Examining immune responses in a mouse model of *Salmonella* infection

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When I set out on this journey called graduate school, I was more than a bit naïve as to what my life would be like. I received my invitation to interview for the Microbiology, Immunology, and Cancer Biology (MICaB) Ph.D. program while I was at Officer Candidate School for the Army and was ecstatic at the prospect of doing something that did not require me to run wherever I went and respond to constant yelling. My first day of school, October 3, 2005, was four days after I finished training to become an Engineer Officer at Fort Leonard Wood. I was already behind the power curve, as my classmates were familiar with one another and had been diligently working for almost a month by this time. Just over a month after that, my new husband and I became pregnant with our first child. I was a new graduate student in the MICaB program, a new Platoon Leader in the Minnesota Army National Guard, and I was going to be a mother.

I believe that God sometimes inserts people into my life whose role is to help me through such trying times. The first individual to step in was my friend and fellow MICaB student, Wynette Dietz. Although I hardly knew anyone in the program, due to my late start, she spoke up immediately when I found out I needed to do a fourth laboratory rotation. She mentioned that Stephen McSorley had a slot available to train a graduate student. So, I interviewed with Stephen, six months pregnant and a member of the National Guard, and it turned out that the lab was a perfect fit. I don't know how many advisors would have taken me on at that point, and for that, I am forever grateful to Stephen. Thank you for being the best advisor I could have asked for, through all of the ups and downs, the thin and the thick. Thank you for your constant patience, your guidance, and your belief in my ability to succeed.

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Dedication

This dissertation is dedicated to my husband, Danny, and to our beautiful boys, Liam and Ewen.

Abstract

Salmonella infections are responsible for significant morbidity and mortality throughout the world. Although extensive research has elucidated the mechanisms of protective immunity following vaccination with live vaccine strains (LVS) of *Salmonella*, very little work has been done to examine immune responses during and following antibiotic treatment of virulent *Salmonella* infections. We have developed a murine model of naturally acquired immunity to *Salmonella*, where susceptible mice are orally infected with virulent *S. typhimurium* and treated with antibiotics for an extended period of time. These mice demonstrate weak protective immunity to rechallenge with virulent *Salmonella*, which is due to Th1 and antibody responses and can be augmented by the administration of a TLR5 agonist.

We have also used antibiotic treatment to examine the development of Th1 responses to LVS *Salmonella*, which are vital for mediating protective immunity to this pathogen. We show that Th1 cells develop after sustained exposure to *Salmonella* antigens. Eradication of the bacteria by antibiotic intervention within one week of primary infection has profound effects on the ability of mice to survive rechallenge with virulent *Salmonella.* We also establish that full effector/memory function of Th1 cells, as determined by robust production of Th1 cytokines, requires two weeks of exposure to *Salmonella* antigens.

Finally, we use short-term antibiotic treatment to establish a model of relapsing virulent *Salmonella* infection where mice appear to have cleared the bacteria soon after they begin treatment but suffer recurrent and fatal *Salmonella* infection upon withdrawal. We demonstrate that *Salmonella* harbored in $CD11b^{+}Gr1^{-}$ resident monocytes in mouse mesenteric lymph nodes (MLNs) are the source of relapsing infection. In addition, the

MLNs appear to act as a filter prohibiting the dissemination of *Salmonella* to systemic tissues.

By using antibiotic treatment to examine immune responses to *Salmonella*, this thesis work may contribute to future research in the development of efficacious therapeutic and/or preventative typhoid vaccines. Moreover, these studies may stimulate future studies using these same tools in other infectious disease models.

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Chapter 1

Introduction

I. Infectious Diseases Past and Present

In a message to Congress in 1969, US Surgeon General William Stewart made a bold statement: "…It is time to close the book on infectious diseases. The war against pestilence is over." Just over a decade later marked the emergence of the human immunodeficiency virus (HIV), the cause of acquired immunodeficiency syndrome (AIDS), which is responsible for 3 million deaths every year, making it the most deadly of any infectious disease[1]. Indeed, lessons learned from emerging and reemerging infectious diseases have taught us some humility since the Surgeon General claimed victory over forty years ago.

Undoubtedly, advances in the understanding, prevention, and treatment of infectious diseases have been significant since Robert Koch, Louis Pasteur, and colleagues began their study. Particularly in developed nations, improvements in sanitation and food preparation aided in the prevention of food-borne illness[2, 3]. In addition, treatment with antibiotics and the development of effective vaccines dramatically improved our ability to combat many infectious diseases[4]. Such progress led to a considerable decline in disease burden, which, in turn, fostered beliefs that humans were capable of ridding the world of infectious disease, as proclaimed by US Secretary of State George Marshall in 1948. One reviewer commented, "…by 1949, the death rate from infectious diseases in the United States was the lowest ever recorded[5]." Due to vaccination against small pox in the United States, only 41 cases were reported in the year 1950[6]. Indeed, a 1970 paper referred to infectious diseases as follows, "In the short space of fifty to a hundred years, man, their chief antagonist, has seriously interfered with their environment, and with his hygiene, his vaccines and his drugs has constantly threatened their very existence[7]."

However, outbreaks of legionellosis, Lyme disease, and Ebola in 1976 provided

evidence that the battle was far from won[1]. Nevertheless, even after HIV began its formidable rampage, the idea that humans were winning the battle against infectious disease prevailed. The "eradicationist" position prevailed for nearly a half-century when, in the early 1990s, a newfound respect for the power of infectious disease began an era of vigilance in the response to several more devastating outbreaks[2]. Notably, in 1992, the National Academy of Science's Institute of Medicine (IOM) published *Emerging Infections: Microbial Threats to Health in the United States*[8]. This landmark publication spurred others to join the cause, as journals dedicated issues to emerging infectious diseases soon thereafter, and January of 1996 was declared 'Emergent Diseases Month'[9]. Publications throughout the 1990s attributed the sudden surge in emerging infectious diseases to, "…changes in human behavior, industrial and economic development, travel and mass movements, civil unrest and wars, but also microbial genomic change and adaptation[10]."

It was clear that the attitude of complacency that dominated the previous fifty years or so had contributed to the grim situation that the world was facing: outbreaks of plague in India, cholera in South and Central America, and Ebola in Zaire[2]. As Western nations basked in euphoric victory, the rest of the world became more and more vulnerable to epidemics. After World War II, populations exploded, and urban slums devoid of resources provided easy targets for emerging infectious diseases. Moreover, the ease of world travel guaranteed that the epidemics raging through developing countries would eventually reach more affluent nations[1, 2, 10]. And when they did, the reality was especially difficult to bear, as infectious disease research was all but nonexistent when the Western world finally realized its susceptibility to epidemics.

Ironically, to a certain extent, this vulnerability was a result of advances in modern medicine. Extending the lives of people contributed to overcrowding, and this

provided another niche for emerging infections, in homes for the elderly and hospitals, where microbes spread like wildfire amongst immunocompromised individuals. Nosocomial infections are an increasing problem, the most frightening being caused by *Staphylococcus aureus*, which is responsible for the most cases of nosocomial pneumonia, bloodstream infections, and infections of surgical wounds[2].

Mention of *S. aureus* leads to the next challenge that has arisen following developments in modern medical science: antimicrobial resistance. This issue was far from new in the early 1990s, but one that had been increasingly more apparent since the first antibiotic, penicillin, was discovered. Alexander Fleming himself who made the Nobel Prize-winning discovery, was wary of the potential for bacteria to acquire resistance when faced with such a strong selective pressure. Less than ten years after Fleming's acceptance speech, one reviewer commented, "Repeated warnings of the danger of…increasing microbic resistance have been given so often by so many authorities that the exhortations are wearying to those who heed them[11]."

Bacteria gain resistance to antimicrobials by various mechanisms. First and foremost, they evolve at an alarming rate under selective pressure, due to the fact that they can replicate many billions of times during the lifetime of a human being[2]. They may develop mutations that increase their ability to resist the effects of antibiotics, or they may acquire resistance genes from other bacteria by horizontal gene transfer[1]. For example, bacteria might produce enzymes that destroy the antibiotics, or they may prevent the penetration of the antibiotics by adding bulky groups to their structure. Furthermore, they may produce enzymes that bind the antibiotics, or they may change porins that would normally allow the entry of antimicrobials. Finally, bacteria might acquire the ability to express pumps that eliminate antimicrobials. Two examples of the most frightening resistant bacteria are methicillin-resistant *S. aureus* (MRSA), which is

also resistant to penicillin, and strains of *Mycobacterium tuberculosis* that are resistant to both first-line and second-line antibiotics[2, 12]. Indeed, tuberculosis is the second most deadly infection, after AIDS[1]. The most recent estimates suggest that there are currently more people suffering from tuberculosis than at any other time in history, of which about 2 million die every year[1, 2].

It is evident by the ever-increasing number of antibiotic resistant microbes that we are behind the power curve when it comes to the treatment of infectious diseases. A better approach, therefore, is perhaps attempting to prevent them altogether. The most successful method has been through mass vaccination, which is credited with the eradication of both small pox and polio. In 1974, one reviewer commented, "Immunology has extended our understanding of many diseases, but its applicability to prevention of control of infection still is its greatest contribution to our health. Prevention of infection by immunization has reduced fear, prolonged life, improved productivity, and enhanced cultural and social improvements for everyone. It becomes almost unimaginable what the impact would be if some of the infectious diseases now so prevalent were similarly controlled[13]."

Vaccination usually consists of the administration of either live, attenuated microorganisms, killed microorganisms, or components of microbes, which stimulate the immune system to develop protective antibodies that will defend the body against challenge with virulent microbes[14, 15]. When the earliest vaccines were administered, it was not known how protection was mediated. Although there are still unanswered questions, research in immunology continues to unfold the mechanisms responsible for protective immunity generated by vaccines.

According to the World Health Organization (WHO), a new infectious disease emerges every year[1]. Severe acute respiratory syndrome (SARS)[16] and influenza A

(H1N1)[17] are two examples of recent pandemics that ensure the world maintains watchful vigilance in the face of infectious diseases. As such, continued research on the prevention of infectious diseases, particularly through vaccination, is not only warranted, but is vital to combating this global threat that persists and grows.

"Our heads are round so that our thinking can change direction." –Francis Picaba

II. Cellular Immunity

A pivotal advance in the field of immunology, which continues to aid our understanding of important concepts such as the mediation of protective immunity by vaccination, was the discovery of cellular immunity. Although George Mackaness published many papers on this subject, beginning in the early 1960s, the concept of cellular immunity was first discovered by Elie Metchnikoff over a half-century earlier, in 1882[18-20]. Metchnikoff was the first to use experimental data to show that phagocytic mononuclear cells from previously infected animals were more efficient at killing bacteria than those from naive animals[21, 22]. In 1908, three years after publishing his work on cellular immunity and the theory of phagocytosis, he shared the Nobel Prize with Paul Erlich, the founder of another significant arm of immunology, humoral immunity, which describes serum antibody mediation of host defense[23, 24].

Between Metchnikoff's acceptance speech and Mackaness' classic work, the research of a handful of scientists began to build on the concept of cellular immunity. Two groups found that what was referred to as tuberculin hypersensitivity, a delayed-type hypersensitivity (DTH) response to antigens of *Mycobacterium tuberculosis* evidenced by the slow development of a skin lesion following intradermal injection[25], could be transferred using either whole blood or spleen cells[26]. Zinsser and Mueller agreed that another mechanism existed in DTH that did not require antibodies[27], but it

was not until the early 1940s that a strong link between cellular immunity and DTH was established by Landsteiner and Chase[26].

Although Metchnikoff and others provided the foundation for the theory of cellular immunity, it was Mackaness who discovered how phagocytic cells such as macrophages acquire the ability to become efficient at killing bacteria. Most research being conducted in the first half of the 20th Century focused on the role of antibody in the defense against infectious disease. Antibody opsonization was known to be important for the lysis of infected cells by complement; it also aided in phagocytosis. However, it was also known that during the course of some infections, the level of antibody did not correlate with the level of protection against future exposure to the disease, which indicated that there existed a form of resistance to microbes that was independent of antibody[28]. In order to investigate this form of resistance, Mackaness infected mice with the facultative intracellular bacteria, *Listeria monocytogenes*, which was known to generate weak antibody responses but strong protective immunity[29]. He found that mice infected with *Listeria* were resistant to rechallenge with the bacteria but had no evidence of protective antibody[29, 30]. Rather, the phagocytic macrophages from resistant mice were capable of inactivating *Listeria in vitro* and were therefore most likely responsible for the protection he observed[29]. In addition, Mackaness later found that during the period of time when bacteria were being cleared from infected mice most rapidly, there was a corresponding increase in the proliferation of macrophages, which further supported his claim that macrophages were responsible for mediating protective immunity to *Listeria*[31].

During his experiments with *Listeria*, Mackaness found that macrophages became “activated” to kill bacteria. He next sought to determine the specificity of this response by conducting a series of co-infection experiments, using *Listeria monocytogenes* and *Brucella abortus*. Mackaness found that if he infected mice with

one species of bacteria, waited until macrophages became activated, and then infected the mice with the other bacteria, the second organism was cleared more rapidly than in naïve animals[32]. The ability of the macrophages to kill unrelated bacteria was enhanced and was therefore non-specific. However, if mice that had fully recovered from primary infection with one organism were infected with the other organism, the mice were not protected, indicating that protective immunity was specific[32].

In an effort to gather additional support for the role of cellular immunity in infection, Mackaness went on to study other model organisms that infected mice, and he and his collegues published several papers on *Salmonella*. For instance, Blanden, Mackaness, and Collins established that the development of acquired immunity to *Salmonella* was dependent on the antibacterial activation of phagocytic cells[33]. They found that macrophages from mice that had been infected with *Salmonella* inactivated nearly all intracellular bacteria, while macrophages from naïve mice were only able to kill about 50 to 60% of them[33]. In a related study, after vaccinating mice with different attenuated strains of *Salmonella*, they found that in order to generate protective immunity, it was necessary that an active infection was established: there was a direct correlation between the number of residual bacteria in the tissues and the level of efficiency of the “antibacterial mechanism” of the host phagocytes[34]. Mackaness and collegues published a third paper on this subject in the same issue of *The Journal of Experimental Medicine*. Here, they provided still more evidence that acquired resistance to *Salmonella* in mice was not due to humoral immunity, as passive transfer of immune serum to infected animals did not aid in the clearance of the organisms from host tissues[35]. Soon after the trio of papers, Blanden and Mackaness showed that delayed-type hypersensitivity could be transferred using spleen cells, but not serum, from

vaccinated mice to naïve mice, which provided further support for the role of cellular immunity in resistance to *Salmonella* infection[36].

Thus far, Mackaness and colleagues had provided solid evidence that immunity to facultative intracellular parasites is mediated by the activation of macrophages capable of killing the bacteria. With McGregor and Koster, Mackaness then went back to using a model of *Listeria* infection and found that other cells took part in resistance to infection. These cells, which they called small short-lived lymphocytes, had a very high rate of turnover and moved rapidly from the blood into the peritoneal cavity following bacterial infection, presumably after stimulation by the bacteria in local lymph nodes[37, 38]. After these studies, Mackaness and collegues published a paper that discussed the role of inflammation in this process. They showed that, following bacterial infection, the short-lived lymphocytes were recruited to inflammatory sites where they could influence macrophages to carry out their microbicidal function[39]. Later work that Mackaness published with McGregor and Hahn, using the *Listeria* model, showed that T cells, or lymphocytes that mature in the thymus, were important mediators of cellular immunity, corroborating the findings of several other groups[40].

Indeed, George Mackaness made great strides in advancing the field of immunology. And, as any scientist does, he left some questions unanswered that provided opportunities for the next investigator to continue his research. One such scientist who was inspired by the work of Mackaness was Carl Nathan. He aimed to identify mechanisms of cellular immunity by exploring how the T lymphocytes were communicating signals to the macrophages that induced them to kill intracellular bacteria. Nathan first showed that it was a soluble mediator generated by antigen-stimulated T cells that provided the necessary stimulus to the macrophages[41]. He later

discovered that the soluble mediator was interferon-gamma (IFN-γ)[42]. These were important discoveries that incited still more questions to be answered.

III. Th1/Th2 Cells

While Mackaness was beginning to show that activated macrophages were essential for defense against facultative intracellular bacteria, other investigators were discerning various subpopulations of lymphocytes based on their effector functions and expression of distinct markers. First, B lymphocytes were found to produce antibody and T lymphocytes to act as "helpers" for the B cells[43, 44]. Then, based on differential expression of Ly alloantigens, T cells were divided into helper cells ($Lyt1^+$ or $CD4^+$) and cytotoxic cells ($Lyt2^+$ or $CD8^+$)[45, 46].

Subdivisions of helper T cells, which express the surface molecule CD4 (L3T4), had been previously described, but concrete distinctions had not been defined until the work of Mosmann and Coffman in 1986[45]. They tested a panel of antigen-specific T cell clones and found that there were two discrete profiles of lymphokine production. In response to antigen stimulation, Type 1 T helper cells (Th1 cells) produced IFN-γ, interleukin-2 (IL-2), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF); Type 2 T helper cells (Th2 cells) produced IL-3, a B cell stimulating factor 1 (BSF1, later known as IL-4), and a mast cell growth factor (IL-5)[47, 48].

Once the two groups of helper T cells were defined, extensive research was conducted with the goal of establishing the functions of Th1 and Th2 cells and their products. For example, it was found that both types of helper cells were able to provide help to B cells in an antigen-specific manner[49]. Th1 and Th2 cells, however, stimulated B cells to produce different antibody, or immunoglobulin (Ig), isotypes. In general, Th1

cells were found to stimulate the production of IgG2a, while Th2 cells induced B cells to make IgE[49].

Since it was known that helper T cells were involved in DTH responses, another line of research was dedicated to defining which type of cell, Th1, Th2, or both, was responsible for arbitrating DTH responses. In order to address this question, Cher and Mosmann injected the footpads of mice with antigen and either Th1 or Th2 cells. Their results showed that the Th1 subset of helper T cells was capable of mediating an antigen-specific DTH reaction, but the Th2 subset was not[50]. It should be noted that cytotoxic T lymphocytes (CTLs) were also found to be capable of inducing a DTH response, as shown by Zinkernagel et al[51], which is consistent with the similar cytokine profiles of CTLs and Th1 cells.

As more and more data was generated, it became increasingly clear that a major role of Th1 cells was to enhance cytotoxic events. Th1 cells produce IFN-γ and lymphotoxin (LT, later also known as tumor necrosis factor-beta [TNF-β]), which activate macrophages to kill intracellular pathogens. These lymphokines also stimulate macrophages to increase expression of Fcγ receptors, which bind the Fc regions of antibodies of the IgG isotype[52]. In addition, IFN-γ increases the level of IgG2a, which can then be bound by macrophages and further increase the cytotoxicity of macrophages; IgG2a can also opsonize pathogenic microbes, which induces lysis via complement, a component of the innate immune system, neutralization, or increased phagocytosis and degradation by macrophages[52].

Th2 cells, in contrast, tend to increase levels of serum IgE and the expression of Fcε on B cells. Mast cell and eosinophil proliferation also occurs in response to Th2 cytokines[49]. These are all features of allergic immune responses and also are important

in the defense against parasitic helminth infections.

There was considerable debate over whether the findings on the T helper cell dichotomy could be applied to humans. It was found that the subsets discovered in the mouse are also true for human T cells[45, 53].

Around the same time, discoveries on the regulation and development of Th1 and Th2 cells were elucidated. First, it was found that Th1 and Th2 cells could promote their own continued expansion in an autocrine manner and could also inhibit the growth of the opposite cell type, via the production of their relative cytokines[54]. Then, several groups began to reveal important aspects pertaining to how the two subsets develop. It was found that IL-4 was necessary for the development of Th2[55, 56] cells and that IL-12 was required for the induction of Th1 cells[57, 58]. Furthermore, researchers established that the two subsets shared a common precursor and were induced to develop into either Th1 or Th2 cells based on the cytokine milieu[59, 60].

Still other important discoveries were made on the transcriptional regulation of Th1 and Th2 cells. Th1 cells differentiate through Stat4 (signal transducer and activator of transcription 4)[61] and the transcription factor T-bet (T-box expressed in T cells)[62]. T-bet exerts its effects by upregulating IFN-γ and downregulating IL-4 and IL-5. On the other hand, Th2 cells differentiate through Stat6 and the activation of Gata3, which has the opposite effects[45].

The classic example of the Th1 and Th2 subsets mediating antagonistic immune responses to an infectious agent is that of murine leishmaniasis. Before these subsets were named, immune responses to *Leishmania major*, an intracellular protozoan parasite, were described by Foo Y. Liew to produce two functionally distinct populations of $CD4^+$ T cells, which mirrored the activities of the later classified Th1 and Th2 helper

cells[63]. In transfer experiments, Liew et al showed that cells transferred from infected resistant mice into syngeneic mice, were protective, while cells transferred from infected susceptible mice exacerbated disease[64].

Several years after these observations were made, Scott et al showed that the immune response to *Leishmania* was dependent on the type of helper T cells that predominated[65]. This group injected either Th1 or Th2 cells specific for *Leishmania* antigens into *Leishmania*-infected mice. Mice that received Th1 cells recovered from the infection, while mice that received Th2 cells became progressively worse[65]. The studies of Heinzel et al corroborated these findings, demonstrating that IFN-γ was protective and IL-4 was damaging during infection with *Leishmania*[66].

There are two models of subcutaneous *Leishmania major* infection in mice, depending on the susceptibility of the strain[67]. Mice such as BALB/c are susceptible to *Leishmania* infection. The immune response in these mice is mediated by Th2 cells[68]. High levels of antibody, namely IgE, and IL-4, and low levels of IFN-γ lead to a progressive disease that ends in death of the animals. In contrast, infection of resistant C57BL/6 mice with *Leishmania* results in a Th1-mediated response: antibody levels remain low, while robust IFN-γ production induces killing of intracellular parasites by activated macrophages[69]. These mice develop a local infection and recover relatively quickly.

Examining the genetic differences between these two strains of mice has shed some light on the genetics of protection against intracellular pathogens. Resistance to *Leishmania* infection depends on the expression of a gene called *Lsh*, which is important for the control of replication of the parasite inside macrophages. This gene is important in the early control of other intracellular pathogens, such as *Salmonella typhimurium* and

mycobacterial infections; however, it is called by different names in each of these disease models[70].

Given the dependence on Th1 cells in mediating protection against *Leishmania*, it makes sense that resistance is controlled by a gene that is important for survival of the parasite within macrophages, its principle host cell. The chief toxins produced by activated macrophages are reactive oxygen species and nitric oxide (NO). It has been found that NO, produced by the inducible NO synthase (iNOS) is indispensable for the microbicidal effects on intracellular *Leishmania*[69]. These observations are exemplified by the two strains of mice with opposing immune responses to *Leishmania*: susceptible BALB/c mice lack significant expression of iNOS following *Leishmania* infection, while resistant C57BL/6 mice maintain high levels[69]. Once again, IFN-γ is essential for the induction of iNOS, which underlines the importance of macrophage activation and the role of Th1 cells in the defense against *Leishmania*.

IV. Immune Responses to *Salmonella* Infection

Like *Leishmania, Salmonella* is a facultative intracellular pathogen. As such, the immune response to infection with *Salmonella* shares many commonalities with that of *Leishmania*. First of all, it is clear that macrophage activation and Th1 cells are essential for defense against *Salmonella* infection.

Survival within macrophages is essential to the virulence of *Salmonella*. As mentioned, susceptibility in mice is determined by the expression of a gene that controls the intracellular replication of *Salmonella* within macrophages, called slc11a1 (formerly known as Nramp1)[71]. In addition, *Salmonella* mutants that are unable to survive and replicate within macrophages are attenuated for virulence[72]. Furthermore, it has been

found that, like *Leishmania*, the production of reactive oxygen and nitrogen intermediates by host macrophages is important in the defense against *Salmonella* infection[73, 74].

During *Salmonella* infection, the production of IFN-γ by Th1 cells activates macrophages that kill the intracellular bacteria[74]. Several laboratories have provided evidence to support this conclusion. For example, in 1992, Mastroeni et al found that after subjecting mice immunized with a live vaccine strain of *S. typhimurium* to CD4 depletion, those administered the CD4 depleting antibody demonstrated impaired immunity to rechallenge with virulent *Salmonella*, as evidenced by increased mortality and higher bacterial loads in organs of the reticuloendothelial system (RES)[75]. In this same study, IFN-γ was also found to be important for protective immunity to *Salmonella*. Others have drawn similar conclusions using IFN-γ-[76] or CD4-depleting antibodies[77]. Furthermore, using gene-targeted immunodeficient mice, Hess et al found that mice lacking $CD4^+$ TCR-αβ cells and mice unable to respond to IFN-γ (deficient in the IFN-γ receptor [IFN-γR]) were both highly susceptible to infection with an attenuated $aroA^-$ live vaccine strain (LVS) of *Salmonella*, which is easily cleared by immunocompetent mice[78]. Still another group showed that IFN-γ is important for clearance of primary infection with LVS *Salmonella*[79].

