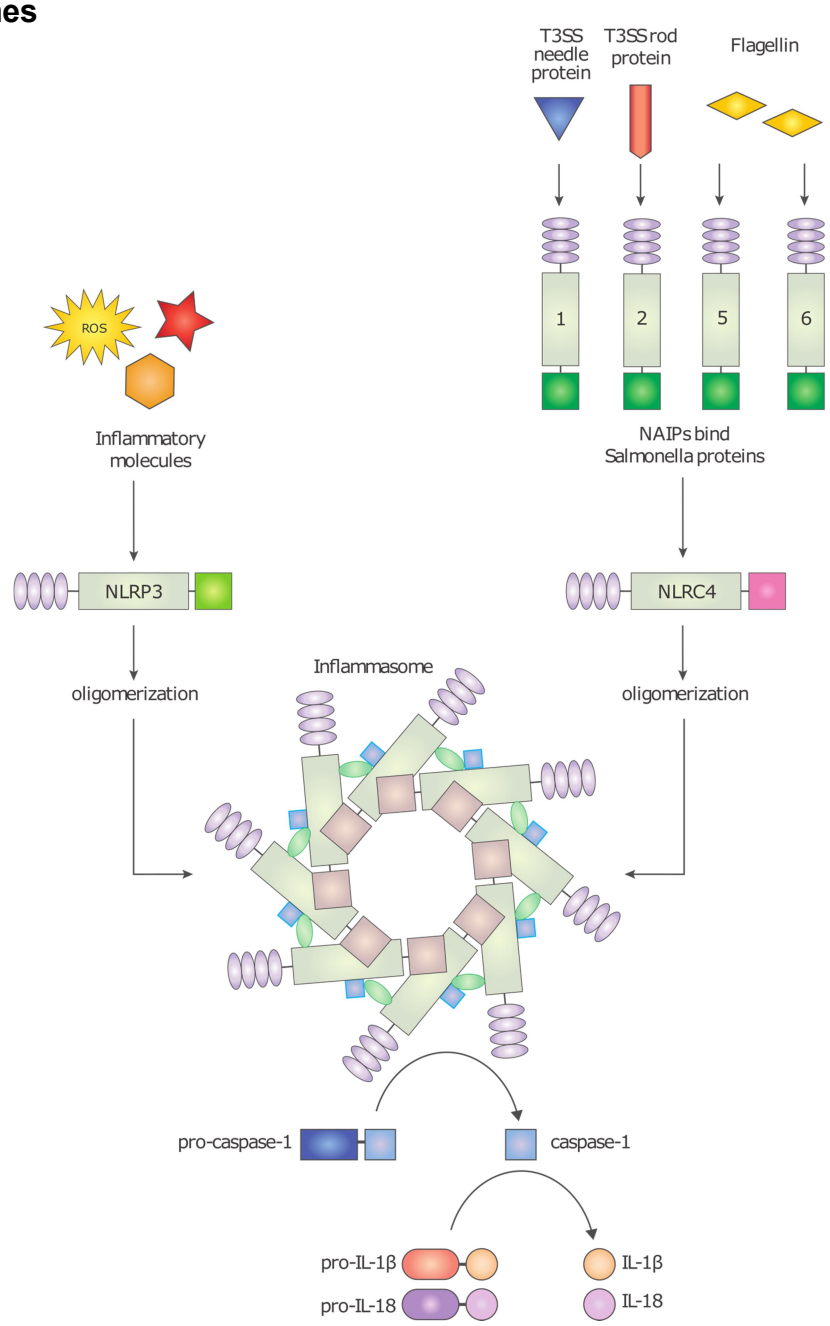

Figure 1.4 Inflammatory molecules are detected by NLRP3, or *Salmonella* T3SS proteins or flagellin are recognized intracellularly by NAIP receptors that bind NLRC4. Activation of one or both NLR receptor pathways results in assembly of an inflammasome complex. This multimeric protein complex activates pro-caspase-1 to the active protease caspase-1, which then cleaves pro-IL-1β and pro-IL-18 into their active forms IL-1β and IL-18, which are subsequently secreted.

1.12 Bacteremia with non-typhoidal *Salmonella*

Salmonella causes a self-limited inflammatory diarrhea in immunocompetent individuals, but patients with certain pre-existing medical conditions are at high risk for systemic infection and extra-intestinal dissemination of the pathogen. Below, we provide a brief summary of three illnesses that predispose patients to systemic disease rather than localized inflammatory diarrhea from non-typhoidal *Salmonella* and what we have learned about the molecular mechanisms of susceptibility.

1.12.1 Chronic Granolumatous Disease

Humans deficient in NADPH oxidase activity, diagnosed as chronic granulomatous disease (CGD), are highly susceptible to prolonged and severe illness after infection with common pathogens (210). These patients have normal neutrophil numbers and recruitment, but the ability of neutrophils to produce superoxide is greatly reduced due to a mutation in one of the subunits of the NADPH oxidase complex. As discussed above, superoxide reacts with other molecules to produce a variety of different ROS, including hydrogen peroxide. Without a functional NADPH oxidase complex, only low levels of hydrogen peroxide are achieved which can be detoxified by catalase-positive microorganisms like *Salmonella*. In these patients, *Salmonella* is thus able to survive, grow, and escape the phagosome of host immune cells.

1.12.2 HIV/AIDS

Human immunodeficiency virus, HIV, infects CD4+ T cells, leading to their destruction through viral-mediated lysis or immune killing of infected cells. Shortly after infection with the virus, HIV patients experience a significant decrease of intestinal $CD4^+$ T cells and even anti-retroviral therapy frequently fails to fully reverse depletion of this

cell type (211, 212). This lack of T cells includes the Th17 subset, an important population in the immune signaling cascade induced by *Salmonella* infection (11). In rhesus macaques infected with the Simian Immunodeficiency Virus, Th17 cell depletion leads to increased *Salmonella* dissemination to the mesenteric lymph nodes and the spleen (11). Moreover, macrophages and other cell types present in whole blood isolated from HIV patients in Africa showed dysfunctional cytokine release (213, 214), which may be caused by viral protein interference with macrophage signaling networks. The HIV protein Nef has been implicated in reducing macrophage expression of CD36, a receptor involved in phagocytosis. Nef also interferes with macrophage oxidative burst (215). Without a fully functioning immune system, HIV patients are susceptible to extreme *Salmonella* infections, such as vertebral osteomyelitis (216), meningitis (217), and septicemia, which are frequently fatal.

The high prevalence of HIV-infected persons in Sub-Saharan Africa resulted in the emergence of distinct *Salmonella* strains. Presumably, the selective pressure to maintaining many virulence genes is reduced for *Salmonella* strains circulating in a population of HIV-infected individuals. In these patients, a less virulent strain of *Salmonella* can cause disease because of their weakened immune system. A strain of non-typhoidal *Salmonella*, ST313, has been identified as causing more typhoid-like disease, exhibiting degradation in genes necessary to initiate intestinal inflammation (218, 219). Infections with this strain result in systemic disease without diarrheal symptoms. Analysis of blood from patients actively infected with invasive non-typhoidal *Salmonella* revealed a lack of IFNγ or TLR-induced NF-κB inflammatory response (214). Cell culture studies show *Salmonella* ST313 invades non-phagocytic cells less

efficiently and stimulates less inflammasome activation in macrophages than normal *Salmonella* isolates, as indicated by lower mature caspase-1 and IL-1β levels (220). In addition to being less inflammatory and more invasive than most non-typhoidal *Salmonella* strains, *Salmonella* ST313 also carries multiple drug resistance genes and at least one isolate has shown resistance to heavy metal toxicity (221).

The high prevalence and severity of *Salmonella* infection in the African population is not just of concern for HIV patients, but also for children. Children have lower levels of stomach acidity and a less-experienced immune system and are therefore more susceptible to *Salmonella* infection, a problem that is exacerbated in areas where modern sanitation (clean water and sewage treatment) are limited. Studies of African children have revealed an important role for complement proteins and anti-*Salmonella* antibodies in controlling *Salmonella* infection (222, 223). The neutrophil oxidative burst was defective unless *Salmonella* was opsonized by serum antibodies and complement proteins (223). Adequate production of immune proteins depends on a diet with sufficient protein content, so children raised without adequate nutrition are highly susceptible to *Salmonella* (224).

Figure 1.5 Compromised immune function and non-typhoidal *Salmonella* susceptibility

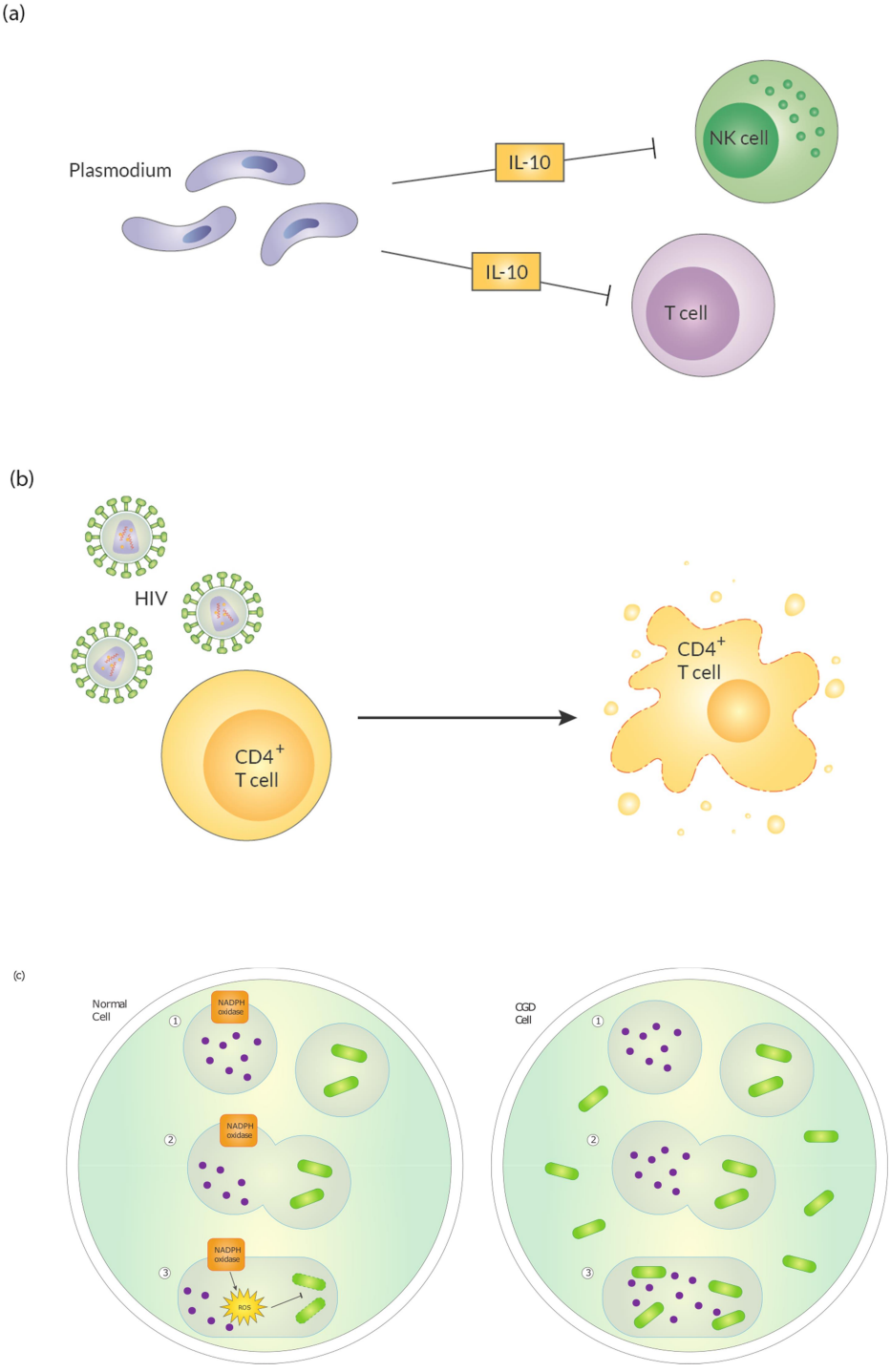

Figure 1.5 Host immunity to non-typhoidal *Salmonella* is impaired in co-infection with (A) malaria because Plasmodium secrete and induce production of the anti-inflammatory cytokine IL-10, or (B) HIV, which causes destruction of intestinal CD4+ cell populations. (C) Neutrophil microbicidal function is impaired without reactive oxygen species (ROS) production by the NADPH oxidase enzyme complex.

1.12.3 Malaria

Malaria is caused by infection with *Plasmodium* species parasites that are transmitted to humans by mosquitoes. Like for HIV infection, there is a strong association between malaria infection and invasive *Salmonella* infections (225), which prompted new investigation on why *Plasmodium* infection would promote *Salmonella* bacteremia. The *Plasmodium* parasite induces red blood cell lysis and the liberated heme activates heme oxygenase-1 (HO-1), an enzyme that breaks down heme. Upregulation of heme oxygenase protects against the effects of hemolysis during malaria infection. However, this response was also shown to increase susceptibility to *Salmonella*. A study of malaria-infected children showed that impaired neutrophil oxidative burst correlated with high levels of HO-1 expression, and as the malaria infection resolved, so did the ability to have a normal oxidative burst response. Neutrophil function was specifically tested against *Salmonella ex vivo* (226), and experiments in mice indicate that inhibition of HO-1 is protective against *Salmonella* bacteremia during malaria co-infection (227). Further studies with mice show that the gut microbiota is altered upon infection with *Plasmodium*, which increases susceptibility to colonization with non-typhoidal *Salmonella* (228).

Independent of red blood cell lysis and HO-1 expression, malaria infection induces production of IL-10, an anti-inflammatory cytokine that interferes with an effective host inflammatory response, including the activation of NADPH oxidase (229), and leads to higher rates of *Salmonella* invasion. Mice lacking receptors for IL-10 on myeloid cells were more resistant to *Salmonella* invasion, demonstrating the importance of myeloid cell activation in maintaining a protective barrier in the intestine (230). In

rhesus macaques infected with *Plasmodium fragile*, *Salmonella* induced lower levels of fluid accumulation, lower expression of neutrophil chemoattractant, and reduced recruitment of neutrophils to the gut in comparison to animals singly infected with *Salmonella* (231). Another effect of malaria infection, observed in humans and mice, is L-arginine deficiency, which contributes to intestinal IL-4 and histamine production, leading to increased intestinal permeability. This permeability likely plays a role in increasing *Salmonella* dissemination during co-infection, because oral supplementation with L-arginine or L-citrulline reduced bacterial loads in mesenteric lymph nodes and enhanced cell junction integrity (232).

1.13 Conclusions

Inflammatory diarrhea caused by non-typhoidal *Salmonella* is the result of the complex interaction between virulence factors expressed by the pathogen and the host response to *Salmonella* infection. Invasion of intestinal epithelial cells, replication in macrophages, and activation of Th17 responses are important steps to induce the recruitment of neutrophils to the gut. The activation of the immune response is overall beneficial to the host because it keeps *Salmonella* localized to the gut and prevents complications like bacteremia, which is observed in individuals with a primary or secondary defect of host immunity.

Unfortunately, host inflammation also promotes *Salmonella* colonization by enabling the pathogen to better compete with the gut microbiota. The microbiota provides initial colonization resistance to *Salmonella*, but the mechanisms are not well understood. Two studies started to shed light on the mechanism and showed that

Salmonella transport to the mesenteric lymph nodes is inhibited by the microbiota (233) and that the microbiota contributes to *Salmonella* clearance from the gut lumen (234). Thus, antibiotic treatment during uncomplicated inflammatory diarrhea caused by *Salmonella* is usually not recommended because it prolongs fecal shedding of the pathogen (235, 236).

As unselective killing by traditional antibiotics does not better the outcome of an infection with *Salmonella*, new strategies to reduce *Salmonella* colonization have to be developed. In this era of increased microbial resistance and failing antibiotics, therapies targeted at only one or one group of bacteria are highly desirable. One such step towards a highly targeted approach to limit *Salmonella* colonization and systemic dissemination is a recently described siderophore-based immunization strategy. Here, mice immunized with siderophores that were rendered immunogenic developed antibodies that selectively starved *Salmonella* for the essential metal iron and induced expansion of beneficial microbes (237). Strategies such as these with limited side effects on the resident microbiota have potential to be further developed to limit *Salmonella* colonization and dissemination also in humans.

A greater understanding of the mechanisms by which *Salmonella* causes inflammatory diarrhea, survives in the gut, and of the mechanisms by which the microbiota provides colonization resistance is essential to design new therapies to control infection with this pathogen.

Chapter 2: Neutrophil depletion exacerbates systemic but not intestinal *Salmonella* Typhimurium burden

2.1 Abstract

Salmonella Typhimurium causes gastroenteritis that is characterized by a massive recruitment of neutrophils to the intestine. Neutrophil deficiency or functional impairment is associated with higher morbidity and mortality in humans. Previous work showed neutrophil defenses are exploited by *S.* Typhimurium to increase its competitive advantage against other microbes in the intestine (166, 167), but the effect of neutrophil recruitment to the intestine on *S.* Typhimurium spread to other tissues has not been fully characterized. To better understand neutrophil contributions to the intestinal immune barrier during *S.* Typhimurium infection, we depleted mice of neutrophils via antibody treatment against a neutrophil surface receptor: Ly6G or CXCR2. In mice depleted of neutrophils, we observed minimal to no difference in total *S.* Typhimurium burden in feces, but higher *S.* Typhimurium colonization in the mesenteric lymph nodes and spleen. Interestingly, neutrophil depletion with anti-CXCR2 antibody treatment led to higher levels of *S.* Typhimurium in Peyer's patches, which was not observed in anti-Ly6G neutrophil depleted mice, suggesting CXCR2-expressing cells other than neutrophils are contributing to defense against *S.* Typhimurium in Peyer's patches. Additionally, we found that neutrophil depletion of C57BL/6 mice with a functional NRAMP1 transporter does not have an impact on tissue colonization by *S.* Typhimurium at 72h post-infection, suggesting that NRAMP1 functionality is more important than neutrophil responses in early stages of *S.* Typhimurium infection. Finally, we highlight the importance of marker selection in detecting cells after antibody depletion, as

depleting antibodies can shield epitopes on cells and misrepresent their presence in tissues.

2.2 Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a foodborne pathogen that causes over a million cases of gastroenteritis in the United States each year. *S.* Typhimurium infection is characterized by fever, cramping, and inflammatory diarrhea, with stools containing a large number of neutrophils. The importance of the neutrophil response is apparent when patients who have few or functionally defective neutrophils, such as those with chemotherapeutic bone marrow suppression, chronic granulomatous disease, or HIV infection, are infected with *S.* Typhimurium. These patients are more susceptible to *S.* Typhimurium infections and have a higher rate of complications like bacteremia and sepsis (238, 239).

Here, we use the streptomycin pretreatment mouse model of *S.* Typhimurium infection to induce gastroenteritis in C57BL/6 mice. Mice infected with non-typhoidal *S.* Typhimurium do not normally develop diarrhea, but treatment with one dose of streptomycin suppresses the normal microbiota of the mice and induces a low level of inflammation. *S.* Typhimurium thrives in inflammatory conditions, especially in comparison to the majority of commensal microbes, and induces cecal inflammation and neutrophil influx into the intestine. Previous research from the Raffatellu Lab and others showed that neutrophil recruitment to the intestine during *S.* Typhimurium infection leads to high levels of neutrophil antimicrobial proteins and oxidative stress in the intestinal lumen, which changes the environment and availability of nutrient sources

for gut bacteria. *S.* Typhimurium has evolved to take advantage of the inflammatory environment by utilizing unique nutrient sources and overcoming metal ion sequestration, thereby allowing it to grow to very high levels in the intestine (166, 167, 240, 241).

In contrast to typical human infection, *S.* Typhimurium gastroenteritis in mice is not contained to the intestine but spreads systemically within two to three days of infection. The dissemination of *S.* Typhimurium from the intestine can be followed temporally from Peyer's patches to mesenteric lymph nodes and then to the spleen and liver (reviewed in (242). The mechanism of spread is still poorly understood, but potential explanations for high tissue levels of *S.* Typhimurium include numerous bacteria disseminating from the gut or a few seeding bacteria disseminating and then proliferating in the tissue. A recent study using mixed populations of *S.* Typhimurium supports dissemination as a seeding event, as less diversity of *S.* Typhimurium strains was detected in tissues than in feces (243). To better understand how neutrophils help combat *S.* Typhimurium gastroenteritis and disseminated infection, as in immunocompromised patients, mice were depleted of neutrophils using antibodies and then infected with *S.* Typhimurium.

Antibody depletion of neutrophils has historically been accomplished using anti-Gr-1 (clone RB6-8C5), which binds both Ly6G and Ly6C proteins. Ly6G expression is considered exclusive to neutrophils, but Ly6C is present on other leukocytes, so Gr-1 antibody has been found to bind to plasmacytoid DCs, macrophages/monocytes, and CD8+ T cells (244). In 2008, an antibody selective for Ly6G (clone 1A8) was generated (245), allowing specific detection and deletion of neutrophils. We and others have also

depleted neutrophils using anti-CXCR2 antibody (167, 246), which was generated in rabbits using a peptide corresponding to the N-terminal 17 amino acids of murine CXCR2 (247). CXCR2 is highly expressed on neutrophils, and is a chemokine receptor that directs neutrophil migration to sites of infection or inflammation. To address the role of CXCR2 in neutrophil recruitment as well as the protective functions of neutrophils during *S.* Typhimurium infection, we depleted mice of neutrophils using either anti-CXCR2 or anti-Ly6G antibody and analyzed dissemination of *S.* Typhimurium to Peyer's patches, mesenteric lymph nodes, liver, and spleen.

2.3 Materials and Methods

Animal experiments: All procedures were approved by the University of California, Irvine Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from Taconic Farms / Envigo. C57BL/6 mice modified with the *Slc11a1* allele from 129S1 strain (NRAMP1+) were provided by Dr. Greg Barton (UC Berkeley) and bred in-house.

Antibody depletion: C57BL/6 female mice 8-12 weeks of age and between 18g and 21g were injected intraperitoneally with 500µg of anti-Ly6G antibody (clone 1A8, BioXCell) or Rat IgG (Jackson ImmunoResearch), or 500µl anti-CXCR2 in rabbit serum 24 hours prior to infection and again 24h post-infection to maintain the depletion.

Salmonella infection: Prior to infection, streptomycin (Calbiochem) was administered via oral gavage at 1mg per gram mouse weight. A dose of 10^9 *Salmonella enterica* serovar Typhimurium strain IR715 bearing a plasmid for streptomycin resistance was administered via oral gavage 24 hours after streptomycin treatment.

Intestinal colonization was monitored every 24 hours post-infection by quantifying *S.* Typhimurium present in fecal samples. Mice were sacrificed at 72 hours post-infection. At sacrifice, *S.* Typhimurium levels were determined for Peyer's patches, mesenteric lymph nodes, liver and spleen. All *S.* Typhimurium levels were normalized to colony-forming units (cfu) per mg of fecal sample or tissue.

Cell isolation & preparation: Intestinal cells were isolated from approximately one third of the cecum and the entire colon by first cutting the cylindrical tissue lengthwise and cleaning the tissue of fecal matter by vigorously shaking the tissue in wash buffer (HBSS, 15mM HEPES, 1% antibiotic/antimycotic [Ab/Am]). The intraepithelial layer was removed by gently shaking in EDTA solution (HBSS, 15mM HEPES, 10% FBS, 1% Ab/Am, 5mM EDTA) in a 37°C water bath for 15 minutes. Saving the EDTA solution on ice, the tissue was removed and cut into small pieces and transferred into a solution of IMDM with digestive enzymes (IMDM, 10% FBS, 1% Ab/Am, 20µg/mL liberase [Roche], 1mg/mL Collagenase and 250µg/mL DNase [Sigma]). Tissue pieces were disrupted using a gentleMACS tissue dissociator (program spleen_04, Miltenyi Biotech), incubated with shaking at 37°C for 15 minutes, and again disrupted with the tissue dissociator. Cells were filtered, along with the intraepithelial cells, and collected by centrifugation at 800rcf for 10 minutes. Cells were suspended in IMDM media without digestive enzymes and counted prior to flow staining.

Flow cytometry: Tissues were analyzed by flow cytometry for the presence of neutrophils [CD11b (eBioscience), Ly6G (1A8 BioLegend)]. Calprotectin expression was determined by using an S100A9 antibody (R&D Systems) conjugated in-house to the

Pacific Blue fluorophore (LifeTechnologies). All flow cytometry data was obtained using a BD LSR-II or ACEA Novocyte and analyzed using FlowJo (Treestar).

Statistics: Statistical analysis was conducted using Prism (GraphPad). A two-tailed t-test was performed to compare immune cell numbers per million live cells (as determined by flow cytometry viability staining), the log of cfu *S.* Typhimurium per mg sample, and the log of the median fluorescence intensity between groups. * p value < 0.05, ** < 0.01, *** < 0.001.

2.4 Results and Discussion

S. Typhimurium activates a cytokine signaling cascade when it penetrates the intestinal epithelial barrier via transcytoplasmic migration through Peyer's patch M-cells (248), active invasion of the epithelium using a type-III secretion system (21), or dendritic cell phagocytosis (reviewed in (249)). Once across the intestinal barrier, *S.* Typhimurium interacts with antigen presenting cells like dendritic cells and macrophages, which leads to expression of cytokines that stimulate resident T cells to release IL-17 and IL-22 (17, 148). These cytokines then activate epithelial cell secretion of antimicrobial proteins and the neutrophil-attracting chemokines CXCL1 and CXCL2 (149), which are detected by the receptor CXCR2 in neutrophils (169). To test whether CXCR2 is important for neutrophil recruitment to the intestine during *S.* Typhimurium infection, and to better understand the role of neutrophils in reducing *S.* Typhimurium dissemination, mice were depleted of neutrophils using either anti-Ly6G or anti-CXCR2 antibody. Mice treated with IgG showed neutrophil recruitment to the intestinal mucosa at 72 hours post-infection, but this accumulation of neutrophils was prevented in mice

treated with anti-CXCR2 antibody (Figure 2.1). The reduction in neutrophils did not cause a significant increase in fecal *S.* Typhimurium levels (Figure 2.2), but led to significantly higher *S.* Typhimurium burden in Peyer's patches, mesenteric lymph nodes, and spleen, with a trend toward higher *S.* Typhimurium in liver (Figure 2.3). These data suggests that intestinal neutrophils are an important component of the immune barrier against *S.* Typhimurium spread to extra-intestinal tissues.

