Eliciting Th1 effector functions: A mechanism and role for innate amplification of the Th1 response during infection

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Dedication

This work is dedicated to all of the friends whose encouragement kept me going, to my family for their love and understanding, and to the teachers and mentors who have over the years cultivated my own love and understanding of science.

Abstract

Innate and adaptive immunity have classically been considered as two distinct categories of cells and responses. Recently, however, an increasing appreciation for the dynamic interactions between these responses has developed. In this dissertation, this overlap is explored in the context of Th1 CD4 T cell production of IFN-γ (interferon-gamma) in response to innate stimuli. In particular, we first asked what triggers innate stimulation of Th1 cells, examining multiple ligands, infections and time points to show that this response occurred within broad contexts of intracellular infection. We then asked how T cells are able to recognize innate stimuli, focusing on whether the T cell intrinsic response relied upon direct LPS (lipopolysaccharide) recognition or indirect recognition of secondary signals. T cell intrinsic requirements were examined in mixed bone marrow chimeras that allowed T cells to be compared within the same environment. Upon demonstrating that the innate Th1 cell response required IL-18 (interleukin-18) receptor signaling, we next explored how T cell extrinsic PRRs (pattern recognition receptors) elicit effector functions through IL-18 secretion. Here, we showed a dual requirement for both TLR4 (toll-like receptor 4) and inflammasome pathways after LPS stimulation during *Salmonella* infection. The convergence of these pathways was required for increased IL-18 secretion, suggesting a dual level of control in production of such a pro-inflammatory cytokine. Finally, we asked whether the innate stimulation of Th1 cells is a required response pathway during clearance of *Salmonella*. Using Lck-cre x MyD88-loxP crossed mice, we demonstrated a deficiency in bacterial clearance in the absence of the signaling molecule MyD88 within T cells, which impairs the ability of Th1 cells to respond to IL-18, but not classical antigen stimulation. Together, this data suggests that the Th1 cell response utilizes a pathway of innate stimulation to amplify IFN-γ production under situations of severe inflammation, in which the classical adaptive response pathway may not be sufficient to mediate a strong and rapid response. Future work may explore additional infectious contexts, other CD4 T cell subsets, memory responses, and circumstances of immunopathology.

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Overview

In the earliest studies of immunology the categorical distinction between innate and adaptive immunity was a critical framework for further understanding the mechanisms of each. However, as we expand our understanding of the complex networks of cells and cytokines, transcription factors and molecular memory, interactions and responses, it becomes clear that any framework we place upon the immune system is an approximation just waiting for an exception. Some of these exceptions are nominal; artifacts of culture systems or the tools available, that may ultimately prove interesting but meaningless. However, many of these exceptions prove to be details in a bigger picture we simply haven't uncovered yet. We now know that the innate and adaptive immune responses are not separate systems. They are an interwoven and often overlapping series of events that have evolved around the central goal of a simultaneously rapid, effective and controlled defense against self damage.

During an infection, pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and nod-like receptors (nucleotide-binding oligomerization domain, NLRs), recognize conserved molecular patterns- that is, molecules or motifs that could be produced during many different types of infection. These molecular patterns can be generated by the microbe itself or by the damage it inflicts. Microbe-associated molecular patterns, or MAMPs, include many common constitutents of pathogens, such as the Gram negative cell wall component lipopolysaccharide (LPS). On the other hand, damage-associated molecular patterns (DAMPs) are less well described, but include cellular products typically only found in a particular location under cellular stress or death, for example DNA in the cytoplasm or extracellular space is recognized by several PRRs as a sign of host cell damage (Tang et al., 2012). Together, these conserved molecular patterns are the first indication of a potential problem for the host, and their broad pattern recognition is a defining characteristic of the innate immune response, shown in the bottom box of Figure A-1. These innate responses are often thought of in the context of innate cell activation or early inflammatory cytokine production, for example, dendritic cell (DC) activation and production of interleukin-1 (IL-1). However, it has been

observed under various conditions that sometimes these innately recognized molecules trigger traditionally "adaptive" responses, both for TLR- (Dillon et al., 2004; Manicassamy and Pulendran, 2009; Tough et al., 1997) and NLR-recognized ligands (Dunne et al., 2010; Ritter et al., 2010; Trunk and Oxenius, 2012).

While innate immunity can be defined by its recognition of broadly conserved microbial patterns, the adaptive immune response is distinguished by the specificity and simultaneous breadth of its antigen receptors. On T cells, the T cell receptors (TCRs) rearrange during initial development to recognize a different peptide sequence for each different T cell. These peptides are presented on the surface of an interacting cell within the major histocompatability complex (MHC). TCR interaction with the specific peptide sequence and MHC recognized by it is also called cognate or antigen-specific activation, illustrated in the top panel of Figure A-1. This recognition of a specific peptide sequence/ MHC pair makes activation of an individual T cell relatively uncommon, while the recognition of many different peptide sequences by the full T cell repertoire gives T cells a powerful capacity to respond to a broad variety of potential challenges. Much of this work will focus on T cell interactions that occur subsequent to and independent of these specific TCR interactions, thus to distinguish TCR-dependent and –independent signals these interactions are often referred to as non-cognate.

Here, we focus on a classical example of adaptive immunity, the effector response of CD4 T helper subset 1 (Th1) cells during intracellular infection. CD4 T cells may differentiate into various subsets with distinct functions, but during infections with endosomally-enclosed pathogens, the ability of Th1 cells to produce IFN-γ and thereby activate macrophages is a critical requirement of pathogen control and clearance. Th1 cell activation, differentiation, stimulation, effector function, and memory formation will be discussed in greater detail in later sections, but it is important to note here that all of these topics are classically considered in the discussion of adaptive immune responses because T cells are primarily described as an adaptive, cognate-antigen specific cell type. The Th1 subset can be identified by its expression of the transcription factor T-bet, and the production of effector cytokines that include IFN-γ, TNF-α and IL-2. These functions are

typically attributed to direct interactions between T cells and APCs presenting cognate antigen, which prevents unnecessary or off-target inflammation. It has been observed, however, that T cells can also respond in a variety of different ways to non-cognate stimuli (Berg and Forman, 2006; Freeman et al., 2012; Pien et al., 2002).

Characterizing these "innate-like" responses is critical to a full understanding of T cell function, including clearance of infection and autoimmune pathologies. In this work, we discuss what is currently known about innate T cell responses, and then focus on elicitation of the critical effector cytokine IFN-γ by LPS to further elucidate a kinetic, mechanism, and role for this non-traditional Th1 response during intracellular bacterial infections.

