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Inhibition of Mammalian T cells by *Salmonella enterica* serovar Typhimurium

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ABSTRACT OF THE DISSERTATION

Inhibition of Mammalian T cells by *Salmonella enterica* serovar Typhimurium

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Bacterial pathogens must avoid clearance by the immune system to establish infection, yet many processes of bacterial immune subversion remain undefined. T cells are a key component of the mammalian immune system and are required for protective immunity against many bacterial pathogens. *Salmonella enterica* serovar Typhimurium avoid clearance by the host immune system by suppressing T cell responses, yet the mechanisms mediating this immunosuppression are still unknown.

We previously showed that *S.* Typhimurium act directly on T cells to inhibit their proliferation and down-modulate T cell receptor (TCR) expression. Based on these results, we have used a genetic approach to identify the *S.* Typhimurium genes required for T cell inhibition. In these studies, we show that S. Typhimurium inhibit T cell

responses by producing L-asparaginase II, which catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia. L-asparaginase II is necessary and sufficient to suppress T cell blastogenesis, cytokine production and proliferation, and to down-modulate expression of the TCR. Purified L-asparaginase II alters TCR levels and cytokine profiles of T cells *in vitro.* Furthermore, *S*. Typhimurium-induced inhibition of T cells *in vitro* is prevented upon addition of L-asparagine. *S*. Typhimurium lacking the L-asparaginase II gene (*STM3106*) are unable to inhibit T cell responses and exhibit attenuated virulence *in vivo*. L-asparaginases are used to treat acute lymphoblastic leukemia through mechanisms that likely involve amino acid starvation of leukemic cells, and these findings indicate that pathogens similarly use L-asparagine deprivation to limit T cell responses.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

6xHis	hexahistidine
Ab	antibody
ALL	acute lymphoblastic leukemia
APC	allophycocyanin or antigen presenting cell
APC-Cy7	allophycocyanin-cyanine 7
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming units
CO_2	carbon dioxide
DCs	dendritic cells
DNA	deoxyribonucleic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable
FITC	fluorescein isothiocynate
FSC	forward scatter
GFP	green fluorescence protein
GMFI	geometric mean fluorescence intensity
IFN	interferon
IL	interleukin

L-Asnase II	L-asparaginase II
Kb	kilobase
LB	Luria-Bertani
LPS	lipopolysaccharide
μg	microgram(s)
μl	microliter(s)
μm	micrometer(s) or micron(s)
MHC	major histocompatibility complex
ml	milliliters
MLN	mesenteric lymph node(s)
mM	millimolar
MOI	multiplicity of infection
nm	nanometer
NK	natural killer
NLRs	NOD-like receptors
NTS	nontyphoidal *Salmonellae*
OD	optical density
OVA	ovalbumin
PacBlue	Pacific blue
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll

PE-Cy7	phycoerythrin cyanine 7
RNA	ribonucleic acid
RPM	rotations per minute
SCV	*Salmonella*-containing vacuole
SEM	standard error of the mean
SSC	side scatter
STm	*S*. Typhimurium
TCR-β	T cell receptor β-chain
TLRs	Toll-like receptors
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
T_H	T helper cell
TNF-α	tumor necrosis factor α
T reg	T regulatory
TTSS	type three secretion system
UI	uninfected
WT	wild-type

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INTRODUCTION

I. Introduction to the immune system

In order to combat or prevent infection, mammalian hosts have developed a variety of defense strategies. The front line of defense is the physical and chemical barriers, which prevent microbes from entering the body. When microbes are able to penetrate or evade these barriers, the immune system springs into action. The immune system is an extremely dynamic and diverse entity, allowing it to be exceedingly effective at protecting an individual from invading pathogens. The first responders are cells of our innate immune system, which is considered fast-acting, but relatively unspecific (180). Innate immune cells express pattern recognition receptors (PRRs) on their surface, and these receptors detect evolutionary conserved pathogen-associated molecular patterns (PAMPs) on the invading microbes (9, 55, 105, 142, 157, 180). Additionally, many of these cells are phagocytes (e.g., neutrophils, macrophages and dendritic cells (DCs)), capable of engulfing and killing microbes.

Macrophages and DCs are professional antigen presenting cells (pAPCs) that after internalization and subsequent killing of the pathogen, process bacterial proteins (via the proteasome or acidified endocytic vesicles), generating pathogen-derived peptides or antigens (46, 105, 169). These antigens get attached by peptide loading complexes (PLCs) and are subsequently bound by class I and II major histocompatibility (MHC) molecules; these latter complexes are transported to the cell surface (46, 168, 169). T cells of the adaptive immune system may recognize these antigen-MHC complexes on the surface of macrophages and DCs. An immunological synapse is

formed at the cell surface between a T cell and APC; it consists of the molecules that contribute to adhesion and signaling between these two cells (52, 163). This communication between T cells and members of the innate immune system is important for initiating a cell-mediated adaptive immune response (105).

The adaptive immune response is highly specific because it relies on the keen recognition ability of lymphocytes, but takes days or weeks to develop (105). Lymphocytes have receptors on their surface, which recognize and respond to an individual antigen of microbes. There are billions of lymphocytes present in a host at any given moment, recognizing a wide repertoire of highly diverse antigens. Once a lymphocyte comes across an antigen its receptor recognizes, there is clonal expansion of this engaged lymphocyte, and all of the newly expanded cells are now specific for this antigen. After the adaptive immune response successfully clears the pathogen, there is contraction of this phase of the immune response (184). A subset of these lymphocytes will become memory cells. These memory cells elicit a faster and larger response if these cells encounter the same antigen again.

Innate and adaptive immune responses work together to effectively control and clear infections with invading pathogens. When cells of the innate immune system are activated, they work to eliminate the pathogen and produce cytokines or chemokines that recruit and activate cells of the adaptive immune system (9). Cytokines are a family of small but potent polypeptides that influence the immune response. Chemokines are a subset of cytokines functioning as chemoattractants to direct leukocyte trafficking and positioning within tissues. The adaptive immune system also provides positive feedback

to the innate immune system through production of cytokines that can activate cells of the innate immune response or boost cell numbers (114).

A. The innate immune system

The quick-responding cells of the innate immune response serve two main purposes: 1) limit microbial dissemination and 2) clear or control the infection until the host engages a specific, adaptive immune response (11, 180). The cells composing the innate immune system include: antigen-presenting DCs, phagocytic macrophages and neutrophils, natural killer (NK) cells and γδ T cells (70, 111). These cells sense bacterial pathogens by recognizing PAMPs using Toll-like receptors (TLRs), NOD-like receptors (NLRs), and complement (9, 55, 105, 142, 157, 180). Macrophages and DCs are important cells of the innate immune system that express TLRs (70, 111).

PAMPs are invariant, evolutionary conserved entities spanning entire classes of pathogens that allow the host to distinguish the pathogen from self; examples of PAMPs include peptidoglycan, flagellin, LPS, ds RNA, CpG DNA, etc. (111). PRRs convert PAMP recognition into activation of signal transduction and cellular response pathways (19, 111). Detection of bacteria by TLRs and NLRs triggers a cytokine and chemokine response resulting in amplification of danger responses, enhancement of the bactericidal capacity of immune cells, and ultimately generation of a more specific, adaptive immune response (19, 111, 140, 180). NF-κB, AP1, and IRF3/7 activate many of these responses; they are transcription factors able to initiate production of pro-inflammatory mediators, such as cytokines and interferons (111). TLRs span a cellular membrane, whether it is at the cell surface or the membranes of endo-lysosomal compartments, while NLRs are soluble and found in the cytoplasm (28, 89, 111). TLRs

survey the extracellular environment and phagolysosomal compartments and NLRs monitor the cytosol (70, 89, 111).

Phagocytosis is used by the innate immune system to stop and kill invading pathogens (67, 163). It is an active process consisting of “receptor binding, internalization, and phagosome maturation” (140). A phagocyte is able to recognize and bind PAMPs on the invading pathogen using its germline-encoded PRR. Subsequently, the phagocyte is able to engulf and ingest the microbe, which is contained in a membrane-bound vesicle, called a phagosome (175). The phagosome becomes acidified and matures into a digestive organelle after it fuses with lysosomes forming a phagolysosome (67, 140, 175). The lysosome releases its arsenal of destruction to the invading microbe: acidic pH, reactive O_2 species, proteolytic enzymes, and antibacterial peptides (76, 163, 175). Many of these molecules interrupt the integrity of the bacterial cell membrane, ultimately killing the bacteria (163).

Neutrophils and NK cells are important innate effector cells. They can kill both invading microbes as well as infected cells (111). Neutrophils are typically the first responder cell to the site of infection and can be activated by virtually all PAMPs and have mRNA for all known TLRs (111). This allows them to be ready to attack and engulf a variety of invading pathogens from viruses to bacteria to fungi (111). Neutrophils utilize various mechanisms to kill invading pathogens, including the oxidative burst and neutrophil-extracellular-traps (NETs) (145). They are short-lived cells (1-3 days) that die after they have accomplished a round of phagocytosis. NK cells release their cytoplasmic granules, containing granzyme and perforin, into the infected, bound cell. NK cells express invariant receptors making them fast-acting cells.

They do not require activation in order to kill infected cells or invading pathogens, but their cytotoxicity is increased 20-100 fold upon activation with interferon (IFN)-α, IFN-β or IL-12. Activated NK cells secrete IFN-γ, enhancing the ability of macrophages to kill pathogens (111).

Macrophages are versatile cells that play many roles and are found in non-lymphoid tissues as well as lymphoid organs. They are key players in the innate immune system, help orchestrate the immune response, and clear dead cells and cellular debris (163). They can change their function depending on the environmental signals they receive. Macrophages are longer-lived cells (surviving for months) and are very efficient at ingesting and killing microbes by producing various lethal molecules (e.g., NO, O_2^-, and H_2O_2) and toxic degradative enzymes (171). Macrophages detect microbial pathogens and sense their viability using receptors (e.g., TLRs and NLRs) on their surface, in phagosomal vacuoles, and in the cytosol (140, 163). Additionally, they help induce inflammation by secreting cytokines to activate and recruit other immune cells to the site of infection. Macrophages also can act as APCs, displaying peptide on their surface for recognition by T cells.

DCs are an important part of the innate immune response and provide an essential link between the innate and adaptive immune response (17, 18, 27, 159, 168). When DCs are in an immature state, they have an enhanced phagocytic capacity and have lower expression of co-stimulatory molecules (19). After DCs recognize PAMPs on pathogens, they undergo activation, maturation, and migration to secondary lymphoid organs (17, 18, 159). As a part of this process, DCs have decreased phagocytic capabilities and display increased antigen-presenting MHC molecules and

co-stimulatory molecules (like CD80, CD86, and CD40) on their surface, allowing them activate naïve T cells (18, 19, 111, 159). This results in an immune response specific to a particular antigen (19). Activated DCs produce and secrete cytokines (e.g., IL-2) and induce activation and proliferation of lymphocytes, cells of the adaptive immune system (111).

DCs have different mechanisms by which they take up, process, and present antigenic peptides. Uptake can occur by receptor-mediated phagocytosis or macropinocytosis; processing can occur via the proteasome or acidified endocytic vesicles; and presentation can occur by the traditional class I and II pathways (described above), cross-presentation, or peptide transfer (111). Cross-presentation occurs when exogenous antigens are taken into the endocytic pathway and delivered into the cytosol for the eventual delivery to MHC class I molecules for presentation to $CD8^+$ T cells (117). Peptide transfer occurs when an antigen is transmitted from one DC to another for presentation to T cells (117). DCs are “the most efficient” APCs, which internalize microbes, selectively process antigen, and present pathogen-derived peptides on their surface (6, 9, 105, 114, 165, 180).

$\gamma\delta$ T cells are a fast-acting subset of lymphocytes, producing cytokines resulting in pathogen clearance, inflammation, and homeostasis (11). They can also kill infected cells by engagement of death-inducing receptors as well utilizing effector molecules like granzymes and perforin (11). $\gamma\delta$ T cells release powerful chemoattractants for both neutrophils and macrophages, contributing to the early recruitment of these key players in the innate immune response (11). $\gamma\delta$ T cells are able to recognize conserved non-peptide antigens as well antigens displayed by MHC (11). Furthermore, certain subsets

of γδ T cells have a more memory or pre-activated phenotype which allows them to elicit a robust and fast-acting response without the requirement of lymphocyte amplification (11).

B. The adaptive immune system

Lymphocytes compose the cells of the adaptive immune system. There are two classes of lymphocytes: B lymphocytes (B cells) and T lymphocytes (T cells) (6, 11, 70). In the absence of infection, lymphocytes are small, inactive cells and, in the case of naïve T cells, can survive for years (184). Both B cells and T cells express highly diverse, antigen-specific receptors on their surface enabling them to recognize countless foreign and self antigens (6, 11, 70, 184). Each lymphocyte expresses many antigen receptors on its surface, but these receptors are all specific for a single antigen (6, 70). Upon the proper recognition of the cognate antigen, lymphocytes can clonally expand, resulting in a population of lymphocytes all recognizing the same antigen (11). This contributes to the specificity of the adaptive immune response, which is initiated in a peripheral lymphoid organ, such as the lymph nodes or spleen. Only those lymphocytes encountering their specific antigen will be activated to proliferate and differentiate into effector cells. Clonally expanded lymphocytes acquire effector functions, which are tailored to clear that particular invading pathogen (11).

After the antigen-specific lymphocytes have successfully cleared the pathogen, the adaptive immune response goes through a contraction phase where many of these antigen-specific lymphocytes die by programmed cell death (apoptosis), and only a small fraction differentiates into memory cells (184). Apoptosis involves cell shrinkage, chromatin condensation, and nuclear fragmentation; ultimately the cell breaks into

smaller pieces, which can be phagocytosed by neighboring APCs (10, 181). Establishment of immunological memory is perhaps the most important task of the adaptive immune system; a second encounter with the same antigen induces a more rapid and robust immune response (184).

i. Primary adaptive immune response

During infection, the immune response is constantly changing; both its nature and potency adapt over time, depending on when and where pathogens are encountered. The primary immune response occurs the first time a pathogen is encountered and infection established. As cells of the innate immune system via intercellular communication engage lymphocytes of the adaptive immune response, the innate immune cells and their antibacterial mechanisms are the prime responders to the pathogen. Several days to weeks are needed for clonal expansion and differentiation of naïve lymphocytes into effector T cells and antibody-secreting B cells (95, 156).

The immune response starts with the fairly nonspecific, fast-acting responses of the innate immune system and becomes highly specialized and stronger against the invading microbe as the adaptive immune response is activated and quickly develops. Many microbes are controlled or killed by the innate immune response. However, if the invading pathogen load exceeds a certain threshold, the innate immune response engages cells of the adaptive immune system. The co-stimulatory ligands and antigen displayed in MHC molecules and the secretion of cytokines by cells of the innate immune system are essential for priming and activating the adaptive immune response.

The resulting adaptive immune response is antigen-specific and likely multiple antigens trigger the expansion of multiple T cell clones, all capable of recognizing the

invading pathogen. The pathogen is eventually cleared by co-ordination of antibodies and the actions of effector T cells. Subsequently, there is contraction of the adaptive immune response returning to a quiescent state, though a subpopulation of the antigen-experienced lymphocytes will persist in the form of memory cells (Figure 1).

B cells become activated when the B cell receptor (BCR) recognizes its specific antigen; an additional important component of B cell activation is a second signal delivered by $CD4^+$ T helper cells (6). The BCR recognizes both soluble and membrane-bound antigens; furthermore, the BCR has the capacity to recognize antigens in their unprocessed, native state (6). After antigen engagement, B cells acquire the ability to process and present antigen via MHC class II to $CD4^+$ T helper cells, which in turn induce B cell proliferation and differentiation (6). This is a requirement when it is a thymus-dependent antigen. When the antigen is thymus-independent (e.g., bacterial polysaccharides), there is no need for co-stimulation from $CD4^+$ T helper cells. Activated B cells can differentiate into plasma cells, which secrete high-affinity antibodies, or into memory B cells, which provide long-term protection in case of subsequent exposure to the same antigen (6). This initial exposure to the antigen results in secretion of IgM, which is generally followed by secretion of IgG, due to class switching, as the response develops (156). These high-affinity, antigen-specific antibodies contribute to the specificity of the adaptive immune response and effective pathogen clearance.

There is a very low precursor population of T cells that recognize a particular antigen; only an estimated 1 in 100,000 or 200 T cells in the entire lymphoid system of a mouse have this capability (95, 143, 184). During the expansion phase, a single

antigen-specific T cell can go through more than 15 consecutive rounds of division, resulting in ~10,000 fold amplification, and these antigen-specific T cells are key in pathogen clearance (95, 184).

An important component of inducing T cell-mediated immunity is T cell priming: the activation, proliferation, and differentiation of naïve T cells into effector T cells (Figure 2 (166)). The development of the T cell response is an antigen-driven process coupled to proliferation and differentiation (20). T cells increase in size and granularity, a process known as blastogenesis, before proliferating, resulting in a lengthy and metabolically demanding activation process (47, 95, 130, 184).

Following the capture of antigen and presentation in the context of an MHC molecule, the APC (e.g., DC, macrophage, or B cell) migrates to a peripheral lymphoid organ, where it presents the antigen to naïve T cells. T cell activation generally requires two signals. The first signal transduced is when the TCR and co-receptor (e.g., CD4 or CD8) recognize their cognate antigen in the context of MHC expressed on the surface of an APC (Figure 3 (46, 52,117)). The second signal comes from co-stimulatory molecules on the surface of the same APC signaling through CD28 (Figure 3 (46, 52, 117)). CD28-mediated co-stimulation promotes generation of IL-2, further stimulating T cells (130). As T cells become activated, their expression profile of numerous surface markers changes; CD69 and CD25 are considered early activation markers, while CD62L and CD44 are considered later activation markers (92, 104, 184). During the differentiation process, T cells often get an additional stimulation by detecting key cytokines.

The co-receptor, either CD4 or CD8, contributes to the type of antigen that is recognized by the TCR and also helps to determine their effector function. $CD4^+$ T cells typically recognize peptides derived from extracellular (exogenous) proteins displayed in the context of class II MHC (52). When $CD4^+$ T cells recognize infected cells, they can acquire the ability to produce and secrete cytokines that activate the intracellular killing mechanisms of the infected antigen presenting cells. $CD8^+$ T cells typically recognize peptides derived from cytoplasmic (endogenous) proteins displayed in the context of class I MHC (52). When $CD8^+$ T cells recognize infected cells, they can also acquire the ability to produce and secrete cytokines, but additionally have the ability to directly lyse infected cells, thereby disrupting the intracellular life cycle of the pathogen.

$CD4^+$ T cells are considered to have a greater repertoire of effector functions and can be further classified into several subsets of T helper cells: T_H1, T_H2, and T_H17 or T regulatory (T reg) cells, which contribute to the immune response to distinct subsets of invading pathogens (31, 90). T_H1 cells play a role in the inflammatory response to intracellular pathogens and contribute to cell-mediated immunity by producing IFN-γ, TNF-α, and IL-2 (11, 31, 121). IL-2 signals the continuation of T cell proliferation and differentiation (95). Production of IL-12 and IL-18 by macrophages drives expansion of the T_H1 cell response (172). T_H2 cells help clear extracellular organisms and parasites by producing IL-4, IL-5, IL-10 and IL-13 and contribute to humoral immunity along with assisting in IgG1 and IgE class switching (11, 31, 121). IL-4, STAT-6, and Gata-3 drive the T_H2 cell response (121). T_H17 cells contribute in the clearance of fungi and extracellular bacteria by recruiting and activating neutrophils; they produce IL-17A, IL-17F, IL-21 and IL-22 (11, 31). T reg cells suppress the activation and development of T

cells, thereby dampening the T cell response and ultimately leading to contraction of the response (74). Additionally, they suppress the immune response to self-antigens, ensuring immune tolerance (11, 74). The commitment to a specific T helper cell lineage is irreversible and occurs by the fourth round of cell division (31).

Naïve $CD8^+$ T cells require more co-stimulatory activity to become activated effector cells than naïve $CD4^+$ T cells. $CD4^+$ T cells contribute to the maturation of $CD8^+$ T cells by producing IL-2 that promotes $CD8^+$ T cell differentiation and stimulate APCs to express higher levels of co-stimulatory molecules (117). Some $CD8^+$ T cells are termed 'cytotoxic' T cells because they have cytotoxic granules, containing perforin, granzymes, and granulysin, which they utilize to kill infected cells (52). This antigen-specific, targeted cell lysis is an important function of $CD8^+$ T cells (107). Effector T cells can enter the bloodstream, allowing them to travel to the infection site.

ii. Immunological memory and the secondary immune response

a. Establishment of immunological memory

After the primary infection is resolved, there is contraction of the antigen-specific T and B cell populations. Approximately 95% undergo apoptosis and the remaining small percentage of both populations contributes to establishment of immunological memory (90, 95, 184). These memory lymphocytes are distinct from their predecessors during the primary adaptive immune response (95, 156). These surviving antigen-experienced lymphocytes go through a transient refractory state where they dampen their response to antigen, ultimately diminishing their proliferative capacity (60). As antigen exposure lowers, these cells gradually return to an activation-competent resting

state (60). Memory cells are long-lived cells that are maintained in a resting state, but upon exposure to the same antigen again provide an accelerated, more robust response (Figure 4) (184).

b. Recall responses of B and T cells

The response by B cells in a recall response is both qualitatively and quantatively different from the response in a primary adaptive immune response (156), since there are more antigen-specific memory B cells. Memory B cells can quickly differentiate into plasma cells upon subsequent antigen exposure; additionally, they continuously express co-stimulatory molecules (CD80 and CD86) on their surface (6, 156). Further, the secondary antibody response has low levels of IgM and much higher amounts of IgG, along with some IgA and IgE (6, 156). Memory B cells are the main source of these antibodies; as these cells had already switched from IgM to the more mature isotypes, thus more are produced during the secondary response (6, 156). Furthermore, the affinity of IgG antibodies increases during the primary antibody response and it continues to increase during the secondary response (6,156). This results in higher affinity antibodies in the secondary response (6, 156). Reactivated B cells with high-affinity antigen receptors efficiently engage antigen and subsequently interact with helper T cells, resulting in their quick activation and robust proliferation (6, 156).

Memory T cells are a heterogeneous population that are different from their naïve counterparts (95). Memory T cells display unique surface molecules allowing them to migrate and home to nonlymphoid tissues (92). Likewise, these surface molecules (e.g., adhesion molecules) make memory T cells more sensitive to antigen stimulation,

allowing them to respond quicker (since they no longer require co-stimulation) and have an enhanced production of cytokines (95). Antigen-experienced memory T cells also prevent the activation of naïve T cells to the same antigen.

Memory T cells can be further classified into central memory (T_{CM}) and effector memory (T_{EM}) T cells; originally defined on their distinct migration patterns (60, 95). T_{CM} cells are $CD62L^+$ and $CCR7^+$, allowing them to retain their migratory properties and circulate through the secondary lymphoid organs (181), while T_{EM} cells are $CD62L^-$ and $CCR7^-$ and express receptors allowing them to home to sites of infection (181). Additionally, T_{CM} cells maintain the ability to proliferate robustly and secrete IL-2, IFN-γ, and TNF-α abundantly upon re-stimulation (95, 181). However, they still require a brief period (only minutes, as compared to days) of differentiation to acquire specific effector functions (e.g., directly lysing infected cells) (95). In contrast, T_{EM} cells do not undergo much proliferation after re-stimulation, but can still secrete large amounts of effector cytokines (like IFN-γ and TNF-α) as well as elicit other effector molecules like perforin and granzyme B (95, 181). Thus, the fast-acting and robust responses of both T_{EM} and T_{CM} cells allow for quicker clearance when an immunized host re-encounters a pathogen.

II. Bacterial subversion of the host immune response

Numerous pathogens subvert pathways of the innate immune systems, but how bacterial pathogens overcome pathways of the adaptive immune systems is not as well understood. To survive and replicate in the mammalian host, bacterial pathogens must adapt to unique and often hostile environments. Thus, they have evolved sophisticated

molecular mechanisms to evade recognition by their host or manipulate their host. Bacterial pathogens must: gain entry to the body; acquire nutrients; survive or grow; and evade and modulate both innate and adaptive immune responses. Many bacterial pathogens utilize protein secretion systems to produce and export effector proteins (e.g., toxin) to manipulate host cells and host cell function into an environment that is conducive to bacterial entry, survival, and growth.

A. Subversion of the innate immune response

Innate immune cells have many strategies to kill invading pathogens. Phagocytes engulf invading pathogens and subsequently kill them by phagocytosis. Another important mechanism used by the innate immune response to eliminate pathogens is to elicit help from the adaptive immune system. In order to survive, bacterial pathogens have developed countering strategies to avoid innate cells or their effector functions. These abilities include (though are not limited to): 1) inhibiting uptake by phagocytes; 2) interfering with phagocytosis; 3) killing innate immune cells; 4) delaying host cell death; and 5) dampening host inflammatory responses.

i. Inhibition of uptake by phagocytes

A number of pathogens inhibit uptake by phagocytes by secreting effector proteins that disrupt host cell signal transduction pathways or by producing an antiphagocytic capsule. For example, *Yersinia spp., Pseudomonas aeruginosa,* and enteropathogenic *Escherichia coli* (EPEC) use protein secretion systems to inject effector proteins into the host cell cytosol (175). These effector proteins inhibit bacterial uptake. They interact with host proteins responsible for actin polymerization to inhibit the cell surface modifications required for phagocytic internalization (175). Other

pathogens (e.g., *Streptococcus pneumoniae*) form a capsule, which offers them protection from phagocytes (66).

ii. Interfering with phagocytosis

While some pathogens interfere with phagocyte uptake, a number of intracellular pathogens interfere with different aspects of the phagocytic pathway to survive. *Legionella pneumophila, Mycobacterium tuberculosis* and *Brucella abortus* prevent the phagolysosome from maturing (175). If the antimicrobial molecules from the lysosome do not enter the phagosome, the membrane-bound compartment can be used for bacterial survival and growth (175). *L. pneumophila* utilize a type IV secretion system to deliver over 200 effector proteins into the host cytosol; some of these proteins have distinct biochemical properties to manipulate the host vesicular transport system, preventing acidification of the *Legionella*-containing vacuole (46, 53). *M. tuberculosis* deplete H^+ ATPase molecules from the vacuolar membrane, preventing its acidification, thereby inhibiting phagolysosome maturation (66, 67). Other intracellular pathogens (e.g., *Rickettsia spp., Shigella spp.* and *Listeria spp.*) escape killing mechanisms by disrupting the phagosomal membrane and escaping before it fuses with the lysosome, allowing the bacteria to enter the cytosol where they may survive and replicate (66, 127, 129, 171, 175).

iii. Killing of phagocytes

To inhibit clearance, many pathogens have strategies to avoid being killed; other pathogens avoid being killed by actually killing the host cell. The killing of host cells can benefit the pathogen in several ways. Pathogens can kill macrophages or DCs to evade the host's immune system. *Salmonella spp., Shigella spp., Yersinia spp., S.*

pneumoniae, L. pneumophila and *P. aeruginosa* induce apoptosis in host inflammatory cells, preventing the release of pro-inflammatory molecules (30, 53, 66, 175). Some of these organisms use secretion systems to inject effector proteins, which bind and activate host caspase-1, triggering apoptosis, ultimately resulting in host cell death (30, 66, 168, 175). Other pathogens (e.g., *Bacillus anthracis* and *Staphylococcus aureus*) use toxins to activate caspase-1 to kill macrophages, inhibiting the defense of these phagocytes (10). Yet other pathogens (e.g., *Neisseria gonorrhoeae*) translocate pore-forming proteins into host membranes, generating holes, ultimately killing the host cell (49).

iv. Delaying host cell death

Obligate intracellular pathogens require intact host cells for their survival and delay the dying of host cells. *Anaplasma phagocytophilum* target neutrophils and, upon uptake, inhibit apoptosis of the cell (by up-regulating anti-apoptotic proteins) for hours or even days until the bacterium successfully finishes its developmental cycle (10). *Chlamydia trachomatis* is another example of an obligate, intracellular pathogen that requires viable, intact host cells for its own replication (10). *C. trachomatis* target members of the apoptotic machinery for proteasomal degradation as well up-regulate anti-apoptotic proteins (10). Also, by delaying host cell death there is not the immediate release of pro-inflammatory cytokines.

v. Dampening of host inflammatory responses

Some pathogens survive by dampening the host inflammatory responses. This can be facilitated by evading stimulation of TLRs or NLRs as well as inhibiting or manipulating cytokine production, which will impact the activation and recruitment of

other immune cells. Other microbes (e.g., *Helicobacter pylori, Porphyromonoas gingivalis,* and *L. pneumophila*) modify their PAMPs to avoid recognition by TLRs and prevent triggering an immune response (111). *Y. enterolitica* injects effector proteins, including *Yersinia* outer proteins (Yops), to interfere with antigen presentation by DCs to T cells (8). *Yersinia spp.* utilize another set of virulence factors, V antigens, during infection (111). These stimulate an anti-inflammatory response by inducing IL-10 production and suppressing TNF-α production (66, 111). IL-10 is a potent suppressor of macrophage activation and function (66). By stimulating production of immunosuppressive cytokines, such as IL-10, pathogens dampen the inflammatory response.

B. Subversion of the adaptive immune response

A number of bacterial pathogens avoid clearance by subverting pathways of the adaptive immune system, yet many of these mechanisms are not well understood. Some pathogens avoid clearance by evading aspects of the humoral immune response or the cell-mediated immune response. When there is a thymus-dependent antigen, B cells need co-stimulation from a $CD4^+$ T helper cell; however, if there is not a proper T cell response established, there is a subsequent failure to initiate a proper B cell response.

i. Evasion of the humoral immune response

a. Inhibition of BCR signaling

Several bacterial pathogens inhibit the responses of B cells by interfering with BCR signaling. By reducing BCR signaling, there is suppression of the overall B cell response resulting in reduced antibody and cytokine production. *Y. pseudotuberculosis*

use their type three secretion system (TTSS) effector, YopH, to dephosphorylate key components of the BCR after stimulation by antigen (66, 172). This inhibits up-regulation of the co-stimulatory molecules, CD86 and CD69 (66, 172). CagA expression by *H. pylori* inhibits IL-3-dependent B-cell proliferation by inhibiting JAK-STAT signaling (1).

b. Evasion of the antibody-mediated immune response

Evasion of the antibody-mediated immune response is one mechanism pathogens exploit to prevent clearance by the adaptive immune response. Antigenic variation is used by *S. pneumoniae* and *N. gonorrhoeae* to evade the antibody response (22). There are more than 80 strains of *S. pneumoniae*. Each strain is antigenically distinct due to differences in the structure of its polysaccharide capsule. *N. gonorrhoeae* use several mechanisms of evading the humoral response. It has a highly variable pilin structure; pilin is the major subunit of *Neisseria spp.* type IV pilus apparatus, which is critical for infection. The variability is generated by non-reciprocal DNA rearrangements of its multiple *pilS* silent storage loci and the pilin expression locus (22). This process generates enormous antigenic variation and allows the bacterium to evade neutralization by secretory IgA (22). Additionally, *Neisseria spp.* can avoid the effector functions of secretory IgA by secreting proteases which cleave it at the hinge region (62). The resulting Fab and Fc fragments have a shortened half-life in mucous secretions and cannot agglutinate the bacteria. *Treponema pallidum* are camouflaged because their surface is coated with host proteins (126, 135). This avoids recognition by antibodies until the bacteria have invaded tissues, like the central nervous system, where they are less accessible to antibodies (126, 135).

ii. Subversion of the cell-mediated response

a. Down-modulation of MHC class I or II

Many pathogens inhibit antigen processing and presentation by down-modulating expression of MHC class I or II, which ultimately allows them to avoid clearance because the T cell response is dampened. Mycobacteria and *C. trachomatis* are pathogens capable of down-modulating MHC class II surface expression and preventing the transcription of IFN-γ responsive genes, cooperatively inhibiting the $CD4^+$ T cell response (66). Also, *C. trachomatis* can block MHC class I surface expression (66). *S.* Typhimurium also interfere with antigen presentation by both MHC class I and II, suppressing the response of both $CD4^+$ and $CD8^+$ T cells (18, 27, 71, 133).

b. Inhibition of TCR signaling

Other pathogens avoid clearance by T cells by reduced signaling through the TCR. This results in a dampened T cell response, which can be observed by a lack of T cell proliferation. *N. gonorroheae* utilize opacity-associated proteins (OPA) to inhibit the activation and proliferation induced by ligation of the TCR on $CD4^+$ T cells (66). *Y. pseudotuberculosis* use its effector, YopH, to dephosphorylate important proteins of the TCR after it is engaged by antigen (66, 172). This prevents TCR-mediated IL-2 production and ERK activity (66, 172). YopH can also obstruct Ca^{2+} signaling, which is important in T cell activation (172).

c. Manipulation of cytokine production

Some bacteria manipulate cytokine production by T cells to avoid clearance. Mycobacteria manipulate the host's production of cytokines and stimulate production of immunosuppressive cytokines, which inhibits the immune response (66). Mycobacteria-

infected macrophages produce IL-6, inhibiting T cell activation, and the immunosuppressive cytokines IL-10 and TGF-β (66). Additionally, the production of these cytokines can trigger the generation of T reg cells, which also dampens the immune response (66).

