Protective B cell Responses During *Salmonella* Infection

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Dedication

This dissertation is dedicated to my parents, Keithroy A. S. Nanton and Romelia C. Nanton, for sacrificing everything so that I may have the chance to dream big.

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Introduction

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Salmonella Overview

The *Salmonella enterica* species of bacteria are a major cause of morbidity and mortality worldwide. They are gram-negative, facultative intracellular anaerobes that cause a wide range of disease in many animal hosts. Specifically, *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi (*S.* Typhi and *S.* Paratyphi, respectively) are restricted to human hosts and are the causative agents of enteric fever. Other *Salmonella enterica* serovars such as *S.* Typhimurium, cause disease in a broad host range from humans to birds, cattle and rodents. In humans, *S.* Typhimurium is mainly known to cause a self-limiting gastroenteritis in developed countries such as the United States, while a non-typhoidal bacteremia is prevalent in developing regions such as sub-Saharan Africa (1), (2). *S.* Typhi alone causes a staggering 21 million cases of typhoid and an under-estimated 200,000 deaths per year worldwide (3). In light of this burden of disease, it is important to understand its pathogenesis and examine the most effective ways to prevent it.

Enteric Fever

Typhoid fever is transmitted via the fecal-oral route, usually in conditions of poor sanitation, lack of access to clean drinking water and contaminated food. Following ingestion of *S.* Typhi, there is an incubation period of about 7-14 days, after which fever becomes apparent with the onset of bacteremia. In this early symptomatic phase, the fever may be low-grade and elevate to high-grade fever over a couple of weeks. Accompanying the fever are flu-like symptoms, dull headache, abdominal pain, muscle weakness, and dry cough. While children and the immune-compromised may have

diarrhea, adults generally experience constipation. Clinical signs include hepatomegaly and splenomegaly upon palpation of the abdomen. If left untreated, symptoms can progress rapidly, with the most severe cases resulting in GI bleeding, intestinal perforation, typhoid encephalopathy and death. Complications from typhoid are seen in 10-15% of cases (4, 5), and relapse of disease has been reported in 10-15% of patients two to three weeks after the initial fever has resolved. However, the relapsing disease is usually milder in severity. Reinfection with different strains of *S.* Typhi does occur, suggesting a lack of protective immunity. Additionally, up to 4% of patients can become carriers of *S.* Typhi (4). Enteric fevers caused by Paratyphi infections have similar symptoms and signs with less disease severity.

Treatment of infection is complicated by two factors. The first issue is that the symptoms listed above may be mistaken for many other febrile illnesses, namely malaria, which have vastly different treatment regimens. The definitive diagnostic test for typhoid is blood culture of the organism. The sensitivity of this test varies depending on when during illness the blood is drawn and how much blood is sampled (5-7). The gold standard of diagnosis, however, remains bone marrow culture, with sensitivity of these cultures reaching 30X that of culture from the same amount of blood (8). Of note, culture of bone marrow seemed to also correlate better with clinical indicators of disease such as white blood cell and platelet counts (negative correlation) and time to fever clearance after treatment (positive correlation) (8). Unfortunately, the logistics and technical laboratories needed for blood culture and especially bone marrow collection and culture are not available in many resource-poor settings. Thus, diagnosis using these methods is impractical and not widely used in regions where typhoid burden is highest (7).

The second issue lies in the growing emergence of antibiotic resistance among *S.* Typhi strains. The first line of antibiotics for treatment of enteric fever in the 1940s

was chloramphenicol. Antibiotic resistance began to emerge in the 1970s. Since the 1980s antibiotic resistance has been seen for all of the first line drugs including trimethoprim, sulfamethoxazole and ampicillin. Currently, fluoroquinolones and cephalosporins are used for treating enteric fever. Partial antibiotic resistance to fluoroquinolones has been seen in certain parts of Asia, complicating treatment . But, fully fluoroquinolones-resistant *S.* Typhi is still uncommon (1, 9-12).

While typhoid was prevalent in the U.S. and Northern Europe in the 1800s, improvement in sanitation has made enteric fever a rare occurrence in these regions. (1). Today, most of the disease burden falls on south and south-east Asia, sub-Saharan Africa and central and south America. In 2004 the World Health Organization Bulletin reported that *S. Typhi*, is responsible for 21,650,974 cases of typhoid and 216,510 deaths worldwide. People most affected range from infancy to adolescence, with children under 5 years of age group having the highest incidence of disease. Of note, these figures came from an analysis of 22 different studies from 13 countries that used blood culture to confirm cases, with data from an additional 4 regions that had national typhoid fever surveillance systems, spanning 1.8 person-years of typhoid surveillance. The sensitivity of blood culture was accounted for by adjusting the estimated incidence upward by a factor of 2. As no studies in this analysis were large enough to determine accurate case-fatality rates, the authors applied a 1% case-fatality rate to be conservative in their estimates. Importantly, data are absent for much of eastern, middle and western Africa, and extrapolation of burden in these regions came from Egypt (low incidence) and South Africa (high incidence) (3, 13). Taken together, these facts indicate that we do not have an accurate estimate of the burden of disease, especially the mortality rate, which is more than likely higher than the estimates reported in the latest WHO Bulletin.

Non-typhoid Salmonelloses

In contrast to enteric fever, gastroenteritis due to non-typhoid *Salmonella* infections is prevalent in all regions of the world resulting in an estimated 93.8 million cases and 155,000 deaths per year with over 80% of cases due to contaminated food (14, 15). However, the clinical picture in the developed regions is quite different than that in developing regions. In places such as the United States of America NTS cause a self-limiting gastroenteritis that is characterized by diarrhea, nausea and vomiting in immune-competent individuals. In such regions invasive bacteremia due to NTS can occur in up to 5% of individuals, and mainly in the immune-compromised (16).

In sub-Saharan Africa, NTS infections are a major cause of acute febrile illness and bacteremia in children and adults, with most cases attributed to *S. Typhimurium* and *S. enteritis's*. Clinical symptoms and signs include fever, lower respiratory tract infection and hepato-splenomegaly and diarrhea. NTS bacteremia in this region is more prevalent than typhoid fever, has a high correlation with the rainy season and is associated with a high case-fatality rate of 22-47%, even in treated individuals. Diagnosis and treatment of NTS bacteremia is plagued by many of the same issues of typhoid diagnosis- the symptoms and signs are non-specific leading to inappropriate treatment measures and blood culture is impractical and not systematically used in resource-poor settings (16-18).

Children 3 years old and younger are at increased risk of NTS bacteremia resulting in sepsis, pneumonia and meningitis. Several factors have been shown to correlate with NTS bacteremia in children including malnutrition, severe malaria infection, sickle cell anemia, HIV infection and intestinal schistosomiasis. In adults, the risk factor is mainly advanced HIV infection. In contrast, HIV and malaria infections do not correlate with an increased incidence of typhoid fever. Many strains that cause

infection are multi-drug resistant (to first line antibiotics), but for the most part remain sensitive to fluoroquinolones (16, 19-21). In many respects, at least in sub-Saharan Africa, NTS infections are far more prevalent and deadly than typhoid fever and increased resources must be dedicated to improved diagnostics, treatment and prevention.

II: Vaccines against Typhoid Fever

Paramount to improving morbidity and mortality due to *Salmonella* infections, are improved sanitation, access to clean drinking water and food safety in areas of endemicity. However, as current politics and capital are limiting factors to achieving these goals, cost-effective vaccines are still necessary to decrease the burden of disease (22). There are currently two commercially available vaccines against typhoid fever: the live attenuated Ty21a vaccine and the subunit Vi capsular polysaccharide vaccine (ViCPS). Notably, there are no commercially available vaccines specifically targeting *neither S. Paratyphi nor* NTS strains. Determining the efficacy of a vaccine is imperative for public health ministries and governments to set priorities for vaccination of their populations. In 2007, Fraser *et al*. published a meta-analysis to determine the efficacy of the Ty21a and ViCPS vaccines. A meta-analysis published by Engels *et al*. in 1998 included data on the efficacy and toxicity of the whole cell vaccines no longer on the market (23). Below, the results of the meta-analyses and specific clinical studies are described.

Killed Whole-Cell Vaccine

Killed whole-cell vaccines for typhoid were in use since the late 1800s, but efficacy studies were not published until the 1960s. The meta-analysis by Engels et al, included 10 studies in Yugoslavia, USSR, Poland, Guyana and Tonga published between 1962 and 1975. In these studies, the vaccine strain was killed by acetone, formal, heat or alcohol. The cumulative efficacy of a two-dose regimen was 73% for up to 5 years. This efficacy is greater than those of the Ty21a and ViCPS vaccines, as described in later sections. However, these killed whole cell vaccines posed risk in terms of toxicity, with nearly 16% of participants reporting fever (only 2% for Ty21a), and 20% reporting swelling at the injection site (only 3.7% for ViCPS). Furthermore, 10% of participants reported having to miss work or school, a parameter not examined in the Ty21a trials. Thus, although the efficacy of the whole-cell vaccines is greater than for Ty21a or the ViCPS and for longer duration, the toxicity has precluded its use in large-scale vaccination programs (23, 24).

Ty21a

Ty21a is a non-capsulated, attenuated chemically mutated strain of *S.* Typhi derived from the Ty2 strain. In the United States and Canada, the Ty21a vaccine is currently licensed as a four-dose oral regimen, with a booster dose suggested every 5 years. Other countries use a three-dose regimen with boosting every 1-3 years depending on whether one lives in an endemic or non-endemic area. This vaccine is only licensed for children 5 years and older, with some formulations targeting children as young as 2 years of age. This misses the key target population of infants and toddlers under the age of 2. Fraser et al. completed a meta-analysis of clinical trials to determine

the overall efficacy of Ty21a in liquid, enteric capsule and gel capsule formulations. Five studies conducted in Egypt, Chile and Indonesia were included in the meta-analysis.

One such clinical trial included in the meta-analysis was published by Black, et al in 1990. In a double-blinded study in Santiago, Chile in 1982, children ages 5-22 were enrolled to receive 1 dose, 2 doses or placebo. Education of the doctors and nurses in the area health centers and hospitals as well as parents was conducted to increase awareness of the signs and symptoms of typhoid, and therefore surveillance. Suspected cases of typhoid were confirmed by blood and bone marrow culture. Only confirmed cases were included in the efficacy results. Efficacy was determined as the number of study participants with confirmed cases of typhoid in the placebo versus one-dose and two-dose groups. In this particular study, the placebo group showed 227 cases per 100,000 after the first year, compared to 170 per 100,000 in the one-dose group and 109 per 100,000 in the two-dose group. This meant that the efficacy after one year of the two-dose regimen was 52% and the efficacy of the one-dose regimen was only 25%. Two years after vaccination, the efficacy was 59% for the two-dose regimen and 29% for the one-dose regimen. Three to five years following vaccination, there was no efficacy of the immunization. This study revealed that although Ty21a affords some limited protection in the first two years with only two doses, a more optimized dosing regimen was needed in order to make use of the vaccine feasible (25).

Further studies included in the meta-analysis by Fraser et al. evaluated the efficacy of a 3-dose regimen. The cumulative efficacy of a three-dose regimen of Ty21a over three years was 51%, including liquid formulations and enteric capsules. However, when the different formulations were examined separately, the enteric capsules had a seven-year efficacy of 62% and the liquid formulation had a five-year efficacy of 79% compared with a three-year efficacy of only 25% for gel capsules. Taken together, these

analyses showed that the Ty21a vaccine is moderately effective over 5-7 years (26). The challenge still remains that this vaccine is not approved for use in the under five-age group which, arguably, needs the vaccine the most.

ViCPS

The Vi capsular polysaccharide vaccine is the purified capsule of the Ty2 strain of *S.* Typhi. It is licensed for use in many countries as a single injected dose given. The meta-analysis by Fraser et al includes four studies on efficacy spanning South Africa, Nepal and China. Efficacy of a single dose was 68% after the first year but decreased to 50% by year three after vaccination (26). Unfortunately, although the ViCPS vaccine is safe, it stimulates an arm of the adaptive immune system that does not maintain immunological memory. This vaccine stimulates thymus-independent, short-lived antibody responses that do not maintain life-long immunity. Thus, the vaccine cannot be given to children under 2, as they make very poor responses to thymus-independent antigens such as capsule polysaccharides. Conjugation of the ViCPS to a protein antigen, as in other childhood vaccines, would offer T cell help to produce long-lived antibody responses. While adults make adequate antibodies in response to thymus-independent antigens, these antibodies are short-lived and it is recommended that people be re-vaccinated every two years.

Other Typhoid Vaccines

In 2008 the World Health Organization released a position paper on Typhoid Vaccines:

> "In view of the continued high burden of typhoid fever and increasing antibiotic resistance, and given the safety, efficacy,

> feasibility and affordability of 2 licensed vaccines (Vi and Ty21a), countries should consider the programmatic use of typhoid vaccines for controlling endemic disease." (27)

Additionally, the WHO acknowledged the gap in coverage for children less than 2 years of age:

> "Although the Vi and the Ty21a vaccines provide appreciable levels of protection and have a good record of safety, improved vaccines against typhoid fever are desirable. Such vaccines should confer higher levels and more durable protective immunity in all age groups, including those aged <2 years, preferably without the need for booster doses." (27)

Indeed, next generation vaccines are in the pipeline, with many focusing on conjugating the ViCPS to a protein carrier that would elicit a more robust response with greater potential for immunological memory. There are a number of these Vi-conjugate vaccines in various stages of clinical trials. One such Vi vaccine conjugated to tetanus toxoid (Vi-TT) is already licensed in India- PedaTyph (Biomed). Other vaccines in Phase 2 and 3 stages of development use a recombinant ExoProtein A from *Pseudomonas aeruginosa* (Vi-rEPA; NIH/Lanzhou Institute in China), a mutant diphtheria toxoid (Vi-CRM_{197}; Novartis), and diphtheria toxoid (Vi-DT; International Vaccine Institute/Shanta Biotech, Biofarma in Indonesia and SK Pharma in Korea). Vi-DT vaccines are in preclinical trials in Cuba (Finlay Institute) and Vietnam (DAVAC). Another Vi vaccine conjugated to *S.* Typhi outer membrane protein OmpC (OmpC-Vi) is in preclinical trials in India. New live attenuated vaccines are also coming through the pipeline. These vaccines use targeted mutation of specific genes in Ty2, compared to the non-specific chemical mutagenesis used to create Ty21a: M01ZH09 (ΔaroCΔSsaV) and CVD909 (ΔaroC/D ΔhtrA constitutive expression of Vi antigen) (28). It remains to be seen whether these new

generation vaccines will provide greater immunogenicity and efficacy in all target age groups, including infants. Once these new vaccines are license, the Gavi Alliance which funds vaccines in the developing world will help to usher in the use of these vaccines in national immunization programs (28).

III: Mouse Models of Invasive Salmonelloses

In 1892 it was discovered that *S.* Typhimurium causes a systemic typhoid-like illness in mice (29). Since then, *S.* Typhimurium infection of mice has become a well-established and accepted model of typhoid fever. In fact, many of the features of *Salmonella* pathogenesis that we know about today have been characterized in the murine typhoid model and correlate with human clinical and pathology data. A distinct advantage of using the mouse model of infection is the ease of manipulating both host and bacterial genes to determine the factors necessary to drive pathogenesis and control infection. As such, this model has informed the strategies for producing next-generation subunit and live attenuated vaccines for typhoid.

Nramp1/Slc11a1 and Mouse Strain Susceptibility

There are two main mouse models for invasive *Salmonella* infection. These two models can be distinguished by mouse expression of a protein called Natural resistance-associated macrophage protein 1 (Nramp1) encoded by the gene Slc11a1. Nramp1 is a proton/divalent cation antiporter located on endosomal membranes within phagocytic cells called macrophages. They transport cations such as Fe^{2+}, Zn^{2+} and Mn^{2+} into the acidified endosome, aiding in the production of reactive oxygen species with antimicrobial properties. As *Salmonella* create a niche and grow inside of these

endosomes, called *Salmonella*-containing vacuoles, this cation antiporter functions to help kill intra-phagosomal bacteria in activated macrophages (30). Nramp susceptible ($Nramp^s$) mouse strains, such as BALB/c and C57BL/6, have a non-functional protein and succumb to infection with a low dose of virulent *S.* Typhimurium within one week. Mice with a functional Nramp1 ($Nramp^r$) such as CBA and 129Sv, however, can survive low dose virulent *S.* Typhimurium challenge, but bacteria can establish a persistent infection. This resistant mouse model is thought to mirror the persistent typhoid observed in humans, and is also used to study the protective B cell response to infection, which will be discussed in a later section (31, 32). $Nramp^s$ strains of mice can survive infection with attenuated vaccine strains of *S.* Typhimurium, and infection with this vaccine strain provides protection against a secondary virulent *S.* Typhimurium infection. This particular model has been used extensively to study the protective T cell response to infection, which will be discussed in a later section (2, 33, 34).

Salmonella Pathogenesis and the Early Immune Response

After introduction of *Salmonella* via oral gavage of mice, the bacteria enter the gastrointestinal tract. Once in the small intestine (SI), *Salmonella* traverse this barrier via two different mechanisms. The first mechanism is via active invasion through specialized epithelial micro fold (M) cells that are scattered throughout the epithelial layer but are particularly numerous around Payer's patches. These M cells usually function to sample particulate antigens in the lumen of the gut and translocate them to antigen-presenting cells in the underlying secondary lymphoid tissue (35). Once *Salmonella* pass through these M cells, they are taken up by professional phagocytic cells- monocytes, macrophages and dendritic cells- within the Peyer's Patch sub-epithelial dome (36, 37). Indeed, a key feature of infection in the mouse and in humans is the enlargement of the

Peyer's Patches as inflammation due to infection causes an influx of phagocytic cells to this site of infection (38). In some cases, *Salmonella* can actively trigger programmed cell death of macrophages in a caspase 1-dependent manner, and thus be released into the extracellular space (37) (39). Thereafter, *Salmonella* gain access to the systemic circulation via migration within phagocytic cells throughout the lymphatic system or by extracellular spread and access to the blood vessels. The second mechanism by which *Salmonella* can gain access to the mammalian host is via sampling of the gut lumen within the lamina propria by professional phagocytic antigen-presenting cells called dendritic cells (40). Once inside of these dendritic cells, *Salmonella* gain access to the systemic circulation through the lymphatic drainage.

