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Host-Microbe Competition for Zinc in the Inflamed Gut

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Author
Liu, Janet Zhen-Yi

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Host-Microbe Competition for Zinc in the Inflamed Gut

DISSEPTION

Submitted in partial satisfaction for the requirement for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Janet Zhen-Yi Liu

Dissertation Committee:
Professor Manuela Raffatellu
Professor Ming Tan
Professor Alan G. Barbour
Professor Marian L. Waterman
Professor Robert A. Edwards

2014
DEDICATION

To

My parents, and my grandparents.

爸爸，妈妈，

阿公，阿婆，

爷爷，奶奶，

之所以有今日的我是因为有您们的关爱与呵护。

我想从心里说一声：谢谢您们！
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CURRICULUM VITAE

Janet Zhen-Yi Liu

Education

2008-2014 Doctor of Philosophy in Biomedical Sciences
Department of Microbiology and Molecular Genetics
University of California, Irvine

2004-2008 B.S. in Biological Sciences (minor in Chinese)
University of California, Davis

Honors and Awards

Graduated with Honors at University of California, Davis

2nd Prize 2010 Immunology Fair Poster Award winner from University of California, Irvine, Institute for Immunology

Research experience

2008 Internship – Epidemiology of vector-borne diseases
Supervisor, Dr. Janet Foley, UC Davis School of Veterinary Medicine

2008 Graduate Rotation – Fall Quarter
Supervisor, Dr. Ulrike Luderer

2009 Graduate Rotation – Winter Quarter
Supervisor, Dr. Haoping Liu

2009 Graduate Rotation – Spring Quarter
Supervisor, Dr. Manuela Raffatellu

Teaching experience

2007 Tutor for General Microbiology laboratory (MIC102L)
Supervisor, Dr. Eric Mann, UC Davis

Past employment

2007-2008 Assistant Lab Technician - Comparative Pathology Laboratory
Graduate school activities

2010 – 2011  Student Seminar Committee

2010 – 2011  Graduate Student Representative

Foreign languages

Chinese: speak, read and write fluently

Publications


   • Selected as a Featured Article in the May 2011 issue of *Cell Metabolism*
   • Selected as Research Highlight in *Nature Reviews Immunology* (2011) June 11: 368-369
   • Evaluated by *Faculty of 1000* as “Must Read”

   • Selected as a Featured Article in the March 2012 issue of *Cell Host & Microbe*
   • Featured in Editor’s Choice in *Science* (2012) Mar 30: 1546
   • Selected as Research Highlight in *Nature Reviews Microbiology* (2012) May 10: 309
   • Evaluated by *Faculty of 1000* as “Must Read”


   • J. Z. Liu created the cover illustration for the (2013) Jul 17, volume 14 issue of *Cell Host & Microbe*
• Selected as a Featured Article in the July 2013 issue of Cell Host & Microbe
• Preview by Guenther Weiss “Intestinal irony: how probiotic bacteria outcompete bad bugs”, Cell Host and Microbe. (2013) July 17(14) pg3-4
• Selected for discussion by the This Week In Microbiology podcast, August 7th 2013
• Selected as Editor’s Choice in the August 2013 edition of Science Translational Medicine


Book chapters


Presentation abstracts

Immunology Fair, 2009 – University of California, Irvine. The creation and characterization of a zinc uptake mutant in Salmonella Typhimurium as a new tool for studying the role of calprotectin-mediated zinc withholding in the inflamed gut mucosa.
Immunology Fair, 2010 – University of California, Irvine. Resistance to calprotectin-mediated zinc withholding promotes *Salmonella Typhimurium* growth in the inflamed gut.

Midwinter Conference of Immunologists, 2011 – Asilomar, CA. Resistance to calprotectin-mediated zinc withholding promotes *Salmonella* Typhimurium growth in the inflamed gut.

FASEB Microbial Pathogenesis: Mechanisms of Infectious Disease, 2011 – Snowmass Village, CO. Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut.

Immunology Fair, 2011 – University of California, Irvine. Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut.


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ABSTRACT OF THE DISSERTATION

Host-Microbe Competition for Zinc in the Inflamed Gut

By
Janet Zhen-Yi Liu

Doctor of Philosophy in Biomedical Sciences
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Professor Manuela Raffatellu, Chair

Salmonella enterica serovar Typhimurium is a common food-borne pathogen that causes localized enteritis in healthy individuals. A general host strategy to limit the growth of bacterial pathogens is to produce factors to limit the availability of metal nutrients such as iron and zinc, a concept termed “nutritional immunity”. Calprotectin is one such host-produced antimicrobial protein that binds and sequesters zinc and manganese ions from resident and invading microbes. It has been shown in previous studies that the two subunits of calprotectin, S100a8 and S100a9, are highly up-regulated during S. Typhimurium gastroenteritis, suggesting calprotectin is highly expressed during S. Typhimurium infection.

Despite this, S. Typhimurium replicates to high numbers in the inflamed gut. We therefore hypothesize S. Typhimurium is resistant to calprotectin-mediated zinc withholding, and that this is one of the mechanisms exploited by this pathogen to thrive in the inflamed gut. Using a mouse model of S. Typhimurium gastroenteritis, we found that calprotectin induction does not inhibit S. Typhimurium proliferation in the inflamed gut. S. Typhimurium overcomes calprotectin-mediated zinc chelation by expressing a high affinity zinc transporter, ZnuABC, thereby promoting its own growth over competing microbes. A znuA mutant exhibited a severe
colonization defect in competition with wild-type *S*. Typhimurium. This colonization defect is diminished in the absence of either calprotectin or inflammation.

ZnuABC is also expressed by *E. coli* Nissle 1917 (*E. coli* Nissle), a probiotic that has been shown to be able to reduce *S*. Typhimurium levels in the inflamed gut. We hypothesized that zinc transporters such as ZnuABC are also important for *E. coli* Nissle probiotic activity and demonstrated that a zinc transporter mutant *E. coli* Nissle was unable to reduce *S*. Typhimurium colonization. Furthermore, the ability of *E. coli* Nissle to reduce *S*. Typhimurium colonization was dependent on the presence of host calprotectin protein, as *E. coli* Nissle was unable to reduce *S*. Typhimurium colonization in calprotectin-deficient mice. These findings have broadened our understanding about the role of zinc in health and disease and have important implications for understanding the mechanisms of probiotic activity and pathogen colonization.
Chapter 1

Introduction to Non-typoidal *Salmonella* Infection

1.1 Biology of *Salmonella*

*Salmonella* is a genus of rod-shaped Gram negative, facultative anaerobe bacteria in the family Enterobacteriaceae, a group that also includes the commonly used laboratory bacterium, *Escherichia coli*. *Salmonella* is a predominantly motile bacterium, with peritrichous flagella providing propulsion. It was first isolated in 1885 by Dr. Theobald Smith of the US Department of Agriculture. The genus was subsequently named after his supervisor, Dr. Daniel Elmer Salmon, in 1900 (1). *Salmonella* nomenclature and classification can be confusing, as some strains were historically recognized as species rather than serovars. Currently the *Salmonella* genus is divided into two species: *Salmonella bongori* and *Salmonella enterica*. The *S. enterica* species is organized into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (103). The subspecies are in turn classified into more than 2500 serovars based on the lipopolysaccharide O antigen and the flagellar H antigen. Despite the diversity in phylogenetic composition, the majority of human pathogenic *Salmonella* is found within *Salmonella enterica* subspecies *enterica* (*S. enterica ssp. enterica*). Fig. 1.1 shows a cladogram of the *Salmonella* genus, including some prominent human pathogenic serovars in subspecies *enterica*. Many *S. enterica ssp. enterica* serovars infect a wide range of warm-blooded animals, though some serovars preferentially infect a specific host such as pigs, which are the common host for serovar Choleraesuis. Other *S. enterica ssp. enterica* serovars are host specific, and will only naturally infect one species of host. For example, serovar Gallinarum infects only chickens, while serovar Typhi is a human specific pathogen.
**Figure 1.1 Cladogram of the genus *Salmonella*.** The genus is composed of two species, *S. bongori* and *S. enterica*. Within enterica there are six subspecies; most human pathogenic *Salmonella* strains are found within the subspecies enterica, including serovars *S. Typhi*, *S. Paratyphi* A and B, and *S. Typhimurium*. Image modified from Porwollik et al., 2002 (71).
Figure 1.1

- E. coli
- S. bongori
- S. enterica ssp. houtenae
- S. enterica ssp. arizonae
- S. enterica ssp. diarizonae
- S. enterica ssp. salamae
- S. enterica ssp. indica
- S. enterica ssp. enterica serovar Paratyphi A
- S. enterica ssp. enterica serovar Typhi
- S. enterica ssp. enterica serovar Paratyphi B
- S. enterica ssp. enterica serovar Typhimurium
Serovars Typhi and Paratyphi are, respectively, the causative agents of the systemic diseases typhoid fever and paratyphoid fever, and are collectively known as enteric fever. The localized inflammatory diarrheal infection that the public commonly associates with *Salmonella* is a very different disease than enteric fever. To distinguish the two types of diseases in the clinic, *Salmonella* serovars are divided into typhoidal and nontyphoidal serovars. Typhoidal *Salmonella* serovars include the previously discussed Typhi and Paratyphi, while nontyphoidal *Salmonella* (NTS) serovars include Typhimurium and Enteritidis. Unlike the host restricted typhoidal serovars, NTS serovars can infect a wide range of animals, including humans and domesticated animals. Patients infected with NTS serovars will develop fever, a localized intestinal infection with a strong inflammatory response, and diarrhea with large number of neutrophils in the stool. A fever is also commonly seen during typhoid infection, however typhoidal *Salmonella* infection affects multiple organs and typhoidal *Salmonella* can colonize the gallbladder in a small fraction (approximately 4%) of patients, leading to a chronic carrier state that can persist for years (97). Diarrhea is observed in less than 60% of typhoid patients, and unlike NTS infection, mononuclear cells are the predominant immune cells in the stool (14, 105). Furthermore, the magnitude of the inflammatory response during typhoidal *Salmonella* infection is lower compared to NTS (77). One reason why typhoidal *Salmonella* such as *S. Typhi* elicits a dampened inflammatory response in comparison to NTS is due to the expression of certain virulence factors that prevents the immune system from recognizing *S. Typhi*. One key virulence factor is the Vi-antigen, which is found in *S. Typhi* but not in NTS, including *S. Typhimurium* (21). The Vi-antigen forms a capsule that helps to cloak the bacterium and prevent activation of host Toll-like receptor 4, which recognizes bacterial lipopolysaccharide (110). Despite having certain virulence factor differences such as the Vi-antigen, *S. Typhi* and *S. Typhimurium* are
nearly 90% identical in their genome sequence. However, genomic studies have found that more than 200 genes that function in S. Typhimurium are inactivated or disrupted in S. Typhi (65). Some of the inactivated genes include those involved in anaerobic metabolism, and suggests that as S. Typhi became more host adapted as a systemic human pathogen it lost function in genes that NTS such as S. Typhimurium would need to survive in the oxygen-poor inflamed gut (69).

Due to significant differences in clinical manifestation and molecular pathogenesis, infections caused by NTS and typhoidal Salmonella are very distinct from one another, and it is important to distinguish between the two types of Salmonella infection in the clinic as well as in a research setting. This dissertation will focus on NTS, specifically S. Typhimurium, which has been used for many years to explore functions of the host immune response, and is a leading model pathogen in the study of host-pathogen interaction.

1.2 S. Typhimurium and Disease

Salmonella is found worldwide and is estimated to be responsible for 3 billion infections every year all over the world (17). However, data regarding NTS is hard to obtain, as most patients with NTS infections do not report to hospitals. It is estimated that globally there are around 93.8 million cases of NTS infections, with 155,000 deaths every year (64). In the United States an estimated 1.4 million people become infected with NTS, and NTS are the leading cause of death from foodborne illness (66, 87). While many NTS strains are capable of causing gastroenteritis, S. Typhimurium and S. Enteritidis are the most commonly isolated serovars from patients with Salmonella related food poisoning (73).

A major source of S. Typhimurium infection is from contaminated food sources, such as undercooked meat, poultry, eggs, as well as tainted water sources and contact with natural
carriers of NTS, such as reptiles. The incubation period for S. Typhimurium infection is usually less than 48 hours (35). The infectious dose for healthy adults is ≥10⁶ cells (8). However, this number can be lower for individuals with reduced stomach acidity, such as those taking antacids or drugs that inhibit stomach acid production, as well as for the very young, the elderly, and individuals with immunocompromised immune systems (19). The typical symptoms of S. Typhimurium infection are fever, headache, abdominal pain, malaise, nausea, and inflammatory diarrhea with stool containing high numbers of neutrophils. The severe intestinal inflammation and neutrophil influx differentiate inflammatory diarrhea from secretory diarrhea caused by viral infection or bacterial toxin such as cholera toxin, where there is mild or no fever and the absence of white blood cells in the stool. S. Typhimurium infection in immunocompetent patients will typically resolve in a week without the need of medical treatment, however bacteria may continue to be shed for up to a month in the stool (12). Oral rehydration therapy can be used in patients exhibiting dehydration due to severe fluid loss from diarrhea (22). Antibiotic treatment is not recommended unless in instances of bacteremia or in high-risk patients, as antibiotic resistance is widespread and antibiotics may prolong Salmonella shedding (68). HIV patients with NTS infection have higher rates of developing bacteremia (34). In sub-Saharan Africa, where AIDS prevalence is high, invasive NTS, predominantly S. Typhimurium or S. Enteritidis, is one of the leading causes of febrile infection in HIV patients (78). The emergence of multidrug resistant NTS (i.e. resistance to ampicillin, cotrimoxazole, and chloramphenicol) has complicated treatment and necessitated the use of third generation fluoroquinolone antibiotics (39, 50).

Diagnosis of NTS infection is performed by isolating the pathogen from stool samples of inflammatory diarrhea patients, or from the blood of bacteremic patients. Salmonella are non-lactose fermenting and appear colorless on MacConkey agar. Salmonella is also capable of
producing hydrogen sulfide gas, and in triple sugar iron (TSI) agar. S. Typhimurium produces ferrous sulfide that appear as a black precipitate. MacConkey and TSI agars are commonly used for the selection of a number of enteric bacteria and are not specific for identification of *Salmonella*. For more specific isolation of *Salmonella*, tetrahionate (TT) broth, Hektoen enteric (HE) agar, *Salmonella-Shigella* (SS) agar, and xylose-lysine-deoxycholate (XLD) agar can be used. Serovar can be determined using agglutination assay in which antiserum for a specific O or H antigen is mixed with a sample of bacteria. Granular clumping of the cells indicates a positive result (51).

**1.3 Animal Models of S. Typhimurium Infection**

Much of what is known about *S. Typhimurium* pathogenesis in humans was discovered in *S. Typhimurium* infected patients who have immunodeficiencies. The importance of phagocytes in limiting *S. Typhimurium* infection to the gut was gleaned from patients with chronic granulomatous disease (CGD). CGD patients have defective phagocyte NADPH oxidase (PHOX), an enzyme associated with phagocytes such as neutrophils and macrophages. PHOX plays a central role in the generation of reactive oxygen species, which the host uses to kill bacteria, including *S. Typhimurium*, during infections. Patients with CGD are deficient in bacteria killing, and have recurrent infections as well as increased susceptibility to developing septicemia from NTS infections (39). Another important pathway in the control of NTS like *S. Typhimurium* is the IL-12/IL-23 axis. IL-12 and IL-23 are cytokines secreted by antigen presenting cells (APCs) upon contact with bacteria, and are important for stimulating T cells. Patients with Mendelian susceptibility to mycobacterial disease (MSMD) have defects in the *IL12RB1* gene or the *IL12B* gene, both of which are shared between the IL-12 and IL-23
pathways, and have increased susceptibility to non-tubercular *Mycobacteria* and invasive NTS infections (107). Defects in IL-23 signaling abrogated the secretion of IL-17, a key cytokine that is necessary for the prevention of *S. Typhimurium* dissemination (37, 60). IL-17 also has an important role in the susceptibility of HIV patients to NTS infections. HIV patients are highly vulnerable to invasive NTS infections, as discussed in section 1.2, but the mechanism for increased susceptibility to NTS in this group of immunocompromised patients was not clear. To study HIV and NTS infection in real-time, an animal model was needed that is permissive to both kinds of infections.

Rhesus macaques, which are genetically 93% identical to humans (33), are naturally susceptible to *S. Typhimurium* inflammatory diarrhea and can be infected with SIV, an immunodeficiency virus related to HIV, making them an ideal animal model for studying NTS/HIV coinfection (20, 53). Using this animal model it was found that a preexisting SIV infection alters the host immune response to *S. Typhimurium*. During SIV infection the virus targets and depletes CD4⁺ T cells, including T helper (Th) 17 cells that produce the cytokines IL-17 and IL-22. IL-17 and IL-22 signaling is necessary for neutrophil recruitment and antimicrobial peptide expression, and help to maintain the mucosal barrier against bacterial infections (4, 60). In SIV and *S. Typhimurium* coinfected macaques the expression of IL-17, IL-22, the CXC chemokine IL-8, and the antimicrobial protein lipocalin-2 are attenuated compared to rhesus macaques that were infected with only *S. Typhimurium*. The resulting defects in intestinal mucosal barrier thereby contribute to the accelerated dissemination of *S. Typhimurium* to mesenteric lymph nodes, and mimic the disseminating NTS infections seen in HIV patients (76).
While rhesus macaques are an ideal model for HIV-NTS coinfection, many of the molecular details about early S. Typhimurium inflammatory diarrhea were elucidated in the calf model. Calves orally infected with S. Typhimurium develop a gastrointestinal inflammatory disease that closely resembles human inflammatory diarrhea. Infected animals will exhibit fever, diarrhea, profuse neutrophil infiltration, and pseudomembrane formation due to necrosis of the intestinal upper mucosa (104). Oral infections in calves have contributed to understanding the importance of S. Typhimurium virulence factors in the induction of inflammatory diarrhea. The type III secretion system (T3SS) is a needle-like, macromolecular structure that allows Salmonella to inject protein effectors into the host cell cytosol. S. Typhimurium encodes two T3SS: T3SS-1, which translocates effectors that lead to host cell actin rearrangement and bacterial invasion (30); and T3SS-2, which is necessary for survival in the macrophage phagosome (13). Using the calf model, it was found that a T3SS-1 deficient mutant S. Typhimurium could not induce inflammation and diarrhea, and that a T3SS-2 deficient mutant induced less inflammation in calves (104).

In addition to oral infection, the study of ligated ileal loops in calves has allowed for the study of early S. Typhimurium-induced pathology, as well a detailed assessment of the roles different effectors and virulence factors play in inducing inflammation and diarrhea. To conduct an ileal loop experiment, anesthetized animals first undergo laparotomy, after which the ileum is exposed and small sections are ligated using surgical threads to form loops (109). Each loop is injected with an inoculum of Salmonella strains, and tissue damage, neutrophil infiltration, and fluid secretion can be assessed (84, 85). Using the ligated ileal loop model Santos et al. were able to establish the rapid development of gross pathology in the early minutes to hours of S. Typhimurium pathogenesis (85). Additionally, the specific contribution of various S.
Typhimurium virulence factors to pathogenesis can be assessed by the injection of different mutants into individual loops within the same animal. A reduction in inflammation indicates, for example, that a specific T3SS-1 effector plays a role in inducing gastrointestinal disease (117, 118). Furthermore, the calf model revealed a role for flagellin in inflammation, as animals infected with flagellin deficient mutants of S. Typhimurium induced less fluid secretion (a criterion that is used as a measurement of diarrhea) and neutrophil infiltration (88, 112). However, despite their significant contributions to the study of S. Typhimurium pathogenesis, large animal models like macaques and calves also have severe limitations. This includes the lack of transgenic animals and the limited availability of facilities capable of housing large animals. Mice, which are more permissive for genetic manipulation and more readily bred and housed, are the most commonly used vertebrate model for S. Typhimurium infection.

In mice S. Typhimurium infection causes a systemic disease (mouse typhoid) that is similar to human typhoid fever, with dissemination of the bacteria to the spleen and liver, and pathology such as follicular hyperplasia, ulceration of the Peyer’s patches, and a predominantly mononuclear leukocyte infiltration to the small intestine (86). The similarities in disease phenotype have led to the establishment of S. Typhimurium infection in mice as a mouse model for human typhoid. Many seminal discoveries in S. Typhimurium pathogenesis were made using the mouse typhoid model. These include the identification of the key virulence factor T3SS-2 in a signature-tagged mutagenesis screen in 1995 (46), and the discovery of the avirulent, immunogenic aroA mutant in 1981, which contributed to human S. Typhi vaccine developments (47, 98) and our understanding of the adaptive immune response to S. Typhimurium infection (82, 93). More recently the mouse typhoid model has been used to understand the chronic carrier
state of typhoid disease and *S.* Typhimurium transmission in naturally resistant 129X/1/SvJ mice (10, 56, 57).

The 129X/1/SvJ mice are resistant to *S.* Typhimurium infection because the animals are able to control the infection at systemic sites (49, 80). In contrast, many commonly used mouse lines, (such as C57BL/6 and BALB/C) are predisposed to lethal *S.* Typhimurium infection. The difference in susceptibility to *S.* Typhimurium infection is due to a single nucleotide mutation in *Slc11a1*, which encodes the divalent ion transporter NRAMP1 found on macrophage phagosome membranes (6). The mutation changes amino acid 169 from a Gly to an Asp, which renders NRAMP1 nonfunctional and renders the animal extremely susceptible to a number of intracellular pathogens, including *S.* Typhimurium (108). Mouse lines with functional NRAMP1 (such as 129Sv) are therefore resistant to *S.* Typhimurium, while mouse lines with nonfunctional NRAMP1 (such as C57BL/6) are highly susceptible to *S.* Typhimurium infection. However, many genetically modified mouse lines are in the C57BL/6 background, making it difficult to study long-term *S.* Typhimurium infection in transgenic mice in the C57BL/6 background. To overcome this, a functional NRAMP1 (*Slc11a1*) allele was transferred from 129Sv mice into C57BL/6 mice, which reestablished resistance to *S.* Typhimurium in these animals (40).