H. pylori deploy extensive mechanisms to inhibit T cells. Co-culture of *H. pylori* with T cells results in T cell inhibition; this is characterized by decreased IL-2 production, decreased CD25 surface expression, inhibition of proliferation and cell-cycle arrest (1). Many of these effects are the result of VacA (1, 54, 154, 161). *H. pylori* inhibit induction of T_H1-biased $CD4^+$ T cells by hindering IL-12 secretion by DCs, dampening their function (8).

d. Inhibition of T cell migration

Shigella flexneri inhibit the migration of activated T cells. Furthermore, they actively invade activated $CD4^+$ T cells (81). Invasion is dependent on the bacteria's TTSS; after *S. flexneri* invades the T cell, there is TTSS effector, IpgD-dependent inhibition of chemokine-induced T cell migration (81). *S. flexneri* also utilize their TTSS to skew the T cell response to a T_H2 response by inhibiting production of IL-12 (127).

e. Inhibition of T cell proliferation

Some pathogens avoid clearance by inhibiting T cell proliferation. *H. pylori* block antigen-dependent proliferation of T cells through secretion of VacA into the extracellular space and CagA into the host cell (48, 54, 66, 154, 161). Additionally, *H. pylori* utilize arginase to inhibit T cell proliferation by down-modulating expression of the CD-3ζ chain of the TCR (1). Further, *Yersinia spp.* use YopH to block the

"phosphatidylinositol 3-kinase-dependent T cell activation pathway required for cell proliferation" (172).

f. Killing T cells

To avoid T cell effector functions, some pathogens induce T cell apoptosis. *L. monocytogenes* triggers lymphocyte apoptosis using the pore-forming toxin listeriolysin O (24). *Trypanosoma cruzi* induce apoptosis of $CD4^+$ T cells both *in vitro* and *in vitro* (49). By triggering apoptosis in T cells, the bacteria actively inhibit the T cell response.

In conclusion, bacterial pathogens utilize many different strategies to overcome pathways of the mammalian immune response to avoid clearance and establish infection. *Salmonellae* are pathogenic bacteria that cause acute immunosuppression and delay the onset of protective immune responses, yet these mechanisms are not well understood. This dissertation focuses on elucidating mechanisms of T cell-mediated immune subversion used by *Salmonellae.*

III. *Salmonellae*

A. Taxonomy

Salmonellae are facultative intracellular Gram-negative bacterial pathogens that are members of the *Enterobacteriacae* family (35, 50, 71, 88, 157). It is thought that *Salmonellae* diverged from the closely related genus *Escherichia coli* about 100-150 million years ago (35, 142). *Salmonellae* genomes consist of around 4500 genes and over 100 have been linked to virulence (35). The genus *Salmonella* is divided into two species, *S. bongori* and *S. enterica* (50, 84). *S. bongori* has only one subspecies

(subspecies V) whereas *S*. *enterica* are divided into six subspecies (I, II, IIIa, IIIb, IV and VI) (35, 50, 84). S*almonella* serotypes are differentiated by biochemical and antigenic characteristics as well as genome phylogeny. *S. bongori* is mainly found in cold-blooded animals and is considered an opportunistic pathogen (35). *S. enterica* subspecies I is restricted to warm-blooded animals, such as mammals, and thus includes important human pathogens, while the others mainly infect cold-blooded animals (50, 84). Serotypes of *S*. *enterica* subspecies I include the important human pathogens: *S*. Typhi, *S*. Typhimurium and *S*. Enteritidis. *S. enterica* subspecies I genomes are >90% homologous and these differences may contribute to host specificities, which is an important factor of *Salmonella* biology and pathogenicity (17). The further classification into serovars based on the flagellar antigen expands the number of *Salmonella* variants to greater than 2,500 (34, 50, 84).

B. Epidemiology

Salmonellae are a significant cause of worldwide morbidity and mortality in humans (55, 162). *Salmonellae* are able to survive in the environment (35). Hence, they flourish in parts of the world where drinking water quality and sewage treatment are inadequate (112, 114, 138). *Salmonellae* serotypes can be divided into two categories: Typhoidal *Salmonellae* (e.g., *S*. Typhi, *S*. Paratyphi), which cause enteric (typhoid) fever, and nontyphoidal (NTS) *Salmonellae* (e.g., *S*. Typhimurium, *S*. Enteritidis), which cause enteritis. NTS salmonellosis is characterized by massive influx of neutrophils in the colon and ileum (50). In contrast, neutrophils are largely absent from the gut in typhoidal salmonellosis (50).

Annually, there are an estimated 28 million cases of enteric fever (typhoid and paratyphoid) reported worldwide, resulting in 500,000 to 600,000 deaths (77, 96). Additionally, there are 93.8 million cases of nontyphoidal gastroenteritis each year, and approximately 155,000 of these cases are fatal (34, 149, 162). In sub-Saharan Africa, *S.* Typhimurium and *S.* Enteritidis are increasing causes of lethal disease (e.g., bacteremia and meningitis) (149, 162). Recently, emergence of multidrug-resistant *S.* Typhi and NTS serotypes has been increasing, which is limiting treatment options (57, 112, 138, 162).

There are around 40,000 cases of salmonellosis reported in the United States each year, leading to 400 deaths. Moreover, NTS serotypes are the primary cause of death from food-borne illness in the US and cost $0.5-2.3 billion in medical care and lost productivity (142, 162). This does not even factor in the losses in the animal and poultry sector due to salmonellosis.

C. *Salmonellae* host range and specificity

The host specificity of different *Salmonellae* serotypes often determines the nature of the clinical illness, but the molecular basis for these host specificities is not well understood. *S*. Typhi and *S*. Paratyphi are highly adapted for humans and cause enteric fever; they do not colonize or cause disease in animals (77, 97). *S*. Typhimurium and *S*. Enteritidis have broad host ranges and may colonize or cause gastroenteritis in humans, mice and fowl; they are most frequently associated with gastroenteritis in humans because of the large reservoirs of bacteria in domestic animals (35, 84, 149, 162). Approximately 90% of genes in *S.* Typhi and *S.*

Typhimurium are identical, suggesting that the remaining 10% of genes contribute to host specificity (34).

D. Symptoms and pathogenesis of infection with *Salmonellae*

There is a range of disease caused by *Salmonellae*: typhoid fever (caused by *S.* Typhi and *S.* Paratyphi), gastroenteritis (mostly caused by *S.* Typhimurium and *S.* Enteritidis), invasive disease or bacteremia (can be caused by all *Salmonellae* serotypes), and asymptomatic carriage (77). Gastroenteritis is characterized by nausea, vomiting, non-bloody diarrhea, fever, cramps, and headache (77, 96, 112). The symptoms of typhoid fever include sepsis and bacteremia, with continued fever for more than a week before abdominal pain and gastrointestinal symptoms (77, 96, 112).

An estimated 4-6% of typhoid patients become chronic, asymptomatic carriers of *S.* Typhi (57, 112, 114, 138, 148, 162). Typhoid carriers are of particular concern because they are reservoirs of infection and disease, which can occur over decades (57, 138, 148). The most infamous case of a typhoid carrier is 'Typhoid' Mary Malon who was a cook on Long Island, NY and accountable for at least 26 deaths and dozens of infections in others in the United States (57, 77, 138).

Salmonellae are acquired by oral ingestion of contaminated food or water and transmitted through fecal shedding of bacteria (35, 57, 112, 114, 138). *Salmonellae* penetrate the mucus barrier and adhere to and invade into epithelial cells of the terminal small intestine and subepithelial tissue, where the bacteria can be internalized by macrophages (180).

When nontyphoidal *Salmonellae* cause self-limiting gastroenteritis, the infection generally does not progress beyond the lamina propria (114, 180). Nontyphoidal

Salmonellae infection results in the induction of acute phase, exudative inflammatory changes in the intestine. The exudative inflammation is initiated in part by pro-inflammatory cytokines (180). Bacterial detection by TLRs and NLRs initiates the cytokine cascade (180). Exudative inflammation is characterized by increased vascular permeability, neutrophil recruitment, and the formation of tissue exudates above surfaces or within spaces (180). The neutrophils recruited to the site of infection help confine the bacteria to the gastrointestinal tract because these phagocytes are equipped with antibacterial host defenses (180). However, in some instances, nontyphoidal *Salmonellae* may spread hematogenously to other sites in the body (180). Additionally, the resulting inflammatory response mediates the release of prostaglandins, which stimulate cAMP and active fluid secretion with loose diarrheal stools (180). Another important component of exudative inflammation is the epithelial release of antimicrobial factors (180). In NTS salmonellosis, there is an adequate inflammatory response that results in the eventual bacterial clearance.

In contrast, when *Salmonellae* infection causes enteric fever, the bacteria penetrate the host epithelium and there is interstitial inflammation, but a lack of neutrophil recruitment (180). Typhoidal *Salmonellae* are able to break through the intestinal barrier through the Peyer's patches, preferentially entering through M cells, specialized antigen sampling cells, which sample luminal contents for uptake by phagocytic immune cells (114). *Salmonellae* interact with DCs after passage through M cells (27). After penetrating the intestinal epithelium, *Salmonellae* can also be found in macrophages in the lamina propria (56). From the Peyer's patches, *Salmonellae*

disseminate to the mesenteric lymph nodes (MLNs), allowing the bacteria to enter the blood stream, leading to transient bacteremia (114).

Salmonellae are readily cleared from the blood by phagocytes in the spleen and liver. However, a small number of bacteria can survive and replicate inside these host cells. The ability of *Salmonellae* to survive and replicate inside professional phagocytes is important for virulence (114). *Salmonellae* use phagocytes to gain entry into lymph nodes, spleen and liver, allowing the bacteria to establish long-term infections (148). More than 80% of *Salmonellae* found in these tissues are inside pAPCs, but the majority of infected pAPCs present in MLNs, spleen, and liver contain only very few bacteria (97, 148). After dissemination into these distal organs, *Salmonellae* thrive because they adjust to the stressful environment produced by the innate immune system utilizing various virulence factors (107).

Following phagocytosis of *Salmonellae* by pAPCs, the host cells can secrete cytokines to recruit lymphocytes to the site of infection (26). Additionally, the pAPCs travel to local lymph nodes to present *Salmonellae* antigens to T cells, activating adaptive immunity (148). The recruited lymphocytes can also secrete cytokines to enhance the antibacterial functions of phagocytes and also contribute to lysis of infected host cells (96, 107, 114). After the adaptive immune system is engaged, *Salmonellae* are eventually cleared (107). Antibiotic therapy is often recommended to fully clear a typhoidal *Salmonellae* infection and to avoid the carrier state.

E. Mouse models to study acute and chronic *Salmonella* infection

S. Typhimurium are associated with gastroenteritis in humans, but can cause typhoid-like systemic infections in mouse models (50, 112, 142, 162). Our laboratory

uses *S.* Typhimurium to study typhoid-like systemic infections in mouse models. Laboratory mouse strains available to study *Salmonella* vary in their susceptibility to the bacteria (often based on the expression of a functional Nramp allele). In susceptible strains of mice, *S.* Typhimurium induce acute immunosuppression and delay the onset of a protective immune responses. Infections of mice with S. Typhimurium have served as useful models to study the human disease caused by S. Typhi (34, 35, 88). These susceptible strains of mice, such as C57BL/6J, are good models for studying the acute disease (14, 114, 148). After infection with *S.* Typhimurium, susceptible strains of mice die from uncontrolled bacteremia and organ failure within a week using an intravenous dose as low as 100 CFU (14, 92, 139, 148). Furthermore, the C57BL/6J strain of mouse has important 'immunological tools' available (e.g., creating gene deficient mice, generating mouse strains where all the TCRs recognize one specific antigen, etc.), allowing researchers to study specific aspects of a *Salmonella* infection (107). The more resistant strains of mice, such as 129X1/SvJ, are useful models for studying *Salmonella* persistent/chronic infection and transmission (14, 34, 87, 114, 148). This chronic mouse model of salmonellosis has been used to identify genes required for *Salmonella* persistence. Both murine models have contributed in understanding salmonellosis, including identifying virulence factors, mechanisms of pathogenicity, and the host immune response to infection (25, 34).

F. *Salmonella* virulence factors

Salmonellae have a large arsenal of virulence factors and it has been estimated that approximately 4% of its genome is required for fatal infection in mice (12, 34). The virulence factors listed below are only a handful of virulence factors that *Salmonella*

deploy in order to gain access to their host and thrive as a pathogen. Additional studies need to be conducted in order to have a comprehensive understanding of how *Salmonella* are successful pathogens, focusing on how they are able to subvert the immune response, particularly the T cell response.

During the course of evolution, *Salmonella* acquired numerous pathogenicity islands from other bacterial species by repeated events of horizontal gene transfer. The pathogenicity islands are identifiable because they generally have a different GC content from the rest of the genome (120). Numerous genes involved with *Salmonella* virulence are found in these pathogenicity islands, and to date there have been 12 pathogenicity islands identified in *Salmonella* (19, 84). Uniquely present in *S.* Typhi is *Salmonella* Pathogenicity Island (SPI)-7, which encodes the Vi capsular polysaccharide that is extremely immunogenic (5, 34, 57). Vi capsule reduces recognition of *S.* Typhi by PRRs and promotes survival inside macrophages (180). The Vi antigen of *S.* Typhi also stimulates IL-10 production, inducing an immunosuppressive environment in the intestinal mucosa (111).

The *S.* Typhimurium genome contains SPI-1 and SPI-2, which play important, but distinct, roles in helping the bacteria thrive as a pathogen (19, 84, 174). SPI-1 and SPI-2 expression is regulated by *S.* Typhimurium's ability to sense extracellular cues in the environment, and both encode TTSSs (19, 174). TTSSs are needle-like, multi-protein structures that can inject bacterial proteins into host cells as they span from the bacteria's outer membrane to the host cell's membrane (45). These systems function at well-defined phases of *S.* Typhimurium's pathogenic cycle. SPI-1 genes are mainly expressed when *S.* Typhimurium is extracellular, like in the intestinal lumen (19, 185).

Expression of SPI-1-encoded proteins enable *S.* Typhimurium to invade epithelial cells (19, 88). Conversely, SPI-2 genes are primarily expressed when *S.* Typhimurium are intracellular and aid in survival and replication inside host cells, especially macrophages (19, 174).

Once *S.* Typhimurium attach to epithelial cells in the intestinal lumen, they inject (via a TTSS) the SPI-1-encoded effector proteins, SipA, SopE, SopE2, and SopB, into the host cytoplasm (15, 21, 125, 179). These proteins trigger actin polymerization, membrane ruffling and ultimately bacterial uptake into epithelial and M cells, which are non-phagocytic cells (21, 125, 179). Additional important proteins are SipB, SipC, and SipD, which are components of the translocon as they insert into the host cell membrane (125, 185). SipB can induce apoptosis and SipC induces actin polymerization (125, 185). Briefly, SopE and SopE2 are G-nucleotide exchange factors (GEFs) and activate Cdc42 and/or Rac1, which are key regulators of host cell actin polymerization; this contributes to stimulating and energizing actin polymerization (125, 179). Activation of Cdc42 and Rac1 leads to the recruitment and activation of WASP and with the Arp2/3 complex initiates actin polymerization (84). *S.* Typhimurium strains deficient for either SopE or SopE2 exhibit only a minor reduction in cellular invasion, indicating some functional redundancy between the two proteins (125). SopE works with SipA, which binds F actin, “stimulating actin polymerization, actin filament stabilization, and bundling” (179). SopB is an inositol phosphatase (15, 125).

SptP halts this process, as it has a tyrosine phosphatase domain and a GTPase activating domain, restoring the normal cytoskeleton structure within 1-2 hours post-injection (21, 179). The GTPase activating domain is able to inactivate Rho GTPases,

like Cdc42 and Rac1 (179). SptP assists in generation of a permissive environment for bacterial growth. Both SopE and SopE2 get ubiquinated and rapidly targeted for degradation, less than 30 minutes post-injection (125). Further, it has been shown that there is 'hierarchy' in SPI-1 TTSS effector protein injection and function. The injection of SipA and SopE is completed within seconds (90-120 seconds), while injection of SptP is completed significantly later (up to 370 seconds) (179). The 'injection hierarchy' likely helps to circumvent functional interference between effector proteins and to optimize host cell manipulation (179).

Expression of SPI-2-encoded genes contributes to various aspects of intracellular survival: interfering with endocytic trafficking, avoiding NADPH oxidase-dependent killing, altering host immune signaling, delaying apoptosis-like cell death, and controlling *Salmonella*-containing vacuole (SCV) dynamics (45, 174). *S.* Typhimurium deficient for SPI-2 TTSS are highly attenuated, showing a requirement of SPI-2 TTSS for intracellular survival and replication (88). *S.* Typhimurium SPI-2 mutants fail to avert antigen presentation to T cells by infected DCs (19, 159).

SPI-2 gene expression is controlled by the two-component regulatory system SsrA/SsrB, also encoded inside the pathogenicity island (171). *S.* Typhimurium deficient for the effector SpiA cannot assemble a TTSS secretion needle encoded by the SPI-2 TTSS, preventing the subsequent translocation of other effector proteins (18). SpiC is required to translocate other effector proteins by the SPI-2 TTSS (18). Additionally, SpiC obstructs vesicular trafficking in host cells, inhibiting SCV-lysosome fusion by targeting phosphoinositide 3-kinase (PI3K) activity (18, 19, 159). Specifically, SPI-2 inhibits trafficking of inducible nitric oxide synthase (iNOS) to the SCV, thus

increasing *S.* Typhimurium survival (84). Many of the SCVs only contain one bacterium and as *S.* Typhimurium replicates within an SCV, the vesicle divides along (40). SPI-2 also prevents co-localization of the SCV and NADPH oxidase (84). SifA maintains SCV integrity inside macrophages and induces formation of *Salmonella*-induced filaments (Sifs) (19, 174). SseI is recruited to the SCV and affects the migration of infected phagocytes (45). SseJ is an acyltransferase contributing to vacuole integrity and esterification of cholesterol in infected cells (45). *S.* Typhimurium use SseF and SseG to direct SCV positioning (30, 45). Collectively, the effector proteins encoded by SPI-2 allow *S.* Typhimurium to survive inside host cells.

S. Typhimurium flagella contribute to their virulence. Flagella enable *S.* Typhimurium to travel to intestinal epithelia after ingestion (34, 68). Since flagella participate in early stages of infection, flagellar gene expression gets down-regulated once *S.* Typhimurium are intracellular; this topic will be discussed in more detail later (8, 34, 68).

Additionally, fimbrial and non-fimbrial adhesins help *S.* Typhimurium to adhere, invade, and colonize the intestinal epithelia; furthermore, they contribute to persistence of the bacteria in mice (7, 164).

A few serovars of *Salmonella* contain serovar-specific virulence plasmids (34, 68, 94). *S.* Typhimurium and other medically relevant strains, generally harbor these plasmids (94). In *S.* Typhimurium, such a plasmid contributes to rapid growth and survival inside the host (94). Further, expression of plasmid-encoded genes inhibits cytokine production and release from infected cells, inducing an immunosuppressive environment (94, 101).

S. Typhimurium virulence gene expression is highly regulated. Expression of many *S.* Typhimurium virulence genes is regulated by the PhoP/PhoQ two-component regulatory system. This system controls the expression of more than 200 genes, and approximately 20% are required for virulence and stress resistance (119). PhoQ is a sensor of acidic pH and antimicrobial peptides, and PhoP is the transcriptional regulator (119). The PhoP/PhoQ regulon controls genes contributing to survival inside macrophages, importantly SPI-2 genes, and participates in structural changes of LPS, increasing the bacterium's resistance to macrophage killing (84, 119). Macrophages infected with PhoP$^-$ *S.* Typhimurium are more efficient at presenting antigens to T cells than macrophages infected with wild-type *S.* Typhimurium (178). *S.* Typhimurium lacking a functional PhoP/PhoQ system are highly attenuated *in vivo* (136).

G. Host response to *Salmonella*

Much of what is known about immunity to *Salmonellae* comes from experimental infection of mice with *S*. Typhimurium. Infection occurs when *S*. Typhimurium successfully uses its virulence factors to overcome clearance mechanisms of the host's immune system. The intestinal epithelium is the major physical barrier in the gut against *S.* Typhimurium infection, and Paneth cells produce anti-microbial peptides to prevent infection (84). If *S.* Typhimurium penetrates host intestinal epithelia, transcriptional re-programming occurs, and pro-inflammatory cytokines and chemokines are expressed (19, 84). These pro-inflammatory molecules recruit phagocytes (e.g., neutrophils, macrophages, and DCs) to the infected lamina propria (19). These early responding cells help to control the initial growth and spread of *S.* Typhimurium (35, 122, 142, 180). However, *S.* Typhimurium dampen the inflammatory response early

after infection (74). Resolution of a primary infection with *S.* Typhimurium is a synergistic effort of innate and T cell-mediated effects (35). T cells, particularly IFN-γ-producing $CD4^+$ T cells, play a key role in clearing infection of *S.* Typhimurium (59).

i. The innate immune response to *S.* Typhimurium

If *S.* Typhimurium invade the host epithelium on their basal-lateral surface, these cells secrete IL-8, attracting neutrophils to the infection site (36, 37, 44, 102). Accordingly, at early stages of infection, neutrophils and macrophages are important for controlling the initial growth and systemic spread of *S.* Typhimurium (35, 122, 142, 180). Specifically, neutrophils and macrophages control *S.* Typhimurium growth by utilizing reactive oxygen and nitrogen species inside their phagosomes (44, 97). *S.* Typhimurium may lose their flagella once the bacteria are bound to host cells, which may be ingested by DCs, leading to DC activation (8, 177). Once DCs are activated, they produce IL-8 and MIP-1α, further recruiting neutrophils and macrophages (177).

Neutrophils (characteristic in NTS) produce several pro-inflammatory cytokines, including TNF-α and IL-1β, that recruit (innate) immune cells to the site of infection (44). IL-18 and IL-1β help limit *S.* Typhimurium replication during early stages of infection by stimulating IFN-γ production from NK and NKT cells (148). IL-12 and IL-18 are secreted by activated macrophages, and these cytokines act independently and synergistically on NK, NKT, $CD4^+$ T, and $CD8^+$ T cells to further enhance production of IFN-γ; this in turn activates macrophages resulting in a positive feedback loop (35, 107, 148). DCs are important APCs during *S.* Typhimurium infection that efficiently engage cells of the adaptive immune response and are essential for initiating the primary immune response (182, 183).

Macrophage activation contributes to protective immunity against *S.* Typhimurium, and IFN-γ enhances the bactericidal mechanisms of macrophages (107). IL-12 production also contributes to polarization of T helper cells toward the T_H1 response, and these T_H1 cells are the main source of IFN-γ later in infection and in a recall response (35, 107). Since TNF-α together with IFN-γ activate macrophages and IL-12 is important in the T cell response, cumulatively these are important cytokines in resistance to wild-type *S.* Typhimurium infection (136). Additionally, host production of iNOS can be activated after sensing microbial products or in response to cytokines (i.e. IL-1, TNF-α, and IFN-γ) (84).

Many components of the innate immune system recognize *S.* Typhimurium by its PAMPs. The O-antigen of lipopolysaccharide (LPS) of *S.* Typhimurium is a PAMP recognized by complement component 3 (C3), which activates the alternative pathway of complement activation; additionally, LPS includes a lipid A moiety, which stimulates TLR4 (55, 157, 180). The flagellum, an appendage responsible for motility, of *S.* Typhimurium activate TLR5 (105, 157, 180). TLR5 is found on the basolateral surface of human intestinal epithelial cells and TLR4 is expressed on plasma membranes (180). Signaling through both TLR4 and TLR5 activates myeloid differentiation primary response gene 88 (MYD88), which is a common adaptor protein, to engage mitogen activated protein (MAP) kinase signal transduction pathways to initiate expression of pro-inflammatory genes (157).

ii. The adaptive immune response to *S.* Typhimurium

The immunity that eventually develops against *S*. Typhimurium requires both humoral and cell-mediated immune responses. Both B and T cells contribute to the

adaptive immune response to *S.* Typhimurium; moreover, both participate in control and clearance of the primary infection and in protection against a secondary infection. Much of what is known about the role of B and T cells in a *S.* Typhimurium infection was established by experiments where various lymphocyte populations were depleted via antibodies or adoptively transferred into naïve animals, or by studying *S.* Typhimurium infection in a gene-deficient mouse (107). While these experiments have provided valuable insight into the dynamics of the immune response in a *S.* Typhimurium infection, they may not accurately mimic a natural infection.

Studies conducted in mice deficient for various components of the T cell response including TCR-β-/- (lacking αβ T cells); TCR-δ-/- (lacking γδ T cells); H-2I-Aβ-/- (MHC class II deficient, lacking $CD4^+$ αβ T cells); and β2m-/- (MHC class I deficient, lacking $CD8^+$ T cells) demonstrated that $CD4^+$ αβ T cells play the largest role in resistance to *S.* Typhimurium infection (136). Further, mice deficient for $CD4^+$ or $CD8^+$ T cells, B cells, or IFN-γ succumb to infection, even with attenuated strains of *S.* Typhimurium (18, 39, 75, 97, 110).

Importantly, immunization studies conducted in susceptible strains of mice using a live, attenuated strain of *S.* Typhimurium have shown that both humoral and adaptive immunity contribute to protection (59). *S.* Typhimurium-specific $CD4^+$ T_H1 cells are crucial for the establishment of protective immunity (59). Further, $CD4^+$ T_H1 memory cells, $CD8^+$ T cells and anti-*S.* Typhimurium antibodies contribute to the resistance to *S.* Typhimurium challenge in immunized animals (97, 98). Additionally, adoptively transferring $CD4^+$ T cells, when compared to transfer of $CD8^+$ T cells, from immunized mice into naïve hosts results in better protection when the resulting mice are challenged

with virulent *S.* Typhimurium (107). However, depletion of either $CD4^+$ or $CD8^+$ T cells *in vivo* reduces the protection of immunized mice to subsequent challenge (110).

B cells are important in forming and establishing a T cell response to *S.* Typhimurium (97). Additionally, B cells are vital to the expansion of *S.* Typhimurium-specific memory T cells and a significant source of inflammatory cytokines, including IFN-γ (35, 110, 118). The communication between T and B cells helps develop a strong *S.* Typhimurium-specific antibody response (97). The specificity of the antibodies can be directed to both protein antigens and non-protein antigens, like LPS (107).

Furthermore, *S.* Typhimurium-specific immune sera can transfer protection to naïve mice from challenge with virulent *S.* Typhimurium; interestingly, neither IgA nor mucosal Ig are required (18, 75, 118). B cells are essential for the quick clearance of *S.* Typhimurium in challenged mice (118). While it has been established that B cells are fundamental for protective immunity (110, 118), it was recently shown that another important role of B cells in *S.* Typhimurium immunity is independent of antibody secretion but rather involved in the development of T cell protective immunity (118).

T cells are critical for effective control and eventual clearance of *S.* Typhimurium in a primary infection as well as in a secondary infection (35, 92, 107, 108, 166). Both $CD4^+$ and $CD8^+$ T cells are required for effective clearance of *S.* Typhimurium, and an enhanced T cell response is associated with a decrease in bacterial burden (74, 92, 97, 107). Stimulation via CD28 results in a protective immune response through IFN-γ secretion and antibody production (107). IFN-γ is an important cytokine in clearance of a *S.* Typhimurium infection and can be secreted by NK cells, macrophages, B cells, and T cells (107). Additionally, antibody depletion of $CD4^+$ T cells results in significantly

increased bacterial burden (74). Indeed, these results demonstrate a clear role for $CD4^+$ T cells for effectively combating a *S.* Typhimurium infection.

T cells are a major contributor in amplifying the immune response to *S.* Typhimurium infection, and T cell depletion results in a diminished inflammatory response (56). In the cecal mucosa, T cells are stimulated (by macrophages and DCs) to produce IL-22, IL-17, and IFN-γ (56). Even after production of IFN-γ by T cells, it takes a number of days to clear the *S.* Typhimurium infection (107).

Numerous studies document that *S.* Typhimurium infection results in a T_H1 response, illustrated by abundant IFN-γ secretion; interestingly, a few imply that attenuated *S.* Typhimurium can cause a T_H2 response, illustrated by IL-4 and IgE production (107). Conversely, mice deficient for IL-4 are more resistant to *S.* Typhimurium infection than wild-type control mice (107). This suggests that production of IL-4 may not be protective, but instead it could hinder control of the infection (107).

$CD8^+$ T cells contribute to clearance of *S.* Typhimurium because they are able to lyse infected cells, releasing the bacteria into the extracellular space, where *S.* Typhimurium become susceptible to ingestion by phagocytes (107). $CD8^+$ T cells secrete cytokines, which also contribute to *S.* Typhimurium clearance (107).

T cell responses to *S.* Typhimurium are not well characterized and pathogen-derived epitopes recognized by T cells are only now being identified. $CD4^+$ T cells recognize different antigens of *S.* Typhimurium throughout the course of a primary infection (90). During early stages of infection, $CD4^+$ T cells, specific for FliC, secreting IFN-γ are observed (90). These T cells are found in the intestinal tissues, and interestingly these T cells do not appear to control the bacterial colonization and are not

found in the spleen or liver (90). During later stages of infection, there are even more $CD4^+$ T cells secreting IFN-γ, and these T cells recognize the TTSS effectors SseI and SseJ and fail to detect flagellin (90). The $CD4^+$ T cells specific for SseJ and SseI are found in the systemic sites of spleen and liver (90). This correlates with *S.* Typhimurium continually changing its gene expression profile during distinct phases of infection. This may be explained because once *S.* Typhimurium is intracellular, it no longer expresses flagellin and begins to up-regulate its SPI-2 TTSS (8, 17-19, 90). Since *S.* Typhimurium continually modifies its expression profile to adapt to different environments it encounters, the specificity of the immune response must also evolve, though it may lag behind that of the bacterium, resulting in a delayed response.

H. Manipulation of the mammalian immune responses by *Salmonella*

In susceptible strains of mice, *S*. Typhimurium induce acute immunosuppression and delay onset of protective adaptive immune responses, yet the mechanisms of this immunosuppression remain largely unknown. The bacteria are able to blunt the inflammatory response shortly after infection. *S*. Typhimurium are able to evade killing by phagocytes and inhibit cell-mediated adaptive immunity; this aids in their long-term survival and persistence. Additionally, it takes six weeks to clear a vaccine strain of *S.* Typhimurium and two or three months for immunological memory to form (75, 107, 165).

i. Manipulation of the innate immune response

a. *S.* Typhimurium evade recognition by phagocytes

Following internalization by host cells, *S.* Typhimurium remodel the LPS on their surface by shortening the length of the O-antigen and changing the number of acyl

chains; additionally, there are changes in protein composition in both the outer and inner membrane during growth inside macrophages (71). Furthermore, *S.* Typhimurium alter the composition of their peptidoglycan during intracellular growth (134), which allows *S.* Typhimurium to avoid recognition by TLR2. *S.* Typhimurium swap between two non-allelic forms of their surface flagellin protein by genetic phase variation, allowing them to switch between two antigenically distinct proteins (18).

b. *S.* Typhimurium survive and replicate inside phagocytes

S. Typhimurium survive and replicate in both macrophages and DCs (16, 137, 155, 183). *S.* Typhimurium alter their gene transcription profile once inside phagocytes allowing them to express genes required for intracellular survival and turn off genes that are no longer required (18, 71). A large number of genes, including the genes encoded by SPI-2, are required for *S.* Typhimurium to survive inside phagocytes. SPI-2 effectors injected into the cytoplasm of host cells prevent the fusion of the SCVs with lysosomes, resulting in increased intracellular bacterial survival (159, 160). *S.* Typhimurium not only inhibit SCV fusion with lysosomes, but also inhibit lysosome biogenesis to prevent the generation of this degradative organelle (40, 83). The inhibition of phagosome-lysosome fusion and evasion of endolysosomal proteases are strategies used by *S.* Typhimurium to interfere with antigen processing and presentation by professional antigen presenting cells (160). Recently, it was shown that SifA reduces the potency of the lysosomes by subverting the hydrolytic lysosomal enzymes while retaining lysosomal membrane glycoproteins in the SCV membrane, thereby attenuating lysosome function, which increases *S.* Typhimurium growth (103). SPI-2 effectors also

inhibit host NADPH oxidase delivery to the SCV (51). *S.* Typhimurium use the effector SrfH to increase phagocyte migration (18). DCs have been described as 'perfect Trojan horses' in which *S.* Typhimurium can survive, move to deeper tissues, and actively suppress the adaptive immune response (27).

