

THE UNIVERSITY OF CHICAGO

THE LYMPHOTOXIN PATHWAY MEDIATES PROTECTIVE IMMUNITY  
AGAINST A BACTERIAL PATHOGEN

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## **Table of Contents**

List of Figures .....	vi
List of Tables .....	viii
List of Abbreviations .....	ix
Acknowledgements .....	xi
Abstract .....	xiii
Chapter 1: Introduction .....	1
Disease caused by <i>Salmonella enterica</i> .....	1
Asymptomatic carriage as a disease reservoir of <i>Salmonella</i> .....	4
Mouse models of <i>Salmonella</i> infection and disease .....	6
Anti- <i>Salmonella</i> memory responses .....	16
The lymphotoxin pathway .....	20
The lymphotoxin pathway's role in infectious disease .....	26
The role of lymphotoxin in <i>Salmonella</i> infection .....	32
Chapter 2: Materials and Methods .....	34
Mice .....	34
Bacterial growth .....	34
Infections .....	35
Bacterial CFU in fecal or tissue samples .....	35
<i>Salmonella</i> antigen preparation .....	36

ELISA.....	36
<i>In vitro</i> and <i>in vivo</i> restimulation .....	37
Fusion protein and functional antibody blockade.....	38
Flow cytometry.....	38
Statistical Analysis .....	39
Chapter 3: The role of the lymphotoxin pathway in resolution of <i>Salmonella</i>	
gastrointestinal infection .....	40
Similar kinetics of disease after oral exposure to virulent <i>Salmonella</i> between <i>Ltbr</i> <sup>+/-</sup>	
and <i>Ltbr</i> <sup>-/-</sup> littermates. ....	40
Attenuated <i>Salmonella</i> does not cause death or weight loss in <i>Ltbr</i> <sup>+/-</sup> or littermate <i>Ltbr</i> <sup>-</sup>	
<sup>-/-</sup> mice.....	40
LTβR is required for clearance of attenuated <i>Salmonella</i> from the gut lumen and	
resolution of gut inflammation.....	42
LTβR is required to drive microbiota-mediated clearance of <i>Salmonella</i> from the gut.	
.....	45
<i>E. coli</i> levels are not a predictor of clearance .....	47
LTβR's role in <i>Salmonella</i> clearance depends on direct signaling .....	49
Chapter 4: The role of the lymphotoxin pathway in anti- <i>Salmonella</i> immunity .....	
LTβR is essential to generate protective immunity to secondary challenge with	
virulent <i>Salmonella</i> following vaccination with attenuated <i>Salmonella</i> .....	50

The role of LT $\beta$ R in mediating protection against <i>Salmonella</i> is independent of gut persistence. ....	51
LT $\beta$ R drives generation of anti- <i>Salmonella</i> class switched antibody. ....	52
LT $\beta$ R's role in generation of anti- <i>Salmonella</i> class switched antibody is independent of gut persistence and GALT priming. ....	53
Active LT $\beta$ R signaling drives generation of anti- <i>Salmonella</i> class switched antibody. ....	54
LT $\beta$ R contributes to IFN $\gamma$ and TNF $\alpha$ cytokine recall responses. ....	55
T cell responses to <i>Salmonella</i> are impaired in <i>Ltbr</i> <sup>-/-</sup> mice. ....	56
<i>Salmonella</i> infection of <i>Ltbr</i> <sup>-/-</sup> causes impairments in TCR driven proliferation. ....	57
Granulocytes accumulate after <i>Salmonella</i> infection in <i>Ltbr</i> <sup>-/-</sup> mice but do not mediate T cell suppression <i>in vitro</i> . ....	59
Purified CD4 <sup>+</sup> T cells from <i>Salmonella</i> vaccinated mice have an intrinsic defect in proliferation. ....	61
Anti- <i>Salmonella</i> CD4 <sup>+</sup> IFN $\gamma$ recall responses after <i>in vivo</i> restimulation ....	62
Late phase IFN $\gamma$ blockade impairs survival at challenge but does not impact <i>Salmonella</i> gut clearance or anti- <i>Salmonella</i> IgG. ....	65
Chapter 5: Cellular sources of lymphotoxin in anti- <i>Salmonella</i> responses ....	67
Membrane LT $\alpha$ 1 $\beta$ <sub>2</sub> is the ligand for LT $\beta$ R that mediates primary and secondary immune responses to <i>Salmonella</i> .....	67
Lymphotoxin from $\alpha\beta$ T cells is not required for anti- <i>Salmonella</i> responses ....	68

Lymphotoxin from Rorc <sup>+</sup> ILC3s and T cells does not contribute to anti- <i>Salmonella</i> antibody responses, but has a partial impact on survival after challenge.....	69
Lymphotoxin from B cells is essential for anti- <i>Salmonella</i> IgG and contributes to protective immunity to <i>Salmonella</i> . .....	70
Discussion .....	73
Overall summary of findings .....	73
Lymphotoxin's role in <i>Salmonella</i> gut clearance.....	74
Antibody responses to <i>Salmonella</i> .....	78
Protective Immunity to <i>Salmonella</i> .....	81
Concluding remarks .....	88
References .....	89

## **List of Figures**

Figure 1. Ligands and receptors of the lymphotoxin pathway. ....	21
Figure 2. Role of lymphotoxin in lymph node remodeling during infection. ....	29
Figure 3. LT $\beta$ R does not impact survival following oral administration of virulent <i>Salmonella</i> .....	40
Figure 4. LT $\beta$ R does not impact survival following oral administration of attenuated <i>Salmonella</i> in the streptomycin model of <i>Salmonella</i> .....	41
Figure 5. LT $\beta$ R is required for clearance of attenuated <i>Salmonella</i> from the gut lumen and resolution of inflammation. ....	43
Figure 6. LT $\beta$ R impacts CFU of intracellular <i>Salmonella</i> during late phase of infection.	44
Figure 7. LT $\beta$ R signaling drives microbiota mediated clearance of <i>Salmonella</i> .....	46
Figure 8. <i>E. coli</i> abundance does not correlate with <i>Salmonella</i> clearance and is not altered by LT $\beta$ R.....	48
Figure 9. Lymphotoxin signaling drives fecal clearance of <i>Salmonella</i> . ....	49
Figure 10. LT $\beta$ R is essential for mediating protective immunity to <i>Salmonella</i> . ....	50
Figure 11. The role of LT $\beta$ R in mediating protection against <i>Salmonella</i> is independent of gut persistence .....	51
Figure 12. LT $\beta$ R is required for anti- <i>Salmonella</i> IgG responses. ....	52
Figure 13. The role of LT $\beta$ R in IgG responses against <i>Salmonella</i> is independent of gut priming and gut persistence. ....	53
Figure 14. Lymphotoxin signaling drives generation of anti- <i>Salmonella</i> IgG responses .....	54
Figure 15. LT $\beta$ R contributes to IFN $\gamma$ and TNF $\alpha$ cytokine recall responses.....	55

Figure 16. T cell proliferative responses to <i>Salmonella</i> antigens are impaired in <i>Ltbr</i> <sup>-/-</sup> mice.....	56
Figure 17. <i>Salmonella</i> -mediated suppression of T cells in <i>Ltbr</i> <sup>-/-</sup> mice in response to anti-CD3/CD28 .....	57
Figure 18. Lymphocyte survival after 48 hours in culture.....	58
Figure 19. Granulocytes accumulate in <i>Ltbr</i> <sup>-/-</sup> mice after <i>Salmonella</i> infection but do not mediate lymphocyte suppressing <i>in vitro</i> . .....	59
Figure 20. <i>Salmonella</i> induced CD4 intrinsic proliferation defect in <i>Ltbr</i> <sup>-/-</sup> mice.....	61
Figure 21. anti- <i>Salmonella</i> CD4 <sup>+</sup> IFN $\gamma$ recall responses after <i>in vivo</i> restimulation. ....	63
Figure 22. IFN $\gamma$ blockade during late phase of attenuated <i>Salmonella</i> infection. ....	65
Figure 23. Lymphotoxin is required for protective immunity to <i>Salmonella</i> and clearance from the gut lumen. ....	67
Figure 24. Lymphotoxin from $\alpha\beta$ T cells is dispensible for anti- <i>Salmonella</i> immunity, gut clearance, and antibody production .....	68
Figure 25. Lymphotoxin from T cells and innate lymphoid type 3 cells is dispensible for anti- <i>Salmonella</i> gut clearance, and antibody production and has a partial impact on protection at challenge .....	70
Figure 26. Lymphotoxin from B cells is requires for anti- <i>Salmonella</i> IgG.....	71

**List of Tables**

Table 1. Antibody responses to model antigens in lymphotoxin deficient mouse strains.  
.....25

## **List of Abbreviations**

AFC .....	antibody forming cell
APC .....	antigen presenting cell
CFU .....	colony forming unit
CG .....	chicken gamma globulin
DC .....	dendritic cell
ELISA .....	enzyme-linked immunosorbent assay
FDC .....	follicular dendritic cell
FRC .....	fibroblastic reticular cell
FSC .....	forward scatter
GALT .....	gut associated lymphoid tissue
GC .....	germinal center
GI.....	gastrointestinal
HEV .....	high endothelial venule
HSV .....	herpes simplex virus
i.p.....	intraperitoneal
ILC3.....	innate lymphoid type3 cells
ILF .....	isolated lymphoid follicle
iNTS.....	invasive non typhoidal <i>Salmonella</i>
KO .....	knock out
LCMV.....	lymphocytic choriomeningitis virus
LN .....	lymph node
LT .....	lymphotoxin

LTi..... lymphoid tissue inducing cell  
LT $\alpha$  ..... lymphotoxin alpha  
LT $\beta$  ..... lymphotoxin beta  
LT $\beta$ R..... lymphotoxin beta receptor  
MCMV..... murine cytomegalovirus  
MLN ..... mesenteric lymph node  
MSMD..... Mendelian susceptibility to mycobacterial disease  
NK..... natural killer  
NP..... 4-hydroxy-3-nitrophenyl acetyl  
NTS ..... non typhoidal *Salmonella*  
OVA..... ovalbumin  
PP..... Peyer's patch  
*S. typhi* ..... *Salmonella typhi*  
SCV ..... *Salmonella*-containing vacuole  
SPF..... specific pathogen free  
SRBC..... sheep red blood cells  
SSC ..... side scatter  
T3SS ..... type 3 secretion system  
TNF..... tumor necrosis factor  
TNFR..... tumor necrosis factor receptor  
VSV ..... vesicular stomatitis virus  
WT ..... wild type

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## **Abstract**

How some bacteria evade the immune system to cause persistent infection is unclear. Oral vaccination against bacterial pathogens could limit lethal infection but the immune pathways that generate protective immunity are poorly understood. Using streptomycin pretreatment and oral vaccination with live, attenuated *Salmonella* to establish persistent colonization of mice, we demonstrate that the lymphotoxin pathway is essential for the generation of protective immunity to *Salmonella* infection. Lymphotoxin beta receptor (LT $\beta$ R) deficient mice have normal initial colonization of attenuated *Salmonella* in the gut but fail to achieve clearance of *Salmonella* from the feces, and die following challenge with virulent *Salmonella*. Using a gnotobiotic approach we show that even with the same input microbiota, LT $\beta$ R signaling drives microbiota-mediated clearance of *Salmonella*. The role of LT $\beta$ R in *Salmonella* protective responses is not limited to mucosal infection, as LT $\beta$ R deficient mice vaccinated and challenged intraperitoneally also succumb to challenge. Antibody responses to *Salmonella* occurring in the spleen are extrafollicular and contribute to protection. To examine whether antibody responses were deficient in LT $\beta$ R<sup>-/-</sup> mice, we measured serum concentrations of anti-*Salmonella* IgM and IgG. Although LT $\beta$ R<sup>-/-</sup> mice have levels of serum anti-*Salmonella* IgM similar to LT $\beta$ R sufficient controls, they fail to produce anti-*Salmonella* IgG, implying a role for lymphotoxin in class switching. Surprisingly, we find that lymphotoxin (LT) plays an essential role in *Salmonella* immunity independent of its role in secondary lymphoid tissue development; treatment of WT mice with LT $\beta$ R-Ig is sufficient to reduce the formation of anti-*Salmonella*

antibody responses and increase persistence of *Salmonella* in the gut. Using mice conditionally deficient in LT beta (LT $\beta$ ), we find that B cell (CD19<sup>Cre</sup>) derived lymphotoxin is essential for anti-*Salmonella* IgG responses. Finally, we show that multiple cellular sources of lymphotoxin, especially B cells and likely ILC3 (Rorc<sup>Cre</sup>), but not T cells (CD4<sup>Cre</sup>), contribute to protection against challenge with virulent *Salmonella*. Together these data demonstrate the essential role of the LT/LT $\beta$ R signaling pathway in the generation of immunity to *Salmonella* infection.

## **Chapter 1: Introduction**

### *Disease caused by Salmonella enterica*

*Salmonella enterica* is a widespread and diverse pathogen of both humans and animals. Infection can result in responses ranging from self-limiting gastroenteritis to wide spread systemic infection depending on bacterial serovar and host immune health. Although there are more than 2000 known serovars, or subspecies, of *Salmonella enterica*, they can be broadly separated by the type of disease they cause; non-typhoidal strains cause gastrointestinal diarrheal disease while typhoidal strains disseminate systemically and cause typhoid fever.

Non typhoidal strains of *Salmonella* (NTS), such as *Salmonella typhimurium* and *Salmonella enteritidis* are the causative agent of diarrheal disease (Havelaar et al. 2015). Non-typhoidal strains make up the vast majority of *Salmonella* serovars and are considered infection “generalists”: they can infect a wide range of host species including mammals, reptiles and birds (Bäumler and Fang 2013). It is estimated that NTS causes 94 million infection per year and 115,000 deaths (“Salmonellosis (Nontyphoidal) - Chapter 3 - 2016 Yellow Book | Travelers’ Health | CDC” 2016). This number is likely an underestimate as few individuals submit to stool tests. Gastrointestinal disease manifests with bloating, cramping and diarrhea that is not bloody and fever that lasts for 4 to 7 days (“Salmonellosis (Nontyphoidal) - Chapter 3 - 2016 Yellow Book | Travelers’ Health | CDC” 2016).

Recently, non typhoidal strains of *Salmonella* have been developing increasing antibiotic resistance. In sub-Saharan Africa between 50 to 70% of non typhoidal strains are reported to have multi drug resistance (Kariuki et al. 2015). This figure is especially

alarming considering that invasive non typhoidal *Salmonella* is on the rise in immunocompromised individuals, especially children and adults with AIDS. During invasive NTS (iNTS), *Salmonella* is no longer restricted to the GI tract and spreads into systemic tissue, causing fever, anemia, pneumonia, splenomegaly, and hepatomegaly. In iNTS, diarrhea may be present but is not a prominent feature of disease (Feasey et al. 2012). The estimated global burden of iNTS is 3.4 million, with 2/3 of the burden being carried by children. Shockingly, mortality rates due iNTS are 10-30% (Kariuki et al. 2015; Feasey et al. 2012).

These striking figures highlight the importance of understanding *Salmonella* disease susceptibility and progression. HIV status is the most important determinant of susceptibility to iNTS (Feasey et al. 2012). Immunodeficiencies caused by genetic mutation can also predispose certain individuals to developing iNTS versus gastrointestinal disease (Gilchrist, MacLennan, and Hill 2015). Mendelian susceptibility to mycobacterial disease (MSMD) mutations are highly associated with increased susceptibility to iNTS. MSMD mutations all effect portions of the IL12/IFN $\gamma$  response including IFN $\gamma$ R, STAT1, IL12, IL12R, IRF8, TYK2, and NEMO. Other important factors that predispose to iNTS include TH17 recruitment of neutrophils, and neutrophil oxidative burst capacity (Gilchrist, MacLennan, and Hill 2015). These findings also highlight the importance of developing a vaccine to non typhoidal strains. Currently there are no FDA approved vaccines against non typhoidal *Salmonella* (“Salmonellosis (Nontyphoidal) - Chapter 3 - 2016 Yellow Book | Travelers’ Health | CDC” 2016).

Typhoidal strains of *Salmonella* such as *Salmonella typhi* (*S. typhi*) are the causative agents of typhoid fever. This disease is characterized by long term fever,

general malaise, abdominal pain and diarrhea, with symptoms lasting 6 to 30 days (“Typhoid & Paratyphoid Fever - Chapter 3 - 2016 Yellow Book | Travelers’ Health | CDC” 2016). Each year *S. typhi* is responsible for 21 million infections and 200,000 deaths (“Typhoid & Paratyphoid Fever - Chapter 3 - 2016 Yellow Book | Travelers’ Health | CDC” 2016). *S. typhi* is estimated to be at least 10,000 years old and as many as 50,000 to 71,000 years old (Roumagnac et al. 2006; Lan, Reeves, and Octavia 2009) splitting off from its generalist gastrointestinal *Salmonella* relatives to become a human specialist.

Because *S. typhi* infection is restricted to humans, human to human transmission is the only infection route. With the rise of modern sanitation *S. typhi* infections have been dramatically reduced in the developed world. In the developing world, especially Africa and India, infections are common (Kariuki et al. 2015). Although two FDA approved vaccines against typhoid fever are currently available, current vaccination strategies have not proven highly effective and are especially underperforming in endemic regions where sanitation is poor. Neither the injectable typhoidal Vi polysaccharide (ViPS, trade name Typhim VI<sup>®</sup>) or live attenuated oral (Ty21a strain, trade name Vivotif<sup>®</sup>) vaccines are recommended for children under 2 years of age. Both vaccines require multiple administrations and have short terms of protection (2-3 years for ViPS and 5-7 years for Ty21a) and efficacy rates have varied widely ranging from 33-77% (Date et al. 2015). Ongoing development of vaccines to *S. typhi* is crucial, as are continued improvements to water sanitation that limit pathogen spread.

### *Asymptomatic carriage as a disease reservoir of Salmonella*

Understanding disease reservoirs is important in limiting spread of pathogens. In typhoidal *Salmonella* one important disease reservoir consists of previously infected individuals who were never, or are no longer, symptomatic but still carry and shed bacteria. These asymptomatic long term carriers can act as disease reservoirs, propagating illness in an otherwise healthy area. This case is best demonstrated in the unfortunate story of Mary Malone, more commonly referred to as Typhoid Mary.

The bacterial nature of typhoid was first suspected in 1873 when William Bund found infection to be transmitted via the fecal-oral route. In 1880 Karl Joseph Eberth visualized the bacteria within the Peyer's patches (PP) and spleens of typhoid patients. By 1884 the pathogen was grown in culture by Georg Gaffky and in 1885 after isolation of a variant causing "hog typhoid" it was formally named by Theobald Smith, a pathologist working in the laboratory of Daniel Elmer Salmon (Kinney 2009). In 1906 when a wealthy family in New York's Oyster Island was stricken with disease, sanitary engineer and Ph.D. George Soper knew that in order to determine why six of the 11 members of the household were ill, he needed to find the source of the contaminating bacteria (Soper 1907). Soper was intrigued that at the time of the first case of Typhoid in the household, no other cases of the disease were reported in the Oyster Bay area and no other cases followed. Soper tracked the food and water sources into the household and systematically eliminated each as a possible source of bacteria. With careful epidemiology Soper narrowed the timeline of the initial infection. By tracking the movements of the family members he finally centered his attention on a cook who had been hired shortly before the household fell ill, and left the family three weeks after the

outbreak. While common convention at the time held that the cook could not have been a source of disease because she herself had not been ill, Soper proceeded to track down this woman, whose name was Mary Malone.

Mary Malone was deeply offended by Soper's suggestion that she may have spread disease, as she herself was perfectly healthy. She violently refused Soper's suggestion that she donate fecal, blood and urine samples for analysis. Soper instead took an epidemiological approach and carefully tracked Malone's previous employment. He quickly discovered that where Mary Malone went, Typhoid followed. Soper convinced the New York department of health to take Malone into custody. Subsequent stool analysis revealed that Malone was shedding high bacterial titers of *Salmonella* on a regular basis, despite being in pristine health. She was an asymptomatic carrier (Soper 1907).

Malone's condition became a life long ordeal resulting in two forced quarantines, the last until the end of her life. As a single chronic carrier Typhoid Mary was responsible for the known infection of 51 individuals resulting in 3 deaths, although this number is likely a gross underestimate. Typhoid Mary's predicament may be the most famous but it is by far rare. An estimated 2-5% of *S. Typhi* infected patients go on to become long term carriers (Gunn et al. 2014) and it is estimated that 20% of infected individuals are super shedders, contributing to 80% of the transmission of disease (Woolhouse et al. 1997). Chronic carriers are often difficult to identify as at least 25% of carriers never report any clinical disease (Gonzalez-Escobedo, Marshall, and Gunn 2011). Understanding the biological basis of why certain individuals go on to become

long term carriers or super shedders is therefore of utmost public health importance. Unfortunately, little is known about the host components that mediate persistence.

In humans, chronic carriage of disease has been strongly associated with *Salmonella* infection of the biliary tract and the presence of gallstones, and as many as 90% of chronic carriers develop gall stones (Schjøler et al. 1983; Gunn et al. 2014). *Salmonella* is capable of infecting the gall bladder epithelium in a mouse model of infection (Menendez et al. 2009). Gallstones, brought on by diet, predispose mice to gallbladder infection by *Salmonella* (Crawford et al. 2010) and this unique niche seems to protect *Salmonella* against antibiotic elimination (Gunn et al. 2014). The gallbladder is not the only niche for persistent *Salmonella* infection as removal of the gallbladder from long term carriers does not always cure disease (Ristori et al. 1982). The biliary tree, mesenteric lymph nodes (LNs) and the liver have also been found to be bacterial reservoirs. One possible mechanism for bacterial persistence is *Salmonella*'s ability to remain dormant within infected cells (Núñez-Hernández et al. 2014). Slow growing bacteria within cecal lymph node CD103<sup>+</sup> dendritic cells (DCs) are sufficient to allow relapse after antibiotic treatment (Kaiser et al. 2014). Additional work into understanding the host pathways responsible for long term carriage of *Salmonella* continues.

#### *Mouse models of Salmonella infection and disease*

After ingestion of contaminated food and water *Salmonella* travel through the gastrointestinal (GI) track, surviving stomach acidity to gain access to the small intestine. The generation of typhoidal disease requires that *Salmonella* gains access into host systemic tissue. *Salmonella* has two main routes of entry into the host, the first

is via M cells, specialized epithelial cells in the follicle associated epithelium on Peyer's patches (PP) in the small intestine, and the second via infection of non-phagocytic enterocytes. A third mechanism of invasion, via uptake by CX3CR1<sup>+</sup> CD11c<sup>+</sup> cells has also been described (Kaiser et al. 2012; Broz, Ohlson, and Monack 2012). Infection of mice with *S. typhimurium* revealed that M cells are preferentially infected by *Salmonella* early following infection compared to other epithelial cells (Clark et al. 1994; Jones, Ghori, and Falkow 1994). The ability of *Salmonella* to form long polar fimbria, as coded for by the *lpf* operon is critical in the ability to attach to PP but not to non-absorptive enterocytes (Bäumler, Tsolis, and Heffron 1996). Active infection requires injection of effector molecules into host cells with a type 3 secretion system (T3SS) called Spi-1 (Hansen-Wester and Hensel 2001). Although Spi-1 promotes invasion via M cells (Clark, Hirst, and Jepson 1998), it is not absolutely essential for entry (Martinez-Argudo and Jepson 2008). Spi-1 is however essential for infection of non-phagocytic epithelial cells (Jepson et al. 1995). Using Spi-1, *Salmonella* injects a complex array of effector molecules into the cell that mediate invasion and trigger host inflammation and immune responses (LaRock, Chaudhary, and Miller 2015). Following infection bacteria translocated through the M cell to gain access to the DCs and macrophages located in the pocket below.

Being a facultative intracellular pathogen, once *Salmonella* has gained access into a host cell, such as a macrophage, it induces changes to the actin cytoskeleton to form *Salmonella*-containing vacuoles (SCVs). Using another T3SS, Spi-2, *Salmonella* manipulate host cell biology to maintain and alter the SCV to allow for replication (Hansen-Wester and Hensel 2001; Hapfelmeier et al. 2005; LaRock, Chaudhary, and

Miller 2015). The pathogen uses Spi-2 effectors to move the vacuole towards the cell membrane releasing bacteria into the extracellular space which results in the infection of new cells. In some cells, *Salmonella* does not replicate within the SCV but is in a dormant, but persistent state. These cells act as a protective niche for the bacteria (Kaiser et al. 2014). The ability of *Salmonella* to replicate and survive intracellularly protects it from both the innate and adaptive immune system. Antibodies and neutrophils can only kill *Salmonella* in its extracellular phase (Cheminay, Chakravorty, and Hensel 2004). Although immune cell rich PP are considered preferential sites for *Salmonella* entry, infection of enterocytes is possible. Once *Salmonella* has gained access to the lamina propria or PP and infected DCs and macrophages, these cells can spread the bacteria into further systemic tissues, such as the mesenteric lymph nodes (MLN) or spleen, as they migrate into these organs.

Many of the details of this invasion mechanism have been elucidated using both *in vitro* cell culture models and *in vivo* models of infection. As addressed above *S. typhi* is a human specialist and therefore not a natural pathogen of rodents. Therefore, most rodent models of *Salmonella* infection use *S. typhimurium* or *S. enteritidis* strains. Unlike in humans, oral infection of mice with *S. typhimurium* results in systemic infection and does not cause gastrointestinal disease in most animals. While this model is sufficient to study systemic immune responses to typhoidal disease, it is not useful for the dissection of non typhoidal gut inflammation. Furthermore, most common strains of mice, especially those that are most popular in immunological research such as BALB/c and C57BL/6 have a mutated version of the *S1c11a* gene coding for the Nramp1 protein (Stecher et al. 2006; Mastroeni 2002). The Nramp1 protein is targeted to the SCV in

infection where it functions to restrict iron transport thereby limiting pathogen replication (Wessling-Resnick 2015). Therefore animals on the C57BL/6 and BALB/c background are highly susceptible to infection with *Salmonella*, succumbing to disease within one to two weeks (Mittrücker and Kaufmann 2000). Two experimental concerns must therefore be addressed in order to use mice as models for *Salmonella* infection. The first is how to study immune responses beyond the innate phase of infection. The second, how to study non typhoidal disease. To address these concerns, several strategies have been developed. The use of common strains such as C57BL/6 infected with fully virulent *Salmonella* proved to be a highly effective tool to study infection initiation and innate immune responses (McSorley 2014). To study immune responses and bacterial behavior at later time points experimentalists can choose to study fully virulent *Salmonella* in a strain with intact Nramp1 such as the 129x1/SvJ strain (Stecher et al. 2006; Mastroeni 2002). From a microbiological perspective this strategy allows many mutants of *Salmonella* to be studied and the changes in immune response measured. The benefit is that the host has an intact innate response and the pathogen is fully virulent. Since *Salmonella* interacts with the host via virulence factors this can lead to an understanding that is more physiologically relevant. Unfortunately, the SvJ strain is limited in its potential to fully understand immunological responses due to a lack of sufficient genetic knockout mice on this background. Another approach is to use attenuated *Salmonella* mutants such as those that have limited intracellular replication in susceptible strains of mice. Use of attenuated strains of *Salmonella* that have limited intracellular replication but retain virulence factors responsible for initiation of infection (such as the *aroA* or *aroD* mutants) cause self-limiting disease without killing the host

(Barthel et al. 2003; Hapfelmeier et al. 2005). These strains therefore mimic many components of human disease and are amenable to the study of resolution of primary infection and generation of immunological memory. One caveat is that intracellular replication may be an important component for initiation of the immune responses. By lacking the *Nramp1* gene we must also take into account that the C57BL/6 strain has impaired innate immunity and therefore other compensatory mechanisms that may not exist in *Nramp1* sufficient strains. Therefore, by using attenuated mutants in susceptible strains, or virulent *Salmonella* in *Nramp1* sufficient backgrounds, late phases of host and bacterial interactions can be studied. Indeed, these strategies have proven very useful in understanding the requirements for bacterial clearance, and development of immunological memory.

Another experimental consideration in using mouse models of *Salmonella* disease in mice is that even though *S. typhimurium* is a human non typhoidal strain, it causes typhoidal disease in mice without gastrointestinal symptoms. An important observation is that in both the resistant 129x1/SvJ strain and susceptible strains, infection with the same dose of inoculum will result in variable levels of colonization in the gut. While all resistant 129x1/SvJ mice become infected systemically a small percentage of animals will also become colonized with *Salmonella* in the GI tract, develop colitis and become super shedders (Gopinath et al. 2013). Similarly, 5% of susceptible mice become colonized with *Salmonella* in the gut lumen and develop gastrointestinal inflammation (Stecher et al. 2010).

One feature that distinguishes mice that naturally become colonized from those that do not is microbiota composition. Infection of a variety of strains led to the

observation that a positive correlation exists between *E. coli* abundance and the ability of *Salmonella* to colonize the gut (Stecher et al. 2010). Indeed, a complex microbiota is required to confer resistance to *Salmonella*. Mice that are germ free or those colonized with a simplified microbiota (altered Schaedler's flora) all become colonized with *Salmonella* in the gut and develop robust colonic and cecal inflammation (Endt et al. 2010; Stecher et al. 2005).

The ability of the microbiota to directly compete with a pathogen for space or nutrients, thereby reducing susceptibility of the host to pathogen infection is called colonization resistance (Yurist-Doutsch et al. 2014). One well-developed strategy to turn *S. typhimurium* into a gastrointestinal disease is to reduce colonization resistance by pretreating animals with streptomycin. Streptomycin is an aminoglycoside antibiotic that binds to the 30S ribosomal subunit causing faulty protein production. This class of antibiotics are most effective against most gram-negative aerobes and facultive anaerobic bacilli but have poor efficacy against anaerobes and gram-positive bacteria ("Aminoglycosides" 2016). *Salmonella* carry two separate genetic loci, *strA* and *strB* that mediate resistance to streptomycin (Yamada and Davies 1971). In both susceptible stains and resistant strains of mice, pretreatment of animals with streptomycin prior to oral administration of *Salmonella* results in a reduction of colonization resistance by the microbiota and allows for colonization of the pathogen in the gut (Barthel et al. 2003; Que and Hentges 1985; Stecher et al. 2006). Single dose treatment with streptomycin causes a significant reduction in the order Bacteroidales and an expansion of Deferribacteria and Proteobacteria phyla (Stecher et al. 2007). Once *Salmonella* is introduced, within 4 days 90% of the of the cecal microbiota consists of Proteobacteria,

of which 70% is *Salmonella*. Other commensal Proteobacteria such as *E. coli*, which were low abundance species prior to antibiotic treatment and infection, now become significantly represented (Stecher et al. 2007). Since *E. coli* abundance correlates with *Salmonella* gut colonization, one theory suggests that *Salmonella* is best suited to complete with members of the gut microbiota that are its closest relatives (Stecher et al. 2010).

Although a single high dose (20mg) of streptomycin is the most widely used and well defined antibiotic to reduce colonization resistance and induce *Salmonella* gut colonization, other antibiotics such as vancomycin and metronidazole, even at low doses, can also be utilized with similar effects (Kaiser et al. 2012). A direct comparison between streptomycin and vancomycin shows that while each antibiotic perturbs the microbiota differently (low dose streptomycin led to a reduction of Firmicutes while vancomycin treatment greatly expanded Firmicutes and  $\gamma$ -Proteobacteria) either was capable of reducing colonization resistance and allowing for *Salmonella* colonization and gut inflammation (Sekirov et al. 2008). Because low dose antibiotic treatment did not alter overall bacterial abundance, these results suggest that a disruption of microbiota diversity, rather than abundance, is sufficient reduce colonization resistance (Yurist-Doutsch et al. 2014).

Streptomycin pretreatment of C57BL/6, 129Sv/Ev, and DBA/2 mice results in indistinguishable levels of colonic and cecal inflammation. Mice from the 129Sv/Ev strain go on to develop persistent infection including of the gall duct epithelium, mirroring chronic infection in humans (Stecher et al. 2006). On susceptible backgrounds, infection with a virulent strain results in death, but use of intracellular

replication attenuated *Salmonella* results in colonization that is self-limiting and cleared within 4 - 9 weeks in most animals (Endt et al. 2010).

Triggering of inflammation is especially important in allowing *Salmonella* colonization of the gut. Effective competition of *Salmonella* with the microbiota is essential to establishing colonization in the gut and this competition is achieved by induction of inflammation by the pathogen. Avirulent *Salmonella* deficient in type III effector proteins or Spi-1 are unable to trigger gut inflammation or sustain colonization in the gut, despite pretreatment with streptomycin (Stecher et al. 2007; Barthel et al. 2003). In animals in which another trigger of inflammation exists, such as in IL10 deficiency or ongoing VILLIN-HA T cell-transfer mediated colitis, no streptomycin is required to facilitate *Salmonella* colonization even with avirulent *Salmonella* (Stecher et al. 2007). Therefore, triggering of inflammation is a key step in initiation of colonization in the gut by limiting competition from the microbiota.

