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L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses through hydrolysis of L-asparagine and suppression of metabolic reprogramming

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Abstract of the Dissertation

L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses through hydrolysis of L-asparagine and suppression of metabolic reprogramming

By

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Salmonella enterica serovar Typhimurium (*S.* Typhimurium) are pathogenic bacteria that suppress T cell responses to avoid clearance by the immune system, yet the mechanisms that mediate this immunosuppression remain largely unknown. We previously showed that L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence. Here, we found that L-Asparaginase II of *S.* Typhimurium exhibits L-asparagine hydrolase activity and that this activity is required for the inhibition of T cells. Furthermore, we found that exogenous L-asparagine is a resource important for T cell function and that L-Asparaginase II-mediated deprivation of exogenous L-asparagine inhibits T cell responses through suppression of metabolic reprogramming. The suppression of T cell metabolism was characterized by the inhibition of mTOR signaling, autophagy, c-Myc expression and new protein synthesis required for the activation, proliferation and differentiation of naïve T cells into effector T cells. These findings advance knowledge of a mechanism used by *S.* Typhimurium to delay onset of protective immune responses and thus have fundamental implications for understanding host interactions with bacterial pathogens.

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List of Abbreviations

PRR- Pattern Recognition Receptor

PAMP-Pathogen-associated molecular pattern

MBL- Mannose binding lectin

LPS- Lipopolysaccharide

APC- Antigen presenting cell

CTL- Cytotoxic T lymphocytes

PMN- Polymorphonuclear leukocyte

CD- Cluster of differentiation

TLR- Toll-like receptor

NLR- Nod-like receptor

TCR- T cell receptor

BCR- B cell receptor

MHC- Major histocompatability complex

TGF-β- Transforming growth factor beta

SPI- *Salmonella* pathogenicity island

SCV- *Salmonella* Containing vacuole

T3SS- Type three secretion system

TNF-α- Tumor necrosis factor alpha

IFN-γ- Interferon gamma

mTOR- Mammalian target of rapamycin

ATG- Autophagy-related proteins

TCA- Tricarboxylic acid cycle

IL-1β- Interleukin 1 beta

IL-2- Interleukin 2

IL-4- Interleukin 4

IL-6- Interleukin 6

IL-10- Interleukin 10

IL-12- Interleukin 12

IL-18- Interleukin 18

IL-23- Interleukin 23

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Chapter 1: Introduction

1. The Immune System and Infection

The mammalian immune response is typically discussed in two parts, the innate response and the adaptive response. Though these two systems interact considerably, each has specific, critical functions in protecting the host from potential pathogens. The innate immune system responds quickly and non-specifically to foreign organisms, recognizing conserved structures and/or proteins shared by many pathogens (1-3). In contrast, the adaptive immune system develops responses to infection that are specific to the invading pathogen. Adaptive immune responses can also lead to immunological memory, which is the ability to respond to a second encounter with a pathogen, quicker and more efficiently than when first infected. Adaptive immune responses must be initiated by cells of the innate immune system and take longer to develop than innate immune responses. Together, these systems provide the host critical protection from infectious disease.

1.1 The Innate Immune System

The hallmarks of the innate immune response are its ability to respond quickly and non-specifically to foreign organisms. The innate immune response is usually initiated within minutes or hours and does so by recognizing conserved structures and/or proteins shared by many pathogens (1-3). The innate immune system is characterized by three important functions, which it uses to defend the host: professional phagocytes, antimicrobial peptides, and the complement system (4). Each of these components play a critical role in eliminating pathogens

by recognizing conserved features shared by many pathogens, known as pathogen-associated molecular patterns (PAMPs).

1.1.1 Antimicrobial Peptides

Antimicrobial peptides are a group of molecules produced by a wide variety of organisms from insects to plants to mammals. Antimicrobial peptides are an integral part of the innate immune system and are usually made up of less than 100 amino acid residues that can exert bactericidal, fungicidal and even tumoricidal effects (5). Antimicrobial peptides are typically produced by granulocytes, but can also be produced by intestinal paneth cells, skin epithelial cells, monocytes and some lymphocytes (5). Defensins, cathelicidins and protegrins are among the best-characterized antimicrobial peptides. Antimicrobial peptides have been found to exist in a variety of structures and this likely is an important reason for how they are able to exert such a wide spectrum of activity (6). Additionally, since these peptides are so small they are able to quickly diffuse, allowing for quick defense against an invading pathogen (6). The mechanism by which antimicrobial peptides work is still an active area of investigation, however antimicrobial peptides can be divided into two subsets: membrane acting peptides and nonmembrane acting peptides (6). Membrane acting peptides can permeablize the membrane of the target cell by forming pores, whereas nonmembrane acting peptides translocate across the target cell membrane without permeablizing (6). Upon reaching its target, antimicrobial peptides can destroy by depolarizing or otherwise disrupting the membrane, whereas others work by translocating across the target cell membrane and inhibiting essential cellular processes like protein synthesis and cell wall synthesis (6).

1.1.2 The Complement System

The complement system is a key effector mechanism of the innate immune system and also plays an important role in tailoring the adaptive immune response. In fact, the complement system's name is derived from its function in "complementing" the antibody response (2, 7). In addition to its function in controlling infection as part of the innate immune response, the complement system has been shown to have roles in clearance of apoptotic cells and tissue regeneration (7, 8).

The complement system is made up of over 35 proteins that circulate in the blood (2, 9). Activation of the complement system can occur through three distinct pathways: the classical pathway, the alternative pathway and the lectin pathway. Each pathway is stimulated by specific ligands, but all three pathways eventually converge and form the same effector molecule known as C3 (2, 7, 9, 10). The binding of one of the complement proteins to its ligand sets off an enzyme cascade, where one complement protein cleaves another, activating that protein which then goes on to cleave and activate another protein. This repeating cycle leads to activation and amplification of the complement response.

The classical pathway is activated by the binding of C1q, a component of the complement system, to its ligand. C1q has several ligands such as certain proteins found on bacterial cell walls as well as immune complexes found on the surface of the pathogen such as antibody: antigen complexes (2, 7). The lectin pathway is initiated by the binding of carbohydrate-binding proteins, such as mannose binding lectin (MBL), to carbohydrates on the surface of the pathogen. The alternative pathway is activated when spontaneously activated components of the complement system bind at the surface of pathogens (2, 7).

Activation of any of these three pathways leads to the eventual production of an enzyme known as C3 convertase. This enzyme is produced on the surface of the pathogen and its function is to cleave another complement protein, C3. The cleavage of C3 yields two important products, C3a and C3b (2, 11). C3a is an initiator of inflammation and C3b binds the pathogen thereby making it a target for phagocytosis. Additionally, C3b can bind to C3 convertase itself, forming another effector molecule, C5 convertase. The enzymatic activity of C5 convertase leads to the production of C5a, a chemotactic protein that can activate certain phagocytic cells, and C5b which initiates another aspect of the complement system, known as the membrane-attack complex.

1.1.3 Professional Phagocytes

Professional phagocytes such as macrophages, monocytes and neutrophils (also known as polymorphonuclear granulocytic neutrophilic leukocytes, PMNs) accumulate quickly at sites of infection, tissue damage or inflammation and provide an early line of defense. Phagocytes detect and ingest pathogens without aid from other cells of the immune system and do this by using cell surface pattern recognition receptors (PRRs). PRRs detect invading pathogens by recognizing conserved features shared by many pathogens, known as pathogen –associated molecular patterns (PAMPs)(2, 12, 13) discussed in detail below. PAMPS recognized by PRRs include microbial components such as peptidoglycan, teichoic acids, lipopolysaccharide (LPS), lipoproteins and flagellin (4, 14).

Recognition of PAMPs by professional phagocytes is made by a variety of PRRs and are an effective mechanism used by the host to quickly recognize and ultimately control an infection (1, 4, 14-19). PRRs are constitutively expressed by host cells and can be divided into three groups,

each recognizing distinct PAMPs and have particular functions (1, 2, 19, 20). The first group is secreted PRRs. This group circulates in blood and lymph and is involved in opsonization, activation of the complement system, agglutination, and can regulate inflammation (1, 2, 21). The second group of PRRs is surface receptors expressed by phagocytes that bind pathogens for phagocytosis, discussed in more detail below (1, 2). The third group of PRRs is surface receptors that bind molecules derived from pathogens and induce signaling which leads to the production of anti-microbial peptides, chemokines and pro-inflammatory cytokines (1, 2). Cytokines produced in response to induction of this class of PRRs also play a key role in development of an adaptive immune response (1). Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are important examples of PRRs that make up this third class. TLRs are important in detecting PAMPs located in the extracellular milieu and NLRs recognize intracellular PAMPS (1, 4, 14, 20, 22).

Following the recognition and binding of the pathogen to cell surface receptors, phagocytosis can occur. Phagocytosis is the process of engulfing the pathogen into a vesicle known as the phagosome. Following engulfment, phagosomes become acidified and this harsh environment can kill most pathogens (2). Phagosomes can also undergo a maturation process that leads to its fusion with a lysosome, which are intracellular vesicles that contain antimicrobial enzymes and proteins, capable of destroying many pathogens (2, 13, 23). These antimicrobial proteins and peptides can inhibit pathogen growth or destabilize the pathogen, as discussed earlier (2, 12, 23). Phagocytosis can also activate phagocytes to produce reactive oxygen and nitrogen species (2). Reactive oxygen and nitrogen species are highly toxic to many pathogens and lead to their destruction within the phagosome (23).

Another important function of phagocytes is to produce and secrete cytokines and chemokines, which play a vital role in host defense against infection (2, 24)}. Cytokines are proteins that are produced in response to a variety of signals and can act on many different cell types by binding to specific receptors (2). Phagocytes produce a variety of different types of cytokines, which can be pro-inflammatory or anti-inflammatory. Chemokines are attractants important for the recruitment of immune cells to sites of infection. The particular types of cytokines and chemokines produced by phagocytes depend on which cell surface receptors are stimulated by a particular pathogen (2). Additionally, cytokines and chemokines play an important part in mediating adaptive immune responses.

Some professional phagocytes (e.g. macrophages, dendritic cells) are professional antigen-presenting cells (APCs). An important function of professional antigen presenting cells is to process and present antigen to T cells (see below). During infection, professional antigen presenting cells may process products produced by pathogens and present the resulting breakdown products on their surface for the recognition by T cells (see below) (23). Antigen presentation is a key step in activating the adaptive immune response. Additionally, stimulation of certain PRRs on APCs induces the expression of molecules required for co-stimulation of T cells. Therefore, phagocytes, in addition to controlling infection as discussed above, they also play an important role in intracellular communication and inducing adaptive immune responses.

1.2 The Adaptive Immune Response

The innate immune system encompasses many mechanisms to quickly respond to an invading pathogen. Many times an infection is efficiently contained by this system, however, there are pathogens that are able to evade or overpower the innate immune response. It is in

these cases where the adaptive immune response is needed. In addition to providing additional help to control and clear infection, one of the most important consequences of adaptive immune system engagement is the establishment of immunological memory. Immunological memory refers to the ability of the immune system to respond more quickly and effectively to pathogens that have been encountered previously.

The adaptive immune system is made up of B and T lymphocytes. B and T lymphocytes are defined phenotypically by the expression of membrane bound antigen receptors, whose principal function is to detect antigen. These antigen receptors are clonally distributed; each lymphocyte clone has a particular specificity and expresses a unique receptor that is different from all other clones. These unique antigen receptors allow lymphocytes to recognize and bind a diverse array of antigens. In the absence of infection the number of circulating B and T lymphocytes is very low but there is always at least one that can recognize any foreign antigen (2). Upon antigen recognition, naïve lymphocytes (mature cells which have not yet encountered antigen) are activated, which induces their clonal expansion and differentiation into effector cells. A fraction of these effector cells can become memory cells. Memory cells can be reactivated quicker than naïve cells upon reinfection and provide immunological memory (2).

1.2.1 T Lymphocytes

T cells originate in the bone marrow, but develop and mature in the thymus. Mature T cells exit the thymus and enter the periphery, where they may become activated by antigen. The presentation of antigen to T cells usually occurs in lymphoid organs and may lead to induction of a T cell response. T cells can be broadly divided into two major subsets based on the expression of the co-receptors, CD4 and CD8. $CD4^+$ T cells express the CD4 co-receptor whereas CD8+ T cells express the CD8 co- receptor. Although $CD4^+$ and $CD8^+$ T cells are the two major subsets

of T cells, there are other subsets of T cells such as gamma delta T cells. $CD4^+$ T and $CD8^+$ T cells differ in that they can acquire different effector functions. $CD4^+$ T cells produce and secrete cytokines while $CD8^+$ T cells can also directly lyse infected cells. Additionally, $CD4^+$ and $CD8^+$ T cells recognize different types of antigens. $CD4^+$ T cells recognize antigen derived from vacuolar compartments and $CD8^+$ T cells recognize cytosolic antigens (2, 25). However, T cells do not recognize the antigen alone; it must be presented by a complex known as the major histocompatability complex (MHC) by antigen presenting cells. Therefore, in order for a T cell response to be induced, professional antigen presenting cells (APC) must first internalize, process and present antigen to T cells. Following the recognition of antigen, T cells undergo a process known as priming (2).

T cell priming refers to the activation, proliferation and differentiation of naïve T cells into effector cells. Induction of the priming process requires three signals to be transmitted to the T cell. The first signal is initiated when antigen bound to a major histocompatibility complex (MHC) molecule on an antigen presenting cell binds to the T cell's antigen recognition receptor, called the T cell receptor (TCR) (2, 26). MHC molecules are membrane glycoproteins and are divided into two classes: MHC class I molecules and MHC class II molecules. Though both types of MHC molecules serve the same general purpose- to present antigen- they differ in the types of antigen they present. MHC class I molecules are expressed by all nucleated cells and present antigen derived from cytosolic pathogens (26). Professional antigen-presenting cells, including B cells, almost exclusively express MHC class II molecules. These molecules present antigen derived from pathogens contained in vacuolar, or endocytic, compartments. These two MHC molecules allow for the presentation of pathogen-derived peptides from just about all sub-cellular compartments (26).

Signal two required for the priming of T cells, is also provided by the antigen presenting cells (27). The B7 molecules are the most well known co-stimulatory molecules and it interacts with the cell surface receptor CD28 on the T cell. The ligation of this receptor is required for clonal expansion and proper function of T cells. This co-stimulation contributes to the production of a cytokine, interleuken-2 (IL-2), which is produced by the T cells and is required for their proliferation and differentiation (2, 28). IL-2 is a key part of clonal expansion; without it, T cells do not proliferate. The third signal required for T cell activation comes from cytokines produced by antigen presenting cells. The cytokine environment that a T cell is in at the time priming is initiated influences the differentiation of T cells to a particular subset, such as T_H1 or T_H2 (2).

Upon activation, naïve T cells differentiate into different types of effector cells. The $CD8^+$ T cells recognize MHC class I molecules displaying antigen derived from a cytosolic pathogen and differentiate into CTLs (2, 25). CTLs have the ability to directly lyse infected cells and secrete the cytokines interferon gamma (IFN-γ) and tumor necrosis factor (TNF)-α, which play important roles in stimulating other cells of the immune system (29). $CD4^+$ T cells, however, recognize class II MHC containing peptides derived from antigens in vacuolar compartments (25). $CD4^+$ T cells can differentiate into at least four types of effector cells; T_H1, T_H2, T_H17 and regulatory T cells (T_{regs}). These subsets are characterized by the types of cytokines they secrete, which define the effector functions they possess. The two major types of $CD4^+$ T cells are T_H1 and T_H2; they have the ability to interact with B cells and enhance the antimicrobial activities of other immune cells.

T_H1 cell differentiation is induced in the presence of the cytokines IFN-γ and interleuken-12 (IL-12) (2, 30). These cytokines come predominantly from cells of the innate immune system,

typically dendritic cells and natural killer (NK) cells. T_H1 cells have the ability to stimulate macrophages, which activates their microbicidal capabilities, allowing it to kill intracellular bacteria (2). T_H1 cells also have the ability to stimulate B cells to induce the production of specific antibodies. T_H1 cells carry out these effector functions by secreting the cytokines IL-2 and IFN-γ.

The T_H2 cell population is induced in the presence of the cytokine interleuken-4 (IL-4) where the initial source of this cytokine is not yet clear, but T_H2 cells themselves do produce IL-4, which can influence the differentiation of other T cells (30). T_H2 cells share an effector function with T_H1 cells in that it can also stimulate B cells to produce specific antibodies, but of different types. T_H2 cells produce the cytokine interleuken-5 (IL-5), which plays a role in the activation of other immune cells (2, 30).

The last two subsets of T cells, T_H17 and T_{reg} cells are not as well characterized as T_H1 and T_H2 cells, but it is clear that they play important roles in immunity. T_H17 cells are activated by the cytokines interleuken-6 (IL-6) and transforming growth factor (TGF)-β, but in the absence of IL-4 and IL-12 (2). T_H17 cells produce the cytokines interleuken-17A and F (IL-17A and IL-17F) that have been shown to aid in the clearance of extracellular pathogens by activating neutrophils (31). These cells can also stimulate B cells to produce specific antibodies (31). T_{regs} have been suggested to have immunosuppressive effector functions. These cells can be induced in the presence of TGF-β, but also produce this cytokine and another, interleukin-10 (IL-10). Both of these cytokines can inhibit the response of T cells and therefore likely play an important role in regulating T cell responses (2, 32, 33).

In addition to these subsets of effector T cells, there is another class of T cells that is an important part of host immunity, memory T cells. Unlike effector T cells, which quickly die

once they have exerted their effector functions, memory T cells persist in the host following activation. Memory T cells remain in circulation and protect the host against repeated exposure to the same pathogen (34). These memory cells respond more quickly and robustly to secondary infections because of increased sensitivity to stimulation (2). Both $CD4^+$ and $CD8^+$ T cells can become memory cells but the precise signaling events required to induce a T cell to become a memory cell is not completely understood (34, 35). There are also two distinct types of memory T cells, effector memory T cells and central memory T cells, which differ in their response times, function and location within the host. These cells can persist for the life of the host and play a critical part in preventing disease.

1.2.2 B Lymphocytes

B lymphocytes originate and mature in the bone marrow. Following maturation, B lymphocytes enter the bloodstream and circulate within lymphoid tissue as mature naïve lymphocytes (2). The antigen receptor on the B lymphocyte surface is the B cell receptor (BCR). Similar to T cells, B cells must also be activated in order to respond to infection (2). B cells also require multiple signals to induce activation; the first signal comes from stimulation of the BCR via antigen (2). T cells provide the second signal, in two parts. However, in order for a B cell to receive this second signal, it must be displaying antigen in the context of an MHC class II molecule as described above for the T cell to recognize the B cell (2). T cells that recognize this peptide-MHC class II complex then deliver part of the second signal needed for B cell activation which is the interaction between the CD40 ligand of the T cell with the CD40 receptor on the B cell. Other signals provided by the T cell are B cell stimulation cytokines including IL-10, IL-4, IL-5 and IL-6 which drive B cell proliferation and differentiation (2). Once the B cell has received the proper activating signals the BCR serves two main functions, to initiate intracellular

signaling which leads to proliferation and differentiation into plasma cells, the effector form of B cells (2). Plasma cells produce antibodies, the soluble form of the BCR. The second important function that antigen binding to the BCR leads to is the internalization of the bound antigen for processing and eventual presentation on its surface for T cells (2).

Antibodies play a critical role in protecting the host from extracellular pathogens, and the spread of intracellular pathogens. Antibodies can bind to pathogens, or pathogen derived particles like toxins. The binding of antibodies to foreign molecules can lead to three outcomes beneficial to the host. First the binding of antibodies to a pathogen makes it recognizable by professional phagocytes, particularly in cases where PAMPS are not detectable by the innate immune system, and enables them to ingest and destroy the pathogen. Next, antibodies can bind to pathogen-derived particles like toxins produced by many bacterial pathogens. The antibody-toxin complex cannot engage host cells, which the toxins usually target for destruction or infection and eventually is detected and phagocytosed (2). Finally, antibodies can boost activation of the complement system. Specific regions of antibodies that are bound to bacterial surfaces form receptors for proteins of the complement system. This can lead to the direct killing of bacteria or ingestion by professional phagocytes (2).

An important aspect of B cells is their ability to interact with T cells. The BCR can take up and internalize antigen, process it and display it on the B cell surface in the context of an MHC class II molecule. B cells express high levels of MHC class II molecules, as compared to other antigen presenting cells, allowing for high levels of peptide-MHC class II complexes to be displayed on its surface (2). Once this peptide-MHC complex is displayed, a $CD4^+$ T cell can recognize it, stimulating the T cell. Stimulated T cells can then go on to produce proteins that induce the B cell to proliferate, and differentiate into antibody-secreting B cells (2). Although

there are microbial antigens that can stimulate B cells independent of T cells, the response to a large variety of pathogens requires the B cell interaction with $CD4^+$ T cells (2).

Another important role of B cells in immunity is providing immunological memory. Similar to T cells, B cells also develop into memory cells that provide long last immunity. The recall response generate by memory B cells is distinct from that of the primary B cell response in several ways. First, a small population of high-affinity B cells that have already undergone clonal expansion generates the secondary response. Therefore, the receptors expressed by memory B cells and the antibodies they produce are of high affinity for specific antigen leading to a more effective response when compared to a primary B cell response (2). Memory B cells also express higher levels of MHC class II and co-stimulatory molecules, than naïve B cells, which leads to increased antigen uptake, presentation and ultimately initiation of T cell responses (2). The increased affinity of memory B cells for antigen also allows for B cells to induce T cell responses at lower doses of antigen suggesting that B cell differentiation and antibody production begins sooner following antigen stimulation than naïve B cells in during a primary response (2). Overall this demonstrates that the recall response provided by B cells is crucial in both providing protection for the host but also initiating T cell responses quickly and efficiently that also contribute to protection.

1.3 Host Response To *Salmonella*

Although the immune system has multiple strategies to detect, control and destroy pathogens as described above, it is not infallible. Many pathogens have mechanisms to subvert the immune system and allow them to survive and establish infection. Though it is clear that pathogens use a variety of strategies to evade the immune system, the mechanisms are not completely understood. Understanding how and why the immune system fails to protect from

certain pathogens as well as how pathogens manipulate the immune system to establish infection is a critical area of research. A better understanding of host-pathogen interactions will allow for the development of new and improved treatments for infectious disease as well as provide a better understanding of how the immune system works.

As described briefly above, the immune system is made up of many different cells and in order to protect the host from infection, intracellular communication between all parts of the immune system is vital. Due to this complexity, I have focused my dissertation work on a particular area of host-pathogen interactions. Specifically, my work examines the interaction between the bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and T cells.

2. *Salmonella*

1.4 *Salmonella* species classification

Salmonellae are gram-negative, facultative, rod-shaped intracellular bacteria belonging to the *Enterobacteriaceae* family (36, 37). The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori* (38, 39). However, the species *Salmonella enterica*, which is responsible for causing nearly all *Salmonella*-related disease in humans, can be divided further into six subspecies and over 2,500 serovars (36, 38, 40, 41). The subspecies and serovar classification is based on the differential expression of surface antigens, in particular the O antigen associated with lipopolysaccharide (LPS) and the H antigen that is associated with flagellar proteins (36, 41). This system of classification is called the White-Kauffmann Scheme (41).

1.5 *Salmonella* Epidemiology

Salmonella enterica can cause a range of diseases, from a self-limiting gastroenteritis to a systemic disease known as typhoid (enteric) fever (40, 42-44). Non-typhoidal *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) causes gastroenteritis, while the typhodial *Salmonella enterica* serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi) are responsible for causing Typhoid fever (38, 40, 42). The diseases caused by both the non-typhoidal and typhoidal serovars of *Salmonella* are a significant cause of concern all over the world. It is estimated that there are over 27 million cases of Typhoid fever every year that leads to over 200,000 deaths. Furthermore, cases of disease caused by non-typhoidal *Salmonella* affect over 93 million people and results in 155,000 deaths (42). Antibiotics are available for treatment, but there is a growing concern about the emergence of antibiotic resistant strains of *Salmonella*, thus, there is a need for alternative forms of treatment (38, 40, 45). Additionally, because *Salmonella* can be used to infect large numbers of people by contaminating food and water supplies the Centers for Disease Control classifies *Salmonella* as a category B agent of bioterrorism (http://www.bt.cdc.gov/agent/agentlist-category.asp).

1.6 *Salmonella* Pathogenesis

The causative agent of the systemic disease typhoid fever, *S.* Typhi, only causes disease in humans, however, *S.* Typhimurium, which causes gastroenteritis in humans, causes a typhoid fever-like systemic disease in mice (46). *S.* Typhi and *S.* Typhimurium share many of the same virulence factors, including flagella, SPI-1 and SPI-2 type three secretion systems (38). However, since there is not a mouse model of infection for *S.* Typhi it is difficult to determine exactly why these two cause different diseases in humans, but it has been suggested that differences in their genetic make up made be responsible (38). *S.* Typhi has a *viaB* locus that

encodes for genes involved in export and regulation of the Vi capsular polysaccaride and has been suggested to be a virulence determinant that can suppress the inflammatory response of the host and allow for dissemination (38). *S.* Typhi also has a cluster of toxin-like genes that can be delivered into host cells leading to DNA damage that could result in cell death (38). Since *S.* Typhimurium does not have either of these potential virulence factors its is predicted that these genetic differences may, at least in part, contribute to the differences in the diseases each causes in humans (38). Despite these differences, infection of mice with *S.* Typhimurium serves as a useful model for typhoid fever in humans (42, 47, 48).

Following oral-gastric infection, typically through ingestion of contaminated food or water, *Salmonella* must adhere to the gut epithelium and cross the intestinal epithelium in order to establish infection (49). One of the first barriers *Salmonella* encounters within the gut is the resident microbiota, however it has been suggested that *Salmonella* can induce intestinal inflammation, via its virulence factors, in order to gain a growth advantage and survive the harsh environment of the stomach (49). Once it has established adherence to the gut epithelium, *Salmonella* is able to invade the intestinal mucosa. *Salmonella* preferentially invades via M cells, which are specialized epithelial cells located in Peyer's Patches of the distal ileium (small intestine) (43). From there, the bacteria are taken up by host cells and survive and replicate by inducing the formation of a special vacuolar compartment known as the *Salmonella* containing vacuole (SCV) (36, 43, 49-51). *Salmonella* invasion of host cells is a key part of invasion because *Salmonella* unable to survive intracellularly are avirulent (51-54). Once inside host cells *Salmonella* can disseminate via the mesenteric lymph nodes to the liver and spleen, where the bacteria survive and replicate inside host cells. Eventually, *Salmonella* gain re-entry into the

bloodstream, leading to septic shock, multiple organ failure and death of the host (46, 49-51, 55-57).