The first major site of replication of bacteria is in the draining lymph node of the gut, the mesenteric lymph node. From here, bacteria spread to systemic sites of the host, namely the spleen, liver and bone marrow. Just as in infection of humans, infection in mice causes hepatomegaly and splenomegaly. Splenomegaly is the result of a major increase in all immune cells. While there is a 3-5-fold increase in monocytes, macrophages, NK cells, neutrophils, T cells and B cells, there is a 10-fold increase in red blood cells (41). *S.* Typhimurium, like other gram-negative bacteria, have pathogen associated molecular patterns (PAMPs), lipopolysaccharide (LPS) and flagellant, that bind pathogen recognition receptors (PRRs) such as toll-like receptors (TLR) 4 and 5, respectively. Binding of these PRRs activates innate immune cells such as macrophages and dendritic cells, causing them to increase the expression of surface molecules such as Major Histocompatibility Complex II, and secrete inflammatory cytokines. Inflammatory cytokines such as IL-1, IL-6 and IL-8 help to recruit more innate immune cells to the site of infection (2). Cytokines such as IL-12 and IL-18 help to instruct natural killer cells and activated CD4+ T cells to produce another inflammatory

cytokine, interferon gamma (IFNγ). IFNγ is a key cytokine in controlling the spread and replication of intracellular bacteria during early and late stages of infection, by stimulating the antimicrobial activity of macrophages. Indeed people who have deficiencies in IFNγ or its receptor are more susceptible to non-typhoidal *Salmonella* infections (42). While the innate immune response is able to control infection for 2-3 weeks, without adaptive immunity, mice succumb to primary infection, even with attenuated strains of *Salmonella*.

The next two sections will describe the adaptive immune response to *Salmonella* infection and the mechanisms by which these immune responses clear infection and protect against re-infection.

IV: CD4+ T cell responses to *Salmonella* infection

As intra-phagosomal pathogens that have multiple mechanisms to evade the innate immune system, *Salmonella* require activation of the adaptive immune system for clearance. Specifically, CD4+ T cells are essential for protection against *Salmonella* infection. This is most evident in HIV+ adults whose CD4+ T cells reach below 200 cells/all. These individuals are more susceptible to disseminated *Salmonella* infections. Although there is no correlation between HIV/AIDS and an increased susceptibility to typhoid fever, there is a major prevalence of non-typhoidal Salmonelloses among HIV-infected adults with mortality between 20-59%, depending on the area of endemicity (20, 21).

The activation, proliferation and differentiation of a naïve CD4+ T cell require three signals. The first signal is the presentation of a peptide antigen by a professional antigen presenting cell, usually the dendritic cell, since these cells reside in the T cell

area of lymphoid organs where naïve T cells are also located (43, 44). Other antigen presenting cells that reside outside of the T cell area of the lymphoid tissue are macrophages and B cells (44). Dendritic cells process proteins and cleave them into peptides within endosomal compartments. These peptides, which can contain specific sequences from *Salmonella* proteins are then loaded onto the antigen presentation molecule, the Major Histocompatibility Complex II (MHCII). T cells recognize their cognate peptide-MHCII antigens via their T cell receptors. If the T cell encounters its cognate antigen, a signal is transduced via adaptor and signaling molecules within the T cell that lead to activation. The second signal required is a co-stimulatory signal achieved by the engagement of CD28 on the T cell and B7-1 or B7-2 (also known as CD80 and CD86, respectively) on the antigen-presenting cell. B7-1 is only expressed on antigen presenting cells after activation by TLR and other PRR signals. Signal two instructs the T cell that this antigen stimulation is against non-self, by being restricted to antigen presenting cells that have encountered non-self, such as LPS (45, 46).

The third signal is a particular cytokine profile secreted by the antigen presenting cell or local environment that instructs the activated T cell to differentiate along a defined pathway. For example, antigen-presenting cell secretion of IL-12 instructs the newly activated CD4+ T cell to express the transcription factor T-bet, the lineage-defining transcription factor for T_H1 cells, and produce IFNγ. Indeed, susceptible mice that lack MHCII, CD4 cells, T-bet, or IFNγ eventually succumb to infection with an attenuated *S.* Typhimurium (47, 48). In mucosal tissues where TGFβ is highly expressed, antigen-presenting cells can be activated to produce IL-6 which instructs activated CD4+ T cells to express the transcription factor RORγt and produce IL-17 (49). Thus, these helper cells become T_h17 cells, which have been shown to be important in fighting extracellular microbes and in defense at mucosal ports of entry into the body. Alternatively, in an

environment where IL-4 is expressed, antigen-presenting cells will initiate CD4+ T cell differentiation into T_H2 cells that express the transcription factor GATA3 and produce IL-4, IL-5 and IL-13. These T_H2 cytokines are important in driving IgE production and protection against helminthes, and also play a key role in atopic and allergic responses (50, 51). As a mucosal and intracellular pathogen, *Salmonella* primarily induces protective T_H17 and T_H1 responses. Thus, understanding how the CD4+ T cell, and specifically the T_H1 response forms during infection, how they develop memory and which *Salmonella* antigens promote protective CD4+ T cell responses is of utmost importance in developing novel, effective vaccines.

Salmonella-*specific CD4+ T cell Response*

As early as three days following primary infection of susceptible mice with a vaccine strain of *S.* Typhimurium, there is a major polyclonal expansion of CD4+ T cells in the spleen and lymph nodes (52). In the spleen, the overall CD4+ T cell numbers increase 2-4 fold, and most of this increase can be attributed to activated cells that have encountered cognate antigen, and express CD44 and CD11a, markers of antigen-experience, and CD25 and CD69, markers of recent activation. In systemic sites, most of these activated T cells express the lineage-defining transcription factor T-bet, and a fraction of these cells produce T_h1 cytokines IFNγ and TNFα. This expansion occurs early and plateaus until about 3 weeks post-infection, before beginning a contraction phase as the bacterial burden begins to decrease. It is apparent that protective T_h1 effector cells form during this period, because if one depletes CD4+ T cells prior to challenge with virulent *S.* Typhimurium mice are no longer able to control the infection and succumb around this 3 week time point (53). Although studying the polyclonal CD4+ T cell response during infection gives us some clues about protection, it is difficult

to know if this response of polyclonal CD4 T cells is completely *Salmonella* specific or may also contain non-specific CD4 T cells that are activated in a bystander manner. Also, it is important to differentiate among the protective and non-protective responses and determine the characteristics of those responses that are protective.

Most of the detailed work studying *Salmonella*-specific CD4+ T cell responses has been accomplished using adoptive transfer of SM1 transgenic CD4+ T cells into wild type susceptible C57BL/6 mice. SM1 T cells express a TCR that recognizes a peptide of *Salmonella* flagellant in the context of I-A^b, the MHCII molecule expressed in susceptible C57BL/6 mice. When mice are infected orally with *Salmonella*, CCR6+ dendritic cells in Peyer's Patches initially activate SM1s as early as 3 hours after infection, causing SM1 T cells to express CD69 and secrete TNFα (54). SM1 T cell activation can subsequently be detected in the mesenteric lymph nodes and five days later can also be found in the spleen. SM1 T cells differentiate into T_h1 cells, expressing T-Bet and secreting IFNγ. The distribution of the SM1 response following oral infection is skewed to the early intestinal sites of entry, the Peyer's Patches and mesenteric lymph nodes. This is likely due to the fact that flagellin is down-regulated when *Salmonella* enter the intraphagosomal compartment. This early intestinal focus on flagellin also holds true when analyzing polyclonal CD4+ T cell responses by ELISPOT after repeated oral immunization with an attenuated *S.* Typhimurium. CD4+ T cells responding in the mesenteric lymph nodes and lamina propria were heavily focused on flagellin as a target antigen, while flagellin-specific T cells were a smaller component of the response in systemic sites such as the spleen (55). Interestingly, these intestinal flagellin specific cells also produced both IFNγ and IL-17. In contrast, CD4+ T cell responses to *Salmonella* proteins expressed during intracellular growth occurred later in the response, were localized to systemic sites of infection, and only produced IFNγ (55).

Unfortunately, memory CD4+ T cell responses to *Salmonella* cannot be studied using the SM1 adoptive transfer system. Although there is robust activation and expansion of these cells at day 3 of infection, by day 6 post-infection, most of these activated T cells are no longer detectable in circulation. This loss of T cells is an active process that requires the presence of live bacteria and has been referred to as “culling”. Initial studies suggested that *Salmonella* may actively, whether directly or indirectly, kill activated CD4+ T cells in a *Salmonella* Pathogenicity Island II-dependent manner (56, 57). However, it was also possible that observation may be an artifact of using TCR transgenic cells in an infectious disease model. A more direct methodology to visualize the polyclonal response to particular *Salmonella* antigens makes use MHCII-peptide tetramers bound to a fluorochrome such as phycoerythrein, which can in turn, be used to detect these cells via flow cytometry (58). Using this technique, it was apparent that *Salmonella* cull activated CD4+ T cell responses, whether the cognate antigen was expressed by *Salmonella* or a different pathogen, such as *Listeria monocytogenes*. Culling of CD4+ T cells is thought to preferentially target T cells that bind to their target antigens with higher avidity, as measured by the MFI of tetramer staining (57). Thus, *Salmonella* have an incompletely defined capacity to eliminate responding CD4 T cells and this functionality may have evolved to combat effective adaptive immunity from host Th1 cells.

Further examination of the endogenous CD4+T cell response during *S.* Typhimurium infection using MHCII tetramers revealed that the kinetics of the response depended on the temporal expression of the antigen. The flagellin $_{427\text{-}441}$ -specific response peaked at day 7 after a 12.9-fold expansion and started to contract and express markers of memory CCR7 and CD27, as early as 10 days post-infection, while bacterial loads were still high. In contrast, the $SseJ_{329\text{-}341}$ – specific response peaked with

a 210-fold expansion 52 days post-infection after bacteria had cleared, followed by a gradual contraction phase. Thus, although the naïve precursor frequency for flagellin$_{427-441}$–specific and SseJ$_{329-341}$–specific were similar, sustained antigen stimulation appears to be necessary for robust CD4 T cell expansion (55). This observation is solidified by the fact that treatment with antibiotics before the peak of infection also results in sub-optimal protective immunity to subsequent virulent infection and this correlated with decreased CD4+ T cell recall responses. Thus, antibiotic-treated mice had a reduced percentage of CD4+ T cells expressing CD11a and producing IFNγ in response to restimulation with heat-killed *Salmonella* (59). Unfortunately, although there are now several known CD4+ T cell epitopes known in the *Salmonella* model system, flagellin, PagC, SseI and SseJ, among these, only flagellin is known to function as a protective antigen (55, 60, 61). Further work is necessary to determine additional protective antigens that can elicit a robust CD4+ T cell response that mediates bacterial killing. Furthermore, the mechanisms by which Salmonella can inhibit the responding Th1 population require further investigation.

V: B cell Responses to *Salmonella* infection

The second arm of the adaptive immune system consists of B cells. B cells are best known as the cells that differentiate into plasma cells and secrete antibodies. As pathogens with an extracellular phase of infection, *Salmonella* induce robust antibody responses in humans and in mice. In fact, the ViCPS vaccine is thought to mediate its protective effect by stimulating short-lived protective antibodies. Unlike conventional αβ T cells, B cells can recognize pathogens directly with surface immunoglobulin that form the B cells receptor (BCR). The full B cell repertoire also has the potential to recognize

any three-dimensional structure via BCRs, whether the molecule be protein, carbohydrate, lipid, DNA, etc. Most vaccines on the market today elicit high-affinity, class-switched antibodies for their protective effects. It is important to understand how B cell responses are produced to specific pathogens in order to take advantage of these protective responses for next generation vaccines.

B cell subsets, Activation and Antibody Production

The three main mature B cell subsets in the periphery are follicular B cells, marginal zone B cells, and B1 B cells. All three subsets respond to stimulation via signals delivered via the BCR and innate receptors such as TLRs. In total, the BCR consists of several protein subunits including the surface antigen-binding immunoglobulin, Igα, Igβ, CD19, CD21 and CD81. However, these three different subsets of B cells have seemingly evolved to provide distinct functions in mediating host defense (62).

Follicular B cells first develop in the bone marrow and mature through transitional stages either in the bone marrow or out in the peripheral lymphoid tissue. As with naïve T cells, naïve B cells circulate throughout the lymph nodes, spleen and Peyer's Patches where they localize to the follicular zones. These naïve, mature B cells display IgM^{lo} and IgD^{hi} BCRs on their surface that can recognize a diverse array of antigens. Cross-linking of the B cell receptor via binding their cognate antigens activates follicular B cells. B cell cross-linking alone may lead to activation-induced cell death. However, B cells can also receive "danger" signals via stimulation of their TLRs by pathogen-associated molecular patterns. These activated B cells upregulate MHCII and co-stimulatory molecules such as CD80 and CD86. In the lymph nodes and spleen, activated follicular B cells can either

differentiate into short-lived plasma cells without T cell help (T-independent responses) or migrate towards the T cell zones or periarteriolar sheaths (PALS) where they interact with activated CD4+ T cells by presenting cognate peptide antigens on MHCII (T-dependent responses). These activated CD4+ T cells express surface CD40L, which binds to CD40 on the activated B cell and instructs the B cell to proliferate. After this interaction, follicular B cells can quickly differentiate into IgM^+ memory B cells or short-lived plasmacytes that secrete low-affinity IgM, which can be important to curb the spread of infection during this early stage of the response. They can also migrate back to the follicle to establish the germinal center response (63).

It is within the germinal centers that B cells undergo massive proliferation, differentiation into antibody-secreting cells, somatic hypermutation of the variable regions of their BCRs and class-switch recombination of the constant regions of their BCRs. CD40 signaling provided by CD40L on T_{FH} cells is crucial for the germinal center reaction. Somatic hypermutation of the antigen-binding domains of the BCRs allows for affinity maturation to cognate antigen, while class-switch recombination allows for secreted antibody to diversify its antimicrobial function. It is also usually from the germinal center that long-lived plasma cells and memory B cells arise. The long-lived plasma cells home to the bone marrow where they produce high-affinity antibody, possibly for the remainder of life. Memory B cells reside in secondary lymphoid organs as well as bone marrow, with receptors that have higher affinity to cognate antigen. Memory B cells can respond to activation by proliferating and differentiating into antibody-secreting cells more quickly than naïve B cells (62-64).

The cytokine signals that activated B cells receive dictate the class of antibody that is produced. In the context of BCR and TLR stimulation, IL-4 promotes the expression of IgG1 and IgE classes of antibody. IFNγ promotes expression of IgG2a,

IgG2b and IgG3. TGFβ in mucosal tissues promotes the production of IgA. Thus, during a parasite infection, IL-4 produced by T_H2 cells promotes the production of IgE, whose receptors are abundant on innate cells such as mast cells that secrete effectors that protect against these parasites. Likewise, IFNγ produced by T_H1 cells promotes the production of IgG subclasses that specialize in opsonization and targeting to Fcγ receptors on phagocytes that in turn kill bacteria such as *Salmonella* (62, 63).

In addition to differentiating into antibody-secreting plasma cells, activated follicular B cells can produce pro- and anti-inflammatory cytokines that modulate the immune response. Similar to the pattern of cytokines that define T_H1 and T_H2 cells, B effector 1 cells (B_e1) produce T_H1-type cytokines such as IFNγ, while B_e2 cells produce IL-4 (65).

B-1 cells are thought to have their origins in the fetal liver as well as the adult bone marrow. They tend to reside in the peritoneal cavity and the pleural space of the lung, and when activated, can migrate from the peritoneum to the mesenteric lymph node or the lamina propria of the gut. Unlike follicular B cells, the BCR repertoire of B-1 cells is not as diverse and tends to recognize conserved patterns on microbial pathogens such as phosphorylcholine. There are two specific populations of B1 cells- $CD5^+$ B-1a and $CD5^-$ B-1b cells. Very much unlike follicular B-2 cells, these B-1 cells do not require BCR stimulation to become antibody-secreting cells. Upon stimulation with TLR ligands, such as LPS, B-1 cells can differentiate into short-lived IgM-secreting plasma cells, and thus act in a T-independent manner. In the gut, B-1 cells can be stimulated by TLR ligands to produce IgA. While B-1 cells can quickly contribute antibody as a first line of defense against pathogens, they do not generally contribute to long-lived high affinity antibody responses like follicular B cells do (62).

Marginal zone B cells (MZ B cells) arise from a precursor in the bone marrow and reside in the marginal sinus of the spleen. Like B-1 cells, marginal zone B cells can quickly become activated and produce IgM in the absence of BCR stimulation. As these MZ B cells express CD1d, they are thought to recognize lipid antigens. MZ B cells also function to present antigen to follicular B cells. They express high levels of CD21 on which they capture antigen in immune complexes and shuttle the antigen to follicular B cells in the follicle (62).

B cell responses during Salmonella *infection*

Although it is well established that CD4+ T cells are required for immunity to *Salmonella* infection, it is less appreciated that B cells also play a significant role. However, it is apparent that via the moderately effective ViCPS vaccine that induces a T-independent antibody response, B cells can offer protection against *Salmonella* infection in humans. A recent study found that in mice, B-1b B cells that differentiate into short-lived plasma cells and contribute to bacterial clearance mediate the protective response induced by the ViCPS. The presence of *Salmonella*-specific antibodies in the serum of Malawian children along with functional complement kills *Salmonella* isolates *in vitro.* However, serum from children 16 months of age and younger that lacked antibody was not able to kill *Salmonella*, even in the presence of functional complement (66). This indicates a key role for antibodies in controlling the extracellular infection in the blood. Indeed, a similar observation was made in mice. The early IgG2a extrafollicular antibody response during *Salmonella* infection in mice curtails extracellular infection, although it does not completely resolve it (67).

Determining the role for B cells in *Salmonella* immunity has mostly been studied in the mouse model of typhoid. In 1993, Mastroeni et al established that protection against

oral challenge with virulent *Salmonella* required adoptive transfer of both immune spleen cells and immune serum from mice immunized with attenuated bacteria (68). Later studies using B cell-deficient mice (Igμ -/- or Igh-6 -/- mice) on a susceptible background strain demonstrated that although these mice are able to survive and clear a primary attenuated infection, they succumb to a secondary virulent infection. However, these studies conflict as to whether transfer of immune serum is capable of restoring protective immunity in B cell deficient mice. It is also clear from these studies that the absence of B cells during primary infection impairs the secondary T_H1 response to infection (69-71).

Additional studies in mice have shown that the protective effects of B cells during *Salmonella* infection may not simply be limited to antibody production. Ugrinovic *et al*, showed that T_H1 responses in B-cell deficient mice are impaired in bacterial clearance and that this impairment can be detected early after infection (72). This study suggested a key role for B cells as antigen-presenting cells during primary infection. Further studies have shown that MyD88 signaling in B cells is required for initial priming of the T_H1 response, while BCR-specificity and B cell production of IFNγ is important for establishing the memory T_H1 response (73, 74). Interestingly, the germinal center B cell response, which produces high affinity BCRs and antibodies as well as long-lived plasma cells and memory B cells is delayed during *Salmonella* infection (67). Although these studies demonstrate that antigen-specific B cells and secreted antibody may be important for protective immunity to *Salmonella* infection, it is unclear which mechanisms of protection are more important.

Further work must still be done to determine the protective antigens of the follicular B cell response. Some studies have shown that LPS-specific antibodies inhibit *Salmonella* clearance, especially apparent in HIV+ adults (75). A mouse study showed that rapidly induced extrafollicular antibodies against *Salmonella* outer membrane proteins can

impede the spread of primary infection in the blood (67). However, it is unknown which antigen-specific B cells secrete cytokines and present antigen to influence the T_H1 response.