While the mouse typhoid model has been important in expanding our understanding of *S.* Typhimurium pathogenesis, it does not emulate the strong gastrointestinal inflammatory response that is a hallmark of inflammatory diarrhea seen in humans. Orally infected mice do not develop inflammation of the intestine, and do not exhibit diarrhea. This is in part due to the fact that the gut is colonized by a diverse, microbial community termed the intestinal microbiota. The microbiota helps to protect the host by resisting colonization of exogenous bacteria. This protective effect results in poor colonization of *S.* Typhimurium in the gut of mice following oral
infection. However, this colonization resistance can be overcome by oral gavage of streptomycin, an antibiotic that disrupts the microbiota and allows *S. Typhimurium* to colonize to high levels in the gut (9, 72, 95). In addition to altering the microbiota, streptomycin induces mild inflammation in the gut, which is beneficial to the growth of Enterobacteriaceae, including *S. Typhimurium* (91) (the mechanisms by which inflammation benefits *S. Typhimurium* will be discussed in detail in section 1.5). Although streptomycin treatment does not replicate the diarrhea phenotype of human patients in mice, and the animals will eventually develop a systemic infection, the antibiotic treatment is able to replicate the strong intestinal inflammatory response seen in inflammatory diarrhea. Wolf-Dietrich Hardt and his group showed in 2003 that in mice pretreated with streptomycin *S. Typhimurium* infection induced neutrophil infiltration and intestinal pathology similar to that observed in human gastroenteritis in the mouse cecum, and to a lesser degree, in the colon as well (3). The physiological relevance of this model was further supported by work from various groups that confirmed the roles of T3SS-1, T3SS-2, and flagellin in the streptomycin treated mice, which had previously been observed in the calf model (15, 42, 94). The streptomycin-pretreated model of mouse gastroenteritis is now commonly used to study *S. Typhimurium* gastroenteritis, and is the primary animal model of *S. Typhimurium* pathogenesis discussed in this dissertation.

### 1.4 Molecular Pathogenesis of *S. Typhimurium*

The following section will discuss the current model of *S. Typhimurium* pathogenesis and the host immune response following infection. *S. Typhimurium* transmission is carried out via the fecal-oral route. After ingestion and passage through the stomach, *S. Typhimurium* comes into contact with host intestinal epithelial cells and M cells. *S. Typhimurium* use T3SS-1 to inject a
variety of effector proteins into the host cell and induce actin cytoskeleton remodeling, resulting in characteristic ruffling of the cell membrane and internalization of S. Typhimurium (27, 92). Once inside intestinal epithelial cells, S. Typhimurium translocates from the apical to the basolateral side of the cell, where it then enters the lamina propria. APCs in the lamina propria can phagocytose S. Typhimurium cells, and the bacteria are contained within vacuoles termed Salmonella containing vacuoles (SCV) (Fig 1.2). To avoid being killed by the host lysosome pathway, S. Typhimurium express T3SS-2 and secrete effectors into the cell cytosol that disrupts the fusion of the SCV with lysosomes (96). Once the SCV is established, S. Typhimurium can replicate within the vacuole, and is transported to systemic sites via the infected phagocyte.

Although S. Typhimurium is able to subvert killing by lysosome fusion, it is detected by Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which are able to recognize pathogen-associated molecular patterns (PAMPs) that are shed or secreted by S. Typhimurium (111). TLRs (such as TLR4 and TLR5, which detect LPS and flagellin respectively) are expressed on the outer membrane of host cells (44, 52, 99). On intestinal epithelial cells TLR5 are localized to the basolateral side of the cell in order to differentiate between commensal bacteria and invasive bacteria like S. Typhimurium (32). In contrast, NLRs (such as NLRC4/Ipaf, which recognizes flagellin) are present in the cytosol (26). Binding of TLRs and NLRs to their respective ligands result in the activation of proinflammatory pathways, and the production of neutrophil attracting chemokines and inflammatory cytokines such as IL-1β, IL-18, TNF-α and IL-23, which serve to amplify the immune response (11, 101).

IL-23 acts as a mediator in the amplification of the host immune response, as it promotes the development and function of proinflammatory Th17 cells (120) (Fig. 1.2). Th17 cells are a distinct lineage from Th1 and Th2 cells, and are characterized by the release of a subset of
Figure 1.2 *S. Typhimurium* pathogenesis. During inflammation caused by *S. Typhimurium* infection, cytokine signals released by T cells promote the secretion of CXC chemokines. This leads to infiltration of neutrophils and the release of antimicrobial proteins by neutrophils and epithelial cells into the lumen of the intestine. The microbiota is susceptible to these antimicrobial peptides and their numbers become diminished, but *S. Typhimurium* is resistant to some of these antimicrobial peptides and is able to colonize the inflamed gut.
Figure 1.2

Salmonella

Microbiota

Intestinal Lumen

Antimicrobial Proteins

APC

Neutrophils

CXC Chemokines

IL-23

T cell

IL-17

IL-22

Lamina Propria
cytokines, including IL-17 and IL-22. Although Th17 cells were initially found in rhesus macaques to be the primary source of IL-17 and IL-22 during S. Typhimurium infection, recent studies have shown that in the mouse NK cells and γδ T cells produce IL-17 and IL-22, and that innate lymphoid cells are another source of IL-22 (7, 90). Receptors for IL-17 are found on several cell types, including APCs and epithelial cells, while IL-22 receptor is restricted to non-myeloid cells of the epithelium (29, 115, 116). IL-17 and IL-22 expression increase in the intestinal mucosal of streptomycin pretreated mice, calves, and rhesus macaques following S. Typhimurium infection (36, 75, 76).

The two cytokines orchestrate the mucosal immune response to S. Typhimurium in a number of ways. One important function of the IL-17/IL-22 pathway is to induce expression of granulocyte colony stimulating factor (G-CSF) (2, 70, 101). G-CSF stimulates granulopoiesis in the bone marrow and enhances neutrophil function (79). Another important function of IL-17 and IL-22 is to stimulate and enhance the production of CXC chemokines such as CXCL1 by mucosal epithelial cells (81). The chemokines form a concentration gradient that guides the expanded neutrophil population to the intestinal mucosa where they are able to clear S. Typhimurium from the tissue (83) (Fig. 1.2). This neutrophil barrier is impaired in neutropenic and HIV patients, who are therefore highly susceptible to S. Typhimurium dissemination and developing bacteremia (106). While neutrophils have an important protective effect, neutrophil influx is a double-edged sword for the host, as it also disrupts the intestinal epithelial cell layer, resulting in increased inflammation and tissue necrosis during S. Typhimurium infection (67, 104). Activated neutrophils also release 5’-AMP that can stimulate the secretion of chloride ions from epithelial cells, thereby contributing to the development of diarrhea during infection (63). Thus, while the neutrophil stimulating and recruiting arm of the IL-17/IL-22 pathway is
important for defense against *S. Typhimurium* dissemination, the resulting collateral damage to the host contributes to the disease phenotype associated with inflammatory diarrhea.

In addition to their role in neutrophil infiltration, IL-17 and IL-22 also induce several antimicrobial responses in the mucosa that are important for limiting the growth of microbes. Expression of the C-type lectin Reg3γ is dependent on IL-22, and Reg3γ and has been found to be important in controlling *Clostridium rodentium* infection in mice (119). β-defensins, which form a major family of antimicrobial peptides in mammals, can be induced by IL-17 during *Candida albicans* infection, and β-defensin 3 has been shown to have candidacidal activity *in vitro* (16). Meta analysis of IL-22 stimulated human T84 cells with ileal loop tissue from *S. Typhimurium* infected rhesus macaques has also shown that there is a significant overlap in the expression of antimicrobial genes, including *Muc4*, *Nos2*, and *Lcn2*, which encodes the antimicrobial protein lipocalin-2 (76). Another antimicrobial protein that is highly induced in the mucosa by IL-17 and IL-22 is calprotectin, which is a heterodimer of the S100A8 and S100A9 peptides (4, 59, 119) (Fig. 1.2). Calprotectin is highly abundant in neutrophils, and can constitute up to 40% of neutrophil content (100). Furthermore, it is expressed by epithelial cells upon stimulation with IL-17 and IL-22 (59, 119). Both lipocalin-2 and calprotectin exert their antimicrobial activity by limiting the availability of metal nutrients to microbes (18, 25).

However, despite the induction of antimicrobial proteins and clearance of *S. Typhimurium* from intestinal tissue by neutrophils, *S. Typhimurium* is still able to highly colonize the lumen of the inflamed intestine (83). This finding is surprising, because conventionally inflammation is thought of as an antibacterial response that suppresses bacterial growth. This is evidenced by the significant reduction in Bacteroidetes and Firmicutes, two groups that normally form the bulk of the endogenous microbiota, during *S. Typhimurium* infection (Fig. 1.2). The reduction in
Bacteroidetes and Firmicutes in the inflamed gut is accompanied by an increase in Enterobacteriaceae, the group of bacteria that includes *E. coli* and *S. Typhimurium*, and which normally constitute only a small portion of the microbiota (62, 95). This observation suggests that *S. Typhimurium* and other closely related bacteria benefit from inflammation, and have evolved mechanisms that allow them to overgrow in the inflamed gut environment. Some of these mechanisms have recently been discovered, and will be summarized in the next section.

### 1.5 *S. Typhimurium* in the Inflamed Gut

As I alluded to in the previous chapter, the inflamed gut is a hostile environment for most microbes and the host immune response leads to dramatic changes in the intestinal environment. One of the major changes that occur is the clearance of luminal content due to diarrhea. The absence of luminal content means there are few nutrients available for the remaining bacteria to use for growth. *S. Typhimurium* and related bacteria such as *E. coli* have evolved to utilize host mucus as the primary source of carbon and nitrogen (54). *S. Typhimurium* is also able to utilize alternative electron acceptors that become available only during inflammation. Under normal conditions the intestinal microbiota is composed chiefly of obligate anaerobic bacteria that perform fermentation (43, 89). The fermentation end product hydrogen sulfide is highly toxic, and is detoxified by conversion to thiosulfate by host epithelial cells (28, 58). During inflammation reactive oxygen species (ROS) are released into the lumen by neutrophils, and ROS oxidizes thiosulfate to tetrathionate. The *ttr* gene cluster of *S. Typhimurium* allows it to use tetrathionate as an alternative electron acceptor for respiration (113). Tetrathionate respiration also allows *S. Typhimurium* to utilize ethanolamine as a carbon source. Ethanolamine is a host-derived nutrient and is a poor fermentation substrate. However, *S. Typhimurium* is able to use
ethanolamine as its sole carbon source by using tetrathionate as an electron acceptor (102). In addition to tetrathionate respiration, some NTS strains also carry a bacteriophage-encoded effector, SopE, that enhances host production of nitrate during inflammation. Nitrate is an energetically superior terminal electron acceptor compared to tetrathionate, and horizontal gene transfer of the SopE bacteriophage effector enhanced luminal colonization of a lysogenized S. Typhimurium strain (61). The ability to utilize alternative carbon sources and perform respiration therefore provides S. Typhimurium with a competitive advantage over the obligate anaerobic members of the microbiota. The subsequent overgrowth of S. Typhimurium in the intestinal lumen in turn contribute to enhanced transmission of the pathogen to new hosts via the fecal-oral route (56).

In addition to utilizing alternative metabolites, S. Typhimurium is also able to acquire other nutrients from the inflamed host environment. Metal ions such as iron are necessary for the growth of both prokaryotes and eukaryotes, and bacteria have evolved iron-chelating molecules termed siderophores to scavenge iron in iron-poor environments such as the inflamed gut. In turn, the host has evolved antimicrobial proteins that can prevent metal ion uptake by bacteria. This concept that the host restricts bacterial growth by limiting the availability of metal ions is termed “nutritional immunity” (48). An example of nutritional immunity is the secretion of lipocalin-2 during S. Typhimurium infection. Lipocalin-2 binds to enterochelin, a small molecular weight siderophore secreted by many bacteria in the family Enterobacteriaceae, and thereby limits the growth of enteric bacteria by depriving them of iron (25, 38). S. Typhimurium is able to overcome lipocalin-2 mediated iron sequestration by producing a C-glucosylated derivative of enterochelin, called salmochelin, by expressing the iro gene cluster (24).
expression of salmochelin confers a colonization advantage for \( S. \) Typhimurium over bacteria that are susceptible to lipocalin-2 iron chelation in the inflamed gut (74).

\( S. \) Typhimurium is not the only bacterium that can take advantage of the inflammatory condition in the gut. Studies have shown that other Enterobacteriaceae bloom in the inflamed gut (62, 95), and that host derived ethanolamine and nitrate also enhances \( E. \) coli growth during inflammation (5, 114). Since \( E. \) coli and \( S. \) Typhimurium are both able to successfully colonize the inflamed gut, one strategy to reduce \( S. \) Typhimurium colonization is to use a probiotic, nonpathogenic \( E. \) coli as a competitor, and one such probiotic is the \( E. \) coli strain Nissle 1917 (\( E. \) coli Nissle). \( E. \) coli Nissle was first discovered from a healthy soldier during a \textit{Shigella} outbreak in World War I. In the decades that followed its discovery, \( E. \) coli Nissle has been used to reduce the duration of diarrhea in children, and has been found to be effective in maintaining remission in patients with ulcerative colitis (45, 55). While \( E. \) coli Nissle is a beneficial probiotic bacteria, like \( S. \) Typhimurium, it too is able to resist lipocalin-2 iron sequestration by the expression of the \textit{iro} gene cluster (23). In addition to the \textit{iro} gene cluster, \( E. \) coli Nissle also express other iron acquisition systems, including aerobactin, yersiniabactin, and ChuA heme receptor (41). Multiple iron transporters have been shown to enhance colonization of the host by bacteria. In a study by Garcia et al., wild-type uropathogenic \( E. \) coli strain CFT073 expressing multiple iron transport systems is better able to colonize the urinary tract than iron transporter mutants (31). \( E. \) coli CFT073 is closely related to \( E. \) coli Nissle(41), and our group wondered if multiple iron acquisition systems would allow \( E. \) coli Nissle to effectively compete with \( S. \) Typhimurium during colonization of the inflamed gut.

In a 2013 paper published by Dr. Elisa Deriu et al., which I co-authored, we found that the multiple iron acquisition systems confer \( E. \) coli Nissle a competitive advantage over \( S. \).
Typhimurium, and *E. coli* Nissle was able to reduce *S.* Typhimurium numbers in both acute and chronic models of *S.* Typhimurium gastrointestinal colonization. In addition, we found that host lipocalin-2 is needed in order for *E. coli* Nissle to reduce *S.* Typhimurium colonization, as the probiotic activity of *E. coli* Nissle is diminished in lipocalin-2 deficient mice (23). This study demonstrated the importance of iron acquisition for *E. coli* Nissle probiotic function, and that lipocalin-2 can enhance *E. coli* Nissle probiotic activity. However, while these studies provided a better understanding of the role of iron during *S.* Typhimurium and *E. coli* Nissle colonization of the inflamed gut, there was still an open question about the potential role of other metal ions in *S.* Typhimurium pathogenesis and *E. coli* Nissle probiotic activity. Lipocalin-2 is one of many antimicrobial proteins produced during *S.* Typhimurium infection in the gut. Another antimicrobial protein that is highly expressed in the inflamed gut during *S.* Typhimurium infection is calprotectin (74). Calprotectin has been shown to reduce the growth of microbes by binding to and sequestering zinc and manganese ions. While little is known about the role of zinc in microbial pathogenesis, the high level of calprotectin expression in the inflamed gut during *S.* Typhimurium infection suggests a role for zinc during *S.* Typhimurium pathogenesis. Understanding the role of zinc will be important to determining whether *S.* Typhimurium resistance to nutritional immunity is limited to iron, or if it is a colonization strategy that can be applied more broadly to other metal nutrients. For my dissertation project my aim is therefore to develop a better understanding of the role of zinc during *S.* Typhimurium pathogenesis, and to determine whether zinc acquisition contributes to *E. coli* Nissle probiotic activity. In the next chapter I will review what is currently known about the role of zinc in biology, specifically with regards to host immune function and bacterial systems of zinc transport.
1.6 References


Chapter 2

The Role of Zinc in Health and Disease

2.1 Zinc Availability and Distribution

Zinc is the 23rd most common element in the earth crust, making it one of the most common trace metals on earth. In biological systems it is, after iron, the second most abundant heavy metal ion found in living organisms (10). The adult human body contains 2-4 g of zinc, much of it is found in muscles and bones, with approximately 1% found in plasma, bound to albumin, transferrin and α-macroglobulin (39). In mammalian cells, the total cellular concentration of zinc is in the order of several hundred micromolar; however free zinc ions are available only in the picomolar range (29). In bacteria, zinc has been estimated to be in the millimolar range, with free zinc ions being in the femtomolar range (33). The reason why the vast majority of cellular zinc is not found in its free form is because most of it is bound to proteins.

The interaction of zinc and protein can generally be divided into structural and catalytic roles, though in some proteins (e.g. alcohol dehydrogenase) zinc may serve both functions. As a structural component, zinc helps to induce peptide folding and stabilize protein structure. As a catalyst, zinc can serve as a Lewis acid and facilitate a number of enzymatic reactions. Unlike many other transition metals such as iron, zinc has a full d shell, and therefore does not undergo redox reaction. In addition to its stability, zinc is highly flexible in its coordination geometry, making it a common cofactor found in more than 300 enzymes in living organisms (31). In fact, it has been estimated that approximately 10% of the human proteome, and 5% of prokaryotic proteomes bind to zinc (3, 4). In the human genome, zinc-bound transcription factors (most of
which are zinc fingers) account for ~40% of all zinc-associated proteins (3). The other 60% of zinc-associated proteins are, for the most part, enzymes and transport proteins (3). In bacteria only about 7% of bacterial zinc-bound proteins are involved in gene expression, with the majority being bound to enzymes such as β-lactamase, metallophosphatases, and peptidases (4). In fact, zinc is so ubiquitous that zinc-binding enzymes from all six enzyme families (i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases) have been found. The function of these zinc-binding proteins are in turn involved in many biological processes, including nucleic acid and protein synthesis, stress response, cell division, second messenger signal transduction, and apoptosis (49).

While all cells require zinc, sufficient zinc nutrient is particularly important for proper immune function. Even a marginal zinc deficiency can reduce the number of peripheral lymphocytes in humans and mice (41). Cells have evolved a variety of proteins to maintain zinc homeostasis, and the function of some of these zinc regulatory proteins (with an emphasis on their role in immune response) will be discussed in the following section.

2.2 Zinc and the Immune System

Inadequate intake of zinc has serious health consequences. In humans signs of zinc deficiency include stunted growth, diarrhea, dermatitis, and increased susceptibility to infections (14, 38, 41). In severe cases, such as in patients with the zinc malabsorption disease acrodermatitis enteropathica, insufficient zinc absorption can be fatal (49). One of the consequences of zinc deficiency is impaired immune function, which may be the cause for increased susceptibility to infection in zinc-deficient patients. Mice fed a zinc deficient diet develop thymus gland atrophy and have reduced number of T cells and T cell subsets, as well as
decreased proliferation in response to specific antigens and mitogens (15, 16, 32). While zinc is undoubtedly important for proper cellular function and metabolism, excess zinc also has adverse effects on the body. Excess zinc intake can lead to nausea, vomiting, diarrhea, abdominal cramps, and headaches (1). To prevent extremes in cellular zinc concentration, a system of zinc transport and regulatory proteins has evolved to help to maintain homeostasis.

Znt of the SLC30 protein family, and Zip from the SLC39 protein family are transporters expressed on cellular membranes. In vertebrate cells Znt proteins mediate the transport of zinc into extracellular fluid or into intracellular vesicles, thereby reducing cytosolic concentration of zinc. Zip proteins, in contrast, transport zinc from extracellular space or intracellular compartments into the cytosol (23). While Znt and Zip mediate the transport of zinc, the intracellular regulation of zinc is mediated by metallothioneins, a class of low molecular weight proteins that can bind up to seven zinc ions (20). Metallothioneins are thought to act as a “zinc buffer”, supplying zinc to other enzymes and transcription factors during zinc limiting conditions (24). In addition to metallothioneins, another zinc binding protein is calprotectin. Calprotectin is a heterodimer of the EF-hand calcium-binding proteins, S100A8 and S100A9. It has two metal binding sites, capable of binding zinc and manganese (12). Mazzatti and colleagues have suggested that calprotectin, like metallothionein, has a role in the regulation of intracellular zinc concentration (30). The most extensively studied function for calprotectin however, has been its role as a marker of inflammation and antimicrobial protein.

Calprotectin is highly secreted during inflammation and has been associated with a number of inflammatory conditions, including inflammatory bowel disease (44, 50). It is found in high concentrations in the cytosol of neutrophils, and studies have shown that it is also expressed in monocytes and activated macrophages, as well as in endothelial and epithelial cells
The antimicrobial function of calprotectin stems from its metal binding capability (12, 43). Just as host cells require zinc to grow, pathogens also require zinc to proliferate. To limit pathogen access to zinc, serum zinc level decrease following infection, and zinc becomes concentrated in the liver (42, 46). Calprotectin further reduces the availability of zinc at the site of infection by binding to and sequestering zinc and manganese, effectively starving pathogens of these vital metal ions (11, 26, 48). This concept of limiting pathogen growth by withholding metal nutrient availability is termed “nutritional immunity”, a term first coined to describe iron sequestration from microbes (21). A number of microbes, including *E. coli, Staphylococcus aureus, Borrelia burgdorferi, Listeria monocytogenes*, and *Candida albicans*, have been shown to be susceptible to calprotectin antimicrobial activity (11, 27, 28, 43, 44, 48, 53). However, given the comparatively rapid generation time of bacteria compared to vertebrates, it is not surprising that bacteria have mechanisms to sense zinc availability and evolved systems to acquiring this precious metal from zinc limiting environments. Many of these mechanisms were first discovered and characterized in *E. coli*, but are also present in *S. Typhimurium*. The following section will review the currently known mechanisms of zinc homeostasis in *S. Typhimurium*.

### 2.3 Bacterial Response to Zinc

Bacteria are highly sensitive to changes in free zinc ions, and expression of zinc import or export genes are trigged by very minute changes in free zinc concentration (33). In *E. coli* and *S. Typhimurium* zinc uptake is regulated by the Fur family protein, Zur, and zinc export is regulated by the MerR family protein, ZntR. Zur and ZntR respond to femtomolar changes in intracellular free zinc concentrations (33). It is thought that the reason for such stringent control
of intracellular zinc level is because zinc has a tendency to bind tightly to metalloproteins, and misregulation of zinc concentration will disrupt protein function (51). To prevent zinc toxicity, S. Typhimurium expresses zinc exporters to remove excess zinc. The ZntA P-type ATPase zinc exporter is expressed when the transcriptional regulator ZntR is bound to zinc ions and is capable of initiating ZntA transcription (5, 34). Another zinc export protein is the cation diffusion facilitator ZitB, which is expressed constitutively in E. coli and expels zinc using a transmembrane proton gradient (9, 52).