Further evidence for the role of Th1 cells in mediating protective immune responses to *Salmonella* has been gathered by studies on molecules essential for the development of this helper T cell subset. For example, IL-12 is involved in the differentiation of naïve T cells into Th1 cells, as previously mentioned[57, 58]. Depletion of this cytokine resulted in decreased resistance of immunized mice to infection with virulent *Salmonella*[80]. Another study, by Ravindran et al, demonstrated the importance

of T-bet, the master transcription regulator Th1 cells, in mediating protective immunity to *Salmonella*[81]. Mice deficient in T-bet succumbed to infection with LVS *Salmonella* and failed to generate IFN-γ-producing $CD4^+$ T cells[81].

Conclusions drawn from studies in mice are supported by evidence of these same mechanisms being important for protection against *Salmonella* in human disease. For example, Jouanguy et al found that humans with deficiencies in IL-12 or IFN-γR signaling demonstrated increased susceptibility to *Salmonella*[82].

In addition to Th1 responses being essential for immunity to typhoid, the role of B cells has been to shown to be important. McSorley and Jenkins showed that B cell-deficient mice on the resistant BALB/c background effectively cleared primary infection with LVS *Salmonella* but succumbed to rechallenge[83]. Mastroeni et al and Mittrucker et al both found the same to be true in B cell-deficient mice on the innately susceptible C57BL/6 background[84, 85]. One review of the role of B cells suggested that antibody was important in preventing cell-to-cell transmission of *Salmonella* after apoptosis of infected macrophages[86]. Alternatively, it has been put forward that antibody may play a role in processing *Salmonella* antigens and augmenting antigen presentation to $CD4^+$ T cells, which then could boost cellular immunity[87, 88]. Finally, Wijburg et al proposed that *Salmonella*-specific IgA in the intestinal mucosa is secreted into the lumen and prevents the bacteria from penetrating gut epithelial cells and/or reduces bacterial loads[89]. Although it is unclear as to how B cells are protective against *Salmonella*, the data fit with what we know about human vaccines against *Salmonella*, as these are known to generate protective antibody[90].

As for $CD8^+$ T cells, their role in immune responses directed against *Salmonella* infection appears to be a minor one. The most compelling evidence showed that mice

deficient in $CD8^+$ T cells were able to recover from infection with *Salmonella.* For example, Hess et al showed that mice that did not have MHC class I-restricted T cells were as resistant to infection with LVS *Salmonella* as their littermate controls[78]. Another group found that mice lacking $CD8^+$ T cells showed only a minor reduction in their ability to resist infection[91].

It is evident that adaptive immunity to *Salmonella* is mediated by macrophages, Th1 cells, and B cells. In addition, since *Salmonella* is an enteric pathogen that infects its host via the fecal-oral route, many detailed studies have outlined the activation of *Salmonella*-specific immune responses in the intestinal mucosa. *Salmonella* enters the host by crossing intestinal epithelial cells of the gut. The classical route of infection is via microfold, or M cells, which are interspersed amongst the enterocytes in the follicle-associated epithelium of Peyer's patches[92]. In the subepithelial dome (SED) of Peyer's patches, *Salmonella* can infect phagocytic cells, such as monocytes, macrophages, and dendritic cells (DCs). From this initial site of infection, bacteria travel via the lymphatics to the mesenteric lymph nodes, spleen, and liver, presumably within circulating infected phagocytes[93]. Some studies have suggested that *Salmonella* can gain access to the gut via CX3CR1-expressing DCs in the lamina propria that extend processes between epithelial cell tight junctions into the intestinal lumen and sample its contents[94]. However, more evidence is required to solidify the validity of this alternative route of infection.

Once *Salmonella* penetrates the intestinal barrier and is phagocytosed, innate immune mechanisms are activated. *Salmonella* express pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin, which are recognized by the host's pattern-recognition receptors (PRRs). For example, LPS is recognized by Toll-like receptor 4 (TLR4), and flagellin is recognized by TLR5[95]. Ligation of TLRs induces the activation of antigen-presenting cells (APCs) such as DCs, evidenced by

upregulation of the co-stimulatory molecules B7-1 (CD80), B7-2 (CD86), and CD40 ligand (CD40L). Also, TLR5 is expressed basolaterally on intestinal epithelial cells, and upon ligation, flagellin can trigger the production of pro-inflammatory cytokines, such as IL-8[96]. Moreover, flagellin has also been shown to stimulate the secretion of the chemokine CCL20, which is important for recruiting immature DCs to sites of infection[97].

CCL20 is the ligand for CCR6, which is expressed on a subset of DCs that reside in the SED of the Peyer's patches. Salazar-Gonzalez et al reported that these DCs activate *Salmonella*-specific T cells following infection and are therefore instrumental in the defense against this pathogen[98]. In addition, Ravindran et al found that recruitment of blood phagocytes mediated by CCR6 was critical for the initiation of *Salmonella*-specific immune responses[99].

Some interesting data have been generated by investigating the activation of *Salmonella*-specific T cells. Srinivasan et al developed an assay for examining *Salmonella*-specific T cells that involved pulsing *Salmonella*-infected mice with heat-killed bacteria and then analyzing cytokine production of CD4 T cells. It was found that, following vaccination of C57BL/6 mice with LVS *Salmonella*, about 50% of the CD4 T cells became activated, as evidenced by their rapid production of IFN-γ[100]. These cells persisted in the memory pool and could be detected several weeks after clearance of the bacteria. This group later reported that *Salmonella*-specific T cells could be activated by innate stimuli, and that IL-18 was involved in this antigen-independent production of IFN-γ[101].

V. Antigen-Specific Responses to *Salmonella*

Although considerable research has contributed to the understanding of immune responses to *Salmonella*, the lack of available antigens with which to study specific adaptive immune responses has been a hindrance. Thus, the identification of antigens recognized by host T and B lymphocytes is of critical importance.

One of the reasons that identification of *Salmonella*-specific epitopes has been so challenging is that the genomes of the bacteria are so large. For instance, the genome of the commonly studied *S. enterica* Serovar Typhimurium (oftentimes referred to as *S. typhimurium* for simplicity), which causes a typhoid-like disease in susceptible mice and acute gastroenteritis in humans, contains around 4300 open reading frames[102]. Each of these has the ability to encode many peptide epitopes that could be recognized by T cells. Another obstacle in identifying antigenic epitopes is that during the course of *Salmonella* infection, protein expression is not temporally or spatially constant[103]. However, despite these impediments, a few *Salmonella*-specific epitopes have been painstakingly identified.

Although the current consensus is that CD8 T cells are not required for the generation of protective immunity to *Salmonella*, early studies set out to identify epitopes that are presented on MHC class-I molecules. For instance, Lo et al identified an immunodominant epitope for CTL responses as the 192-200 peptide of the GroEL protein. Two additional MHC class-I restricted peptide epitopes were identified by another group who found that I-K^b-restricted peptide residues 73-80 and 132-139 of a *Salmonella* porin, OmpC were immunodominant targets of CTLs from infected mice[104].

As with epitopes recognized by CD8 T cells, very few MHC class-II restricted epitopes have been defined, despite the early establishment of the importance of CD4 T cell-mediated immunity in *Salmonella* infection. The first epitope found to be recognized by CD4 T cells was the I-A^k-restricted FliC 339-350 peptide, which lies within the

Salmonella flagellin protein[105]. As previously mentioned, flagellin is the ligand for TLR5, an innate immune receptor that provides an important link between innate and adaptive immunity: flagellin ligation of TLR5 is known to activate DCs, which can then present antigen to T cells. In addition, flagellin in the main structural protein of flagella; about 8% of the protein synthesized by *Salmonella* is utilized to construct flagellin[105]. Therefore, flagellin is a likely candidate for the induction of adaptive immune responses.

It was soon found that flagellin is also targeted by CD4 T cells in C57BL/6 mice. McSorley et al generated FliC-specific CD4 T cell clones that were specific for the 427-441 peptide in the context of I-A^b MHC class-II molecules[106]. Bergman et al confirmed the immunodominance of the two flagellin epitopes identified thus far and added two more to the list: I-E^k-restricted FliC 80-94 and I-A^b-restricted FliC 455-469[107]. It was clear from these studies that FliC-specific CD4 T cell responses were important mediators of protective immunity to *Salmonella*. This conclusion was supported by work that showed that flagellin could stimulate protective immune responses in mice[106]. One other CD4 T cell epitope was identified by Musson et al as the I-A^d-restricted SipC 381-394[108].

A different method for identifying CD4 T cell antigenic epitopes was derived by Rollenhagen et al who employed a quantitative *in vivo* screening method that involved infecting mice with different *Salmonella* strains, each expressing different amounts of a fluorescent model antigen[109]. This group found that highly expressed antigens were the preferred targets of CD4 T cells *in vivo*. After testing the ability of candidate antigens to induce protective immunity, they concluded that Mig-14 and SseB were the most effective and might be useful in the generation of subunit vaccines to *Salmonella*[109].

It is clear that epitope discovery is an arduous process that has yielded limited results. As such, efforts to study *Salmonella*-specific immune responses mediated by

various cell populations often used model antigens that provided some evidence for the roles of these cells but did little to advance the field as far as determining *in vivo* responses of epitope-specific T cells[103].

However, Chen and Jenkins made significant progress by using T cell receptor (TCR) transgenic mice, which contain CD4 T cells that are all specific for a single epitope, to study the immune response to *Salmonella*[110]. Although others had tried to monitor the response in the transgenic mice themselves, this system is somewhat contrived. Chen and Jenkins used the DO11.10 TCR transgenic mouse, whose CD4 T cells are specific for ovalbumin (OVA) peptide, in a manner that brought the field closer to a physiologically relevant system. They were the first to adoptively transfer cells from a TCR transgenic mouse into congenic wild-type recipient mice, infect the mice with *Salmonella* expressing recombinant OVA peptide, and track the transgenic cells, using an antibody specific for the TCR, amongst the polyclonal endogenous population[110]. They found that the TCR transgenic CD4 T cells expanded in susceptible mice, but that they did not maintain their ability to produce IFN-γ at later time points during the infection. Therefore, the susceptibility of the mice to *Salmonella* infection was due to the function and not the frequency of *Salmonella*-specific T cells[110].

Although this system is useful for studying *in vivo* antigen-specific T cell responses following infection, it is limited in some respects. For one, the system does not accurately depict the regulation of proteins generated by microbes during the course of infection. *In vivo*, both spatial and temporal changes in protein expression levels can occur, and this is not modeled in the recombinant proteins expressed by pathogens used in this system[103]. Another complication is that forced expression of a recombinant protein by the pathogen of interest can have profound effects on its virulence and

therefore make it difficult to interpret results[103]. Finally, this system does not provide information on adaptive T cell-specific responses, since the CD4 or CD8 transgenic T cells rarely contribute to protective immunity[103]. Despite these drawbacks, however, in situations when epitopes have not yet been identified, the over-expression of model antigens may be the only option for gathering information about *in vivo* antigen-specific T cell responses.

In order to increase the relevance of this system, investigators set out to create a TCR transgenic mouse whose CD4 T cells were specific for an immunodominant epitope of *Salmonella*, therefore modeling CD4 T cells that presumably were part of the endogenous repertoire of a wild-type mouse. The SM1 mouse, generated by McSorley et al, did just that: SM1 CD4 T cells are specific for FliC 427-441 in the context of I-A^{b}[111]. When transferred into naïve wild-type mice that were subsequently orally infected with *Salmonella*, the SM1 cells rapidly expanded and became activated in the local lymph nodes[111]. However, the response was contained in the Peyer's patches and mesenteric lymph nodes, while the bacteria spread to the spleen and liver. Although it is unclear why this is the case, one study indicated that competition for antigen from endogenous *Salmonella*-specific T cells could explain it[100]. However, it has been found that the frequency of endogenous FliC-specific CD4 T cells is very low[112], so it is possible that SM1 cells are competing for access to antigen-presenting cells and/or cytokines[103]. Other possibilities are that flagellin is differentially expressed in different organs, or that SM1 cells simply do not have access to it[103].

As with the use of surrogate antigens, the SM1 system is limited, most notably because the response to other epitopes may not mirror the response to FliC. In addition, changing the precursor frequency of naïve T cells can have a variety of negative consequences, from altering T cell half-life and activation kinetics to hampering memory

development[103]. Therefore, a better approach is to study endogenous antigen-specific T cell responses in an intact animal. The construction of peptide:MHC tetramers, along with the development of methodology for examining low-frequency endogenous populations, has been successfully employed[112, 113]. For example, Moon et al has used this technology to examine endogenous FliC-specific CD4 T cells, which consist of a minute population in C57BL/6 mice, only about 20 cells per mouse[112]. Future research applying these techniques in the context of *Salmonella* infection will shed new light on endogenous T cell responses.

VI. Immune Evasion, Antibiotic Resistance, and Bioterrorism

One aspect of the SM1 TCR transgenic T cell system that was not mentioned is that the expansion and activation of SM1 cells is highly dose-dependent, meaning that SM1 cells fail to expand and become activated following low-dose *Salmonella* infection[114]. It is possible that this is an example of an important immune evasion strategy of *Salmonella*, as flagellin has been shown to be heavily downregulated *in vivo*[115]. Since it appears that the SM1 response is localized to mucosal sites, despite the dissemination of the bacteria to systemic tissues, one theory might be that *Salmonella* actively regulates the expression of flagellin to evade detection by SM1 cells.

There have been many studies conducted with the goal of examining immune evasion mechanisms employed by *Salmonella*. Most of these studies have investigated the ability of *Salmonella* to inhibit antigen presentation and therefore T cell activation. For example, one study showed that the *Salmonella yej* operon encodes a transporter system that hinders macrophages in antigen presentation of MHC class-I epitopes[116]. Tobar et al demonstrated that *Salmonella* could prevent antigen processing and presentation by DCs, by avoiding lysosomal degradation[117]. This group did a follow-up

study to show that these mechanisms were also active *in vivo*, and that they prevented the development of adaptive immunity[118]. In addition, they found that this immune evasion tactic was dependent on the expression of a type three secretion system (TTSS) and effector proteins encoded on the *Salmonella* pathogenicity island 2 (SPI-2)[118]. Similar results were obtained in another study, which found that the subversion of DC functions was dependent on the induction of NO synthase by DCs, in addition to virulence factors encoded on SPI-2[119]. Furthermore, Srinivasan et al found that SM1 cells proliferated and became activated in response to *Salmonella* infection, but they failed to survive long term[100]. This group later found that *Salmonella* virulence factors caused the SM1 cells to die by apoptosis[120], which lends support to the findings of previous investigators. Finally, still another study reported that *Salmonella* inhibited T cell proliferation by a mechanism requiring direct contact of the bacteria with T cells[121]. These data clearly show that *Salmonella* is capable of inhibiting the function of Th1 cells *in vivo*.

Besides its ability to evade the immune responses of its host, another reason that *Salmonella* is a formidable pathogen is its ability to develop antibiotic resistance. Multi-drug resistant (MDR) strains have been around for many years[122], and as new antibiotics are introduced, *Salmonella* is able to adapt and become resistant. Indeed, it has been shown that all of the main metabolic enzymes of *Salmonella* are currently being targeted by antibiotics[123]. That future antibiotic development is at such an impasse is a sobering concept.

The threat of *Salmonella* moves beyond antibiotic resistance. *Salmonella* was used as a weapon of bioterrorism on U.S. soil in 1984, in the largest bioterrorism attack in U.S. history[124]. The Rajneeshee cult planned to sabotage the town elections in the Dalles, Oregon, by poisoning the water supply with *Salmonella*. Fortunately, the plan

was never carried out, as some of the members turned informant when the cult collapsed[124]. However, several hundred individuals became ill following some of the cult's initial use of the bacteria, which consisted of poisoning several restaurant salad bars with *Salmonella*.

VII. Prevention of *Salmonella* infection

Salmonella infections are responsible for significant morbidity and mortality throughout the world. Around 217,000 people die every year from systemic typhoid, caused by *S. enterica* Serovar Typhi, and many suffer acute gastroenteritis, caused by the other 2000 Serovars of *S. enterica* that commonly contaminate food sources in developed countries[122, 125, 126].

All of the above mentioned reasons call for appropriate prevention measures. The most successful attempts at typhoid prevention have been through mass vaccination[126]. The first typhoid vaccine was introduced in 1896 and utilized in field trials in the 1960s[126]. It was found to be moderately effective but was a whole-cell vaccine that was highly uncomfortable and caused significant swelling at the injection sight. It also led to systemic side effects in a large proportion of individuals vaccinated[126].

It has been known since 1957 that attenuated strains of *Salmonella* can induce strong protective immunity in mice[127]. Such research led to the development of one of two vaccines against *S.* Typhi that are currently available in the United States, called Ty21a. Ty21a is a live, attenuated oral vaccine that has been shown to be very effective at protecting individuals against typhoid. Unfortunately, since it is live, it is not available for immunocompromised individuals and children under the age of six[122]. The other available vaccine is a subunit vaccine based on the Vi antigen of *S.* Typhi. It is safe for individuals age two and older[122]. This vaccine is effective, but it requires a booster every

two years, since it is a polysaccharide and therefore does not induce switched antibody responses.

Although the available vaccines have their limitations, they have been well-studied and their mechanisms for generating protective immunity are established. In particular, much research has been performed on LVS *Salmonella*, as discussed so far in this review. However, very little research has been conducted on situations where individuals are infected with virulent *S.* Typhi and treated with antibiotics. Unlike those immunized with LVS *Salmonella* who develop strong protective immunity, these individuals develop protection against future infection that is weak at best. In addition, many typhoid patients are treated with antibiotics, and this is certainly not modeled in LVS *Salmonella* infection models. Furthermore, other issues with virulent infection can develop. For example, around 5-10% of patients that recover from typhoid suffer from relapse of the infection[126]. Also, some individuals, such as the infamous Mary Malone, known as Typhoid Mary, become chronic shedders of *Salmonella*. Shedding of *Salmonella* in the stool seems to correlate with antibiotic treatment[128].

Thesis Statement

In this thesis, we set out to test the hypothesis that antibiotic treatment of murine typhoid can answer questions related to re-infection, the timing of Th1 development, and relapse of infection. In the first chapter, we discuss the issue of re-infection. We show that treatment with antibiotics interferes with the ability of *Salmonella*-infected mice to develop strong protective immunity[129]. We also show that the weak acquired immunity these mice develop is mediated by Th1 cells and serum antibody[129]. In the second chapter, we examine the kinetics of Th1 development following infection with LVS *Salmonella* and antibiotic treatment. We demonstrate that these cells require prolonged

exposure to *Salmonella* antigens in order to develop into robust memory cells. Finally, in the third chapter, we determine that relapse in antibiotic-treated mice infected with virulent *Salmonella* is mediated by intracellular bacteria harbored in $CD11b^{+}Gr1^{-}$ monocytes. We also show that the mesenteric lymph nodes act as a barrier in preventing the systemic spread of *Salmonella.*

Chapter 2

Successful treatment of bacterial infection hinders development of acquired immunity

Griffin, A, Baraho-Hassan, D and McSorley SJ. 2009. Successful treatment of bacterial infection hinders development of acquired immunity. *J Immunol* 183:1263-1270.

Antibiotics are routinely used to control bacterial infection but the acquisition of acquired immunity following successful treatment has rarely been examined. We developed a model that allows visualization of acquired immunity during and following antibiotic treatment of typhoid. Pathogen-specific humoral and cellular immune responses were activated rapidly in antibiotic-treated mice but were not sustained after successful antibiotic treatment and did not confer protection to secondary infection. In marked contrast, pathogen-specific Th1 and antibody responses matured over several weeks following immunization with a live vaccine strain (LVS). The deficiency in protective immunity following antibiotic treatment could be overcome by administering flagellin during antibiotic therapy. Thus, development of protective immunity is hindered by rapid therapeutic elimination of bacteria but can be overcome by providing additional inflammatory and/or antigenic stimuli.

Introduction

Live attenuated vaccines allow the transient colonization of an immunized host and induce robust humoral and cellular immune responses[2]. Successful treatment of an active bacterial infection also results in transient colonization of the host, but it is unclear whether a similar protective response is induced. Induction of acquired immunity following successful antibiotic treatment is particularly important for individuals in endemic areas where re-infection is likely to occur and antibiotic therapy is widely used.

Human typhoid, caused by infection with *Salmonella enterica* serovar typhi (*S.* typhi) is a public health concern in many developing nations[130], and is also recognized as a potential bioterrorism agent in the US[131]. *S.* typhi, does not infect other mammals[132], but several *Salmonella* serovars cause a typhoid-like disease in mice, sometimes referred to as murine typhoid[133]. It has been demonstrated that immunization with a live vaccine strain (LVS) of *Salmonella* confers robust protective immunity to secondary infection in both murine and human typhoid[134, 135], and both CD4 Th1 cells and antibody are required[74, 79].

Much less is known about the induction of acquired immunity during successful treatment of bacterial infections, including typhoid. In theory, antibiotic treatment should liberate bacterial antigens and Pathogen Associated Molecular Patterns (PAMPs) from dead bacteria and therefore allow efficient activation of pathogen-specific T and B cell responses. However, antibiotic treatment of primary typhoid failed to induce significant immunity to secondary infection in a study of human volunteers[136]. Other clinical studies indicate that acquired immunity after recovery from primary typhoid is insufficient to prevent re-infection. Relapse of primary infection is reported in 5-10% of typhoid patients and 1-4% can become long-term carriers of disease[137]. Indeed, re-infection with a molecularly distinct strain of *Salmonella* has been reported in patients that have

previously resolved typhoid[126]. Overall, these clinical observations suggest that acquired immunity following clearance of primary typhoid may differ substantially from protective immunity induced by transient colonization with LVS-*Salmonella*.

We decided to examine this issue directly by generating a mouse model that allows visualization of *Salmonella*-specific immunity during and after resolution of typhoid with antibiotic therapy. These experiments demonstrate that antibiotic-mediated resolution of murine typhoid elicits a limited acquired immune response that is insufficient to protect against secondary infection. Although CD4 Th1 cells were efficiently activated during the early stage of antibiotic treatment, they failed to fully develop into an effective Th1 memory pool. In marked contrast, effective Th1 memory matured over a period of several weeks in mice administered LVS-*Salmonella*. However, the weak acquired immunity evident in antibiotic-treated mice could be markedly enhanced by administration of a TLR5 agonist during the period of antibiotic treatment.

Materials and Methods

Mouse strains.

RAG-deficient SM1 TCR transgenic mice expressing the CD90.1 allele have been described[111, 114]. C57BL/6 mice were purchased from NCI (Frederick, MD) and used at 6-12 weeks of age. IFN-γ-deficient, Rag-deficient, B cell-deficient (Igh-6^{tm1Cgn}), and MHC class-II-deficient mice were purchased from The Jackson Laboratory. All mice were housed in specific pathogen-free conditions and cared for in accordance with Research Animal Resources (RAR) practices at the University of Minnesota.

Salmonella infection and antibiotic treatment.

S. typhimurium strains BRD509 ($AroA^-AroD^-$) and SL1344 were grown overnight in Luria-Bertani broth without shaking and diluted in PBS after determining bacterial concentrations using a spectrophotometer. Mice were infected orally by gavage with $5x10^9$ bacteria, immediately following administration of 100ul of a 5% $NaHCO_3$ solution. In all infection experiments, the actual bacterial dose was confirmed by plating serial dilutions onto MacConkey's agar plates and incubating overnight at 37°C. Mice infected with SL1344 were treated with Enrofloxacin (Baytril) at 2mg/ml in their drinking water for five weeks, beginning 2 days post-infection. Five days after antibiotic withdrawal, mice were re-challenged with $5x10^7$ SL1344 and monitored daily for survival. When moribund, mice were euthanized by cervical dislocation as stipulated by our animal care protocol.

Bacterial colonization in vivo.

Spleens and mesenteric lymph nodes from infected mice were removed and homogenized in Eagle's Hanks Amino Acids (EHAA, Biofluids, Rockville, MD) containing 2% fetal bovine serum. Serial dilutions were plated on MacConkey's agar plates, incubated overnight at 37°C, and bacterial counts calculated for each organ.

Adoptive transfer of SM1 T cells.

Spleen and lymph nodes (cervical, axillary, brachial, inguinal, periaortic, and mesenteric) of RAG-deficient, CD90.1 congenic, SM1 TCR transgenic mice were harvested. After generating a single-cell suspension, the percentage of SM1 cells was determined using a small aliquot of this suspension and antibodies to CD4, CD90.1, and Vβ2 (eBioscience, San Diego, CA). A FACS Canto (BD Biosciences) was used to determine the percentage of $CD4^+$ $V\beta2^+$ SM1 cells, and the total number of SM1 cells calculated. SM1 cells were then incubated with CFSE at 37°C for 8 minutes with shaking every 2-3 minutes. Cells were washed two times in cold HBSS before adjusting the concentration and injecting 1-3x10^6 SM1 T cells into the lateral tail vein of recipient C57BL/6 mice.

Flow cytometry.