VI. Thesis Statement

In this thesis, we aim to further define the protective role of B cells during *Salmonella* infection. Understanding how B cells contribute to protective immunity is essential for designing more efficacious next generation vaccines against Typhoid and new vaccines against Paratyphoid and Non-Typhoid *Salmonella* strains. In the second chapter we test the hypothesis that systemic IgG production is an essential mechanism by which B cells protect against virulent infection. Surprisingly, we found that unlike B cells deficient mice, mice that lack the ability to secrete IgG or all antibodies are still largely protected from virulent *Salmonella* following resolution of infection with a vaccine strain. We further show that B cells, in the absence of antibody, are required for establishing robust memory T_h1 responses.

That B cells are important for protective immunity during secondary infection, suggests that we should better understand how this protection develops during a primary infection. In the third chapter, we aimed to define the kinetics and characteristics of the primary endogenous ovalbumin-specific B cell response during live vaccination with attenuated *Salmonella* expressing ovalbumin and OVA-specific B cell tetramers. We show that much like the report from Cunningham *et al*., which showed a delay in germinal center response using antibody ELISAs and histology, there was a delay in the OVA-specific B cell responses until the infection had cleared. Additionally, we observed a delay in germinal center formation in these OVA-specific B cells, in contrast to

immunization with OVA and adjuvant alone. Upon co-administration of OVA with *Salmonella* infection, there was a decrease in the expansion and germinal center formation of OVA-specific B cells compared to mice that received OVA alone. We further discovered that, unlike culling of T cells during *Salmonella* infection, this inhibition of the B cell might not be linked to expression of *Salmonella* Pathogenicity Island II proteins.

Chapter 2

B cells are Essential for Protective Immunity against *Salmonella* Independent of Antibody Secretion

Nanton MR, Way SS, Shlomchik MJ and McSorley SJ. 2013. Cutting Edge: B cells are essential for protective immunity against *Salmonella* independent of antibody secretion. *J Immunol*. 189(12): 5503-7.

Abstract

Typhoid fever and non-typhoidal bacteremia caused by *Salmonella* remain critical human health problems. B cells are required for protective immunity to *Salmonella* but the mechanism of protection remains unclear. Here, we immunized WildType, B cell deficient, antibody-deficient and class-switched antibody-deficient mice with attenuated *Salmonella* and examined protection against secondary infection. As expected, WildType mice were protected and B cell deficient mice succumbed to secondary infection. Interestingly, mice with B cells but lacking secreted antibody or class-switched antibody had little deficiency in resistance to *Salmonella* infection. The susceptibility of B cell deficient mice correlated with marked reductions in CD4 T cell IFN-γ production after secondary infection. Together, these data suggest that the primary role of B cells in acquired immunity to *Salmonella* is via the development of protective T cell immunity.

Introduction

Typhoid fever is caused by infection with *Salmonella* Typhi and is a serious health concern worldwide, causing an estimated 21 million cases and 216,000 deaths per year (3). Non-typhoidal salmonellosis (NTS) is caused by other *Salmonella* serovars and is a growing problem among HIV-infected adults and HIV-negative children in Africa and Asia (18, 20, 66, 75). Currently, there are two vaccines for typhoid fever that each provide limited protection but are not widely used in endemic areas (25, 26). There is no available vaccine for NTS, although numerous target antigens have recently been defined (76). The development of novel, effective vaccines for typhoid and NTS requires greater understanding of *Salmonella*-specific T and B cell responses (77).

Immunity to *Salmonella* is studied using a well-established murine model of typhoid, in which *Salmonella* Typhimurium causes fatal disseminated disease in susceptible, $Nramp^s$ mice (33, 78). After oral infection, *Salmonella* can gain access to the mammalian host by invading M cells in the Peyer's Patches of the small intestine (33). *Salmonella* subsequently disseminate via the lymphatic system and replicate within phagocytic cells of the spleen, liver and bone marrow. *Salmonella* actively inhibit phagolysosomal fusion and infected macrophages require activation via IFN-γ to kill bacteria (37). *Salmonella*-specific Th1 cells that produce IFN-γ are essential for controlling bacterial growth, and mice lacking αβ CD4 T cells, Th1 cells, or IFN-γ eventually succumb to primary infection with attenuated bacteria (47, 48). Patients with primary genetic deficiencies in IL-12 or IFN-γ receptor signaling suffer from repeated disseminated *Salmonella* infections (42, 79). Thus, Th1 cells play an important role in mediating protective immunity in both human and murine Salmonellosis.

The resolution of primary *Salmonella* infection confers robust protective immunity against secondary challenge. CD4 T cells are essential for this acquired resistance and

depletion of CD4 T cells eliminates the protective effect of vaccination with attenuated *Salmonella* (80). More surprisingly for an intra-macrophage infection, B cells are also essential for acquired immunity to *Salmonella,* and immunized B cell-deficient mice display enhanced susceptibility to secondary infection (69-71). However, the protective role of antibody in secondary immunity is somewhat controversial. Passive transfer of antibody is reported to be protective in some studies, while others have observed no protective effect (68-70). Furthermore, neither IgA, nor mucosal immunoglobulins are required for protective immunity to *Salmonella* (76, 81). B cells can contribute to protective immunity via antigen presentation to *Salmonella*-specific Th1 cells (69, 72), or as an important source of inflammatory cytokines during infection (73, 74). However, it remains unclear whether the contribution of B cells to protective immunity is largely mediated by antibody-dependent or antibody-independent mechanisms.

Here, we examined the role of B cells in protection against infection with virulent *Salmonella* using transgenic mouse strains that lack B cells, class-switched antibody, or antibody secretion and demonstrate that antibody production is largely dispensable for protection against secondary *Salmonella* infection. In contrast, B cells are required for optimal priming of *Salmonella*-specific Th1 cells that mediate bacterial clearance.

Materials and Methods

Mice

Balb/c (WildType) and JhD/Balb/c (B cell Deficient) mice (National Cancer Institute, Frederick, MD) were used at 6-12 weeks of age. Transgenic m+s IgM and mIgM use the B1-8 heavy chain, have a restricted BCR repertoire, were maintained on a JhD/Balb/c background (82) and were provided by Dr. Shlomchik (Yale University, New Haven, CT). Transgenic mice were intercrossed with JhD/Balb/c mice and used at 6-12 weeks of age. Homozygosity at the JHD locus was maintained by interbreeding with JhD mice and PCR screening of mIgM heavy chain was done using the following primers (Vh186.2 5' 216 CTACTGGATGCACTGGGTGA and Vh186.2 3' 459 TTGGCCCCAGTAGTCAAAGTA). All mice were housed in specific pathogen free conditions for breeding and experimentation.

Bacteria and Infection

Attenuated *S.* Typhimurium BRD509 (ΔaroA/ΔaroD) and parental virulent strain SL1344 were grown overnight in Luria-Bertani broth and diluted in PBS after estimating bacterial counts by spectrophotometry. Mice were immunized IV with $5x10^5$ BRD509 and challenged orally with $5x10^7$ SL1344 after oral administration of 100μl 5% $NaHCO_3$. Infection doses were confirmed by plating serial dilutions onto MacConkey agar plates. Any moribund infected mice were euthanized as stipulated in our IACUC protocol. Bacterial growth *in vivo* was calculated by plating serial dilutions of organ homogenates onto MacConkey agar and bacterial counts were determined after overnight incubation at 37ºC.

Detection of in vivo cytokine production and Flow Cytometry

Salmonella-specific CD4 and CD8 T cell responses were visualized, as previously described (83). Immunized mice were injected IV with $1x10^8$ Heat-Killed *Salmonella* Typhimurium (HKST) and spleens harvested three or five hours later. A single cell suspension was surface stained using FITC-, PE-, PE-Cy5-, PE-Cy7-, APC-, eF450-, AF700- and APCeF780-conjugated antibodies to CD3, CD4, CD8, Gr-1, CD11c, CD11b, F4/80, B220, and CD44 in Fc block (spent 24G2 supernatant, 2% rat serum, 2% mouse serum). Cells were fixed, permeabilized, and stained intracellularly using PE conjugated anti- IFN-γ. All staining reagents were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Samples were analyzed by flow cytometry using a FACSCanto and data analyzed using FlowJo Software (Tree Star).

Salmonella-specific antibody response

Blood was collected by retro-orbital bleeding and sera prepared and stored at -20°C. *Salmonella*-specific IgM and IgG antibodies were measured by ELISA, as previously described (83).

Statistical Analysis

Statistical analysis was performed using unpaired t tests and one-way ANOVA (Prism 4, GraphPad Software, Inc., La Jolla, CA). Survival data was compared using a Log-rank (Mantel–Cox) test (Prism 4). Statistical differences between groups are highlighted with *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

Results and Discussion

Class-switched antibody is not required for secondary protection against Salmonella

Defining protective immune responses to *Salmonella* infection is a prerequisite for development of new effective vaccines against typhoid and NTS (33). Although CD4 T cells are critical for protective immunity to *Salmonella*, the contribution of B cells has not been clearly defined. *Salmonella*-specific antibody production, inflammatory cytokine production, and direct antigen presentation to T cells have each been proposed as mechanisms to explain the protective role of B cells during secondary infection (67, 69, 70, 72, 74). We sought to investigate whether B cells provide secondary protective immunity against *Salmonella* primarily in an antibody dependent or independent manner. Given previous data showing that serum transfer can protect (70), but that neither IgA nor mucosal immunoglobulin is required (76), we hypothesized that systemic IgG is essential for secondary clearance of bacteria. To test this hypothesis, we examined immunity in B cell deficient mice (JhD), transgenic mice with B cells that cannot class switch or secrete antibody (membrane IgM- mIgM), and mice with B cells that cannot class switch but are able to secrete IgM, (membrane + secretory IgM- m+s IgM) (82). Although the mIgM and m+s IgM transgenic mice have a restricted BCR repertoire, they do not have significant deviations in naive B cell and T cell subsets (Fig. 2-4A – 2-4D and (84)). All four strains (WildType, B cell deficient, mIgM, and m+s IgM mice) survived vaccination with attenuated *S.* Typhimurium and had largely cleared bacteria from the spleen 44 days later (Fig. 4E). This confirmed previous reports that resolution of primary infection with attenuated *Salmonella* does not require B cells (69, 70).

To examine acquired immunity to secondary *Salmonella* infection, naive and immunized mice from all four strains were challenged orally with virulent *S.* Typhimurium (Fig. 2-1A). Regardless of the B cell compartment, all naïve mice succumbed to primary

infection with virulent *Salmonella* at a similar rate (Fig. 2-1A). In contrast, immunized WildType mice resisted secondary infection with virulent *Salmonella*, while B cell deficient mice succumbed to secondary challenge (Fig. 2-1A). Surprisingly, m+s IgM mice that lack class-switched antibody also survived secondary infection with *Salmonella*, demonstrating a similar degree of protective immunity to wild-type mice (Fig. 2-1A). Furthermore, most mIgM mice that lack all secreted antibodies were resistant to secondary *Salmonella* infection. However, approximately 25% of these mice eventually died from infection, and this was statistically different from the survival of WildType and B cell deficient mice (Fig. 2-1A). Together, these data confirm that B cells are essential for resistance to secondary infection with virulent *Salmonella*, and surprisingly demonstrate that production of class-switched antibodies is not required for protective immunity. Additionally, although secreted IgM antibodies may contribute to secondary protection, the mechanism of B cell-mediated protection against secondary *Salmonella* infection is largely antibody-independent in this vaccination and rechallenge model.

Given these findings, it was important to confirm the absence of circulating *Salmonella*-specific antibody in each B cell deficient strain examined above. Serum was collected nine days after secondary infection, and *Salmonella*-specific antibody responses were examined. Nine days after secondary infection, both WildType mice and IgM Ab Only (m+s IgM) mice had modest levels of circulating *Salmonella*-specific IgM (Fig. 2-1B), but only WildType mice developed *Salmonella*-specific IgG (Fig. 2-1B). These results confirm that only WildType mice produced a class-switched antibody response to *Salmonella*, but that IgM Ab Only mice developed low *Salmonella*-specific IgM responses during secondary infection.

Secondary bacterial clearance does not require class-switched antibody

Given the fact that mice lacking all antibodies had a 25% death rate following virulent challenge, it seemed likely that bacterial clearance was hindered at late time points in these mice, perhaps because IgM is required for clearance from a particularly persistent anatomical site such as the mesenteric lymph nodes (85). Thus, we examined the rate of bacterial clearance in immunized mice lacking B cells, class-switched antibody or all antibody. Three days after secondary infection, WildType mice had lower bacterial loads in the spleen than B cell deficient mice (Fig. 2-2A), demonstrating that B cells are required for rapid secondary clearance of bacteria. At this early time point, no significant differences were apparent between antibody deficient strains and B cell deficient mice, but antibody deficient mice had a trend towards lower CFUs in the spleen (Fig. 2-2A). No significant differences were detected in liver CFUs at this same early time point (Fig. 2-2B). Nine days after secondary infection, mice lacking B cells had much higher bacterial loads in both the spleen and liver compared to WildType mice (Fig. 2-2A and 2-2B). In marked contrast, mIgM and m+s IgM mice had lower CFUs in both spleen and liver (Fig. 2-2A and 2-2B). Together these data demonstrate that the rate of bacterial clearance during secondary infection is largely unaffected by the absence of antibody, despite a requirement for B cells. This finding contrasts with prior studies that showed a protective effect of serum transfer (68, 70). However, these studies were not designed to test an antibody-independent role of B cells and both described protection against low dose challenge. Our finding has broad implications since the measurement of circulating immunoglobulin is often used as an indicator of vaccine efficacy.

B cell-deficient mice have reduced CD4 T cell responses to Salmonella

It is clear from previous work that secretion of IFN-γ by Th1 cells is critical for the resolution of *Salmonella* infections (47, 53). We confirmed this by depleting CD4 and CD8 T cells in immunized WildType mice and challenging with a virulent strain of *Salmonella*. T cell depletion caused a significant increase in bacterial loads during secondary infection (Fig. 2-5A). It has been suggested that antibody can enhance T cell responses to *Salmonella* by allowing bacterial uptake via Fc receptors on dendritic cells (86). B cells also can present antigen and secrete cytokines that shape the development of protective T cell responses. Thus, we examined the effect of B cell or antibody deficiency on the generation of *Salmonella*-specific Th1 cells.

WildType, B cell deficient, m+s IgM and mIgM mice were immunized with attenuated *Salmonella* and *Salmonella*-specific CD4 T cell responses examined 42 days later. As previously reported (52, 59), immunized WildType mice had a large population of CD4 T cells that produced IFNγ in response to HKST stimulation (Fig. 2-3A and Fig. 2-5B). In marked contrast, immunized B cell deficient mice had lower numbers of IFNγ-producing Th1 cells in response to HKST (Fig. 2-3A and Fig. 2-5B). This difference was antibody-independent since immunized m+s IgM and mIgM mice had similar levels of IFNγ-producing CD4 T cells as WildType mice (Fig. 2-3A and Fig. 2-5B). In fact, mIgM mice, which lack all secreted antibodies, had a larger population of *Salmonella*-specific IFNγ-producing CD4 T cells. Interestingly, IFNγ-producing CD8 T cells were also slightly reduced in immunized B cell deficient mice, but this was not statistically significant (Fig. 2-3B and Fig. 2-5B). Taken together, these data indicate that B cells, but not antibody, are required for shaping the development of protective CD4 Th1 responses to *Salmonella*. A similar role for B cells has been reported in other infection models such as LCMV and Pneumocystis (87, 88). Although B cells may directly present antigen and

drive *Salmonella*-specific Th1 responses, a recent study demonstrated that B cell production of IL-6 is important for maximal Th17 responses, and B cell production of IFNγ contributed to Th1 development (74). A recent study has also shown that B cells can affect secondary responses to *Salmonella* infection via a MyD88 and IL-10-dependent mechanism (89). Thus, B cells likely contribute to protective CD4 responses, via antigen presentation and production of specific cytokines that drive effector lineage commitment during primary responses. It is not yet clear whether these required B cells are necessarily *Salmonella*-specific, however the limited B cell repertoire in IgM Only and No Antibody mice did not affect protective immunity. We also attempted to address this issue using *in vitro* re-stimulation and B cell tetramer pull-down experiments in previously infected mice, but did not detect an elevated frequency of *Salmonella*-specific B cells using either of these approaches. However, it remains possible that expanded *Salmonella*-specific B cells contribute to immunity to secondary infection.

Together, our data demonstrate that antibody production plays only a minor role in *Salmonella* immunity while B cells are required for the development of protective T cell immunity. These findings will be important for the development of new effective vaccines against typhoid and NTS.

FIGURE 2-1. Class-switched antibody is not necessary for immunity to Salmonella. (**A**) Naïve WildType, JhD, m+s IgM and mIgM mice were infected orally with $5x10^7$ *S.* Typhimurium (SL1344) and survival monitored. WildType, JhD, m+s IgM, and mIgM mice were immunized IV with $5x10^5$ *S.* Typhimurium (BRD509 ΔaroA/ΔaroD). Forty-two to Sixty-six days later, mice were challenged orally with $5x10^7$ *S.* Typhimurium (SL1344) and survival was monitored. Data are pooled from three separate experiments and show the percentage of surviving mice in each group. The total number of mice is indicated. Survival of immunized WildType, m+s IgM, and mIgM mice was statistically different ($p>0.05$) from JhD mice using a Log-rank (Mantel-Cox) test. Survival of mIgM was also statistically different by Log-rank test when compared to WildType but not when compared to m+s IgM mice. (**B**) Mice were immunized IV with $5x10^5$ BRD509, at 42 days they were challenged orally with $5x10^7$ SL1344 and serum collected 9 days later. Data show levels of heat-killed *Salmonella*-specific IgM and IgG as determined by ELISA. (n= 4-5 mice per group).