*Salmonella* can trigger inflammation via Spi-1 or Myd88/Spi-2 dependent mechanisms (Kaiser et al. 2012). Using the T3SS Spi-1 *Salmonella* injects effector molecules, such as the SopA and SopE, into a cell (LaRock, Chaudhary, and Miller 2015; Hapfelmeier et al. 2004). SopE is a guanine-nucleotide exchange factor that can activate the host Rho GTPases Rac1 and Cdc42 (Hardt et al. 1998). Activation of these molecules leads to actin remodeling and caspase-1 activation through a yet unknown pathway (Müller et al. 2009). Activated caspase-1 cleaves pro-IL1 $\beta$  and proIL18, and these cytokines are key mediators of inflammation. Mice that are deficient in caspase 1 do not recruit macrophages, dendritic cells, or T cells to the mucosa following *Salmonella* infection (Müller et al. 2009). *Salmonella* can also trigger inflammation in a

Myd88 dependent, Spi-2 dependent pathway (Hapfelmeier et al. 2005). Recognition of *Salmonella* by TLR2 and TLR4 and leads to the upregulation of bacterial Spi-2 and is required for intracellular bacterial growth (Arpaia et al. 2011).

*Salmonella* uses many mechanisms to co-opt pathways of commensal metabolism as well as the host inflammatory response to compete with the gut microbiota and facilitate colonization. During early invasion, *Salmonella* consumption of a microbiota metabolite H<sub>2</sub>, enhances *Salmonella* colonization (Maier et al. 2013). Molecular hydrogen, H<sub>2</sub>, is produced by primary fermenters, including Bacteroides and Clostridia, in the gut (Fischbach and Sonnenburg 2011; Dabrock, Bahl, and Gottschalk 1992; Macy and Probst 1979). This molecule can act as an electron sink in the anaerobic environment of the gut and is used as such by non-primary fermenters including acetogens, methanogens, and sulfate-reducing bacteria (Fischbach and Sonnenburg 2011). *Salmonella* with mutations in the *hyb* operon, that codes for the hydrogenase that utilizes H<sub>2</sub>, are impaired in their ability to compete with wildtype *Salmonella* for colonization in the gut. In germ free animals, in which there is no source of H<sub>2</sub>, WT *Salmonella* loses its competitive colonization advantage over *hyb* mutant *Salmonella* (Maier et al. 2013).

Active triggering of inflammation also works to the advantage of *Salmonella*. Once the host senses infection it seeks to limit pathogen replication by limiting iron availability. The host produces lipocalin-2, a protein that limits the growth of pathogens such as *E. coli* by sequestering iron (Flo et al. 2004; Berger et al. 2006). *Salmonella* can bypass this protective mechanism because it produces a variant of a common bacterial iron scavenging system that is not bound by lipocalin-2 (Raffatellu et al. 2009).

Therefore, while lipocalin-2 limits the growth of commensals by limiting iron availability, *Salmonella* can replicate freely.

*Salmonella* also outcompetes commensals via its use of sugar substrates, including mucins, during inflammation (Raffatellu et al. 2009). Host immune cells sense infection and transmigrate into the lumen to aid in anti-bacterial inflammatory responses. One mechanism by which these cells seek to limit pathogen growth is by producing reactive oxygen species that react with luminal thiosulfate to produce tetrathionate (Winter et al. 2010). *Salmonella* encode the *ttr* operon which allows for tetrathionate to be used as a terminal electron acceptor in anaerobic respiration (Winter et al. 2010). In the presence of a functional *ttr* *Salmonella* undergo anaerobic respiration utilizing fermentation end products that would otherwise be unavailable for further metabolism to commensals (Winter and Bäumler 2011).

Infection with *Salmonella* also triggers the IL22/IL23 pathway (Godinez et al. 2009). This pathway leads to the generation of anti-microbial peptides such as RegIII $\beta$  and RegIII $\gamma$  that are essential to limiting *Citrobacter* infection (Zheng et al. 2008). RegIII $\gamma$  preferentially targets gram positive bacteria while RegIII $\beta$  inhibits growth of both gram positive and gram negative microbes (Stelzer et al. 2011; Cash et al. 2006). Infection with *Salmonella* results in the upregulation of RegIII $\beta$  in the cecum (Stelzer et al. 2011), but anti-microbial peptides such as RegIII $\beta$  and RegIII $\gamma$  do not kill *Salmonella* *in vitro* (Cash et al. 2006; Stelzer et al. 2011). One mechanism by which *Salmonella* protects itself from RegIII $\beta$  killing is through O-antigen mediated modification to LPS that limit RegIII $\beta$  access to its target peptidoglycan; O-antigen mutants of *Salmonella* have increased susceptibility to RegIII $\beta$  killing *in vitro* (Stelzer et al. 2011). The activation

of the IL22/IL23 pathway and subsequent upregulation of RegIII $\beta$  by *Salmonella* does have an impact on commensals as administration of recombinant RegIII $\beta$  to specific pathogen free (SPF) mice increased *Salmonella*'s ability to initiate colonization of the gut (Stelzer et al. 2011).

Using these mechanisms *Salmonella* is highly effective at triggering and using host inflammatory responses to gain a foothold and colonize the colon and cecum of mice. Less is known about how *Salmonella* is ultimately cleared from the gut, as is the case in the majority of infected mice and humans. Clearance of *Salmonella* from the gut requires a complex microbiota; germ free animals or those colonized with a simplified microbiota are not capable of clearing the pathogen from the gut (Endt et al. 2010; Stecher et al. 2005). Addition of a complete microbiota to *Salmonella* monocolonized animals is sufficient to drive *Salmonella* clearance (Endt et al. 2010). Which specific members of the gut microbiota are required to mediate clearance is unknown. Another outstanding question is if, and how, the host manipulates this clearance. Production of IgA after *Salmonella* infection is robust, but this molecule is not required to drive clearance from the gut (Endt et al. 2010). IgA deficient and heterozygous littermates have equal rates of clearance. B cells and T cells are independently also not required to mediate clearance from the gut (Endt et al. 2010). What, if any, host components contribute to microbiota-driven clearance of *Salmonella* remains an open question.

#### *Anti-Salmonella memory responses*

Understanding the requirements for the formation of anti-*Salmonella* memory responses could aid in vaccine development. Currently, vaccines against *Salmonella*

*typhi*, including oral vaccines, have limited efficacy, especially in endemic regions (MacLennan, Martin, and Micoli 2014). Both T and B cell responses contribute to protection against *Salmonella* (Mittrücker and Kaufmann 2000; Bao et al. 2000). CD4<sup>+</sup> T cell responses, especially production of IFN $\gamma$ , are an important determinant in *Salmonella* memory protective responses. Nude mice and mice lacking  $\alpha\beta$  T or CD4<sup>+</sup> T cells are highly susceptible to *Salmonella* infection, succumbing even when inoculated with attenuated strains (Hess et al. 1996; Sinha et al. 1997). TCR $\delta$  deficient mice tolerate attenuated *Salmonella* as well as wild type mice (Hess et al. 1996). IFN $\gamma$  is also essential: IFN $\gamma$ R<sup>-/-</sup> succumb to attenuated *Salmonella* (Hess et al. 1996) and IFN $\gamma$  deficient mice, or those in which IFN $\gamma$  or TNF $\alpha$  is blocked during primary infection with *Salmonella*, have higher bacterial burdens in systemic organs (Nauciel and Espinasse-Maes 1992; Bao et al. 2000). Predictably, mice deficient in CD4<sup>+</sup> Th1 cells also share a similar phenotype; *Tbet* deficient mice succumb to attenuated *Salmonella* (Ravindran et al. 2005). CD4 T cells produced IFN $\gamma$  seems to be most important in early responses to primary infection and early memory responses (Pie et al. 1997). IFN $\gamma$  deficiency actually leads to higher titers of anti-*Salmonella* IgA and IgG (Bao et al. 2000). The role of CD8 T cells is less well defined, but it seems that they contribute modestly to the late phase of primary infection (Lee, Dunmire, and McSorley 2012). Th17 cells may play a role in *Salmonella* responses at the mucosa, as IL17 $\alpha$  deficient mice have increased bacterial dissemination (Schulz et al. 2008).

The role of T cell responses to *Salmonella* has been largely studied in the absence of streptomycin pretreatment where colonization of the gut is not a long term event. Therefore, the role of T cells in the mucosa is less well defined. For example,

gamma delta ( $\gamma\delta$ ) T cells or IL17 cells, which are enriched in the gut associated lymphoid tissue (GALT) do not appear to play a major role in *Salmonella* infection (Schulz et al. 2008). These cells do however mobilize within hours of sensing *Salmonella* infection in the gut (Edelblum et al. 2015).

B cells have been found to contribute in both antibody dependent and independent ways. B cell antibody-dependent contributions limit pathogen entry into host cells while B cell antibody independent mechanisms, including as APCs or cytokine producers, contribute to robust memory T cell formation (McSorley and Jenkins 2000; Nanton et al. 2012; Barr et al. 2010; Mastroeni, Villarreal-Ramos, and Hormaeche 1993). IgG, predominantly the IgG2c isotype in the C57BL/6 strain, is generated against *Salmonella* outer membrane proteins as early as three days post infection. Transfer of anti-*Salmonella* IgG2c, and to some extent IgM, impedes the ability of *Salmonella* to infect macrophages (Cunningham et al. 2007). Similarly, IgA formed against the O antigen limits bacterial entry into epithelial cells in the gut, and prevents secondary inflammation (Endt et al. 2010). Antibody, including IgA, is dispensable for clearance of *Salmonella* in the gut tissue (Endt et al. 2010). Under most circumstances, transfer of immune serum is also insufficient to provide protection of naïve mice against challenge (Mastroeni et al. 2000; Mastroeni, Villarreal-Ramos, and Hormaeche 1993). However co-transfer of both T cells and immune serum has some protective effect in naïve animals (Mastroeni, Villarreal-Ramos, and Hormaeche 1993).

Recently it has been demonstrated that high affinity antibody production during *Salmonella* infection occurs largely outside of the germinal center (GC) (Cunningham et al. 2007; Di Niro et al. 2015). Germinal center like structures do not appear in WT mice

vaccinated with *aroA* attenuated *Salmonella* until one month following infection, and even then, these structures are small and immature; they express low levels of PNA positive cells and are found at atypical sites between the T and B cells zones. Class switching and affinity maturation occur at the same rate in extrafollicular locations and these germinal center like structures (Di Niro et al. 2015). Whether this is a pathogen strategy to limit host responses, or a host driven adaptive response is not yet known, but earlier formation of GC-like structures can be induced if bacterial burdens are lowered by treatment with antibiotics (Cunningham et al. 2007). Both of the studies published on the subject use the *aroA* mutant strain of *Salmonella*, that is deficient in intracellular replication (Cunningham et al. 2007; Di Niro et al. 2015). It will be important to determine in Nramp1 sufficient animals if infection with fully virulent *Salmonella* also delays GC formation, or if this phenotype is attributable to the lack of intracellular replication of the *aroA* mutants. Host contributions to extra-follicular antibody responses, especially during infection, are poorly understood.

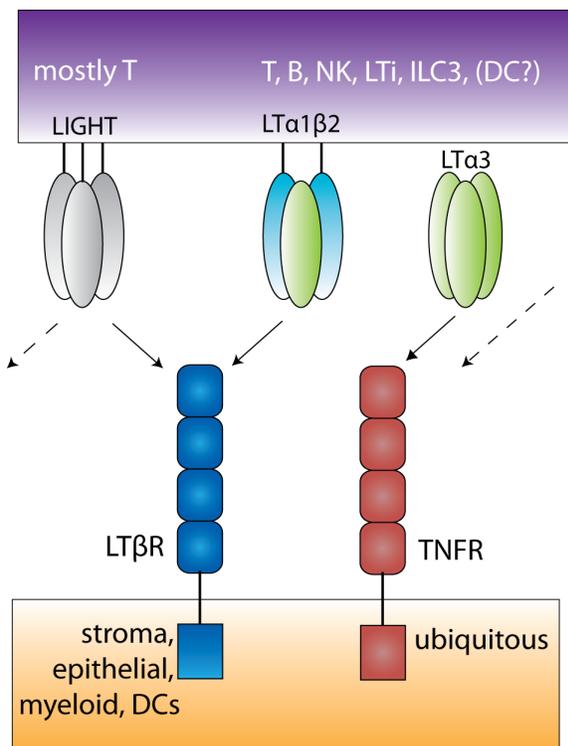
Antibody independent functions of B cells have also been shown to play an essential role in anti-*Salmonella* memory, but not primary, responses. B cell deficient mice tolerate infection with attenuated *Salmonella* and achieve systemic clearance of attenuated *Salmonella* with equal kinetics to WT mice (McSorley and Jenkins 2000; Mastroeni et al. 2000). Similarly, B cell deficient mice clear attenuated *Salmonella* from the gut in the streptomycin pretreatment model (Endt et al. 2010). B cells may be more critical for the memory response. B cell deficient mice previously infected with attenuated *Salmonella* have higher loads of bacteria in the spleen and liver when later challenged with a virulent strain (McSorley and Jenkins 2000; Mastroeni et al. 2000)

and nearly 100% of mice succumb to infection, although with slower kinetics than naïve animals (Nanton et al. 2012). This role of B cells seems to be largely antibody independent. When the same experiment is performed in animals that retain B cells but cannot produce antibody, the majority of mice survive challenge (Nanton et al. 2012).

B cell deficiency results in a reduction in the number of IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells following attenuated *Salmonella* infection (McSorley and Jenkins 2000). Activation of MyD88 signaling in B cells drives the induction of highly responsive IFN $\gamma$  producing T cells early after infection but does not impact memory IFN $\gamma$  CD4 T cell responses. On the other hand, while B cell presentation of antigen via MHCII does not have a role in early effector T cell responses to *Salmonella*, it does significantly contribute to memory IFN $\gamma$  responses 7 weeks post infection. Mice in which B cells are absent or in which B cells lack MHCII, have significant impairments in CD4 IFN $\gamma$  and IL-2 production in response to *Salmonella* (Barr et al. 2010).

### *The lymphotoxin pathway*

Lymphotoxin signaling plays a role in many processes that are foundational to the generation of an appropriate immune response. First and foremost, lymphotoxin-producing lymphoid tissue inducing cells (LTi) are indispensable for the formation of peripheral lymph nodes and PP during development (De Togni et al. 1994; Mariathasan et al. 1995; Mebius et al. 1996; Adachi et al. 1997; M. Matsumoto et al. 1997). Beyond that, lymphotoxin signaling within lymphoid organs organizes lymphoid architecture and promotes lymph node expansion and germinal center formation. The lymphotoxin pathway also drives innate cytokine responses during infection.



**Figure 1. Ligands and receptors of the lymphotoxin pathway.**

Lymphotoxin refers to two molecules within the TNF superfamily: LTα and LTβ. These molecules exist in two forms, as a soluble homotrimer LTα3, or as a membrane bound heterotrimer, LTα1β2 (Browning, Androlewicz, and Ware 1991; Browning et al. 1993; Browning et al. 1995). As members of the TNF superfamily, these molecules are biologically and structurally related to other family members especially TNF and LIGHT (Fu and Chaplin 1999). Lymphocytes, especially B and T cells are the primary producers of both membrane LTα1β2 and

soluble LTα3, but expression of these molecules has also been found on natural killer (NK) cells and innate lymphoid 3 cells (ILC3s) (Ware et al. 1992; Tumanov et al. 2011). The expression of LT on DCs has also been reported (Moussion and Girard 2011). TNF, LTα, and LTβ are genetically linked, and are found closely grouped together within the class III portion of the MHC/HLA on chromosome 6 (Inoko and Trowsdale 1987; Spies et al. 1986; Müller et al. 1987).

Two receptors exist for the LT ligands. LTα is the ligand for TNFRI and TNFRII, sharing its receptors with TNF (Fu and Chaplin 1999). These TNF receptors are widely expressed on almost all cell of the body (Ware et al. 1995). The cell-surface heterotrimer LTα1β2 signals exclusively through a different receptor, LTβR (Crowe et al.

1994). Expression of  $LT\beta R$  is less promiscuous. Epithelial cells, stromal cells, and myeloid cells including macrophages and DCs are the predominant expressers of  $LT\beta R$  (Ware et al. 1995). Binding of  $LT\alpha$  to the TNFR triggers activation of the classical NF- $\kappa B$  pathway while signaling by  $LT\alpha 1\beta 2$  via  $LT\beta R$  preferentially triggers activation of the alternative NF- $\kappa B$  signaling cascade (Dejardin et al. 2002; Oeckinghaus, Hayden, and Ghosh 2011). Collectively, the lymphotoxin pathway is a central organizer of lymphoid tissues.

The essential role of the lymphotoxin pathway in lymphoid tissue development was first identified by the Chaplin group when they generated the  $LT\alpha$  deficient mouse (De Togni et al. 1994). These animals lacked lymph nodes and PPs and had disrupted splenic architecture. A single mesenteric lymph node was detected in only 2-5% of mice (Fu, Huang, et al. 1997). Since neither TNFR I or TNFR II had these same defects in lymphoid development, it suggested that  $LT\beta R$  is required for lymphoid tissue ontogeny (Pfeffer et al. 1993; Erickson et al. 1994). This was later confirmed when  $LT\beta R$  deficient mice were generated (Fütterer et al. 1998). Further evidence suggesting that membrane  $LT\alpha 1\beta 2$  is the primary driver of lymphoid tissue development came with the generation of  $LT\beta$  deficient mice (Koni et al. 1997).  $LT\beta$  deficient mice lack  $LT\alpha 1\beta 2$  but retain soluble  $LT\alpha$ . Peripheral lymph nodes and PPs in  $LT\beta$  deficient mice are lacking, but mesenteric and cervical lymph nodes are intact, suggesting that  $LT\alpha$  signaling via the TNFRs can compensate to some degree for  $LT\alpha 1\beta 2$  deficiency.

The generation of lymph nodes occurs within a fixed window during fetal development (Rennert et al. 1996). Disruption of  $LT\beta R$  signaling via administration of  $LT\beta R$ -Ig fusion protein to pregnant mice at or before day 12 of gestation results in an

absence of PP and most lymph nodes, with mesenteric and some cervical lymph nodes retained (Rennert et al. 1996). Administration of the same fusion protein by day 16 of pregnancy causes PP and popliteal LNs to be absent while others remains (Rennert et al. 1996). Giving the fusion protein at day 18, all PP were absent but LN were present. Indeed later studies reveal that Ror $\gamma$ <sup>+</sup> LTi (now a member of the ILC3 family of innate lymphoid cells) leave the fetal liver to seed sites of lymph node development (van de Pavert and Mebius 2010). These cells are drawn via their expression of CCR5 to sites rich in CXCL13 (van de Pavert et al. 2009). Once clustered at sites of future lymph node development, LTi via LT $\alpha$ 1 $\beta$ 2 interact with LT $\beta$ R<sup>+</sup> stromal cells. This interaction begins a positive feedback loop of chemokine, adhesion molecule, and cytokine expression, drawing lymphoid cells into the site (van de Pavert and Mebius 2010; Vondenhoff et al. 2009). Lymphoid cells, especially B cells drawn to the newly forming lymph node also express LT $\alpha$ 1 $\beta$ 2. Interactions between these new arrivals and different subsets of stromal cells, via LT $\beta$ R sets up the distinctive micro-domains associated with fully formed lymphoid structures (Bénézech et al. 2010; Mebius et al. 1996).

Although the development of lymph nodes and PPs occurs in fetal ontogeny, lymphotoxin/LT $\beta$ R signaling continues to have a role in the maintenance of splenic and lymphoid structure throughout adult life. Tonic lymphotoxin signaling helps to maintain the follicular dendritic cell (FDC) network within the spleen and lymph node (Koni et al. 1997; Alimzhanov et al. 1997). B cell production of lymphotoxin helps to maintain numbers of subscapular macrophages important in viral responses (Moseman et al. 2012). Lymphotoxin signaling in the steady state is also needed to maintain the phenotypic features of the high endothelial venule (HEV) that allows the migration of

naïve lymphocytes into lymph nodes, and blockade of signaling with the LT $\beta$ R-Ig caused a reduction in lymph node size (Browning et al. 2005). Treatment of newborn mice (in which lymph nodes are formed) with LT $\beta$ R-Ig leads to a loss of T/B segregation and an absence of follicles (Ettinger et al. 1996). During immunization and infection, lymphotoxin contributes to expansion of the fibroblastic reticular cell (FRC) network (Katakai et al. 2004).

All of these functions make lymphotoxin an important mediator in high affinity antibody responses. The role of LT $\beta$ R in high affinity antibody responses was revealed with the use of model antigens such as haptenated proteins and sheep red blood cells (SRBC). When LT $\alpha$ , LT $\beta$  or LT $\beta$ R deficient mice were immunized with these antigens, especially at low doses or in the absence of adjuvant, little to no class switched antibody was produced (Matsumoto et al. 1997; Koni et al. 1997; Banks et al. 1995; Matsumoto et al. 1996) (**Table 1**). Although these animals make robust antigen specific IgM responses, they are unable to form germinal centers and make IgG. These findings support the importance of B cell follicles and germinal centers as the sites for class switching and affinity maturation. It was therefore surprising that lymphotoxin deficient strains are capable of both class switching and some affinity maturation when antigen is adjuvanted, given at high dose, or administered repeatedly (Matsumoto et al. 1996; Koni et al. 1997; Fütterer et al. 1998; Koni and Flavell 1999; Wang et al. 2000) (**Table 1**). The production of high affinity IgG occurred in the absence of well-structured follicles and germinal centers, suggesting that although lymphotoxin is essential for the maintenance of lymphoid tissue architecture, the presence of this architecture is not absolutely necessary for the formation of high affinity, class switched antibody.

**Table 1. Antibody responses to model antigens in lymphotoxin deficient mouse strains.**

In this figure data is summarized into findings in which poor antibody responses were noted (top) or in which lymphotoxin deficient strains made partial or normal levels of class switched antibody responses. \* As measured by low concentration coated ELISA

Model Antigen	Mouse genotype	GC?	IgM	IgG	High affinity?*	reference
<b>Conditions with poor class switched antibody responses</b>						
i.p. SRBC	<i>Lta</i> <sup>-/-</sup>	no	+	-		(Y. X. Fu, Molina, et al. 1997a; Koni et al. 1997)
	<i>Ltb</i> <sup>-/-</sup>		++	-		(Koni et al. 1997; Tumanov et al. 2003)
irradiated HSV	<i>Lta</i> <sup>-/-</sup>			-		(Banks et al. 1995)
KLH in alum 2x	<i>Lta</i> <sup>-/-</sup>			-		
Low dose NP-OVA	<i>Lta</i> <sup>-/-</sup>	no	++	-	-	(Mitsuru Matsumoto et al. 1996)
<b>Conditions with normal or partial class switched antibody responses</b>						
NP-SRBC 2x	<i>Lta</i> <sup>-/-</sup>			+	+/-	(Y. Wang et al. 2000, 200)
NP-SRBC 4x	<i>Lta</i> <sup>-/-</sup>			+	+	
High dose NP-OVA (200ug)	<i>Lta</i> <sup>-/-</sup>	no	++	+	+/-	(Mitsuru Matsumoto et al. 1996)
High dose NP-OVA (200ug) + alum	<i>Lta</i> <sup>-/-</sup>	no	++	+	+	
NP <sub>19</sub> -CG (5ug) + alum	<i>Ltbr</i> <sup>-/-</sup>	no	+	+/-	-	(Agnes Fütterer et al. 1998)
NP <sub>19</sub> -CG (200ug) + alum	<i>Ltbr</i> <sup>-/-</sup>		+	+	+	
NP <sub>16</sub> CG (50ug) + alum	<i>Lta</i> <sup>-/-</sup>		+	+	+/-	(Koni et al. 1997)
	<i>Ltb</i> <sup>-/-</sup>		+	+/-	+/-	
NP <sub>13</sub> CG (50ug) + alum	<i>Lta</i> <sup>-/-</sup>	somewhat	+	+	+/-	(Koni and Flavell 1999)

It should be noted that while the study of the mammalian immune system has closely tied the role of lymph nodes with adaptive immune responses, evolutionarily, T and B cell responses are 300 million years older than lymph nodes (Boehm, Hess, and Swann 2012). Lymph nodes like structures only appear in birds and mammals some 100 million years ago (Boehm, Hess, and Swann 2012) and true lymph nodes, as we study them in the mouse and human only appear in therian mammals (monotremes such as the platypus do not have true lymph nodes while marsupials do) (Lane et al. 2010). Incidentally, the evolutionary appearance of true lymph nodes coincided with a gene duplication event in the TNF locus that resulted in the generation of *Ltbr* (Lane et al. 2010). Although monotreme mammals lack encapsulated lymph nodes, they have isolated lymphoid follicles (ILFs) where lymph nodes would be, and have organized germinal centers (Diener and Ealey 1965). However, it seems that these animals do not efficiently produce memory B cell responses (Wronski, Woods, and Munday 2003). From an evolutionary perspective, and from observations in mice with  $LT\beta R/LT$  deficiencies, it seems that the lymphotoxin pathway greatly enhances the ability to efficiently produce high affinity antibody.

#### *The lymphotoxin pathway's role in infectious disease*

The role of the lymphotoxin pathway in infectious disease has been characterized for a variety of pathogens including viruses, bacteria and parasites. In general, defects within the pathway result in impaired immunity although the specific components of the pathway and the mechanism by which they control pathogens vary greatly. Overall, we can separate the role of the lymphotoxin in infectious disease into

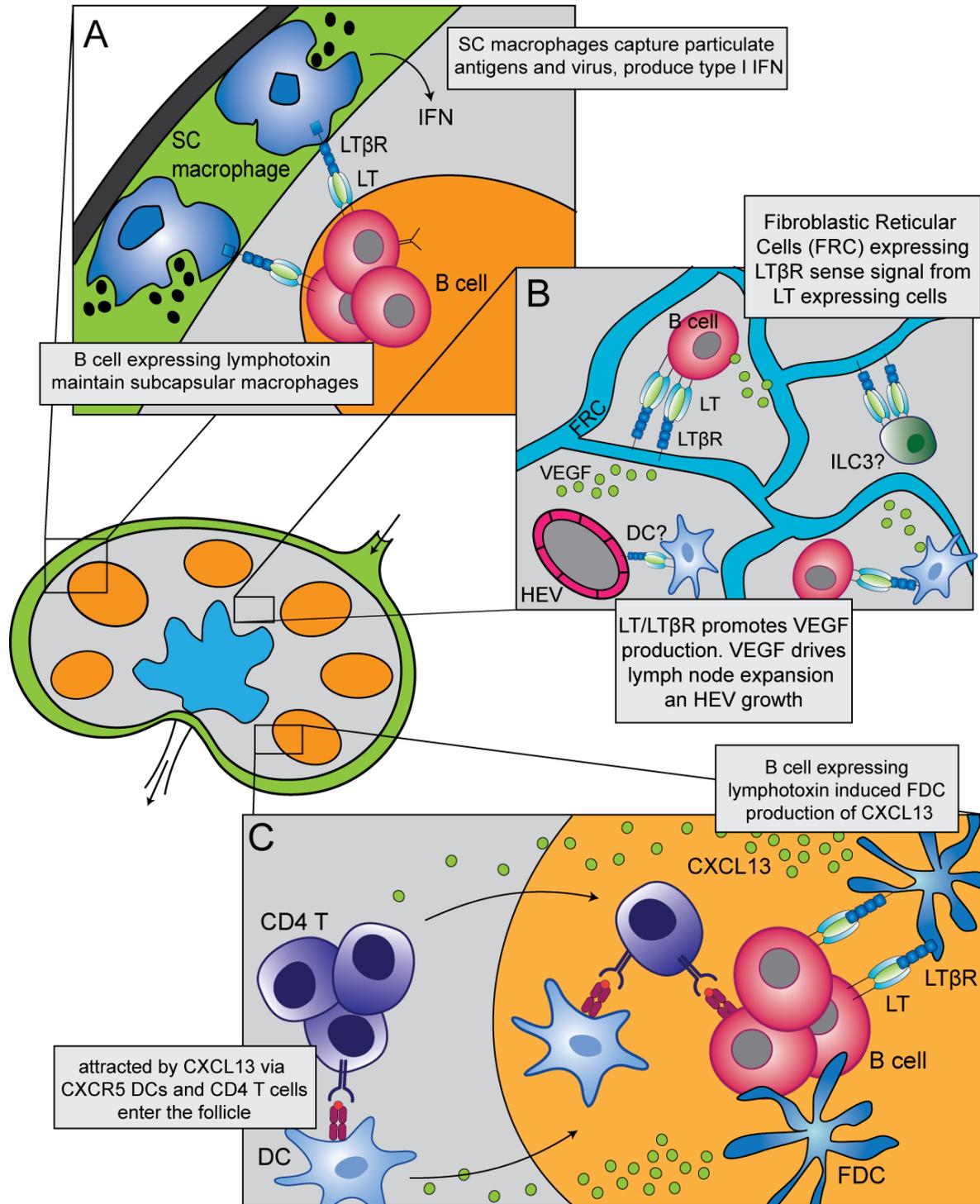
three general categories: importance in lymphoid structures, contributions to lymphoid tissue micro-architecture, and contributions to inflammatory cytokine production. Of course, in many disease models all three of these functions work together to mediate productive immune responses.

Lymphoid structures act as organizational centers for immune responses, coordinating appropriate interactions between cells. Development of lymph nodes occurs early in embryonic development and blocking LT $\beta$ R/TNFR or LTi cell signaling during this window results in the birth of animals that lack lymphoid structures but retain a spleen and active lymphotoxin signaling. These mice are therefore important tools to study the necessity of lymph node structures in disease. Removal of draining lymph nodes can also serve this purpose. The role of draining lymph nodes or localized structures such as PPs has been an ongoing area of study. In some instances, the absence of draining lymph nodes is compensated for by other lymphoid structures. For example, oral infection of LT $\alpha$  deficient mice with rotavirus results in prolonged viral shedding (Lopatin et al. 2013). But despite a lack of mesenteric lymph nodes and PPs, anti-viral IgA develops, although in a delayed fashion, and leads to viral clearance. The appearance of IgA corresponds with an increase in B cells within the lamina propria (Lopatin et al. 2013). On the other hand, the removal of draining lymph nodes can have a significant impact on disease progression. LT $\beta$ <sup>-/-</sup> and LT $\beta$ R<sup>-/-</sup> mice have increased parasitic load following infection with *Leishmania* (Ehrchen et al. 2008; Xu et al. 2007). This same phenotype can be recapitulated in lymph node less animals created by administration of LT $\beta$ R-Ig or anti IL7R to pregnant mothers (Rennert et al. 1996). Despite having functional ongoing lymphotoxin signaling, lymph node less animals

infected with *Leishmania* have increased parasitic loads, and reduced T cell IFN $\gamma$  responses (Ehrchen et al. 2008). In addition to the importance of draining lymph nodes, active lymphotoxin signaling also appeared to have a role in responses to *Leishmania*. Irradiated C57BL/6 mice reconstituted with LT $\beta^{-/-}$  bone marrow had an intermediate disease phenotype compared to WT or LT $\beta^{-/-}$  mice; they were more resistant than LT $\beta^{-/-}$  animals but more susceptible than B6, with reduced IFN $\gamma$  responses (Xu et al. 2007).

These results highlight that fact that lymphotoxin signaling contributes to more than just lymph node development. During active infection lymphoid organs undergo structural changes in which cells of different types are geographically organized within the tissue to promote appropriate interaction between cells (**Figure 2**). The interaction between static stromal cells and actively moving lymphoid cells often relies on lymphotoxin. Animals that are deficient in LT $\alpha$ , LT $\beta$  or LT $\beta$ R all have a disorganization of lymphoid tissues to some degree (Fu and Chaplin 1999). Blocking lymphotoxin signaling during infection with LT $\beta$ R-Ig also results in a disruption of lymphoid tissue microarchitecture (Ettinger et al. 1996). Unsurprisingly, many of the impacts of lymphotoxin in infectious disease center on its role in lymphoid tissue remodeling. Indeed one of the consequences of CD4 T cell loss in HIV infection may be a reduction of LT $\alpha$ 1 $\beta$ 2 and a disruption of lymphoid structure that further prevents the regeneration of T cell responses (Zeng et al. 2012).