A single-cell suspension was generated from harvested mouse spleens, mesenteric lymph nodes and Peyer's Patches, and samples incubated on ice in the dark for 30 minutes in F_c block (spent culture supernatant from the 24G2 hybridoma, 2% rat serum, 2% mouse serum, and 0.01% sodium azide) containing primary antibodies. FITC-, PE-, PE-Cy5-, or APC-conjugated antibodies specific for CD4, CD11a, CD90.1, Vβ2, TNF-α, and IFN-γ were purchased from eBioscience and BD Bioscience. After staining, cells

were analyzed by flow cytometry using a FACS Canto and data analyzed using FlowJo software (Tree Star, San Carlos, CA).

Tracking SM1 cells in vivo.

After infection with *Salmonella*, spleens, mesenteric lymph nodes, and Peyer's Patches were harvested on days 3, 5, 10, and 20 into EHAA medium containing 2% fetal bovine serum. Cells were stained as described above. The percentage of SM1 cells per organ was determined, as well as the activation and expansion of SM1 cells using the cell-surface marker CD11a and CFSE dye dilution, respectively, using flow cytometry.

Salmonella-specific antibody.

Mice were infected with *Salmonella* as described above. Each week post-infection, for five weeks, mice were bled retro-orbitally and serum was prepared by centrifugation and collection of supernatant. In addition, stool was collected, weighed, and suspended at 10% weight/volume in a fecal diluent (10mM Tris, 100mM NaCl, 1mM $CaCl_2$, 0.05% Tween 20, 5mM sodium azide, 1ug of aprotinin/ml, 1mM benzamidine, 10ug of leupeptin/ml, 10ug of pepstatin A/ml [pH 7.4])[138] before centrifugation to remove fecal solids. Serum and processed stool samples were stored at -20°C before direct use in antibody ELISA. High protein binding plates were coated with heat-killed *S. typhimurium* diluted in 0.1M $NaHCO_3$ and incubated overnight at 4°C. After incubation in F_c block for one hour at 37°C, plates were washed twice in PBS/0.05% Tween 20. Samples were added in serial dilutions, diluted in 10% FCS/PBS, and incubated for two hours at 37°C. Plates were washed four times before biotin-conjugated antibody specific for the desired isotype was added. After incubation for one hour at 37°C, plates were washed six times.

Finally, plates were incubated for one hour at 37°C in alkaline phosphatase diluted in 10% FCS/PBS. Plates were washed eight times and a substrate containing sodium phosphate, citric acid, O-phenylenediamene, and H_2O_2 was added. After sufficient color-change was observed, 2N H_2SO_4 was added to stop the reaction before plates were analyzed using a spectrophotometer.

Detection of in vivo cytokine production.

Mice were infected with *Salmonella* as described above. Each week post-infection, for six weeks, mice were injected i.v. with bacterial antigens to activate *Salmonella*-specific T cells (10^8 heat-killed *S. typhimurium*). Six hours later, spleens and mesenteric lymph nodes were harvested into EHAA containing 2% fetal bovine serum and a single-cell suspension generated. Following rapid surface staining on ice, cells were fixed with formaldehyde, permeabilized using saponin (Sigma-Aldrich), and stained intracellularly using cytokine-specific antibodies.

Flagellin immunization.

Mice were infected with virulent *S. typhimurium* and treated with antibiotics as described above. On the first day of antibiotic treatment, mice were injected i.v. with 100ug of highly purified purified *Salmonella* flagellin[139]. Injections were repeated each week for the duration of antibiotic treatment. After rechallenge with virulent *S. typhimurium*, as previously described, mice were monitored daily for signs of morbidity and euthanized when moribund.

Results

Effective treatment of murine typhoid with antibiotics

Ciprofloxacin is a fluoroquinolone antibiotic commonly used to treat human typhoid[140]. We examined whether the veterinary fluoroquinolone derivative Enrofloxacin would allow resolution of fatal primary typhoid in C57BL/6 mice[141]. Indeed, simply adding Enrofloxacin to the drinking water two days after oral infection allowed 100% of mice to survive a fatal infectious dose (Fig. 2-1A). Although *Salmonella* could not be detected in the spleen 72 hours after Enrofloxacin treatment (Fig. 2-1B, and data not shown), a much longer period of treatment was required to prevent recurrence of bacterial shedding following antibiotic withdrawal (data not shown). Similar reports of chronic bacterial shedding following apparent resolution of primary typhoid have been reported[142].

Recovery from primary infection confers limited protective immunity to re-infection

Mice that completely resolved primary infection using antibiotic treatment were re-infected to determine whether acquired immunity developed during this period of transient bacterial colonization. Indeed, naïve mice succumbed to infection with virulent *Salmonella* (Fig. 2-2A), whereas antibiotic-treated mice were protected against secondary infection, as demonstrated by increased survival time (Fig. 2-2A), and lower bacterial loads in the spleen and liver one week after secondary infection (data not shown). However, despite evidence of acquired immunity, almost all antibiotic-treated mice eventually succumbed to secondary typhoid (Fig. 2-2A), thus demonstrating that immunity following antibiotic treatment is incomplete. This weak protective immunity was

absent in uninfected mice treated with antibiotics and therefore not attributable to residual antibiotics in tissues (Fig. 2-2B). When compared directly with effective LVS-*Salmonella* immunization, immunity in antibiotic-treated mice was considerably less efficient at protecting against secondary infection (Fig. 2-2C). Since the protective effect of LVS-*Salmonella* immunization correlated with a longer period of host colonization (Fig. 2-1C), it was of interest to determine whether antibiotic clearance of LVS-*Salmonella* would also result in incomplete acquired immunity. Indeed, treatment of mice immunized with LVS-*Salmonella* resulted in an inability of these mice to resolve secondary infection with virulent *Salmonella* (Fig. 2-3).

Acquired immunity following antibiotic therapy is mediated by CD4 T cells and antibody

CD4 Th1 cells and antibody are required for protective immunity conferred by LVS-*Salmonella* immunization[78, 79, 143]. We examined the basis of weak acquired immunity in antibiotic-treated mice by resolving primary infection in several gene-deficient strains and then examining secondary immunity to typhoid. As expected, naïve wild-type (Wt) mice succumbed rapidly to typhoid whereas antibiotic-treated Wt mice survived longer (Fig. 2-4). Interestingly, antibiotic-treated IFN-γ-deficient mice (Fig. 2-4A), Rag-2-deficient mice (Fig. 2-4B), B cell-deficient mice (Fig. 2-4C), or mice lacking CD4 T cells (Fig. 2-4D) displayed no evidence of acquired immunity to secondary infection, demonstrating that both Th1 cells and antibody are required for limited acquired immunity following antibiotic treatment of primary typhoid.

Early activation of pathogen-specific T cells in antibiotic-treated mice

The failure of antibiotic-treated mice to resist secondary infection suggested a deficiency in the initial activation or development of *Salmonella*-specific CD4 T cells. We used *Salmonella*-specific SM1 T cells to compare the initial activation of *Salmonella*-specific CD4 T cells after infection and antibiotic treatment versus immunization with LVS-*Salmonella*. SM1 cells were activated to increase surface expression of CD11a, underwent several rounds of cell division, and accumulated in the Peyer's patch and mesenteric lymph nodes (MLN) of mice administered LVS-*Salmonella* or treated with antibiotics to resolve primary typhoid (Fig. 2-5). Therefore, the initial activation of *Salmonella*-specific CD4 T cells was similar after oral immunization with LVS-*Salmonella* or antibiotic treatment of typhoid.

Lack of sustained effector Th1 and antibody response following antibiotic treatment

Next we examined the effector Th1 response at the time of secondary challenge. In mice immunized with LVS-*Salmonella*, a large proportion of spleen and mesenteric lymph node $CD11a^{Hi}$ CD4 T cells produced IFN-γ in response to re-stimulation (Fig. 2-6A). In contrast, a lower percentage of Th1 cells was detected in antibiotic-treated mice (Fig. 2-6A). Furthermore, Th1 cells in antibiotic-treated mice produced less IFN-γ on a per cell basis than Th1 cells recovered from LVS-*Salmonella* immunized mice (Fig. 2-6A). Therefore, weak protective immunity in antibiotic treated mice correlates with a deficiency in the number and activity of effector/memory Th1 cells.

Recent reports have suggested that CD4 T cells require sustained antigen presentation for maximal proliferation and development of effector function[144, 145]. We therefore examined the maturation of effector/memory Th1 responses over several

weeks in mice immunized with LVS-*Salmonella* and mice resolving primary typhoid with antibiotic therapy. There was no difference in CD4 IFN-γ production between antibiotic-treated and LVS-*Salmonella* mice at 1 week (Fig. 2-6B), in broad agreement with the detection of efficient early activation of SM1 cells in antibiotic-treated mice (Fig. 2-5), and suggest that initial priming conditions are similar in both models. However, at later time points, Th1 cell responses increased dramatically in LVS-immunized mice but actually declined in mice administered antibiotics (Fig. 2-6B). Together, these data demonstrate a remarkable difference in the development of Th1 cells in LVS-vaccinated and antibiotic-treated mice.

A similar deficiency was noted in the *Salmonella*-specific antibody response. Rising titers of *Salmonella*-specific IgG2c were detected in the serum of LVS-*Salmonella* immunized mice (Fig. 2-7A). A smaller increase in *Salmonella*-specific IgM (Fig. 2-7B) and fecal IgA (Fig. 2-7C) titers was detected, but there was no increase in IgG1 (data not shown). In antibiotic-treated mice, lower titers of *Salmonella*-specific IgG2c were detected and a significant IgM response was absent (Fig. 2-7A, B). However, *Salmonella*-specific fecal IgA responses developed normally in antibiotic treated mice (Fig. 2-7C), perhaps because intestinal IgA class switching can occur in the absence of CD4 T cell help[146]. Together, these data demonstrate that resolution of primary typhoid with antibiotic therapy causes a deficiency in the maturation of *Salmonella*-specific CD4 Th1 and serum antibody responses.

Recovery of protective immunity by co-administration of flagellin

Incomplete development of Th1 and antibody responses following antibiotic treatment might be due to very rapid elimination of bacterial antigen and PAMPs,

causing CD4 T cell to mature in the absence of sustained inflammation. Therefore, we examined whether acquired immunity could be enhanced by administration of the TLR5 agonist, flagellin, which has been shown to function as an effective adjuvant and is also an immunodominant antigen recognized by CD4 T cells[147]. As expected, naïve wild-type (Wt) mice succumbed rapidly to infection but mice that had previously resolved typhoid using antibiotics survived longer (Fig. 2-8). Weekly administration of bacterial flagellin during the period of antibiotic treatment allowed mice that resolve primary infection to resist re-infection with typhoid (Fig. 2-8). This protective effect was not simply due to the immunizing effect of flagellin itself as flagellin administration failed to induce significant protective immunity in uninfected mice (Fig. 2-8). Thus, administration of a simple TLR5 agonist and antigen during the period of antibiotic therapy allowed recovery of robust protective immunity to typhoid.

Discussion

Immunization with live attenuated organisms can induce cellular and humoral immunity responses and effective protection against numerous vial and bacterial pathogens. One unique aspect of these live vaccines is that they involve a short period of host colonization with a weakened microbe. Much less is known about the development acquired immunity following antibiotic treatment of an infection with virulent bacteria, despite the fact that this is a common occurrence in endemic areas.

Unlike several other antibiotics (Griffin et al, unpublished), we found that Enrofloxacin is highly effective at clearing primary typhoid in the mouse model. Indeed, other investigators have recently used Enrofloxacin to resolve *Salmonella* infection in mice[148]. Although Enrofloxacin rapidly eliminated bacteria from the spleen and liver, mice recommenced the shedding live bacteria in fecal pellets if treatment was withdrawn before 30 days of treatment. Under these circumstances spleens and livers were re-colonized by rapidly growing bacteria and all mice succumbed to recurrent infection (data not shown). The anatomical site where persistent *Salmonella* survive during treatment is not yet clear, but this issue is under investigation in our laboratory. If mice were treated with Enrofloxacin in drinking water for at least 30 days, no recurrent infection developed, no bacteria were shed in fecal pellets, and these mice survived in our animal facility for over 6 months. Prolonged Enrofloxacin treatment is therefore effective at resolving murine typhoid in highly susceptible mice.

Mice that fully resolved primary infection with antibiotic treatment displayed evidence of acquired immunity to re-infection in that they survived longer and had lower bacterial burdens. This protective immunity required production of IFN-γ and the presence of class-II-restricted T cells indicating an important protective role for Th1 cells,

similar to the protective immunity induced by LVS-*Salmonella*[75-77, 80]. Our experiments also demonstrate a prominent role for B cells in resistance following antibiotic treatment, consistent with previous studies of immunity mediated by live attenuated *Salmonella*[83-85]. However, it should be noted that B cell deficient mice have also been reposted to have a deficiency in the development of Salmonella-specific Th1 cells[84, 88], complicating interpretation of this particular experiment. The presence of specific antibody may simply reduce the initial challenge dose before intracellular infection takes place or prevent cell-to-cell transmission after macrophage apoptosis. However, a role for B cells and/or antibody in the enhancement of antigen presentation to CD4 T cells by dendritic cells has also been suggested[84, 88, 117].

Surprisingly, the adaptive immune response induced following antibiotic therapy was considerably less robust than LVS-*Salmonella* immunization and was insufficient to protect against re-infection. The weak response was not due to an intrinsic difference in virulent versus LVS strains since antibiotic treatment of LVS-immunization severely hindered the development of protective immunity. Failure to develop robust acquired immunity after antibiotic treatment is consistent with clinical reports suggesting weak protective immunity in typhoid endemic areas and the lack of protective immunity in studies using human volunteers[126, 136, 137]. It is unlikely that incomplete immunity was due to a lack of antigen, since at the time of antibiotic administration bacterial loads are very high in the spleen and liver of infected mice. Furthermore, *Salmonella*-specific CD4 T cells were effectively activated and initially developed effector Th1 responses that were similar to mice immunized with LVS-Salmonella. These data suggest that the initial priming of bacterial-specific responses occurs normally in antibiotic treated-mice.

In marked contrast, we detected a deficiency in Th1 and antibody responses at later time points after antibiotic treatment. This immune deficiency correlated with the

inability of these mice to resist secondary infection. We propose that the rapid elimination of bacterial antigen and PAMPs under antibiotic therapy disrupts the normal development of Th1 immunity that is detected in LVS-*Salmonella* immunized mice. Indeed, it has previously been noted that the full proliferative capacity and effector development of CD4 T cells requires prolonged antigen stimulation[144, 145]. However, unlike these previous reports, our experiments have been completed in an infectious disease model that requires CD4 T cells for protective immunity. Together, our data indicate that an effective therapy that rapidly eliminates bacteria from the host may actually be detrimental to the subsequent acquisition of CD4-mediated protective immunity to secondary infection. If this is the case, it represents an important challenge to antibiotic therapy in endemic areas since successful treatment may therefore leave the individual susceptible to re-infection.

There are two aspects of bacterial persistence that might be required for the full development of CD4 responses. First, antigen persistence may allow pathogen-specific T cells to gradually acquire full effector function through sequential restimulation of responding clones. Second, the presence of prolonged inflammatory stimuli could provide non-TCR mediated maturation signals that allow Th1 development. Our data show that intervention with flagellin, which unusually happens to be an antigenic target of *Salmonella*-specific T cells and a TLR agonist, can rescue the development of effective protective immunity in antibiotic-treated mice. We are currently examining whether intervention with other adjuvants or *Salmonella* antigens would also have a similar effect. Addition of adjuvant delivery or therapeutic vaccination to antibiotic therapy may therefore be an important requirement for optimal development of CD4 Th1 responses and protection from secondary challenge. Therefore, these studies may aid

the design of attenuated vaccines or the development of a novel therapy against bacterial infection.

Figure 2-1. Enrofloxacin effectively resolves active murine typhoid. C57BL/6 mice were orally infected with $5x10^9$ virulent *S. typhimurium* (SL1344) and some mice treated with enrofloxacin drinking water. (A) Date show percent survival of antibiotic-treated and untreated mice and is representative of three similar experiments. (B) Spleens were harvested from infected mice at various time-points after infection and bacterial loads were determined by plating organ homogenates on MacConkey's agar. All untreated infected mice were dead by day 7. Data show mean bacterial load +/- SD for 3-5 mice per time point. (N.D. = no bacteria detected in antibiotic-treated mice).

Figure 2-1

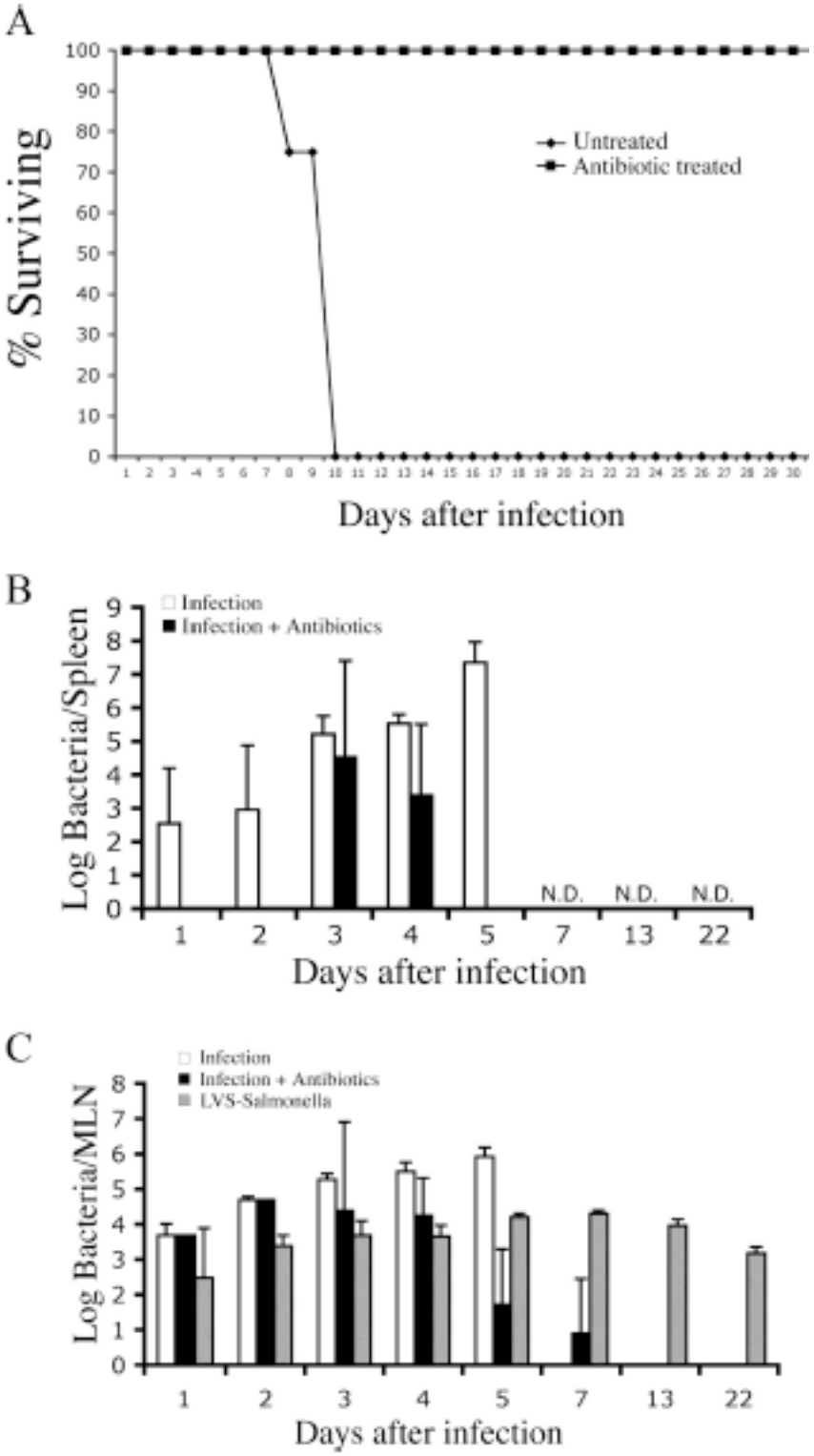

A
% Surviving
Untreated
Antibiotic treated
Days after infection
B
Infection
Infection + Antibiotics
Log Bacteria/Spleen
N.D.
N.D.
N.D.
Days after infection
C
Infection
Infection + Antibiotics
LVS-Salmonella
Log Bacteria/MLN
Days after infection

Figure 2-2. Incomplete protective immunity to typhoid in antibiotic-treated mice. (A) C57BL/6 mice were orally infected with $5x10^9$ virulent *S. typhimurium* and treated with enrofloxacin for 35 days to resolve primary infection. Five days after antibiotic withdrawal, these treated mice and a group of naïve C57BL/6 mice were orally infected with $5x10^7$ virulent *S. typhimurium*. (B) Naïve C57BL/6 mice were administered enrofloxacin in drinking water for 30 days before oral infection with virulent *S. typhimurium* five days after antibiotic withdrawal (C) Some C57BL/6 mice were immunized orally with $1x10^{10}$ LVS-*Salmonella* (BRD509) while other mice were treated exactly as in A. Forty two days later, both group of mice were challenged orally with $5x10^7$ virulent *S. typhimurium*. Graphs show percent survival of infected mice and are representative of 2-5 similar experiments.

Figure 2-2.

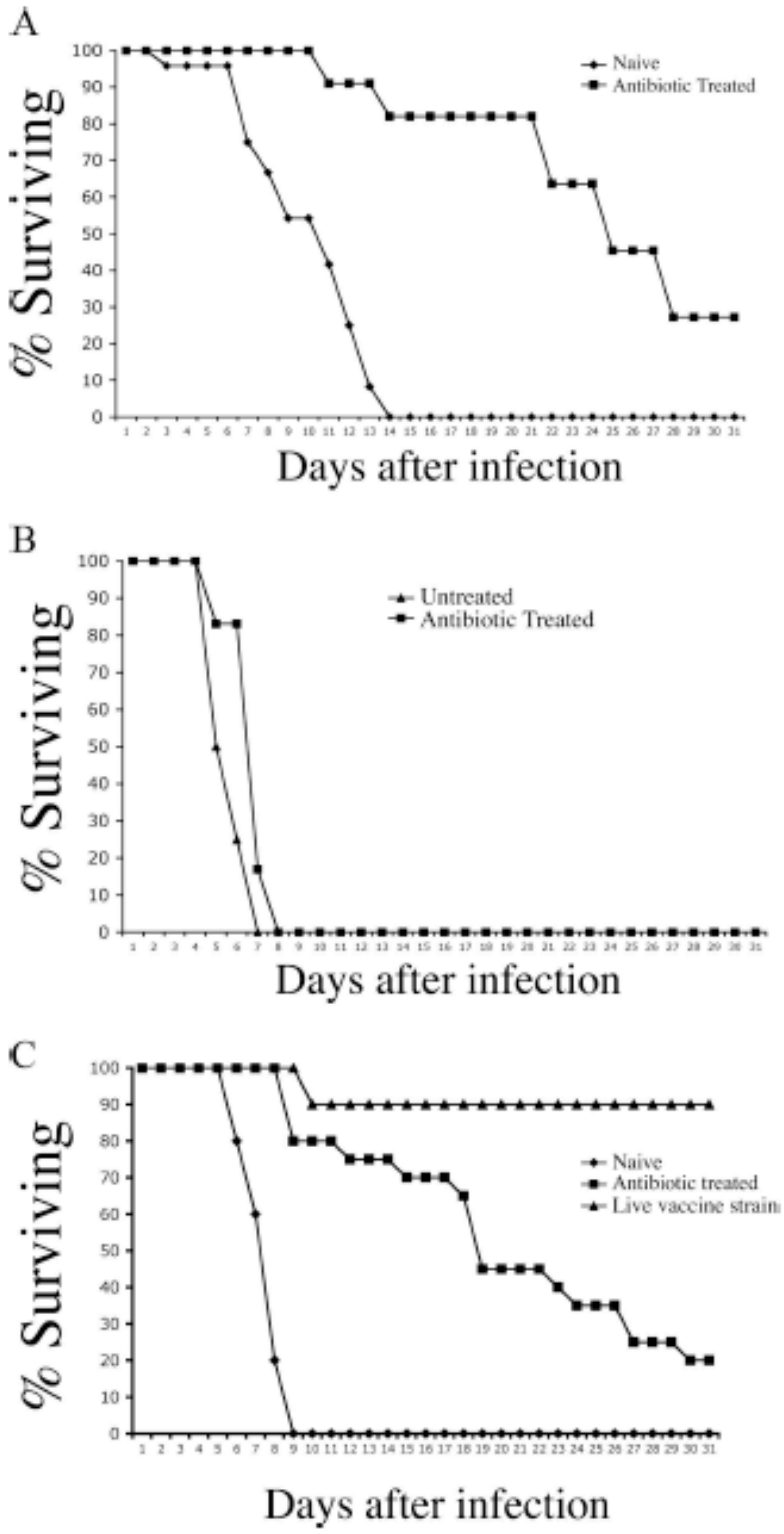

A
% Surviving
Days after infection
Naive
Antibiotic Treated
B
% Surviving
Days after infection
Untreated
Antibiotic Treated
C
% Surviving
Days after infection
Naive
Antibiotic treated
Live vaccine strain

Figure 2-3. Incomplete protective immunity in antibiotic-treated mice immunized with LVS *Salmonella*. (A) C57BL/6 mice were orally infected with $5x10^9$ LVS-Salmonella and some mice were treated with enrofloxacin for 35 days to resolve primary infection. Five days after antibiotic withdrawal, these mice and a group of naïve C57BL/6 mice were orally infected with $5x10^7$ virulent *S. typhimurium*.