FIGURE 2-1

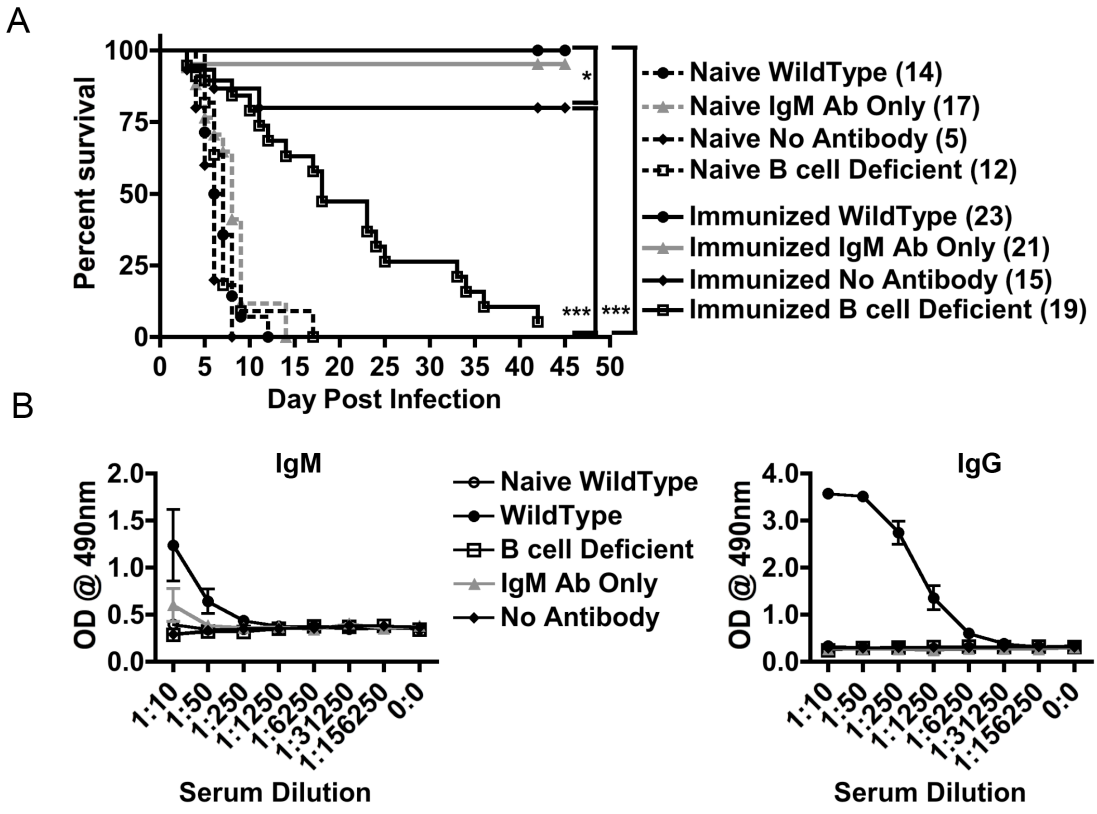

A
Percent survival
Day Post Infection
Naive WildType (14)
Naive IgM Ab Only (17)
Naive No Antibody (5)
Naive B cell Deficient (12)
Immunized WildType (23)
Immunized IgM Ab Only (21)
Immunized No Antibody (15)
Immunized B cell Deficient (19)
B
IgM
IgG
OD @ 490nm
Serum Dilution
Naive WildType
WildType
B cell Deficient
IgM Ab Only
No Antibody

FIGURE 2-2. Rapid bacterial clearance does not require antibody.

(**A-B**) Mice were immunized IV with $5x10^5$ BRD509 and 42-66 days later challenged orally with $5x10^7$ SL1344. Three and nine days later bacterial counts were determined in (**A**) spleen and (**B**) liver. Data show mean Log_{10} CFUs per organ and n= 6-26 mice per group/time point. One-way ANOVA comparing all groups in the spleen at day 3 yielded a p value of 0.0409 (*), at day 9 yielded a p value of <0.0001 (***). One-way ANOVA comparing all groups in the liver at day 3 yielded a p value of 0.2026 (ns), and at day 9 a p value of <0.0001 (***).

FIGURE 2-2

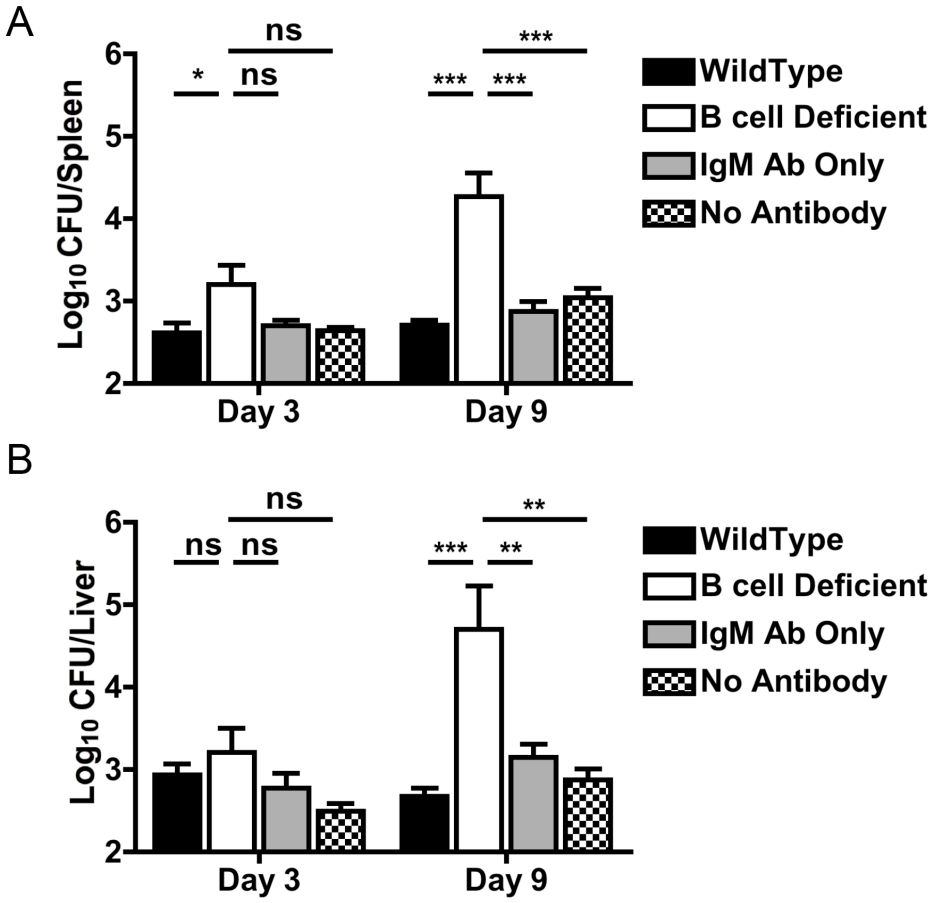
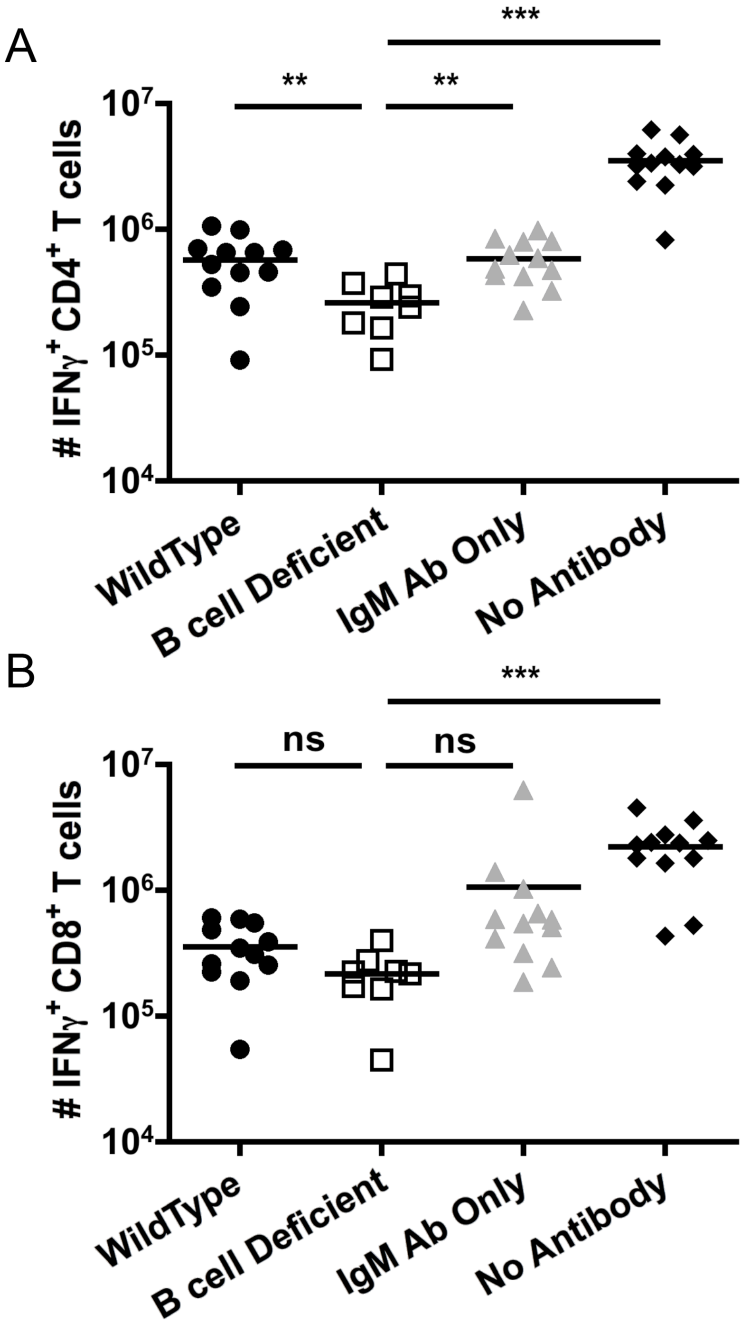

A
ns
***
*
ns
***
***
WildType
B cell Deficient
IgM Ab Only
No Antibody
Log10 CFU/Spleen
6
5
4
3
2
Day 3
Day 9
B
ns
**
ns
ns
***
**
WildType
B cell Deficient
IgM Ab Only
No Antibody
Log10 CFU/Liver
6
5
4
3
2
Day 3
Day 9

FIGURE 2-3. Antibody is not required for optimal *Salmonella*-specific Th1 cells.

(**A-B**) Mice were immunized IV with $5x10^5$ BRD509 and 42-47 days later injected IV with 10^8 HKST to stimulate T cell responses. Bar graphs showing mean number of (**A**) CD4 or (**B**) CD8 T cell producing IFN-γ after stimulation with HKST. One way ANOVA comparing all groups of CD4+ T cell IFNγ production yielded a p value of <0.0001 (***), and comparing CD8+ T cell IFNγ production yielded a p value of 0.0002 (***).

FIGURE 2-3


A
***
**
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10^7
10^6
10^5
10^4
IFNγ+ CD4+ T cells
WildType
B cell Deficient
IgM Ab Only
No Antibody
B
***
ns
ns
10^7
10^6
10^5
10^4
IFNγ+ CD8+ T cells
WildType
B cell Deficient
IgM Ab Only
No Antibody

FIGURE 2-4. **Transgenic mIgM and m+s IgM have intact B and T cell compartments and resolve attenuated *Salmonella* infection.** (**A-D**) Naïve WildType, B cell Deficient (JhD), IgM Ab Only (m+s IgM), and No Antibody (mIgM) mice were sacrificed and spleen, lymph nodes, peritoneal cavity and Peyer's Patches were harvested. Tissues were homogenized to a single cell suspension and stained to determine the number and percentage of B220+CD3- B cells (**A**), CD3+B220- T cells (**B**), CD3+CD4+CD8- T cells (**C**), and CD3+CD8+CD4- T cells (**D**). B cell Deficient mice contained no Peyer's Patches. All other mice had 5-8 Peyer's Patches. (**E**) WildType, B cell Deficient, IgM Ab Only and No Antibody mice were immunized IV with $5x10^5$ BRD509. At the 7, 28 and 44 days post live vaccination, mice were sacrificed and spleen, liver and bone marrow from one femur and tibia per mouse were harvested. Bacterial growth *in vivo* was calculated by plating serial dilutions of organ homogenates onto MacConkey agar and bacterial counts were determined after overnight incubation at 37ºC.

FIGURE 2-4 A-D

A

Tissue	Spleen		Lymph Nodes		Peritoneal Cavity		Peyer's Patches	
B cells (Live→ Singlet→ B220+CD3- cells)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)
WildType	44.90 (1.70)	$3.01x10^7$ ($2.85x10^6$)	19.27 (6.34)	$3.81x10^6$ ($4.44x10^6$)	14.97 (2.23)	$1.46x10^5$ ($6.56x10^4$)	27.70 (4.37)	$2.74x10^5$ ($1.78x10^5$)
B cell Deficient	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
IgM Ab Only	34.03 (1.76)	$9.51x10^6$ ($1.61x10^6$)	18.43 (7.53)	$3.33x10^6$ ($3.10x10^6$)	20.27 (4.66)	$3.11x10^4$ ($2.18x10^4$)	25.07 (7.96)	$2.46x10^5$ ($3.05x10^5$)
No Antibody	37.60 (1.75)	$3.37x10^7$ ($1.04x10^7$)	21.60 (1.42)	$1.30x10^7$ ($1.57x10^6$)	28.37 (0.70)	$1.78x10^6$ ($2.83x10^5$)	39.93 (11.52)	$1.16x10^6$ ($1.32x10^6$)

B

Tissue	Spleen		Lymph Nodes		Peritoneal Cavity		Peyer's Patches	
T cells (Live→ Singlet→ CD3+B220- cells)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)
WildType	22.80 (4.58)	$1.56x10^7$ ($5.03x10^6$)	72.03 (9.52)	$1.26x10^7$ ($1.28x10^7$)	5.10 (1.37)	$4.74x10^4$ ($1.79x10^4$)	4.48 (1.62)	$4.59x10^4$ ($4.12x10^4$)
B cell Deficient	22.73 (4.73)	$2.12x10^6$ ($9.51x10^5$)	87.37 (3.32)	$3.55x10^6$ ($2.13x10^6$)	7.53 (6.89)	$1.87x10^4$ ($1.81x10^4$)	n/a	n/a
IgM Ab Only	28.80 (4.92)	$8.20x10^6$ ($2.59x10^6$)	72.83 (6.77)	$1.17x10^7$ ($7.56x10^6$)	27.23 (14.17)	$4.18x10^4$ ($2.86x10^4$)	6.59 (2.68)	$6.11x10^4$ ($7.00x10^4$)
No Antibody	23.67 (2.25)	$2.10x10^7$ ($5.01x10^6$)	64.73 (4.46)	$3.87x10^7$ ($2.69x10^6$)	2.41 (14.17)	$3.35x10^5$ ($2.86x10^4$)	8.57 (2.95)	$2.51x10^5$ ($2.90x10^5$)

C

Tissue	Spleen		Lymph Nodes		Peritoneal Cavity		Peyer's Patches	
CD4 T cells (Live→ Singlet→ CD3+B220- cells→ CD4+CD8-)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)
WildType	67.23 (2.06)	$1.05x10^7$ ($3.59x10^6$)	70.63 (2.67)	$8.82x10^6$ ($8.81x10^6$)	74.17 (7.11)	$3.55x10^4$ ($1.48x10^4$)	83.13 (4.35)	$3.59x10^4$ ($3.02x10^4$)
B cell Deficient	65.97 (4.58)	$1.42x10^6$ ($7.03x10^5$)	77.90 (8.01)	$2.60x10^6$ ($1.71x10^6$)	76.67 (4.81)	$2.56x10^4$ ($1.45x10^4$)	n/a	n/a
IgM Ab Only	67.97 (0.31)	$5.58x10^6$ ($1.78x10^6$)	66.73 (2.25)	$7.83x10^6$ ($5.11x10^6$)	77.10 (2.49)	$3.27x10^4$ ($2.30x10^4$)	74.63 (19.61)	$5.06x10^4$ ($5.98x10^4$)
No Antibody	75.37 (0.64)	$1.58x10^7$ ($3.67x10^6$)	69.33 (1.85)	$2.69x10^7$ ($2.53x10^6$)	81.33 (1.31)	$2.74x10^5$ ($1.42x10^5$)	85.73 (3.01)	$2.16x10^5$ ($2.50x10^5$)

D

Tissue	Spleen		Lymph Nodes		Peritoneal Cavity		Peyer's Patches	
CD8 T cells (Live→ Singlet→ CD3+B220- cells→ CD8+CD4-)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)	% (+/- SD)	Abs # (+/- SD)
WildType	28.63 (1.23)	$4.43x10^6$ ($1.33x10^6$)	25.93 (2.03)	$3.36x10^6$ ($3.48x10^6$)	19.47 (6.29)	$8.86x10^3$ ($3.96x10^3$)	4.28 (1.93)	$2.49x10^3$ ($3.10x10^3$)
B cell Deficient	23.53 (2.20)	$4.84x10^5$ ($1.85x10^5$)	24.13 (3.84)	$8.04x10^5$ ($3.75x10^5$)	14.39 (6.23)	$4.08x10^3$ ($1.94x10^3$)	n/a	n/a
IgM Ab Only	25.33 (0.64)	$2.09x10^6$ ($6.94x10^5$)	27.67 (2.02)	$3.29x10^6$ ($2.20x10^6$)	18.40 (1.55)	$7.51x10^3$ ($5.04x10^3$)	4.26 (1.26)	$3.14x10^3$ ($4.02x10^3$)
No Antibody	21.07 (0.61)	$4.43x10^6$ ($1.13x10^6$)	25.17 (0.25)	$9.73x10^6$ ($2.53x10^6$)	15.13 (0.55)	$5.01x10^4$ ($2.34x10^4$)	4.64 (1.66)	$1.48x10^4$ ($2.04x10^4$)