Additionally, *S.* Typhimurium mutants unable to survive within macrophages quickly get cleared from mice, indicating intracellular survival is important for virulence (148). Intracellular *S.* Typhimurium are protected from reactive oxygen and nitrogen radicals inside macrophages (43, 171). *S.* Typhimurium up-regulate arginase, the competing enzyme of iNOS; this removes the substrate (L-arginine) from iNOS inside the macrophage reducing the ability these cells to kill *S.* Typhimurium (84). *S.* Typhimurium induces immunosuppression by stimulating NO production in a PhoP-dependent manner by phagocytes and NK cells, ultimately dampening the T cell response (18, 93, 170). Additionally, viable, intracellular *S.* Typhimurium induce iNOS expression and production of NO by DCs, ultimately suppressing T cell proliferation after antigen presentation by MHC molecules via a SPI-2-dependent mechanism (27, 159).

c. *S.* Typhimurium kill phagocytes

S. Typhimurium utilize their TTSSs to actively kill phagocytes. Early studies demonstrated that mutants deficient for invasion genes, *sipD* and *hilA*, failed to kill macrophages, implicating these genes in "early and rapid cytotoxicity" of macrophages (61, 91, 116). Later studies showed a requirement of the SPI-1 effector SipB, as well caspase-1, for *S.* Typhimurium-induced macrophage killing (13, 61). Now it is accepted that *S.* Typhimurium kill macrophages by at least two separate mechanisms, activating

caspase-1 and caspase-2 which cooperatively induce apoptosis (61, 72, 91, 113, 115, 116, 167). Caspase-1 and -2 are part of the cysteine-containing aspartate-specific proteases, which contribute to apoptosis and secretion of pro-inflammatory cytokines (80, 146). Similarly, *S.* Typhimurium kill DCs using the SPI-1-encoded TTSS to induce caspase-1 inside host cells (168). Indeed, *S.* Typhimurium contribute to whether the host cell lives or dies, depending on the cell type and stage of infection.

d. *S.* Typhimurium inhibit antigen presentation

S. Typhimurium can also inhibit antigen presentation by MHC class I and class II molecules (133, 160). The proteins encoded by the *yej* operon interfere with antigenic peptides being loaded on phagosomal MHC class I (133). Mice infected with *yej* mutant *S.* Typhimurium have an enhanced CD8+ T cell response compared to mice infected with wild-type *S.* Typhimurium, suggesting that *yej* mutant *S.* Typhimurium may be a better vaccine strain (133).

S. Typhimurium suppress surface expression of and antigen presentation by MHC class II molecules via a SPI-2-dependent mechanism (27, 106, 160). *S.* Typhimurium use their effector proteins to inhibit endosomal trafficking and lysosomal fusion, which reduces the expression of MHC molecules on the surface of DCs (18, 19). *S.* Typhimurium targets mature, but not immature, MHC class II from the cell surface for ubiquitination, thereby tagging it for proteasomal degradation (85). Further, *S.* Typhimurium deficient for SPI-2 cannot inhibit antigen presentation by DCs to MHC class II-restricted T cells (27, 159). SPI-2 contributes to decreasing the availability of bacterial antigens in the context of MHC molecules resulting in a poor T cell response (19).

Furthermore, the subunit of flagellin of *S.* Typhimurium may be "bait" for DCs, as it has been suggested that flagellin is a surface protein that may also be shed or sheared from the bacterium (8, 104). DCs ingest, process, and present flagellin-derived antigen (FliC subunit) in the context of MHC class II; this results in $CD4^+$ T cells that recognize FliC peptide (8, 104). Also contributing to this phenomenon is the fact that once *S.* Typhimurium are inside host cells, there is reduced production of flagellin because the bacterium no longer needs to be motile (8). This contributes to *S.* Typhimurium evading detection by the FliC-specific $CD4^+$ T cells.

ii. Manipulation of the T cell Immune response

A significant contribution to understanding *S.* Typhimurium-induced T cell immunosuppression was made in 2010. Johanns et al. demonstrated that T reg cells have increased suppressive capabilities, by expressing Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), early in *S.* Typhimurium infection, which allows the bacteria to establish infection (74). Eventually, an effective T cell response is mounted, which correlates with both the diminished suppressive abilities of T reg cells and decreased bacterial burden (74). Further, the authors showed that depletion of T reg cells, or blocking CTLA-4 with antibodies, early in infection increases T cell activation allowing for efficient bacterial clearance (74). CTLA-4 is a protein receptor that can dampen the immune response. These results suggest that the capacity of the T reg cells to inhibit the T cell response dictates the dynamics of a *S.* Typhimurium infection from establishment to persistence or clearance (74). This study provides insight into how *S.* Typhimurium inhibit the T cells response, but yet not much has been described about what bacterial genes are required for T cell inhibition.

a. *S.* Typhimurium cause immunosuppresion of T cells

A useful tool to study how *S.* Typhimurium inhibit T cells involves the use of T cells from transgenic mice, which have a TCR specific for one antigen. Conducting adoptive transfer studies using these T cells generates an 'artificial' setting because generally there is observation of this manipulated population, without characterizing the endogenous T cell population. However, these studies have allowed for important, but limited, insights into the complex process of *S.* Typhimurium-induced immunosuppression.

Short exposure to a *S.* Typhimurium-infected environment *in vivo* is sufficient to suppress an adoptively transferred population of naïve T cells (104, 151). In another study, which used adoptively transferred T cells specific for ovalbumin (OVA) peptide displayed by either MHC I or MHC II, there was decreased activation of these T cells when the peptide was expressed by *S.* Typhimurium as compared to a OVA peptide control (18, 150, 151, 159). Increased intracellular growth of *S.* Typhimurium increases the number of $CD8^+$ T cells, but decreases their effector function (139). Additionally, *S.* Typhimurium eradicate adoptively transferred, antigen-specific $CD4^+$ T cells via a SPI-2-dependent mechanism after their expansion, during the establishment of immunological memory (39, 152). The bacteria specifically target those $CD4^+$ T cells with the highest avidity (39).

S. Typhimurium limit the *in vivo* proliferation of both $CD4^+$ and $CD8^+$ T cells, even though the T cells may be activated (108). A delayed T cell response correlates with an increase in bacterial burden (74). Additionally, the suppressive functions of T reg cells

contribute to the overall *S.* Typhimurium-induced immune suppression at early time points (74).

Furthermore, there is inhibition of the $CD4^+$ T cell responses in cultures of splenic cells taken from *Salmonella*-infected mice (38). *S.* Typhimurium LPS can inhibit T cell function *in vivo* by decreasing IL-2 and TNF-α production; interestingly, this is not observed in uninfected mice co-injected with LPS and OVA peptide, suggesting that another factor also contributes to T cell inhibition (151).

S. Typhimurium derived inhibitor of T cell proliferation (STI) was described to be a potent inhibitor of T cells (3, 99, 100). STI prevents proliferation of activated T cells as well as production of IL-2 and IFN-γ (99, 100).

Collectively, these results indicate *S.* Typhimurium induce a potent immunosuppressive environment, which leads to lymphocyte depletion, reduced activation, and diminished effector function, but many of the mechanisms by which *S.* Typhimurium cause immunosuppression remain largely undefined.

b. *S.* Typhimurium directly inhibit T cells

Previously we have shown, as have others, that *S.* Typhimurium are able to kill APCs, thereby preventing antigen presentation to T cells (13, 72, 91, 113, 115, 165, 168). However, when mutant *S.* Typhimurium (unable to kill DCs) is cultured with DCs, there is still no T cell proliferation (165). This implies that *S.* Typhimurium-induced DC death alone is not responsible for the lack of T cell proliferation. Additionally, our laboratory demonstrated that when T cells are stimulated through ligation of the TCR, the T cell proliferation that generally occurs does not happen when *S.* Typhimurium are present (Figure 5 (165)). Furthermore, this inhibition of T cell proliferation correlates

with down-modulation of TCR-β, a receptor required for antigen recognition and T cell function (Figure 5 (166)). We determined that a proteinaceous factor, which is present in the supernatant from the co-culture of T cells and *S.* Typhimurium, is responsible for T cell inhibition (165, 166). This potentially novel inhibitor may be produced and secreted by *S.* Typhimurium or induced by *S.* Typhimurium but produced and secreted by the T cells. The studies of this dissertation characterize the identity, origin, and mechanism of this inhibitory factor.

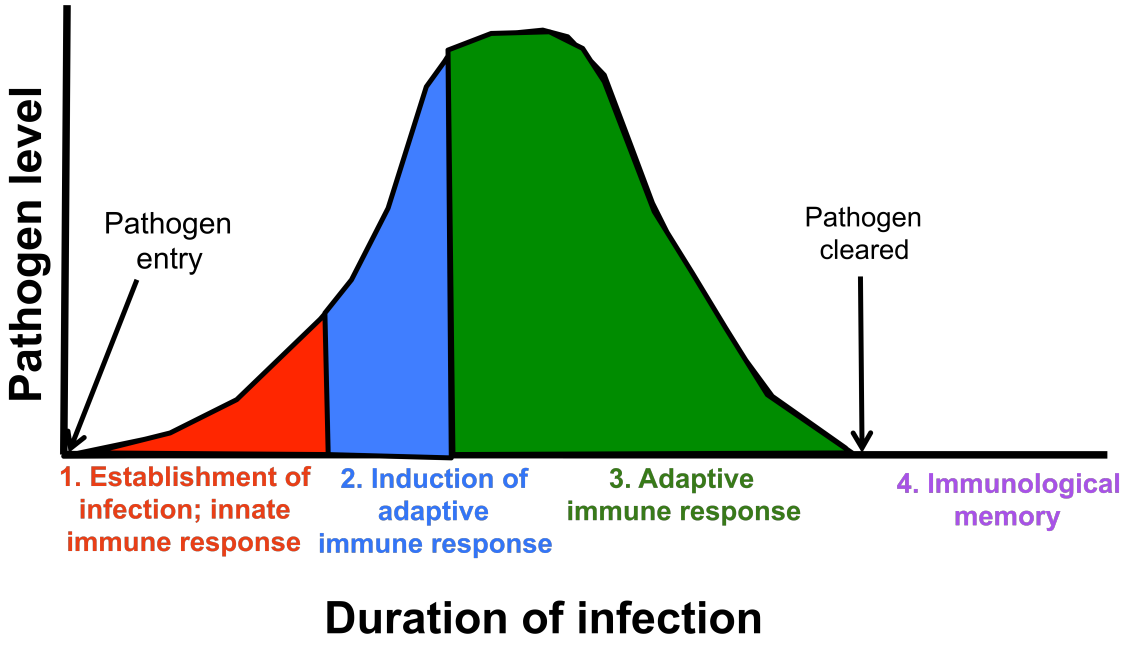

Figure 1 Dynamics of a primary adaptive immune response

When a pathogen successfully gains access to its host, the immune system starts its response against the invader. The first responding cells are those of the innate immune system. If the innate immune system is not able to clear the pathogen, there is establishment of infection. During this the process, cells of the innate immune system engage the lymphocytes of the adaptive immune and there is antigen-specific clonal expansion of these cells. As the adaptive immune system eradicates the infection, there is contraction of the adaptive immune. A small population of antigen-specific lymphocytes persists and contributes to immunological memory.

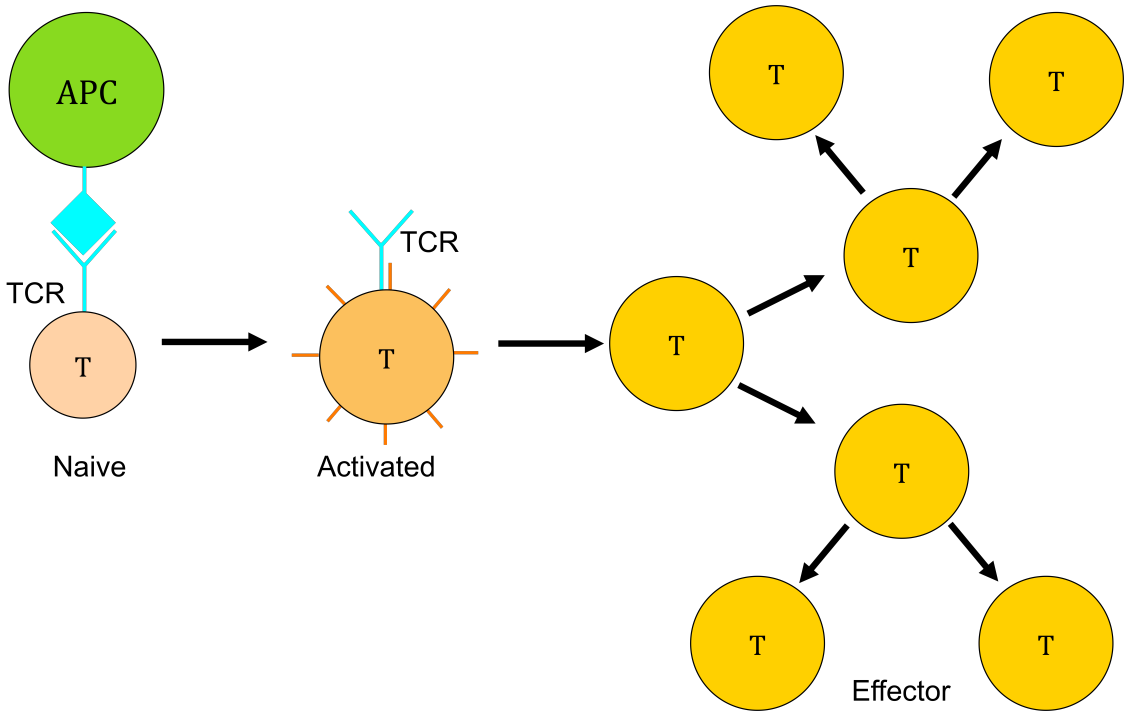

Figure 2 Steps of T cell priming

T cell priming is an important component of inducing T cell-mediated adaptive immunity. It includes the activation, proliferation, and differentiation of naïve T cells into effector T cells. A pathogen-derived peptide is presented by an antigen presenting cell through either MHC class I or class II to a naïve T cell. This engagement between the antigen and the T cell receptor activates the T cell. This is the first step in T cell clonal expansion; activated T cells increase in size and granularity, a process known as blastogenesis, before proliferating (47, 95, 184). The activated T cell can proliferate and differentiate into effector T cells, which recognize a specific antigen and may be capable of producing cytokines or directly lysing infected cells.

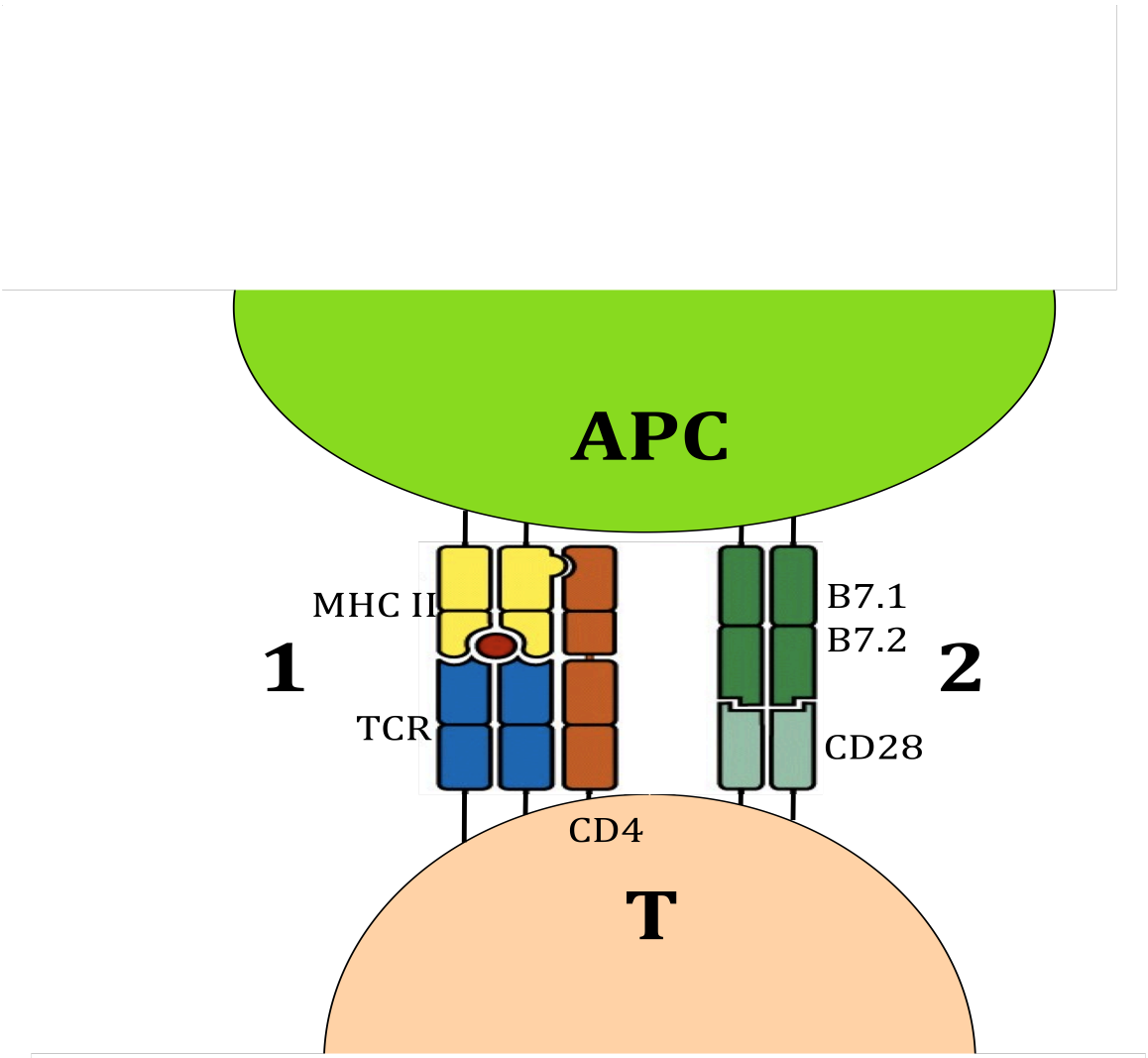

Figure 3 T cells need two signals for their activation

The first signal is stimulation of the TCR, and in this illustration also a CD4 co-receptor, through the binding of the cognate antigen (red circle) in the context of MHC. The second signal comes from co-stimulatory molecules on the surface of the same APC; in this example CD28 on the surface of the naïve T cell engages B7 molecules. The delivery of the second signal allows for optimal activation and proliferation of the T cell.

Figure adapted from Janeway's Immunobiology, 7th edition (117).

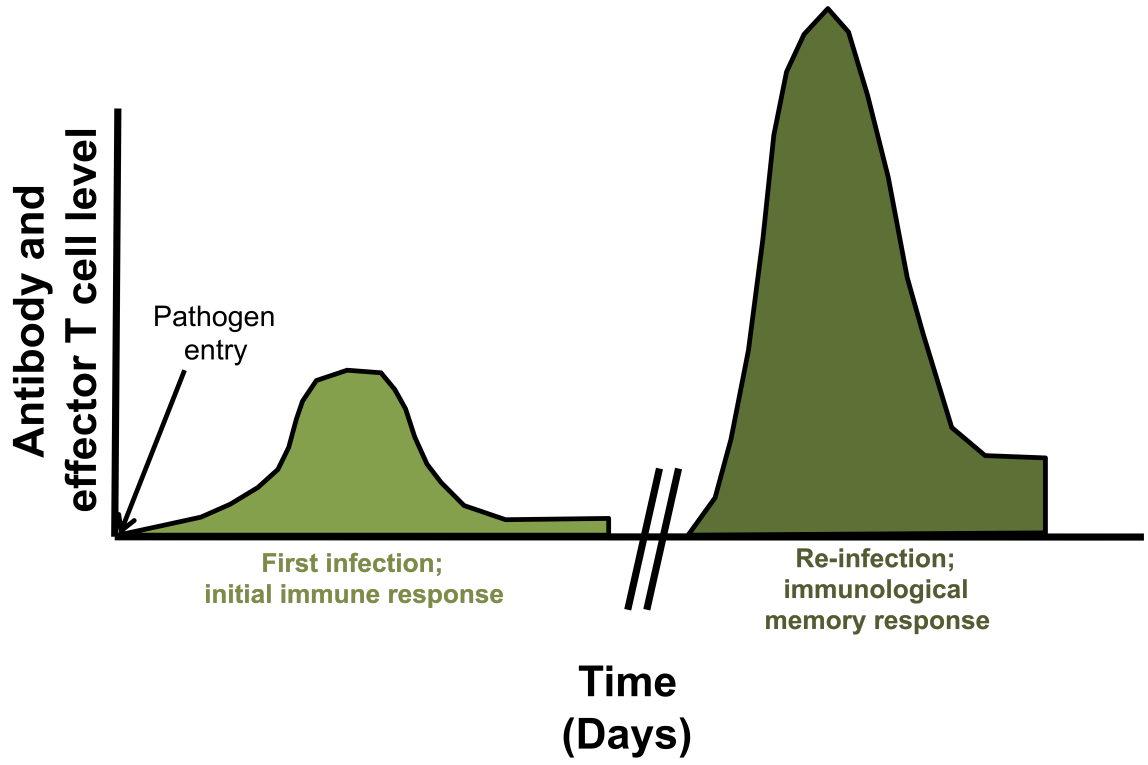

Figure 4 A recall response is a faster, more robust response than the initial response

When the adaptive immune response first encounters an antigen, it takes days to weeks to develop an antigen-specific response. Upon subsequent re-exposure, which can be years later, antigen-experienced lymphocytes initiate a quicker, more robust immune response. This leads to faster clearance of the pathogen.

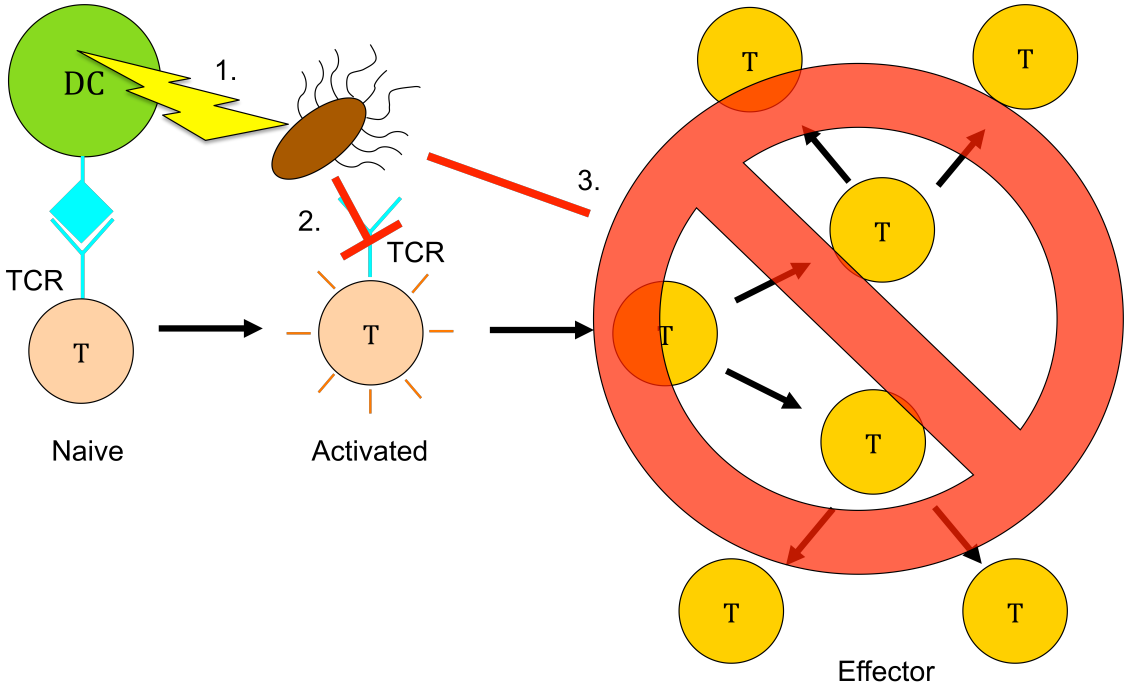

Figure 5 Aspects of *Salmonella*-induced T cell inhibition

S. Typhimurium are able to inhibit T cells. Some of these mechanisms include: 1) killing antigen presenting cells by triggering programmed cell death and thereby inhibiting antigen presentation by the dendritic cells and subsequent T cell proliferation (168); 2) down-modulating TCR-b surface expression on T cells (166); and 3) inhibiting T cell proliferation (165).

SPECIFIC AIMS

Salmonellae are a leading and increasing cause of worldwide morbidity and mortality in humans. Infections with *Salmonellae* range in severity from self-limiting gastroenteritis to typhoid fever. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) cause gastroenteritis in humans. In mice, *S.* Typhimurium cause a systemic infection and enteric fever (107). Much of what is known about immunity to *Salmonellae* comes from experimental infection of mice with *S.* Typhimurium.

T cells play an important role in adaptive immunity to *Salmonella.* However, T cell-mediated immunity to *Salmonella* takes months to develop and has been described as 'dampened and delayed'. Thus, a greater understanding of T cell-mediated immunity to *Salmonella* is needed to develop new and more effective interventions. Therefore, we have been analyzing the role of T cells in immunity to *Salmonella*.

We previously showed that *S.* Typhimurium act directly on T cells to inhibit their proliferation and down-modulate TCR expression. Additionally, we found that a proteinaceous inhibitor of T cells was secreted into the medium when T cells were co-cultured with *S.* Typhimurium. Based on these results, we sought to identify if this inhibitory factor was produced or induced by *S.* Typhimurium. We used a genetic approach to identify the *S.* Typhimurium genes required for T cell inhibition. This research characterizes the identity and function of this inhibitory factor.

MATERIALS AND METHODS

I. Culture of bacteria

A. Live *Salmonella enterica* serovar Typhimurium

S. Typhimurium strain 14028 (American Type Culture Collection) and isogenic, spontaneous nalidixic acid-resistant derivative *S*. Typhimurium strain IR715 (153) were used as WT strains. *S*. Typhimurium were grown aerobically at 37ºC in Luria-Bertani (LB) broth or on LB agar.

Targeted, kanamycin-resistant multi- and single-gene deletion mutants were constructed using the lambda red recombinase method (33), as described previously (141). Multi-gene deletion mutants were designed to avoid genes known to be essential for growth on rich medium (unpublished resource, M. McClelland and H. Andrews-Polymenis). Each mutation removed a variable number of genes, depending on the nature of the surrounding genes. For mouse infection experiments, mutations were moved from *S*. Typhimurium strain 14028 into *S*. Typhimurium strain IR715 by P22-mediated transduction.

To generate bacterial growth curves, *S*. Typhimurium were grown aerobically at 37ºC in LB broth for 18 hours. The resulting overnight cultures were diluted 1:100 in LB broth or tissue culture medium and grown aerobically at 37ºC to stationary phase (~7 hours). The optical density at 600 nm (OD_{600nm}) was measured every 30 minutes.

B. Treated *Salmonella enterica* serovar Typhimurium

Where noted, *S*. Typhimurium were grown aerobically at 37ºC in LB broth or on LB agar as above. Prior to infection, *S*. Typhimurium were fixed with Fixation Buffer

(BioLegend) for 30 minutes and washed to remove any residual fixative. Additionally, prior to infection, some *S*. Typhimurium were treated with chloramphenicol (100 μg/ml) for 2 hours.

C. Plasmid construction and use

Plasmids encoding *STM3106* were constructed to complement the phenotype of *ΔSTM3106 S*. Typhimurium or to purify C-terminal, hexahistidine (6xHis)-tagged L-asparaginase II from *ΔSTM3106 S*. Typhimurium. The *STM3106* open reading frame was amplified from the chromosome of *S*. Typhimurium strain 14028 by PCR using primers 5'-GCTAGCGAAGAGATATACCATGGAGTTTTTCAGGAAAACGGC-3' and 5'-TCTAGATTAATACTGATTGAACATCGTCTGG-3' or 5'-GCTAGCGAAGAGATATACCATGGAGTTTTTCAGGAAAACGGC-3' and 5'-TCTAGATTATTAGTGGTGGTGGTGGTGGTGATACTGATTGAACATCGTCTGG-3', respectively. Restriction sites for endonucleases *NheI* and *XbaI* were incorporated into the primer sequences to facilitate cloning via pCR®2.1 (Invitrogen) into expression plasmid pBAD18-Cm (American Type Culture Collection), immediately downstream of the inducible arabinose promoter. The resulting constructs (pBAD-*STM3106* and pBAD-*STM3106*His) or empty vector were introduced into *ΔSTM3106 S*. Typhimurium by electroporation. Transformants were selected using LB agar supplemented with chloramphenicol (30 μg/ml). To trans-complement the phenotype of *ΔSTM3106 S*. Typhimurium *in vivo*, the *STM3106* open reading frame with 200 base pair upstream region was amplified from the chromosome of *S*. Typhimurium strain 14028 by PCR using primers 5'-GCGCGCAGCCTGAAGTAATCTTTCC-3' and 5'-CGCGCGTTAATACTGATTGAACATCGTCTGG-3' and cloned into pCR®2.1. The

resulting plasmid was digested with *EcoRI* and a ~1.2 kb fragment was cloned into pWSK29 (173). The resulting construct (pWSK29-*STM3106*) was introduced into *ΔSTM3106 S*. Typhimurium by electroporation. Transformants were selected using LB agar supplemented with carbenicillin (100 mg/ml).

II. Immune cell enrichment, purification and isolation

A. T cell enrichment

Splenocytes harvested from C57BL/6J mice were used as a source of T cells. Following treatment of splenocytes with ACK lysing buffer (Invitrogen) to lyse red blood cells, CD90.2-conjugated MACS microbeads and magnetic separation columns (Miltenyi Biotec) were used for the enrichment of T cells. Enriched populations of T cells were suspended in RP-10 (i.e., RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, HEPES, and 50 μM 2-mercaptoethanol) and used in T cell assays.

B. T cell purification

All of the antibodies used in this section were from BioLegend. Splenocytes harvested from C57BL/6J mice were stained in the presence of Fc block (clone 93) using allophycocyanin (APC)-conjugated anti-mouse CD90.2 (clone 30-H12), and/or peridinin chlorophyll (PerCP)-conjugated anti-mouse CD4 (clone RM4-5), and/or PE-Cy7-conjugated anti-mouse CD8α (clone 53-6.7). $CD90.2^+$ cells were purified using a FACSAria flow cytometer (BD Biosciences). Additionally, $CD90.2^+$ cells that also were $CD4^+$ or $CD8^+$ were purified. Purified populations of T cells were suspended in RP-10 and used in T cell assays.

C. Macrophage isolation

Bone marrow-derived macrophages were cultured from C57BL/6J mice and 129X1/SvJ mice as described previously (132). Briefly, bone marrow cells isolated from the femurs of C57BL/6J mice and 129X1/SvJ mice were suspended in medium (i.e., DMEM with high glucose (4,500 μg/ml) (Invitrogen) supplemented with 20% heat-inactivated FBS (HyClone), 30% L-cell conditioned medium, 2 mM L-glutamine, and 1 mM sodium pyruvate) and seeded into 100 mm diameter petri dishes at $4x10^6$ cells per plate. After 5 days of incubation at 37ºC/5% CO_2, macrophages were collected and used for infection assays.

III. T cell assays

A. Analysis of TCR-β expression

S. Typhimurium-induced or L-asparaginase II-induced down-modulation of TCR-β, suppression of T cell blastogenesis and inhibition of cytokine production were measured by flow cytometry. Briefly, T cells were seeded at $1x10^5$ cells per well (96-well format) in tissue culture plates coated with 5 μg/ml of anti-CD3ε (clone 145-2C11, BioLegend). T cells were cultured in the absence or presence of bacteria at a multiplicity of infection of ~50 or purified L-asparaginase II from either *S.* Typhimurium or *E. coli* (Sigma). After 2 hours of incubation at 37ºC/5% CO_2, T cells were pelleted by centrifugation and resuspended in medium supplemented with penicillin/streptomycin (200 units/ml for penicillin and 200 μg/ml for streptomycin) and gentamicin (50 μg/ml), killing all bacteria within two hours. After an additional 18-20 hours of incubation at 37ºC/5% CO_2, T cells were harvested, stained and analyzed by flow cytometry. T cells

treated with conditioned medium or purified L-asparaginase II were incubated for 18-20 hours in the presence of antibiotics, as above.

Where noted, medium was supplemented with chloramphenicol (100 μg/ml), L-asparagine (10 mM), L-aspartate (10 mM), or L-glutamine (10 mM).