1.7 *Salmonella* virulence factors

Both *S.* Typhi and *S.* Typhimurium use a number of virulence factors in order to evade the host immune system and establish infection. It has been suggested that at least 4% of the *Salmonella* genome is required for virulence in mice (58). For *Salmonella,* almost all of the genes encoding these virulence factors are found in distinct genetic regions, known as *Salmonella* Pathogenicity Islands (SPIs) and some are found on a virulence plasmid (36). Five SPI regions have been identified in *Salmonella* so far; SPI-1 to SPI-5 and all have been implicated in contributing to virulence (36). SPI-1 and SPI-2 have been well studied and both encode for type three secretion systems (T3SS)(38, 52, 57, 59). These T3SSs are needle like structures that secrete over 30 effector proteins directly into host cells and play critical roles in *Salmonella* virulence (60). Each T3SS has largely distinct roles, though there is some overlap, to enable the bacteria to establish infection (52). Much less is understood about how the other three SPIs contribute to virulence. Below is an overview of some of the most important virulence factors.

The primary barrier of the gut is a layer of epithelial cells that line the intestine. These cells are used for nutrient uptake and blocking the entry of pathogens both physically and by secreting antimicrobials and mucus (49). In order for *Salmonella* to establish infection, it must breach this barrier. Following oral ingestion of *Salmonella*, it reaches the intestinal epithelium where it encounters the antimicrobial activities of the epithelial cells. To counteract this, *Salmonella* has a virulence mechanism called the PhoP-PhoQ system. In addition to other functions, this system senses the antimicrobial peptides and activates transcription of genes that lead to the resistance of

Salmonella to these toxic peptides (49, 57, 61, 62). Additionally, *Salmonella* are able to induce an inflammatory response, which when combined with its ability to evade destruction by antimicrobials provides *Salmonella* with a growth advantage over the resident microflora as well as the ability to adhere to the epithelial cells (49). Once *Salmonella* adhere to this cell layer they are able to cross the barrier via specialized cells known as M cells as well as non-phagocytic cells by inducing its own uptake, via its SPI-1 T3SS (49, 63-65).

Although cells such as macrophages can use phagocytosis to take up *Salmonella*, the bacteria can actively induce their uptake, by non-phagocytic cells, particularly into epithelial cells of the intestinal epithelium, and to do so they use the SPI-1 T3SS. The SPI-I encoded T3SS is activated very early during infection and the effector proteins it encodes for are necessary for the bacteria to invade host cells (37, 38, 60, 66, 67). The SPI-1 region encodes for a variety of proteins with specific functions. A number of these proteins have been identified and their primary function is to modulate host cell processes. However there are genes that are used to assemble the needle structure of the T3SS, deliver the effectors into the host cell and others that function as chaperones, which protect the effector proteins from degradation by the host (36, 52, 66, 67).

The SPI-2 T3SS is activated later during infection and its primary purpose is for survival and replication in host cells within the SCV (36, 37, 52, 68). The SPI-2 region has four sets of genes: those involved in the T3SS machinery, those encoding for the regulators, those encoding the chaperones and those encoding the effector proteins which get injected into the host cell (36). Gene products of the SPI-2 T3SS are involved in maturation of the SCV and manipulation of endocytic trafficking (38, 66).

In vivo macrophages seem to be the main cell *Salmonella* invades and lives within the host. The importance of the macrophage for *Salmonella* to successfully establish an infection is highlighted by the work demonstrating that *Salmonella* mutants unable to survive in macrophages are avirulent (54, 69). *Salmonella* is able to kill macrophages by at least two different mechanisms: a SPI-1 T3SS dependent mechanism and SPI-2 T3SS dependent mechanism (39, 46, 70, 71)}. SPI-1 T3SS induced macrophage death occurs quickly upon engulfment of the bacteria and is dependent on Caspase-1 activation, resulting in a type of cell death known as pyroptosis (discussed below) (39, 72). However, the exact type of cell death triggered by the SPI-2 T3SS within macrophages is still debated. Some suggest it is a Caspase-1 dependent pyroptosis while others suggest it is Caspase-1 independent and results in a form of cell death more closely resembling apoptosis (39, 53, 72, 73). In both situations, SPI-2 T3SS induced macrophage death is delayed, taking place 18-24 hours post infection and results in lysis of the infected cell (39, 72). Additionally it has been suggested that *Salmonella* live within macrophages in a dormant-like state, persisting but not replicating (43). *Salmonella* may be actively entering this "latent" state as a mechanism to evade both the innate and adaptive immune system.

Some strains of *Salmonella* harbor a virulence plasmid, found mostly in a few clinically important strains such as *S.* Typhimurium; of these, the best-characterized plasmid is pSLT (36). This plasmid has a conserved region that contains five genes, known as the *spvRABCD* locus (36). These genes encode for a regulator, two effector proteins, a cytotoxic protein, and several proteins of unknown function (36). Although the precise roles of the spv genes are not well understood, the operon contributes to Salmonella virulence in mouse models of infection (36, 74).

Salmonella also has several other mechanisms for virulence including a Type 1 Secretion System (T1SS), fimbriae, flagella and transporters for iron uptake that *Salmonella* need for survival (36, 52). The T1SS has two effector proteins that have been shown to play a role in adhesion and invasion, while fimbriae are required for biofilm production and attachment to eukaryotic cells, which is required for the SPI-1 T3SS to inject its effectors into the host (36, 52). Flagella are utilized by *Salmonella* for motility and likely contributes to its ability to establish infection, but the components of the flagella, flagellin, seem to induce immune responses that can lead to cell death. The role of flagella and flagellin in virulence is still a widely studied area (38, 47, 75). Finally, since iron is limiting within the host *Salmonella* encode ion transporters that it uses to import iron (52).

Thus, *Salmonella* use a wide array of virulence factors to invade, survive and replicate within the host. There is some redundancy among the virulence factors that *Salmonella* encode; however, the complete collection of these factors allows the bacteria to cause a systemic lethal disease.

3. The Host Response to *Salmonella*

1.8 The Innate Immune Response to *S.* Typhimurium

Salmonella does not go completely undetected by the immune system. Upon invasion of the epithelium, *Salmonella* can be detected and/or take up by cells such as macrophages, inflammatory monocytes and dendritic cells. *Salmonella* that are extracellular can be detected by TLRs, whereas intracellular *Salmonella* can be detected by NLRs and both can lead to the production and expression of the pro-inflammatory cytokine IL-13 (49, 76, 77). Additionally, stimulation of NLRs by intracellular *Salmonella*, via the SPI-1 and SPI-2 T3SSs, can lead to the

activation of a complex called the inflammasome (49, 63). This complex includes a cysteine protease, pro-Caspase-1, which upon activation is cleaved and goes on to activate, via cleavage, two important inflammatory cytokines, IL-1β and IL-18 (37, 49, 78). These cytokines play important roles in mediating the host immune response to *Salmonella.* Mice deficient for these cytokines are much more susceptible to *Salmonella* infection (78, 79). IL-1β recruits other immune cells, like neutrophils, to the site of the infection, while IL-18 can induce the release of interferon-γ (IFN-γ) by T cells and nitric oxide synthesis (49, 80-82). IL-18 and IL-23 also stimulate an increase in the production of antimicrobial peptides as well as chemokines that recruit more neutrophils to the site of infection (49).

Activated Caspase-1, also induces a type of programmed cell death known as pyroptosis. This form of cell death is dependent on Caspase-1 and leads to lysis of infected cells. Pyroptosis leads to amplification of the inflammatory response because pores form in the dying cells allowing both the bacteria and cellular contents to leak out (49, 78). It has been noted that mice deficient for Caspase-1 expression are more susceptible to *Salmonella* infection; these mice have higher bacterial loads in both the spleen and liver and succumb to infection quicker than wild-type mice (49, 78, 83). This suggests that activating Caspase-1 dependent cell death may provide an advantage to the host by destroying the intracellular niche of *Salmonella*. However, it has also been suggested that *Salmonella* effector proteins may activate Caspase-1, particularly during the acute phase of infection, as a means to stimulate inflammation and gain a growth advantage in the gut or even as a means of delaying antigen presentation to the adaptive immune system (37, 40, 49, 84). Therefore, Caspase-1 is clearly a player in the innate immune response to *Salmonella* infection and is still an active area of research.

Inflammasome activation is just one of the ways the innate immune system responds to *Salmonella* infection; other aspects of innate immunity also play important roles. Early during *Salmonella* infection, two of the first cells recruited are neutrophils and immature monocytes. These cells are the main source of the important inflammatory cytokines, TNF-α and IL-1β, which recruits more immune cells to the site of the infection as well as enhance certain immune responses (76, 85). Upon phagocytosis, these cells are efficient killers of *Salmonella* using both reactive oxygen and nitrogen species, helping to confine the pathogen in an attempt to limit its replication and dissemination to distal organs (65, 85). Neutrophils are also thought to be the main cell that takes up and kills extracellular bacteria and bacteria that are released from infected cells dying via pyroptosis (49).

Dendritic cells are also quickly recruited early during infection, take up the bacteria and are considered the cell responsible for transporting *Salmonella* from the intestinal tract to the mesenteric lymph nodes (43). Just like macrophages dendritic cells are also susceptible to *Salmonella*-induced Caspase-1 dependent cell death (46). However, probably the most important role that dendritic cells play during a *Salmonella* infection is activating the adaptive immune response. It has been demonstrated *in vivo* that dendritic cells are required for the priming of the T cell response to *Salmonella* (85-87). In order for a dendritic cell to present antigen, it must first undergo a maturation process. This process can be stimulated either directly by *Salmonella* or indirectly by cytokines, such as TNF-α and IL-1β, produced during the inflammatory response (85). Maturation of dendritic cell results in increased expression of co-stimulatory molecules, increased antigen sampling via MHC II as well as increased antigen processing and presentation (85, 88). However, only direct stimulation leads to the ability to induce T cell activation (85, 88).

1.9 The Adaptive Response Immune Response to *S.* Typhimurium

1.9.1 B Cells

Although, the role of B cells in primary infection is not clear, antibody-mediated protection is important for protection against secondary infection (89). Wild-type B cell deficient mice survive infection with an attenuated strain of *S.* Typhimurium, but succumb to secondary infection with an attenuated strain, suggesting an important role for B cells during secondary infection (51, 90). Interestingly, the susceptibility of B cell deficient to secondary infection correlated with decreases in production of IFN-Y by $CD4^+$ T cells (89). Furthermore, mice containing B cells but unable to secrete antibodies survive vaccination but show little defects following secondary challenge (89). TLR activation of B cells during *Salmonella* infection has been shown to optimize the generation of the T_H1 cell response by the secretion of cytokines (43). Additionally, B cells were shown to be required for the development of T_H1 memory cells, in an antibody-independent manner (43, 91). Collectively, Taken together this work suggests that B cells play an important role in resolution of secondary infection with *S.* Typhimurium that is independent of antibody secretion and likely has to do with development of the T cell response (47, 89, 90).

Protective immunity against *Salmonella* could also be transferred. The transfer of both serum and T cells from *S.* Typhimurium vaccinated animals into naïve animals provided protection against virulent *S.* Typhimurium (92-94). This suggests a role for antibodies in the development of a protective immune response to *S.* Typhimurim (90, 92). Although the precise role of antibodies is not clear, *S.* Typhimurium can induce cell death of the host cell it infects, leading to a brief time before *S.* Typhimurium invades a neighboring cell when it can be found extracellularly (65). Opsonization of bacteria by *S.* Typhimurium-specific antibodies lead to a

decrease in bacterial colonization, suggesting antibodies could play a role in preventing bacterial dissemination (65).

Recent work has suggested that B cells may play a suppressive role during primary infection with *S.* Typhimurium (95). B cells lacking an important adaptor molecule, MyD88, are more resistant to *S.* Typhimuirum infection than wild-type mice (95). This increased survival was attributed to the secretion of IL-10 by B cells during infection (95). IL-10 is a known immunosuppressive cytokine that can suppress responses of T cells and neutrophils (95). Taken together this work demonstrates important roles for B cells during infection with *S.* Typhimurium but highlights the need for further investigation to elucidate the precise role B cells and antibodies play.

1.9.2 T cells

Both $CD4^+$ and $CD8^+$ T cells participate in the immune response to *S.* Typhimurium. In experiments using either T cell deficient mice or mice where T cells were depleted by antibodies, it was found that T cells were required for control and recovery from a primary *S.* Typhimurium infection using either virulent or attenuated strains (50, 51, 96). $CD4^+$ T cells play a more crucial role in clearance of *S.* Typhimurium than CD8+ T cells because depletion of $CD4^+$ T cells alone resulted in an inability to clear the infection even with an attenuated *Salmonella* strain (51). In contrast, depletion of $CD8^+$ T cells alone led to a modest impairment in clearance against attenuated strains of *S.* Typhimurium, while vaccine-induced protection with wild-type *S.* Typhimurium was significantly reduced (50, 97, 98).

T cells, particularly interferon (IFN)-γ-producing $CD4^+$ T cells, play a critical role in clearance of *S.* Typhimurium (55, 64). IFN-γ activates and stimulates the antimicrobial activities

of macrophages as well as the secretion of the cytokines IL-12 and IL-18, which induce helper T cells to continue to produce IFN-γ (64). IFN-γ is particularly important in the host defense to *S.* Typhimurium because mice either treated with IFN-γ antibodies or lacking the IFN-γ receptor are impaired in their ability to clear infection with a sub-lethal dose of wild-type *S.* Typhimurium (64). Additionally, a number of immunodominant antigens recognized by *S.* Typhimurium-specific T cells have been identified, but not all have been shown to be protective (47).

Work has also demonstrated an important role for the $CD4^+$ T cell subset, T_H17 cells, during the host response to *S.* Typhimurium. T_H17 cells have been suggested to be important for preventing the dissemination of *S.* Typhimurium from the intestine to systemic sites, early in infection (99). This protective function of T_H17 cells is attributed to the cytokines it produces, such as IL-17 and IL-22. These cytokines stimulate intestinal epithelial cells to produce antimicrobial proteins and chemokines that can recruit neutrophils and immature dendritic cells to the site of infection (99).

It has also been suggested that *Salmonella* may suppress the response of T cells. Mittrucker et al showed that following infection with virulent *S.* Typhimurium *in vivo*, both activated $CD4^+$ and $CD8^+$ T cells were detectable and many of those cells were capable of secreting INF-γ, however, these cells did not proliferate extensively (50). Furthermore, the peak of the T cell response, in terms of IFN-γ production, did not correlate with a decrease in bacterial titers in these animals; it was not until several weeks later that bacterial titers decreased. However, when these experiments were repeated using an attenuated strain of *Salmonella* the peak of the T cell response and reduction in bacterial load did correspond (50). This work demonstrated that a T cell response could develop during the course of a *S.* Typhimurium infection, indicating that the levels of IFN-γ produced by T cells was not sufficient to control the infection (50). Additionally,

this work suggested that *S.* Typhimurium might actively prevent an effective T cell response by affecting their ability to properly activate and therefore their ability to proliferate.

Further work has been done demonstrating that the response of T cells is delayed in mice infected with *S.* Typhimurium. This work suggested that another subset of T cells might play an important role, T regulatory cells (T_{regs}). T_{regs} are a subset of $CD4^+$ T cells that have been typically associated with maintaining tolerance to self-antigens, but more recently have been associated with immune responses to infection, particularly *S.* Typhimurium (100). Early during a *S.* Typhimurium infection, T_{regs} were found to highly express CTLA-4, a cell surface molecule known to be associated with T cell suppressive potency, while at later times post infection there was a drastic reduction in expression of CTLA-4 (100). This decrease in CTLA-4 expression corresponded with the activation of T cells and eventual clearance of the infection. Therefore, this work suggested that the T_{reg} population induced during early stages of the infection could delay the development of protective T cell responses, allowing for increased bacterial burden and the decreased clearance (100). This work along with experimental data from several groups has suggested that *Salmonella* may actively interfere with the response of T cells during infection (47, 50, 63, 65, 75, 100, 101).

1.10 *S.* Typhimurium induced immunosuppression

Although the mechanisms by which *S.* Typhimurium interfere with T cell responses are not completely understood, multiple ideas have been proposed. These mechanisms include: the killing of APCs, avoidance of phagosome-lysosome fusion, recruitment of immunosuppressive immature myeloid cells, down modulation of MHC-I and MHC-II and modulation of antigen availability (46, 76, 102-104).

As discussed earlier, *S.* Typhimurium can kill APCs via a Caspase-1 dependent mechanism, but it has also been shown that *S.* Typhimuiurm can prevent the fusion of the SCV with lysosomes, via a SPI2 dependent mechanism (105, 106). It has also been demonstrated that immature myeloid cells that have suppressive effects on T cells are recruited to sites of *S.* Typhimurium infection (102)(Tam et al. unpublished observation). Additionally, it has been shown that *S.* Typhimurium actively down modulates expression of both MHC-I and MHC-II (103, 104, 107). *S.* Typhimurium can also modulate the amount of antigen available for processing. It has been shown that *S.* Typhimurium can modify expression of over 40 different genes once inside host cells, as well as modify its own proteins in order prevent recognition, via mechanisms involving effector proteins from both the SPI-1 and SPI-2 type three secretion systems (108-110). Finally, as discussed above, *S.* Typhimurium has been shown to induce suppressive functions of T_{reg} cells (100).

Each of these mechanisms could lead to reduced antigen processing and presentation to T cells, thereby inhibiting the T cell response. These mechanisms all demonstrate effects on cells of the immune system that could potentially affect the T cell response. However, other than work done by the Way group demonstrating that *S.* Typhimurium infection induces immunosuppressive functions of T_{reg} cells, and by van der Velden et al, demonstrating *S.* Typhimurium has direct inhibitory effects on T cells, little is understood about the mechanisms by which *S.* Typhimurium directly modulates the response of T cells (100, 111, 112). Therefore, a focus of the research in our laboratory is on the direct inhibitory effects of *S.* Typhimurium on T cells.

4. *S.* Typhimurium directly inhibits the response of T cells

1.11 *S.* Typhimurium mediated killing of APCs alone is not responsible for T cell inhibition

A number of studies have shown that *Salmonella* interfere with development of the T cell response (50, 51, 96, 101, 106, 113, 114). However, the mechanism by which Salmonellae suppress T cell responses is not well understood. Since a number of studies have shown that *Salmonella* can kill APCs via a Caspase-1 dependent mechanism, it would be logical to infer that the lack of APCs to present antigen would prevent development of the T cell response (39, 40, 46, 53, 71, 73, 76, 107, 115). To determine whether *Salmonella's* ability to kill APCs was responsible for the inhibition of T cell responses, dendritic cells that were genetically deficient in Caspase-1 were used in an in vitro antigen presentation assay (112). In this assay, T cells and peptide-pulsed Caspase-1 deficient dendritic cells were co-cultured with *S.* Typhimurium and the T cells were monitored for proliferation. The T cells did not proliferate and similar results were obtained when wild-type dendritic cells were infected with mutant S. Typhimurium (that could not kill the dendritic cells) to stimulate the T cells (112). This work indicated that S. Typhimurium-induced dendritic cell death alone was not responsible for the observed inhibition of T cells. This study also suggested that the inability of T cells to proliferate might be the result of a direct effect of *Salmonella* on the T cells (112).

1.12 *S.* Typhimurium has a direct immunosuppressive effect on T cells

To determine whether *S.* Typhimurium was capable of directly inhibiting T cells, T cells were co-cultured with *S.* Typhimurium in the presence of anti-CD3 and anti-CD28. These two antibodies are used to stimulate T cells, similarly to APCs, and induce T cell proliferation. This

allows T cells to be stimulated in the absence of APCs. Following TCR engagement, T cells left untreated, proliferated as expected whereas T cells cultured with live *S.* Typhimurium did not (112). Additionally, it was shown that *S.* Typhimurium down-modulated expression of the β-chain of the TCR (111). The TCR, as described earlier, is a key part of T cell priming which leads to proliferation and differentiation of T cells. Thus, this work indicated that S. Typhimurium has a direct inhibitory effect on T cells.

1.13 A soluble factor that is likely a protein is responsible for *S.* Typhimurium-induced T cell inhibition

Following the observation that *S.* Typhimurium could act directly on T cells and prevent their proliferation and expression of the TCR the next question was whether direct contact between *S.* Typhimurium and the T cells was required for *S.* Typhimurium induced T cell inhibition. Using a transwell system that prevented physical contact between the T cells and *S.* Typhimurium, it was shown that contact was required in order for *S.* Typhimurium to prevent T cell proliferation (112). Further use of this system lead to the discovery that when cultured supernatant from stimulated *S.* Typhimurium infected T cells was transferred to uninfected, stimulated T cells, these T cells also had a reduced ability to proliferate (112). This work suggested that contact between *S.* Typhimurium and the T cells might result in the release of a soluble factor, or factors, capable of inhibiting T cell proliferation and expression of the TCR (112). Additionally, heat or trypsin treatment destroyed the inhibitory activity of the conditioned medium, indicating that the soluble factor was likely a protein (111). Therefore, it was of interest to determine the identity of the T cell inhibitory factor(s), produced or induced by *S.* Typhimurium.

1.14 *S.* Typhimurium-induced inhibition of T cells does not require SPI1, SPI2, *phoP* or STI

Since contact was required between *S.* Typhimurium and the T cells for inhibition, it was next examined whether *S.* Typhimurium used a contact dependent mechanism to induce T cell inhibition. To do so, *S.* Typhimurium mutants deficient for *invA* and *spiB*, essential genes of the SPI1 and SPI2 type three-secretion systems, respectively, were examined for their ability to induce T cell inhibition (111). Both of these mutants, as well as an *invA spiB* double mutant, were able to induce T cell inhibition, suggesting that neither type three secretion system was required for *S.* Typhimurium induced T cell inhibition (111)

Similar results were obtained for several other known *S.* Typhimurium virulence mechanisms, including: First a *phoP* null mutant and a $phoP^C$ mutant, which constitutively expresses *phoP* were still able to inhibit T cells (111). The *phoP* gene product has been shown to be important in regulating expression of multiple *S.* Typhimurium virulence factors (103). Next, STI, *Salmonella* Typhimurium-derived T cell inhibitor, has been suggested to play a role in *Salmonella* induced T cell inhibition via its gene product (116). A mutant lacking this gene was also tested and found to be capable of inhibiting T cell proliferation (111). Finally, a *Salmonella* mutant cured of its virulence plasmid was also tested for its ability to inhibit T cell proliferation and it was found to still inhibit T cell proliferation (111). Collectively this work demonstrates that the direct immunosuppressive effects *Salmonella* has on T cells is not due to known *Salmonella* virulence effectors.

1.15 The T cell inhibitory soluble factor is not IL-10 and does not require TGF-β signaling

The cytokines IL-10 and TGF-β are known to have immunosuppressive effects on T cells (117). In order to determine if these mammalian produced proteins were required for T cell

inhibition induced by *S.* Typhimurium, a TCR-β down modulation assay was used as a readout for inhibition (111). When cultured in the presence of *S.* Typhimurium, T cells from mice deficient for IL-10 or mice transgenic for a dominant negative form of TGF-βRII (cells unable to respond to TGF-β) expressed similar levels of TCRβ as wild-type T cells (111). This work indicated that IL-10 or TGF-β signaling does not contribute to *S.* Typhimurium induced T cell inhibition (111).

1.16 Hypothesis and Rationale

The goal of this dissertation was to identify the inhibitory factor(s) and characterize the mechanism of inhibition. I imagined the factor could be produced by *S.* Typhimurium, or induced by *S.* Typhimurium, but produced by the T cells and knowing the identify of the inhibitory factor(s) would help in my effort to determine the mechanism of inhibition. Therefore, given that the inhibitory factor(s) was likely a protein, I used standard biochemical techniques to purify the inhibitor. Another student in the laboratory took a parallel approach that involved a genetic screen to identify the *S.* Typhimurium genes required for T cell inhibition. The identification of the factor is described in Chapter 2 and the characterization of the mechanism is described in Chapters 3-7.

Chapter 2. L-Asparaginase II produced by *Salmonella* inhibits the response of T cells

2.1 Chapter Summary

The first aim of my dissertation research was to purify and identify the secreted factor responsible for *S.* Typhimurium-induced T cell inhibition. Results published by my advisor had

suggested that the secreted inhibitor present in conditioned medium harvested from T cells cultured with *S.* Typhimurium was heat- and trypsin sensitive, and thus was likely a protein. Therefore, I used standard biochemical fractionation and chromatography techniques as well as mass spectrometry to purify and identify the secreted inhibitor. I had made substantial progress towards the purification of the inhibitor when another Ph.D. student in the laboratory, Amy Kullas, identified a *S.* Typhimurium gene required for T cell inhibition. She and I collaborated to show that the gene product, a putative L-Asparaginase, was necessary and sufficient to cause T cell inhibition. The results of this work were published (Kullas AL, McClelland M, Yang HY, Tam JW, **Torres A**, Porwollik S, Mena P, McPhee JB, Bogomolnaya L, Andrews-Polymenis H, van der Velden AWM. 2012. L-Asparaginase II produced by *Salmonella* inhibits T cell responses and mediates virulence. *Cell Host & Microbe*. **12**:791-8).

2.2 Introduction

Previous work suggested that the factor, or factors, responsible for T cell inhibition was likely a protein and that it was secreted into the extracellular environment. However, it was unclear whether the inhibitory factor(s) was produced by *S.* Typhimurium or induced by *S.* Typhimuirum, but produced by the T cells. Since, this inhibitory factor(s) was present in the conditioned medium, I decided to use a biochemical approach to determine the identity and source of the inhibitory factor(s). Given that the factor(s) is likely a protein, I pursued classic purification techniques such as ammonium sulfate precipitation and ion exchange chromatography.

2.3 Experimental Methods

Bacterial Strains and Culture Conditions

S. Typhimurium strain 14028 (American Type Culture Collection) was used as the wild-type strain. Bacteria were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar using standard microbiological techniques.

Protein Purification

Briefly, enriched populations of T cells suspended in medium supplemented with 1 μg/ml of anti-CD28 antibody (clone E18; BioLegend) were seeded at 1 x 10^5 cells per well into flat-bottom 96-well tissue culture plates coated with 5 μg/ml of anti-CD3ε antibody (clone 145-2C11; BioLegend). The T cells were then cultured in the absence or presence of wild-type *S.* Typhimurium at a multiplicity of infection of about 60. After 2 hours of incubation at 37°C in 5% CO_2, the T cells were pelleted by centrifugation and resuspended in medium supplemented with 50 μg/ml gentamicin and 2% penicillin and streptomycin, killing all remaining bacteria within 2 hours (data not shown). After an additional 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected, filtered and ammonium sulfate was slowly mixed in at 4°C for 2 hours to overnight. The samples were then subjected to ultracentrifugation and the resulting supernatants were removed and the pellets were resuspended in PBS. The resuspended pellets and supernatants were dialyzed overnight into cell culture medium in order to remove salt. Saturated ammonium sulfate (Sigma) was used at concentrations ranging from 30-80%.