FIGURE 2-4 E

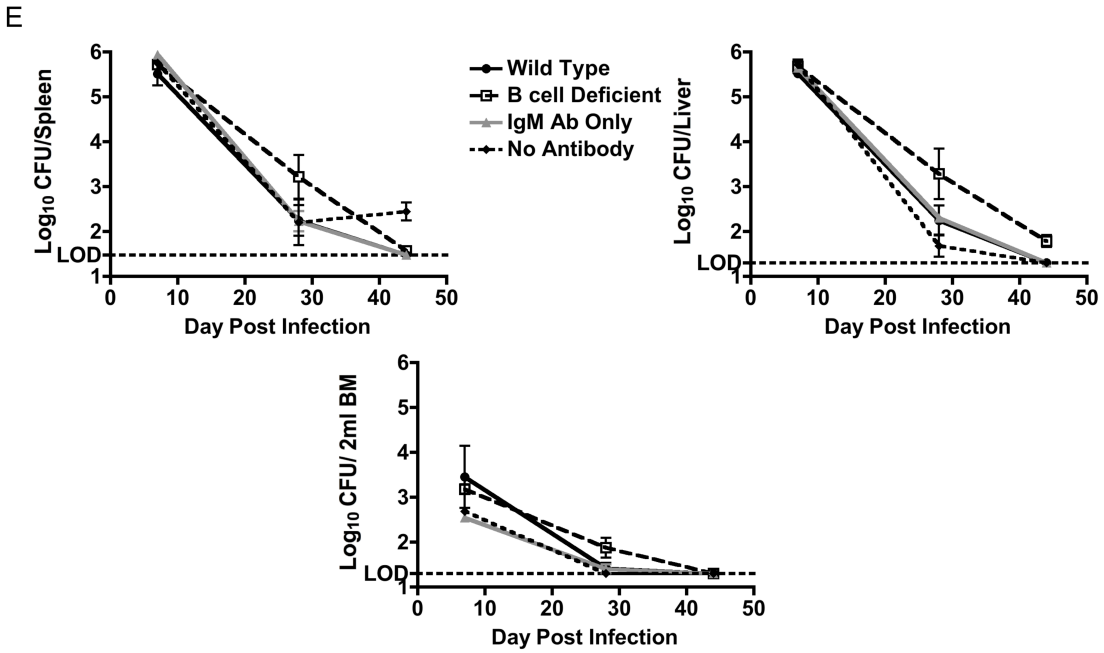
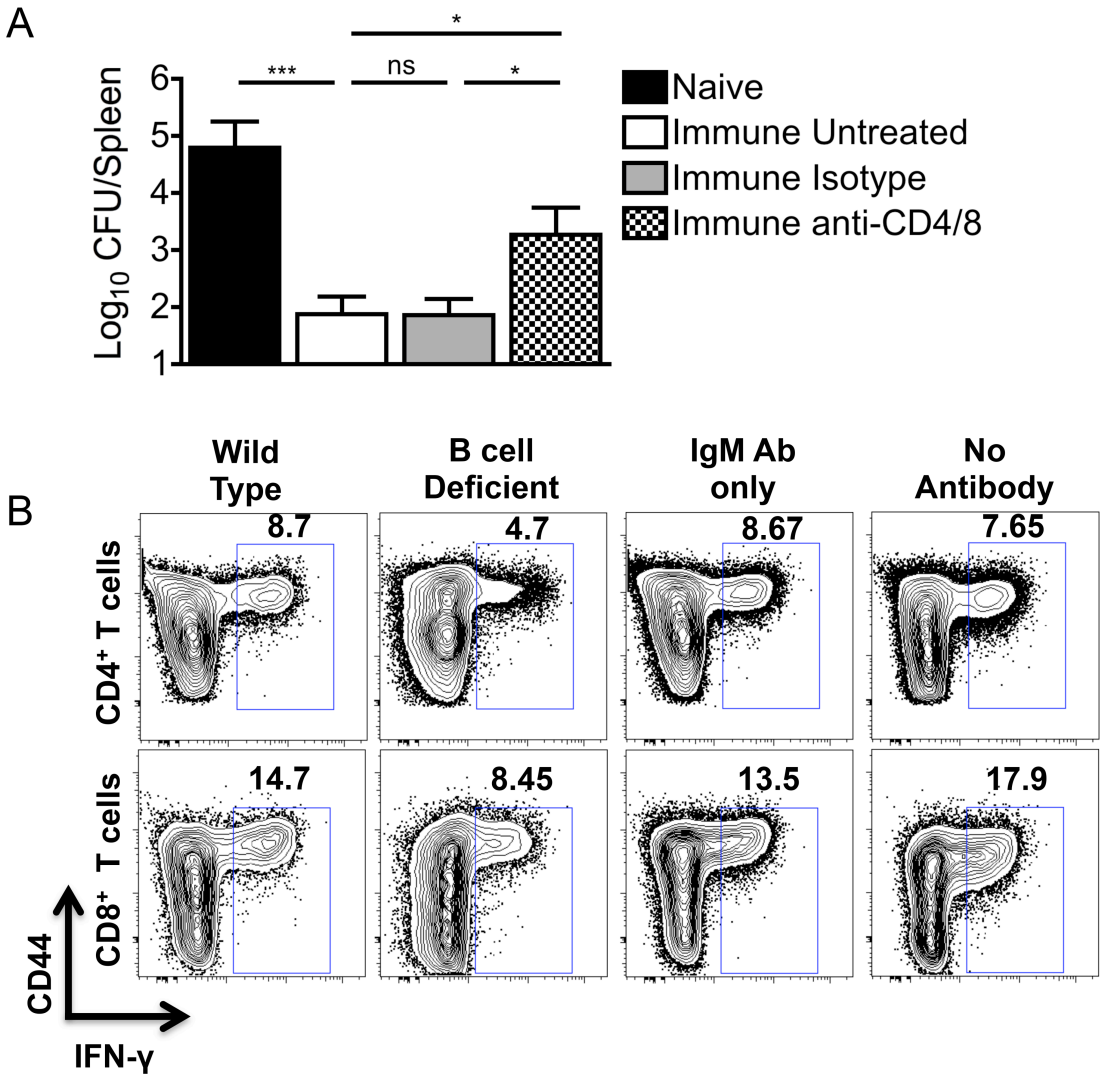

E
Log10 CFU/Spleen
Log10 CFU/Liver
Log10 CFU/ 2ml BM
LOD
Day Post Infection
Wild Type
B cell Deficient
IgM Ab Only
No Antibody

FIGURE 2-5. Robust IFN-γ production by T cells does not require antibody secretion. (**A**) Mice were immunized IV with $5x10^5$ BRD509 and 42 to 85 days later were administered 750 µg of depleting anti-CD4 and anti-CD8 or isotope control antibodies before oral challenge with $5x10^7$ SL1344. CFUs were enumerated in the spleen of infected mice, 3 days later. (**B**) Mice were immunized IV with $5x10^5$ BRD509 and 42 days later injected IV with 10^8 HKST to stimulate T cell responses. FACS plots show intracellular IFN-γ production and CD44 surface expression after gating on CD4 and CD8 T cells.

FIGURE 2-5


A
*
***
ns
*
Log10 CFU/Spleen
6
5
4
3
2
1
Naive
Immune Untreated
Immune Isotype
Immune anti-CD4/8
B
Wild Type
B cell Deficient
IgM Ab only
No Antibody
CD4+ T cells
8.7
4.7
8.67
7.65
CD8+ T cells
14.7
8.45
13.5
17.9
CD44
IFN-γ

Chapter 3

B cell expansion and germinal center formation are delayed during *Salmonella* infection independent of *Salmonella* Type-III secretion system effector proteins

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Abstract

New vaccines against NTS and typhoid are urgently required and human and mouse infection models indicate a critical role for CD4+ T cells and B cells in protective immunity. Previous studies also suggest that live *Salmonella* can actively inhibit host protective immunity, but the mechanism remains poorly defined. Here, we utilized a novel OVA-B cell tetramer approach to examine the antigen-specific B cell response to live *Salmonella*-OVA vaccination. OVA-B cell expansion and germinal center formation were delayed until 45 days in vaccinated mice, suggesting active inhibition by bacteria. The *Salmonella* Pathogenicity Island II locus has been implicated in culling CD4+T cell responses during live vaccination. Thus, we examined the effect of the SPI2 locus on B cell responses by concurrently infecting mice with a ΔSPI2 mutant, immunizing with OVA protein and measuring the expansion and germinal center formation of OVA-B cells. WildType, and not ΔSPI2, *Salmonella* inhibited expansion of OVA-specific B cells 7 days after immunization. Although infection with single SPI2 effector mutants ΔsifA and ΔsseF showed a partial rescue of OVA B cell responses at day 7, this directly correlated with a decrease in bacterial load compared to WildType infection. The results of this work suggest two dilemmas for *Salmonella* vaccination. 1. SPI2 effectors cause a sub-optimal *Salmonella*-specific B cell response during live vaccination, which directly correlates with bacterial load. 2. Subunit vaccination, in conjunction with active *Salmonella* infection, may elicit a weaker immune response to the administered subunit vaccine.

Introduction

Infection with different *Salmonella* serovars causes Typhoid and Non-typhoidal Salmonelloses (NTS) and both these diseases are major causes of morbidity and mortality worldwide (3). The heaviest burden of disease falls upon children under 5 years of age in south and southeastern Asia and sub-Saharan Africa. Invasive NTS infections are a growing problem among HIV-infected adults, malaria-infected children and immune-compromised individuals, primarily in sub-Saharan Africa (18-20). Although there are currently two licensed Typhoid vaccines, they offer only limited (20-70%) efficacy for up to 5 years (25). The first of these, Ty21a (Vivotif®), is a safe, live attenuated strain of *S.* Typhi that requires four doses for efficacy. The second licensed typhoid vaccine, ViCPS (Typhim Vi®), is a purified capsule polysaccharide that is effective at curtailing typhoid outbreaks and providing short-term protection to travelers. However, these typhoid vaccines are not licensed for use in children less than 2 years of age, and neither is routinely utilized in endemic areas.

The limited protection afforded by vaccination with ViCPS is attributed to the induction of a short-lived T-independent antibody response (90). Improved Vi Capsular vaccines are being produced that utilize conjugation to diphtheria toxoid or tetanus toxoid in order to prime T cell responses to produce long-lasting protective antibodies (28). The mechanism of protection mediated by the live attenuated Ty21a vaccine is generally assumed to involve cellular immunity, and indeed, *Salmonella*-specific T cell responses have been detected in immunized patients (91, 92). Antibody responses are also induced following immunization with live attenuated *Salmonella* vaccines, but the role of these antibodies in protective immunity has not been examined (92, 93). The generation of improved vaccines for typhoid and NTS will require deeper understanding

of adaptive immunity to *Salmonella* infection and knowledge of how *Salmonella* are able to subvert a host protective response.

Infection of inbred mice with *Salmonella* Typhimurium is a well-established model of typhoid and invasive Salmonellosis. Susceptible Nramp^{s} strains such as C57BL/6 and Balb/c mice are unable to resolve primary infection with virulent *Salmonella* but can be immunized successfully with live attenuated *Salmonella* strains. The resolution of primary infection with attenuated bacteria requires CD4+ Th1 cells, IL-12 and IFN-γ (48, 80). Acquired immunity to secondary virulent infection similarly requires Th1 cells but surprisingly B cells and/or antibodies are also essential for protection (69-71, 94). In resistant Nramp^{r} mouse strains such as 129/SvJ, primary infection with virulent *S.* Typhimurium can be resolved and antibodies, but not memory CD4+ T cells, are required for control of secondary infection (32). Thus, in both susceptible and resistant mouse models B cells play an essential role in resolution of infection. These observations in a mouse model of invasive *Salmonella* infection also mirror findings in human disease. Individuals with a primary genetic deficiency in IL-12 or IFN-γ signaling are more susceptible to NTS (42, 79), demonstrating the importance of Th1 cells in bacterial clearance. However, the absence of *Salmonella*-specific antibodies in young children correlates with increased susceptibility to NTS (75), suggesting that B cell responses are critical for protection. While some progress has been made in understanding the CD4+ T cell response to infection, the B cell response during infection with *Salmonella* is not clearly understood.

Like many successful pathogens, *Salmonella* have evolved sophisticated mechanisms to evade and subvert protective host immune responses. *Salmonella* are able to subvert macrophage phagocytosis and can survive and proliferate within *Salmonella*-containing vacuoles (SCVs), which have been adapted to suit bacterial

growth. Genes within the *Salmonella* Pathogenicity Island 2 (SPI2) are essential for survival within macrophages and encode a Type III secretion system (T3SS) that allows transfer of bacterial proteins to the host cell cytosol (95) . Once inside the acidic environment of the macrophage endosome, *Salmonella* express SPI2 genes that together form a needle complex that penetrates the SCV membrane. Using this needle complex, *Salmonella* inject SPI2 effector proteins that help to maintain the SCV structure by affecting filament formation and actin polymerization surrounding the SCV (96). In addition, some of the proteins injected into the cytosol can affect the induction and maintenance of *Salmonella*-specific immune responses. Several in vitro studies have shown that SPI2 effector proteins can inhibit the antigen presentation activity of bone marrow-derived dendritic cells (BMDCs) to CD4 and CD8 T cells (97). Importantly, SPI2 effectors have also been implicated in eliminating *Salmonella*-specific CD4 T cells after activation and clonal expansion has occurred, a process referred to as “culling”. Previous work in our laboratory demonstrated that an intact SPI2 T3SS is required for culling *Salmonella*-specific TCR transgenic CD4+ T cells during infection of Nramp^{s} mice (56). Subsequently, Ertelt *et al* have demonstrated that SPI2 genes are required for the culling of the highest avidity activated CD4+ T cells during infection of an Nramp^{r} strain. In this model, culling occurred irrespective of whether cognate antigen was expressed by *Salmonella* or exogenously by a different pathogen. Although these studies point to the importance of SPI2 genes in the regulation of CD4+ T cell effector responses, they were unable to identify the individual effector proteins responsible for this effect. Furthermore, neither of these previous studies examined whether SPI2 effector proteins were able to affect the generation of B cell responses to *Salmonella* infection. Previous work by Cunningham *et al* has demonstrated that antibody responses to *Salmonella* LPS, flagellin and outer membrane proteins can be detected early after infection, however, the

antibody response to other *Salmonella* antigens is delayed (67). Furthermore, the germinal center response to infection was also delayed, suggesting that *Salmonella* can directly or indirectly inhibit B cell responses.

In this current study we have directly visualized the endogenous *Salmonella*-specific B cell response generated during primary infection of Nramp^r mice. We chose the Nramp^r infection model because it allows for analysis of B cell responses in a model where antibodies are required for protective immunity. Using newly developed tetramer reagents and a methodology devised to study OVA-specific B cells (98), we have visualized primary OVA-specific B cell expansion and germinal center formation during infection with *Salmonella*-OVA. Surprisingly, *Salmonella*-specific B cell expansion was delayed until bacterial clearance had already occurred. In addition, *Salmonella* infection inhibited B cell expansion and germinal center formation after immunization of mice with OVA and the SPI2 effector proteins sifA and sseF mediated this inhibitory effect. The inhibitory effect of sifA and sseF correlated directly with the ability of these proteins to influence bacterial load in mouse spleen and liver. Together, our data visualize endogenous pathogen-specific B cell responses to infection and demonstrate that unlike for culling of activated CD4+ T cells, inhibition of B cell responses during *Salmonella* infection may be less SPI2-dependent and more bacterial load-dependent.

Materials and Methods

Bacteria Strains and Plasmids

Bacterial strains and plasmids utilized in this study are listed in Table 1 below. The χ4550 strain and the pYA3149 plasmid were kindly provided by Roy Curtiss III. The χ4550/pYA3149-OVA strain was kindly provided by Marc Jenkins. The SPI2 type three secretion system (T3SS-2) single effector-deficient mutants were kindly provided by Michael Hensel. For the strains generated in this study, the high frequency generalized transducing mutant of bacteriophage P22 (*HT105/1 int-201*) was used to transfer mutations between strains; as strain SR-11 is naturally resistant to P22 infection, cleaning transductants of phage was not necessary. A *Salmonella* Pathogenicity Island 2 type three secretion system (T3SS-2) deficient mutant of χ4550 was generated by transducing the Δ*spiB*::KSAC deletion of SPN450 into χ4550/pYA3149-Empty and selecting for kanamycin resistant transductants, yielding SPN524/pYA3149-Empty. PCR amplification (PCR Supermix, Invitrogen) was utilized to confirm null amplification of *spiB* (Primers: 5'-TGGCTGAATGAAGGTAACC-3' and 5'-CTCAGATGGACAATTTCTCC-3') and to confirm the location of the KSAC cassette as previously described (99). An SPN524 derivative that had titrated out pYA3149-Empty was obtained by growing SPN524/pYA3149-Empty in LB + DAP, plating dilutions on LB agar + DAP, and then screening colonies for DAP auxotrophy on LB agar. Electrocompetent cells of SPN524 were then generated as previously described(100). Purified pYA3149-OVA (Miniprep spin kit, Qiagen) from χ4550/pYA3149-OVA was then electroporated into SPN524 and electroporants selected for by plating on LB agar. Presence of pYA3149-OVA was checked by purifying the plasmid and comparing band size to pYA3149-Empty after BamHI (New England Biolabs) restriction digestion and agarose gel electrophoresis.

Designation	Relevant genotype	Source
S. Typhimurium strains		
χ4550	SR-11 *gyrA1816* Δ*asdA1*[*zhf-4*::Tn10] Δ*crp-1* Δ*cya-1*	(101)
SPN450	IR715 Δ*spiB*(+25 to +1209)::KSAC	(102)
SPN524 (ΔSPI2)	χ4550 Δ*spiB*(+25 to +1209)::KSAC	This study
NCTC 12023	Wild Type	
HH104	Δ*sseC::aphT*	(95)
HH107	Δ*sseF::aphT*	(95)
HH108	Δ*sseG::aphT*	(95)
MvP373	Δ*sscB sseFG::aph*	(103)
MvP376	Δ*sspH2::aph*	(104)
MvP389	Δ*sifB*	(104)
MvP390	Δ*sspH1*	(104)
MvP392	Δ*sseJ*	(104)
MvP393	Δ*sseI*	(104)
MvP394	Δ*slrP*	(104)
MvP498	Δ*pipB2::aph*	(97)
MvP505	Δ*sopD2::aph*	(97)
MvP509	Δ*sifA::aph*	(97)
MvP570	Δ*sseK1::aph*	(97)
MvP571	Δ*sseK2::aph*	(97)
MvP873	Δ*gogB*	(97)
MvP874	Δ*pipB*	(97)
PhoP^c	Constitutive PhoP/Q expression	(105)
Plasmids		
pYA3149-Empty	*asdA*+	(101)
pYA3149-OVA	*asdA*+, OVA expression	(106)

Mice and Infections

C57Bl/6J mice (Jackson Laboratories) were intercrossed with 129Sv/J mice (Jackson laboratories). F1 mice from this cross were used in these studies. All mice were housed in specific pathogen free conditions at the University of Minnesota-Twin Cities and University of California Davis for breeding and experimentation. All *S.* Typhimurium strains were grown overnight in Luria-Bertani broth and diluted in PBS after estimating bacterial counts by spectrophotometry. Mice were immunized IV with 10μg ovalbumin with 10μg LPS and/or IV with $5x10^5$ χ4550-OVA, χ4550-comp and ΔSPI2; or IV with $5x10^3$ of the NCTC 12023 and SPI2 effector mutant strains and/or 10μg ovalbumin with

10^8 Heat-Killed *Salmonella* (HKST). Infection doses were confirmed by plating serial dilutions onto MacConkey agar plates. Any moribund infected mice were euthanized as stipulated in our IACUC protocol. Bacterial growth *in vivo* was calculated by plating serial dilutions of organs homogenized in 0.01% TritonX /1XPBS onto MacConkey agar and bacterial counts were determined after overnight incubation at 37ºC.

OVA and Decoy B cell Tetramer Production

Ovalbumin and Decoy tetramers were produced as previously described (98). Briefly, ovalbumin (Sigma Aldrich) was biotinylated using an EZ-link Sulfo-NHS-LC-Biotinylation kit (Thermo Fisher Scientific) using a 7:1 molar ratio of biotin to ovalbumin. Free biotin was removed via desalting columns (GE Healthcare). The molar amount of biotinylated ovalbumin was measured via Western blot by incubating a fixed amount of biotinylated ovalbumin with differing amounts of SA-PE (Prose) at room temperature, followed by SDS-Page (Bio-Rad Laboratories). The gel was transferred onto PVDF membrane and probed with SA-AP to determine the point at which there was free biotin on the ovalbumin to bind to the SA-AP. The ratio of SA-PE to biotinylated ovalbumin was then used to calculate the molar concentration of ovalbumin actually biotinylated. Biotinylated ovalbumin was incubated with SA-PE in a 6:1 ratio for 30 minutes. OVA- B cell tetramer was concentrated and excess biotinylated ovalbumin removed via centrifugation in a 100-kDa molecular weight cutoff Amicon Ultra filter (Millipore). Tetramer was stored at 4°C at a concentration of 1μM. The nonspecific “decoy” tetramer was produced by conjugation of SA-PE to AF647 (Invitrogen) for 60 minutes at room temperature. Free AF647 was removed via centrifugation in a 100-kDa molecular weight cutoff Amicon Ultra filter (Millipore). The SA-PE*AF647 was incubated with 20 molar

excess of biotin for at least 30 minutes at room temperature and decoy tetramer was stored at 1μM at 4°C.