Although ZntA and ZitB play a role in preventing zinc toxicity, their role in S. Typhimurium pathogenicity has not been characterized. In contrast, much more is known about the role of zinc uptake systems and their role in S. Typhimurium pathogenesis. This is because during infections zinc concentrations in the tissue and serum are reduced (11, 46), and therefore zinc acquisition is of great importance to pathogens. The Zur protein, which regulates a number of zinc import systems, is bound to zinc ions when zinc is plentiful in the environment. This zinc-bound form of Zur binds to DNA and prevents RNA polymerase binding to downstream zinc transporter genes. When cellular zinc concentration decreases and zinc is no longer bound to Zur, Zur can no longer bind DNA and transcription of zinc import systems is derepressed (7).

In E. coli and S. Typhimurium there are at least two known zinc import systems that are regulated by Zur: ZinT and ZnuABC (17, 36). ZinT is a lipocalin-like protein found in the periplasm (13). It was shown to contribute to bacterial growth in zinc-replete media, but ZinT did not make a significant contribution to S. Typhimurium virulence in mouse infection experiments (37). Instead, evidence suggests ZinT plays an auxiliary role in zinc transport by forming a complex with, and enhancing the zinc recruiting ability of the high affinity zinc transporter, ZnuABC (22, 37). ZnuABC is a member of the ATP-binding cassette family of
transporters, and similar to ZinT, it is also located in the periplasm of *E. coli* and *S.* Typhimurium. It consists of three proteins: the periplasmic ZnuA component that binds to zinc ions; the inner membrane permease ZnuB; and the ATPase ZnuC that provides energy for zinc ion translocation. ZnuA binds to zinc in the periplasm, and then delivers it onto ZnuB to be imported into the cell. Inactivation of ZnuABC results in significant growth impairment of *S.* Typhimurium in zinc-deficient conditions (2, 6). In addition, loss of ZnuABC function decreases *S.* Typhimurium survival in human macrophage and epithelial cell lines, and diminishes its virulence in mice (2, 6, 35).

In addition to ZnuABC, *E. coli* and *S.* Typhimurium also transport zinc via the permease ZupT. ZupT belongs to the Zip family of proteins, and in *E. coli* it was shown to be expressed constitutively at a low level (18). ZupT takes up a variety of different divalent ions, including zinc, iron, manganese, and copper, though it has a preference for zinc over other metals (18, 19, 47). In a strain of uropathogenic *E. coli* ZnuABC and ZupT mediate zinc uptake, however ZnuABC but not ZupT was shown to be important for colonization in a murine model of urinary tract infection (40). While ZupT may not play a role in *E. coli* urinary tract infection, ZupT has been shown to play a role in the intraperitoneal (i.p.) infection model of *S.* Typhimurium in mice. A ZupT mutant *S.* Typhimurium exhibited diminished virulence in i.p. infection of functional NRAMP1 mice (25). In addition, Cerasi et al. recently demonstrated in the absence of the ZnuABC transporter ZupT also contributed to *S.* Typhimurium competitive fitness in i.p. infected, nonfunctional NRAMP1 mice (8).

While these studies have demonstrated the importance of ZnuABC and ZupT for *S.* Typhimurium and *E. coli* growth and colonization, the interaction between bacterial zinc acquisition and host zinc withholding mechanism is not clear. The antimicrobial protein
calprotectin is able to inhibit the growth of microbes by sequestering zinc ions, and is highly expressed in the inflamed gut during S. Typhimurium infection, as discussed in Chapter 1. However, given that S. Typhimurium and E. coli Nissle are both able to highly colonize the inflamed gut when calprotectin is expressed, I hypothesize that these bacteria are resistant to calprotectin-mediated zinc withholding, and that zinc transporters such as ZnuABC and ZupT may contribute to S. Typhimurium colonization and E. coli Nissle probiotic activity. I will discuss the results of my research on the role of calprotectin during S. Typhimurium gastroenteritis, and my work on the contribution of bacterial zinc transporters to S. Typhimurium colonization and E. coli Nissle probiotic activity in the inflamed gut in this dissertation.
2.4 References


Chapter 3

Zinc Sequestration by the Neutrophil Protein Calprotectin Enhances Salmonella Growth in the Inflamed Gut.

3.1 Summary

Neutrophils are an important constituent of the innate immune response to infection with bacterial or fungal pathogens. The release of antimicrobial proteins that reduce microbial growth is one means by which neutrophils mediate their killing activity. The neutrophil protein calprotectin exerts its antimicrobial activity by sequestering essential micronutrient metals such as zinc, thereby limiting their availability to microbes, a process termed nutritional immunity. Here we show that calprotectin is induced during infection with the gut pathogen Salmonella enterica serovar Typhimurium and its metal sequestration does not inhibit S. Typhimurium proliferation. Remarkably, S. Typhimurium overrides calprotectin-mediated zinc chelation by expressing a high affinity zinc transporter (ZnuABC), thereby promoting its own growth over that of competing microbes. In summary, our findings describe a novel mechanism by which Salmonella thrives in the inflamed gut by overcoming the zinc sequestration of calprotectin and highlight the importance of zinc acquisition as a mechanism of intestinal colonization.

3.2 Introduction

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a gut pathogen that causes an acute gastroenteritis characterized by inflammatory diarrhea. S. Typhimurium encodes two type III secretion systems (T3SS-1 and T3SS-2) that are important for eliciting intestinal inflammation (15, 48). Initiation of the inflammatory response in the gut requires interaction
with host cells, including epithelial cells and antigen-presenting cells (APCs) like macrophages and dendritic cells. APCs infected with *S. Typhimurium* secrete several cytokines, including interleukin (IL-) 23 and IL-18, which stimulate T cells in the intestinal mucosa to produce IL-17 and IL-22 (12, 13, 38, 43). These cytokines subsequently induce responses in the tissue that results in the influx of neutrophils to the gut mucosa, a hallmark of inflammatory diarrhea.

Neutrophils control *S. Typhimurium* dissemination, as inferred from clinical observations in patients with defects in neutrophil killing mechanisms (for instance, Chronic Granulomatous Disease patients) or neutropenia (54). In these groups of patients, *S. Typhimurium* infection often disseminates from the gut, resulting in bacteremia and high mortality (35). In response to *S. Typhimurium* infection, both neutrophils and epithelial cells secrete antimicrobial proteins into the intestinal lumen that may be responsible for the dramatic changes in the composition of the microbiota observed during *S. Typhimurium* infection (3, 29, 44). Furthermore, several recent studies have demonstrated that the host inflammatory response favors *S. Typhimurium* growth in the gut and its transmission to a naïve host (3, 25, 29, 37, 44). Within this inflammatory environment, *S. Typhimurium* must acquire essential nutrients and anaerobically respire tetrathionate to successfully outgrow the resident microbiota (37, 55). *S. Typhimurium* must also be resistant to the inhibitory and lethal activities of antimicrobial proteins released into the lumen in response to infection, some of which may actually promote the growth of this pathogen over competing microbes that are susceptible to their activity.

The identity of many antimicrobial proteins secreted during *S. Typhimurium* infection, and whether these peptides are protective or promote pathogen growth, is largely unknown. Because the influx of neutrophils is a hallmark of *S. Typhimurium* diarrhea, antimicrobial proteins released by neutrophils are likely major players in the host response to *S. Typhimurium*.
Calprotectin, a heterodimer of the two EF-hand calcium-binding proteins S100A8 and S100A9, is one of the most abundant antimicrobial proteins in neutrophils, constituting approximately 40% of cytoplasmic neutrophil content (47). This protein complex is secreted to high levels during inflammation (45) and is associated with extracellular traps released by apoptotic neutrophils to kill microbes (49). Mucosal and skin epithelial cells stimulated with the cytokine IL-22 also express S100a8 and S100a9, indicating that epithelial cells may also be a source of this antimicrobial protein (27, 57). Calprotectin has antimicrobial activity against many microorganisms, including *Escherichia coli*, *Borrelia burgdorferi*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Candida albicans* (9, 28, 30, 42, 45, 49, 56). This activity is dependent on the ability of calprotectin to bind nutrient metals such as zinc and manganese, thereby starving microbes of these essential metal ions (9, 20, 49). With regard to the intestine, fecal calprotectin levels are used clinically to monitor the severity of intestinal inflammation in patients with inflammatory bowel diseases (24). Despite these observations, the role of calprotectin in the intestinal inflammatory response during infection with gut pathogens is largely unknown. In this study, we set out to determine the role of the antimicrobial protein calprotectin during infection with *S. Typhimurium* as a model for inflammatory diarrhea.

### 3.3 Results

**Calprotectin expression in the cecum during infection with *S. Typhimurium***

Our previous studies indicated that the two subunits of calprotectin, *S100a8* and *S100a9*, are among the highest up-regulated genes during infection with *S. Typhimurium* in the ileum of rhesus macaques and in the cecum of mice (12, 38). To further confirm these observations, we employed the streptomycin-pretreated mouse colitis model of *S. Typhimurium* infection, which
results in acute inflammation of the cecal mucosa characterized by an influx of neutrophils (4, 37). Using this model, we found that calprotectin was highly induced in the cecum and fecal samples of mice infected with S. Typhimurium compared to mock-infected mice (Fig. 3.1). The mRNA for the two subunits of calprotectin, \textit{S100a8} and \textit{S100a9}, was significantly up-regulated at both day 3 (Fig. 3.1A) and day 4 post-infection (Fig. 3.1B). We next extracted total protein from fecal samples (Fig. 3.1C) and the large intestine (Fig. 3.1D) of mock- or S. Typhimurium-infected mice and then determined calprotectin concentration by ELISA. We detected a significant increase in the expression of calprotectin in both the fecal samples and in the tissue of the large intestine after S. Typhimurium infection, with average concentrations being 130 mg/ml and 793 mg/ml respectively. Because intestinal epithelial cells may express antimicrobial peptides, we next determined whether crypt colonocytes could be a source of calprotectin.

Mice were infected with either S. Typhimurium or mock and we isolated crypt cells from the large intestine (Appendix A1). As a positive control, we confirmed that crypt colonocytes isolated from mice infected with S. Typhimurium exhibited an increase in the expression of the antimicrobial peptide lipocalin-2, which is induced in epithelial cells during infection (37). In addition, we detected increased expression of the \textit{S100a8} and \textit{S100a9} subunits, indicating that these cells may also be a source of calprotectin (Fig. 3.2). As the increase in calprotectin correlated with the increased inflammatory response and the influx of neutrophils in these mice, we questioned to what extent neutrophils and epithelial cells contribute to the increase of calprotectin expression observed during S. Typhimurium infection.

To test this, we infected mice treated with an antibody against the neutrophil receptor CXCR2 (Fig. 3.3). The CXCR2 receptor binds to the chemokine \textit{Cxcl-1} expressed in the inflamed gut, thereby promoting the transmigration of neutrophils to the site of infection.
Figure 3.1 Expression of calprotectin in the cecum of mice infected with *S. Typhimurium*. (A, B) *S100a8* and *S100a9* were detected by RT-PCR at 72 h (A) and 96 h (B) after infection with wild-type *S. Typhimurium*. Data are expressed as fold increase over mock-infected wild-type mice. Bars represent the geometric means ± standard error. (C, D) The concentration of calprotectin detected by ELISA (top panel) and Western Blot (bottom panel) in the fecal samples (C) and the large intestine with content (D) of mice 96 hours post infection with *S. Typhimurium* (n=4) or mock control (n=4). Bars represent the geometric mean ± standard deviation. ** *P* value ≤ 0.01.
Figure 3.1

A

mRNA (fold change)

1

10

100

1000

10000

S100a8  S100a9

3 days p.i.

B

mRNA (fold change)

1

10

100

1000

S100a8  S100a9

4 days p.i.

C

Fecal Calprotectin (μg/ml)

0

50

100

150

200

250

300

α-S100A9

α-S100A8

S. Typhimurium  mock

**

D

Intestinal Calprotectin (μg/ml)

0

500

1000

1500

2000

α-S100A9

α-S100A8

S. Typhimurium  mock

**
Figure 3.2 Expression of calprotectin in colon crypts of mice infected with S. Typhimurium.

Expression of the \( S100a8 \) and \( S100a9 \) mRNAs was detected in crypt colonocytes isolated from the colon of mice infected with \( S. \) Typhimurium (\( n=3 \)) and expressed as fold increase over mock-infected mice (\( n=3 \)). Expression of \( Lcn2 \) was detected as a positive control, and of the T cell cytokine \( Il-17a \) and the neutrophil marker \( Ly6g \) as a negative control. ND = not detected. Data represents the geometric mean ± standard error. A significant increase over mock control is indicated by * (\( P \) value ≤ 0.05) and ** (\( P \) value ≤ 0.01). (See also Appendix A1).
Figure 3.2
Figure 3.3 Depletion of neutrophils in α-CXCR2 treated *S. Typhimurium* infected mice.

Mice were injected intraperitoneally with either normal rabbit serum (NRS) or a rabbit polyclonal antibody blocking the CXCR2 receptor (α-CXCR2) 24 hours prior to infection with *S. Typhimurium* and sacrificed at 72 hours post-infection. (A) Representative dot plot (FSC=forward scatter; SSC=side scatter) of blood cells gated on leucocytes from mice treated with NRS (top) and α-CXCR2 (bottom) (B) Representative dot plot of blood leucocytes gated on neutrophils expressing Ly6g and CD11b from mice treated with NRS (top) and α-CXCR2 (bottom).
Figure 3.3
Blocking the CXCR2 receptor reduces neutrophil transmigration to the infected tissue and depletes neutrophils from the blood, as previously described by Hosking et al (17). As a control, mice were injected with normal rabbit serum (NRS). To test the effectiveness of our neutrophil depletion, we detected the Cd11b^+ Ly6g^{high} cells (neutrophils) in the blood collected from the NRS-treated and the anti-CXCR2-treated mice by flow cytometry. As expected, treatment with the anti-CXCR2 antibody reduced the amount of neutrophils in the blood (Fig. 3.3B). To determine the contribution of neutrophils and colonocytes to the expression of calprotectin during infection, we extracted both RNA and protein from the cecum (Fig. 3.4A and B) and the crypt colonocytes (Fig. 3.4C and D) of the NRS-treated and the anti-CXCR2-treated mice. Our results indicate that in mice treated with the anti-CXCR2 antibody, there was only a minor reduction in the expression of S100a8 and S100a9 mRNA in comparison to the NRS-treated mice in the cecum (Fig. 3.4A) and no reduction in the crypt colonocytes (Fig. 3.4C). Thus, neutrophils appear to be dispensable for the induction of S100a8 and S100a9 transcription in crypt colonocytes. In contrast, mice treated with the anti-CXCR2 antibody exhibited a marked reduction in the expression of calprotectin at the protein level, which correlated with the reduction of the neutrophil marker myeloperoxidase (MPO) in these mice (Fig. 3.4B). Moreover, we did not detect high levels of calprotectin expression at the protein level in crypt colonocytes – the low level of expression was likely due to contamination with neutrophils, as indicated by the detection of MPO (Fig. 3.4D). Therefore, although transcription of S100a8 and S100a9 is highly induced in colonocytes, neutrophils appear to be the major source of calprotectin protein. Taken together, our results indicate that calprotectin is expressed at high levels in the large intestine during S. Typhimurium infection, suggesting that it may play an important role in the host response to this infection.
**Figure 3.4 Expression of calprotectin in mice following neutrophil depletion.** (A, B) Cecal expression of the \textit{S100a8} and the \textit{S100a9} subunits of calprotectin were detected by Real-time RT PCR (A) and Western blot (B). (C, D) Crypt expression of the \textit{S100a8} and the \textit{S100a9} subunits of calprotectin was detected by Real-time RT PCR (C) and Western blot (D); MPO=myeloperoxidase. Data represents the geometric mean ± standard error. A significant difference in expression between NRS-treated and α-CXCR2-treated mice is indicated by ** \((P \text{ value} \leq 0.01)\).
Figure 3.4

**A**

![Bar graph showing mRNA fold change for S100a8 and S100a9 in Cecum.](image)

**B**

![Western blots showing protein expression in Cecum.](image)

**C**

![Bar graph showing mRNA fold change for S100a8 and S100a9 in Crypts.](image)

**D**

![Western blots showing protein expression in Crypts.](image)
Growth of S. Typhimurium in rich media supplemented with calprotectin

To investigate the role of calprotectin during S. Typhimurium infection, we next determined whether calprotectin was able to reduce the growth of S. Typhimurium. We performed a growth assay of S. Typhimurium in rich media supplemented with purified calprotectin (Fig. 3.5). When calprotectin was added at a concentration typical of a tissue abscess (500 mg/ml) (19), S. Typhimurium growth was inhibited. In contrast, lower concentrations of calprotectin, comparable to that measured in the fecal samples of infected animals (Fig. 3.1C), only minimally reduced S. Typhimurium growth (Fig. 3.5A), indicating that Salmonella could potentially defend against this insult in the intestinal lumen. We then looked to determine the mechanism by which S. Typhimurium grew in the media supplemented with calprotectin.

Because calprotectin is known to chelate zinc ions, we hypothesized that S. Typhimurium growth was dependent on the high affinity zinc transporter encoded by the znuABC operon, which is induced in zinc-limiting conditions (2, 6). We subsequently deleted the gene encoding the periplasmic zinc-binding component, znuA, and confirmed that our znuA mutant had a growth defect in minimal media that could be rescued by supplementation with zinc sulfate, as previously shown (2) (Appendix A2). When we grew the znuA mutant in rich media supplemented with calprotectin, we found that its growth was impaired at concentrations of calprotectin where wild type is only minimally inhibited (approximately 250 mg/ml) (Fig. 3.5B). Thus, the ability to acquire zinc through the ZnuABC transporter renders S. Typhimurium more resistant to the antimicrobial activity of calprotectin in vitro. Our results thus far suggested that the calprotectin level encountered by S. Typhimurium in the intestinal lumen has only minimal activity against the organism and that zinc acquisition through ZnuABC may render this pathogen resistant to this protein in vivo.
Figure 3.5 Growth of *S. Typhimurium* in rich media supplemented with calprotectin. *S. Typhimurium* wild-type (A) or the *znuA* mutant (B) were grown in LB media supplemented with calprotectin at the indicated concentrations. Growth was determined by reading the OD$_{600}$ in a microplate reader at the indicated times. Data represent the geometric mean of 4 biological replicates ± standard error. A significant difference in growth between wild-type and the *znuA* mutant is indicated by ** (P value ≤ 0.01).
Figure 3.5

A

wild-type

OD₆₀₀

Time (h)

B

znuA mutant

OD₆₀₀

Time (h)
The ZnuABC zinc transporter promotes *S. Typhimurium* colonization of the inflamed cecum

To assess whether high affinity zinc transporters may provide an advantage in the gut environment, we first determined the concentration of zinc in the feces of mice infected with *S. Typhimurium* or mock by inductively coupled plasma mass spectrometry (ICP-MS) (**Fig. 3.6**). We found that the concentration of fecal zinc in the absence of infection was about 220 mg/kg, while it was reduced to 56 mg/kg in infected mice (**Fig. 3.6**). Therefore, the lower levels of zinc in the inflamed gut suggested that the expression of a high affinity zinc transporter may provide a growth advantage for colonization. To test this, we next investigated whether inactivation of the *znuA* gene would impair the ability of *S. Typhimurium* to colonize the intestine. *S. Typhimurium* infection results in intestinal inflammation, which is facilitated by the actions of two type III secretion systems encoded by *Salmonella* pathogenicity islands (SPI)-1 and SPI-2 (41). Inactivation of both secretion systems through deletion of the *invA* and *spiB* genes, respectively, renders *S. Typhimurium* unable to elicit colitis in mice (37, 44). We thus compared groups of mice infected with *S. Typhimurium* wild-type, the *znuA* mutant, or a mutant lacking both the *invA* and *spiB* genes. The *znuA* mutant triggered similar levels of inflammation and expression of calprotectin and cytokines in the cecum as observed with the wild-type strain, indicating that this mutant retained virulence (**Fig. 3.7 and Appendix A3**). However, colonization of the *znuA* mutant was reduced approximately 200 fold at 96 h post-infection to levels similar to an avirulent *S. Typhimurium* strain lacking *invA* and *spiB* (**Fig. 3.8A**).

In recent years, several studies have demonstrated that *S. Typhimurium* and other gut pathogens benefit from inflammation because they have to compete with the resident microbiota to colonize the inflamed gut (3, 25, 29, 44, 55). It is known that the *invA spiB* mutant does not
Figure 3.6 Wild-type *S. Typhimurium* reduces intestinal zinc concentration in infected mice. (A) The concentration of zinc was measured by ICP-MS in fecal samples collected from mock-infected (n=4) or *S. Typhimurium*-infected (n=4) mice four days post-infection. Bars represent geometric means ± standard deviation. A significant difference is indicated by ** (*P* value ≤ 0.01).
Figure 3.6

![Bar graph showing fecal Zn²⁺ levels (mg/kg) for mock and wild-type treatments.](image)
**Figure 3.7 Inflammation and calprotectin expression in cecum of infected mice.** (A, B) Histopathology of cecal samples were collected from mice four days after infection with *S. Typhimurium* wild-type, the *znuA* mutant, or the *invA spiB* mutant. (A) H&E stained cecal sections from representative animals in each group. An image at lower magnification (10x) and one at higher magnification (40x) from the same section are shown. L=lumen; M=mucosa; SM=submucosa. Note marked edema in the submucosa and inflammation in mice infected with both *S. Typhimurium* wild-type and the *znuA* mutant. (B) Blinded histopathology score indicating the score of individual mice (circles), and the average score for each group (bars). The grey quadrant includes scores indicative of moderate to severe inflammation. (C) S100A8, S100A9, myeloperoxidase (MPO) and tubulin were detected by immunoblot in the cecum of mice infected with *S. Typhimurium* wild-type or the *znuA* mutant.
Figure 3.7

A

10x

40x

S. Typhimurium wild-type
S. Typhimurium znuA
S. Typhimurium invA spiB

B

Pathology Score

C

wild-type
znuA

α-S100A8
α-S100A9
α-MPO
α-tubulin

62
Figure 3.8 The ZnuABC zinc transporter promotes *S. Typhimurium* colonization of the inflamed cecum. (A) Enumeration of *S. Typhimurium* in the colon content (wild-type n=11, *znuA* mutant n=11, *invA spiB* mutant n=6; *znuA*+ZnSO$_4$ n=9; wild-type+ZnSO$_4$ n=6) (B) Analysis of the cecal microbiota using 16S rRNA gene qRT-PCR (wild-type n=10, *znuA* mutant n=9, *invA spiB* mutant n=6; *znuA*+ZnSO$_4$ n=9). Bars represent geometric means ± standard error. (A) A significant difference in comparison to wild-type infected mice is indicated by ** (*P* value ≤ 0.01). (B) Significant differences between groups are indicated by * (*P* value ≤ 0.05) and ** (*P* value ≤ 0.01). (Also see Appendix A3).
Figure 3.8

A

Bacteria in colon contents (CFU/mg)

wild-type  znuA  invA  znuA  wild-type
spib  (ZnSO₄)  (ZnSO₄)

wild-type  znuA  invA  znuA  wild-type
spib  (ZnSO₄)  (ZnSO₄)

B

16S copy number/μl of DNA

Eubacteria  Clostridiales  Lactobacillales  Bacteroidetes  Salmonella  Other Enterobacteriaceae

invA  spib  wild-type  znuA  znuA + ZnSO₄
colonize as well as wild-type because it does not trigger an inflammatory response and it is therefore outcompeted by the microbiota (25, 37, 44, 55). Because the \textit{znuA} mutant showed a striking colonization defect despite eliciting inflammation, we hypothesized that this strain is less fit than wild type to sustain competition with the microbiota.