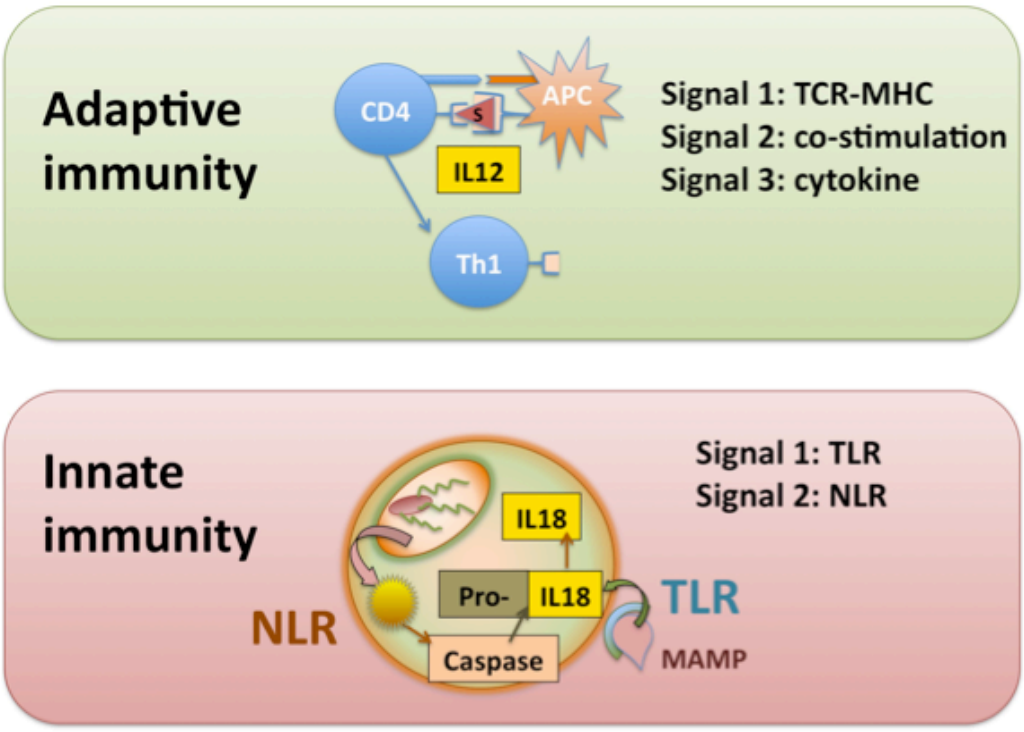

Figure A-1: Signals of conventional innate and adaptive immunity

The immune response is traditionally divided into 2 arms: the rapid, germ-line encoded innate immune responses, and the RAG-optimized adaptive immune responses. Adaptive immunity (top panel) includes the T cell response. Activation of T cells relies upon 3 key signals: TCR recognition of peptide presented in the MHC, co-stimulatory interactions, and cytokines that instruct differentiation. Innate immunity (bottom panel) may also involve multiple signals: TLRs to upregulate IL-1 family cytokine precursor proteins, and NLRs to activate the caspases that cleave 2 of the IL-1 family members, IL-1 and IL-18, prior to secretion. We reconsider this model in the discussion.

Salmonella **and other intracellular bacterial infections**

While most bacteria live and replicate extracellularly, entering the host cell only when engulfed and destroyed by phagocytes, some bacteria have adapted unique survival strategies to allow a protected life cycle within host cells. Some of these bacteria are obligate intracellular pathogens, like *Chlamydia*, that cannot replicate outside of the cell, but many bacteria are capable of occupying either space. The immune system has, in turn, developed a number of ways to recognize pathogens within cells, pathways which the pathogen actively attempts to thwart (Thi et al., 2012).

This work primarily focuses on systemic infection of mice with *Salmonella*, a gram negative enteric pathogen that resides predominantly within the phagosomes of macrophages located in the spleen, liver, and bone marrow. In humans, there are two forms of systemic salmonelloses: typhoid fever and non-typhoidal salmonellosis, or NTS. Typhoid and paratyphoid fevers are caused by the human-specific pathogens *Salmonella enterica* serovar Typhi and Paratyphi, still occur endemically in developing countries, and can cause severe systemic disease even in healthy individuals. Estimates range as high as 27 million annual infections with either Typhi or the clinically indistinguishable Paratyphi. In contrast, NTS occurs only in immunocompromised individuals, but can originate from any of the ~2000 *Salmonella* serovars capable of causing foodborne illness in humans and harbored in a wide variety of animal reservoirs (Griffin and McSorley, 2011). Thus, both systemic infections remain a source of concern for public health officials worldwide.

Although antibiotics effective against *Salmonella* are available, the options are relatively limited for intracellular pathogens as compared to more accessible extracellular pathogens. Additionally, among those antibiotics currently available there is a growing incidence of drug resistance, including multi-drug resistance to the first-line treatments, and resistance to the now standard fluoroquinolones. Further, decreased susceptibility to the fluoroquinolone ciprofloxacin has been associated in enteric fever patients with prolonged fever and increased rates of treatment failure (Crump and Mintz, 2010). Finally, an analysis of the *Salmonella* metabolic pathways has suggested that most of the

major or non-redundant pathways have already been targeted or considered for drug inhibition, suggesting a limitation to prospective future development of new antibiotic treatments (Becker et al., 2006). Together, these studies emphasize the need for alternative treatment options and for improved vaccination strategies that could lessen the need for, and consequently the selective pressure upon, traditional antibiotic therapy.

Currently, two vaccinations are commercially available in the U.S. for travelers to typhoid endemic countries. One is a Vi capsular polysaccharide (ViCPS) vaccine administered intramuscularly as one dose at least one week prior to exposure. The second is an oral, attenuated Ty21a vaccine available under several formulations, typically administered every other day as 3 separate doses two weeks prior to exposure. Both vaccinations suffer from limitations that impair their practicality in typhoid endemic regions, not the least of which is the need for regular re-vaccination, and the low reported efficacy at 3 years of 51-55% (Fraser et al., 2007). The ViCPS vaccine is approved in children over the age of two years old, and the oral vaccine for children over the age of 5 years, while repeated exposure before the age of 5 in endemic areas has been shown. In addition, while evidence suggests that Ty21a may be cross-protective for paratyphoid, the ViCPS vaccine targets an antigen that does not exist in Paratyphi and some strains of Typhi (Crump and Mintz, 2010; Pakkanen et al., 2012). Further, because the oral vaccine is a live, attenuated *Salmonella* strain, it is not suitable for use in immune-compromised patients, posing a challenge to widespread use in areas co-endemic for HIV. Thus, currently available vaccination strategies are not adequate to allow control of systemic typhoidal disease (Fraser et al., 2007). Whether currently available vaccines mediate any protection to non-typhoidal systemic diseases has not been thoroughly characterized. This data emphasizes a need to better understand the immune response during systemic *Salmonella* infections, to inform better vaccine design.