Figure 2-3.

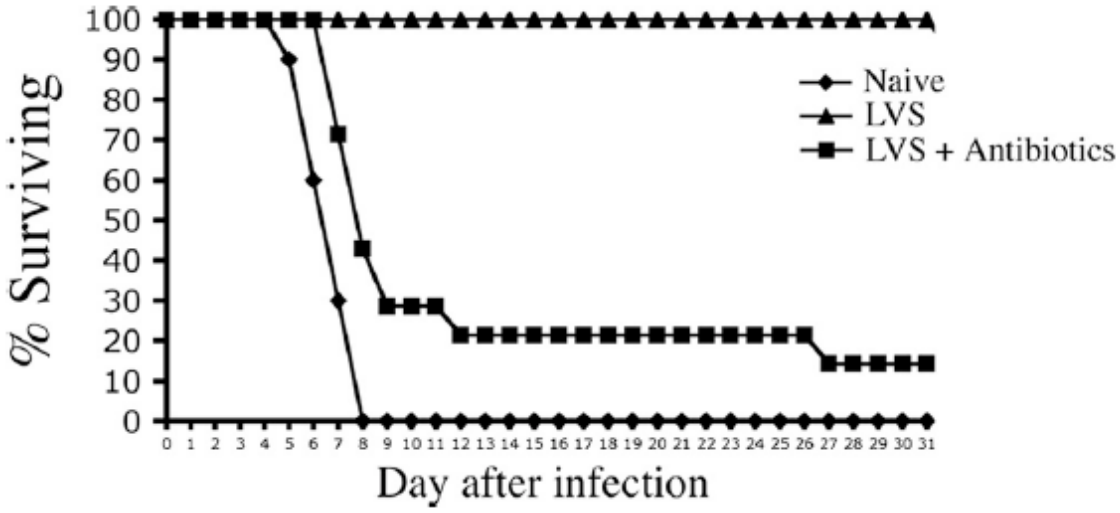

% Surviving
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Day after infection
Naive
LVS
LVS + Antibiotics

Figure 2-4. Acquired immunity after antibiotic therapy of typhoid requires class-II restricted T cells and antibody. C57BL/6 (Wt) and, (A) IFN-γ-deficient, (B) Rag-deficient, (C) B cell deficient, or (D) MHC class-II deficient mice, were infected orally with $5x10^9$ virulent *S. typhimurium* and treated with antibiotics in drinking water for 35 days. Antibiotic-treated and naïve Wt mice were re-infected orally with $5x10^7$ virulent *S. typhimurium*. Graphs show the percent survival of infected mice and are representative of 2-3 similar experiments.

Figure 2-4.

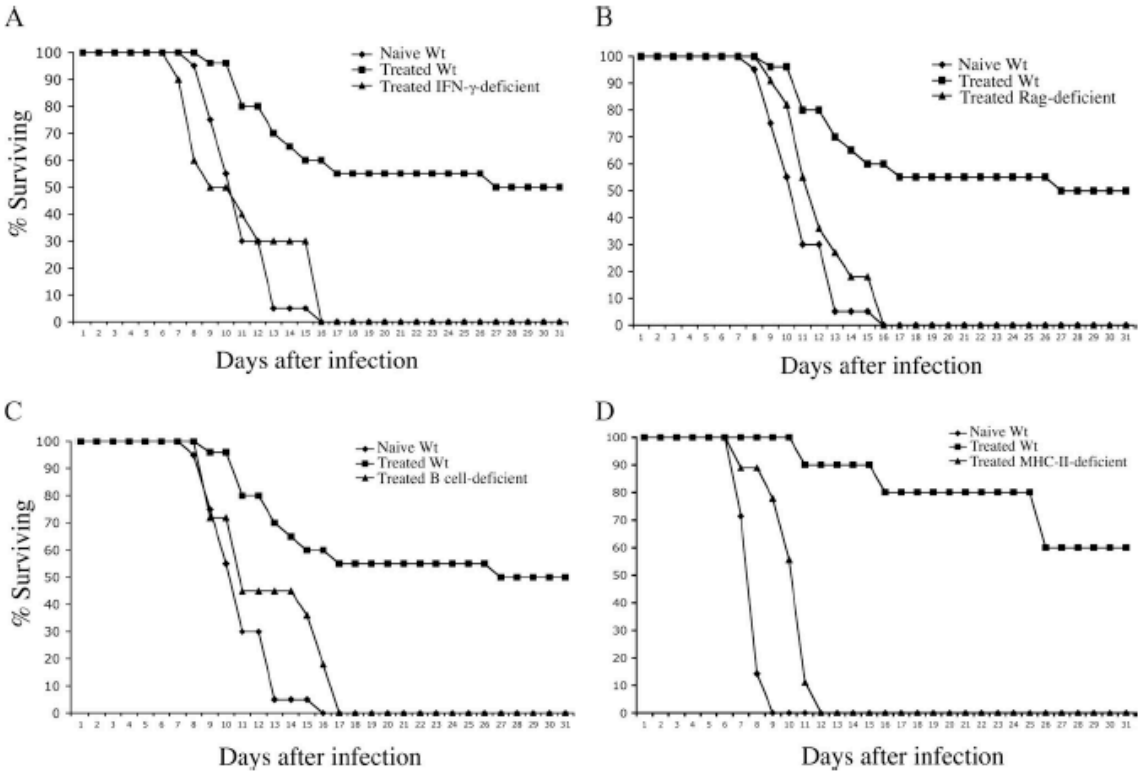

A
% Surviving
Days after infection
Naive Wt
Treated Wt
Treated IFN-γ-deficient
B
Days after infection
Naive Wt
Treated Wt
Treated Rag-deficient
C
% Surviving
Days after infection
Naive Wt
Treated Wt
Treated B cell-deficient
D
Days after infection
Naive Wt
Treated Wt
Treated MHC-II-deficient

Figure 2-5. Rapid activation of *Salmonella*-specific CD4 T cells following antibiotic-cure or LVS-*Salmonella* immunization. C57BL/6 mice were adoptively transferred with $2x10^6$ CFSE-stained CD90.1+ SM1 T cells before oral infection with $5x10^9$ LVS-*Salmonella* (BRD509) or oral infection with $5x10^9$ virulent *S. typhimurium* (SL1344) followed by antibiotic treatment 2 days later. Three days after infection Peyer's Patches and mesenteric lymph nodes (MLN) were harvested and SM1 T cells identified by flow cytometry staining for CD4 and CD90.1 (A) CD11a surface staining and CFSE dye dilution, and (B) the percentage of SM1 T cells from antibiotic-treated and LVS-*Salmonella* immunized mice.

Figure 2-5.

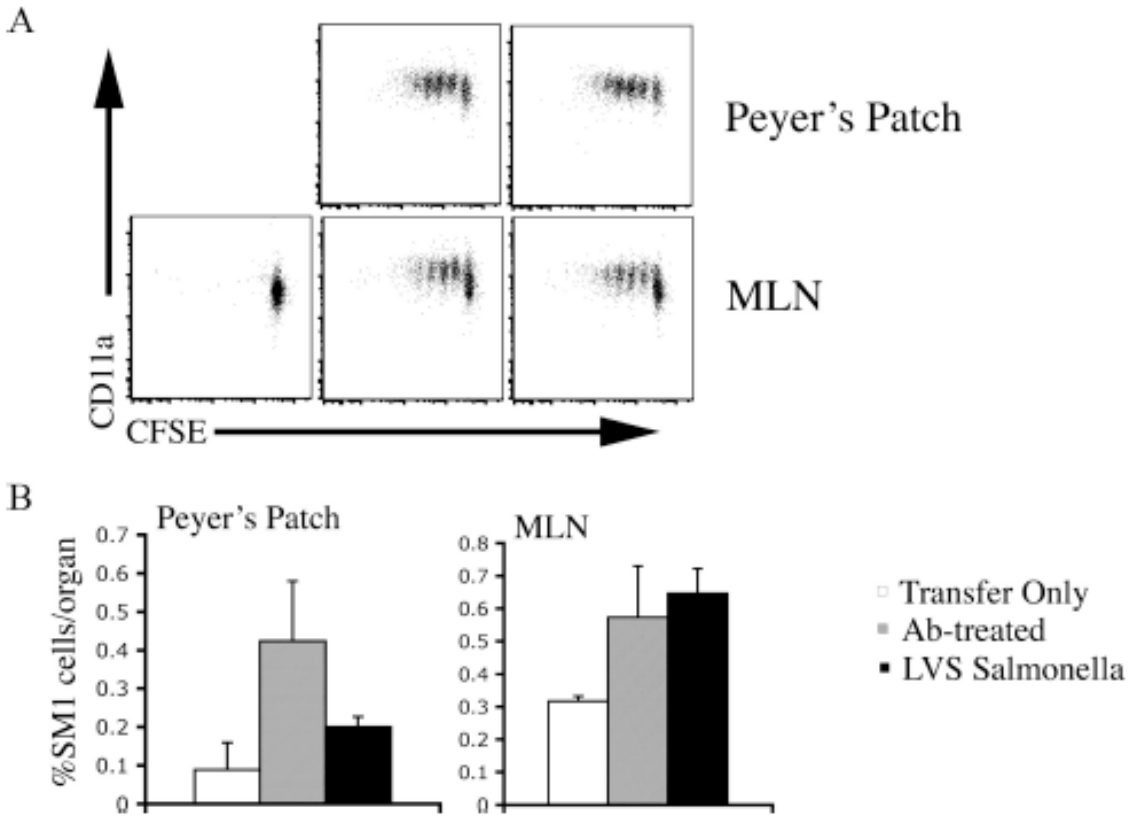

A
Peyer's Patch
MLN
CD11a
CFSE
B
Peyer's Patch
MLN
%SM1 cells/organ
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Transfer Only
Ab-treated
LVS Salmonella

Figure 2-6. Deficient maturation of Th1 responses in antibiotic-treated mice. C57BL/6 mice were immunized with LVS-*Salmonella* or orally infected with virulent *S. typhimurium* and treated with antibiotics for 35 days. (A) Forty-two days after immunization or infection, CD4 Th1 cell responses in the spleen were examined by re-stimulation with *Salmonella* lysate and direct ex-vivo detection of intracellular cytokine production. FACS plots show IFN-γ production by gated CD4 T cells in response to six hours of stimulation with *Salmonella* lysate. Bar graphs show (middle) mean percentage of IFN-γ^+ CD4 T cells +/- SD, and (right) mean-fluorescence intensity +/- SD of IFN-γ producing cells from 4 mice per group. Data are representative of three similar experiments. (B) At weekly time points, CD4 Th1 responses were examined exactly as described in A. Bar graphs show mean percentage of IFN-γ^+ CD4 T cells +/- SD in the spleen and MLN of 3-5 mice per group and are representative of 2 similar experiments.

Figure 2-6.

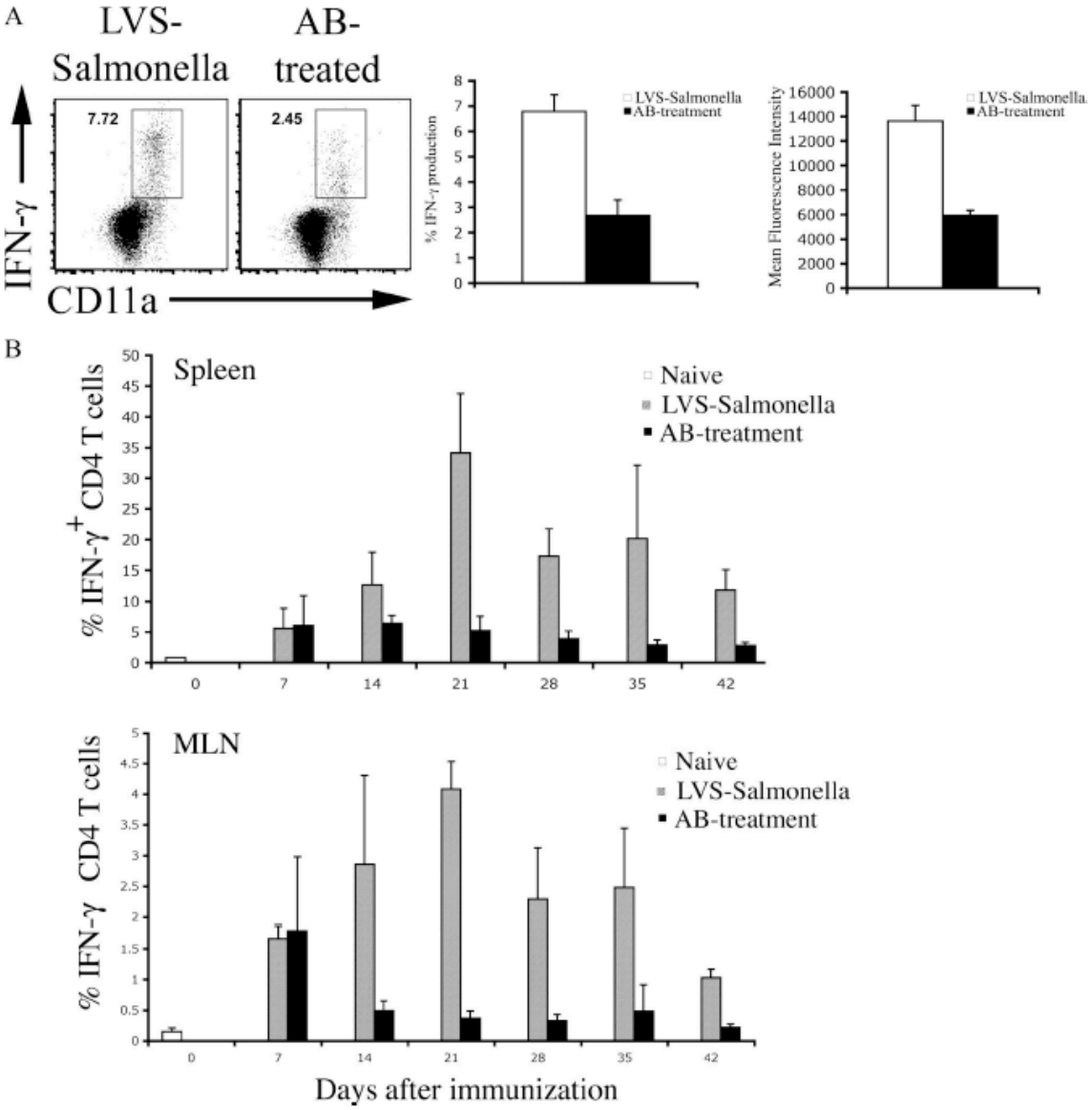

A
LVS-Salmonella
AB-treated
7.72
2.45
IFN-γ
CD11a
% IFN-γ production
LVS-Salmonella
AB-treatment
Mean Fluorescence Intensity
B
Spleen
% IFN-γ+ CD4 T cells
Naive
LVS-Salmonella
AB-treatment
MLN
% IFN-γ CD4 T cells
Days after immunization

Figure 2-7. Deficient development of *Salmonella*-specific serum antibody responses in antibiotic-treated mice. C57BL/6 mice were immunized with LVS-*Salmonella* or orally infected with virulent *S. typhimurium* and treated with antibiotics for 35 days. (A-B) Blood was collected at weekly time points and *Salmonella*-specific antibody responses examined using isotype-specific antibody ELISAs (C) Stool was collected, weighed, suspended in a fecal diluent, and the presence of *Salmonella*-specific IgA determined using an antibody ELISA.

Figure 2-7.

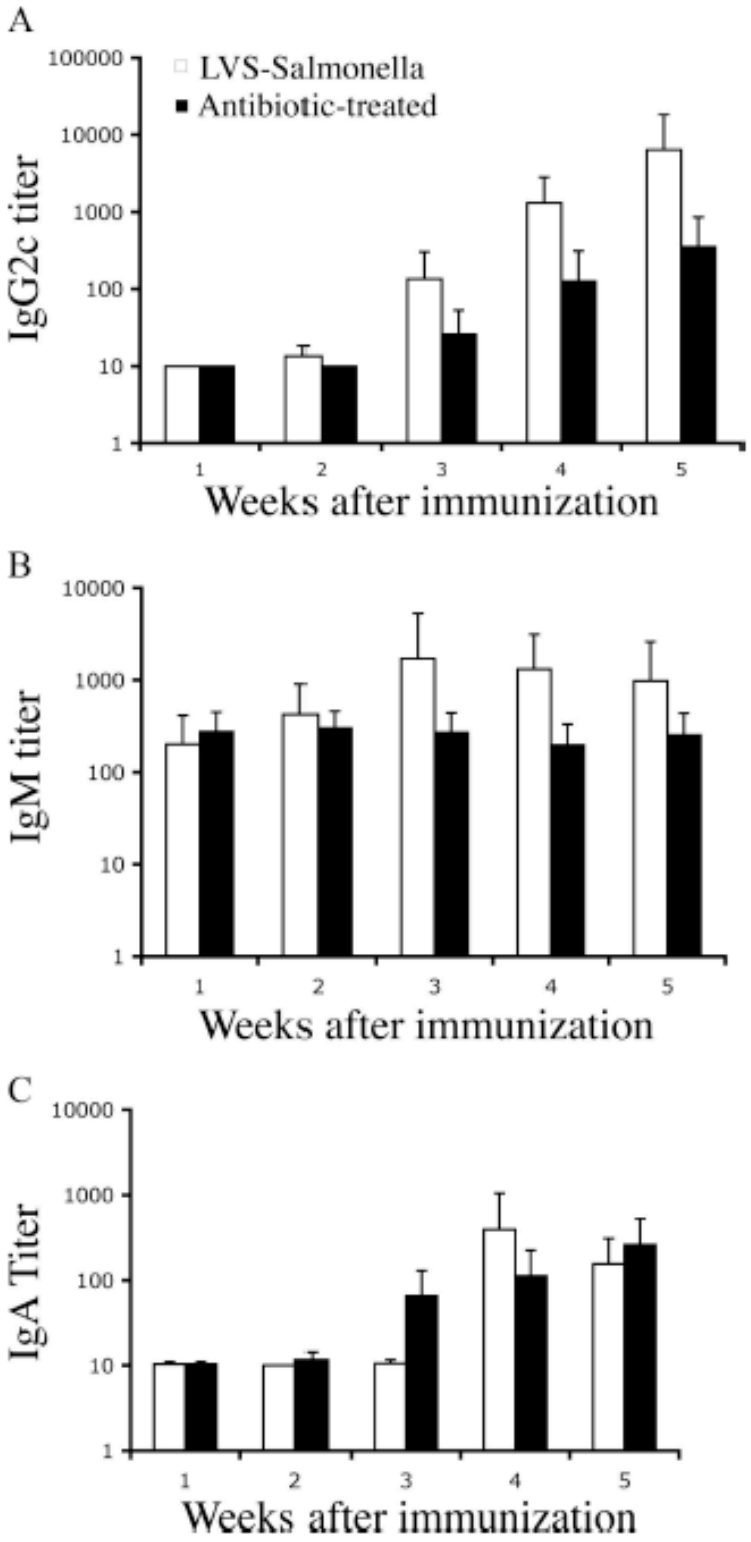
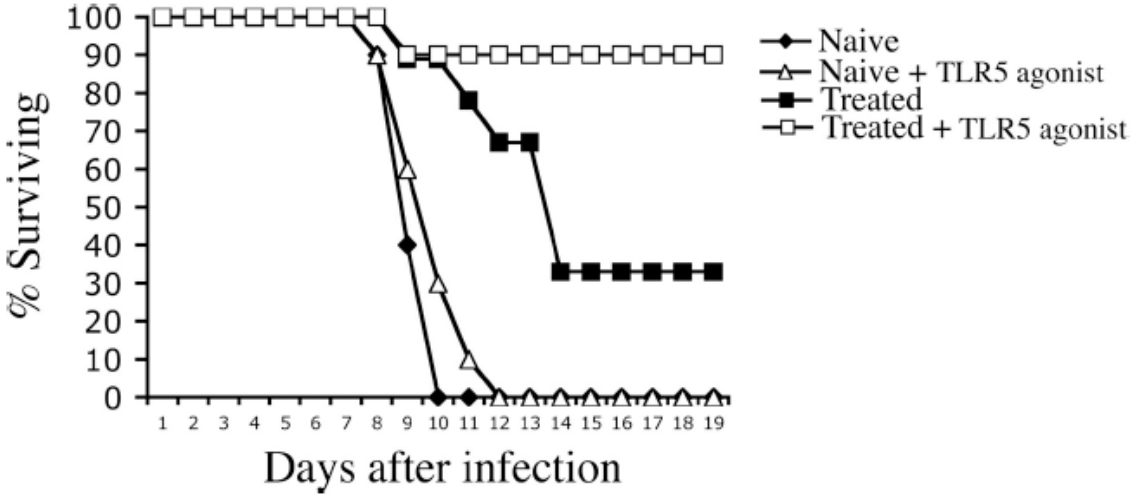

A
LVS-Salmonella
Antibiotic-treated
IgG2c titer
100000
10000
1000
100
10
1
1
2
3
4
5
Weeks after immunization
B
IgM titer
10000
1000
100
10
1
1
2
3
4
5
Weeks after immunization
C
IgA Titer
10000
1000
100
10
1
1
2
3
4
5
Weeks after immunization

Figure 2-8. Treatment with flagellin enhances protective immunity in antibiotic-treated mice. C57BL/6 mice were orally infected with virulent *S. typhimurium* and treated with antibiotic drinking water for 35 days. Groups of infected and naïve mice were injected i.v. with 100 μg purified flagellin at weekly intervals during antibiotic treatment. Five days after antibiotic treatment was halted, naïve and antibiotic-treated mice were infected orally with virulent *S. typhimurium*. Data shows the percent survival of infected mice and is representative of 2 similar experiments.

Figure 2-8.


% Surviving
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Days after infection
Naive
Naive + TLR5 agonist
Treated
Treated + TLR5 agonist

Chapter 3

Generation of *Salmonella*-specific Th1 cells requires sustained *in vivo* stimulation

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The administration of live attenuated oral vaccines for human typhoid has proven to be an effective way to generate protective immunity. Several studies have shown that this protection is partly mediated by *Salmonella*-specific Th1 cells, however the timing and stimulatory requirements to generate optimal Th1 effector/memory responses have not been carefully examined. We used antibiotic interruption of vaccination with live attenuated *Salmonella* to determine the stimulatory requirements for *Salmonella*-specific Th1 development and protective immunity. Optimal development of protective immunity to *Salmonella* infection required one week of exposure to a live attenuated *Salmonella* strain. In contrast, optimal development of *Salmonella*-specific Th1 effector/memory cells required two weeks of in vivo colonization. Thus, sustained in vivo stimulation with a live vaccine strain is required for the development of robust *Salmonella*-specific Th1 effector/memory cells.

Introduction

Salmonella enterica serovar Typhi (hereafter referred to as *S. typhi*), the causative agent of human typhoid, is responsible for significant morbidity and mortality throughout the world, particularly in developing countries. The most recent estimate is that *S. typhi* infects 21.7 million people and causes over 200,000 fatalities, every year[125]. *Salmonella* infections are typically transmitted via the fecal-oral route, most commonly by contaminated food or water. As such, typhoid outbreaks tend to concentrate in geographical areas lacking adequate sanitation or access to clean drinking water[149]. For infected individuals with access to medical treatment, antimicrobial resistance is also a growing concern[126] and our current reliance on antibiotic treatment of typhoid is not a realistic long-term strategy to combat this disease. The most obvious solution to endemic typhoid in developing nations would be the wide scale development of sanitation and water treatment infrastructure, however this strategy would impose huge financial burdens and logistical difficulties on already impoverished nations[150]. Therefore, the development of an effective, safe, and inexpensive typhoid vaccine that can be administered in areas where typhoid is endemic is urgently required[90, 122, 151].

Two typhoid vaccines are available in the US and other developed nations and are widely used by foreign travelers to endemic areas[90, 151]. The first of these is a parenteral subunit vaccine consisting of the Vi capsular polysaccharide of *S.* Typhi, and the second is a live attenuated oral vaccine, which has been shown to be a safe and effective way to induce strong protective immunity in immunocompetent individuals[90, 134, 135]. Much of our knowledge about the mechanism of protective immunity to typhoid comes from the development and study of animal models of *Salmonella* infection[135]. In particular, laboratories have studied acquired protective immunity to *Salmonella* after vaccination of mice with attenuated strains of *S. enterica* serovar Typhimurium.

Immunization of susceptible mice with these live vaccine strains confers solid protective immunity to challenge with virulent *Salmonella*, and this protection is known to require both cellular and humoral immunity[77, 78, 81, 83, 148]. Many of these studies have emphasized an essential role for *Salmonella*-specific $CD4^+$ Th1 cells in the acquisition protective immunity by vaccination. Importantly, these *Salmonella*-specific Th1 cells secrete the cytokine IFN-γ, which is capable of activating infected macrophages to kill intracellular bacteria.