Analysis of the microbiota in stool samples of mice infected with the \textit{znuA} mutant confirmed this prediction (\textbf{Fig. 3.8B}). Infection with \textit{S. Typhimurium} wild-type, but not the \textit{invA spiB} mutant, induced a loss of \textit{Bacteroidetes} and \textit{Clostridiales}, as previously described (3, 25, 29, 44, 55). Therefore, \textit{S. Typhimurium} wild-type but not the \textit{invA spiB} mutant was able to outcompete the microbiota (\textbf{Fig. 3.8}). Remarkably, the \textit{znuA} mutant induced a loss of \textit{Bacteroidetes} and \textit{Clostridiales} similar to wild-type. However, the \textit{znuA} mutant exhibited a markedly reduced ability to take advantage of the decrease in competing microbes and it colonized to similar levels as the \textit{invA spiB} mutant (\textbf{Fig. 3.8}). Intriguingly, while administration of zinc to mice in the form of zinc sulfate enhanced the growth of \textit{S. Typhimurium} wild-type, it did not rescue the \textit{znuA} mutant (\textbf{Fig. 3.8}). One plausible explanation is that administration of zinc sulfate may promote the growth of other bacterial species that are more fit than the \textit{znuA} mutant to colonize this environment. To test this possibility, we analyzed the microbiota in stool samples of mice supplemented with zinc sulfate infected with the \textit{znuA} mutant. While we observed low levels of \textit{Bacteroidetes} and \textit{Clostridiales}, we also detected a significant increase in Enterobacteriaceae other than \textit{Salmonella} spp, which may account at least partly for the reduced ability of the \textit{znuA} mutant to proliferate in this environment. Moreover, we did not detect an increase in other Enterobacteriaceae in mock-infected or wild-type infected mice supplemented with zinc sulfate (data not shown). These results indicate that the ZnuABC transporter aids in competing with the microbiota and promotes \textit{S. Typhimurium} colonization of the cecum.
Resistance to calprotectin-mediated zinc sequestration provides a growth advantage to *S. Typhimurium* in the gut.

To test if zinc acquisition and resistance to calprotectin would increase *S. Typhimurium* fitness *in vivo*, we infected mice with an equal mixture of *S. Typhimurium* wild-type and the *znuA* mutant (Fig. 3.9-3.13 and Appendix A4-5). In this experimental setting, the ability of the *znuA* mutant and of wild type to colonize the intestine is compared in each individual animal (i.e. in the same gut environment), thereby reducing the effect of animal-to-animal variation observed in single infections. *S. Typhimurium* infection resulted in increased cecal inflammation and an influx of neutrophils (Fig. 3.9 and Appendix A4), which correlated with increased transcript levels of the neutrophil chemoattractant *Cxcl-1* (Fig. 3.10A) and the neutrophil marker *Ly6g* (Fig. 3.10B). Moreover, the transcript levels of the pro-inflammatory cytokines *Il-17a* and *Il-22* were also increased (Appendix A4). These data confirmed that *S. Typhimurium* infection was associated with acute cecal inflammation and neutrophil influx. The transcript levels of the two subunits of calprotectin *S100a8* and *S100a9* were also determined to be highly induced (Fig. 3.11). While no calprotectin was detected in the cecal mucosa of mock-infected mice, the protein complex was highly abundant after *S. Typhimurium* infection, and it correlated with the increase in MPO levels (Fig. 3.12A). In this gut environment with high levels of inflammation and calprotectin, we observed a marked increase in *S. Typhimurium* wild-type over the *znuA* mutant at days 3 and 4 post-infection (average of 87-fold and 690-fold respectively), suggesting that zinc acquisition through the ZnuABC transporter enhances *S. Typhimurium* growth in the inflamed gut (Fig. 3.13).
Figure 3.9 Histopathology results of mice infected with \textit{S. Typhimurium} (wild-type + \textit{znuA mutant}) or mock. Histopathology of the cecum. Upper panels, H&E stained cecal sections from representative animals in each group. An image at lower magnification (10x) and one at higher magnification (40x) from the same section are shown. L= lumen; M= mucosa; SM= submucosa. Note marked edema in the submucosa and inflammation in infected mice. Lower panel, blinded histopathology scores, indicating the score of individual mice (circles), and the average score for each group (bars).
Figure 3.9

Mouse genotype: wild-type, S100a9−/−, wild-type, wild-type, wild-type

Pathology Score

- wild-type versus znuA
- wild-type versus znuA
- invA spiB versus invA spiB znuA
- mock
- wild-type versus znuA (ZnSO₄)
Figure 3.10 Quantification of neutrophil-associated markers in the cecum by quantitative real-time PCR. Transcript levels of Cxcl-1 (A) and Ly6g (B), were determined in wild-type mice (white columns), S100a9−/− mice (dark grey columns), and wild-type mice supplemented with zinc sulfate (light grey columns). Mice were either mock-infected or infected with S. Typhimurium as indicated. Data are expressed as fold increase over mock-infected wild-type mice. Bars represent the geometric mean of at least 4 replicates ± standard error. Significant differences in gene expression in comparison to wild-type infected C57BL/6 mice (first group) are indicated by ** (P value ≤ 0.01). (Also see Appendix A4).
Figure 3.10

A

- **Wild-type vs znuA**
  - WT
  - S100a9<sup>−</sup>

- **invA spiB vs invA spiB znuA**
  - WT
  - S100a9<sup>−</sup>
  - WT (ZnSO<sub>4</sub>)

B

- **Wild-type vs znuA**
  - WT
  - S100a9<sup>−</sup>

- **invA spiB vs invA spiB znuA**
  - WT
  - S100a9<sup>−</sup>
  - WT (ZnSO<sub>4</sub>)

**Cxcl-1 mRNA (fold change)**

**Ly6g mRNA (fold change)**
Figure 3.11 Calprotectin expression in the cecum detected by quantitative real-time PCR.

Transcript levels of \( S100a8 \) (A) and \( S100a9 \) (B) were determined in wild-type mice (white columns), \( S100a9^{-/-} \) mice (dark grey columns), and wild-type mice supplemented with zinc sulfate (light grey columns). Mice were either mock-infected or infected with \( S. \) Typhimurium as indicated. Bars represent the geometric mean of at least 4 replicates ± standard error. Significant differences in gene expression in comparison to wild-type infected C57BL/6 mice (first group) are indicated by * (\( P \) value ≤ 0.05) and ** (\( P \) value ≤ 0.01).
Figure 3.11

A

<table>
<thead>
<tr>
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<th>S100a8 mRNA (fold change)</th>
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<td>wild-type vs znuA</td>
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<td>wild-type</td>
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<td>S100a9^-</td>
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Figure 3.12 Calprotectin expression in the cecum detected by Western blot. (A-D) S100A8, S100A9, myeloperoxidase (MPO) and tubulin were detected by immunoblot in the cecum of mice infected with *S. Typhimurium*. Strain and mouse genotypes are indicated. PC = positive control.
Figure 3.12

A. Wild-type mice

- α-S100A8
- α-S100A9
- α-MPO
- α-tubulin

Wild-type vs znuA

B. Wild-type mice

- α-S100A8
- α-S100A9
- α-MPO
- α-tubulin

PC vs invA spiB vs invA spiB znuA

C. S100a9+ mice

- α-S100A8
- α-S100A9
- α-MPO
- α-tubulin

PC, mock, wild-type vs znuA

D. Wild-type mice (ZnSO₄)

- α-S100A8
- α-S100A9
- α-MPO
- α-tubulin

Wild-type vs znuA
Figure 3.13 Resistance to calprotectin-mediated zinc sequestration provides a growth advantage to *S. Typhimurium*. Cecal samples were collected from wild-type and *S100a9*−/− mice after infection with *S. Typhimurium*. Competitive index was calculated by dividing the output ratio (CFU of the wild-type /CFU of the mutant) by the input ratio (CFU of the wild-type /CFU of the mutant). (A, B) Competitive indices of *S. Typhimurium* strains in the colon contents of mice (n≥6/group) at four days (A) or three days (B) post infection. Strain and mouse genotypes are indicated. NS = not significant. Bars represent geometric mean ± standard error. Significant differences are indicated by * (P value ≤ 0.05) and ** (P value ≤ 0.01).
Figure 3.13

A

Competitive Index in cecal contents (96 h p.i.)

- ** wild-type vs S100a9^{-/-} mice
- * wild-type mice vs wild-type mice (ZnSO_{4})

B

Competitive Index in cecal contents (72 h p.i.)

- ** wild-type vs S100a9^{-/-} mice
- * wild-type mice vs wild-type mice (ZnSO_{4})

N.S.
We next sought to determine whether zinc acquisition through the ZnuABC transporter was significant in the absence of gut inflammation. To determine if the ZnuABC transporter provides a growth advantage in the absence of inflammation, we infected mice with an equal mixture of an *invA spiB* mutant and an *invA spiB znuA* mutant (Fig. 3.9-3.13 and Appendix A4-A5). As expected, these mice exhibited minimal or no intestinal inflammation (Fig. 3.9 and Appendix A4), and the transcript levels for the neutrophil chemoattractant *Cxcl*-1, the neutrophil marker *Ly6g* and the pro-inflammatory cytokines *Il*-17 and *Il*-22 were increased only minimally when compared to mock-infected animals (Fig. 3.10 and Appendix A4). Increased expression of the two subunits of calprotectin, *S100a8* and *S100a9*, was not detectable by quantitative real-time PCR and little calprotectin and MPO expression was found by Western blot (Fig. 3.12B). Importantly, in this mixed infection, the *invA spiB* mutant and the *invA spiB znuA* mutant were recovered at nearly equal ratios at both 72 h and 96 h post infection, indicating that the *znuA* mutant is still capable of growing to similar levels as wild type in the intestinal lumen under non-inflammatory conditions (Fig. 3.13). Because neutrophils are a major source of calprotectin, we infected mice treated with an antibody against CXCR2 with a mixture of *S. Typhimurium* wild-type and the *znuA* mutant. As a control, mice were injected with normal rabbit serum (NRS). In the NRS-treated mice, where calprotectin was expressed to similar levels as wild-type mice, we recovered 80 fold more *S. Typhimurium* wild-type than the *znuA* mutant at 72 hours post-infection (Fig. 3.14), which is comparable to what we observed in wild-type (not NRS-treated) mice at the same time point (Fig. 3.13B). In contrast, the growth disadvantage of the *znuA* mutant was reduced in mice treated with an antibody against CXCR2 (Fig. 3.14), which showed reduced calprotectin expression (Fig. 3.4). These data indicate that only during inflammation is zinc acquisition through the ZnuABC transporter seen to provide a growth advantage.
Figure 3.14 Neutrophil influx during inflammation provides a growth advantage to *S. Typhimurium.* Competitive index in cecal content of mice treated with either normal rabbit serum (NRS) or a rabbit polyclonal antibody blocking the CXCR2 receptor (a-CXCR2) at 72 hours post-infection. Competitive index was calculated by dividing the output ratio (CFU of the wild-type /CFU of the mutant) by the input ratio (CFU of the wild-type /CFU of the mutant). Bars represent geometric mean ± standard error. Significant differences are indicated by * (*P value ≤ 0.05) and ** (*P value ≤ 0.01). (Also see Appendix A5).
Figure 3.14

[Bar chart showing competitive index in cecal contents (72 h p.i.) for NRS and α-CXCR2, with wild-type vs znuA comparison.]

** and * indicate significant differences.
Next, we investigated whether the growth benefit provided by intestinal inflammation to S. Typhimurium wild-type was dependent on the expression of calprotectin. To assess this, we employed $S100a9^{-/-}$ mice, which lack the expression of both the S100A8 and S100A9 subunits of calprotectin due to decreased stability of S100A8 at the protein level in the absence of its binding partner S100A9 (31) (Fig. 3.12B). The $S100a9^{-/-}$ mice were infected with an equal mixture of S. Typhimurium wild-type and the $znuA$ mutant (Fig. 3.9-3.13 and Appendix A4-A5), resulting in similar levels of colonization, inflammatory changes and MPO expression in tissue equivalent to wild-type mice (Fig. 3.9-3.12 and Appendix A5). Moreover, infection of $S100a9^{-/-}$ mice resulted in an increase in expression of the pro-inflammatory markers Cxcl-1, Ly6g, Il-17 and Il-22 comparable to wild-type mice (Fig. 3.10 and Appendix 4). Importantly, S. Typhimurium wild-type and the $znuA$ mutant were recovered at nearly equal ratios at 72 h and 96 h post infection (Fig. 3.13). Taken together, these results indicate that sequestration of zinc ions by calprotectin provides a growth advantage to S. Typhimurium over an isogenic strain lacking the ability to acquire zinc via the ZnuABC transporter.

Next, we tested whether we could rescue the $znuA$ mutant by administering zinc sulfate to mice infected with an equal mixture of S. Typhimurium wild-type and the $znuA$ mutant. Infected mice receiving zinc sulfate via oral gavage through the course of the infection displayed similar levels of inflammation as wild-type mice (Fig. 3.9 and Appendix A4). Furthermore, zinc supplementation did not result in significant changes in the expression of the pro-inflammatory genes Il-17, Il-22 or Ly6g, with the exception of the basal expression of Cxcl-1 (Fig. 3.10 and Appendix A4). Similar to what we observed in the single infection in (Fig. 3.8A), zinc administration promoted a significant increase in overall S. Typhimurium colonization at both 72 h and 96 h post-infection (Appendix A5). Importantly, administration of zinc sulfate
significantly reduced the growth disadvantage of the $znuA$ mutant at 96 hours post-infection (Fig. 3.13A). Combined with our results of zinc supplementation in mice infected with the $znuA$ mutant alone (Fig. 3.7C and 3.8A), it emerges that the administration of zinc sulfate was able to rescue the $znuA$ mutant in vivo only when the wild-type was also present (Fig. 3.13A). These results suggest that wild-type may provide some cross-protection to the mutant in the mixed infection, either directly or indirectly by reducing the growth of competing microbes, and further underline the importance of an intact ZnuABC transporter during $S. Typhimurium$ infection. Overall, our findings indicate that resistance to calprotectin, mediated by the capacity to acquire zinc through the ZnuABC transporter, promotes $S. Typhimurium$ competition in the inflamed gut.

**The ZupT permease contributes to competitive advantage of $S. Typhimurium$ in the spleen of streptomycin pre-treated mice.**

In addition to ZnuABC, $S. Typhimurium$ also expresses the ZupT permease. The ZupT permease can take up a number of divalent metal ions, though it has a preference for zinc. While the ZnuABC transporter appear to be the primary zinc transporter in zinc limiting conditions (40), ZupT has been shown to contribute to intraperitoneal infection of $S. Typhimurium$ susceptible (i.e. mice with nonfunctional NRAMP1) and $S. Typhimurium$ resistant mice (i.e. mice with functional NRAMP1) (7, 23). However, the role of ZupT in a gastroenteritis infection of mice had not previously been studied. To determine the contribution of ZupT to $S. Typhimurium$ colonization fitness during gastroenteritis, we infected streptomycin-pretreated C57BL/6 mice with a mixture of $S. Typhimurium$ wild-type and $znuA$ mutant, wild-type and
Figure 3.15 The ZupT permease contributes to systemic dissemination of *S. Typhimurium* in mice. Cecum and spleen samples were collected from mice three or four days after infection with wild-type and *znuA* mutant *S. Typhimurium*, wild-type and *zupT* mutant *S. Typhimurium*, wild-type and *znuA zupT* mutant *S. Typhimurium*, and *znuA* mutant and *znuA zupT* mutant *S. Typhimurium*. Competitive index was calculated by dividing the output ratio (CFU of the wild-type /CFU of the mutant) by the input ratio (CFU of the wild-type /CFU of the mutant). (A, B) Competitive indices of *S. Typhimurium* strains in the colon contents of mice at 72 hr and 96 hr post infection (A) and in the spleen at 96 hr post infection (B).
zupT mutant, wild-type and znuA zupT mutant, or znuA mutant and znuA zupT mutant, and examined colonization of each strain in the cecum and spleen (Fig. 3.15).

Consistent with our previous observation (Fig. 3.13), S. Typhimurium strains with mutation in znuA were significantly diminished in their ability to colonize the cecum at days 3 and 4 post-infection (Fig. 3.15A), and were also deficient in colonizing the spleen (Fig. 3.15B). In contrast, the S. Typhimurium zupT mutant had no colonization defect in comparison to S. Typhimurium wild-type in the cecum of infected mice (Fig. 3.15A). However, the zupT mutant did exhibit a small, but significant defect in colonization of the spleen (Fig. 3.15B). These results reinforce our earlier finding that the ZnuABC transporter provides an important advantage in colonizing the cecum of streptomycin treated mice, and confirms a previous study that showed a znuA mutant exhibited decreased colonization fitness in a systemic infection model of S. Typhimurium (36). In addition, we have found that ZupT function enhances S. Typhimurium colonization of the spleen in C57BL/6 mice, which express the nonfunctional NRAMP1 metal transporter. This result is in line with work done by Cerasi et al., who showed ZupT contributes to S. Typhimurium zinc acquisition during systemic infection in nonfunctional NRAMP1 mice.

ZnuABC confers a competitive advantage to S. Typhimurium during systemic infection.

In our oral infection experiments we have found that calprotectin levels are elevated in the inflamed gut (Fig. 3.1), and that a S. Typhimurium znuA mutant that is sensitive to calprotectin-mediated zinc withholding has a colonization defect in the gut (Fig. 3.8). We also recovered significantly less znuA mutant than wild-type S. Typhimurium in the spleen of orally infected C57BL/6 mice (Fig. 3.15). However, it is not clear if the decreased colonization fitness of the znuA mutant in the spleen is due to calprotectin zinc sequestration, or due to the znuA
mutant being outcompeted by the microbiota (Fig. 3.8B), and is attenuated in dissemination to systemic sites.

To assess the role of calprotectin in the colonization of systemic organs by S. Typhimurium, we will bypass the gut mucosal barrier, and use a systemic infection model of S. Typhimurium infection. We infected C57BL/6 and S100a9⁻/⁻ mice using intraperitoneal injection with either wild-type or znuA mutant S. Typhimurium, and recovered similar numbers of wild-type S. Typhimurium in C57BL/6 and S100a9⁻/⁻ mice in the spleen (Fig. 3.16A) and liver (Fig. 3.16B) 96 h post-infection. We also recovered similar numbers of znuA mutant S. Typhimurium in both C57BL/6 and S100a9⁻/⁻ mice. To reduce the effect of animal-to-animal variation, we performed a mixed infection of wild-type and znuA mutant S. Typhimurium in C57BL/6 and S100a9⁻/⁻ mice. We recovered a significant increase in the number of wild-type S. Typhimurium over znuA mutant in both C57BL/6 and S100a9⁻/⁻ mice in the spleen (Fig. 3.17A) and liver (Fig. 3.17B) 96 h post-infection. There was no significant difference in competitive index in C57BL/6 mice and S100a9⁻/⁻ mice, indicating that zinc sequestration during systemic infection may be dependent on other factors besides calprotectin.

3.4 Discussion

One strategy a host employs in response to bacterial infection is to inhibit bacterial growth by limiting the availability of essential metal ions, a process known as nutritional immunity (21). However, with the exception of iron, the role of metal sequestration in response to pathogens is not thoroughly understood; evidence for the importance of zinc acquisition at the host-pathogen interface comes largely from studies on high affinity zinc transporters in bacteria.
Figure 3.16 The ZnuABC transporter is crucial for colonization of systemic organs. Wild-type and $S100a9^{-/-}$ mice were injected intraperitoneally with $1 \times 10^2$ CFU of wild-type or $znuA$ mutant $S$. Typhimurium (n≥9). (A) Spleen and (B) liver samples were collected at 96 hr post infection and enumerated on selective media to determine CFU. Circles represent wild-type mice, and triangles represent $S100a9^{-/-}$ mice; filled symbols represent animals infected with wild-type $S$. Typhimurium, and open symbols represent animals infected with $znuA$ mutant $S$. Typhimurium. Bar represents geometric mean. Significant differences are indicated by ** ($P$ value ≤ 0.01).
Figure 3.16

A

Bacteria in spleen (CFU/g) 96h p.i.

- Wild-type mice
- $S100a9^{-/-}$ mice

B

Bacteria in liver (CFU/g) 96h p.i.