Ion exchange chromatography was done using supernatants collected as described above. Supernatants were run on a DEAE-Sepharose CL-6B column (Amersham Biosciences) equilibrated with 20mM Tris buffer (pH 7.6-8) and eluted with a linear gradient of 0 to 1.0 M NaCl. Fractions resulting from ammonium sulfate precipitation and ion exchange chromatography were analyzed for inhibitory activity using the T cell assay described below.

T Cell Enrichment and T Cell Assays.

Splenocytes harvested from naïve C57BL/6J mice (females, 6 to 8 weeks of age) were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell separation technology (Miltenyi Biotec) was used to enrich for $CD90.2^+$ T cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enrichment resulted in 75-85% enrichment of $CD90.2^+$ T cells. Enriched populations of T cells were suspended in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) and used in T cell assays.

In T cell assays aimed at measuring the effect of fractions resulting from ammonium sulfate precipitations and ion exchange chromatography, enriched populations of T cells suspended in medium supplemented with 1 μg/ml of anti-CD28 antibody (clone E18; BioLegend) were seeded at 1 x 10^5 cells per well into flat-bottom 96-well tissue culture plates coated with 5 μg/ml of anti-CD3ε antibody (clone 145-2C11; BioLegend). The T cells were then left untreated or treated with supernatant from protein purification described above. After 20 hours of incubation at 37°C in 5% CO_2, the T cells were harvested, stained and analyzed using flow cytometry.

Cell Staining and Analysis by Flow Cytometry.

All antibodies and reagents described in this section were purchased from BioLegend. Routinely, cells were stained in the presence of Fc block (clone 93) using allophycocyanin (APC)-conjugated anti-mouse CD90.2 antibody (clone 30-H12), phycoerythrin (PE)-conjugated anti-mouse CD25 antibody (clone PC61), and fluorescein isothiocyanate (FITC)-conjugated anti-

mouse TCR-β antibody (clone H57-597). Where indicated, cells were stained in the presence of Fc block using FITC-conjugated anti-mouse CD90.2 antibody, APC-conjugated anti-mouse CD25 antibody and propidium idodide to assess cell viability. Live cells expressing CD90.2, CD25 and TCR-β were identified as anti-CD3ε- and anti-CD28-stimulated T cells. Size and granularity of live cells were analyzed using forward scatter and side scatter. Data were acquired and analyzed using a FACSCalibur with CellQuest™ Pro software (BD Biosciences).

2.4 Results

Partial Purification of the Inhibitory Factor

The first attempt at purification of the inhibitory factor(s) utilized ammonium sulfate precipitation, also known as salting out. This process is used to purify proteins based on their solubility in a high salt solution (ammonium sulfate)(118). This technique involved taking filtered supernatants from T cells co-cultured with *S.* Typhimurium and subjecting it to varying concentrations of ammonium sulfate. In order to track the inhibitory activity, a bioassay was used as a means to determine whether precipitation was successful. T cells were treated with samples resulting from various concentrations of ammonium sulfate precipitations and then TCR-β expression was analyzed by flow cytometry, similar to that used by van der Velden et al (111). This first attempt at purification resulted in the inhibitory factor(s) salting out at an 80% ammonium sulfate concentration (Figure 2.1A). The inhibitory factor did not salt out at any ammonium sulfate concentrations below 80%. Silver stain analysis revealed that there was no detectable change in the overall protein content of the sample when comparing untreated supernatant to supernatants subjected to an 80% ammonium sulfate precipitation. Therefore, in

an attempt to decrease the overall amount of protein in the sample, ammonium sulfate precipitation was done sequentially. The supernatant was first subjected to a 60% ammonium sulfate precipitation and the supernatant resulting from this, which contained the inhibitory factor(s), was then subjected to an 80% ammonium sulfate precipitation. This sequential fractionation resulted in a decrease in the overall protein content of the sample, as visualized by silver stain analysis, but a large amount of protein still remained. This indicated that ammonium sulfate precipitation alone would not be sufficient to purify and identify the inhibitory factor(s). However, successful precipitation of the inhibitory factor was further evidence that the factor(s) was a protein and therefore further biochemical purification techniques could be used.

The next technique used to purify and identify the inhibitory factor(s) was ion exchange chromatography, which separates proteins based on their charge (119). A DEAE-Sepharose column was used which is an anion-exchanger. Using this technique, the inhibitory factor(s) was successfully eluted from a single fraction, in the range of 23-41% salt, as demonstrated by TCR-β down-modulation (Figure 2.1B and C). This fraction was also analyzed by silver stain and very little protein was detectable. This fraction was subjected to mass spectrometry analysis and over 200 proteins, derived from both *S.* Typhimurium and T cells, were found in the sample. Of the proteins detected by mass spectrometry, none stood out as possible candidates for the inhibitory factor(s). Therefore, further purification was needed and a strategy using multiple purification techniques in sequence, which included ammonium sulfate precipitation, ion exchange chromatography and hydrophobic interaction chromatography, began to be optimized.

L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence.

As I continued to make progress towards the purification of the inhibitor, another Ph.D. student in the laboratory, Amy Kullas, identified a *S.* Typhimurium gene required for T cell inhibition. She and I collaborated to show that the gene product, a putative L-Asparaginase, was necessary and sufficient to cause T cell inhibition. The results were published in a paper that I co-authored (Kullas AL, McClelland M, Yang HY, Tam JW, **Torres A**, Porwollik S, Mena P, McPhee JB, Bogomolnaya L, Andrews-Polymenis H, van der Velden AWM. 2012. L-Asparaginase II produced by *Salmonella* inhibits T cell responses and mediates virulence. *Cell Host & Microbe*. **12**:791-8). Below is the summary of the paper.

> "Microbial pathogens must avoid clearance by the immune system to establish infection, yet many mechanisms of microbial immune subversion remain undefined. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) inhibit the response of T cells directly, but the factor responsible for this inhibition has not been identified. We report that production of L-Asparaginase II by *S.* Typhimurium is necessary and sufficient to cause inhibition of T cells. The mechanism of inhibition involves suppression of T cell blastogenesis, cytokine production and proliferation, and down-modulation of the T cell receptor. *S.* Typhimurium lacking the L-Asparaginase II gene (*STM3106*) are attenuated for virulence, which may be due to a reduced ability of these bacteria to inhibit the response of T cells *in vivo*. L-Asparaginase II is used clinically to treat acute lymphoblastic leukemia, yet production of L-Asparaginase II by pathogenic bacteria has been unrecognized as a mechanism of microbial immune subversion."

2.5 Discussion

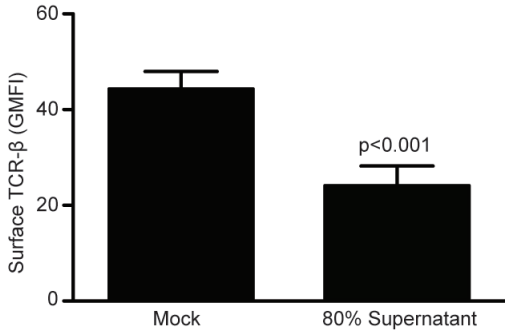
Here, through a combination of biochemical and genetic approaches the factor responsible for T cell inhibition was identified as L-Asparaginase II, encoded by the *S.*

Typhimurium gene *ansB*. The identification of this factor completes the first aim of this dissertation. Purified L-Asparaginase II that resulted from this work enabled me to pursue the characterization of the mechanism by which L-Asparaginase II inhibits the response of T cells.

2.6 Figures

Figure 2.1. Partial purification of the T cell inhibitor. (A) Conditioned medium from T cells cultured in the presence of wild-type *S.* Typhimurium was harvested, filtered and added to an ammonium sulfate solution (80%) to precipitate proteins based on solubility. The resulting fraction was dialyzed to remove all salts and to resolubilize the proteins. T cells were then treated with the dialyzed solution for 20 hours and then harvested, stained and analyzed by flow cytometry for expression of surface TCR-β. Data pooled from three independent experiments and analyzed used a paired student T test. **(B)** Representative elution profile. Large amounts of the T cell inhibitor were obtained in the form of serum-free conditioned medium from EL-4 cells cultured in the presence of *S.* Typhimurium. EL-4, a commercially available T cell line was established from a lymphoma induced in a C57BL/6J mouse. Conditioned medium was harvested, filtered, and dialyzed using Tris-HCl buffers (5-20 mM, pH=8) and 10kDa MWCO dialysis tubing. After 3 days of dialysis, samples were removed from tubing, filtered, and loaded onto a DEAE (weak anion) column to separate proteins based on charge. A Tris-HCl buffer (20 mM, pH=8) containing NaCl (1M) was used to elute proteins off the column. Resulting elution profiles were used to identify fractions containing protein. **(C)** Fractions containing protein were tested for inhibitory activity using the T cell assay. Briefly, fractions that corresponded to peaks were combined, dialyzed and concentrated using cell culture medium, and then added to freshly isolated T cells. After 20 hours of incubation, T cells were harvested, stained and analyzed by flow cytometry for TCR-β expression. Data are pooled from three independent experiments and show mean with SD of duplicate samples. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons post test; p values < 0.05 were considered to be statistically significant.

A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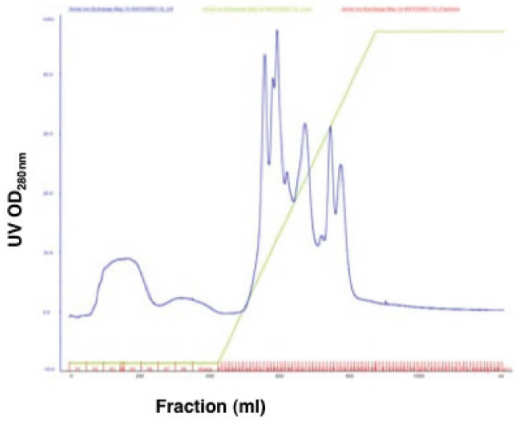
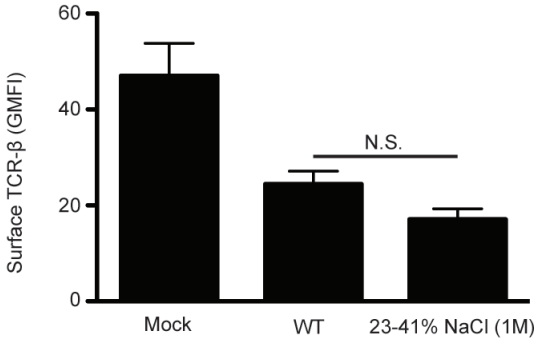
40
20
0
Surface TCR-β (GMFI)
p<0.001
Mock
80% Supernatant

B


UV OD280nm
Fraction (ml)

C


60
40
20
0
Surface TCR-β (GMFI)
N.S.
Mock
WT
23-41% NaCl (1M)

Chapter 3. L-Asparaginase II produced by *S.* Typhimurium exhibits L-asparagine hydrolase activity

3.1 Chapter Summary

Little is known about the L-Asparaginase II protein produced by *S.* Typhimurium. However, Kullas et al. have demonstrated that L-Asparaginase II of *S.* Typhimurium is both necessary and sufficient to cause T cell inhibition (130). L-Asparaginase II of *S.* Typhimurium is a putative L-asparagine hydrolase. To characterize the mechanism by which L-Asparaginase II of *S.* Typhimurium causes T cell inhibition, I set out to determine whether L-Asparaginase II of *S.* Typhimurium exhibits L-asparagine hydrolase activity. A coupled enzyme assay was used to demonstrate that L-Asparaginase II of *S.* Typhimurium is a L-asparagine hydrolase. Using the coupled enzyme assay, we calculated the basic kinetic parameters of the enzyme.

3.2 Introduction

L-Asparaginase proteins are found in most organisms including bacteria, yeast, plants, algae, actinomycetes and even humans (120, 121). L-Asparaginases are enzymes that catalyze the hydrolysis of L-asparagine to aspartic acid and ammonia. Gram-negative bacteria may encode two types of L-Asparaginases, L-Asparaginase I and L-Asparaginase II. L-Asparaginase I proteins are located in the cytosol, while L-Asparaginase II proteins are located in the periplasm and have been suggested to be secreted (122). In the 1950's it was first discovered that treating lymphomas in rats and mice with guinea pig serum lead to a significant regression of the cancers (123). Later, it was discovered that the component of the guinea pig serum responsible for the anti-cancer effect was L-Asparaginase II (124). Following this discovery, the

effects of L-Asparaginase II on normal cells were characterized. It was demonstrated that L-Asparaginase II had immunosuppressive effects on both humoral and cellular immune responses, and that L-Asparaginase II treatment inhibited the growth of stimulated lymphocytes characterized by a significant decrease in DNA synthesis (125, 126). These results suggested that the anti-cancer effects could be due to the enzymatic activity of L-Asparaginase II (122, 125). However, experimental data from this time suggested that the addition of L-asparagine alone could not fully rescue the inhibitory effects of L-Asparaginase II (125). Further work suggested that, in addition to depleting the cells of L-asparagine, L-Asparaginase II altered the surfaces of lymphocytes, possibly by cleaving membrane glycoproteins required for T cell activation (126-128). It was also shown that L-Asparaginase II treatment inhibited RNA and protein synthesis and uptake of radiolabeled fucose, suggesting that L-Asparaginase II had global effects on lymphocytes (127). Following this work, mostly done in the 1970's, little research was done to advance knowledge of the effects L-Asparaginase II on lymphocytes. Despite not knowing the mechanism of action by which L-Asparaginase II exerts its effects on lymphocytes, L-Asparaginase II is now used to treat acute lymphoblastic leukemia (ALL), a T cell cancer. It has been hypothesized that L-Asparaginase II may deplete the environment of exogenous L-asparagine required by the cancer cells, via its enzymatic activity, thereby killing the cancer cells by starvation (120, 123, 129, 130).

The *S.* Typhimurium gene *STM3106* encodes a putative L-Asparaginase II protein that has not yet been characterized. Here, we investigated the ability of *STM3106* gene product to hydrolyze L-asparagine.

3.3 Experimental Methods

Alignment of Protein Sequences

L-Asparaginase II protein sequences from *S.* Typhimurium strain 14028 and *Escherichia coli* (*E. coli*) strain K-12 substrain W3110 were aligned using T-Coffee Multiple Sequence Alignment Tools program.

Purification of L-Asparaginase II

Nickel-affinity chromatography was used to purify His-tagged L-Asparaginase II from *Escherichia coli* strain LMG194 (Invitrogen) carrying pBAD-*STM3106*HisT89A, as described previously (131). 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 His-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 of Tris (pH=8), 500 mM of NaCl and 20 mM imidazole (buffer A). The column was washed with 100 ml of buffer A and His-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 His-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 His-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 using *Limulus* amebocyte lysate gel clot assay (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. In order to obtain more concentrated L-Asparaginase II to be used in the kinetic assays, the above protocol was followed with the following additional steps: following induction the bacteria were kept at 37°C for 4 hours to overnight. The bacteria were pelleted by centrifugation and a periplasmic extraction was performed immediately on the pellet. Briefly, the pellet was resuspended in 400ml of 200mM Tris (pH=8), 500mM sucrose, 0.5mM EDTA and mixed at 4°C for at least 30 minutes. Next, 400ml of 2mM ice cold $MgCl_2$ was added and then mixed overnight at 4° for 30 minutes to overnight. The mixture was then spun down by centrifugation and the supernatant was diluted 1:1 in 20mM Tris, 500mM NaCl and 20mM imidazole (pH=8). This resulting periplasmic extraction was then used in purification as described above.

Kinetic Analysis of Coupled Enzyme Assay

A coupled enzyme assay was used to measure the ability of L-Asparaginase II of *S.* Typhimurium to hydrolyze L-asparagine. The assay used is based on the principle that L-asparagine hydrolysis results in the production of ammonia, which, in the presence of NADPH and Glutamate Dehydrogenase, reacts with α-ketogluterate to form L-glutamate and $NADP^+$. The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia generated as a result of L-asparagine hydrolysis. Thus, the oxidation of NADPH measured by the decrease in absorbance at 340 nm provides a functional readout for L-asparagine hydrolase activity. Briefly, steady-state kinetic assays were performed at 25°C in 2 mM sodium phosphate buffer containing

5 nM purified wild-type or mutant L-Asparaginase II, 1 mM EDTA, 10 mM α-ketogluterate, 250 μM NADPH, and 0.224 mg/ml Glutamate Dehydrogenase (Sigma). Reactions were initiated by the addition of L-asparagine, with concentrations ranging from 4.25 μM to 60 μM. We monitored enzyme activity by measuring the absorbance at 340 nm (ε = 6,300 $M^{-1}cm^{-1}$) using a Cary 100 Bio UV-Vis spectrophotometer (Varian). Data were analyzed and kinetic parameters were calculated using KaleidaGraph 4.1.3 (Synergy Software). Initial velocities were calculated using the following equation: m1-0*x- (m3)*(1-exp(-m4*x))/m4; m1 = 2; m2 = 0.003; m3 = 0.008; m4 = 0.01 (132).

3.4 Results

L-Asparaginase II of *S.* Typhimurium exhibits L-asparagine hydrolase activity.

Previously, we showed that *STM3106* is required for *S.* Typhimurium to inhibit T cell responses and mediate virulence (131). *STM3106* encodes a putative L-Asparaginase that is 96% identical to L-Asparaginase II of *E. coli* at the amino acid level (Figure 3.1A). To determine the ability of L-Asparaginase II of *S.* Typhimurium to hydrolyze L-asparagine, we used a coupled enzyme assay (133, 134)(Figure 3.1B). This assay is based on the principle that L-asparagine hydrolysis results in the production of ammonia, which, in the presence of NADPH and Glutamate Dehydrogenase, reacts with α-ketogluterate to form L-glutamate and $NADP^+$. The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia generated as a result of L-asparagine hydrolysis. Thus, the oxidation of NADPH provides a functional readout for L-asparagine hydrolase activity. We performed steady-state kinetic assays to measure L-Asparaginase II-catalyzed NADPH oxidation and plotted the initial velocity of the reaction as a function of L-asparagine substrate concentration (Figure 3.1C). The calculated values of the

kinetic parameters K_m, k_{cat} and k_{cat}/K_m were 11.8 ± 1.8 μM, 7587 ± 190 min^{-1}, and 642 μM^{-1} min^{-1}, respectively (Figure 3.1C). Thus, L-Asparaginase II of *S.* Typhimurium exhibits L-asparagine hydrolase activity.

3.5 Discussion

I have found that L-Asparaginase II of *S.* Typhimurium exhibits L-asparagine hydrolase activity. An immediate implication of this result is that this enzymatic activity may be required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells. This work is important for several reasons; first it established an assay that will be helpful in further characterizing the mechanism by which L-Asparaginase II inhibits T cells. Second, L-Asparaginase II produced by *S.* Typhimurium has not been characterized and this represents the first kinetic analysis done on this enzyme and demonstrates that it exhibits similar kinetics as the *E.coli* L-Asparaginase II (135).

3.6 Figures

Figure 3.1 L-Asparaginase II of S. Typhimurium exhibits L-asparagine hydrolase activity. (***A***) Alignment of primary amino acid sequences of L-Asparaginase II of *S.* Typhimurium and L-Asparaginase II of *E. coli* were done using T-Coffee Multiple Sequence Alignment Tools program. Amino acid residues in black boxshades are identical. Amino acid residues in grey boxshades are similar. (***B***) Coupled enzyme assay used to measure the ability of L-Asparaginase II of *S.* Typhimurium to hydrolyze L-asparagine. The assay is based on the principle that L-asparagine hydrolysis results in the production of ammonia (1), which, in the presence of NADPH and Glutamate Dehydrogenase, reacts with α-ketogluterate to form L-glutamate and $NADP^+$ (2). The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia generated as a result of L-asparagine hydrolysis, providing a functional readout for L-asparagine hydrolase activity. (***C***) Michaelis-Menten plot and K_m, k_{cat} and k_{cat}/K_m values for L-Asparaginase II of *S.* Typhimurium. Data are representative of three independent experiments and show mean with SD of duplicate samples.

A

```
STm       1 LPNITILATGGTIAGGGDSATKSNYTAGKVGVENLVDAVPQLKDIAVVKGEQVVNIGSQD
Ecoli     1 LPNITILATGGTIAGGGDSATKSNYTVGKVGVENLVNAVPQLKDIANVKGEQVVNIGSQD

STm      61 MNDEVWLTLAKKINTECDSTDGFVITHGTDTMEETAYFLDLTVKCNKPVVLVGAMRPSTS
Ecoli    61 MNDNVWLTLAKKINTDCDKTDGFVITHGTDTMEETAYFLDLTVKCDKPVVMVGAMRPSTS

STm     121 MSADGPFNLYNAVVTAADKQSANRGVLVVMNDTVMDGRDVTKTNTTDVATFKAVNYGPLG
Ecoli   121 MSADGPFNLYNAVVTAADKASANRGVLVVMNDTVLDGRDVTKTNTTDVATFKSVNYGPLG

STm     181 YIHNGKIDYQRTPERKHTTSTPFDVSKLTALPKVGIVYNYANASDLPAKALVDAGYDGIV
Ecoli   181 YIHNGKIDYQRTPARKHTSDTPFDVSKLNELPKVGIVYNYANASDLPAKALVDAGYDGIV

STm     241 SAGVGNGNLYKTVFDTLATAAHNGTVVVRSSRVPTGATTQDAEVDDAKYGFVASGTLNPQ
Ecoli   241 SAGVGNGNLYKSVFDTLATAAKTGTAVVRSSRVPTGATTQDAEVDDAKYGFVASGTLNPQ

STm     301 KARVLLQLALTQTKDPKQIQTMFNQY
Ecoli   301 KARVLLQLALTQTKDPQQIQQIFNQY
```

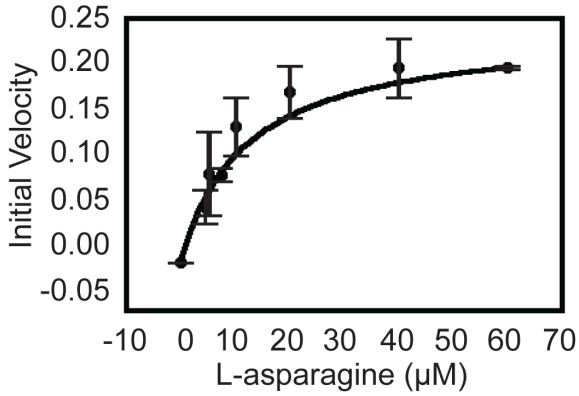
B

Coupled Enzyme Assay

(1) L-asparagine + H_2O $\xrightarrow{\text{L-Asnase II}}$ L-aspartate + NH_4^+

(2) NH_4^+ + α-ketogluterate + NADPH $\xrightarrow{\text{GDH}}$ L-glutamate + NAD^+ + H_2O

C



K_m = 11.8 ± 1.8 μM
k_{cat} = 7587 ± 190 min^{-1}
k_{cat}/K_m = 642 μM^{-1} min^{-1}

Chapter 4. L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells

4.1 Chapter Summary

A primary focus of my dissertation research has been to characterize the mechanism by which L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses. In this chapter, I provide evidence indicating that the ability of L-Asparaginase II of S. Typhimurium to cause inhibition of T cells can be destroyed by heat treatment and blocked by adding L-asparagine to T cells cultured with L-Asparaginase II. Furthermore, I provide evidence indicating that enzymatic activity is required for L-Asparaginase II of S. Typhimurium to inhibit T cells.

4.2 Introduction

Very little is known about L-Asparaginase II of *S.* Typhimurium. However, my kinetic analysis of the protein indicates not only that it exhibits L-asparagine hydrolase activity, but also that its kinetic parameters are highly similar to those of L-Asparaginase II of *E. coli* (135).

Enzymatic and characterization of the *E.coli* L-Asparaginase II has been performed and it has been shown that the *E.coli* L-Asparaginase II can catalyze the hydrolysis of L-asparagine and it has been proposed this reaction occurs via a ping-pong mechanism (122, 136-138). A ping-pong mechanism produces two products and is characterized by two steps; the first step involves the change of the enzyme into a temporary intermediate form and the second step requires that one product is formed and released before the second substrate can bind (122, 132)

Structural studies have suggested that L-Asparaginase II functions as a tetrameric protein complex, also referred to as “a dimer of dimers” because it is made up of four identical subunits,

creating four active sites (122, 136). Work done on the *E.coli* L-Asparaginase II has demonstrated that the threonine residue at position 89 is important for L-asparagine hydrolase activity of L-Asparaginase II. This residue seems to play a role in the interactions between dimers required for catalytic activity (122, 135, 136, 139, 140). Site-specific mutagenesis experiments showed that a single amino acid substitution at threonine 89 resulted in catalytically inactive L-Asparaginase II protein (139). As described in Chapter 1, L-Asparaginase II of *S.* Typhimruium is 96% identical to L-Asparaginase II of *E.coli* at the amino acid level, and the threonine residue at position 89 is conserved.

Here, I used a combination of biochemical and genetic approaches to investigate whether L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cell responses.

4.3 Experimental Methods

Bacterial Strains and Culture Conditions

S. Typhimurium strain 14028 (American Type Culture Collection) was used as the wild-type strain. *S.* Typhimurium lacking the L-Asparaginase II gene *STM3106* (*ΔSTM3106 S.* Typhimurium) and *ΔSTM3106 S.* Typhimurium complemented with a plasmid encoding *STM3106* (pBAD-*STM3106*) have been published (131). Generation of *S.* Typhimurium expressing enzymatically inactive L-Asparaginase II is described below. Bacteria were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar using standard microbiological techniques. Chloramphenicol (30 μg/ml) and L-arabinose (0.1% (w/v)) were added to the culture medium to maintain plasmid pBAD-*STM3106 in vivo* and induce expression of L-Asparaginase II, respectively.

Site-Directed Mutagenesis of *STM3106*

Site-directed mutagenesis was used to introduce a site-specific mutation into *STM3106*, resulting in an alanine substitution for threonine at position 89. The mutagenesis was performed on plasmid pBAD-*STM3106* using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent) and primers 5' GGTTTCGTGATCACCCACGGTGCGGATACGATGGAAGAGACCGC-3' and 5'-GCGGTCTCTTCCATCGTATCCGCACCGTGGGTGATCACGAAACC-3' or 5'-GCTAGCGAAGAGATATACCATGGAGTTTTTCAGGAAAACGGC-3' and 5'-TCTAGATTATTAGTGGTGGTGGTGGTGGTGATACTGATTGAACATCGTCTGG-3' respectively. The resulting constructs, pBAD-*STM3106*T89A and pBAD-*STM3106*HisT89A (used for purification), were verified by DNA sequencing and introduced into *ΔSTM3106 S.* Typhimurium or *E. coli* by electroporation. Transformants were selected using LB agar supplemented with chloramphenicol (30 μg/ml). An isolated single colony was picked and the presence of the plasmid was confirmed. The resulting strains grew normally in LB broth and, following induction with L-arabinose, expressed mutant L-Asparaginase II at levels that were comparable to the levels expressed by *ΔSTM3106 S.* Typhimurium carrying pBAD-*STM3106*.