This importance of lymphotoxin in infection driven lymphoid tissue remodeling and immune responses is best exemplified in studies involving viral infection. For



**Figure 2. Role of lymphotoxin in lymph node remodeling during infection.**

Lymphotoxin plays a major role in lymph node maintenance and remodeling. **A.** B cell expressing LT maintains LTβR<sup>+</sup> subcapsular macrophages. These cells capture viral and particulate antigens and produce type I IFN. **B.** Lymphotoxin promotes lymph node expansion and HEV growth during infection by promoting VEGF production. B cells are one known source of LT but other sources may also be involved. **C.** LT on B cells interacting with LTβR on follicular dendritic cells (FDC) promotes release of CXCL13. This chemokine draws T cells and DCs into the B cell zone to promote GC reactions.

example,  $LT\alpha^{-/-}$  mice infected with herpes simplex virus (HSV) prime efficient CD8 T cell responses but their effector functions are impaired. Although the percentage of antigen-specific, tetramer positive CD8 T cells was the same between C57BL/6 WT and  $LT\alpha^{-/-}$  mice following HSV infection, there were marked defects in ex vivo killing and IFN $\gamma$  production of CD8 T cells derived from  $Lta^{-/-}$  mice (Kumaraguru et al. 2001). During LCMV infection,  $Lta^{-/-}$  mice also have defects in CD8 responses, including a reduction in virus specific killing and intracellular T cell IFN $\gamma$  (Suresh et al. 2002). Similarly, infection of  $Ltb^{-/-}$  or  $Ltbr^{-/-}$  mice with *Leishmania* resulted in the development of persistent lesions and impairments in CD4 IFN $\gamma$  production at 9 weeks and 10 weeks after infection but not at 2 weeks (Xu et al. 2007; Wilhelm et al. 2002).

The mechanism by which lymphotoxin drives T cells responses during viral infection appears to be related to the role of  $LT\alpha 1\beta 2$  in lymph node architecture. During LCMV infection B cells expressing lymphotoxin reorganize draining lymph nodes (Kumar et al. 2010) When T cells from  $Lta^{-/-}$  mice were transferred into B6 mice and infected with LCMV, their function was normal while the reciprocal transfer of CD8 T cells from WT mice into  $Lta^{-/-}$  mice resulted in CD8 impairment (Suresh et al. 2002).

Some of this effect is due to LT's role in maintenance of cells involved with infection. This is most clear in the case of VSV infection where B cells producing lymphotoxin are essential to maintain subcapsular sinus macrophages within the draining lymph node. Subcapsular macrophages act as sentinels of viral infection, capturing incoming viral particles and producing robust type I interferon (Moseman et al. 2012). In the absence of B cell LT, subcapsular sinus macrophages are depleted, type I interferon responses are reduced, and viral load increases. Similarly,  $LT\beta R$  mice

infected with MCMV had reduced type I IFN $\gamma$  responses (Schneider et al. 2008), suggesting that the role of the lymphotoxin pathway in subcapsular macrophage responses to virus is fairly broad.

A role for B cell lymphotoxin in T cell responses has been demonstrated in a chronic helminth infection model. In this system, LT from B cells is essential to the expression of CXCL13 by stromal cells and the recruitment of T cells into the B cells zone (León et al. 2012). In the absence of this pathway Th2 responses are impaired (León et al. 2012). These active processes related to lymphoid tissue organization seem to be key mediators in organizing immune responses, and lymphotoxin is at the center of this reorganization.

Finally, lymphotoxin mediated regulation of chemokine and cytokine production is critical in both viral and bacterial infections. Infection of *Rag*<sup>-/-</sup> mice with HSV results in robust inflammation of the central nervous system and death (Liang et al. 2015). Blocking the lymphotoxin pathway with LT $\beta$ R-Ig reduces chemokine production and recruitment of monocytes into the CNS, lowers cytokine production in the serum and CNS, and improves animal survival (Liang et al. 2015). During *Citrobacter* infection, early production of antimicrobial peptides limits bacterial spread (Tumanov et al. 2011). This defense pathway is initiated in the innate phase by ILC production of IL-22 after interaction with IL-23 producing DCs or epithelial cells. Lymphotoxin is essential in the interaction of DCs and epithelial cells with ILC3s and the production of robust IL-22 responses (Wang et al. 2010; Tumanov et al. 2011; Macho-Fernandez et al. 2015). When lymphotoxin is blocked or when ILC3s selectively lack LT $\beta$ , IL22 production is impaired and animals succumb to infection (Tumanov et al. 2011). The lymphotoxin

dependent IL22/23 pathway is also present during chemically induced colitis. Administration of DSS also induced the IL-22 pathway of barrier protection in a lymphotoxin dependent mechanism, and a deficiency in LT $\beta$  results in reduced survival (Koroleva et al. 2016).

### *The role of lymphotoxin in Salmonella infection*

Relatively little is known about the role of lymphotoxin in *Salmonella* infection. Previously published work in the streptomycin model of *Salmonella* reported that at two or three days following inoculation with 10<sup>8</sup> CFU of virulent *Salmonella* there was no difference in fecal bacterial load between WT and LT $\beta$ R deficient mice (Barthel 2003, Hapfelmeir 2005). At these time points, colonic pathology was also comparable. However, fecal bacterial burden at later time points was not analyzed as virulent *Salmonella* kills hosts on the C57BL/6 background. Interestingly, by day 3 (but not day 2) following oral *Salmonella* administration LT $\beta$ R deficient mice showed higher levels of bacteria in the liver suggesting that the lymphotoxin pathway could have a role in *Salmonella* responses at later time points (Barthel et al. 2003).

Hashizume and colleagues published two papers that address the role of various gut associated lymphoid tissues in mediating antibody responses to attenuated *Salmonella* vaccine strains (Hashizume et al. 2008; Hashizume et al. 2007). This group utilized an *aroA aroD* attenuated *Salmonella* carrying a plasmid with the non-toxic fragment C of tetanus toxin under the *nirB* promoter to orally vaccinate mice in the absence of streptomycin (Hashizume et al. 2008; Hashizume et al. 2007). By generating lymph node-less mice with injection of anti-IL7 receptor to pregnant mothers this group

found that lymph nodes, PP, and MLNs are dispensable for the formation of tetanus toxin specific IgG following oral immunization with vaccine *Salmonella* (Hashizume et al. 2008). They did however report that preformed PPs, but not active lymphotoxin signaling is essential for the generation of vaccine-specific IgA. These studies suggest that lymphotoxin's role in GALT formation is dispensable for the formation of IgG, at least when *Salmonella* vaccine does not actively colonize the gut. Second, the result that treatment with LT $\beta$ R-Ig during the course of vaccination does not alter IgA antibody responses to a *Salmonella* carried plasmid (tetC) suggests that in the absence of streptomycin pretreatment active lymphotoxin signaling is not essential for IgA formation (Hashizume et al. 2008; Hashizume et al. 2007). These publications do not address the importance of IgA, lymphoid tissue, or lymphotoxin signaling in protective immunity against *Salmonella* or *Clostridium tetani* (the bacteria which produces tetanus toxin).

Lymphotoxin had been demonstrated to play a role in IL22 responses at the gut (Spahn et al. 2004; Tumanov et al. 2011), and the IL22 pathway has been shown to enhance *Salmonella* colonization (Behnsen et al. 2014). The impact of LT $\beta$ R in late phases of *Salmonella* infection or in protective immunity to *Salmonella* have not been explored. Therefore, using an attenuated *Salmonella* strain and the streptomycin model we sought to explore the role of the lymphotoxin pathway in anti-*Salmonella* immune responses.

## **Chapter 2: Materials and Methods**

### *Mice*

*Ltbr*<sup>+/-</sup>, *Ltbr*<sup>-/-</sup>, *Ltb*<sup>+/-</sup>, *Ltb*<sup>-/-</sup>, *Ltb*<sup>fl/fl</sup>, *Cd4*<sup>Cre+/wt</sup> *Ltb*<sup>fl/fl</sup>, *Rorc*<sup>Cre+/wt</sup> *Ltb*<sup>fl/fl</sup>, *Cd19*<sup>Cre+/wt</sup>

*Ltb*<sup>fl/fl</sup> were bred and maintained under specific pathogen free conditions at the University of Chicago. Littermate controls were used throughout and generated by breeding heterozygous mice to knockout mice with the dam being the knockout or cre+ animal in the breeding scheme. *Cd19*<sup>Cre+/Cre+</sup> animals were purchased from Jackson and bred to *Ltb*<sup>fl/fl</sup> animals. All other strains were maintained within the same facility for more than five years. Germ-free *Ltbr*<sup>-/-</sup> mice were rederived by Taconic, breeders were shipped under germ-free conditions, and maintained in the gnotobiotic facility at the University of Chicago. Germ-free *Ltbr*<sup>+/+</sup> mice were generated by breeding germ-free *Ltbr*<sup>-/-</sup> mice to germ-free C57BL/6 mice also purchased from Taconic Bioscience. Mice were maintained according to the standards set by the University of Chicago's Institutional Animal Care and Use Committee (protocol #71866).

### *Bacterial growth*

*Salmonella enterica* serovar *typhimurium* SL3261 *aroA* attenuated strain (generously provided by Dr. Cathryn Nagler) or a virulent parental strain SL1344, was grown in LB broth from a single colony in a low volume (4mL) starter culture. After 8-10 hours of growth, a large volume of LB was inoculated with the starter culture at a 1:100 dilution and grown overnight. CFU was estimated by spectrophotometry based on a previously determined standard curve.

## *Infections*

All infections were performed in littermate-controlled mice in BSL2 conditions at 6-15 weeks of age. Animals were treated with 20 mg/mouse streptomycin (US Biological) by gavage. 24 hours later animals were inoculated by gavage with of  $1 \times 10^9$  CFU *aroA*, SL3261 diluted in PBS. For virulent challenge, naive mice or those that had 45 to 50 days previously been inoculated with attenuated *Salmonella* were orally challenged with a dose of  $1 \times 10^6$  SL1344 in the absence of streptomycin. Dosage was confirmed by serial dilution and plating on MacConkey agar plates.

## *Bacterial CFU in fecal or tissue samples*

To determine bacterial CFU in feces, pellets were collected, weighed, and resuspended in PBS at 100mg/mL. Samples were briefly homogenized. The homogenate was spun down for 10min at 800 RPM and the supernatant was collected. To determine CFU in spleen and liver tissue organs were collected, weighed and resuspended in PBS with 0.01% Triton X-100 at 100mg/mL and briefly homogenized. To determine *Salmonella* load within gut tissues, colon and cecum was collected, opened longitudinally, washed with ice cold PBS, and weighted. Tissues were then incubated for 30 min at 37 degrees in RPMI with 10% FCS plus gentamycin (400 $\mu$ g/mL) then washed three times with 10 mL cold PBS. Tissues were resuspended in PBS with 0.01% Triton and briefly homogenized. After serial dilution in PBS, CFU of all samples was determined by plating on MacConkey agar with streptomycin (50 $\mu$ g/ml).

### *Salmonella antigen preparation*

*Salmonella* protein extract from SL3261 was produced by overnight culture of bacteria (as described above) followed by centrifugation at 4000 RPM. The bacterial pellet was frozen at -80 degrees and subsequently freeze-shocked in liquid nitrogen and thawed in a 37-degree water bath 3 times. It was then sonicated on ice 3 times for 1 min. The protein fraction was extracted by addition of B-PER® Bacterial Protein Extract reagent (Thermo Scientific) according to the manufacturer's instructions. Briefly, the sample was incubated for 30 min with slow rotation at room temperature and centrifuged at 1400 RPM at 4 degrees for 20 minutes. The clear supernatant was stored at -80 until further use.

Heat killed *Salmonella typhimurium* was prepared by overnight growth in LB broth. OD was determined as previously described and the sample was spun down. After washing twice with PBS, the loosened bacterial pellet was incubated for 1 hour at 65 degrees and then resuspended in PBS at  $1 \times 10^9$  CFU/mL. Samples were stored at -80 until further use.

### *ELISA*

ELISA plates were coated with *Salmonella* protein extract at 10ug/mL in PBS overnight. After washing 3x with PBS plus 0.05% Tween 20, plates were blocked with 3% FCS for 2-4 hours. After washing 3x, serum samples were diluted in PBS + 1% FCS and, added to the plate, and incubated overnight at 4 degrees. A standard curve derived from hyperimmunized serum was added onto each plate. After washing, detection antibodies for anti-IgG AP (Southern Biotech 1031-04) or anti-IgM AP (Southern Biotech

1021-04) were diluted in PBS + 1% FCS and incubate at room temperature for 1 hour. After a final wash, anti-*Salmonella* antibody responses were detected by addition of *p*NPP (KPL 50-80-00).

Hyperimmunized serum was generated by repeated oral immunization of C57BL/6 mice with attenuated SL3261 *Salmonella*. To quantify total antibody of a specific isotype serum was added in serial dilution to anti-IgG or anti-IgM coated plates. Unlabeled mouse IgG or IgM was used as a standard. To quantify *Salmonella*-specific antibody serum was added in serial dilution to *Salmonella* antigen coated or BSA coated plates. The total Ig ELISA was used to quantify the amount of *Salmonella*-specific antibody that was *Salmonella* reactive and BSA unreactive.

#### *In vitro and in vivo restimulation*

A single cell preparation of splenocytes was aliquoted at  $5 \times 10^5$  cells/ well in a round bottom 96 well plate in RPMI with 10% FCS, pen/strep, and HEPES. Cells were stimulated with *Salmonella* antigen at a dose of 50 ng/well to 50 $\mu$ g/well. For anti-CD3/CD28 re-stimulation, plates were coated with anti-CD3 (eBioscience, clone 17A2, 16-0032-85) at 2 $\mu$ g/mL in PBS overnight, then washed three times with PBS. Soluble anti-CD28 (eBioscience, clone 37.51, 16-0281-85) was added at a concentration of 2 $\mu$ g/mL. 48 hours later the supernatants were collected and cytokine production was measured with the mouse inflammation cytokine bead array (Becton, Dickinson and company, 552364) according to manufacturer's instructions. For proliferation studies, cells were stained with CellTrace Violet (ThermoFisher C34557) according to manufacturer's instructions.

For *in vivo* stimulation animals were injected i.v. with  $3 \times 10^8$  CFU of heat killed *Salmonella*. Four or 24 hours later animals were sacrificed and spleens were collected into ice cold PBS. A single cell suspension was made by mashing through a 0.5um filter. Cells were stained as described below.

#### *Fusion protein and functional antibody blockade*

LT $\beta$ R-Ig (Rennert et al. 1996) was produced in house. 100 $\mu$ g LT $\beta$ R-Ig was administered i.p. every 7 days starting at day 22 and ending on day 43 after oral administration of attenuated *Salmonella* to *Ltbr*<sup>+/-</sup> animals.

Anti-IFN $\gamma$  blocking antibody (clone R4-6A2) was purchased from BioXCell. Polyclonal rat IgG (BioXCell, Catalog # BE0094) was used as an isotype control. 200 $\mu$ g of antibody was administered i.p. once a week starting at day 28 and ending on day 42 after oral administration of attenuated *Salmonella*.

#### *Flow cytometry*

Cultured or fresh splenocytes were washed once with PBS and stained for 30 minutes at 4°C for 30 minutes with live dead dye (BioLegend). Cells were washed twice with 1% FCS in PBS. Anti-CD16/CD32 Fc Block (in house, clone 2.4G2) was added to cells for 15 min at room temperature. Without washing, surface antibodies in 1% FCS were added at previously established dilutions and incubated for 30 min at 4°C. Surface antibodies include anti-CD4 (PEcy7, Biolegend, clone GK1.5), anti-CD3 (percpCy5.5, Biolegend, clone 145-2c11), and anti-CD8 (APCcy7, Biolegend, clone 53-6.7). Cells were washed twice in 1% FCS. For intracellular staining cells were fixed for 30 minutes

in IC Fixation Buffer (eBioscience, 00-8222-49) followed by a wash in permeabilization buffer (eBioscience, 00-8333-56). Intracellular antibodies were added in permeabilization buffer for 30 min. They include anti-IFN $\gamma$  (APC, Pierce Antibodies, clone XMG1.2), anti TNF $\alpha$  (PE, eBioscience clone, MP6-X722). Fluorescence minus one controls for IFN $\gamma$  and TNF $\alpha$  were used to set positive gates.

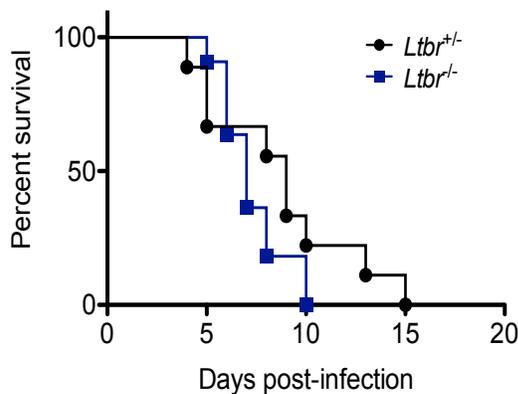
### *Statistical Analysis*

Statistical analysis was performed with GraphPad Prism version 5.0d and analyzed through statistical tests available in the software. Data is representative of Mann Whitney test when normal distribution of the data could not be confirmed and unpaired Student's t test when data was distributed normally. The following symbols indicate the order of significance: \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ , and \*\*\* indicates  $P < 0.001$ .

### Chapter 3: The role of the lymphotoxin pathway in resolution of *Salmonella* gastrointestinal infection

Similar kinetics of disease after oral exposure to virulent *Salmonella* between *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> littermates.

Oral infection of mice on the C57BL/6 background with virulent *Salmonella*



**Figure 3. LTβR does not impact survival following oral administration of virulent *Salmonella*.**

Virulent *Salmonella* strain SL1344 was administered orally to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice and survival was monitored. Percent survival following infection. Three pooled experiments *Ltbr*<sup>+/-</sup> n = 9, *Ltbr*<sup>-/-</sup> n = 11

*typhimurium* strain SL1344 is fatal

(Mastroeni 2002; Stecher et al. 2006). To

determine if LTβR plays a role in

progression of disease littermate *Ltbr*<sup>+/-</sup> and

*Ltbr*<sup>-/-</sup> mice were orally infected with 1 x 10<sup>6</sup>

CFU of SL1344. Both strains of mice were

highly susceptible to virulent *Salmonella*

and 100% of animals succumbed to

infection after 15 days. No difference in

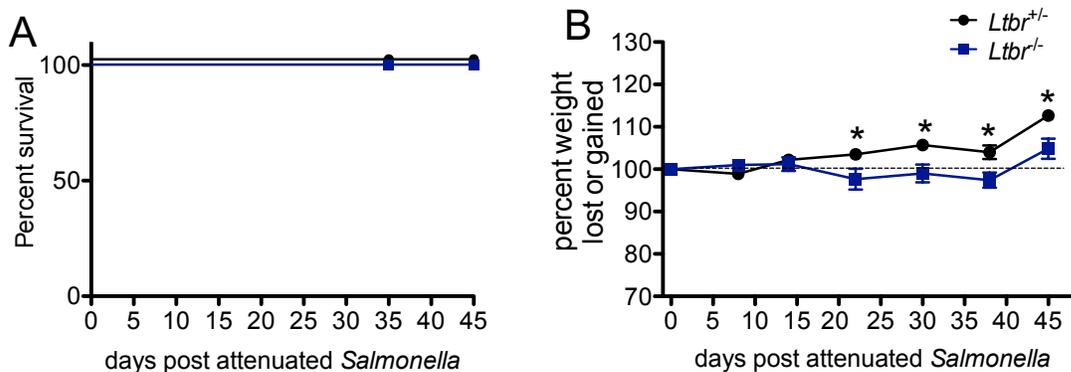
survival was observed between LTβR<sup>+/-</sup> and

LTβR<sup>-/-</sup> (Figure 3).

Attenuated *Salmonella* does not cause death or weight loss in *Ltbr*<sup>+/-</sup> or littermate *Ltbr*<sup>-/-</sup> mice.

Because the C57BL/6 strain is highly susceptible to virulent *Salmonella*, a possible role for LTβR could be masked by the rapid death observed. Attenuated strains of *Salmonella* can reproduce many aspects of human disease pathology while not killing

the host. This is especially true in the streptomycin model of *Salmonella* gastrointestinal disease where infection with a replication attenuated mutant can cause self-limiting gut inflammation and systemic dissemination (Endt et al. 2010). Attenuated strains of *Salmonella* can also act as live attenuated vaccines that mediate protection to virulent *Salmonella* (Hormaeché et al. 1990). Because LT $\beta$ R plays a role in immune responses in the colon after *Citrobacter rodentium* infection (Tumanov et al. 2011; Zheng et al. 2008) we hypothesized that its role in the mucosa of *Salmonella* infected mice would be revealed using the streptomycin treatment model. To test if LT $\beta$ R plays a role in *Salmonella* diarrhea, littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> animals were pretreated orally with streptomycin one day prior to oral infection with 1 x 10<sup>9</sup> CFU attenuated *Salmonella aroA* strain SL3261. Although this protocol leads to inflammation of the colon and cecum and mild diarrhea it did not lead to death or weight loss in either *Ltbr*<sup>+/-</sup> or littermate *Ltbr*<sup>-/-</sup> mice (**Figure 4A**). However, *Ltbr*<sup>+/-</sup> animals did maintain a higher growth rate over the course of infection (**Figure 4B**). These data suggest that an absence of



**Figure 4. LT $\beta$ R does not impact survival following oral administration of attenuated *Salmonella* in the streptomycin model of *Salmonella***

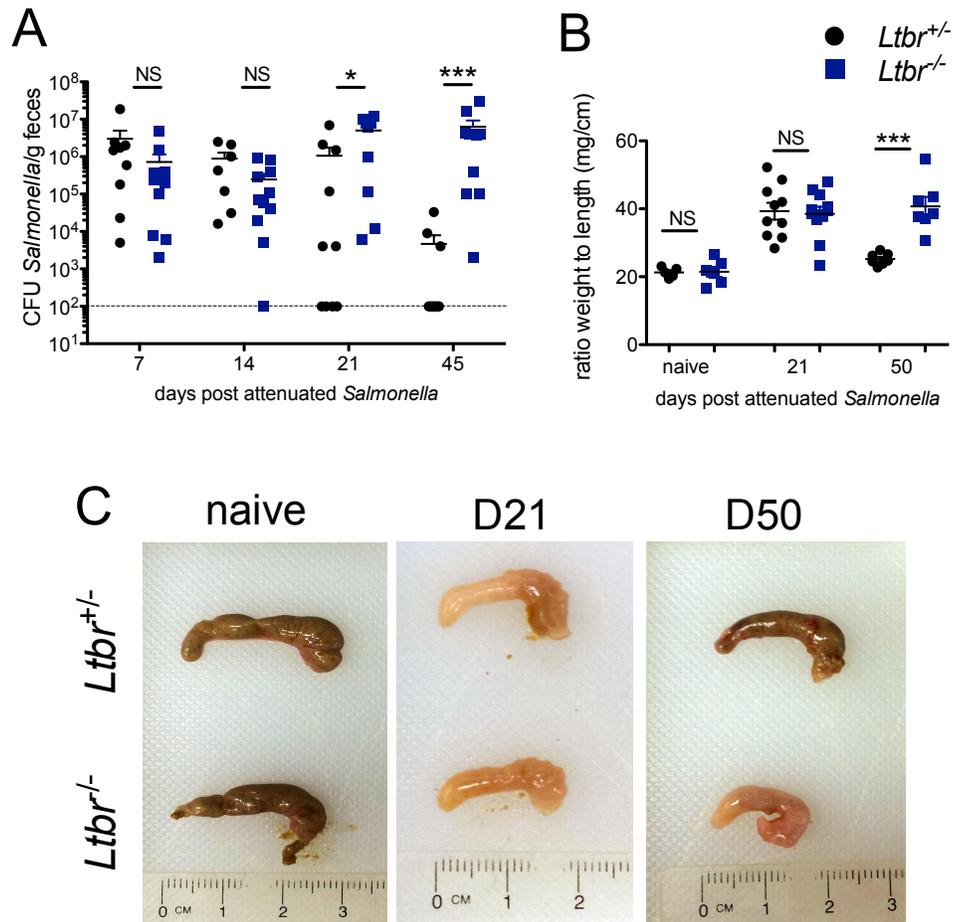
Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. **A.** Survival following attenuated *Salmonella*. **B.** Percent weight lost or gained of mice in **A.** *Ltbr*<sup>+/-</sup> n = 10, *Ltbr*<sup>-/-</sup> n = 11 from two pooled independent experiments. In B, each point represents group average, bars represent  $\pm$  SEM and \*  $P < 0.05$  by unpaired t test.

LT $\beta$ R does not lead to overt disease pathology during infection with attenuated *Salmonella* in the streptomycin model of gastrointestinal disease.

*LT $\beta$ R is required for clearance of attenuated Salmonella from the gut lumen and resolution of gut inflammation.*

Oral pretreatment of mice with streptomycin prior to administration of attenuated *Salmonella* causes a loss of colonization resistance and allows for outgrowth of the pathogen in the lumen of the cecum and colon, and initiation of host inflammation is a key, pathogen-driven event that is required to initiate gut colonization (Stecher et al. 2007). To determine if LT $\beta$ R plays a role in pathogen gut colonization, levels of *Salmonella* in the feces were measured in littermate *Ltbr*<sup>+/-</sup> or *Ltbr*<sup>-/-</sup> mice. Initial colonization, as determined by fecal CFU at day 7 was comparable between both strains of mice and remained similar until day 14 post infection (**Figure 5A**). *Salmonella*-induced colonic inflammation as measured by colon weight to length ratio was comparable between littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice at day 21 post infection (**Figure 5B**). Cecal inflammation, as reflected by morphological changes to the organ, at this time point was also equivalent between both strains of mice (**Figure 5C**). Together these data suggest that initiation of gut pathology and bacterial colonization is not impacted by an absence of LT $\beta$ R.

Gut inflammation and colonization of attenuated *Salmonella* resolves in the C57BL/6 strain by 3 to 9 weeks of infection (Endt et al. 2010). By day 45 post infection the majority of *Ltbr*<sup>+/-</sup> animals had no detectable CFU of *Salmonella* in the feces, indication that they had cleared infection (**Figure 5A**). As bacterial clearance was



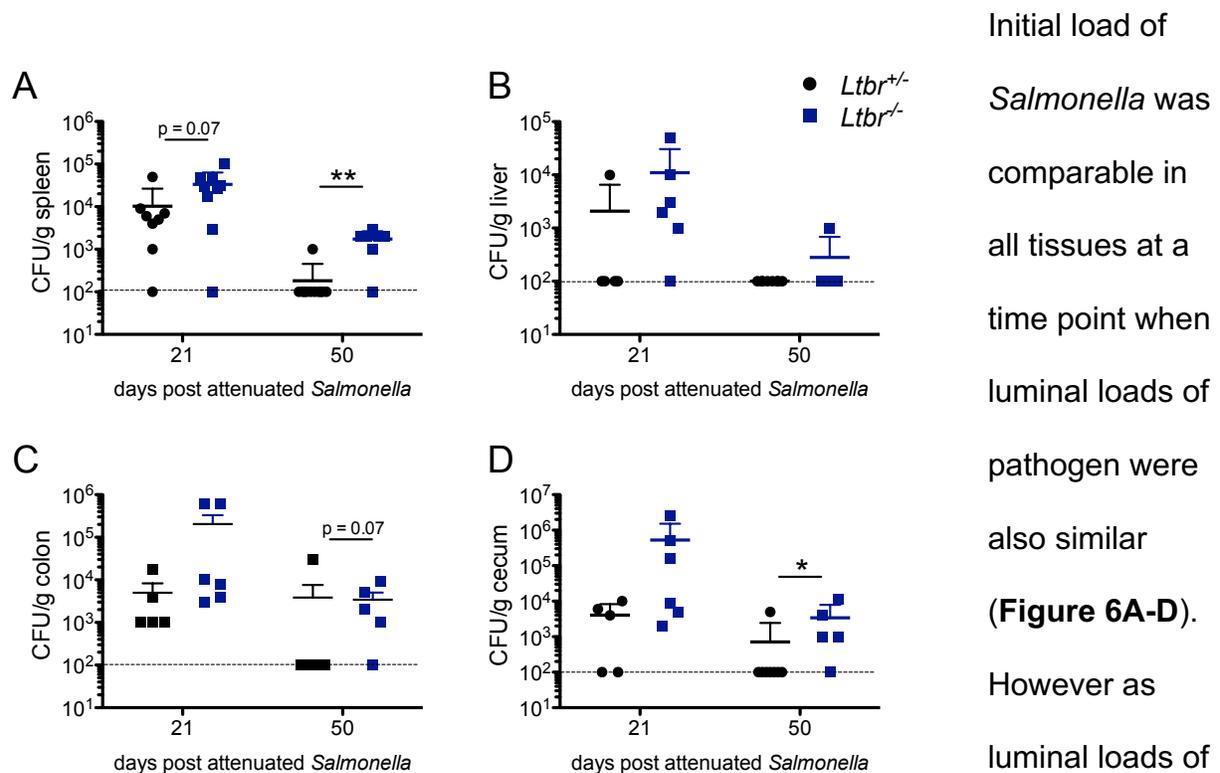
**Figure 5. LT $\beta$ R is required for clearance of attenuated *Salmonella* from the gut lumen and resolution of inflammation.**

Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. **A**. CFU of *Salmonella* in the feces. **B**. Colon weight to length ratio. **C**. Representative image of ceca following infection. For **A**. *Ltbr*<sup>+/-</sup> n = 10, *Ltbr*<sup>-/-</sup> n = 11 from two pooled independent experiments. For **B**. n = 5 to 10 animals per group representing four pooled experiments. \*  $P < 0.05$ , \*\*\*  $P < 0.005$  by Mann-Whitney test. Bars represent mean + SEM.

achieved, *Ltbr*<sup>+/-</sup> mice also resolved colonic inflammation as indicated by a return of colon weight to length ratio to pre-infection status (**Figure 5B**). Similarly, cecal morphology returned to pre-infection state in *Ltbr*<sup>+/-</sup> mice 50 days following infection (**Figure 5C**). Unlike their *Ltbr*<sup>+/-</sup> sufficient littermates, *Ltbr*<sup>-/-</sup> animals were unable to clear *Salmonella* from the gut lumen and maintained high levels of *Salmonella* CFU in the

feces at day 45 post infection (**Figure 5A**). Persistence of pathogen in the gut lumen was reflected by persistent gut inflammation in *Ltbr*<sup>-/-</sup> mice at day 50 post infection (**Figure 5B**) and disrupted cecal morphology (**Figure 5C**).

To determine if an abundance of *Salmonella* in the lumen corresponded to increased pathogen load in the tissues, the abundance of *Salmonella* in systemic organs and within gut tissues were determined in *Ltbr*<sup>+/+</sup> or *Ltbr*<sup>-/-</sup> mice. To measure the amount of *Salmonella* within gut tissues, rather than attached luminal bacteria, colon and cecal tissues were washed with gentamicin to remove any attached bacteria and intracellular *Salmonella* was liberated from tissues by homogenization with a detergent.