- Wild-type mice
- $S100a9^{-/-}$ mice

S. Typhimurium wild-type
S. Typhimurium $\Delta znuA$
Figure 3.17 Competitive index of wild-type to znuA mutant S. Typhimurium is similar between in C57BL/6 wild-type mice and S100a9−/− mice during systemic infection. Competitive indices of wild-type (white columns) and S100a9−/− mice (gray columns) injected intraperitoneally with 1x10^2 CFU of wild-type and znuA mutant S. Typhimurium (n≥9). (A) Spleen and (B) liver samples were collected at 96 hr post infection and enumerated on selective media to determine CFU. Competitive index was calculated by dividing the output ratio (CFU of the wild-type /CFU of the mutant) by the input ratio (CFU of the wild-type /CFU of the mutant). NS = not significant. Bars represent geometric mean ± standard error. Significant differences are indicated by ** (P value ≤ 0.01).
Figure 3.17

A

Competitive index in spleen 96h p.i.

wild-type mice S100a9<sup>−/−</sup> mice

wild-type vs ΔznuA

B

Competitive index in liver 96h p.i.

wild-type mice S100a9<sup>−/−</sup> mice

wild-type vs ΔznuA
Furthermore, only a few studies have investigated the contribution of zinc transporters to bacterial pathogenesis.

The ZnuABC zinc transporter and the zinc uptake regulator Zur were first described in *Escherichia coli* as a system for acquiring zinc under zinc-limiting conditions, subsequently being identified in several species of Gram negative bacteria (14). ZnuABC mutants are attenuated in mice that develop a systemic disease when infected with pathogens including *Brucella abortus, Pasteurella multocida*, and the typhoid model of *S. Typhimurium* (2, 6, 11, 22). ZnuABC has also been found to play an important role in the pathogenesis of a variety of localized infections: In the rabbit model for chancroid, a *Haemophilus ducreyi* znuA mutant is less virulent and is rapidly cleared from lesions (26); In mice infected with uropathogenic *E. coli* and *Proteus mirabilis*, while the ZnuABC transporter is not required for colonization of the bladder in single infections, it does provide an advantage in competitive experiments (34, 40); In chickens infected with *Campylobacter jejuni*, gastrointestinal colonization is dependent on a zinc transporter whose periplasmic component is an ortholog of *E. coli* znuA (10). However, despite experimental evidence indicating that the ZnuABC zinc transporter may promote colonization by several pathogens, little is known about the nutritional immune responses that induce zinc starvation *in vivo*.

The antimicrobial protein calprotectin, whose activity is dependent on zinc and manganese binding, is induced during bacterial and fungal infections in response to the cytokines IL-17 and IL-22 (8, 9, 20, 27, 49, 57). Our research shows that calprotectin is up-regulated in the intestine in response to acute infection with *S. Typhimurium* (colitis model) and can be detected in the feces, consistent with findings that calprotectin is present in the feces of patients with intestinal inflammatory conditions, including inflammatory bowel diseases and colon cancer (19,
24). Remarkably, whereas with other pathogens calprotectin-mediated zinc sequestration is necessary to suppress microbial growth (9, 20, 49), the growth of S. Typhimurium is actually enhanced over competing microbes by calprotectin expression. Importantly, we found that the high affinity ZnuABC zinc transporter, and not the more promiscuous ZupT permease, conferred a significant advantage to S. Typhimurium in colonizing the gut when calprotectin is highly expressed (i.e., when the gut is inflamed). Consistent with this, in the absence of an inflammatory response or in mice lacking calprotectin, the ZnuABC transporter did not provide a colonization advantage. More however, ZnuABC confers a competitive advantage to S. Typhimurium in the presence of calprotectin in the gastroenteritis infection model, but not in the systemic infection model. Therefore, our results indicate that zinc acquisition via the ZnuABC transporter facilitates S. Typhimurium growth in the inflamed gut by overcoming calprotectin-mediated zinc starvation (Fig. 3.15). This is the first time that calprotectin has been shown to enhance the competitive advantage of a pathogen and our work provides a mechanistic link between the ZnuABC transporter and a host zinc-limiting factor.

In conjunction with these insights, our study also underlines the acquisition of the micronutrient zinc in the inflamed gut as an important means of growth and competition between microbes. It is becoming more and more apparent that, in order to survive in a host, pathogens must contend with the resident microbiota for nutrients, often acquiring and evolving specialized systems to gain an advantage (39). In addition to overcoming calprotectin-mediated zinc sequestration, S. Typhimurium can also bypass lipocalin-2-mediated iron starvation in the inflamed gut by employing modified siderophores (37). Together, these findings indicate that resistance to metal withholding responses may represent a common theme for pathogens to compete with the intestinal microbiota when colonizing the inflamed gut mucosa. Moreover,
some species of commensal microbes in the gut may utilize metal transporters to grow under metal limiting conditions. Future analyses of the distribution and function of metal transporters may provide useful insight into the life of microbial communities in the inflamed gut environment.

As the inflammatory host response is also essential for controlling the dissemination of *S. Typhimurium*, therapeutic interventions to limit intestinal inflammation and thus reduce the growth and transmission of this pathogen are not feasible. However, targeting a variety of bacterial metal acquisition systems may represent a promising strategy to limit infections with *S. Typhimurium* and other pathogens.

### 3.5 Materials and Methods

**Bacterial strains and growth conditions**

Bacterial strains and plasmids are listed in Appendix A6. IR715 is a fully virulent, nalidixic acid resistant derivative of *S. Typhimurium* wild-type isolate ATCC 14028 (46). Cultures of *S. Typhimurium* and *E. coli* were routinely incubated either aerobically at 37°C in Luria-Bertani (LB) broth (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl) or on LB agar plates (1.5% Difco agar) overnight. Growth was also determined in M9 minimal media (per liter; 7.5g Na$_2$HPO$_4$, 3g KH$_2$PO$_4$, 0.5g NaCl, 1g NH$_4$Cl, 0.1mM CaCl$_2$, 0.5mM MgSO$_4$, 0.2% glucose) and M9 supplemented with 5 µM ZnSO$_4$. Antibiotics and other chemicals were added at the following concentrations (mg/l) as needed: carbenicillin (Carb), 100; chloramphenicol (Cm), 30; kanamycin (Km), 100; nalidixic acid (Nal), 50; 5-bromo-4-choloro-3-indoyl-B-D-galactopyranoside (Xgal), 40.
Allelic exchange deletion of znuA

Primers used are detailed in Appendix A7. Primers 7 and 8 were used to PCR amplify the region upstream of znuA (flanking region 1) of S. Typhimurium. Primers 4 and 5 were used to PCR amplify the region downstream of znuA (flanking region 2). The PCR products were ligated into pCR2.1 using the TOPO TA cloning kit (Invitrogen), heat shocked into E. coli TOP10 and plated on LB+Km+Xgal. White colonies were screened by EcoRI digestion for the appropriate length of linearized plasmid construct. Positive clones were sequence confirmed by using M13 forward and M13 reverse universal primers. Accurate clones were designated pJL2 (pCR2.1, ΔznuA flanking region 1 cassette) and pJL1 (pCR2.1, ΔznuA flanking region 2 cassette). Flanking region 2 cassette was digested out of pJL1 using SacI and EcoRI double-digestion, ligated into SacI and EcoRI double-digested pGP704 (33), heat shocked into E. coli CC118λpir (16), and plated on LB+Carb, generating pJL3. Flanking region 1 cassette was digested out of pJL2 using SalI and XbaI double-digestion, ligated into SalI and XbaI double-digested pJL3, heat shocked into E. coli CC118λpir, and plated on LB+Carb, generating pJL4.

The chloramphenicol acetyl transferase (CAT) cassette from pCMXX (5) was excised using SacI digestion and ligated into the compatible SacI site of pJL4, generating pJL5 (pGP704, ΔznuA::CAT). pJL5 was purified and heat shocked into E. coli S17-1 λpir (16), then conjugated separately into S. Typhimurium IR715. Transconjugants were selected for using Nal and Cm and colonies with double crossover events were screened for by loss of Carb resistance. Confirmation of mutant was performed using Southern blot analysis. Primers 13 and 14 were used to PCR amplify a probe designed to flanking region 2. The PCR product was ligated into pCR2.1, generating pJL8. The probe encoded on pJL8 was used to confirm the ΔznuA::CAT mutant and the S. Typhimurium ΔznuA::CAT strain was designated JZL3. For complementation
of JZL3, primers 10 and 11 were used to PCR amplify znuA from S. Typhimurium IR715. The PCR product was ligated into pCR2.1 using the TOPO TA system and heat shocked into E. coli TOP10 to generate pJL6. The znuA gene was excised from pJL6 using XhoI and EcoRV double-digestion and ligated into XhoI and EcoRV digested pWSK29 (52), heat shocked into E. coli, DH5α and plated on LB+Carb+Xgal, generating pJL7. pJL7 was purified and electroporated into JZL3. The ΔinvA::tetRA ΔspiB::KSAC ΔznuA::CAT triple mutant was generated by conjugating the pJL5 plasmid in E. coli S17-1 λpir with SPN452 (IR715 ΔinvA::tetRA ΔspiB::KSAC) (37). This strain was confirmed by Southern blot with the same probe as before to flanking region 2 and was designated JZL2.

**Allelic exchange deletion of zupT**

Primers used are detailed in Appendix A7. Primers 15 and 16 were used to PCR amplify the region upstream of zupT (flanking region 1) of S. Typhimurium. Primers 17 and 18 were used to PCR amplify the region downstream of zupT (flanking region 2). The PCR products were ligated into pCR2.1 using the TOPO TA cloning kit (Invitrogen), heat shocked into E. coli TOP10 and plated on LB+Km+Xgal. White colonies were screened by EcoRI digestion for the appropriate length of linearized plasmid construct. Positive clones were sequence confirmed by using M13 forward and M13 reverse universal primers. Accurate clones were designated pJL9 (pCR2.1, ΔzupT flanking region 1 cassette) and pAP1 (pCR2.1, ΔzupT flanking region 2 cassette). Flanking region 1 cassette was digested out of pJL9 using BamHI and XbaI double-digestion, ligated into BamHI and XbaI double-digested pBluescript II SK (+) (1), heat shocked into E. coli DH5α, and plated on LB+Carb, generating pAP2. Flanking region 2 cassette was digested out of pAP1 using BamHI and SacI double-digest, ligated into SacI and XbaI double-
digested pAP2, heat shocked into *E. coli* DH5α, and plated on LB+Carb, generating pAP3. The combined flanking regions cassette is digested from pAP3 with BamHI and ligated into BamHI digested pRDH10 (23), heat shocked into *E. coli* CC118λpir, and plated on LB+Cm, generating pAP4. The kanamycin resistance KSAC cassette from pBS34 (37) was excised using XbaI digestion and ligated into the compatible XbaI site of pAP4, generating pAP7 (pRDH10, ΔzupT::KSAC). pAP7 was purified and heat shocked into *E. coli* S17-1 λpir, then conjugated separately into wild-type *S. Typhimurium* IR715 or the *S. Typhimurium* ΔznuA mutant strain JZL3. Transconjugants were selected for using Nal and Km and colonies with double crossover events were screened for by loss of Cm resistance. Confirmation of mutant was performed using Southern blot analysis. Primers 19 and 20 were used to PCR amplify a probe designed to flanking region 1. The PCR product was ligated into pCR2.1, generating pAP6. The probe encoded on pAP6 was used to confirm the ΔzupT::KSCA mutation. The *S. Typhimurium* ΔzupT::KSAC strain was designated AJP3, and the *S. Typhimurium* ΔznuA::CAT ΔzupT::KSCA strain was designated AJP4.

**Growth in media supplemented with calprotectin**

Recombinant calprotectin was produced as described elsewhere (18). Growth in media supplemented with calprotectin was performed as described by Kelhl-Fie et al with minor modifications (20) Briefly, wild type and ΔznuA *S. Typhimurium* were grown overnight in M9 minimal media at 37°C with agitation. OD₆₀₀ was determined and used to calculate the volume of overnight culture needed to obtain 1x10⁸ cells/ml. The calculated volume of culture was then spun down at 20,000g for 10 minutes in a tabletop centrifuge. The supernatant was discarded and the pellet resuspended in 1 ml of LB media. 1x10⁵ cells/ml was obtained with serial dilution and
10 µl were used to inoculate the wells of a 96-well Nunclon Surface plate (Nunc). Each well contained a 62:28:10 ratio of calprotectin buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM BME, 3 mM CaCl₂) to LB media to inoculum. Two-fold dilutions of calprotectin stock were prepared, starting from 500 µg/ml and diluting down to 15.6 µg/ml. Triplicates were made for each concentration of calprotectin. The 96-well plate was incubated inside a Tupperware container with a moistened piece of paper towel and incubated at 37°C with 5% CO₂. OD₆₀₀ were taken at 0, 2, 4, 5, 6, 7, 8, 12, and 24 hours on a Microplate Reader (Bio Rad) and graphed on a semi-logarithmic scale. The experiment was repeated four times.

Mouse infection experiments

C57BL/6 mice and S100a9⁻/⁻ mice were used in our study. C57BL/6 mice were purchased from Taconic Farms and S100a9⁻/⁻ mice were generated as previously described (31). For studies in the S. Typhimurium gastroenteritis model, C57BL/6 mice and S100a9⁻/⁻ mice were infected orally as previously described (37). Briefly, mice were gavaged with 0.1 ml of a 200 mg/ml streptomycin/sterile water solution one day prior to mock-infection with LB or oral infection with 1×10⁹ CFU of S. Typhimurium in LB. For the zinc supplementation experiment, C57BL/6 mice were gavaged with 0.1 ml of a 10 mg/ml of zinc sulfate/sterile water solution starting a day before streptomycin treatment and were gavaged once a day for the length of the experiment. The cecum was harvested for mRNA, protein, and histopathology at 48-96h post-infection. The cecal contents were collected, serially diluted, and plated on appropriate antibiotic LB agar plates to determine bacterial counts. Groups of 5-11 mice were used for each oral infection experiment. For the typhoid model of S. Typhimurium infection, C57BL/6 mice and S100a9⁻/⁻ mice were injected intraperitoneally (i.p.) with 1×10² CFU of S. Typhimurium in 0.1 ml sterile PBS. The
spleen and liver was harvested at 96h post-infection, homogenized, serially diluted and plated on appropriate antibiotic LB agar plates to determine bacterial counts. Groups of 7-10 mice were used for each i.p. infection experiment. In the mixed infections, competitive indices were calculated by dividing the output ratio (CFU of wild-type / CFU of the mutant) by the input ratio (CFU of wild-type / CFU of the mutant). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

**Isolation of colon crypts**

Streptomycin-treated C57BL/6 mice were infected with S. Typhimurium or mock and sacrificed at 72 hours post-infection. Crypt isolation from colon and cecum was performed as described (53). Briefly, cecum plus colon tissue was collected, flushed, and opened to expose mucosa. Tissue was then incubated at room temperature with a 3 mM EDTA and 0.5 mM DTT solution. After 90 minutes of incubation the tissue was transferred into PBS, shaken, and the detached crypts were decanted into a 15 ml falcon tube and spun down at 4°C 200 g for 5 minutes (see Appendix A1). RNA and protein were isolated using Tri-Reagent (Molecular Research Center) as per the manufacturer’s instructions.

**CXCR2 antibody blocking of neutrophils**

A murine-specific CXCR2 blocking antibody was raised in rabbits following immunization with a 17 amino acid peptide corresponding to the amino terminus of CXCR2 (32). Mice were treated with a-CXCR2 or normal rabbit serum 24 hours prior to infection via intraperitoneal (i.p.) injection as previously described (51). Mice were treated with anti-CXCR2
or normal rabbit serum via intraperitoneal (i.p.) injection 24 hours prior to infection (i.e. at the same time as streptomycin treatment).

**Cell isolation from whole blood**

At 72 hours post infection, mice were sacrificed and 300 to 500 µl of whole blood was collected in sodium heparin tubes (BD Vacutainer) by cardiac puncture using an insulin syringe (BD) and stored on ice until further processing. Red blood cells were lysed by incubating whole blood with 5 ml of ACK Lysing Buffer (GIBCO) for 5 min at room temperature. To stop the lysis reaction, 7 ml of medium (RPMI-1640, 10% FCS, 1% Pen/Strep – GIBCO) was added and the suspension was centrifuged at 1500 g for 7 min at room temperature. Thereafter the pellet was resuspended in 150 µl medium and cells were transferred to a round bottom 96-well plate for extracellular staining.

**Extracellular staining and flow cytometry analysis**

Blood neutrophils were detected with phycoerythrin (PE)-conjugated Ly6G (clone RB6-8C5, eBioscience) and PE-Cy7-conjugated CD11b (eBioscience) monoclonal antibodies as described previously (17). Briefly, cells were incubated with purified anti-mouse CD16/32 antibody (1 in 100 dilution, eBioscience) in FACS-staining buffer (phosphate-buffered saline pH 7.45, 0.5% bovine serum albumin, and 0.02% sodium azide) for 30 min at room temperature in order to block Fc gamma receptors. Subsequently, cells were stained for the above mentioned surface markers for 30 min on ice in the dark (1 in 50 dilution). Cells were washed, resuspended in staining buffer and fixed in 1% paraformaldehyde/phosphate-buffered saline (pH 7.45). Data was acquired on a FACSCalibur (BD Biosciences, San Jose, CA) and further analyzed with
FlowJo software (TreeStar, Ashland, OR). Leucocytes were gated using forward (FSC) and sight scatter (SSC) criteria of cells followed by identifying Ly6G\textsuperscript{high} CD11b\textsuperscript{+} neutrophils.

**Measurement of zinc in fecal samples**

C57BL/6 mice were treated with streptomycin and infected with \textit{S. Typhimurium} wild-type or mock as described. At 96 hours post-infection, fecal pellets were collected with plastic forceps and placed in glass containers that were previously cleaned with nitric acid to remove metal contamination. The fecal pellets were then autoclaved to kill all bacteria. Sample analysis was performed by Applied Speciation (Bothell, WA) as previously described by Corbin et al (9) and detailed in Supplemental methods. Briefly, the fecal samples from 4 infected and 4 uninfected mice were digested by boiling in nitric acid and hydrochloric acid. The samples were then resuspended in water and analyzed by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS). Aliquots of each sample are introduced into a radio frequency (RF) plasma where energy-transfer processes cause desolvation, atomization, and ionization. The ions were extracted from the plasma through a differentially-pumped vacuum interface and traveled through a pressurized chamber (DRC) containing a specific reactive gas which preferentially reacts with interfering ions of the same target mass to charge ratios (m/z). A solid-state detector detected ions transmitted through the mass analyzer, on the basis of their mass-to-charge ratio (m/z), and the resulting current was processed by a data handling system. The results were reported as mg of zinc per kg of dry weight.

**Analysis of the microbiota**
Composition of the bacterial microbiota was analyzed as described earlier (55). Briefly, the colon content was collected from mice 96 hours post-infection, and snap frozen in liquid nitrogen. The DNA was subsequently extracted using the QIAamp DNA stool kit (Qiagen). Two µl of extracted bacterial DNA was used as a template for the q-PCR reaction with the primer pairs developed by Barman et al (3) and presented in Appendix A8. The 16S gene copy numbers per µl of DNA from each sample (one fecal pellet collected from each colon) was determined using the plasmids described in supplementary table 1. To estimate the copy number of Enterobacteriaceae other than *Salmonella*, for each sample the *Salmonella* 16S gene copy number was subtracted from the total Enterobacteriaceae 16S gene copy number.

**Western blot**

Total protein was extracted from mouse cecum using Tri-Reagent (Molecular Research Center). 15 µg of total protein were analyzed using 15% SDS-PAGE gels and transferred to PVDF membranes. Detection of mouse tubulin was performed with a primary rabbit polyclonal antibody (Cell Signaling Technology) while detection of calprotectin was performed with a polyclonal goat anti-mouse S100A8 and a polyclonal goat anti-mouse S100A9 (R&D Systems). Myeloperoxidase was detected using a primary polyclonal goat anti-human and mouse antibody (R&D Systems). As secondary antibody, a goat-anti-rabbit or rabbit-anti-goat conjugate to horseradish peroxidase (HRP) (Jackson) were used.

**Detection of intestinal and fecal calprotectin using ELISA**

Streptomycin treated wild-type C57BL/6 mice were mock or *S. Typhimurium IR715* infected. At 72 hours post-infection mice were sacrificed and the cecum, the colon and fecal
samples were collected. Cecum and colon tissue were placed in 3 ml sterile PBS and homogenized. The homogenate was spun down at 4,000g at 4°C for 20 minutes; supernatant was then used for Western blot and ELISA analysis. Extraction buffer (0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl2, 0.1 M citric acid monohydrate, 5 g/l BSA and 0.25 mM thimerosal (pH 8.0)) adopted from Hycult Biotech’s H305 Human Calprotectin ELISA kit was added to fecal samples. The fecal samples were incubated on ice for 30 minutes and were homogenized at 4°C. Samples were then spun down at 10,000g at 4°C for 20 minutes and the supernatant was used for Western blot and ELISA analyses. Murine S100A8/S100A9 was determined by an in-house established ELISA as described (50). Wells were coated with rabbit polyclonal anti-S100A8 (4 µg/ml). The detection antibody rabbit polyclonal anti-S100A9 (2 µg/ml) was coupled to biotin according the manufactures’ instructions. Streptavidin coupled HRP and TMB as substrate was used and absorbance at 405 nm was recorded using a MRX microplate reader (Dynex Technologies; USA). Recombinant prepared murine S100A8/S100A9 heterodimer was used as standard in the calibration curve. Data were normalized taking into account the weight of the collected fecal samples and tissues and assuming a density of 1g/ml.

**Quantitative real-time PCR**

Total RNA was extracted from mouse cecal tissue using Tri-Reagent (Molecular Research Center). Reverse transcription of 1 µg of total RNA was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche). Quantitative real-time PCR (qRT-PCR) for the expression of Actb, Il-17, Il-22, S100a8, S100a9, Lcn2, Cxcl-1, and Ly6G (see Appendix A8) were performed using the LightCycler 480 SYBR Green Master on the LightCycler 480 II (Roche). Conditions for qRT-PCR were 95°C for 5 minutes, then 45 cycles of 95°C for 10
seconds 60°C for 10 seconds and 72°C for 15 seconds. Gene expression was normalized to β-actin and fold changes in gene expression was relative to uninfected controls and calculated using the ΔΔ Ct method.

**Histopathology**

Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. The pathology score of cecal samples was determined by blinded examinations of cecal sections from a board certified pathologist using previously published methods (4, 37). Each section was evaluated for the presence of neutrophils, mononuclear infiltrate, submucosal edema, surface erosions, inflammatory exudates and cryptitis. Inflammatory changes were scored from 0 to 4 according to the following scale: 0=none; 1=low; 2=moderate; 3=high; 4=extreme. The inflammation score was calculated by adding up all the scores obtained for each parameter and interpreted as follows: 0-2= within normal limit; 3-5= mild; 6-8=moderate; 8+=severe.