B cell Tetramer Staining, Enrichment and Flow Cytometry

Tetramer staining and enrichment were performed as previously described (98, 107). At the indicated times after infection or immunization mice were sacrificed and the spleen and all lymph nodes (inguinal, popliteal, brachial, axillary, cervical, lumbar, caudal, renal, pancreatic and mesenteric) were harvested and homogenized. Cells were incubated with Decoy Tetramer for 10 minutes at room temperature followed by OVA-Tetramer for 15 minutes on ice. After washing excess tetramer, stained samples were incubated for at least 30 minutes on ice with anti-PE microbeads (Miltenyi) and enriched by passing through a magnetized column. Both the tetramer-bound and flow-through samples were subsequently stained with CD11c-, CD11b-, F4/80-, CD4-, CD8-, Gr-1-APC-Cy7, B220- eF450, CD38- AF700, GL7- FITC, and IgM- PE-Cy7. All antibodies were purchased from eBioscience, BD Biosciences or Biolegend. Tetramer-specific cells were analyzed via flow cytometry (BD Canto and Fortessa) after gating on lymphocytes, singlets and B220+Dump- cells. The number of OVA-tetramer positive cells was determined by acquiring the entire bound fraction of enriched cells. In cases where there was an excess of OVA Tetramer + cells that spilled into the flow-through, 1/5 of the flow-through samples were acquired on the flow cytometer and collected numbers multiplied by 5 and added to bound fraction numbers. Flow cytometry data was analyzed using FlowJo Software (TreeStar, Inc).

OVA-specific Antibody Responses

Blood was collected by retro-orbital bleeding and sera prepared and stored at -20°C. OVA-specific IgM and IgG antibodies were measured by ELISA, as previously described (83).

Statistical Analysis

Statistical analysis was performed using unpaired t tests (Prism 4, GraphPad Software, Inc., La Jolla, CA). Statistical differences between groups are highlighted with *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

Results and Discussion

Ovalbumin B cell tetramers can be used to directly visualize Salmonella-specific B cell responses

We sought to identify and define the endogenous B cell response to an antigen expressed by *Salmonella* in vivo. To achieve this goal, we made use of χ4550, an attenuated Δasd auxotrophic mutant strain that is unable to grow in the absence of the pYA3149 plasmid containing the complementing *asd* gene. This plasmid rescues the lethal mutation and also provides a stable vector for constitutive expression of heterologous proteins in *Salmonella* (101). We utilized a previously described χ4550 strain containing the pYA3149-OVA vector with chicken ovalbumin expressed under the Ptrc promotor, (St-OVA; (106)). As previously reported, this strain expresses approximately 70 μg of OVA per 10^8 Salmonella in the periplasmic space while an χ4550 strain containing the control pYA3149 plasmid (St-Comp) does not.

The endogenous B cell response to OVA can be directly identified by flow cytometry using OVA B cell tetramers, "decoy" tetramers and magnetic bead enrichment, as previously described (98, 107). Briefly, the full-length protein ovalbumin is biotinylated and subsequently tetramerized to a Streptavidin core conjugated to phycoerythrein (SA-PE). Endogenous OVA-specific B cell receptors can bind OVA tetramers and anti-PE magnetic beads are used to enrich for the tetramer-bound OVA-specific B cells. Any B cells specific for the SA-PE core are gated out using a decoy tetramer containing a complementary SA-PE core conjugated to AlexaFluor 647 (AF647). Using this methodology, SA-PE-, OVA- and AF647-specific B cells can be distinguished by flow cytometry (Figure 3-1A). Our gating strategy for identifying OVA-specific B cells consisted of an initial lymphocyte gate, followed by a singlet gate and a

dump channel containing CD11c, CD11b, F4/80, CD4, CD8, Gr-1. By gating on B220+/Dump- cells, OVA-specific B cells that bind the OVA-tetramer can be clearly identified (Fig. 3-1B) At the peak of clonal expansion, a 6-fold expansion of OVA tetramer+ B cells was detected in the spleen and lymph nodes of St-OVA infected mice compared to mice infected with St-Comp (Figure 3-1C).

The peak of OVA-specific B cell expansion and germinal center formation occurs after Salmonella-OVA infection has resolved

To visualize the kinetics of antigen-specific B cell expansion during *Salmonella* infection, C57BL6x129/sv F1 Nramp^r mice were infected IV with St-OVA and sacrificed at various timepoints to examine OVA-tetramer staining in harvested secondary lymphoid tissues. Livers were also collected, homogenized and plated onto MacConkey agar to examine bacterial growth in the same mice. During the first 19 days of infection, Salmonella expanded in the liver of infected mice, however, bacterial growth was significantly reduced by day 20 and bacteria fell below the level of detection in all mice by day 37 (Figure 3-2A). Surprisingly, OVA-specific B cell expansion was minimal during the period of active infection but peaked at day 45, with levels declining back down to baseline by 75 days post-infection (Figure 3-2A). By tetramer staining, naïve mice were calculated to have an average of 600 OVA-specific B cells, while mice infected with St-OVA had an average of 3100 OVA-specific B cells at day 45, thus constituting a 5-fold expansion of these cells due to *Salmonella*-OVA infection. The delayed expansion of OVA-specific B cells was also consistent with the late development of OVA-specific antibody responses in *Salmonella*-OVA-infected mice (Fig. 3-2B). Although previous studies have shown that the expansion of OVA-specific B cells to OVA immunization can peak as early as day 7 post immunization, a delay in *Salmonella*-specific B cell

responses is consistent with previous studies that described a delay in antibody responses to some *Salmonella* antigens (67). In a separate set of experiments, St-OVA mice showed a peak of OVA-specific B cell expansion at day 30 post-infection (Figures 3-4A). Notably, this peak is still after the bacteria in most mice have reached undetectable levels in the liver.

We next examined the phenotype of expanded OVA-specific B cells in *Salmonella*-OVA infected mice by examining expression of CD38 and GL7 (Fig. 3-3A). CD38+GL7- cells are described as naïve/memory cells, whereas a CD38-GL7+ phenotype defines germinal center B cells. In addition, an intermediate CD38+GL7+ population has also been reported that may represent activated B cells that have not yet committed to a germinal center or memory cell fate. In our analysis, we have defined this intermediate population as “Activated” cells and the overall gating scheme used is summarized in Fig. 3-3B. In uninfected mice, OVA-specific B cells were CD38+GL7-, typical for naïve B cells. As described above, peak expansion of OVA-specific B cells during St-OVA infection occurred at day 45, after the active infection had been cleared. However, the germinal center reaction was first detected at day 37, since at this time point 18% of OVA tetramer+ B cells had a CD38-GL7+ phenotype (Fig. 3-3 A-D). This germinal center CD38-GL7+ response peaked at day 45 when the percentage and absolute number of OVA tetramer+ cells expressing a CD38-GL7+ phenotype had increased substantially. By day 75 post-infection, all of the surviving B cells had returned to expressing a CD38+GL7- surface phenotype (Fig. 3-3 A, C, D). The increase in number and percentage of cells expressing germinal center phenotype was not observed when gating on OVA tetramer+ B cells in mice infected with St-Comp (Fig. 3-3 E & F), indicating that the surface phenotype changes detected on expanded B cells was due to antigen expression and not simply a non-specific consequence of *Salmonella*

infection. In a separate set of experiments, the peak of the OVA-specific B cell germinal center response was at day 30 instead of day 45, which is still after bacteria in most mice have reached undetectable levels (Fig. 3-4 A-B).

OVA-specific B cell expansion and activation during immunization are inhibited by Salmonella

The marked delay in the clonal expansion and activation of OVA-specific B cells during *Salmonella*-OVA infection contrasted with the rapid expansion of OVA-specific B cells that was reported to occur after OVA immunization (98). We therefore examined whether *Salmonella* infection can inhibit the antigen-specific B cell expansion and activation that occurs during OVA-immunization. In mice immunized with OVA and LPS, a 100-fold expansion of OVA tetramer+ B cells was detectable by day 7 post-immunization, and the vast majority of these B cells expressed a CD38-GL7+ surface phenotype (Fig. 3-5A and B). Consistent with our data above, there was no detectable expansion of OVA-specific B cells in St-OVA infected mice at this same time point and OVA-specific B cells were CD38+GL7- (Fig. 3-5 A and B). In mice immunized with OVA and infected with St-OVA, there was a marked reduction in clonal expansion as measured by percentage and absolute number of OVA-specific B cells when compared to OVA immunization alone (Fig. 3-5 A and B). Similarly, while over 80% of OVA-specific B cells in OVA/LPS immunized mice expressed a germinal center phenotype, only 40% of OVA-specific B cells had this phenotype in the presence of St-OVA (Fig. 3-5A, C and D). Thus *Salmonella* infection can effectively block the expansion of OVA-specific B cells responding to antigen and limit the acquisition of a germinal center phenotype. Interestingly, *Salmonella* infection also caused an increase in the percentage of

“activated” CD38+GL7+ B cells (Fig. 3-5 A, C, & D), suggesting that these cells had been blocked during the transition to a germinal center CD38-GL7+ phenotype.

A functional SPI2 needle is required for Salmonella to inhibit B cell expansion

The marked reduction in B cell expansion and germinal center formation of OVA-specific B cells during *Salmonella* infection suggested that *Salmonella* may directly or indirectly inhibit B cell responses. In fact, several previous studies have demonstrated that a functional SPI2 T3SS is essential for the inhibitory effects of *Salmonella* on T cell responses in vivo and in vitro (56, 57, 97). To examine whether the SPI2-encoded T3SS was required for inhibition of OVA-specific B cells, we introduced a chromosomal deletion at the SPI2 locus in the parental strain χ4550 (ΔSPI2). When OVA-immunized mice were infected with ΔSPI2 there was a significant rescue of OVA-specific B cell expansion (Figure 3-5A & B), and germinal center surface phenotype on responding B cells (Figure 3-5A, C & D). Therefore a functional SPI2 T3SS is required for *Salmonella* to inhibit germinal center formation and antigen-specific B cell expansion.

SPI2 effector proteins sifA and sseF inhibit expansion and germinal center formation of OVA-specific B cells

The SPI2-encoded T3SS is responsible for injecting up to 30 effector proteins into the host cell cytosol (96). In order to identify individual effector proteins involved in B cell inhibition, we utilized a SPI2 mutant library that was previously generated using the virulent NCTC 12023 (WT) strain (97). Mice were immunized with OVA and co-infected with WT *Salmonella*, strains lacking a functional SPI2 T3SS (Δ*ssaV* and Δ*sseC*), or strains with lesions in individual T3SS effector genes. As expected, OVA-specific B cells expanded in mice immunized with OVA, while B cell clonal expansion and germinal

center phenotype development were effectively blocked by concurrent infection with WT *Salmonella* (Fig. 3-6A). In marked contrast, concurrent infection with a Δ*ssaV* mutant in immunized mice allowed a 14-fold expansion of OVA-specific B cells and complete rescue of a germinal center phenotype in expanded B cells (Figure 3-6A). In a screen of 15 individual effector mutants, most of these bacterial strains fully inhibited OVA-specific B cell clonal expansion and the generation of a germinal center phenotype. However, infection with Δ*sifA Salmonella* allowed an 8-fold expansion of OVA-specific B cells and 45% of these cells expressed a germinal center phenotype (Figure 3-6A, B & C). A similar loss of inhibitory capacity was observed when using a Δ*sseF Salmonella* strain (Figure 3-6A, B & C), indicating that at least 2 T3SS effector proteins are required for maximal inhibition of B cell responses.

SifA and SseF-dependent inhibition of OVA- B cell responses correlates with control of bacterial load in mice

Since SPI2 effector proteins are known to regulate the ability of *Salmonella* to replicate in vivo (95), we sought to determine if the inhibition of OVA-specific B cell responses was indeed due to SPI2 effectors or a decrease in bacterial load. To test this possibility we infected mice with a PhoP^c mutant of *S.* Typhimurium and co-administered exogenous OVA. PhoP^c is a strain of *S.* Typhimurium with constitutive expression of the PhoP/Q, a two-component regulon independent of the SPI2 T3SS responsible for sensing the endosomal environment and regulating expression of genes involved in intraphagosomal survival. Constitutive expression of PhoP-activated genes (pag) and constitutive repression of PhoP-repressed genes leads to decreased survival within mouse macrophages (105). Seven days following infection and immunization, spleens and lymph nodes were harvested and OVA-specific B cells enumerated. Mice infected

with PhoP^{c} were able to mount robust OVA-specific B cell expansion and germinal center formation, equivalent to Δ*ssaV* and Δ*sseC* mutants with the functionally deficient SPI2 T3SS (Figure 3-6 A-C).

Since infection with Δ*sseF* and Δ*sifA* showed an intermediate OVA B cell response compared with the robust response in Δ*ssaV*- and Δ*sseC*-infected mice, and the low response in WildType- and Δ*sseK2*-infected mice, we wanted to explore whether the degree of bacterial replication correlated with the level of the B cell response. Mice were infected and given exogenous OVA. Seven days later the spleen (data not shown) and livers of mice were harvested, homogenized and plated on MacConkey agar for enumeration of recoverable CFUs. Indeed, those mice with low OVA-specific B cell responses had the highest bacterial load in the liver (WildType, Δ*sseK2*, Δ*sspH2* and Δ*gogB*; Figures 3-6 and 3-7B). Those mice with the most robust OVA-specific B cell response had the lowest bacterial loads (Δ*ssaV*, Δ*sseC*, PhoP^{c}; Figures 3-6 and 3-7B). Finally, those mice with an intermediate OVA-specific B cell response had intermediate bacterial loads (Δ*sifA*, Δ*sscBsseFG* and Δ*sseF*; Figures 3-6 and 3-7B). Even when mice were infected with the attenuated St-OVA or ΔSPI2 and exogenous OVA, a higher bacterial load in the St-OVA infected mice correlated with a decreased OVA-specific B cell response (Figure 3-7A).

Using direct visualization of endogenous antigen-specific B cells with B cell tetramers, our data clearly show that the B cell response during *Salmonella* infection is delayed. Germinal center formation is also inhibited until mice have cleared bacteria. This delay in B cell responses is not due to a lack of antigen, as antigen availability would be greatest at the peak of infection. Although the inhibition of B cell, and specifically germinal center, responses seem to correlate directly with bacterial load, it is still remains possible that the same *Salmonella* factors controlling bacterial load also

influence the B cell response. One explanation for this delay in B cell responses could be that there is a lack of CD4+ T cell help earlier during infection. Previous data has demonstrated that although SM1 transgenic CD4+ T cells are rapidly activated during infection, they are culled to undetectable levels by 7 days following infection (56). This culling of activated CD4+ T cells was attributed to expression of the SPI2 T3SS and not merely the result of decreased bacterial load, as infection with a PhoP^{c} mutant still mediated the culling of activated CD4+ T cells (56). Thus, it is possible that culling of activated CD4+ T cells and inhibition of the B cell response are mediated by separate *Salmonella*-intrinsic factors.

A second explanation is that *Salmonella* directly inhibit B cell function. Although we did not examine this in our system, others have shown that *Salmonella* can actively infect B cells (108, 109). It is still not known which *Salmonella* proteins affect B cells directly, but our study suggests that they may not be restricted to SPI2 effectors. The third and most intriguing possibility is that *Salmonella*'s effects on macrophages hinder the formation of the germinal center and B cell proliferation. A recent study by Nikbakht, *et al* showed that in the absence of marginal zone macrophages, activated B cells that migrate to the T-B border and receive help from activated CD4+ T cells fail to further migrate to the follicular perimeter, proliferate and form germinal centers in response to a T-dependent antigen (110). Although there is clearly an increase in macrophages during *Salmonella* infection, it is possible that they are rendered functionally incapable of promoting germinal center formation. Further work needs to be done to determine the mechanism by which macrophages promote germinal center formation and the strategies *Salmonella* use to inhibit this response.

FIGURE 3-1. Ovalbumin B cell tetramers can be used to directly visualize Salmonella-specific B cell responses.

(**A**) Description of the flow cytometry plot distinguishing OVA Tetramer+ cells from SA-PE-specific and AF647-specific B cells. (**B**) The gating strategy for identifying OVA-specific B cells consisted of an initial lymphocyte gate, followed by a singlet gate and a dump channel containing CD11c, CD11b, F4/80, CD4, CD8, Gr-1. After gating on B220+/Dump- cells, OVA-specific B cells are identified by gating on OVA-Tetramer^{+} Decoy Tetramer^{-} cells. (**C**) Mice were infected with $5x10^{5}$ St-OVA or St-Comp and the number of OVA-specific B cells at the peak of the response was enumerated.