B. Analysis of T cell proliferation

S. Typhimurium-induced inhibition of T cell proliferation was measured by flow cytometry. Briefly, T cells enriched from mouse spleens were labeled with 5 μM CFSE (Invitrogen) and seeded at $1x10^5$ cells per well (96-well format) in tissue culture plates coated with 5 μg/ml of anti-CD3ε. CFSE is distributed evenly among daughter cells with each round of cell division, resulting in a measurable reduction in cell fluorescence (123). T cells were left untreated or infected with bacteria at a multiplicity of infection of ~50. After 2 hours of incubation at 37ºC/5% CO_2, T cells were pelleted by centrifugation and resuspended in medium supplemented with penicillin/streptomycin (200 units/ml for penicillin and 200 μg/ml for streptomycin) and gentamicin (50 μg/ml). After an additional 3 days of incubation at 37ºC/5% CO_2, T cells were harvested, stained and analyzed by flow cytometry.

IV. Cell staining and analysis by flow cytometry

All antibodies and reagents described in this section were purchased from BioLegend. Data were acquired and analyzed using a FACSCalibur or FACSAria flow cytometer (both BD Biosciences) with CellQuest™ Pro (BD Biosciences) or FlowJo (Tree Star) software. Forward scatter (FSC) and side scatter (SSC) analysis was used to determine size and granularity of live cells.

A. Staining surface markers

Routinely, cells were stained in the presence of Fc block (clone 93) using allophycocyanin (APC)-conjugated anti-mouse CD90.2 (clone 30-H12), phycoerythrin (PE)-conjugated anti-mouse CD25 (clone PC61), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse TCR-β (clone H57-597). Live cells expressing CD90.2, CD25 and TCR-β were identified as anti-CD3ε-stimulated T cells, unless otherwise noted. Additionally, cells were stained in the presence of Fc block using APC-conjugated anti-mouse CD90.2, APC-cyanine 7 (APC-Cy7)-conjugated anti-mouse CD25, pacific blue (PacBlue)-conjugated anti-mouse CD69 (clone H1.2F3), peridinin chlorophyll (PerCP)-conjugated anti-mouse CD4 (clone RM4-5), PE-Cy7-conjugated anti-mouse CD8α (clone 53-6.7) and FITC-conjugated anti-mouse TCR-β. Cells analyzed directly ex vivo were stained in the presence of Fc block using APC-conjugated anti-mouse CD90.2, PE-conjugated anti-mouse CD44 (clone IM7), FITC-conjugated anti-mouse CD62L (clone MEL-14), PerCP-conjugated CD4 or APC-conjugated anti-mouse CD8α.

B. Staining intracellular cytokines

To detect production of intracellular cytokines, cells were cultured for 6 hours in the presence of the intracellular protein transport inhibitor brefeldin A, which causes intracellular accumulation of proteins in the rough endoplasmic reticulum or Golgi complexes. Next, cells were stained as described above, fixed, permeabilized and stained again in the presence of Fc block using PE-conjugated anti-mouse IFN-γ (clone XMG1.2) or IL-2 (clone JES6-5H4).

C. Analysis of T cell blastogenesis

Blastogenesis was calculated by determining the percentage of live cells that, in response to treatment with anti-CD3ε, had transformed from small lymphocytes into larger, more granular cells resembling blast cells, as assessed by flow cytometry.

V. Immunodepletion of L-asparaginase II from supernatant

Conditioned medium (1.75 ml) from T cells cultured in the absence or presence of *S*. Typhimurium was passed through a 0.22 μm filter. Resulting samples were aliquoted into 250 μl fractions that were pre-cleared using 25 μl of protein A-coupled sepharose beads (Sigma), with agitation. After 1 hour of incubation at 4ºC, beads were pelleted by centrifugation and supernatants were collected. Where noted, 22.5 μg of polyclonal rabbit anti-*Escherichia coli* L-asparaginase II antibody or rabbit anti-mouse IgG control antibody (both Abcam) was added to the precleared supernatants. L-asparaginase II of *E. coli* is 96% identical to L-asparaginase II of *S*. Typhimurium at the amino acid level. After overnight incubation at 4ºC, with rotation, 25 μl of protein A-coupled sepharose beads were added to each sample. After an additional 3 hours of incubation at 4ºC, with agitation, beads were pelleted by centrifugation and the resulting supernatants were used in T cell assays.

VI. *In vitro* invasion assays

A. T cell invasion assay

Bacterial invasion of T cells was evaluated using a standard gentamicin protection assay. Briefly, T cells were cultured in the absence or presence of bacteria as described above. After 2 hours of incubation at 37ºC/5% CO_2, T cells were pelleted by centrifugation and resuspended in medium supplemented with gentamicin (25 μg/ml)

to kill all extracellular bacteria. After an additional hour of incubation at 37ºC/5% CO_2, T cells were washed three times with 1 ml of PBS and lysed using 0.5 ml of Triton X-100 (0.1%) to release intracellular bacteria. These bacteria were enumerated by plating onto LB agar.

Bacterial invasion was calculated by determining the percentage of live *S*. Typhimurium recovered from the wells. To determine the percentage of T cells infected with *S*. Typhimurium, T cells were cultured in the absence or presence of green fluorescent protein-expressing *S*. Typhimurium as above. After 2 hours of incubation at 37ºC/5% CO_2, T cells were pelleted by centrifugation, washed and resuspended in medium supplemented with gentamicin (25 μg/ml). After an additional hour of incubation at 37ºC/5% CO_2, T cells were harvested, stained and analyzed by flow cytometry.

B. Macrophage infection assay

Macrophage infections were performed using a standard gentamicin protection assay. Briefly, bone marrow-derived macrophages or RAW264.7 macrophage-like cells were suspended in medium (same as IIC except that concentrations of FBS and L-cell conditioned medium were reduced to 10% and 15%, respectively) and seeded at $1.5x10^5$ cells per ml per well for the primary macrophages into a 24-well tissue culture plate or $2.5x10^5$ cells per ml per well for the RAW264.7 cells. After overnight incubation at 37ºC/5% CO_2, the medium was replaced with 0.5 ml of fresh medium and macrophages were infected with bacteria at a multiplicity of infection of 1. Upon addition of bacteria, the plate was centrifuged for 5 minutes at 1,000 rpm to facilitate bacterial contact with macrophages. After 20 minutes of incubation at 37ºC/5% CO_2,

the wells were washed three times with 1 ml of PBS to remove non-cell-associated bacteria, and fresh medium supplemented with gentamicin (25 μg/ml) was added to each well to kill extracellular bacteria. This is referred to as the 0 hour time point. After 1, 3, 6, 9 and 24 hours of incubation at 37ºC/5% CO_2, the wells were washed three times with 1 ml of phosphate-buffered saline (PBS), and the macrophages were lysed using 0.5 ml of Triton X-100 (0.1%) to release intracellular bacteria. These bacteria were enumerated by plating onto LB agar. To determine the number of cell-associated bacteria at the 0 hour time point, macrophages were lysed immediately following the washes with PBS, after which bacteria were enumerated by plating onto LB agar.

VII. Polyacrylamide gel electrophoresis and Western blot analysis

Conditioned medium harvested from T cells cultured in the absence or presence of WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium or L-asparaginase II was subjected to trichloroacetic acid precipitation for 1 hour (on ice). The resulting samples were centrifuged for 15 minutes at 13,500 rpm (4ºC), after which supernatants were removed and pellets were washed three times with 500 μl of ice-cold acetone. Next, samples were suspended in 50 μl of Laemmli sample buffer (2x), boiled for 15 minutes, centrifuged for 10 minutes and loaded onto an 8% SDS-polyacrylamide gel. Following gel electrophoresis, proteins were transferred onto a nitrocellulose membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell wet-transfer system (Biorad). L-asparaginase II was detected by Western blot using polyclonal rabbit anti-*E. coli* L-asparaginase II antibody diluted 1:10,000 (Abcam), goat anti-rabbit IgG-horse radish peroxidase (HRP) secondary antibody (Jackson) and chemiluminescence (Biorad). The chemiluminescence was detected using film.

Bacterial whole cell lysates were used to determine if L-asparaginase II is expressed under conditions used here for extracellular growth of *S*. Typhimurium. Briefly, a 1 ml aliquot of an overnight culture was subjected to centrifugation (1 minute at 13,500 rpm), after which supernatants were removed and bacterial pellets were frozen at -80ºC. The resulting samples were thawed on ice, suspended in 50 μl of Laemmli sample buffer (2x), boiled for 15 minutes, centrifuged for 10 minutes at 13,000 RPM and loaded onto a 12% Mini-PROTEAN TGX gel (Biorad). Following gel electrophoresis, proteins were transferred onto a nitrocellulose membrane as described above. L-asparaginase II was detected by Western blot using polyclonal rabbit anti-*E. coli* L-asparaginase II antibody diluted 1:3,000, anti-rabbit IgG-infrared (IR) Dye 800 secondary antibody (Rockland) and an Odyssey infrared imager (Li-COR Biosciences).

VIII. Purification of L-asparaginase II

Nickel-affinity chromatography was used to purify C-terminal, 6xHis-tagged L-asparaginase II from *E. coli* strain LMG194 (Invitrogen) carrying plasmid pBAD-*STM3106*His. Briefly, bacteria were grown in Terrific Broth supplemented with chloramphenicol (34 μg/ml) until the OD_{600} reached 1.0, when L-arabinose was added to the culture at a final concentration of 0.1% (w/v) to induce expression of 6xHis-tagged L-asparaginase II. After 20 hours of induction, bacteria were pelleted by centrifugation and the supernatant was harvested and stored at 4°C. The supernatant was diluted 1:1 with water and applied onto a 5 ml HisTrap FF column (GE Healthcare) equilibrated with 20 mM Tris (pH=8), 500 mM NaCl and 20 mM imidazole (buffer A). The column was washed with 100 ml of buffer A, and 6xHis-tagged L-asparaginase II was eluted from the column using a linear gradient over 100 ml of buffer A containing an additional 480

mM imidazole, collecting 2.5 ml fractions. Fractions containing 6xHis-tagged L-asparaginase II were pooled, concentrated using a 50 kDa cut-off Vivacell 70 unit (Vivapure) and applied to a S-200 26/60 column (GE Healthcare) equilibrated with PBS. Fractions containing 6xHis-tagged L-asparaginase II were pooled, concentrated and applied three times onto a 1 ml EndoTrap Blue column (Hyglos) using PBS supplemented with 100 μM of $CaCl_2$ as the equilibration buffer and the supplied buffer for regeneration. A Superdex 10/300GL 200 column (GE Healthcare) was used at each step to ensure the protein remained in a tetrameric structure. Endotoxin concentrations were determined with *Limulus* amebocyte lysate gel clot assays (Associates of Cape Cod). Buffers were either purchased as certified endotoxin-free or tested prior to use with gel clot assays to ensure that endotoxin levels were below 0.12 EU/ml (detection limit). Prior to use, columns and chromatography equipment were soaked in 1 N NaOH and tested to ensure that endotoxin levels were below 0.12 EU/ml.

IX. Mouse experiments

The Institutional Animal Care and Use Committee at Stony Brook University approved all animal studies.

A. Primary infection

Survival assays and organ burden assays were performed using 8 to 10 week-old C57BL/6J and 129X1/SvJ mice (The Jackson Laboratory). These strains of mice have been used as model hosts to study acute and persistent infections with *Salmonellae*, respectively (162). Mice were inoculated intragastrically with either WT *S*. Typhimurium strain IR715 or isogenic, *ΔSTM3106 S*. Typhimurium [$5x10^5$ CFU for C57BL/6J mice and $5x10^7$ CFU for 129X1/SvJ mice] suspended in 0.1 ml of PBS. Mice

were monitored for survival and were euthanized when moribund, or at the termination of the experiment (30 days and 60 days after infection for C57BL/6J mice and 129X1/SvJ mice, respectively). At various times after infection, target organs were harvested from 129X1/SvJ and homogenized by stomacher or MACS gentle-dissociator. The bacterial burden per gram of tissue was quantified by plating for CFU on LB agar containing nalidixic acid (50 μg/ml) or kanamycin (60 μg/ml).

B. *In vivo* complementation of *ΔSTM3106*

F1 (C57BL/6J x 129X1/SvJ) hybrid mice were used to determine whether the presence of *STM3106 in trans* complemented the colonization defect of *ΔSTM3106 S*. Typhimurium in liver because 129X1/SvJ mice were not commercially available in a timely fashion. To minimize concerns about plasmid loss over time, mice were inoculated intravenously with $5x10^3$ CFU of WT *S*. Typhimurium strain IR715, isogenic *ΔSTM3106 S*. Typhimurium or isogenic *ΔSTM3106 S*. Typhimurium carrying plasmid pWSK29-*STM3106*, and bacterial loads per liver were determined after 10 days of infection. Ten-fold serial dilutions of the inoculum were plated on LB agar to determine the inoculum titer. At day 11 post infection, target organs were harvested from F1 (C57BL/6J x 129X1/SvJ) hybrid mice and the bacterial burden per gram of tissue was quantified by plating for CFU on LB agar containing nalidixic acid (50 μg/ml), kanamycin (60 μg/ml) or carbenicillin (100 μg/ml).

C. Challenge experiments

Immunization studies were performed using 8 to 10 week-old C57BL/6J mice. C57BL/6J mice were immunized intragastrically with approximately $5x10^8$ CFU of the prototypical, live-attenuated *S*. Typhimurium vaccine strain CL1509 (14028 *aroA*-) (38,

79, 108). Ninety days after immunization mice were challenged with about $1x10^7$ CFU of IR715 or isogenic Δ*STM3106 S.* Typhimurium. At various times after infection, target organs were harvested and homogenized using the MACS gentle-dissociator. The bacterial burden per gram of tissue was quantified by plating for CFU on LB agar containing nalidixic acid (50 μg/ml) or kanamycin (60 μg/ml). Mice from each group were euthanized at 3, 5, 7, and 11 days post-challenge. The T cells were harvested and analyzed from each of the organs as above.

X. Statistical analysis

Statistical analysis on survival assays and organ burden assays was performed using Log-rank Test and one-tailed, nonparametric Mann-Whitney Test or two-way ANOVA, respectively. Statistical analysis on all other assays was performed using a two-tailed paired t-test (invasion assay) or Repeated Measures (randomized-block) one-way ANOVA with Bonferroni's Multiple Comparison Post Test, comparing selected pairs of columns. Statistical analysis was performed using Prism 5.0b (GraphPad Software). *P* values of less than 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences: *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

RESULTS

I. Characterization of *S*. Typhimurium-induced T cell inhibition *in vitro*

A. Only viable *S*. Typhimurium and those capable of synthesizing new proteins inhibit T cells, but not by invasion

i. *S.* Typhimurium inhibit purified T cells

As we previously described, *S*. Typhimurium have a direct, inhibitory effect on T cells (165, 166). Specifically, *S*. Typhimurium-induced inhibition of T cell proliferation correlates with down-modulation of TCR-β, a receptor required for antigen recognition and T cell function (165, 166). In these studies, we used enriched T cells in our inhibition assays (166). This mechanism of selection is not 100% efficient (data not shown). Hence, we wanted to determine if the small percentage of non-T cells could contribute to T cell inhibition (18).

Cells expressing high levels of CD90.2, a pan-T cell marker, were selected using a FACS Aria. The resulting FACS-purified T cells (>99% pure) were cultured in the absence or presence of wild-type *S*. Typhimurium. Importantly, after 20 hours of incubation, *S*. Typhimurium down-modulated expression of TCR-β on purified T cells, indicating *S*. Typhimurium is indeed causing T cell inhibition (Figure 6A).

Since it has been shown that both $CD4^+$ and $CD8^+$ T cells contribute to the effective clearance of *S*. Typhimurium, we wanted to examine whether *S*. Typhimurium is able to effectively inhibit both T cell subsets (107). FACS-purified $CD4^+$ T cells or $CD8^+$ T cells were cultured in the absence or presence of wild-type *S*. Typhimurium, as above. Similar to the purified total T cell population, after 20 hours of incubation, both

$CD4^+$ and $CD8^+$ T cells displayed significantly less surface expression of TCR-β after culture with *S*. Typhimurium (Figures 6B and 6C). Collectively, these results demonstrate that *S*. Typhimurium are responsible for T cell inhibition and can suppress both $CD4^+$ and $CD8^+$ T cells.

ii. Viable *S*. Typhimurium down-modulate T cell receptor expression and suppress T cell blastogenesis

We next examined if *S*. Typhimurium were actively suppressing the T cell response. If the bacteria actively cause inhibition, it is likely that *S*. Typhimurium have to be alive. To determine if down-modulation of TCR-β by *S*. Typhimurium requires viable microorganisms, T cells were left uninfected or cultured with live or formaldehyde-fixed *S*. Typhimurium. After 20 hours of incubation, T cells cultured with viable *S*. Typhimurium expressed significantly less surface TCR-β than T cells left uninfected (Figures 7A and 7B). In contrast, T cells cultured with formaldehyde-fixed *S*. Typhimurium expressed levels of surface TCR-β that were similar to the levels expressed by T cells left uninfected (Figures 7A and 7B).

As part of their activation process, T cells increase in size and granularity, a process known as blastogenesis, before proliferating (47, 95, 184). To evaluate various aspects of *S*. Typhimurium-induced T cell inhibition, we evaluated if T cell blastogenesis is inhibited by culture with viable *S*. Typhimurium. Indeed, blastogenesis of T cells cultured with viable *S*. Typhimurium was significantly reduced when compared to blastogenesis of uninfected T cells or T cells cultured with formaldehyde-fixed *S*. Typhimurium (Figures 7C and 7D). Blastogenesis was calculated by determining the percentage of live cells that, in response to treatment with anti-CD3ε, had transformed

from small lymphocytes into the larger, more granular cells. Together, these results and prior observations from our laboratory indicate that *S*. Typhimurium have to be alive to inhibit T cells (165, 166).

iii. *S*. Typhimurium capable of synthesizing new proteins down-modulate T cell receptor expression and suppress T cell blastogenesis

Given that *S*. Typhimurium have to be alive to inhibit T cells, we next wanted to establish if the bacteria also have to be capable of synthesizing new proteins to cause T cell inhibition. To determine if new protein synthesis by *S*. Typhimurium is required for down-modulation of TCR-β, *S*. Typhimurium were pre-treated for 2 hours with chloramphenicol prior to infection, and 100 µg/ml chlorampenicol remained in the medium throughout the course of the infection to prevent any bacterial protein synthesis. After 20 hours of incubation, T cells cultured with chloramphenicol-treated *S*. Typhimurium expressed levels of surface TCR-β similar to the levels expressed by uninfected T cells (Figures 8A and 8B). Additionally, blastogenesis of T cells cultured with *S*. Typhimurium was significantly increased when chloramphenicol was present (Figures 8C and 8D). Thus, *S*. Typhimurium must be viable and able to synthesize new proteins to down-modulate TCR-β expression and suppress T cell blastogenesis.

B. *STM3106* is required for *S*. Typhimurium to inhibit T cells

i a. *STM3106* is required for *S*. Typhimurium to down-modulate T cell receptor expression, suppress T cell blastogenesis and block cytokine production

Given that *S*. Typhimurium must be alive and able to synthesize new proteins to suppress the T cell response, we wanted to identify the bacterial genes required for inhibition of T cells. In earlier studies, we took a targeted approach and tested *S*. Typhimurium mutants deficient for genes that contribute to pathogenesis or have been shown to cause immunosuppression. These include *invA* (SPI-1 TTSS), *spiB* (SPI-2 TTSS), *invA* and *spiB* (SPI-1 and SPI-2 TTSS), *phoP, sti,* or *S.* Typhimurium cured of the virulence plasmid and determined that none of these were required for T cell inhibition (166). Here, we looked at *S.* Typhimurium deficient for *fliC,* since FliC-specific $CD4^+$ T cells have been studied extensively during *S.* Typhimurium infection, and analyzed its ability to inhibit T cells. *S.* Typhimurium deficient for *fliC*, an important component of flagellin, down-modulate TCR-β (Figure 9).

Next, we took a randomized, genetic approach. A library of 156 mutants (generous gift from the McClelland and Andrews-Polymenis groups) was screened to characterize any mutants that would be unable to down-modulate TCR-β surface expression. Each mutant in this library carries a targeted deletion of multiple linked, non-essential genes (a total of ~1,700 genes are deleted in the library). One mutant, *ΔSTM3104-7*, completely failed to down-modulate TCR-β (Figure 10A). To identify the gene(s) deleted in this mutant required for *S*. Typhimurium to down-modulate TCR-β, strains with targeted deletions of *STM3104*, *STM3105*, *STM3106* or *STM3107* were generated and tested for their ability to cause T cell inhibition (Figures 10B and 10C). Only *ΔSTM3106 S*. Typhimurium were unable to down-modulate TCR-β (Figure 10C). The presence of *STM3106 in trans*, but not the empty vector control, complemented the phenotype of *ΔSTM3106 S*. Typhimurium (Figures 11A and 11B).

We next explored if *STM3106* contributed to other aspects of T cell inhibition, such as suppression of T cell blastogenesis and inhibition of cytokine production. Indeed, *S*. Typhimurium lacking *STM3106* also failed to suppress T cell blastogenesis when compared to wild-type bacteria (Figures 11C and 11D). As previously reported by our laboratory, wild-type *S.* Typhimurium inhibited the ability of T cells to produce protective cytokines, such as interferon (IFN)-γ (Figure 12A and 12B) and interleukin (IL)-2 (Figures 12C and 12D) (165, 166). Using multiparametric flow cytometry, we determined that T cells expressing low levels of TCR-β on their surface were the same T cells that also contained low amounts of intracellular cytokines. Importantly, *ΔSTM3106 S*. Typhimurium were no longer able to inhibit production of interferon (IFN)-γ and interleukin (IL)-2, resembling uninfected T cells (Figures 12A-D).

i b. *STM3106* is required for *S*. Typhimurium to inhibit T cell proliferation

To determine if *STM3106* is required for *S.* Typhimurium to inhibit T cell proliferation, T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with wild-type (WT) or *ΔSTM3106 S*. Typhimurium. After 3 days of incubation, T cells cultured with WT *S*. Typhimurium had not proliferated in response to stimulus (Figures 13A and 13B). In contrast, T cells cultured with *ΔSTM3106 S*. Typhimurium, like T cells left uninfected, had proliferated extensively (Figures 13A and 13B). Collectively, these results demonstrate that *STM3106* is required for *S*. Typhimurium to down-modulate TCR-β expression, suppress T cell blastogenesis, block cytokine production, and inhibit T cell proliferation.

ii a. Comprehensive analysis of the population of T cells inhibited by *S.* Typhimurium

We wanted to further analyze the inhibited T cell population. We previously reported that *S*. Typhimurium were only able to inhibit activated $CD25^+$ T cells, but not resting T cells (166). Here, we looked at $CD69^+$ T cells, an additional T cell activation marker, and analyzed the ability of *S*. Typhimurium to cause inhibition (184). In fact, we did find that wild-type *S*. Typhimurium, but not *ΔSTM3106 S*. Typhimurium, are able to down-modulate TCR-β on $CD69^+$ T cells (Figures 14A and 14B) and inhibit their cytokine production (Figures 15A-D).

ii b. *STM3106* is required to inhibit both $CD4^+$ and $CD8^+$ T cells

As described above, we determined that *S*. Typhimurium inhibit both $CD4^+$ and $CD8^+$ T cells (Figures 6B and 6C). We next wanted to identify if *STM3106* contributed to both their inhibition. Using multiparametric flow cytometry, we examined both $CD4^+$ T cells and $CD8^+$ T cells in the context of expressing the different activation markers CD25 and CD69. We found that S. Typhimurium inhibits all $CD25^+$ $CD4^+$ T cells and $CD25^+$ $CD8^+$ T cells as well as $CD69^+$ $CD4^+$ T cells and $CD69^+$ $CD8^+$ T cells and that *STM3106* is required for inhibition (Figures 16A-D).

C. *S.* Typhimurium likely do not invade T cells to cause T cell inhibition

Considering *S*. Typhimurium are actively suppressing the T cell response, we wanted to evaluate if bacterial association or invasion is required for T cell inhibition. *S*. Typhimurium invasion of T cells was determined using a standard gentamicin protection assay.

Briefly, T cells were cultured in the absence or presence of green fluorescent protein-expressing *S*. Typhimurium. After all the extracellular bacteria were killed, the T cells were harvested, stained, and analyzed by flow cytometry. After infection with GFP-expressing bacteria, approximately 0.1% of T cells were GFP^+, only slightly greater than uninfected T cells, where 0.02% of T cells were GFP^+ (Figure 17A). Thus, it is unlikely that *S*. Typhimurium associate or invade with T cells to cause inhibition.

Furthermore, we compared the ability of *ΔSTM3106 S*. Typhimurium to invade T cells that of wild-type *S.* Typhimurium using a CFU assay. We determined that neither wild-type *S*. Typhimurium nor *ΔSTM3106 S*. Typhimurium efficiently invade T cells (Figure 17B).

D. *S.* Typhimurium deficient for *STM3106* do not have a generalized growth defect

It was plausible that *ΔSTM3106 S*. Typhimurium fail to inhibit T cells because they have a general growth defect. We conducted growth curve experiments in bacterial culture medium as well as in RP-10 tissue culture medium. *S*. Typhimurium lacking *STM3106* grew normally in both media (Figures 18A and 18B). Additionally, we saw equal survival of both bacterial strains inside 129X1/SvJ and C57BL/6J bone-marrow-derived macrophages as well as inside RAW264.7 macrophage-like cells (Figures 19A-C). Cumulatively, these findings indicate that *ΔSTM3106 S*. Typhimurium do not have a generalized growth defect.

E. L-asparaginase II encoded by *STM3106* is necessary and sufficient to cause inhibition of T cells

i. L-asparaginase II encoded by *STM3106* is necessary for T cell inhibition

STM3106 (*ansB*) encodes L-asparaginase II, an extracytoplasmic enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Figure 20A). We showed previously that a proteinaceous factor capable of down-modulating TCR-β was present in medium harvested from T cells cultured with *S*. Typhimurium (166). To identify if this inhibitory factor could be L-asparaginase II produced by *S*. Typhimurium, medium harvested from T cells cultured with *S*. Typhimurium was subjected to Western blot analysis. The presence of L-asparaginase II in medium could not be detected, even if the supernatant was concentrated 100X (data not shown and Figure 21A). However, L-asparaginase II production was observed under the bacterial growth conditions used (Figure 21B).

Simultaneously, we were conducting immunodepletion experiments on the supernatants from the co-culture of *S*. Typhimurium and T cells. Intriguingly, the capacity of the medium to down-modulate TCR-β was lost when L-asparaginase II-specific antibody was used in these experiments (Figure 20B). In contrast, the capacity of the medium to down-modulate TCR-β was retained when control antibody or no antibody was used (Figure 20B). Hence, L-asparaginase II is necessary for *S*. Typhimurium to cause down-modulation of TCR-β.

ii. L-asparaginase II encoded by *STM3106* is sufficient for T cell inhibition

To determine if L-asparaginase II of *S*. Typhimurium is sufficient to cause down-modulation of TCR-β, T cells were treated with purified L-asparaginase II. After 20 hours

of incubation, T cells treated with purified L-asparaginase II expressed significantly less surface TCR-β than T cells left untreated (Figure 22A). Furthermore, T cells treated with purified L-asparaginase II expressed levels of surface TCR-β that were comparable to the levels expressed by T cells cultured with wild-type *S*. Typhimurium (Figure 22A). This again was observed for both $CD4^+$ and $CD8^+$ T cells (Figures 22B and 22C). T cells treated with purified L-asparaginase II also expressed significantly less intracellular IFN-γ and IL-2 than T cells left untreated (Figures 22D and 22E). Thus, L-asparaginase II of *S*. Typhimurium is sufficient to cause inhibition of T cells.

iii. L-asparaginase II-mediated depletion of L-asparagine contributes to T cell inhibition

To find out if depletion of L-asparaginase II substrate is responsible for *S*. Typhimurium-induced inhibition of T cells, L-asparagine was added to T cells cultured with *S*. Typhimurium. Exogenous L-asparagine, but not L-aspartate or L-glutamine, prevented *S*. Typhimurium-induced down-modulation of TCR-β (Figure 23). Furthermore, culturing uninfected T cells in excess of these amino acids, as well as in the presence of ammonia, did not affect TCR-β expression (Figure 23 and A. Torres Gersch and A. van der Velden, unpublished data). Additional experiments conducted in our laboratory, demonstrated that when uninfected T cells were cultured in medium lacking L-asparagine, they expressed levels of TCR-β similar to T cells cultured in the presence of *S*. Typhimurium in rich medium (A. Torres Gersch and A. van der Velden, unpublished data). Further, we showed that a mutation in the catalytic site of the purified L-asparaginase II enzyme resulted in the failure to down-modulate TCR-β, block cytokine production, and inhibit T cell proliferation (A. Torres Gersch, A. Kullas, and A.

van der Velden, unpublished data). Collectively, these results suggest that L-asparaginase II-mediated depletion of L-asparagine contributes to *S*. Typhimurium-induced T cell inhibition.

iv. T cell inhibition is specific to L-asparaginase II

S. Typhimurium encodes both an L-asparaginase I and L-asparaginase II. Since both enzymes have the same function (though L-asparaginase I is a low-affinity, cytoplasmic enzyme) we tested the ability of *S*. Typhimurium deficient for L-asparaginase I to down-modulate TCR-β. Only *S*. Typhimurium lacking L-asparaginase II, but not L-asparaginase I, failed to cause T cell inhibition (Figure 24).

Additionally, we tested if L-glutaminase would be sufficient to cause T cell inhibition. Purified L-glutaminase was added at the same concentration as L-asparaginase II as well as 10-fold higher concentration to uninfected T cells. After 20 hours, only T cells that were treated with L-asparaginase II showed lower levels of TCR-β (Figure 25).

v. Regulation of L-asparaginase II

We next wanted to gain insight into the regulation of L-asparaginase II in *S*. Typhimurium. We again took a genetic approach. We screened a select panel of targeted deletion mutants of known virulence regulators and found that none appeared to contribute to L-asparaginase II-mediated T cell inhibition (Figure 26). We also took a random approach and screened a transposon-insertion library (generous gift from the Bäumler and Tsolis groups). Here, we found one potential mutant of interest; an insertion in *tufB*, encoding an elongation factor thermo unstable (EF-Tu), produced an intermediate phenotype (Figure 27). Studies in other organisms have shown that their

respective EF-Tu interact with their host and immune cells. *Mycoplasma pneumonia* EF-Tu binds fibronectin; the authors speculate that surface localization of EF-Tu provides *M. pneumonia* a distinct mechanism to colonize tissues and gain access to intracellular niches (32). *Lactobacillus johnsonii* mediates its attachment to human intestinal cells and mucins through EF-Tu (58). Interestingly, it has been described that *Pseudomonas aeruginosa* evades human complement attack by using EF-Tu to bind complement regulator Factor H and plasminogen (82). Indeed it is intriguing to think that EF-Tu can function beyond its canonical role in bacterial protein synthesis and participate in bacterial binding to host cells and immune evasion.

More multi-gene deletions were generated and screened for their ability to cause TCR-β down-modulation. Subsequently, we found that *dsbA* appears to contribute to T cell inhibition by a L-asparaginase II-dependent mechanism (P. McLaughlin and A. van der Velden, unpublished data). These are exciting preliminary results, and more work needs to be done to further understand the regulation of L-asparaginase II.

F. Summary of L-asparaginase II-mediated T cell inhibition *in vitro*

As summarized in Figure 28, we report that *S.* Typhimurium use L-asparaginase II to inhibit the response of T cells. Further, we show that production of L-asparaginase II by *S*. Typhimurium is both necessary and sufficient to cause inhibition of T cells. An immediate implication of our results is that production of L-asparaginase II by *S*. Typhimurium causes depletion of exogenous L-asparagine, leading to down-modulation of TCR-β expression, suppression of T cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T cell proliferation.

G. Acknowledgements

Thank you to the members of the flow cytometry core facility in the hospital for their assistance in purifying the various populations of T cells and expertise with conducting the seven-color cytometry. I would like to thank the members of the Dr. Michael McClelland and Dr. Helene Andrews-Polymenis laboratories for the construction and verification of the targeted, multi-gene deletion library and sharing this valuable resource with us. I appreciate: Dr. Ando van der Velden for assistance with the T cell invasion experiments; Galina Romanov for her assistance with the macrophage infections and Western blots; Dr. Joseph McPhee for assistance with the immunodepletion experiments; Karen Chave for purification of L-asparaginase II from *S.* Typhimurium; and AnnMarie Torres Gersch for titrating the amount required for T cell inhibition and for helping to take this part of the project to the next level. Thank you to Dr. Andreas Bäumler and Dr. Renee Tsolis for sharing the transposon insertion library.