The role of Th1 cells and IFN-γ in intracellular infections

As mentioned earlier, some bacteria and other pathogens have developed the capacity to reside within cells and effectively hide from extracellular immune recognition. Often, these pathogens enter the cells initially using the cells' own phagocytic capacity, but then

are able to escape phagolysosomal degradation or escape the phagosome entirely, by a wide range of different mechanisms (Pluddemann et al., 2011; Thi et al., 2012). Given this unique lifestyle, intracellular pathogens require a special type of immune response designed to recognize infected phagocytes and mediate either killing of the infected cell or internal pathogen killing mechanisms. CD8 T cells have cell-specific cytotoxic capacity, allowing directed killing of infected cells, while both CD8 and CD4 T cells can secrete pro-inflammatory cytokines that activate phagocytes to initiate internal mechanisms of pathogen destruction.

CD8 T cells have multiple cytolytic capacities initiated by TCR interactions, including release of secretory granules and death receptor-mediated apoptosis. However, while these responses have a critical role in anti-viral defenses, their role against other intracellular pathogens is limited. (Lewinsohn et al., 2011). Of more importance to intracellular bacterial infections, CD8 can produce pro-inflammatory cytokines such as IFN-γ, which activates macrophages to undergo changes that alter the intracellular environment to become less hospitable to the invading pathogen. While CD8 T cells respond to MHC-I that mostly present antigen processed from the cytosol, CD4 T cells respond to MHC-II presented antigen on special antigen-presenting cells derived from the endocytic pathway. This allows CD4 T cells to recognize antigens from pathogens hiding inside of cells within endosomes, as well as antigens taken up from outside of the cell (Neefjes et al., 2011).

Among the various CD4 T cell subsets, the CD4 Th1 cells are the primary response to intracellular pathogens. CD4 differentiation and the other subsets will be discussed in more detail later, but in discussing the critical role of Th1 during intracellular infection it is important to first briefly define them. After CD4 T cells are activated they receive a differentiation signal that determines their cytokine profile. IL-12 upregulates the transcription factor T-bet, which is required by Th1 for IFN-γ production. (Geginat et al., 2013). Once activated, Th1 cells are programmed to secrete pro-inflammatory cytokines that include IFN-γ, TNF-α, and IL-2 upon restimulation.

IFN-γ is especially important during intracellular infection, as mentioned above, because of its critical capacity to activate macrophages to become M1, or classically activated, macrophages. M1 macrophages modify their internal environment to become as inhospitable as possible, including production of anti-microbial compounds like reactive oxygen and nitrogen species, as well as themselves secreting pro-inflammatory cytokines (Murray and Wynn, 2011). The importance of IFN-γ-mediated macrophage activation is highlighted by the effects resulting from the loss of IFN-γ or IFN-γ-inducing cytokines and transcription factors (de Jong et al., 1998; Nyirenda et al., 2010; Ravindran et al., 2005). In both mice and humans, loss of IFN-γ results in an inability to effectively clear intracellular pathogens (Jouanguy et al., 1999). Further, in humans with chronic granulomatous disease or mycobacterial granulomas, IFN-γ is an effective, albeit toxic, therapeutic (Ezekowitz et al., 1988; Holland et al., 1994; Marciano et al., 2004). Combined, these studies clearly demonstrate the requirement of IFN-γ for effective clearance of intracellular pathogens.

Innate Immunity

The innate immune system is classically defined by an early, rapid inflammatory response to broad molecular patterns (MAMPs and DAMPs). This response does not utilize recombinase-activated gene (RAG) mediated lymphocyte receptor rearrangement to improve diversity or specificity within its recognition repertoire, as is the case for the adaptive immune response. Instead, innate immune responses rely on conserved, germline-encoded receptors. As outlined in the overview, the response to MAMPs and DAMPs is mediated by a group of pattern recognition receptors (PRRs), of which the best-described examples include TLRs and NLRs, but which also includes RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and others (Broz and Monack, 2013). Many types of cells have been found to express some type of PRR and participate in innate immune responses, but of particular importance to the early immune response are the phagocytes, including macrophages, DCs, mast cells and neutrophils. During an infection, professional phagocytes engulf pathogens and dead or dying cells to mediate

pathogen killing and wound healing, and are able to utilize PRRs to become rapidly activated (Pluddemann et al., 2011).

Recognition of these patterns has evolved as an early warning system, a way for the immune system to recognize the presence of evolutionarily identified harmful situations. This allows these cells to act as the first responders to a potentially harmful situation, like an infection, but at the same time allows relatively liberal production of potentially harmful cytokines by the innately responding cell. To counterbalance this problem, these responses are generally self-limiting in strength and duration. In many situations the innate immune response will be sufficient to quickly resolve the infection, while in others it merely serves to minimize the host damage until the adaptive immune responses have had time to take over.

The role of TLRs and NLRs in the early immune response to intracellular pathogens has been well defined (Broz and Monack, 2013; Franchi, 2011). TLRs are expressed on membranes, and as such generally responsible for the recognition of extracellular pathogens and cellular debris on the cell surface or intracellular pathogens and phagocytosed material within the endosome. Endosomal TLRs, including TLR3, 7 and 9, recognize foreign nucleic acids. Cell surface TLRs recognize a variety of foreign motifs that are generally conserved across many microbes, such as bacterial cell wall and membrane components. NLRs are cytoplasmic sensors that can recognize pathogen-derived molecules or signs of cellular stress.

Many different types of molecules can be ligands for PRRs, and these can originate from bacterial, viral, or parasitic pathogens, as well as from the host itself. Upon cell damage or necrotic cell death, endogenous host ligands can be released into inappropriate locations, e.g. nuclear contents into the cytosol or cytoplasmic contents into the extracellular space. Some common examples of PRR ligands recognized during *Salmonella* infection include the recognition of LPS by TLR4 or flagellin by either TLR5 or NLRC4 (Broz and Monack, 2013). However, NLRP3 has also been shown to respond during *Salmonella*, possibly to LPS, or possibly to cell damage signals, since NLRP3 recognizes a very broad list of ligands (Broz et al., 2010).

TLRs initiate a signaling cascade via the toll-IL-1 receptor (TIR) domain containing adaptor molecules MyD88 or TRIF that ultimately result in, among other things, the upregulation of the pro-inflammatory transcription factor NFκb . While most NLR family members are involved in assembly or signaling of a cytoplasmic sensor complex known as the inflammasome, a few exceptions exist that can also promote NFκb signaling or transcriptional activation independent of inflammasome complex formation, including NOD1 and NOD2. Historically, inflammasomes are assembled by NLRs to activate caspases that process IL-1, which can promote inflammation or lead to pyroptosis, a form of programmed cell death that allows internal pathogens to be re-exposed to external recognition receptors. These responses are part of the "canonical" inflammasome pathway, however "non-canonical" inflammasomes are also described that activate other caspases. (Broz and Monack, 2013).