Although Th1 cells are essential for immunity to *Salmonella* infection, the timing and development of *Salmonella*-specific effector/memory Th1 cells has not been carefully examined in vivo. Studies of T cell memory development in other disease models suggest that sustained access to antigen can be an important variable for the optimal maturation and development of long-term memory responses in some situations, but not in others[145, 152, 153]. For example, in a mouse model of systemic *Listeria monocytogenes* infection, CD8 T cells require a very short time of antigen exposure in order to differentiate and develop memory cells[152]. However, in another mouse model, sustained engagement of the T cell receptor by cognate ligand was required throughout the expansion phase for the development of CD4 memory cells[145]. It is not yet clear whether this requirement for sustained TCR stimulation represents an intrinsic difference between CD4 and CD8 T cell effector/memory development or whether a period of prolonged antigenic stimulation might be specifically required for memory T cell development in some disease models, but not in others.

As CD4 Th1 cells are essential for the induction of acquired immunity following vaccination of mice with attenuated *S.* Typhimurium, we sought to determine the length of time that mice would need to be colonized with a live vaccine strain in order for

optimal Th1 development and vaccine-mediated protection to be induced. Our data demonstrate that the induction of robust protective immunity requires at one week of antigenic exposure while maximal development of Th1 responses takes considerably longer.

Materials and Methods

Mouse strains.

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD) and used at 6-12 weeks of age. All mice were housed in specific pathogen-free conditions and cared for in accordance with Research Animal Resources (RAR) practices at the University of Minnesota.

Salmonella infection and antibiotic treatment.

S. typhimurium strain BRD509 ($AroA^-D^-$) was grown overnight in Luria-Bertani broth without shaking and diluted in PBS after determining bacterial concentrations using a spectrophotometer. Mice were infected orally by gavage with $5x10^9$ bacteria, immediately following administration of 100ul of a 5% $NaHCO_3$ solution. In all infection experiments, the actual bacterial dose was confirmed by plating serial dilutions onto MacConkey's agar plates and incubating overnight at 37°C. Some mice were treated with Enrofloxacin (Baytril) at 2mg/ml in their drinking water, beginning on various days post-infection. Five days after antibiotic withdrawal (six weeks after primary infection), mice were re-challenged with $5x10^7$ SL1344 and monitored daily for survival. When moribund (displaying no movement when gently prodded with an index finger), mice were euthanized by cervical dislocation as stipulated by our animal care protocol.

Bacterial colonization in vivo

On days two, three, four, and seven, spleens and mesenteric lymph nodes (MLN) from infected mice were removed and homogenized in Eagle's Hanks amino acids (EHAA;

Gibco) containing 2% FBS. Serial dilutions were plated on MacConkey's agar plates and incubated overnight at 37°C, and bacterial counts were calculated for each organ.

Adoptive transfer of SM1 T cells

Spleen and lymph nodes (cervical, axillary, brachial, inguinal, and mesenteric) of RAG-deficient, CD90.1 congenic, SM1 TCR transgenic mice were harvested. After generating a single-cell suspension, the percentage of SM1 cells was determined using a small aliquot of this suspension and Abs to $CD4^+V\beta2^+$ SM1 cells, and the total number of SM1 cells was calculated. SM1 cells were then incubated with CFSE at 37°C for eight minutes, with shaking every 2-3 minutes. Cells were washed twice in cold HBSS before adjusting the concentration and injecting 1 x 10^6 SM1 T cells into the lateral tail vein of recipient C57BL/6 mice.

Flow cytometry

A single-cell suspension was generated from harvested mouse MLNs and spleens. Samples were incubated on ice in the dark for 30 minutes in FACS staining buffer (Hank's Balanced Salt Solution containing 2% FBS and 0.1% sodium azide) containing primary Abs. FITC-, PE-, Cy5-PE- or APC-conjugated Abs specific for CD4, CD11a, CD90.1, Vβ2, IFN-γ, TNF-α, CD3, and CD8 were purchased from eBiosciences and BD Biosciences. After staining, cells were analyzed by flow cytometry using a FACS Canto, and data were analyzed using FlowJo software (Tree Star).

Tracking SM1 cells in vivo

After i.v. injection of 200ug of flagellin peptide 427-441 (VQNRFNSAITNLGNT), spleens and MLNs were harvested on days 3 and 10 into EHAA medium containing 2% FBS. Cells were stained, as described above. The percentage of SM1 cells per organ was determined, as well as the activation and expansion of SM1 cells, using the cell surface marker CD11a and CFSE dye dilution, respectively, using flow cytometry.

Detection of in vivo cytokine production

Mice were infected with BRD509 and some were treated with antibiotics beginning at different time points following infection, as described above. Six weeks post-infection, and five days after antibiotic withdrawal, mice were injected i.v. with 10^8 heat-killed BRD509 to activate *Salmonella*-specific T cells. Six hours later, spleens and mesenteric lymph nodes were harvested into EHAA containing 2% FBS, and a single-cell suspension was generated. Following rapid surface staining on ice, cells were fixed with formaldehyde, permeabilized using saponin (Sigma-Aldrich), and stained intracellularly using cytokine-specific Abs.

CD4 T cell depletion

Mice were infected with BRD509 as described above. Some mice were treated with antibiotics beginning two days post-infection. Antibiotics were withdrawn 37 days post-infection. Forty-two days post-infection (after the infection had been cleared), mice were injected i.p. with 500ug anti-CD4 (GK1.5) or IgG2b (LTF-2) isotype control antibody. Mice were rechallenged with SL1344, as described, one day following injection. Mice were injected with another round of antibody three days post-infection. Each day, tails were bled and cells stained for FACS analysis to determine percent depletion of CD4 T cells, which was greater than 99.7% throughout the experiment. On days three and six

post-infection, spleens, livers, and MLNs were analyzed for bacterial colonization, as described above.

Results

Elimination of live vaccine strain colonization using antibiotic treatment

Fluoroquinolones, such as ciprofloxacin, are antibiotics commonly used to treat typhoid in endemic areas[140]. Enrofloxacin is a veterinary fluoroquinolone derivative known to act on the type-II topoisomerase DNA gyrase of Gram-negative bacteria, thereby inhibiting DNA synthesis[154]. Our laboratory previously reported that antibiotic treatment of susceptible mice with enrofloxacin, rapidly eradicated the growth of virulent bacteria from the spleen and mesenteric lymph nodes (MLNs)[129]. Antibiotic treatment of mice immunized with a live vaccine strain of *S*. Typhimurium hindered the development of protective immunity to rechallenge with a virulent strain[129]. We therefore decided to examine the clearance of this vaccine strain in more detail and hypothesized that early treatment of vaccinated mice with antibiotics rapidly eliminated the attenuated bacteria, and thus limited the available antigen that was required to induce protective immunity.

Initially, we examined whether antibiotic treatment actually led to the efficient removal of this live attenuated strain of *Salmonella*, that grows slowly in vivo due to an inability to generate essential metabolites. C57BL/6 mice were immunized orally with an $AroA^-D^-$ live vaccine strain (LVS) of *S.* Typhimurium and treated with enrofloxacin in drinking water beginning two days post-immunization. After 24 hours, bacteria were undetectable in the MLNs of antibiotic-treated mice but were still present in the MLN of mice not treated with antibiotics (Fig. 3-1). Therefore, enrofloxacin rapidly eliminates a vaccine strain of *Salmonella* in vivo and can be used as a simple intervention to modulate the period of antigen exposure during live vaccination.

Seven days of live vaccine colonization is required for the development of robust protective immunity to *Salmonella*

Live vaccine strains of *Salmonella* typically colonize the host for 3-5 weeks following immunization and can mediate protective immunity against *Salmonella* infection. We used antibiotic treatment to determine the minimum period of live vaccine colonization that is required to develop protective immunity. Groups of mice were vaccinated orally with LVS *S.* Typhimurium and then administered antibiotics starting 2, 7, 14, or 21 days later. All antibiotic treated mice continued treatment until 37 days post-infection followed by five days of untreated drinking water[129]. At 42 days post-vaccination, each group of mice was challenged with a virulent strain of *S*. Typhimurium and protection against infection was evaluated. Mice that received antibiotics beginning 7, 14, and 21 days post-immunization displayed marked resistance to *Salmonella* infection when compared to naïve mice (Fig. 3-2A). However, mice that received antibiotics starting 2 days after live vaccination displayed a reduced ability to resist subsequent *Salmonella* infection (Fig. 3-2A). Therefore, the development of protective immunity to *Salmonella* infection requires between 2 and 7 days of colonization with a live vaccine strain.

In a second series of experiments, we attempted to further define this time period by intervening with antibiotic treatment at 1, 2, 3, 5, or 7 days following live vaccination. Groups of mice were orally immunized with LVS *S.* Typhimurium, treated with antibiotics, challenged with virulent *S*. Typhimurium, and subsequently monitored for the ability to resist infection. Vaccinated mice that were treated with antibiotics as early as day 1, 2, 3, or 5 displayed markedly reduced ability to resist *Salmonella* infection (Fig. 3-2B). In contrast, the vast majority of mice that were antibiotic treated beginning 7 days post vaccination were able to resist *Salmonella* infection (Fig. 3-2B). Together, these data

demonstrate that mice require colonization for around 7 days with a live vaccine strain in order to develop robust protective immunity to *Salmonella* infection.

Antibiotic treatment does not affect initial CD4 T cell activation and expansion

These results demonstrated that antibiotic treatment during vaccination can have a profound detrimental effect on the development of protective immunity. Rather than limiting access to antigen, it was possible that the antibiotics were directly impeding initial CD4 T cell activation or expansion in vivo. In order to examine this issue, we monitored the effect of antibiotic treatment on *Salmonella*-specific CD4 T cell expansion following peptide immunization. C57BL/6 mice were adoptively transferred *Salmonella*-specific SM1 CD4 T cells and immunized with the relevant cognate antigen (flagellin peptide 427-441)[106, 111] while also being treated with antibiotics. SM1 T cells were activated to clonally expand in mice that were treated with antibiotics (Fig. 3-3), indicating that antibiotic treatment alone does not hinder the ability of CD4 T cells to be activated or expand in vivo.

Two weeks of live vaccine colonization is required for optimal development of *Salmonella*-specific Th1 responses

Endogenous *Salmonella*-specific CD4 T cell responses can be visualized directly ex vivo following brief stimulation with Heat-killed *Salmonella* typhimurium (HKST) in vivo. In order to examine the effect of vaccine strain colonization on Th1 development, we also monitored the development of *Salmonella*-specific IFN-γ-producing T cells in parallel to the protection experiments described above. Mice that received a live vaccine strain of *Salmonella* without antibiotic treatment generated an expanded population of

$CD11a^{Hi}$ cells that produced IFN-γ following stimulation with HKST (Fig. 3-4A, No Abx). In marked contrast, vaccinated mice that were treated with antibiotics beginning at day 1 had an impaired *Salmonella*-specific Th1 response (Fig. 3-4A, Day 1). A weak *Salmonella*-specific Th1 response was also detected in mice vaccinated for 2-5 days before antibiotic intervention (Fig. 3-4A, Day 2, 3, and 5). However, a large increase in this Th1 response was noted in groups of mice that were exposed to live *Salmonella* for 7 days before antibiotic treatment started (Fig. 3-4A, Day 7), although this response was still substantially lower than the maximal response noted without antibiotic treatment (Fig. 3-4A, No Abx). In addition to the lower percentage of IFN-γ-producing cells in antibiotic-treated mice, CD4 Th1 cells from these mice also produced a lower amount of IFN-γ produced per cell (Fig. 3-4A).

The notable recovery of Th1 responses in mice that were colonized with a vaccine strain for 7 days, also correlated with the ability of these mice to resist *Salmonella* infection (Fig. 3-3). However, as this response was still lower than vaccinated mice that did not receive antibiotics, we examined whether further delaying treatment would amplify the *Salmonella*-specific CD4 response. Indeed, maximal Th1 effector function was observed if antibiotic treatment was delayed for 14 days, as the IFN-γ-producing CD4 T cells from this group were equivalent in percentage and MFI to those observed the untreated group (Fig. 3-4B). Therefore, although one week of live vaccine colonization was required to develop protective immunity against *Salmonella* infection, up to 2 weeks of exposure was required for optimal *Salmonella*-specific Th1 development.

CD4 cells mediate protective immunity after vaccination

Our data correlate the development of *Salmonella*-specific Th1 cells with the generation of protective immunity against infection. In order to demonstrate that CD4 T cells actually mediate protective immunity in this model, we vaccinated mice with LVS *Salmonella* and examined protective immunity after depletion of CD4 T cells. Bacterial loads were high in naïve mice but were substantially lower in mice that were previously vaccinated with LVS *Salmonella* (Fig. 3-5). In contrast, mice that were treated with anti-CD4 depleting antibody had increased bacterial colonization (Fig. 3-5).

Discussion

Vaccination is currently the best option for combating infectious diseases[90, 122, 151], and live attenuated vaccine strains have proven to be an effective means at inducing strong protective immunity against many pathogens[90, 134, 135]. While live attenuated vaccines are widely used they are not always approved for all age groups and multiple doses of live attenuated typhoid vaccine are required due to poor immunogenicity[122]. Human typhoid remains a considerable problem in many developing countries and development of an improved live attenuated vaccine will require greater understanding of how *Salmonella* strains induce a protective immune response.

Our laboratory recently used antibiotic treatment of virulent *Salmonella* strains to examine the development of immunity to *Salmonella*. In completing these studies we noticed that antibiotic treatment during immunization with a live vaccine strain severely reduced the development of protective immunity. This was somewhat surprising, given previous work where early interruption of *Listeria* vaccination did not significantly reduce CD8 T cell development. Our current experiments have examined this process in more detail and demonstrate that prolonged colonization with a live vaccine strain of *Salmonella* is required to develop immunity to challenge infection. The most likely explanation for this finding is that antibiotic treatment hinders Th1 effector/memory development by rapidly eliminating a source of bacterial antigens. Indeed, our TCR transgenic adoptive transfer experiments show that antibiotics do not directly affect the ability of CD4 T cells to proliferate and become activated in vivo. Furthermore, the development of protective immunity in this model is dependent on CD4 T cells during challenge infection. Thus, our data are most consistent with a model where optimal development of protective *Salmonella*-specific Th1 cells requires between 1-2 weeks of antigen exposure.

Other studies have noted that sustained antigen exposure can enhance the survival of CD4 T cells into the memory pool or allow greater development of effector potential[152, 153]. In experiments examining CD4 Th1 development directly, we found that antibiotic interruption up to 2 weeks could hinder the maturation of these cells. The longer time period for optimal development of Th1 cells versus protective immunity is most likely a function of the challenge dose used in our studies, i.e. one week of stimulation allows sufficient Th1 development to protect against the challenge dose used in our experiments, while longer exposure to antigen is required for maximal Th1 development.

The finding that protective immunity requires at least one week of colonization with a live vaccine strain of *Salmonella* and that optimal Th1 development requires two weeks, is important with respect to typhoid vaccination, and provides an explanation for why multiple doses of live attenuated Salmonella strains are often required to generate robust immunity. Indeed, if attenuation of Salmonella strains shortens the time period of colonization significantly then such strains would be expected to generate weak Th1 responses and poor immunity. Thus, development of highly attenuated strains with sustained in vivo colonization should be a goal of future vaccine design. In conclusion, our data suggest a surprisingly long time period of CD4 T cell stimulation is required for anti-Salmonella immunity. These studies should aid in the design of effective vaccines that can be widely distributed in developing nations where the burden of typhoid is most detrimental.

Figure 3-1. Treatment with enrofloxacin rapidly eliminates LVS *Salmonella* from mouse MLNs. C57BL/6 mice were orally infected with 5 x 10^9 attenuated *S.* Typhimurium (BRD509). Some mice were treated with enrofloxacin in their drinking water beginning 2 dpi. MLNs were harvested from infected mice at various time points after infection, and bacterial loads were determined by plating organ homogenates on MacConkey's agar. Data show mean bacterial load +/- SD for three to five mice per time point.

Figure 3-1

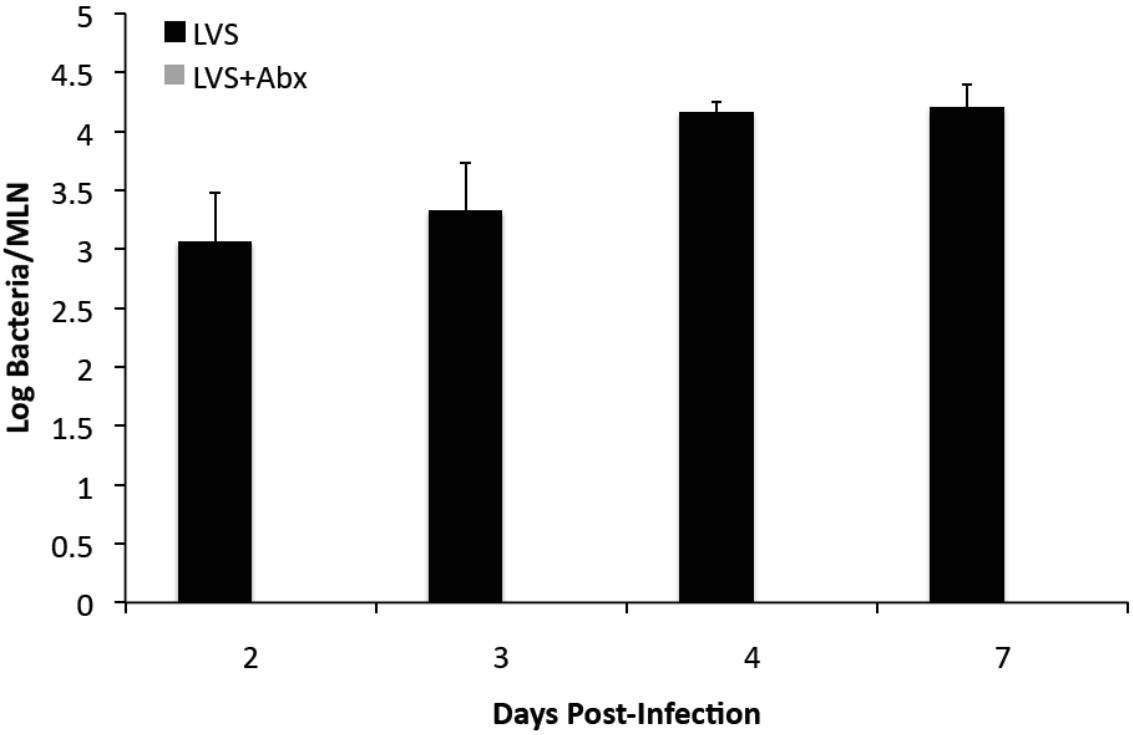

LVS
LVS+Abx
Log Bacteria/MLN
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
2
3
4
7
Days Post-Infection

Figure 3-2. One week of exposure to bacterial antigens leads to protective immunity. C57BL/6 mice were orally infected with 5 x 10^9 attenuated *S.* Typhimurium (BRD509). Some mice were treated with enrofloxacin in their drinking water beginning at various time points after infection. Five days after antibiotic withdrawal (six weeks post-infection), mice were rechallenged with 5 x 10^7 virulent *S.* Typhimurium. Data show the percentage of survival of infected mice.

Figure 3-2

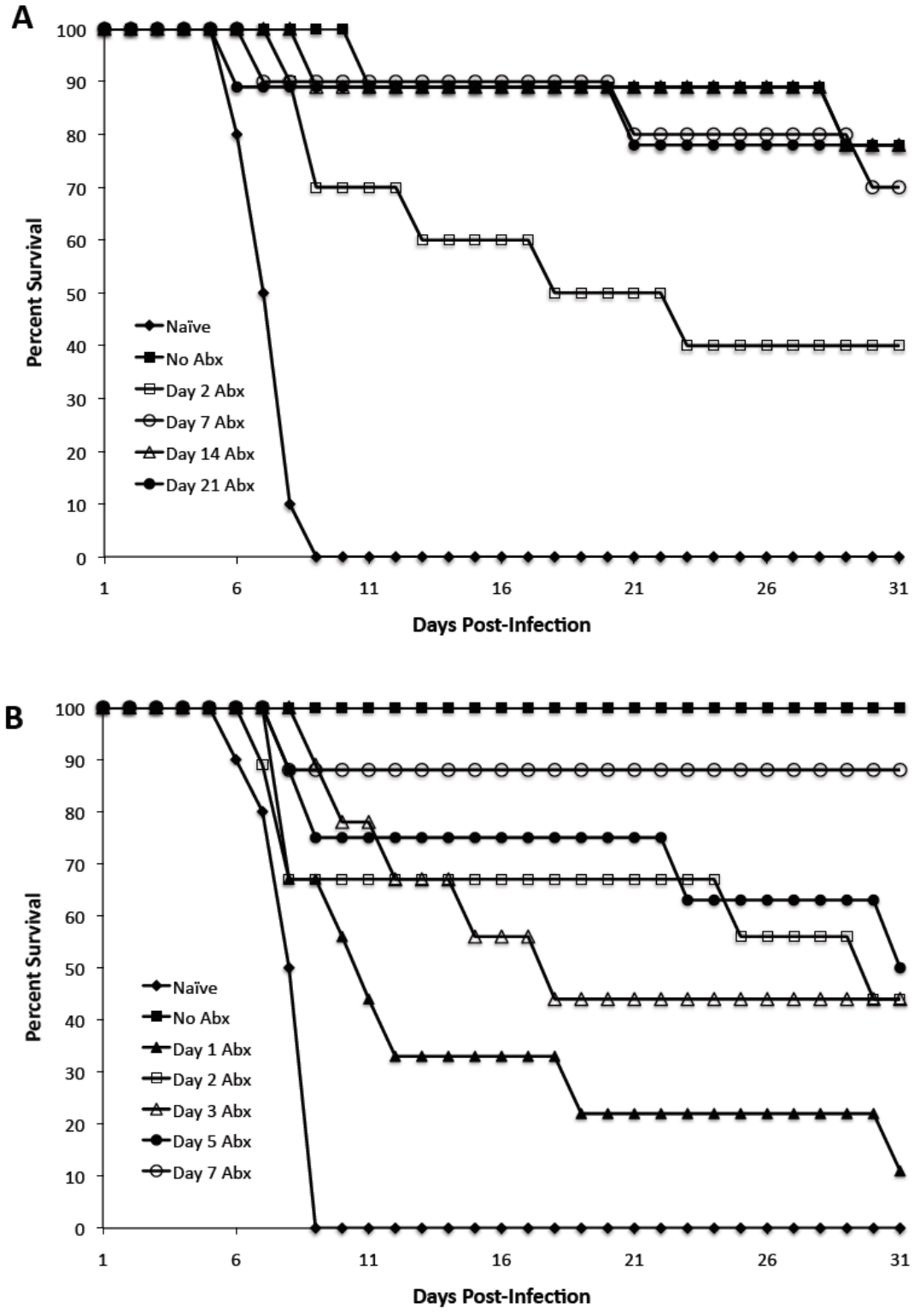

A
Percent Survival
Days Post-Infection
Naïve
No Abx
Day 2 Abx
Day 7 Abx
Day 14 Abx
Day 21 Abx
B
Percent Survival
Days Post-Infection
Naïve
No Abx
Day 1 Abx
Day 2 Abx
Day 3 Abx
Day 5 Abx
Day 7 Abx

Figure 3-3. CD4 T cells expand and become activated in the presence of antibiotics. C57BL/6 mice were adoptively transferred with 1 x 10^6 CFSE-stained CD90.1$^+$ SM1 T cells. Some of the mice were administered antibiotics in their drinking water. The following day, mice were injected i.v. with 200ug flagellin peptide 427-441. Three days after injection, MLNs and spleens were harvested, and SM1 T cells were identified by flow cytometry staining for CD4 and CD90.1. Plots show CD11a surface staining and CFSE dye dilution (*A*), and the percentage of SM1 T cells from antibiotic-treated and flagellin-injected mice (*B*).