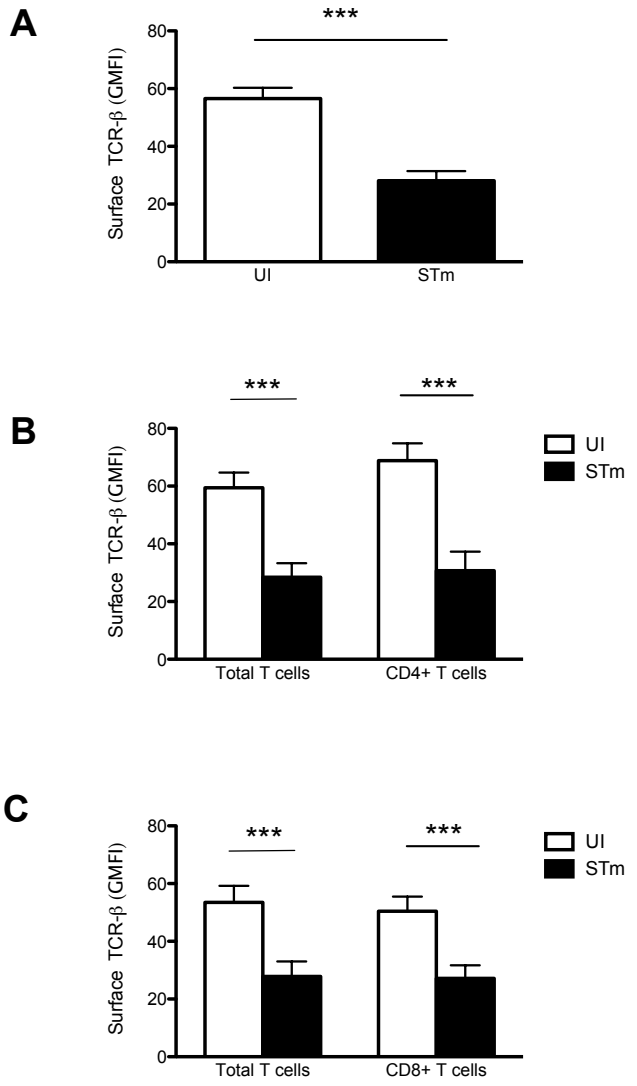

Figure 6 ***S.* Typhimurium down-modulate T cell receptor expression on purified CD4$^+$ and CD8$^+$ T cells**

(A) Expression of surface TCR-β by CD90.2$^+$CD25$^+$ T cells left uninfected (UI) or cultured with *S.* Typhimurium (STm), *P*=0.0004. **(B)** Expression of TCR-β by CD90.2$^+$CD25$^+$ T cells (Total T cells) left UI or cultured with STm; or by CD90.2$^+$CD4$^+$CD25$^+$ T cells (CD4$^+$T cells) left UI or cultured with STm, *P*=0.0002. **(C)** Expression of TCR-β by CD90.2$^+$CD25$^+$ T cells (Total T cells) left UI or cultured with STm; or by CD90.2$^+$CD8$^+$CD25$^+$ T cells (CD8$^+$T cells) left UI or cultured with STm, *P*=0.0008. Data show mean with SEM from at least three independent experiments.

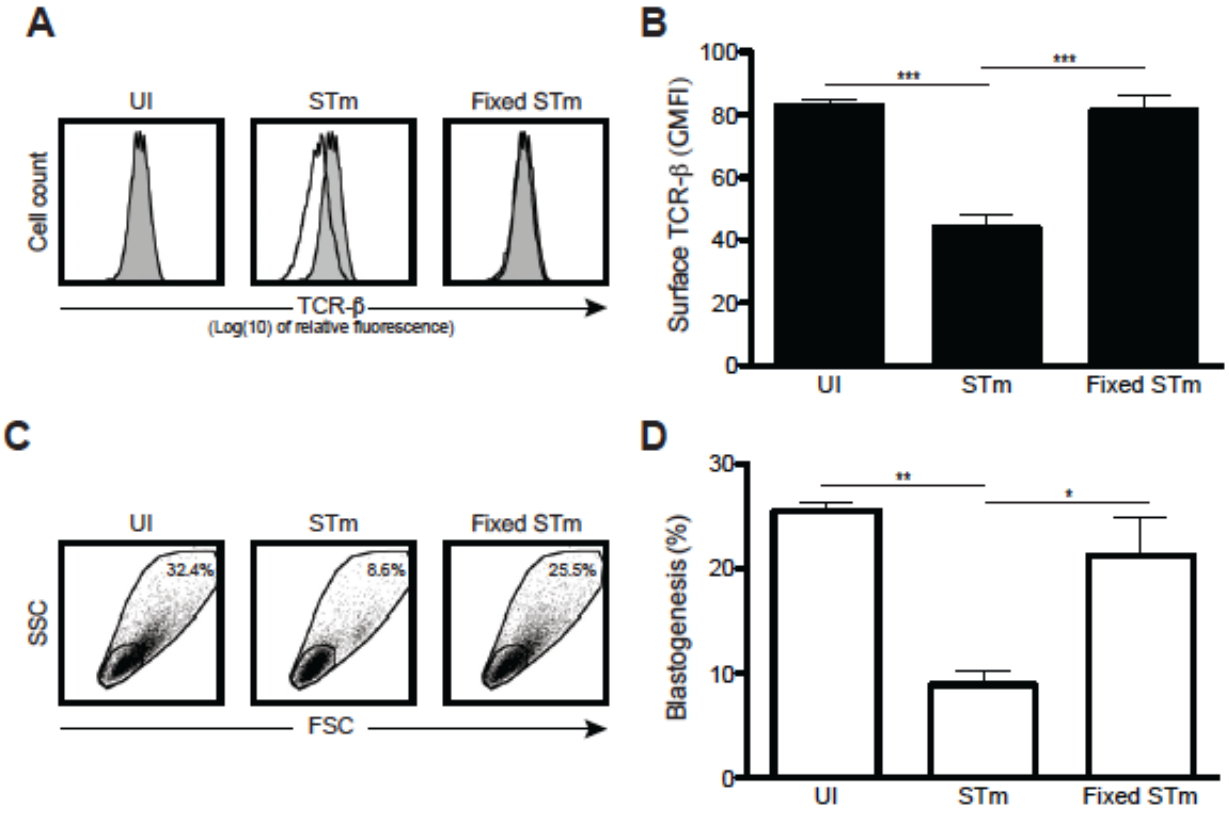

Figure 7 Only viable *S.* Typhimurium are able to down-modulate T cell receptor expression and suppress T cell blastogenesis

(A and B) Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI, grey in histograms) or cultured with viable *S*. Typhimurium (STm) or formaldehyde-fixed *S*. Typhimurium (Fixed STm), *P*=0.0002. **(C and D)** Blastogenesis of $CD25^+$ T cells treated as in (A), *P*=0.0054. Data are representative of (A and C) or show mean with SEM (B and D) from at least three independent experiments.

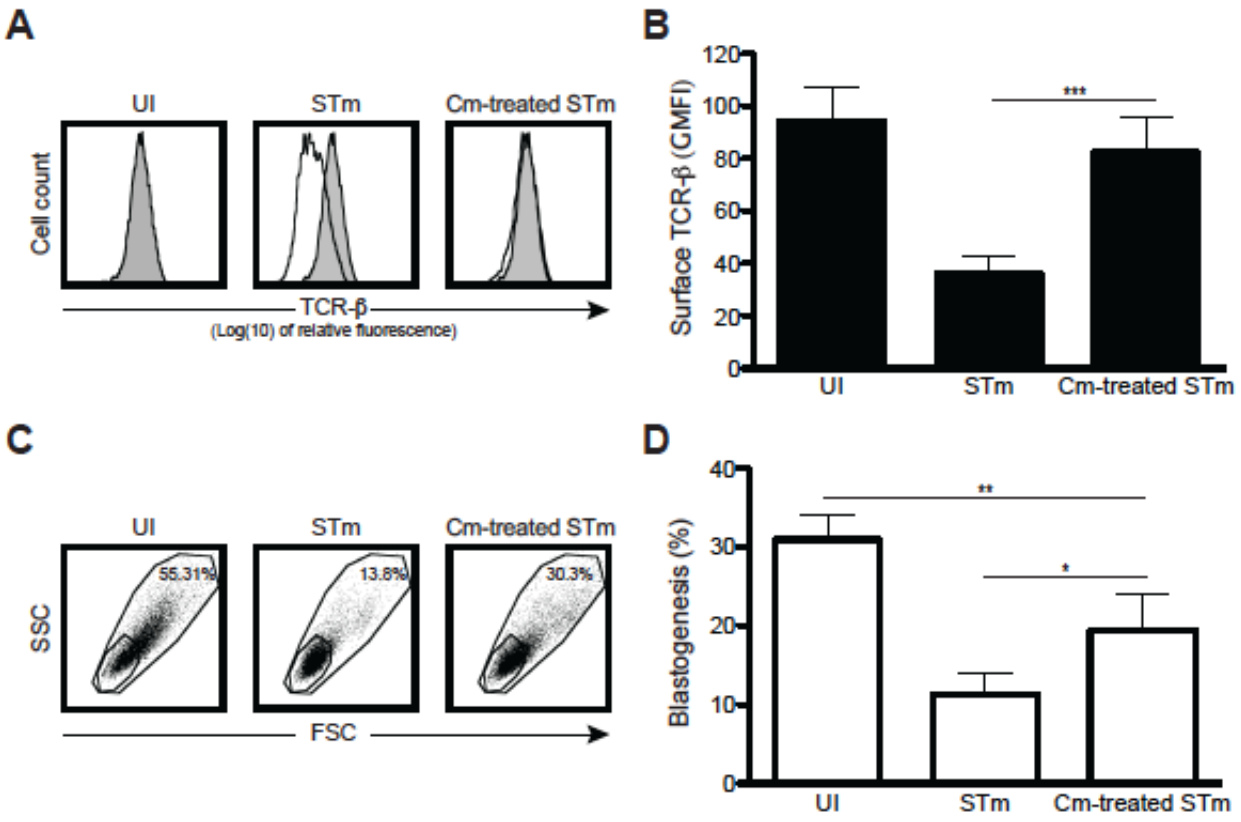

Figure 8 **Only *S.* Typhimurium capable of synthesizing new proteins are able to down-modulate T cell receptor expression and suppress T cell blastogenesis**

(A and B) Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI, grey in histograms) or cultured with untreated *S*. Typhimurium (STm) or chloramphenicol-treated *S*. Typhimurium (Cm-treated STm), $P<0.0001$. **(C and D)** Blastogenesis of $CD25^+$ T cells treated as in (A), $P=0.0004$. Data are representative of (A and C) or show mean with SEM (B and D) from at least three independent experiments.

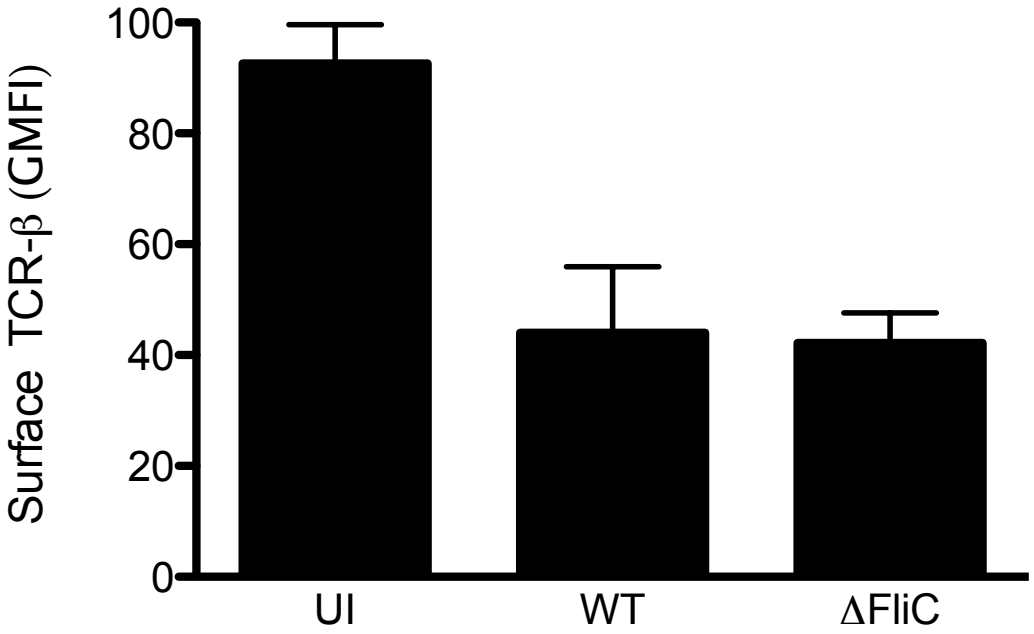

Figure 9 ***S.*** **Typhimurium deficient for** ***fliC*** **down-modulate T cell receptor expression**

Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI), or cultured with wild-type *S*. Typhimurium (WT), or cultured with *S*. Typhimurium deficient for *fliC* (ΔFliC). There was not a statistical difference in TCR-β expression on T cells cultured with WT *S*. Typhimurium or Δ*fliC S.* Typhimurium. Data show mean with SEM from at least three independent experiments.

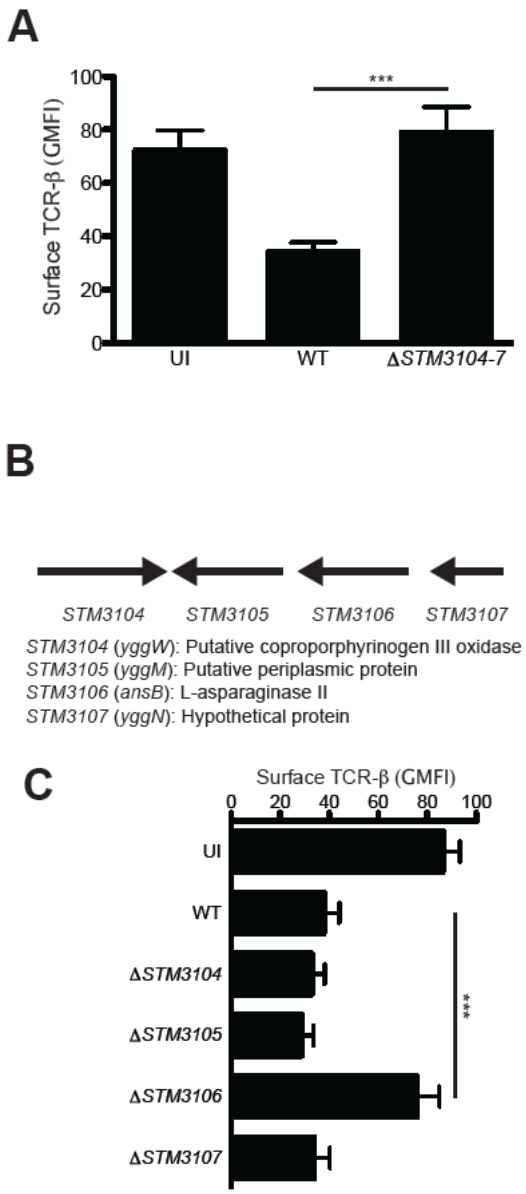

Figure 10 *STM3106* is required for *S*. Typhimurium to down-modulate T cell receptor expression

(A) Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI) or cultured with either WT or *ΔSTM3104-7 S*. Typhimurium, *P*<0.0001. **(B)** Genetic organization of the chromosomal region deleted in *ΔSTM3104-7 S*. Typhimurium. **(C)** Expression of surface TCR-β by $CD25^+$ T cells left UI or cultured with WT, *ΔSTM3104*, *ΔSTM3105*, *ΔSTM3106* or *ΔSTM3107 S*. Typhimurium, *P*<0.0001. Data show mean with SEM from at least three independent experiments.

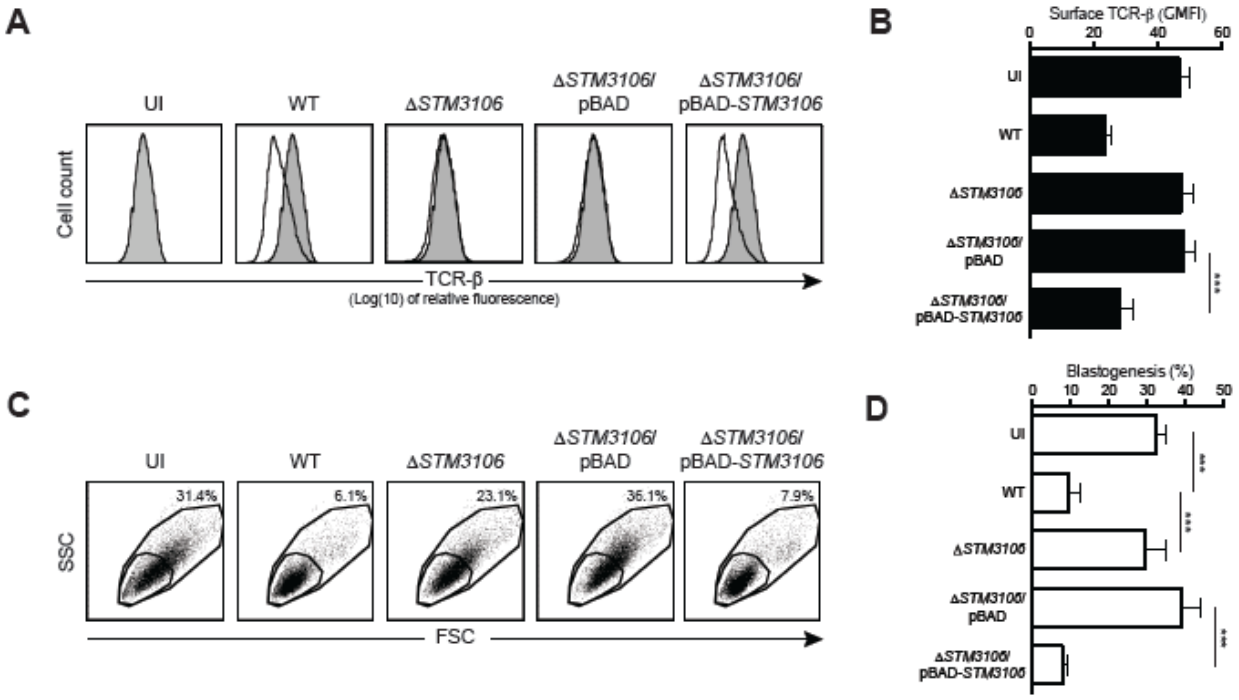

Figure 11 *STM3106* is required for *S*. Typhimurium to down-modulate T cell receptor expression and suppress T cell blastogenesis

(A and B) Expression of surface TCR-β by $CD25^+$ T cells left UI (grey in histograms) or cultured with WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium, *ΔSTM3106 S*. Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD) or *ΔSTM3106 S*. Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*), $P<0.0001$. **(C and D)** Blastogenesis of $CD25^+$ T cells treated as in (A), $P<0.0001$. Data are representative of (A and C) or show mean with SEM (B and D) from at least three independent experiments.

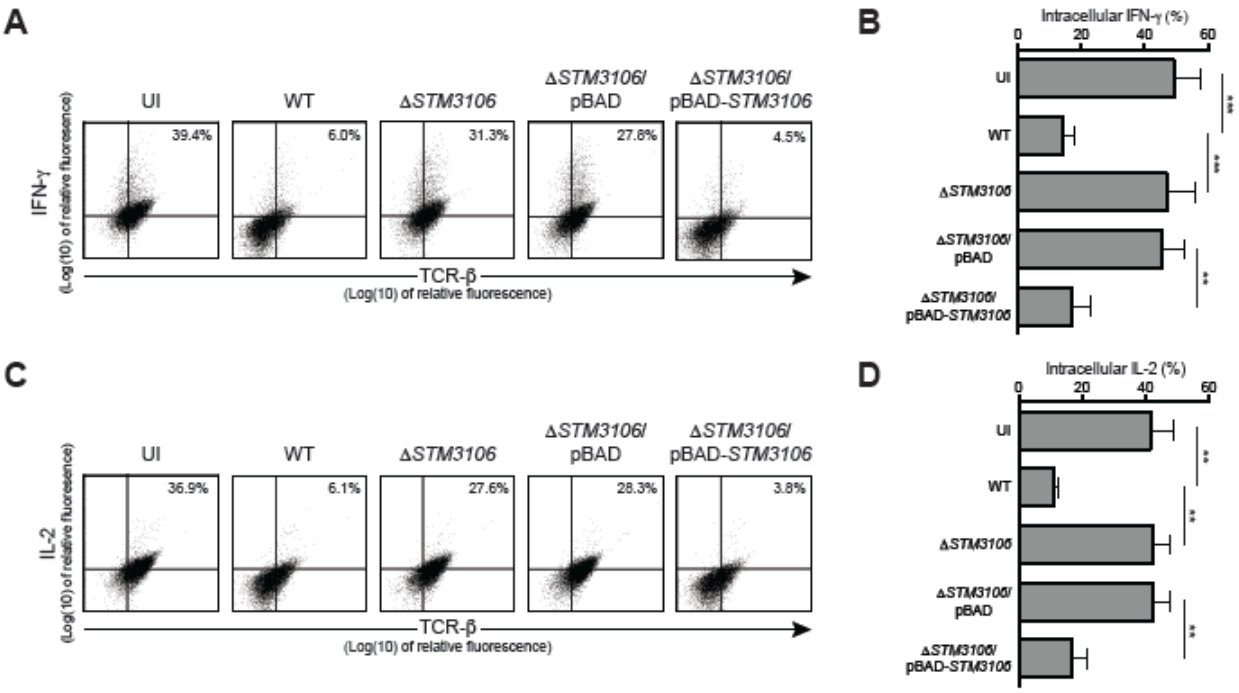

Figure 12 *STM3106* is required for *S*. Typhimurium to block cytokine production in T cells

(A and B) Expression of intracellular IFN-γ by $CD25^+$ T cells left UI or cultured with WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium, *ΔSTM3106 S*. Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD) or *ΔSTM3106 S*. Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*), $P<0.0001$. **(C and D)** Expression of intracellular IL-2 by $CD25^+$ T cells treated as in (A), $P=0.0001$. Data are representative of (A and C) or show mean with SEM (B and D) from at least three independent experiments.

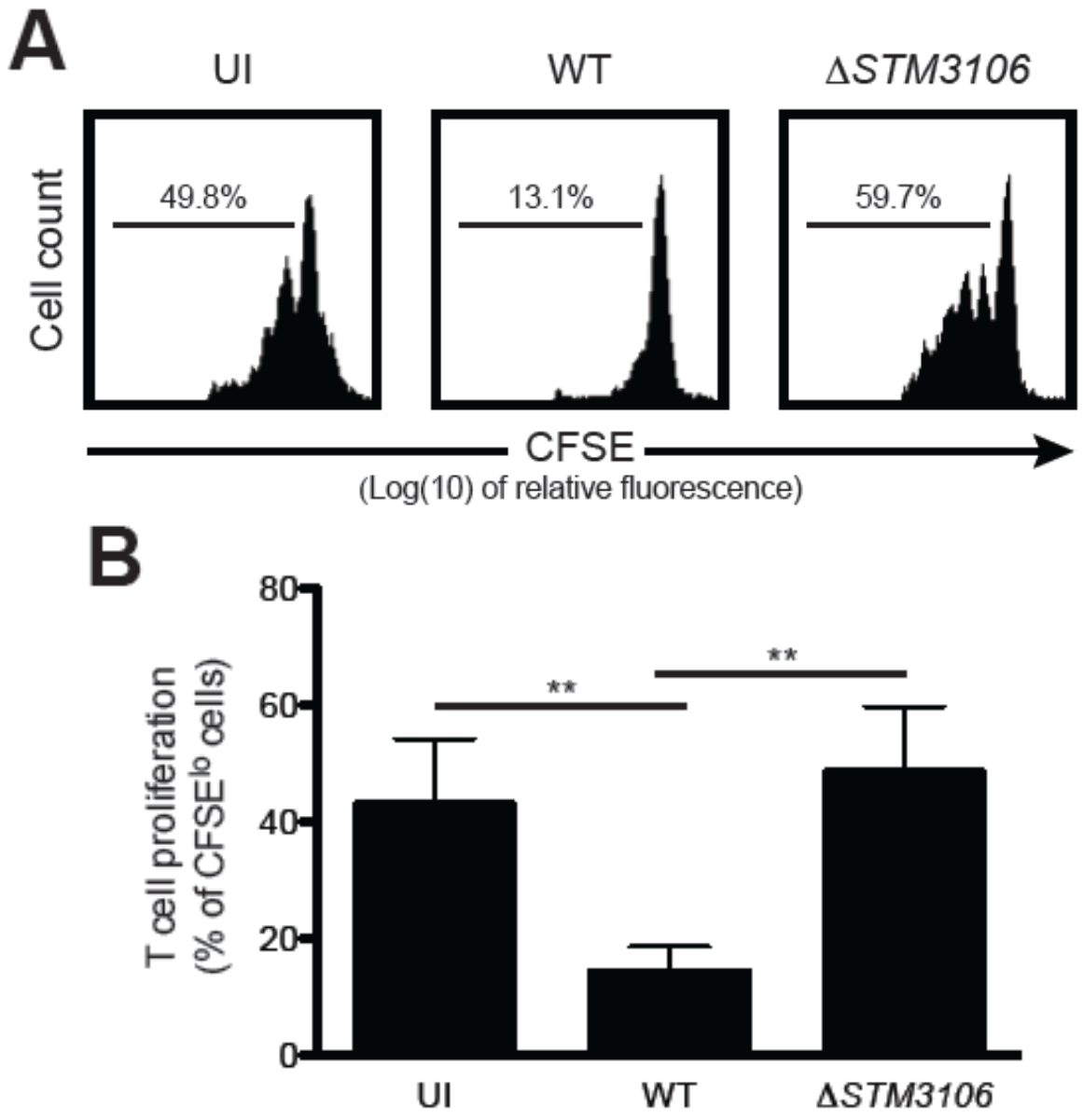

Figure 13 *STM3106* is required for *S*. Typhimurium inhibit T cell proliferation

(A and B) Proliferation of CFSE-labeled T cells left UI or cultured with WT or *ΔSTM3106 S*. Typhimurium, *P*=0.0021. Data are representative of (A) or show mean with SEM (B) from at least three independent experiments.

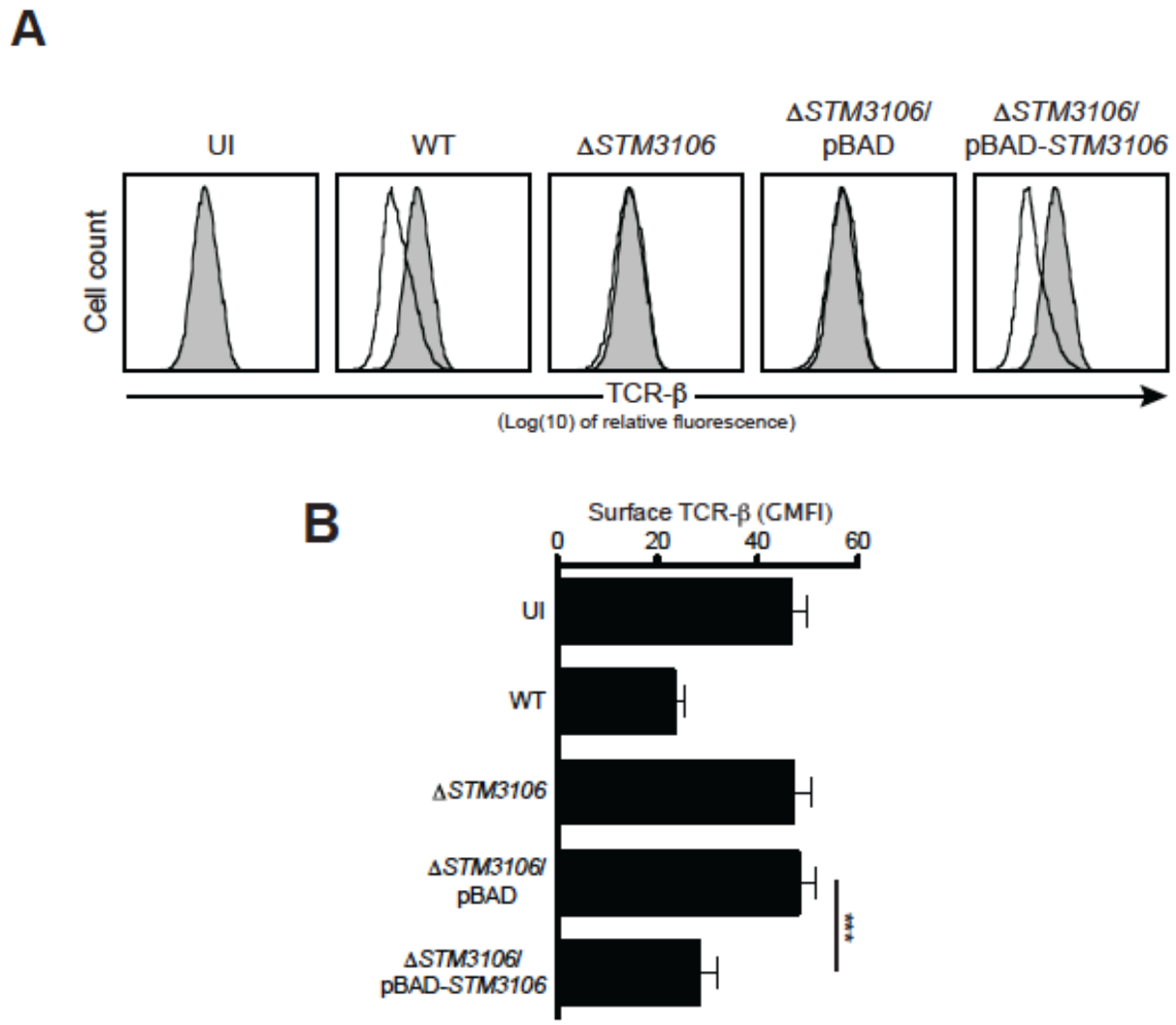

Figure 14 ***STM3106*** **is required for *S*. Typhimurium to down-modulate T cell receptor expression on CD69$^+$ T cells**

(A and B) Expression of surface TCR-β by CD69$^+$ T cells left UI (grey in histograms) or cultured with WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium, *ΔSTM3106 S*. Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD) or *ΔSTM3106 S*. Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*), $P<0.0001$. Data are representative of (A) or show mean with SEM (B) from at least three independent experiments.

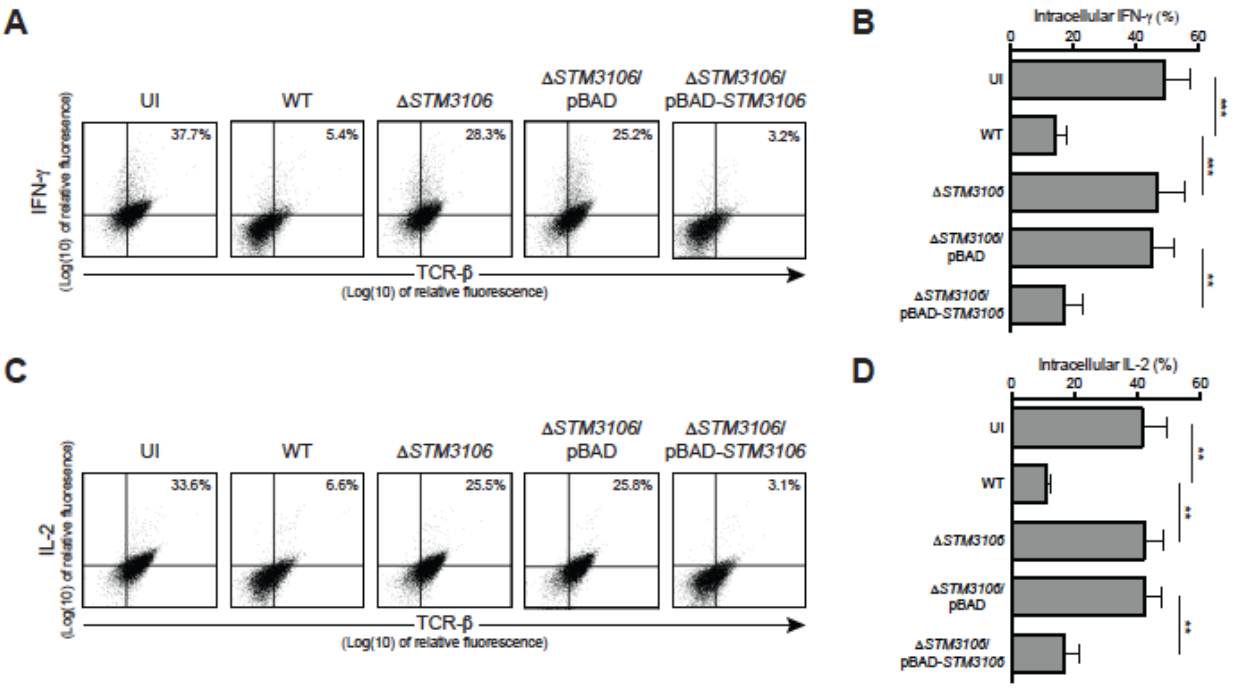

Figure 15 ***STM3106* is required for *S.* Typhimurium to inhibit cytokine production of CD69$^+$ T cells**

(A and B) Expression of intracellular IFN-γ by CD69$^+$ T cells left UI or cultured with WT *S.* Typhimurium, *ΔSTM3106 S.* Typhimurium, *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD) or *ΔSTM3106 S.* Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*), $P<0.0001$. **(C and D)** Expression of intracellular IL-2 by CD69$^+$ T cells left UI or treated as in (A), $P=0.0002$. Data are representative of (A and C) or show mean with SEM (B and D) from at least three independent experiments.