In particular, recent evidence has elaborated upon the interactions required for the assembly and function of the canonical NLRC4 inflammasome, which, as mentioned above, is a major PRR during *Salmonella* infection. A family of proteins called NAIPs (neuronal apoptosis inhibitory proteins) was shown to act as innate ligand sensors for flagellin and T3SS (type III secretion system) proteins. These NAIPs are then able to activate NLRC4 by an unknown mechanism, which facilitates its interaction with the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), resulting in the cleavage of pro-caspase 1 into the active caspase 1. Caspase 1 can cleave the pro-inflammatory cytokines IL-1 and IL-18 to allow secretion, or result in pyroptosis. The use of different NAIPs as sensors for different ligands as occurs in the NLRC4 inflammasome complex may apply to other inflammasomes, but remains to be explored (Broz and Monack, 2013).

While the inflammatory effects of TLR sensing of conserved microbial patterns are typically studied as a single interaction, it has been described for some inflammasomes that NLR sensing of cellular damage is not sufficient on its own to result in pro-inflammatory effects, instead requiring a priming signal from a toll agonist (Latz et al., 2013). This may be due to a requirement for toll-mediated upregulation of transcription

of the unprocessed form of inflammatory cytokines that can then be processed by NLR-induced caspases. Together, these pathways allow a rapid inflammatory response to be initiated upon recognition of generic indicators of potential danger.

Later, the concepts of "innate-like" T cell subsets and non-specific activation of conventional T cells will be addressed as innate immune functions in adaptive immune cells. There is also evidence of T cells responding directly to PRR signaling, a mechanism we will not explore in any detail here simply because it does not appear to be involved in the pathways we have studied (Reynolds and Dong, 2013). Further, it is important to note that the term "non-specific" is itself somewhat misleading, as most innate immune receptors recognize specific ligands, these ligands simply aren't specific to a single pathogen (Lanier, 2013). Overall, it seems the innate immune response has come to mean any rapid immune response not attributable to direct recognition of a rearranged lymphocyte receptor. While this makes categorization of an adaptive response simple, it disproportionately groups many complex and diverse pathways under an ill-defined umbrella. As we begin to understand these innate and "innate-like" pathways better we are likely to gain an appreciation for their critical roles throughout the immune response.

CD4 T cell development and priming

As mentioned in the overview, adaptive immune responses develop to recognize a broad range of very specific antigens, rather than a small set of conserved patterns. This development of specificity relies on RAG-mediated recombination of the recognition sequences in the lymphocyte receptors of both B and T cells. For T cells, these initial development stages take place in the thymus, at which stage these T cells are also called 'thymocytes'. In brief, thymocytes enter the thymus as double-negative for both the CD4 and CD8 co-receptors, with germline encoded TCR sequences. Genes for the γ, δ, and β TCR arms rearrange first by RAG mediated splicing and recombination of their variable regions. This results in either a strong Erk signal through a completed γδ TCR, resulting in development of a γδ T cell (discussed later), or a weak Erk signal through a pre-TCR complex of rearranged β-chain coupled to a surrogate, un-rearranged pre-TCR α chain.

Commitment to the αβ T cell lineage results in α chain rearrangement.

Rearrangement of the TCR specificity is designed to provide recognition to a wide array of antigens. However, this random process also has the potential to result in T cells that either cannot interact with the MHC on which antigens will be presented, or T cells that will recognize and react to self-peptides, resulting in autoimmunity. These problems are dealt with by positive and negative selection, respectively. Thymocytes upregulate both CD4 and CD8, becoming double-positive (DP), express low levels of TCR, and require positive selection by an interaction with an MHC. This interaction will then inform expression of the transcription factor ThPOK, which commits thymocytes to the CD4 lineage and represses Runx3, a transcription factor required for the CD8 lineage and cytolytic capacity (Cheroutre and Husain, 2013). At this stage T cells may also express Foxp3, thus committing to the natural regulatory T cell (nTreg) lineage, as opposed to the induced Treg (iTreg) subset of CD4 T cells that can differentiate in the periphery (discussed in the next section). Negative selection, on the other hand, occurs when a thymocyte is able to interact too strongly with a self antigen, and results in apoptosis. A thymocyte that successfully passes positive and negative selection will then leave the thymus, trafficking to peripheral lymph tissues as a naïve T cell.

Conventional CD4 T cell activation is initiated when a naïve CD4 T cell encounters an antigen presenting cell (APC) within secondary lymphoid tissue, such as lymph nodes or spleen. This APC may be a dendritic cell (DC) that has encountered and taken up antigen in the periphery, including sites of infection, or more likely a circulating DC that has encountered a peripheral DC and 'acquired' antigen (Reis e Sousa, 2006). Antigen sampling by DCs occurs in the periphery constitutively, and circulation within the lymphatics is continuous, regardless of infection or inflammation. Thus, it is critical that T cells be able to distinguish between the self peptides typically collected by the DCs, and peptides collected from a potentially harmful source. Since DCs collect the antigens they present somewhat indiscriminately and display many of the PRRs mentioned in the last section that allow them to recognize danger, they are a key component of the innate immune response. However, their ability to patrol the periphery and then inform T cells

of the presence of both inflammation and particular antigens makes these cells critical to the initiation of adaptive immunity as well. In this way, DCs are the first key link between innate and adaptive immune responses.

An APC first binds to a T cell by a weak interaction of cell-adhesion molecules, particularly the binding of ICAM-1 on the APC to LFA-1 on the T cell, and this interaction is usually non-productive. However, if the APC has engulfed and processed antigen, peptide will be presented on the cell surface within the MHC. Occasionally, the T cell receptor (TCR) will recognize the peptide presented in the MHC during these casual encounters and form either a stable interaction as a synapse, or a transient interaction, known as a kinapse (Dustin, 2008). This antigen-specific, or cognate antigen, interaction is known as signal 1 of T cell activation, and is illustrated in Figure A-2. While CD4 T cells interact with peptide MHC-II complexes presented on APCs, it is important to remember that CD8 TCRs recognize peptide on MHC-I instead, and that most cells have the ability to present MHC-I. The specific TCR:MHC interaction, while required, is not sufficient to induce differentiation or effector functions of the T cell on its own.

In addition, evidence has shown that the nature of this initial TCR:MHC interaction can have significant downstream effects on the differentiation pathway (Gottschalk et al., 2010; Turner et al., 2009), effector capacity, response to stimulation, and memory formation of T cells that occurs after this initial priming event. In particular, studies have examined the influence of the strength of this interaction (Moran et al., 2011; Zehn et al., 2014), the duration (Miskov-Zivanov et al., 2013; Yarke et al., 2008), the number of successive TCR:MHC interactions (Celli et al., 2005; Moreau and Bousso, 2014), the effects of antigen concentration, and the role of inflammation and other stimuli on the nature of this interaction (Malherbe et al., 2008; Zehn et al., 2014).