Figure 2.1

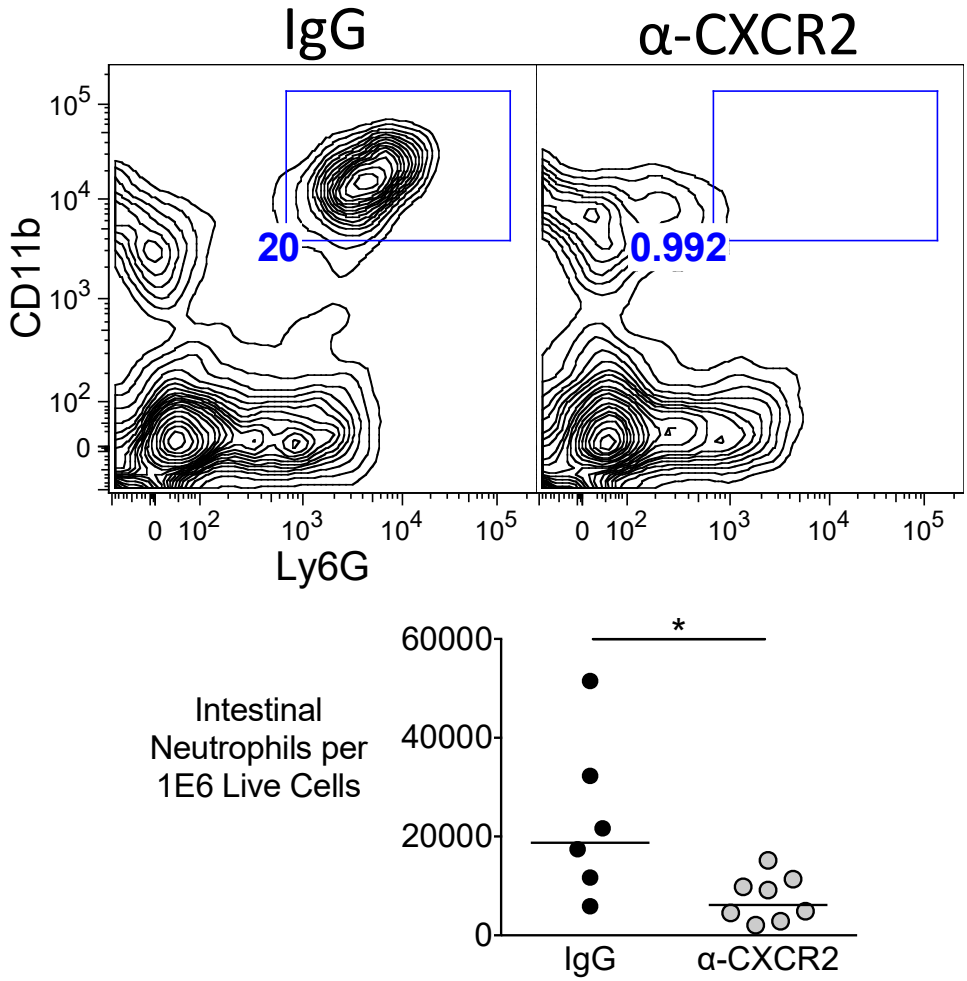

Figure 2.1 Intestinal neutrophils in mice treated with IgG or anti-CXCR2 antibody 72 hours post-infection with *S.* Typhimurium

Figure 2.2

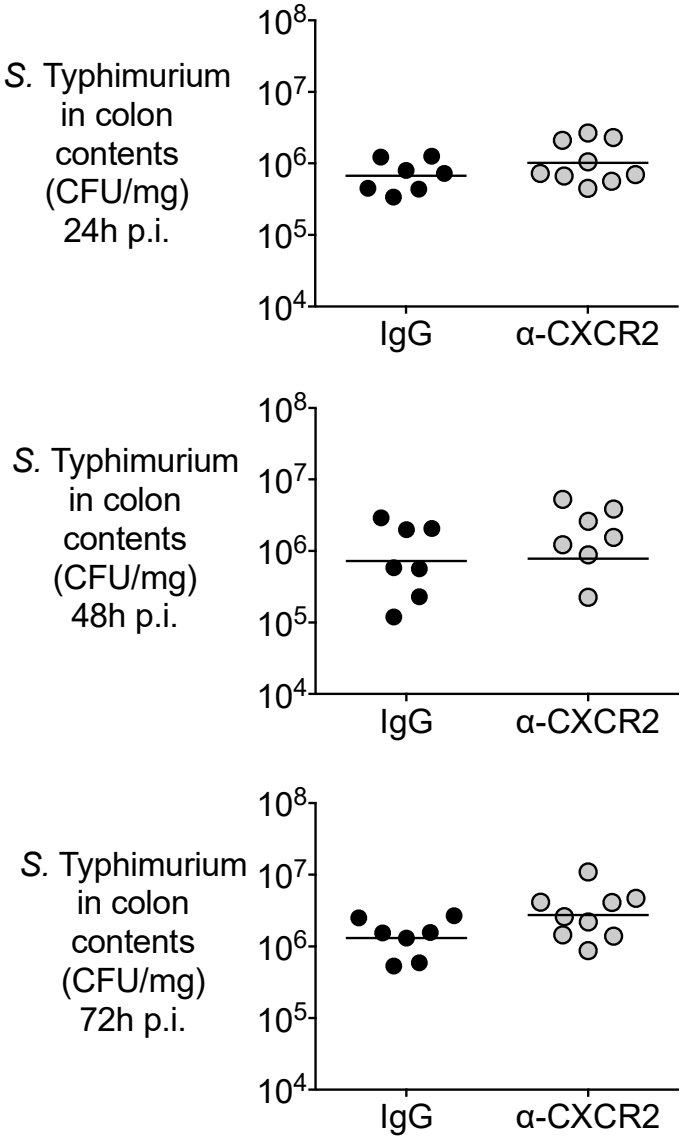

Figure 2.2 Fecal *S.* Typhimurium at 24, 48, and 72 hours post-infection in mice treated with IgG or anti-CXCR2 antibody

Figure 2.3

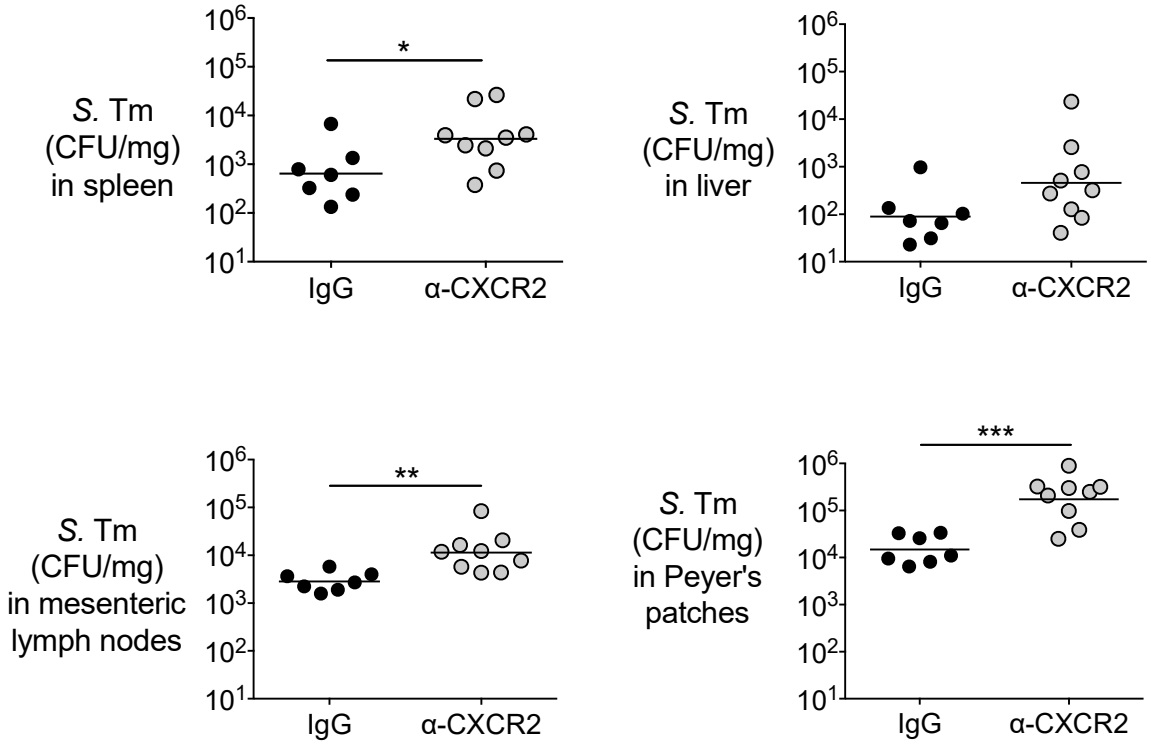

Figure 2.3 *S.* Typhimurium burden in tissues of mice treated with IgG or anti-CXCR2 antibody, 72h post-infection

Although CXCR2 is dominantly expressed by neutrophils, and anti-CXCR2 treatment significantly reduced intestinal neutrophils, Ly6G is a more specific marker for neutrophils, so we repeated our depletion using anti-Ly6G (clone 1A8) antibody. As with anti-CXCR2 treatment, intestinal neutrophils were significantly lower in anti-Ly6G treated mice compared to IgG treated mice after 72 hours of *S.* Typhimurium infection (Figure 2.4). Fecal *S.* Typhimurium levels were similar at 24 and 48 hours post-infection, but higher in anti-Ly6G treated mice at 72 hours (Figure 2.5). To test whether this represents a true difference in fecal colonization compared to anti-CXCR2 treated mice, a direct comparison of treatments would be required because absolute *S.* Typhimurium numbers vary between infections. However, it may indicate that the cellular targets of anti-CXCR2 antibody differ enough from those of anti-Ly6G antibody that the intestinal lumen environment affects *S.* Typhimurium growth significantly. Tissue burden of *S.* Typhimurium in anti-Ly6G treated mice was higher in mesenteric lymph nodes and spleen, with a trend toward higher *S.* Typhimurium in liver (Figure 2.6), as observed in anti-CXCR2 treated mice, further supporting the importance of neutrophils in protecting against *S.* Typhimurium infection in these tissues. In contrast, *S.* Typhimurium levels were similar in Peyer's patches of IgG and anti-Ly6G treated mice. These results indicate that *S.* Typhimurium levels in Peyer's patches are not dependent on neutrophils, but on a different type of CXCR2-expressing cell.

Figure 2.4

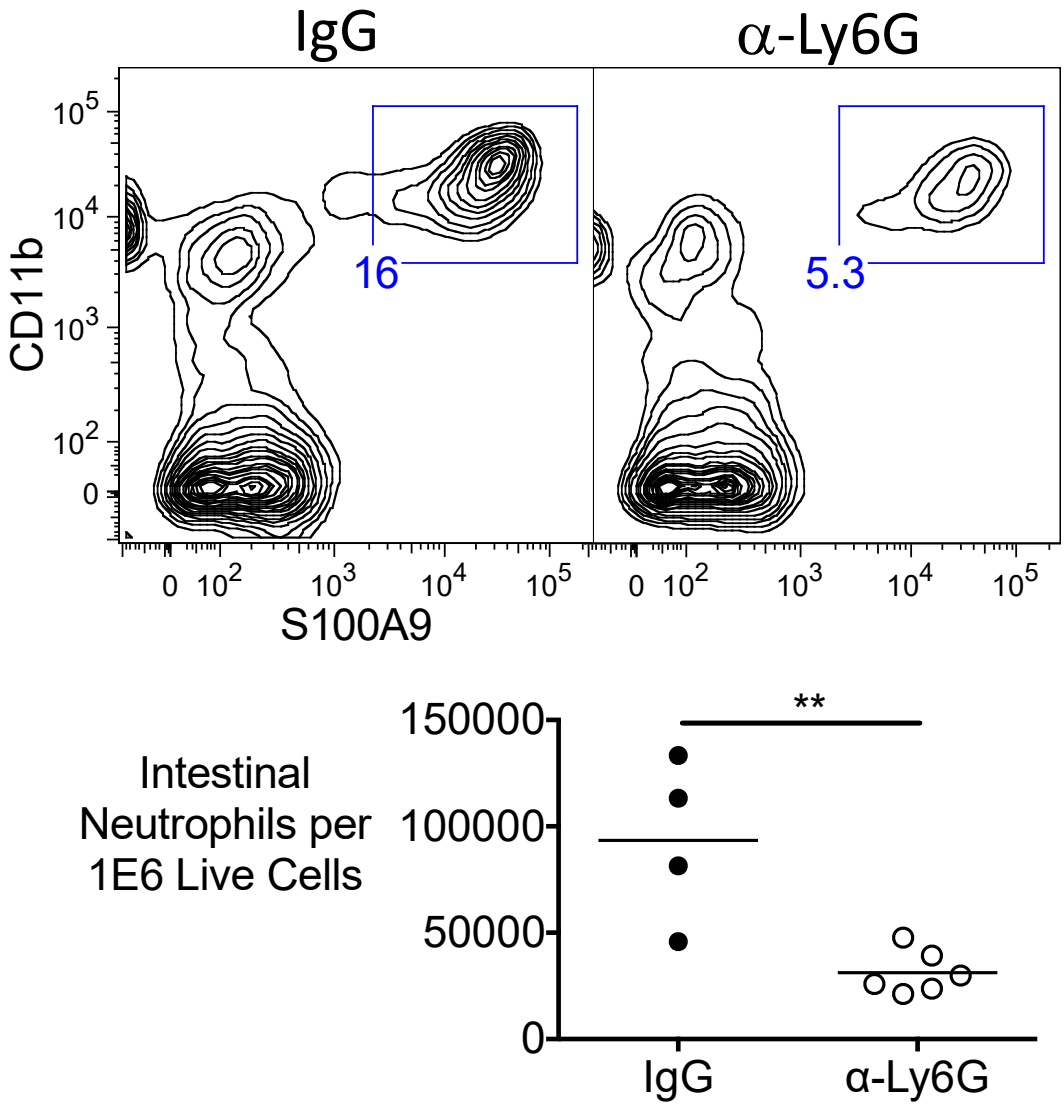

Figure 2.4 Intestinal neutrophils in mice treated with IgG or anti-Ly6G antibody 72h post-infection with *S.* Typhimurium

Figure 2.5

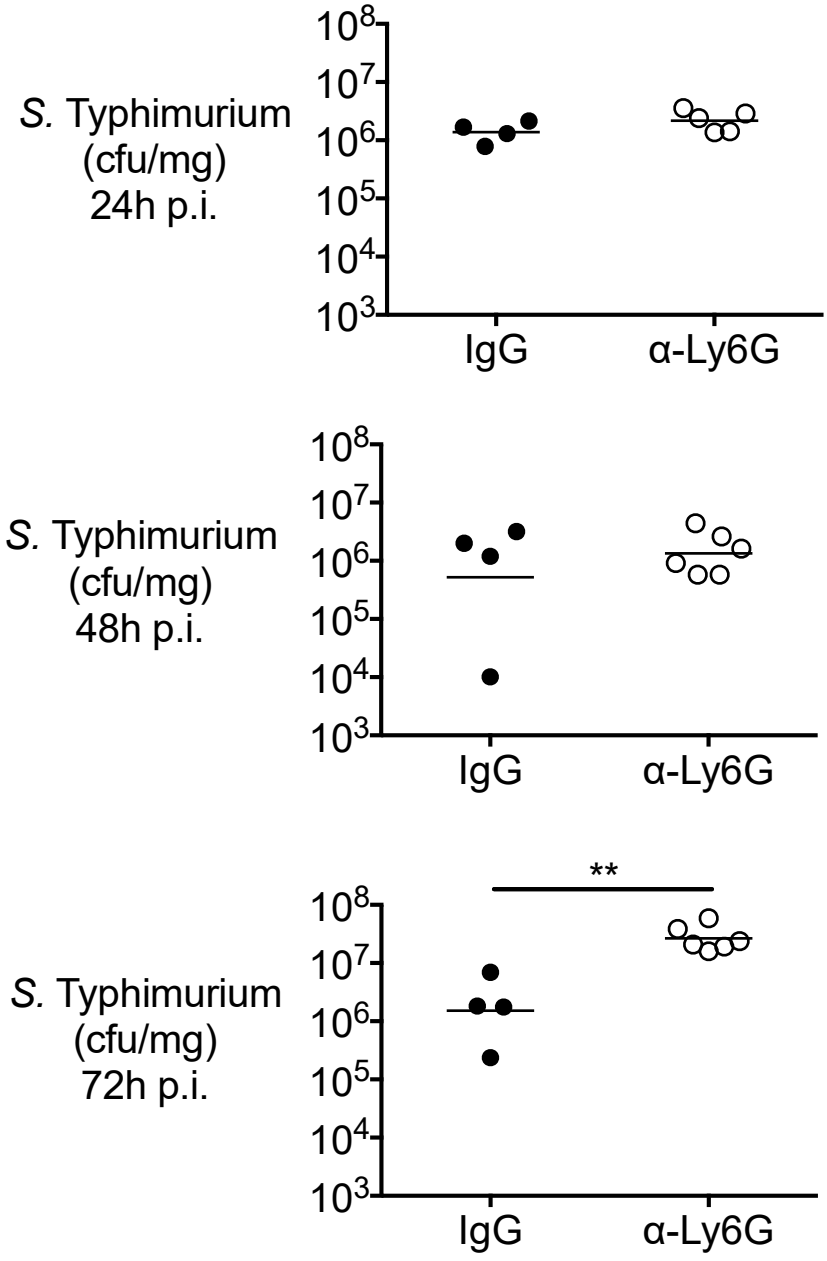

Figure 2.5 Fecal *S.* Typhimurium at 24, 48, and 72 hours post-infection in mice treated with IgG or anti-Ly6G antibody

Figure 2.6

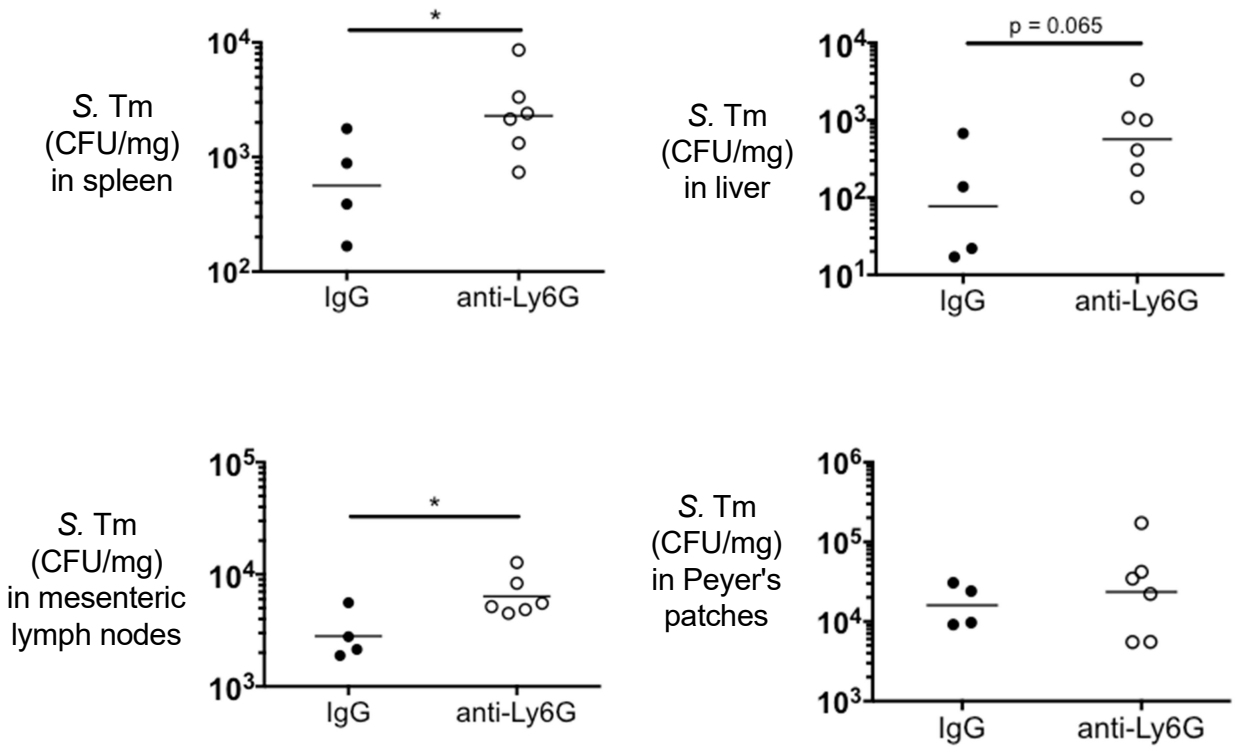

Figure 2.6 *S.* Typhimurium burden in tissues of mice treated with IgG or anti-Ly6G antibody, 72h post-infection

Together, these data indicate that intestinal neutrophils are important for defense against *S.* Typhimurium in mesenteric lymph nodes and spleen, and appear to have a minor role in liver. In Peyer's patches, CXCR2-expressing cells limit *S.* Typhimurium invasion and/or growth, but the failure to reproduce this phenotype in anti-Ly6G treated mice suggests that these CXCR2-expressing cells are not neutrophils. A detailed analysis of the cell types in Peyer's patches before and after antibody treatment and correlation with *S.* Typhimurium burden will be important for deciphering any additional roles for CXCR2-expressing cells in defense against *S.* Typhimurium infection. If an additional target of anti-CXCR2 treatment is identified, that cell type may be contributing to the intestinal lumen environment such that co-depletion with neutrophils supports *S.* Typhimurium growth at similar levels as in IgG treated mice.

The above experiments were conducted with wild-type C57BL/6 mice, which are known to harbor a glycine to aspartate mutation in the *Slc11a1* gene that renders the encoded ion transporter, NRAMP1, nonfunctional. NRAMP1 has been identified as a key player in immunity to pathogens with an intracellular phase of infection, like *S.* Typhimurium. Therefore, wild-type C57BL/6 mice, with a non-functional transporter (NRAMP1-), are more susceptible to *S.* Typhimurium infection than 129 mouse strains, which have a functional NRAMP1 transporter (NRAMP1+). To test the importance of neutrophils in the context of NRAMP1 functionality, C57BL/6 mice modified with the *Slc11a1* allele from 129S1 strain were treated with anti-Ly6G antibody or IgG and infected with *S.* Typhimurium. Intestinal neutrophils were reduced by anti-Ly6G treatment (Figure 2.7), but the absolute number of neutrophils present at 72 hours post-

infection was notably fewer in NRAMP1+ mice than that observed in NRAMP1- mice (Figures 2.4, 2.7). Fecal colonization of *S.* Typhimurium was similar between the treatment groups (Figure 2.8), as well as *S.* Typhimurium burden in Peyer's patches, mesenteric lymph nodes, spleen, and liver (Figure 2.9). These data indicate that neutrophils do not significantly contribute to control of *S.* Typhimurium infection at 72h post-infection in NRAMP1+ mice. Although the number of intestinal neutrophils is statistically lower after anti-Ly6G treatment, there is no evidence to support a biological significance to the decrease in neutrophils at this time point. Peak neutrophil recruitment in NRAMP1+ mice is likely delayed in comparison to NRAMP1- mice, and prolonged antibody treatment would be required to determine if neutrophil depletion affects *S.* Typhimurium burden in NRAMP1+ mice.

Figure 2.7

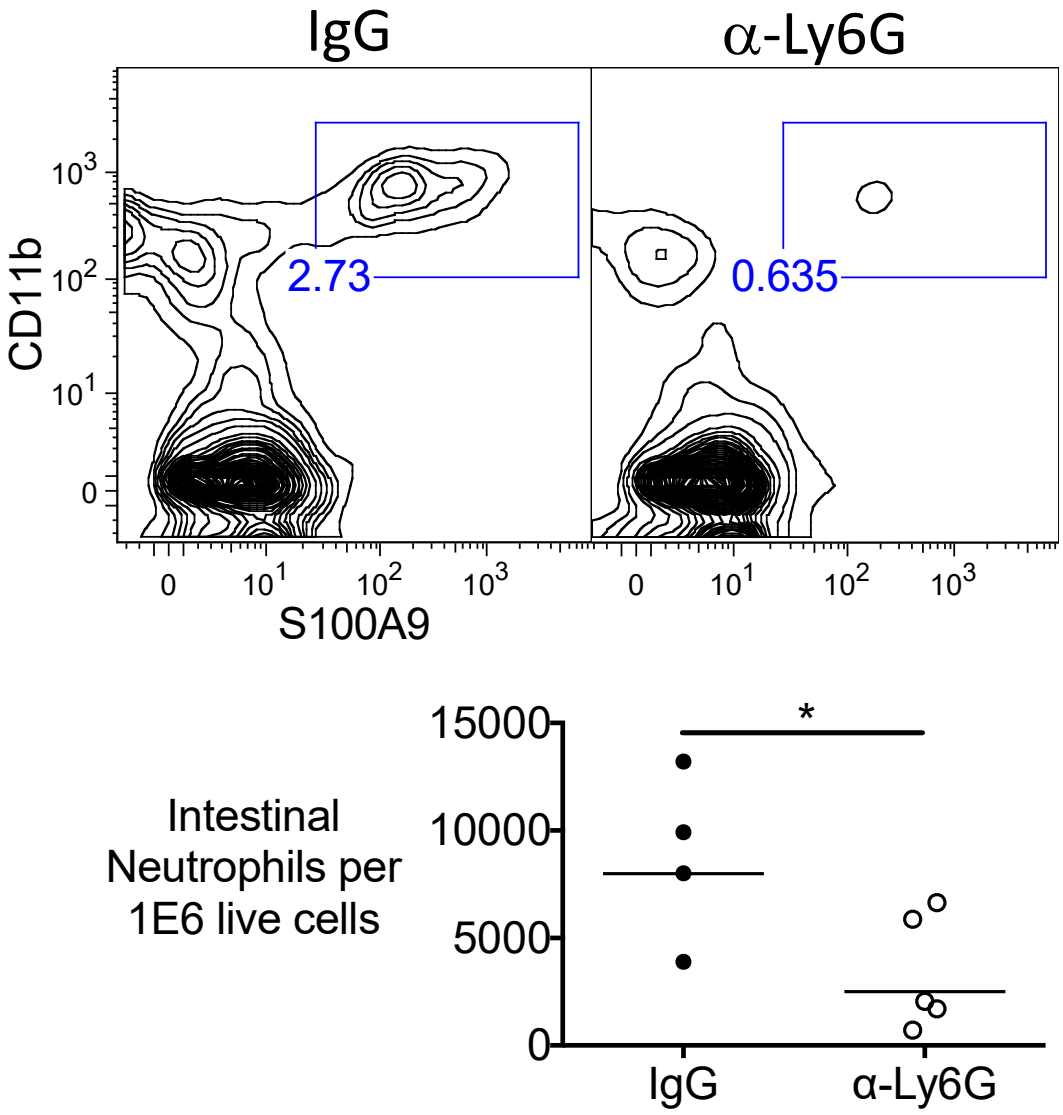

Figure 2.7 Intestinal neutrophils in *Slc11a1 G169* (NRAMP1+) mice treated with IgG or anti-Ly6G antibody 72h post-infection with *S.* Typhimurium

Figure 2.8

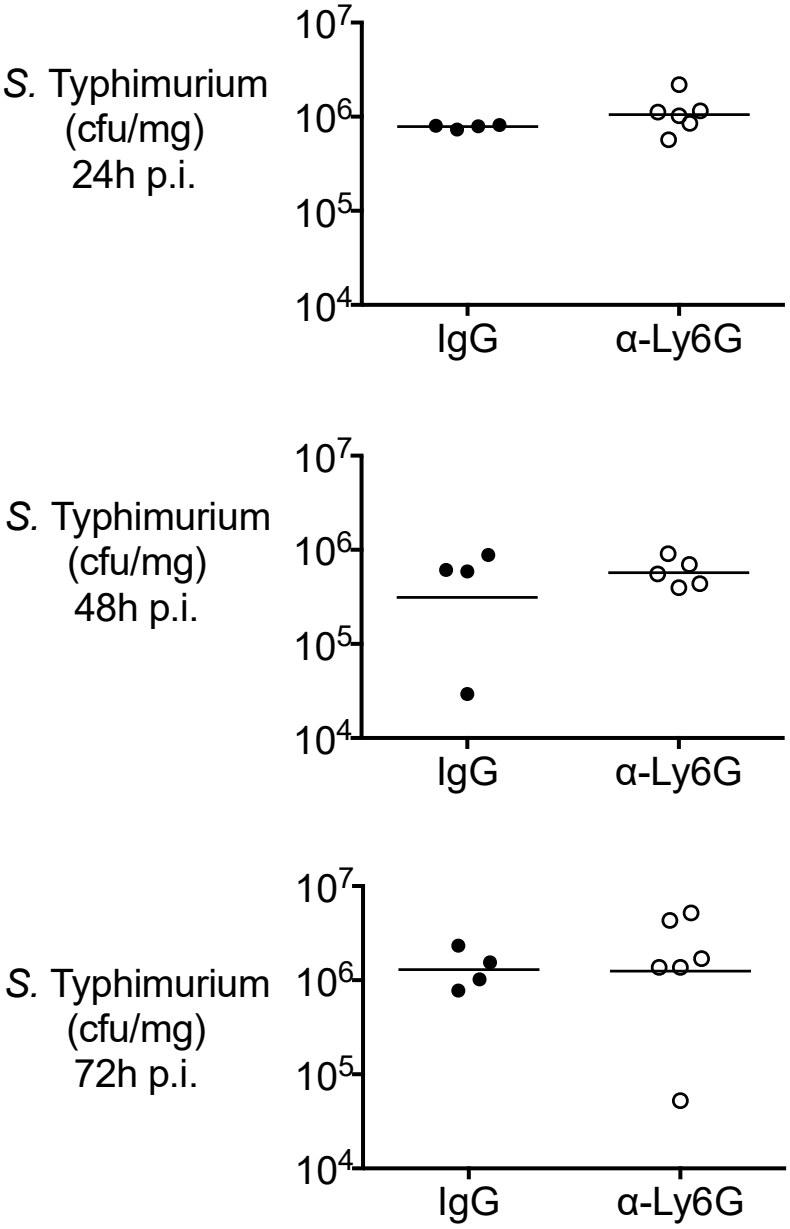

Figure 2.8 Fecal *S.* Typhimurium at 24, 48, and 72 hours post-infection in *Slc11a1 G169* (NRAMP1+) mice treated with IgG or anti-Ly6G antibody

Figure 2.9

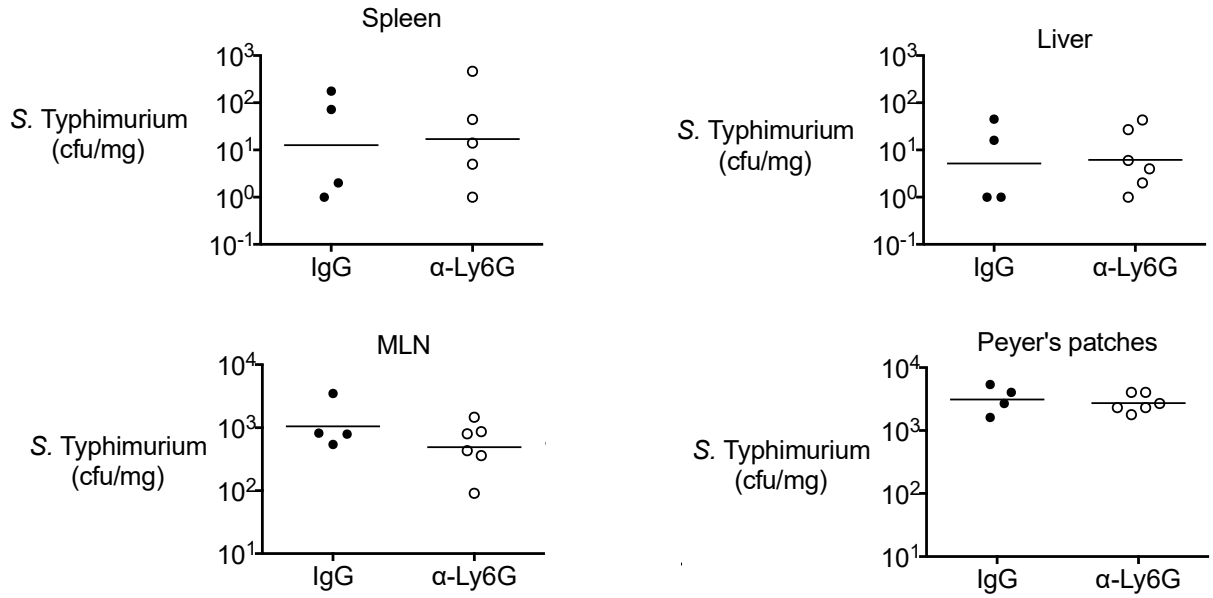

Figure 2.9 *S.* Typhimurium burden in tissues of *Slc11a1 G169* (NRAMP1+) mice treated with IgG or anti-Ly6G antibody, 72h post-infection

Antibody based cell depletion is a quick and increasingly affordable method to identify the roles of cell types in a complex system. Confirming the cell depletion using antibody-based cell analysis (flow cytometry) can be challenging, because the molecular target of the fluorescently-labeled antibody should be different than that of the depleting antibody, but still specific for the cell type of interest. Neutrophils are typically identified by the co-expression of CD11b and Ly6G. The RB6-8C5 Gr-1 antibody that binds both Ly6C and Ly6G has largely been replaced by the 1A8 clone specific for Ly6G, making it easier to differentiate neutrophils from inflammatory monocytes. This specificity, however, presents the challenge of identifying neutrophils after depletion with the anti-Ly6G 1A8 antibody, as all the Ly6G epitopes that may be present are bound by unconjugated antibody. CD11b is widely expressed by immune cells, so a second marker is necessary to identify neutrophils. In the absence of a better alternative, researchers have used the less-specific anti-Gr-1 antibody, but this, too, would be blocked from Ly6G epitope sites bound by the depleting antibody in addition to detecting Ly6C-expressing cells.