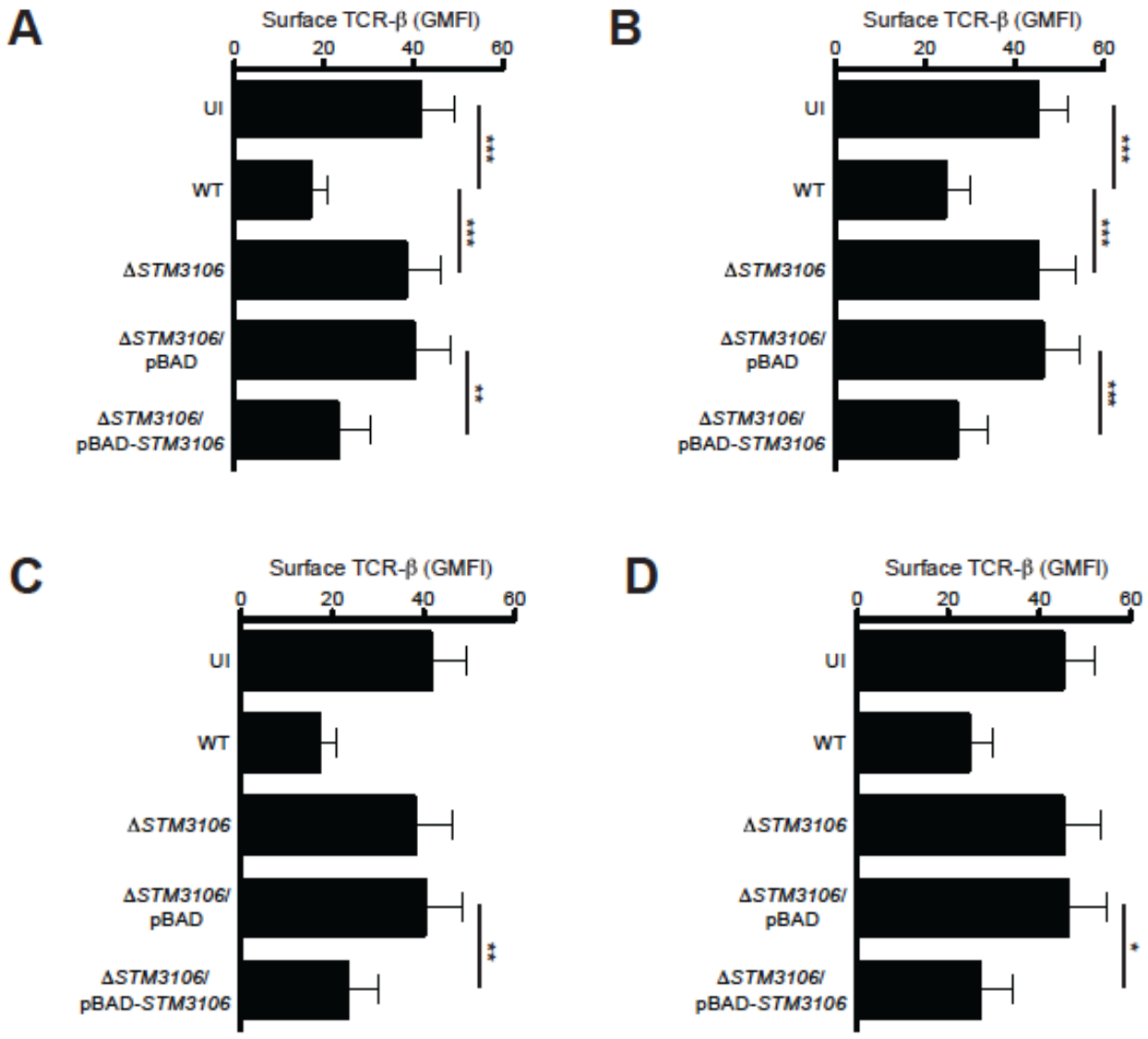

Figure 16 *STM3106* is required to inhibit both CD4+ and CD8+ T cells

(A and B) Expression of surface TCR-β by $CD4^+$ (A) or $CD8^+$ (B) $CD25^+$ T cells left uninfected (UI) or cultured with WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium, *ΔSTM3106 S*. Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD) or *ΔSTM3106 S*. Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*), $P<0.0001$ for both $CD4^+$ and $CD8^+$ T cells. **(C and D)** Expression of surface TCR-β by $CD4^+$ (C) or $CD8^+$ (D) $CD69^+$ T cells left uninfected or treated as in (A), $P<0.0001$ for $CD4^+$ and $P=0.0061$ for $CD8^+$ T cells. Data show mean with SEM from at least three independent experiments.

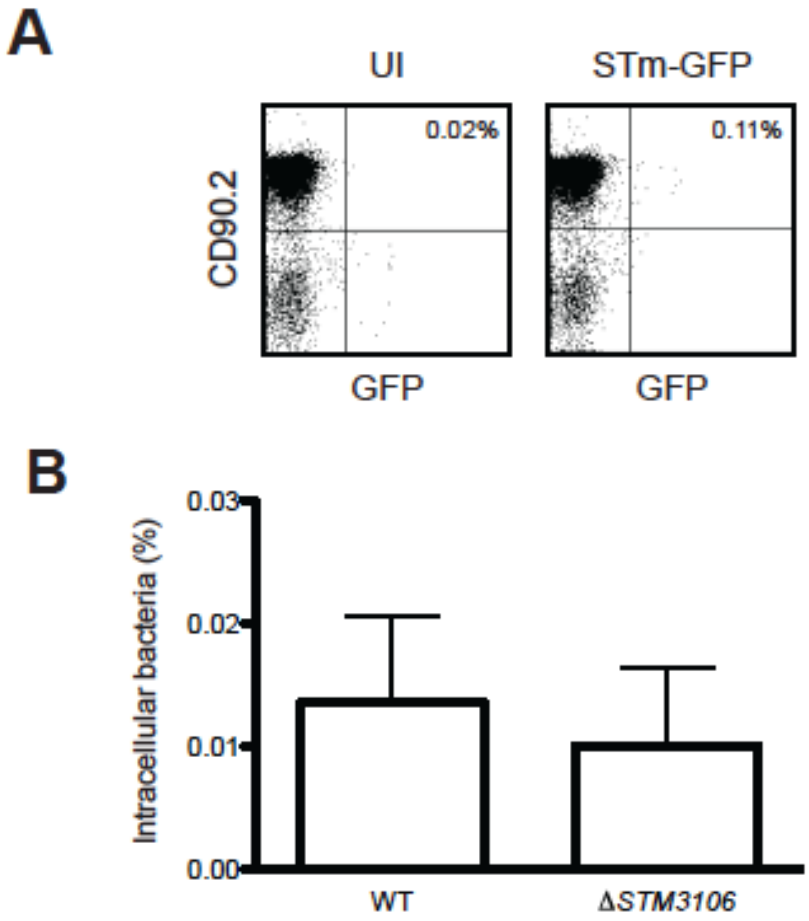

Figure 17 *S*. Typhimurium likely do not invade T cells to cause T cell inhibition

(A) Detection of green fluorescent protein (GFP) associated with T cells left uninfected (UI) or cultured with WT *S*. Typhimurium expressing GFP. **(B)** Invasion of T cells by WT or *ΔSTM3106 S*. Typhimurium as assessed by a CFU assay, *P*=0.3774. Differences (A and B) were not statistically significant. Data are representative of (A) or show mean with SEM (B) from at least three independent experiments.

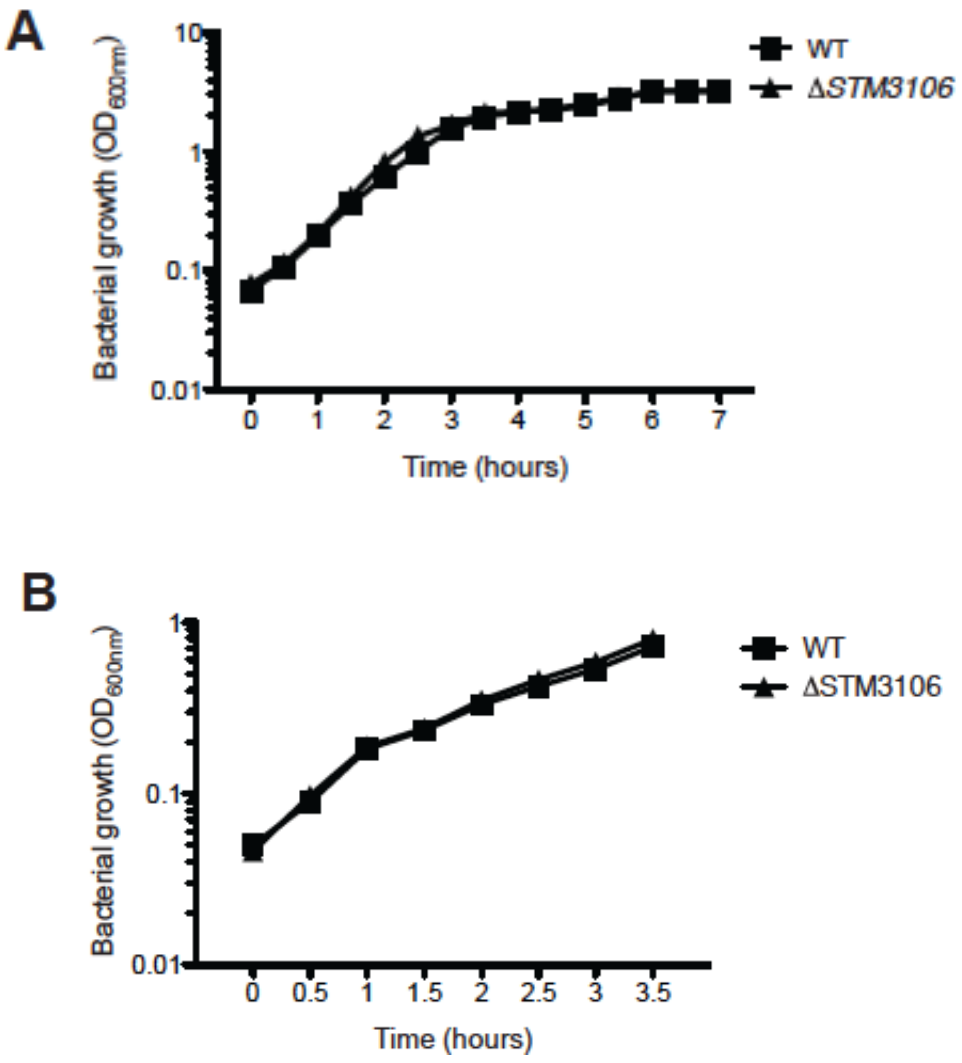

Figure 18 Δ*STM3106 S.* Typhimurium do not have a general growth defect

(A) Growth of WT (squares) and *ΔSTM3106* (triangles) *S*. Typhimurium in LB broth. **(B)** Growth of WT (squares) and *ΔSTM3106* (triangles) *S*. Typhimurium in tissue culture medium. Data are representative of at least three independent experiments.

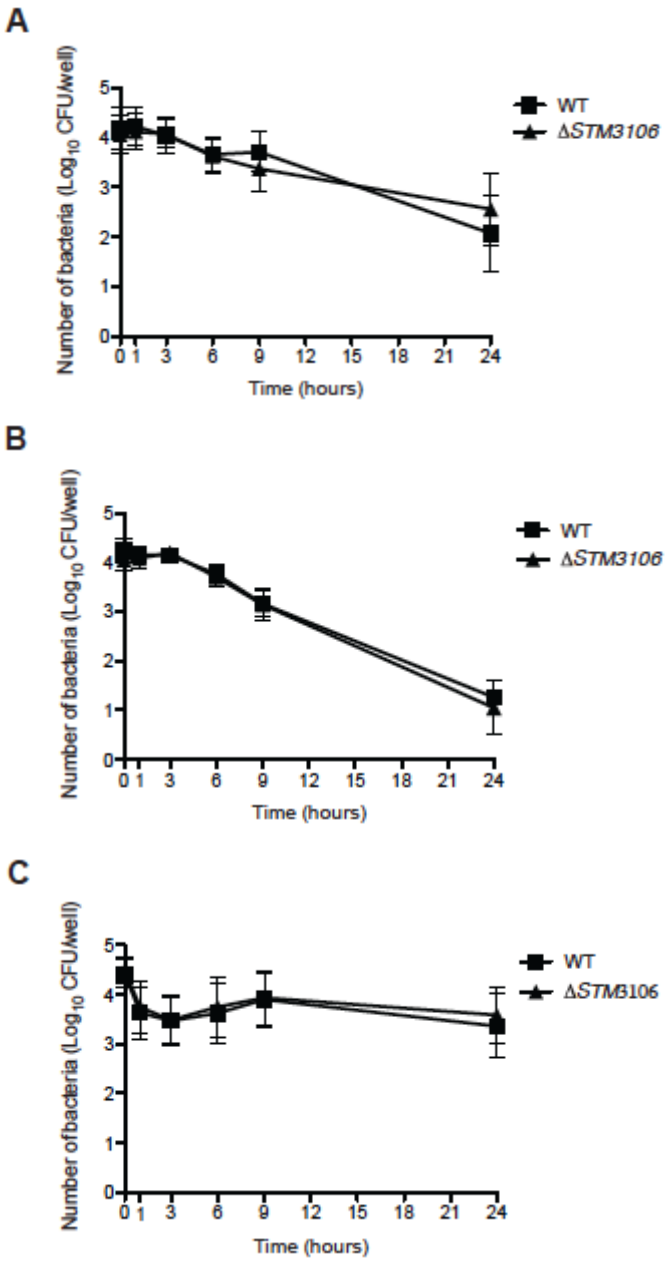

Figure 19 **Δ*STM3106 S.* Typhimurium survive similarly to wild-type *S.* Typhimurium inside macrophages**

(A) Survival and growth of WT (squares) and *ΔSTM3106* (triangles) *S*. Typhimurium inside bone marrow-derived macrophages cultured from 129X1/SvJ mice. **(B)** Survival and growth of WT (squares) and *ΔSTM3106* (triangles) *S*. Typhimurium inside bone marrow-derived macrophages cultured from C57BL/6J mice. **(C)** Survival and growth of WT (squares) and *ΔSTM3106* (triangles) *S*. Typhimurium inside RAW264.7 macrophage-like cells. Data are representative of at least three experiments.

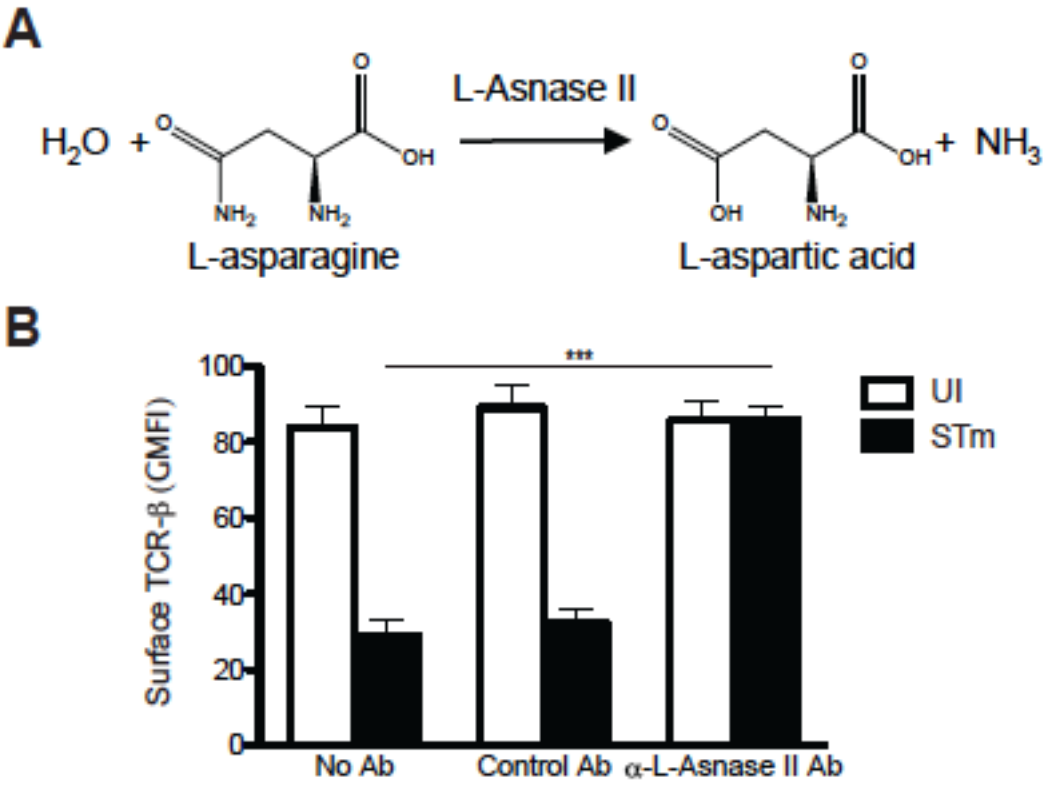

Figure 20 L-asparaginase II encoded by *STM3106* is necessary to cause down-modulation of the T cell receptor

(A) *STM3106* encodes L-asparaginase II (L-Asnase II), an extracytoplasmic enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. **(B)** Expression of surface TCR-β by $CD25^+$ T cells incubated with filtered medium harvested from T cells left uninfected (UI) or infected with WT *S*. Typhimurium (STm). This medium had been subjected to immunodepletion using L-asparaginase II-specific antibody (α-L-Asnase II Ab), control antibody (Control Ab) or no antibody (No Ab), *P*<0.0001. Data show mean with SEM from at least three independent experiments.

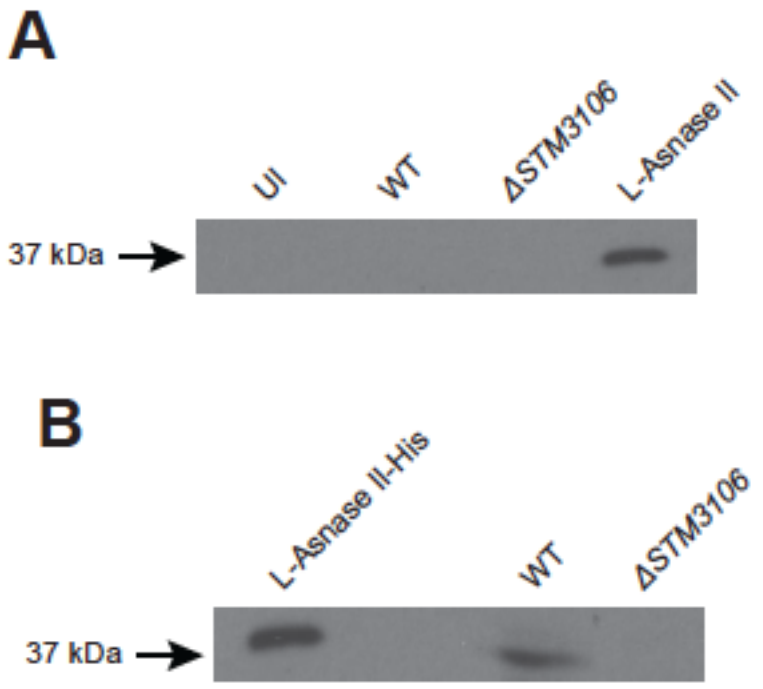

Figure 21 L-asparaginase II is detected under bacterial growth conditions, but not in supernatant taken from T cells cultured with *S.* Typhimurium

(A) Western blot analysis of conditioned medium harvested from T cells left UI or cultured with WT or *ΔSTM3106 S*. Typhimurium. Polyclonal rabbit anti-*E. coli* L-asparaginase II antibody was used to detect L-asparaginase II. Purified L-asparaginase II of *E. coli* was used as a positive control. **(B)** Western blot analysis of whole cell lysates of WT or *ΔSTM3106 S*. Typhimurium grown in LB broth. Polyclonal rabbit anti-*E. coli* L-asparaginase II antibody was used to detect L-asparaginase II. Purified C-terminal, 6xHis-tagged *S*. Typhimurium L-asparaginase II was used as a positive control.

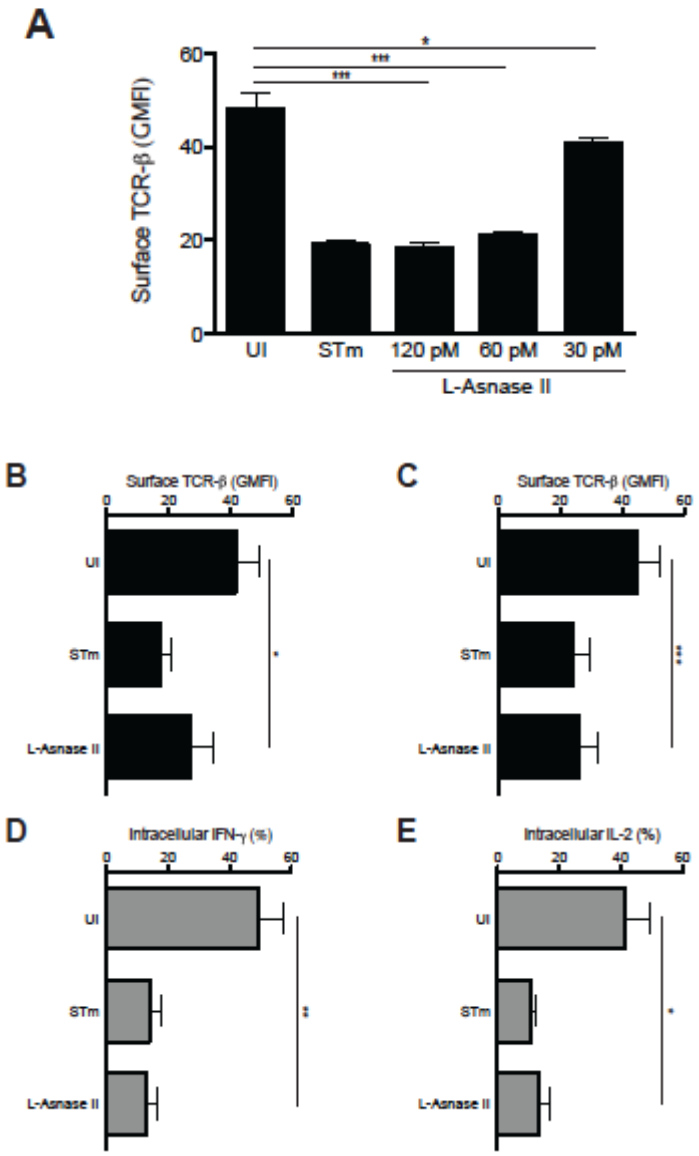

Figure 22 L-asparaginase II is sufficient to cause down-modulation of the T cell receptor and block cytokine production

(A) Expression of surface TCR-β by CD90.2$^+$ CD25$^+$ T cells left UI, cultured with STm or treated with the indicated concentrations of purified L-asparaginase II (L-Asnase II), *P*=0.0018. **(B and C)** Expression of surface TCR-β by CD4$^+$ (B) and CD8$^+$ (C) CD25$^+$ T cells left uninfected (UI) or treated as in (A), cultured with WT *S*. Typhimurium (STm) or treated with 60 pM of purified L-asparaginase II (L-Asnase II), *P*=0.0012 for CD4$^+$ T cells and *P*<0.0001 for CD8$^+$ T cells. **(D and E)** Expression of intracellular IFN-γ (D) and IL-2 (E) by CD25$^+$ T cells left uninfected or treated as in (A), *P*=0.0015 for IFN-γ-producing T cells and *P*=0.0070 for IL-2-producing T cells. Data show mean with SEM from at least three independent experiments.

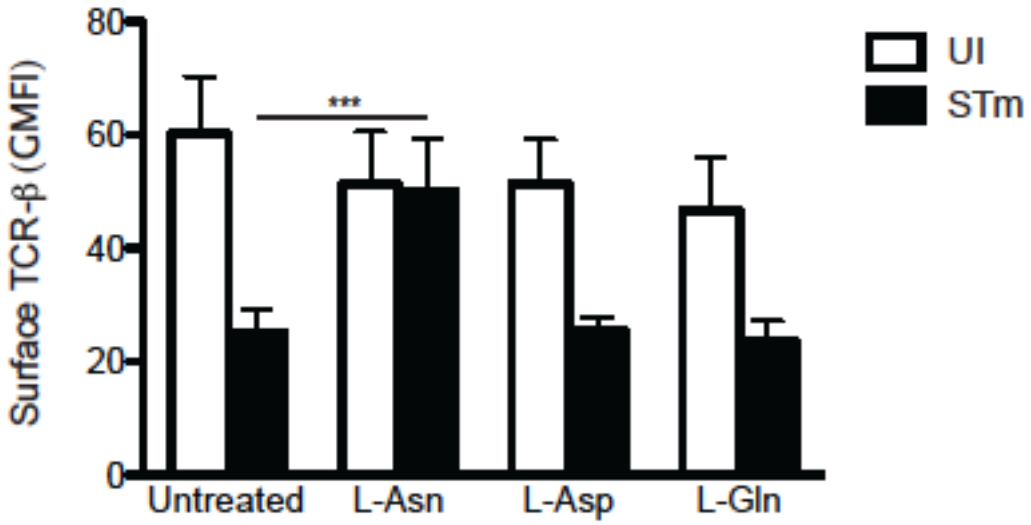

Figure 23 Supplementation with L-asparagine blocks *S.* Typhimurium-induced T cell inhibition

Expression of surface TCR-β by untreated, L-asparagine-treated (L-Asn), L-aspartate-treated (L-Asp) or L-glutamine-treated (L-Gln) $CD25^+$ T cells left UI or cultured with STm, $P<0.0001$. Data show mean with SEM from at least three independent experiments.

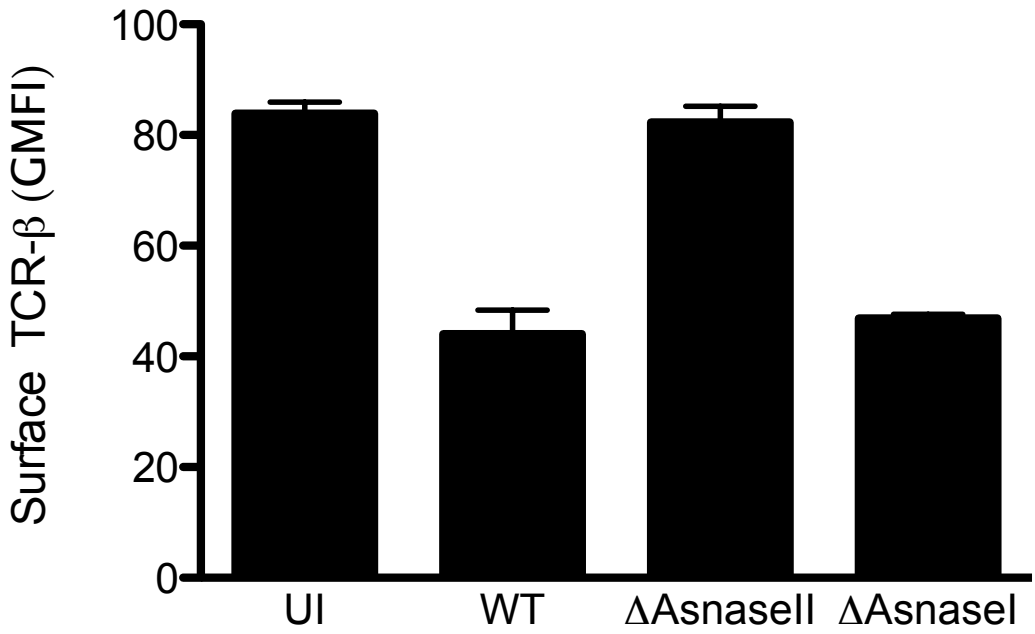

Figure 24 *S.* Typhimurium deficient for L-asparaginase I inhibit T cells

Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI), cultured with wild-type (WT) *S*. Typhimurium, *S*. Typhimurium deficient for L-asparaginase II (ΔAsnase II), or *S*. Typhimurium deficient for L-asparaginase I (ΔAsnase I). Data show mean with SEM from at least three independent experiments. The difference between WT and ΔAsnaseI was not statistically significant.

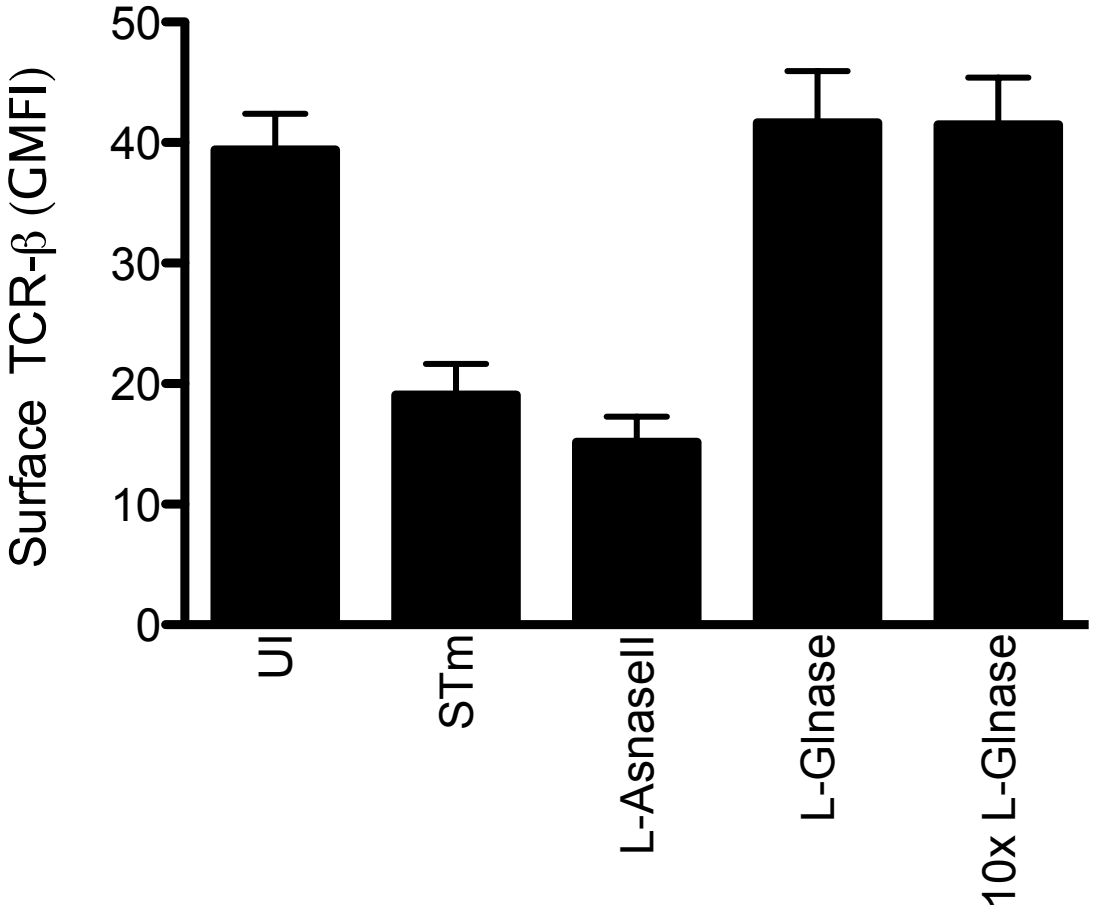

Figure 25 Purified L-glutaminase does not inhibit T cells

Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI), cultured with wild-type *S.* Typhimurium (STm), treated with 10 mU of purified L-asparaginase II (L-Asnase II), treated with 10 mU of purified L-glutaminase (L-Glnase), or treated with 100 mU of purified L-glutaminase (10X L-Glnase). Data show mean with SEM from at least three independent experiments. Differences between UI, L-Glnase, and 10x L-Glnase were not statistically significant.