Figure 3-3

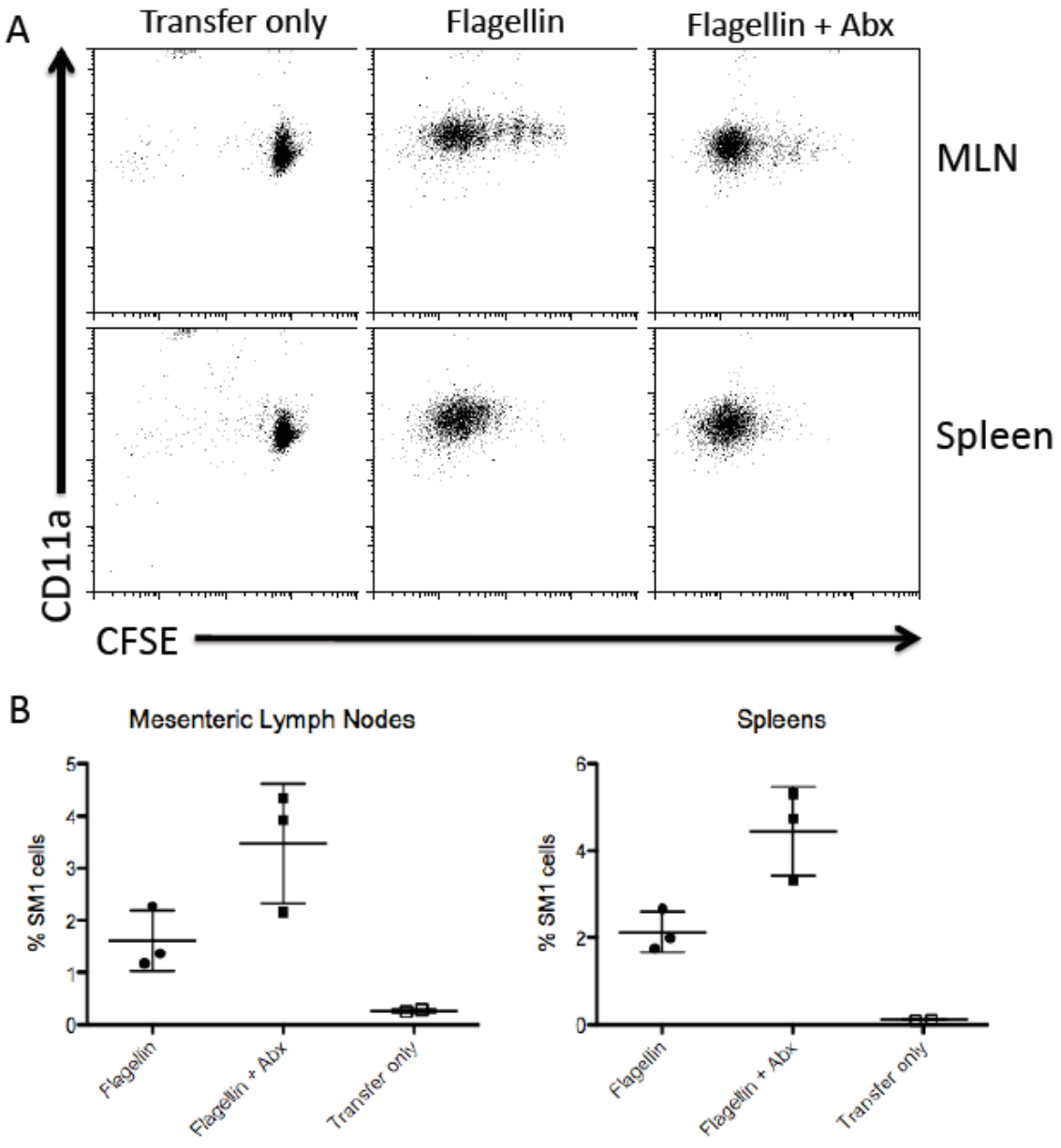

A
Transfer only
Flagellin
Flagellin + Abx
MLN
Spleen
CD11a
CFSE
B
Mesenteric Lymph Nodes
Spleens
% SM1 cells
Flagellin
Flagellin + Abx
Transfer only

Figure 3-4. Exposure to bacterial antigens correlates with development of *Salmonella*-specific Th1 development. C57BL/6 mice were orally infected with 5 x 10^9 attenuated *S.* Typhimurium (BRD509). Some mice were treated with enrofloxacin in their drinking water beginning at various time points after infection. Five days after antibiotic withdrawal (six weeks post-infection), CD4 Th1 cell responses in the spleen were examined by restimulation with *Salmonella* lysate and direct ex vivo detection of intracellular cytokine production. FACS plots show IFN-γ production by gated CD4 T cells in response to 6 h of stimulation with *Salmonella* lysate. Graphs show mean percentage of IFN-γ^+ CD4 T cells +/- SD (*left*) and mean fluorescence intensity +/- SD of IFN-γ-producing cells (*right*) from four mice per group.

Figure 3-4

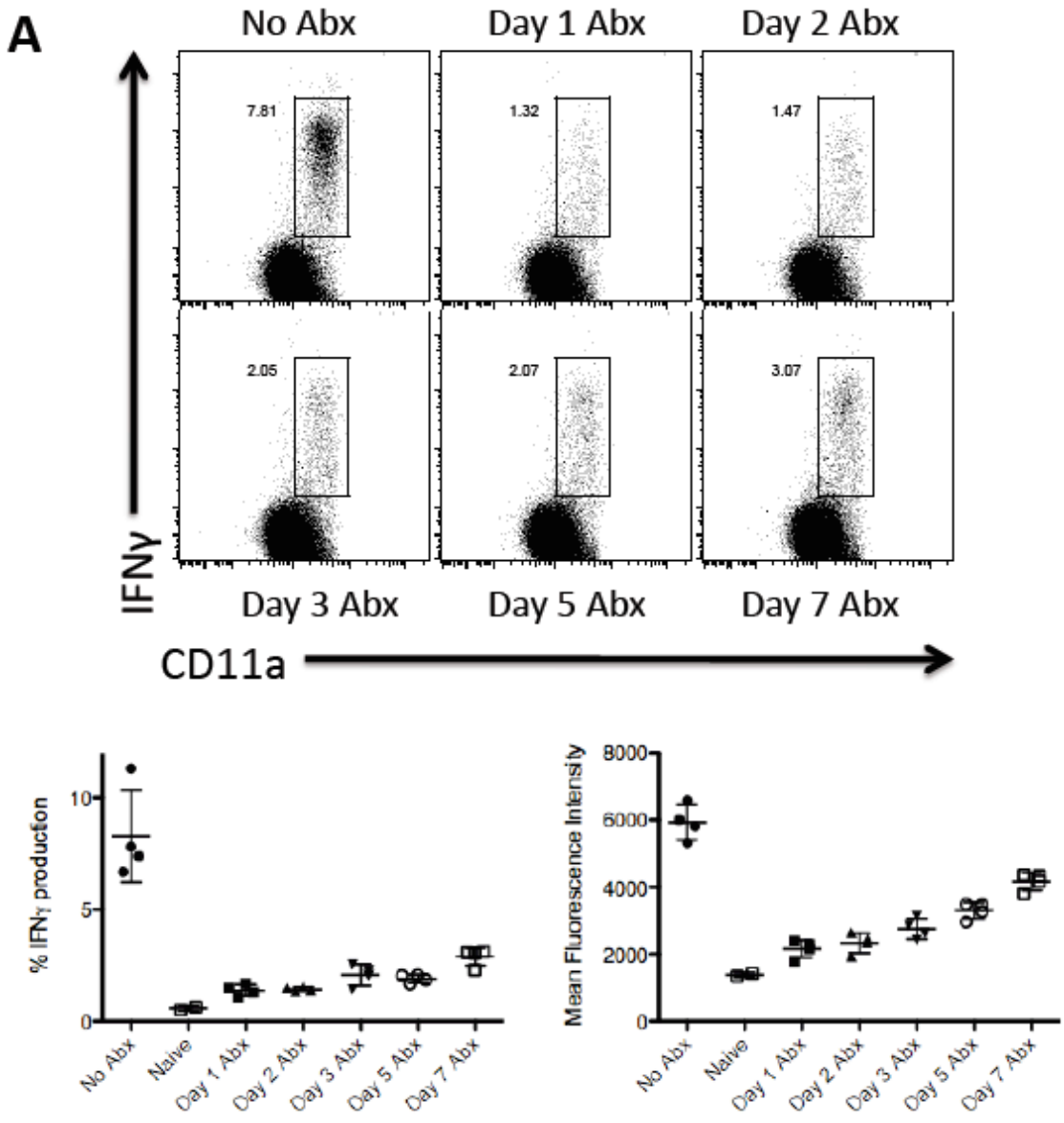

A
No Abx
Day 1 Abx
Day 2 Abx
7.81
1.32
1.47
2.05
2.07
3.07
IFNγ
Day 3 Abx
Day 5 Abx
Day 7 Abx
CD11a
% IFNγ production
10
5
0
No Abx
Naive
Day 1 Abx
Day 2 Abx
Day 3 Abx
Day 5 Abx
Day 7 Abx
Mean Fluorescence Intensity
8000
6000
4000
2000
0
No Abx
Naive
Day 1 Abx
Day 2 Abx
Day 3 Abx
Day 5 Abx
Day 7 Abx

Figure 3-4

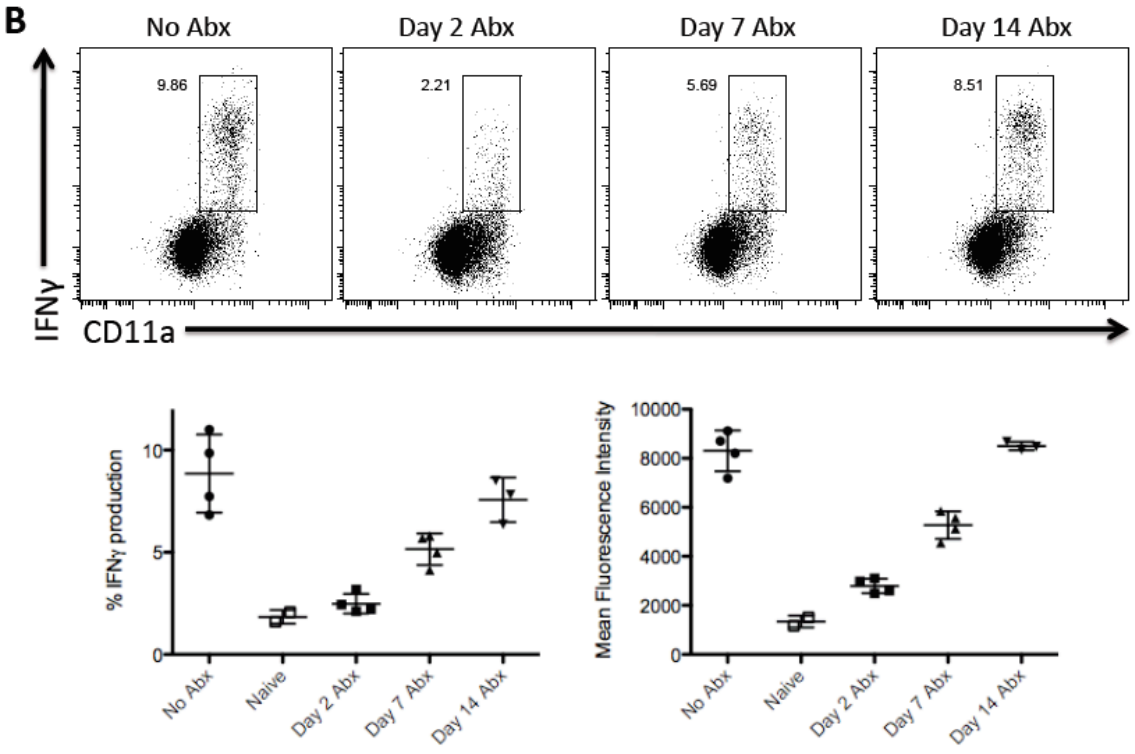
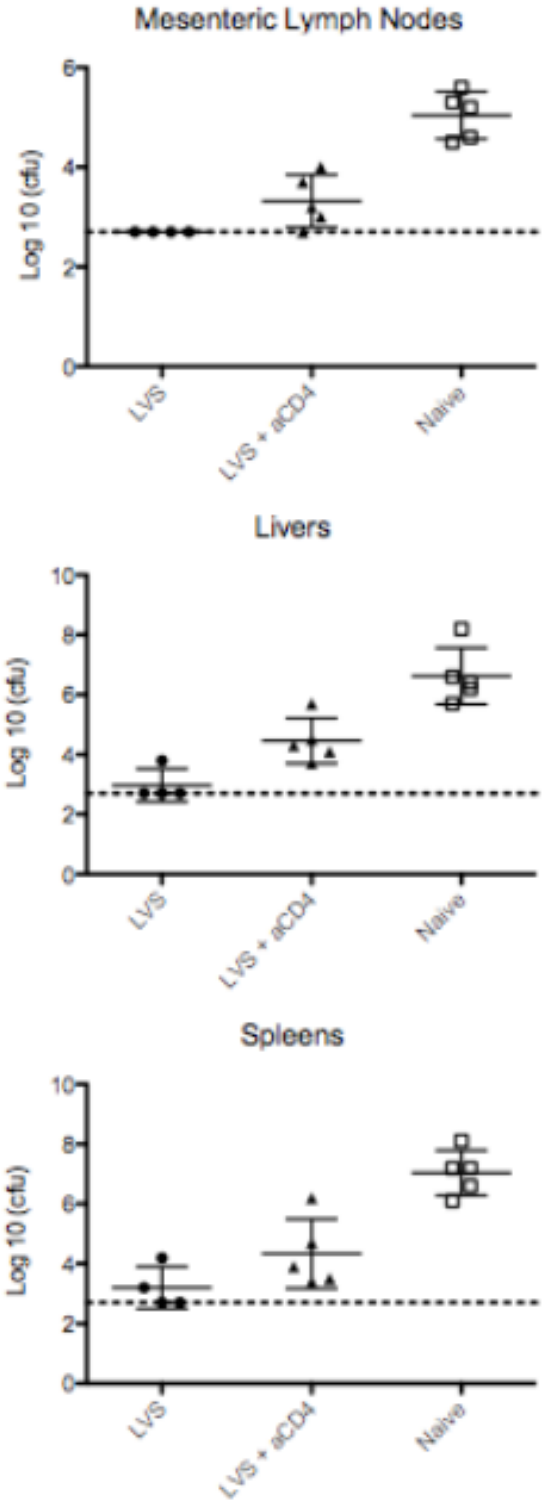

B
No Abx
Day 2 Abx
Day 7 Abx
Day 14 Abx
9.86
2.21
5.69
8.51
IFNγ
CD11a
% IFNγ production
Mean Fluorescence Intensity
No Abx
Naive
Day 2 Abx
Day 7 Abx
Day 14 Abx

Figure 3-5. Protective immunity is mediated by Th1 cells. C57BL/6 mice were orally infected with 5 x 10^9 attenuated *S.* Typhimurium (BRD509). Six weeks post-infection, mice were injected with 500ug anti-CD4 depleting or isotype control antibody and then rechallenged with 5 x 10^7 virulent *S.* Typhimurium the following day. Mice were administered a second dose of antibody three days post-infection. On day six following rechallenge, MLNs, livers, and spleens were harvested from infected mice, and bacterial loads were determined by plating organ homogenates on MacConkey's agar. Data show mean bacterial load +/- SD for three to five mice per time point.

Figure 3-5


Mesenteric Lymph Nodes
Log 10 (cfu)
0
2
4
6
LVS
LVS + αCD4
Naive
Livers
Log 10 (cfu)
0
2
4
6
8
10
LVS
LVS + αCD4
Naive
Spleens
Log 10 (cfu)
0
2
4
6
8
10
LVS
LVS + αCD4
Naive

Chapter 4

Dissemination of persistent intestinal bacteria via the mesenteric lymph nodes causes typhoid relapse

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Enteric pathogens can cause relapsing infections in a proportion of treated patients but greater understanding of this phenomenon is hindered by the lack of appropriate animal models. We report here, a robust model of relapsing primary typhoid that initiates following apparently successful antibiotic treatment of susceptible mice. Four days of enrofloxacin treatment was sufficient to reduce bacterial loads below detectable levels in all major organs and mice appeared otherwise healthy. However, any interruption of further antibiotic therapy allowed renewed fecal shedding to occur, renewed bacterial growth in systemic tissues, and mice eventually succumbed to relapsing infection. In vivo imaging of luminescent *Salmonella* identified the mesenteric lymph nodes (MLNs) as the major reservoir of relapsing infection. A magnetic-bead enrichment strategy isolated MLN $CD11b^{+}Gr\text{-}1^{-}$ resident monocytes that were associated with low numbers of persistent *Salmonella*. However, the removal of MLNs increased the severity of typhoid relapse demonstrating that this organ serves as a protective filter to restrain dissemination of bacteria during antibiotic therapy. Together, these data describe a robust animal model of typhoid relapse and identify an important intestinal phagocyte subset involved in protection against systemic spread of enteric infection.

Introduction

Salmonella contamination of food or fresh produce is responsible for several recent outbreaks of gastroenteritis in the US[155-157]. Aside from the health problems experienced by affected individuals, these outbreaks cause economic hardship for food manufacturers and erode public confidence in the safety of the US food supply[131, 158]. Certain *Salmonella* serovars can also cause a systemic infection of the reticuloendothelial system, resulting in a disease known as typhoid or paratyphoid fever[159]. As with other enteric diseases, typhoid is typically transmitted via the fecal-oral route and is concentrated in communities without access to clean water and/or basic sanitation[149, 160]. At present, one in six individuals (1.1 billion people) has no access to clean water and 40% of the world's population (2.6 billion people) lack primitive sanitary facilities[150]. The cost of improving this infrastructure is prohibitive and these figures are predicted to increase by the year 2025 (to 2.9 billion and 4.2 billion respectively)[150]. The most recent estimates indicate that typhoid and paratyphoid fever affect approximately 27.1 million people and cause 217,000 deaths annually, with most of these cases localized to south, and southeast, Asia[125]. Therefore, *Salmonella* infections are a health concern in developing nations lacking basic societal infrastructure, but are also an important cause of gastrointestinal infection in developed nations where contaminated food and produce are rapidly and widely distributed.

Salmonella can cause a chronic carrier condition in a proportion of typhoid patients or exposed asymptomatic individuals[126, 161]. This carrier state is associated with persistent infection of the gall bladder and the shedding of bacteria in stools over a prolonged period of time[126, 161]. A recent report suggests that such chronic carriage is associated with bacterial biofilm formation on gallstones during the initial exposure to *Salmonella*[162]. Non-human primate studies indicate that *Salmonella* can also persist

within the mesenteric lymph nodes (MLNs) of infected animals[163]. Indeed, persistent *Salmonella* infection has been widely studied using inbred mice that survive primary infection with *Salmonella* due to expression of the wild type allele of the Slc11a1 gene (formerly known as Nramp-1)[161]. Experiments using this resistant mouse model confirm that *Salmonella* persist in the gallbladder[162], and within F4/80$^+$, MOMA-2$^+$ hemophagocytic macrophages in the MLNs of infected mice[142, 164].

A related but distinct feature of human typhoid is the relapse of primary infection, which is observed in 5-15% of patients after apparent resolution of disease[126, 165]. These clinical relapses can occur in untreated typhoid patients, but are more commonly observed after apparently successful antibiotic treatment of primary infection[166-169]. It remains unclear why relapsing typhoid occurs in some patients, or if there are therapeutic strategies that could prevent recurrence of primary enteric infection. Indeed, it is not known how *Salmonella* are able to evade killing during antibiotic therapy, or even where the persistent bacteria are located during treatment. Furthermore, given the rapid induction of an adaptive immune response to oral *Salmonella* infection[98], it is perplexing that relapse of systemic infection can occur at all. Although the resistant mouse model of typhoid has allowed careful study of long-term *Salmonella* carriage[142, 164, 170, 171], there are currently no good animal models of relapsing infection. Thus, the dynamics of bacterial growth, the location of persistent bacteria, the virulence factors involved in this process, as well as the nature of the host immune response during relapsing disease, have not yet been examined.

Inbred strains of mice expressing a mutant allele of Slc11a1 are extremely susceptible to *Salmonella* and rapidly succumb to overwhelming infection before any examination of bacterial persistence and/or relapse is possible[161]. Although the susceptible mouse model is often used to examine adaptive immunity to *Salmonella*,

such studies typically use *Salmonella* strains of significantly reduced virulence[86, 103]. As an alternative to examining immunity to attenuated bacteria in susceptible mice, we recently reported an antibiotic treatment model that allows examination of the immune response to virulent bacteria in susceptible mice[129]. This model may be more relevant for understanding the process of naturally acquired immunity to *Salmonella* in endemic areas since it allows examination of immunity to bacteria that are fully virulent. However, during these studies, it was noted that resolution of primary typhoid required an extremely long period of antibiotic compliance[129].

Here, we report the relapse of primary typhoid in antibiotic-treated susceptible mice and suggest that this model may be useful for understanding relapsing clinical typhoid in humans[166-169]. Relapse of primary typhoid occurred in all antibiotic-treated mice, despite the apparent clearance of bacteria from systemic tissues using conventional laboratory detection methods. Live *in vivo* imaging of luminescent *Salmonella* highlighted the MLNs as the major source of relapsing primary infection. Indeed, individual MLNs maintained a low number of persistent *Salmonella* throughout the period of antibiotic treatment as detected by a sensitive culture methodology. Persistent bacteria were associated with a relatively rare population of $CD11b^{+}Gr\text{-}1^{-}$ MLN resident monocytes. Removal of MLNs greatly increased susceptibility to relapsing typhoid, demonstrating that this lymphoid organ actually functions to prevent the spread of systemic bacteria during antibiotic treatment. Together, these data describe a robust animal model for examination of relapsing typhoid and characterize an important source of relapsing enteric infection during antibiotic therapy.

Materials and Methods

Ethics Statement

All mice were cared for in accordance with University of Minnesota Research Animal Resource guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota.

Mouse strains.

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and NCI (Frederick, MD) and used at 6-12 weeks of age. All mice were housed in specific pathogen-free conditions and cared for in accordance with Research Animal Resources (RAR) practices at the University of Minnesota.

Salmonella infection and antibiotic treatment.

S. typhimurium strains BRD509 ($AroA^-AroD^-$) and SL1344 were grown overnight in Luria-Bertani broth without shaking and diluted in PBS after determining bacterial concentrations using a spectrophotometer. Mice were infected orally by gavage with $5x10^9$ bacteria, immediately following administration of 100ul of a 5% $NaHCO_3$ solution. In all infection experiments, the actual bacterial dose was confirmed by plating serial dilutions onto MacConkey's agar plates and incubating overnight at 37°C. Mice infected with SL1344 were treated with Enrofloxacin (Baytril) at 2mg/ml in drinking water, beginning 2 days post-infection. When moribund (displaying no movement when gently prodded with an index finger), mice were euthanized by cervical dislocation as stipulated by our animal care protocol.

Bacterial colonization in vivo – plating method

Mice were infected orally by gavage with $5x10^9$ cfu SL1344 and treated with antibiotics for one week, beginning 2 days post-infection. The following organs were removed and homogenized in Eagle's Hanks amino acids (EHAA; Gibco) containing 2% FBS: Peyer's patches, mesenteric lymph nodes (MLN), spleen, liver, bone marrow, and gall bladder. Serial dilutions of these tissues were plated on MacConkey's agar and incubated overnight at 37°C. Bacterial counts were calculated for each organ. In some experiments, mice were treated with antibiotics for one, two, or three weeks. Once mice resumed shedding *Salmonella* in their feces, as determined by plating fecal pellets homogenized in PBS on MacConkey's agar, spleens and livers were homogenized and plated as described above.

Bacterial colonization in vivo – culture method

Mice were infected orally by gavage with $5x10^9$ cfu SL1344 and treated with antibiotics for one week. The following organs were removed, homogenized in 0.05% Triton X PBS, serially diluted in Luria-Bertani broth, and incubated overnight at 37°C: spleen, liver, brain, kidney, stomach, small intestine, colon, bone marrow, and mesenteric lymph nodes. In some experiments, mesenteric lymph nodes were homogenized and cells stained for T cell, B cell, and phagocyte markers (CD3, B220, and Gr-1, respectively) and sorted using a FACS Aria. Populations were then serially diluted and incubated as described. To confirm *Salmonella* growth, 10ul from each culture tube were plated on MacConkey's agar and incubated overnight at 37°C. Bacterial counts were then calculated for each organ based on the last dilution where growth was detected.

Determination of fecal shedding

Mice were infected orally by gavage with $5x10^9$ cfu of either SL1344 or *S. typhimurium* strain x4700, which is deficient in lipopolysaccharide, and treated with antibiotics for one week. Fecal pellets were collected daily, beginning the day of antibiotic withdrawal, homogenized in PBS, plated on MacConkey's agar, and incubated overnight at 37°C. The growth of *Salmonella*/fecal shedding was recorded until mice became moribund, at which time the mice were euthanized and deaths recorded.

Live in vivo imaging

Mice were infected orally by gavage with $5x10^9$ cfu *S. typhimurium*-Xen26 (Caliper Life Sciences, Hopkinton, MA), which stably expresses the full *Photorhabdus luminescens lux* operon on the bacterial chromosome. Mice were anesthetized and imaged daily in a Xenogen IVIS Lumina (Caliper Life Sciences) until they became moribund, at which time they were euthanized. Some mice were treated with antibiotics, beginning two days post-infection, and imaged daily. In other groups, antibiotics were withdrawn after one week of treatment and mice were imaged until they became moribund. A third group of mice that had previously been immunized with BRD509 ($AroA^-AroD^-$) was infected with $5x10^9$ cfu *S. typhimurium*-Xen26 and imaged alongside the naïve and antibiotic-treated groups. One mouse per group was sacrificed each day and the following organs were imaged directly: liver, kidney, spleen, lungs, stomach, mesenteric lymph nodes, and intestines.