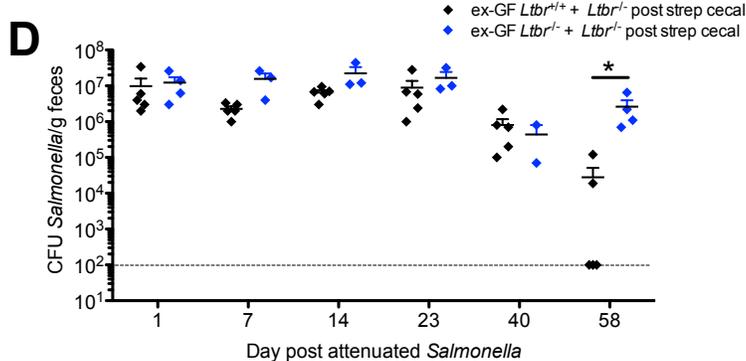
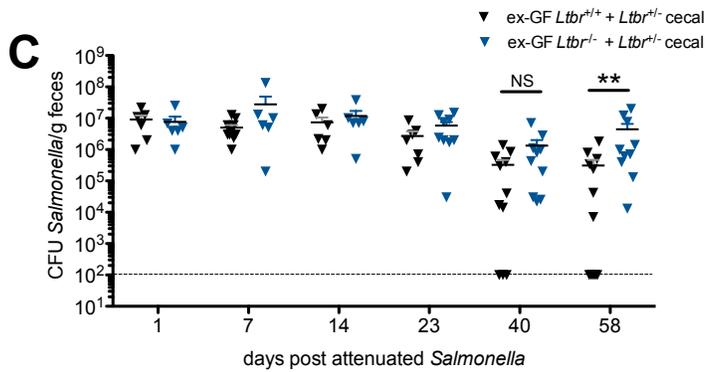
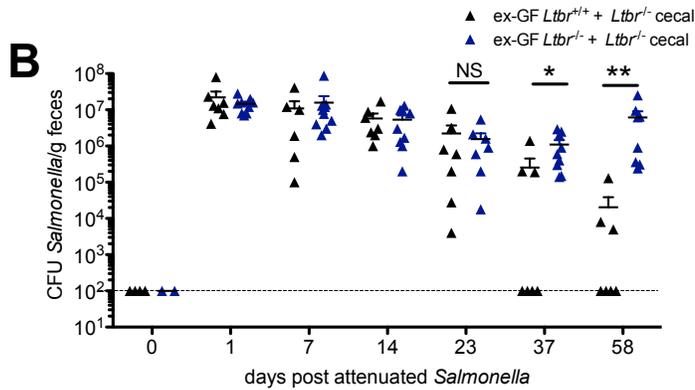
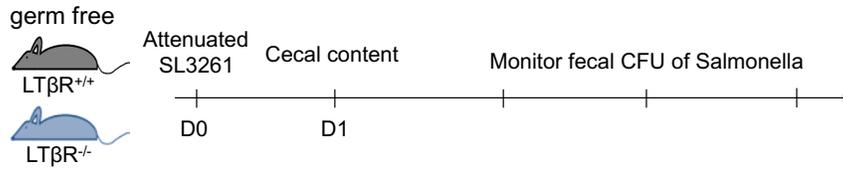
**Figure 6. LTβR impacts CFU of intracellular *Salmonella* during late phase of infection.**

Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration with attenuated *Salmonella*. CFU of intracellular *Salmonella* in **A.** spleen **B.** liver **C.** colon **D.** cecum. n = 5-8 per group \* *P* < 0.05, \*\* *P* < 0.01 by Mann-Whitney test

mice, so did the abundance of bacteria within tissues; *Ltbr*<sup>-/-</sup> mice harbored higher loads of *Salmonella* within the spleen and cecum compared to *Ltbr*<sup>+/-</sup> littermates (**Figure 6A, D**). As the *aroA* strain is inefficient at intracellular replication, we interpret the increased abundance of intracellular *Salmonella* in systemic tissues of *Ltbr*<sup>-/-</sup> mice as a result of continued persistence in the gut lumen.

*LTβR* is required to drive microbiota-mediated clearance of *Salmonella* from the gut.

Clearance of *Salmonella* from the gut requires a complete microbiota (Endt et al. 2010; B. Stecher et al. 2005). It was possible that *Ltbr*<sup>-/-</sup> animals lacked an essential component of the gut microbiota necessary for competition with, and clearance of, *Salmonella*. To test this hypothesis, germ free *Ltbr*<sup>+/+</sup> or *Ltbr*<sup>-/-</sup> animals were monocolonized with attenuated *Salmonella*. Monocolonization led to robust growth of *Salmonella* in the feces in both strains of mice. One day later, both groups of mice were conventionalized by oral administration of the cecal content from the same SPF *Ltbr*<sup>-/-</sup> donor (**Figure 7A**). Addition of bacteria from SPF *Ltbr*<sup>-/-</sup> donors was sufficient to drive clearance of *Salmonella* from the gut lumen of *Ltbr*<sup>+/+</sup> animals; most *Ltbr*<sup>+/+</sup> mice had no detectable *Salmonella* CFU in the feces by day 58 post attenuated *Salmonella* (**Figure 7B**). However, the same SPF donor microbiota was unable to mediate *Salmonella* clearance in

**A**

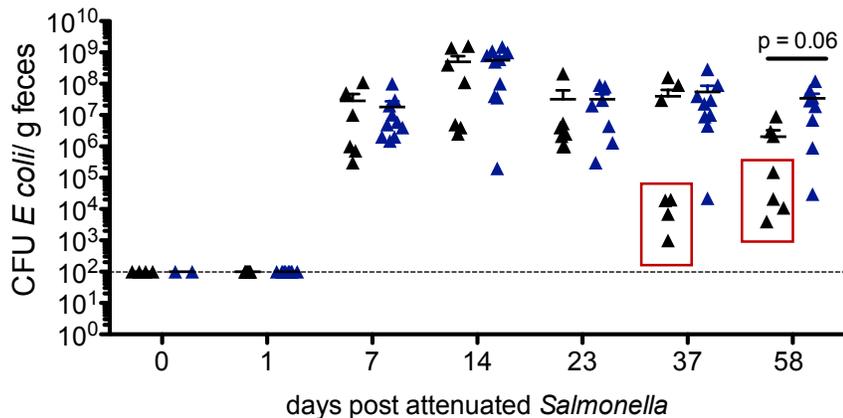
### Figure 7. LTβR signaling drives microbiota mediated clearance of *Salmonella*.

Germ free *Ltbr*<sup>+/+</sup> or *Ltbr*<sup>-/-</sup> animals were orally colonized with attenuated *Salmonella*. One day later animals were conventionalized by oral gavage with cecal contents from SPF donors. **A.** Schematic of experiment. CFU of *Salmonella* in the feces of mice colonized with cecal content from **B.** *Ltbr*<sup>-/-</sup> SPF donor, **C.** *Ltbr*<sup>+/+</sup> SPF and **D.** SPF *Ltbr*<sup>-/-</sup> donor treated with streptomycin one day prior to harvest of cecal content. **A.** *Ltbr*<sup>+/+</sup> n = 7, *Ltbr*<sup>-/-</sup> n = 10, pooled from two independent experiments. **B.** n = 5-12 per group, pooled from two independent experiments. **C.** n = 4-5 mice per group, one experiment. Each dot represents an individual mouse, bars represent mean ± SEM, NS is not significant, \* *P* < 0.05, \*\* *P* < 0.01 by Mann-Whitney test.

*Ltbr*<sup>-/-</sup> hosts which maintained near peak levels of *Salmonella* colonization (**Figure 7B**). Similar results were obtained when donor cecal material came from *Ltbr*<sup>+/-</sup> donors (**Figure 7C**). It was possible that the abundance of *Salmonella* competitors in our colony was low, and that treatment of animals with streptomycin removed these species. To test this question SPF *Ltbr*<sup>-/-</sup> mice were treated with streptomycin one day prior to collection of cecal material. This cecal content was then used to conventionalize *Salmonella* monocolonized mice. As with previous data, streptomycin-treated SPF cecal content was sufficient to mediate *Salmonella* clearance in *Ltbr*<sup>+/+</sup> animals but not *Ltbr*<sup>-/-</sup> (**Figure 7D**). These data indicate that while *Ltbr*<sup>-/-</sup> mice do indeed harbor a microbiota capable of clearing *Salmonella* from the gut, LTβR signaling or LTβR-dependent lymphoid structures are required to drive this clearance.

#### *E. coli* levels are not a predictor of clearance

The abundance of *E. coli* species prior to colonization with *Salmonella* has been positively correlated with the ability of *Salmonella* to colonize the host gut even in the absence of streptomycin treatment (Stecher et al. 2010). It has also been suggested that *Salmonella* utilize the IL-22 pathway to compete with members of the microbiota, especially *E. coli*, to mediate colonization (Behnsen et al. 2014). It is not known if *E. coli* abundance correlates with clearance of *Salmonella*. Since LTβR is an important driver of the IL-22 pathway during *Citrobacter rodentium* infection (Tumanov et al. 2011), it was possible that *Salmonella* persistence in *Ltbr*<sup>-/-</sup> mice is associated with an inability to control IL-22 sensitive gut bacteria such as *E. coli*. To determine if levels of *E. coli*



**Figure 8. *E. coli* abundance does not correlate with *Salmonella* clearance and is not altered by LTβR.**

Germ free *Ltbr*<sup>+/+</sup> or *Ltbr*<sup>-/-</sup> animals were orally colonized with attenuated *Salmonella*. One day later animals were conventionalized by oral gavage with cecal contents from *Ltbr*<sup>-/-</sup> donors. *E. coli* CFU in feces was measured by plating on MacConkey agar in the absence of streptomycin. Boxed triangles correspond to mice with undetectable levels of *Salmonella*. *Ltbr*<sup>+/+</sup> n = 7, *Ltbr*<sup>-/-</sup> n = 10, pooled from two independent experiments, each dot represents an individual mouse, bars represent mean ± SEM, NS is not significant, \* P < 0.05, \*\* P < 0.01 by Mann-Whitney test.

correlated with clearance of *Salmonella*, *E. coli* levels were measured in feces of germ free *Ltbr*<sup>+/+</sup> or *Ltbr*<sup>-/-</sup> mice, monocolonized with *Salmonella* and then conventionalized with cecal contents from *Ltbr*<sup>-/-</sup> donors. Both *Ltbr*<sup>+/+</sup> and *Ltbr*<sup>-/-</sup> mice harbored similar levels of *E. coli* following conventionalization and abundance of this species peaked at day 14 after conventionalization (**Figure 8**). Levels of *E. coli* decreased as *Salmonella* abundance decreased, and in *Ltbr*<sup>+/+</sup> mice those with the lowest level of *E. coli* achieved clearance earlier than those with high levels of *E. coli* (boxed triangles in Figure 8) suggesting that both strains may be controlled by similar mechanisms. However, there was not a significant difference in the level of *E. coli* between *Ltbr*<sup>+/+</sup> and *Ltbr*<sup>-/-</sup> mice during either the initiation or clearance phase. Therefore, although *E. coli* levels may correlate with *Salmonella* colonization and abundance, they do not appear to drive *Salmonella* clearance.

## LTβR's role in *Salmonella* clearance depends on direct signaling

Active lymphotoxin signaling can be blocked in lymph node sufficient mice by

administration of LTβR-Ig fusion

protein. To determine if the role of

lymphotoxin in clearance of

*Salmonella* from the gut is through

active lymphotoxin signaling, *Ltbr*<sup>+/-</sup>

animals were treated with blocking

LTβR-Ig fusion protein once a week

for four weeks starting at day 22

following attenuated *Salmonella*

vaccination. CFU of bacteria in the

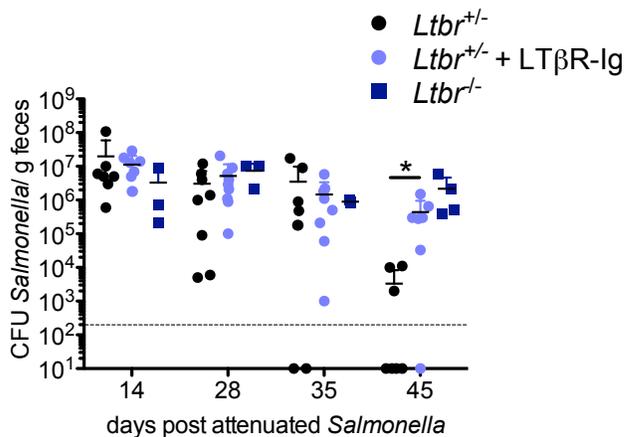
feces was monitored. Transient

blockade of lymphotoxin receptor

signaling was sufficient to delay

**Figure 9. Lymphotoxin signaling drives fecal clearance of *Salmonella*.**

100μg LTβR-Ig was administered i.p. every 7 days starting at day 22 and ending on day 43 after oral administration of attenuated *Salmonella* to *Ltbr*<sup>+/-</sup> animals. Littermate *Ltbr*<sup>-/-</sup> and untreated *Ltbr*<sup>+/-</sup> are included as a positive control. CFU of *Salmonella* in the feces. *Ltbr*<sup>+/-</sup> n = 8, *Ltbr*<sup>+/-</sup> + LTβR-Ig n = 9, *Ltbr*<sup>-/-</sup> n = 4, pooled from two independent experiments. \* *P* < 0.05 by Mann-Whitney test. Each dot represents an individual mouse, bars represent mean + SEM



bacterial clearance from the gut compared to untreated *Ltbr*<sup>+/-</sup> mice, and treated animals

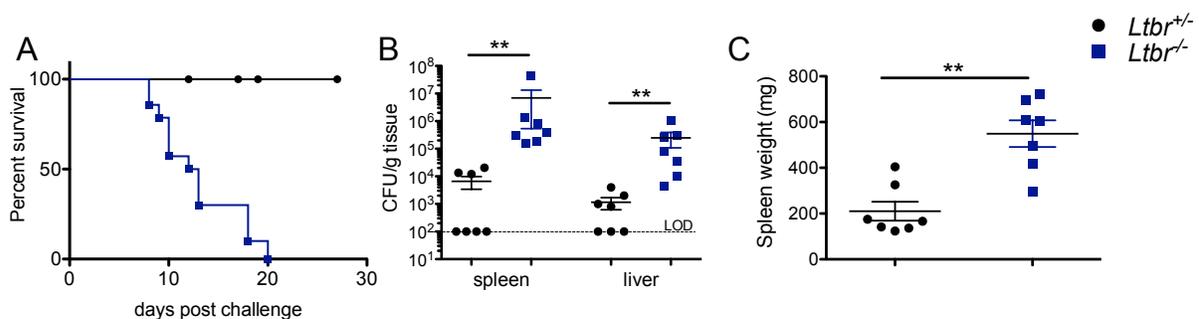
had *Salmonella* levels comparable to *Ltbr*<sup>-/-</sup> mice (**Figure 8**). These data suggest that

active signaling of LTβR drives clearance of *Salmonella*.

## Chapter 4: The role of the lymphotoxin pathway in anti-*Salmonella* immunity

*LTβR* is essential to generate protective immunity to secondary challenge with virulent *Salmonella* following vaccination with attenuated *Salmonella*

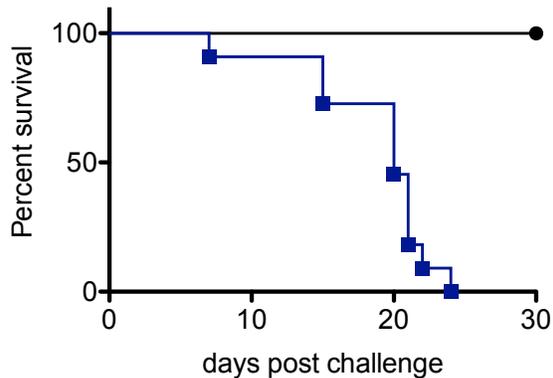
The strain of attenuated *Salmonella* utilized can act as a live-attenuated vaccine and mediate protection to challenge with virulent *Salmonella* (Cardenas and Clements 1992). To determine if *LTβR* plays a role in acquisition of protective immunity, littermate *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>* animals were pretreated with streptomycin 24 hours prior to oral administration of attenuated *Salmonella* and challenged 45 days later with the virulent strain SL1344 in the absence of streptomycin. While all *Ltbr<sup>+/-</sup>* mice were fully protected against challenge, none of the *Ltbr<sup>-/-</sup>* littermate mice survived (Figure 10A). Death of *Ltbr<sup>-/-</sup>* mice was preceded by high *Salmonella* burden in systemic organs (Figure 10B) as well as profound splenomegaly (Figure 10C). These data suggested that *LTβR* is essential for mediating protective immunity to *Salmonella* following oral vaccination with an attenuated strain.



**Figure 10. *LTβR* is essential for mediating protective immunity to *Salmonella*.**

Oral challenge with  $1 \times 10^7$  CFU virulent *Salmonella* of littermate *Ltbr<sup>+/-</sup>* or *Ltbr<sup>-/-</sup>* mice. 45 days prior to challenge, animals were pretreated with streptomycin followed one day later by oral attenuated *Salmonella*. **A.** Survival post challenge. **B.** CFU of *Salmonella* in the spleen and liver 10 days post challenge. **C.** Spleen weight 10 days post challenge. **A.** *Ltbr<sup>+/-</sup>* n = 10, *Ltbr<sup>-/-</sup>* n = 14, pooled from 3 independent experiments. **B** and **C** *Ltbr<sup>+/-</sup>* n = 7, *Ltbr<sup>-/-</sup>* n = 7, pooled from two independent experiments. \*\*  $P < 0.01$  using Mann-Whitney test. Each dot represents an individual mouse, bars represent mean  $\pm$  SEM.

The role of  $LT\beta R$  in mediating protection against *Salmonella* is independent of gut persistence.



**Figure 11. The role of  $LT\beta R$  in mediating protection against *Salmonella* is independent of gut persistence**

Attenuated *Salmonella* was administered by intraperitoneal (i.p.) injection to littermate *Ltbr*<sup>+/-</sup> or *Ltbr*<sup>-/-</sup> animals. 45 days later animals were challenged with virulent *Salmonella* by i.p. injection. Survival post challenge with virulent *Salmonella*. *Ltbr*<sup>+/-</sup> n = 8 *Ltbr*<sup>-/-</sup> n = 11, representative of two independent experiments.

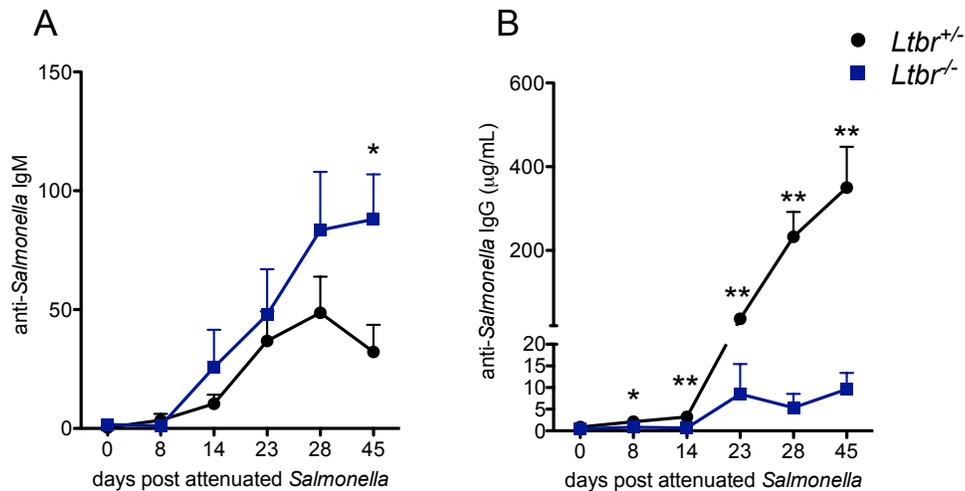
Persistent infections have been shown to impair the innate and adaptive immune system through a variety of mechanisms (Zajac et al. 1998; Monack, Mueller, and Falkow 2004; Ng et al. 2013; Zuniga et al. 2015). It is possible that long term persistence of attenuated *Salmonella* in gut tissue results in tolerance or exhaustion that can lead to impaired immune responses. To test this hypothesis, *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice were given

attenuated *Salmonella* by i.p. injection. Attenuated *aroA Salmonella* is impaired in intracellular replication and the presence of *Salmonella* was undetectable in feces.

Upon i.p. challenge with virulent *Salmonella*, *Ltbr*<sup>+/-</sup> mice were fully protected indicating the attenuated *Salmonella* can induce protective immune responses in the absence of growth in the gut. Unlike their *Ltbr*<sup>+/-</sup> littermates *Ltbr*<sup>-/-</sup> mice died after i.p. challenge with virulent *Salmonella* (**Figure 11**). These data suggest that persistence of attenuated *Salmonella* in the gut is not the cause of impaired immunity at challenge, and that both gut persistence and impaired immunity are downstream effects of  $LT\beta R$  deficiency.

*LTβR* drives generation of anti-*Salmonella* class switched antibody.

Protective memory responses against *Salmonella* comprise both B and T cell responses (Mittrücker and Kaufmann 2000). To determine if B cell immunity to *Salmonella* requires *LTβR*, anti-*Salmonella* antibody responses in the serum of *Ltbr*<sup>+/-</sup> and littermate *Ltbr*<sup>-/-</sup> mice were measured following oral administration of streptomycin and attenuated *Salmonella*. While both *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice made robust anti-*Salmonella* IgM (Figure 12A), *Ltbr*<sup>-/-</sup> were unable to mount anti-*Salmonella* IgG responses (Figure 12B). This suggests that while *Ltbr*<sup>-/-</sup> do mount antibody responses against *Salmonella*, *LTβR* plays a role in class switching to IgG.

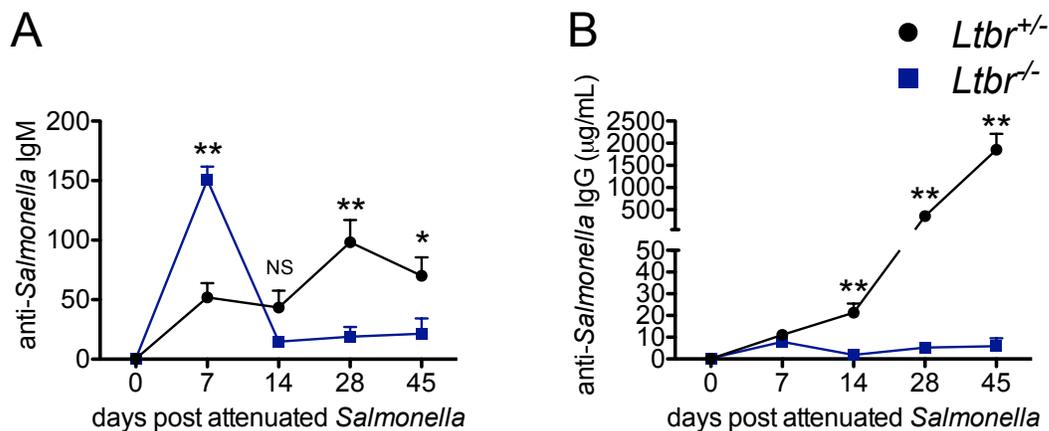


**Figure 12. *LTβR* is required for anti-*Salmonella* IgG responses.**

Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. **A.** Anti-*Salmonella* IgM as measured by ELISA. **B.** Anti-*Salmonella* IgG. Values are standardized to hyper-immunized serum. *Ltbr*<sup>+/-</sup> n = 12 *Ltbr*<sup>-/-</sup> n = 14, pooled from four independent experiments, \* *P* < 0.05, \*\* *P* < 0.01 by Mann-Whitney test, each dot represents group mean, bars represent mean + SEM.

*LTβR's role in generation of anti-Salmonella class switched antibody is independent of gut persistence and GALT priming.*

Because the previous experiment was performed with oral *Salmonella* it is possible that the lack of GALT structures and priming in *Ltbr<sup>-/-</sup>* mice resulted in the lack of anti-*Salmonella* IgG. To determine if this class switch defect in *Ltbr<sup>-/-</sup>* mice was GALT specific, littermate *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>* mice were vaccinated with attenuated *Salmonella* by i.p. injection. As with oral *Salmonella*, i.p. administration caused a robust anti-*Salmonella* IgM and IgG antibody response in *Ltbr<sup>+/-</sup>* mice. While *Ltbr<sup>-/-</sup>* mice were able to mount anti-*Salmonella* IgM at day 7 post infection (**Figure 13A**), they were unable to generate anti-*Salmonella* IgG (**Figure 13B**). Levels of IgM in *Ltbr<sup>-/-</sup>* mice were low at



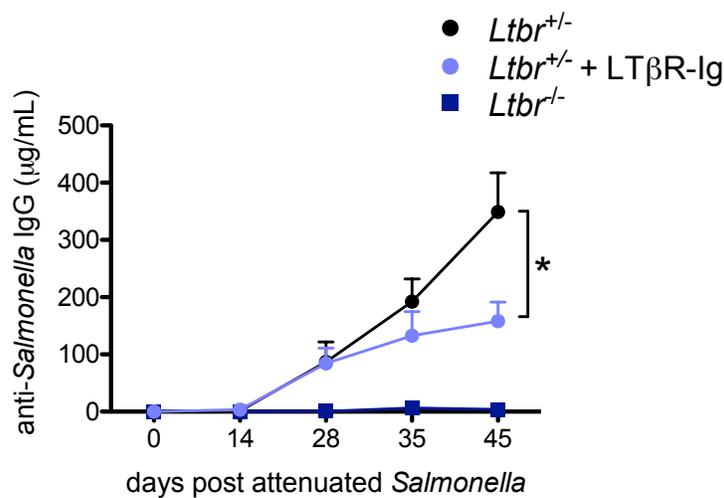
**Figure 13. The role of  $LT\beta R$  in IgG responses against *Salmonella* is independent of gut priming and gut persistence.**

Attenuated *Salmonella* was administered by intraperitoneal (i.p.) injection to littermate *Ltbr<sup>+/-</sup>* or *Ltbr<sup>-/-</sup>* animals. 45 days later animals were challenged with virulent *Salmonella* by i.p. injection. A. Anti-*Salmonella* IgM as measured by ELISA. B. Anti-*Salmonella* IgG. A and B values are standardized to hyper-immunized serum. *Ltbr<sup>+/-</sup>* n = 8 *Ltbr<sup>-/-</sup>* n = 11, representative of two independent experiments, NS is not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$  by Mann Whitney test, each dot represents group mean, bars represent + SEM.

later time points during infection suggesting, perhaps, that sustained antigen in the gut promotes IgM production. Overall, these data reveal that the role of LT $\beta$ R in class switching of antibody is not limited to the GALT.

*Active LT $\beta$ R signaling drives generation of anti-Salmonella class switched antibody.*

To further determine if the role of LT $\beta$ R in the generation of anti-*Salmonella* IgG is through GALT structure or active lymphotoxin signaling, *Ltbr*<sup>+/-</sup> animals were treated



**Figure 14. Lymphotoxin signaling drives generation of anti-*Salmonella* IgG responses**

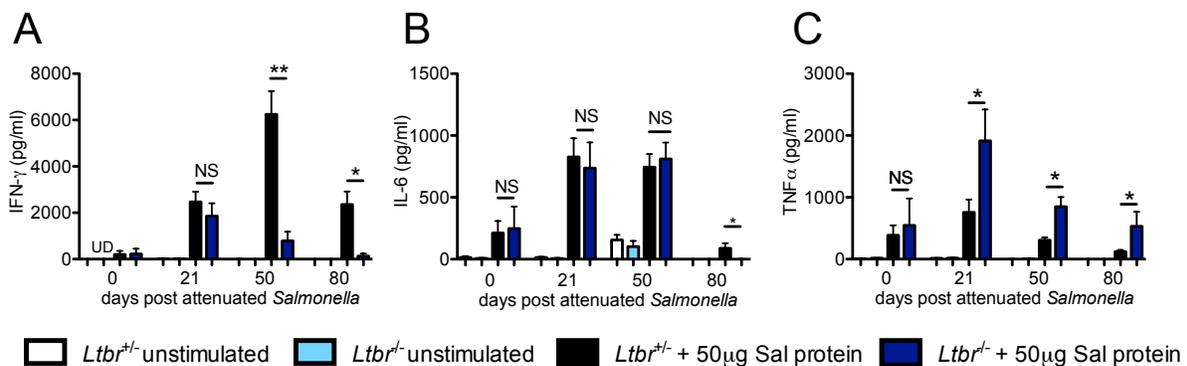
100 $\mu$ g LT $\beta$ R-Ig was administered i.p. every 7 days starting at day 22 and ending on day 43 after oral administration of attenuated *Salmonella* to *Ltbr*<sup>+/-</sup> animals. Littermate *Ltbr*<sup>-/-</sup> and untreated *Ltbr*<sup>+/-</sup> are included as a positive control. Anti-*Salmonella* IgG, *Ltbr*<sup>+/-</sup> n = 8, *Ltbr*<sup>+/-</sup> + LT $\beta$ R-Ig n = 9, *Ltbr*<sup>-/-</sup> n = 4, pooled from two independent experiments \* *P* < 0.05 by Student's t test. Each dot represents group mean, bars represent + SEM.

with blocking LT $\beta$ R-Ig fusion protein during oral attenuated *Salmonella* vaccination. This treatment was sufficient to decrease anti-*Salmonella* IgG responses to levels that were intermediate between untreated *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice (**Figure 14**). However, this transient blockade did not impact survival of the mice at challenge (data not

shown). These data suggest that active lymphotoxin signaling contributes to anti-*Salmonella* IgG responses.

*LTβR contributes to IFNγ and TNFα cytokine recall responses.*

Although antibody does contribute to protection against *Salmonella*, it is neither sufficient to mediate protection nor strictly required (Mastroeni, Villarreal-Ramos, and Hormaeche 1993; Mittrücker and Kaufmann 2000). IFNγ and TNFα production is a major determinant of protective immunity. To determine if LTβR contributes to cytokine responses against *Salmonella*, splenocytes from naïve or attenuated *Salmonella*-infected *Ltbr*<sup>+/-</sup> or *Ltbr*<sup>-/-</sup> mice were re-stimulated in vitro with *Salmonella* protein extract. Splenocytes from both *Ltbr*<sup>+/-</sup> or *Ltbr*<sup>-/-</sup> mice were able to mount anti-*Salmonella* IFNγ recall responses 21 days after receiving attenuated *Salmonella*. However, while *Ltbr*<sup>+/-</sup> mice continued to make robust IFNγ recall responses to *Salmonella* 50 and 80 days post infection, *Ltbr*<sup>-/-</sup> had severely impaired IFNγ responses at these time points (**Figure 15A**). These impairments were limited to IFNγ as splenocyte IL-6 production was similar between the two strains (**Figure 15B**) and TNFα production was enhanced in *Ltbr*<sup>-/-</sup> mice, even as early as day 21 following *Salmonella* infection (**Figure 15C**). These data



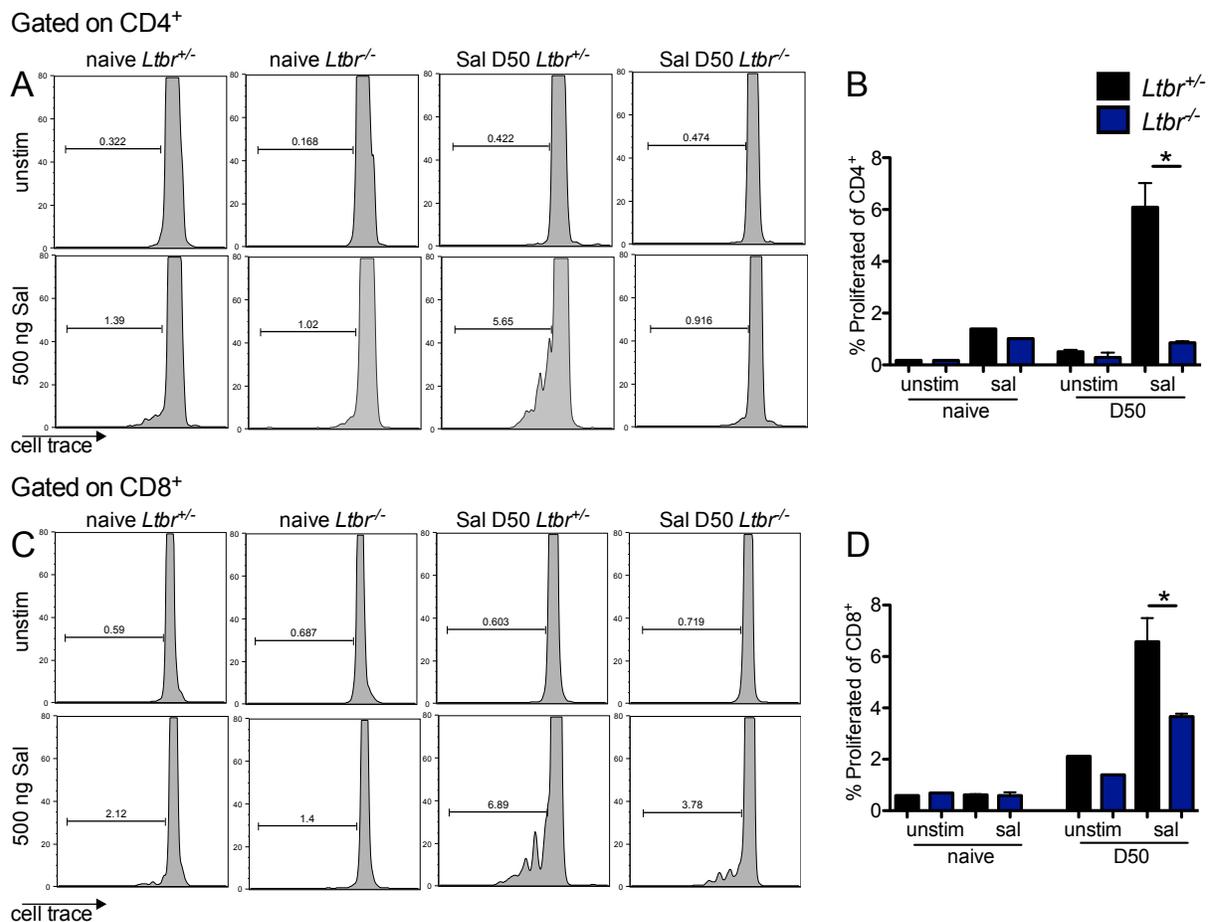
**Figure 15. LTβR contributes to IFNγ and TNFα cytokine recall responses.**

Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. Splenocyte cultures were restimulated or not for 48 hours in vitro with *Salmonella* protein extract **A**. IFN-γ, **B**. IL-6, and **C**. TNF-α measured in culture supernatant. n = 3 to 8 per group, pooled from three independent experiments. Bars represent mean +SEM, \* P < 0.05, \*\* P < 0.01 by Mann-Whitney test.

suggest that  $LT\beta R$  contributes to late phase  $IFN\gamma$  and  $TNF\alpha$  recall responses following infection with attenuated *Salmonella*.