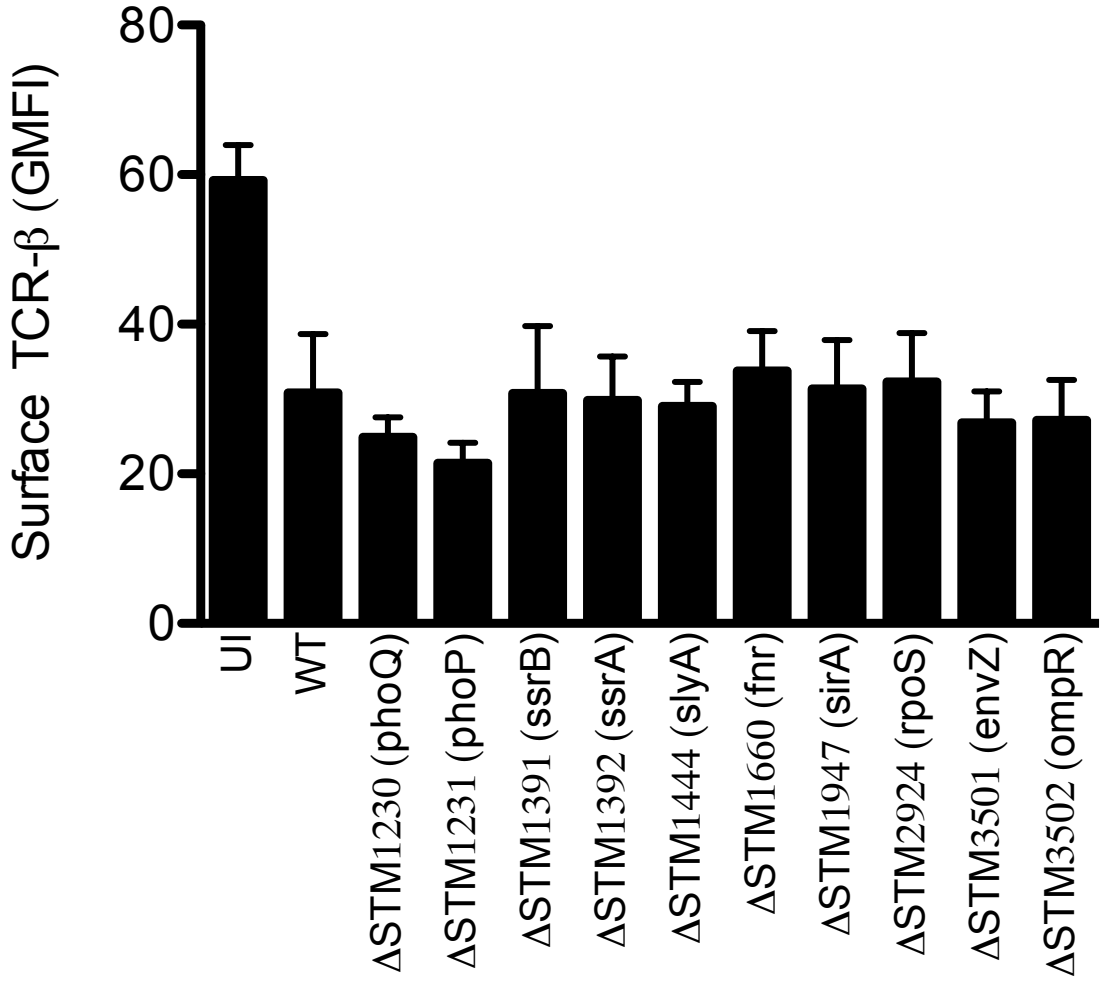

Figure 26 Several known virulence regulators do not contribute to T cell inhibition

Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI) or cultured with wild-type (WT), *ΔSTM1230*, *ΔSTM1231*, *ΔSTM1391*, *ΔSTM1392, ΔSTM1444*, *ΔSTM1660*, *ΔSTM1947*, *ΔSTM2924, ΔSTM3501*, or *ΔSTM3502 S*. Typhimurium. Data show mean with SEM from at least three independent experiments.

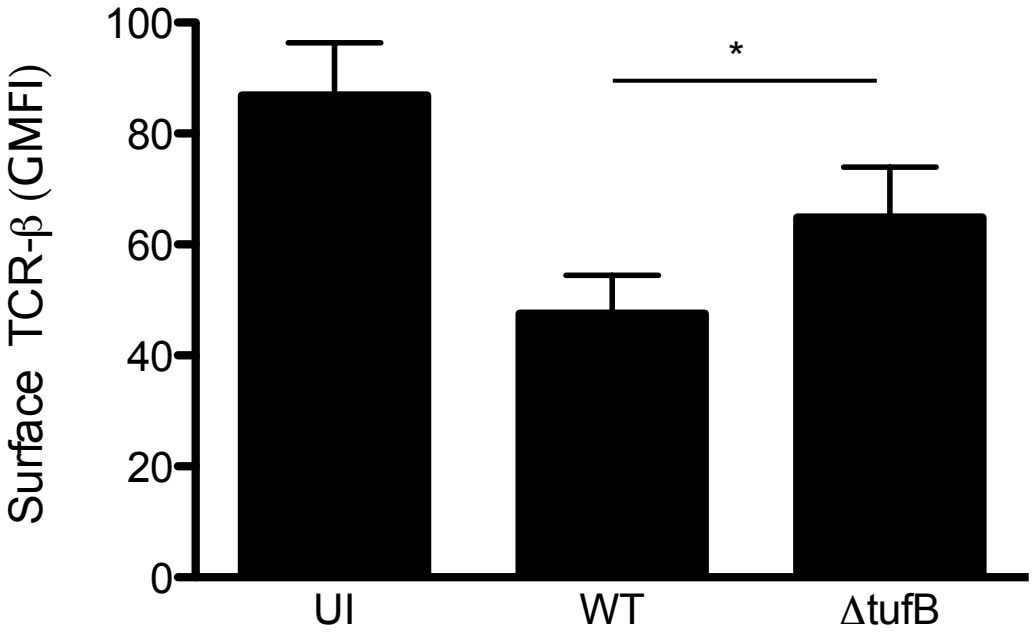

Figure 27 ***S.*** **Typhimurium deficient for** ***tufB*** **do not inhibit T cells as efficiently as wild-type** ***S.*** **Typhimurium**

Expression of surface TCR-β by $CD25^+$ T cells left uninfected (UI), cultured with wild-type *S.* Typhimurium (WT) or *S.* Typhimurium deficient for *tufB* (ΔtufB), *P*=0.035. Data show mean with SEM from at least three independent experiments.

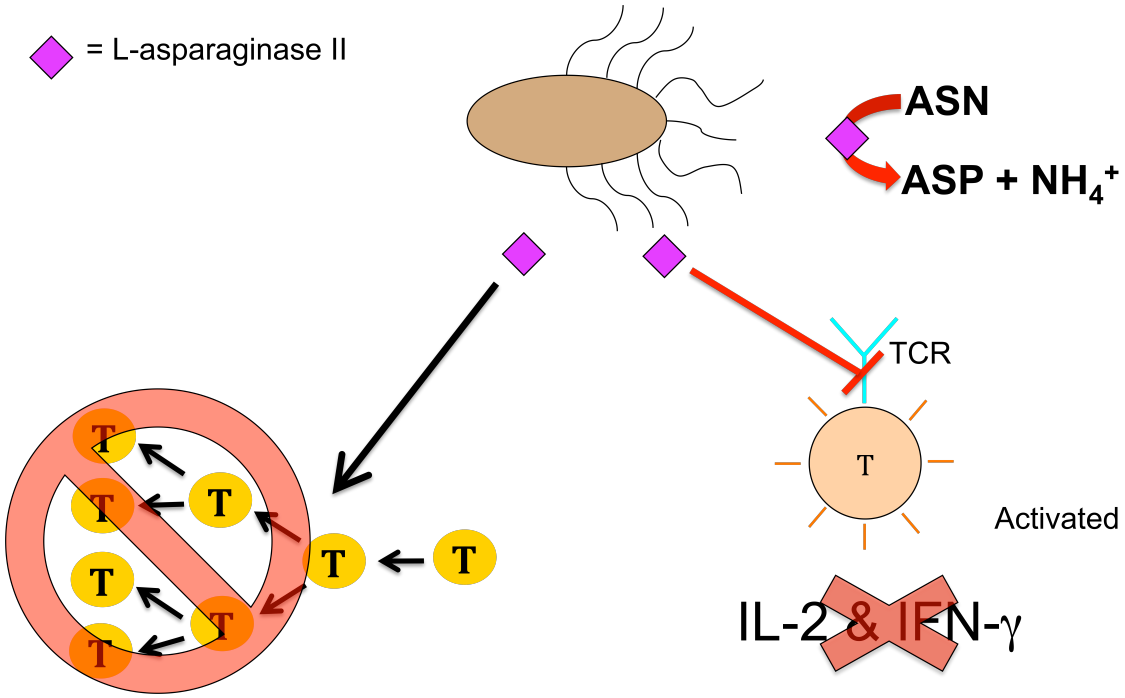

Figure 28 ***S.*** **Typhimurium use L-asparaginase II to inhibit the response of T cells**

Production of L-asparaginase II by *S*. Typhimurium is both necessary and sufficient to cause inhibition of T cells. *S*. Typhimurium use L-asparaginase II to hydrolyze L-asparagine, leading to down-modulation of TCR-β expression, suppression of T cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T cell proliferation.

II. Role of L-asparaginase II in *S.* Typhimurium infection

A. Mice infected with *ansB* mutant *S*. Typhimurium survive better than those mice infected with wild-type *S*. Typhimurium

As *ΔSTM3106 S*. Typhimurium have a profound defect *in vitro*, we wanted to determine whether *STM3106* (*ansB*), and therefore L-asparaginase II, is required for virulence of *S*. Typhimurium. Survival assays were performed using 129X1/SvJ mice, which have served as a useful model to study persistent salmonellosis (14, 112, 138, 149). Significantly more mice survived infection with *ΔSTM3106 S*. Typhimurium than with wild-type *S*. Typhimurium (Figure 29A*)*. A similar trend was observed for C57BL/6J mice, which are more susceptible to infection with *S*. Typhimurium (Figures 29B and 29C) (34, 35, 87). These data illustrate that the *S.* Typhimurium *ansB* mutant unable to express L-asparaginase II is attenuated for virulence in both acute and chronic mouse models of infection.

B. *S*. Typhimurium lacking L-asparaginase II are deficient for liver colonization

Since there was a statistical difference in the time-to-death studies using the 129X1/SvJ mice, organ burden assays were performed on the surviving mice to determine bacterial loads. After 60 days of infection, a long-term time point used to assess bacterial persistence (73, 86, 109), significantly fewer *ΔSTM3106 S*. Typhimurium than wild-type *S*. Typhimurium were recovered from livers, but not spleens and mesenteric lymph nodes (Figure 30A).

Additional organ burden assays were performed to determine the kinetics of primary wild-type and *ΔSTM3106 S*. Typhimurium infection. Similar numbers of wild-

type and *ΔSTM3106 S*. Typhimurium were recovered from spleens and livers during early stages of infection (Figures 30B and 30C). In contrast, significantly fewer *ΔSTM3106 S*. Typhimurium than wild-type *S*. Typhimurium were recovered from livers, but not spleens, during later stages of infection (Figures 30B and 30C). The presence of *STM3106 in trans* complemented the colonization defect of *ΔSTM3106 S*. Typhimurium in liver (Figure 31). Cumulatively, these results implicate L-asparaginase II contributes to the ability of *S*. Typhimurium to persist in the liver and is required for virulence.

C. *S*. Typhimurium deficient for L-asparaginase II elicit a more robust T cell response

Given that *ΔSTM3106 S*. Typhimurium fail to inhibit T cells *in vitro,* we examined whether there is a difference in the T cell response during the course of *S.* Typhimurium infection. Multiparametric flow cytometry was used to determine if *S*. Typhimurium utilize L-asparaginase II to inhibit the response of T cells *in vivo*. We analyzed the total T cell population, activation markers, and effector function. More T cells were recovered from livers and spleens of mice infected with *ΔSTM3106 S*. Typhimurium than from livers and spleens of mice infected with wild-type *S*. Typhimurium (Figures 32A and 32B). Furthermore, at the peak of the T cell response (Day 21), more activated T cells and IFN-γ-producing T cells were recovered from livers and spleens of mice infected with *ΔSTM3106 S*. Typhimurium than from livers and spleens of mice infected with wild-type *S*. Typhimurium (Figures 33A-D). However, these differences did not reach statistical significance. Interestingly, an enhanced T cell response did correlate with decreased bacterial burden in the liver (Figure 30B and Figure 32A).

Together, these results indicate that upon eliciting a productive adaptive immune response, characterized by an increase in activated, IFN-γ-secreting T cells, there is a decrease in bacterial burden in mice infected with *ΔSTM3106 S*. Typhimurium.

D. Contribution of L-asparaginase II in a secondary challenge with *S.* Typhimurium

Seeing as we have data implicating L-asparaginase II in T cell inhibition during a primary *S.* Typhimurium infection, we wanted to investigate whether L-asparaginase II would be utilized to dampen the T cell recall response during a secondary infection with *S.* Typhimurium. Here, C57BL/6J mice were immunized with the prototypical, live-attenuated *S.* Typhimurium vaccine strain CL1509 (14028 *aroA*-) (38, 79, 108). Approximately 100 days after immunization, mice were challenged with $5x10^6$ CFU of wild-type *S*. Typhimurium or *ΔSTM3106 S*. Typhimurium. Mice from each group were euthanized 3 days, 5 days, 7 days and 11 days after the challenge.

More wild-type *S*. Typhimurium than *ΔSTM3106 S*. Typhimurium were recovered from livers during early stages of infection (Figure 34A). In contrast, similar numbers of wild-type *S*. Typhimurium and *ΔSTM3106 S*. Typhimurium were recovered from spleens during the course of infection (Figure 34B).

Similar numbers of T cells were recovered from livers of mice infected with *ΔSTM3106 S*. Typhimurium and with wild-type *S*. Typhimurium (Figure 35A). However, on Day 11 post-challenge, there were significantly more T cells recovered from the spleens of mice infected with *ΔSTM3106 S*. Typhimurium than with wild-type *S*. Typhimurium (Figure 35B). Moreover, at Day 11 post-challenge, there were more activated T cells ($CD69^+$ or $CD25^+$) recovered from spleens of mice infected with

ΔSTM3106 S. Typhimurium than from spleens of mice infected with wild-type *S*. Typhimurium (Figures 35C and 35D). Together, these results suggest that wild-type *S*. Typhimurium use L-asparaginase II to dampen the robust, rapidly proliferating T cell recall response.

Collectively, these results demonstrate that *STM3106* (*ansB*), and therefore L-asparaginase II, contributes to virulence of *S*. Typhimurium in both acute and chronic mouse models of infection, and that *S*. Typhimurium utilize L-asparaginase II to colonize and persist in liver. Furthermore, these results suggest that the attenuation in virulence of *ΔSTM3106 S*. Typhimurium may be due to a reduced ability of these bacteria to inhibit the response of T cells *in vivo*.

E. Model of L-asparaginase II-mediated T cell inhibition in *S.* Typhimurium infection

Our data show that the *S.* Typhimurium utilize L-asparagine II to suppress T cells directed against it. Interestingly, L-asparaginases are used clinically to treat acute lymphoblastic leukemia (ALL) and have been shown to inhibit blastogenesis and proliferation by lymphocytes by a mechanism involving depletion of exogenous L-asparagine. Here, we suggest that *S.* Typhimurium cause immunosuppression by utilizing L-asparaginase II to dampen the T cell response *in vivo* by depleting L-asparagine from the localized environment, as L-asparagine deprivation has been shown to induce immunosuppression.

F. Acknowledgements

I would like to thank: Patricio Mena for his assistance and expertise in performing the animal work; Jason Tam for his help in analyzing the dynamics of the T cell

response and generating the F1 mice for the *in vivo* complementation study; Dr. James Bliska for the use of the MACS gentle-dissociator; and Patrick McLaughlin for continuing to pursue the studies examining the dynamics of the T cell recall response. In preparation of the animal harvest days, Joanna Luke did a tremendous job of making sure all the reagents we needed were available and these days would not have gone as smoothly without her. Also, thank you to our lab aide(s) for helping fill dilution tubes and making the plates.

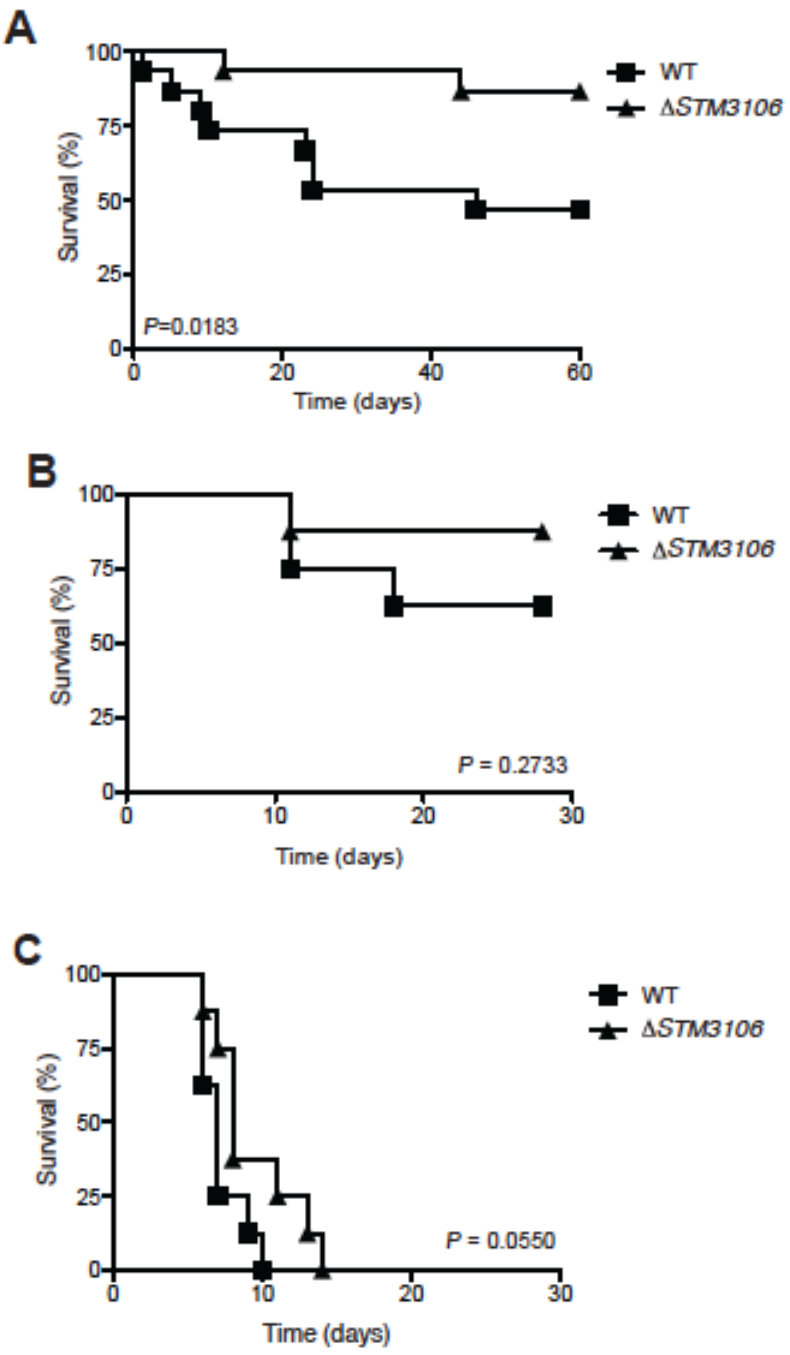

Figure 29 A *S.* Typhimurium *ansB* mutant unable to express L-asparaginase II is attenuated for virulence in mouse models of infection

(A) Survival of 129X1/SvJ mice (n=15 per group) inoculated intragastrically with $5x10^7$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, *P*=0.0183. **(B and C)** Survival of C57BL/6J mice (n=8 per group) inoculated intragastrically with $5x10^5$ CFU (B) or $5x10^6$ CFU (C) of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, *P*=0.2733 (B) and *P*=0.0550 (C).

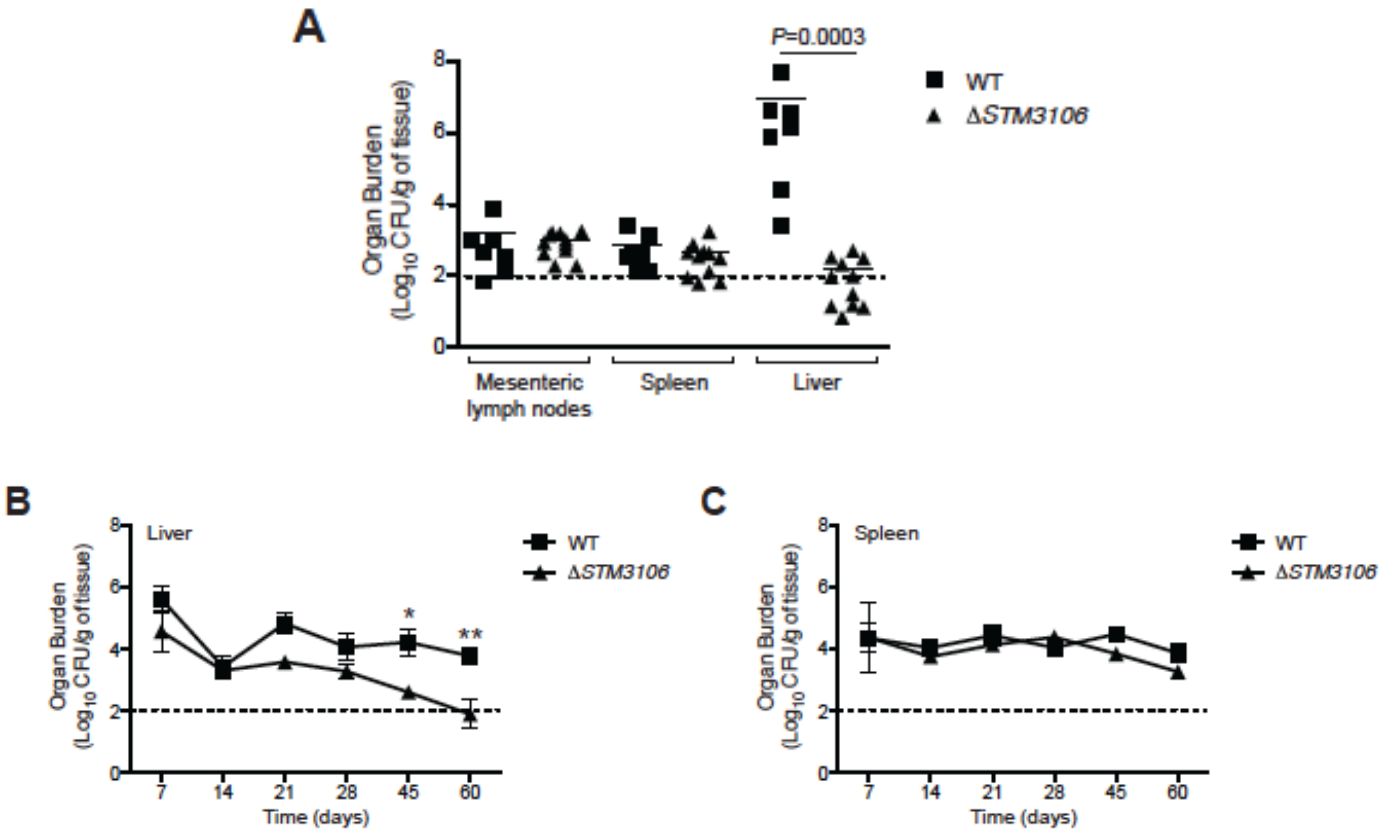

Figure 30 ***S.* Typhimurium lacking L-asparaginase II are deficient for liver colonization**

(A) Bacterial loads per gram of tissue harvested from 129X1/SvJ mice (n=15 per group) 60 days after intragastric inoculation with $5x10^7$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, *P*=0.0003 for liver. At the time of harvest, 8 mice infected with WT *S*. Typhimurium and 4 mice infected with *ΔSTM3106 S*. Typhimurium had succumbed. **(B and C)** Bacterial loads per gram of liver (C) and spleen (D) tissue harvested from 129X1/SvJ mice (n=5 per group per time point) at various times after intragastric inoculation with $5x10^7$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, *P*=0.0001 for liver and *P*=0.2905 for spleen. Data show mean with SEM.

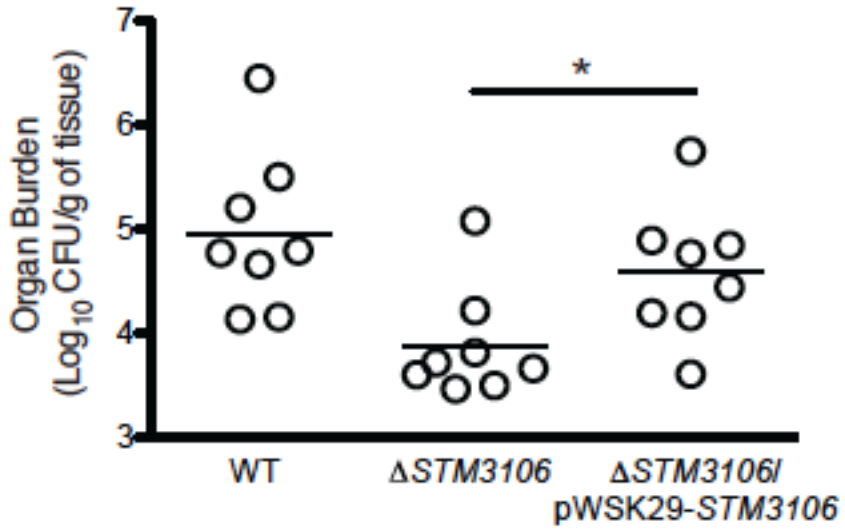

Figure 31 Expression of *STM3106 in trans* in *S*. Typhimurium lacking L-asparaginase II restores their ability to colonize the liver

Bacterial loads per gram of liver tissue harvested from F1 (C57BL/6J x 129X1/SvJ) hybrid mice (n=8 per group) 10 days after intravenous inoculation with $5x10^3$ CFU of WT *S*. Typhimurium, *ΔSTM3106 S.* Typhimurium or *ΔSTM3106 S.* Typhimurium carrying a derivative of pWSK29 encoding *STM3106* (*ΔSTM3106*/pWSK29-*STM3106*), *P*=0.0111. Data show mean with SEM.

ΔSTM3106 S. Typhimurium carrying a derivative of pWSK29 encoding *STM3106* (*ΔSTM3106*/pWSK29-*STM3106* were able to down-modulate surface expression of TCR-β (Data not shown).

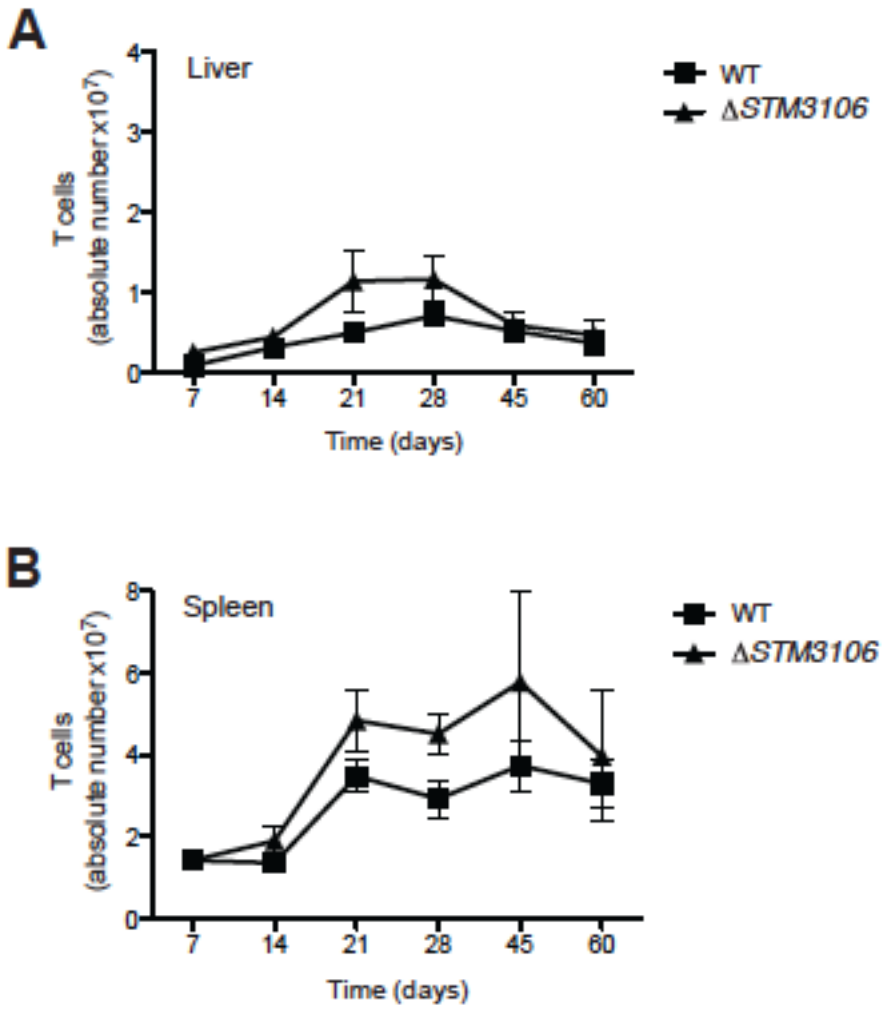

Figure 32 ***S*. Typhimurium deficient for L-asparaginase II elicit a more robust T cell response during primary infection**

(A and B) Absolute numbers of T cells present in livers (A) and spleens (B) harvested from 129X1/SvJ mice (n=5 per group per time point) at various times after intragastric inoculation with $5x10^7$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, *P*=0.1917 for liver and *P*=0.2934 for spleen. Data show mean with SEM.

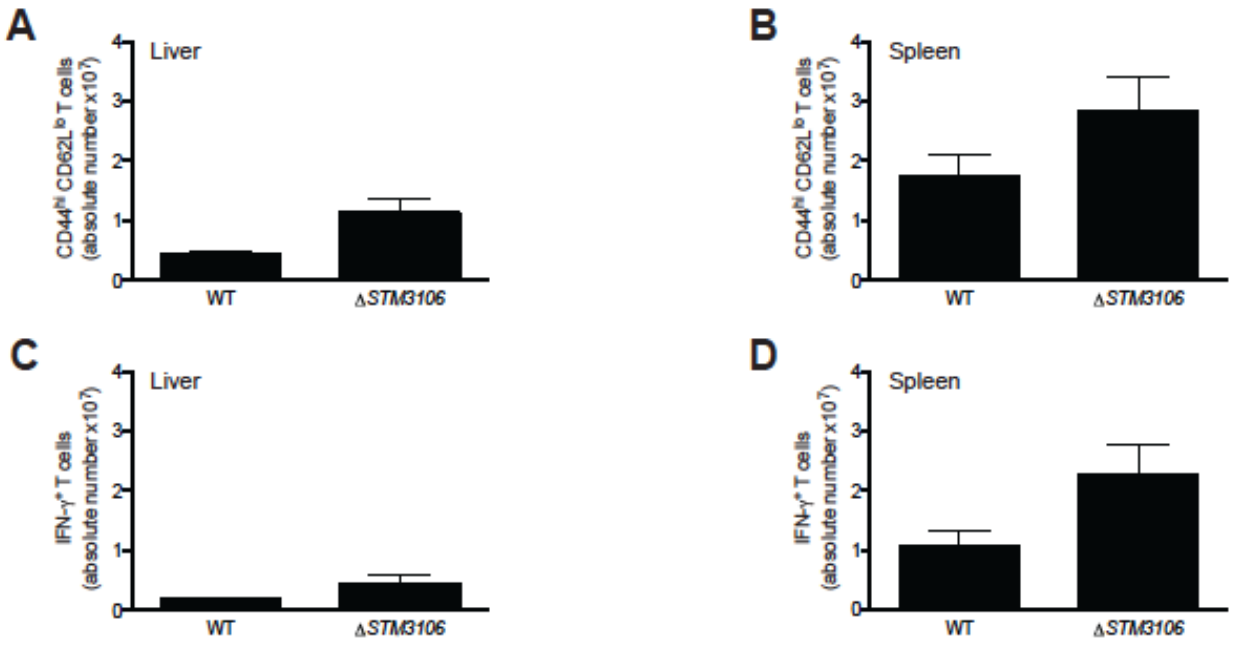

Figure 33 More activated T cells secreting IFN-γ were found in spleens and livers of mice infected with *S.* Typhimurium deficient for L-asparaginase II than with wild-type *S.* Typhimurium

(A and B) Absolute numbers of $CD44^{hi}$ $CD62L^{lo}$ T cells present in livers (A) and spleens (B) of 129X1/SvJ mice (n=5 per group per time point) at day 21 after intragastric inoculation with $5x10^7$ CFU of WT or *ΔSTM3106 S*. Typhimurium. Differences in the number of activated T cells in mice infected with WT or *ΔSTM3106 S*. Typhimurium were not statistically significant. **(C and D)** Absolute numbers of IFN-γ-producing T cells present in livers (C) and spleens (D) of 129X1/SvJ mice (n=5 per group per time point) at day 21 after treatment as in (A). Differences in the number of T cells secreting IFN-γ in mice infected with WT or *ΔSTM3106 S*. Typhimurium were not statistically significant. Data show mean with SEM.