**Statistical analysis**

Differences between treatment groups were analyzed by ANOVA followed by Student’s t test. A P value equal or below 0.05 was considered statistically significant.

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3.6 References


microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe 2:204.


Chapter 4

Probiotic Bacteria Compete with *Salmonella* for Zinc.

4.1 Summary

*Salmonella enterica* serovar Typhimurium is a leading cause of food borne illness in the world, and colonizes the human large intestine to high numbers after infection. The probiotic *Escherichia coli* Nissle 1917 has been shown to be effective in ameliorating *S.* Typhimurium infection. However, the mechanism by which *E.* coli Nissle is able to limit *S.* Typhimurium growth is not well understood. Recent work has shown that *E.* coli Nissle is more efficient at acquiring iron metal ions than *S.* Typhimurium. In this study we uncovered that zinc acquisition by *E.* coli Nissle is necessary for effective competition against *S.* Typhimurium. Mutation of the two zinc transporters ZnuABC and ZupT diminished the ability of *E.* coli Nissle to reduce *S.* Typhimurium colonization, and reduced the probiotic’s competitive fitness against *S.* Typhimurium. Additionally, the host zinc sequestering peptide calprotectin is necessary for the reduction of *S.* Typhimurium colonization, as *E.* coli Nissle was unable to reduce *S.* Typhimurium colonization in calprotectin-deficient mice. Our findings suggest that zinc acquisition plays an important part in the competition between *E.* coli Nissle and *S.* Typhimurium, and that host zinc sequestration is required for *E.* coli Nissle reduction of *S.* Typhimurium colonization.

4.2 Introduction

*Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is one of the most common causes of bacterial foodborne gastroenteritis, an infection characterized by a strong intestinal
immune response and by neutrophil infiltration to the lumen (23). The inflammatory response elicited by S. Typhimurium is dependent on the expression of two type III secretion systems, T3SS-1 and T3SS-2, which act as molecular syringes for the delivery of effector proteins to host cells (22, 56). To initiate inflammation S. Typhimurium need to first come into contact with host cells, which include intestinal epithelial cells and antigen presenting cells (APCs) such as macrophages and dendritic cells. Infected APCs detect S. Typhimurium through Toll-like and NOD-like receptors and secrete a number of cytokines, including IL-23, IL-1β, and IL-18 (17, 30, 50). These cytokines in turn stimulate T cells in the gut to produce cytokines including IL-17 and IL-22, signals that in turn induce intestinal epithelial cells to secrete neutrophil chemoattractants such as CXC chemokines, resulting in the influx of neutrophils to the infected gut (16, 17, 45).

Activated neutrophils and intestinal epithelial cells respond to S. Typhimurium infection by secreting antimicrobial proteins into the lumen (35, 44). As a result, the commensal microbiota, consisting largely of Bacteroidetes and Firmicutes, is dramatically reduced and altered during inflammation (3, 33, 38, 51). Paradoxically, the inflammatory response favors the growth of S. Typhimurium and other Enterobacteriaceae, which are able to overgrow in the inflamed gut mucosa (5, 38, 51). The bloom of S. Typhimurium in the inflamed gut is possible because the pathogen is able to migrate towards and utilize sources of alternative electron acceptors in the form of nitrate and tetrathionate, which are produced only during inflammation (37, 46, 59). Additionally, S. Typhimurium is able to utilize host-derived nutrients in the form of mucin and ethanolamine as carbon sources (51, 55).

Metal ions are another important form of nutrient for S. Typhimurium as well as many other pathogens, and as such the host has evolved antimicrobial proteins that sequester metals
such as iron, zinc, and manganese from microbes to limit their growth, in a process termed “nutritional immunity” (26). However, many pathogens including S. Typhimurium have evolved specialized transporters to overcome nutritional immunity and successfully colonize the host. One example of a host mechanism that sequesters metal nutrients is the secretion of the antimicrobial protein lipocalin-2, which binds to and sequesters the iron-laden form of the bacterial siderophore enterochelin (15). To elude lipocalin-2 iron withholding, S. Typhimurium produces a C-glucosylated derivative of enterochelin called salmochelin, which is not bound by lipocalin-2 (14). Thus, internalization of the iron-laden salmochelin promotes evasion of lipocalin-2 sequestration of enterochelin and enhances S. Typhimurium colonization of the inflamed gut (44).

S. Typhimurium evasion of host nutritional immunity is not limited to iron, as we have previously shown that S. Typhimurium is also able to overcome host zinc sequestration in the gut (35). Zinc is an essential nutrient required by both prokaryotic and eukaryotic life. Zinc ions coordinate the structure of zinc finger transcription factors, and serve as the catalytic core in enzymes such as metalloproteases, superoxide dismutases, and metallo-β-lactomases. In bacteria it is estimated that zinc serves as a cofactor for 5-6% of all protein (2). During zinc limiting conditions, S. Typhimurium expresses the high-affinity zinc transporter ZnuABC (1, 7, 35). In addition, S. Typhimurium also expresses the ZupT permease, which transports zinc and other divalent metal ions in a constitutive manner (19, 53). Both ZnuABC and ZupT have been shown to contribute to S. Typhimurium virulence in the mouse model of infection (1, 7, 8, 28, 35). Although very little is known about host antimicrobial mechanisms that sequester metals other than iron, recent studies have shown that during infection the host secretes the antimicrobial protein calprotectin, a heterodimer of the S100A8 and S100A9 peptides that binds and sequesters
away zinc and manganese ions (9, 11, 24, 29, 57). Calprotectin constitutes up to 40% of neutrophil cytosolic content (54) and the expression of its two subunits is also induced in epithelial cells by stimulation with IL-17 and IL-22 (5, 34, 61). Therefore, during S. Typhimurium infection both epithelial cells and neutrophils contribute to the high levels of calprotectin observed in the inflamed gut (5, 35). However, by expressing ZnuABC, S. Typhimurium overcomes calprotectin-mediated zinc sequestration, thereby overgrowing the microbiota and thriving in the inflamed gut (5, 35). These studies highlight the importance of zinc acquisition in the inflamed gut as means of colonization and competition between S. Typhimurium and the microbiota, and suggest that other bacterial strains may also be able to compete with S. Typhimurium for zinc in this environment.

A bacterium that colonizes the inflamed gut is the probiotic *Escherichia coli* strain Nissle 1917 (*E. coli* Nissle, serotype O6:K5:H1). The strain was first isolated in WWI when it was recovered from the stool of a soldier who did not develop gastroenteritis during a *Shigella* outbreak (41). Since then, *E. coli* Nissle has been proven effective in the treatment and prevention of intestinal disorders including chronic constipation, ulcerative colitis, and infantile diarrhea (32, 36, 40). Despite being used as a probiotic for nearly a century, the mechanisms through which *E. coli* Nissle exerts its protective effect are still not well understood. Nevertheless, we have recently shown that *E. coli* Nissle utilizes multiple iron uptake systems to outcompete and reduce *S. Typhimurium* colonization in mouse models of gastroenteritis (13). Loss of multiple iron transport systems diminished *E. coli* Nissle probiotic activity, as mutants deficient in iron uptake were unable to reduce *S. Typhimurium* colonization (13). Additionally, *E. coli* Nissle also needed inflammation and iron limitation by lipocalin-2 to better colonize the gut and reduce *S. Typhimurium* colonization (13), demonstrating that this probiotic strain
requires host iron sequestration mechanism to serve as an effective competitor against *S. Typhimurium*. As *S. Typhimurium*, in addition to iron, also requires zinc to successfully replicate in the inflamed gut (8, 35), we set out to determine whether *E. coli* Nissle can also reduce *S. Typhimurium* colonization by competing with this pathogen for zinc.

4.3 Results

**Zinc transporters contribute to *E. coli* Nissle growth in zinc-deplete media.**

To determine whether *E. coli* Nissle could also compete with *S. Typhimurium* for zinc in the inflamed gut, we first constructed deletion mutants in ZnuABC and ZupT of *E. coli* Nissle. Although the function of these two transporters in *E. coli* Nissle is not known, their disruption significantly diminishes the capacity of the uropathogenic *E. coli* CFT073, a closely related strain, to grow in zinc-deplete culture media and to cause urinary tract infection (47). After generating an *E. coli* Nissle *znuA*, and a *znuA zupT* mutant strain, we studied the growth kinetics of the mutants and the wild type in both metal-replete and metal-deficient media, in comparison to *S. Typhimurium* wild-type, *znuA* and *znuA zupT* strains (Fig. 4.1). In nutrient-rich LB medium, there was no difference in growth between the zinc transporter mutants compared to their isogenic wild-type *E. coli* Nissle (Fig 4.1A) or *S. Typhimurium* (Fig. 4.1B) over a 24 hours period. In contrast, in zinc-deplete M9 minimal medium *E. coli* Nissle zinc transporter mutants (Fig. 4.1C) and *S. Typhimurium* zinc transporter mutants (Fig. 4.1D) exhibited a severe growth defect. Addition of 5 µM ZnSO₄ to M9 minimal medium was sufficient to restore the growth of *S. Typhimurium* *znuA* and *znuA zupT* mutants to wild type levels (Fig. 4.1F). *E. coli* Nissle *znuA* was also rescued to wild-type levels in 5 µM ZnSO₄ supplemented M9 minimal medium, whereas *E. coli* Nissle *znuA zupT* mutant was partially rescued at this concentration of ZnSO₄.
Figure 4.1 Zinc transporters are necessary for growth in zinc-deplete media. Wild-type (WT), ΔznuA, and ΔznuA ΔzupT E. coli Nissle strains (EcN, white symbols) and wild-type (WT), ΔznuA, and ΔznuA ΔzupT S. Typhimurium strains (STM, gray symbols) grown in LB media (A and B), M9 minimal media (C and D), or M9 supplemented with 5 µM ZnSO₄ (E and F). Growth was quantified by enumeration of bacterial CFU on selective media at each time point. Data are representative of three independent experiments. Bars representing geometric mean ± standard error. ** (P value ≤ 0.01).
Figure 4.1

- EcN WT
- EcN znuA
- EcN znuA zupT
- STM WT
- STM znuA
- STM znuA zupT

A

B

C

D

E

F

CFU/ml

Time (h)

CFU/ml

Time (h)

CFU/ml

Time (h)

CFU/ml

Time (h)

CFU/ml

Time (h)

CFU/ml

Time (h)

CFU/ml

Time (h)

LB Media

M9 Minimal Media

M9 Minimal Media + 5 µM ZnSO₄
(Fig. 4.1E). Of note, we confirm that ZnuABC appears to be the primary zinc transporter in both *E. coli* Nissle and *S. Typhimurium* (1, 7, 8, 35), although ZupT may play a role if zinc limitation is further enhanced (8). These results demonstrate that the growth defect of the *E. coli* Nissle and *S. Typhimurium* zinc transporter mutants in M9 minimal media was largely due to zinc limitation, and support our hypothesis that zinc acquisition by *E. coli* Nissle via the ZnuABC and ZupT transporters could be important in the inflamed gut, where this metal is limited (35).

**E. coli** Nissle resistance to the antimicrobial peptide calprotectin is mediated by zinc transporters.

One of the host proteins that limit the concentration of zinc in the inflamed gut is the antimicrobial protein calprotectin (35). As we have found that *E. coli* Nissle colonize the inflamed gut and that it is resistant to the iron sequestration activity of the antimicrobial protein lipocalin-2 (13), we next sought out to determine whether *E. coli* Nissle is also resistant to calprotectin, and whether its resistance is dependent on zinc transporters. To this end, we grew wild-type, *znuA*, and *znuA zupT* *E. coli* Nissle strains in rich media (LB) supplemented with calprotectin and compared the growth to their equivalent genotypes in *S. Typhimurium*. In the absence of calprotectin, all *E. coli* Nissle and *S. Typhimurium* strains grew to similar levels after 16 hours, while the addition of 125 µg/ml of calprotectin significantly reduced the growth of *E. coli* Nissle and *S. Typhimurium* *znuA* and *znuA zupT* mutants (Fig. 4.2). Notably, all three *E. coli* Nissle strains grew to a higher amount than *S. Typhimurium* strains in media supplemented with calprotectin, and no difference in growth between *E. coli* Nissle wild-type and the zinc transporter mutants was observed in media supplemented with 250 µg/ml of calprotectin (Fig. 4.2A). We even recovered approximately 10 fold more *E. coli* Nissle wild type than *S.*
Figure 4.2 Resistance to calprotectin is mediated by zinc transporters. (A) Wild-type (WT), ΔznuA, and ΔznuA ΔzupT E. coli Nissle (EcN) strains and (B) wild-type, ΔznuA, and ΔznuA ΔzupT S. Typhimurium (STM) strains were grown in LB media without calprotectin or in LB media supplemented with 125 µg/ml or 250 µg/ml calprotectin. Growth was quantified by enumeration of bacterial CFU on selective media at each time point. Data are representative of three independent experiments. Bars represent geometric mean ± standard error. ** (P value ≤ 0.01).
Typhimurium wild-type in media supplemented with either 125 µg/ml or 250 µg/ml of calprotectin (**Fig. 4.2A and 4.2B**). These results indicate that *E. coli* Nissle appears to be resistant to calprotectin, as its growth was only minimally impaired even at the highest concentration. Furthermore, the zinc transporters, and in particular ZnuABC as we previously shown for *S. Typhimurium* (8, 35), contribute to the *E. coli* Nissle resistance to zinc sequestration by calprotectin, although additional undiscovered mechanisms also likely play a role.

**Zinc transporters enhance *E. coli* Nissle competition against *S. Typhimurium.**

As our results suggested that *E. coli* Nissle is resistant to concentrations of calprotectin similar to those we found in the inflamed gut (35), we next sought to determine whether zinc acquisition via the ZnuABC and ZupT zinc transporters is important for the beneficial activity of *E. coli* Nissle during *S. Typhimurium* infection. To this end, we infected streptomycin pre-treated C57BL/6 mice with *S. Typhimurium* (colitis mouse model (4)), and co-administered either *E. coli* Nissle wild-type or the *znuA zupT* mutant (**Fig. 4.3**). As a control, groups of mice treated with streptomycin were infected with *S. Typhimurium* alone, or administered only *E. coli* Nissle, either the wild-type or the *znuA zupT* mutant. The colonization levels of both *E. coli* Nissle and *S. Typhimurium* were then determined at 72 hours and 96 hours post-infection (**Fig. 4.3**), and intestinal inflammation was assessed by histology (**Fig. 4.5A**). We recovered high levels of *E. coli* Nissle wild-type and *znuA zupT* mutant in the colon content of all groups of mice at 72 hours post infection (**Fig. 4.3A**), indicating that *E. coli* Nissle intestinal colonization was not affected by the absence of ZnuABC and ZupT, or by the presence of *S. Typhimurium*, as we previously shown (13). At 96 hours post infection, *E. coli* Nissle colonization levels remained
Figure 4.3 Deletion of zinc transporter diminished *E. coli* Nissle capacity to reduce *S. Typhimurium* colonization. C57BL/6 mice were pretreated with streptomycin and infected with *S. Typhimurium* (STM) alone, *E. coli* Nissle (EcN) wild-type (WT) alone, *E. coli* Nissle ΔznuA ΔzupT alone, or co-administered with *S. Typhimurium* and *E. coli* Nissle wild-type, or *S. Typhimurium* and *E. coli* Nissle ΔznuA ΔzupT. CFU was determined at (A and B) 72h and (C and D) 96h post-infection. *S. Typhimurium* is represented in black circles while *E. coli* Nissle strains are represented in white circles. * (P value ≤ 0.05), ** (P value ≤ 0.01).
Figure 4.3

A

EcN in C57BL/6 mice colon content (CFU/mg) 72h p.i.

EcN WT znuA zupT

B

STM in C57BL/6 mice colon content (CFU/mg) 72h p.i.

STM + +

EcN EcN znuA zupT

C

EcN in C57BL/6 mice colon content (CFU/mg) 96h p.i.

EcN WT znuA zupT

D

STM in C57BL/6 mice colon content (CFU/mg) 96h p.i.

STM + +

EcN EcN znuA zupT
Figure 4.4 Calprotectin is required for reduction of *S. Typhimurium* colonization by *E. coli* Nissle. *S100a9*<sup>−/−</sup> mice were pretreated with streptomycin and infected with *S. Typhimurium* (STM) alone, *E. coli* Nissle (EcN) wild-type (WT) alone, or co-administered with *S. enterica* serovar Typhimurium and *E. coli* Nissle wild-type. CFU was determined at (A and C) 72h and (B and D) 96h post-infection. *S. Typhimurium* is represented in black circles while *E. coli* Nissle strains are represented in white circles. ** (*P* value ≤ 0.01).
Figure 4.4

A

EcN in S100a9<sup>−/−</sup> mice colon content (CFU/mg) 72h p.i.

B

STM in S100a9<sup>−/−</sup> mice colon content (CFU/mg) 72h p.i.

C

EcN in S100a9<sup>−/−</sup> mice colon content (CFU/mg) 96h p.i.

D

STM in S100a9<sup>−/−</sup> mice colon content (CFU/mg) 96h p.i.
Figure 4.5 Histopathology of C57BL/6 and $S100a9^{-/-}$ mice in single and mixed infections. Total pathology score with geometric mean (bar) of individual C57BL/6 (A) and $S100a9^{-/-}$ mice (C), and pathology criteria score of individual C57BL/6 (B) and $S100a9^{-/-}$ mouse (D) in the indicated infection groups. The gray region includes scores indicative of moderate to severe inflammation.
Figure 4.5

A

Pathology score (cecum) 96 h p.i.

0 2 4 6 8 10 12 14

EcN WT STM EcN STM STM Mock

znuA znuA znuA

zupT zupT

C57BL/6 mice

B

Pathology score (cecum) 96 h p.i.

0 2 4 6 8 10 12 14

EcN WT STM STM STM Mock

znuA znuA

zupT

zupT

S100a9−/− mice
high in mice that were infected with *S. Typhimurium*, but were decreased in mice administered with *E. coli* Nissle alone (Fig. 4.3C). The decreased colonization of *E. coli* Nissle could be attributed to the absence of inflammation in *E. coli* Nissle single infection mice, as *E. coli* Nissle strains maintained high levels of colonization in co-administrated mice, which were inflamed (Fig. 4.5A). These results are consistent with a prior study that suggests that *E. coli* strains benefit from intestinal inflammation by utilizing host-derived nitrate to respire in the inflamed gut (60). With regards to *S. Typhimurium* intestinal colonization, we confirmed that the pathogen was recovered at high levels in the colon content at both 72 and 96 hours post-infection in single infected mice (Fig. 4.3B and 4.3D), as we and others have previously shown in this model. Moreover, consistent with our recent findings (13), co-administration of *E. coli* Nissle wild-type reduced colonization levels of *S. Typhimurium* at both 72 and 96 hours post-infection (Fig. 4.3B and 4.3D). In particular, we detected two orders of magnitude less *S. Typhimurium* in groups of mice that received *E. coli* Nissle wild-type, compared to mice infected with *S. Typhimurium* only (Fig. 4.3D). Additionally, while *S. Typhimurium* alone induced high levels of inflammation, administration of *E. coli* Nissle wild-type resulted in slightly lower levels of inflammation, as observed by histopathology (Fig. 4.5A), confirming previous results (13).

In contrast to what we observed for *E. coli* Nissle wild-type, administration of the *E. coli* znuA zupT mutant did not reduce *S. Typhimurium* colonization at 72 hours post-infection (Fig. 4.3B). At 96 hours post-infection, the *E. coli* Nissle znuA zupT mutant was able to significantly reduce *S. Typhimurium* colonization by an average of 3 fold. However, this was much less than the two log reduction made by wild-type *E. coli* Nissle (Fig. 4.3D). Together with the observation that the *E. coli* Nissle znuA zupT mutant is relatively resistant to calprotectin (Fig 4.2A), these results raise the possibility that unknown mechanisms may either contribute to
import zinc or compensate for zinc limitation in the absence of ZnuABC and ZupT. Moreover, the level of inflammation in mice infected with S. Typhimurium that were administered the E. coli Nissle znuA zupT mutant was comparable to what was observed in mice infected with S. Typhimurium alone (Fig. 4.5A). Altogether, these results demonstrate that zinc uptake via specialized transporters contributes to the probiotic effect of E. coli Nissle during S. Typhimurium infection by reducing the intestinal colonization of the pathogen.

**E. coli Nissle requires host antimicrobial protein calprotectin to reduce S. Typhimurium colonization.**

One of the mechanisms by which the host limits zinc in the host is by the secretion of the antimicrobial protein calprotectin, which chelates this essential metal ion, thereby limiting its availability to pathogens (9, 29, 57). We have previously shown that wild-type S. Typhimurium evade zinc sequestration by calprotectin because it expresses the ZnuABC zinc transporter. Moreover, S. Typhimurium exploited calprotectin to gain a competitive advantage against an isogenic *znuA* mutant and the microbiota in the inflamed gut (35). As E. coli Nissle is also resistant to calprotectin and achieves greater colonization when the intestine is inflamed (Fig 4.3C) (13, 60), we hypothesized that E. coli Nissle would lose its competitive advantage over S. Typhimurium in the absence of calprotectin. To test this hypothesis, *S100a9*−/− mice that do not produce calprotectin (39) were infected with S. Typhimurium after streptomycin treatment, and administered *E. coli* Nissle wild-type. As a control, groups of mice treated with streptomycin were infected with S. Typhimurium, or administered only *E. coli* Nissle wild-type. Colonization levels in the colon content of both S. Typhimurium and *E. coli* Nissle were determined at 72 hours and 96 hours post-infection, and intestinal inflammation was assessed by histology (Fig.
4.4 and 4.5B). Similar to wild-type mice (Fig. 4.3A and 4.3C), colonization of wild-type *E. coli* Nissle alone decreased from 72 hours to 96 hours post-infection in *S100a9*/* mice (Fig. 4.4A, 4.4C), likely because of the absence of inflammation in these mice (Fig. 4.5B). However, in contrast to wild-type mice (Fig. 4.3B), administration of wild-type *E. coli* Nissle failed to reduce *S. Typhimurium* colonization at 72 hours post-infection in *S100a9*/* mice (Fig. 4.4B). At 96 hours post-infection there was a trend towards decreased number of *S. Typhimurium* in mice that were administered *E. coli* Nissle, which however was not statistically significant (Fig. 4.4D). Of note, although administration of *E. coli* Nissle to *S100a9*/* mice failed to reduce *S. Typhimurium* intestinal colonization, the probiotic still had a beneficial effect as it reduced intestinal inflammation (Fig. 4.5B). Altogether, these results demonstrate that *E. coli* Nissle requires the expression of calprotectin to reduce *S. Typhimurium* colonization of the inflamed gut.