During this initial interaction, the naïve T cell needs to encounter additional cell-to-cell signals, called costimulatory signals, which serve as a second signal to promote naïve T cell activation. A naive T cell that receives only this first TCR signal, but not costimulation, will undergo anergy or deletion as a mechanism of peripheral self-

tolerance. Anergic T cells are generally unable to become activated upon subsequent TCR:MHC interactions, even in the presence of co-stimulation. This form of tolerance is crucial to preventing autoimmune responses from T cells that either escaped negative selection, or for self-ligands that were not able to be presented in the thymus during T cell development.

Costimulatory signals are believed to usually come from the same APC that initially presented antigen to the T cell, but this requires that the APC itself be activated, which results in the upregulation of the necessary interacting molecules on the APC. In the absence of disease DCs generally arrive in the lymph tissue in an unactivated state. In the context of infections, these DCs become activated by the innate inflammatory signals and conserved microbial patterns present at the sites of infection where the foreign antigen is collected. Activation of DCs, also sometimes referred to as 'licensing' or 'maturation', allows these cells to upregulate many of the surface molecules and chemokines required for a productive interaction with T cells, including increased MHC and LFA in addition to co-stimulatory molecules. (Reis e Sousa, 2006). Most often the activated APC upregulates B7.1 and B7.2 (also known as CD80 and CD86) to provide costimulation by interacting with CD28 on the T cell, but other activating and inhibitory co-stimulatory interactions can also occur (Chen and Flies, 2013). These first 2 signals allow T cells to survive and proliferate, but do not provide sufficient instructions for the T cells to determine a differentiation pathway. For this to occur a third signal is required, as shown in Figure A-3. This signal is typically believed to be a cytokine secreted by the same interacting APC as a result of its own activation, but potentially any cytokine present in the inflammatory milieu in sufficient quantity (Cui et al., 2009).

CD4 T helper cell subset differentiation

A number of cytokines are capable of causing differentiation, and thus delivering the 3rd and final signal for T cell activation. For CD4 T cells, different cytokines or combinations of cytokines will influence a different differentiation pathway, leading to different effector T cell subsets with distinct functions. While the differentiation resulting from signal 3 is sometimes simplified to suggest a solely cytokine-dependent decision

mechanism, it is of interest to note that costimulatory molecules, such as ICOS (Simpson et al., 2010), have been implicated in aspects of differentiation, effector function, cell survival, and memory formation. Further, as mentioned above, aspects of the initial TCR:MHC interaction can influence differentiation and effector function. These caveats suggest that this division of labor may not be as clear-cut as first appeared (Chen and Flies, 2013).

For the purposes of this work, we will focus primarily on the differentiation of the Th1 subset of CD4 T cells. When signal 1 and signal 2 are delivered to a naïve CD4 T cell, followed by interleukin-12 (IL-12), this 3rd signal causes differentiation toward a Th1 phenotype. Further, Schulz et al demonstrated that interferon-gamma (IFN-γ) can upregulate both T-bet and the IL-12Rβ during activation, but that TCR signaling actually inhibits IL-12Rβ expression, therefore the timing of this 3rd signal is tightly controlled (Schulz et al., 2009). However, while IFN-γ may promote the Th1 cell lineage by improving the capacity for IL-12 response, it is neither necessary nor sufficient on its own to cause Th1 differentiation (Haring and Harty, 2006; O'Donnell et al., 2014).

While our work focuses on Th1 cell production of IFN-γ, numerous other CD4 T cell subsets have been identified, as outlined in Figure A-4. While Th1 cell differentiation requires IL-12, Th2 cells differentiate in response to IL-4, Tfh (T follicular helper) cells to IL-6 and IL-21, iTreg (induced T regulatory) cells to TGF-β and IL-2, and Th17 cells to a combination of TGF-β, IL-6, IL-21, and IL-23 (Lazarevic et al., 2013). Another, less understood, subset of CD4 are able to acquire the cytotoxic capacity typically associated with CD8 T cells (also called CTL, or cytotoxic lymphocytes), and are thus named CTL CD4. This differentiation is believed to result from very strong TCR signaling rather than a particular signal 3 cytokine, but this requires further investigation (Cheroutre and Husain, 2013).

Each subset has a lineage defining transcription factor associated with its differentiation, but in fact each differentiation pathway results in the upregulation or suppression of many different transcription factors and cytokines. Briefly, Th2 differentiation is defined by GATA3, Tfh by BCL-6, iTreg by Foxp3, and Th17 by ROR-γt. CTL CD4 must

downregulate the thymic CD4 lineage determining transcription factor ThPOK and upregulate Runx3, typically expressed by CD8 T cells, in order to acquire cytolytic capacity (Cheroutre and Husain, 2013). These transcription factors allow expression of key effector cytokines for each subset, shown in Figure A-4. (Geginat et al., 2013; Lazarevic et al., 2013).

Th1 differentiation is characterized by the upregulation of the hallmark transcription factor T-bet, but involves the upregulation or suppression of many other genes as well. In particular, T-bet is known to suppress the transcription factors required for other T helper lineages by epigenetic modification (Lazarevic et al., 2013). These changes allow production of effector cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor- alpha (TNF-α), and IL-2, as well as inhibiting the responses typical of other CD4 T cell subsets, such as IL-10 or IL-4 production. Although this differentiation signal is required to complete activation, there is now considerable evidence that these differentiation pathways are not permanent, a capacity known as plasticity.

The process of differentiation during the initial priming events drastically alters the T cell, affecting not only cascades of transcription factors but also miRNA regulation and the epigenetic signature (Sallusto and Monticelli, 2013). All of these changes are geared toward allowing the T cell to specialize and more rapidly respond when called into action. Despite this, T cell effector function after activation requires continued regulation, both to prevent unnecessary inflammation at off-target locations and to conserve resources. Thus, both effector and memory T cells must continuously walk a fine line between a rapid and a harmful response. The secondary signals that elicit effector functions after Th1 cell activation will be the subject of a later section.

Differences between CD4 and CD8 T cell activation

When considering any T cell intrinsic mechanism or function it is important to remember that what is true for CD4 T cells is not always the case for CD8 T cells, and vice versa. This is an important distinction when considering what is or is not ‘known’ for a given T cell response. To complicate this issue, often these differences are context dependent, with different infections or inflammatory circumstances altering the relative response of

each population differently. While signal 1 in CD4 T cells occurs in response to extracellular or phagosomal antigen that has been engulfed and presented by MHC-II on APCs, CD8 T cell signal 1 is mediated by MHC-I presented antigen derived from cytosolic processing and presented on the surface of nearly all cell types. The result is a strong cytotoxic CD8 response to infections which occur inside of cells, except those contained within vesicles, which trigger MHC-II presentation and thereby CD4 responses. This allows for a partial division of labor between T cell lineages, although some critical functions overlap or provide redundancy, such as IFN-γ production.