Magnetic bead column enrichment

Mice were infected with SL1344 and treated with antibiotics for one week. MLNs were extracted from 10 mice, pooled, and treated with collagenase D (Roche, Indianapolis, IN) for five minutes at room temperature with constant mashing. The single-cell suspension generated was washed and filtered before incubating with MACS beads (Miltenyi Biotech) conjugated to anti-CD11c, -CD11b, or -mPDCA-1 antibodies for 15 minutes at 4°C. In other experiments, cells were incubated with PE-conjugated Abs specific for CD103, Ly6C, or Gr-1 for 30 minutes on ice before incubation with PE-conjugated MACS beads. Cell suspensions were washed and passed through two subsequent MACS columns (both bound and unbound fractions were passed through two columns, and the bound fraction from the second column's unbound fraction was passed through another column). All bound fractions were combined and cells were counted using a hemocytometer. A small aliquot from each fraction was used for staining for flow cytometry to determine purity, which was 92-98% for CD11b, CD103, Ly6C, and Gr-1. Purity was 90% for CD11c and 78% for mPDCA-1. Cell fractions were then incubated in serial dilutions of Luria-Bertani broth overnight at 37°C and the following day, 10ul of culture from each tube was plated on MacConkey's agar and incubated overnight at 37°C.

Flow cytometry

A single-cell suspension was generated from harvested mouse MLNs and samples were incubated on ice in the dark for 30 minutes in FACS staining buffer (Hank's Balanced Salt Solution containing 2% FBS and 0.1% sodium azide) containing primary Abs. FITC-, PE-, PE-Cy5, or APC-conjugated Abs specific for CD3, B220, Gr-1, CD11b, CD11c, CD103, CD16/32, CD206, MHC II, CCR6, and CD115 were purchased from

eBiosciences and BD Biosciences. Purified CCR2 Ab was purchased from Abcam and conjugated to DyLight 488 (Thermo Scientific). mPDCA-1 Ab conjugated to APC was purchased from Miltenyi Biotech. CD64 Ab conjugated to FITC was purchased from R&D Systems. After staining, cells were analyzed by flow cytometry using a FACS Canto, and data were analyzed using FlowJo software (Tree Star). Cell sorting was performed using a FACS Aria. Sorted cell populations were more than 99% pure.

Surgical removal of MLNs

C57BL/6 mice were anaesthetized and the small intestine and cecum together with MLN were exteriorized through incision along the abdomen. Mesenteric lymphadenectomy was performed by microdissection along the length of the superior mesenteric artery to the aortic root[172]. After surgery the small intestine and cecum were reintroduced into the abdomen, the lesion of the abdominal wall was stitched with degradable thread and the outer skin sealed with wound clips. The animals were infected with *Salmonella* 6 to 8 weeks after surgery and compared to sham operated age matched controls.

Results

Relapse of *Salmonella* infection following antibiotic withdrawal

Ciprofloxacin is a fluoroquinolone antibiotic that is commonly used to treat human typhoid[140]. Enrofloxacin is a veterinary fluoroquinolone derivative that can resolve murine typhoid in highly susceptible C57BL/6 mice, if administered in drinking water for 35 days[129]. Such antibiotic-treated mice have been followed for over a year after the resolution of primary typhoid, during which time they displayed no discernable evidence of infection and did not resume excretion of bacteria in feces (data not shown).

In an attempt to determine the minimum time period of compliance for resolving murine typhoid, we examined shorter periods of antibiotic therapy. C57BL/6 mice were infected orally with virulent *Salmonella* (strain SL1344) and administered antibiotics in drinking water, beginning two days after oral challenge. All mice appeared to have resolved primary infection after only four days of antibiotic treatment, bacteria were no longer excreted in feces (data not shown), and *Salmonella* were undetectable by conventional plating of intestinal (Peyer's patch and MLN) or systemic tissues (spleen, liver, bone marrow, and gall bladder) (Fig. 4-1). However, despite this rapid response to antibiotic therapy, a full week of antibiotic treatment was insufficient to eradicate primary infection. If antibiotic therapy was halted after a week, mice resumed shedding *Salmonella* in feces 3-8 days later (Fig. 4-2A). Shortly after renewed fecal shedding was detected, these mice developed obvious symptoms of typhoid and eventually succumbed to relapsing infection (Fig. 4-2B). Lipopolysaccharide (LPS) expression is essential for *Salmonella* to adapt to an intracellular environment and persist within phagocytic cells[173]. Consistent with this role for LPS, LPS-deficient *Salmonella* were unable to induce relapsing typhoid after one week of antibiotic treatment (Fig. 4-2).

In order to examine whether a proportion of these mice had actually contracted a secondary infection from a small cohort of relapsing individuals, a group of *Salmonella*-infected and antibiotic-treated mice were placed in individual cages. All individually housed mice developed relapsing disease following one week of antibiotic treatment (data not shown). Therefore, although dissemination of bacteria via fecal shedding is likely to occur among co-housed animals, it does not explain the high incidence of typhoid relapse in this mouse model.

Next, we examined whether extending the length of antibiotic treatment would reduce the incidence of typhoid relapse in these infected mice. Groups of C57BL/6 mice were infected orally with virulent *Salmonella* and administered antibiotic drinking water for either, 1, 2, or 3 weeks. Mice in each of these treatment groups eventually developed relapsing primary typhoid as determined by renewed fecal shedding and bacterial growth in the spleen and liver after fecal shedding was detected (Fig. 4-3). As previously reported, antibiotic treatment for five weeks was required in order to fully resolve typhoid and prevent relapsing infection[129].

Mesenteric lymph nodes are the primary site of typhoid relapse

The source of this relapsing infection was perplexing since bacteria were undetectable in multiple tissues shortly following antibiotic treatment. In order to visualize bacterial colonization of multiple anatomical sites simultaneously, we decided to infect mice orally with virulent *Salmonella* containing a chromosomal copy of the *Photorhabdus luminescens lux* operon and used in vivo luminescent imaging to detect emitted photons in live anesthetized mice and among various harvested organs. In the first few days after oral infection, *Salmonella* were consistently detected in the intestines, Peyer's patches, mesenteric lymph nodes (MLN), and stomach of infected mice (Fig. 4-4, Infected).

Colonization of intestinal tissues was anticipated, as these are known sites of initial bacterial entry and initial replication[174]. However, detection of a prominent signal from the stomach was unexpected, especially as this persisted throughout the course of infection and therefore does not simply represent a signal from initial dose administered (Fig. 4-4, Infected).

Salmonella were occasionally detected in the cervical lymph nodes or lungs of infected mice at early stages of infection (Fig. 4-4, Infected day 2 lungs, and data not shown). Cervical lymph node infection most likely represents bacterial entry across an abrasion within the oral cavity or esophagus, perhaps caused by the gavage needle itself during the infection procedure. Similarly, very early detection of bacteria in the lungs likely indicates that a proportion of the gavage suspension inadvertently entered the airways during delivery. However, the frequency of signal detection in each of these anatomical locations was infrequent and therefore did not allow more detailed analysis. At later stages of infection, bacteria were frequently detected in the spleen, liver, and lungs of infected mice (Fig. 4-4, Infected), representing the transition of initial mucosal infection to systemic disease of the reticuloendothelial system.

In parallel, we examined bacterial colonization of immune mice that were previously vaccinated with an attenuated strain of *Salmonella*, thus conferring complete protection against secondary infection[129]. Although colonization of the spleen and liver was transiently detected in these immune mice, a low, but persistent, signal from the MLN was consistently detected in and this particular tissue was typically the last to be cleared (Fig. 4-4, Immune). Imaging of antibiotic-treated mice largely confirmed our previous culture experiments and indicated that bacteria are undetectable within a few days of exposure to antibiotics (Fig. 4-4, Antibiotics). Interestingly, as noted during

imaging of immune mice, the final organ to display a detectable signal of infection were typically the MLNs (Fig. 4-4, Antibiotic-treated, day 4).

Next, we examined typhoid relapse in antibiotic-treated mice following antibiotic withdrawal. Although bacterial growth eventually progressed to include multiple systemic tissues, most notably the spleen, liver, and lungs (Fig. 4-5, day 12), the earliest detection of bacteria consistently occurred in the MLN and was noted at time points when signal was undetectable in any other organ (Fig. 4-5, day 5-9). Taken together, these data highlight MLNs as an important site of bacterial accumulation during typhoid relapse. The detection of bacteria in this lymphoid tissue might indicate long-term and low level bacterial persistence in the presence of antibiotics or suggest that small numbers of bacteria are consistently migrating to this tissue during antibiotic therapy.

Low numbers of bacteria are present in MLN of mice during antibiotic treatment

The in vivo imaging approach above allowed for simultaneous analysis of multiple infected tissues and an overview of important infection sites during relapsing typhoid. However, in vivo detection of bacteria using this methodology is relatively insensitive and is likely to underestimate low-level persistent infection in tissues. We therefore developed more sensitive culture methodology to confirm and extend our imaging analysis. Our experimental approach involved harvesting, homogenizing, and incubating serial dilutions of whole organs in overnight broth cultures in order to amplify low numbers of bacteria, followed by plating of these cultures on MacConkey agar to confirm the presence of *Salmonella*.

In an initial experiment, multiple tissues from two mice that had received antibiotic treatment for typhoid but had not yet resumed fecal shedding were examined.

No bacteria could be detected in the bone marrow, gall bladder, liver, brain, lungs, stomach or large intestine of these mice, but *Salmonella* were successfully cultured from MLNs of both mice, and the spleen, small intestine, and kidney of a single mouse (data not shown). In a second experiment, the spleen, MLNs, stomach, kidney, lungs and brain were chosen for further analysis. Again, *Salmonella* were cultured from the MLNs of all antibiotic-treated mice, and also in the spleen, lungs, and stomach of a minority of mice (Table 4-1, Experiment 1). Next, a larger study was completed, focusing only on the spleen and MLNs of antibiotic-treated mice. Consistent with previous experiments, *Salmonella* were cultured from the MLNs of all antibiotic treated mice examined and also found in a minority of whole spleen cultures (Table 4-1, Experiment 2). This sensitive culture methodology is therefore in broad agreement with in vivo imaging studies above, and demonstrates that the MLNs represent a major site of bacterial infection after antibiotic treatment of murine typhoid.

In the experiments above we examined mice that had halted antibiotic treatment two days previously. This approach was based on the assumption that detection of very low numbers of bacteria during the period of antibiotic treatment would be challenging. However, this assumption proved to be incorrect and whole organ broth cultures also allowed direct detection of *Salmonella* in the MLN of mice that were actively undergoing treatment with antibiotics for a period of one week. By plating out dilutions of organ homogenates in these experiments, MLNs from a single mouse were found to contain 10.9 +/- 4.3 (mean +/- SEM) bacteria following one week of apparently successful antibiotic therapy. In all subsequent experiments persistent bacterial colonization was examined in mice during the period of active antibiotic treatment.

Persistent *Salmonella* are associated with MLN CD11b^{+}Gr-1^{-} resident monocytes

As *Salmonella* are facultative intracellular bacteria it was of some interest to determine whether persistent bacteria were cell-associated or extracellular in vivo. Therefore, MLNs from individual antibiotic-treated mice were homogenized and lightly centrifuged before pellets and supernatants from whole organs were cultured separately. From an examination of five individual mice, bacteria were predominantly associated with MLN pellets rather than supernatants (data not shown), indicating that persistent bacteria are unlikely to persist extracellularly in the MLNs.

Next, we attempted to identify the cell population that was associated with persistent MLN bacteria by using a *Salmonella* strain expressing GFP and detection of fluorescent cells. However, this approach was unsuccessful, because the low number of persistent bacteria falls below the frequency required for reliable detection by flow cytometry even when using pooled MLNs from multiple mice. As an alternative approach FACS cell sorting and/or magnetic-bead enrichment followed by broth culture of sorted or enriched cell populations was used to identify the location of persistent MLN bacteria. In order to narrow down the target cell population, we initially FACS sorted three cell prominent populations from MLNs of antibiotic-treated mice, B cells ($B220^+$), T cells ($CD3^+$), and phagocytes ($Gr\text{-}1^+$). Although *Salmonella* infect cells of the reticuloendothelial system, bacterial association with B and T cells has previously been reported[175]. However, this approach revealed that no bacteria could be cultured from any of these three FACS sorted populations, while *Salmonella* were readily detected in unsorted cells taken from the same infected mice (data not shown). Magnetic bead column enrichment is an extremely sensitive approach for examining low frequency cell populations and has recently been used to detect as few as 10-20 antigen-specific CD4 T cells from secondary lymphoid tissues of individual mice[176]. We therefore used this strategy to enrich MLN populations that could potentially contain persistent *Salmonella*.

Dendritic cells are known to harbor persistent organisms in other intracellular infection models[177, 178], and *Salmonella* have also been reported to associate with CD11c intermediate cells in the MLN during primary infection[179]. However, after enrichment of MLN $CD11c^+$ cells, *Salmonella* were cultured from the unbound (CD11c-), but not bound (CD11c+) fraction, indicating that bacteria do not persist within $CD11c^+$ dendritic cells during antibiotic treatment (Table 4-2). $CD11c^-$ plasmacytoid dendritic cells were also enriched from MLN of antibiotic-treated mice and similarly *Salmonella* were not found to be associated with this population (Table 4-2). $CD103^+$ cells in the MLN are thought to represent dendritic cells that have migrated from the intestinal lamina propria[180], and could therefore be infected in the intestinal tissue or MLN. However, after enrichment, *Salmonella* were again cultured from the unbound fraction, but were not detected in the enriched MLN $CD103^+$ population (Table 4-2). Together, these enrichment experiments suggest that persistent *Salmonella* in the MLN of antibiotic-treated mice are not associated with migrating or resident dendritic cell populations.

Studies using the Slc11a1 resistant mouse model have demonstrated that *Salmonella* are able to persist within $F4/80^+MOMA\text{-}2^+$ hemophagocytic macrophages in the MLNs of infected mice[142, 164]. Therefore, we examined the association of persistent *Salmonella* with monocyte/macrophage populations by enriching $CD11b^+$ cells from the MLN of antibiotic treated mice. Indeed, when $CD11b^+$ cells were enriched, *Salmonella* were detected in the column-bound fraction, and were either reduced or entirely depleted from the unbound fraction (Table 4-2). Thus, *Salmonella* persisting in the MLN during antibiotic treatment are primarily associated with $CD11b^+$ phagocytes. The majority of $CD11b^+$ cells in the MLN of *Salmonella*-infected mice express Ly6C or Ly6G[179, 181], and both of these molecules are detected by an antibody specific to Gr-1[182]. However, when $Gr\text{-}1^+$ cells were enriched from the MLN by magnetic beads, *Salmonella*

were not found within the Gr-1$^+$ fraction but were associated with the unbound fraction (Table 4-2). Indeed, this result confirms our previous FACS sorting experiments using Gr-1$^+$ MLN cells, which also failed to detect bacteria in this population. Together, these data demonstrate that the persistent bacteria causing relapsing typhoid are found associated with a rare population of CD11b$^+$Gr-1$^-$ phagocytes within MLNs of antibiotic-treated mice.

Surgical removal of MLNs increases the severity of typhoid relapse

Previous studies using the resistant mouse model of typhoid suggested that *Salmonella* can persist in the MLN[142, 164]. Such view is consistent with observations in our typhoid relapse model described above. However, the MLNs also serve as an important firewall, preventing the access of enteric floral bacteria to systemic tissues such as the spleen and can limit the systemic spread of Salmonella in acute Salmonella infections[183]. It was therefore possible that the low numbers of bacteria in the MLNs of antibiotic-treated mice actually represented the capture of *Salmonella* that were persistently transiting into this organ from other intestinal sites. In order to test this hypothesis directly, we examined typhoid relapse in mice where MLNs had been surgically removed. In mice lacking MLNs, much greater bacterial burdens were detected 7 days after antibiotic withdrawal, compared to mice with intact MLNs (Fig. 4-6). These data demonstrate that MLNs are not required for relapsing typhoid to occur and that this organ serves an important function in preventing the spread of systemic infection after cessation of antibiotic therapy.

Discussion

Persistent *Salmonella* infection is usually studied using resistant mice that express the wild type allele of Slc11a1[161]. These mice develop chronic infection of the gall bladder and/or MLNs[142, 162, 164], and are similar to a proportion of typhoid patients who develop chronic carriage of *Salmonella*[126, 161]. Importantly, these resistant mice do not develop relapsing clinical disease and appear otherwise healthy even while excreting virulent bacteria in fecal pellets[142, 162, 164].

Relapse of primary *Salmonella* infection is a common but distinct feature of human typhoid and involves the recurrence of clinical disease in patients who had previously resolved symptoms, usually following antibiotic treatment[126, 165]. Such recurrence of primary infection is difficult to study in the murine model, as resistant mice do not appear to develop relapsing clinical disease unless they are administered immunosuppressive therapy[142]. Our data demonstrate the presence of relapsing primary infection in antibiotic-treated susceptible mice and thus offer an alternative mouse model to examine an important relapsing enteric infection. This robust model should be amenable to dissection of the bacterial virulence factors that are associated with relapsing enteric infections, and indeed our data already demonstrate that LPS expression is required. In addition, this model should allow for in depth analysis of the innate and adaptive immune response during relapsing infection. Our previously published data suggests that repeated stimulation of *Salmonella*-specific CD4 T cells is required in order to generate an effective Th1 response that can eradicate secondary infection[129]. Thus, relapsing primary infection may represent a failure in CD4 memory cell development resulting from abbreviated CD4 stimulation in antibiotic-treated mice and patients.

Our data clearly demonstrate that although Enrofloxicin treatment rapidly eliminates bacteria from infected tissues, a small population is detectable in the MLN and remains present even after several weeks of antibiotic treatment. This finding might indicate a unique function of the MLN itself, or suggest that bacteria inhabiting the MLN are somehow altered to resist antibiotic treatment. This latter possibility is unlikely, as bacteria recovered from the MLN retained normal sensitivity to fluoroquinolone treatment in vitro (data not shown), demonstrating that acquisition of an antibiotic resistant phenotype had not occurred. Furthermore, bacterial colonies recovered from the MLNs did not display any increased virulence when compared to the parental strain, SL1344 (data not shown). Furthermore, it seems unlikely that the MLN represents an unusual anatomical location where antibiotic concentrations would be particularly low, especially since the majority of *Salmonella* are rapidly cleared from this tissue and the remaining bacteria fall below the level of detection by conventional methods (Fig. 4-1). It remains possible that persisting bacteria are situated in an unusual anatomical niche within the MLN that encourages long-term residence in the presence of antibiotics. Indeed, previous studies in the resistant mouse model indicate that *Salmonella* persist within MLN hemophagocytic macrophages that have phagocytosed another infected cell[164]. Therefore, it is possible that this unusual intracellular location could confer survival advantages in the face of circulating antibiotics. However, our MLN explant studies above suggest an alternative model. The increased growth of relapsing systemic *Salmonella* in the absence of MLNs demonstrates that this organ is not essential for bacterial persistence during antibiotic treatment. Therefore, *Salmonella* most likely persist at another gastrointestinal site, which we could not detect even by sensitive culture methods. One potential site would be solitary intestinal lymphoid tissues (SILT), since these are poorly studied lymphoid cell clusters that are known to be infected with

Salmonella following oral infection[184]. Still such low level of persistence is more rapidly manifest in adenectomized mice, which lack the protective firewall of the MLNs to reduce systemic spread of bacteria after antibiotics have been removed. Therefore, we propose that the detection of *Salmonella* in MLN of chronically infected mice[142, 162, 164], and in our relapse model most likely reflects captured bacteria that are dynamically entering the host intestinal tissue while antibiotic therapy is ongoing.

Intestinal dendritic cells containing commensal bacteria are known to traffic to the MLN and this unique organ therefore represents an important inductive site for mucosal immune responses to enteric flora following penetration of the epithelial layer or after bacteria have been captured by lamina propria dendritic cells sampling the intestinal lumen[94, 172, 185]. The MLN usually functions as a firewall for such bacteria and surgical removal of the organ allows enteric bacteria access to systemic tissues[172]. The small number of *Salmonella* in the MLN of antibiotic-treated mice would therefore be consistent with low-level trafficking of *Salmonella* from infected epithelial cells or from the luminal bacteria. It seems likely that the intestinal source of persistent bacteria would have reduced exposure to systemic antibiotics and therefore the lumen would appear to be the most likely primary site of persistent infection. If our model is correct, previous detection of *Salmonella* within hemophagocytic macrophages in resistant mice also represents the capture of bacteria that have migrated to the MLN. The requirement for five weeks of antibiotic compliance in our model indicates that the primary site of intestinal bacteria is finally exhausted at this point and trafficking of *Salmonella* into the MLN subsides.

The detection of *Salmonella* associated with $CD11b^{+}Gr\text{-}1^{-}$ phagocytic cells but not dendritic cells was unexpected and interesting, especially as dendritic cells are

known to harbor other persistent pathogens. Blood monocytes are divided into inflammatory and resident populations expressing Gr-1^{+}CCR2^{+}CX$_3$CR1^{low} or Gr-1^{low}CCR2^{low}CX$_3$CR1^{+} [186, 187]. During *Salmonella* infection, a large number of inflammatory monocytes are recruited to the MLN to control initial bacterial replication[181]. The fact that low numbers of persistent *Salmonella* were found within Gr-1^{-}CD11b^{+} cells suggests that this is a resident monocyte population that was located within the MLN prior to infection. Alternatively, as Gr-1^{-} monocytes are known to migrate into inflamed tissues prior to the infiltration of Gr-1^{+} monocytes[188], this population may have migrated from the blood after initial infection. Either way, this is a relatively minor population within the MLN since the vast majority of CD11b^{+} cells express Gr-1 in the MLN and may represent a particular population of phagocyte that prevents systemic spread of bacteria from intestinal lymph drainage. Further characterization of this population should lead to greater understanding of *Salmonella* persistence and typhoid relapse and may suggest interventions that can eradicate remaining bacteria from antibiotic treated patients and prevent relapsing enteric disease.

Table 4-1. *Salmonella* is consistently found in mouse mesenteric lymph nodes following virulent infection and one week of antibiotic treatment. C57BL/6 mice were orally infected with $5x10^9$ cfu SL1344 and treated with enrofloxacin in their drinking water for 7 days. Two days after antibiotic withdrawal, organs were harvested, homogenized, and incubated in serially diluted broth cultures overnight at 37°C. The following day, 10ul of each culture was plated on MacConkey's agar and incubated overnight at 37°C. Data represent the number of mice who showed growth of *Salmonella* out of the total number examined.

Table 4-1.

Experiment 1	Undiluted	1:10	1:100	1:1000
Spleen	2/5	2/5	0/5	0/5
MLN	5/5	5/5	2/5	0/5
Stomach	1/5	1/5	1/5	1/5
Kidney	0/5	0/5	0/5	0/5
Lungs	2/5	1/5	1/5	0/5
Brain	0/5	0/5	0/5	0/5
Experiment 2	Undiluted	1:10	1:100	1:1000
Spleen	3/10	3/10	1/10	0/10
MLN	10/10	10/10	6/10	2/10

Table 4-2. *Salmonella* are harbored in CD11b^{+}Gr-1^{-} cells in mouse mesenteric lymph nodes during antibiotic treatment. C57BL/6 mice were orally infected with 5x10^{9} cfu SL1344 and treated with enrofloxacin in their drinking water for 7 days. Mesenteric lymph nodes were enriched for either CD11c, mPDCA-1, CD103, CD11b, or Gr-1 using magnetic bead separation. Bound and unbound fractions were incubated in serially diluted broth cultures overnight at 37°C. The following day, 10ul of each culture was plated on MacConkey's agar and incubated overnight at 37°C. Data show the number of times a particular fraction was positive for the growth of *Salmonella* out of the total number of times the experiment was performed.

Table 4-2.

	Unbound fraction	Bound fraction
CD11c	4/4	0/4
mPDCA-1	1/1	0/1
CD103	2/2	0/2
CD11b	1/2	2/2
Gr-1	3/3	0/3

Figure 4-1. *Salmonella* are undetectable by conventional plating methods in intestinal and systemic tissues shortly after the start of antibiotic treatment. C57BL/6 mice were orally infected with $5x10^9$ cfu virulent *S. typhimurium* (SL1344) and some mice treated with enrofloxacin in drinking water starting two days later. Organs were harvested from infected mice at the indicated time points after infection, and bacterial loads were determined by plating organ homogenates on MacConkey's agar. Data show mean bacterial load +/- SD for five mice per time point. (N.D. = no bacteria detected in antibiotic-treated mice.)

Figure 4-1.