Purification of L-Asparaginase II

Nickel-affinity chromatography was used to purify His-tagged L-Asparaginase II from *Escherichia coli* strain LMG194 (Invitrogen) carrying plasmid pBAD-*STM3106*HisT89A, as described previously (131). 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 His-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 of Tris (pH=8), 500 mM of NaCl and 20 mM imidazole (buffer A). The column was washed with 100 ml of buffer A and His-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 His-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 His-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 using *Limulus* amebocyte lysate gel clot assay (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. In order to obtain more concentrated L-Asparaginase II to be used in the kinetic assays, the above protocol was followed with the following additional steps: following induction the bacteria were kept at 37°C for 4 hours to overnight. The bacteria were pelleted by centrifugation and a periplasmic extraction was performed immediately on the pellet. Briefly, the pellet was resuspended in 400ml of 200mM Tris (pH=8), 500mM sucrose, 0.5mM EDTA and mixed at 4°C for at least 30 minutes. Next, 400ml of 2mM ice cold $MgCl_2$ was added and then mixed overnight at 4° for 30 minutes to overnight. The mixture was then spun down by centrifugation and the supernatant

was diluted 1:1 in 20mM Tris, 500mM NaCl and 20mM imidazole (pH=8). This resulting periplasmic extraction was then used in purification as described above.

Kinetic Analysis of Coupled Enzyme Assay

A coupled enzyme assay was used to measure the ability of L-Asparaginase II of *S.* Typhimurium to hydrolyze L-asparagine. The assay used is based on the principle that L-asparagine hydrolysis results in the production of ammonia, which, in the presence of NADPH and Glutamate Dehydrogenase, reacts with α-ketogluterate to form L-glutamate and $NADP^+$ (Figure 1B). The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia generated as a result of L-asparagine hydrolysis. Thus, the oxidation of NADPH measured by the decrease in absorbance at 340 nm provides a functional readout for L-asparagine hydrolase activity. Briefly, steady-state kinetic assays were performed at 25°C in 2 mM sodium phosphate buffer containing 5 nM purified wild-type or mutant L-Asparaginase II, 1 mM EDTA, 10 mM α-ketogluterate, 250 μM NADPH, and 0.224 mg/ml Glutamate Dehydrogenase (Sigma). Reactions were initiated by the addition of L-asparagine, with concentrations ranging from 4.25 μM to 60 μM. We monitored enzyme activity by measuring the absorbance at 340 nm (ε = 6,300 $M^{-1}cm^{-1}$) using a Cary 100 Bio UV-Vis spectrophotometer (Varian). Data were analyzed and kinetic parameters were calculated using KaleidaGraph 4.1.3 (Synergy Software). Initial velocities were calculated using the following equation: m1-0*x- (m3)*(1-exp(-m4*x))/m4; m1 = 2; m2 = 0.003; m3 = 0.008; m4 = 0.01 (132).

T Cell Enrichment and T Cell Assays

Splenocytes harvested from naïve C57BL/6J mice (females, 6 to 8 weeks of age) were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell

separation technology (Miltenyi Biotec) was used to enrich for $CD90.2^+$ T cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enrichment resulted in 75-85% enrichment of $CD90.2^+$ T cells. Unless indicated otherwise, enriched populations of T cells were suspended in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) and used in T cell assays.

In T cell assays aimed at measuring the effect of L-Asparaginase II on TCR-β surface expression, blastogenesis and IL-2 secretion, enriched populations of T cells suspended in medium supplemented with 1 μg/ml of anti-CD28 antibody (clone E18; BioLegend) were seeded at 1 x 10^5 cells per well into flat-bottom 96-well tissue culture plates coated with 5 μg/ml of anti-CD3ε antibody (clone 145-2C11; BioLegend). The T cells were then left untreated or treated with 5 ng/ml of purified wild-type L-Asparaginase II or mutant L-Asparaginase II. After 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using enzyme-linked immunosorbent assay (ELISA). Additionally, the T cells were harvested, stained and analyzed using flow cytometry.

In T cell assays aimed at measuring the effect of L-Asparaginase II produced by *S.* Typhimurium on TCR-β surface expression, blastogenesis and IL-2 secretion, enriched populations of T cells were seeded into tissue culture plates, as described above. The T cells were then cultured in the absence or presence of wild-type *S.* Typhimurium, *ΔSTM3106 S.* Typhimurium, *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* or *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*T89A at a multiplicity of infection of ~60. L-arabinose was added to the cultures at a final concentration of 0.1% (w/v) to induce expression of His-tagged L-Asparaginase II by L-arabinose-pretreated bacteria (see above). After 2 hours of

incubation at 37°C in 5% CO_2, the T cells were pelleted by centrifugation and resuspended in medium supplemented with 50 μg/ml gentamicin and 2% penicillin and streptomycin, killing all remaining bacteria within 2 hours (data not shown). After an additional 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using ELISA. Additionally, the T cells were harvested, stained and analyzed using flow cytometry. Where noted L-Asparaginase II was heat treated at 95°C for 10 minutes and medium was supplemented with L-asparagine (10mM; Sigma).

Cell Staining and Analysis by Flow Cytometry

All antibodies and reagents described in this section were purchased from BioLegend. Routinely, cells were stained in the presence of Fc block (clone 93) using allophycocyanin (APC)-conjugated anti-mouse CD90.2 antibody (clone 30-H12), phycoerythrin (PE)-conjugated anti-mouse CD25 antibody (clone PC61), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse TCR-β antibody (clone H57-597). Where indicated, cells were stained in the presence of Fc block using FITC-conjugated anti-mouse CD90.2 antibody, APC-conjugated anti-mouse CD25 antibody and propidium idodide to assess cell viability. Live cells expressing CD90.2, CD25 and TCR-β were identified as anti-CD3ε- and anti-CD28-stimulated T cells. Size and granularity of live cells were analyzed using forward scatter and side scatter. Data were acquired and analyzed using a FACSCalibur with CellQuest™ Pro software (BD Biosciences). Blastogenesis was calculated by determining the percentage of live cells that, in response to treatment with anti-CD3ε and anti-CD28, had transformed from small lymphocytes into larger, more granular cells resembling blast cells.

IL-2 Enzyme-Linked Immunosorbent Assay

IL-2 concentrations in collected T cell supernatants were determined using Mouse IL-2 ELISA MAX Deluxe kit (BioLegend).

Statistical Analysis

Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using a two-tailed, paired Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

4.4 Results

The Inhibitory Activity of L-Asparaginase II can be destroyed by heat treatment

To determine the effect of heat treatment on the ability of L-Asparaginase II to inhibit T cell responses, I incubated purified L-Asparaginase II at 95°C for ten minutes and then added it to T cell cultures at a final concentration of 5 ng/ml. T cells left untreated and T cells treated with purified L-Asparaginase II not subjected to heat treatment were used as controls. After 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using ELISA. Additionally, the T cells were harvested, stained and analyzed using flow cytometry. T cells incubated with heat-treated L-Asparaginase II expressed significantly more surface TCR-β than did T cells incubated with L-Asparaginase II left untreated (Figure 4.1A). Furthermore, T cells incubated with heat-treated L-Asparaginase II expressed levels of surface TCR-β that were comparable to the levels expressed by T cells left untreated (Figure 4.1A). Similar results were obtained when we evaluated the ability of T cells to

blast (Figure 4.1B). Thus, L-Asparaginase II must be in its native conformation to cause inhibition of T cells.

Exogenous L-asparagine can prevent L-Asparaginase II-mediated inhibition of T cells.

Previous studies from our laboratory have indicated that exogenous L-asparagine, but not L-aspartate or L-glutamate, can prevent S. Typhimurium-induced inhibition of T cells (131). To determine the effect of exogenous L-asparagine on the ability of purified L-Asparaginase II to cause inhibition of T cells, I added L-asparagine to T cells cultured with L-Asparaginase II. T cells left untreated and T cells treated with L-Asparaginase II were used as controls. After 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using ELISA. Additionally, the T cells were harvested, stained and analyzed using flow cytometry. Exogenous L-Asparagine prevented L-Asparaginase II-mediated down modulation of surface TCR-β (Figure 4.2A). Similar results were obtained when we evaluated the ability of T cells to blast (Figure 4.2B). Thus, hydrolysis of exogenous L-asparagine may contribute to L-Asparaginase II-mediated inhibition of T cells.

L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells.

To determine whether enzymatic activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells, we substituted alanine for threonine at position 89, a conserved amino acid residue important for enzymatic activity of *E. coli* L-Asparaginase II (139). Using the coupled enzyme assay, we detected oxidation of NADPH with wild-type L-Asparaginase II but not mutant L-Asparaginase II (Figure 4.2A), indicating that the alanine substitution for threonine at position 89 (T89A) rendered L-Asparaginase II of *S.* Typhimurium enzymatically inactive.

We next treated T cells with purified wild-type L-Asparaginase II or enzymatically inactive L-Asparaginase II. T cells left untreated were used as a control. After 20 hours of incubation, T cells treated with enzymatically inactive L-Asparaginase II expressed significantly more surface TCR-β than did T cells treated with wild-type L-Asparaginase II (Figure 4.3B). Furthermore, T cells treated with enzymatically inactive L-Asparaginase II expressed levels of surface TCR-β that were comparable to the levels expressed by T cells left untreated (Figure 4.3B). Similar results were obtained when we evaluated the ability of the T cells to blast (Figure 4.3C) or secrete IL-2 (Figure 4.3D). Consistent with these results, we found that *S.* Typhimurium expressing enzymatically inactive L-Asparaginase II (*ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106:*T89A) were unable to down modulate TCR-β (Figure 4.3E), suppress T cell blastogenesis (Figure 4.3F), and inhibit secretion of IL-2 (Figure 4.3G). As expected, we found that wild-type *S.* Typhimurium and Δ*STM3106 S.* Typhimurium carrying the plasmid pBAD-*STM3106* (compliment) were able to inhibit the response of T cells, where *ΔSTM3106 S.*Typhimurium (lacking the L-Asparaginase II gene) were unable to inhibit the response of T cell (Figure 4.3E-G). Induction assays revealed *S.* Typhimurium expressing enzymatically inactive L-Asparaginase II (Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*:T89A) expressed similar levels of L-Asparaginase II to Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* and Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*:T89V, an uncharacterized mutant. Additionally, the inability of *S.* Typhimurium expressing enzymatically inactive L-Asparaginase II to suppress T cells was not due to differences in growth defects since growth curves comparing wild-type *S.* Typhimurium (14028), Δ*STM3106 S.* Typhimuirum, *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* and *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*:T89A all grew similarly (Figure

4.4). Collectively, these results indicate that L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells.

4.5 Discussion

Using a combination of biochemical and genetic approaches, I found that L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to cause inhibition of T cells. Here, we have demonstrated that in addition to down modulating expression of TCR-β, that L-Asparaginase II treatment also suppresses T cell blastogenesis and secretion of IL-2, two key aspects of T cell activation. An immediate implication of this result is that L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses by depleting exogenous L-asparagine. The result further suggests that exogenous L-asparagine is a resource important for T cell function.

4.6 Figures

Figure 4.1. The inhibitory activity of L-Asparaginase II can be destroyed by heat treatment. (A) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left untreated (Mock), treated with wild-type L-Asparaginase II (WT), or treated with wild-type L-Asparaginase II that had been heat-treated (95°C, 10 min) before addition to T cells; p=0.0190. **(B)** Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as in (A); p=0.0029. Data show mean with SEM from three independent experiments. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons post test; p values < 0.05 were considered to be statistically significant.

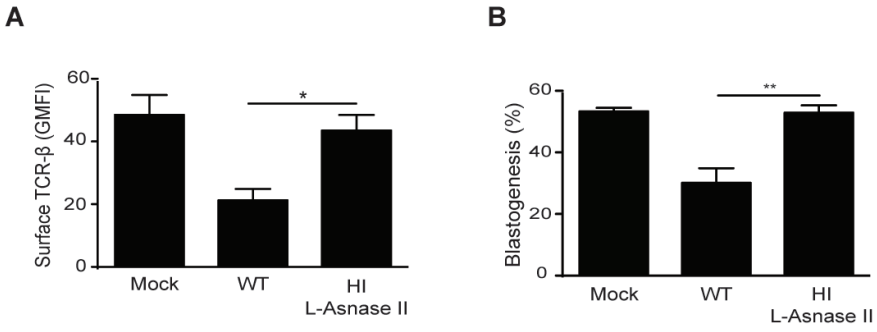

A
Surface TCR-β (GMFI)
60
40
20
0
*
Mock
WT
HI
L-Asnase II
B
Blastogenesis (%)
60
40
20
0
**
Mock
WT
HI
L-Asnase II

Figure 4.2. Exogenous L-asparagine can prevent L-Asparaginase II-mediated inhibition of T cells. (A) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left untreated (Mock), treated with wild-type L-Asparaginase II (WT), or treated with wild-type L-Asparaginase II plus 10mM L-asparagine; p=0.0053. **(B)** Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as in (A); p=0.0067. Data show mean with SEM from three independent experiments. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons post test; p values <0.05 were considered to be statistically significant.

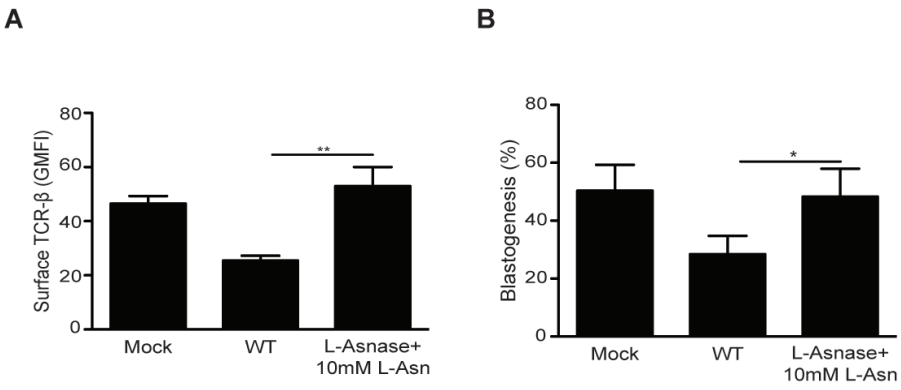
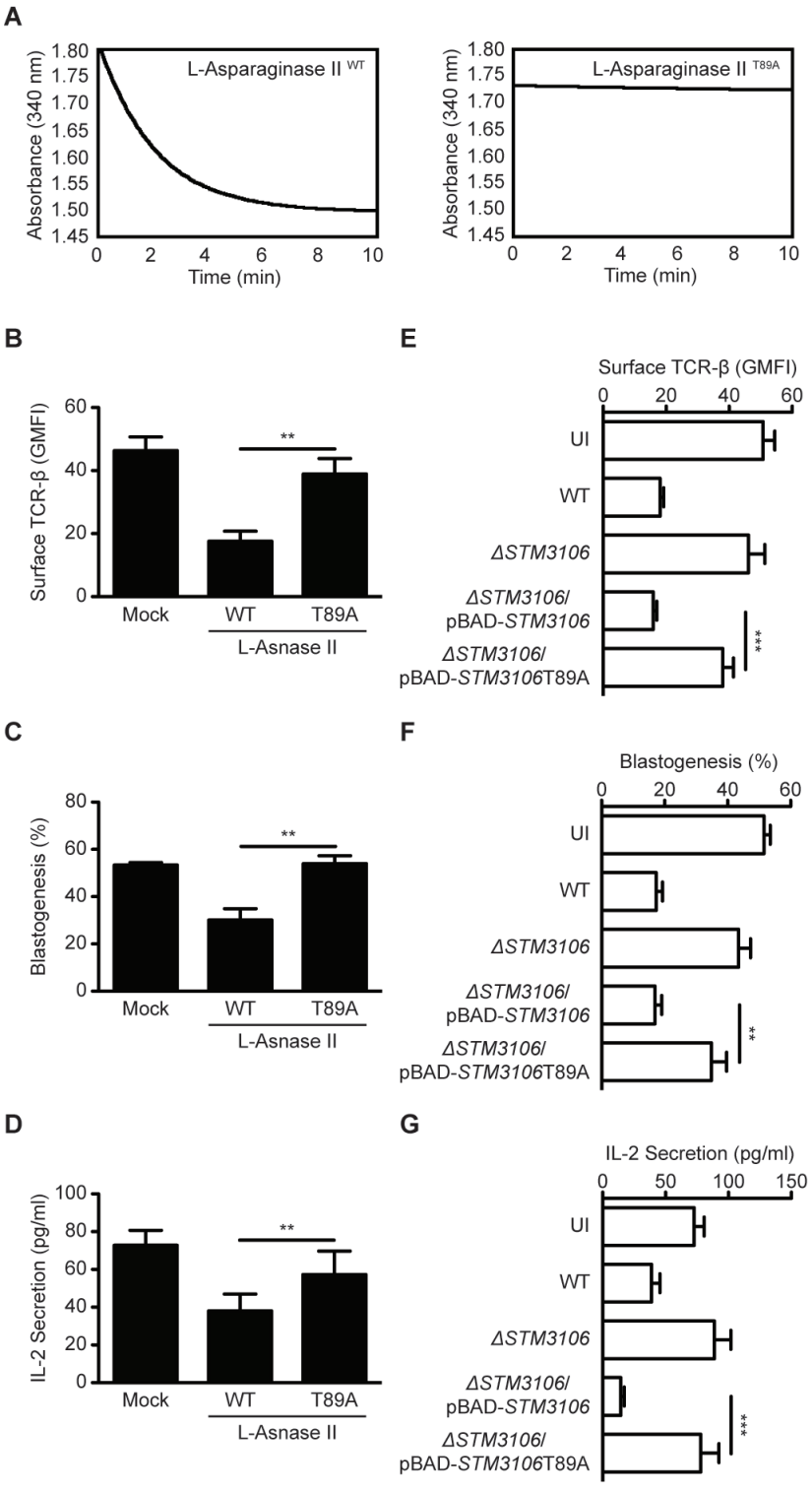

A
B
Surface TCR-β (GMFI)
Blastogenesis (%)
80
60
40
20
0
**
*
Mock
WT
L-Asnase+
10mM L-Asn

Figure 4.3. L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells. (***A***) Reaction progress of coupled enzyme assays using wild-type L-Asparaginase II (L-Asparaginase II^{WT}; left) or mutant L-Asparaginase II (L-Asparaginase II^{T89A}; right). (***B***) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left untreated (Mock) or treated with wild-type (WT) or enzymatically inactive (T89A) L-Asparaginase II (L-Asnase II); $p = 0.0002$. (***C***) Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as in (B); $p = 0.0035$. (***D***) IL-2 secretion by anti-CD3ε/CD28-stimulated T cells treated as in (B); $p = 0.0021$. (***E***) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with wild-type *S.* Typhimurium (WT), *S.* Typhimurium lacking the L-Asparaginase II gene *STM3106* (*ΔSTM3106*), *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* encoding wild-type L-Asparaginase II (*ΔSTM3106*/pBAD-*STM3106*) or *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*T89A encoding enzymatically inactive L-Asparaginase II (*ΔSTM3106*/pBAD-*STM3106*T89A); $p < 0.0001$. (***F***) Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as in (E); $p < 0.0001$. (***G***) IL-2 secretion by anti-CD3ε/CD28-stimulated T cells treated as in (E); $p < 0.0001$. Data are representative of (A), or show mean with SEM from (B-G), at least three independent experiments. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons post test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$).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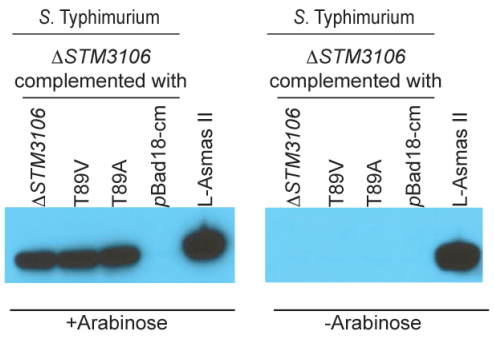
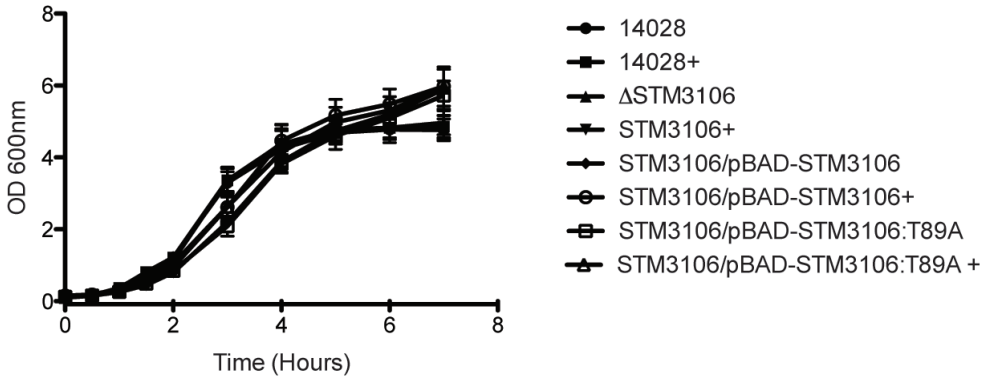
L-Asparaginase II WT
L-Asparaginase II T89A
Absorbance (340 nm)
Time (min)
B
Surface TCR-β (GMFI)
Mock
WT
T89A
L-Asnase II
C
Blastogenesis (%)
D
IL-2 Secretion (pg/ml)
E
Surface TCR-β (GMFI)
UI
WT
ΔSTM3106
ΔSTM3106/ pBAD-STM3106
ΔSTM3106/ pBAD-STM3106T89A
F
Blastogenesis (%)
G
IL-2 Secretion (pg/ml)

Figure 4.4. Characterization of *S.* Typhimurium carrying plasmid pBAD-*STM3106T89A*. **(A)** Induction assay. Overnight cultures of Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*, Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106T89V*, Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106T89A* and Δ*STM3106 S.*Typhimurium carrying plasmid pBAD-18cm (empty vector) were diluted, grown to an OD_{600} = 0.6 and then were supplemented with or without 10mM arabinose. Following 4 hours at 37°C/5%CO_2, bacteria were pelleted and resuspended in 4x SDS-PAGE buffer and boiled for 30 minutes. Lysates were analyzed for L-Asparaginase II expression by western blot analysis using a polyclonal rabbit anti-*E.coli* L-Asparaginase II antibody. Purified L-Asparaginase II was used as a positive control. Data are representative of two independent experiments. Data are representative of two independent experiments. **(B)** Overnight cultures of wild-type *S.* Typhimurium (14028), Δ*STM3106 S.* Typhimurium (Δ*STM3106*), Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* (Δ*STM3106* /pBAD-STM3106), and Δ*STM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106*:T89A (Δ*STM3106*/pBAD-STM3106:T89A) were diluted, and supplemented with or without arabinose (10mM) and grown at 37°/5%CO_2. Samples were taken over a 7 hours and analyzed at by spectrophotometer at 600nm. Data are representative of three independent experiments.

A


S. Typhimurium
ΔSTM3106 complemented with
ΔSTM3106
T89V
T89A
pBad18-cm
L-Asmas II
S. Typhimurium
ΔSTM3106 complemented with
ΔSTM3106
T89V
T89A
pBad18-cm
L-Asmas II
+Arabinose
-Arabinose

B


OD 600nm
Time (Hours)
0
2
4
6
8
14028
14028+
ΔSTM3106
STM3106+
STM3106/pBAD-STM3106
STM3106/pBAD-STM3106+
STM3106/pBAD-STM3106:T89A
STM3106/pBAD-STM3106:T89A +

Chapter 5. L-asparagine is a resource important for T cell function

5.1 Chapter Summary

Having found that L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to cause inhibition of T cells, I hypothesized that L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses by depleting exogenous L-asparagine and, furthermore, that L-Asparagine is a resource important for T cell function. In this chapter, I provide evidence indicating that T cells cultured in medium lacking L-asparagine are inhibited, as if they were cultured with *S.* Typhimurium. Furthermore, under the conditions tested, culturing T cells in medium lacking L-asparagine does not adversely affect T cell viability. Collectively, these results indicate that L-asparagine is a resource important for T cell function.

5.2 Introduction

Amino acids are important building blocks needed for protein synthesis and metabolism within cells (141). Therefore the availability of amino acids can have significant, mostly detrimental, effects on cells. This is especially true in the case of T cells where it has been shown that upon stimulation T cells up regulate the synthesis and uptake up of amino acids considerably (142). T cell priming (activation, proliferation and differentiation) is a metabolically demanding process; therefore, there is a dramatic increase in uptake and synthesis of amino acids following T cell stimulation in order to support the metabolic needs of these rapidly growing cells (142). Although some work has been performed to define the role of specific amino acids such as L-glutamine in T cell activation, the role of most amino acids,

including L-asparagine, in T cell function is unclear. Here, I examined the importance of exogenous L-asparagine in T cell function.

5.3 Experimental Methods

Bacterial Strains and Culture Conditions.

S. Typhimurium strain 14028 (American Type Culture Collection) was used as the wild-type strain. Bacteria were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar using standard microbiological techniques.

T Cell Enrichment and T Cell Assays.

Splenocytes harvested from naïve C57BL/6J mice (females, 6 to 8 weeks of age) were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell separation technology (Miltenyi Biotec) was used to enrich for $CD90.2^+$ T cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enrichment resulted in 75-85% enrichment of $CD90.2^+$ T cells. Unless indicated otherwise, enriched populations of T cells were suspended in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) and used in T cell assays.

In T cell assays aimed at measuring the effect of L-Asparaginase II produced by *S.* Typhimurium on TCR-β surface expression, blastogenesis and IL-2 secretion, enriched populations of T cells were seeded into tissue culture plates, as described above. The T cells were then cultured in the absence or presence of wild-type *S.* Typhimurium at a multiplicity of infection of ~60. After 2 hours of incubation at 37°C in 5% CO_2, the T cells were pelleted by

centrifugation and resuspended in medium supplemented with 50 μg/ml gentamicin and 2% penicillin and streptomycin, killing all remaining bacteria within 2 hours (data not shown). After an additional 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using ELISA. Additionally, the T cells were harvested, stained and analyzed using flow cytometry.