**E. coli** Nissle requires zinc transporters and the expression of calprotectin to outgrow *S. Typhimurium*.

Our results suggest that *E. coli* Nissle and *S. Typhimurium* compete for the same niche in the inflamed gut. We thus quantified the competitive advantage of *E. coli* Nissle over *S. Typhimurium* by calculating the competitive index in mice that were administered a 1:1 mixture of the strains (Fig. 4.3 and 4.4). Consistent with our prior study (13), we found that wild-type *E. coli* Nissle significantly outcompeted *S. Typhimurium* at both 72 hours and 96 hours post-infection in C57BL/6 mice (Fig. 4.6). In contrast to wild-type *E. coli* Nissle, we found a near 1:1 ratio of the *E. coli* Nissle *znuA zupT* mutant and *S. Typhimurium* wild-type at 72 hours post-infection (Fig. 4.6A), and we even recovered slightly more *S. Typhimurium* than the *E. coli* Nissle *znuA zupT* mutant at 96 hours post-infection (Fig. 4.6B). Contrary to wild-type mice,
Figure 4.6 *E. coli* Nissle exhibits reduced competitive advantage over *S. Typhimurium* in the absence of zinc transporters or host calprotectin. Colon content samples from co-administration experiments in C57BL/6 and *S100a9*−/− mice (Figure 4.3 and 4.4) were collected (A) 72h and (B) 96h post-infection. Competitive index (CI) was calculated by dividing the output ratio (CFU of *E. coli* Nissle strain /CFU of *S. Typhimurium*) by the input ratio (CFU of *E. coli* Nissle strain /CFU of *S. Typhimurium*). Bars represent geometric mean ± standard error. * (P value ≤ 0.05); ** (P value ≤ 0.01).
Figure 4.6
wild-type *E. coli Nissle* was unable to significantly outcompete *S. Typhimurium* in *S100a9−/−* mice (Fig. 4.6), indicating that wild-type *E. coli Nissle* requires functional calprotectin to successfully outgrow *S. Typhimurium* in the inflamed gut.

### 4.4 Discussion

*E. coli Nissle* has been in use as a probiotic since the early 20th century, but only in the beginning of the 21st century have the mechanisms for its probiotic activity been gradually elucidated. Much of the work that has been performed thus far has focused on the immunomodulatory effects of *E. coli Nissle*, including reduction of mucosal proinflammatory cytokine secretion (18), increased production of antibodies (10), induction of β-defensin 2 (58), and activation of γδ T cells (21). These immunomodulatory effects of *E. coli Nissle* on the gut mucosa can have important consequences for host immune function, as the gut-associated lymphoid tissue (GALT) is the largest lymphoid organ in the body (48). In addition to the many immune cells residing in the gut, there are also trillions of resident bacteria in the gastrointestinal tract, collectively termed the gut microbiota. The host gut provides niches for the microbiota to colonize, and in exchange, the microbiota has evolved a symbiotic relationship with the host by synthesizing and breaking down nutrients, and training the immune system (42, 49). Another protective role of the microbiota is to act as a barrier and resist colonization of exogenous bacteria by occupying the available niches in the intestine, a concept termed “colonization resistance”. Inflammation, such as the type induced by *S. Typhimurium*, disrupts the intestinal microbiota and diminishes the colonization resistance against enteric pathogens (6, 43, 51). *E. coli Nissle* is able to efficiently colonize the inflamed intestinal environment, and in addition to its immunomodulatory effects, can also be used as a substitute for the disrupted microbiota to
resist enteric infections. However, the interaction of *E. coli* Nissle with other bacteria, such as
the pathogen *S. Typhimurium*, in the context of competition has not been well examined.

Recent studies have unveiled how certain bacteria, especially members of the
Enterobacteriaceae group that include *S. Typhimurium* and *E. coli*, are able to take advantage of
the host’s inflammatory state. This includes the ability to utilize alternative electron acceptors
and nitrogen sources that become available following the production of reactive oxygen and
nitrogen species by activated host cells (59, 60). In addition to taking advantage of new
metabolic resources, *S. Typhimurium* has evolved to overcome host inflammation mediated
metal ion starvation, a mechanism known as nutritional immunity. This includes the production
of salmochelin, a siderophore that is resistant to lipocalin-2 mediated iron sequestration, and the
expression of ZnuABC, a high affinity zinc transporter that confers resistance to zinc
sequestration by calprotectin (35, 44). Paradoxically, these same “virulence traits” are also found
in the probiotic *E. coli* Nissle, and lipocalin-2 resistance is in fact essential for the strain’s
probiotic effect (13). Both *S. Typhimurium* and *E. coli* Nissle express the *znuABC* and *zupT* zinc
transporters, and our work here demonstrates that *E. coli* Nissle is also resistant to calprotectin
due to the expression of zinc transporters. The subversion of the host immune response is
therefore not unique to pathogens alone, and can be utilized by closely related, probiotic bacteria.

In *in vivo* competition between *S. Typhimurium* and *E. coli* Nissle, the latter’s expression
of multiple iron transporters gave the probiotic an edge over its competitor (13). In our study, we
found that *E. coli* Nissle mutant defective for the two known zinc transporters is unable to reduce
*S. Typhimurium* at 72 hours, but is still able to significantly reduce *S. Typhimurium* colonization
levels by 96 hours, although not as much as wild-type *E. coli* Nissle. These results suggest that
during late acute *S. Typhimurium* gastroenteritis, *E. coli* Nissle is able to reduce *S. Typhimurium
colonization in a manner that is independent of the two known zinc transporters, ZnuABC and ZupT. Given that E. coli Nissle possesses multiple iron transporters (i.e. enterochelin, salmochelin, aerobactin, yersiniabactin, and ChuA heme receptor) (20), E. coli Nissle may possess additional zinc transporter(s) that has not yet been identified. It is also possible that iron acquisition at the early acute stage is more important for E. coli Nissle metabolism than zinc acquisition, which would give it an advantage over closely related bacteria such as S. Typhimurium. Altogether, our study further supports the notion that the ability of E. coli Nissle to acquire metal ions more efficiently than S. Typhimurium is beneficial to the host, and that E. coli Nissle acts as an antimicrobial peptide supplement, reducing the pool of available metal ions that S. Typhimurium would otherwise use for its own growth. Identifying the unknown zinc transporter(s) in E. coli Nissle will be important in furthering our understanding of its probiotic effects, as well as aid in the design of new probiotics that can be used to treat a range of intestinal disorders.

4.5 Materials and Methods

Bacterial strains, plasmid, and growth conditions

Bacterial strains and plasmids are listed in Appendix B1. Cultures of S. Typhimurium IR715 (52) and E. coli Nissle (ArdeyPharm) were routinely incubated either aerobically at 37°C in Luria-Bertani (LB) broth (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl) or on LB agar plates (1.5% Difco agar) overnight. Antibiotics and other chemicals were added at the following concentrations (mg/l) as needed: carbenicillin (Carb), 100; chloramphenicol (Cm), 30; kanamycin (Km), 50; nalidixic acid (Nal), 50; 5-bromo-4-choloro-3-indoyl-B-D-galactopyranoside (Xgal), 40; sucrose, 20.
Allelic exchange deletion of znuA in E. coli Nissle

To construct an E. coli Nissle znuA mutant, DNA regions flanking the znuA gene of approximately 600-800 bp in length were amplified by PCR with primers znuA FR1-Fw and znuA FR1-Rv (upstream flanking region, FR1) and primers znuA FR2-Fw and znuA FR2-Rv (downstream flanking region, FR2). A BamHI restriction site was added to the 5’ end of primers znuA FR1-Fw and znuA FR2-Rv, and a XbaI restriction site was added to the 5’ end of primers znuA FR1-Rv and znuA FR2-Fw (Appendix B2). Phusion High Fidelity DNA polymerase (New England Biolabs) amplified the flanking regions from wild-type E. coli Nissle, generating blunt-end PCR products. The blunt-end flanking region PCR products were digested with XbaI restriction enzyme to expose compatible sticky ends on the 3’ end of FR1 PCR product and on the 5’ end of FR2 PCR product. The digested PCR products were then purified using MinElute Reaction Cleanup kit (Qiagen) and ligated using the Quick Ligation kit (New England Biolabs). Phusion High Fidelity DNA polymerase (New England Biolabs) and primers znuA FR1-Fw and znuA FR2-Rv were used to amplify FR1 and FR2 in tandem (FR1-FR2). A PCR product of the predicted size was gel purified and ligated into pCRBlunt II-TOPO using the ZERO Blunt cloning kit (Invitrogen). The construct was heat shocked into E. coli TOP10 and plated on LB+Kan. Single colonies were screened by EcoRI digestion for the appropriate length of linearized plasmid construct and insert fragment sizes. Positive clones were sequence confirmed by using M-13 Fw and M-13 Rv universal primers. Accurate clones were designated pNM3 (pCRBlunt II-TOPO::FR1-FR2). The plasmid pNM3 was digested with BamHI and the FR1-FR2 fragment was gel purified and ligated to BamHI digested suicide vector pRDH10 (31) to generate pNM4 (pRDH10::FR1-FR2). E. coli CC118 λpir cells (25) were transformed with
pNM4 and transformants were plated on LB+Cm agar. To carry out conjugation, purified pNM4 was heat shocked into *E. coli* S17-1 λpir (25). Wild-type *E. coli* Nissle carrying the temperature sensitive pSW172 plasmid (37) was conjugated with *E. coli* S17-1 λpir containing pNM4 on LB agar. Transconjugants were selected for using Carb and Cm. Sucrose selection was used to clean the pNM4 plasmid from the genome. Conjugation involving wild-type *E. coli* Nissel (pSW172) was performed at 30°C to allow replication of the temperature sensitive pSW172 plasmid. pSW172 was cured by growth at 37°C. Confirmation of mutant was performed using PCR. The *E. coli* Nissle *znuA* mutant strain (*E. coli* Nissle Δ*znuA*(-82 to +1000)::scar) was termed JZL4.

**Construction of *E. coli* Nissle *zupT* mutant**

Generation of mutant in *E. coli* Nissle carrying a deletion in *znuA* and *zupT* was constructed using the lambda red recombinase system (12). Briefly, primers (*zupT*-Fw and *zupT*-Rv, Appendix B2) homologous to sequences within the 5′ and 3′ ends of the target regions were designed and a nonpolar kanamycin resistance cassette derived from plasmid pKD4 was used to replace the target gene in JZL4. Kanamycin was used for selection of the deletion construct and the mutation was confirmed using PCR. The resulting strain was termed SET2 (*E. coli* Nissle Δ*znuA*(-82 to +1000)::scar, Δ*zupT*(-42 to +774)::Kan).

**Bacterial growth in LB and M9 minimal media**

*S. Typhimurium* and *E. coli* Nissle wild-type, *znuA* mutant, and *znuA zupT* mutant strains were tested for their ability to grow under nutrient rich (LB) and nutrient limiting conditions (M9 minimal media per liter; 7.5g Na$_2$HPO$_4$, 3g KH$_2$PO$_4$, 0.5g NaCl, 1g NH$_4$Cl, 0.1mM CaCl$_2$, 0.5mM MgSO$_4$, 0.2% glucose). Inocula were prepared from overnight cultures of the strains
grown aerobically in LB at 37°C with agitation. Absorbance (\(\lambda=600\text{nm}\)) of the overnight cultures was determined by spectrophotometry and used to calculate the volume required to obtain 10\(^9\) cells. To prepare LB inoculum, 10\(^9\) cells were harvested by centrifugation, resuspended in 1 ml of LB, and 10 \(\mu\)l was used to inoculate 20 ml of LB. To prepare M9 minimal media inoculum, 10\(^9\) cells were washed twice in M9 minimal media, resuspended in 1 ml of M9 minimal media, and 10 \(\mu\)l was used to inoculate 20 ml of M9. Growth was monitored by determining the number of colony forming units (CFUs) per ml of culture at 0, 2, 5, 8, 16, and 24 hours of incubation at 37 \(\degree\)C with agitation. The growth of the wild-type, \(znuA\) mutant, and \(znuA\ zupT\) mutant \(S.\) Typhimurium and \(E.\) coli Nissle strains were also tested in M9 minimal media supplemented with 5 \(\mu\)M ZnSO\(_4\).

**Bacterial growth in LB supplemented with calprotectin**

\(S.\) Typhimurium and \(E.\) coli Nissle wild-type, \(znuA\) mutant, and \(znuA\ zupT\) mutant strains were tested for their ability to grow in LB supplemented with calprotectin (CP). To prepare inocula, all strains were grown in M9 minimal media at 37\(\degree\)C for 20 hours with agitation. Absorbance (\(\lambda=600\text{nm}\)) of the overnight cultures was determined by spectrophotometry and used to calculate the volume required to obtain 10\(^9\) cells. Cells were harvested at 15,000 rpm for 5 min in a tabletop centrifuge. The supernatant was discarded and the pellet was resuspended in 1 ml of M9 minimal media. Resuspended cells were serially diluted 10,000 fold in M9 minimal media and 10 \(\mu\)l were the used to inoculate the wells of a 96-well Nunclon Surface plate (Nunc). Each well contained 100 \(\mu\)l of a 10:28:62 ratio of inoculum to LB media to CP buffer (20 mM Trish pH 7.5, 100 mM \(\beta\)-mercaptoethanol, 3 mM CaCl\(_2\)). Recombinant CP was produced as described elsewhere (27). CP was added to the media to final concentrations of 0, 62.5, 125, 250, and 500
µg of the protein per ml prior to inoculation. The 96-well plate was placed inside a moist chamber and incubated at 37°C with 5% CO₂. Growth of S. Typhimurium and E. coli Nissle strains was monitored by determining the CFUs per ml of culture at 2, 5, 8, and 16 hours of incubation. Each experiment was repeated a minimum of three times.

**Mouse Experiments**

C57BL/6 mice and S100a9⁻/⁻ mice were used in our study. C57BL/6 mice were purchased from Taconic Farms and S100a9⁻/⁻ mice were generated as previously described (39). Mice were given streptomycin (1 mg per gram of mouse) by oral gavage one day before S. Typhimurium infection or E. coli Nissle administration, as described in the streptomycin pretreatment model (4). Twenty-four hours after streptomycin treatment, mice were infected with either 1x10⁹ CFU of S. Typhimurium alone, or administered 1x10⁹ CFU of E. coli Nissle wild-type alone, E. coli Nissle znuA zupT mutant alone, or co-administered with 1x10⁹ CFU of a mixture of strains at a 1:1 ratio as indicated. Colon contents were collected at 72 and 96 hours post-infection, serially diluted with sterile PBS, and plated on selective media to determine the numbers in the colon of S. Typhimurium and each strain of E. coli Nissle used in the experiment. In the mixed administration, competitive indices were calculated by dividing the output ratio (CFU of E. coli Nissle /CFU of S. Typhimurium) by the input ratio (CFU of E. coli Nissle /CFU of S. Typhimurium). Groups of 5-8 mice were used for each experiment.

**Histopathology**

Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 µm, and stained with hematoxylin and eosin. The pathology
score of cecal samples was determined by blinded examinations of cecal sections from a board certified pathologist as previously described (4, 44). Each section was evaluated for the presence of neutrophils (PMN), mononuclear infiltrate, submucosal edema, surface erosions, inflammatory exudates, and cryptitis. Inflammatory changes were scored from 0 to 4 according to the following scale: 0 = none; 1 = low; 2 = moderate; 3 = high; 4 = extreme. The inflammation score was calculated by adding up all of the scores obtained for each parameter and interpreted as follows: 0–2 = within normal limit; 3–5 = mild; 6–8 = moderate; 8+ = severe.

**Statistical Analysis**

Differences between treatment groups were analyzed by ANOVA followed by Student’s t test. A $P$ value equal to or below 0.05 was considered statistically significant.
4.6 References


Chapter 5
Discussion and Future Directions

The idea of nutritional immunity, in which the host restricts nutrient availability to limit pathogen growth, has been around since the 1970s (31). For many years, the focus of nutritional immunity research has been on the role of iron, and only within the last 20 years have other transition metal nutrients and their role in pathogenesis begun to be unraveled. Calprotectin, which was first described as an antimicrobial protein in the early 1990s, contributes to nutritional immunity by binding zinc and manganese ions, and it has been shown to limit the growth of a number of microbes, including *E. coli*, *C. albicans*, *B. burgdorferi*, and *S. aureus* by restricting access to zinc (4, 27, 28). To overcome nutritional immunity, some pathogens have evolved mechanisms to acquire essential metals during nutrient-limiting conditions. The high affinity ZnuABC zinc transporter system helps microbes acquire zinc in zinc-deficient environments, such as the host environment during infection (4). We demonstrated in Chapter 3 that ZnuABC is important for overcoming calprotectin-mediated zinc-sequestration during *S. Typhimurium* gastroenteritis. Wild-type *S. Typhimurium* is able to colonize the inflamed gut despite the expression of calprotectin by neutrophils and intestinal epithelial cells, while a *znuA* mutant strain is susceptible to calprotectin and shows reduced colonization levels (Fig. 3.1-3.14).

The importance of the ZnuABC transporter for *S. Typhimurium* colonization of the inflamed gut was demonstrated in a mouse model of acute *S. Typhimurium* gastroenteritis. In this model, the C57BL/6 mice we used are highly susceptible to *S.
Typhimurium infection due to a functional deletion mutation in the Slc11a1 gene that encodes the NRAMP1 divalent metal ion transporter and the animals will typically succumb to systemic infection within 5 days post-infection (11). In contrast, mice with functional NRAMP1 are resistant to S. Typhimurium infection (30). Work by Denise Monack and her group have shown that the functional NRAMP1 inbred mouse line 129SvJ develop a persistent S. Typhimurium infection following oral inoculation, and about 27% of infected mice can shed S. Typhimurium at high numbers for months (12, 13, 16). These highly colonized mice, termed “supershedders”, can be used to study the transmission of S. Typhimurium to naïve hosts by cohousing the supershedder mice with naïve mice (12). Transmission to a new host is a key step in the infection cycle of a pathogen, and in order to determine if host calprotectin and ZnuABC expression also play a role in S. Typhimurium transmission we will need to use mice that have functional NRAMP1.

Many transgenic mouse lines, including the S100a9 knockout line used in this dissertation to elucidate the role of calprotectin in S. Typhimurium infection, are on a C57BL/6 background (15). By introducing the functional NRAMP1 (Slc11a1) allele from 129Sv mice into the C57BL/6 background, functional NRAMP1 C57BL/6 mice were generated that are better able to control S. Typhimurium dissemination (9). In our lab, these functional NRAMP1 C57BL/6 mice are able to survive for up to 11 days post-infection. The functional NRAMP1 C57BL/6 mice will be crossed with S100a9−/− mice to generate animals that are functional NRAMP1, S100A9 knockout on a C57BL/6 background. These functional NRAMP1, S100A9 knockout mice generated can then be
used to determine whether ZnuABC-mediated resistance to calprotectin is important for S. Typhimurium transmission.

Transmission of S. Typhimurium is also dependent on the composition of the microbiota in the host animal. In the supershedder transmission model, only a fraction of all mice infected with S. Typhimurium became supershedders. However, by treating mice with the antibiotic streptomycin, and thus disrupting the microbiota, all infected mice became supershedders (12). Alterations in the microbiota therefore can increase the susceptibility of the host to colonization by exogenous bacteria. In this dissertation we demonstrated that ZnuABC-mediated resistance to calprotectin zinc sequestration allowed S. Typhimurium to outcompete the calprotectin sensitive microbiota (Chapter 3). Competition between a pathogen and the microbiota over zinc nutrient is not unique to S. Typhimurium infection, and has also been shown to be important in Campylobacter jejuni colonization of the avian gastrointestinal tract. Expression of ZnuABC by C. jejuni in the gut of infected chicken allows the pathogen to outcompete the intestinal microbiota for zinc (7). However, it is important to note that C. jejuni is considered a commensal bacterium in birds, and it does not induce inflammation in chicken (10). It remains to be determined whether the expression of the ZnuABC transporter also benefit C. jejuni in a gastroenteritis model of infection, and whether resistance to host zinc sequestration and competition over the microbiota for zinc nutrient is a strategy utilized by other bacterial agents that cause inflammatory diarrhea, such as Shigella ssp. and Clostridium difficile.

In addition to its role as a barrier against pathogen colonization, the microbiota also has a profound effect on host biology, including providing essential nutrients, modulating the immune response, contributing to intestinal tissue development, and
influencing host metabolism (21). However, little is known about the effect of zinc in maintaining a healthy microbiota. Given the ubiquitous need for zinc in biological processes, it is likely that zinc availability will also have an important effect on the microbiota and its composition. In our study we found that a \textit{znuA} mutant \textit{S. Typhimurium} exhibited a significantly reduced ability to colonize the inflamed gut relative to the isogenic wild-type strain. Zinc supplementation did not rescue the colonization phenotype of the \textit{znuA} mutant, and instead increased the abundance of non-\textit{Salmonella} Enterobacteriaceae (\textbf{Fig. 3.8}). This increase of other Enterobacteriaceae species may partly account for the inability of the \textit{znuA} mutant to take advantage of the excess zinc and would suggest that non-\textit{Salmonella} Enterobacteriaceae species have a competitive advantage over the \textit{znuA} mutant, possibly due to the ability to up-regulate functional zinc transport systems, including ZnuABC. However, it is unclear which species specifically are benefiting from the zinc supplementation. Identifying the members of a complex group of microorganisms is possible using DNA sequencing, and sequencing large microbial communities has recently become more feasible through advances in data processing, improved phylogenetic resolution, and reductions in cost of sequencing technology. By using deep-sequencing technology, we will be able to reveal the identity of the members of the microbiota benefiting from the zinc supplementation and inform future experiments elucidating the role zinc plays in shaping the microbiota.

We have demonstrated in Chapter 4 that \textit{in vitro} zinc supplementation of zinc-deplete M9 minimal media is able to rescue the growth of \textit{S. Typhimurium} and \textit{E. coli} Nissle zinc transporter mutants (\textbf{Fig. 4.1}). While zinc supplementation was unable to rescue the growth of the \textit{S. Typhimurium} \textit{znuA} mutant \textit{in vivo}, in wild-type \textit{S. Typhimurium}. 