FIGURE 3-1

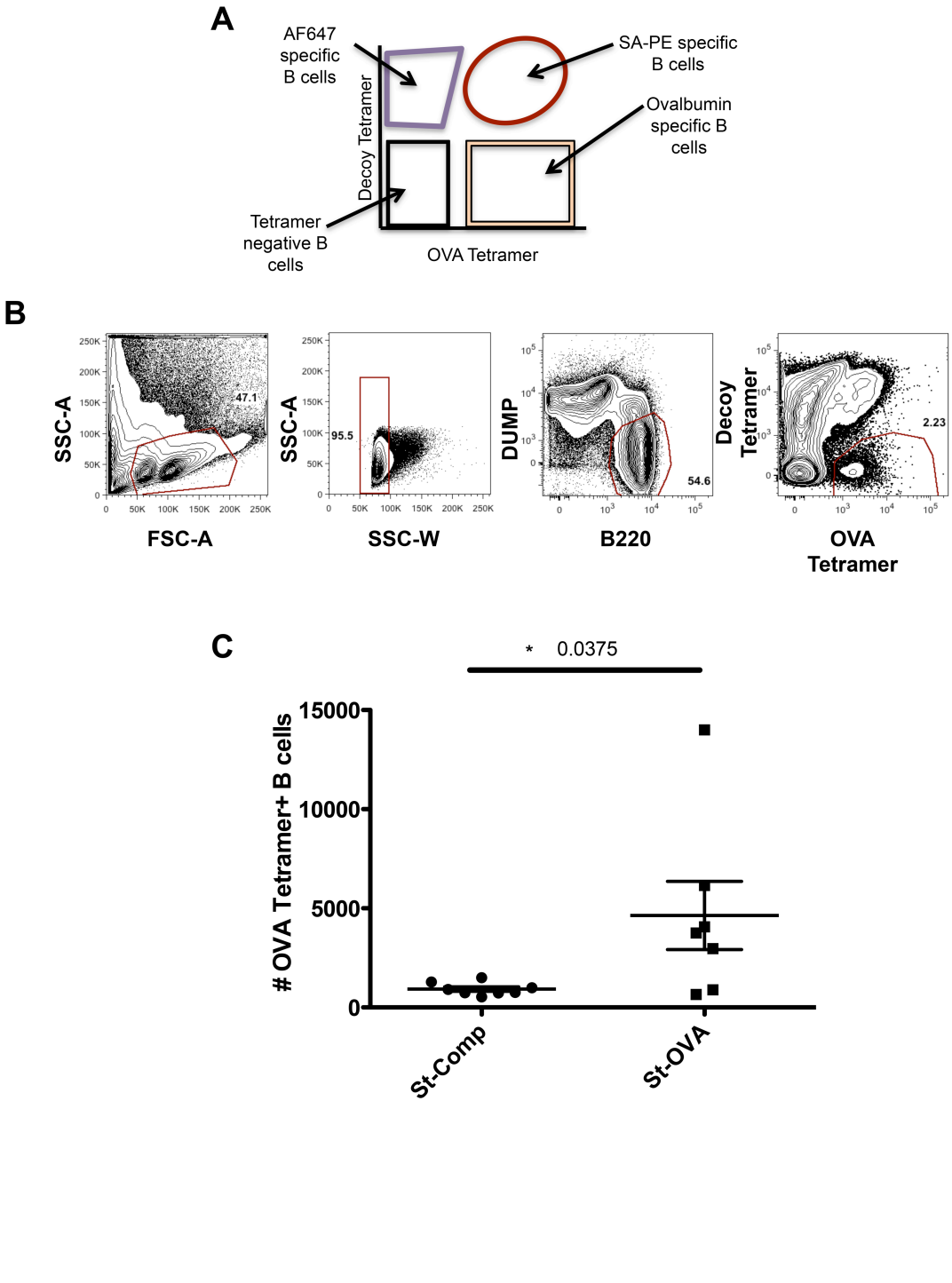
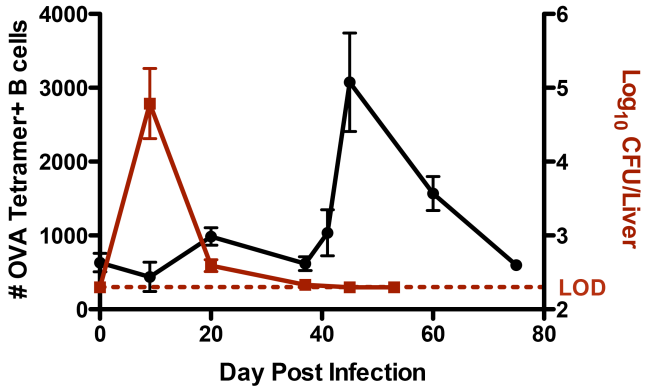

A
AF647 specific B cells
SA-PE specific B cells
Ovalbumin specific B cells
Decoy Tetramer
Tetramer negative B cells
OVA Tetramer
B
SSC-A
FSC-A
47.1
SSC-A
SSC-W
95.5
DUMP
B220
54.6
Decoy Tetramer
OVA Tetramer
2.23
C
* 0.0375
# OVA Tetramer+ B cells
15000
10000
5000
0
St-Comp
St-OVA

FIGURE 3-2. The peak of OVA-specific B cell expansion occurs after *Salmonella*-OVA infection has resolved. (**A**) Mice were infected IV with $5x10^5$ St-OVA and at the indicated times after infection the spleen and lymph nodes were harvested and OVA-Tetramer staining and flow cytometry was performed as described in Figure 3-1B. Numbers of OVA-specific B cells are enumerated here (black line). Livers of infected mice were collected, homogenized and plated on MacConkey agar to determine bacterial load at the indicated times post infection. (**B**) At the indicated times post IV infection with $5x10^5$ St-OVA serum was collected via retro-orbital eye bleed and IgM, IgG1, IgG2a, IgG2b and IgG3 antibody titer determined via ELISA.

FIGURE 3-2

A


OVA Tetramer+ B cells
4000
3000
2000
1000
0
6
5
4
3
2
Log_{10} CFU/Liver
LOD
0
20
40
60
80
Day Post Infection

B

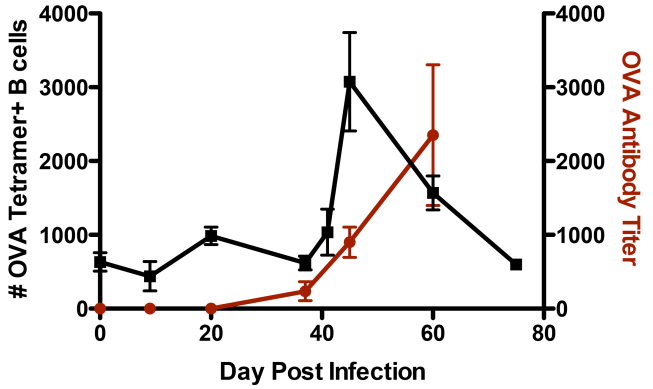

OVA Tetramer+ B cells
4000
3000
2000
1000
0
OVA Antibody Titer
0
20
40
60
80
Day Post Infection

FIGURE 3-3. The peak of OVA-specific germinal center formation occurs after *Salmonella*-OVA infection has resolved. (**A**) Mice were infected with $5x10^5$ St-OVA. Spleen and lymph nodes were harvested at the indicated times following infection and stained with OVA- and Decoy- tetramers to identify OVA-specific B cells as described in Figure 3-1B. Cells were further stained with CD38 and GL7. Flow cytometry plots are displayed (**B**) $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells are activated B cells and $CD38^{lo}GL7^+$ cells represent B cells in a germinal center reaction. (**C**) Samples treated as in (A). Graph shows numbers of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype. (**D**) Samples treated as in (A). Graph shows the percent of OVA-specific cells with naïve/memory, activated or germinal center phenotype. (**E**) Mice treated as in (A) or infected with $5x10^5$ St-Comp. Graph shows the number of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype 45-49 days post-infection. P values reflect statistical analysis via unpaired t test between numbers of germinal center phenotype cells. (**F**) Mice treated as in (A) or infected with $5x10^5$ St-Comp. Graph shows the percent of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype 45-49 days post-infection. P values reflect statistical analysis via unpaired t test between percents of germinal center phenotype cells.

FIGURE 3-3

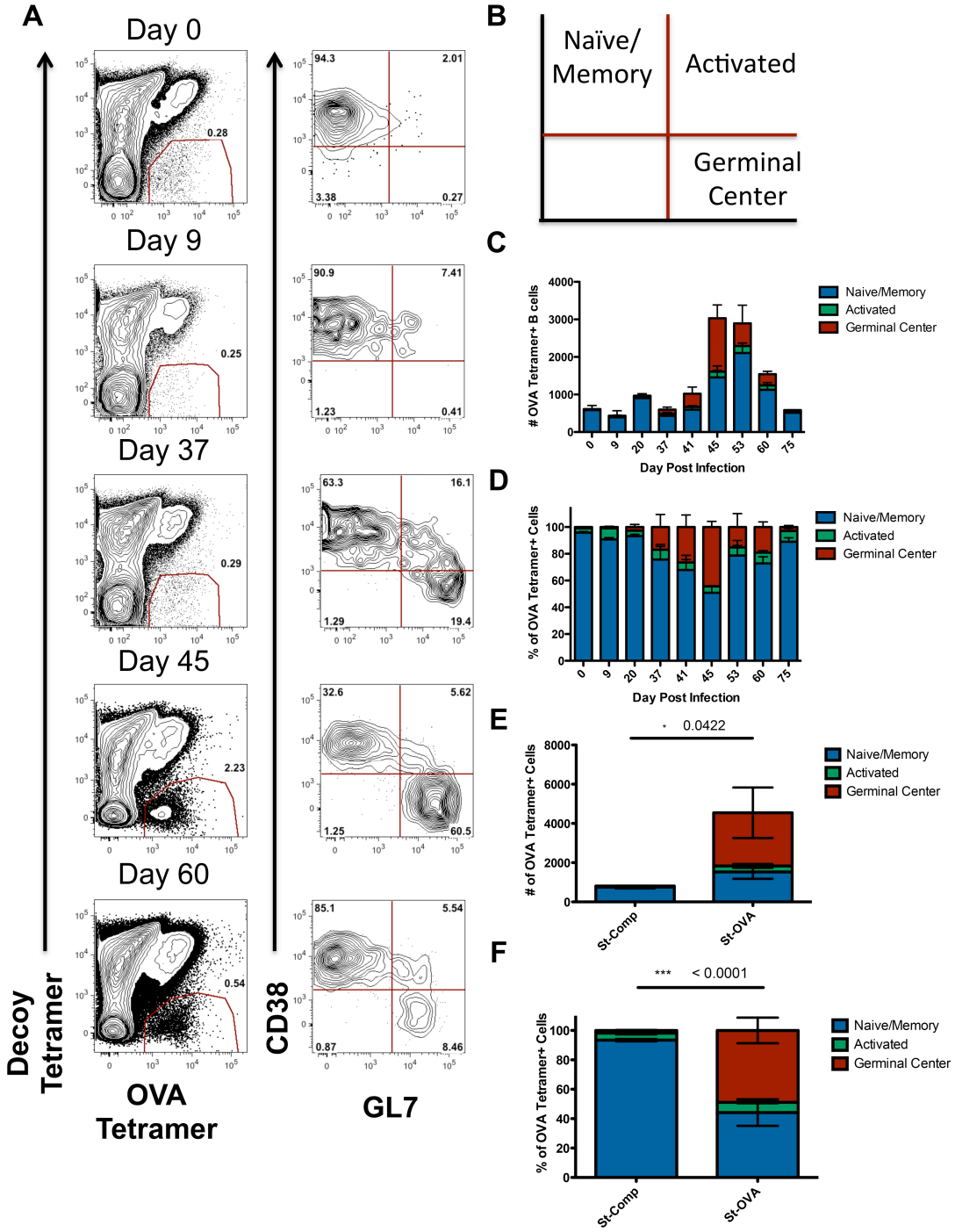

A
Day 0
Day 9
Day 37
Day 45
Day 60
0.28
94.3
2.01
3.38
0.27
0.25
90.9
7.41
1.23
0.41
0.29
63.3
16.1
1.29
19.4
2.23
32.6
5.62
1.25
60.5
0.54
85.1
5.54
0.87
8.46
Decoy Tetramer
OVA Tetramer
CD38
GL7
B
Naïve/ Memory
Activated
Germinal Center
C
OVA Tetramer+ B cells
Day Post Infection
Naive/Memory
Activated
Germinal Center
D
% of OVA Tetramer+ Cells
Day Post Infection
Naive/Memory
Activated
Germinal Center
E
0.0422
# of OVA Tetramer+ Cells
St-Comp
St-OVA
Naive/Memory
Activated
Germinal Center
F
*** < 0.0001
% of OVA Tetramer+ Cells
St-Comp
St-OVA
Naive/Memory
Activated
Germinal Center

FIGURE 3-4. The peak of OVA-specific B cell expansion and germinal center formation occurs at day 30 after Salmonella-OVA infection has resolved. A separate group of experiments identical to those described in Figure 3-3. (**A-B**) Number of OVA-specific B cells at the indicated times following infection with $5x10^5$ St-OVA. (**C**) Graph shows the number of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype at the indicated days post-infection. (**D**) Graph shows the percent of OVA-specific B cells with a naïve/memory, activated or germinal center phenotype at the indicated days post-infection.

FIGURE 3-4

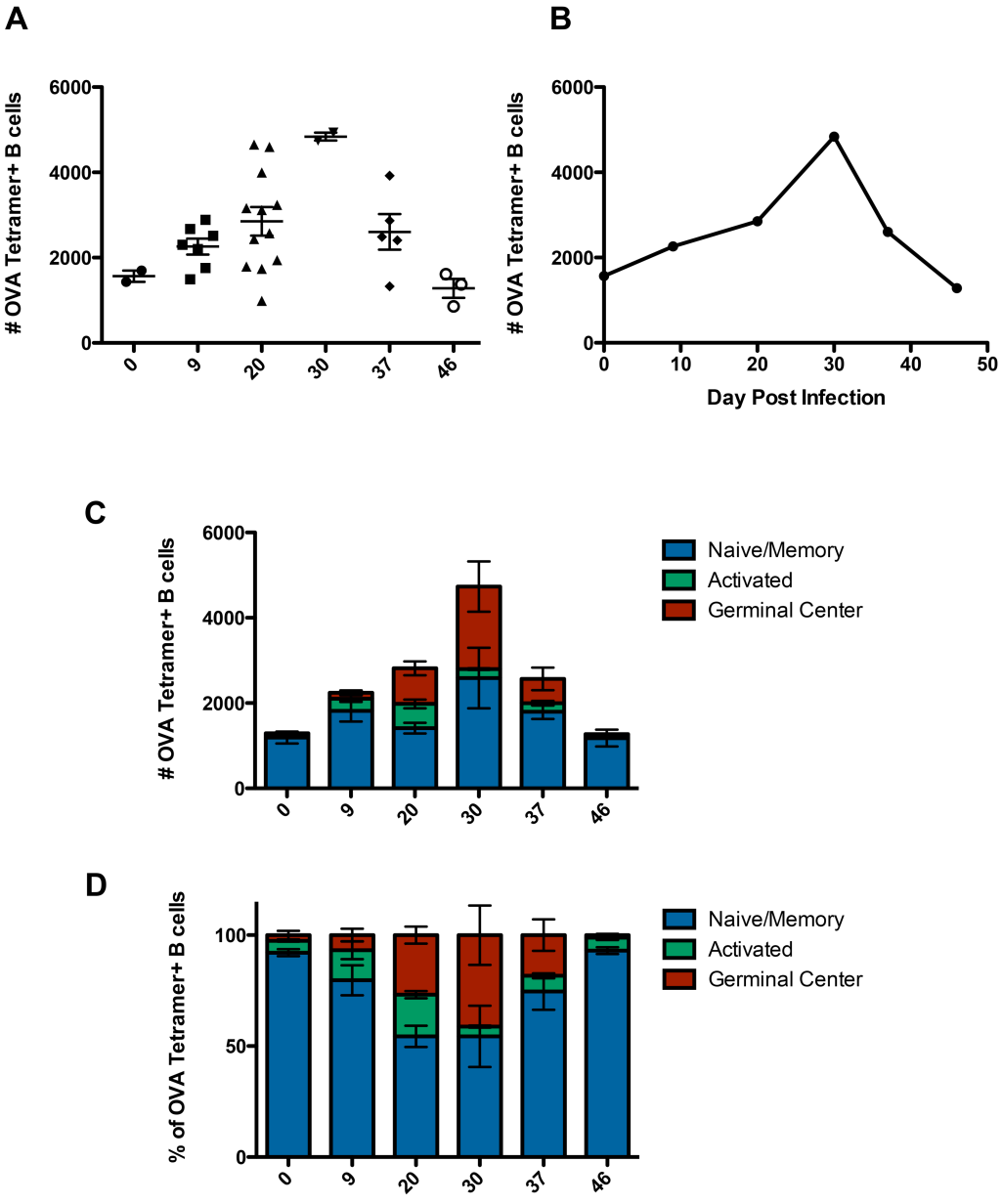

A
OVA Tetramer+ B cells
6000
4000
2000
0
0
9
20
30
37
46
B
OVA Tetramer+ B cells
6000
4000
2000
0
0
10
20
30
40
50
Day Post Infection
C
OVA Tetramer+ B cells
6000
4000
2000
0
0
9
20
30
37
46
Naive/Memory
Activated
Germinal Center
D
% of OVA Tetramer+ B cells
100
50
0
0
9
20
30
37
46
Naive/Memory
Activated
Germinal Center

FIGURE 3-5. OVA-specific B cell expansion and activation during immunization are inhibited by *Salmonella* and a functional SPI2 needle is required for *Salmonella* to inhibit B cell expansion. Mice were infected IV with $5x10^5$ St-OVA or ΔSPi2 and/or immunized with 10μg OVA and 10μg LPS. Seven days post infection/immunization the spleen and lymph nodes were harvested and OVA-Tetramer staining and flow cytometry was performed as described in Figure 3-1B. $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells are activated B cells and $CD38^{lo}GL7^+$ cells represent B cells in a germinal center reaction. N= 6 mice per group. (**A**) Representative flow cytometry plots of the indicated infection/immunization strategies are shown. (**B**) Graph shows numbers of OVA-specific B cells in the indicated infection/immunization groups. (**C**) Graph shows the percent of OVA-specific B cells in the indicated infection/immunization groups with naïve/memory, activated or germinal center phenotype. P values reflect statistical analysis via unpaired t test between percents of germinal center phenotype cells. (**D**) Graph shows the number of OVA-specific B cells in the indicated infection/immunization groups with naïve/memory, activated or germinal center phenotype. P values reflect statistical analysis via unpaired t test between numbers of germinal center phenotype cells.

FIGURE 3-5

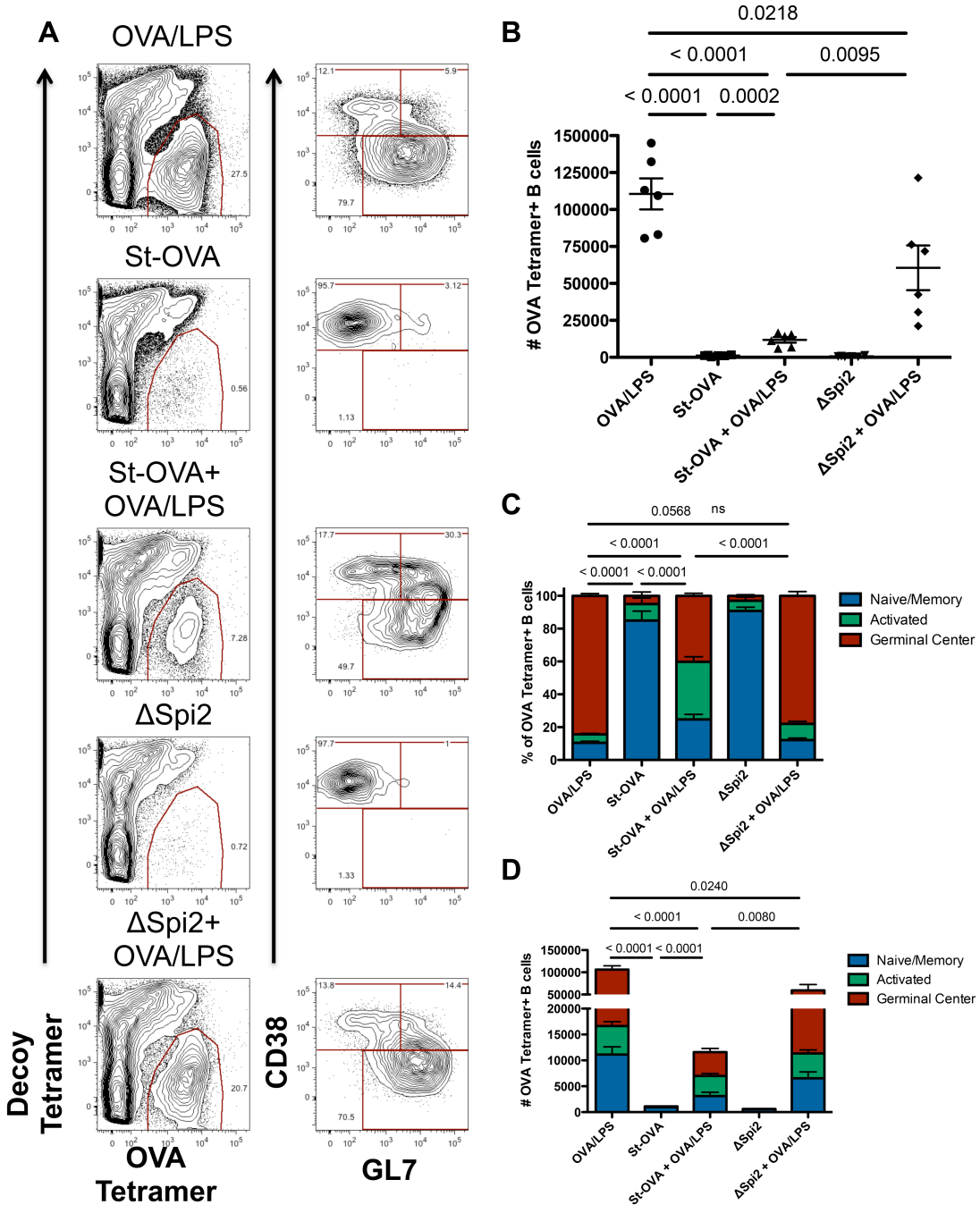

A
OVA/LPS
St-OVA
St-OVA+ OVA/LPS
ΔSpi2
ΔSpi2+ OVA/LPS
Decoy Tetramer
OVA Tetramer
CD38
GL7
B
0.0218
< 0.0001
0.0095
< 0.0001
0.0002
OVA Tetramer+ B cells
150000
125000
100000
75000
50000
25000
0
OVA/LPS
St-OVA
St-OVA + OVA/LPS
ΔSpi2
ΔSpi2 + OVA/LPS
C
0.0568
ns
< 0.0001
< 0.0001
< 0.0001
< 0.0001
% of OVA Tetramer+ B cells
100
80
60
40
20
0
Naive/Memory
Activated
Germinal Center
OVA/LPS
St-OVA
St-OVA + OVA/LPS
ΔSpi2
ΔSpi2 + OVA/LPS
D
0.0240
< 0.0001
0.0080
< 0.0001
< 0.0001
OVA Tetramer+ B cells
150000
100000
50000
20000
15000
10000
5000
0
Naive/Memory
Activated
Germinal Center
OVA/LPS
St-OVA
St-OVA + OVA/LPS
ΔSpi2
ΔSpi2 + OVA/LPS