In T cell assays aimed at measuring the effect of L-asparagine deprivation on TCR-β surface expression, blastogenesis, IL-2 secretion and viability, enriched populations of T cells were suspended in medium lacking L-asparagine (RPMI1640 medium lacking L-asparagine, L-lysine and L-arginine (Caisson Laboratories) supplemented with 0.218998 mM (47.98467 mg/L) of L-lysine (Cambridge Isotope Laboratories), 1.150242 mM (242.31 mg/L) of L-arginine (Cambridge Isotope Laboratories), 10% dialyzed fetal bovine serum (Caisson Laboratories), 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) supplemented with 1 μg/ml of anti-CD28 antibody and seeded into tissue culture plates, as described above. Where indicated, L-asparagine (Sigma) was added to the medium at a final concentration of 0.378472 mM (50.0037 mg/L) to make complete RP-10 medium. After 20 hours of incubation at 37°C in 5% CO_2, culture supernatants were collected and IL-2 concentrations were determined using ELISA. Additionally, the T cells were harvested, stained and analyzed using flow cytometry.

Cell Staining and Analysis by Flow Cytometry.

All antibodies and reagents described in this section were purchased from BioLegend. Routinely, cells were stained in the presence of Fc block (clone 93) using allophycocyanin (APC)-conjugated anti-mouse CD90.2 antibody (clone 30-H12), phycoerythrin (PE)-conjugated anti-mouse CD25 antibody (clone PC61), and fluorescein isothiocyanate (FITC)-conjugated anti-

mouse TCR-β antibody (clone H57-597). Where indicated, cells were stained in the presence of Fc block using FITC-conjugated anti-mouse CD90.2 antibody, APC-conjugated anti-mouse CD25 antibody and propidium idodide to assess cell viability. Live cells expressing CD90.2, CD25 and TCR-β were identified as anti-CD3ε- and anti-CD28-stimulated T cells. Size and granularity of live cells were analyzed using forward scatter and side scatter. Data were acquired and analyzed using a FACSCalibur with CellQuest™ Pro software (BD Biosciences). Blastogenesis was calculated by determining the percentage of live cells that, in response to treatment with anti-CD3ε and anti-CD28, had transformed from small lymphocytes into larger, more granular cells resembling blast cells.

IL-2 Enzyme-Linked Immunosorbent Assay.

IL-2 concentrations in collected T cell supernatants were determined using Mouse IL-2 ELISA MAX Deluxe kit (BioLegend).

Statistical Analysis.

Statistical analysis was performed using Prism 5.0b (GraphPad Software). Data were analyzed using a two-tailed, paired Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post-test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

5.4 Results

Exogenous L-asparagine is a resource important for T cell function.

An immediate implication of our results showing that L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells is that L-Asparaginase II

inhibits T cell function by depleting exogenous L-asparagine. To determine the importance of exogenous L-asparagine in T cell function, we cultured T cells in medium lacking L-asparagine. T cells cultured in medium containing L-asparagine were used as a control. After 20 hours of incubation, we found that T cells cultured in medium lacking L-asparagine expressed less surface TCR-β than T cells cultured in medium containing L-asparagine (Figure 5.1A, white bars). The absence of exogenous L-asparagine reduced the ability of T cells to blast and secrete IL-2 (Figures 5.1B and C, white bars), but did not adversely affect T cell viability (Figure 5.1D). Interestingly, T cells cultured in medium lacking L-asparagine expressed levels of surface TCR-β that were comparable to the levels expressed by T cells cultured with *S.* Typhimurium (Figures 5.1A,B, and C; black bars). These results indicate that exogenous L-asparagine is a resource important for T cell function.

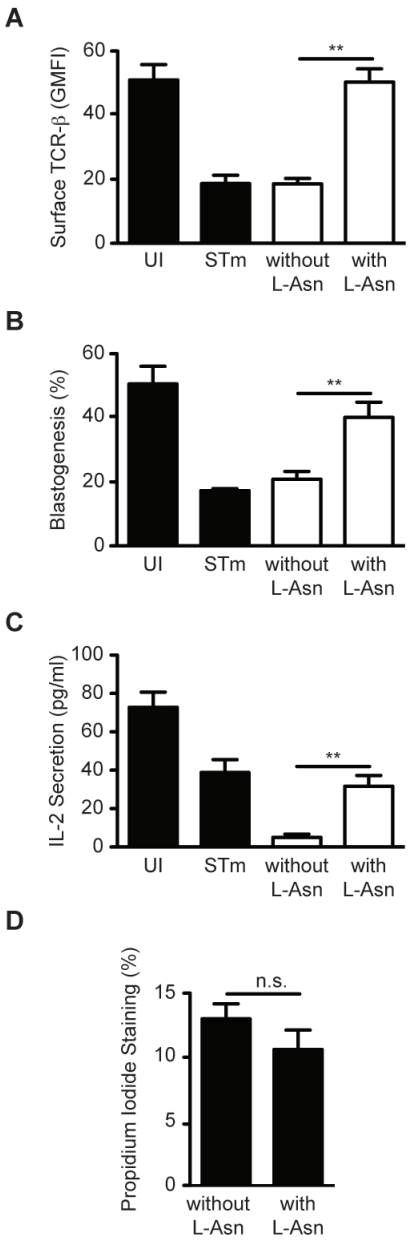
5.5 Discussion

It has been suggested that the amino acid availability, in particular the non-essential amino acid glutamine, is important for the process of T cell activation (143). Other work has shown that deprivation of tryptophan and arginine also has deleterious effects on T cells, such as preventing proliferation (144, 145). However, little work has been done on the effects other amino acids, particularly non-essential amino acids have on T cells. The work done here demonstrates that L-asparagine is an important resource for T cell function. This is important because L-Asparaginase II is currently used to treat ALL by depleting the exogenous L-asparagine that the cancer cells need to survive. However, few studies have looked at effects L-asparagine deprivation may have on T cells. This is because most L-Asparaginase II proteins have glutaminase activity associated with them, though it is usually only between 3-9% of the asparaginase activity (123). The primary source of L-Asparaginase II used clinically is the *E.coli*

L-Asparaginase II, which has significant glutaminase activity, and therefore most of its detrimental side effects have been attributed to this attribute (146). However, little to no work has been done on what affects, if any L-asparagine deprivation has on healthy cells, T cells in particular. Therefore, this work represents one of the first pieces of evidence suggesting that L-asparagine may be "conditionally essential" for T cells, especially during activation.

5.6 Figures

Figure 5.1. Exogenous L-asparagine is a resource important for T cell function. (***A***) Expression of surface TCR-β by anti-CD3ε/CD28-stimulated T cells left uninfected (UI), infected with *S*. Typhimurium (STm) or cultured in medium with (+) or without (-) L-asparagine (L-Asn); $p < 0.0001$. (***B***) Blastogenesis of anti-CD3ε/CD28-stimulated T cells treated as in (A); $p < 0.0001$. (***C***) IL-2 secretion by anti-CD3ε/CD28-stimulated T cells treated as in (A); $p < 0.0001$. (***D***) Propidium Iodide staining of anti-CD3ε/CD28-stimulated T cells treated as in (A); $p = 0.0784$. Data show mean with SEM from three independent experiments. Data were analyzed using a one-way ANOVA with Tukey's multiple comparisons post test; p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (** $p < 0.01$).


A
Surface TCR-β (GMFI)
60
40
20
0
**
UI
STm
without L-Asn
with L-Asn
B
Blastogenesis (%)
60
40
20
0
**
UI
STm
without L-Asn
with L-Asn
C
IL-2 Secretion (pg/ml)
100
80
60
40
20
0
**
UI
STm
without L-Asn
with L-Asn
D
Propidium Iodide Staining (%)
15
10
5
0
n.s.
without L-Asn
with L-Asn

Chapter 6. L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of mTOR signaling, autophagy and c-Myc expression

6.1 Chapter Summary

Upon recognition of a foreign peptide in the context of an MHC molecule, T cells undergo a process called priming, which refers to the activation, differentiation and proliferation of naïve T cells into effector T cells. T cell priming is a metabolically demanding process characterized by enormous changes in gene and protein expression and cell metabolism. Here, I investigated the effect of L-Asparaginase II-mediated L-asparagine deprivation on mTOR signaling, autophagy and c-Myc expression. I found that all three processes were inhibited in T cells treated with L-Asparaginase II or cultured in medium lacking L-asparagine.

6.2 Introduction

In order for T cells to become fully activated and differentiate into effector cells they must undergo metabolic reprogramming. Little work has been done to determine the effect of amino acid deprivation on T cell function and none has focused on L-asparagine. Following stimulation of T cells there are dramatic changes in cell metabolism such as increased use of glycolysis, glutaminolysis and the pentose phosphate pathway (142, 147, 148). T cell metabolic reprogramming has been suggested to include up regulation of the serine/threonine kinase mTOR as well as up regulation of autophagy (147, 149).

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that is evolutionarily conserved from yeast to man (147). mTOR integrates many different signals that control important cellular processes including cellular growth, proliferation, protein synthesis, cellular survival and metabolism (142, 147). Although multiple factors control mTOR activity; from amino acid availability, oxygen levels, energy status and growth factors, the mechanism(s) of mTOR activation are not completely understood (147, 150). Most of the work done on understanding mTOR activation has been in the context of cancer, since disruption in mTOR activity has been found in multiple cancers. However, recent evidence indicates that mTOR plays a critical role in T cell function (147, 151, 152). In addition to integrating environmental signals, mTOR activity may play a key role in integrating activating signals from dendritic cells and cytokines during T cell activation (152). Upon TCR engagement, mTOR signaling is quickly activated, which may be important for the switching of naïve T cells from a quiescent state to a fully activated state (153). Amino acid availability regulates mTOR signaling, and also has significant effects on autophagy and c-myc, a positive regulator of T cell metabolism (142, 147, 151, 153-156).

Autophagy is a cellular process used to degrade proteins, sugars, nucleotides, lipids, whole organelles and even pathogens (157, 158). Autophagy is implicated in multiple cellular processes, including organ development, aging, cell death, immune defense, and cellular differentiation (157). Dysregulation of autophagy is linked to a number of pathological conditions, including cancer as well as inflammatory and neurodegenerative disorders (157). There are three major classes of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy. Each type of autophagy leads to lysosomal degradation of cellular components, but each differs in the process by which the cellular components are brought to the

lysosomes. Chaperone-mediated autophagy leads to the degradation of cellular components that contain a pentapeptide motif recognized by the chaperone heat shock cognate 70 (Hsc70) (157, 159). Microautophagy is a process whereby cytoplasmic components are directly engulfed and degraded by lysosomes (157). Macroauotphagy involves the sequestration of cytosolic constituents into double membrane structures known as autophagosomes, which fuse with lysosomes to promote the degradation of cargo (157, 159, 160). Although microautophagy and macroautophagy are generally considered to be nonspecific with respect to the type of cellular components that they target for degradation, there is mounting evidence to suggest that cellular components are specifically targeted for degradation by these two processes (158).

Macroautophagy (referred to as just autophagy) is active at a basal level in almost all cell types and functions in the turnover of cellular proteins. However, autophagy is upregulated in response to different stressors or stimuli, such as nutrient deprivation. Autophagy is particularly important in times of metabolic stress because it serves as an alternative energy source for the cell (158). The autophagic process is regulated by a set of proteins known as Autophagy-related (Atg) proteins. These proteins are required for the formation and maturation of the autophagosome, and the fusion of the autophagosome with the lysosome (161, 162). Atg8, also known as LC3, is the only protein known to specifically associate with the autophagosome. During autophagy, the cytoplasmic form of microtubule-associated protein 1 light chain 3 (LC3-I) is processed and modified into a lipidated form (LC3-II) that is recruited to autophagosomal membranes (160). LC3-II is considered a reliable marker for monitoring autophagy (159, 161, 163).

Basal levels of autophagy are found in T cells of all developmental stages, ranging from thymocytes to peripheral naïve T cells to activated T cells. However, the role of autophagy in T

cell function is not well understood (157). T cells deficient for autophagy display increased apoptosis and impaired ability to proliferate following TCR stimulation, suggesting autophagy is essential for T cell survival and proliferation (164, 165). Furthermore, autophagy may play an important role in regulating organelle homeostasis and turn over of mitochondria in T cells (166). Mitochondrial turnover in T cells appears to be developmentally regulated; thymocytes have high mitochondrial content (proteins) whereas activated T cells have significantly lower mitochondrial content. T cells deficient for autophagy have increased levels of mitochondrial content, reactive oxygen species and pro-apoptotic protein expression, indicating that autophagy serves an important regulatory role in T cells (167, 168).

T cell activation is a metabolically demanding process that involves naïve T cells switching from oxidative phosphorylation to glycolysis to meet the demands of rapid growth, proliferation and protein synthesis (157, 169). Interestingly, naïve T cells express a basal level of autophagy that, following TCR engagement, is upregulated substantially (149, 157). This upregulation of autophagy during T cell activation seems to be required for proliferation and cytokine production. T cells unable to upregulate autophagy in response to TCR stimulation have reduced ATP levels, which, in turn, have been linked to impaired proliferation and transcription and production of IFN-γ and IL-2 (149, 170, 171). Thus, autophagy may be a process important for T cells to gain access to energy needed for activation before glycolysis has begun.

Mature, naïve T cells in the periphery are considered quiescent cells because they are small and have a low level of metabolic activity (153). These T cells utilize catabolic forms of metabolism, primarily the tricarboxylic acid cycle (TCA) cycle and oxidative phosphorylation (172). In contrast, activated T cells use anabolic forms of metabolism such as aerobic glycolysis,

glutaminolysis and glucose metabolism (151). This switch in metabolism likely occurs because upon stimulation T cells need to rapidly grow and proliferate in order to differentiate into functional effector cells, such that the number of cells can increase exponentially every 4-6 hours (147). Although glycolysis is not the most efficient way to produce ATP, it does allow for the production and therefore use of important building blocks for lipid, protein and DNA synthesis needed during activation (147, 172). mTOR and the transcription factor c-Myc have both been implicated as important regulators of T cell metabolism. Blockade of mTOR signaling has been shown to prevent T cell proliferation, while increased mTOR signaling also results in increased amino acid uptake (172, 173). Interestingly, expression of the transcription factor c-Myc is up regulated significantly in T cells that have been stimulated, which has been shown to be critical for the metabolic reprogramming of these cells (156). Although what regulates these distinct metabolic profiles as well as what regulates the switch from oxidatative phosphorylation to glycolsyis is not completely understood, it is clear that metabolic reprogramming is important for T cell activation and thus development of a T cell response.

As described in Chapters 4 and 5, I have shown that L-asparagine hydrolase activity is required for L-Asparaginase II of *S.* Typhimurium to inhibit T cells and that exogenous L-asparagine is a resource important for T cell function. Here, I examined the effects L-Asparaginase II and L-asparagine deprivation on mTOR signaling, autophagy and c-Myc expression in T cells.

6.3 Experimental Methods

T Cell Enrichment and T Cell Assays.

Splenocytes harvested from naïve C57BL/6J mice (females, 6 to 8 weeks of age) were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer (0.15

M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell separation technology (Miltenyi Biotec) was used to enrich for $CD90.2^+$ T cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enrichment resulted in 75-85% enrichment of $CD90.2^+$ T cells. Unless indicated otherwise, enriched populations of T cells were suspended in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) and used in T cell assays.

In T cell assays aimed at measuring the effect of L-Asparaginase II on the levels of S6, phosphorylated S6, LC3 and c-Myc, enriched populations of T cells suspended in medium supplemented with 1 μg/ml of anti-CD28 antibody were seeded at 1.0×10^6 cells per well into 24-well tissue culture plates coated with 5 μg/ml of anti-CD3ε antibody. The T cells were then left untreated or treated with 50 ng/ml of purified wild-type L-Asparaginase II or mutant L-Asparaginase II. Where indicated, leupeptin (Fisher), a protease inhibitor, and ammonium chloride (Acros Organics), an inhibitor of lysosomal proteolysis, were added to the cultures three hours prior to harvest at final concentrations of 100 μM and 20 mM, respectively, to prevent intralysosomal degradation of LC3-II. Pilot experiments were done to determine the optimal time to analyze samples and it was found that 12 hours post stimulation was ideal because it is during the crucial time during activation where changes in gene expression and metabolic reprogramming have been documented (174). After 12 hours of incubation at 37°C in 5% CO_2, the T cells were harvested and lysed using RIPA buffer (Sigma) supplemented with 1x cocktails of protease and phosphatase inhibitors (Roche Applied Science). The protein concentrations of the resulting whole cell lysates were determined using Bio-Rad Protein Assay (Bio-Rad), after 5 to 9 μg of each sample was subjected to polyacrylamide gel electrophoresis and Western blot

analysis. Parallel T cell assays aimed at measuring the effect of L-Asparaginase II produced by *S*. Typhimurium or L-asparagine deprivation on the levels of S6, phosphorylated S6, LC3 and c-Myc were performed, essentially as described above.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis.

Aliquots of whole cell lysates were mixed with SDS-PAGE sample buffer (1x final), boiled for 15 minutes, centrifuged briefly to pellet debris and loaded onto a 12% Tris-Glycine polyacrylamide gel (Bio-Rad). Following gel electrophoresis, proteins were transferred onto PVDF membranes (Bio-Rad) using Trans-blot SD Semi-Dry Transfer Cell system (Bio-Rad). Expression of LC3 was detected using polyclonal antiserum (diluted 1:3,000) from rabbits immunized with recombinant human LC3 (Medical & Biological Laboratories). Expression of S6 ribosomal protein was detected using a rabbit IgG mAb (diluted 1:1,000) specific for a synthetic peptide corresponding to residues of human S6 (Cell Signaling). Expression of phophorylated S6 was detected using polyclonal antiserum (diluted 1:1,000) from rabbits immunized with a synthetic phosphopeptide corresponding to residues surrounding Ser240 and Ser244 of human S6 (Cell Signaling). Expression of c-Myc was detected using polyclonal antiserum (diluted 1:3,000) from rabbits immunized with a synthetic peptide corresponding to amino-terminal residues of c-Myc (Cell Signaling). Expression of β-Actin was detected using polyclonal antiserum (diluted 1:5,000) from rabbits immunized with a synthetic peptide corresponding to amino-terminal residues of human β-Actin (Cell Signaling). For detection, we used horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Cell Signaling) and ECL Western Blotting Detection Reagent (GE Healthcare).

6.4 Results

L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of mTOR signaling

Active mTOR signaling can be measured by monitoring the phosphorylation status of ribosomal protein S6 (S6), a known mTOR substrate (153, 175). To define the effect of L-asparagine deprivation on mTOR signaling, we monitored the phosphorylation status of S6 in T cells cultured in medium lacking L-asparagine. Naïve T cells and T cells cultured in medium containing L-asparagine were used as controls. Since mTOR activity is upregulated quickly following stimulation we analyzed expression of mTOR activity 12 hours following stimulation. We found that naïve, unstimulated T cells expressed low levels of phosphorylated S6 over total S6, but that these levels increased considerably following TCR engagement (Figure 6.1A). Furthermore, we found that T cells cultured in medium lacking L-asparagine expressed substantially reduced levels of phosphorylated S6 over total S6 when compared to T cells cultured in medium containing L-asparagine (Figure 6.1A). Thus, L-asparagine deprivation causes T cell inhibition through suppression of mTOR signaling.

L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of autophagy

A functional consequence of reduced mTOR signaling may be induction of autophagy (176). During autophagy, the cytoplasmic form of microtubule-associated protein 1 light chain 3 (LC3-I) is processed and modified into a lipidated form (LC3-II) that is recruited to autophagosomal membranes (159, 166, 171). LC3-II is a reliable marker for monitoring autophagy (161, 163). To define the effect of L-Asparaginase II-mediated L-asparagine deprivation on autophagy, we measured LC3-II levels in T cells treated with wild-type L-

Asparaginase II or enzymatically inactive L-Asparaginase II. Naïve T cells or mock-treated T cells were used as controls. Where indicated, leupeptin, a protease inhibitor, and ammonium chloride, an inhibitor of phagosome-lysosome fusion, were added to the cultures to prevent intralysosomal degradation of LC3-II. Consistent with published evidence, naïve T cells exhibited a basal level of LC3-II expression that increased considerably in response to TCR engagement (mock-treated T cells) (Figure 6.1B)(149). T cells treated with wild-type L-Asparaginase II expressed levels of LC3-II that were substantially lower than the levels expressed by mock-treated T cells, but similar to the levels expressed by naïve T cells (Figure 6.1B). Furthermore, T cells treated with enzymatically inactive L-Asparaginase II expressed levels of LC3-II that were comparable to the levels of LC3-II expressed by mock-treated T cells (Figure 6.1B). Similar results were obtained when we cultured T cells with live *S*. Typhimurium (Figure 6.1C). T cells left uninfected displayed an upregulation of autophagy that was suppressed when we infected cells with wild-type or *S.* Typhimurium carrying plasmid pBAD-*STM3106*. Additionally, this suppression was not observed when T cells were infected with Δ*STM3106* or *S.* Typhimurium carrying plasmid pBAD-STM3106:T89A. Furthermore, we obtained similar results when we suspended the T cells in medium lacking L-asparagine, demonstrating that naïve, unstimulated T cells display a low level of autophagy that is dramatically upregulated following stimulation, but this upregulation is not longer observed when T cells are cultured in the absence of L-asparagine (Figure 6.1D). Thus, L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of autophagy.

L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of c-Myc expression

Another functional consequence of reduced mTOR signaling may be suppression of metabolic reprogramming. Naïve T cells utilize catabolic metabolism to meet metabolic needs (172). Following TCR engagement, T cells up regulate glycolysis, glutaminolysis and glucose metabolism (142, 172, 177, 178). This metabolic switch is important for T cells to activate, proliferate and differentiate (142). These and other cellular processes have been linked to c-Myc, a transcription factor that positively controls activation-induced metabolic reprogramming (156). To define the effect of L-Asparaginase II-mediated L-asparagine deprivation on metabolic reprogramming, we measured expression of c-Myc in T cells treated with wild-type L-Asparaginase II or enzymatically inactive L-Asparaginase II. Naïve T cells or mock-treated T cells were used as controls. We found that naïve T cells expressed low levels of c-Myc, but that these levels increased considerably following TCR engagement (Figure 6.1E). T cells treated with wild-type L-Asparaginase II expressed levels of c-Myc that were substantially lower than the levels expressed by mock-treated T cells, but similar to the levels expressed by naïve T cells (Figure 6.1E). Furthermore, T cells treated with enzymatically inactive L-Asparaginase II expressed levels of c-Myc that were comparable to the levels of c-Myc expressed by mock-treated T cells (Figure 6.1E). Similar results were obtained when we suspended the T cells in medium lacking L-asparagine (Figure 6.1F). Thus, L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of metabolic reprogramming.

6.5 Discussion

The results described in this Chapter are evidence that L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of mTOR signaling, autophagy and c-Myc expression. Collectively, these results suggest that L-Asparaginase II-

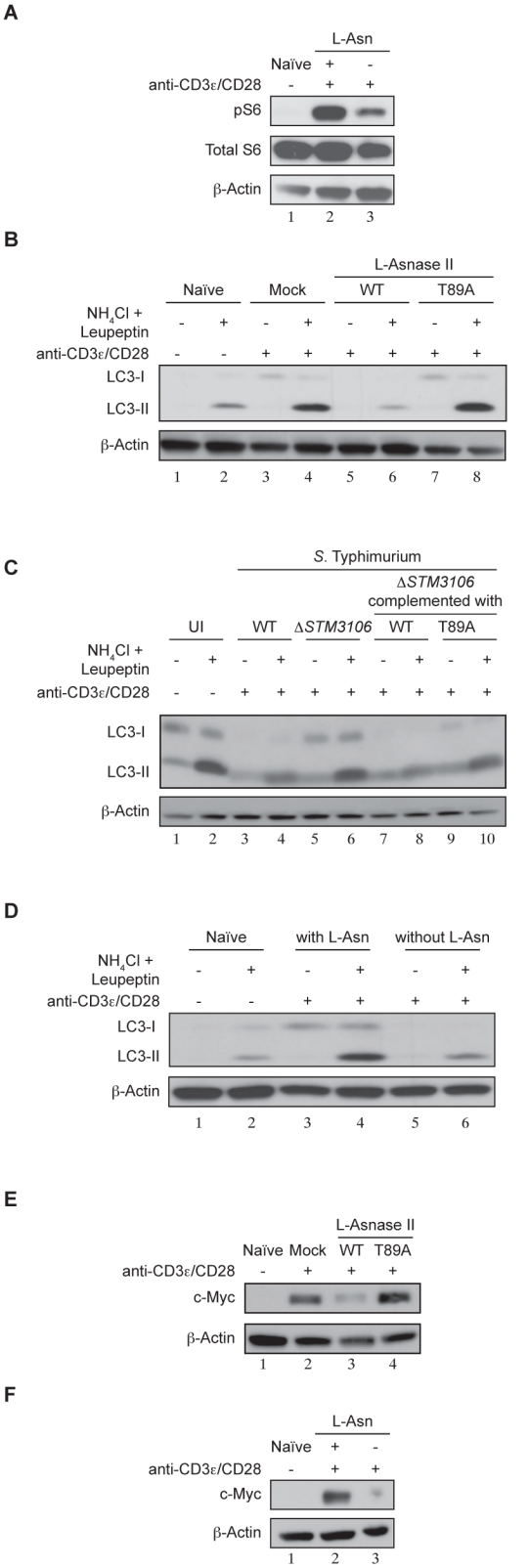
mediated L-asparagine deprivation causes inhibition of T cell function through suppression of metabolic reprogramming.

Activation induced metabolic reprogramming is characterized by increased nutrient uptake and a switch to aerobic glycolysis, glutamine oxidation and glucose metabolism from oxidative phosphorylation (148, 169). It has been noted that mTOR, autophagy and c-Myc are important players in this process of metabolic reprogramming during activation. mTOR has been shown to play a key role in T cell activation, and the work shown here suggests an important role for mTOR in regulating T cell activation through amino acid availability (153, 172). Autophagy also has been suggested to play an important role in T cell activation, because autophagy deficient T cells are defective in proliferation, IL-2 and IFN-γ secretion (149, 171). My work further suggests an important role for autophagy, particularly during the process of activation. It is clear that upon activation there is an increase in autophagy, but this increase is inhibited in T cells treated with L-Asparaginase II or cultured in medium lacking L-asparagine. This suggests that activation-induced autophagy may be an important part of the priming process. Although the role of autophagy in T cell activation is not well understood, my results are consistent with the notion that autophagy may provide T cells with access to nutrients that are needed for this metabolically demanding process by an mTOR independent mechanism (149). Additionally, the inability of T cells to up regulate mTOR signaling following L-Asparaginase II treatment, or in medium lacking L-asparagine, may prevent up regulation of c-Myc expression. It has been suggested that mTOR activity may have effects on c-Myc expression, but little is known about this potential connection (156). However, it is clear that c-Myc up regulation is a key aspect in the metabolic switch during T cell activation (156).

Therefore, since the T cells cannot switch their metabolic profile, they are unable to complete activation and become effector cells, rendering them useless in the fight against the *S.* Typhimurium infection.