The 3rd signal in CD8 T cell priming is not usually thought of in terms of differentiation of particular subsets with distinct functions, as seen for CD4, although functional subsets have been described. Termed Tc1, Tc2, and Tc17 in reference to their Th counterparts, these cells are found in relatively low frequency under normal circumstances, and the mechanisms driving the development of these alternative CD8 T cells remain poorly understood (Geginat et al., 2013). Signal 3 was initially identified in CD4 and CD8 T cells simply as the inflammatory cytokine(s) required to induce proliferation and differentiation to effector capacity (Curtsinger et al., 1999). Type I interferons and IL-12 have both extensively been shown to result in the survival, expansion, and differentiation of CD8 T cells, and are critical for the conventionally described CD8 T cell effector responses, including cytolytic activity and IFN-γ production. (Curtsinger et al., 1999; Haring et al., 2006; Mescher et al., 2006; Pham et al., 2011).

Additional differences between CD4 and CD8 T cell activation include, but are not limited to: a shorter required duration of antigenic stimulation in CD8 T cells (Celli et al., 2005; Obst et al., 2005; van Stipdonk et al., 2001), different transcriptional regulation, including partial redundancy for the transcription factors T-bet and Eomes in IFN-γ production in CD8, but not CD4 (Lazarevic et al., 2013), and differences in cellular trafficking and antigen surveillance (Mandl et al., 2012). Further, evidence suggests that CD4 T cells can help to initiate CD8 T cell priming (Beuneu et al., 2006; Castellino et al., 2006), and may be required for optimal CD8 memory formation, a regulatory interaction that argues against mechanistic redundancy in the activation process. Thus, while T cells

have evolved to share many similar pathways, CD4 and CD8 T cells are distinct cell types with different functions and rules to govern them.

Non-cognate activation of conventional T cells

While the strict rules governing conventional T cell activation are important to prevent unnecessary inflammation, under severe circumstances, such as occur during a rapidly dividing or systemic infection, the immune response must engage in a carefully-controlled race in which the conventional rules become a hindrance to achieving the necessary strength and rapidity of the effector response. For this reason, it may not be surprising that non-cognate interactions with conventional T cells have also been described. This is often referred to as "bystander activation" (Bou Ghanem and D'Orazio, 2011; Kamath et al., 2005; Lertmemongkolchai et al., 2001; McNally and Welsh, 2002), seemingly in reference to the idea that these are cells which happen to be in proximity to the necessary stimuli, perhaps intended for other cognate-specific cells engaged in direct interactions, thus making the non-cognate cell an unintended 'bystander' of the conventional response. However, it is equally possible that this bystander response is not incidental, but an integral functional capacity of T cells to recognize extreme stress.

In considering sources of activation for T cells, it is important to recognize that the ambiguous use of the word "activation" in reference to interactions encountered by T cells is a source of confusion in the literature. Activation is sometimes used to describe: 1) the initial process undergone by naive T cells in secondary lymphoid organs that results in proliferation and emigration to sites of infection, as described above 2) the secondary stimulation of previously activated effector T cells that occurs upon arrival at sites of infection, which will be explored in more detail in the next section and 3) the re-activation of resting memory T cells, which will be discussed later. Figure A-5 looks at each of these circumstances and compares conventional T cell interactions to bystander or non-cognate and innate-like T cell interactions. While the word activation has been applied to each circumstance, significant differences exist between each of these types of T cell interaction. Further exploration of the inherent requirements of each type of T cell interaction requires a more detailed understanding of how the existing evidence applies to

each circumstance separately.

The earliest descriptions of bystander activation focus on cytokine or innate stimuli inducing T cell proliferation in the absence of antigen (Kamath et al., 2005; McNally and Welsh, 2002; Tough et al., 1996). However, most of this work was done in viral infection models, focuses on CD8 T cells, and does not differentiate between naïve and previously activated T cells. Further, these data must be interpreted with caution, since cytokine-induced proliferation may not always lead to an effector T cell state, and especially given the evidence that these same signals can induce apoptosis (McNally and Welsh, 2002). Transient expression of the early activation marker CD69 was observed in naïve CD8 after Type I IFN stimulation, but this activation was not maintained nor was it shown to induce effector functional capacity (Kamath et al., 2005).

There is also considerable work describing elicitation of effector functions from CD8 T cells by non-cognate stimuli, also often referred to as bystander activation or stimulation (Freeman et al., 2012; Lertmemongkolchai et al., 2001; Raué et al., 2004). However, it is important to note that this work generally describes stimulation of previously activated T cells, or makes no distinction between activated and naive T cells, thus this is not activation in the sense of initial T cell priming. Bystander stimulation of effector and memory T cells will be discussed in more detail in a later section, and the intrinsic differences between naive and activated T cells was discussed briefly already.

Even less evidence for TCR-independent stimulation exists specific to CD4 T cells, and most show that bystander responses occur only in previously activated or memory CD4 T cells (Bangs et al., 2009; Chapman et al., 2005; Di Genova et al., 2010). There is, however, limited *in vitro* data showing that very high doses of IL-2 can make naïve CD4 T cells responsive to IL-12 or IL-18 without TCR signaling (Chakir et al., 2003). Considering these distinctions, as well as the inability of many of these studies to rule out innate-like populations that were not yet identified at the time the studies were conducted, the question of whether naive T cells can be primed in a non-cognate manner under sufficient inflammatory stimulation, and whether these cells would be functional as effectors *in vivo*, lacks a definitive answer. The work described herein does not attempt to

address this question, but rather examines non-cognate secondary stimulation at sites of infection, which for clarity we will not refer to as activation.

Secondary stimulation of T cells

Conventional T cell activation in secondary lymphoid tissues, as outlined above, results in trafficking of activated T cells to the sites of infection, where their effector functions are needed. A T cell that has been activated is now ready to respond rapidly upon additional stimulation, but does not constitutively secrete cytokines. At the site of infection, an activated T cell may encounter additional cognate antigen presented by APCs there, and this second specific interaction will trigger a robust effector response. Unlike the initial activation process, this antigen-specific interaction alone is sufficient to induce cytokine production, because of the T cell's activated state (Honda et al., 2014).

Several works in the past five years have examined this requirement for additional stimulation at the site of infection, focusing on the necessity of local cognate antigen presentation to induce effector responses. Particularly, McLachlan et al showed in vivo that T cells activated in response to an antigen and adjuvant will produce effector cytokines when antigen is readministered in one ear, but do not produce cytokines to irrelevant antigen given in the other ear. Further, the effector response can be halted by blocking the TCR interaction with the antigen. (Egen et al., 2011; McLachlan et al., 2009).While these studies demonstrate that T cells do not arrive in the periphery still producing cytokines from their initial activation, and that secondary antigen interactions are sufficient to promote effector responses, they do not conclusively rule out other mechanisms for effector T cell stimulation. In addition, the T cells are activated by an antigen and adjuvant combination, which models the low inflammatory conditions of a vaccine rather than the highly inflammatory environment of an infection. The impact of inflammation on T cell programming at every stage is still an area poorly understood, but it is possible that as we learn more about these interactions we will uncover some of the pathways that have so long separated vaccine efficacy from the immunological memory that follows infection.