Through our studies of the neutrophil protein calprotectin (166, 167), we have found that high expression of the S100A9 subunit in CD11b+ cells is a specific marker for neutrophils. As an extremely abundant intracellular neutrophil protein, calprotectin is also easily detectable with a dim fluorophore. To demonstrate the effect of the antibody used for detection of neutrophils on data analysis for mice treated with anti-Ly6G antibodies, we used the following panel: CD11b, Ly6G, and S100A9. We analyzed for the presence of neutrophils using either CD11b+ and Ly6G+ or CD11b+ and S100A9+. We observed that the depletion appears falsely more significant when the masked Ly6G

epitope is used for detection of neutrophils in mice treated with anti-Ly6G antibody (Figure 2.10). A larger error would be made with data from bone marrow, where depleting antibodies are bound to the epitope, but the antibody-mediated cell destruction is not active (Figure 2.11). Lastly, to show that S100A9 expression is the differentiating factor between Gr-1+ cells and Ly6G+, two aliquots from the same mouse intestinal samples were stained with CD11b, S100A9, and either Gr-1 or Ly6G. Neutrophils defined as CD11b+ Gr-1+ contain both S100A9- and S100A9+ cells, but when neutrophils are defined using CD11b+ Ly6G+, only the S100A9+ cells remain (Figure 2.12). We propose, therefore, that CD11b+ S100A9+ staining is more specific for identifying neutrophils after anti-Ly6G antibody treatment.

Figure 2.10

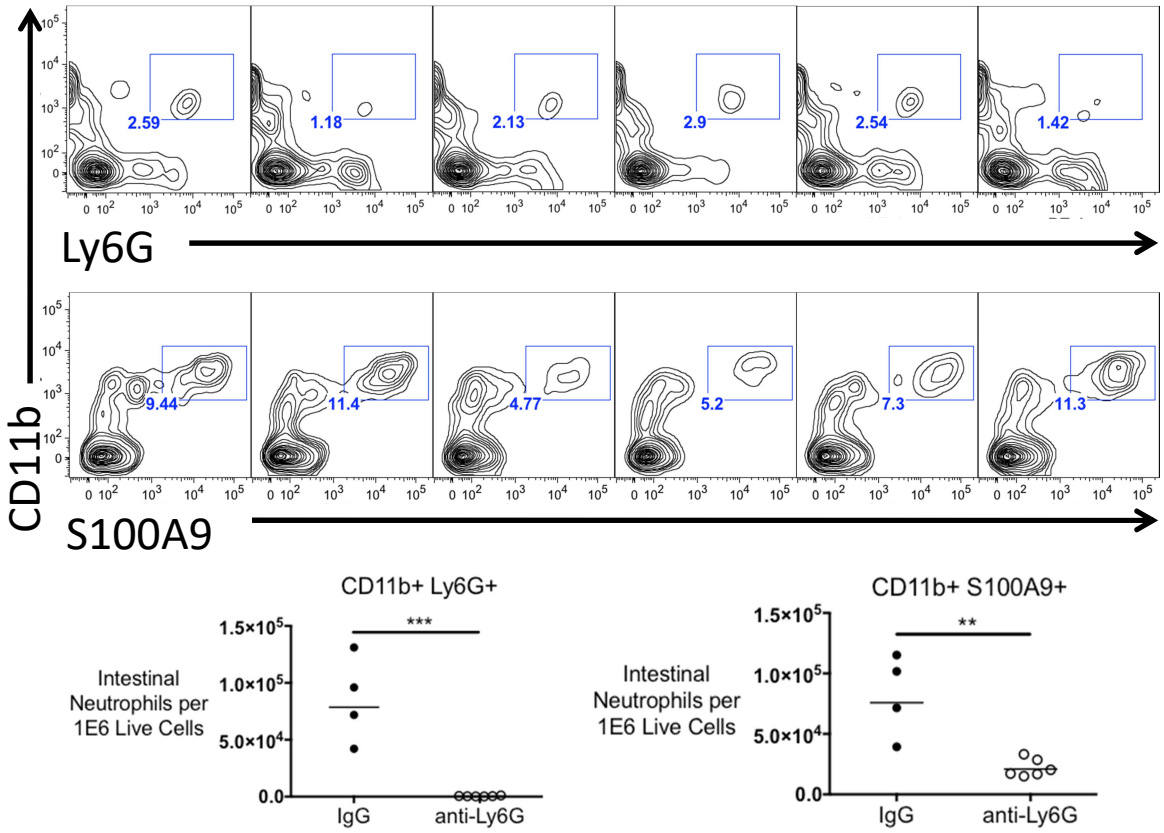

Figure 2.10 Intestinal neutrophils as detected by CD11b+ Ly6G+ or CD11b+ S100A9+ flow staining in the same samples from six mice treated with anti-Ly6G antibody. Flow plots from IgG treated mice not shown, but quantified in the graphs below.

Figure 2.11

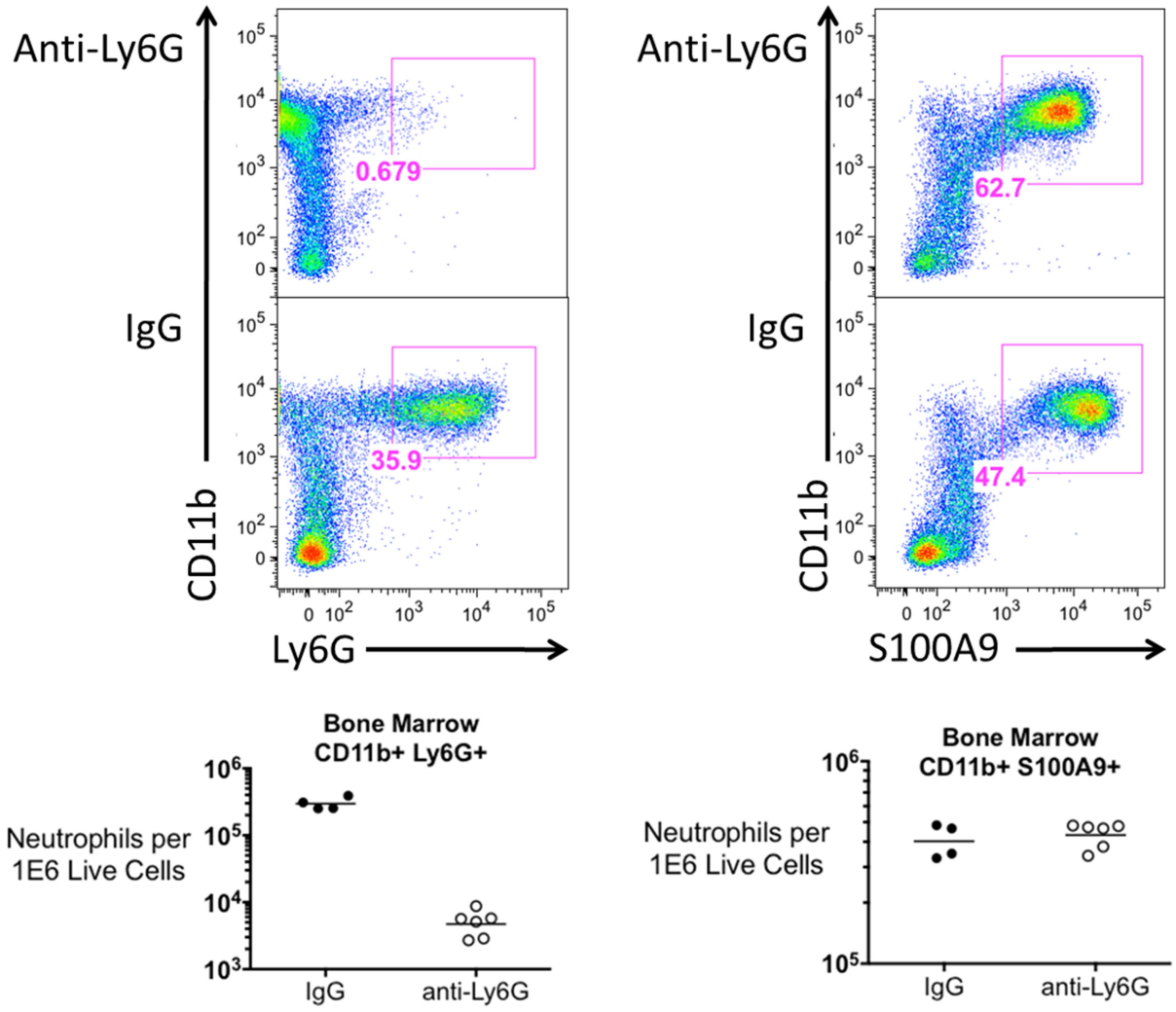

Figure 2.11 Representative flow plots of bone marrow neutrophils as detected by CD11b+ Ly6G+ or CD11b+ S100A9+ flow staining from IgG or anti-Ly6G treated mice. The data on the left is artifactual, as the Ly6G epitope is shielded by the depleting antibody.

Figure 2.12

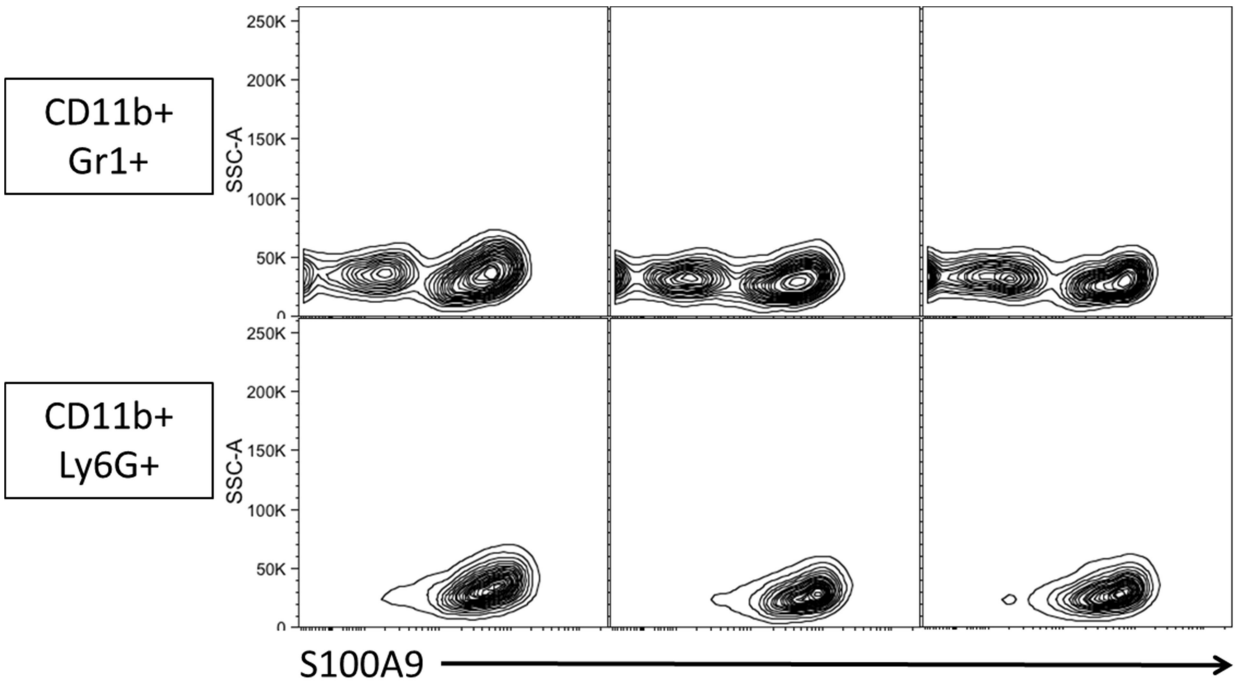

Figure 2.12 Top: Intestinal neutrophils as identified by and gated on CD11b+ Gr1+ have populations positive and negative for S100A9 expression. Bottom: the same three samples, with neutrophils identified by and gated on CD11b+ Ly6G+ have only S100A9+ cells, showing that S100A9 is a better surrogate marker for Ly6G+ neutrophils than Gr1.

In summary, neutrophil depletion with anti-CXCR2 or anti-Ly6G antibody leads to higher *S.* Typhimurium burden in mesenteric lymph nodes and spleen of C57BL/6 (*Slc11a1* G169D) mice at 72 hours post-infection, supporting a protective role of intestinal neutrophils in preventing *S.* Typhimurium spread from the intestine. Neutrophil infiltration of the intestine was also reduced by anti-Ly6G treatment of NRAMP1+ C57BL/6 mice, but did not yield a significant increase in *S.* Typhimurium levels in fecal samples or tissue. Extra-intestinal neutrophils may also be important for killing *S.* Typhimurium at the sites of dissemination, and future studies using migration-deficient neutrophils could begin to address their role. *S.* Typhimurium burden in Peyer's patches was not affected by anti-Ly6G neutrophil depletion, but was significantly higher with anti-CXCR2 neutrophil depletion, suggesting that another CXCR2-expressing cell type is contributing to host defense against *S.* Typhimurium in Peyer's patches. Antibody-mediated cell depletion is a great tool to generate short-term, targeted immunodeficiency, but specific, non-overlapping epitopes must be used to detect the cell type of interest after depletion. Our study indicates that intracellular S100A9 is a superior marker to Gr-1 after anti-Ly6G antibody depletion.

Chapter 3: CXCR2-dependent leukocyte migration to intestinal mucosa confers protection against infection with *Salmonella* Typhimurium

3.1 Abstract

Immune cells reside in or migrate through intestinal tissue as part of homeostatic surveillance. In response to infection, resident epithelial and immune cells release chemotactic cytokines to recruit additional leukocytes to the intestinal mucosa. *Cxcl2* is the most upregulated transcript in mouse cecal tissue during *Salmonella*-induced colitis; however, the contribution of this chemokine to the mucosal response during *Salmonella* infection is not completely understood. Here we show for the first time that *Cxcr2*$^{-/-}$ mice exhibit fewer B cells in Peyer's patches, as well as nearly 100-fold higher *Salmonella* burden. Furthermore, we demonstrate that B cells migrate in response to CXCR2 chemokines, and that the role of Peyer's patch B cells in protection against *Salmonella* is independent of intestinal IgA. Collectively, our data support a role for CXCR2 in directing B cell migration and a role for Peyer's patch B cells in IgA-independent defense against *Salmonella* intestinal colonization. In agreement with previous studies, we also found that neutrophil recruitment to the intestinal mucosa was greatly impaired in mice lacking CXCR2. Moreover, we found a corresponding increase in *Salmonella* dissemination to the mesenteric lymph nodes (MLN) and the liver, consistent with a role of CXCR2 in enhancing the mucosal barrier during *Salmonella* infection. To better understand the specific contribution of neutrophil recruitment in protection against *Salmonella,* we generated mice selectively lacking *Cxcr2* in granulocytes, and they exhibited similar defects in neutrophil migration and *Salmonella* burden in MLN and liver. However, *Salmonella* colonization and B cell numbers in Peyer's patches no

longer differed from controls. Altogether, this work expanded our understanding of CXCR2-mediated neutrophil recruitment in influencing outcomes of *Salmonella* infection and uncovered an important role for CXCR2 in B cell homing to Peyer's patches and in host defense against *Salmonella* intestinal infection.

3.2 Introduction

The intestinal mucosa contains an immune surveillance network that is responsible for maintaining the barrier between trillions of intestinal microbes and host tissues. Immune cell migration into the intestinal mucosa during development and homeostasis is guided by cytokines produced by stromal cells. In the event of a microbial breach, intestinal epithelial cells and resident immune cells produce chemokines to induce migration of circulating leukocytes into the mucosa and pro-inflammatory cytokines to activate lymphocytes (250). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), the leading cause of bacterial gastroenteritis, invades intestinal tissue and induces a cytokine signaling cascade through the IL-23 Th17 axis that culminates in massive production of CXCL1 and CXCL2 by epithelial cells (149). These chemokines recruit cells expressing the receptor CXCR2, which is most highly expressed on neutrophils (ImmGen.org).

CXCR2 is a G-protein coupled receptor that binds multiple ligands (CXCL1, 2, 5, and 15 in mice), has been shown to affect migration of neutrophils and oligodendrocytes (251-253), and has been detected on endothelial cells and subsets of NK cells (254, 255). Upon certain inflammatory stimuli, such as TLR4 activation, CXCR2 expression on circulating blood neutrophils decreases (256), and reduction in CXCR2 expression

levels correlates to reduced neutrophil migration to sites of inflammation (257). Neutrophil migration into the intestine is important to prevent bacteremia and dissemination during *S.* Typhimurium infection, as evidenced by patients with neutrophil immunodeficiency, who are more likely to be infected with *S.* Typhimurium and have higher rates of complicated illness (238, 239, 258-260). However, the mechanisms by which neutrophils control *S.* Typhimurium infections and the consequences of CXCR2 expression on outcomes *S.* Typhimurium infection are not completely understood. Therefore, we hypothesized that CXCR2 on neutrophils is important for directing neutrophils to the intestinal mucosa during *S.* Typhimurium infection, and that these neutrophils would be important for reducing systemic spread of *S.* Typhimurium.

S. Typhimurium infection in mice lacking CXCR2 has been partially characterized. Marchelletta et al. observed fewer neutrophils in the mouse cecum and corresponding higher *S.* Typhimurium cecal colonization and dissemination to the MLN in *Cxcr2*$^{-/-}$ mice (199). The transmigration of neutrophils across the intestinal epithelium has been shown to be a destructive process that contributes to protein loss and development of diarrhea (12), but even without neutrophil migration in *Cxcr2*$^{-/-}$ mice, fecal water increased through epithelial ion transporters whose expression is independent of CXCR2 and neutrophil influx (199).

To build on this work and better understand the role of CXCR2 in the protective immune response to *S.* Typhimurium gastroenteritis, we used the streptomycin pretreated *S.* Typhimurium gastroenteritis model and compared CXCR2-deficient mice to their wild-type littermates. We found that neutrophil recruitment via CXCR2 was critical for host defense against *S.* Typhimurium in mesenteric lymph nodes and liver.

But importantly, the role for CXCR2 in host defense extended beyond neutrophil migration. Specifically, we uncovered a previously undiscovered role for CXCL1-CXCR2 signaling in mediating B cell recruitment to Peyer's patches, as well as a role for B cell defenses against *S.* Typhimurium in Peyer's patches that is independent of IgA production.

3.3 Materials & Methods

Animal experiments: All procedures were approved by the University of California, Irvine Institutional Animal Care and Use Committee. C57BL/6 mice harboring a deletion in *Cxcr2* were obtained from Dr. Tom E. Lane and Dr. Richard Ransohoff and crossed with C57BL/6 mice modified with the *Slc11a1* allele from 129S1 strain (courtesy of Dr. Greg Barton). C57BL/6 *LoxP-Cxcr2* mice were generously provided by Dr. Richard Ransohoff and crossed with C57BL/6 *LysM-Cre* mice (The Jackson Laboratory) to generate *Cxcr2*$^{fl/fl}$ *LysM-Cre*+ and *Cxcr2*$^{fl/fl}$ *LysM-Cre*- control mice. *Cxcr2*$^{fl/fl}$ *LysM-Cre*+ females were subsequently bred with a *Cxcr2*$^{-/-}$ male to generate *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice and *Cxcr2*$^{fl/-}$ *LysM-Cre*- control mice. Mice lacking B cells, *muMT*, were purchased from The Jackson Laboratory. C57BL/6 mice treated with anti-CXCR2 antibody were purchased from Taconic Farms / Envigo.

Breeding *Cxcr2*$^{-/-}$ mice in sufficient numbers to complete the experiments described herein presented numerous challenges. *Cxcr2*$^{-/-}$ female mice are not fertile under normal animal husbandry conditions. Breeding *Cxcr2*$^{-/-}$ males and females with delivery of live pups has been achieved with very limited success when the mice are fed a high-fat diet (observation from the Lane laboratory, not published). As *Salmonella* infection is

sensitive to the microbiota, and the microbiota is altered from a high-fat diet, we chose to breed heterozygous (*Cxcr2*$^{+/-}$) males and females on normal chow to generate *Cxcr2*$^{-/-}$ mice and their *Cxcr2*$^{+/+}$ littermates. *Cxcr2*$^{-/-}$ mice were expected to represent 25% of the pups born, but our observed rate of birth and survival to genotyping at 3 weeks of age was 13%. Of these mice, another 40% died before achieving a size that enabled us to include them in experiments (minimum weight of 16g for infection). To increase the *Cxcr2*$^{-/-}$ mice generated, we bred *Cxcr2*$^{+/-}$ females with *Cxcr2*$^{-/-}$ males. From these breeding pairs, *Cxcr2*$^{-/-}$ mice represented 46% of the pups alive at weaning. Unfortunately, 40% of these mice also died without growing sufficiently to be included in an experiment. The *Cxcr2*$^{+/+}$ control mice were bred from littermates of the parental heterozygous mice.

B cell transplant: Two million B cells purified from Peyer's patches and peritoneal cavity were transferred to B cell deficient mice (muMT) in 400µl sterile PBS via intraperitoneal injection. Purity >90% of the injected cells was confirmed by flow cytometry. Mice were monitored daily for any sign of distress and sacrificed 10 days after transplant. Blood, peritoneal cells, spleen, and mesenteric lymph nodes were harvested for detection of B cells by flow cytometry. The ileum was collected, cut longitudinally, rolled, and formalin fixed for microscopic examination of Peyer's patches or lymphoid aggregates.

Salmonella infection: Prior to infection, streptomycin (Calbiochem) was administered via oral gavage at 1mg per gram mouse weight. A dose of 10^9 *Salmonella enterica* serovar Typhimurium strain IR715 (261) bearing a plasmid for streptomycin resistance was administered via oral gavage 24 hours after streptomycin treatment (14).

Intestinal colonization was monitored every 24 hours post-infection by quantifying *S.* Typhimurium present in fecal samples. Mice were sacrificed after 96 hours or after weight loss of nearly 20% of original body weight, which occurred at 7-9 days post-infection for mice with the *Slc11a1* allele from 129S1 strain. At sacrifice, *S.* Typhimurium levels were determined for Peyer's patches, terminal ileum, mesenteric lymph nodes, liver and spleen. All *S.* Typhimurium levels were normalized to colony-forming units (cfu) per mg of fecal sample or tissue.

Cell isolation & preparation: Blood was obtained post-CO_2 asphyxiation via cardiac puncture and immediately mixed with heparin to prevent clotting. Bone marrow cells were obtained from mouse femurs by flushing the bone with HBSS and dissociating cell clumps by drawing up and expelling them through a 19-gauge needle. Spleen, mesenteric lymph node, and Peyer's patch cells were isolated by grinding tissue pieces between frosted glass slides until a single-cell suspension was obtained. Intestinal cells were isolated from approximately one third of the cecum and the entire colon by first cutting the cylindrical tissue lengthwise and cleaning the tissue of fecal matter by vigorously shaking the tissue in wash buffer (HBSS, 15mM HEPES, 1% antibiotic/antimycotic [Ab/Am]). The intraepithelial layer was removed by gently shaking in EDTA solution (HBSS, 15mM HEPES, 10% FBS, 1% Ab/Am, 5mM EDTA) in a 37°C water bath for 15 minutes. Saving the EDTA solution on ice, the tissue was removed and cut into small pieces and transferred into a solution of IMDM with digestive enzymes (IMDM, 10% FBS, 1% Ab/Am, 20μg/mL liberase [Roche], 1mg/mL Collagenase and 250μg/mL DNase [Sigma]). Tissue pieces were disrupted using a gentleMACS tissue dissociator (program spleen_04, Miltenyi Biotech), incubated with

shaking at 37°C for 15 minutes, and again disrupted with the tissue dissociator. Cells were filtered, along with the intraepithelial cells, and collected by centrifugation at 800rcf for 10 minutes. Cells were suspended in IMDM media without digestive enzymes and counted prior to flow staining. Cells isolated for transplant were collected by peritoneal lavage with 5mL PBS containing 2% FBS and by dissection of Peyer's patches from the ileum of uninfected *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice. Single cells were obtained from Peyer's patch tissue by briefly grinding tissue pieces between ground glass slides. B cells were purified using negative selection (see below).

Flow cytometry: Tissues were analyzed by flow cytometry for the presence of neutrophils [CD11b, Ly6G (1A8 BioLegend)], T cells [CD3, CD4, CD8], B cells [CD19, B220], dendritic cells [CD11b, CD11c], macrophages [CD11b, F4/80], and expression of CXCR2 (R&D Systems) (antibodies from eBioscience except where indicated). Calprotectin expression was determined by using an S100A9 antibody conjugated in-house to the Pacific Blue fluorophore (R&D Systems, LifeTechnologies). All flow cytometry data was obtained using a BD LSR-II or ACEA Novocyte and analyzed using FlowJo (Treestar).

EasySep® B cell isolation technique: Negative selection by magnetic beads using the EasySep protocol (Stemcell Technologies), for isolating pan-murine B cells was performed as per the manufacturer's instructions on cells isolated from Peyer's patches.

Transwell Migration Assay: Migration of B cells toward CXCL1 or CXCL2 were tested using Transwell inserts (Corning Inc., 5.0-μm pore size). Purified B cells from Peyer's patches of 2 mice were combined and suspended in RPMI 1640 supplemented

with 0.5% BSA. B cells (3.0 x 10^5 in 100µl) were placed on top of the filter above each well. Media containing no chemokine, CXCL1 (KC), CXCL2 (MIP-2) (250 ng/ml each), or CXCL12α (SDF-1α) (50ng/ml) was added to the bottom chamber (600µl, all chemokines from PeproTech). Cells were allowed to migrate for 3 hours at 37°C and 5% CO_2. After incubation, the cells in the lower chambers were counted using a flow cytometer. The number of cells in the bottom chamber of the media-only control was subtracted from the number of migrated cells in each chemokine well. Migration toward CXCL1 or CXCL2 is expressed as a percentage of migration toward CXCL12α.

Fecal IgA measurement: Fecal samples were collected from uninfected mice or mice 4 days post-infection and dissociated by 30 minutes of vigorous shaking in 400µl PBS with protease inhibitor cocktail (cOmplete tablet mini, EDTA-free; Roche). Fecal solids were removed via centrifugation at 7200g for 20 minutes. Supernatant was collected and stored at -20°C until IgA was measured using the mouse IgA ELISA Ready-SET-Go (eBioscience). IgA levels were normalized per milligram of fecal sample.

Statistics: Statistical analysis was conducted using Prism (GraphPad). A two-tailed t-test was performed to compare immune cell numbers per million live cells (as determined by flow cytometry viability staining), the log of cfu *S.* Typhimurium per mg sample, and the log of the median fluorescence intensity between groups. * p value < 0.05, ** < 0.01, *** < 0.001. B cell number and *S.* Typhimurium levels in Peyer's patches were analyzed using Pearson's correlation and the data fit with a linear regression model.

3.4 Results

Mouse susceptibility to *S.* Typhimurium varies between mouse strains, and one major determinant of susceptibility is the functionality of the NRAMP1 transporter, encoded by the gene *Slc11a1*. C57BL/6 and BALB/c mice harbor a G169D mutation in *Slc11a1,* which renders the NRAMP1 protein non-functional, and mice with this mutation are more susceptible to *S.* Typhimurium infection (262). Knockout animals used to be frequently generated using SvJ/129 mice, which have a functional NRAMP1 transporter and thus are more resistant to infections with *S.* Typhimurium. *Cxcr2*$^{-/-}$ mice were generated in SvJ/129 mice (263), and despite many generations of crosses with C57BL/6 mice over the last 20 years, we and others found that the proximity of the *Cxcr2* and *Slc11a1* genes has led to the retention of a functional NRAMP1 transporter in *Cxcr2*$^{-/-}$ mice ((199) and data not shown).

Briefly, sequencing of our C57BL/6 *Cxcr2* colony confirmed that the SvJ/129 *Slc11a1* locus was intact in the *Cxcr2*$^{-/-}$ mice. Heterozygotes for *Cxcr2* were also heterozygous for *Slc11a1* G169, and the *Cxcr2*$^{+/+}$ littermates were homozygous for the *Slc11a1* G169D mutation, rendering them more susceptible to *S.* Typhimurium infection and therefore unsuitable as controls. To generate suitable controls, we crossed our *Cxcr2*$^{+/-}$ mice with C57BL/6 mice with the *Slc11a1* G169 restored (courtesy of Greg Barton, UC Berkeley). All the mice presented herein as *Cxcr2*$^{+/+}$ are C57BL/6 mice with a functional NRAMP1 transporter (*Slc11a1* G169).

To test the role of CXCR2 during *S.* Typhimurium infection, mice were orally infected with *S.* Typhimurium after treatment with streptomycin. On the second day of infection, *S.* Typhimurium fecal colonization was transiently lower in *Cxcr2*$^{+/+}$ mice than

Cxcr2$^{-/-}$ mice, but returned to similar levels for the remainder of the infection (Figures 3.1, 3.2). In the mouse tissues at 4 days post-infection, *S.* Typhimurium levels were significantly higher in Peyer's patches, terminal ileum, mesenteric lymph nodes (MLN), and liver of *Cxcr2*$^{-/-}$ mice compared to *Cxcr2*$^{+/+}$ mice (Figure 3.3). *S.* Typhimurium dissemination to the spleen was not significantly different between *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice (Figure 3.5), possibly because *Cxcr2*$^{-/-}$ mice have numerous neutrophils in the spleen as previously described (263). Notably, the colonization of Peyer's patches was nearly 100-fold higher in *Cxcr2*$^{-/-}$ mice, a much larger difference than the 10-fold increase observed in most other tissues. After 7-9 days of infection, *Cxcr2*$^{-/-}$ mice no longer showed significantly higher levels of *S.* Typhimurium in the terminal ileum, MLN, and the liver compared to *Cxcr2*$^{+/+}$ mice (Figure 3.4). Peyer's patch colonization, however, remained 100-fold higher in *Cxcr2*$^{-/-}$ mice (Figure 3.4).