*T cell responses to Salmonella are impaired in  $Ltbr^{-/-}$  mice.*

To understand if the reduction of  $IFN\gamma$  observed in splenocytes cultured from  $Ltbr^{-/-}$  mice reflected a T cell defect, proliferation of T cells in re-stimulated splenocyte



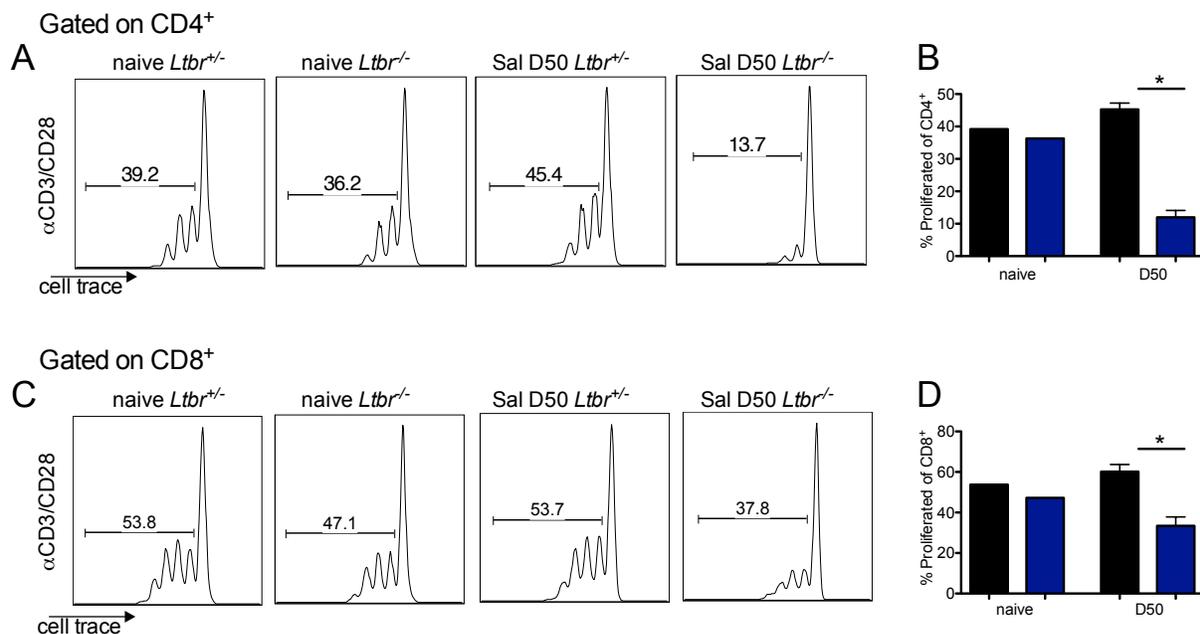
**Figure 16. T cell proliferative responses to *Salmonella* antigens are impaired in  $Ltbr^{-/-}$  mice.**

Oral streptomycin was administered to littermate  $Ltbr^{+/-}$  and  $Ltbr^{-/-}$  one day prior to oral administration of attenuated *Salmonella*. Splenocytes were labeled with CellTrace, stimulated or not with *Salmonella* antigen, and cultured for 48 hours. **A.** Representative flow plot of CD4 T cell proliferation as measured by CellTrace dilution. **B.** Quantification of A. **C.** Representative flow plot of CD8 T cell proliferation. **D.** Quantification of C. One representative experiment of two. Naïve mice n = 1, infected mice n = 4. Bars represent mean +SEM \*  $P < 0.05$ , by Mann-Whitney test.

cultures derived from naïve mice or those infected 50 days earlier with attenuated *Salmonella* was measured. Stimulation with *Salmonella* protein extract caused robust proliferation of CD4 and CD8 T cells derived from *Salmonella*-infected *Ltbr*<sup>+/-</sup> mice but not naïve mice (**Figure 16**). However, CD4 and CD8 T cells derived from *Ltbr*<sup>-/-</sup> mice infected 50 days earlier with attenuated *Salmonella* had a significant defect in antigen driven proliferation (**Figure 16**). These data suggest that exposure of *Ltbr*<sup>-/-</sup> mice to attenuated *Salmonella* results in impairments in antigen-specific CD4 and CD8 T cell proliferation.

### *Salmonella* infection of *Ltbr*<sup>-/-</sup> causes impairments in TCR driven proliferation

To determine if the observed impairment in T cell proliferation was antigen

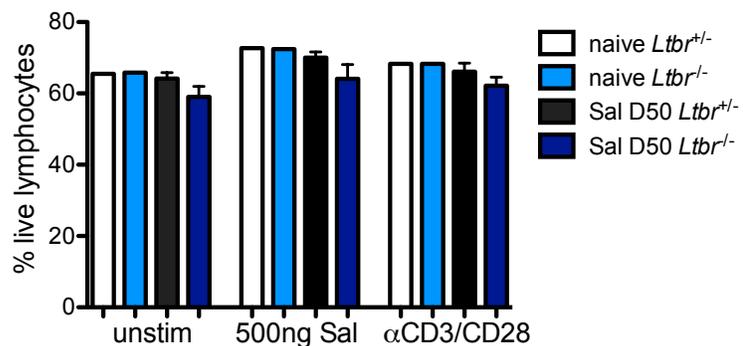


**Figure 17. *Salmonella*-mediated suppression of T cells in *Ltbr*<sup>-/-</sup> mice in response to anti-CD3/CD28** Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. Splenocytes were labeled with CellTrace, stimulated or not for 48 hours with plate bound anti-CD3 and soluble anti-CD28 **A**. Representative flow plots of CD4<sup>+</sup> T cell proliferation **B**. Quantification of **A**. **C**. Representative flow plots of CD8<sup>+</sup> T cell proliferation. **D**. Quantification of **C**. One representative experiment of two. Naïve mice n = 1, infected mice n = 4. \* P < 0.05, by Mann-Whitney test.

specific or more broad, proliferation of T cells in response to restimulation with plate bound anti-CD3 plus soluble anti-CD28 was measured in mixed splenocyte cultures. Splenocytes derived from naïve *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice had comparable levels of CD4 and CD8 T cell proliferation when re-stimulated with anti-CD3/CD28 (**Figure 17**). However, *Salmonella*-exposed *Ltbr*<sup>-/-</sup> mice had impairments in CD4 and CD8 T cell proliferation when re-stimulated with anti-CD3/CD28 (**Figure 17**). This impairment in CD2/CD28 driven proliferation was most pronounced in CD4 T cells of *Ltbr*<sup>-/-</sup> mice. These data suggest that exposure of *Ltbr*<sup>-/-</sup> mice to attenuated *Salmonella* results in broad, antigen-nonspecific impairments in CD4 and CD8 T cell proliferation.

One possible explanation for these data is that T cells from *Ltbr*<sup>-/-</sup> mice had a defect in survival after stimulation with either *Salmonella* antigen or anti-CD3/CD28. This does not seem to be the case as the percentage of live splenocytes in culture was unchanged between *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice under the same stimulation conditions

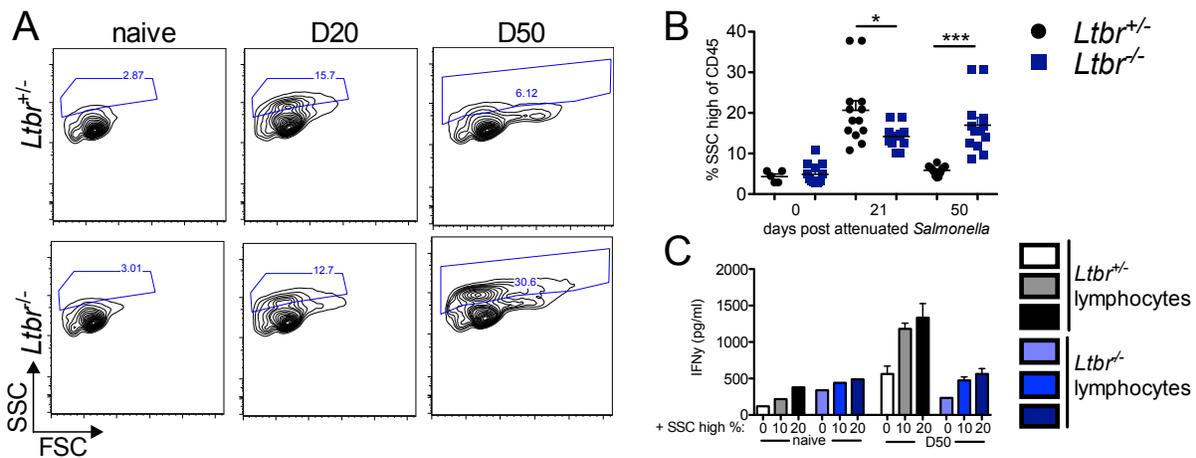
(**Figure 18**). These data suggested that exposure of *Ltbr*<sup>-/-</sup> mice to live, attenuated *Salmonella* causes profound CD4 T cell impairments in both antigen specific and non-specific populations that are not due to cell death following stimulation.



**Figure 18. Lymphocyte survival after 48 hours in culture.** Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. Splenocytes were cultured for 48 hours with indicated stimulus. Percent of live lymphocytes was measured as % of live/dead dye negative FSC/SSC gated lymphocytes. One representative experiment of two. Naïve mice n = 1, infected mice n = 4

*Granulocytes accumulate after Salmonella infection in Ltbr<sup>-/-</sup> mice but do not mediate T cell suppression in vitro.*

As the observed T cell impairments in *Ltbr<sup>-/-</sup>* mice extended to antigen non-specific responses, and occurred in mixed splenocyte cultures, a possible explanation for the observed impairment was that *Ltbr<sup>-/-</sup>* mice had increased numbers of suppressive cells. It has previously been observed that suppressive GR1<sup>+</sup> monocytes accumulate following *Salmonella* infection and that these cells can suppress antigen specific T cells responses and impair CD3/CD28 driven T cell proliferation in vitro (Tam et al. 2014). To determine if these innate cells play a role in suppression of immune responses in *Ltbr<sup>-/-</sup>* mice, the proportion of granulocytic cells within the spleens of littermate *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>*



**Figure 19. Granulocytes accumulate in *Ltbr<sup>-/-</sup>* mice after *Salmonella* infection but do not mediate lymphocyte suppressing *in vitro*.**

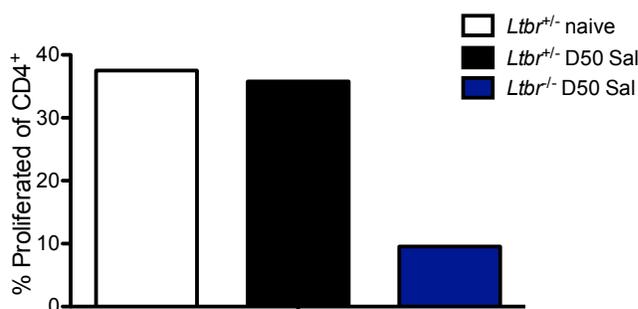
Oral streptomycin was administered to littermate *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>* one day prior to oral administration of attenuated *Salmonella*. **A**. Representative flow plot of granulocytes in the spleen of naïve or *Salmonella* infected animals. **B**. Quantification of A. For **C** granulocytes were sorted based of SSC<sup>high</sup> gating as in A. Lymphocytes were sorted by tight gating of SSC<sup>low</sup>FSC<sup>int</sup> cells. Cells were cultured for 48 hours with plate bound anti CD3 and soluble anti CD28 as previously described. SSC<sup>high</sup> cells from the same mouse were added at time 0 as a percentage of lymphocytes. **C**. IFN $\gamma$  in the supernatant after 48 hours in culture. A and B representative of three pooled experiments, n = 5 -14 per group. \* P < 0.05, \*\*\* P < 0.005 by Mann-Whitney test. **C**. One experiment, average of duplicate wells per mouse, per data point. For naïve mice n=1, *Salmonella* infected mice n = 3-4 per group.

mice was measured following oral *Salmonella* infection. As expected, oral infection with *Salmonella* increased the proportion of granulocytic (SSC<sup>hi</sup>) cells within the spleen of both *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice, and at day 20 following infection both strains had elevated proportions of these cells (**Figure 19A, B**). At 50 days following *Salmonella* infection, the percentage of granulocytes within the spleen of *Ltbr*<sup>+/-</sup> mice had returned to pre-infection levels. At this same time point, the proportion of these cells remained elevated in *Ltbr*<sup>-/-</sup> mice (**Figure 19A, B**).

To determine if these cells were indeed leading to suppression of *in vitro* cultures, granulocyte free lymphocytes were sorted from the spleens of naïve or day 50 *Salmonella* infected *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice and restimulated *in vitro* with anti-CD3/CD28. Even in the absence of granulocytic cells, lymphocytes of *Ltbr*<sup>-/-</sup> mice were suppressed in their ability to produce IFN- $\gamma$  compared to *Ltbr*<sup>+/-</sup> littermates (**Figure 19C**). To further determine if granulocytes had suppressive ability, granulocytes sorted from spleens of the same mice were added back into the lymphocyte cultures at an increasing percentage. The addition of granulocytes into lymphocyte cultures enhances IFN- $\gamma$  production in both *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice. Despite an increase in IFN- $\gamma$  production following addition of granulocytes, cultures from *Ltbr*<sup>-/-</sup> mice still had an impairment in IFN- $\gamma$  production compared to cultures from *Ltbr*<sup>+/-</sup> mice (**Figure 19C**). These data suggest that while granulocytes do indeed accumulate in the spleens of *Ltbr*<sup>-/-</sup> mice following *Salmonella* infection, they do not lead to the suppression of T cell responses observed *in vitro*.

*Purified CD4<sup>+</sup> T cells from Salmonella vaccinated mice have an intrinsic defect in T cell proliferation.*

Because purified lymphocytes (containing predominantly T cells and B cells) derived from *Ltbr<sup>-/-</sup>* mice had reduced production of IFN- $\gamma$  following stimulation with anti-CD3/CD28 it was possible that T cell suppression was T cell intrinsic. To test this hypothesis, CD4<sup>+</sup> T cells were sorted from the spleens of naïve *Ltbr<sup>+/-</sup>* mice, or *Salmonella* infected *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>* mice and restimulated with anti-CD3/CD28. As in the mixed splenocyte cultures T cells derived from naïve or *Salmonella* infected *Ltbr<sup>+/-</sup>* mice proliferated robustly in response to TCR stimulation while purified CD4 T cells sorted from *Salmonella* infected *Ltbr<sup>-/-</sup>* mice had a marked defect in proliferation (**Figure 20**). These data suggest that *Salmonella* infection leads to CD4<sup>+</sup> T cell intrinsic defects in *Ltbr<sup>-/-</sup>* but not *Ltbr<sup>+/-</sup>* mice. In this preliminary experiment cells from naïve *Ltbr<sup>-/-</sup>* mice were not included and therefore this experiment does not definitively demonstrate that the observed impairment is due to *Salmonella* infection.



**Figure 20. *Salmonella* induced CD4 intrinsic proliferation defect in *Ltbr<sup>-/-</sup>* mice.**

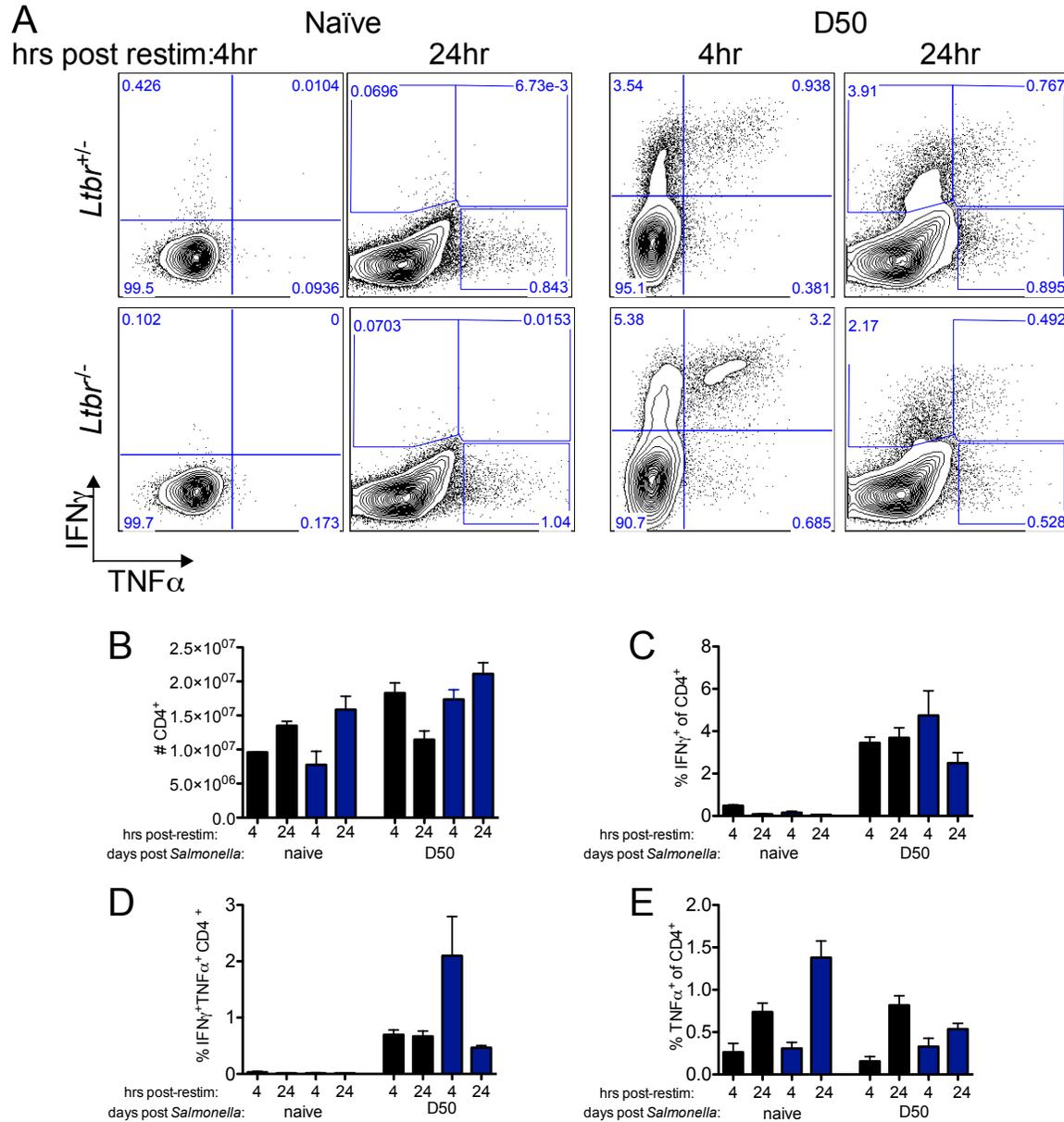
Oral streptomycin was administered to littermate *Ltbr<sup>+/-</sup>* and *Ltbr<sup>-/-</sup>* one day prior to oral administration of attenuated *Salmonella*. CD4 T cells were sorted from naïve or day 50 infected spleens, stained with cell trace dye and cultured for 48 hours with plate bound anti CD3 and soluble anti CD28. Proliferation as measured by cell trace dilution in live, CD4 T cells after 48 hours in culture is shown. Each bar represents average of triplicate wells representing CD4 T cells from sorted from two spleens of pooled mice.

### *Anti-Salmonella CD4<sup>+</sup> IFN $\gamma$ recall responses after in vivo restimulation*

To determine if the T cell defect observed following *in vitro* stimulation occurred *in vivo*, naïve or *Salmonella* infected *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice were injected *in vivo* with heat killed *Salmonella*, a potent stimulus of memory recall responses (Ravindran et al. 2005), and intracellular production of cytokines from T cells was measured 4 hours after restimulation. Naïve *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice restimulated *in vivo* with heat killed *Salmonella* and sacrificed 4 hours later did not produce measurable quantities of intracellular IFN $\gamma$  or TNF $\alpha$ . *Ltbr*<sup>+/-</sup> mice infected 50 days earlier with oral attenuated *Salmonella* produced high levels of intracellular IFN $\gamma$  four hours after *in vivo* restimulation with heat killed *Salmonella* indicating a robust memory recall response. Like their *Ltbr*<sup>+/-</sup> littermates, CD4 T cells from *Salmonella* experienced *Ltbr*<sup>-/-</sup> mice produced significant levels of intracellular IFN $\gamma$ , and the percentage of IFN $\gamma$ <sup>+</sup> CD4 T cells was comparable between *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice (**Figure 21 A, C**). Unlike their *Ltbr*<sup>+/-</sup> littermates, *Salmonella* experienced *Ltbr*<sup>-/-</sup> mice also had a population of IFN $\gamma$ , TNF $\alpha$  double positive cells CD4 T cells. These data indicated that *in vivo* *Ltbr*<sup>-/-</sup> mice retained a population of *Salmonella* specific CD4 T cells 50 days post infection and had an additional population of multifunctional *Salmonella*-specific T cells (**Figure 21 A, D**). This result appeared to be in direct contrast with the results obtained when splenocytes were restimulated with *Salmonella* antigens *in vitro*.

Several key differences between *in vitro* experiments and *in vivo* experiments could explain this discrepancy. While both experiments were performed with complex antigens that require APC processing, the *in vitro* experiments were performed with a protein rich *Salmonella* extract while *in vivo* experiments used heat killed *Salmonella*

which is a more complex mixture of antigens. Secondly, while analysis of T cells responses after *in vitro* stimulation occurred after 48 hours, analysis of *in vivo*



**Figure 21. anti-*Salmonella* CD4<sup>+</sup> IFN $\gamma$  recall responses after *in vivo* restimulation.**

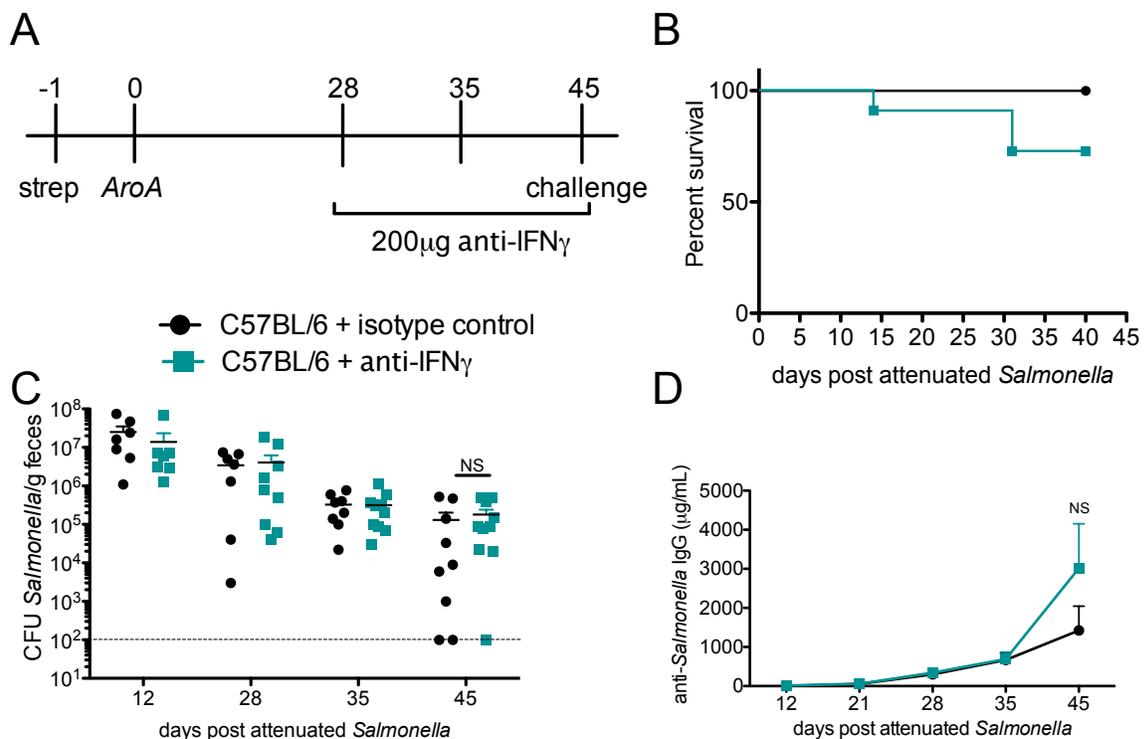
Oral streptomycin was administered to littermate *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> one day prior to oral administration of attenuated *Salmonella*. Naïve or infected mice were restimulated by i.v. injection of heat killed *Salmonella*. At 4 or 24 hours post restimulation animals were sacrificed and spleens were collected and stained for IFN $\gamma$  and TNF. **A**. Representative flow plot, gated on CD4 T cells. **B**. Absolute number of CD4 T cells in the spleen. **C**. Percentage of IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> of all CD4<sup>+</sup>. **D**. Percentage of IFN $\gamma$ <sup>+</sup> TNF $\alpha$ <sup>+</sup> CD4<sup>+</sup> of all CD4<sup>+</sup>. **E**. Percentage of TNF $\alpha$ <sup>+</sup> CD4<sup>+</sup> of all CD4<sup>+</sup>. Representative of three pooled experiments. n = 4-6 per group.

responses occurred only 4 hours after restimulation. This could be significant if the mechanism of T cell suppression required time to take effect. If for example the suppressive mechanism reduced T cell proliferation, as observed *in vitro*, but not T cell activation, no differences could be observed between the two strains of mice when the animals were sacrificed after only 4 hours.

To test this hypothesis, naïve or *Salmonella* infected *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice were injected *in vivo* with heat killed *Salmonella* and intracellular production of cytokines from T cells was measured 24 hours after restimulation to allow time for T cell proliferation. CD4 T cells from naïve *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice restimulated *in vivo* with heat killed *Salmonella* and sacrificed 24 hours later did not produce measurable quantities of intracellular IFN $\gamma$  but did produce TNF $\alpha$  (**Figure 21 A, C, E**). CD4 T cells from *Ltbr*<sup>+/-</sup> mice that had 50 days previously been infected with attenuated *Salmonella* continued to produce robust IFN $\gamma$  recall responses that were similar in magnitude to recall responses after 4 hours of restimulation. CD4 T cells from *Salmonella* experienced *Ltbr*<sup>+/-</sup> mice had a reduction of IFN $\gamma$ <sup>+</sup> 48 hours after restimulation, although this reduction did not reach statistical significance. These data suggest that one possible explanation for the discrepancy between the *in vivo* and *in vitro* data is that the mechanism of suppression acting in *Ltbr*<sup>-/-</sup> mice requires additional time to be apparent *in vivo*. Tracking antigen specific T cell proliferation and survival in *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice following *in vivo* restimulation could potentially answer this question.

Late phase IFN $\gamma$  blockade impairs survival at challenge but does not impact *Salmonella* gut clearance or anti-*Salmonella* IgG.

If indeed the reduction of IFN $\gamma$  and T cell responses observed *in vitro* extended *in vivo*, it would predict that a reduction of IFN $\gamma$  would have an impact on progression of disease. To test this question, C57BL/6 mice were treated with blocking anti-IFN $\gamma$  antibody or isotype control starting at day 28 post attenuated *Salmonella* (**Figure 22A**). This time point was chosen because splenocyte cultures from *Ltbr*<sup>-/-</sup> mice show defects in IFN $\gamma$  only at later time point after infection. At day 45 post infection both sets of mice



**Figure 22. IFN $\gamma$  blockade during late phase of attenuated *Salmonella* infection.**

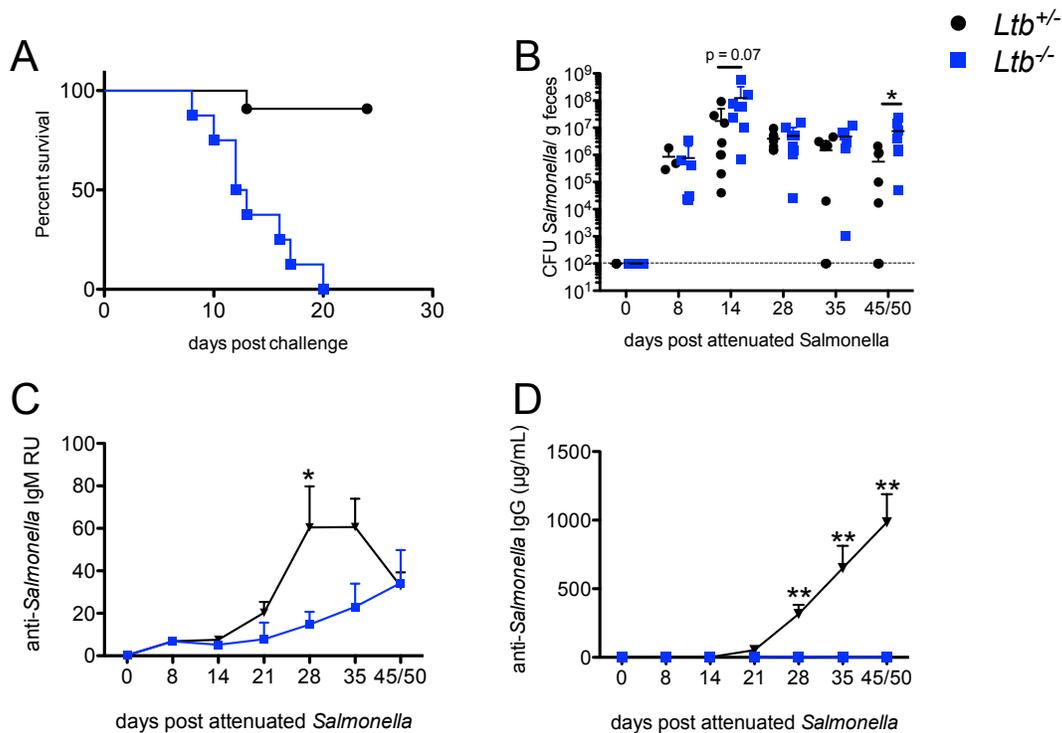
Oral streptomycin was administered to in house bred C57BL6 mice one day prior to oral administration of attenuated *Salmonella*. 200 $\mu$ g Anti-IFN $\gamma$  or isotype control was administered by IP injection at day 28, 35, and 45 following infection. Mice were challenged with virulent *Salmonella* at day 45 post attenuated *Salmonella*. **A**. Experimental schematic. **B**. Survival post virulent challenge. **C**. Fecal CFU of *Salmonella*. **D**. Anti-*Salmonella* IgG. Two pooled experiments. Isotype control n = 9, anti-IFN $\gamma$  n = 9. Statistics by Mann Whitney Test.

were challenged with virulent *Salmonella*. Blocking of IFN $\gamma$  at a late time point following attenuated *Salmonella* resulted in a minor increased mortality at virulent challenge (**Figure 22B**). IFN $\gamma$  blockade did not alter clearance of attenuated *Salmonella* from the gut (**Figure 22C**) or anti-*Salmonella* IgG responses (**Figure 22D**). These data suggest that while the IFN $\gamma$  defect observed in *Ltbr*<sup>-/-</sup> mice could explain the failure to survive challenge, other functions of the lymphotoxin pathway drive *Salmonella* gut clearance and the generation of anti-*Salmonella* IgG responses.