For a number of reasons discussed later, an activated T cell may never encounter its

cognate antigen at the site of infection. In fact, Egen et al demonstrate that T cells in mycobacterial granulomas have limited encounter with their cognate antigen, and suggest that this is the reason for their limited production of effector cytokines. They further show that providing additional antigen leads to increased production of IFN-γ, and deduce that antigen presentation is a limiting factor within granulomas that prevents T cell activation (Egen et al., 2011). Again, while this data provides evidence that antigen at sites of infection can elicit T cell production of effector cytokines, it does not rule out other mechanisms of T cell stimulation. In fact, as mentioned earlier, administration of T cell stimulatory cytokines or IFN-γ can resolve granulomas. Thus, if antigen presentation is limited within granulomas, it stands to reason that the known effects of these treatments may indeed be antigen-independent.

Further, it has been shown that myeloid cells deficient for MHC II have higher mycobacterial loads than their MHC II- sufficient counterparts within the same host (Srivastava and Ernst, 2013). This suggests that the resolution of infection within these myeloid cells requires contact-dependent activation. However, as in the works mentioned above, this study cannot differentiate between the requirement for an initial cognate activation interaction and subsequent interactions, although in this case the signals in question are those to the infected cells, not the T cells. Thus, it is possible that myeloid cells, once activated in a contact-dependent manner, can then respond to CD4 T cell cytokine production at the site of infection. Intravital imaging in mice during *Leishmania* infection supports this idea, showing that macrophages within an 80 micrometer radius of CD4 T cells producing IFN-γ contained higher levels of iNOS (inducible nitric oxide synthase) without requiring direct contact with the T cells (Muller et al., 2012). However, it is also important to note that in the latter study a significant difference in pathogen burden was not observed in MHC II- deficient phagocytes, supporting the suggestion of Srivastava and Ernst that different pathogens may elicit different response requirements. Taken together, the above studies demonstrate a role for cognate-specific interactions, but do not rule out a role for non-cognate interactions.

Additionally, when time is a limiting factor, waiting for repeated APC: T cell interactions

to result in a productive encounter in a chaotic inflammatory environment may not be practical. For these reasons, a non-cognate mechanism of stimulation for T cells that have already been activated in a conventional, cognate-specific manner is both logical and probable. As detailed in a separate section below, this response is well recognized for many types of lymphocytes described as "innate-like", but for conventional T cells the concept of an innate response is not well described as such. These cell sets and their responses to different stimuli are outlined in Figure A-5.

As mentioned earlier, much of the literature pertaining to "bystander activation" describes responses of previously activated T cells, particularly CD8 T cells due to the predominant use of viral infections within this area of study. Much of this work has focused on the proliferative capacity of T cells, largely in response to common gamma chain cytokines or Type I IFN, which is an important point in the early amplification of an effector response . However, it is also important to define whether these proliferating cells actually go on to be functional effectors in an *in vivo* setting, and to consider the rate of apoptosis alongside proliferation (Curtsinger et al., 2003; McNally and Welsh, 2002). The work herein will focus on bystander stimulation during a later phase of the effector response: the elicitation of the effector functions. In CD8 T cells this can mean a variety of things, from secretion of effector cytokines, to the production of granzyme and cytolytic functions. For CD4 Th1 cells, elicitation of the effector response is determined by production of the effector cytokines IFN-γ, TNF-α, and IL-2.

As discussed above, T cells are believed to secrete IFN-γ at the site of infection while directly engaged by an infected APC, allowing this cytokine to be released in a targeted manner, thus containing the inflammatory effects at the synapse. In fact, *in vitro* evidence shows that secretion of certain cytokines like IFN-γ, but not others like TNF-α, is polarized toward the TCR/ MHC synapse (Huse et al., 2006). However, a large body of *in vitro* literature now supports the idea that a previously activated T cell can also be stimulated without direct cognate-interaction in the presence of sufficient inflammatory signals. In particular, IL-12 and IL-18 are known to stimulate IFN-γ production, and indeed IL-18 was originally called interferon-gamma inducing factor (IGIF).

Nonetheless, a multitude of cytokines in various combinations have now been shown to elicit IFN-γ production from T cells. Most of this literature looks at CD8 T cells and *in vitro* stimulations (Berg and Forman, 2006; Freeman et al., 2012). Alternatively, previous work in our laboratory explored the role of IL-18 in LPS stimulation of Th1 cells (Srinivasan et al., 2007). While all of this work together suggests the possibility of a mechanism wherein T cells are innately stimulated in order to quickly amplify the adaptive response, an exact mechanism and role for this innate-like CD4 Th1 response has not been well characterized.

Innate stimulation of memory versus effector T cells

Thus far, we have discussed how naïve CD4 T cells develop in the thymus, travel to secondary lymphoid tissue, encounter APCs there, and require 3 specific signals for conventional activation. After a naïve CD4 T cell 1) recognizes a specific peptide sequence presented on MHC-II with its TCR, 2) receives co-stimulatory signals such as the CD28 interaction with CD80/86, and 3) receives IL-12 signaling this CD4 T cell will proliferate and differentiate into a Th1 cell. These cells upregulate surface markers and chemokine receptors that allow them to leave the secondary lymphoid tissues and travel to sites of infection, as well as altering their expression profiles for a myriad of transcription factors and cytokines. These changes render the effector cell capable of rapid response upon secondary stimulation at the site of infection, limiting the production of cytokines to the locations they are most needed. While this secondary stimulation is usually thought to be TCR-dependent, there is evidence for a TCR-independent mechanism as well.

This expansion of specific effector Th1 cells typically takes a few days, and in prolonged infections like *Salmonella* this T cell response can take a few weeks to reach the peak of expansion. During this time T cells are responding to a complex network of signals: IL-7 to survive, IL-2 to proliferate, pro-inflammatory cytokines to strengthen responses, anti-inflammatory cytokines to dampen responses, and potentially TCR signals. The combination of these encounters does more than just stimulate T cells to produce effector cytokines- it establishes their fate. While T cell responses are critical to pathogen

clearance in many cases, they are also highly toxic. For this reason, most T cells will ultimately be instructed to die. After the peak of expansion T cells undergo a contraction phase in which most T cells receive apoptotic signals.