Figure 3.1

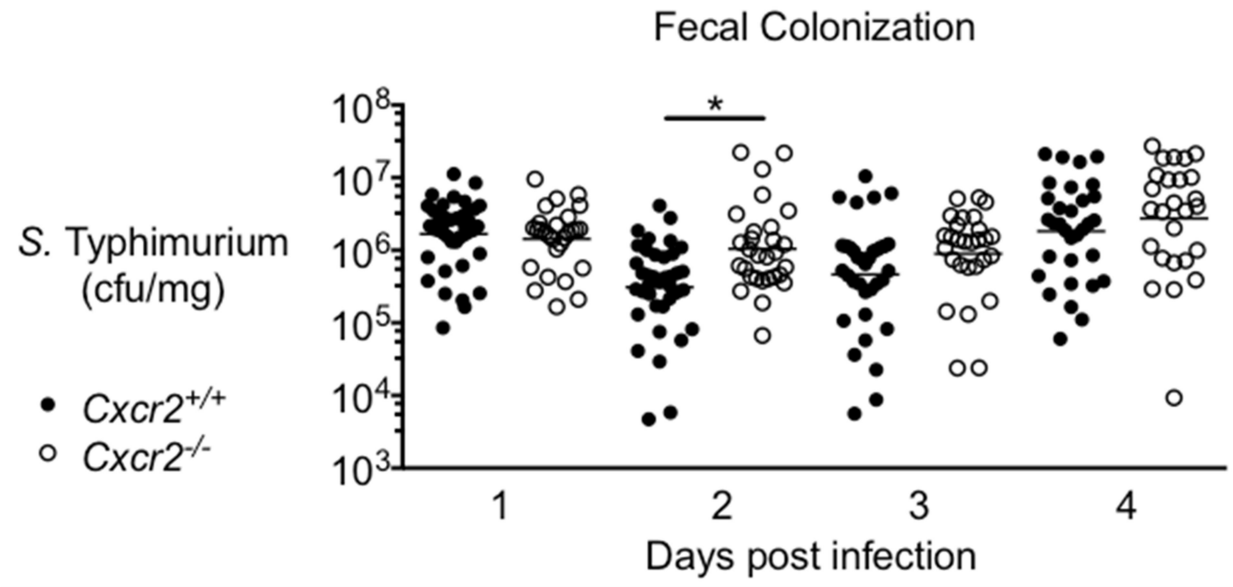

Figure 3.1 *Salmonella* Typhimurium fecal colonization of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice over 4 days of infection

Figure 3.2

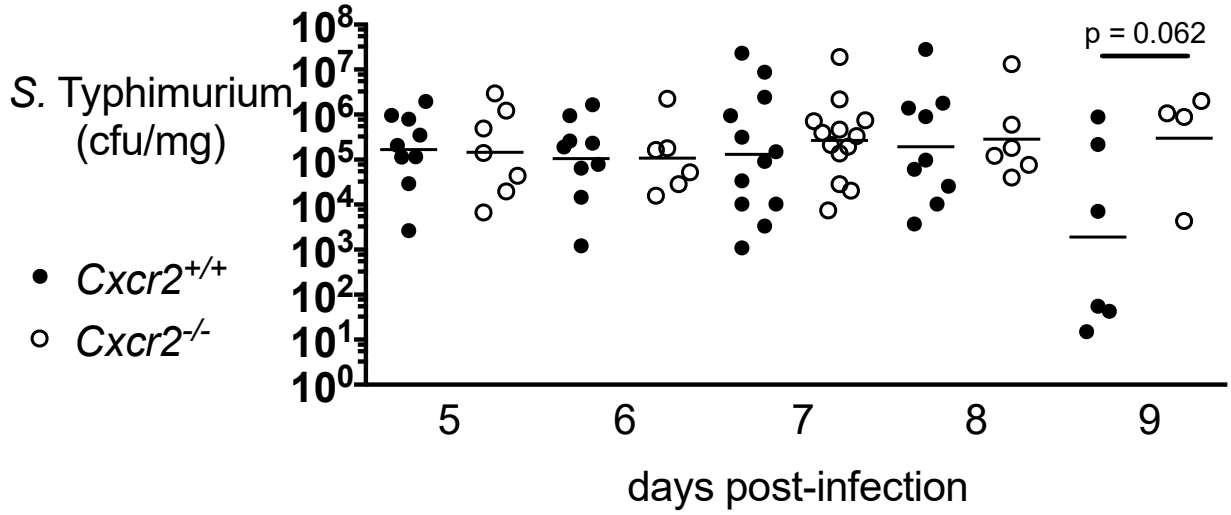

Figure 3.2 *Salmonella* Typhimurium fecal colonization of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice over days 5-9 post-infection

Figure 3.3

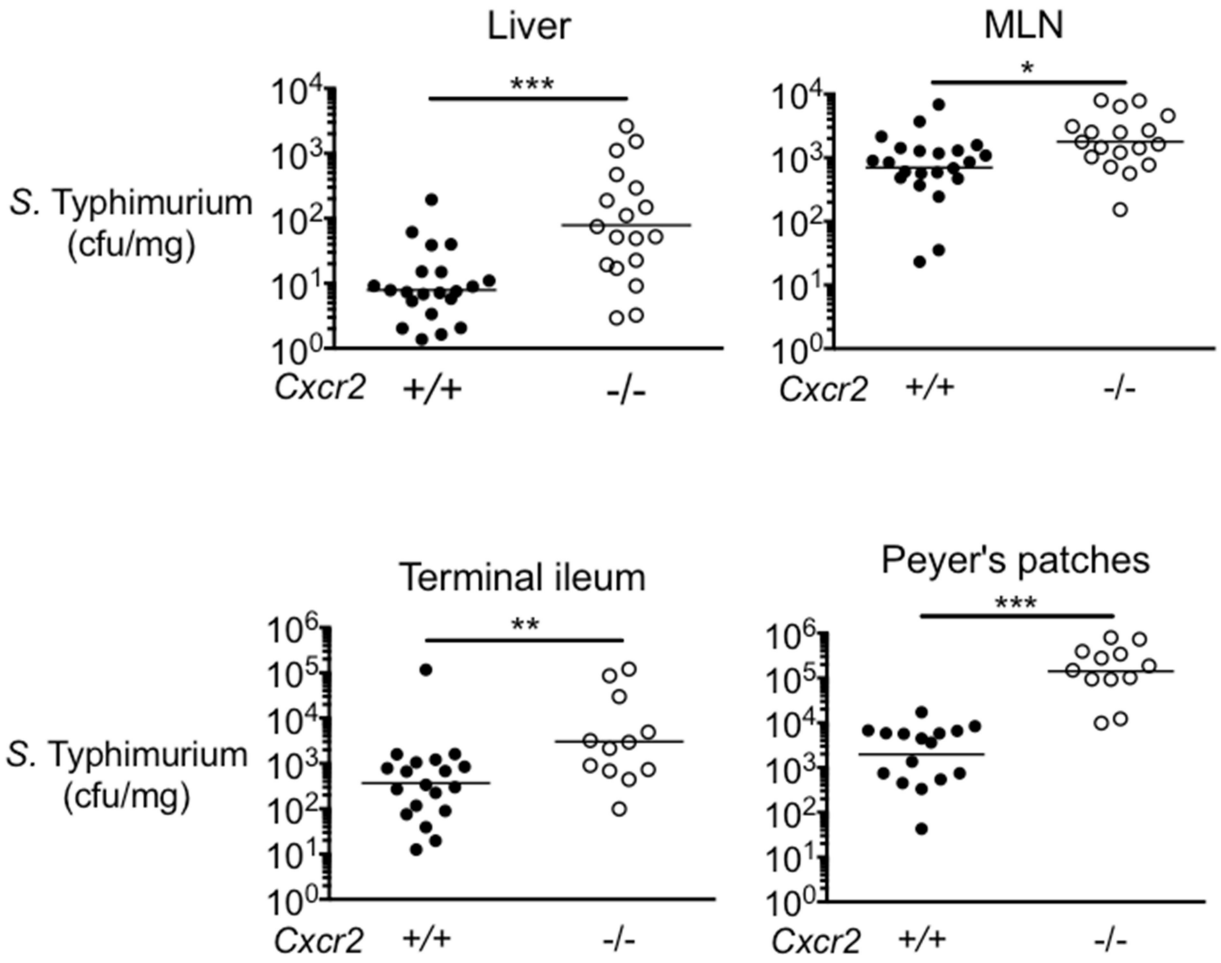

Figure 3.3 *Salmonella* Typhimurium tissue burden in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice at 4 days post-infection

To understand the mechanism of increased tissue colonization and dissemination in *Cxcr2*$^{-/-}$ mice, we quantified the immune cell infiltration in the cecum and colon at day 4 post-infection by physically and enzymatically dissociating intestinal tissue and analyzing the single-cell suspension using flow cytometry. Neutrophils in circulation highly express CXCR2, and several studies show defective neutrophil migration in *Cxcr2*$^{-/-}$ mice (257, 264-266), so we expected *Cxcr2*$^{-/-}$ mice to have fewer neutrophils in the large intestine during *S.* Typhimurium infection. Indeed, *Cxcr2*$^{-/-}$ mice exhibited significantly fewer neutrophils in the intestine compared to *Cxcr2*$^{+/+}$ controls at both 4 days and 7-9 days post-infection (Figure 3.6), supporting the conclusion that neutrophil recruitment to intestinal tissues during *S.* Typhimurium infection is at least partly dependent on CXCR2. A preliminary characterization of the other immune cells in the large intestine revealed fewer dendritic cells and B cells, and more T cells in *Cxcr2*$^{-/-}$ mice compared to *Cxcr2*$^{+/+}$ mice (Figure 3.7). Expression of CXCR2 on dendritic cells and B cells has not been reported in mice, so the mechanism behind the intestinal deficiency in these cell types is unclear, but this data suggests a role for CXCR2 in dendritic and B cell migration to the intestine, whether through direct expression or secondary signals. The increase in T cells may be a relative change in proportion of total cells secondary to the decrease in neutrophils, dendritic cells, and B cells, or may be from increased recruitment or expansion of T cells in *Cxcr2*$^{-/-}$ mice. These data suggest that a deficient immune response in the intestine of *Cxcr2*$^{-/-}$ mice contributes to increased tissue colonization and dissemination, but future studies will be essential to clarify any role for CXCR2 in B cell, dendritic cell, and T cell population of the intestine during *S.* Typhimurium infection. We hypothesize that the deficiency in neutrophil

recruitment in *Cxcr2*$^{-/-}$ mice was responsible for the increase in *S.* Typhimurium burden because neutrophils are the dominant immune cell recruited after *S.* Typhimurium infection, and they utilize several antimicrobial strategies to kill pathogens like *S.* Typhimurium.

Figure 3.4

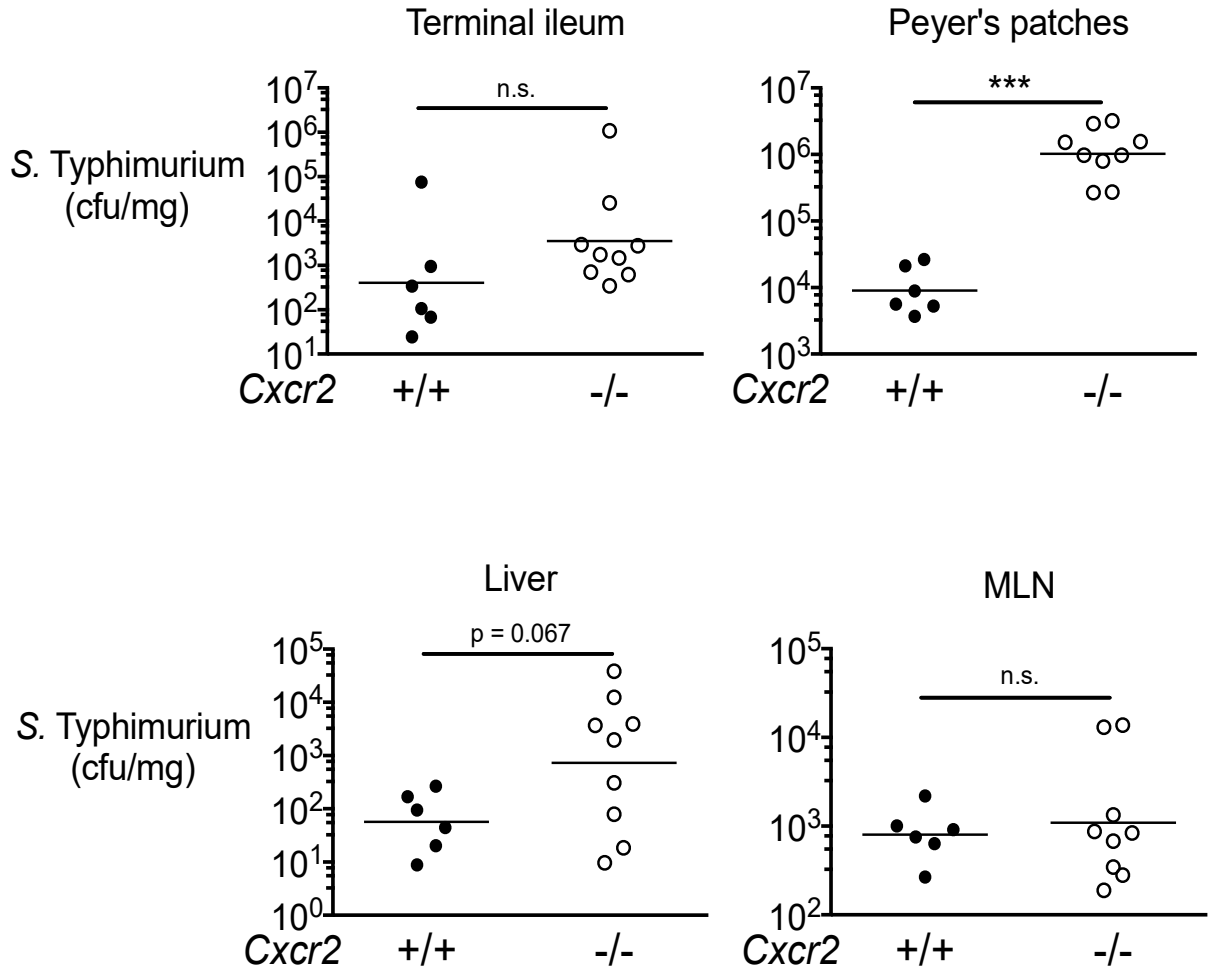

Figure 3.4 *Salmonella* Typhimurium tissue burden in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice at 7-9 days post-infection

Figure 3.5

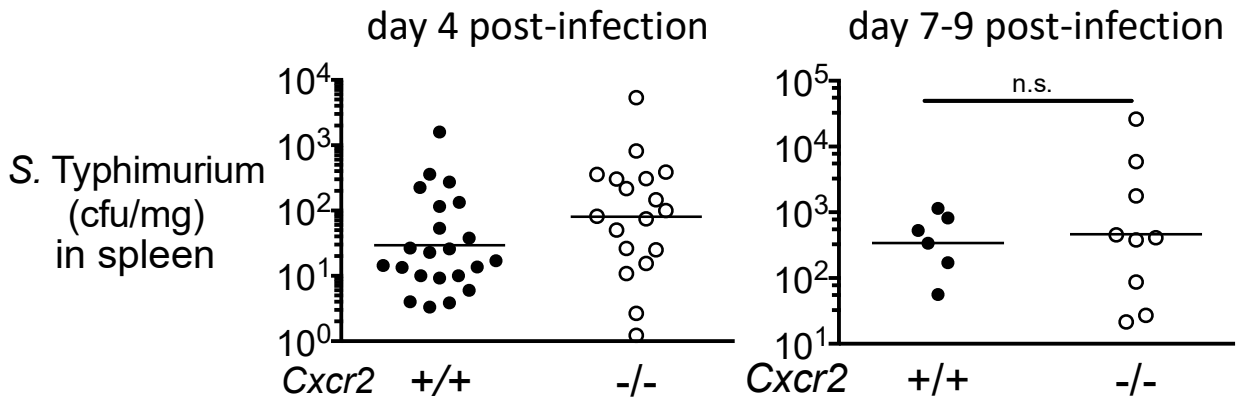

Figure 3.5 *Salmonella* Typhimurium dissemination to spleen in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice at 4 and 7-9 days post-infection

Figure 3.6

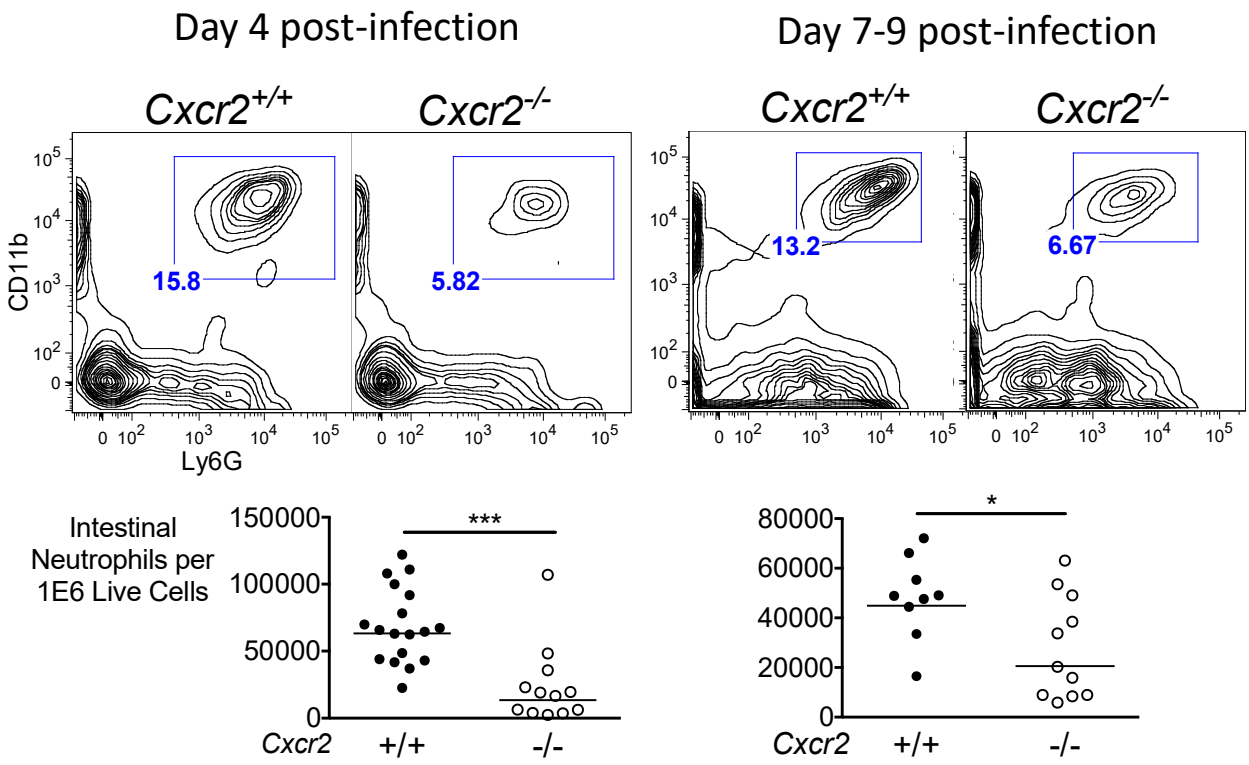

Figure 3.6 Neutrophils present in cecal and large intestinal tissue of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice after 4 or 7-9 days of *Salmonella* Typhimurium infection

Figure 3.7

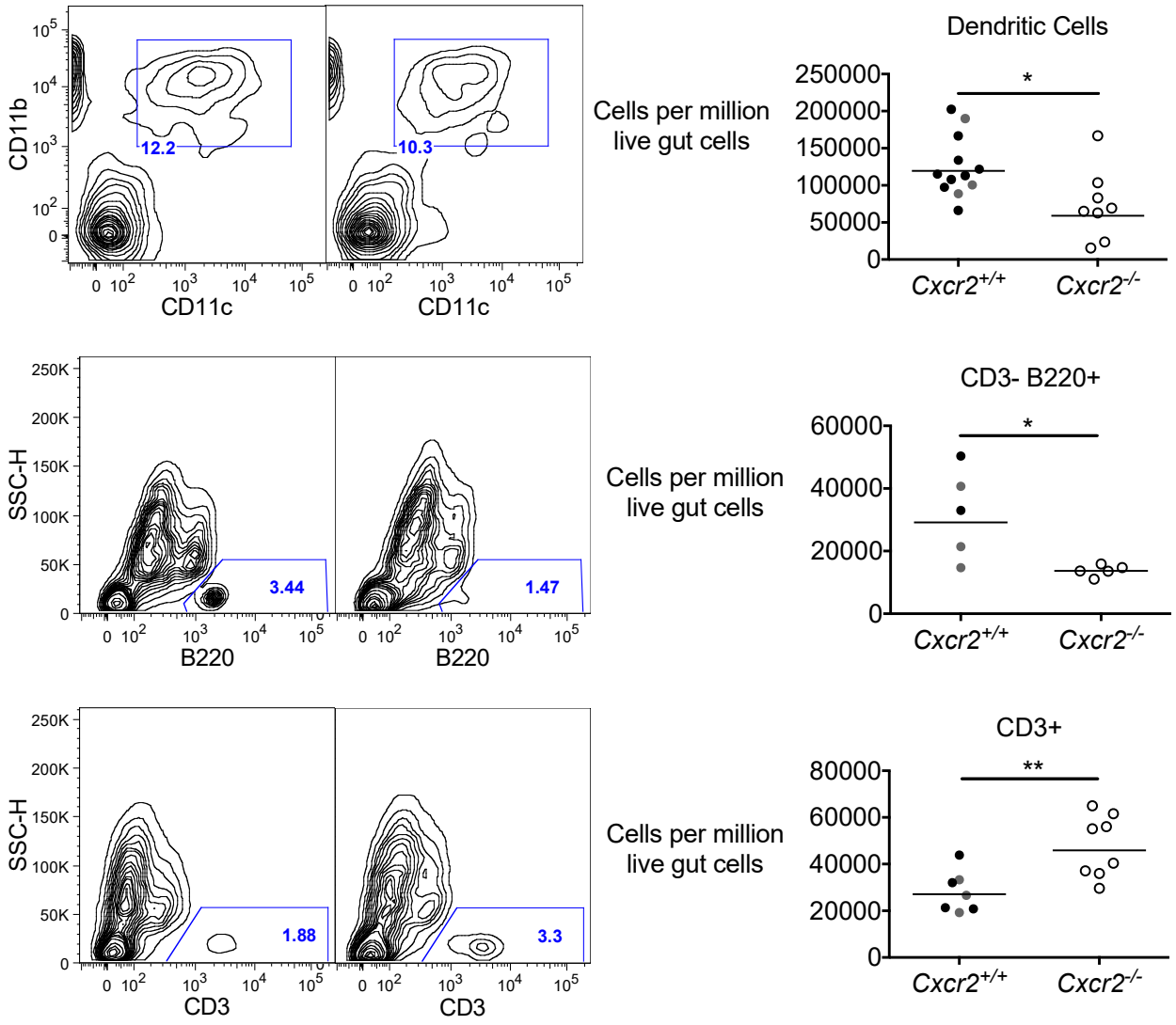

Figure 3.7 Dendritic cells, B cells, and T cells present in cecal and large intestinal tissue of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice after 4 days of *Salmonella* Typhimurium infection.

To specifically study the role of CXCR2 in myeloid lineage cells (granulocytes), we generated *Cxcr2*$^{fl/fl}$ *lysozyme M* (*LysM)-Cre* mice (Figure 3.8). If the phenotype observed in *Cxcr2*$^{-/-}$ mice is mostly from neutrophils, we expected deletion of *Cxcr2* from granulocytes (which include neutrophils) would decrease neutrophil migration to intestinal tissue, leading to increased dissemination of *S.* Typhimurium. Characterization of uninfected *Cxcr2*$^{fl/fl}$ mice revealed a reduction in neutrophil CXCR2 expression due to the LoxP sites flanking *Cxcr2,* and the presence of *LysM-Cre* did not result in a further decrease in neutrophil CXCR2 compared to *Cxcr2*$^{fl/fl}$ mice, as measured by flow cytometry (data not shown). Because lysozyme M expression may be induced during *S.* Typhimurium infection, we hypothesized that expression of the lysozyme-driven Cre recombinase may also be upregulated during *S.* Typhimurium infection, driving deletion of *Cxcr2*. To test this hypothesis, *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *LysM-Cre* mice were infected with *S.* Typhimurium, and neutrophil infiltration of the intestine and bacterial burden were quantified. Neutrophil expression of CXCR2 was measured for evidence of gene deletion. After 4 days of *S.* Typhimurium infection, we observed similar *S.* Typhimurium burden for *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *LysM-Cre* mice in fecal (Figure 3.9) and tissue samples (Figure 3.10). Neutrophil infiltration into the cecum and colon, measured by flow cytometry analysis of dissociated tissue, was equivalent between *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *LysM-Cre* mice, and the same degree of CXCR2 expression was present on bone marrow neutrophils from *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *LysM-Cre*+ mice (Figure 3.11, 3.12). As controls for CXCR2 expression, bone marrow from infected *Cxcr2*$^{+/+}$ and *Cxcr2*$^{fl/+}$ *LysM-Cre*+ mice was also analyzed.

Figure 3.8

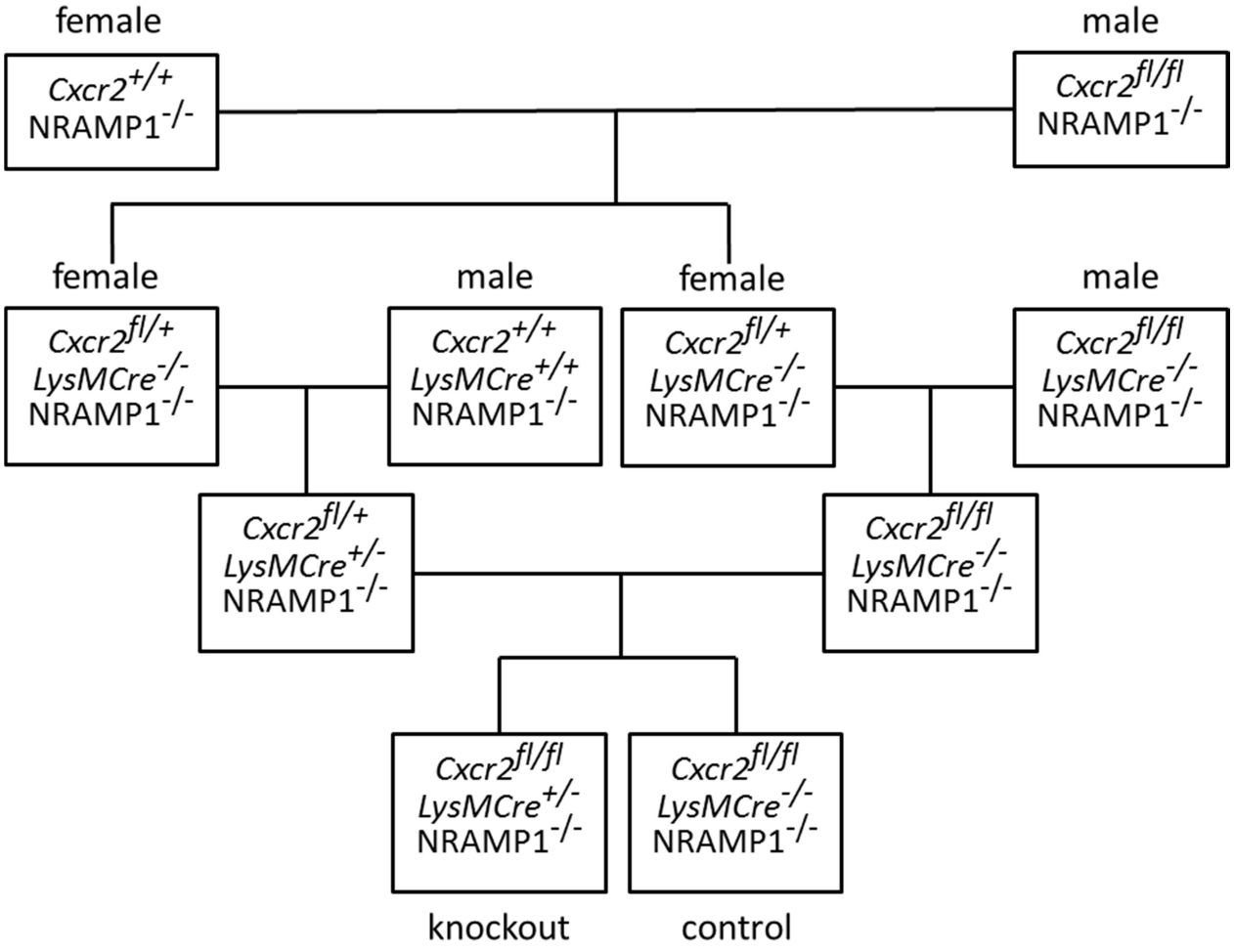

Figure 3.8 Diagram depicting the generation of littermate *Cxcr2*$^{fl/fl}$*-LysM-Cre*- and *Cxcr2*$^{fl/fl}$*-LysM-Cre*+ mice in our colony. NRAMP1$^{-/-}$ indicates a nonfunctional NRAMP1 transporter from *Slc11a1* allele with the G169D mutation that is present in C57BL/6 mice.