6.6 Figures

Figure 6.1. L-Asparaginase II-mediated L-asparagine deprivation causes T cell inhibition through suppression of mTOR signaling, autophagy and c-Myc expression. (***A***) Western blot analysis of whole cell lysates of naïve T cells (cultured for 3 hours without stimulation) or anti-CD3ε/CD28-stimulated T cells cultured for 12 hours in medium with (+) or without (-) L-asparagine (L-Asn) using anti-S6 or anti-phospho-S6 (pS6) antibody. (***B***) Western blot analysis of whole cell lysates of naïve T cells (cultured 3 hours without stimulation), anti-CD3ε/CD28-stimulated T cells (Mock) or anti-CD3ε/CD28-stimulated T cells treated with wild-type (WT) or enzymatically inactive (T89A) L-Asparaginase II (L-Asnase II) (all cultured for 12 hours) using anti-LC3 antibody. Where indicated, ammonium chloride and leupeptin were added to the cultures. (***C***) Western blot analysis of whole cell lysates of anti-CD3ε/CD28-stimulated T cells left uninfected (UI) or cultured with wild-type *S.* Typhimurium (WT), *S.* Typhimurium lacking the L-Asparaginase II gene *STM3106* (*ΔSTM3106 S.* Typhimurium) or *ΔSTM3106 S.* Typhimurium carrying plasmid pBAD-*STM3106* encoding wild-type L-Asparaginase II (*ΔSTM3106* complemented with WT) or pBAD-*STM3106*T89A encoding enzymatically inactive L-Asparaginase II (*ΔSTM3106* complemented with T89A) cultured for 12 hours, using anti-LC3 antibody. Where indicated, ammonium chloride and leupeptin were added to the cultures. (***D***) Western blot analysis of whole cell lysates of naïve T cells (cultured 3 hours without stimulation) or anti-CD3ε/CD28-stimulated T cells cultured in medium with or without L-asparagine (L-Asn) for 12 hours, using anti-LC3 antibody. Where indicated, ammonium chloride and leupeptin were added to the cultures. (***E***) Western blot analysis of whole cell lysates of naïve T cells (cultured 3 hours without stimulation), anti-CD3ε/CD28-stimulated T cells left untreated (Mock) or anti-CD3ε/CD28-stimulated T cells treated with wild-type (WT) or enzymatically inactive (T89A) L-Asparaginase II (L-Asnase II) cultured for 12 hours, using anti-c-Myc antibody. (***F***) Western blot analysis of whole cell lysates of naïve T cells (cultured 3 hours without stimulation) or anti-CD3ε/CD28-stimulated T cells cultured in medium with (+) or without (-) L-asparagine (L-Asn) for 12 hours, using anti-c-Myc antibody. β–Actin was used a loading control. Data are representative of at least three independent experiments.


A
L-Asn
Naïve + -
anti-CD3ε/CD28 - + +
pS6
Total S6
β-Actin
1 2 3
B
L-Asnase II
Naïve Mock WT T89A
NH4Cl + Leupeptin - + - + - + - +
anti-CD3ε/CD28 - - + + + + + +
LC3-I
LC3-II
β-Actin
1 2 3 4 5 6 7 8
C
S. Typhimurium
ΔSTM3106 complemented with
UI WT ΔSTM3106 WT T89A
NH4Cl + Leupeptin - + - + - + - + - +
anti-CD3ε/CD28 - - + + + + + + + +
LC3-I
LC3-II
β-Actin
1 2 3 4 5 6 7 8 9 10
D
Naïve with L-Asn without L-Asn
NH4Cl + Leupeptin - + - + - +
anti-CD3ε/CD28 - - + + + +
LC3-I
LC3-II
β-Actin
1 2 3 4 5 6
E
L-Asnase II
Naïve Mock WT T89A
anti-CD3ε/CD28 - + + +
c-Myc
β-Actin
1 2 3 4
F
L-Asn
Naïve + -
anti-CD3ε/CD28 - + +
c-Myc
β-Actin
1 2 3

Chapter 7. L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses by depleting exogenous L-asparagine T cells need for protein synthesis

7.1 Chapter Summary

Our work demonstrates that exogenous L-asparagine is a resource important for T cell function. However, it has not been shown whether T cells actually take up and utilize this amino acid. Therefore, the goal of the work presented in this chapter was to determine whether T cells take up and utilize L-asparagine and what the potential effects L-Asparaginase II induced L-asparagine deprivation had on L-asparagine utilization by T cells. Here, we used a proteomics approach and found that T cells can incorporate exogenous L-asparagine into newly synthesized proteins, but T cells treated with L-Asparaginase II had significantly less incorporation of exogenous L-asparagine.

7.2 Introduction

Within the cell, mTOR signaling plays an important role in transmitting environmental signals, such as amino acid availability that can lead to affects on protein synthesis (179). We have shown that depleting exogenous L-asparagine reduces mTOR signaling in T cell and we speculated that exogenous L-asparagine is also needed for protein synthesis to support T cell proliferation. Although L-asparagine can be made by T cells and is not considered an essential amino acid, we hypothesized that during the priming of T cells, exogenous L-asparagine is required in order to meet the metabolic demands of rapid growth and proliferation. Furthermore, we reasoned that L-Asparaginase II might interfere with the T cells ability to utilize this resource. To test this hypothesis, I examined the incorporation of exogenous L-asparagine into

newly synthesized proteins. To do so, I used a proteomic approach that relies on the metabolic labeling of proteins using stable, non-radioactive isotope labeled amino acids. Using medium containing isotope labeled L-asparagine, I was able to analyze the effects of L-Asparaginase II treatment on L-asparagine uptake and utilization.

7.3 Experimental Methods

T Cell Enrichment and T Cell Assays.

Splenocytes harvested from naïve C57BL/6J mice (females, 6 to 8 weeks of age) were used as a source of T cells. Following treatment of the splenocytes with ACK lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA) to lyse red blood cells, magnetic cell separation technology (Miltenyi Biotec) was used to enrich for $CD90.2^+$ T cells. CD90.2 is a pan-T cell marker expressed by both $CD4^+$ and $CD8^+$ T cells. Enrichment resulted in 75-85% enrichment of $CD90.2^+$ T cells. Unless indicated otherwise, enriched populations of T cells were suspended in RP-10 medium (RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and streptomycin) and used in T cell assays.

In T cell assays aimed at measuring incorporation of exogenous L-asparagine, enriched populations of T cells were suspended in medium containing heavy, $^{13}C_6$-labeled L-asparagine and$^{13}C_6{}^{15}N_4$-labeled L-arginine (RPMI1640 medium lacking L-asparagine, L-lysine and L-arginine (Caisson Laboratories) supplemented with 0.378472 mM (50.0037 mg/L) of $^{13}C_6$-L-asparagine (Cambridge Isotope Laboratories), 0.218998 mM (47.98467 mg/L) of L-lysine (Cambridge Isotope Laboratories), 1.150242 mM (242.31 mg/L) of and$^{13}C_6{}^{15}N_4$-labeled L-arginine (Cambridge Isotope Laboratories), 10% dialyzed fetal bovine serum (Caisson Laboratories), 0.2 M L-glutamine, 0.1 M HEPES, 50 μM 2-ME, and 1% penicillin and

streptomycin) supplemented with 1 μg/ml of anti-CD28 antibody and seeded at 1.5 x 10^6 cells per well into 24-well tissue culture plates coated with 5 μg/ml of anti-CD3ε antibody. The T cells were then left untreated or treated with 50 ng/ml of purified wild-type L-Asparaginase II or mutant L-Asparaginase II. T cells were analyzed after 24 hours of incubation in order to allow for enough time for detection of new protein synthesis following stimulation. After 24 hours of incubation at 37°C in 5% CO_2, the T cells were harvested and lysed using a mass spectrometry-compatible lysis buffer (50 mM $(NH_4)HCO_3$, 4 M urea, 1x Invitrosol (Invitrogen) and 1x cocktails of protease and phosphatase inhibitors). The protein concentrations of the resulting lysates were determined using EZQ protein quantitation kit (Invitrogen), after which 30 μg of each sample was diluted in 50 mM $(NH_4)HCO_3$ for trypsin digestion. Sequencing Grade Modified Trypsin (Promega) was added to each of the samples at a ratio of 1:30 (trypsin to protein), as was $CaCl_2$ at a final concentration of 2 mM. After 16 hours of incubation at 37°C, the samples were acidified with 90% formic acid (2% final) to stop proteolysis and centrifuged for 30 minutes at 14,000 rpm to remove insoluble material. Supernatants containing mixtures of soluble peptides were collected and subjected to multidimensional chromatography and tandem mass spectrometry.

Multidimensional Chromatography and Tandem Mass Spectrometry.

Peptide mixtures were pressure-loaded onto a 250 μm inner diameter fused-silica capillary packed first with 3 cm of 5 μm strong cation exchange material (Partisphere SCX, Whatman), followed by 3 cm of 10 μm C18 reverse phase particles (Aqua, Phenomenex). Loaded and washed microcapillaries were connected *via* a 2 μm filtered union (UpChurch Scientific) to a 100 μm inner diameter column, which had been pulled to a 5 μm inner diameter tip using a P-2000 CO_2 laser puller (Sutter Instruments), then packed with 13 cm of 3 μm C18

reverse phase particles and equilibrated in 5% acetonitrile, 0.1% formic acid (Buffer A). This split-column was then installed in-line with a NanoLC high-performance liquid chromatography (HPLC) pump (Eskigent). The flow rate of channel 2 was set at 300 nl/min for the organic gradient. The flow rate of channel 1 was set to 0.5 μl/min for the salt pulse. Fully automated 11-step chromatography runs were carried out. Three different elution buffers were used: 5% acetonitrile, 0.1% formic acid (Buffer A); 98% acetonitrile, 0.1% formic acid (Buffer B); and 0.5 M ammonium acetate, 5% acetonitrile, 0.1% formic acid (Buffer C). In such sequences of chromatographic events, peptides are sequentially eluted from the SCX resin to the reverse phase resin by increasing salt steps (increase in Buffer C concentration), followed by organic gradients (increase in Buffer B concentration). The last chromatography step involved a high salt wash with 100% Buffer C followed by acetonitrile gradient. The application of a 2.5 kV distal voltage electrosprayed the eluting peptides directly into an LTQ-Orbitrap XL mass spectrometer equipped with a nano-LC electrospray ionization source (ThermoFinnigan). Full mass spectrometry (MS) spectra were recorded on the peptides over a 400 to 2000 m/z range by the Orbitrap, followed by five tandem mass spectrometry (MS/MS) events sequentially generated by LTQ in a data-dependent manner on the first, second, third, and fourth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFinnigan).

Database Search and Interpretation of Tandem Mass Spectrometry Datasets.

Tandem mass spectra were searched against a concatenated target-decoy database containing the forward and reverse sequences of the target database using the Andromeda search engine (180, 181). The target sequence was downloaded from a mouse uniprot database (database released on March, 06, 2013)(182), and 124 common contaminant proteins. In

Andromeda, we specified $^{13}C_6$-L-asparagine as the SILAC label for the database search. The quantitative proteomics software package MaxQuant was used to report a list of identified proteins and ratios of heavy and light L-asparagine (183). The peptide mass search tolerance was set to 10 ppm. No differential modifications were considered. Tryptic status was imposed on the database search. The discriminant score was set such that a false positive rate of 1% was determined based on the number of accepted decoy database peptides.

7.4 Results

L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses by depleting the exogenous L-asparagine T cells need for protein synthesis.

T cells were cultured in medium containing ^{13}C-labeled L-asparagine and $^{3}C_6{}^{15}N_4$-labeled L-arginine, then harvested for proteomic analysis after 24 hours. Using the stable isotope labeled L-asparagine and L-arginine together allows for better resolution of proteins affected by L-Asparaginase II treatment, since incorporation of L-asparagine into newly synthesized proteins may be low and difficult to detect. I observed proteome-wide incorporation of labeled asparagine and arginine and identified these proteins using a high-resolution mass spectrometer, LTQ-Orbitrap. This is an excellent strategy to perform global surveillance of exogenous asparagine usage in cells without radioactive labeling. We were able to observe the global trend of asparagine incorporation in T cells and identify these newly synthesized proteins incorporated with exogenous asparagine (Figure 7.1).

Next, I examined the effect of L-Asparaginase II on the utilization of exogenous L-asparagine in T cells. I cultured T cells in the presence of either wild type L-Asparaginase II or mutant L-Asparaginase II in media containing ^{13}C-labeled L-asparagine. Mock-treated T cells

cultured in the same media were used as controls. After 24 hours, I found that T cells cultured in the presence of the wild-type L-Asparaginase II had significantly less incorporation of exogenous ^{13}C-labeled L-asparagine than T cells cultured without L-Asparaginase II (Figure 7.1). Furthermore, T cells cultured in the presence of enzymatically inactive L-Asparaginase II allowed a comparable level of exogenous L-asparagine incorporation as T cells cultured without L-Asparaginase II (Figure 7.1). Thus, L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses by depleting the exogenous L-asparagine required for protein synthesis in T cell proliferation.

When we studied proteins affected in T cells cultured in the presence of the wild-type or mutant L-Asparaginase II, we found these proteins are enriched in cell metabolism, protein translation, and immune response (data not shown). Functional enrichment of these proteins supports the effect of L-Asparaginase II we have observed on T cells, and future studies examining the role of these proteins in T cells will provide mechanistic insights on how to reverse the effect of S. Typhimurium on T cell inhibition.

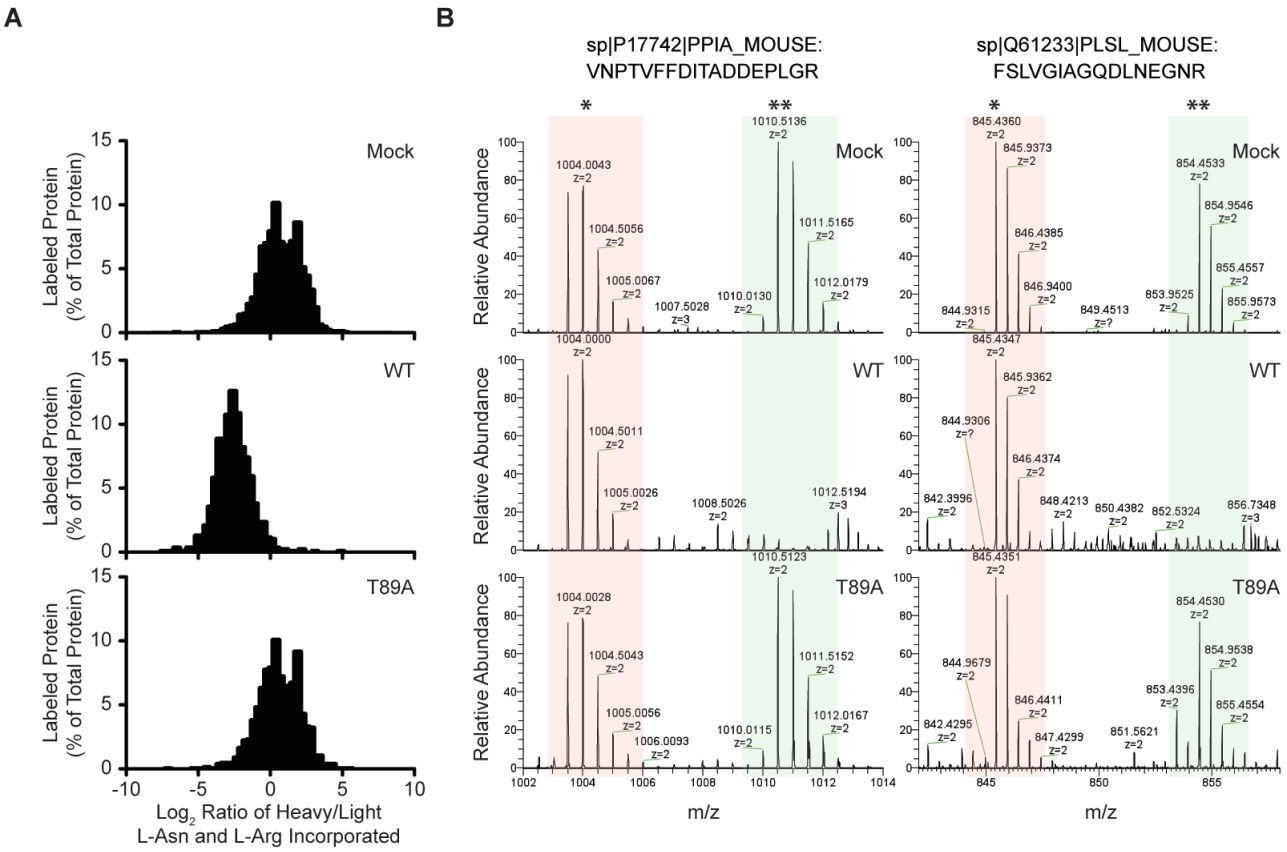
7.5 Discussion

The results described in this Chapter are evidence that T cells take up exogenous L-asparagine and incorporate it into newly synthesized proteins. Additionally, this work demonstrated that wild-type but not enzymatically inactive L-Asparaginase II can interfere with the utilization of exogenous L-asparagine by T cells. We are currently pursuing pathway analysis and our preliminary results suggest that the pathways most affected by L-Asparaginase II treatment are those involved in metabolism. Collectively, these results suggest that interference with the T cell's ability to utilize L-asparagine has global effects on protein synthesis. Following stimulation naïve T cells must up regulate a large number of genes in order

to quickly grow, proliferate and reprogram their metabolism, therefore we hypothesize that in the absence of L-asparagine, naïve T cells are unable to start up this process.

7.6 Figures

Figure 7.1. L-Asparaginase II of *S.* Typhimurium inhibits T cell responses by depleting the exogenous L-asparagine T cells need for protein synthesis. (*A*) Multidimensional chromatography and tandem mass spectrometry analysis of mixtures of soluble peptides prepared from whole cell lysates of anti-CD3ε/CD28-stimulated T cells left untreated (Mock) or treated with wild-type (WT) or enzymatically inactive (T89A) L-Asparaginase II. The T cells were enriched from spleens of naïve C57BL/6J mice and cultured for 24 hours in medium containing heavy, $^{13}C_6$-labeled L-asparagine and$^{13}C_6{}^{15}N_4$-labeled L-arginine. Heavy L-arginine was used to increase the number of different peptides that could be analyzed. For metabolically labeled proteins, the ratio of heavy to light (natural) L-asparagine and L-arginine incorporated was calculated and reported. (*B*) Mass spectrometry spectra for two representative peptides from samples prepared and analyzed as in (A); one asterisk indicates unlabeled peptide and two asterisks indicate corresponding isotope-labeled peptide. Data are representative of two independent experiments.


A
B
Mock
WT
T89A
Labeled Protein (% of Total Protein)
Log2 Ratio of Heavy/Light L-Asn and L-Arg Incorporated
sp|P17742|PPIA_MOUSE: VNPTVFFDITADDEPLGR
sp|Q61233|PLSL_MOUSE: FSLVGIAGQDLNEGNR
Relative Abundance
m/z

Chapter 8. Proposed Mechanism for L-Asparaginase II induced inhibition of T cells

The results of this Dissertation demonstrate that *S.* Typhimurium-induced L-asparagine deprivation limits T cell responses, providing an understanding of a mechanism used by *S.* Typhimurium to establish infection in the mammalian host and avoid clearance by the immune system. Thus, we propose the following model (Figure 8.1) for the mechanism of *S.* Typhimurium-induced L-asparagine deprivation of T cells:

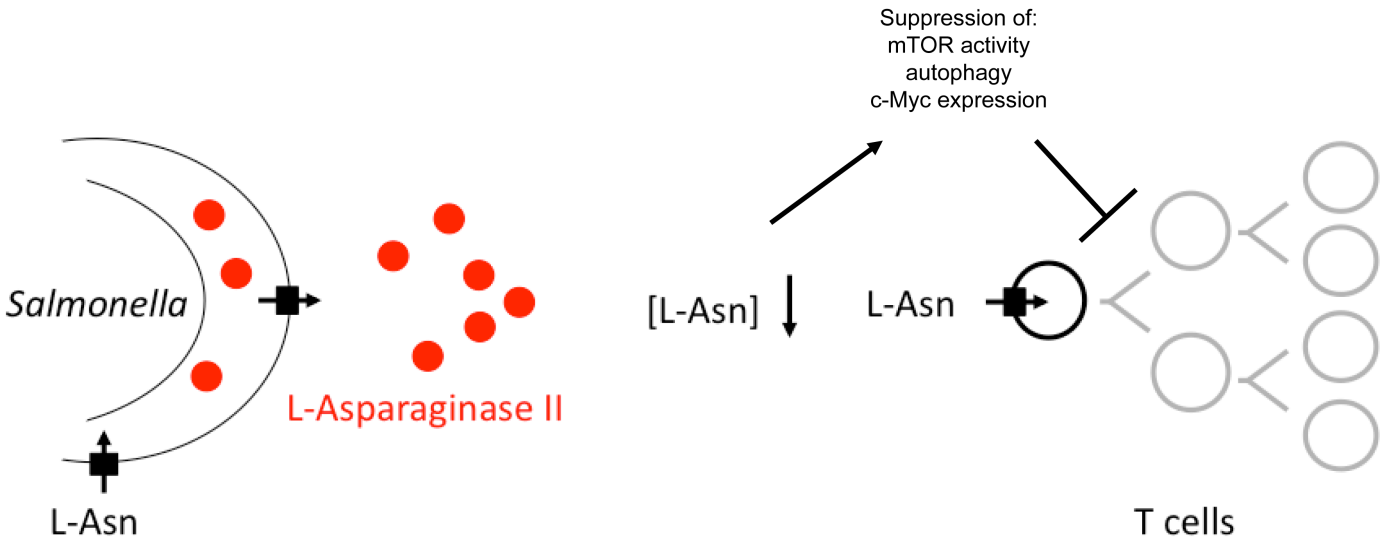
1. L-Asparaginase II is produced by *S.* Typhimurium. L-Asparaginase II may be secreted into the environment or the bacteria may import L-asparagine for its own metabolic needs. These two possibilities are not necessarily mutually exclusive.

2. L-Asparaginase II hydrolyzes L-asparagine into aspartic acid and ammonia, limiting the amount available in the environment.

3. The limiting amount of L-asparagine creates an unsuitable environment for T cell activation, proliferation and differentiation. T cells sense the nutrient limiting environment, which leads to defects in mTOR signaling. Decreased mTOR signaling leads to suppression of protein synthesis needed for autophagy and metabolic reprogramming, processes required for T cells to proliferate and differentiate into effector cells. Thus, T cells, which are required to clear a *S.* Typhimurium infection, are unable to respond and clear the infection.

8.1 Figure

Figure 8.1. Model of T cell inhibition induced by *S.* Typhimurium L-Asparaginase II. We propose that L-Asparaginase II produced by *S.* Typhimurium inhibits T cell responses and mediates virulence through depletion of L-asparagine, preventing the T cells from fully activating and reprogramming their metabolism.


Salmonella
L-Asparaginase II
L-Asn
Suppression of:
mTOR activity
autophagy
c-Myc expression
[L-Asn]
L-Asn
T cells

Chapter 9. Dissertation Discussion and Future Directions

Discussion

Here, we have shown that L-Asparaginase II produced by *S.* Typhimurium is an L-asparagine hydrolase (Figure 3.1C). We report that L-asparagine hydrolase activity is required for L-Asparaginase II-mediated *S.* Typhimurium-induced direct inhibition of T cells (Figure 4.3). We have also shown that L-asparagine is a resource important for T cell function, which is characterized by suppression in mTOR activity, autophagy, c-Myc expression and protein synthesis (Figures 5.1, 6.1 and 7.1). An immediate implication of our results is that the L-Asparaginase II activity of *S.* Typhimurium leads to the depletion of exogenous L-asparagine, depriving T cells of a nutrient important for its functions in clearing infection. Thus, suggesting an important role for L-Asparaginase II in the pathogenesis of *S.* Typhimurium.

Taken together, we propose a model where *S.* Typhimurium produces L-Asparaginase II, and via its L-asparagine hydrolase activity mediates depletion of exogenous L-asparagine that leads to suppression of protein synthesis and metabolic reprogramming required for T cells to become effector cells. *S.* Typhimurium may require L-asparagine for its own survival and import it creating competition for the resource between the host and bacteria or *S.* Typhimurium may secrete L-Asparaginase II directly into the environment. Both scenarios create an environment where L-asparagine is limiting and this deprivation is sensed by the T cells resulting in decreased mTOR activity and ultimately suppression of protein synthesis. Suppression of protein synthesis prevents the T cells from upregulating autophagy and c-Myc, thereby leaving the cells "stuck", unable to proliferate, differentiate and switch their metabolism to become effector cells. This model is supported by our mass spectrometry data demonstrating that L-

Asparaginase II treated T cells are defective in their ability to synthesize new proteins (Figure 7.1).

The process of activation, proliferation and differentiation of a naïve, quiescent T cell into an effector cell is a metabolically demanding process (174, 184). This process requires quiescent T cells to switch their metabolism from primarily oxidative phosphorylation to aerobic glycolysis as well as increase protein synthesis, which together allows for rapid growth, proliferation and differentiation into effector cells (143, 156, 172, 184-186). In order to complete this process T cells must rely on uptake and use of exogenous nutrients such as amino acids (142, 143, 147). mTOR activity is an important regulator of T cell activation, differentiation, metabolism and protein synthesis because it integrates and transmits a variety of environmental signals, including amino acid availability (147, 151, 154, 186). Naïve, unstimulated T cells have very low level of mTOR activity that upon stimulation is dramatically upregulated in order to activate, proliferate and differentiate (Figure 6.1A)(147, 153, 187). We have shown here that in the absence of L-asparagine there is suppression of mTOR activity in stimulated T cells (Figure 6.1A). Thus, indicating that exogenous L-asparagine is a resource important for optimal mTOR activity.

mTOR activity has been shown to regulate autophagy (148, 150, 151, 188). However, we have shown, as well as others, that naïve T cells display a low level of autophagy that upon stimulation is dramatically upregulated (Figure 6.1B-D) (149). Interestingly, T cells unable to upregulate autophagy upon stimulation are defective in their ability to proliferate and produce cytokines, suggesting that autophagy is a required process used by T cells to become effector cells (149, 171). Here, we found that T cells either treated with L-Asparaginase II or cultured in

medium lacking L-asparagine do not upregulate autophagy upon stimulation (Figure 6.1B-D). Thus, indicating that L-Asparaginase II-mediated L-asparagine deprivation suppresses the ability of T cells to upregulate autophagy. We also show here that stimulated T cells display significant levels of both mTOR activity and autophagy indicating T cells utilize both processes in order to become effector cells (Figure 6.1). This work suggests that the regulation of autophagy, in T cells, may be mTOR independent. This is in contrast to how mTOR has been thought to regulate autophagy, where upregulation of mTOR leads to decreased levels or inhibition of autophagy (148, 189). This difference likely has to do with the important role autophagy plays in T cell activation, likely as a source of energy, whereas other cells do not rely on autophagy for this purpose under normal conditions (149, 190, 191).

mTOR activity has also been shown to regulate expression of the transcription factor c-Myc (151, 156). C-Myc is a critical regulator for the metabolic reprogramming that must take place in T cells (156). T cells unable to make this metabolic shift, such as seen in T cells deficient for the transcription factor c-Myc, are inhibited in their ability to blast, proliferate and secrete IFN-γ, indicating the importance of this process for T cells to become effector cells (156). Consistent with previously published work, we found that naïve T cells do not express detectable levels of c-Myc, but following stimulation there is an increase in c-Myc expression (Figure 6.1E and F) (156). However, we show here that when stimulated T cells were treated with L-Asparaginase II or cultured in medium lacking L-asparagine there was no upregulation in expression of c-Myc (Figure 6.1E and F). Thus, L-Asparaginase II-mediated L-asparagine depletion may prevent T cells from making the metabolic shift required to become effector cells.