FIGURE 3-6. SPI2 effector proteins sifA and sseF inhibit expansion and germinal center formation of OVA-specific B cells. Mice were infected IV with $5x10^3$ of the indicated *S*. Typhimurium WildType or SPI2 effector mutant strains along with 10µg OVA/10^8 HKST. Seven days post infection/immunization the spleen and lymph nodes were harvested and OVA-Tetramer staining and flow cytometry was performed as described in Figure 3-1B. $CD38^+GL7^-$ cells represent the naïve and memory B cells, $CD38^+GL7^+$ cells are activated B cells and $CD38^{lo}GL7^+$ cells represent B cells in a germinal center reaction. N= 3-11 mice per group. (**A**) Graph shows numbers of OVA-specific B cells in the indicated infection/immunization groups. (**B**) Graph shows the number of OVA-specific B cells in the indicated infection/immunization groups with naïve/memory, activated or germinal center phenotype. (**C**) Graph shows the percent of OVA-specific B cells in the indicated infection/immunization groups with naïve/memory, activated or germinal center phenotype.

FIGURE 3-6

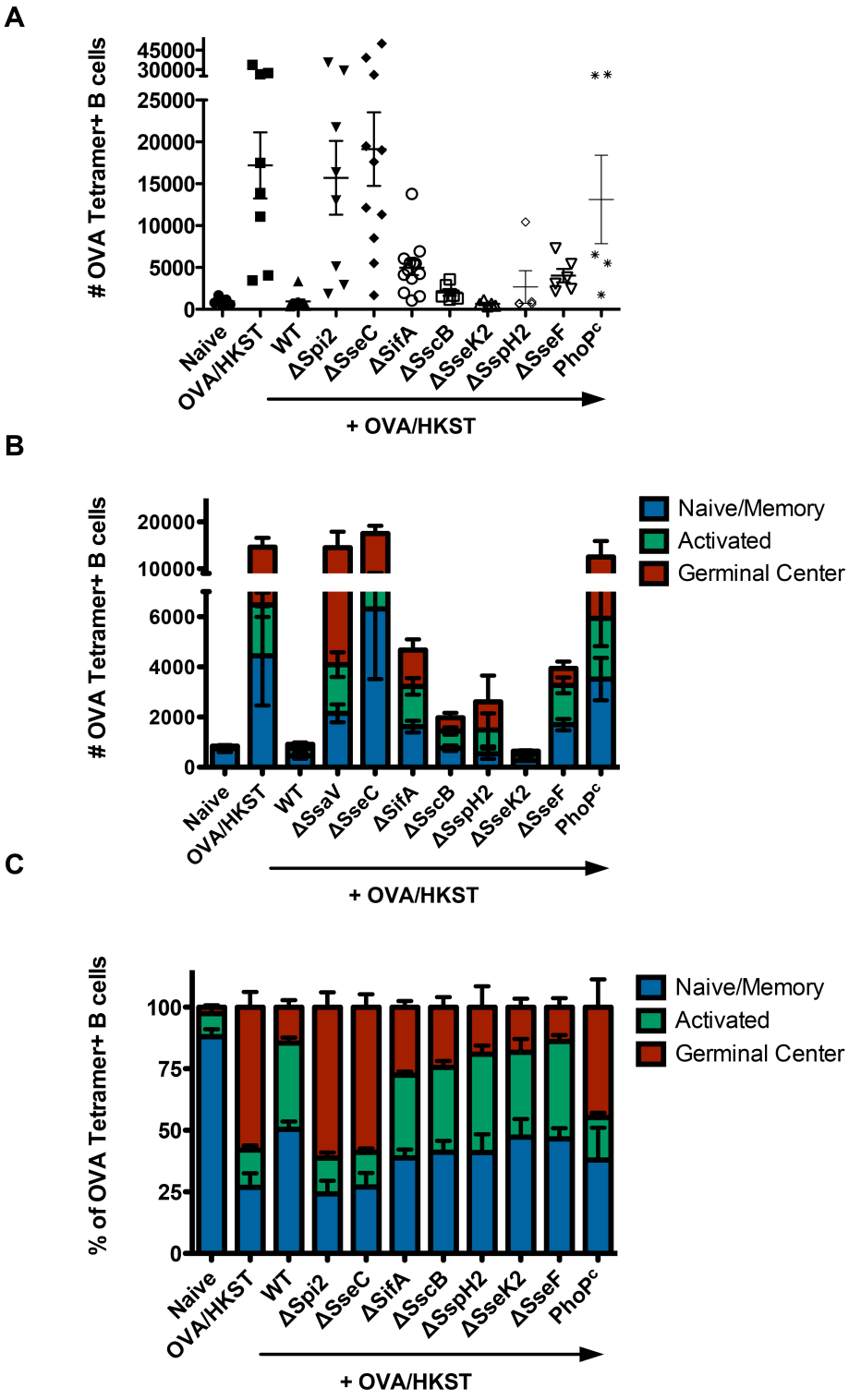
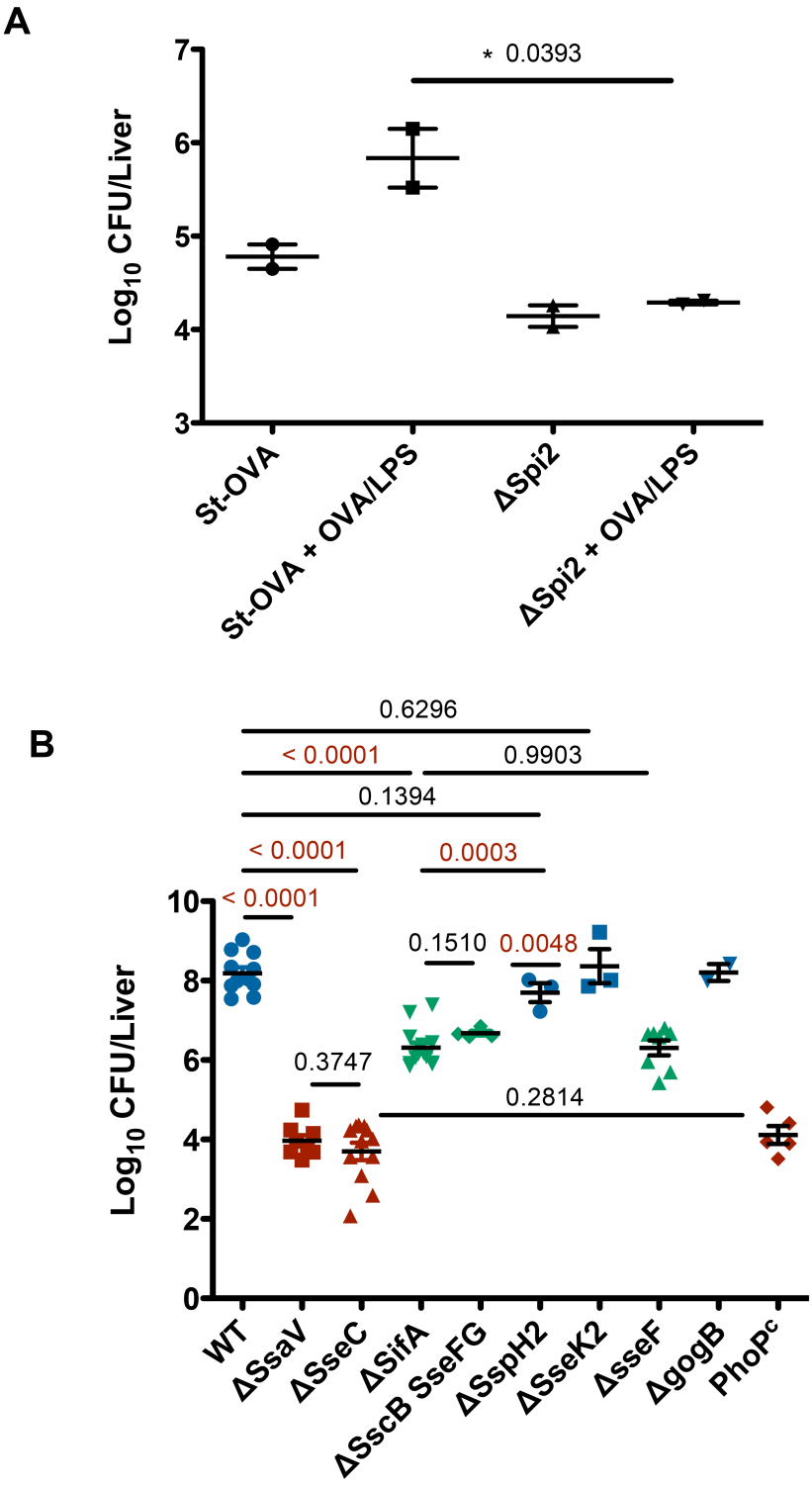

A
OVA Tetramer+ B cells
45000
30000
25000
20000
15000
10000
5000
0
Naive
OVA/HKST
WT
ΔSpi2
ΔSseC
ΔSifA
ΔSscB
ΔSseK2
ΔSspH2
ΔSseF
PhoPc
+ OVA/HKST
B
OVA Tetramer+ B cells
20000
10000
6000
4000
2000
0
Naive
OVA/HKST
WT
ΔSsaV
ΔSseC
ΔSifA
ΔSscB
ΔSspH2
ΔSseK2
ΔSseF
PhoPc
+ OVA/HKST
Naive/Memory
Activated
Germinal Center
C
% of OVA Tetramer+ B cells
100
75
50
25
0
Naive
OVA/HKST
WT
ΔSpi2
ΔSseC
ΔSifA
ΔSscB
ΔSspH2
ΔSseK2
ΔSseF
PhoPc
+ OVA/HKST
Naive/Memory
Activated
Germinal Center

FIGURE 3-7. SifA and SseF-dependent inhibition of OVA- B cell responses correlates with control of bacterial load in mice. (**A**) Mice were infected with $5x10^5$ St-OVA or ΔSPI2 and/or immunized with 10μgOVA/ 10μg LPS. Seven days post infection livers of infected mice were collected, homogenized and plated on MacConkey agar to determine bacterial load. N = 2 mice per group. (**B**) Mice were infected IV with $5x10^3$ of the indicated *S*. Typhimurium WildType or SPI2 effector mutant strains along with 10μg OVA/10^8 HKST. Seven days post infection livers of infected mice were collected, homogenized and plated on MacConkey agar to determine bacterial load. N= 3-11 mice per group. Statistical significance was determined by unpaired t test between the indicated groups. P values are as displayed.

FIGURE 3-7


A
* 0.0393
Log10 CFU/Liver
7
6
5
4
3
St-OVA
St-OVA + OVA/LPS
ΔSpi2
ΔSpi2 + OVA/LPS
B
0.6296
< 0.0001
0.9903
0.1394
< 0.0001
0.0003
< 0.0001
0.1510
0.0048
0.3747
0.2814
Log10 CFU/Liver
10
8
6
4
2
0
WT
ΔSsaV
ΔSseC
ΔSifA
ΔSscB SseFG
ΔSspH2
ΔSseK2
ΔsseF
ΔgogB
PhoPc

Chapter 4

Concluding Remarks

Salmonella infections are a major cause of morbidity and mortality worldwide. The current estimates for Typhoid alone are 21 million cases and over 200,000 deaths per year. Given the difficulty of diagnosis and the limited studies used to derive these figures, these numbers are likely an underestimate of the true burden of disease (3). Additionally, Paratyphoid infections are a rising concern in some countries in Southeast Asia (13). Meanwhile, Non-Typhoid Salmonelloses cause a staggering case-fatality rate among children and the immune-compromised in sub-Saharan Africa (17, 20). As *Salmonella* infections are transmitted via the fecal oral route, usually by ingestion of contaminated water and food, it is imperative to improve sanitation and access to clean water in endemic regions (16, 22, 111). However, many endemic areas lack the infrastructure, funds and/or political backing to undertake such large-scale improvements (7). Even in countries such as the United States, which have great sanitation and access to clean water, suffer from over 1.4 million cases of *Salmonella* gastroenteritis per year. This is largely due to the mass production of our foods, including meat and vegetables, which can easily become contaminated with fecal matter from *Salmonella*-infected livestock, that is then rapidly distributed across the country (112-114).

As with any other infections, *Salmonella* can effectively be treated with antibiotics. However, *Salmonella* have acquired resistance to first-line antibiotics, and *Salmonella* strains resistant to fluoroquinolones, the current antibiotics of choice, have started to emerge (9-11). As new antibiotic production is not underway, we face the frightening possibility of not being able to control these infections in the near future. It is due to these facts that the World Health Organization has made vaccination for Typhoid a major priority in endemic regions. The WHO recommends adopting vaccination of at-risk groups with the current Typhoid vaccines on the market- the live-attenuated Ty21a and the subunit Vi polysaccharide vaccine (27). Both vaccines suffer from only

moderate, short-lived immunity, and neither target the demographic most in need of protection- children under 2 years of age (23). While next generation vaccines seek to conjugate the Vi polysaccharide to protein carriers to increase immunogenicity, it remains to be seen whether increased immunogenicity will translate into increased protection with this particular antigen. The protein carriers are not *Salmonella*-specific and, thus, will not induce *Salmonella*-specific T_h1 responses (28). Data from mouse models of infection suggest that the ideal vaccine would elicit protective T and B cell responses, but it is unclear if T cells and B cells must recognize the same antigens for greatest protective efficacy. What is clear from past and current Typhoid vaccine development, is that the immunology of *Salmonella* infections and the bacteria's effect on immune cells must be taken into account.

Our work in this thesis aimed to further define the role of B cells in protection against *Salmonella*. While previous literature showed that antibodies can be protective in secondary *Salmonella* infection (70, 71), it was unclear if antibodies were the main protective mechanisms of B cells. We showed that even mice with B cells that lack the capacity to produce systemic antibody were largely protected against a secondary challenge to virulent *Salmonella*. While this does not suggest that antibodies are not protective, it does suggest that antibody-independent mechanisms of protection are sufficient for B cells to mediate their protective effects. Specifically, we show that even in the absence of antibodies, B cells are able to promote a robust memory T_h1 response. B cell deficient mice succumbed to a secondary infection and had decreased numbers of CD4+ T cells producing IFNγ. This work points to the necessity to determine which *Salmonella* antigens elicit this protective B cell response that also promotes memory T_h1 development. A subunit vaccine that targets both of these arms of the immune system would likely be more protective than the next-generation Vi-conjugate vaccines. Our

work also highlights the dilemma of using antibody titer as a readout for protection. Although antibodies to particular antigens may form during infection or immunization, these antibodies may not be protective or the antibody-mediated protection may not be long-lived. Further work must also be done to determine when B cells are protective. Do they play a role early during primary infection? Late during primary infection? Or early during secondary infection?

Determining how B cells protect during challenge is complicated by the paucity of data describing the primary B cell response during infection. In this thesis, for the first time, we described the use of B cell tetramers to characterize the endogenous *Salmonella*-specific B cell response during infection. We showed that in contrast to immunization with OVA alone, infection of mice with *Salmonella* expressing OVA resulted in delayed expansion and germinal center formation of OVA-specific B cells. These data highly suggest that, even during infection with an attenuated strain, active infection can inhibit certain aspects of the B cell response. Because *Salmonella* cull the CD4+ T cell response during infection in a SPI2-dependent manner, we hypothesized that these effectors also affect the B cell response. Using SPI2 deficient strains of *Salmonella* we showed that OVA-specific B cell expansion and germinal center formation can be rescued. Surprisingly, however, this recovery of B cell responses correlated with the ability of the bacteria to establish intracellular infection, as infection with a $PhoP^c$ mutant also rescued the OVA-specific B cell response. This result is in contrast to previous data that showed that activated CD4+ T cells were still culled during infection with a $PhoP^c$ mutant indicating that *Salmonella* can inhibit T and B cell responses via separate mechanisms (56).

Our data suggest that during active infection, *Salmonella* maintain mechanisms that dampen the adaptive immune response. Coupled with the findings that CD4+ T cells

require more long-term exposure to antigens during infection in order to mount an adequate memory response (59, 83), our data highlight the difficulty of generating effective live attenuated vaccines. On the one hand, the bacteria must be attenuated enough so as not to cause disease; on the other hand the bacteria can not be too attenuated such that the immune system clears the bacteria before adequate adaptive immunity is primed and memory responses developed. Our work has further implications on how and when the current live attenuated vaccine is given. As even attenuated strains of *Salmonella* showed inhibition of B cell responses to exogenous antigens not expressed by *Salmonella*, it is reasonable to be concerned that co-administration of Ty21a with other vaccines could result in sub-optimal responses to these additional vaccines. Further work deciphering the *Salmonella*-specific effectors that cull CD4+ T cell responses and inhibit B cell responses while not completely attenuating the growth of the bacteria would enable rational live attenuated vaccine design.

In summary, this thesis provides evidence for the decisive role of B cells in protection against secondary virulent *Salmonella* in an antibody-independent manner. We also show that B cell expansion and germinal center formation are inhibited during live infection. This *Salmonella*-mediated inhibition of B cell responses may be independent of the bacterium's ability to cull CD4+ T cell responses.

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