Figure 3.9

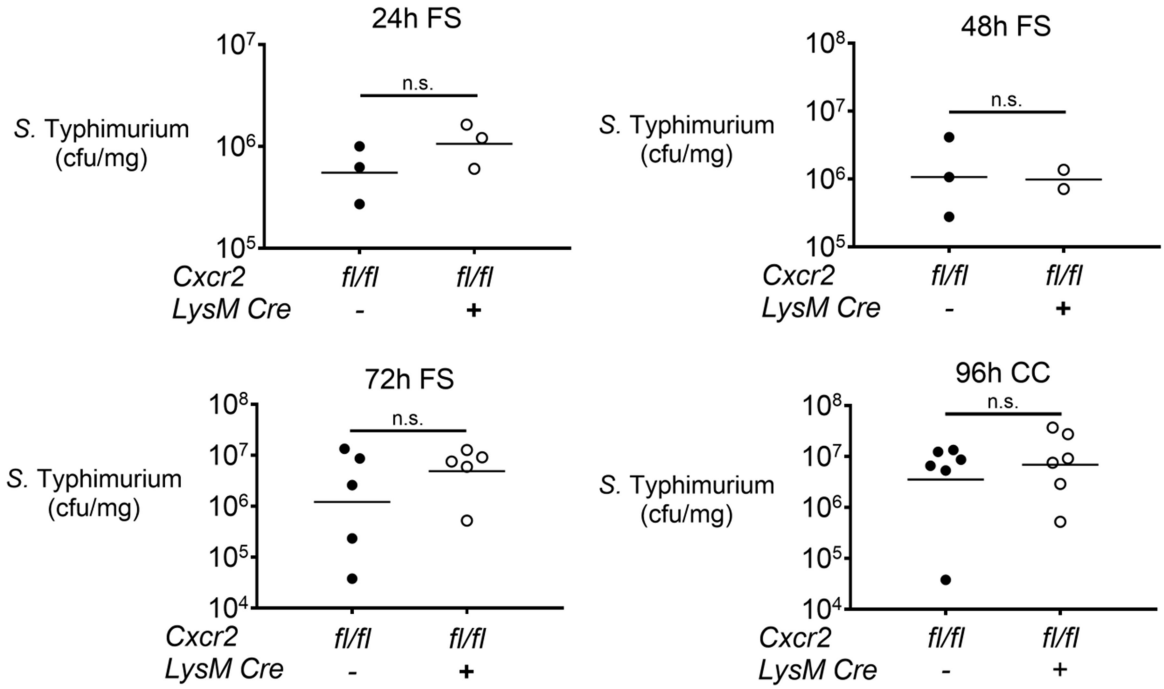

Figure 3.9 *Salmonella* Typhimurium fecal colonization of *Cxcr2*$^{fl/fl}$ *and Cxcr2*$^{fl/fl}$-*LysM-Cre*+ mice over 4 days of infection

Figure 3.10

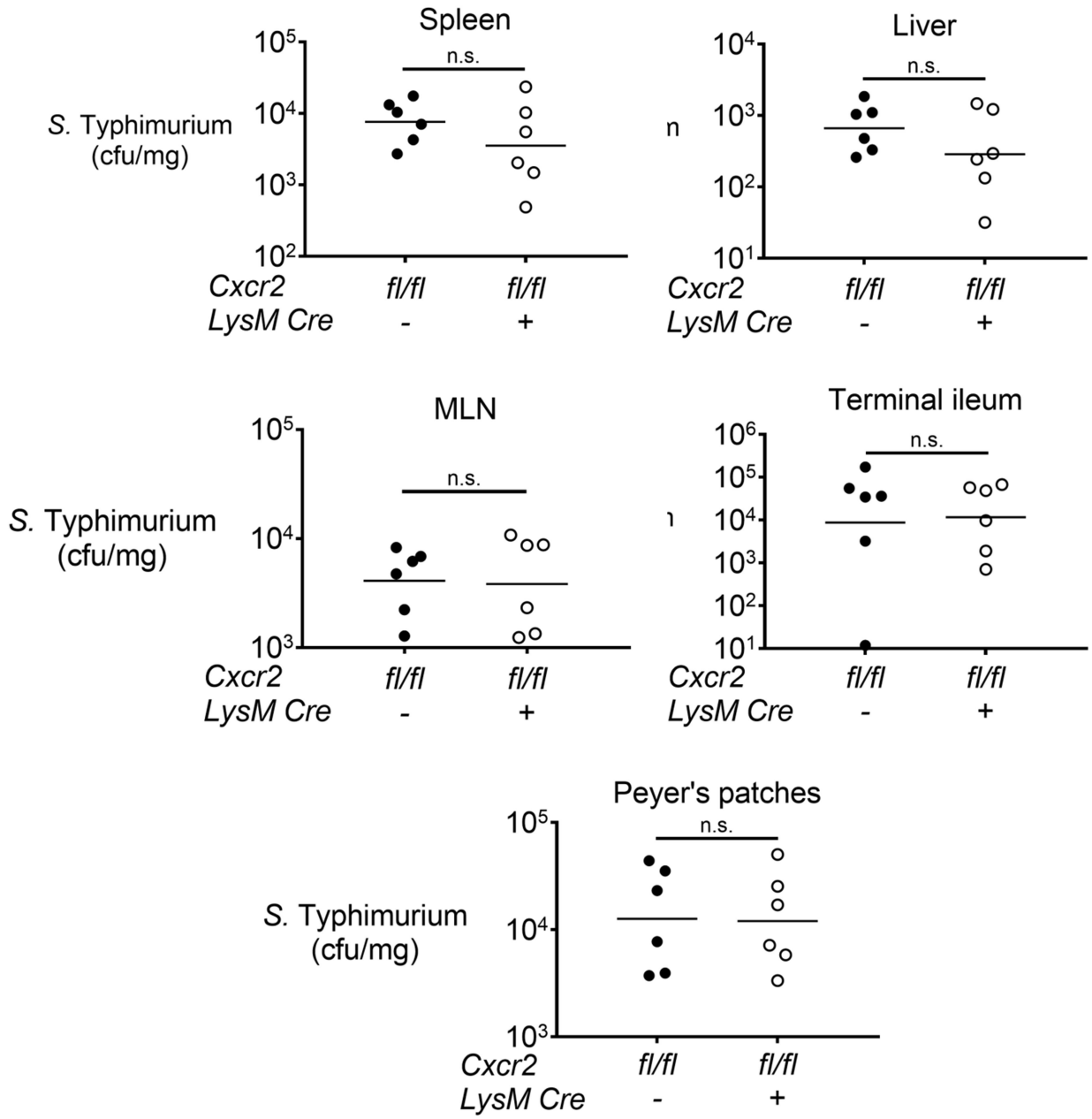

Figure 3.10 *Salmonella* Typhimurium tissue burden in *Cxcr2*$^{fl/fl}$ *lysozyme M* (*LysM)-Cre - or* + mice at 4 days post-infection

Figure 3.11

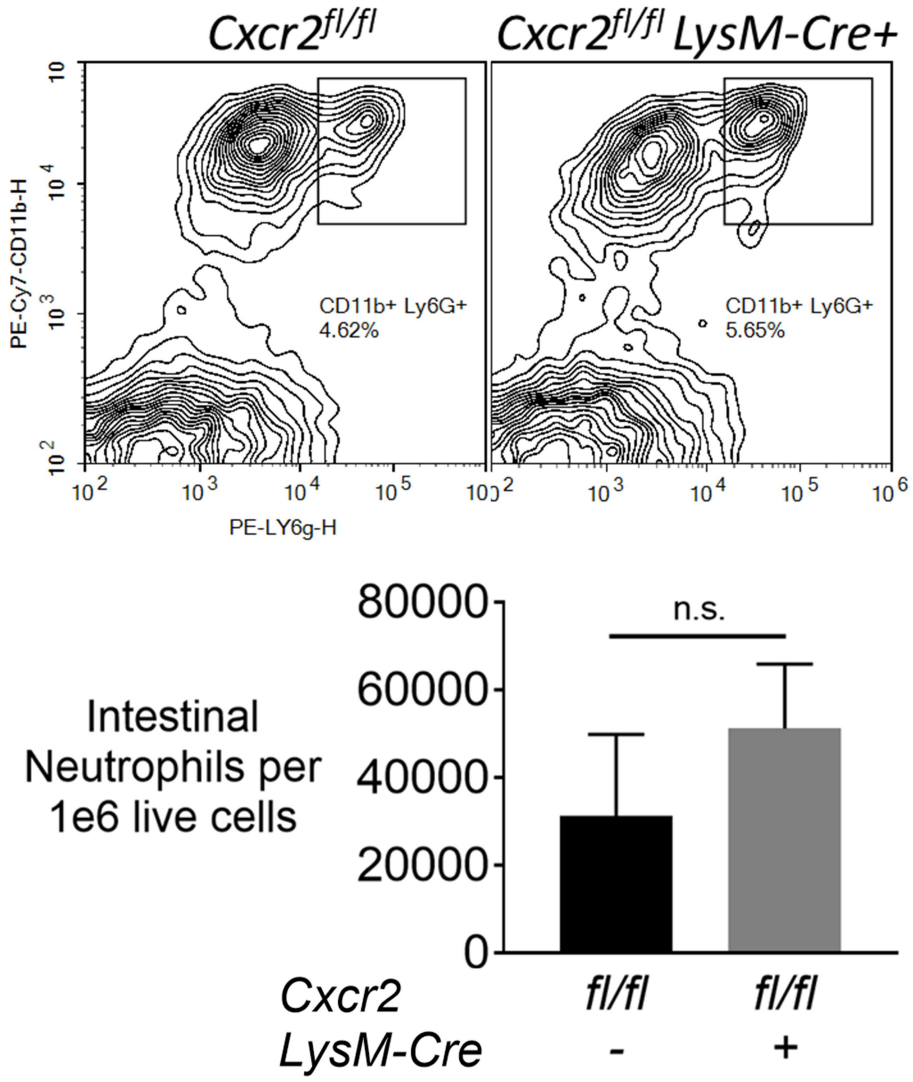

Figure 3.11 Neutrophils present in cecal and large intestinal tissue of *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$*-LysM-Cre*+ mice after 4 days of *Salmonella* Typhimurium infection

Figure 3.12

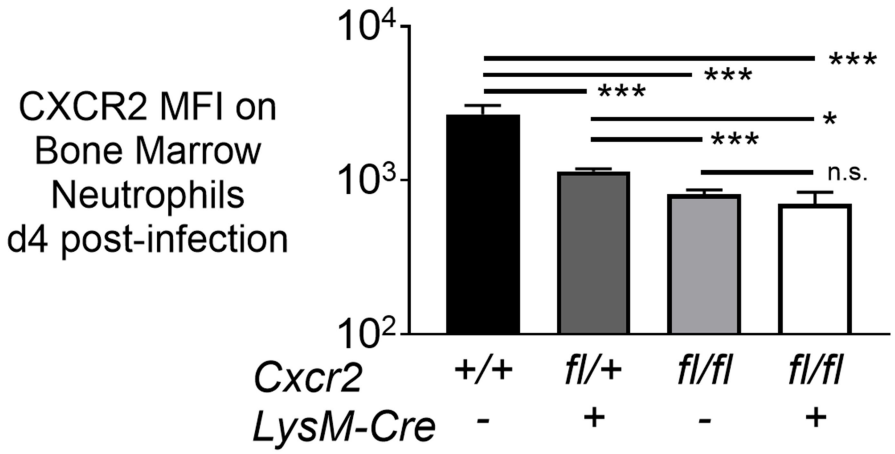

Figure 3.12 Relative CXCR2 expression on neutrophils from infected *Cxcr2*$^{+/+}$, *Cxcr2*$^{fl/+}$ *LysM-Cre*+, *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *LysM-Cre*+ mice. MFI, Median fluorescence intensity

Neutrophils have a short half-life, so they are continually generated in the bone marrow. The LysM-Cre recombinase appears to be insufficiently active to delete both copies of *Cxcr2* from this rapidly replaced population. Inefficient deletion using the LysM-Cre recombinase has been observed by another research group (267). To improve deletion of *Cxcr2* by *LysM-Cre*, we generated mice with one deleted and one floxed *Cxcr2* allele (*Cxcr2*$^{fl/-}$ *LysM-Cre*+ and *Cxcr2*$^{fl/-}$ control mice) (Figure 3.13). In contrast to *Cxcr2*$^{-/-}$ mice, which are undersized (Figure 3.14) and exhibit profound neutrophilia (263), *Cxcr2*$^{fl/-}$ *LysM-Cre* mice grow at a normal rate (Figure 3.15) and do not exhibit neutrophilia (Figure 3.16). Characterization of neutrophils from *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice showed CXCR2 similar to *Cxcr2*$^{-/-}$ mice, but only in 67% of mice genetically positive for LysM-Cre. *Cxcr2*$^{fl/-}$ mice also showed decreased CXCR2 expression on neutrophils compared to *Cxcr2*$^{+/+}$ mice, but more than *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice (Figures 3.12 and 3.17).

Figure 3.13

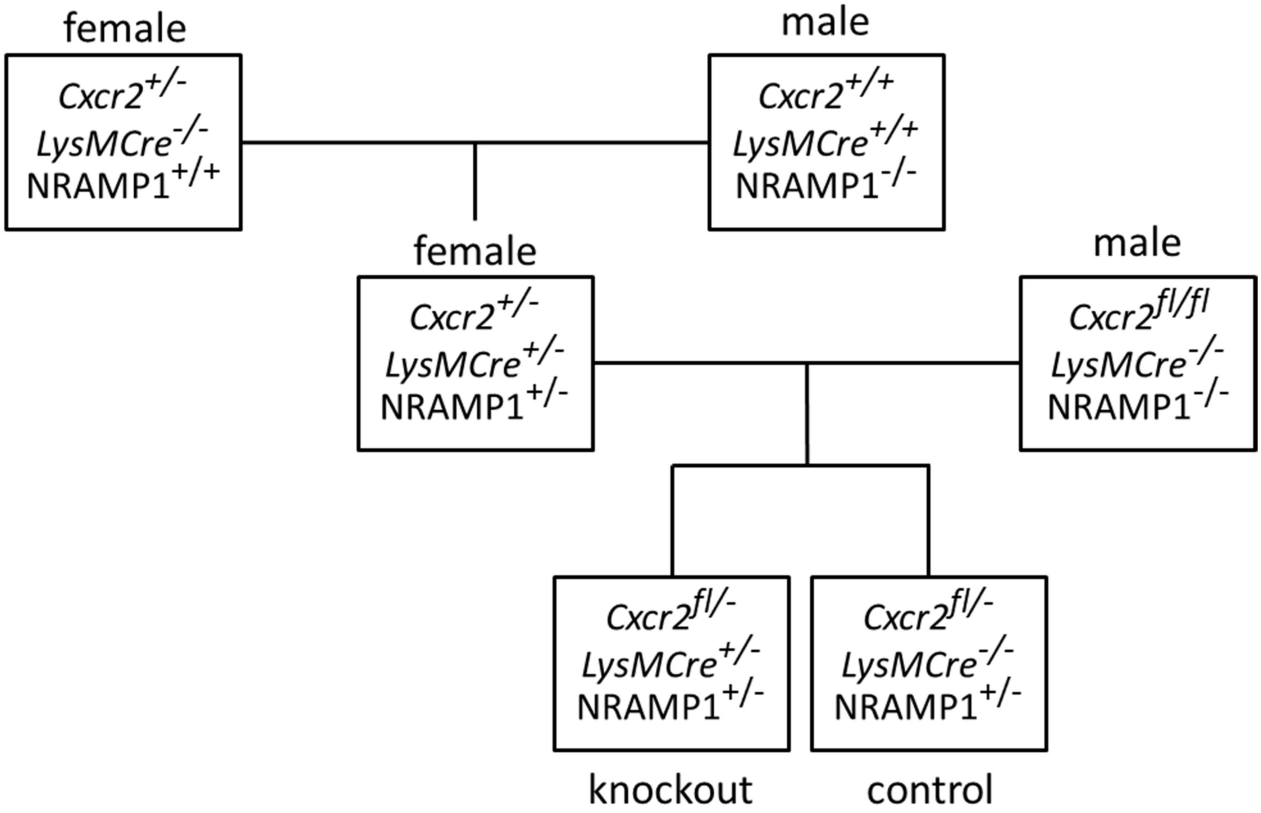

Figure 3.13 Diagram depicting the generation of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre+* mice. NRAMP1$^{+/-}$ indicates one copy of the *Slc11a1* allele encoding for a functional NRAMP1 transporter (linked to the deleted *Cxcr2* allele) and one copy containing the inactivating G169D mutation

Figure 3.14

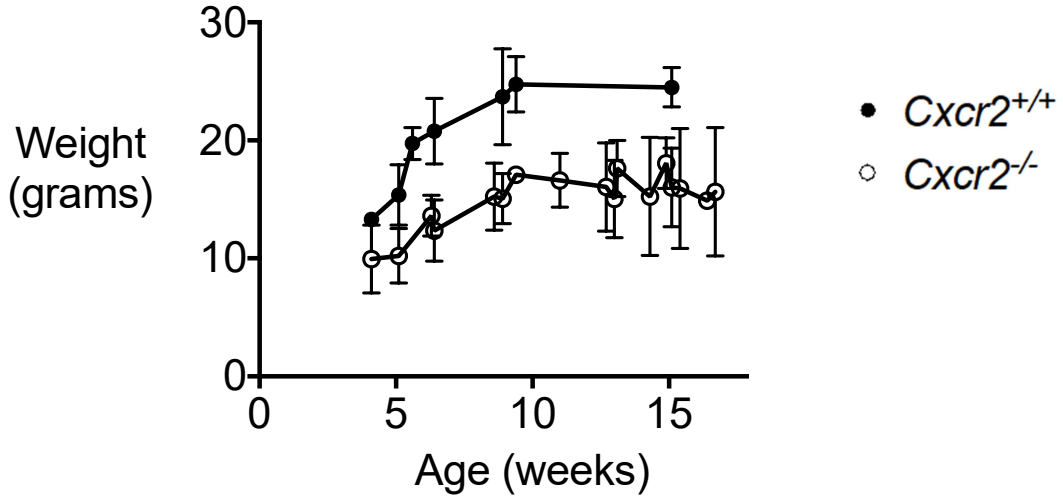

Figure 3.14 Weight of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice from our colony by age in weeks. *Cxcr2*$^{+/+}$ n=4-7 mice per age point; *Cxcr2*$^{-/-}$ n=1-10 mice per age point, mean and median n=4.5 per age point

Figure 3.15

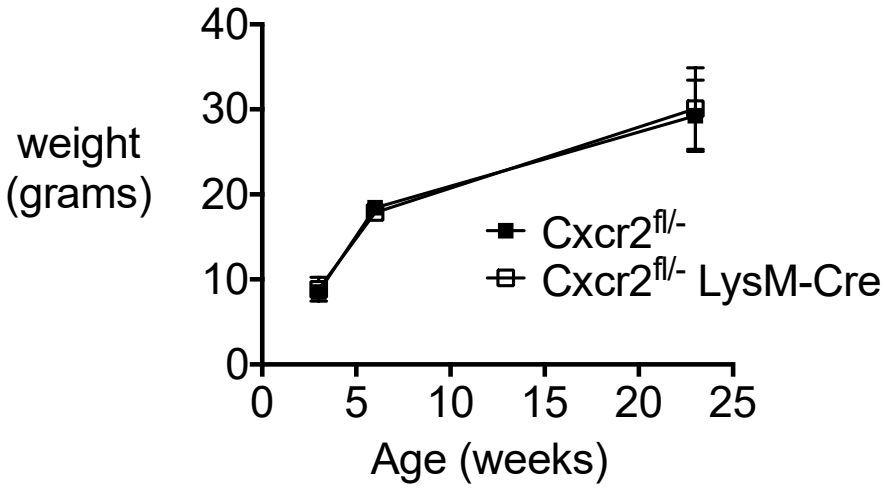

Figure 3.15 Weight of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice by age in weeks. *Cxcr2*$^{fl/-}$ n=3 per age point; *Cxcr2*$^{fl/-}$ *LysM-Cre*+ n=4 per age point

Figure 3.16

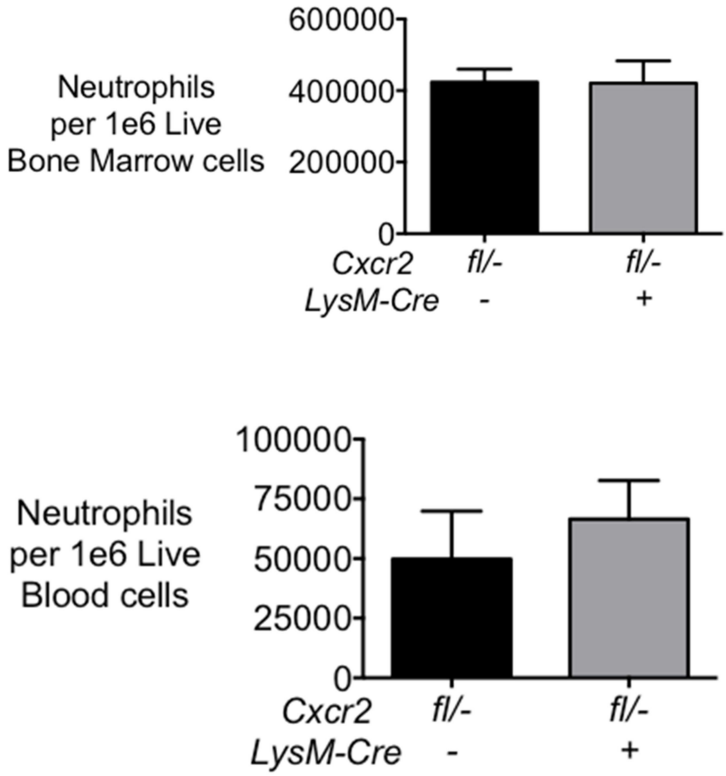

Figure 3.16 Neutrophil percentage in bone marrow and blood of uninfected *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ LysM-Cre+ mice

Figure 3.17

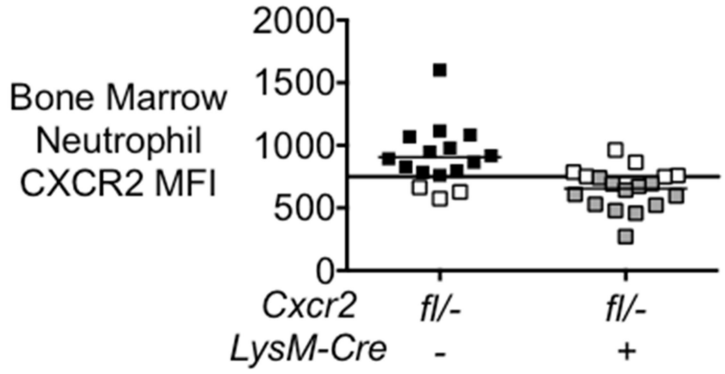

Figure 3.17 CXCR2 expression on bone marrow neutrophils from day 4 post-infection *Cxcr2$^{fl/-}$ and Cxcr2$^{fl/-}$ LysM-Cre*+ mice, with threshold of inclusion and exclusion from experimental data (empty boxes indicated excluded data points)

During *S.* Typhimurium infection, *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice did not exhibit any differences in intestinal colonization levels with *S.* Typhimurium compared to *Cxcr2*$^{fl/-}$ *LysM-Cre*- mice (Figure 3.18), but showed higher *S.* Typhimurium dissemination to the mesenteric lymph nodes and liver (Figure 3.19). Similar to *Cxcr2*$^{-/-}$ mice, the number of neutrophils recruited to the intestine was lower in *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice as compared to controls (Figure 3.20), supporting the conclusion that a large neutrophil response to *S.* Typhimurium infection is important for preventing *S.* Typhimurium dissemination to mesenteric lymph nodes and liver. The granulocyte-specific deletion of *Cxcr2* did not clearly impact *S.* Typhimurium colonization of Peyer's patches or terminal ileum, although levels of *S.* Typhimurium were highly variable in these tissues between individual *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice (Figure 3.19). When compared to the significantly higher colonization of terminal ileum and Peyer's patches of *Cxcr2*$^{-/-}$ mice, these data suggest that *S.* Typhimurium colonization in Peyer's Patches and terminal ileum may be partially limited by granulocytes, but is likely limited by other immune cells.

Figure 3.18

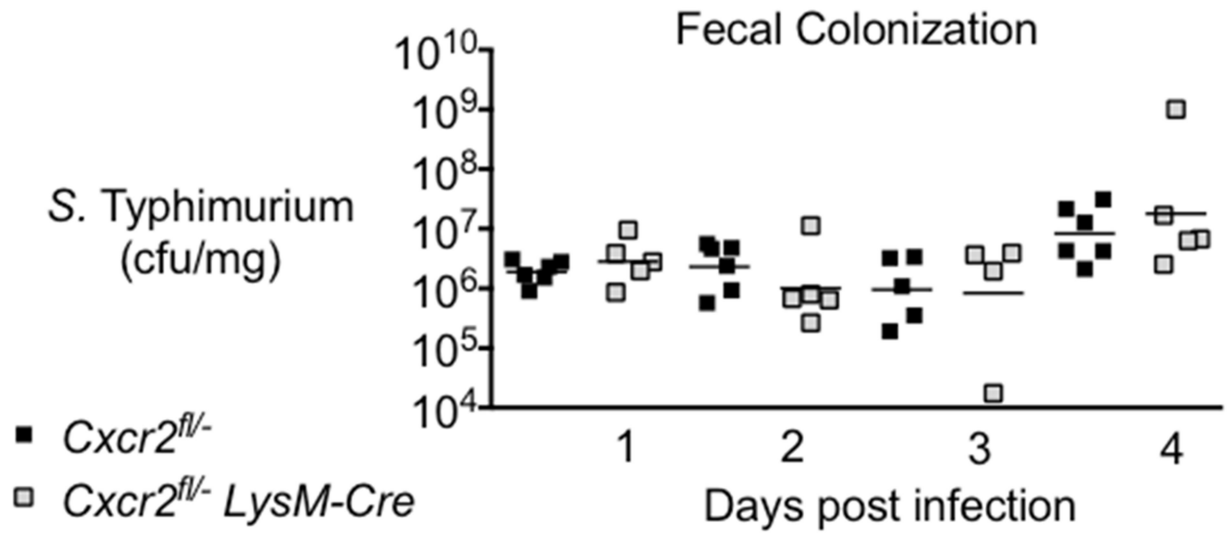

Figure 3.18 *Salmonella* Typhimurium fecal colonization of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice over 4 days of infection

Figure 3.19

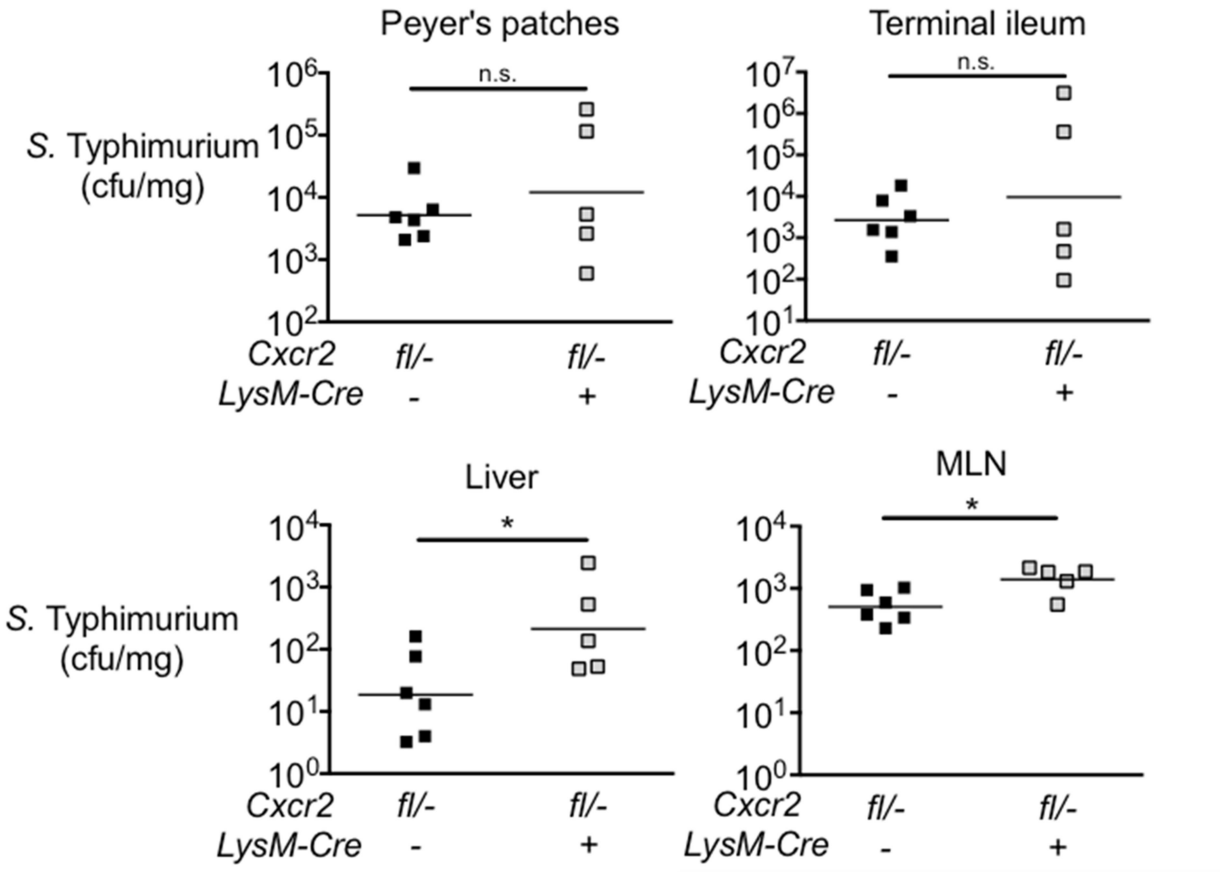

Figure 3.19 *Salmonella* Typhimurium tissue burden in *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice at 4 days post-infection

Figure 3.20

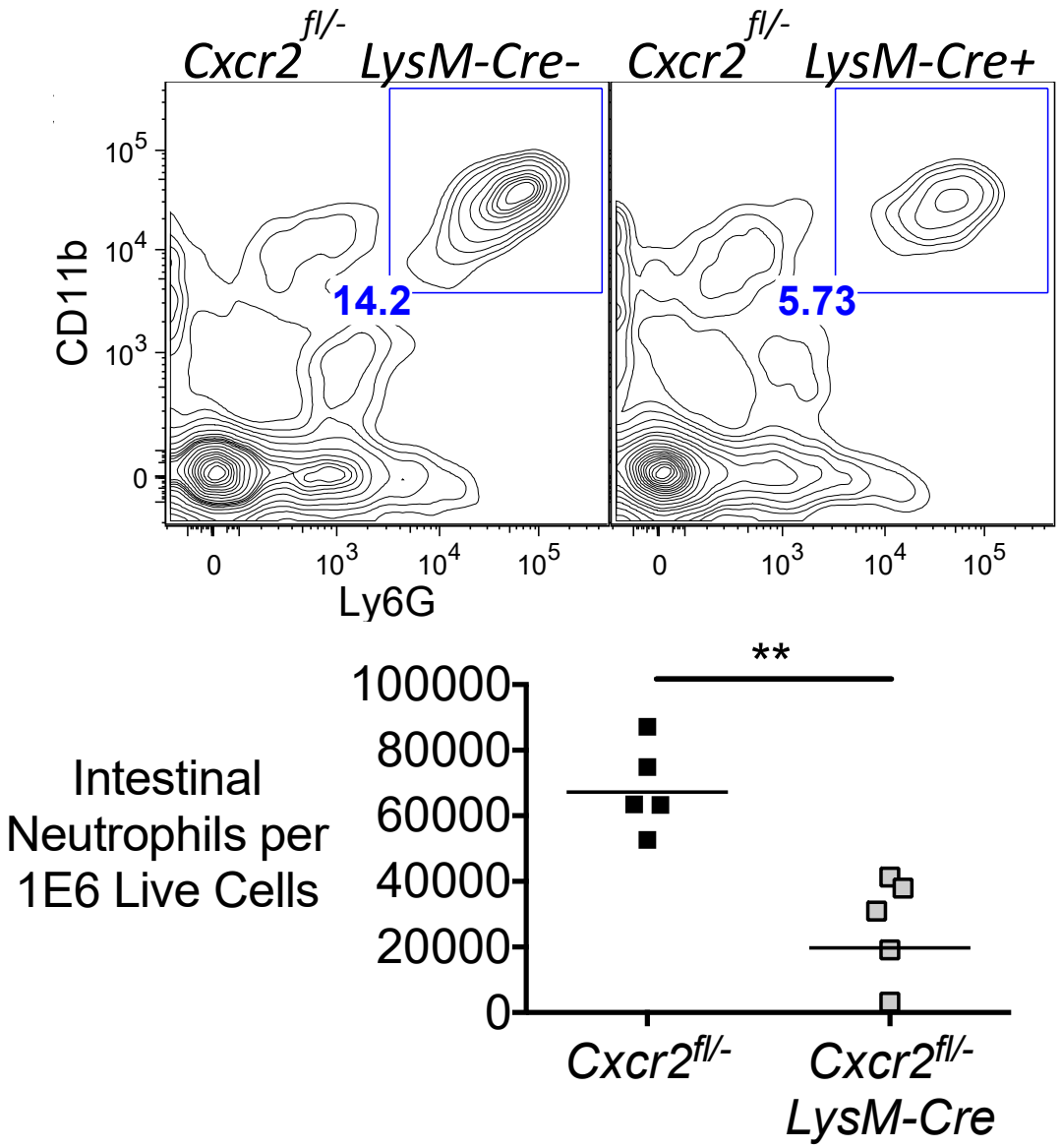

Figure 3.20 Intestinal neutrophils from *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice after 4 days of *Salmonella* Typhimurium infection

While performing these experiments, we noticed some variability and issues with reproducibility. Namely, successive generations of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre+* mice yielded data that showed an inversion of the phenotype in *S.* Typhimurium tissue burden at 4 days post-infection (Figure 3.21), and pooling the data negated the difference between *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre+* mice in all sample types with the sole exception of neutrophil migration to the intestine (Figure 3.22). The variability observed between generations suggested that one or more other genes in this transgenic mouse line was influencing *S.* Typhimurium tissue burden and was inadvertently selected for or against in the course of breeding.