The observations that L-Asparaginase II mediated L-asparagine deprivation led to the suppression of mTOR activity, autophagy and c-Myc expression, suggested that protein synthesis might be affected. We found that stimulated, untreated T cells take up exogenous L-asparagine and incorporate it into newly synthesized proteins, however L-Asparaginase II treated T cells had barely detectable levels of incorporation (Figure 7.1). T cells treated with enzymatically inactive L-Asparaginase II incorporated levels of label similar to that of untreated T cells (Figure 7.1). Furthermore, preliminary bioinformatic analysis of the proteins affected by L-Asparaginase II treatment revealed that these proteins were parts of pathways involved in T cell activation, gene expression and metabolism (Data not shown). Taken together this work demonstrates that L-Asparaginase II-mediated L-asparagine deprivation has detrimental effects on protein synthesis.

We suggest that *S.* Typhimurium produces L-Asparaginase II, which hydrolyzes L-asparagine, either following its secretion into the environment or upon uptake by the bacterium, creating an environment where L-asparagine is limiting. T cells sense this nutrient deprivation and via the central regulator of signaling mTOR, transmit these signals, leading to suppression of protein synthesis. Suppression of protein synthesis results in the inability to upregulate key processes such as autophagy and metabolic reprogramming required for T cells to activate, proliferate and differentiate. Thus *S.* Typhimurium uses L-Asparaginase II as a mechanism to modulate the host response. How T cells sense this specific form of nutrient deprivation and transmit it to mTOR as well as how L-asparagine is taken up by T cells are important points that require further investigation. It is also of interest to determine the role of L-Asparaginase II in *S.* Typhimurium growth and metabolism.

Here we have provided insight into the mechanism by which a metabolic enzyme produced by pathogenic bacteria can impair host immune responses. We have also demonstrated the importance of nutrient availability for the metabolically demanding process that a naïve T cell must undergo to become an effector cell. However, the mechanism by which *S.* Typhimurium utilizes L-Asparaginase II to modulate host responses is not unique to *S.* Typhimurium. The L-Asparaginase II gene, *ansB*, is highly conserved among Gram-negative bacteria like *H. pylori*, where it has been shown that L-Asparaginase II contributed to the virulence of both these pathogens (192, 193). This suggests that L-Asparaginase II may be an important virulence determinant utilized by a variety of pathogens and represents a potential a novel target for treatment. Thus, this work and continued work will improve our understanding of bacterial pathogenesis that could lead to improved therapeutics in infectious disease. But, this work also highlights a new way that pathogens can modulate the host response to establish infection.

Future Directions

This work has provided significant insight into the mechanism by which a metabolic enzyme produced by pathogenic bacteria can impair host immune responses. We have demonstrated the importance of nutrient availability for the function of T cells, however continued work is needed to fully understand this process. First, it is unclear exactly how T cells sense changes in nutrient availability. Although it is clear mTOR plays an important role in integrating these environmental signals, the upstream components that signal to mTOR are not well understood, but two pathways have been suggested to regulate mTOR activity: the PI3K/AKT pathway and the RAS/MAPK pathway (194). Therefore it would be would be interesting to determine if either of these pathways are affected by L-Asparaginase II-mediated

L-asparagine depletion. Knowing which of these upstream components are affected may help determine which cell surface receptors are signaling to mTOR.

Additionally, other work has demonstrated that T cells have amino acid importers that are up regulated in response to TCR stimulation. These transporters are heterodimers made of a heavy chain, CD98, and one of several different light chains (142, 195). However, it is not clear if these transporters import L-asparagine, therefore the mechanism(s) by which L-asparagine is sensed and imported into T cells remains to be determined. We could begin by analyzing expression of a previously characterized amino acid transporter made up of CD98 and the light chain Slc7a5, which has been shown to be the main amino acid transporter upregulated in response to T cell stimulation (195). We could look at expression of this transporter in both L-Asparaginase II treated T cells and T cells cultured in medium lacking L-asparagine, to see if this importer is upregulated. Interestingly, Slc7a5 expression was shown to be required for c-Myc expression and therefore metabolic reprogramming of T cells (195). Cells lacking this importer are defective in their ability to blast, proliferate and secrete IFN-Y, similar phenotypes we observe when T cells are treated with L-Asparaginase II (195). It would be of interest to obtain Slc7a5 deficient T cells and determine whether there is uptake of L-asparagine, using medium containing the stable isotope labeled L-asparagine used in Chapter 7 of this dissertation. If this particular importer was not responsible for L-asparagine uptake, we could then begin to investigate the role of the other light chains that dimerize with CD98 to import amino acids: Slc7a8, Slc7a7 and Slc7a6 (195). Though these importers are not well characterized, we could generate T cells lacking these particular importers and determine their role in L-asparagine uptake.

Another point that was not addressed by this dissertation is the physiologic role of L-Asparaginase II during infection. Work done by fellow labmate, Amy Kullas, and myself has attempted to provide insight into this question. When using supernatants collected from T cells co-cultured with *S.* Typhimuirum, L-Asparaginase II was not detectable by western blot analysis. Concentration of this supernatant, by two different methods, also did not allow for detection of L-Asparaginase II by western blot. However, we know some amount of L-Asparaginase II is present in the supernatant because transfer of the supernatant to freshly isolated T cells results in T cell inhibition and also L-Asparaginase II could be depleted from supernatants using beads coated with a L-Asparaginase II antibody (111, 131). This suggests that the L-Asparaginase II present in supernatants is at levels below detection and this concentration is sufficient for T cell inhibition (131). However, we are also investigating the possibility, as suggested in the discussion of this dissertation, that *S.* Typhimurium may import L-asparagine for its own metabolic purposes, creating a situation where L-Asparaginase II is utilized within the bacterium to hydrolyze L-asparagine. The import of L-asparagine by *S.* Typhimurium still creates an environment where L-asparagine is limiting and possibly even depleted, inducing T cell inhibition. Since we already know that *S.* Typhimurium lacking the L-Asparaginase II gene are attenuated for virulence *in vivo,* it would be of interest to determine if this is due to the inability of *S.* Typhimurium to hydrolyze L-asparagine or to secrete L-Asparaginase II. One way this is could be addressed is by investigating potential L-asparagine importers of *S.* Typhimurium and generating mutants lacking these potential importers. If *S.* Typhimurium unable to take up L-asparagine are defective in their ability to grow and survive, or are attenuated for virulence *in vivo*, it would indicate an important role for L-asparagine uptake in *S.* Typhimurium during infection. Also, since the *ansB* gene contains a secretion signal, we could determine what affects

disrupting this signal would have on the ability of *S.* Typhimurium to inhibit the response of T cells. These two points do not need to be mutually exclusive, there could be a combination of secretion of L-Asparaginase II by *S.* Typhimurium as well as uptake of L-asparagine. It would be important to more directly determine how much L-Asparaginase II is in the supernatant. One potential method to do so could be to generate a monoclonal antibody to L-Asparaginase II produced by *S.* Typhimurium and use that to generate an ELISA based method of detection.

Additionally, it would of interest to show changes in L-asparagine concentration following infection with *S.* Typhimurium or with purified L-Asparaginase II. Therefore, we must first determine the concentration of L-asparagine in the tissue culture medium, we do know how much is present at the beginning of the experiment based on the manufacturer's recipe, but initial experiments aimed at determining L-asparagine concentrations following infection with *S.* Typhimurium or with purified L-Asparaginase II were not successful. Perhaps revisiting these assays and trying to determine exactly why they did not work would be helpful. However, a mass spectrometry based approach could be used to determine L-asparagine concentrations in supernatants. This would be helpful to do *in vitro*, but also *in vivo* following infection. Collecting serum from animals before and at various times post infection would allow us to asses if overall L-asparagine concentrations change and begin to better understand the physiological role of L-asparagine during infection. Being able to measure L-asparagine concentrations both *in vitro* and *in vivo* would be helpful when studying possible effects on other cells types and interactions between other cells and T cells. It would be interesting to determine if nutrient deprivation in the form of L-asparagine depletion had deleterious effects on antigen presenting cells, that may prevent them from presenting antigen to T cells. Perhaps when antigen

presenting cells are stressed they do not take up and/or process antigen, this would be interesting points to examine.

It would also be of interest to determine whether L-Asparaginase II-mediated L-asparagine deprivation affects other cell types. Specifically, we would like to examine effects of L-Asparaginase II on B cells. B cells are similar to T cells in that they must also rapidly clonally expand in response to stimuli, therefore I would predict nutrient availability is important during this time for B cells. B cells could be treated with L-Asparaginase II or cultured in medium lacking L-asparagine, left either unstimulated or stimulated, and then evaluated for their ability to proliferate. However, it would also be worth examining the affects of L-Asparaginase II on other cells types, such as macrophages. Although other cells types do not go through a process of priming like T cells do, they may also be sensitive to nutrient deprivation. The phenotypes displayed by cells like macrophages following L-Asparaginase II treatment or after being cultured in medium lacking L-asparagine may be different than what we observe in T cells. For example, we may observe upregulation of autophagy, indicative of a stress response, when a macrophage is deprived of L-asparagine or treated with L-Asparaginase II (196). Additionally, it has been suggested that L-Asparaginase II can bind to and/or cleave cell surface proteins (128, 197). If so, L-Asparaginase II treatment could have effects on many different cell types that could have significant effects on cell-to-cell interactions, *in vivo*, particularly antigen presentation to T cells. Therefore, it would be interesting to examine cells such as macrophages and dendritic cells, which present antigen to T cells, to see if L-Asparaginase II treatment effects cell surface molecules such as MHC molecules or co-stimulatory molecules.

We showed that T cells treated with L-Asparaginase II or cultured in medium lacking L-asparagine, do not upregulate autophagy, as seen in untreated T cells. Since we are measuring

autophagy 12 hours post stimulation, early in the process of activation, it is likely we are looking at activation-induced autophagy. This phenomenon has been described previously, and suggests that during the process of activation, T cells upregulate autophagy (149). Although, the precise role of autophagy during activation has not been determined, it has been suggested to be an mTOR-independent event and that it may be part of regulating the metabolic changes that T cells undergo during activation (149). It would be interesting to further explore the role of activation-induced autophagy in T cells, particularly, the connection, if any, of mTOR signaling and autophagy. Autophagy deficient T cells are defective in their ability to proliferate and secrete cytokines, which suggests a link to mTOR, since mTOR signaling is required for both of these functions (147, 171). Therefore, one way to examine this issue would be to use autophagy deficient T cells and determine the effects on mTOR signaling, both upstream and downstream. Also, one could use pharmacological inhibitors of the mTOR pathway to determine if inhibition of mTOR signaling at various points affects autophagy.

Another important point to be addressed is the effects on protein synthesis. Although we demonstrate that L-Asparaginase II-mediated L-asparagine deprivation has deleterious effects on protein synthesis, further use of this mass spectrometry based approach will allow us to look at not just newly synthesized proteins, but proteins that may be produced by untreated, stimulated T cells, but not by cells treated with L-Asparaginase II, and vice versa. This would allow us to further our understanding about which aspects of T cell function are inhibited. Additionally this work could be bolstered by more direct evidence, such as ribosome stalling experiments.

We have also demonstrated that L-Asparaginase II treatment of T cells leads to suppression of c-Myc expression, suggesting that these T cells are unable to switch to a glycolytic form of metabolism. Therefore, it would be helpful to clearly demonstrate that L-

Asparaginase II treated T cells are metabolically stuck. This could be done by measuring lactate production of T cells treated with L-Asparaginase II, which is produced in abundance by glycolytic T cells. It would be interesting to determine the type of metabolism utilized by L-Asparaginase II treated T cells; since it is clear they are still viable. Work by others has suggested that T cells unable to switch their metabolism are able to survive by using oxidatative phosphorylation, but do not differentiate into efficient effector T cells (185).

Furthermore, it would be helpful to understand the role of L-asparagine in T cell function. First it would be helpful to take a more global look at how T cells are affected by L-Asparaginase II-mediated L-asparagine deprivation. Looking at how the secretion profile of untreated, L-Asparaginase II treated and T cells cultured in medium lacking L-asparagine differ using a cytokine array, may provide insight into effects L-Asparaginase II has on T cells other than those studied so far. Additionally, this could be done with T cells purified from uninfected animals, animals infected with wild-type *S.* Typhimurium and animals infected with *S.* Tyhimurium lacking the L-Asparaginase II gene. This could also be done on specific subsets of T cells, which would allow us to determine how and if particular subsets of T cells may be affected. I would suspect that a majority of the cytokines potentially produced by T cell would be effected by L-Asparaginase II-mediated L-asparagine deprivation because T cells need to upregulate expression of many genes in order to produce these cytokines, therefore if protein synthesis is suppressed T cells would be unable to produce cytokines (185). Additionally, studying the expression of the gene asparagine synthetase, which is responsible for production L-asparagine in mammalian cells, would allow us to further dissect the importance of L-asparagine at different times during T cell activation as well as post activation. Since we believe that L-asparagine may be conditionally required during T cell activation, it would be important to

determine the expression pattern of asparagine synthetase from a naïve T cell through activation. Once the expression pattern for asparagine synthetase has been established we can determine whether its expression is affected following L-Asparaginase II treatment or following culture in medium lacking L-asparagine. Asparagine synthetase could be measured both at the RNA and protein level. We can also begin to dissect the importance of L-asparagine during T cell activation by stimulating T cells for various times prior to treatment with L-Asparaginase II and determining the effects of T cells by measuring cytokine production, proliferation and asparagine synthetase. This could also be done by stimulating T cells in medium containing L-asparagine for various times and then switching them to medium lacking L-asparagine. By varying the time before the addition of L-Asparaginase II or switching to L-asparagine lacking medium, would allow us to see if and for how long T cells need exogenous L-asparagine, or if there is a point where they are able to survive only on what it can produce. Asparagine synthetase expression should also be evaluated in T cells from animals left untreated, treated with wild-type *S.* Typhimurium and *S.* Typhimurium lacking the L-Asparaginase II gene.

Finally, an important question to follow up on would be whether the L-Asparaginase II protein produced by *S.* Typhimurium possesses glutaminase activity. The L-Asparaginase II protein produced by *E.coli* has both asparaginase and glutaminase activities. This glutaminase activity has been reported to be the cause of cytotoxic effects associated with L-Asparaginase II treatment observed in cancer patients being treated with the *E.coli* L-Asparaginase II protein (198). However, the L-Asparaginase II protein produced by the bacterium *Wolinella succinogenes* (formerly classified as *Vibrio succinogenes*) has asparaginase activity, but no detectable glutaminase activity, and is now also used for cancer treatment (199). Interestingly, the amino acids suggested to play important roles in both the asparaginase and glutaminase

catalytic activity in *E.coli* are conserved in the *W. succinogenes* and *S.* Typhimurium L-Asparaginase II proteins (200). Therefore, it would be interesting to determine whether the L-Asparaginase II protein produced by *S.* Typhimurium possesses glutaminase activity by mutating the amino acids suggested to play a role in gluatminase activity in *E.coli*. Initial experiments to disrupt the potential glutaminase activity resulted in a mutant still capable of inhibiting T cell responses, suggesting either the gluatminase activity does not contribute to L-Asparaginase II-induced T cell inhibition or that the glutaminase activity was not destroyed. Therefore, we need an assay that would allow us to measure glutaminase activity, similar to what we used to measure L-Asparaginase II activity in Chapter 3 of this dissertation. Additionally, if the L-Asparaginase II protein produced by *S.* Typhimurium does have glutaminase activity, it would be important to determine whether this activity contributes, in any way, to the T cell inhibition we observe. Particularly, since glutamine is the nitrogen donor for purine nucleobases as well as the amino donor for synthesis of L-asparagine in the reaction catalyzed by asparagine synthetase (201).

References

1. Schenten D, Medzhitov R. 2011. The control of adaptive immune responses by the innate immune system. In *Advances in Immunology*, pp. 87-124
2. Murphy K, Travers P, Walport M. 2008. *Janeway's Immunobiology*. New York : Garland Science, Taylor and Francis Group. LLC
3. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. *Molecular Biology of the Cell*. New York: Garland Science
4. Bardoel BW, Strijp JAG. 2011. Molecular battle between host and bacterium: recognition in innate immunity. In *J Mol Recognit*, pp. 1077-86
5. Reddy KVR, Yedery RD, Aranha C. 2004. Antimicrobial peptides: premises and promises. In *Int J Antimicrob Agents*, pp. 536-47
6. Pushpanathan M, Gunasekaran P, Rajendhran J. 2013. Antimicrobial peptides: versatile biological properties. In *International Journal of Peptides*, pp. 675391
7. Trouw LA, Daha MR. 2011. Role of complement in innate immunity and host defense. In *Immunol Lett*, pp. 35-7
8. Nauta AJ, Roos A, Daha MR. 2004. A regulatory role for complement in innate immunity and autoimmunity. In *Int Arch Allergy Immunol*, pp. 310-23
9. Sunyer JO, Boshra H, Lorenzo G, Parra D, Freedman B, Bosch N. 2003. Evolution of complement as an effector system in innate and adaptive immunity. In *Immunol Res*, pp. 549-64
10. Rus H, Cudrici C, Niculescu F. 2005. The role of the complement system in innate immunity. In *Immunol Res*, pp. 103-12
11. Mastellos D, Morikis D, Isaacs SN, Holland MC, Strey CW, Lambris JD. 2003. Complement: structure, functions, evolution, and viral molecular mimicry. In *Immunol Res*, pp. 367-86
12. Rabinovitch M. 1995. Professional and non-professional phagocytes: an introduction. In *Trends Cell Biol*, pp. 85-7
13. Blasius AL, Beutler B. 2010. Intracellular toll-like receptors. In *Immunity*, pp. 305-15
14. Kumar H, Kawai T, Akira S. 2011. Pathogen recognition by the innate immune system. In *Int Rev Immunol*, pp. 16-34
15. Arpaia N, Barton GM. 2013. The impact of Toll-like receptors on bacterial virulence strategies. In *Curr Opin Microbiol*, pp. 17-22
16. Martínez VG, Moestrup SK, Holmskov U, Mollenhauer J, Lozano F. 2011. The conserved scavenger receptor cysteine-rich superfamily in therapy and diagnosis. In *Pharmacological Reviews*, pp. 967-1000
17. Roy CR, Mocarski ES. 2007. Pathogen subversion of cell-intrinsic innate immunity. In *Nat Immunol*, pp. 1179-87
18. Palm NW, Medzhitov R. 2009. Pattern recognition receptors and control of adaptive immunity. In *Immunol Rev*, pp. 221-33
19. Crozat K, Vivier E, Dalod M. 2009. Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. In *Immunol Rev*, pp. 129-49
20. Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. In *Cell*, pp. 783-801

21. Gupta G, Surolia A. 2007. Collectins: sentinels of innate immunity. In *Bioessays*, pp. 452-64
22. Finlay BB, McFadden G. 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. In *Cell*, pp. 767-82
23. Flannagan RS, Cosío G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. In *Nat Rev Microbiol*, pp. 355-66
24. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. In *Nat Rev Immunol*, pp. 958-69
25. Cookson BT, Cummings LA, Rassoulian Barrett SL. 2001. Bacterial antigens elicit T cell responses via adaptive and transitional immune recognition. In *Curr Opin Microbiol*, pp. 267-73
26. Neefjes J, Jongsma MLM, Paul P, Bakke O. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. In *Nat Rev Immunol*, pp. 823-36
27. Smith-Garvin JE, Koretzky GA, Jordan MS. 2009. T Cell Activation. In *Annu. Rev. Immunol.*, pp. 591-619
28. Boyman O, Sprent J. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. In *Nat Rev Immunol*, pp. 180-90
29. Northrop JK, Shen H. 2004. CD8+ T-cell memory: only the good ones last. In *Current Opinion in Immunology*, pp. 451-5
30. Sokol CL, Barton GM, Farr AG, Medzhitov R. 2008. A mechanism for the initiation of allergen-induced T helper type 2 responses. In *Nat Immunol*, pp. 310-8
31. Basu R, Hatton RD, Weaver CT. 2013. The Th17 family: flexibility follows function. In *Immunol Rev*, pp. 89-103
32. Jiang S, Dong C. 2013. A complex issue on CD4(+) T-cell subsets. In *Immunol Rev*, pp. 5-11
33. Yamane H, Paul WE. 2013. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. In *Immunol Rev*, pp. 12-23
34. Lefrançois L. 2006. Development, trafficking, and function of memory T-cell subsets. In *Immunol Rev*, pp. 93-103
35. Stockinger B, Bourgeois C, Kassiotis G. 2006. CD4+ memory T cells: functional differentiation and homeostasis. In *Immunol Rev*, pp. 39-48
36. Fàbrega A, Vila J. 2013. Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. In *Clin Microbiol Rev*, pp. 308-41
37. Coburn B, Grassl GA, Finlay BB. 2007. Salmonella, the host and disease: a brief review. In *Immunol Cell Biol*, pp. 112-8
38. Andrews-Polymenis HL, Bäumler AJ, McCormick BA, Fang FC. 2010. Taming the elephant: Salmonella biology, pathogenesis, and prevention. In *Infect Immun*, pp. 2356-69
39. Ramos-Morales F. 2012. Impact of Salmonella enterica Type III Secretion System Effectors on the Eukaryotic Host Cell. In *ISRN Cell Biology*, pp. 1-36
40. Valdez Y, Ferreira RBR, Finlay BB. 2009. Molecular mechanisms of Salmonella virulence and host resistance. In *Curr Top Microbiol Immunol*, pp. 93-127
41. Grimont PW, FX. 2007. Antigenic Formulae of the *Salmonella* serovars, 9th revision. pp. 1-167. Paris, France: World Health Organization Collaborating Center for Reference and Research on Salmonella

42. De Jong HK, Parry CM, Van Der Poll T, Wiersinga WJ. 2012. Host–Pathogen Interaction in Invasive Salmonellosis. In *PLoS Pathog*, pp. e1002933
43. Ruby T, Mclaughlin L, Gopinath S, Monack D. 2012. Salmonella's long-term relationship with its host. In *FEMS Microbiol Rev*, pp. 600-15
44. Steele-Mortimer O. 2008. The Salmonella-containing vacuole: moving with the times. In *Curr Opin Microbiol*, pp. 38-45
45. Chiu H-C, Kulp SK, Soni S, Wang D, Gunn JS, Schlesinger LS, Chen C-S. 2009. Eradication of intracellular Salmonella enterica serovar Typhimurium with a small-molecule, host cell-directed agent. In *Antimicrob Agents Chemother*, pp. 5236-44
46. van der Velden AWM, Velasquez M, Starnbach MN. 2003. Salmonella rapidly kill dendritic cells via a caspase-1-dependent mechanism. In *J Immunol*, pp. 6742-9
47. Moon JJ, McSorley SJ. 2009. Tracking the dynamics of salmonella specific T cell responses. In *Curr Top Microbiol Immunol*, pp. 179-98
48. Monack DM, Mueller A, Falkow S. 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. In *Nat Rev Microbiol*, pp. 747-65
49. Broz P, Ohlson MB, Monack DM. 2012. Innate immune response to Salmonella typhimurium, a model enteric pathogen. In *gutmicrobes*, pp. 62-70
50. Mittrücker H-W, Köhler A, Kaufmann SHE. 2002. Characterization of the murine T-lymphocyte response to Salmonella enterica serovar Typhimurium infection. In *Infect Immun*, pp. 199-203
51. Mittrücker HW, Kaufmann SH. 2000. Immune response to infection with Salmonella typhimurium in mice. In *J Leukoc Biol*, pp. 457-63
52. Ibarra JA, Steele-Mortimer O. 2009. Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival. In *Cell Microbiol*, pp. 1579-86
53. van der Velden AW, Lindgren SW, Worley MJ, Heffron F. 2000. Salmonella pathogenicity island 1-independent induction of apoptosis in infected macrophages by Salmonella enterica serotype typhimurium. In *Infect Immun*, pp. 5702-9
54. Fields PI, Swanson RV, Haidaris CG, Heffron F. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. In *Proc Natl Acad Sci USA*, pp. 5189-93
55. Nauciel C, Espinasse-Maes F. 1992. Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection. In *Infect Immun*, pp. 450-4
56. Eisele NA, Ruby T, Jacobson A, Manzanillo PS, Cox JS, Lam L, Mukundan L, Chawla A, Monack DM. 2013. Salmonella Require the Fatty Acid Regulator PPARδ for the Establishment of a Metabolic Environment Essential for Long-Term Persistence. In *Cell Host Microbe*, pp. 171-82
57. Haraga A, Ohlson MB, Miller SI. 2008. Salmonellae interplay with host cells. In *Nat Rev Microbiol*, pp. 53-66
58. Bowe F, Lipps CJ, Tsolis RM, Groisman E, Heffron F, Kusters JG. 1998. At least four percent of the Salmonella typhimurium genome is required for fatal infection of mice. In *Infect Immun*, pp. 3372-7
59. Bakowski MA, Braun V, Brumell JH. 2008. Salmonella-containing vacuoles: directing traffic and nesting to grow. In *Traffic*, pp. 2022-31