## Chapter 5: Cellular sources of lymphotoxin in anti-*Salmonella* responses

Membrane  $LT\alpha 1\beta_2$  is the ligand for  $LT\beta R$  that mediates primary and secondary immune responses to *Salmonella*

$LT\beta R$  is robustly expressed on stromal cells, epithelium, and myeloid cells (Tumanov et al. 2003). The ligands for  $LT\beta R$  include LIGHT and  $LT\alpha 1\beta_2$ . To determine which  $LT\beta R$  ligand is required for anti-*Salmonella* immunity,  $Ltb^{+/-}$  or  $Ltb^{-/-}$  littermate mice were vaccinated with attenuated *Salmonella* and challenged with virulent *Salmonella* 45 to 50 days later. As with  $Ltbr^{-/-}$  mice,  $Ltb^{-/-}$  mice were unable to survive



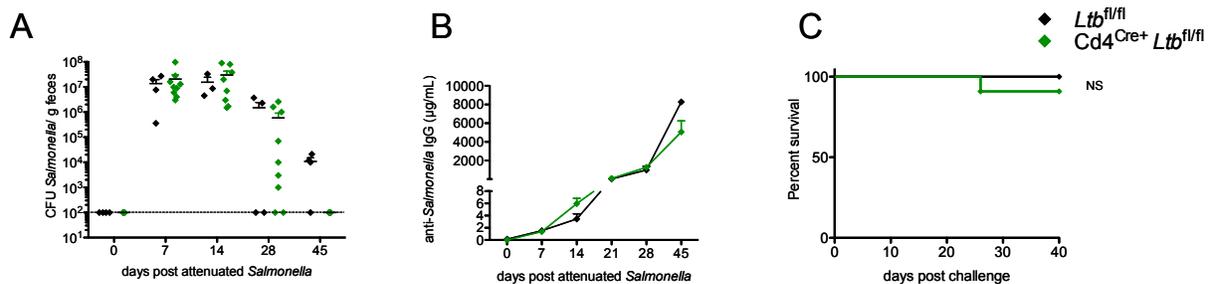
**Figure 23. Lymphotoxin is required for protective immunity to *Salmonella* and clearance from the gut lumen.**

Oral streptomycin was administered to littermate  $Ltb^{+/-}$  and  $Ltb^{-/-}$  one day prior to oral administration with attenuated *Salmonella*. Animals were orally challenged with virulent *Salmonella* 45 to 50 days later. **A**. Survival following challenge. **B**. CFU of attenuated *Salmonella* in the feces. **C**. Anti-*Salmonella* IgM. **D**. Anti-*Salmonella* IgG.  $Ltb^{+/-}$   $n = 11$ ,  $Ltb^{-/-}$   $n = 8$ , two pooled experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$  by Mann-Whitney test. For **B**, each dot represents individual mouse, bars represent mean  $\pm$  SEM. **C** and **D** were standardized to hyper-immune serum and each dot represents mean of group, bars represent + SEM.

virulent challenge, clear attenuated *Salmonella* from gut lumen, or mount anti-*Salmonella* IgG responses (**Figure 23 A-C**). These data suggest that LT $\alpha$ 1 $\beta$ 2 is the ligand for LT $\beta$ R required to mount anti-*Salmonella* immune responses. Since *Ltb*<sup>-/-</sup> mice retain a MLN (Tumanov et al. 2003), it suggests that the absence of this lymphoid structure is not the reason for the impairment in anti-*Salmonella* immunity observed in *Ltbr*<sup>-/-</sup> mice.

### Lymphotoxin from $\alpha\beta$ T cells is not required for anti-*Salmonella* responses

Many lymphoid cells including T cells, B cells, NK cells, and ILCs express membrane lymphotoxin both at steady state and during infection. To determine which cell type provides the LT signal that is sensed by LT $\beta$ R to provide anti *Salmonella* immunity, we employed mice in which the floxed *Ltb* gene was excised by conditional expression of a Cre recombinase. To determine if lymphotoxin expressed in T cells contributed to *Salmonella* responses we used *Cd4*<sup>Cre+/WT</sup> *Ltb*<sup>fl/fl</sup> animals. Because the CD4 gene is upregulated during the development of all  $\alpha\beta$  T cells these mice lack



**Figure 24. Lymphotoxin from  $\alpha\beta$  T cells is dispensable for anti-*Salmonella* immunity, gut clearance, and antibody production**

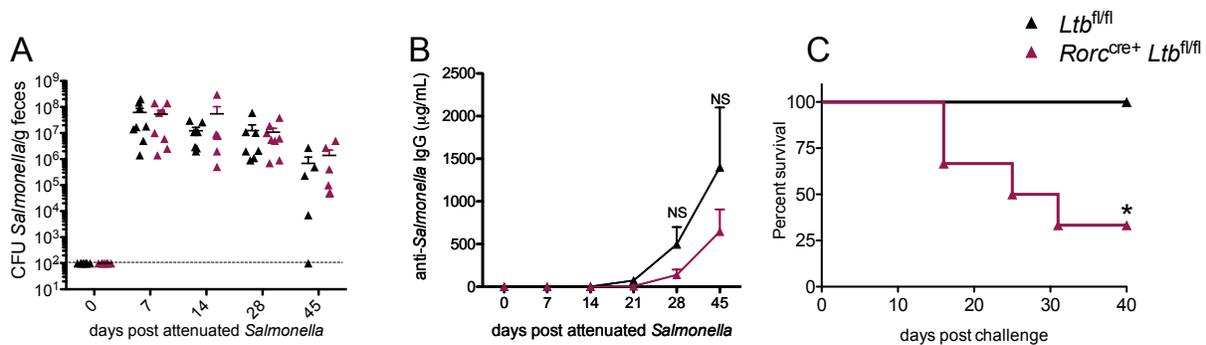
Oral streptomycin was administered to littermate *Ltb*<sup>fl/fl</sup> or *Cd4*<sup>Cre+</sup> *Ltb*<sup>fl/fl</sup> animals one day prior to oral administration with attenuated *Salmonella*. Animals were orally challenged with virulent *Salmonella* 45 to 50 days later. **A**. Fecal CFU of attenuated *Salmonella* from one representative experiment. *Ltb*<sup>fl/fl</sup> n = 4, *Cd4*<sup>Cre+</sup> *Ltb*<sup>fl/fl</sup> n = 9 **B**. Anti-*Salmonella* IgG **C**. Survival after challenge with virulent *Salmonella* 45 days post attenuated *Salmonella*. Two pooled experiments. *Ltb*<sup>fl/fl</sup> n = 9, *Cd4*<sup>Cre+</sup> *Ltb*<sup>fl/fl</sup> n = 11.

lymphotoxin expression on both CD4 and CD8 T cells. Expression of lymphotoxin on  $\gamma\delta$  T cells is not altered in this system.  $Cd4^{Cre+/WT} Ltb^{fl/fl}$  or  $Ltb^{fl/fl}$  littermates were pretreated with streptomycin and infected orally with attenuated *Salmonella*. No differences in fecal CFU or anti-*Salmonella* IgG were observed in these mice (**Figure 24A, B**). When mice were challenged with virulent *Salmonella* 45 days following attenuated *Salmonella* no significant differences in survival were noted (**Figure 24C**). These data indicate that lymphotoxin from  $\alpha\beta$  T cells is dispensable for fecal clearance of *Salmonella*, generation of anti-*Salmonella* IgG, and protective immunity at challenge.

*Lymphotoxin from  $Rorc^+$  ILC3s and T cells does not contribute to anti-*Salmonella* antibody responses, but has a partial impact on survival after challenge.*

Lymphotoxin from ILC3s in the gut plays an important role in early control of *Citrobacter rodentium* in the colon and mice that conditionally lack lymphotoxin from  $Rorc^+$  ILC3s succumb to infection (Tumanov et al. 2011). To determine if lymphotoxin from  $Rorc^+$  ILCs plays a role in *Salmonella* infection,  $Rorc^{Cre+} Ltb^{fl/fl}$  mice and littermate  $Ltb^{fl/fl}$  were orally infected with attenuated *Salmonella* after pretreatment with streptomycin. It should be noted, that because *Rorc* is expressed during  $\alpha\beta$  T cell development, this cre recombinase deletes lymphotoxin expression from both  $\alpha\beta$  T cell as well as all *Rorc* expressing ILC3s including LTi. Innate NK cells and  $\gamma\delta$  T cells retain lymphotoxin expression in this system. Deletion of lymphotoxin from ILC3s and  $\alpha\beta$  T cells did not impact clearance of *Salmonella* from the gut (**Figure 25A**) or generation of anti-*Salmonella* IgG (**Figure 25B**).  $Rorc^{Cre+} Ltb^{fl/fl}$  had a partial defect in survival following challenge with virulent *Salmonella* compared to  $Ltb^{fl/fl}$  littermates. (**Figure**

**25C).** Because *Rorc*<sup>Cre+</sup> *Ltb*<sup>fl/fl</sup> lack expression of lymphotoxin on LTi cells, have all of the structural defects of *Ltb*<sup>-/-</sup> mice (they retain one mesenteric lymph node, but lack all peripheral lymph nodes and Peyer's patches) these data indicate that the absence of these structures does not impact anti-*Salmonella* IgG production or gut clearance. The partial impairment in survival suggests that lymphoid structures may play some, but not an absolute role, in organizing memory responses.



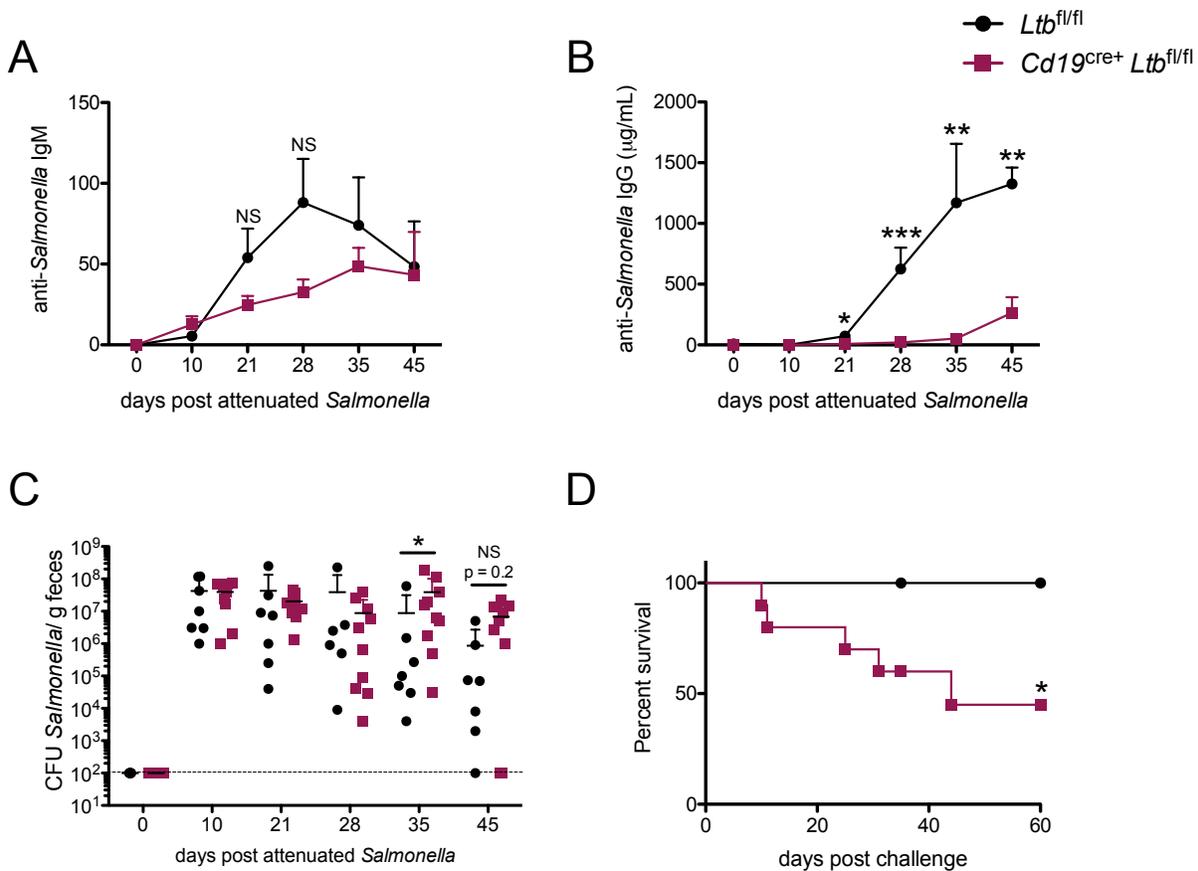
**Figure 25. Lymphotoxin from T cells and innate lymphoid type 3 cells is dispensable for anti-*Salmonella* gut clearance, and antibody production and has a partial impact on protection at challenge**

Oral streptomycin was administered to littermate littermate *Ltb*<sup>fl/fl</sup> or *Rorc*<sup>cre+</sup> *Ltb*<sup>fl/fl</sup> animals one day prior to oral administration with attenuated *Salmonella*. Animals were orally challenged with virulent *Salmonella* 45 to 50 days later. **A.** Fecal CFU of attenuated *Salmonella* from one representative experiment. *Ltb*<sup>fl/fl</sup> n = 8, *Rorc*<sup>cre+</sup> *Ltb*<sup>fl/fl</sup> n = 8 **B.** Anti-*Salmonella* IgG **C.** Survival after challenge with virulent *Salmonella* 45 days post attenuated *Salmonella*, statistics by Mantel-Cox test. Two pooled experiments. *Ltb*<sup>fl/fl</sup> n = 6, *Rorc*<sup>cre+</sup> *Ltb*<sup>fl/fl</sup> n = 6.

*Lymphotoxin from B cells is essential for anti-Salmonella IgG and contributes to protective immunity to Salmonella.*

B cells expressing lymphotoxin organize lymphoid structures and coordinate immune responses during viral and helminth infections (León et al. 2012; Kumar et al. 2010; Moseman et al. 2012; Suresh et al. 2002). To determine if B cell derived lymphotoxin contributes to anti-*Salmonella* immunity, *Cd19*<sup>Cre+/WT</sup> *Ltb*<sup>fl/fl</sup> and *Ltb*<sup>fl/fl</sup>

littermates were orally infected with attenuated *Salmonella* after pretreatment with streptomycin. Anti-*Salmonella* IgM and IgG were measured. Production of anti-*Salmonella* IgM was comparable between  $Cd19^{Cre+/WT} Ltb^{fl/fl}$  and  $Ltb^{fl/fl}$  littermate mice. (Figure 26 A). While  $Ltb^{fl/fl}$  mice generated robust anti-*Salmonella* IgG, B cell-LT deficient mice had a severe impairment in the production of anti-*Salmonella* IgG (Figure 26B).  $Cd19^{Cre+/WT} Ltb^{fl/fl}$  mice also had a minor defect in fecal clearance of *Salmonella*.



**Figure 26. Lymphotoxin from B cells is required for anti-*Salmonella* IgG.**

Oral streptomycin was administered to littermate  $Ltb^{fl/fl}$  and  $Cd19^{Cre+} Ltb^{fl/fl}$  mice one day prior to oral administration with attenuated *Salmonella*. 45 days later animals were challenged orally with virulent *Salmonella*. **A.** Anti-*Salmonella* IgM. **B.** Anti-*Salmonella* IgG. **C.** CFU of *Salmonella* in feces. For A, B and C \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$  by Mann-Whitney test. **D.** Survival post virulent challenge.  $Ltb^{fl/fl}$   $n = 7$ ,  $Cd19^{Cre+} Ltb^{fl/fl}$   $n = 10$ .

At day 35 following oral attenuated *Salmonella*,  $Ltb^{fl/fl}$  mice had lower fecal CFU of

*Salmonella* compared to littermate B cell-LT deficient mice. By day 45 after infection, 3 out of 12  $Cd19^{Cre+/WT} Ltb^{fl/fl}$  mice (all cage mates) had no detectable *Salmonella* in their feces while the remaining mice in the group had high bacterial loads (**Figure 26C**).

When challenged with virulent *Salmonella*, the  $Cd19^{Cre+/WT} Ltb^{fl/fl}$  mice with high fecal *Salmonella* loads were more likely to succumb to infection, resulting in a partial survival defect (**Figure 26D**). Altogether the data suggest that B cell derived lymphotoxin plays a role in production of anti-*Salmonella* IgG and protective responses. The role of B cell derived lymphotoxin in *Salmonella* gut clearance is less clear, as the response in these animals appears bimodal with a noted cage effect. Animals that clear gut infection go on to generate protective responses to virulent *Salmonella*, while those that do not, are susceptible at challenge. This cage effect could be stochastic: If B cell derived lymphotoxin is one component of many that impacts gut clearance of *Salmonella* perhaps in the cage of mice that cleared *Salmonella* compensatory mechanism were stronger. Because clearance is microbiota mediated, these alterations may be passed on to cage mates.

## **Discussion**

### *Overall summary of findings*

Overall this work has explored the role of the  $LT\alpha_1\beta_2/LT\beta R$  pathway in three aspects of immune responses to *Salmonella*: clearance of *Salmonella* from the gut, generation of anti-*Salmonella* memory and immunity, and the cellular sources of lymphotoxin that mediate these effects. On the first point, the role of lymphotoxin in clearance of *Salmonella* from the gut, this work elucidates the first host pathway involved in this process. Previously it was known that a complex microbiota is required for *Salmonella* gut clearance. It was also shown that IgA, B cells, and T cells are not independently necessary or sufficient to mediate this clearance (Endt et al. 2010). It was previously suggested that the microbiota may act independently of the host to resolve *Salmonella* gastrointestinal disease. Here we suggest that the host does indeed play a role in this process.

The second aim of this thesis was to determine the mechanisms by which the lymphotoxin pathway contributes to memory and protective immunity. Our initial findings show that in the absence of  $LT\beta R$ , animals succumb to virulent challenge with *Salmonella* despite previous vaccination. To explain this finding we explored immunization and challenge route, antibody responses, cytokine production, T cell responses, and potential mechanism of T cell suppression. We note significant impairments in anti-*Salmonella* antibody responses as well as potential defects in cytokine and T cell responses. Although no definitive mechanism to explain the lack of immunity in *Ltbr*<sup>-/-</sup> mice was found, defects in multiple arms of the immune response were measured, suggesting a broadly affected immune environment.

Finally, to gain a better understanding of the pathways and mechanisms involved, the cellular sources of lymphotoxin involved in *Salmonella* immunity were analyzed. Using blockade of LT $\beta$ R signaling in WT mice we show that active lymphotoxin signaling contributes to anti-*Salmonella* IgG responses and bacterial clearance from the gut. Using cre/loxp transgenic targeting of the lymphotoxin beta gene, we discovered that multiple cell types expressing lymphotoxin contribute to anti-*Salmonella* immunity. Our findings suggest that lymphotoxin production by B cells is essential for anti-*Salmonella* class switched antibody responses and contributes to protective immunity. This work also demonstrates that lymphotoxin from T cells is dispensable for anti-*Salmonella* responses. Lymphotoxin from T cells and ILC3s did not have an impact on anti-*Salmonella* IgG responses, but had a partial impact on survival at challenge. Since this defect in survival was not observed in lymphotoxin T cell conditionally deficient mice, it suggests that ILC3s and B cells both contribute to anti-*Salmonella* immunity.

Altogether, the findings of this thesis suggest a previously unrecognized role for lymphotoxin in anti-*Salmonella* immunity. In previous studies using fully virulent *Salmonella*, no role for LT $\beta$ R was noted (Barthel et al. 2003). By using an attenuated mutant of *Salmonella* within the streptomycin pretreatment model of diarrhea we were able to uncover previously unknown roles of this pathway.

#### *Lymphotoxin's role in Salmonella gut clearance*

Through several experimental approaches we demonstrate that absence of LT $\beta$ R, LT $\beta$ , or blockade of lymphotoxin signaling significantly impairs *Salmonella* clearance from the gut. In trying to identify the cellular source of lymphotoxin

responsible for this effect we were not able to definitively pinpoint a singular cellular source required for the complete phenotype. Mice in which lymphotoxin was conditionally deficient from B cells showed a bimodal response. Some animals cleared *Salmonella* completely while others maintained high bacterial loads. This result is puzzling in the context of previous literature that describes that B cell deficient mice can clear *Salmonella* (Endt et al. 2010). However, two possible models could explain our findings. The role of lymphotoxin in gut clearance of *Salmonella* could be positive or negative. A positive role for lymphotoxin could be in shaping the microbiota. LT dependent immunological mechanisms like antibody production, the IL22 pathway, antimicrobial peptides, and others could work in concert to help the microbiota outcompete *Salmonella*. In this study we did not explore in detail the effect of lymphotoxin in the gut tissue or in alterations of the microbiota. We did however develop a unique system in which to test how alterations in the microbiota occur over time in hosts that clear *Salmonella* versus those that do not. A potentially fruitful line of future study would be to track the changes in the microbiota (both on the population and transcriptional levels) in germ free *Ltbr*<sup>+/+</sup> and *Ltbr*<sup>-/-</sup> mice monocolonized with *Salmonella* and then conventionalized with the same cecal content. Do different populations of commensals arise in *Ltbr*<sup>+/+</sup> and *Ltbr*<sup>-/-</sup> mice? Which pathways do these commensal populations engage to compete with *Salmonella*? What host pathways are engaged in the gut of *Ltbr*<sup>+/+</sup> and *Ltbr*<sup>-/-</sup> mice? These questions could potentially be very fruitful in understanding the biology of permissive hosts.

The other possibility that could explain the why *Ltbr*<sup>-/-</sup> mice cannot clear *Salmonella* is that the LT pathway acts to modify clearance in a negative way. In WT

mice the lymphotoxin pathway may not directly contribute to clearance, but when it is absent, it may prevent the microbiota from outcompeting *Salmonella*. For example, it is known that triggering host inflammation is an important bacterially-mediated mechanism for initiation of colonization (see introduction pg12). The role of sustained inflammation in maintenance of colonization is not yet known, but it is possible that, as in initiation of colonization, sustained inflammation of the gut allows for a permissive niche for *Salmonella*.

There is some evidence in the literature to support this idea. It has been shown that induction of gall bladder inflammation and gall stones via a high cholesterol diet predisposes mice to *Salmonella* colonization of the gall bladder (Crawford et al. 2010). Perhaps in the absence of the lymphotoxin pathway inflammation in the gut is sustained, allowing for a permissive niche. Although our study did not explore this possibility, some of our results may support this assumption. We noted high TNF levels in re-stimulated splenocyte supernatants from *Salmonella* infected *Ltbr*<sup>-/-</sup> mice. Further we show that granulocytes accumulate and persist in the spleens of *Ltbr*<sup>-/-</sup> and not *Ltbr*<sup>+/-</sup> littermates. Finally, we note that inflammation of the colon and cecum is sustained in these animals. Future work could explore the inflammatory environment in the gut of *Ltbr*<sup>-/-</sup> and *Ltbr*<sup>+/-</sup> littermates following *Salmonella* infection in more detail. Are there increases in inflammatory cytokines and chemokines? What cells infiltrate the colons and cecums of *Ltbr*<sup>-/-</sup> and *Ltbr*<sup>+/-</sup> mice? Barthel et al. noted that B cells are highly enriched in the inflamed colons of *Ltbr*<sup>-/-</sup> mice two days post infection with virulent *Salmonella* in the streptomycin model (Barthel et al. 2003).

In the 129x1/SvJ background some animals become colonized with *Salmonella* in the absence of streptomycin pretreatment and shed high levels of bacteria in the feces. This “super-shedding” phenotype is correlated with an increased number of neutrophils in the spleen, reduced Th1 responses and increased Treg numbers (Gopinath et al. 2013). The reduction of Th1 and increase of neutrophils is a result of high fecal burdens, as animals that are not super shedders can develop the phenotype via administration of streptomycin. This suggests that the observed granulocyte and T cell phenotype may be an effect of high bacterial loads in the gut, and not a cause.

The same study also noted that suppression of inflammation by administration of steroids does not induce mice to become super shedders. The inverse idea is not tested and could be a potentially interesting line of future experimentation. If indeed *Ltbr*<sup>-/-</sup> mice have increased inflammation in the gut, could suppression of inflammation (perhaps via steroids) reduce bacterial loads? Would triggering inflammation at late stages of bacterial infection in WT mice via another method (such as administration of low levels of DSS) promote sustained colonization?

Regardless of which mechanism is in play, our study has implications for individuals who are chronically infected asymptomatic excretors of *Salmonella*. While previously it was proposed that these individuals may lack the required microbiota to mediate clearance (Endt et al. 2010), our findings suggest that these individuals may also lack the necessary host pathways to manipulate this clearance or have maladaptive responses that promote colonization. The conditions that lead to asymptomatic excretion in the case of non typhoidal *Salmonella*, or long term carriage in the case of typhoid are not well understood. In mice and humans, the TNF and

lymphotoxin genes are tandemly arranged between class I and III MHC regions where several SNPs have been found (Nedospasov et al. 1991; Posch et al. 2003). A potentially interesting line of study would be to determine if an association of LT polymorphisms and *Salmonella* carriage exists in human populations.

### *Antibody responses to Salmonella*

While the role of B cell derived lymphotoxin in germinal center formation has been previously well described (Fu et al. 1998; Fu et al. 1997; Matsumoto et al. 1997), recently it has been shown that somatically hypermutated antibody responses to *Salmonella* occur extrafollicularly (Cunningham et al. 2007; Di Niro et al. 2015). Di Niro and colleagues demonstrate that this extrafollicular antibody response is indeed specific and depends on hypermutation. Restricting BCR availability and reversion of acquired mutations both impair the anti-*Salmonella* response (Di Niro et al. 2015). When GC-like structures do form at about one month following infection they are small and immature, expressing low levels of PNA positive cells and are found at atypical sites between the T and B cells zones. Class switching and affinity maturation occur at the same rate in extrafollicular locations and these germinal center like structures (Di Niro et al. 2015).

A limitation of our study is that our observations of B cells are restricted to serum antibody responses. We did not show that the absence of the lymphotoxin pathway impairs extrafollicular antibody responses at the cellular level. However, in the time frame of our experiment, fully formed germinal centers are not expected. One possible avenue for further exploration is to determine the B cell phenotypes following infection in *Ltbr*<sup>+/-</sup> and *Ltbr*<sup>-/-</sup> mice. The number of plasmablasts (TCR<sup>-</sup> F4/80<sup>-</sup> CD44<sup>+</sup>CD138<sup>+</sup>) versus germinal center (B220<sup>+</sup>, CD38<sup>low</sup>, CD95<sup>+</sup>, PNA<sup>+</sup>) B cells could be

determined. I would predict that the number of plasmablasts would not change in *Ltbr*<sup>-/-</sup> mice as these mice produce robust IgM responses. Since we observe a defect in class switching it may be interesting to measure AID expression in B cells from *Ltbr*<sup>-/-</sup> mice. Interestingly, generation of anti-*Salmonella* class switched antibody was also reduced in CD40L deficient mice, while the IgM response is retained. The CD40L/CD40 signaling cascade is similar to LT/LTβR, and these two pathways can work in concert. Engagement of CD40 on B cells upregulates LTαβ expression on these cells to promote FDC maturation (Vu et al. 2008). This response has been characterized for GC responses. Similar mechanisms could be in play for extrafollicular class switching and hypermutation.

CD11c<sup>high</sup> high cells have been implicated in extrafollicular responses and are necessary for plasmablast survival and differentiation into plasma cells (De Vinuesa et al. 1999). Recently, LTβR expression on CD11c<sup>+</sup> DCs has been found to be essential in the survival of fibroblastic reticular cells (FRCs), and an absence of this pathway results in a significant impairment in both GC B cells and antibody forming cells following immunization (Kumar et al. 2015). One possibility is that during extrafollicular antibody formation engagement of CD40 on B cells, via CD40L from T cells, increases cell surface expression of B cell lymphotoxin. Then, lymphotoxin expressing B cells interact with LTβR<sup>+</sup> DCs. These DCs then in turn maintain the FRC network that supports the extrafollicular foci required for class switched IgG responses. One possible way to examine this hypothesis is to utilize available conditionally deficient *Ltbr*<sup>fl/fl</sup> mice crossed to *Cd11c*<sup>Cre</sup>. If this hypothesis is true, it would predict that *Cd11c*<sup>Cre</sup> *Ltbr*<sup>fl/fl</sup> mice will have an impairment in generation of anti-*Salmonella* IgG. Kumar et al. also found that

addition of BAFF and IL-7 rescues FRC survival and increases the number of AFCs following immunization with OVA-alum. Most importantly, addition of agonist anti-LT $\beta$ R has a similar effect (Kumar et al. 2015). Our study shows that transient blockade of LT $\beta$ R reduced anti-*Salmonella* IgG. It is possible that addition of agonist anti-LT $\beta$ R, IL-7, or BAFF to *Ltb*<sup>-/-</sup> could rescue anti-*Salmonella* IgG.

Generation of class switched affinity matured antibody in the absence of germinal centers has been described in LT deficient strains when immunized with high dose of antigen, adjuvanted antigen, or with repeated antigen dosage (Matsumoto et al. 1996; Wang et al. 2000)(**Table 1**). How does the extrafollicular response in *Salmonella* differ from extrafollicular class switching observed to model antigens in lymphotoxin deficient strains? Based on the previous literature with model antigens, *Salmonella* infection meets all of the theoretical requirements to induce some level of high affinity IgG: antigen is persistent and long lasting and provides “adjuvanted” signal one and two. However, our data indicate that despite antigen persistence and no strict requirement for GCs, anti-*Salmonella* antibody responses require LT $\alpha$ 1 $\beta$ 2 production by B cells.

One possible explanation is that *Salmonella* actively suppresses GC formation and that LT-mediated extrafollicular responses are the only remaining avenue for the generation of somatically hypermutated, high affinity antibody in WT mice. There is evidence to support the claim the *Salmonella* actively suppresses GC formation; the appearance of IgG2c correlates with clearance of attenuated *Salmonella* from the spleen and treatment of infected mice with antibiotics reduced the burden of bacteria in the spleen and enhances GC-like formation (Cunningham et al. 2007). An interesting line of experimentation would be to determine what, if any, bacterial virulence factors

are involved. The previous two publications as well as our study, use the *aroA* mutant (Cunningham et al. 2007; Di Niro et al. 2015). While this strain is attenuated it retains both the Spi1 and Spi2 Type 3 secretion systems, machinery with which *Salmonella* injects a wide variety of effector molecules to alter host responses. The requirement of these secretion systems in anti-*Salmonella* GC formation is worthy of further exploration. Another fruitful line of study would be to determine if a reduction in *Salmonella* load in lymphotoxin deficient strains rescues IgG production.

### *Protective Immunity to Salmonella*

A live attenuated *Salmonella* formulation is one of two current FDA approved vaccines for typhoid. Like the parenteral vaccine, the oral formulation has relatively low efficacy rates despite raising both humoral and cellular anti-*Salmonella* responses in vaccinated individuals (Date et al. 2015). Furthermore, this formulation's side effects include cramping and mild diarrhea and it is not recommended to children under 2 years of age. Therefore, understanding the restrictions and limitations of live attenuated vaccines is an important step in improving efficacy. In this study we demonstrated that in the absence of LT $\beta$ R or LT $\beta$  animals do not acquire protective immunity to *Salmonella* following vaccination with an attenuated strain and under circumstances of reduced colonization resistance, become persistently infected. We also show that B cell derived lymphotoxin plays a significant role in the generation of this protection.

Many potential causes for impaired immunity were explored in this study. First, because *Ltbr*<sup>-/-</sup> mice retained high and persistent levels of *Salmonella* in the gut, and persistent infections are known to cause exhaustion and tolerance (Monack, Mueller, and Falkow 2004; Zuniga et al. 2015), we determined if gut persistence was a cause of

poor immunity. We were able to utilize the attenuation of our vaccine strain to our advantage. Because this strain has impaired intracellular replication it cannot freely proliferate outside of the gut lumen. By administering the vaccine strain i.p., we prevent persistent infection, as no bacteria were detected in host feces. We found that the role of lymphotoxin in protective immunity is independent of *Salmonella* persistence; while *Ltbr*<sup>+/-</sup> mice were fully protected by i.p. vaccination, *Ltbr*<sup>-/-</sup> mice all died when challenged with *Salmonella* (also via the i.p. route).

Impaired anti-*Salmonella* IgG responses were noted in this study. Although antibody is not strictly required to mediate protection and gut clearance it contributes to limiting infection. Mice that retain B cells but cannot secrete antibody have a minor (20%) impairment in survival to virulent *Salmonella* after vaccination with attenuated *Salmonella*. Animals that can produce only IgM do not have any vaccination and challenge defects (Nanton et al. 2012). However, it should be noted that in these experiments both attenuated and virulent *Salmonella* was administered intravenously. These studies do not take into account the role of antibody at mucosal surface. Antibody, especially IgA, limits infection at the mucosa (Endt et al. 2010). Therefore at least a portion of the protective role of lymphotoxin could be through antibody production. One way to determine how much of a role antibody plays in protection would be to supplement *Ltbr*<sup>-/-</sup> or *Ltb*<sup>-/-</sup> mice with serum transferred from previously immunized WT or lymphotoxin deficient mice.

Antibody independent functions of B cells have also been shown to be essential to anti-*Salmonella* protective immunity. Mirroring our results with *Cd19*<sup>cre+</sup> *Ltb*<sup>fl/fl</sup> mice, B cell deficient animals, but not antibody deficient animals, succumb to virulent challenge

with *Salmonella* despite previous vaccination with attenuated *Salmonella* (Nanton et al. 2012) and the role of B cells as APCs has been shown to play a role in the generation of anti-*Salmonella* CD4 T cell responses (Barr et al. 2010).

Lymphotoxin is known to alter B cell biology and function to impact T cell responses (León et al. 2012). LT is expressed on B cells and transmits signal through LT $\beta$ R on non-lymphoid cells. Therefore, the impact of LT on B cells cannot be by direct contact with T cells (which also express LT but not LT $\beta$ R). This suggests that the impact of B cell LT on CD4 immunity acts via third party LT $\beta$ R<sup>+</sup> cells. In chronic helminth infection, LT from B cells is essential for the expression of CXCL13 by stromal cells and the recruitment of T cells into the B cells zone (León et al. 2012). In the absence of this pathway, Th2 responses are impaired (León et al. 2012). Although this mechanism did not appear to contribute to anti-*influenza* Th1 responses, it is unknown if a similar mechanism is in play during *Salmonella* infection.