In the course of preparing this dissertation, Mahajan et al (268) published that commercially available wild-type mice, often used for backcrossing, carried an exon duplication in the *Dock2* gene, which results in nonsense-mediated decay of *Dock2* mRNA (269). Dock2 is a guanine-nucleotide exchange factor for Rac1, and plays a vital role in lymphocyte migration after chemokine signaling.

We hypothesized that the presence of this mutation in our colony may explain the variation observed in the generations of mice. We tested the *Cxcr2*^{flox} *LysM-Cre* colony for the presence of the *Dock2* exon duplication and found it to be present in some of the mice, most likely having been introduced from the *Cxcr2*^{flox} line. Frozen tissue samples from mice in the above experiments were tested, and data from mice harboring the mutation were excluded. To provide sufficient numbers for analysis, the colony was cleared of the *Dock2* mutation and the experiments were repeated (work completed by Purnima Sharma and Dr. Maria Valeri). As seen in Figure 3.23, *S.* Typhimurium burden is not significantly different in Peyer's patches or terminal ileum when *Cxcr2* is

selectively deleted from granulocytes, but is significantly higher in liver and mesenteric lymph nodes, again suggesting that *S.* Typhimurium burden is controlled by *Cxcr2*-expressing granulocytes in these extra-intestinal tissues, but local colonization of Peyer's patches and terminal ileum are dominantly regulated by another immune cell type.

Figure 3.21

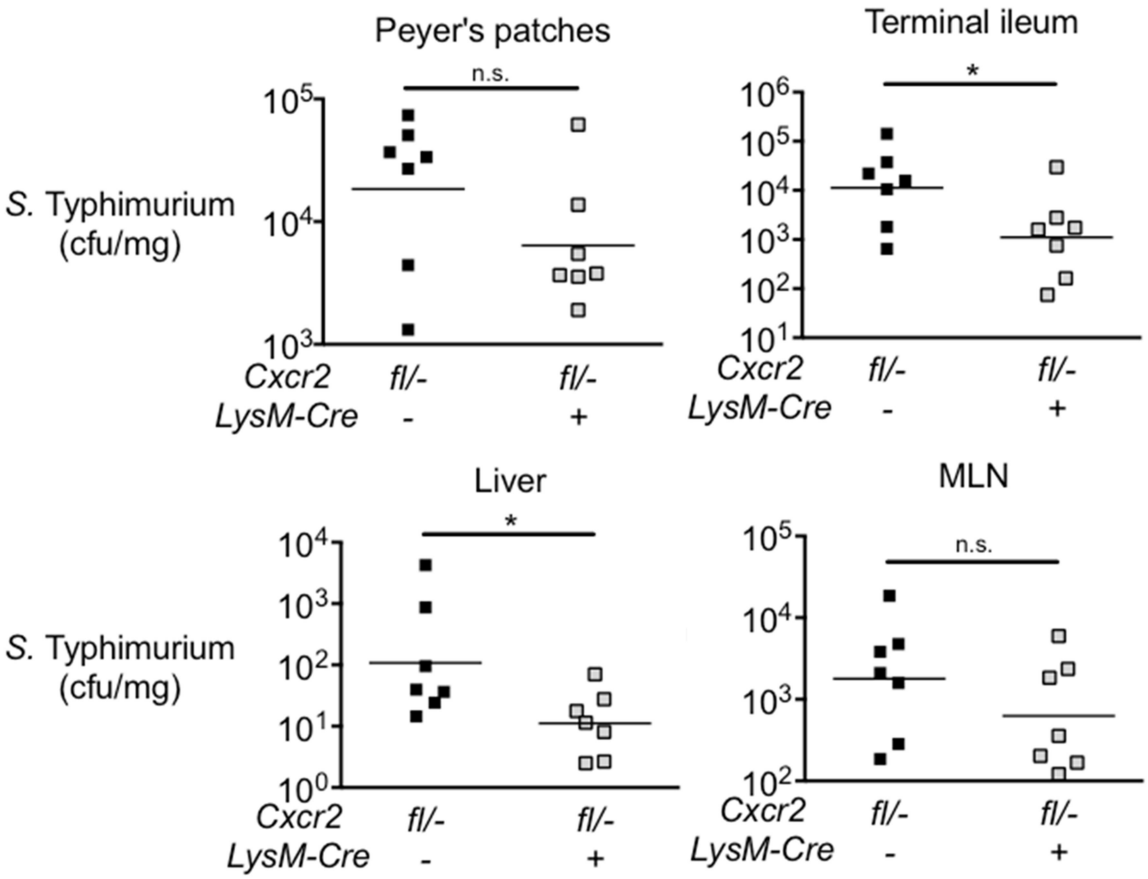

Figure 3.21 *Salmonella* Typhimurium tissue burden in *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice at 4 days post-infection, later generations of mice

Figure 3.22

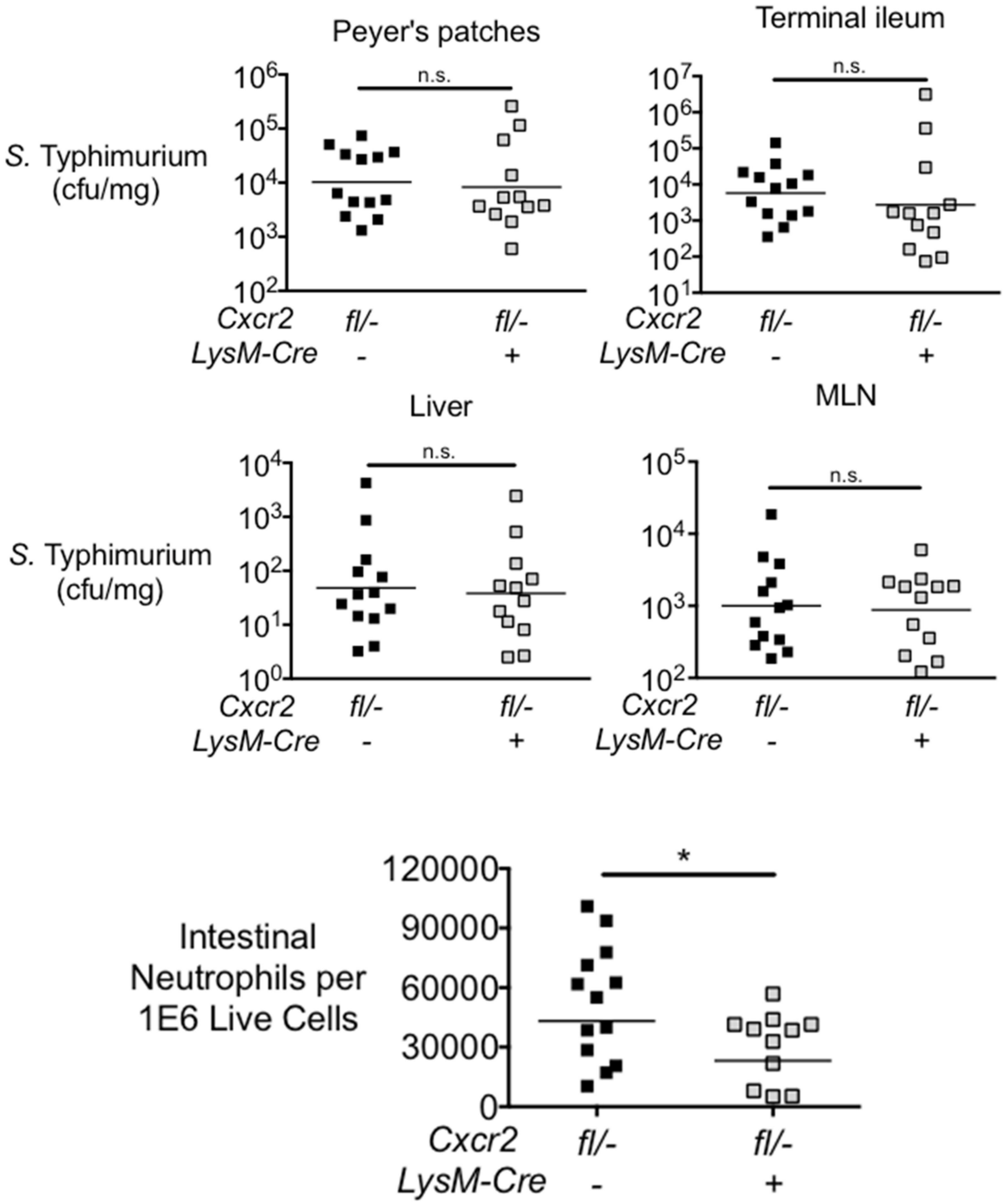

Figure 3.22 *Salmonella* Typhimurium tissue burden and intestinal neutrophils in *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice at 4 days post-infection, pooled data

Figure 3.23

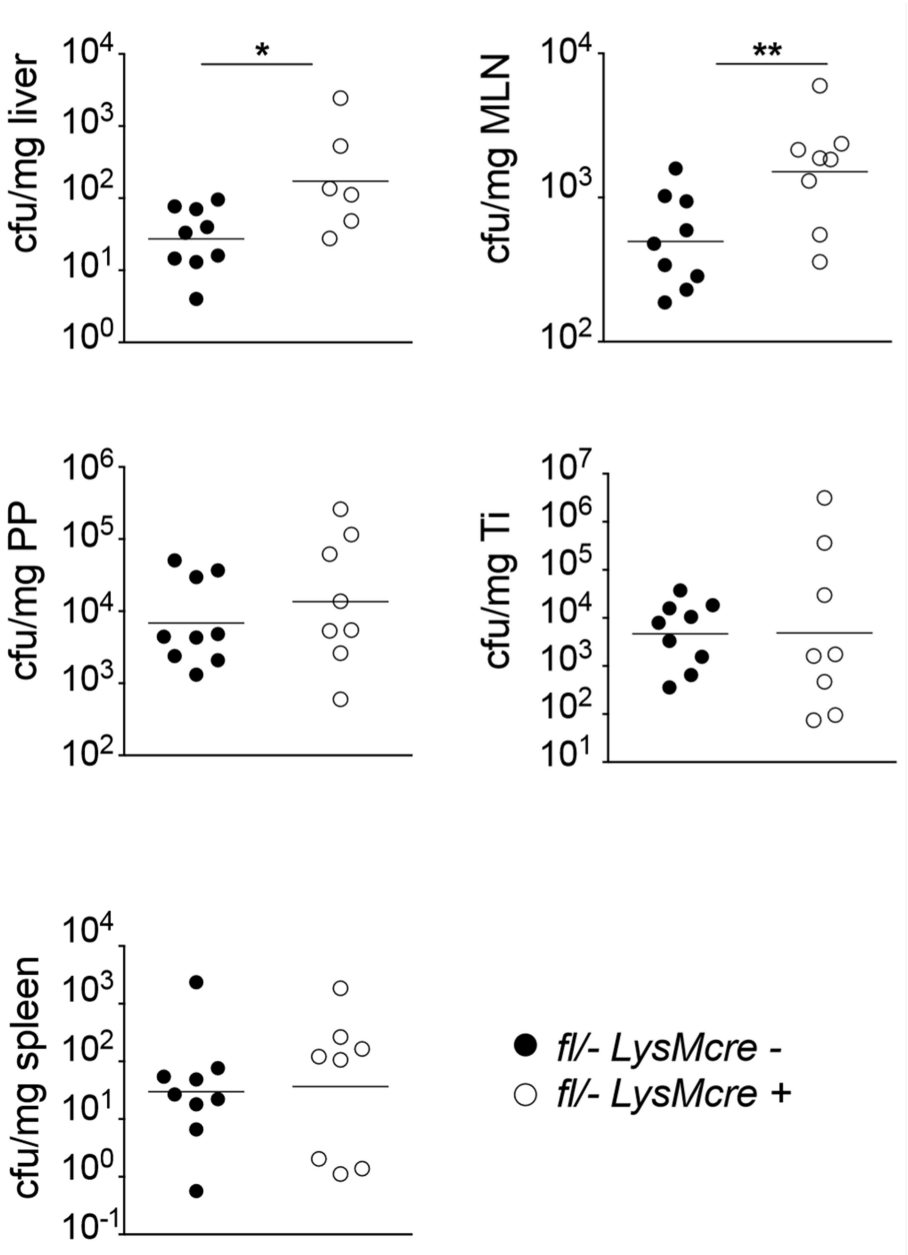

Figure 3.23 *Salmonella* Typhimurium tissue burden and intestinal neutrophils in *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre* mice at 4 days post-infection, without mice bearing *Dock2* mutation (MLN = mesenteric lymph nodes; PP = Peyer's patches; Ti = Terminal ileum); Graphs courtesy of Dr. Maria Valeri

To identify which cells are most important in modulating *S.* Typhimurium burden in Peyer's patches, we isolated Peyer's patches from *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice and identified neutrophils, macrophages, dendritic cells, CD4+ T cells, CD8+ T cells, and B cells using flow cytometry. The only cell type with a significant difference between *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice was B cells (Figures 3.24 and 3.30), the most dominant cell type in Peyer's patches (270). Peyer's patches of *Cxcr2*$^{-/-}$ mice, uninfected or 4 days post- *S.* Typhimurium infection showed deficiency in B cells. Moreover, the number of Peyer's patch B cells in infected mice negatively correlated with the *S.* Typhimurium burden (Figure 3.24), suggesting that B cells are important for resistance to high *S.* Typhimurium colonization of Peyer's patches. In mice selectively lacking CXCR2 on granulocytic cells, Peyer's patches contained similar numbers of B cells as control mice, both in uninfected mice and in mice infected with *S.* Typhimurium for 4 days (Figure 3.25). These data support a granulocyte-independent role for CXCR2 in the B cell population of Peyer's patches.

Peyer's patch B cells are important for initiating production of IgA that is secreted into the intestinal lumen (271). Humans and animals exposed to *S.* Typhimurium can produce *Salmonella*-specific immunoglobulins (222, 272, 273), which provides protection against *S.* Typhimurium (222, 274). In order to determine if *Cxcr2*$^{-/-}$ mice exhibit lower fecal IgA levels, fecal samples were collected from untreated *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice and total fecal IgA was measured. No deficiency in IgA production was found in *Cxcr2*$^{-/-}$ mice. On the contrary, despite their B cell deficiency, *Cxcr2*$^{-/-}$ mice produced significantly higher amounts of IgA compared to their *Cxcr2*$^{+/+}$ counterparts (Figure 3.28). As expected, infection with *S*. Typhimurium led to higher fecal IgA levels

in *Cxcr2*$^{+/+}$ mice, with a 2.4-fold increase compared to uninfected mice. This infection-induced increase in IgA production is significantly higher in *Cxcr2*$^{-/-}$ mice, which exhibited a 12-fold increase over uninfected *Cxcr2*$^{-/-}$ mice. Thus, *S*. Typhimurium infected CXCR2 deficient mice had drastically higher fecal IgA levels compared to *Cxcr2*$^{+/+}$ mice (Figure 3.28). As described on page 98 regarding the *Cxcr2*^{flox} colony, some mice in the *Cxcr2* colony also carried a mutation in the *Dock2* gene which effects lymphocyte trafficking. When the fecal IgA level was re-examined using mice without the *Dock2* mutation, no difference in fecal IgA level was observed (Figure 3.29, provided by Purnima Sharma and Dr. Maria Valeri).

Figure 3.24

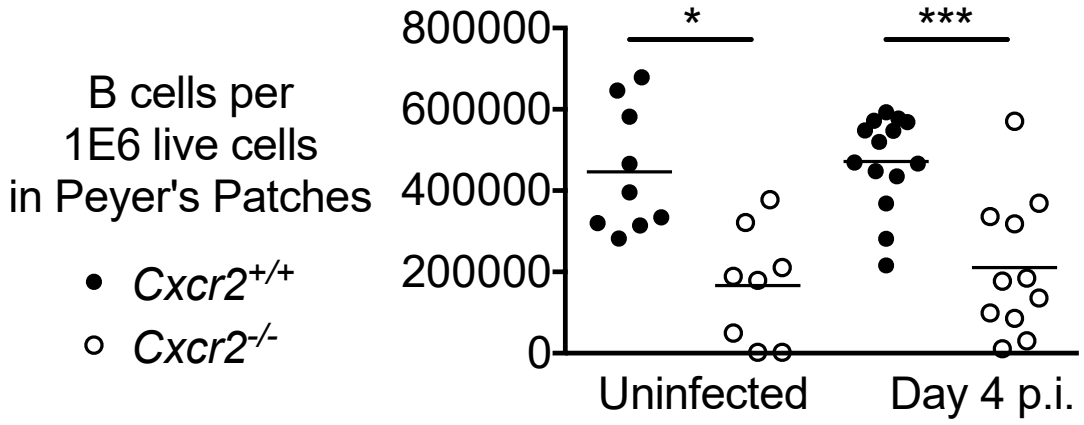

Figure 3.24 B cells in Peyer's patches of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, uninfected and 4 days post-*Salmonella* Typhimurium infection.

Figure 3.25

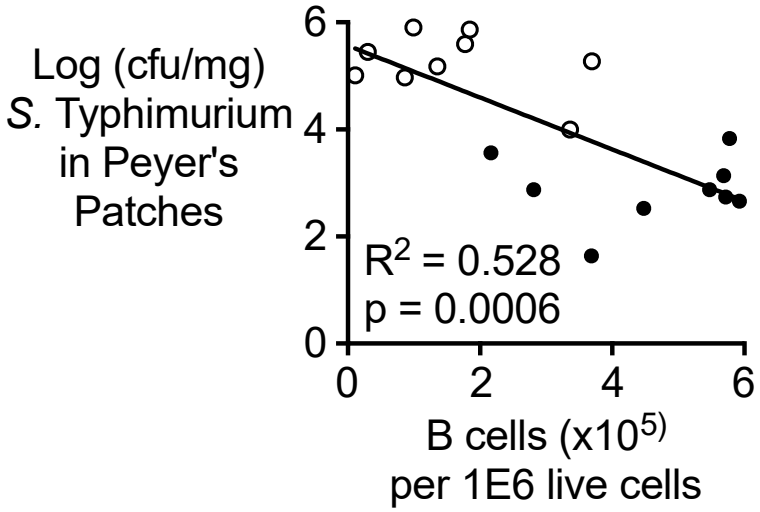

Figure 3.25 Correlation of Peyer's patch B cell numbers and *Salmonella* Typhimurium colonization of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice

Figure 3.26

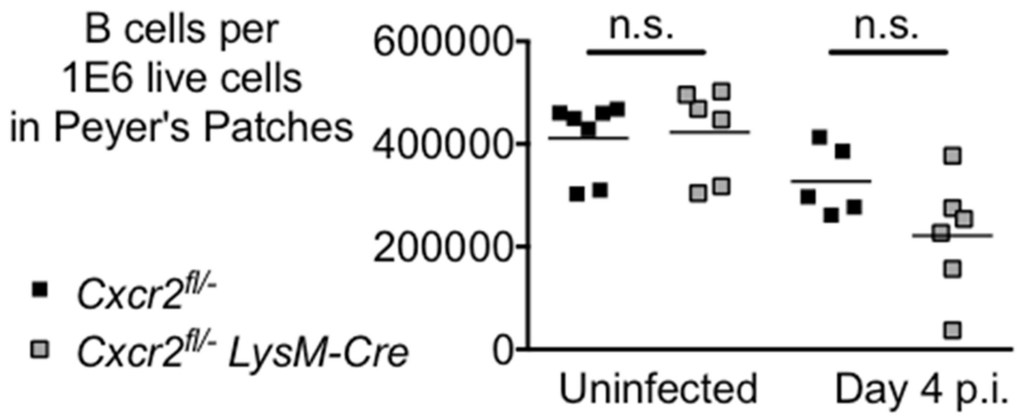

Figure 3.26 B cells in Peyer's patches of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$*LysM-Cre* mice, uninfected and 4 days post-*Salmonella* Typhimurium infection.

Figure 3.27

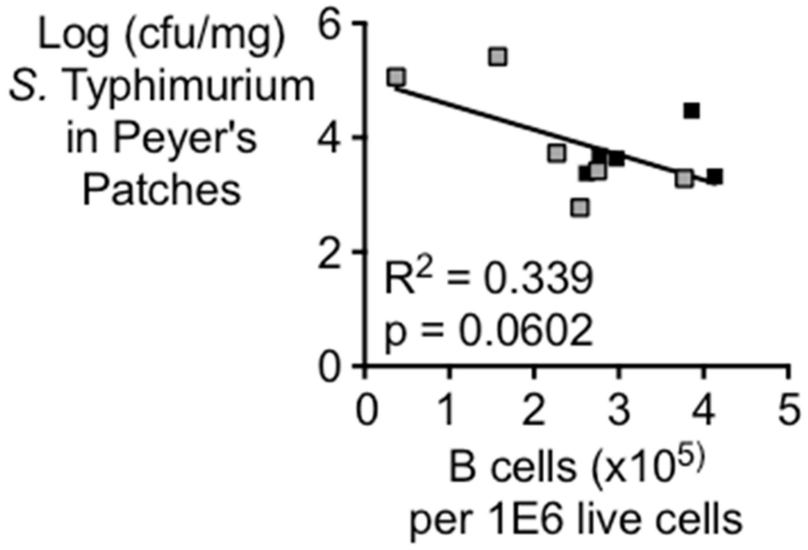

Figure 3.27 Correlation of Peyer's patch B cell numbers and *Salmonella* Typhimurium colonization of *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$*LysM-Cre* mice

Figure 3.28

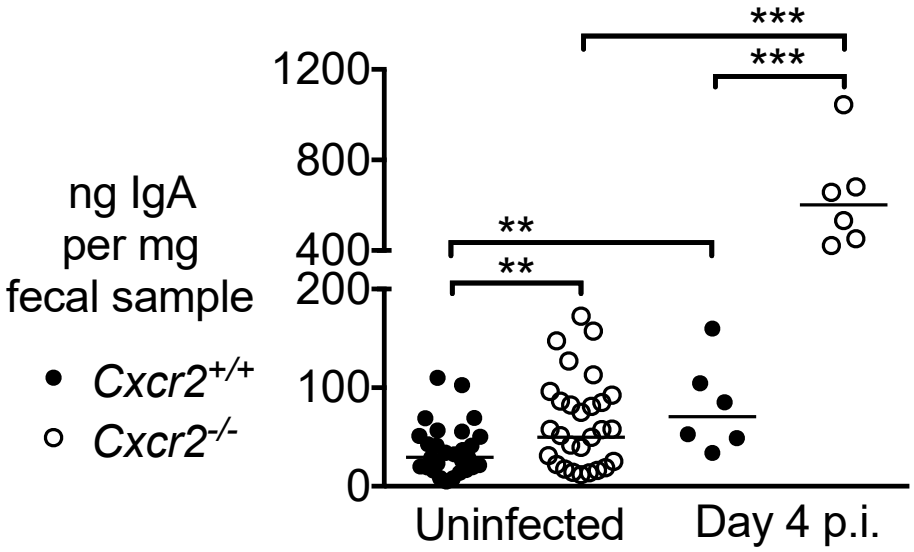

Figure 3.28 Fecal IgA levels in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, uninfected and 4 days post- *Salmonella* Typhimurium infection

Figure 3.29

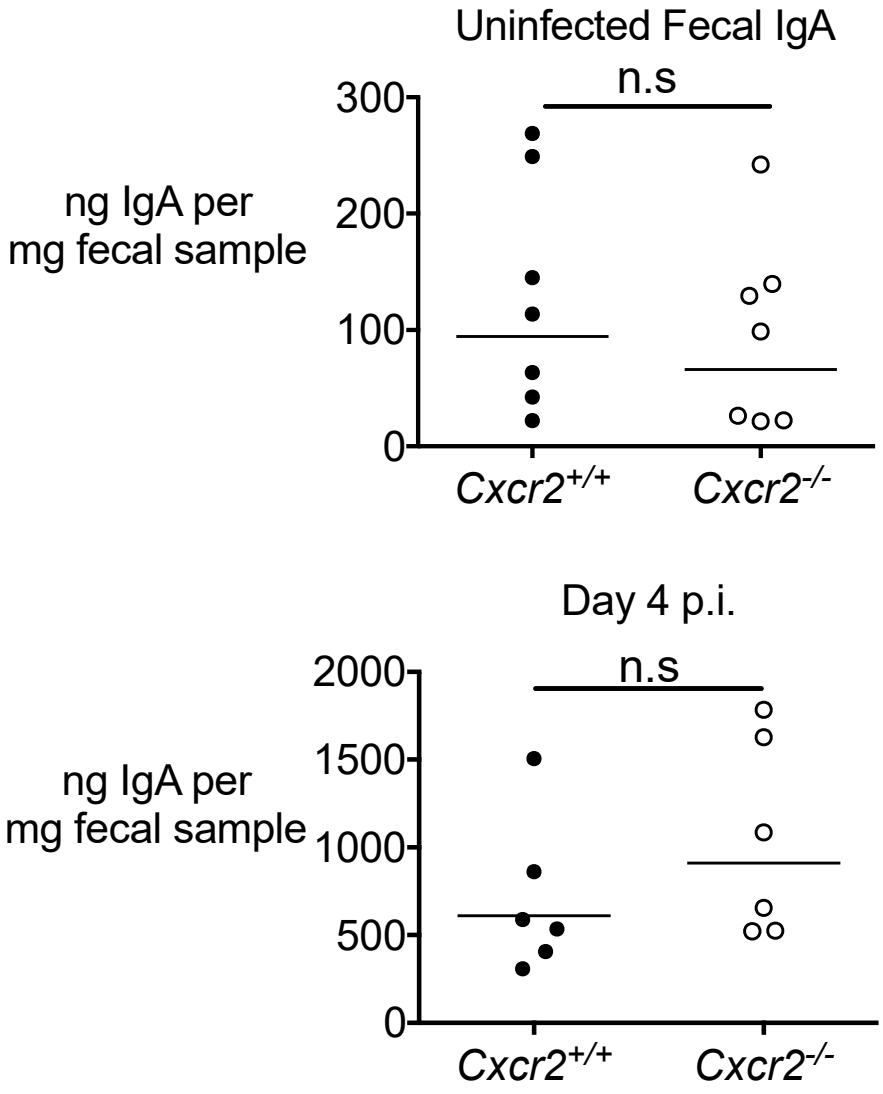

Figure 3.29 Fecal IgA levels in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, uninfected and 4 days post- *Salmonella* Typhimurium infection, mice with *Dock2* mutation excluded; Graphs courtesy of Purnima Sharma and Dr. Maria Valeri