60. Cardenal-Muñoz E, Ramos-Morales F. 2011. Analysis of the Expression, Secretion and Translocation of the Salmonella enterica Type III Secretion System Effector SteA. In *PLoS ONE*, pp. e26930
61. Linehan SA, Holden DW. 2003. The interplay between Salmonella typhimurium and its macrophage host--what can it teach us about innate immunity? In *Immunol Lett*, pp. 183-92
62. Hornef MW, Wick MJ, Rhen M, Normark S. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. In *Nat Immunol*, pp. 1033-40
63. Bueno SM, Riquelme S, Riedel CA, Kalergis AM. 2012. Mechanisms used by virulent Salmonella to impair dendritic cell function and evade adaptive immunity. In *Immunology*, pp. 28-36
64. Dougan G, John V, Palmer S, Mastroeni P. 2011. Immunity to salmonellosis. In *Immunol Rev*, pp. 196-210
65. Griffin AJ, McSorley SJ. 2011. Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. *Mucosal Immunol* 4: 371-82
66. Agbor TA, McCormick BA. 2011. Salmonella effectors: important players modulating host cell function during infection. In *Cell Microbiol*, pp. 1858-69
67. Hansen-Wester I, Hensel M. 2001. Salmonella pathogenicity islands encoding type III secretion systems. In *Microbes Infect*, pp. 549-59
68. García-del Portillo F. 2001. Salmonella intracellular proliferation: where, when and how? In *Microbes Infect*, pp. 1305-11
69. Monack DM, Bouley DM, Falkow S. 2004. Salmonella typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected Nramp1+/+ mice and can be reactivated by IFNgamma neutralization. In *J Exp Med*, pp. 231-41
70. Brennan MA, Cookson BT. 2000. Salmonella induces macrophage death by caspase-1-dependent necrosis. In *Mol Microbiol*, pp. 31-40
71. Monack DM, Navarre WW, Falkow S. 2001. Salmonella-induced macrophage death: the role of caspase-1 in death and inflammation. In *Microbes Infect*, pp. 1201-12
72. Guiney DG, Fierer J. 2011. The Role of the spv Genes in Salmonella Pathogenesis. In *Front. Microbio.*, pp. 129
73. Monack DM, Detweiler CS, Falkow S. 2001. Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. In *Cell Microbiol*, pp. 825-37
74. Matsui H, Bacot CM, Garlington WA, Doyle TJ, Roberts S, Gulig PA. 2001. Virulence plasmid-borne spvB and spvC genes can replace the 90-kilobase plasmid in conferring virulence to Salmonella enterica serovar Typhimurium in subcutaneously inoculated mice. In *Journal of Bacteriology*, pp. 4652-8
75. Srinivasan A, Foley J, Ravindran R, McSorley SJ. 2004. Low-dose Salmonella infection evades activation of flagellin-specific CD4 T cells. In *J Immunol*, pp. 4091-9
76. Wick MJ. 2011. Innate immune control of Salmonella enterica serovar Typhimurium: mechanisms contributing to combating systemic Salmonella infection. In *J Innate Immun*, pp. 543-9
77. Rydström A, Wick MJ. 2007. Monocyte recruitment, activation, and function in the gut-associated lymphoid tissue during oral Salmonella infection. In *J Immunol*, pp. 5789-801

78. Fink SL, Cookson BT. 2007. Pyroptosis and host cell death responses during Salmonella infection. In *Cell Microbiol*, pp. 2562-70
79. Broz P, Monack DM. 2011. Molecular mechanisms of inflammasome activation during microbial infections. In *Immunol Rev*, pp. 174-90
80. Latz E, Xiao TS, Stutz A. 2013. Activation and regulation of the inflammasomes. In *Nat Rev Immunol*, pp. 397-411
81. Kim J-J, Jo E-K. 2013. NLRP3 Inflammasome and Host Protection against Bacterial Infection. In *J Korean Med Sci*, pp. 1415-23
82. Dinarello CA, Novick D, Kim S, Kaplanski G. 2013. Interleukin-18 and IL-18 Binding Protein. In *Front Immunol*, pp. 289
83. Raupach B, Peuschel S-K, Monack DM, Zychlinsky A. 2006. Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against Salmonella enterica serovar Typhimurium infection. In *Infect Immun*, pp. 4922-6
84. Müller AJ, Hoffmann C, Galle M, Van Den Broeke A, Heikenwalder M, Falter L, Misselwitz B, Kremer M, Beyaert R, Hardt W-D. 2009. The S. Typhimurium effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. In *Cell Host Microbe*, pp. 125-36
85. Tam MA, Rydström A, Sundquist M, Wick MJ. 2008. Early cellular responses to Salmonella infection: dendritic cells, monocytes, and more. In *Immunol Rev*, pp. 140-62
86. Jung S, Unutmaz D, Wong P, Sano G-I, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA. 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. In *Immunity*, pp. 211-20
87. Salazar-Gonzalez RM, Niess JH, Zammit DJ, Ravindran R, Srinivasan A, Maxwell JR, Stoklasek T, Yadav R, Williams IR, Gu X, McCormick BA, Pazos MA, Vella AT, Lefrancois L, Reinecker H-C, McSorley SJ. 2006. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. In *Immunity*, pp. 623-32
88. Spörri R, Reis e Sousa C. 2005. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. In *Nat Immunol*, pp. 163-70
89. Nanton MR, Way SS, Shlomchik MJ, McSorley SJ. 2012. Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. In *J Immunol*, pp. 5503-7
90. Mittrücker HW, Raupach B, Köhler A, Kaufmann SH. 2000. Cutting edge: role of B lymphocytes in protective immunity against Salmonella typhimurium infection. In *J Immunol*, pp. 1648-52
91. Barr TA, Brown S, Mastroeni P, Gray D. 2010. TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to Salmonella enterica. In *J Immunol*, pp. 2783-9
92. Mastroeni P, Simmons C, Fowler R, Hormaeche CE, Dougan G. 2000. Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent Salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to Salmonella antigens. In *Infect Immun*, pp. 46-53

93. McSorley SJ, Jenkins MK. 2000. Antibody is required for protection against virulent but not attenuated Salmonella enterica serovar typhimurium. In *Infect Immun*, pp. 3344-8
94. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. In *Infect Immun*, pp. 3981-4
95. Neves P, Lampropoulou V, Calderon-Gomez E, Roch T, Stervbo U, Shen P, Kühl AA, Loddenkemper C, Haury M, Nedospasov SA, Kaufmann SHE, Steinhoff U, Calado DP, Fillatreau S. 2010. Signaling via the MyD88 Adaptor Protein in B Cells Suppresses Protective Immunity during Salmonella typhimurium Infection. In *Immunity*, pp. 777-90
96. Ertelt JM, Johanns TM, Mysz MA, Nanton MR, Rowe JH, Aguilera MN, Way SS. 2011. Selective culling of high avidity antigen-specific CD4+ T cells after virulent Salmonella infection. In *Immunology*, pp. 487-97
97. Lo WF, Ong H, Metcalf ES, Soloski MJ. 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules. In *J Immunol*, pp. 5398-406
98. Nauciel C. 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to Salmonella typhimurium infection. In *J Immunol*, pp. 1265-9
99. McGeachy MJ, McSorley SJ. 2012. Microbial-induced Th17: superhero or supervillain? In *J Immunol*, pp. 3285-91
100. Johanns TM, Ertelt JM, Rowe JH, Way SS. 2010. Regulatory T Cell Suppressive Potency Dictates the Balance between Bacterial Proliferation and Clearance during Persistent Salmonella Infection. In *PLoS Pathog*, pp. e1001043
101. Srinivasan A, McSorley SJ. 2007. Pivotal advance: exposure to LPS suppresses CD4+ T cell cytokine production in Salmonella-infected mice and exacerbates murine typhoid. In *J Leukoc Biol*, pp. 403-11
102. al-Ramadi BK, Greene JM, Meissler JJ, Eisenstein TK. 1992. Immunosuppression induced by attenuated Salmonella: effect of LPS responsiveness on development of suppression. In *Microbial Pathogenesis*, pp. 267-78
103. Wick MJ, Harding CV, Twesten NJ, Normark SJ, Pfeifer JD. 1995. The phoP locus influences processing and presentation of Salmonella typhimurium antigens by activated macrophages. In *Mol Microbiol*, pp. 465-76
104. Qimron U, Madar N, Mittrücker H-W, Zilka A, Yosef I, Bloushtain N, Kaufmann SHE, Rosenshine I, Apte RN, Porgador A. 2004. Identification of Salmonella typhimurium genes responsible for interference with peptide presentation on MHC class I molecules: Deltayej Salmonella mutants induce superior CD8+ T-cell responses. In *Cell Microbiol*, pp. 1057-70
105. Tobar JA, González PA, Kalergis AM. 2004. Salmonella escape from antigen presentation can be overcome by targeting bacteria to Fc gamma receptors on dendritic cells. In *J Immunol*, pp. 4058-65
106. Tobar JA, Carreño LJ, Bueno SM, González PA, Mora JE, Quezada SA, Kalergis AM. 2006. Virulent Salmonella enterica serovar typhimurium evades adaptive immunity by preventing dendritic cells from activating T cells. In *Infect Immun*, pp. 6438-48

107. Cheminay C, Möhlenbrink A, Hensel M. 2005. Intracellular Salmonella inhibit antigen presentation by dendritic cells. In *J Immunol*, pp. 2892-9
108. Bueno SM, González PA, Schwebach JR, Kalergis AM. 2007. T cell immunity evasion by virulent Salmonella enterica. In *Immunol Lett*, pp. 14-20
109. Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI. 1997. Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. In *Science*, pp. 250-3
110. Miller SI, Mekalanos JJ. 1990. Constitutive expression of the phoP regulon attenuates Salmonella virulence and survival within macrophages. In *Journal of Bacteriology*, pp. 2485-90
111. van der Velden AWM, Dougherty JT, Starnbach MN. 2008. Down-modulation of TCR expression by Salmonella enterica serovar Typhimurium. In *J Immunol*, pp. 5569-74
112. van der Velden AWM, Copass MK, Starnbach MN. 2005. Salmonella inhibit T cell proliferation by a direct, contact-dependent immunosuppressive effect. In *Proc Natl Acad Sci USA*, pp. 17769-74
113. Johanns TM, Ertelt JM, Lai JC, Rowe JH, Avant RA, Way SS. 2010. Naturally occurring altered peptide ligands control Salmonella-specific CD4+ T cell proliferation, IFN-gamma production, and protective potency. In *J Immunol*, pp. 869-76
114. Luu RA, Gurnani K, Dudani R, Kammara R, van Faassen H, Sirard J-C, Krishnan L, Sad S. 2006. Delayed expansion and contraction of CD8+ T cell response during infection with virulent Salmonella typhimurium. In *J Immunol*, pp. 1516-25
115. Mitchell EK, Mastroeni P, Kelly AP, Trowsdale J. 2004. Inhibition of cell surface MHC class II expression by Salmonella. In *Eur J Immunol*, pp. 2559-67
116. Matsui K, Nagano K, Arai T, Hirono I, Aoki T. 1998. DNA sequencing of the gene encoding Salmonella typhimurium-derived T-cell inhibitor (STI) and characterization of the gene product, cloned STI. *FEMS Immunol Med Microbiol* 22: 341-9
117. Boyman O, Purton JF, Surh CD, Sprent J. 2007. Cytokines and T-cell homeostasis. In *Current Opinion in Immunology*, pp. 320-6
118. Burgess RR. 2009. Protein precipitation techniques. In *Meth Enzymol*, pp. 331-42
119. Jungbauer A, Hahn R. 2009. Ion-exchange chromatography. In *Meth Enzymol*, pp. 349-71
120. Verma N, Kumar K, Kaur G, Anand S. 2007. L-asparaginase: a promising chemotherapeutic agent. In *Critical Reviews in Biotechnology*, pp. 45-62
121. Nomme J, Su Y, Konrad M, Lavie A. 2012. Structures of Apo and Product-Bound Human l-Asparaginase: Insights into the Mechanism of Autoproteolysis and Substrate Hydrolysis. In *Biochemistry*, pp. 6816-26
122. Michalska K, Jaskolski M. 2006. Structural aspects of L-asparaginases, their friends and relations. In *Acta Biochim Pol*, pp. 627-40
123. Müller HJ, Boos J. 1998. Use of L-asparaginase in childhood ALL. In *Crit Rev Oncol Hematol*, pp. 97-113
124. BROOME JD. 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. II. Lymphoma 6C3HED cells cultured in a medium devoid of L-asparagine lose their susceptibility to the effects of guinea pig serum in vivo. In *J Exp Med*, pp. 121-48

125. Shons AR, Etheredge EE, Najarian JS. 1971. The effect of L-asparaginase in mixed leukocyte culture. In *Transplantation*, pp. 85-7
126. Fidler IJ, Montgomery PC. 1972. Effects of L-asparaginase on lymphocyte surface and blastogenesis. In *Cancer Res*, pp. 2400-6
127. Fidler IJ, Montgomery PC. 1973. Effects of L-asparaginase on lymphocyte's surface leading to decreased binding of alloantibodies. In *Res Commun Chem Pathol Pharmacol*, pp. 627-34
128. Fidler IJ, Montgomery PC, Cesarini JP. 1973. Modification of surface topography of lymphocytes by L-asparaginase. In *Cancer Res*, pp. 3176-80
129. Crowther D. 1971. L-asparaginase and human malignant disease. In *Nature*, pp. 168-71
130. Yu M, Henning R, Walker A, Kim G, Perroy A, Alessandro R, Virador V, Kohn EC. 2012. L-asparaginase inhibits invasive and angiogenic activity and induces autophagy in ovarian cancer. In *J. Cell. Mol. Med.*, pp. 2369-78
131. Kullas Amy L, Mcclelland M, Yang H-J, Tam Jason W, Torres A, Porwollik S, Mena P, Mcphee Joseph B, Bogomolnaya L, Andrews-Polymenis H, Van Der Velden Adrianus WM. 2012. L-Asparaginase II Produced by Salmonella Typhimurium Inhibits T Cell Responses and Mediates Virulence. In *Cell Host and Microbe*, pp. 791-8
132. Copeland RA. 2005. Enzymes
A Practical Introduction to Structure, Mechanism, and Data Analysis. pp. 1-412: John Wiley & Sons, Inc.
133. French JB, Cen Y, Vrablik TL, Xu P, Allen E, Hanna-Rose W, Sauve AA. 2010. Characterization of nicotinamidases: steady state kinetic parameters, classwide inhibition by nicotinaldehydes, and catalytic mechanism. In *Biochemistry*, pp. 10421-39
134. Kincaid VA, Sullivan ED, Klein RD, Noel JW, Rowlett RS, Snider MJ. 2012. Structure and catalytic mechanism of nicotinate (vitamin B3) degradative enzyme maleamate amidohydrolase from Bordetella bronchiseptica RB50. In *Biochemistry*, pp. 545-54
135. Derst C, Henseling J, Röhm KH. 1992. Probing the role of threonine and serine residues of E. coli asparaginase II by site-specific mutagenesis. In *Protein Eng*, pp. 785-9
136. Jackson RC, Handschumacher RE. 1970. Escherichia coli L-asparaginase. Catalytic activity and subunit nature. In *Biochemistry*, pp. 3585-90
137. Wehner A, Harms E, Jennings MP, Beacham IR, Derst C, Bast P, Röhm KH. 1992. Site-specific mutagenesis of Escherichia coli asparaginase II. None of the three histidine residues is required for catalysis. In *Eur J Biochem*, pp. 475-80
138. Aung HP, Bocola M, Schleper S, Röhm KH. 2000. Dynamics of a mobile loop at the active site of Escherichia coli asparaginase. In *Biochim Biophys Acta*, pp. 349-59
139. Palm GJ, Lubkowski J, Derst C, Schleper S, Röhm KH, Wlodawer A. 1996. A covalently bound catalytic intermediate in Escherichia coli asparaginase: crystal structure of a Thr-89-Val mutant. In *FEBS Lett*, pp. 211-6
140. Harms E, Wehner A, Aung HP, Röhm KH. 1991. A catalytic role for threonine-12 of E. coli asparaginase II as established by site-directed mutagenesis. In *FEBS Lett*, pp. 55-8

141. Yan L, Lamb RF. 2012. Amino acid sensing and regulation of mTORC1. In *Seminars in Cell and Developmental Biology*, pp. 621-5
142. Wang R, Green DR. 2012. Metabolic reprogramming and metabolic dependency in T cells. In *Immunol Rev*, pp. 14-26
143. Carr EL, Kelman A, Wu GS, Gopaul R, Senkevitch E, Aghvanyan A, Turay AM, Frauwirth KA. 2010. Glutamine uptake and metabolism are coordinately regulated by ERK/MAPK during T lymphocyte activation. In *J Immunol*, pp. 1037-44
144. Nicholson LB, Raveney BJE, Munder M. 2009. Monocyte dependent regulation of autoimmune inflammation. In *Curr Mol Med*, pp. 23-9
145. Huang L, Baban B, Johnson BA, Mellor AL. 2010. Dendritic cells, indoleamine 2,3 dioxygenase and acquired immune privilege. In *Int Rev Immunol*, pp. 133-55
146. Derst C, Henseling J, Röhm KH. 2000. Engineering the substrate specificity of Escherichia coli asparaginase. II. Selective reduction of glutaminase activity by amino acid replacements at position 248. In *Protein Sci*, pp. 2009-17
147. Powell JD, Delgoffe GM. 2010. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. In *Immunity*, pp. 301-11
148. Rathmell JC. 2012. Metabolism and autophagy in the immune system: immunometabolism comes of age. In *Immunol Rev*, pp. 5-13
149. Hubbard VM, Valdor R, Patel B, Singh R, Cuervo AM, Macian F. 2010. Macroautophagy Regulates Energy Metabolism during Effector T Cell Activation. In *J Immunol*
150. Dodd KM, Tee AR. 2012. Leucine and mTORC1: a complex relationship. In *Am J Physiol Endocrinol Metab*, pp. E1329-42
151. Waickman AT, Powell JD. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. In *Immunol Rev*, pp. 43-58
152. Chi H. 2012. Regulation and function of mTOR signalling in T cell fate decisions. In *Nat Rev Immunol*, pp. 325-38
153. Yang K, Shrestha S, Zeng H, Karmaus PWF, Neale G, Vogel P, Guertin DA, Lamb RF, Chi H. 2013. T Cell Exit from Quiescence and Differentiation into Th2 Cells Depend on Raptor-mTORC1-Mediated Metabolic Reprogramming. In *Immunity*, pp. 1043-56
154. Zheng Y, Collins SL, Lutz MA, Allen AN, Kole TP, Zarek PE, Powell JD. 2007. A role for mammalian target of rapamycin in regulating T cell activation versus anergy. In *J Immunol*, pp. 2163-70
155. Yang K, Neale G, Green DR, He W, Chi H. 2011. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. In *Nat Immunol*, pp. 888-97
156. Wang R, Dillon Christopher P, Shi Lewis Z, Milasta S, Carter R, Finkelstein D, Mccormick Laura L, Fitzgerald P, Chi H, Munger J, Green Douglas R. 2011. The Transcription Factor Myc Controls Metabolic Reprogramming upon T Lymphocyte Activation. In *Immunity*, pp. 871-82
157. Valdor R, Macian F. 2012. Autophagy and the regulation of the immune response. In *Pharmacological Research*, pp. 475-83
158. Sridhar S, Botbol Y, Macian F, Cuervo AM. 2012. Autophagy and disease: always two sides to a problem. In *J Pathol*, pp. 255-73

159. Ravikumar B, Futter M, Jahreiss L, Korolchuk VI, Lichtenberg M, Luo S, Massey DCO, Menzies FM, Narayanan U, Renna M, Jimenez-Sanchez M, Sarkar S, Underwood B, Winslow A, Rubinsztein DC. 2009. Mammalian macroautophagy at a glance. In *J Cell Sci*, pp. 1707-11
160. Mizushima N, Komatsu M. 2011. Autophagy: renovation of cells and tissues. In *Cell*, pp. 728-41
161. Mizushima N, Yoshimori T, Levine B. 2010. Methods in mammalian autophagy research. In *Cell*, pp. 313-26
162. Yano T, Kurata S. 2011. Intracellular recognition of pathogens and autophagy as an innate immune host defence. In *Journal of Biochemistry*, pp. 143-9
163. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, et al. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. In *Autophagy*, pp. 445-544
164. Willinger T, Flavell RA. 2012. Canonical autophagy dependent on the class III phosphoinositide-3 kinase Vps34 is required for naive T-cell homeostasis. In *Proceedings of the National Academy of Sciences*, pp. 8670-5
165. Pua HH, He Y-W. 2007. Maintaining T lymphocyte homeostasis: another duty of autophagy. In *Autophagy*, pp. 266-7
166. Stephenson LM, Miller BC, Ng A, Eisenberg J, Zhao Z, Cadwell K, Graham DB, Mizushima NN, Xavier R, Virgin HW, Swat W. 2009. Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes. In *Autophagy*, pp. 625-35
167. Pua HH, Guo J, Komatsu M, He Y-W. 2009. Autophagy Is Essential for Mitochondrial Clearance in Mature T Lymphocytes. In *The Journal of Immunology*, pp. 4046-55
168. Kovacs JR, Li C, Yang Q, Li G, Garcia IG, Ju S, Roodman DG, Windle JJ, Zhang X, Lu B. 2011. Autophagy promotes T-cell survival through degradation of proteins of the cell death machinery. pp. 144-52
169. MacIver NJ, Michalek RD, Rathmell JC. 2013. Metabolic regulation of T lymphocytes. In *Annu. Rev. Immunol.*, pp. 259-83
170. He IXMaY-W. 2013. Autophagy, a novel pathway to regulate calcium mobilization in T lymphocytes. pp. 1-7
171. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He Y-W. 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. In *J Exp Med*, pp. 25-31
172. Pearce EL. 2010. Metabolism in T cell activation and differentiation. In *Current Opinion in Immunology*, pp. 314-20
173. Jones RG, Thompson CB. 2007. Revving the engine: signal transduction fuels T cell activation. In *Immunity*, pp. 173-8
174. Heikamp EB, Powell JD. 2012. Sensing the immune microenvironment to coordinate T cell metabolism, differentiation & function. In *Seminars in Immunology*, pp. 414-20
175. Jaber N, Dou Z, Chen J-S, Catanzaro J, Jiang Y-P, Ballou LM, Selinger E, Ouyang X, Lin RZ, Zhang J, Zong W-X. 2012. Class III PI3K Vps34 plays an essential role in autophagy and in heart and liver function. In *Proc Natl Acad Sci USA*, pp. 2003-8
176. Stephan JS, Yeh Y-Y, Ramachandran V, Deminoff SJ, Herman PK. 2009. The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. In *Proc Natl Acad Sci USA*, pp. 17049-54

177. Wang R, Green DR. 2012. Metabolic checkpoints in activated T cells. In *Nat Immunol*, pp. 907-15
178. Wahl DR, Byersdorfer CA, Ferrara JLM, Opipari AW, Glick GD. 2012. Distinct metabolic programs in activated T cells: opportunities for selective immunomodulation. In *Immunol Rev*, pp. 104-15
179. Sengupta S, Peterson TR, Sabatini DM. 2010. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. In *Mol Cell*, pp. 310-22
180. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. In *J Proteome Res*, pp. 1794-805
181. Elias JE, Gygi SP. 2007. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. In *Nat Meth*, pp. 207-14
182. Besson MT, Soustelle L, Birman S. 2000. Selective high-affinity transport of aspartate by a Drosophila homologue of the excitatory amino-acid transporters. In *Curr Biol*, pp. 207-10
183. Cox J, Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. In *Nat Biotechnol*, pp. 1367-72
184. Pearce Erika L, Pearce Edward J. 2013. Metabolic Pathways in Immune Cell Activation and Quiescence. In *Immunity*, pp. 633-43
185. Chang C-H, Curtis JD, Maggi LB, Faubert B, Villarino AV, O'Sullivan D, Huang SC-C, van der Windt GJW, Blagih J, Qiu J, Weber JD, Pearce EJ, Jones RG, Pearce EL. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. In *Cell*, pp. 1239-51
186. Cobbold SP. 2013. The mTOR pathway and integrating immune regulation. In *Immunology*
187. Fracchia KM, Pai CY, Walsh CM. 2013. Modulation of T Cell Metabolism and Function through Calcium Signaling. In *Front Immunol*, pp. 324
188. Xu X, Ye L, Araki K, Ahmed R. 2012. mTOR, linking metabolism and immunity. In *Seminars in Immunology*, pp. 429-35
189. Alers S, Loffler AS, Wesselborg S, Stork B. 2012. Role of AMPK-mTOR-Ulk1/2 in the Regulation of Autophagy: Cross Talk, Shortcuts, and Feedbacks. In *Mol Cell Biol*, pp. 2-11
190. McLeod IX, Jia W, He Y-W. 2012. The contribution of autophagy to lymphocyte survival and homeostasis. In *Immunol Rev*, pp. 195-204
191. Gamper CJ, Powell JD. 2012. All PI3Kinase signaling is not mTOR: dissecting mTOR-dependent and independent signaling pathways in T cells. In *Front Immunol*, pp. 312
192. Scotti C, Sommi P, Pasquetto MV, Cappelletti D, Stivala S, Mignosi P, Savio M, Chiarelli LR, Valentini G, Bolanos-Garcia VM, Merrell DS, Franchini S, Verona ML, Bolis C, Solcia E, Manca R, Franciotta D, Casasco A, Filipazzi P, Zardini E, Vannini V. 2010. Cell-cycle inhibition by Helicobacter pylori L-asparaginase. In *PLoS ONE*, pp. e13892
193. Shibayama K, Takeuchi H, Wachino J-I, Mori S, Arakawa Y. 2011. Biochemical and pathophysiological characterization of Helicobacter pylori asparaginase. In *Microbiol Immunol*, pp. 408-17

194. Avruch J, Long X, Ortiz-Vega S, Rapley J, Papageorgiou A, Dai N. 2009. Amino acid regulation of TOR complex 1. In *Am J Physiol Endocrinol Metab*, pp. E592-602
195. Sinclair LV, Rolf J, Emslie E, Shi Y-B, Taylor PM, Cantrell DA. 2013. Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. In *Nat Immunol*, pp. 500-8
196. Deretic V, Saitoh T, Akira S. 2013. Autophagy in infection, inflammation and immunity. In *Nat Rev Immunol*, pp. 722-37
197. Nakayama H, Hayashi T, Salata KF, Notkins AL. 1990. Flow cytometry to identify cell types to which enzymes bind. Effect of lactic dehydrogenase virus on enzyme binding. In *J Biol Chem*, pp. 14355-7
198. Reinert RB, Oberle LM, Wek SA, Bunpo P, Wang XP, Mileva I, Goodwin LO, Aldrich CJ, Durden DL, McNurlan MA, Wek RC, Anthony TG. 2006. Role of glutamine depletion in directing tissue-specific nutrient stress responses to L-asparaginase. In *J Biol Chem*, pp. 31222-33
199. Durden DL, Distasio JA. 1980. Comparison of the immunosuppressive effects of asparaginases from Escherichia coli and Vibrio succinogenes. In *Cancer Res*, pp. 1125-9
200. Lubkowski J, Palm GJ, Gilliland GL, Derst C, Röhm KH, Wlodawer A. 1996. Crystal structure and amino acid sequence of Wolinella succinogenes L-asparaginase. In *Eur J Biochem*, pp. 201-7
201. Cory JG, Cory AH. 2006. Critical roles of glutamine as nitrogen donors in purine and pyrimidine nucleotide synthesis: asparaginase treatment in childhood acute lymphoblastic leukemia. In *In Vivo*, pp. 587-9