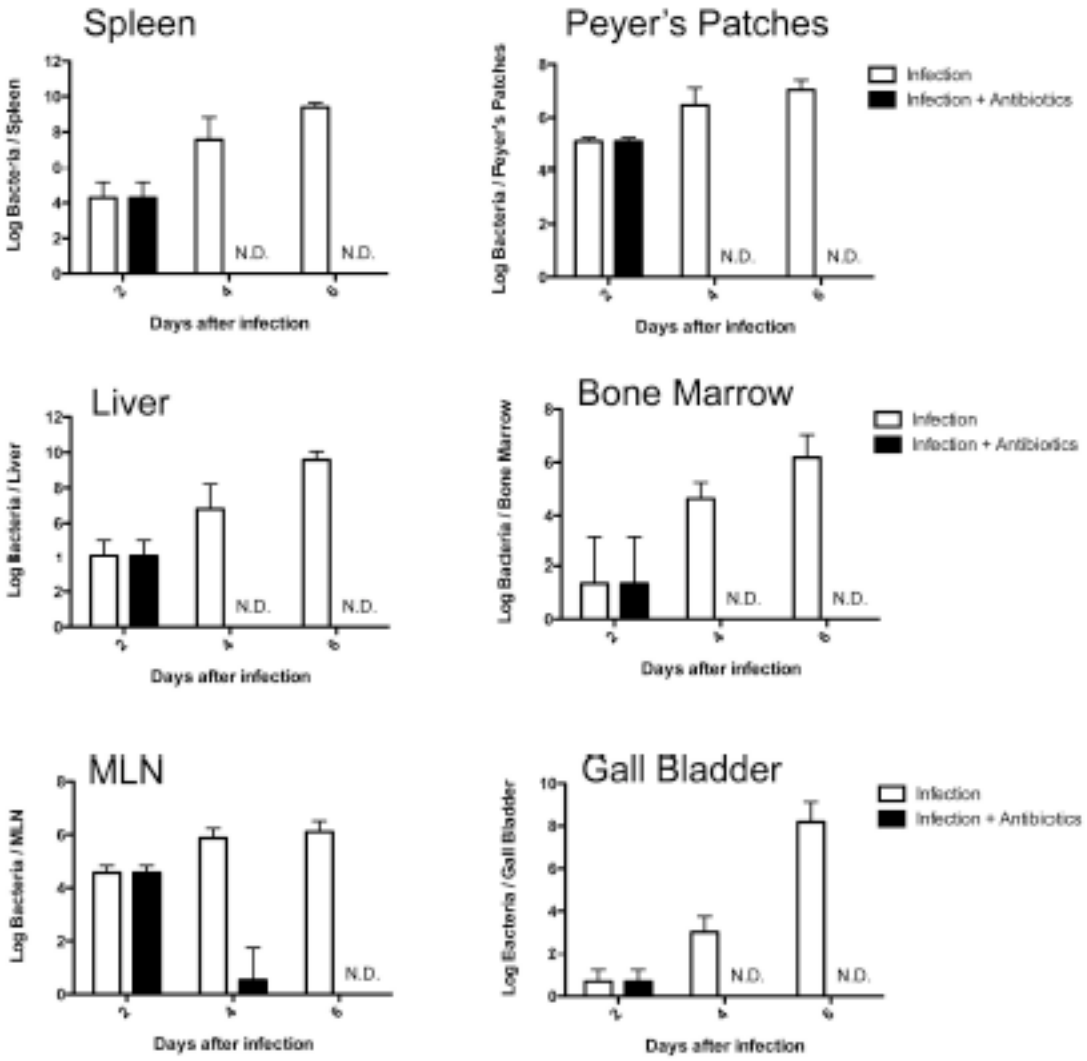

Spleen
Log Bacteria / Spleen
N.D.
N.D.
Days after infection
Peyer's Patches
Log Bacteria / Peyer's Patches
N.D.
N.D.
Days after infection
Infection
Infection + Antibiotics
Liver
Log Bacteria / Liver
N.D.
N.D.
Days after infection
Bone Marrow
Log Bacteria / Bone Marrow
N.D.
N.D.
Days after infection
Infection
Infection + Antibiotics
MLN
Log Bacteria / MLN
N.D.
Days after infection
Gall Bladder
Log Bacteria / Gall Bladder
N.D.
N.D.
Days after infection
Infection
Infection + Antibiotics

Figure 4-2. Relapsing typhoid is detected if antibiotic therapy is halted after one week. C57BL/6 mice were orally infected with $5x10^9$ cfu SL1344 or *S. typhimurium* strain x4700 and treated with enrofloxacin in their drinking water for 7 days. Beginning on the day of antibiotic withdrawal, *A,* fecal pellets were collected, homogenized in PBS, and plated on MacConkey's agar to determine shedding of *Salmonella* in stool. Data show percentage of mice shedding bacteria, and *B,* mice were monitored for signs of relapse and euthanized when moribund (data show percentage survival).

Figure 4-2.

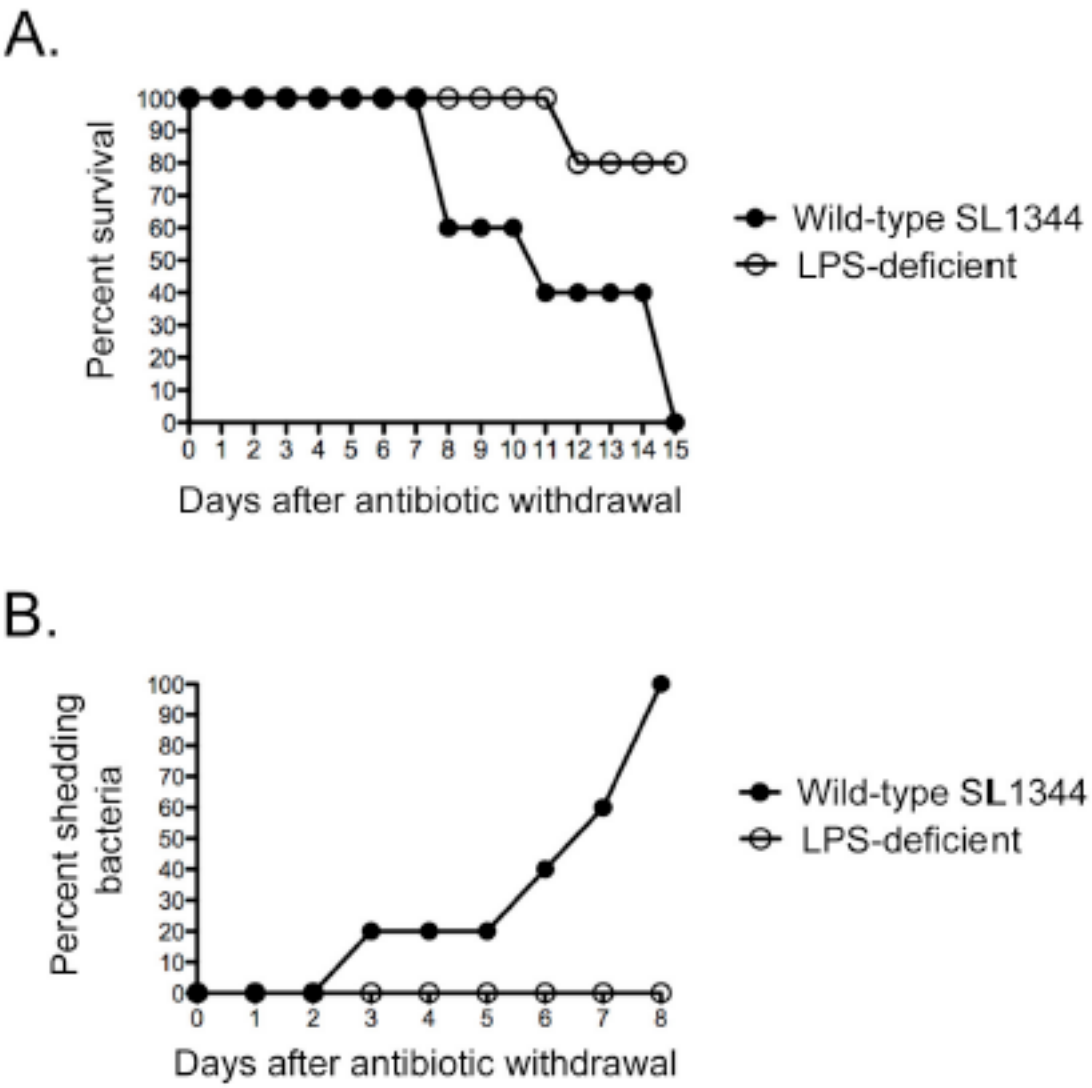

A.
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days after antibiotic withdrawal
Wild-type SL1344
LPS-deficient
B.
Percent shedding bacteria
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8
Days after antibiotic withdrawal
Wild-type SL1344
LPS-deficient

Figure 4-3. Relapsing typhoid still occurs in mice treated for up to three weeks with antibiotics. C57BL/6 mice were orally infected with $5x10^9$ cfu SL1344 and treated with enrofloxacin in their drinking water for *A,* 1 week, *B,* 2 weeks, or *C*, 3 weeks. Mice were then monitored for fecal shedding of *Salmonella*. When mice began shedding bacteria, they were euthanized and homogenates of spleens and livers plated on MacConkey's agar. Data show cfu *Salmonella* for individual mice.

Figure 4-3.

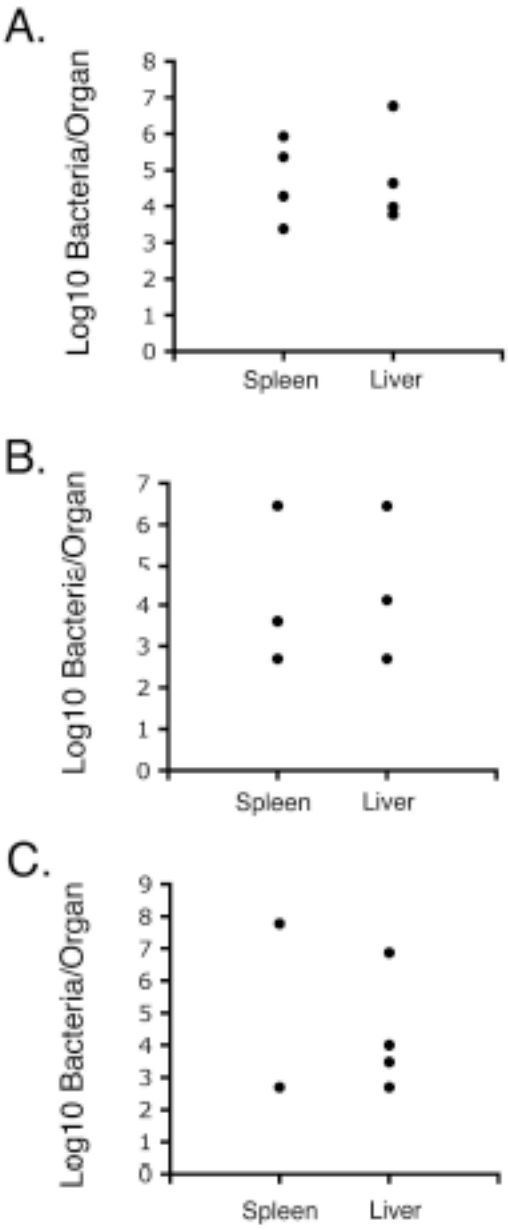

A.
Log10 Bacteria/Organ
0
1
2
3
4
5
6
7
8
Spleen
Liver
B.
Log10 Bacteria/Organ
0
1
2
3
4
6
7
Spleen
Liver
C.
Log10 Bacteria/Organ
0
1
2
3
4
5
6
7
8
9
Spleen
Liver

Figure 4-4. The mesenteric lymph nodes are a major site of bacterial colonization during antibiotic treatment. C57BL/6 mice were orally infected with $5x10^9$ cfu *S. typhimurium*-Xen26 (Infected) and some mice were treated with enrofloxacin in their drinking water beginning 2 days post-infection (Antibiotic-Treated). Immune mice were previously immunized with a live vaccine strain of *S. typhimurium* (BRD509) prior to infection with Xen26. Mice were anesthetized and imaged daily following infection and individual images of live mice 6 days post-infection are shown on the far right. One mouse per group was euthanized each day and organs imaged directly ex vivo. Each image, beginning in the upper left hand corner and moving counter-clockwise, shows the following organs: liver, spleen, kidneys, lungs, MLNs, stomach, and intestines.

Figure 4-4.

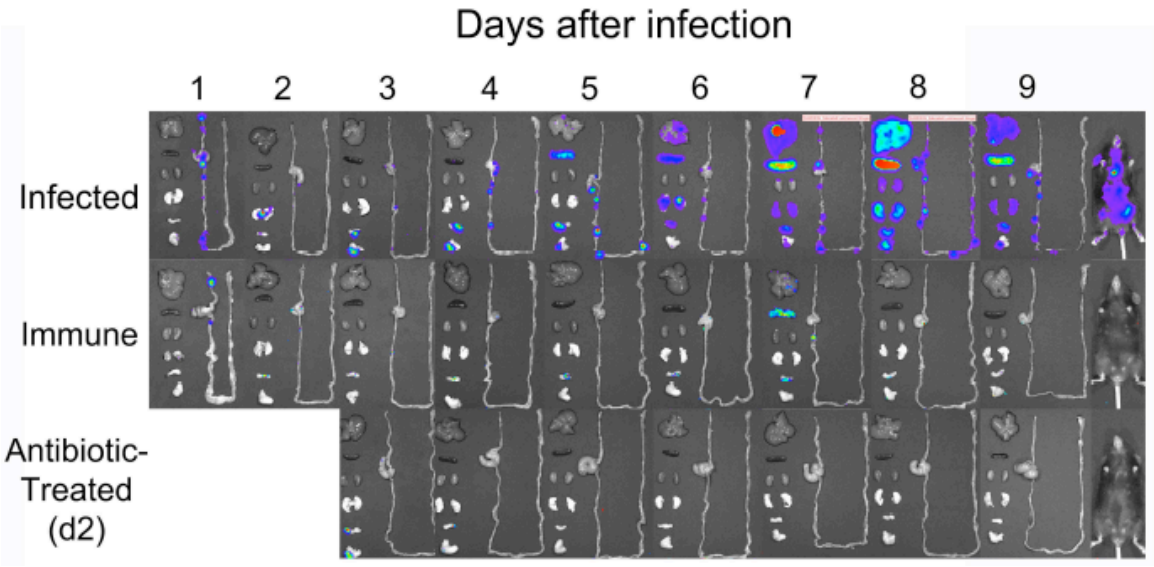

Days after infection
1
2
3
4
5
6
7
8
9
Infected
Immune
Antibiotic-Treated (d2)

Figure 4-5. Relapsing typhoid is initially detected in the mesenteric lymph nodes of infected mice. C57BL/6 mice were orally infected with $5x10^9$ cfu *S. typhimurium*-Xen26 and treated with enrofloxacin in their drinking water for 7 days. Organs were imaged daily, beginning 5 days after antibiotic withdrawal, as described in Figure 4. Organs shown are representative of three imaged mice per day.

Figure 4-5.

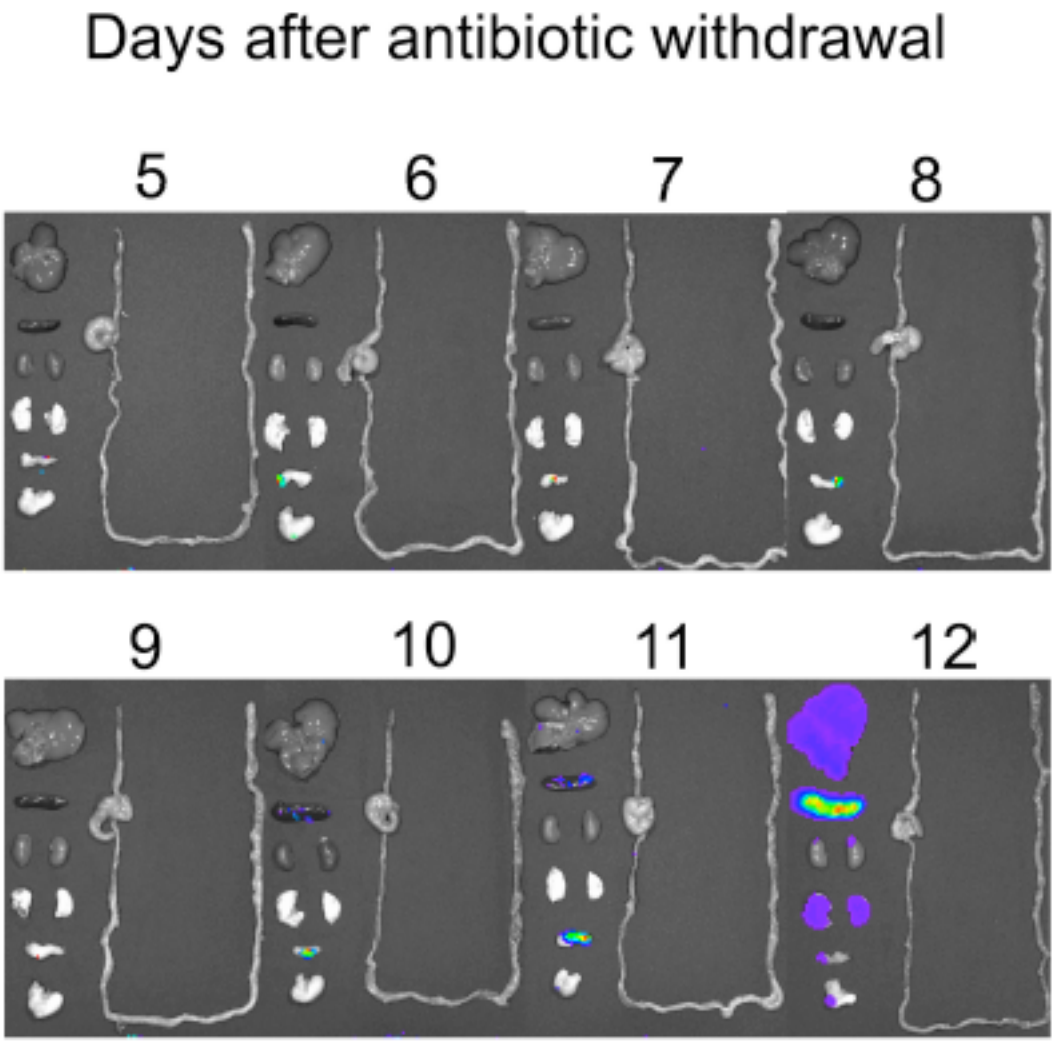
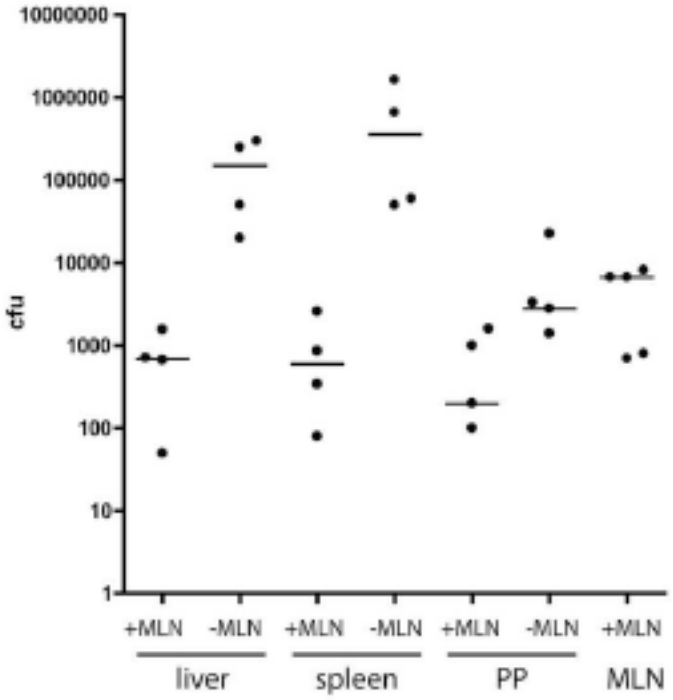


Figure 4-6. Mesenteric lymph nodes prevent dissemination of *Salmonella* during typhoid relapse. Mesenteric lymph nodes of C57BL/6 mice were surgically removed and mice orally infected with $5x10^9$ cfu SL1344 and treated with enrofloxacin in their drinking water for 7 days. After antibiotic withdrawal and fecal shedding of *Salmonella* resumed, mice were euthanized and organ homogenates plated to determine bacterial loads. Graphs show pooled data from three individual MLN explant experiments.

Figure 4-6.


10000000
1000000
100000
10000
1000
100
10
1
cfu
+MLN
-MLN
+MLN
-MLN
+MLN
-MLN
+MLN
liver
spleen
PP
MLN

Chapter 5

Concluding Remarks

In a PubMed search for "*Salmonella*," it appears that there are over 67,000 papers that have been published on *Salmonella* so far. Given the incidence of morbidity and mortality due to *Salmonella* infections, this number will continue to grow. Although great advances have been made in our understanding of immune responses to *Salmonella*, much more remains to be discovered.

One area that is wide open is the study of immune responses to *Salmonella* during and following antibiotic treatment. In areas of the world where infection with *S.* Typhi is common, individuals may recover from typhoid with the help of antibiotics. However, these individuals may suffer relapse of infection and/or become chronic carriers of the disease and shed the bacteria in their stool. In addition, typhoid patients that recover from the disease do not develop adequate protective immunity to future assaults with *Salmonella* and can be re-infected with the same or similar strains.

Vaccination is the most effective method of preventing infectious diseases, and those who are vaccinated with LVS *Salmonella* develop robust protective immunity. Although the immune response to LVS *Salmonella* has been well characterized and very efficient, available vaccines may only be administered to individuals with competent immune systems. In other words, those who are likely to suffer the most from infection with *Salmonella* cannot be vaccinated. As such, research on the immune response during infection and antibiotic treatment is warranted.

This thesis provided insight into the mechanisms of protective immunity that are important during virulent *Salmonella* infection and antibiotic treatment of mice. We first developed a murine model of naturally acquired immunity to *Salmonella* that closely mirrors human typhoid infection, by infecting susceptible mice with virulent *S.* Typhimurium and treating them with antibiotics. We concluded from these studies that the weak protective immunity generated in these mice was due to Th1 and antibody

responses. We also found that adaptive immunity could be boosted by injecting the mice with flagellin protein, a TLR5 agonist. Interestingly, we were unable to repeat these results using other adjuvants, such as CpG DNA (a TLR9 agonist) and Pam3CSK4 (a TLR1/2 agonist). These data suggest that it was processed and presented flagellin epitopes that provided the immunostimulus to augment protective immunity in these experiments, not the whole protein. It is also possible that flagellin was able to act as both an antigen and an adjuvant in these experiments. This could be easily tested by injecting the mice with flagellin peptide instead of whole protein.

In addition to studying the effects of antibiotics in a virulent model of murine typhoid, we extended our application of this methodology to learn about the development of Th1 responses, which are known to mediate strong protective immunity following infection with LVS *Salmonella*. We found that by treating LVS *Salmonella*-infected mice with antibiotics, we could modify the amount of time that CD4 T cells were exposed to *Salmonella* antigens. This, in turn, determined the level of effector/memory function that these cells would develop. We found that the longer we delayed the administration of antibiotics, the more robust the production of IFN-γ by Th1 cells following *in vivo* recall. It was necessary to delay antibiotic treatment for at least one week in order for the mice to generate protective immunity to rechallenge with virulent *Salmonella*. However, for full development of effector/memory function of Th1 cells, antibiotics had to be delayed for two weeks. It is likely that mice that were treated with antibiotics beginning one week post-infection did not succumb to secondary infection because, although their Th1 cells were not fully functional, they had sufficient antibody for defense against rechallenge. In order to determine the protective capacity of the Th1 cells alone, it might be interesting to transfer these cells into naïve recipients before primary infection and compare this to

the transfer both serum and Th1 cells. It is likely that the Th1 cells play a role, but that antibody is also important.

Still another application for antibiotic treatment was found in examining mice who appeared to have cleared virulent infection, but who died of relapsing typhoid following withdrawal of antibiotics before 35 days. We found that the source of relapsing infection was intracellular *Salmonella* that were harbored in a $CD11b^{+}Gr1^{-}$ resident monocyte population in the MLNs. Furthermore, the MLNs seemed to act as a filter for these cells that prevented systemic spread of infection, as removal of this organ resulted in increased susceptibility to relapsing typhoid. More in depth analyses of the $CD11b^{+}Gr1$- cells will provide information on their characteristics and functions. The identification of a novel cell surface marker that could be targeted by a specific antibody could function as a therapeutic agent to be administered during antibiotic treatment. Such methods could be applied to human infection, particularly in individuals who develop chronic carriage of *Salmonella* following antibiotic treatment. Alternatively, antibiotics could be developed that target this particular cell type. New knowledge pertaining to these issues will be instrumental in the development of therapeutic vaccines.

Utilizing antibiotic treatment of *Salmonella* in a mouse model, the data presented in this thesis deepen our understanding of immune responses during this infectious disease. Our model elucidates a prime example of cellular immunity mediated by Th1 cells, which is dependent on antigen exposure, and which results in relapsing infection if not cleared with sufficient treatment with antibiotics. The use of antibiotics has the potential to improve our understanding of other infectious diseases as well. For example, antibiotics are being employed in murine models of *Francisella tularensis* in order to further our understanding of immune responses to this pathogen[189].

It is plausible that the knowledge gained in these and other models will improve strategies of disease control using antibiotics that may enhance protective immunity following secondary exposure. In addition, antibiotic treatment regimens may be altered in order to prevent chronic carrier states, which sometimes occur during treatment of humans suffering from *Salmonella* infection[166-169]. Finally, this work may provide the foundation for future studies in the development of effective vaccines that protect humans against various infections. In summary, we have shown that antibiotic treatment can be an invaluable tool when studying immune responses to infection, and these strategies may be applied to other systems in order to broaden our understanding of a multitude of infectious diseases.

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