Figure 3.30

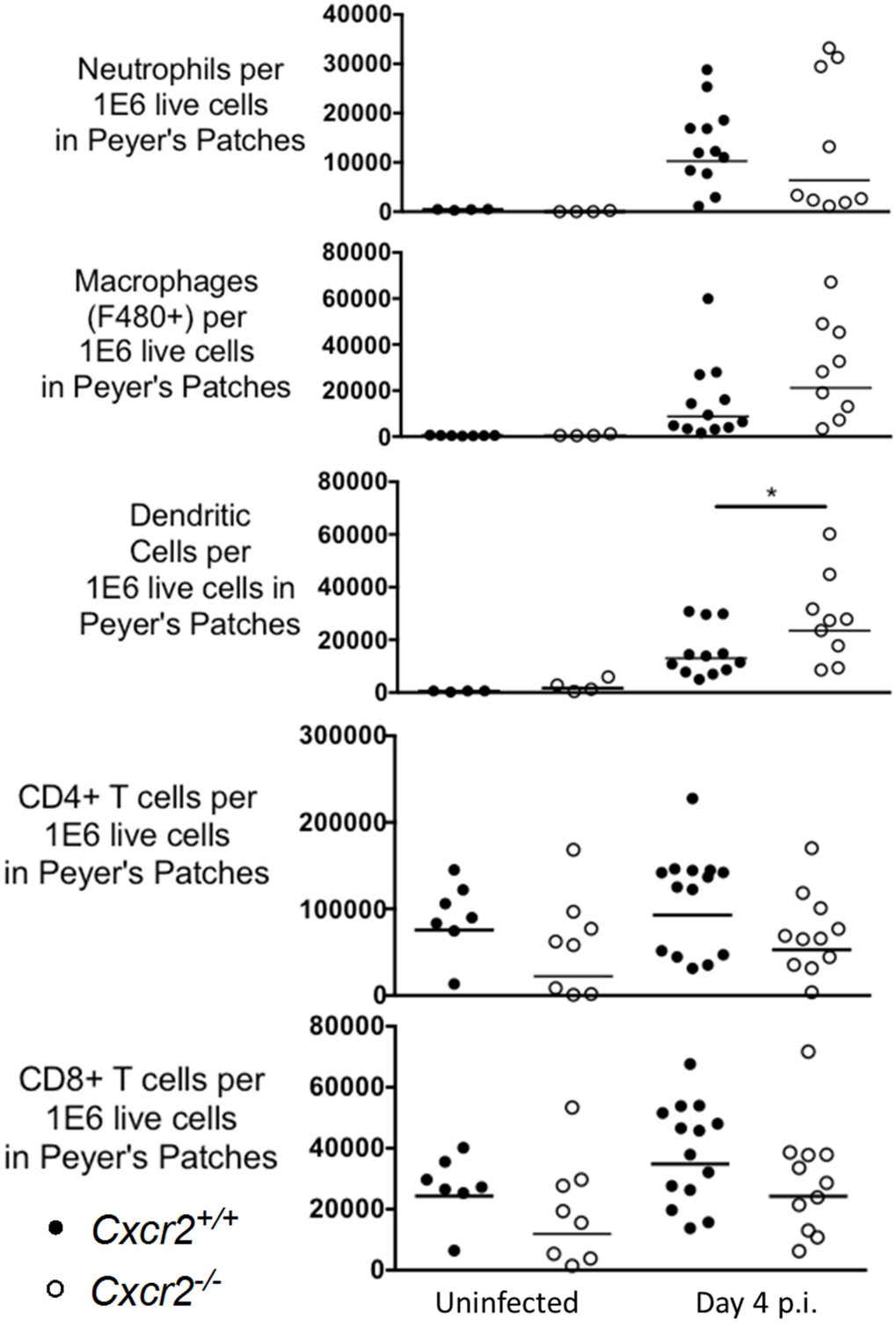

Figure 3.30 Neutrophils, macrophages, dendritic cells, CD4+ and CD8+ T cells in Peyer's patches of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, uninfected and 4 days post-*Salmonella* Typhimurium infection

The deficiency of B cells in Peyer's patches of *Cxcr2*$^{-/-}$ mice suggested that CXCR2 may be expressed by B cells and may be important for their migration into or retention inside Peyer's patches. B cell migration into Peyer's patches is at least partially mediated by CXCR5, and B cell exit is increased by CXCR4 expression (275). To determine whether changes in CXCR4 or CXCR5 expression were the reason for reduced B cell numbers in Peyer's patches of *Cxcr2*$^{-/-}$ mice, we measured CXCR4 and CXCR5 expression on B cells from *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice using flow cytometry analysis. CXCR4 and CXCR5 expression was comparable between *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice for B cells isolated from blood, Peyer's patches, and peritoneal cavity (Figure 3.31). CXCR2 expression was not higher on B cells from uninfected *Cxcr2*$^{+/+}$ mice than from *Cxcr2*$^{-/-}$ mice, as measured by flow cytometry analysis.

As a functional measure of CXCR2 on B cells, we tested the ability of *Cxcr2*$^{+/+}$ B cells to migrate toward CXCL1 and CXCL2, chemokines specific for CXCR2. B cells isolated from Peyer's patches of *Cxcr2*$^{+/+}$ mice migrated toward CXCL1 and CXCL2 at a rate approximately 20% of migration toward CXCL12α, the ligand for CXCR4 (Figure 3.32). Migration was not observed in B cells isolated from the peritoneal cavity (data not shown), supporting Peyer's patches as a unique niche for CXCR2-expressing B cells. To further test the presence of CXCR2 on Peyer's patch B cells, we analyzed B cell numbers in Peyer's patches of mice treated with anti-CXCR2 antibody and found a significant decrease in B cell numbers as a group, but variable response in individual mice (Figure 3.33). To our knowledge, these data are the first demonstration of functional CXCR2 expression on B cells. Of note, not every preparation of B cells showed migration, despite equivalent purity from isolation. Although Peyer's patches

are cleaned of visible fecal contamination after isolation, we could not exclude the possibility of bacterial products in the final B cell suspension. Protocol optimization for more consistent results is underway.

Figure 3.31

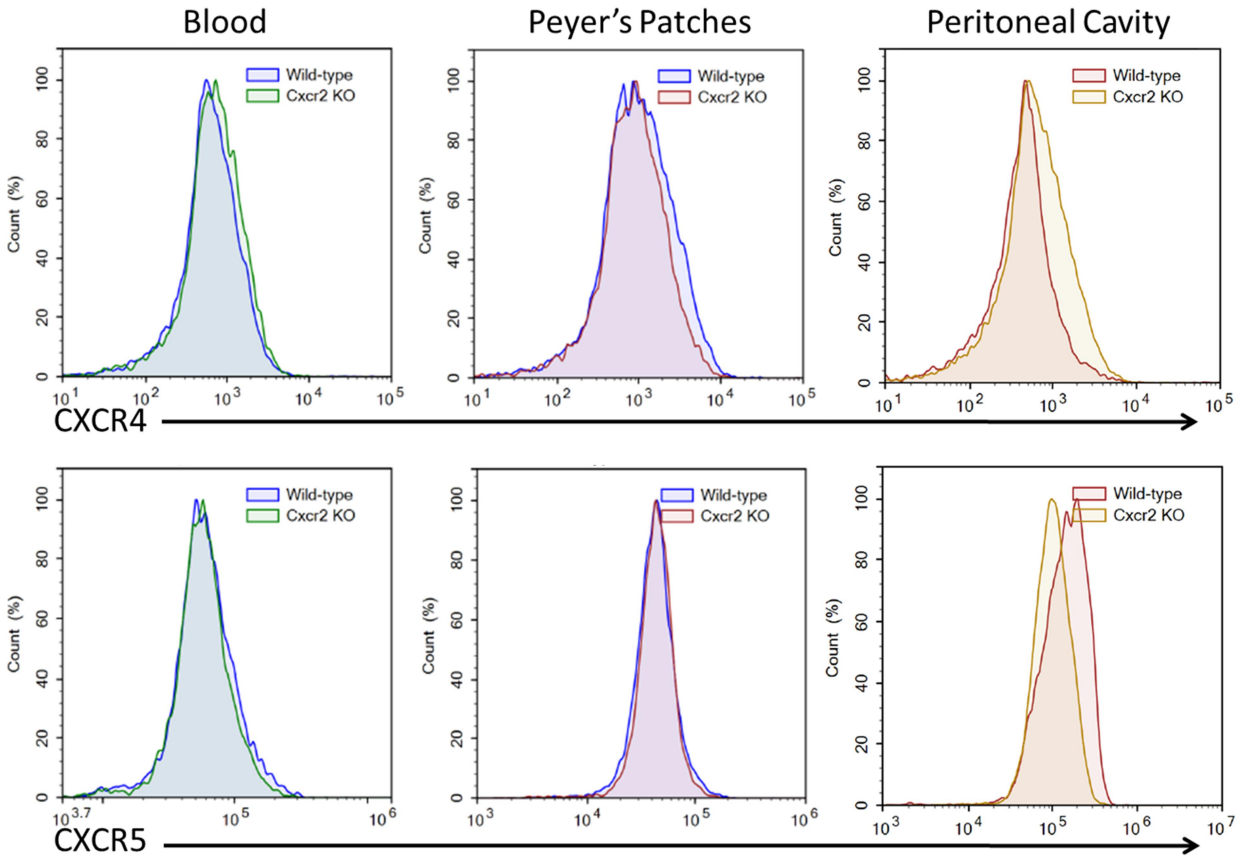

Figure 3.31 Representative plots showing relative CXCR4 and CXCR5 expression on B cells from blood, Peyer's patches, and peritoneal cavity of uninfected *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice

Figure 3.32

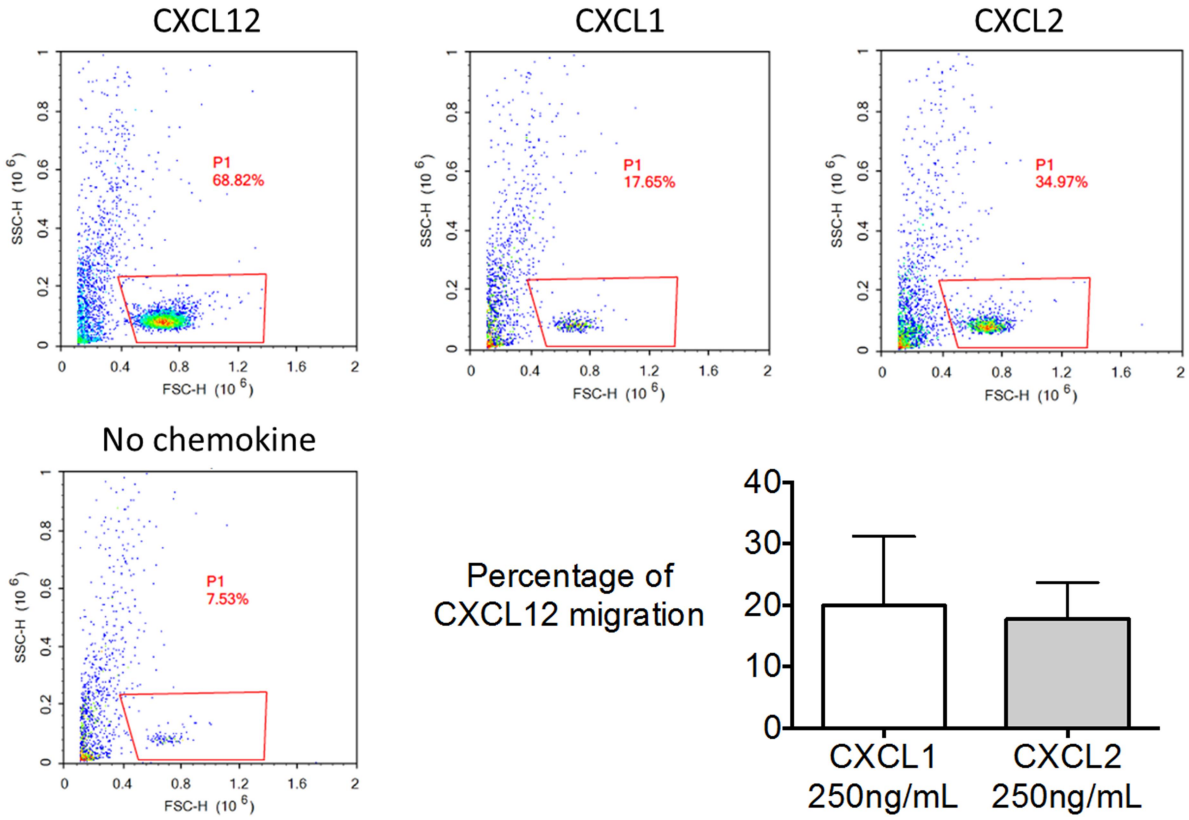

Figure 3.32 Representative plots showing migration of purified B cells from Peyer's patches of *Cxcr2*$^{+/+}$ mice toward the chemokines CXCL1 and CXCL2 and bar graphs of mean and standard deviation from two experiments.

Figure 3.33

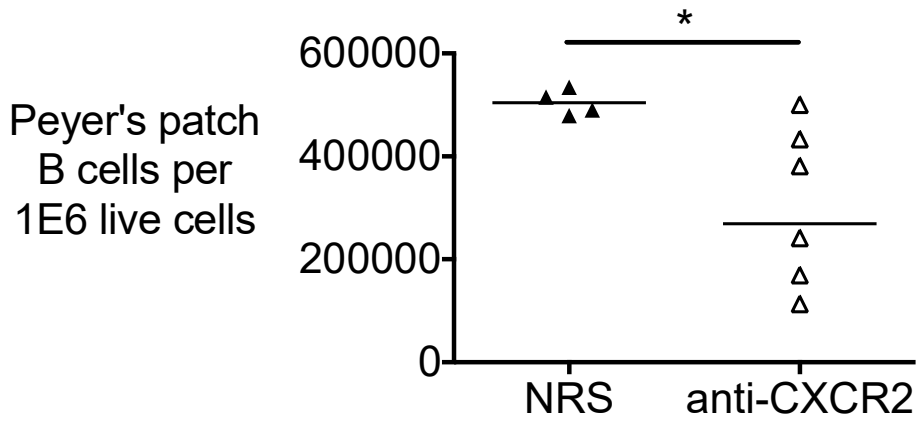

Figure 3.33 Depletion of Peyer's patch B cells in mice treated with anti-CXCR2 antibody compared to mice treated with normal rabbit serum (NRS)

To better understand the mechanism that controls B cell numbers in Peyer's patches of *Cxcr2*$^{-/-}$ mice, mice lacking B cells (muMT) were transplanted intraperitoneally with B cells purified from *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice. If CXCR2 is important for homing B cells to Peyer's patches, we expected to see Peyer's patch colonization with *Cxcr2*$^{+/+}$ B cells, but not with *Cxcr2*$^{-/-}$ B cells. B cells were isolated and purified from the peritoneal cavity and Peyer's patches of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, and two million B cells were injected intraperitoneally into mice lacking B cells. Ten days post-transfer, recipient mice were sacrificed and cells were isolated from blood, spleen, mesenteric lymph nodes, and the peritoneal cavity. Peyer's patches were not grossly visible, so the small intestine was rolled and examined under magnification for the presence of lymphocyte clusters (data not shown). Flow cytometric data showed very few to no B cells in the blood, spleen, or mesenteric lymph node of transplanted animals (Figure 2.34), but B cells accounted for 12-20% of live cells in the peritoneal cavity in recipients of *Cxcr2*$^{+/+}$ B cells and 6-20% in recipients of *Cxcr2*$^{-/-}$ B cells (Figure 2.35). This data suggests that neither *Cxcr2*$^{+/+}$ nor *Cxcr2*$^{-/-}$ B cells migrate into the blood, spleen, or mesenteric lymph nodes from the peritoneal cavity. Upon microscopic examination of the terminal ileum, a single lymphoid cluster was observed in one mouse that received *Cxcr2*$^{+/+}$ cells (data not shown), but as the full thickness of the terminal ileum was not examined via microscopy, the presence of lymphoid clusters in other recipient mice cannot be confidently excluded. B cell transfer via intravenous injection is of interest, as vascular exit of B cells more closely mimics the recruitment of circulating B cells into Peyer's patches.

Figure 3.34

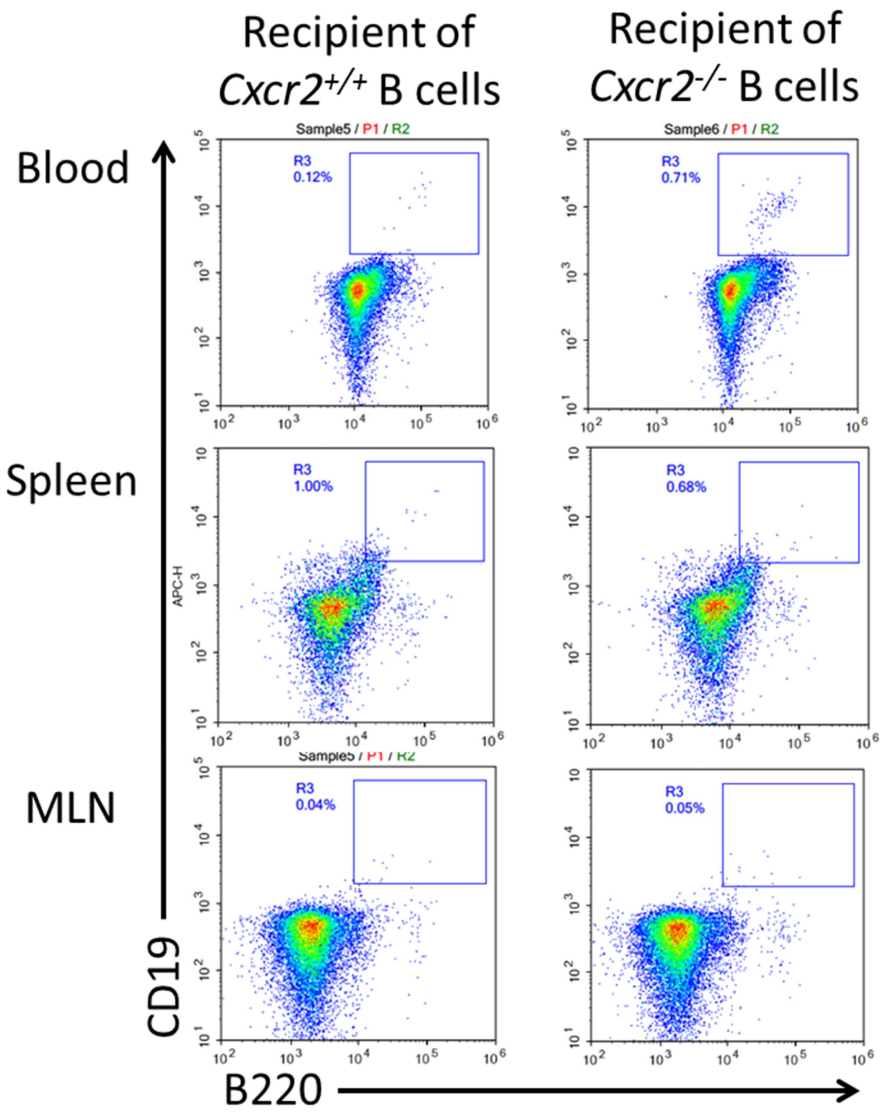

Figure 3.34 Blood, spleen, and mesenteric lymph node analysis for B cells transferred into *muMT*- (B cell deficient) mice

Figure 3.35

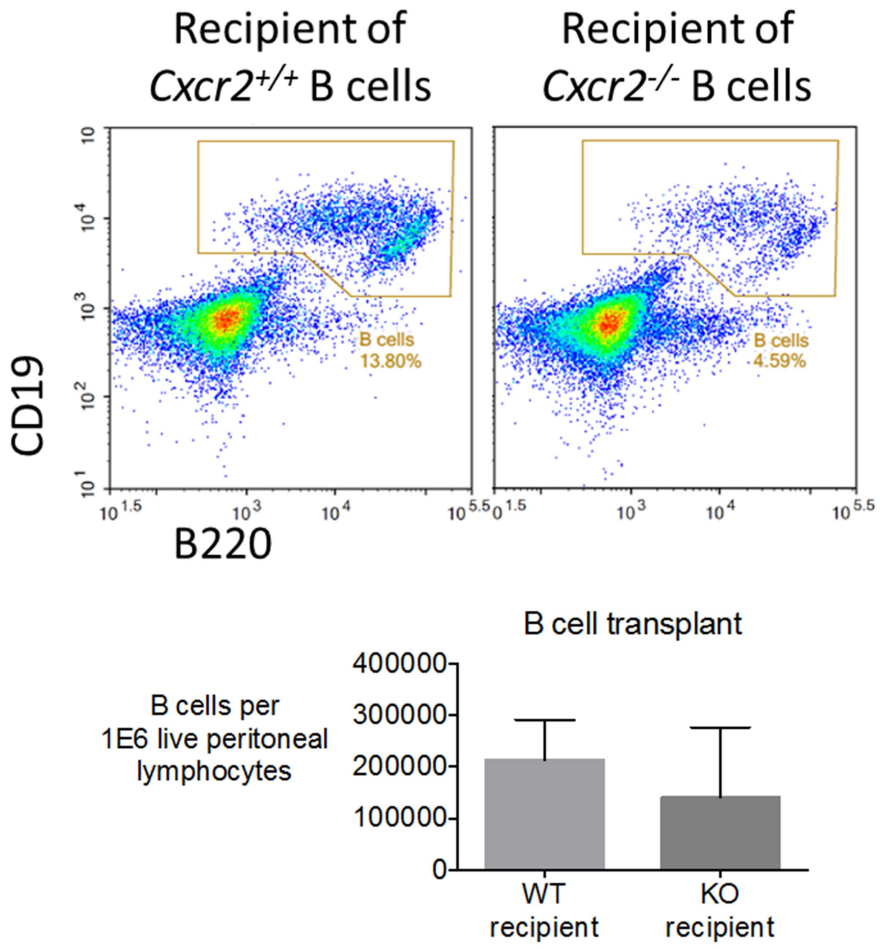

Figure 3.35 Analysis of peritoneal B cells transferred into *muMT-* (B cell deficient) mice (above: example flow cytometry plots; below: mean and deviation of two experiments WT n=2; KO n=2)

To probe the specific role for CXCR2 on B cells, we crossed *CD19-Cre* mice with *Cxcr2*$^{fl/fl}$ mice, but these mice did not show any decrease in B cell population of Peyer's patches in uninfected mice or after 4 days of *S.* Typhimurium infection (Figure 2.36). Furthermore, the mice did not show a defect in defense against *S.* Typhimurium colonization of Peyer's patches (Figure 2.37). These data would suggest that the B cell population of Peyer's patches is dependent on multiple factors, and deletion of *Cxcr2* alone is not sufficient to reduce Peyer's patch B cell numbers.

Figure 3.36

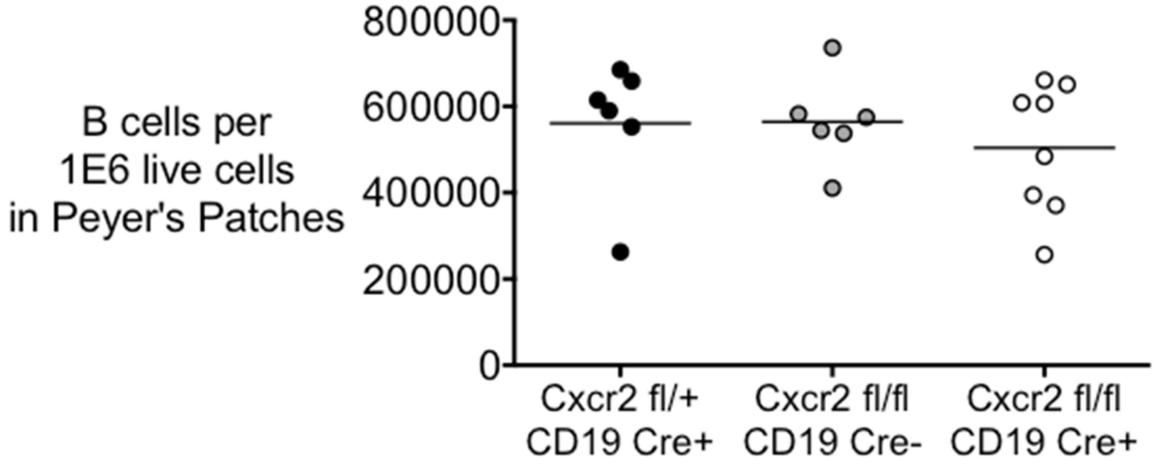

Figure 3.36 B cells in Peyer's patches of *Cxcr2*$^{fl/fl}$ and *Cxcr2*$^{fl/fl}$ *CD19-Cre* mice, 4 days post-*Salmonella* Typhimurium infection

Figure 3.37

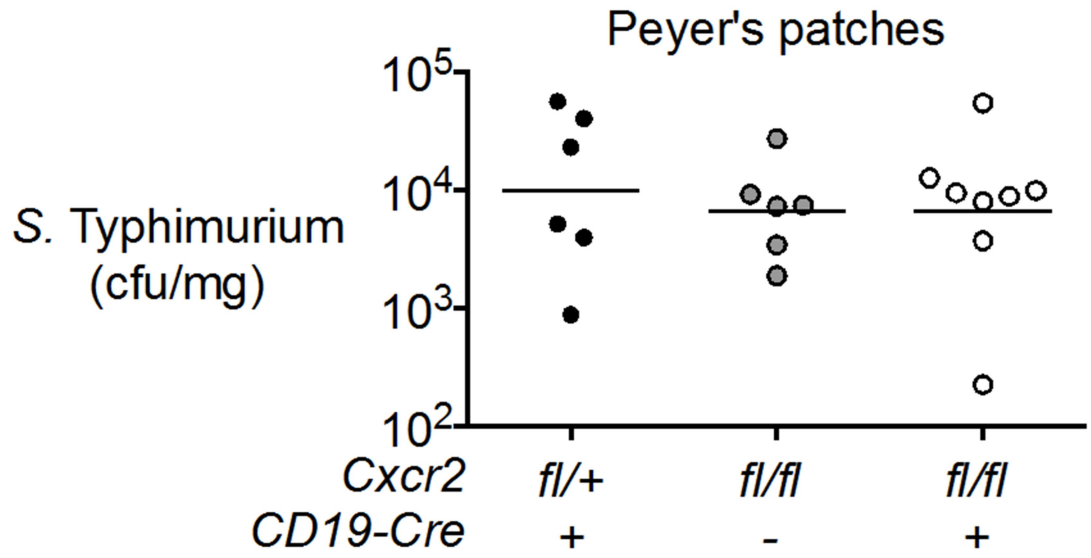

Figure 3.37 *Salmonella* Typhimurium colonization in Peyer's patches of *Cxcr2*$^{fl/+}$ *CD19-Cre+, Cxcr2*$^{fl/fl}$ *CD19-Cre-, and Cxcr2*$^{fl/fl}$ *CD19-Cre+* mice 4 days post-infection

3.5 Discussion & Future Directions

The intestinal mucosa is home to numerous immune cells, and chemokine-mediated migration of leukocytes into the intestinal *lamina propria* and associated lymphoid tissues (e.g. Peyer's patches) is important for homeostatic surveillance of tissue that is in close proximity to trillions of microbes. Immune responses to intestinal bacteria must be carefully regulated, as inappropriate immune activation in response to commensal organisms can cause inflammatory bowel disease, or an insufficient response to an invasive organism, such as *S.* Typhimurium, can lead to bacteremia and dissemination. Here we show that neutrophil infiltration to the intestinal mucosa is crucial for reducing *S.* Typhimurium dissemination to mesenteric lymph nodes and the liver, that *S.* Typhimurium numbers in Peyer's patches correlates with the number of B cells, and that CXCR2 is a key chemokine receptor for both of these processes.

Neutrophil recruitment to the intestine is a characteristic of *S.* Typhimurium gastroenteritis, and although *S.* Typhimurium is able to temporarily thrive in the inflammatory environment produced by infiltrating neutrophils (166, 167, 240, 241), neutrophil antimicrobial activities are essential for combating *S.* Typhimurium systemic dissemination (258, 274, 275). Data presented here and work by others show that mice without CXCR2 have higher *S.* Typhimurium burden in Peyer's patches, terminal ileum, mesenteric lymph nodes, and liver than mice expressing CXCR2, and this increase corresponds to a deficiency in neutrophil recruitment to the intestine. This is in agreement with the study by Marchelletta et al (199), where BALB/c *Cxcr2*$^{-/-}$ mice had higher *S.* Typhimurium levels in the MLN and fewer neutrophils in intestinal tissue compared to *Cxcr2*$^{+/+}$ mice at 3 days post-infection. Therefore, the lack of CXCR2 leads

to an insufficient neutrophil response to the intestine, permitting higher *S.* Typhimurium dissemination.