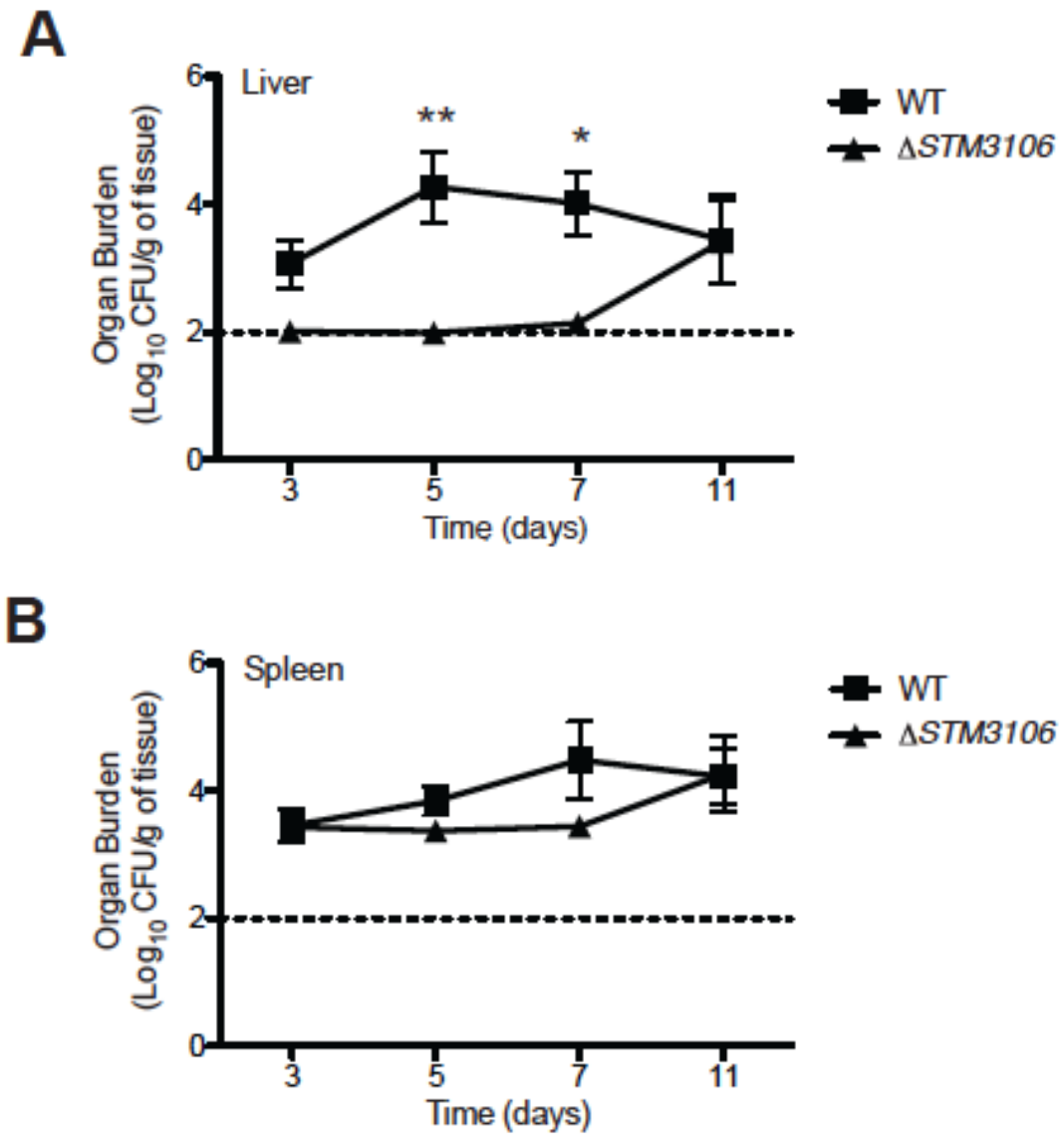

Figure 34 ***S*. Typhimurium lacking L-asparaginase II are deficient for liver colonization during secondary infection**

(A and B) Bacterial loads per gram of liver (A) and spleen (B) tissue harvested from immunized C57BL/6J (n=5 per group) after intragastric inoculation with $5x10^6$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium, ***P*<0.01 and * *P*<0.05, respectively, for liver and there were no differences that reached statistical significance for spleen. Data show mean with SEM.

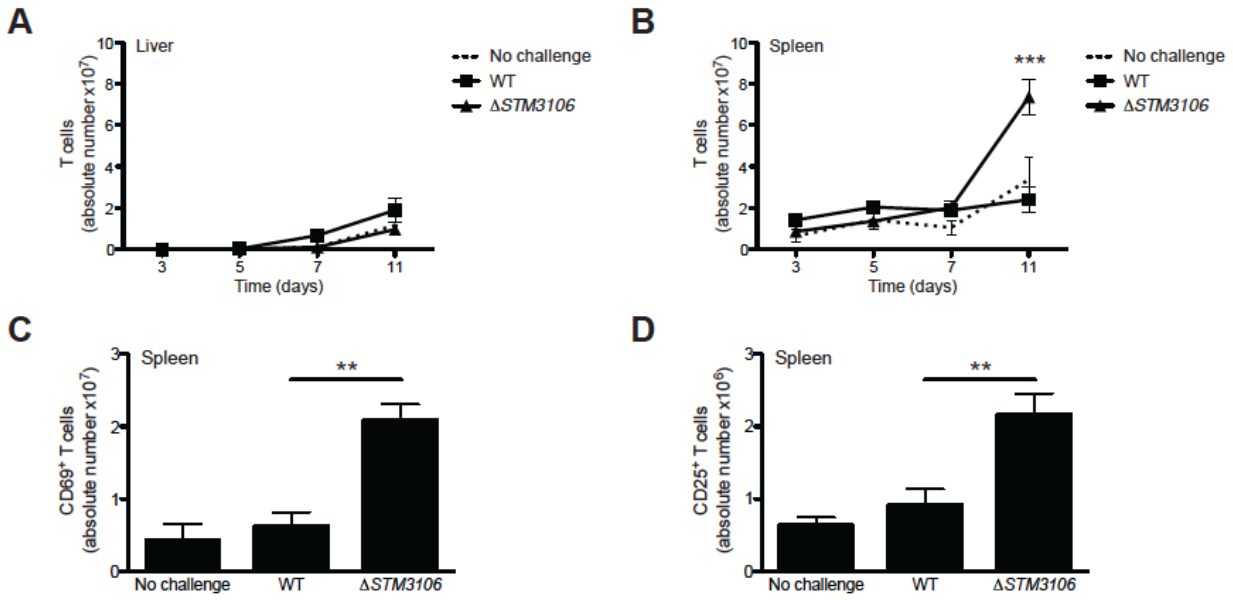

Figure 35 *S*. Typhimurium deficient for L-asparaginase II elicit a more robust T cell response during secondary infection

(A and B) Absolute numbers of T cells present in liver (A) and spleen (B) tissue harvested from immunized C57BL/6J mice (n=5 per group) after intragastric inoculation with $5x10^6$ CFU of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium or mock challenge (PBS, dotted line). **(C and D)** Absolute numbers of activated T cells in the spleen, $CD69^+$ T cells (C) and $CD25^+$ T cells (D) of immunized C57BL/6J mice (n=5 per group per time point) at day 11 after treatment as in (A), $^{**}P<0.01$. Data show mean with SEM.

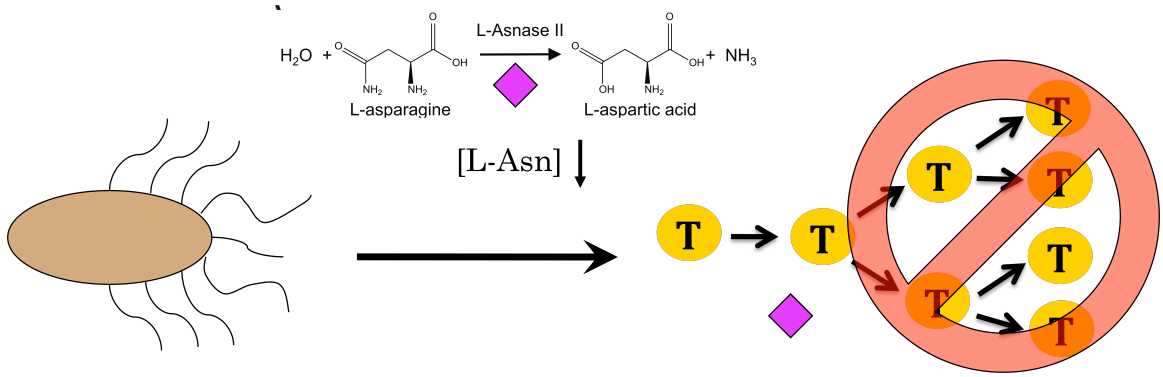

Figure 36 L-asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence through depletion of L-asparagine

Our data show that the *S.* Typhimurium utilize L-asparagine II to suppress T cells directed against it. L-asparaginases are used clinically to treat acute lymphoblastic leukemia (ALL) and have been shown to inhibit blastogenesis and proliferation by lymphocytes by a mechanism involving depletion of exogenous L-asparagine. Here, we suggest that *S.* Typhimurium cause immunosuppression by utilizing L-asparaginase II to dampen the T cell response *in vivo* by depleting L-asparagine from the localized environment, as L-asparagine deprivation has been shown to induce immunosuppression.

DISCUSSION AND FUTURE DIRECTIONS

I. Discussion

These studies demonstrate that *S.* Typhimurium utilize L-asparaginase II to inhibit the response of T cells and that production of L-asparaginase II by *S*. Typhimurium is both necessary and sufficient to cause inhibition of T cells. Our identification of the factor responsible for *S*. Typhimurium-induced T cell inhibition provides an understanding of a previously unrecognized mechanism by which these microorganisms can establish infection in the mammalian host and avoid clearance by the immune system. One implication of our results is that production of L-asparaginase II by *S*. Typhimurium causes depletion of exogenous L-asparagine, leading to down-modulation of TCR-β expression, suppression of T cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T cell proliferation. Another important and more general implication of our work is that of a metabolic enzyme functioning beyond its canonical role in catalyzing the hydrolysis of L-asparagine to aspartic acid and ammonia.

L-asparaginases are used clinically to treat acute lymphoblastic leukemia (ALL) and have been shown to inhibit blastogenesis and proliferation by lymphocytes (41, 42). They function by a mechanism involving depletion of exogenous L-asparagine and, to a lesser extent, L-glutamine (4, 128). In a significant number of patients with ALL, malignant cells exhibit a metabolic defect in L-asparagine synthesis and are dependent on an exogenous source of L-asparagine for survival (4). Normal cells are able to synthesize L-asparagine by up-regulating asparagine synthetase and thus are less

affected by the rapid depletion of this important amino acid following treatment with L-asparaginase (3).

T cells are extremely sensitive to amino acid deprivation, particularly when they are proliferating and differentiating, and it has been shown that APCs regulate T cell proliferation by controlling amino acid availability, specifically releasing cysteine (2, 20, 130). T cells, like malignant cells in ALL, may be dependent on an exogenous source of L-asparagine when undergoing the metabolically demanding process of clonal expansion. Indeed, it has been shown that L-asparagine is required for lymphocyte blastogenesis (131, 176). Furthermore, it has been described that the ability of lymphoid tissues to synthesize L-asparagine is severely restricted, suggesting L-asparagine may be an essential nutrient (131). Here, we suggest that *S.* Typhimurium cause immunosuppression by utilizing L-asparaginase II to dampen the T cell response *in vivo* by depleting L-asparagine from the localized environment, as L-asparagine deprivation has been shown to induce immunosuppression (131).

Our results show that there is a significant difference in colonization of the liver at late stages of infection in mice infected with L-asparaginase II-deficient *S.* Typhimurium when compared to wild-type *S.* Typhimurium. This *in vivo* phenotype could be due to a reduced ability of L-asparaginase II-deficient *S.* Typhimurium to inhibit T cell responses during infection. Consistent with a role for L-asparaginase II in *S*. Typhimurium-induced T cell immunosuppression, it has been previously shown that T cell immunosuppression also involves *S*. Typhimurium genes encoded by the virulence plasmid (63) and *Salmonella* Pathogenicity Island (SPI-2) (39), and that *in vivo* phenotypes reveal themselves when the adaptive immune system is fully engaged. Hence, if *S.*

Typhimurium utilize L-asparaginase II for inhibition of the T cells, it is consistent that an *in vivo* phenotype may reveal itself in a systemic site, such as the liver or spleen, and during later stages of infection.

In agreement with a delay in the onset of the adaptive immune response, we found that the peak of the primary T cell response occurs between days 21 and 28 post-infection, and this correlates with subsequent decrease in bacterial burden in liver of mice infected with Δ*STM3106 S.* Typhimurium. We did not observe a statistically significant difference in the primary T cell response in the spleen and liver of mice infected with wild-type *S.* Typhimurium or L-asparaginase II-deficient *S.* Typhimurium (Figures 32A-33D). However, there is a trend of more activated, IFN-γ-secreting T cells being present in mice infected with *S.* Typhimurium lacking L-asparaginase II than in mice infected with wild-type *S.* Typhimurium (Figures 32A-33D). Given the general properties and functions of cytokines secreted by activated T cells (e.g. cascade induction, paracrine action and attributes of pleiotropy, redundancy, synergism and antagonism), a large and sustained impact on the magnitude of the T cell response may not be needed in order to significantly alter the course of a *S.* Typhimurium infection. Our time-to-death studies are consistent with this idea because more mice survived infection with L-asparaginase II-deficient *S.* Typhimurium than with wild-type *S.* Typhimurium (Figures 29A-C).

If T cells, like malignant cells in ALL, are dependent on an exogenous source of L-asparagine when undergoing the metabolically demanding process of clonal expansion, this may provide insight into the statistically significant difference we observed in the T cell recall response (Figures 35B-D and (131, 176)). Under these

conditions, the antigen-experienced T cells do not require co-stimulation and undergo more rapid clonal expansion when they encounter their cognate antigen. Thus, if *S*. Typhimurium utilize L-asparaginase II to cause T cell inhibition by depriving them of L-asparagine, we would expect to detect a larger difference when there is strong stimulation for them to proliferate and this is indeed what we observed (Figures 35B-D). Collectively, our data indicate that the mechanism by which *S*. Typhimurium inhibit the response of T cells by deploying L-asparaginase II may be similar to the mechanism by which clinically administered L-asparaginases help eliminate human cancers.

In addition to the inhibitory effects on T cells by L-asparaginase II, there may be other effects, such as reduced availability of nutrients to *S.* Typhimurium lacking L-asparaginase II. These effects could be amplified in the liver, since the liver already has lower levels of amino acids (65, 78). It is also possible *S.* Typhimurium specifically target the liver, given that this organ has an important role in the removal of pathogens and antigens (65, 78, 121). Kupffer cells produce IL-10, a potent inhibitory cytokine, in response to LPS (78). Another important function of the liver is to induce peripheral tolerance (158). It is interesting to postulate that wild-type *S.* Typhimurium may modulate IL-10 production or induce tolerance to further amplify the immunosuppressive environment of the liver.

The observations of this study parallel earlier reports which describe *S.* Typhimurium causing T reg-induced immunosuppression, allowing the bacteria to establish infection (74). Eventually, a T cell response is generated, resulting in clearance of the bacteria (74). Thus, it would be of interest to evaluate if there is a difference in T reg cell numbers or their effector function in the livers of mice infected

with Δ*STM3106 S.* Typhimurium or wild-type *S.* Typhimurium. This will allow for interesting insight because the liver has a large resident population of T reg cells, which express enzymes that deplete the environment of essential amino acids (23, 29, 78, 124, 158).

S. Typhimurium have been found to reside primarily inside macrophages of the mouse liver (104). We investigated if a difference in survival inside macrophages may contribute to the difference in liver colonization. We observed no difference in survival between Δ*STM3106 S.* Typhimurium and wild-type *S.* Typhimurium inside macrophages (Figures 19A-C), suggesting that a difference in intracellular survival does not account for the colonization difference. Approximately 80% of macrophages in the liver are Kupffer cells (124). Kupffer cells are liver-specific macrophages with unique endocytic and phagocytic capacities primarily found at sites where blood enters the liver (124). Thus, we need to determine how Δ*STM3106 S.* Typhimurium and wild-type *S.* Typhimurium survive in this specialized population of macrophages as well as in hepatocytes (another abundant cell type of the liver) to fully understand the liver-specific colonization defect.

Our results provide new insight in the mechanism used by *S.* Typhimurium to inhibit T cell responses: down-modulation of TCR-β expression, suppression of T cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T cell proliferation. Our results are consistent with previous studies showing that *Salmonella* T cell inhibitor (STI) produced by *S*. Typhimurium blocks T cell proliferation and cytokine production (3, 99, 100). However, we demonstrated that *sti*-deficient *S*. Typhimurium down-modulated TCR-β on activated T cells, indicating that it was not required for that

aspect of T cell inhibition (166). Additionally, the mechanisms we describe in this study are supported by recent studies demonstrating that purified L-asparaginase II from *S*. Enteritidis and *H. pylori* can inhibit protein synthesis, block cell-cycle progression and suppress replication of cultured cell lines (69, 144, 147). These studies also suggest that L-asparaginase II inhibits proliferation via L-asparagine depletion (69, 147). Additional support for immunosuppression induced by deprivation of L-asparagine comes from studies on the effect of purified *E. coli* L-asparaginase II on endogenous populations of lymphocytes. These studies revealed a reduction in the mature population of T cells, as T cells are most sensitive to L-asparaginase II treatment during early stages of differentiation (21).

Additional reports describe L-asparaginase II-treated cells as having decreased mTOR signaling and phosphorylation of eIF2α (4). The mTOR pathway controls cell growth and division and its activation is required for T cell differentiation (130). Phosphorylation of eIF2α suppresses global protein synthesis, limits the consumption of amino acids, and induces transcription of genes encoding metabolic pathways required for the biosynthesis of amino acids (20, 130). Collectively, these reasons may provide further insight to the mechanism by which L-asparaginase II produced by *S*. Typhimurium inhibits production of a T cell response. It would be interesting to compare the levels of phosphorylation of eIF2α in activated T cells cultured in the presence of L-asparaginase II to the levels in untreated T cells.

We propose that *S.* Typhimurium utilize L-asparaginase II to inhibit induction of a T cell response through depletion of L-asparagine. Additional support for this hypothesis comes from our laboratory. We generated a mutation in the catalytic site of

L-asparaginase II, which subsequently fails to down-modulate TCR-β, block cytokine production, and inhibit T cell proliferation (A. Torres Gersch, A. Kullas, and A. van der Velden, unpublished data). Moreover, we demonstrated that when uninfected T cells were cultured in medium lacking L-asparagine, they expressed levels of TCR-β similar to T cells cultured in the presence of *S*. Typhimurium in rich media (A. Torres Gersch and A. van der Velden, unpublished data). These results indicate that T cells require L-asparagine and are consistent with T cells being extremely sensitive to amino acid deprivation (2, 20, 130). We also have found that treatment of uninfected, activated T cells with L-aspartate or ammonia does not cause T cell inhibition (Figure 23 and A. Torres Gersch and A. van der Velden, unpublished data). Collectively, these results indicate that L-asparaginase II-dependent inhibition is due to depletion of substrate (L-asparagine), not accumulation of product (L-aspartate or ammonia). We are currently in the process of investigating the effect of L-asparagine starvation on T cell function.

We have found that L-asparaginase II down-modulates expression of a number of proteins expressed on the surface of T cells (J. Luke and A. van der Velden, unpublished data). These results are consistent with the hypothesis that L-asparaginase II has a global effect on T cells that may be characterized by starvation and inhibition of protein synthesis. We have begun to analyze expression of asparagine synthetase and preliminary results indicate that there is a difference in transcript levels in activated T cells left uninfected or treated with L-asparaginase II (J. Luke and A. van der Velden, unpublished data). This is analogous to observations made in *H. pylori* where cell-cycle inhibition induced by L-asparaginase correlates to lower expression

levels of asparagine synthetase (144). Collectively, these results suggest that L-asparaginase II may have a global inhibitory effect on T cells.

Interestingly, the L-asparaginase II gene *ansB* is highly conserved in Gram-negative bacteria and has been shown to contribute to the virulence of several important human pathogens. *Campylobacter jejuni* also utilize L-asparaginase II to colonize and persist in liver, and strains expressing L-asparaginase II have increased virulence (64). In addition, asparaginase-deficient *H. pylori* are significantly more attenuated (147).

The studies of L-asparaginase II in *C. jejuni* and *H. pylori* also characterized the importance of a secretion signal in both of these L-asparaginase sequences (64, 144). The secretion signal allowed subcellular localization of L-asparaginase to the periplasm, which allowed the bacteria to efficiently use L-asparagine as a nutrient source. L-asparaginase II of *S.* Typhimurium also has a secretion signal, thus we predict that its localization to the periplasm is also required for its ability to inhibit T cells. We have preliminary data that may support the conclusion that L-asparaginase II is localized to the periplasm. Upon continuing to screen the multi-gene deletion *S.* Typhimurium library to identify additional genes required for T cell inhibition, we found *S.* Typhimurium deficient for *dsbA* were also unable to down-modulate TCR-β (P. McLaughlin and A. van der Velden, unpublished data). Interestingly, in *dsbA*-deficient *S.* Typhimurium, the protein expression levels of L-asparaginase II were reduced when compared to wild-type *S.* Typhimurium (P. McLaughlin and A. van der Velden, unpublished data). DsbA is required for disulfide bond formation of proteins in the periplasm. This suggests that DsbA is required for disulfide bond formation in L-

asparaginase II, allowing for proper protein folding, and may explain why DsbA contributes to T cell inhibition by a L-asparaginase II-dependent mechanism.

Although the canonical function of L-asparaginase II is to catalyze the hydrolysis of L-asparagine to aspartic acid and ammonia, it is striking to consider the convergent role of an enzyme used clinically to suppress T cell cancers with the discovery that the same enzyme serves as a virulence determinant that allows *S*. Typhimurium to suppress T cells directed against it. Our work establishes that L-asparaginase II has important functions in infection and immunity, leaving open the possibility that *S*. Typhimurium produce and secrete L-asparaginase II to utilize L-asparagine and thus compete with the host for nutrients.

Interestingly, L-asparagine may be an important nutrient for bacterial pathogens in the liver, but not in the intestine (64). L-asparagine is continually synthesized in liver and is maintained at approximately 20 μM in mice (124). Given that L-asparagine is found in serum of the liver, it could be an important resource for *S.* Typhimurium at this site (124). Additionally, when growing in minimal medium where only L-asparagine is supplemented, wild-type *S.* Typhimurium grow slightly better than the Δ*STM3106 S*. Typhimurium (P. McLaughlin and A. van der Velden, unpublished data), suggesting that *S.* Typhimurium may utilize L-asparaginase II to use L-asparagine. Since L-asparagine is continually made in the liver, it could be a sustained nutrient source for *S.* Typhimurium. Thus, the bacteria can persist and thrive in the liver when compared to the spleen (Figures 30A-C and Figure 31). In contrast, Δ*STM3106 S*. Typhimurium do not express L-asparaginase II and are not able to use L-asparagine, which is why their persistence in the liver is similar to the bacterial levels found in the spleen (Figures 30A-

C). These results suggest that wild-type *S*. Typhimurium may use L-asparaginase II to effectively compete for the available L-asparagine of the liver and deplete its availability from the T cells.

To determine the importance of T cells in the bacterial colonization difference of the liver, we plan to conduct organ burden studies in T cell-deficient mice. Unfortunately, we have not had the correct strains of mice to do these experiments yet as the required mice are not commercially available. If the colonization difference between wild-type *S.* Typhimurium and L-asparaginase II-deficient *S.* Typhimurium persists in the liver of T cell-deficient mice, this would indicate that T cells are not responsible for the colonization difference. However, if the difference in colonization of the liver is no longer present in T cell-deficient mice, it would suggest that T cells are required for effectively clearing *S.* Typhimurium from the liver. Furthermore, if we observe an intermediate phenotype of the liver colonization in T cell-deficient mice, this would imply that T cells contribute to the difference in liver colonization we identified, but there are other contributing factors as well. This additional mechanism may be due to a reduced ability of L-asparaginase II-deficient *S.* Typhimurium to consume L-asparagine as well as inhibit the T cell response.

L-asparaginases are used to treat acute lymphoblastic leukemia through mechanisms that likely involve amino acid starvation of leukemic cells and these findings indicate that pathogens similarly use L-asparagine deprivation to limit T cell responses. This is the first study to show that a bacterial pathogen, *S.* Typhimurium, utilizes L-asparaginase II to inhibit the mammalian T cell response against it, thereby leading to its increased survival and persistence. Furthermore, the L-asparaginase II

gene *ansB* is highly conserved in bacteria and has been shown to contribute to virulence of several important human pathogens. Thus, L-asparaginase II could prove to be a promising target for development of innovative, broad-spectrum therapeutic approaches and preventive measures to overcome bacterial infections.

II. Future Directions

With these studies, we have gained significant insight into the mechanism by which *S.* Typhimurium inhibit T cell responses and mediate virulence. This has led to new interests of the laboratory and opened new areas of research.

A. Determine how L-asparaginase II gets out of *S.* Typhimurium

We previously reported that there is a proteinaceous factor present in the supernatant harvested from the co-culture of T cells and *S.* Typhimurium that is required for T cell inhibition (166). Here, we report that the identity of the factor is L-asparaginase II. However, how L-asparaginase II gets into the supernatant is elusive. A bioinformatics approach using SignalP4.0 Server predicted the presence and location of a signal peptide cleavage site in the primary amino acid sequence of L-asparaginase II, with a predicted cleavage site between residues 22 and 23. This suggests that L-asparaginase II of *S.* Typhimurium is translocated from the cytoplasm to the periplasm by the Sec system, and then may be secreted into the extracellular environment. Preliminary results have shown the requirement of the Sec-secretion signal of L-asparaginase II for its expression and subsequent T cell inhibition (data not shown). Further experiments need to be conducted to determine how L-asparaginase II gets outside *S.* Typhimurium. We are currently mutating the signal peptide of L-

asparaginase II to inhibit its translocation by the Sec-secretion system. We will then analyze the ability of this mutant L-asparaginase II to cause T cell inhibition. An additional possibility is L-asparaginase II is released by *S.* Typhimurium through outer membrane vesicles (OMVs). To test this hypothesis, we could add purified OMVs from *S.* Typhimurium to isolated T cells and identify if OMVs are sufficient to inhibit T cells. If the OMVs are sufficient for causing T cell inhibition, we could analyze the protein content of OMVs using mass spectrometry.

B. Characterize regulation of L-asparaginase II in *S.* Typhimurium

We determined that deleting *invA* (SPI-1) and/or *spiB* (SPI-2) or *phoP* from *S.* Typhimurium did not alter its ability to inhibit T cells (166). Thus, it is unlikely that any of these directly regulate L-asparaginase II in *S*. Typhimurium. In this study, we screened a select panel of targeted deletion mutants of known virulence regulators and found that none appeared to contribute to L-asparaginase II-mediated T cell inhibition (Figure 26). More multi-gene deletions were generated and screened for their ability to cause TCR-β down-modulation. Subsequently, we found that *dsbA* appears to contribute to T cell inhibition in a L-asparaginase II-dependent mechanism (P. McLaughlin and A. van der Velden, unpublished data). These are exciting preliminary results and more work needs to be done to further understand the regulation of L-asparaginase II and how DsbA contributes to its expression.

C. Identify if L-asparaginase II enzymatic activity is required for T cell inhibition

Here, we show exogenous L-asparagine, but not L-aspartate or L-glutamine, prevented *S*. Typhimurium-induced down-modulation of TCR-β (Figure 23). These

results imply that the enzymatic activity of L-asparaginase II contributes to T cell inhibition. Further, we showed a mutation (T89A) in the catalytic site of L-asparaginase II resulted in the failure to down-modulate TCR-β and inhibit cytokine production (A. Torres Gersch, A. Kullas, and A. van der Velden, unpublished data). Additional studies are underway in our laboratory to quantify the enzymatic activity of the catalytic mutant and wild-type L-asparaginase II. This will allow us to determine if the enzymatic activity of L-asparaginase II is required for T cell inhibition.

D. Examine the effects of L-asparaginase II on T cells

While we have demonstrated L-asparaginase II is sufficient to down-modulate TCR-β surface expression, suppress T cell blastogenesis, and block cytokine production (Figures 22A-E and data not shown), we still do not know the extent of the effects that L-asparaginase II has on T cells. Earlier studies found that L-asparaginase II (from *E. coli*) alters the surface of the lymphocyte, which leads to decreased antigen binding and inhibits the synthesis of membrane glycoproteins (41, 42). Further, we are interested in determining if there is a difference in T cells left uninfected or cultured with wild-type *S.* Typhimurium or Δ*STM3106 S.* Typhimurium in their ability to synthesize their own L-asparagine by analyzing their L-asparagine synthetase levels. This is an active area of research currently underway in our laboratory.

E. Characterize Δ*STM3106 S.* Typhimurium's ability to survive and persist inside hepatocytes and Kupffer cells and induce host cell death

We have seen a remarkable difference in colonization of the liver in mice infected with wild-type *S.* Typhimurium when compared to Δ*STM3106 S.* Typhimurium. An

important experiment is to analyze the histopathology of liver sections from mice infected with wild-type *S.* Typhimurium or Δ*STM3106 S.* Typhimurium. We have observed that there does not appear to be a difference in survival between the two strains inside of macrophages (Figures 19A-C), suggesting that is not responsible for the difference in colonization.

Approximately 80% of macrophages in the liver are Kupffer cells. Kupffer cells are liver-specific macrophages with unique endocytic and phagocytic capacities primarily found at sites where blood enters the liver. Thus, we need to determine how Δ*STM3106 S.* Typhimurium and wild-type *S.* Typhimurium survive in this specialized population of macrophage. Another major type of cell that makes up the liver is the hepatocyte (70-80%, (65)). Thus, it is important to characterize the abilities of Δ*STM3106 S.* Typhimurium and wild-type *S.* Typhimurium to survive and persist inside hepatocytes and Kupffer cells to fully understand the liver-specific colonization defect.

Another possible, though unlikely, explanation for the difference in colonization of the liver is that Δ*STM3106 S.* Typhimurium are unable to cause death of host cells. Wild-type *S.* Typhimurium have been shown to induce host cell death, and this contributes to their ability to be a successful pathogen (13, 61, 72, 91, 113, 115, 116, 137, 167, 168).

F. Examine the contribution of L-asparaginase II to the kinetics and magnitude of the secondary T cell response to *S.* Typhimurium infection

We have promising preliminary results that there appears to be a significant difference in the T cell recall response in immunized mice challenged with wild-type *S.*

Typhimurium or Δ*STM3106 S.* Typhimurium. Unfortunately, it appears that we had not yet reached the peak of the T cell recall response on Day 11 post-challenge. We are in the process of repeating this experiment, taking weekly time points to capture the full dynamics of the recall response of T cells.

G. Determine if expression of L-asparaginase II during primary infection with *S.* Typhimurium affects the ability of the host to respond to secondary challenge with *S.* Typhimurium

These experiments would complement those listed above in the preceding section. If *S*. Typhimurium express L-asparaginase II during infection to inhibit the initial T cell response, mice immunized with a vaccine strain lacking L-asparaginase II may have a greater primary immune response to wild-type *S*. Typhimurium infection than mice immunized with the current vaccine strain. This may result in a larger population of memory T cells, which could translate into an enhanced recall response accompanied by increased T cell proliferation and more effective clearance of wild-type *S*. Typhimurium.

H. Determine the importance of T cells in the bacterial colonization difference of the liver

To determine the importance of T cells in the bacterial colonization difference of the liver, we plan to conduct organ-burden and time-to-death studies in T cell-deficient mice. If the colonization difference between wild-type *S.* Typhimurium and L-asparaginase II-deficient *S.* Typhimurium persists in the liver of T cell-deficient mice, this would indicate that T cells are not responsible for the colonization difference. However, if the difference in colonization of the liver is no longer present in T cell-

deficient mice, it would suggest that T cells are required for effectively clearing *S.* Typhimurium from the liver.

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