B cells' role as antigen presenting cells has been demonstrated as a key driver in optimal Th1 memory formation to *Salmonella* (Barr et al. 2010). One possible explanation for the results observed in our study is that LT on B cells is required to orchestrate the special relationships of T cells and B cells within lymphoid tissue. If T cells and B cells cannot colocalize, B cells cannot act as APC. As discussed previously B cell derived LT promotes the survival of the FCR network, a scaffold upon which cells interact. This is another possible mechanism through which B cells could promote T cell responses.

Our study also explored the impact of *Ltbr* deficiency on cytokine and T cell responses to *Salmonella*. Lacking a TCR transgenic cell system, this study is limited to

observations in the polyclonal T cell response. We noted that long lasting *Salmonella* infection leads to an impairment in splenocyte production of IFN $\gamma$  in response to *in vitro* restimulation with *Salmonella* antigen, and an enhancement in TNF $\alpha$  production. Blocking IFN $\gamma$  in the late phase of infection with attenuated *Salmonella* did not impact IgG production or fecal clearance but did reduce survival to virulent challenge, even when blocking of this cytokine did not continue during the challenge phase. This finding is consistent with the essential role of IFN $\gamma$  in *Salmonella* protective responses (Nauciel and Espinasse-Maes 1992; Bao et al. 2000).

When we sought to determine if this altered cytokine profile could be attributed to T cells, we generated intriguing results. *In vivo* restimulation with heat killed *Salmonella* in *Ltbr*<sup>-/-</sup> mice generated robust IFN $\gamma$  CD4 T cell responses. These results appeared in direct contradiction with *in vitro* findings in which T cells from *Ltbr*<sup>-/-</sup> mice had impaired proliferation or in which splenocytes from these mice had impaired IFN $\gamma$  production. However, when analysis was performed later following *in vivo* stimulation (at 24 hours instead of 4 hours post stimulation) *Ltbr*<sup>-/-</sup> mice no longer had robust IFN $\gamma$  T cell responses, although differences between *Ltbr*<sup>-/-</sup> and *Ltbr*<sup>+/-</sup> mice did not reach statistical significance. It was difficult to resolve these findings as many technical and biological explanations of these data are possible. The source of antigen restimulation (heat killed *Salmonella* versus *Salmonella* protein extract) could be one reason for the disparate results. Heat killed *Salmonella* does not have the same impact on T cell responses as live *Salmonella*. Live, but not heat killed *Salmonella* can reduce *in vivo* antigen specific T cell numbers by inducing apoptosis in a Spi-2 dependent mechanism (Srinivasan et al. 2009). Could persistent infection or lack of lymphoid organization enhance

*Salmonella*-virulent factor driven T cell killing in *Ltbr*<sup>-/-</sup> mice? The effect of *Salmonella* on T cell culling was reported to be most pronounced in antigen specific T cell populations (Srinivasan et al. 2009). Therefore, a useful tool for understanding the impact of lymphotoxin on T cell biology would be the SM1 TCR transgenic cells, specific for *Salmonella* flagellin (Ravindran and McSorley 2005). SM1 T cells could be transferred into *Ltbr*<sup>-/-</sup> mice prior to infection with attenuated *Salmonella* and the number and phenotype of these cells could be tracked.

A further potential interesting, and as of yet unexplored biological mechanism, could be the impact of LTβR on T cell effector, central memory vs effector memory fate decisions. As with B cells, T cells themselves do not express the signal transducing LTβR, but instead express lymphotoxin. Therefore, any impact of LTβR signal transduction on T cells occur through a third party LTβR<sup>+</sup> cell. Signaling of LTβR on DCs can upregulate expression of stimulatory molecules on DCs cells such as OX40L, 4-1BBL, and CD70 (Croft 2014). These stimulatory molecules can in turn contribute to T cell fate decision. For examples, mice lacking OX40 have a selective impairment in CD4 effector memory generation (Soroosh et al. 2007). Another important determinant in the generation of T cell memory responses is removal of antigenic stimulation, as contraction of effector cells does not occur until antigenic load is reduced (Gasper, Tejera, and Suresh 2014). Shorter duration of antigen exposure preferentially leads to generation of central memory while increased duration leads to effector memory responses (Moulton et al. 2006). Indeed the appearance of CD62L<sup>-</sup>CD44<sup>+</sup> effector cells coincides with clearance of *Salmonella* from systemic tissues (Johanns et al. 2010). Tracing of memory versus effector lineages in *Salmonella* infected lymphotoxin deficient

mice could provide evidence of an impact of this pathway on protective responses. Either in a polyclonal setting or using SM1 T cell transgenic system (Ravindran and McSorley 2005) the number of central memory (CD44<sup>+</sup>CD62L<sup>+</sup>CD25<sup>-</sup>CCR7<sup>+</sup>), activated effector (CD44<sup>+</sup>CD62L<sup>-</sup>CCR7<sup>-</sup>CD25<sup>-</sup>CD69<sup>+</sup>) versus effector memory (CD44<sup>+</sup>CD62L<sup>-</sup>CD25<sup>-</sup>CCR7<sup>-</sup>) subpopulations in *Ltbr*<sup>-/-</sup> versus *Ltbr*<sup>+/-</sup> mice could be tracked.

The role of LTβR in organizing lymphoid tissue architecture and the impact of this organization on the phenotype and functions of T cells could also be addressed. LT's role in lymphoid tissue architecture has been shown to impact CD4 and CD8 T cell IFNγ effector function in LCMV and *Leishmania* infections (Suresh et al. 2002; Xu et al. 2007; Wilhelm et al. 2002). In these studies when T cells or bone marrow from lymphotoxin deficient mice were transferred to lymphotoxin sufficient hosts, T cell IFNγ effector function were rescued (Suresh et al. 2002; Xu et al. 2007; Wilhelm et al. 2002). Similarly, T cells derived from *Salmonella* infected *Ltbr*<sup>-/-</sup> mice could be transferred into WT hosts and their response to *Salmonella* infection could be reassessed in an LTβR sufficient setting. This approach could assess the importance of lymphoid tissue architecture on anti-*Salmonella* T cell responses.

Our study also generated preliminary data exploring potential mechanisms of tolerance and suppression. We noted an *in vitro* reduction in the ability of CD4 and CD8 T cells to proliferate in response to *Salmonella* antigen as well as αCD3/CD28 stimulation. This suggested a broad mechanism of T cell suppression. Our initial observation was made in mixed splenocyte cultures and therefore many potential mechanisms of suppression could explain our finding. The first potential mechanism explored was a well-known role for suppressive granulocytic cells during *Salmonella*

infection (Tam et al. 2014). Following infection these immature monocytes accumulate in the spleen, and in *in vitro* cultures are capable of suppressing both antigen specific and  $\alpha$ CD3/CD28 driven CD4 and CD8 T cell proliferation (Tam et al. 2014). Indeed, we noted an accumulation of granulocytic cells in *Ltbr*<sup>-/-</sup> mice, but when these cells were sorted and added back to lymphocyte cultures they did not impact T cell responses. It is possible that we did not sort the correct population of cells, or that better results would be obtained if granulocytes were added to purified T cells. It is also possible that although we did not see an impact of these cells *in vitro*, they mediate *in vivo* effects.

Our final preliminary experiment to address potential T cell suppression in *Ltbr*<sup>-/-</sup> mice noted that purified T cells sorted from spleens of *Salmonella* infected mice had a significant impairment in  $\alpha$ CD3/CD28 driven proliferation. Proliferation of CD4 cells from naïve *Ltbr*<sup>-/-</sup> mice was not measured so it is not known if this experiment represents a *Salmonella* driven phenotype. If, however, this assumption is true, it suggests that the observed T cell suppression is CD4 T cell intrinsic. One intriguing possibility is that T regulatory (Treg) cells accumulate in *Ltbr*<sup>-/-</sup> mice or acquire a more suppressive phenotype. Treg numbers mirror increases in total CD4 expansion following *Salmonella* infection so that the percentage of these cells relative to Foxp3<sup>+</sup> CD4s does not change. However Tregs of persistently infected mice (day 37) are more suppressive, inhibiting  $\alpha$ CD3/CD28 driven proliferation at lower Treg to target ratios than those of naïve animals (Johanns et al. 2010). Deletion of Tregs at early stages following *Salmonella* infection (D5) led to a reduction in bacterial splenic CFU and an enhancement of activated effector cells, but deletion of Tregs from persistently infected (D37) mice did not impact CD4 numbers or *Salmonella* CFU (Johanns et al. 2010). This suggests that

the impact of Tregs has a more significant role early in the T cell response. It is unknown however, if this is also true in a lymphotoxin deficient setting. It is possible that the increased persistence of *Salmonella* in *Ltbr*<sup>-/-</sup> mice could impact Treg numbers and function. This is indeed a possibility as super-shedder mice on the SvJ background have higher levels of Tregs than gut uncolonized mice (Gopinath et al. 2013). The number and suppressive capabilities of Tregs in *Salmonella* infected *Ltbr*<sup>-/-</sup> mice could be analyzed and their suppressive capacity in vitro addressed.

### *Concluding remarks*

Overall, the work presented in this thesis opens many new avenues to explore immune responses to *Salmonella* and other persistent infections as well as the role of the lymphotoxin pathway in infectious disease and vaccination. Although our study may ask more questions than it answers, it also presents viable models and experimental approaches to understanding previously unexplored host pathogen interactions. First, it demonstrates that host immunity plays a vital role in microbiota clearance of *Salmonella* from the gut. Second, it suggests that the lymphotoxin pathway, especially from B cells, could be an integral player in the relatively unexplored field of extrafollicular antibody responses. Finally, we highlight the importance of the lymphotoxin pathway in mediating protective immune responses to oral vaccination with live attenuated bacteria. Development of such vaccines is ongoing, and understanding the host requirements for appropriate responses is essential to ensure both vaccine safety and efficacy.

## References

Adachi, S., H. Yoshida, H. Kataoka, and S. Nishikawa. 1997. "Three Distinctive Steps in Peyer's Patch Formation of Murine Embryo." *International Immunology* 9 (4): 507–14.

Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovsky, K. Rajewsky, S. A. Nedospasov, and K. Pfeffer. 1997. "Abnormal Development of Secondary Lymphoid Tissues in Lymphotoxin Beta-Deficient Mice." *Proceedings of the National Academy of Sciences of the United States of America* 94 (17): 9302–7.

"Aminoglycosides." 2016. *Merck Manuals Professional Edition*. Accessed April 8. <http://www.merckmanuals.com/professional/infectious-diseases/bacteria-and-antibacterial-drugs/aminoglycosides?qt=&sc=&alt=>.

Arpaia, Nicholas, Jernej Godec, Laura Lau, Kelsey E. Sivick, Laura M. McLaughlin, Marcus B. Jones, Tatiana Dracheva, Scott N. Peterson, Denise M. Monack, and Gregory M. Barton. 2011. "TLR Signaling Is Required for Virulence of an Intracellular Pathogen." *Cell* 144 (5): 675–88. doi:10.1016/j.cell.2011.01.031.

Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. 1995. "Lymphotoxin-Alpha-Deficient Mice. Effects on Secondary Lymphoid Organ Development and Humoral Immune Responsiveness." *The Journal of Immunology* 155 (4): 1685–93.

Bao, S., K. W. Beagley, M. P. France, J. Shen, and A. J. Husband. 2000. "Interferon- $\gamma$  Plays a Critical Role in Intestinal Immunity against *Salmonella* Typhimurium Infection." *Immunology* 99 (3): 464–72. doi:10.1046/j.1365-2567.2000.00955.x.

Barr, T. A., S. Brown, P. Mastroeni, and D. Gray. 2010. "TLR and B Cell Receptor Signals to B Cells Differentially Program Primary and Memory Th1 Responses to *Salmonella* Enterica." *The Journal of Immunology* 185 (5): 2783–89. doi:10.4049/jimmunol.1001431.

Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W.-D. Hardt. 2003. "Pretreatment of Mice with Streptomycin Provides a *Salmonella* Enterica Serovar Typhimurium Colitis Model That Allows Analysis of Both Pathogen and Host." *Infection and Immunity* 71 (5): 2839–58. doi:10.1128/IAI.71.5.2839-2858.2003.

Bäumler, A J, R M Tsohis, and F Heffron. 1996. "The Lpf Fimbrial Operon Mediates Adhesion of *Salmonella* Typhimurium to Murine Peyer's Patches." *Proceedings of the National Academy of Sciences of the United States of America* 93 (1): 279–83.

Bäumler, Andreas, and Ferric C. Fang. 2013. "Host Specificity of Bacterial Pathogens." *Cold Spring Harbor Perspectives in Medicine* 3 (12): a010041. doi:10.1101/cshperspect.a010041.

Behnsen, Judith, Stefan Jellbauer, Christina P. Wong, Robert A. Edwards, Michael D. George, Wenjun Ouyang, and Manuela Raffatellu. 2014. "The Cytokine IL-22 Promotes Pathogen Colonization by Suppressing Related Commensal Bacteria." *Immunity* 40 (2): 262–73. doi:10.1016/j.immuni.2014.01.003.

Bénézech, Cécile, Andrea White, Emma Mader, Karine Serre, Sonia Parnell, Klaus Pfeffer, Carl F. Ware, Graham Anderson, and Jorge H. Caamaño. 2010. "Ontogeny of Stromal Organizer Cells during Lymph Node Development." *Journal of Immunology (Baltimore, Md.: 1950)* 184 (8): 4521–30. doi:10.4049/jimmunol.0903113.

Berger, Thorsten, Atsushi Togawa, Gordon S. Duncan, Andrew J. Elia, Annick You-Ten, Andrew Wakeham, Hannah E. H. Fong, Carol C. Cheung, and Tak W. Mak. 2006. "Lipocalin 2-Deficient Mice Exhibit Increased Sensitivity to Escherichia Coli Infection but Not to Ischemia-Reperfusion Injury." *Proceedings of the National Academy of Sciences of the United States of America* 103 (6): 1834–39. doi:10.1073/pnas.0510847103.

Boehm, Thomas, Isabell Hess, and Jeremy B. Swann. 2012. "Evolution of Lymphoid Tissues." *Trends in Immunology*, Special focus: Structure and function of lymphoid tissues, 33 (6): 315–21. doi:10.1016/j.it.2012.02.005.

Browning, Jeffrey L., Norm Allaire, Apinya Ngam-Ek, Evangelia Notidis, Jane Hunt, Steven Perrin, and Roy A. Fava. 2005. "Lymphotoxin-Beta Receptor Signaling Is Required for the Homeostatic Control of HEV Differentiation and Function." *Immunity* 23 (5): 539–50. doi:10.1016/j.immuni.2005.10.002.

Browning, J. L., M. J. Androlewicz, and C. F. Ware. 1991. "Lymphotoxin and an Associated 33-kDa Glycoprotein Are Expressed on the Surface of an Activated Human T Cell Hybridoma." *Journal of Immunology (Baltimore, Md.: 1950)* 147 (4): 1230–37.

Browning, J. L., I. Douglas, A. Ngam-ek, P. R. Bourdon, B. N. Ehrenfels, K. Miatkowski, M. Zafari, A. M. Yampaglia, P. Lawton, and W. Meier. 1995. "Characterization of Surface Lymphotoxin Forms. Use of Specific Monoclonal Antibodies and Soluble Receptors." *Journal of Immunology (Baltimore, Md.: 1950)* 154 (1): 33–46.

Browning, J. L., A. Ngam-ek, P. Lawton, J. DeMarinis, R. Tizard, E. P. Chow, C. Hession, B. O'Brine-Greco, S. F. Foley, and C. F. Ware. 1993. "Lymphotoxin Beta, a Novel Member of the TNF Family That Forms a Heteromeric Complex with Lymphotoxin on the Cell Surface." *Cell* 72 (6): 847–56.

Broz, Petr, Maikke B. Ohlson, and Denise M. Monack. 2012. "Innate Immune Response to Salmonella Typhimurium, a Model Enteric Pathogen." *Gut Microbes* 3 (2): 62–70. doi:10.4161/gmic.19141.

Cardenas, Lucia, and J. D. Clements. 1992. "Oral Immunization Using Live Attenuated Salmonella Spp. as Carriers of Foreign Antigens." *Clinical Microbiology Reviews* 5 (3): 328–42.

Cash, Heather L., Cecilia V. Whitham, Cassie L. Behrendt, and Lora V. Hooper. 2006. "Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin." *Science (New York, N.Y.)* 313 (5790): 1126–30. doi:10.1126/science.1127119.

Cheminay, Cédric, Dipshikha Chakravorty, and Michael Hensel. 2004. "Role of Neutrophils in Murine Salmonellosis." *Infection and Immunity* 72 (1): 468–77. doi:10.1128/IAI.72.1.468-477.2004.

Clark, M. A., B. H. Hirst, and M. A. Jepson. 1998. "Inoculum Composition and Salmonella Pathogenicity Island 1 Regulate M-Cell Invasion and Epithelial Destruction by Salmonella Typhimurium." *Infection and Immunity* 66 (2): 724–31.

Clark, M. A., M. A. Jepson, N. L. Simmons, and B. H. Hirst. 1994. "Preferential Interaction of Salmonella Typhimurium with Mouse Peyer's Patch M Cells." *Research in Microbiology* 145 (7): 543–52. doi:10.1016/0923-2508(94)90031-0.

Crawford, Robert W., Roberto Rosales-Reyes, María de la Luz Ramírez-Aguilar, Oscar Chapa-Azuela, Celia Alpuche-Aranda, and John S. Gunn. 2010. "Gallstones Play a Significant Role in Salmonella Spp. Gallbladder Colonization and Carriage." *Proceedings of the National Academy of Sciences of the United States of America* 107 (9): 4353–58. doi:10.1073/pnas.1000862107.

Croft, Michael. 2014. "The TNF Family in T Cell Differentiation and Function-- Unanswered Questions and Future Directions." *Seminars in Immunology* 26 (3): 183–90. doi:10.1016/j.smim.2014.02.005.

Crowe, P. D., T. L. VanArsdale, B. N. Walter, C. F. Ware, C. Hession, B. Ehrenfels, J. L. Browning, W. S. Din, R. G. Goodwin, and C. A. Smith. 1994. "A Lymphotoxin-Beta-Specific Receptor." *Science* 264 (5159): 707–10. doi:10.1126/science.8171323.

Cunningham, Adam F., Fabrina Gaspal, Karine Serre, Elodie Mohr, Ian R. Henderson, Anthony Scott-Tucker, Sinead M. Kenny, et al. 2007. "Salmonella Induces a Switched Antibody Response without Germinal Centers That Impedes the Extracellular Spread of Infection." *The Journal of Immunology* 178 (10): 6200–6207. doi:10.4049/jimmunol.178.10.6200.

Dabrock, B., H. Bahl, and G. Gottschalk. 1992. "Parameters Affecting Solvent Production by Clostridium Pasteurianum." *Applied and Environmental Microbiology* 58 (4): 1233–39.

Date, Kashmira A., Adwoa Bentsi-Enchill, Florian Marks, and Kimberley Fox. 2015. "Typhoid Fever Vaccination Strategies." *Vaccine, Global Progress on use of Vaccines for Invasive Salmonella Infections*, 33, Supplement 3 (June): C55–61. doi:10.1016/j.vaccine.2015.04.028.

Dejardin, Emmanuel, Nathalie M. Droin, Mireille Delhase, Elvira Haas, Yixue Cao, Constantin Makris, Zhi-Wei Li, Michael Karin, Carl F. Ware, and Douglas R. Green.

2002. "The Lymphotoxin-Beta Receptor Induces Different Patterns of Gene Expression via Two NF-kappaB Pathways." *Immunity* 17 (4): 525–35.

De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shornick, and J. Strauss-Schoenberger. 1994. "Abnormal Development of Peripheral Lymphoid Organs in Mice Deficient in Lymphotoxin." *Science (New York, N.Y.)* 264 (5159): 703–7.

Diener, E., and E. H. M. Ealey. 1965. "Immune System in a Monotreme: Studies on the Australian Echidna (*Tachyglossus Aculeatus*)." *Nature* 208 (5014): 950–53. doi:10.1038/208950a0.

Di Niro, Roberto, Seung-Joo Lee, Jason A. Vander Heiden, Rebecca A. Elsner, Nikita Trivedi, Jason M. Bannock, Namita T. Gupta, et al. 2015. "Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation." *Immunity* 43 (1): 120–31. doi:10.1016/j.immuni.2015.06.013.

Edelblum, Karen L., Gurminder Singh, Matthew A. Odenwald, Amulya Lingaraju, Kamal El Bissati, Rima McLeod, Anne I. Sperling, and Jerrold R. Turner. 2015. "γδ Intraepithelial Lymphocyte Migration Limits Transepithelial Pathogen Invasion and Systemic Disease in Mice." *Gastroenterology* 148 (7): 1417–26. doi:10.1053/j.gastro.2015.02.053.

Ehrchen, J. M., J. Roth, K. Roebrock, G. Varga, W. Domschke, R. Newberry, C. Sorg, et al. 2008. "The Absence of Cutaneous Lymph Nodes Results in a Th2 Response and Increased Susceptibility to *Leishmania Major* Infection in Mice." *Infection and Immunity* 76 (9): 4241–50. doi:10.1128/IAI.01714-07.

Endt, Kathrin, Bärbel Stecher, Samuel Chaffron, Emma Slack, Nicolas Tchitchek, Arndt Benecke, Laurye Van Maele, et al. 2010. "The Microbiota Mediates Pathogen Clearance from the Gut Lumen after Non-Typhoidal *Salmonella* Diarrhea." Edited by C. Erec Stebbins. *PLoS Pathogens* 6 (9): e1001097. doi:10.1371/journal.ppat.1001097.

Erickson, S. L., F. J. de Sauvage, K. Kikly, K. Carver-Moore, S. Pitts-Meek, N. Gillett, K. C. Sheehan, R. D. Schreiber, D. V. Goeddel, and M. W. Moore. 1994. "Decreased Sensitivity to Tumour-Necrosis Factor but Normal T-Cell Development in TNF Receptor-2-Deficient Mice." *Nature* 372 (6506): 560–63. doi:10.1038/372560a0.

Ettinger, R., J. L. Browning, S. A. Michie, W. van Ewijk, and H. O. McDevitt. 1996. "Disrupted Splenic Architecture, but Normal Lymph Node Development in Mice Expressing a Soluble Lymphotoxin-Beta Receptor-IgG1 Fusion Protein." *Proceedings of the National Academy of Sciences of the United States of America* 93 (23): 13102–7.

Feasey, Nicholas A., Gordon Dougan, Robert A. Kingsley, Robert S. Heyderman, and Melita A. Gordon. 2012. "Invasive Non-Typhoidal *Salmonella* Disease: An Emerging and Neglected Tropical Disease in Africa." *Lancet (London, England)* 379 (9835): 2489–99. doi:10.1016/S0140-6736(11)61752-2.

Fischbach, Michael A., and Justin L. Sonnenburg. 2011. "Eating For Two: How Metabolism Establishes Interspecies Interactions in the Gut." *Cell Host & Microbe* 10 (4): 336–47. doi:10.1016/j.chom.2011.10.002.

Flo, Trude H., Kelly D. Smith, Shintaro Sato, David J. Rodriguez, Margaret A. Holmes, Roland K. Strong, Shizuo Akira, and Alan Aderem. 2004. "Lipocalin 2 Mediates an Innate Immune Response to Bacterial Infection by Sequestering Iron." *Nature* 432 (7019): 917–21. doi:10.1038/nature03104.

Fütterer, Agnes, Karin Mink, Arne Luz, Marie H. Kosco-Vilbois, and Klaus Pfeffer. 1998. "The Lymphotoxin  $\beta$  Receptor Controls Organogenesis and Affinity Maturation in Peripheral Lymphoid Tissues." *Immunity* 9 (1): 59–70. doi:10.1016/S1074-7613(00)80588-9.

Fütterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer. 1998. "The Lymphotoxin Beta Receptor Controls Organogenesis and Affinity Maturation in Peripheral Lymphoid Tissues." *Immunity* 9 (1): 59–70.

Fu, Yang-Xin, and David D. Chaplin. 1999. "Development and Maturation of Secondary Lymphoid Tissues." *Annual Review of Immunology* 17 (1): 399–433. doi:10.1146/annurev.immunol.17.1.399.

Fu, Y. X., G. Huang, M. Matsumoto, H. Molina, and D. D. Chaplin. 1997. "Independent Signals Regulate Development of Primary and Secondary Follicle Structure in Spleen and Mesenteric Lymph Node." *Proceedings of the National Academy of Sciences of the United States of America* 94 (11): 5739–43.

Fu, Y. X., G. Huang, Y. Wang, and D. D. Chaplin. 1998. "B Lymphocytes Induce the Formation of Follicular Dendritic Cell Clusters in a Lymphotoxin Alpha-Dependent Fashion." *The Journal of Experimental Medicine* 187 (7): 1009–18.

Fu, Y. X., H. Molina, M. Matsumoto, G. Huang, J. Min, and D. D. Chaplin. 1997a. "Lymphotoxin-Alpha (LTalpha) Supports Development of Splenic Follicular Structure That Is Required for IgG Responses." *The Journal of Experimental Medicine* 185 (12): 2111–20.

García De Vinuesa, C., A. Gulbranson-Judge, M. Khan, P. O'Leary, M. Cascalho, M. Wabl, G. G. Klaus, M. J. Owen, and I. C. MacLennan. 1999. "Dendritic Cells Associated with Plasmablast Survival." *European Journal of Immunology* 29 (11): 3712–21. doi:10.1002/(SICI)1521-4141(199911)29:11<3712::AID-IMMU3712>3.0.CO;2-P.

Gasper, David J., Melba Marie Tejera, and M. Suresh. 2014. "CD4 T-Cell Memory Generation and Maintenance." *Critical Reviews in Immunology* 34 (2): 121–46.

Gilchrist, James J., Calman A. MacLennan, and Adrian V. S. Hill. 2015. "Genetic Susceptibility to Invasive Salmonella Disease." *Nature Reviews Immunology* 15 (7): 452–63. doi:10.1038/nri3858.

Godinez, Ivan, Manuela Raffatellu, Hiutung Chu, Tatiane A. Paixão, Takeshi Haneda, Renato L. Santos, Charles L. Bevins, Renée M. Tsois, and Andreas J. Bäumlner. 2009. "Interleukin-23 Orchestrates Mucosal Responses to Salmonella Enterica Serotype Typhimurium in the Intestine." *Infection and Immunity* 77 (1): 387–98. doi:10.1128/IAI.00933-08.

Gonzalez-Escobedo, Geoffrey, Joanna M. Marshall, and John S. Gunn. 2011. "Chronic and Acute Infection of the Gall Bladder by Salmonella Typhi: Understanding the Carrier State." *Nature Reviews. Microbiology* 9 (1): 9–14. doi:10.1038/nrmicro2490.

Gopinath, Smita, Andrew Hotson, Jennifer Johns, Garry Nolan, and Denise Monack. 2013. "The Systemic Immune State of Super-Shedder Mice Is Characterized by a Unique Neutrophil-Dependent Blunting of T H 1 Responses." *PLOS Pathog* 9 (6): e1003408. doi:10.1371/journal.ppat.1003408.

Gunn, John S., Joanna M. Marshall, Stephen Baker, Sabina Dongol, Richelle C. Charles, and Edward T. Ryan. 2014. "Salmonella Chronic Carriage: Epidemiology, Diagnosis, and Gallbladder Persistence." *Trends in Microbiology* 22 (11): 648–55. doi:10.1016/j.tim.2014.06.007.

Hansen-Wester, Imke, and Michael Hensel. 2001. "Salmonella Pathogenicity Islands Encoding Type III Secretion Systems." *Microbes and Infection* 3 (7): 549–59. doi:10.1016/S1286-4579(01)01411-3.

Hapfelmeier, Siegfried, Kristin Ehrbar, Bärbel Stecher, Manja Barthel, Marcus Kremer, and Wolf-Dietrich Hardt. 2004. "Role of the Salmonella Pathogenicity Island 1 Effector Proteins SipA, SopB, SopE, and SopE2 in Salmonella Enterica Subspecies 1 Serovar Typhimurium Colitis in Streptomycin-Pretreated Mice." *Infection and Immunity* 72 (2): 795–809.

Hapfelmeier, S., B. Stecher, M. Barthel, M. Kremer, A. J. Muller, M. Heikenwalder, T. Stallmach, et al. 2005. "The Salmonella Pathogenicity Island (SPI)-2 and SPI-1 Type III Secretion Systems Allow Salmonella Serovar Typhimurium to Trigger Colitis via MyD88-Dependent and MyD88-Independent Mechanisms." *The Journal of Immunology* 174 (3): 1675–85. doi:10.4049/jimmunol.174.3.1675.

Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galán. 1998. "S. Typhimurium Encodes an Activator of Rho GTPases That Induces Membrane Ruffling and Nuclear Responses in Host Cells." *Cell* 93 (5): 815–26.

Hashizume, Tomomi, Fumiki Momoi, Tomoko Kurita-Ochiai, Shuichi Kaminogawa, Akira Hosono, Kosuke Kataoka, Noriko Shinozaki-Kuwahara, Mi-Na Kweon, and Masafumi Yamamoto. 2007. "Isolated Lymphoid Follicles Are Not IgA Inductive Sites for Recombinant Salmonella." *Biochemical and Biophysical Research Communications* 360 (2): 388–93. doi:10.1016/j.bbrc.2007.06.096.

Hashizume, T., A. Togawa, T. Nochi, O. Igarashi, M.-N. Kweon, H. Kiyono, and M. Yamamoto. 2008. "Peyer's Patches Are Required for Intestinal Immunoglobulin A

Responses to Salmonella Spp.” *Infection and Immunity* 76 (3): 927–34.  
doi:10.1128/IAI.01145-07.

Havelaar, Arie H., Martyn D. Kirk, Paul R. Torgerson, Herman J. Gibb, Tine Hald, Robin J. Lake, Nicolas Praet, et al. 2015. “World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010.” *PLOS Med* 12 (12): e1001923. doi:10.1371/journal.pmed.1001923.

Hess, Jurgen, Christoph Ladel, Diana Miko, and S. H. Kaufmann. 1996. “Salmonella Typhimurium aroA-Infection in Gene-Targeted Immunodeficient Mice: Major Role of CD4+ TCR-Alpha Beta Cells and IFN-Gamma in Bacterial Clearance Independent of Intracellular Location.” *The Journal of Immunology* 156 (9): 3321–26.

Hormaeche, C. E., H. S. Joysey, L. Desilva, M. Izhar, and B. A. Stocker. 1990. “Immunity Induced by Live Attenuated Salmonella Vaccines.” *Research in Microbiology* 141 (7-8): 757–64.

Inoko, H, and J Trowsdale. 1987. “Linkage of TNF Genes to the HLA-B Locus.” *Nucleic Acids Research* 15 (21): 8957–62.

Jepson, M. A., C. B. Collares-Buzato, M. A. Clark, B. H. Hirst, and N. L. Simmons. 1995. “Rapid Disruption of Epithelial Barrier Function by Salmonella Typhimurium Is Associated with Structural Modification of Intercellular Junctions.” *Infection and Immunity* 63 (1): 356–59.

J M Macy, and I. Probst. 1979. “The Biology of Gastrointestinal Bacteroides.” *Annual Review of Microbiology* 33 (1): 561–94.  
doi:10.1146/annurev.mi.33.100179.003021.

Johanns, Tanner M., James M. Ertelt, Jared H. Rowe, and Sing Sing Way. 2010. “Regulatory T Cell Suppressive Potency Dictates the Balance between Bacterial Proliferation and Clearance during Persistent Salmonella Infection.” Edited by Denise M. Monack. *PLoS Pathogens* 6 (8): e1001043. doi:10.1371/journal.ppat.1001043.

Jones, B. D., N. Ghori, and S. Falkow. 1994. “Salmonella Typhimurium Initiates Murine Infection by Penetrating and Destroying the Specialized Epithelial M Cells of the Peyer’s Patches.” *The Journal of Experimental Medicine* 180 (1): 15–23.

Kaiser, Patrick, Médéric Diard, Bärbel Stecher, and Wolf-Dietrich Hardt. 2012. “The Streptomycin Mouse Model for Salmonella Diarrhea: Functional Analysis of the Microbiota, the Pathogen’s Virulence Factors, and the Host’s Mucosal Immune Response.” *Immunological Reviews* 245 (1): 56–83. doi:10.1111/j.1600-065X.2011.01070.x.