Based on preliminary experiments, intestinal B cells and dendritic cells were also significantly lower in *Cxcr2*$^{-/-}$ mice compared to *Cxcr2*$^{+/+}$ mice, but it is not currently known whether this difference is present in uninfected mice. Neutrophils are present in extremely low numbers in the intestine of uninfected *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice (data not shown), but B cells and dendritic cells are part of the normal intestinal immune populations, so a quantitative analysis of these cell types in uninfected mice must be carried out to know if the differences were present under homeostatic conditions or are apparent only during the response to infection. We cannot rule out that these cells may also play a role in intestinal immune defenses that prevent dissemination; however, specific depletion of neutrophils with an anti-Ly6G antibody during *S.* Typhimurium infection also leads to increased dissemination of the pathogen (see chapter 2).

To better understand the contribution of CXCR2-mediated neutrophil recruitment to the higher *S.* Typhimurium levels observed in *Cxcr2*$^{-/-}$ mouse tissues, we deleted *Cxcr2* selectively on cells of the myeloid lineage (*Cxcr2*$^{fl/-}$ *LysM-Cre*+). Unlike *Cxcr2*$^{-/-}$ mice, which exhibit profound bone marrow and blood neutrophilia and weigh significantly less than their heterozygous or wild-type littermates, *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice grow equivalently to their *Cxcr2*$^{fl/-}$ littermates and do not exhibit neutrophilia (Figures 3.15 and 3.16). These mice exhibited reduced neutrophil recruitment to cecal tissues and increased *S.* Typhimurium dissemination to the liver and mesenteric lymph nodes, but *S.* Typhimurium burden in Peyer's patches and terminal ileum were similar between *Cxcr2*$^{fl/-}$ and *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice (Figure 3.23). These results suggest that

intestinal neutrophil recruitment via CXCR2 is important for reducing *S.* Typhimurium burden in the liver and mesenteric lymph nodes, but the *S.* Typhimurium levels in terminal ileum and Peyer's patches are controlled by other immune cells.

Analysis of the cell types in Peyer's patches of *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice showed significantly fewer B cells in Peyer's patches of *Cxcr2*$^{-/-}$ mice, which also had significantly more *S.* Typhimurium. These data support a role for B cells in modulating *S.* Typhimurium levels in Peyer's patches. Furthermore, the *S.* Typhimurium burden in individual *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice negatively correlates with the number B cells in their Peyer's patches, indicating that B cells are important for the defense against *S.* Typhimurium in Peyer's patches. *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice exhibited both similar *S.* Typhimurium levels and B cell numbers as and *Cxcr2*$^{fl/-}$ mice, demonstrating that Peyer's patch B cell numbers are independent of myeloid *Cxcr2* expression. The deficiency of Peyer's patch B cells in *Cxcr2*$^{-/-}$ mice compared to relatively normal B cell numbers in *Cxcr2*$^{fl/-}$ *LysM-Cre*+ mice suggests that CXCR2 expression on non-myeloid cells is responsible for B cell recruitment and retention in Peyer's patches. We show evidence that B cells express CXCR2, which may be important for B cell migration into or retention inside Peyer's patches.

B cell expression of CXCR2 and response to the CXCR2 chemokine IL-8 have been reported for human B cells (278, 279), and microarrays of pre-pro-B and pro-B cells from C57BL/6 mice detected low levels of *Cxcr2* mRNA (ImmGen.org), but to our knowledge, functional expression of CXCR2 has not been previously demonstrated for mouse B cells. A population of CXCR2-expressing B cells was not clearly identified in Peyer's patches of uninfected mice, which may be from expression below the threshold

of detection, a very small population of CXCR2-expressing B cells, or a requirement for infection to induce detectable levels of CXCR2 expression. We tested the ability of B cells from uninfected mice to respond to CXCR2-specific chemokines, CXCL1 and CXCL2 as an indirect means of showing CXCR2 expression on B cells. We found that B cells from Peyer's patches of *Cxcr2*$^{+/+}$ mice migrated toward CXCL1 and CXCL2 at about 20% the frequency of a known B cell chemokine, CXCL12α, which strongly supports the expression of CXCR2 by at least some B cells in Peyer's patches. The mechanism through which CXCR2 affects the number of B cells in Peyer's patches is an area of future investigation.

The increased *S.* Typhimurium burden in Peyer's patches with fewer B cells strongly suggest that B cells are a critical part of the immune response to *S.* Typhimurium in Peyer's patches. One potential mechanism is that B cells produce IgA, which opsonizes *S.* Typhimurium and impairs its ability to invade the intestinal epithelium (280, 281). However, despite their apparent deficiency of B cells, total fecal IgA levels in *Cxcr2*$^{-/-}$ mice were initially found to be higher than in *Cxcr2*$^{+/+}$ mice, both in uninfected mice and mice 4 days after infection with *S.* Typhimurium. After correcting for control mice that were carrying the *Dock2* mutation, fecal IgA levels were equivalent between *Cxcr2*$^{-/-}$ and *Cxcr2*$^{+/+}$ mice. Nevertheless, the lack of B cells in *Cxcr2*$^{-/-}$ mice did not lead to a reduction in IgA levels, potentially indicating that fecal IgA is not protective against *S.* Typhimurium in Peyer's patches, or that total fecal IgA is not indicative of or proportional to the abundance of protective IgA present. Other hypotheses still under consideration for the role fecal IgA may play in *S.* Typhimurium colonization include: *Cxcr2*$^{-/-}$ mice produce low-affinity or otherwise ineffective IgA for protection against *S.*

Typhimurium, and/or IgA defenses against *S.* Typhimurium are subsequently dependent on a cell expressing CXCR2 for bacterial killing. Additionally, B cell defenses against *S.* Typhimurium may be independent of IgA, as recent studies have identified alternative protective roles for B cells in intestinal immunity and inflammation. In a DSS-induced colitis model, B cells promote expansion of the T regulatory subset, which protected against severe tissue damage (282). In an immunization and challenge model of *Salmonella* typhoid, B cells were important for inducing protective T cell subsets (283). Furthermore, work in mice chronically infected with *S.* Typhimurium shows an inverse relationship between the conditions that favor neutrophil expansion (granulopoiesis) and B cell expansion (284). Although this interaction between innate and adaptive cells was observed during infection, and the imbalance in *Cxcr2*$^{-/-}$ mice is present even in uninfected animals, it suggests that the cytokine environment in *Cxcr2*$^{-/-}$ mice, which is known to promote persistent granulopoiesis (152), may also inhibit specific B cell migration or expansion.

3.6 Potential alternative explanations for the data

While considering mechanisms to explain the influence of CXCR2 on B cell numbers in Peyer's patches, it is important to keep in mind that B cells are not absent from Peyer's patches, but rather significantly fewer in number than in mice expressing CXCR2. Additionally, the mechanism controlling Peyer's patch B cell numbers under homeostatic conditions may be different during infection despite the similar degree of deficiency.

If CXCR2-mediated chemotaxis influences B cell populations in Peyer's patches, the deficiency of B cells in *Cxcr2*$^{-/-}$ could be from reduced recruitment of B cells or increased migration out of Peyer's patches. Studies aimed at understanding population dynamics of Peyer's patches during infection showed that the increase in cell number in Peyer's patches was not due to increased recruitment, but rather decreased egress and therefore retention of cells that migrated into Peyer's patches at a normal or steady rate (285). If CXCR2 is mediating B cell retention in Peyer's patches, we would expect whole Peyer's patch tissue to express ligands for CXCR2. Messenger RNA levels for CXCL1 can be quantified from whole Peyer's patches, and protein expression can be detected through Western blot and ELISA, but antibodies sufficient for immunofluorescence detection are not currently available, so we cannot yet visualize expression patterns of CXCL1 within mouse tissue.

Another hypothesis, not exclusive from a role for CXCR2 in B cell retention in Peyer's patches, is that CXCR2 signaling inhibits B cell differentiation into plasma cells. A similar but indirect hypothesis is that *Cxcr2*$^{-/-}$ mice express more IL-21, a cytokine known to promote IgA-producing plasma cell differentiation. Flow staining for plasma cells dissociated from tissue would allow us to quantify the number and proportion of plasma cells present in *Cxcr2*$^{-/-}$ mice compared to *Cxcr2*$^{+/+}$ mice. The mouse plasma cell marker, syndecan-1 (CD138), is also available for immunofluorescent staining, so we can visualize the distribution of plasma cells within Peyer's patches.

A third potential hypothesis is that CXCR2 signaling on B cells inhibits apoptosis, and the lower number of B cells in *Cxcr2*$^{-/-}$ is from B cell death, which could be measured by looking for annexin-V staining in tissue sections. Determining cell death by

flow cytometry is not ideal since dead cells can take up antibodies non-specifically, so B cells would have to first be purified by positive or negative selection, then stained for viability.

A fourth hypothesis to consider is that the dominant effect on B cells populating Peyer's patches is not from their direct expression of CXCR2, but from the differential cytokine and growth factor concentrations present in the *Cxcr2*$^{-/-}$ mice, such as G-CSF or IL-17. Published reports show that *Cxcr2*$^{-/-}$ mice have significantly higher serum G-CSF, which helps promote their continued production of neutrophils despite exhibiting neutrophilia (152).

Lastly, CXCR2 expression on endothelial cells could be impacting the migration of B cells out of circulation and into Peyer's patches. One possible explanation is the influence of CXCL1 on endothelium, upregulating integrin expression to promote B cell adhesion and migration into the tissue. Utilization of an endothelial-specific Cre with *Cxcr2*$^{fl/fl}$ mice may be the best way to assess the specific role of the endothelium, as isolation of these cells or manipulation in cell culture is unlikely to reproduce the tissue organization and functional consequence that we are seeking to understand. Specific effects of CXCR2 ligands on endothelial cell integrin expression or dynamics of B cell adhesion and migration may be teased out using cell-coated flow cells.

In summary, the data presented herein uncovered a new role for CXCR2 in directing B cell migration to Peyer's patches and for B cell defenses against *S.* Typhimurium colonization of Peyer's patches. Furthermore, this work highlighted the specific role for CXCR2-expressing neutrophils in protecting against dissemination of *S*. Typhimurium to liver and mesenteric lymph nodes in mice.

Chapter 4: Discussion and Future Directions

Chemokines in the intestine are important for establishing a homeostatic immune presence and for inducing an inflammatory response in defense against pathogenic organisms. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection induces dramatic enterocyte production of CXCL1 and CXCL2, chemokines specific for the receptor CXCR2. Consistent with previous studies, the data presented herein shows that the chemokine receptor CXCR2 plays an important role in recruiting protective numbers of neutrophils to the intestinal mucosa during infection with *S.* Typhimurium. Furthermore, our work demonstrates a previously unrecognized role for CXCR2 in homeostatic distribution of B cells in intestinal immune tissue, namely Peyer's patches.

S. Typhimurium gastroenteritis has long been associated with a robust neutrophil infiltration to the intestine, such that fecal neutrophils are one of the diagnostic criteria for *Salmonella* associated diarrhea. Previous studies have implicated that the early neutrophil response creates an inflammatory environment that promotes *S.* Typhimurium dominance over commensal intestinal microbes, but ultimately the antimicrobial functions of neutrophils are important for clearance of *S.* Typhimurium from the gut. The enhancement of *S.* Typhimurium growth during inflammation through acquisition of key nutrients and metal ions were elegantly studied by previous members of the Raffatellu Lab and our colleagues. The present studies were initiated with a focus on the protective effects of neutrophil recruitment to the intestine and its dependence on CXCR2.

The role for CXCR2 in neutrophil recruitment was studied using mice with *Cxcr2* gene deletion, either in all cell types or specifically in granulocytes. Mice lacking CXCR2 expression on neutrophils showed a significant reduction in the number of neutrophils in the intestine after four days of *S.* Typhimurium infection (Figures 3.6 and 3.20). The reduction in neutrophil migration to the intestine was similar to that seen when neutrophils were depleted from circulation with antibodies (Figures 2.1 and 2.4), but mice with total or granulocyte-specific *Cxcr2* gene deletion had abundant neutrophils in circulation. The significant reduction in intestinal recruitment of neutrophils lacking CXCR2 leads us to conclude that CXCR2 is the primary receptor guiding neutrophil migration into intestinal tissue during *S.* Typhimurium infection.

Positive evidence that neutrophils expressing CXCR2 were selectively recruited to the intestinal mucosa is complicated by the endocytosis of CXCR2 after stimulation with high levels of its ligands, such that neutrophils that arrive to the intestine following a CXCL1 gradient downregulate their surface expression of CXCR2. Secondly, the current methods used to digest intestinal tissue for single cell analysis involve use of collagenase, which cleaves surface CXCR2. Further evidence for CXCR2-mediated neutrophil recruitment could be shown by transplant of $Cxcr2^{+/+}$ neutrophils into $Cxcr2^{-/-}$ mice and observing a rescue of neutrophil recruitment to the intestine. The $Cxcr2^{+/+}$ donor neutrophils could be more easily tracked if they expressed GFP or were labeled with dyes such as CFSE or BrdU.

A secondary finding from our studies is that CXCR2 is not the only means of neutrophil recruitment to the intestine, as *Cxcr2* deletion was sufficient to significantly decrease neutrophil migration to the intestinal tissue but not to prevent it entirely. While

the neutrophils present in the intestine of antibody-treated mice or the mice with Cre-driven excision of *Cxcr2* can be explained by incomplete deletion of CXCR2-expressing neutrophils, the presence of intestinal neutrophils in *Cxcr2*$^{-/-}$ mice indicates that one or more weaker, CXCR2-independent signals attract neutrophils during *S.* Typhimurium infection. These secondary signals were not strong enough to provide sufficient resistance to *S.* Typhimurium dissemination, but identifying these additional mechanism(s) of recruitment may reveal a ligand and receptor interaction that can be amplified to enhance neutrophil response. We conclude, therefore, that CXCR2 is the primary receptor mediating neutrophil recruitment to the intestine, and there is not an equivalent, redundant chemokine-receptor system to guide neutrophils to the intestine during *S.* Typhimurium infection.

No human disease associated with aberrant CXCR2 expression has been identified, but patients who have bacteremia or sepsis are at risk for a CXCR2-depleted state, as circulating bacterial antigens and high levels of chemokines can cause downregulation of surface CXCR2 on neutrophils. Therefore, neutrophils of a septic patient would no longer be responsive to CXCR2-specific chemotactic signals from infected or damaged tissue loci of infection. This further highlights the importance of prevention and early intervention in patients that are at increased susceptibility to bacteremia. It also indicates that future treatments for bacteremia may inhibit specific immune signals, a seemingly counter-intuitive idea that exchanges a short-term loss for an overall gain. Inflammatory mediators that induce vasodilation are part of the immune response that leads to sepsis, a state that is characterized by low blood pressure and is associated with high rates of mortality.

We utilized two distinct means of reducing neutrophil migration to the intestine during *S.* Typhimurium infection: neutrophil depletion via antibody injection, and deletion of the primary receptor that guides neutrophil migration, CXCR2. Results from both methods yielded the same conclusion: that fewer neutrophils in the intestine in response to *S.* Typhimurium infection corresponded to higher *S.* Typhimurium dissemination to other tissues of the body. Neutrophils have numerous antimicrobial functions, including phagocytosis, reactive oxygen species and antimicrobial protein production, and NETosis, the specialized extravasation of nuclear contents to ensnare microbes and expose them to antimicrobial proteins. Studies of the individual contributions of neutrophil proteins and functions indicate that production of reactive oxygen species is the single largest contributor to *S.* Typhimurium defense, but the collective action of antimicrobial actions and agents produce optimal protection against *S.* Typhimurium dissemination. Although the specific tissue susceptibility varied between antibody target and full versus specific *Cxcr2* deletion, the consistent pattern of mice with unimpaired neutrophil migration to the intestine also having lower *S.* Typhimurium tissue levels than mice with impaired neutrophil response supports the protective role of neutrophils in limiting spread of *S.* Typhimurium outside the intestine.

The increased susceptibility to *S.* Typhimurium dissemination in a neutrophil depleted state is an important consideration for neutropenic patients, as *S.* Typhimurium is among the top causes of gastroenteritis in the United States and worldwide, and *S.* Typhimurium bacteremia can be fatal. Neutropenia is not just a concern for cancer patients treated with chemotherapeutic agents. Common medications for gout, inflammatory bowel disease, hyperthyroidism, and schizophrenia can cause

neutropenia. Patients should be counseled how to reduce their risk of exposure to such common pathogens, and physicians should consider G-CSF or GM-CSF treatment to promote neutrophil production in patients who have known exposure to potentially invasive organisms like *S.* Typhimurium.

In the course of our studies of neutrophil defense against *S.* Typhimurium infection, mice lacking CXCR2 on all cell types were found to have a dramatically higher (approximately 100-fold) *S.* Typhimurium colonization of Peyer's patch tissue compared to mice with wild-type CXCR2 expression. Surprisingly, neutrophils did not appear to be responsible for this phenotype, as the numbers of neutrophils were not significantly different between *Cxcr2*$^{-/-}$ and *Cxcr2*$^{+/+}$ mice. Analysis of immune cells within Peyer's patches showed that *Cxcr2*$^{-/-}$ mice were significantly deficient in B cells, and the *S.* Typhimurium burden in Peyer's patches correlated inversely with B cell number. The deficiency was not a result of *S.* Typhimurium infection, because Peyer's patches from uninfected mice also contained significantly fewer B cells in *Cxcr2*$^{-/-}$ compared to *Cxcr2*$^{+/+}$ mice. These data suggest a previously unknown function for gut B cells: a protective role against *S.* Typhimurium colonization of Peyer's patch tissue.

In an effort to identify the mechanism by which B cells defend against *S.* Typhimurium in Peyer's patches, we measured fecal IgA levels in *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice. Surprisingly, fecal IgA levels were initially found to be higher in *Cxcr2*$^{-/-}$ mice despite having fewer B cells in Peyer's patches (Figure 3.27). After eliminating data from mice harboring the *Dock2* mutation, fecal IgA levels were similar between *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice (Figure 3.29). Although B cells in Peyer's patches are not the only

potential source for fecal IgA (lamina propria B cells could also contribute to IgA secretion), protection against *S.* Typhimurium in *Cxcr2*$^{+/+}$ mice was unlikely to be mediated by fecal IgA levels. An antibody-independent role for intestinal B cells was recently reported in a chemically-induced colitis model, where B cells stimulated expansion of T-regulatory cells and the consequent increase of the anti-inflammatory cytokine IL-10 in the intestine, thereby promoting recovery (282). We did not analyze subsets of CD4+ T cells from Peyer's patches, so it is possible that the B cell deficiency in *Cxcr2*$^{-/-}$ mice corresponds to a T-regulatory cell deficiency. Inflammatory conditions favor *S.* Typhimurium survival in the intestinal lumen, and higher production of IL-10 after treatment with trans-retinoic acid was associated with lower intestinal tissue and mesenteric lymph node *S.* Typhimurium burden (286). Thus, future experiments may reveal an important link between B cells, T regulatory cell induction, and IL-10 production in Peyer's patch defense against *S.* Typhimurium in naïve hosts. Other potential mechanisms of B cell protection against *S.* Typhimurium, including cytokine production, phagocytosis, and cell-cell signaling remain areas of future investigation. We plan to test if there are functional differences in B cells from *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice, which would argue against a simple "critical B cell mass" for protection against *S.* Typhimurium, but would suggest a functional role for CXCR2 in B cell defenses.

Given the known role for CXCR2 as a chemotactic receptor that directs neutrophil migration, our first hypothesis for the role of CXCR2 in B cells was that of directing migration into Peyer's patches or as a retention signal in Peyer's patches. The chemokine receptor CXCR5 is known to be essential for localization of B cells to Peyer's patches, and CXCR4 expression promotes the egress of B cells from Peyer's

patches. In bone marrow, neutrophils express CXCR4 and CXCR2, with CXCR4 signaling acting as the retention signal and CXCR2 signaling inducing egress from the bone marrow. It would not be surprising, then, if B cells have a similar but inverse relationship with CXCR2 and CXCR4 expression, and without CXCR2 present on B cells, they migrate from Peyer's patches, leaving a reduced and possibly transient B cell population behind. Peyer's patch B cells migrated toward CXCR2-specific chemokines *in vitro,* suggesting that some B cell subsets express CXCR2. To our knowledge, this is the first evidence that B cells respond to CXCR2 signals. Two reports studying peripheral blood from humans demonstrated CXCR2 expression and migration toward CXCR2-specific chemokines, but they did not establish a functional role for the receptor on B cells.

We attempted to clarify the role of CXCR2 on B cells using *Cxcr2*$^{fl/fl}$ *CD19-Cre+* mice and *Cxcr2*$^{fl/-}$ *CD19-Cre+* mice, but we were unable to detect aberrations in Peyer's patch B cell numbers nor *S.* Typhimurium burden when compared to controls. Because we did not detect CXCR2 expression using flow cytometry, we were unable to confirm deletion of CXCR2 expression at the protein level. Further analysis of CXCR2 surface expression using flow cytometry and of *Cxcr2* transcript levels in B cells is being conducted to confirm gene deletion. Although the functional role of CXCR2 on B cells is not yet known, our data shows decreased B cells in Peyer's patches of *Cxcr2*$^{-/-}$ mice and a correlation with elevated *S.* Typhimurium colonization, supporting an important role for B cells in creating an immune barrier to *S.* Typhimurium entry or replication in Peyer's patch tissue. Furthermore, we show indirect evidence that B cells express CXCR2, indicating that this receptor may be part of the immune function of B cells or

have an independent role in B cell localization, such as retaining B cells in Peyer's patches.

The importance of B cells in defense against *S.* Typhimurium in humans is most apparent for people with repeated exposure, as they are able to develop *Salmonella*-specific antibodies that help confer resistance to infection (222). Immunization and challenge studies in mice confirm that *Salmonella*-specific antibodies attenuate gastroenteritis upon repeat infection. Experiments comparing B cell-deficient, antibody-deficient, and wild-type mice revealed an antibody-independent role for B cells via inducing T cell immunity to *Salmonella* (287), as detected through splenic lymphocytes. Future experiments will help determine if this protective interaction of B and T cells against *S.* Typhimurium is also true in gut associated lymphoid tissue (GALT), and, furthermore, if there is protective communication between B and T cells during *S.* Typhimurium infection of naïve human hosts.

The implications of B cell CXCR2 expression on human health depends largely on the role for CXCR2 identified by future studies. For example, if CXCR2 directs B cell migration or retention in Peyer's patches, inflammatory conditions such as inflammatory bowel disease may lead to a previously unappreciated redistribution, differentiation, or even death of B cells.

Peyer's patches in humans are mostly understood on an anatomical level, and are not studied functionally in normal adults. Access to Peyer's patch tissue requires an invasive procedure that does not provide routine diagnostic or curative value, so most human Peyer's patch samples come from patients with inflammatory bowel disease. Ulceration of the tissue overlying Peyer's patches has been observed in patients with

Crohn's disease (CD), but the mechanism leading to this localized breakdown of the epithelial barrier is unclear (288). Crohn's disease patients are more likely to have bacterial translocation out of the intestine, so a site that normally samples the intestinal lumen may be a portal for bacterial escape, but damage to Peyer's patch epithelium could either be a means of allowing uncontrolled translocation or a consequence of inflammatory processes stimulated by the GALT detection of microbes in the intestinal mucosa.

Patients with CD exhibit B cell dysfunction compared to healthy control patients, in that B cells spontaneously secrete more antibody (289) or the pro-inflammatory cytokine IL-8, and express higher levels of TLR2 (290). Production of antibodies against intestinal fungal microbes (*Candida* and *Saccharomyces* spp.), is also common in patients with Crohn's disease, demonstrating an overactive B-cell response to a commensal organism. One recent study found that B-cell activating factor (BAFF) was elevated in serum and feces of patients with Crohn's disease or ulcerative colitis (291), further implicating B cell activation in the pathogenesis of inflammatory bowel disease. B-cell depletion clinical trials with rituximab (anti-CD20) did not induce remission for ulcerative colitis patients (292), but this therapy has not yet been tested for Crohn's disease patients. Future studies in humans and animals need to continue analyzing intestinal and lymphoid tissue in conjunction with peripheral blood (or serum) and fecal samples so we can more confidently correlate findings from the easily accessible samples with the immune activation state in tissues. Inflammatory bowel diseases are increasingly prevalent, so further understanding of intestinal health and immune regulation will potentially help millions of patients receive early diagnoses and effective

interventions to prevent chronic inflammatory sequelae such as strictures, fistulas, and neoplastic changes.

Recent progress in understanding another autoimmune disease, multiple sclerosis (MS), revealed an important role for B cells in contributing to damage of the central nervous system (CNS). High levels of antibodies are consistently observed in cerebral spinal fluid of MS patients (compared to serum antibody levels), indicating excessive proliferation and antibody production by B cells. Although B cells are rarely observed near demyelinated lesions at autopsy, recently formed lesions exhibit large numbers of T and B cells (293). It remains unclear whether B cell antibody production, cytokine secretion, or T cell stimulation primarily contributes to demyelinating flares in MS, but B cell depletion using a therapeutic antibody against CD20 decreased relapse rates compared to interferon beta-1 therapy. The benefit of reducing B cell-mediated damage to neural tissue carries an understood risk of weakened immune defenses against microbes, but it remains to be seen if this includes gastrointestinal pathogens such as *S.* Typhimurium. These patients and their physicians must strike a balance between pathogenic B cell activation and dangerous immune deficiency. As we expand our knowledge of the roles of B cells in circulation, lymphoid organs, Peyer's patches, and even the CNS, we can better predict the effect of B cell antigen-targeted therapy on overall health.

My body of work describes the importance of CXCR2 expression on neutrophil migration to the intestine during infection and suggests a homeostatic role for CXCR2 on B cell localization to Peyer's patches. We have not yet probed a relationship

between these two cell types in mediating host defenses against *S.* Typhimurium. Recent work described B-helper neutrophils in spleens of mice that induce antibody class switching and antibody production of marginal zone B cells in the absence of an acute infection (294). A similar neutrophil function for B cell stimulation may exist in Peyer's patches, and in *Cxcr2*$^{-/-}$ mice, without normal neutrophil migration, B cells may be lacking signals important for their protective functions against *S.* Typhimurium.

By its nature, hypothesis-driven research narrows the focus of gene targets, cell types, and disease contexts so the scale of experiments is manageable and data can be clearly interpreted. With that understanding, we acknowledge this data does not exclude roles for CXCR2 on other cell types, even in the context of *S.* Typhimurium infection, but especially in other types of immune challenges. CXCR2 expression has been reported on other cell types, including dendritic cells, NK cells, and endothelial cells, but its role in these cell types is not clear. Furthermore, we analyzed tissues known to be involved in *S.* Typhimurium infection, but other tissues in the body may be home to CXCR2-expressing cells that contribute to host defense against *S.* Typhimurium. Microarray analysis of whole tissue has helped identify numerous important targets, but this broad approach is still biased by the dominating cell populations in the tissue. Detecting a new role for a protein, because of low or conditional expression, or expression by a minor cell type, requires an unbiased approach and analysis of individual cell types. Our identification of a previously unrecognized role for CXCR2 on B cells underscores the likelihood of finding broader expression patterns and more roles for chemokine receptors. Future work focused on

identifying cell-type specific roles for chemokine receptors will add to our appreciation of coordinated immune responses but undoubtedly stimulate more questions.

Despite the extraordinary scientific benefit of transgenic mouse models for identifying the purpose of individual genes and proteins, we must keep in mind the genetic diversity that is present within a mouse line. Even ignoring epigenetic influence on gene expression, increasing reports have identified diversity among mouse lines that are identical by nomenclature. Results from relatively few mice of a single line at one institution must be interpreted with due caution that the phenotype may be from unanticipated changes, whether through chromosomal linkage or chance of breeder mouse selection. Simple crossing of transgenic lines, as is commonplace with flox-Cre deletion systems, likely involves non-target gene variability that introduce random and systematic error into data. Even if we sequenced each animal, we would have to do an epigenome, transcriptome, proteome, and microbiome to truly know the homogeneity of the effects we are observing. In the absence of this overwhelming amount of data, we must settle for multi-pronged approaches to determine the functional roles for individual proteins.

Our study highlights the importance of considering transgenic mice as complex organisms with unpredictable variability in their genome. Using the high standard of co-housed littermates, and consistent C57BL/6J backgrounds, we originally generated data with the opposite conclusion of that shown here. By challenging that unexpected result and more thoroughly genotyping our mice, we found a major *S.* Typhimurium susceptibility gene, *Slc11a1* (NRAMP1), was carried forward from the SvJ/129 mouse line of origin despite more than a decade of crossing with C57BL/6 mice because it is

linked to our gene of interest, *Cxcr2*. Only after generating a *Cxcr2*$^{-/-}$ line with homogeneous expression of *Slc11a1 G169*, were we able to correctly compare *S.* Typhimurium susceptibility between *Cxcr2*$^{+/+}$ and *Cxcr2*$^{-/-}$ mice. In addition, the flox-Cre system used for granulocyte-specific deletion of *Cxcr2* was variably efficient between mice, and the phenotype of *S.* Typhimurium susceptibility changed with subsequent generations of mice, which now appears to be explained by the introduction of a *Dock2* mutation from the *Cxcr2*^{flox} mice. We thank Mahajan et al for their efforts in identifying the likely origin of this mutation and its widespread distribution in mouse colonies around the world.

Intestinal immunity is a challenging field because *in vitro* models are supremely insufficient, the microbial populations in the gut are dynamic and incredibly complex, and the interplay between intestinal health and the rest of the body is just beginning to be appreciated. Our work revealed the importance of neutrophil recruitment to the intestinal mucosa and B cell population of Peyer's patches for defense against *S.* Typhimurium infection in mice, and future experiments will delve deeper into the contribution of B cells in acute inflammatory gastroenteritis. Understanding the B cell population in the intestinal mucosa, whether it is the absolute number, cytokine production potential, immunoglobulin production, or phagocytic activity that is important for defense against pathogens is essential for proper modeling of disease and design of interventions. We anticipate increasing interest in intestinal B cell functions and how cellular dynamics of Peyer's patches influence protective immune responses to intestinal pathogens, and we are eager to participate in the advancement of the field.

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