\textbf{Fig. 3.8}
Typhimurium infected mice, oral zinc supplementation was able to enhance the intestinal colonization and systemic dissemination of the pathogen (Fig. 3.8A and data not shown). This phenotype is surprising, because one of the most well-recognized effects of zinc supplementation in humans is the reduction of diarrhea severity and duration in children (14). In 1988 Sachdev and colleagues conducted a randomized trial in which children with dehydrating diarrhea were given zinc supplements or placebo. They found that zinc supplementation shortened the duration and frequency of diarrhea in children with severe zinc deficiency (24). Since then, it has been shown that zinc supplementation in children with severe diarrhea can reduce the incidence of diarrhea by 18% and reduce prevalence by 25% (2). The WHO and UNICEF have recognized the beneficial effect of zinc in treating diarrhea, and currently recommend 20 mg zinc supplement per day as part of the treatment for children with diarrheal diseases (32).

While zinc supplementation is widely recognized as a safe and cost-effective general treatment for diarrhea, its effectiveness against specific diarrheal agents are not well understood. Zinc supplementation in children with diarrhea caused by *Vibrio cholera* or *Shigella spp.* reduced diarrhea duration in a study in Bangladesh (22, 23), however a clinical trial in Poland showed zinc supplementation had no effect compared to a placebo in children with acute gastroenteritis (18). In a study in India, Patel et al. demonstrated that zinc supplement was beneficial in treating diarrhea caused by *Klebsiella spp.*, had no significant effect in diarrhea patients with *E. coli* or parasites, and even prolonged diarrhea in patients co-infected with *E. coli* and rotavirus (17). The effectiveness of zinc supplementation against diarrhea is therefore not universal. The efficacy of zinc supplementation may depend on the specific etiological agent of diarrhea.
as well as the level of zinc deficiency, a condition that is more prevalent in South Asia than in Europe (3). Currently there is no evidence that zinc supplementation provides any beneficial effects during diarrhea caused by *Salmonella*, nor is there any published data demonstrating an adverse effect of zinc supplementation during *Salmonella* diarrhea.

In our study, the absence of an underlying zinc deficiency may be one reason why we do not see a positive effect of zinc supplementation in *S. Typhimurium* infected mice. A healthy laboratory mouse requires a diet containing 30 mg/kg of zinc (1). However, the mice in our vivarium are fed a zinc-rich diet containing 60 mg/kg of zinc. If our mice are fed a zinc-deficient diet before the start of the experiment, their immune responses will likely be dampened, as it has been shown in previous studies that zinc deficient mice have impaired immune response to infections (5, 26). Zinc supplementation may help to boost the animals’ immune response. However, given that we observed an increase in *S. Typhimurium* colonization level following zinc supplementation in mice fed a zinc rich diet, it is also possible that zinc supplementation may instead favor *S. Typhimurium* colonization. Another important consideration for assessing the effect of zinc supplementation in *S. Typhimurium* infected mice is the fact that, unlike humans, mice do not exhibit diarrhea following *S. Typhimurium* infection. To correlate any potential beneficial effect of zinc supplementation in *S. Typhimurium* infected mice to humans, a different set of criteria, such as luminal colonization level, bacteremia, and expression level of inflammatory markers, must be used.

Our data shows that zinc supplementation promotes the growth of wild-type *S. Typhimurium* in the inflamed gut, but other Enterobacteriaceae benefit from zinc supplementation in the inflamed gut when the *znuA* mutant *S. Typhimurium* is present.
The probiotic *E. coli* Nissle is one such member of Enterobacteriaceae that may benefit from an increase in zinc concentration during inflammation. The idea that zinc and probiotic combination can have a beneficial effect during gastroenteritis is supported by a study from Shamir et al., who demonstrated that an oral supplementation containing zinc and the probiotics *Streptococcus thermophilus*, *Bifidobacterium lactis*, and *Lactobacillus acidophilus* was able to reduce the severity and duration of acute gastroenteritis in young children (25). In our study, wild-type, but not the *znuA* mutant, *S. Typhimurium* was able to take advantage of zinc supplementation, indicating that the ZnuABC zinc transporter is required for enhanced *S. Typhimurium* growth during zinc supplementation. Like *S. Typhimurium*, *E. coli* Nissle also express the ZnuABC zinc transporter and is able to exert its probiotic activity by reducing *S. Typhimurium* colonization in a zinc transporter dependent mechanism (Fig. 4.3). By performing *S. Typhimurium* and *E. coli* Nissle co-administration in zinc supplemented mice we will be able to determine whether zinc supplementation may synergize with *E. coli* Nissle probiotic activity to reduce *S. Typhimurium* colonization, or whether excess zinc diminishes *E. coli* Nissle’s ability to outcompete *S. Typhimurium*. This information will indicate whether zinc co-therapy enhances or counteracts the benefits of *E. coli* Nissle in the treatment of gastroenteritis.

In this study we have shown that the ZnuABC zinc transporter is important for the colonization of *S. Typhimurium*, and for the probiotic activity of *E. coli* Nissle. ZnuABC allows both *S. Typhimurium* and *E. coli* Nissle to overcome calprotectin-mediated zinc sequestration. Calprotectin is an antimicrobial protein, which we showed is expressed mainly by neutrophils in the inflamed gut. However, during the course of our study, we discovered that not all neutrophils present in the inflamed gut express calprotectin. Using
an α-CXCR2 antibody to deplete neutrophils in S. Typhimurium infected mice, we were able to demonstrate that neutrophil-depleted mice exhibited diminished levels of calprotectin expression in whole cecal tissue by Western blot analysis (Fig. 5.1A). When we then examined the pathology evaluation from this experiment, we expected to see a significant decrease in the score for neutrophils from the α-CXCR2 antibody treated mice, as we had been able to demonstrate that neutrophils are the main source of calprotectin during S. Typhimurium infection (Fig. 3.4). However, pathological scores of cecal tissue from infected α-CXCR2 treated animals indicated the presence of a massive neutrophil infiltrate, equal to that seen in infected animals pre-treated with a control normal rabbit serum antibody (Fig. 5.1B). The decrease in calprotectin expression in the ceca of α-CXCR2 antibody treated mice without a corresponding decrease in neutrophil pathology score suggests that there may be different populations of neutrophils with varying capacity of expressing calprotectin and potentially other neutrophil proteins.

This discovery has important implications in the study of S. Typhimurium pathogenesis, as neutrophils play a key role in limiting the infection to the gut. Patients with defective neutrophil function due to infection or genetic mutation are at a higher risk of S. Typhimurium bacteremia (8). Despite the importance of these immune cells to limit infections, it has long been assumed that neutrophils are a homogenous population, and it has only recently been shown that there are distinct subsets of neutrophils. The first subsets were identified in correlation with an animal’s susceptibility to methicillin-resistant S. aureus infection (29). Since then, other studies have shown differences in neutrophil subsets in the context of inflammation, cancer, and B cell development (6, 19, 20).
Figure 5.1 \(\alpha\)-CXCR2 antibody depletion of neutrophils diminish cecal calprotectin expression but does not affect neutrophil pathology score. (A) Western blot detection of S100A8, S100A9, and MPO from cecum of infected, normal rabbit serum (NRS) treated and infected, \(\alpha\)-CXCR2 antibody treated mice. (B) Pathology score of infected NRS treated and infected \(\alpha\)-CXCR2 antibody treated mice. The grey quadrant includes scores indicative of moderate to severe inflammation.
Figure 5.1

A

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<td>α-tubulin</td>
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Cecum

B

Pathology score (cecum) 72 h p.i.

- Cryptitis
- Inflammatory exudate
- Surface erosions
- Submucosal edema
- Mononuclear Infiltrates
- Neutrophils

NRS

α-CXCR2
In our study, we observed a difference in cecal calprotectin expression between α-CXCR2 treated mice compared to control mice, even though both groups of animals showed similar neutrophil pathology scores in the cecum (Fig. 5.1). Since we had shown that neutrophils are the main source of calprotectin, this suggests that the set of neutrophils present in the α-CXCR2 treated mice are expressing less calprotectin, and that there are previously unappreciated subsets of neutrophils present in the inflamed gut during *S. Typhimurium* infection. However, pathology scoring is a qualitative measurement, and the presence of neutrophil subsets must be confirmed using more quantitative methods, such as flow cytometry. Preliminary data generated by Dr. Stefan Jellbauer in our lab using imaging flow cytometry, a technique which combines microscopy with flow cytometry analysis, confirmed the presence of calprotectin positive (CP+) and calprotectin negative (CP-) neutrophils from cells isolated from the gut of *S. Typhimurium* infected mice (Fig. 5.2).

While imaging flow cytometry has validated the existence of CP+ and CP- neutrophils during *S. Typhimurium* gastroenteritis, there is still much to be learned about these important immune cells. In addition to calprotectin protein expression, there are likely other differences in gene and protein expression between the subsets of neutrophils. To determine potential differences between CP+ and CP- neutrophils, we will isolate each subset of cells using fluorescence-activated cell sorting (FACS). FACS sorted cells can then be used for RNAseq and mass spectrometry analysis to determine the transcriptome and proteome profile of each subset. These analyses will give us insight into the expression of surface markers, which can be used to better define each individual neutrophils subset by flow cytometry. Furthermore, the analysis will help to determine
Figure 5.2 Calprotectin positive (CP+) and calprotectin negative (CP-) identified on imaging flow cytometry. Image of two neutrophils (defined as CD11b$^+$ and Gr-1$^+$ cells) isolated from the gut of S. Typhimurium infected mice with differential expression of the S100A9 subunit of calprotectin.
Figure 5.2

CP+ neutrophil

CP- neutrophil

545 µm

Gr-1  CD11b  S100A9
the immunological characteristics, including antimicrobial protein, cytokine, chemokine, and integrin expression profiles between the two subsets.

One current limitation with the FACS sorting approach is that the cells must be fixed and permeabilized for intracellular calprotectin staining, preventing culture and functional analysis of the cells. In order to overcome this limitation and be able to assess the biological function of each subset, GFP-S100A9 tagged transgenic mice can be used. The GFP-tagged S100A9 protein in these mice allows for the sorting of neutrophils based on the expression of calprotectin without the need for fixation and permeabilization. This allows live, functional cells to be sorted and restimulated so that they may be studied in vitro. To assess the function of each subset in vivo, sorted CP+ and CP- neutrophils can be adoptively transferred to neutrophil depleted mice that are infected with S. Typhimurium to determine the role of each subset during S. Typhimurium infection. Our results will contribute to the increasing evidence for neutrophils subsets, and the characterization of these CP+ and CP- neutrophil populations may have a broader impact to other mucosal diseases.
5.1 References


## APPENDIX

<table>
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<th>Page</th>
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<td>Inflammation in S. Typhimurium single infected mice.</td>
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<td>Inflammation in S. Typhimurium mixed infected mice.</td>
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<tr>
<td>Appendix A8</td>
<td>Real-time PCR primers used in Chapter 3.</td>
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Appendix A1 Visual representation of colon crypt isolation. Colon crypts were isolated as described in the Materials and Methods section of Chapter 3. A representative image of isolated colon crypts is shown.
Appendix A1

Isolate crypts with EDTA/DTT

Isolated crypts
Appendix A2 Growth of *S. Typhimurium* in rich and minimal media. *S. Typhimurium* wild-type, the *znuA* mutant (D*znuA*), or the *znuA* mutant complemented with *znuA* expressed on a low-copy plasmid (p*znuA*) were grown in LB, M9, and M9 supplemented with 5 mM ZnSO₄. (A-B) Growth in LB, M9 (A) and M9 supplemented with 5 mM ZnSO₄ (B) was determined by reading the OD₆₀₀ in a microplate reader at the indicated times. Data represent the geometric mean of 4 replicates ± standard error. (C) Generation times in LB and M9. Bars represent the geometric mean of 4 replicates ± standard deviation. * (P value ≤ 0.05) and ** (P value ≤ 0.01).
Appendix A2

A

![Graph A](image)

Log of OD (600nm) vs. Time (hrs)

B

![Graph B](image)

Log of OD (600nm) vs. Time (hrs)

M9 + 5 μM ZnSO₄

C

![Graph C](image)

Doubling Time (min)

WT, znuA, pznuA, WT, znuA, pznuA

LB, M9
Appendix A3 Inflammation in S. Typhimurium single infected mice. (A) Histopathology scores of cecal samples four days after S. Typhimurium infection. Wild-type mice were infected with either S. Typhimurium wild-type or the znuA mutant. Each stacked column represents an individual mouse. A detailed scoring for the animals shown in Figure 4 is provided. (B) Enumeration of S. Typhimurium in the colon content of mice. The colon content was collected and plated 3 days after infection with either S. Typhimurium wild-type or the znuA mutant (wild-type n=11, znuA mutant n=11). * P value ≤ 0.05. (C-D) Transcript levels of S100a8 and S100a9 (C) and Il-17a, Il-22, and Cxcl-1 (D) were determined by real-time RT-PCR in the cecum of mice infected with S. Typhimurium wild-type or the znuA mutant. Data are expressed as fold increase over mock-infected wild-type mice. (B-D) Bars represent the geometric mean of at least 5 replicates ± standard error.
Appendix A3

A

Pathology Score (cecum) 96h p.i.

- Orange: Cryptitis
- Yellow: Edema
- Purple: Inflammatory exudate
- Red: Mononuclear cells
- Green: Surface erosions
- Light blue: Neutrophils

B

Bacteria in colon contents (CFU/mg) 72 h p.i.

- Wild-type
- znuA

C

mRNA (fold change)

- S100a8
- S100a9

D

mRNA (fold change)

- Il-22
- Il-17a
- Cxcl-1

*
Appendix A4 Inflammation in S. Typhimurium mixed infected mice. (A) Histopathology scores of cecal samples four days after S. Typhimurium infection. Wild-type and S100a9−/− mice were infected with mixtures of S. Typhimurium strains as indicated. Each stacked column represents an individual mouse; n = no inflammation. A detailed scoring for the animals shown in Figure 5 is provided. (B-C) Transcript levels of Il-17a (B) and Il-22 (C), were determined in wild-type mice (white bars), S100a9−/− mice (dark grey bars), and wild-type mice supplemented with zinc sulfate (light grey bars). Mice were either mock-infected or infected with S. Typhimurium as indicated. Data are expressed as fold increase over mock-infected wild-type mice. Bars represent the geometric mean of at least 4 replicates ± standard error. Significant differences in gene expression in comparison to wild-type infected C57BL/6 mice (first group) are indicated by ** (P value ≤ 0.01).
Appendix A4

A

Pathology Score (cecum)

Treatment
wild-type versus znuA
wild-type versus znuA
invA spiB versus invA spiB znuA
wild-type versus znuA
mock
mock

Mouse strain
wild-type
S100a9−/−
wild-type
wild-type (ZnSO₄)
wild-type
S100a9−/−

B

Il-17a mRNA (fold change)

wild-type vs znuA
WT
S100a9−/−
WT (ZnSO₄)
invA spiB vs
invA spiB znuA
WT
WT
WT
mock
S100a9−/−
WT (ZnSO₄)

C

Il-22 mRNA (fold change)

wild-type vs znuA
WT
S100a9−/−
WT (ZnSO₄)
invA spiB vs
invA spiB znuA
WT
WT
WT
mock
S100a9−/−
WT (ZnSO₄)
Appendix A5 Enumeration of *S. Typhimurium* in the colon content of mice. Bacterial count of the mixture of *S. Typhimurium* strains in the colon contents of mice (n≥6/group) was determined at four days (A) or three days (B) post infection. (C) Total bacterial count of the mixture of *S. Typhimurium* strains in the colon contents of mice treated with either normal rabbit serum (NRS) or a rabbit polyclonal antibody blocking the CXC2 receptor (a-CXCR2) at 72 hours post-infection. Strain and mouse genotypes are indicated. Filled circles indicate the strain with a wild-type znuA allele, while open circles indicate the strain with a znuA mutation. Colonization of individual strains in each mouse (circles) and the averages (bars) are indicated.
## Appendix A6 Bacterial strains and plasmids used in Chapter 3.

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Appendix A7 Cloning primers used in Chapter 3.

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**Bold:** Extra 5’ DNA; **Underlined:** Restriction site utilized in cloning
Appendix A8 Real-time PCR primers used in Chapter 3.

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<td><em>Mus musculus</em></td>
<td>Lcn2</td>
<td>5’-ACATTTTGTTCCAAAGCTCCAGG-3’</td>
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<td></td>
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<td>5’-CATGGCGAATGTTGTAGTCC-3’</td>
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<tr>
<td><em>Mus musculus</em></td>
<td>Cxcl-1</td>
<td>5’-TGCAACAAAACGGAAGTCA-3’</td>
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<td>5’-TTGTCAGAAGCCAGGTTCAC-3’</td>
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<td><em>Mus musculus</em></td>
<td>Ly6g</td>
<td>5’-TGCGTTGCTCTGGAGATAGA-3’</td>
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<td>5’-CAGAGTAGTGCGACAGTG-3’</td>
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<td><em>Eubacteria</em></td>
<td></td>
<td>UniF340 5’-ACTCCTACGGGAGGCAGT-3’</td>
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<td>(Barman et al., 2008)</td>
<td>16S rRNA</td>
<td>UniR514 5’-ATTACCGCGGTGCTGCC-3’</td>
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<td><em>Salmonella</em></td>
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<td>Sal454 5’-TGTGGGTGTTAAATAACCGCA-3’</td>
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<td>(Barman et al., 2008)</td>
<td>16S rRNA</td>
<td>Uni785R 5’-GACTACAGGCTGATTAATCC-3’</td>
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<td><em>Firmicutes/ Clostridiales</em></td>
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<td>UniF338 5’-ACTCCTACGGGAGGCAGT-3’</td>
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<tr>
<td>(Barman et al., 2008)</td>
<td>16S rRNA</td>
<td>C.cocR491 5’-GCTTCTTTAGTCAAGGTCGATCC-3’</td>
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<td>Kingdom/Class</td>
<td>16S rRNA</td>
<td>Forward Primer</td>
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<td><strong>Firmicutes/Lactobacillales</strong> (Barman et al., 2008)</td>
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<td><strong>Bacteroidetes</strong> (Barman et al., 2008)</td>
<td>16S rRNA</td>
<td>BactF285 5’- GGTTCTGAGAGGAGGTCCC -3’</td>
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<td><strong>Enterobacteriaceae</strong> (Barman et al., 2008)</td>
<td>16S rRNA</td>
<td>Uni515F 5’- GTGCCAGCMGGCGGTAA -3’</td>
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<td>Appendix B</td>
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<td>Appendix B1</td>
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<td>Bacterial strains and plasmids used in Chapter 4.</td>
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<td>Appendix B2</td>
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<td>Cloning primers used in Chapter 4.</td>
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### Appendix B1 Bacterial strains and plasmids used in Chapter 4.

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<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or Reference</th>
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<td><strong>Escherichia coli strains</strong></td>
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<td>CC118 λ&lt;sub&gt;pir&lt;/sub&gt;</td>
<td>F- araD139 Δ(ara, leu)7697 ΔlacX74 phoAΔ20 galE galK thi rpsE rpoB argE&lt;sup&gt;am&lt;/sup&gt; recA1 λ&lt;sub&gt;pir&lt;/sub&gt;</td>
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<td>DH5a MCR</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZD15 Δ(lacZYA-argF)U169 deoR recA1 endA1 phoA supE44 - thi-1 gyrA96 relA1</td>
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<td>S17-1 λ&lt;sub&gt;pir&lt;/sub&gt;</td>
<td>F- recA thi pro rK- mK+ RP4:2-Tc::Mu Km Tn7 λ&lt;sub&gt;pir&lt;/sub&gt;</td>
<td>Herrero et al., 1990</td>
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<tr>
<td>EcN</td>
<td><em>E. coli</em> Nissle 1917 wild-type</td>
<td>ArdeyPharm, Germany</td>
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<tr>
<td>JZL4</td>
<td>EcN ΔznuA(-82 to +1000)::scar</td>
<td>This study</td>
</tr>
<tr>
<td>SET2</td>
<td>EcN ΔznuA(-82 to +1000)::scar ΔzupT(-42 to +774)::Kan</td>
<td>This study</td>
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<td><strong>Salmonella enterica serovar Typhimurium strains</strong></td>
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<tr>
<td>IR715</td>
<td>ATCC 14028 NaI&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Stojiljkovic et al., 1995</td>
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<td>JZL3</td>
<td>IR715, ΔznuA::Cm</td>
<td>Liu et al., 2012</td>
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<td>APJ4</td>
<td>IR715, ΔznuA::Cm ΔzupT::KSAC</td>
<td>Cerasi et al., 2014</td>
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<td><strong>Plasmids</strong></td>
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<td>pACYomega</td>
<td>pH45 omega derivative, Strep&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Takeshi Haneda and Andreas Bäumler</td>
</tr>
<tr>
<td>pCRBlunt II-TOPO</td>
<td>TA-Cloning Vector Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pH45omega</td>
<td>Strep&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Prentki and Krisch, 1984</td>
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<td>pKD4</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Datsenko and Wanner, 2000</td>
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<td>pJK611</td>
<td>pKD46::sacB</td>
<td>Kelly T. Hughes</td>
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<tr>
<td>pNM3</td>
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<td>pRDH10</td>
<td>oriR6K, Cm&lt;sup&gt;R&lt;/sup&gt;, Te&lt;sup&gt;R&lt;/sup&gt;, sacRB</td>
<td>Kingsley et al., 1999</td>
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Appendix B2 Cloning primers used in Chapter 4.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' - 3')</th>
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<td><em>znuA</em></td>
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<td>FR1-Fw</td>
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<td>BamHI</td>
<td>This study</td>
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<tr>
<td>FR1-Rv</td>
<td>TATGCCCTCTAGACAAGTCTGTTTCCTGG</td>
<td>XbaI</td>
<td>This study</td>
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<tr>
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<td>XbaI</td>
<td>This study</td>
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<tr>
<td>FR2-Rv</td>
<td>CACATTGGATCCGGAACTGTTCGCCCCGTC</td>
<td>BamHI</td>
<td>This study</td>
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<tr>
<td><em>zupT</em></td>
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<td>FR-Fw</td>
<td>GTAAGAACCACGGATAACATGATGATGACATCGTTATGTGTAGGCTGGAGCTGCTTC</td>
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<td>This study</td>
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<tr>
<td>FR-Rv</td>
<td>TGTTGCCCTTTAGCAATGGGCAACATCTGTCATTATCGTCTATGGGAAATTAGCCATGGTCC</td>
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<td>This study</td>
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