Kaiser, Patrick, Roland R. Regoes, Tamas Dolowschiak, Sandra Y. Wotzka, Jette Lengefeld, Emma Slack, Andrew J. Grant, Martin Ackermann, and Wolf-Dietrich Hardt. 2014. “Cecum Lymph Node Dendritic Cells Harbor Slow-Growing Bacteria

Phenotypically Tolerant to Antibiotic Treatment.” *PLoS Biology* 12 (2): e1001793. doi:10.1371/journal.pbio.1001793.

Kariuki, Samuel, Melita A. Gordon, Nicholas Feasey, and Christopher M. Parry. 2015. “Antimicrobial Resistance and Management of Invasive Salmonella Disease.” *Vaccine*, Global Progress on use of Vaccines for Invasive Salmonella Infections, 33, Supplement 3 (June): C21–29. doi:10.1016/j.vaccine.2015.03.102.

Katakai, Tomoya, Takahiro Hara, Manabu Sugai, Hiroyuki Gonda, and Akira Shimizu. 2004. “Lymph Node Fibroblastic Reticular Cells Construct the Stromal Reticulum via Contact with Lymphocytes.” *The Journal of Experimental Medicine* 200 (6): 783–95. doi:10.1084/jem.20040254.

Kinney, Erinna Lea. 2009. “Isolation, Identification, and Antimicrobial Susceptibility Analysis of Enterococcus Spp. and Salmonella Spp. from Conventional Poultry Farms Transitioning to Organic Farming Practices.” M.P.H., United States -- Maryland: University of Maryland, College Park. <http://search.proquest.com.proxy.uchicago.edu/docview/304922577/abstract/232F53A9D1494F24PQ/1>.

Koni, Pandelakis A., and Richard A. Flavell. 1999. “Lymph Node Germinal Centers Form in the Absence of Follicular Dendritic Cell Networks.” *The Journal of Experimental Medicine* 189 (5): 855–64.

Koni, Pandelakis A., Rosalba Sacca, Pornsri Lawton, Jeffrey L. Browning, Nancy H. Ruddle, and Richard A. Flavell. 1997. “Distinct Roles in Lymphoid Organogenesis for Lymphotoxins  $\alpha$  and  $\beta$  Revealed in Lymphotoxin  $\beta$ -Deficient Mice.” *Immunity* 6 (4): 491–500. doi:10.1016/S1074-7613(00)80292-7.

Koroleva, Ekaterina, Wayne Muraoka, Cody Spencer, Ekaterina Gubernatorova, Sydney Halperin, Scott Snapper, and Alexei Tumanov. 2016. “O-009 Lymphotoxin Links Microbiota and Group 3 Innate Lymphoid Cells to Protect Against Intestinal Inflammation.” *Inflammatory Bowel Diseases* 22 Suppl 1 (March): S3–4. doi:10.1097/01.MIB.0000480047.19127.68.

Kumaraguru, Udayasankar, Ila A. Davis, Shilpa Deshpande, Satvir S. Tevethia, and Barry T. Rouse. 2001. “Lymphotoxin  $\alpha$ -/- Mice Develop Functionally Impaired CD8+ T Cell Responses and Fail to Contain Virus Infection of the Central Nervous System.” *The Journal of Immunology* 166 (2): 1066–74. doi:10.4049/jimmunol.166.2.1066.

Kumar, Varsha, Dragos C. Dasoveanu, Susan Chyou, Te-Chen Tzeng, Cristina Roza, Yong Liang, William Stohl, Yang-Xin Fu, Nancy H. Ruddle, and Theresa T. Lu. 2015. “A Dendritic-Cell-Stromal Axis Maintains Immune Responses in Lymph Nodes.” *Immunity* 42 (4): 719–30. doi:10.1016/j.immuni.2015.03.015.

Kumar, Varsha, Elke Scandella, Renzo Danuser, Lucas Onder, Maximilian Nitschké, Yoshinori Fukui, Cornelia Halin, Burkhard Ludewig, and Jens V. Stein. 2010. “Global Lymphoid Tissue Remodeling during a Viral Infection Is Orchestrated by a B Cell-

Lymphotoxin-Dependent Pathway.” *Blood* 115 (23): 4725–33. doi:10.1182/blood-2009-10-250118.

Lane, Peter J. L., Fiona M. McConnell, David Withers, Fabrina Gaspal, Manoj Saini, and Graham Anderson. 2010. “Lymphoid Tissue Inducer Cells and the Evolution of CD4 Dependent High-Affinity Antibody Responses.” In *Progress in Molecular Biology and Translational Science*, edited by Adrian Liston, 92:159–74. Development of T Cell Immunity. Academic Press.  
<http://www.sciencedirect.com/science/article/pii/S1877117310920073>.

Lan, Ruiting, Peter R. Reeves, and Sophie Octavia. 2009. “Population Structure, Origins and Evolution of Major Salmonella Enterica Clones.” *Infection, Genetics and Evolution* 9 (5): 996–1005. doi:10.1016/j.meegid.2009.04.011.

LaRock, Doris L., Anu Chaudhary, and Samuel I. Miller. 2015. “Salmonellae Interactions with Host Processes.” *Nature Reviews Microbiology* 13 (4): 191–205. doi:10.1038/nrmicro3420.

Lee, Seung-Joo, Samantha Dunmire, and Stephen J. McSorley. 2012. “MHC Class-I-Restricted CD8 T Cells Play a Protective Role during Primary Salmonella Infection.” *Immunology Letters* 148 (2): 138–43. doi:10.1016/j.imlet.2012.10.009.

León, Beatriz, André Ballesteros-Tato, Jeffrey L Browning, Robert Dunn, Troy D Randall, and Frances E Lund. 2012. “Regulation of TH2 Development by CXCR5+ Dendritic Cells and Lymphotoxin-Expressing B Cells.” *Nature Immunology* 13 (7): 681–90. doi:10.1038/ni.2309.

Liang, Yong, Kaiting Yang, Jingya Guo, Joanna Wroblewska, Yang-Xin Fu, and Hua Peng. 2015. “Innate Lymphotoxin Receptor Mediated Signaling Promotes HSV-1 Associated Neuroinflammation and Viral Replication.” *Scientific Reports* 5: 10406. doi:10.1038/srep10406.

Lopatin, Uri, Sarah E. Blutt, Margaret E. Conner, and Brian L. Kelsall. 2013. “Lymphotoxin Alpha-Deficient Mice Clear Persistent Rotavirus Infection after Local Generation of Mucosal IgA.” *Journal of Virology* 87 (1): 524–30. doi:10.1128/JVI.01801-12.

Macho-Fernandez, E., E. P. Koroleva, C. M. Spencer, M. Tighe, E. Torrado, A. M. Cooper, Y.-X. Fu, and A. V. Tumanov. 2015. “Lymphotoxin Beta Receptor Signaling Limits Mucosal Damage through Driving IL-23 Production by Epithelial Cells.” *Mucosal Immunology* 8 (2): 403–13. doi:10.1038/mi.2014.78.

MacLennan, Calman A., Laura B. Martin, and Francesca Micoli. 2014. “Vaccines against Invasive Salmonella Disease.” *Human Vaccines & Immunotherapeutics* 10 (6): 1478–93. doi:10.4161/hv.29054.

Maier, Lisa, Rounak Vyas, Carmen Dolores Cordova, Helen Lindsay, Thomas Sebastian Benedikt Schmidt, Sandrine Brugiroux, Balamurugan Periaswamy,

et al. 2013. "Microbiota-Derived Hydrogen Fuels Salmonella Typhimurium Invasion of the Gut Ecosystem." *Cell Host & Microbe* 14 (6): 641–51. doi:10.1016/j.chom.2013.11.002.

Mariathasan, S., M. Matsumoto, F. Baranyay, M. H. Nahm, O. Kanagawa, and D. D. Chaplin. 1995. "Absence of Lymph Nodes in Lymphotoxin-alpha(LT Alpha)-Deficient Mice Is due to Abnormal Organ Development, Not Defective Lymphocyte Migration." *Journal of Inflammation* 45 (1): 72–78.

Martinez-Argudo, Isabel, and Mark A. Jepson. 2008. "Salmonella Translocates across an in Vitro M Cell Model Independently of SPI-1 and SPI-2." *Microbiology (Reading, England)* 154 (Pt 12): 3887–94. doi:10.1099/mic.0.2008/021162-0.

Mastroeni, Pietro. 2002. "Immunity to Systemic Salmonella Infections." *Current Molecular Medicine* 2 (4): 393–406.

Mastroeni, Pietro, C. Simmons, R. Fowler, C. E. Hormaeche, and G. Dougan. 2000. "Igh-6  $-/-$  (B-Cell-Deficient) Mice Fail To Mount Solid Acquired Resistance to Oral Challenge with Virulent Salmonella Enterica Serovar Typhimurium and Show Impaired Th1 T-Cell Responses to Salmonella Antigens." *Infection and Immunity* 68 (1): 46–53. doi:10.1128/IAI.68.1.46-53.2000.

Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1993. "Adoptive Transfer of Immunity to Oral Challenge with Virulent Salmonellae in Innately Susceptible BALB/c Mice Requires Both Immune Serum and T Cells." *Infection and Immunity* 61 (9): 3981–84.

Matsumoto, M., Y. X. Fu, H. Molina, and D. D. Chaplin. 1997. "Lymphotoxin-Alpha-Deficient and TNF Receptor-I-Deficient Mice Define Developmental and Functional Characteristics of Germinal Centers." *Immunological Reviews* 156 (April): 137–44.

Matsumoto, Mitsuru, Stanley F. Lo, Cynthia J. L. Carruthers, Jingjuan Min, Sanjeev Mariathasan, Guangming Huang, David R. Plas, et al. 1996. "Affinity Maturation without Germinal Centres in Lymphotoxin- $\alpha$ -Deficient Mice." *Nature* 382 (6590): 462–66. doi:10.1038/382462a0.

McSorley, Stephen J. 2014. "Immunity to Intestinal Pathogens: Lessons Learned from Salmonella." *Immunological Reviews* 260 (1): 168–82. doi:10.1111/imr.12184.

McSorley, Stephen J., and Marc K. Jenkins. 2000. "Antibody Is Required for Protection against Virulent but Not Attenuated Salmonella Entericaserovar Typhimurium." *Infection and Immunity* 68 (6): 3344–48.

Mebius, R. E., P. R. Streeter, S. Michie, E. C. Butcher, and I. L. Weissman. 1996. "A Developmental Switch in Lymphocyte Homing Receptor and Endothelial Vascular Addressin Expression Regulates Lymphocyte Homing and Permits CD4<sup>+</sup> CD3<sup>-</sup> Cells to Colonize Lymph Nodes." *Proceedings of the National Academy of Sciences of the United States of America* 93 (20): 11019–24.

Menendez, Alfredo, Ellen T. Arena, Julian A. Guttman, Lisa Thorson, Bruce A. Vallance, Wayne Vogl, and B. Brett Finlay. 2009. "Salmonella Infection of Gallbladder Epithelial Cells Drives Local Inflammation and Injury in a Model of Acute Typhoid Fever." *The Journal of Infectious Diseases* 200 (11): 1703–13. doi:10.1086/646608.

Mittrücker, Hans-Willi, and S. H. Kaufmann. 2000. "Immune Response to Infection with Salmonella Typhimurium in Mice." *Journal of Leukocyte Biology* 67 (4): 457–63.

Monack, Denise M., Anne Mueller, and Stanley Falkow. 2004. "Persistent Bacterial Infections: The Interface of the Pathogen and the Host Immune System." *Nature Reviews. Microbiology* 2 (9): 747–65. doi:10.1038/nrmicro955.

Moseman, E. Ashley, Matteo Iannacone, Lidia Bosurgi, Elena Tonti, Nicolas Chevrier, Alexei Tumanov, Yang-Xin Fu, Nir Hacohen, and Ulrich H. von Andrian. 2012. "B Cell Maintenance of Subcapsular Sinus Macrophages Protects against a Fatal Viral Infection Independent of Adaptive Immunity." *Immunity* 36 (3): 415–26. doi:10.1016/j.immuni.2012.01.013.

Moulton, Vaishali R., Nicholas D. Bushar, David B. Leiser, Deepa S. Patke, and Donna L. Farber. 2006. "Divergent Generation of Heterogeneous Memory CD4 T Cells." *Journal of Immunology (Baltimore, Md.: 1950)* 177 (2): 869–76.

Moussion, Christine, and Jean-Philippe Girard. 2011. "Dendritic Cells Control Lymphocyte Entry to Lymph Nodes through High Endothelial Venules." *Nature* 479 (7374): 542–46. doi:10.1038/nature10540.

Müller, Andreas J., Claudia Hoffmann, Marlies Galle, Aeke Van Den Broeke, Mathias Heikenwalder, Laura Falter, Benjamin Misselwitz, Marcus Kremer, Rudi Beyaert, and Wolf-Dietrich Hardt. 2009. "The S. Typhimurium Effector SopE Induces Caspase-1 Activation in Stromal Cells to Initiate Gut Inflammation." *Cell Host & Microbe* 6 (2): 125–36. doi:10.1016/j.chom.2009.07.007.

Müller, U., C. V. Jongeneel, S. A. Nedospasov, K. F. Lindahl, and M. Steinmetz. 1987. "Tumour Necrosis Factor and Lymphotoxin Genes Map close to H-2D in the Mouse Major Histocompatibility Complex." *Nature* 325 (6101): 265–67. doi:10.1038/325265a0.

Nanton, M. R., S. S. Way, M. J. Shlomchik, and S. J. McSorley. 2012. "Cutting Edge: B Cells Are Essential for Protective Immunity against Salmonella Independent of Antibody Secretion." *The Journal of Immunology* 189 (12): 5503–7. doi:10.4049/jimmunol.1201413.

Nauciel, CHARLES, and FLORENCE Espinasse-Maes. 1992. "Role of Gamma Interferon and Tumor Necrosis Factor Alpha in Resistance to Salmonella Typhimurium Infection." *Infection and Immunity* 60 (2): 450–54.

Nedospasov, S. A., I. A. Udalova, D. V. Kuprash, and R. L. Turetskaya. 1991. "DNA Sequence Polymorphism at the Human Tumor Necrosis Factor (TNF) Locus. Numerous TNF/lymphotoxin Alleles Tagged by Two Closely Linked Microsatellites in the Upstream

Region of the Lymphotoxin (TNF-Beta) Gene.” *The Journal of Immunology* 147 (3): 1053–59.

Ng, Cherie T., Laura M. Snell, David G. Brooks, and Michael B.A. Oldstone. 2013. “Networking at the Level of Host Immunity: Immune Cell Interactions during Persistent Viral Infections.” *Cell Host & Microbe* 13 (6): 652–64. doi:10.1016/j.chom.2013.05.014.

Núñez-Hernández, Cristina, Ana Alonso, M. Graciela Pucciarelli, Josep Casadesús, and Francisco García-del Portillo. 2014. “Dormant Intracellular Salmonella Enterica Serovar Typhimurium Discriminates among Salmonella Pathogenicity Island 2 Effectors To Persist inside Fibroblasts.” *Infection and Immunity* 82 (1): 221–32. doi:10.1128/IAI.01304-13.

Oeckinghaus, Andrea, Matthew S. Hayden, and Sankar Ghosh. 2011. “Crosstalk in NF- $\kappa$ B Signaling Pathways.” *Nature Immunology* 12 (8): 695–708. doi:10.1038/ni.2065.

Pfeffer, K., T. Matsuyama, T. M. Kundig, A. Wakeham, K. Kishihara, A. Shahinian, K. Wiegmann, P. S. Ohashi, M. Krönke, and T. W. Mak. 1993. “Mice Deficient for the 55 Kd Tumor Necrosis Factor Receptor Are Resistant to Endotoxic Shock, yet Succumb to *L. Monocytogenes* Infection.” *Cell* 73 (3): 457–67.

Pie, Sandrine, Paolo Truffa-Bachi, Marika Pla, and Charles Nauciel. 1997. “Th1 Response in Salmonella Typhimurium-Infected Mice with a High or Low Rate of Bacterial Clearance.” *Infection and Immunity* 65 (11): 4509–14.

Posch, P. E., I. Cruz, D. Bradshaw, and B. A. Medhekar. 2003. “Novel Polymorphisms and the Definition of Promoter ‘alleles’ of the Tumor Necrosis Factor and Lymphotoxin  $\alpha$  Loci: Inclusion in HLA Haplotypes.” *Genes and Immunity* 4 (8): 547–58. doi:10.1038/sj.gene.6364023.

Que, J. U., and D. J. Hentges. 1985. “Effect of Streptomycin Administration on Colonization Resistance to Salmonella Typhimurium in Mice.” *Infection and Immunity* 48 (1): 169–74.

Raffatellu, Manuela, Michael D. George, Yuko Akiyama, Michael J. Hornsby, Sean-Paul Nuccio, Tatiane A. Paixao, Brian P. Butler, et al. 2009. “Lipocalin-2 Resistance Confers an Advantage to Salmonella Enterica Serotype Typhimurium for Growth and Survival in the Inflamed Intestine.” *Cell Host & Microbe* 5 (5): 476–86. doi:10.1016/j.chom.2009.03.011.

Ravindran, Rajesh, Joseph Foley, Thomas Stoklasek, Laurie H. Glimcher, and Stephen J. McSorley. 2005. “Expression of T-Bet by CD4 T Cells Is Essential for Resistance to Salmonella Infection.” *Journal of Immunology (Baltimore, Md.: 1950)* 175 (7): 4603–10.

Ravindran, Rajesh, and Stephen J McSorley. 2005. “Tracking the Dynamics of T-Cell Activation in Response to Salmonella Infection.” *Immunology* 114 (4): 450–58. doi:10.1111/j.1365-2567.2005.02140.x.

Rennert, P. D., J. L. Browning, R. Mebius, F. Mackay, and P. S. Hochman. 1996. "Surface Lymphotoxin Alpha/beta Complex Is Required for the Development of Peripheral Lymphoid Organs." *The Journal of Experimental Medicine* 184 (5): 1999–2006.

Ristori, C., H. Rodríguez, P. Vicent, C. Ferreccio, J. García, H. Lobos, and K. D’Ottone. 1982. "Persistence of the Salmonella Typhi-Paratyphi Carrier State after Gallbladder Removal." *Bulletin of the Pan American Health Organization* 16 (4): 361–66.

Roumagnac, Philippe, François-Xavier Weill, Christiane Dolecek, Stephen Baker, Sylvain Brisse, Nguyen Tran Chinh, Thi Anh Hong Le, et al. 2006. "Evolutionary History of Salmonella Typhi." *Science* 314 (5803): 1301–4. doi:10.1126/science.1134933.

"Salmonellosis (Nontyphoidal) - Chapter 3 - 2016 Yellow Book | Travelers' Health | CDC." 2016. Accessed March 20.

<http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/salmonellosis-nontyphoidal>.

Schiøler, H., E. D. Christiansen, G. Høybye, S. N. Rasmussen, and J. Greibe. 1983. "Biliary Calculi in Chronic Salmonella Carriers and Healthy Controls: A Controlled Study." *Scandinavian Journal of Infectious Diseases* 15 (1): 17–19.

Schneider, Kirsten, Andrea Loewendorf, Carl De Trez, James Fulton, Antje Rhode, Heather Shumway, Sukwon Ha, et al. 2008. "Lymphotoxin-Mediated Crosstalk between B Cells and Splenic Stroma Promotes the Initial Type I Interferon Response to Cytomegalovirus." *Cell Host & Microbe* 3 (2): 67–76. doi:10.1016/j.chom.2007.12.008.

Schulz, Silke M., Gabriele Köhler, Christoph Holscher, Yoichiro Iwakura, and Gottfried Alber. 2008. "IL-17A Is Produced by Th17, Gammadelta T Cells and Other CD4-Lymphocytes during Infection with Salmonella Enterica Serovar Enteritidis and Has a Mild Effect in Bacterial Clearance." *International Immunology* 20 (9): 1129–38. doi:10.1093/intimm/dxn069.

Sekirov, Inna, Nicola M. Tam, Maria Jogova, Marilyn L. Robertson, Yuling Li, Claudia Lupp, and B. Brett Finlay. 2008. "Antibiotic-Induced Perturbations of the Intestinal Microbiota Alter Host Susceptibility to Enteric Infection." *Infection and Immunity* 76 (10): 4726–36. doi:10.1128/IAI.00319-08.

Sinha, K., P. Mastroeni, J. Harrison, R. D. de Hormaeche, and C. E. Hormaeche. 1997. "Salmonella Typhimurium aroA, htrA, and aroD htrA Mutants Cause Progressive Infections in Athymic (nu/nu) BALB/c Mice." *Infection and Immunity* 65 (4): 1566–69.

Soper G. 1907. "The Work of a Chronic Typhoid Germ Distributor." *Journal of the American Medical Association* XLVIII (24): 2019–22. doi:10.1001/jama.1907.25220500025002d.

Soroosh, Pejman, Shouji Ine, Kazuo Sugamura, and Naoto Ishii. 2007. "Differential Requirements for OX40 Signals on Generation of Effector and Central Memory CD4+ T Cells." *Journal of Immunology (Baltimore, Md.: 1950)* 179 (8): 5014–23.

Spahn, Thomas W., Christian Maaser, Lars Eckmann, Jan Heidemann, Andreas Lügering, Rodney Newberry, Wolfram Domschke, Hermann Herbst, and Torsten Kucharzik. 2004. "The Lymphotoxin- $\beta$  Receptor Is Critical for Control of Murine *Citrobacter Rodentium*-induced Colitis." *Gastroenterology* 127 (5): 1463–73. doi:10.1053/j.gastro.2004.08.022.

Spies, T, C C Morton, S A Nedospasov, W Fiers, D Pious, and J L Strominger. 1986. "Genes for the Tumor Necrosis Factors Alpha and Beta Are Linked to the Human Major Histocompatibility Complex." *Proceedings of the National Academy of Sciences of the United States of America* 83 (22): 8699–8702.

Srinivasan, A., M. Nanton, A. Griffin, and S. J. McSorley. 2009. "Culling of Activated CD4 T Cells during Typhoid Is Driven by Salmonella Virulence Genes." *The Journal of Immunology* 182 (12): 7838–45. doi:10.4049/jimmunol.0900382.

Stecher, Bärbel, Samuel Chaffron, Rina Käppeli, Siegfried Hapfelmeier, Susanne Friedrich, Thomas C. Weber, Jorum Kirundi, et al. 2010. "Like Will to like: Abundances of Closely Related Species Can Predict Susceptibility to Intestinal Colonization by Pathogenic and Commensal Bacteria." *PLoS Pathogens* 6 (1): e1000711. doi:10.1371/journal.ppat.1000711.

Stecher, Bärbel, Günther Paesold, Manja Barthel, Marcus Kremer, Jonathan Jantsch, Thomas Stallmach, Mathias Heikenwalder, and Wolf-Dietrich Hardt. 2006. "Chronic Salmonella Enterica Serovar Typhimurium-Induced Colitis and Cholangitis in Streptomycin-Pretreated Nramp1+/+ Mice." *Infection and Immunity* 74 (9): 5047–57. doi:10.1128/IAI.00072-06.

Stecher, Bärbel, Riccardo Robbiani, Alan W Walker, Astrid M Westendorf, Manja Barthel, Marcus Kremer, Samuel Chaffron, et al. 2007. "Salmonella Enterica Serovar Typhimurium Exploits Inflammation to Compete with the Intestinal Microbiota." Edited by Matt Waldor. *PLoS Biology* 5 (10): e244. doi:10.1371/journal.pbio.0050244.

Stecher, B., A. J. Macpherson, S. Hapfelmeier, M. Kremer, T. Stallmach, and W.-D. Hardt. 2005a. "Comparison of Salmonella Enterica Serovar Typhimurium Colitis in Germfree Mice and Mice Pretreated with Streptomycin." *Infection and Immunity* 73 (6): 3228–41. doi:10.1128/IAI.73.6.3228-3241.2005.

Stelter, Christian, Rina Käppeli, Claudia König, Alexander Krah, Wolf-Dietrich Hardt, Bärbel Stecher, and Dirk Bumann. 2011a. "Salmonella-Induced Mucosal Lectin RegIII $\beta$  Kills Competing Gut Microbiota." *PloS One* 6 (6): e20749. doi:10.1371/journal.pone.0020749.

Suresh, M., Gibson Lanier, Mary Katherine Large, Jason K. Whitmire, John D. Altman, Nancy H. Ruddle, and Rafi Ahmed. 2002. "Role of Lymphotoxin Alpha in T-Cell Responses during an Acute Viral Infection." *Journal of Virology* 76 (8): 3943–51.

Tam, J. W., A. L. Kullas, P. Mena, J. B. Bliska, and A. W. M. van der Velden. 2014. "CD11b+ Ly6Chi Ly6G- Immature Myeloid Cells Recruited in Response to Salmonella Enterica Serovar Typhimurium Infection Exhibit Protective and Immunosuppressive Properties." *Infection and Immunity* 82 (6): 2606–14. doi:10.1128/IAI.01590-13.

Tumanov, Alexei V, Sergei I Grivennikov, Alexander N Shakhov, Stanislav A Rybtsov, Ekaterina P Koroleva, Junji Takeda, Sergei A Nedospasov, and Dmitry V Kuprash. 2003. "Dissecting the Role of Lymphotoxin in Lymphoid Organs by Conditional Targeting." *Immunological Reviews* 195 (October): 106–16.

Tumanov, Alexei V., Ekaterina P. Koroleva, Xiaohuan Guo, Yugang Wang, Andrei Kruglov, Sergei Nedospasov, and Yang-Xin Fu. 2011. "Lymphotoxin Controls the IL-22 Protection Pathway in Gut Innate Lymphoid Cells during Mucosal Pathogen Challenge." *Cell Host & Microbe* 10 (1): 44–53. doi:10.1016/j.chom.2011.06.002.

"Typhoid & Paratyphoid Fever - Chapter 3 - 2016 Yellow Book | Travelers' Health | CDC." 2016. Accessed March 20.  
<http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/typhoid-paratyphoid-fever>.

van de Pavert, Serge A., and Reina E. Mebius. 2010. "New Insights into the Development of Lymphoid Tissues." *Nature Reviews Immunology* 10 (9): 664–74. doi:10.1038/nri2832.

van de Pavert, Serge A., Brenda J. Olivier, Gera Goverse, Mark F. Vondenhoff, Mascha Greuter, Patrick Beke, Kim Kusser, et al. 2009. "Chemokine CXCL13 Is Essential for Lymph Node Initiation and Is Induced by Retinoic Acid and Neuronal Stimulation." *Nature Immunology* 10 (11): 1193–99. doi:10.1038/ni.1789.

Vondenhoff, Mark F., Mascha Greuter, Gera Goverse, Dirk Elewaut, Pieter Dewint, Carl F. Ware, Kerim Hoorweg, Georg Kraal, and Reina E. Mebius. 2009. "LTbetaR Signaling Induces Cytokine Expression and up-Regulates Lymphangiogenic Factors in Lymph Node Anlagen." *Journal of Immunology (Baltimore, Md.: 1950)* 182 (9): 5439–45. doi:10.4049/jimmunol.0801165.

Vu, Frances, Umberto Dianzani, Carl F. Ware, Tak Mak, and Jennifer L. Gommerman. 2008. "ICOS, CD40, and Lymphotoxin  $\beta$  Receptors Signal Sequentially and Interdependently to Initiate a Germinal Center Reaction." *Journal of Immunology (Baltimore, Md. : 1950)* 180 (4): 2284–93.

Wang, Y., G. Huang, J. Wang, H. Molina, D. D. Chaplin, and Y. X. Fu. 2000. "Antigen Persistence Is Required for Somatic Mutation and Affinity Maturation of Immunoglobulin." *European Journal of Immunology* 30 (8): 2226–34. doi:10.1002/1521-4141(2000)30:8<2226::AID-IMMU2226>3.0.CO;2-5.

- Wang, Yugang, Ekaterina P. Koroleva, Andrei A. Kruglov, Dmitry V. Kuprash, Sergei A. Nedospasov, Yang-Xin Fu, and Alexei V. Tumanov. 2010. "Lymphotoxin Beta Receptor Signaling in Intestinal Epithelial Cells Orchestrates Innate Immune Responses against Mucosal Bacterial Infection." *Immunity* 32 (3): 403–13. doi:10.1016/j.immuni.2010.02.011.
- Ware, C. F., P. D. Crowe, M. H. Grayson, M. J. Androlewicz, and J. L. Browning. 1992. "Expression of Surface Lymphotoxin and Tumor Necrosis Factor on Activated T, B, and Natural Killer Cells." *Journal of Immunology (Baltimore, Md.: 1950)* 149 (12): 3881–88.
- Ware, C. F., T. L. VanArsdale, P. D. Crowe, and J. L. Browning. 1995. "The Ligands and Receptors of the Lymphotoxin System." *Current Topics in Microbiology and Immunology* 198: 175–218.
- Wessling-Resnick, Marianne. 2015. "Nramp1 and Other Transporters Involved in Metal Withholding during Infection." *The Journal of Biological Chemistry* 290 (31): 18984–90. doi:10.1074/jbc.R115.643973.
- Wilhelm, Patricia, D. Sean Riminton, Uwe Ritter, Frances A. Lemckert, Christina Scheidig, Robert Hoek, Jonathon D. Sedgwick, and Heinrich Körner. 2002. "Membrane Lymphotoxin Contributes to Anti-Leishmanial Immunity by Controlling Structural Integrity of Lymphoid Organs." *European Journal of Immunology* 32 (7): 1993–2003. doi:10.1002/1521-4141(200207)32:7<1993::AID-IMMU1993>3.0.CO;2-F.
- Winter, Sebastian E., and Andreas J Bäumler. 2011. "A Breathtaking Feat." *Gut Microbes* 2 (1): 58–60. doi:10.4161/gmic.2.1.14911.
- Winter, Sebastian E., Parameth Thiennimitr, Maria G. Winter, Brian P. Butler, Douglas L. Huseby, Robert W. Crawford, Joseph M. Russell, et al. 2010. "Gut Inflammation Provides a Respiratory Electron Acceptor for Salmonella." *Nature* 467 (7314): 426–29. doi:10.1038/nature09415.
- Woolhouse, M. E. J., C. Dye, J.-F. Etard, T. Smith, J. D. Charlwood, G. P. Garnett, P. Hagan, et al. 1997. "Heterogeneities in the Transmission of Infectious Agents: Implications for the Design of Control Programs." *Proceedings of the National Academy of Sciences* 94 (1): 338–42.
- Wronski, Eileen V., Gregory M. Woods, and Barry L. Munday. 2003. "Antibody Response to Sheep Red Blood Cells in Platypus and Echidna." *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* 136 (4): 957–63.
- Xu, G., D. Liu, Y. Fan, X. Yang, H. Korner, Y.-X. Fu, and J. E. Uzonna. 2007. "Lymphotoxin 2 (Membrane Lymphotoxin) Is Critically Important for Resistance to Leishmania Major Infection in Mice." *The Journal of Immunology* 179 (8): 5358–66. doi:10.4049/jimmunol.179.8.5358.

Yamada, T., and J. Davies. 1971. "A Genetic and Biochemical Study of Streptomycin- and Spectinomycin-Resistance in *Salmonella Typhimurium*." *Molecular and General Genetics MGG* 110 (3): 197–210. doi:10.1007/BF00337833.

Yurist-Doutsch, Sophie, Marie-Claire Arrieta, Stefanie L. Vogt, and B. Brett Finlay. 2014. "Gastrointestinal Microbiota-Mediated Control of Enteric Pathogens." *Annual Review of Genetics* 48: 361–82. doi:10.1146/annurev-genet-120213-092421.

Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. "Viral Immune Evasion due to Persistence of Activated T Cells without Effector Function." *The Journal of Experimental Medicine* 188 (12): 2205–13.

Zeng, Ming, Mirko Paiardini, Jessica C. Engram, Greg J. Beilman, Jeffrey G. Chipman, Timothy W. Schacker, Guido Silvestri, and Ashley T. Haase. 2012. "Critical Role of CD4 T Cells in Maintaining Lymphoid Tissue Structure for Immune Cell Homeostasis and Reconstitution." *Blood* 120 (9): 1856–67. doi:10.1182/blood-2012-03-418624.

Zheng, Yan, Patricia A Valdez, Dimitry M Danilenko, Yan Hu, Susan M Sa, Qian Gong, Alexander R Abbas, et al. 2008. "Interleukin-22 Mediates Early Host Defense against Attaching and Effacing Bacterial Pathogens." *Nature Medicine* 14 (3): 282–89. doi:10.1038/nm1720.

Zuniga, Elina I., Monica Macal, Gavin M. Lewis, and James A. Harker. 2015. "Innate and Adaptive Immune Regulation During Chronic Viral Infections." *Annual Review of Virology* 2 (1): 573–97. doi:10.1146/annurev-virology-100114-055226.