However, some T cells will receive just the right combination of stimuli and survival signals to transition from an 'armed and ready' effector state to a quiescent memory state. CD4 T cells can actually exist in a number of different memory states, which may ultimately effect their longevity, the areas in which they circulate, and the requirements for re-activation. The best described examples of CD4 memory subsets are the central versus effector memory T cells, which circulate in lymphoid tissue or non-lymphoid tissue, respectively. CD8 T cells are also believed to form these subsets, as well as memory populations called short-lived effector cells (SLECs) or memory precursor effector cells (MPECs) whose formation depends heavily on the inflammatory signals received, but which have not been described for CD4 T cells. Memory T cells are an important component of the rapid response to re-challenge with previously encountered pathogens because of their lowered activation threshold, pre-differentiated state, and extensive modifications that allow for rapid relay of the signals needed to elicit effector function. Understanding how these T cells are formed and are able to respond is especially crucial to vaccine design.

Further complicating the issue of non-cognate T cell stimulation is the lack of understanding as to how this response differs between effector and memory T cell populations, or how this response would impact memory T cell formation. The dramatic changes undergone by T cells in the transition from naïve to activated state, discussed briefly earlier, have been well studied. However, of equal importance and less clarity is the question of the cell-intrinsic changes that differentiate effector and memory T cell populations, how these populations are generated, and how these differences impact response to stimulation. Particularly, while recent work has begun to address these questions in CD8, very little is known about the response threshold of memory CD4 T cells (Lazarevic et al., 2013). Given that much of the work for the CD8 response to inflammation is in the context of the fate decision signals that lead CD8 to become either

SLECs or MPECs, whereas such populations have not been described in CD4 memory T cells, it will be important to examine the effects of non-cognate stimulation of effector CD4 on memory formation separately from studies of CD8 T cells (Plumlee et al., 2013). Further, the mechanisms and requirements for non-cognate stimulation of CD4 memory T cells and CD8 memory T cells is likely to differ, as are the specific signals required to stimulate the different CD4 memory subsets, whether effector versus central memory, or Th1 versus Th17 primed. While CD4 memory T cells retain transcriptional memory of priming, elicitation of their responses may also be susceptible to the plasticity seen in effector CD4 T cells, a fact which is likely to play an important role in the effects seen by non-cognate restimulation of memory CD4 (Lazarevic et al., 2013; Pepper and Jenkins, 2011).

Non-conventional T cells and ILCs

Although conventional T cell activation occurs in the highly regulated, antigen-specific manner described above, there are a number of exceptions to these rules. In particular, a number of cell subsets have been described that have overlapping surface markers, developmental lineages, transcription factor profiles, or effector functions to conventional T cell subsets, but which respond in a non-conventional manner (Lanier, 2013). These cells are often thought of as innate-like cells with adaptive-like functions that can provide critical assistance in the early immune response. Examples include natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and innate lymphoid cells (ILCs), but this list will undoubtedly continue to expand as the sensitivity of cell detection techniques continues to improve.

ILCs are a rapidly expanding group of cells defined predominantly by their lack of lymphocyte antigen receptors (TCR/BCR) or lineage-specific markers (Lanier, 2013). Recently, a uniform nomenclature for ILCs was proposed that divides the various cells into 3 main groups. Much like the CD4 T helper subsets, Group 1 ILCs can be characterized by the expression of T-bet and IFN-γ, Group 2 express GATA3, and Group 3 express ROR-γt and produce IL-17 or IL-22. (Spits et al., 2013). Most ILCs require IL-7R signaling and express the surface marker CD90 and the transcription factor ID2,

although it has been proposed in mice that IL-7 may inhibit transition of ILC3 to ILC1 (Walker et al., 2013). Given the focus of this work on Th1 and IFN-γ production, further elaboration will center on the Group 1 ILC subset.

Natural killer (NK) cells have been included within this nomenclature as a Group 1 ILC, alongside ILC1. While NK cells have been very well described as an early source of IFN-γ and TNF-α, in addition to their cytotoxic functions, very little is known about ILC1. Although there is evidence that they develop from ILC3s after IL-12 stimulation, it was recently shown that ILC1 can also develop independently from a common innate lymphoid progenitor (Klose et al., 2014). However, whether they arise separately or as a consequence of functional plasticity, it remains that there is a group of T-bet^{+} ILCs which can respond to IL-12 and IL-18 signals to produce IFN-γ, but which are not NK cells and do not exhibit cytotoxicity (Spits et al., 2013; Walker et al., 2013). As a whole, the early innate effector responses of the Group 1 ILCs during intracellular infections play a key role in host protection, inflammation and initiation of adaptive responses.

While ILCs lack a TCR, several cell types express unique TCRs that allow for a non-conventional response, known collectively as "innate-like T cells" due to their ability to respond rapidly to innate stimulation. Among these innate-like T cells, one common method allowing for a non-cognate-antigen response is an invariant or semi-invariant TCR. While conventional TCRs undergo RAG (recombination activating gene) dependent rearrangement of their α and β chains during development in the thymus to allow for a broader repertoire with improved specificity, some innate-like cell populations possess TCRs with single α-chain and restricted β-chain specificities. Particular examples of cells with these alternative TCRs include invariant NKT cells (iNKT) and MAIT cells (Lanier, 2013).

NKT cells are perhaps the best described of these innate-like T cell subsets. NKT cells are innate-like T cells in the sense that they develop in the thymus and express a TCR, but they also express NK1.1 and several innate activating or inhibtory receptors typically found on NK cells (Brennan et al., 2013). Two types of NKT cells exist: NKT I are the well-described, invariant NKT cells known to respond to lipids and especially with high

affinity to α-galactosylceramide presented by the MHC-related molecule CD1d, while NKT II are less studied to date, but have a diverse TCR repertoire and fail to respond to α-galactosylceramide (Gapin et al., 2013). While iNKT can be activated by CD1d presentation of foreign lipid antigens, they may also be activated by CD1d presentation of lipid self-antigens and require inflammatory cytokine signals, allowing for more rapid and innate-like responses (Brennan et al., 2013; Brigl et al., 2011; Gapin et al., 2013).

Another semi-invariant T cell population are the recently described MAIT cells, characterized by their localization to mucosal tissue and their recognition of the MHC-related molecule, MR1, which binds the metabolites of B vitamins generated by bacteria and fungi (Lanier, 2013). MAIT cells develop and are pre-programmed in the thymus, but quickly acquire an activated phenotype in the periphery. There is now evidence to suggest that this activation occurs in response to microbiota; in particular, the observation that germ-free mice have diminished numbers of MAIT cells, which can be recovered upon monoculture reconstitution with many bacteria or yeast, but not *Enterococcus faecalis*, which lacks the riboflavin metabolic pathway. Unlike NKT, they seem to respond predominantly to TCR ligation and do not require cytokine stimulation to elicit effector functions, which consist mostly of IFN-γ and TNF-α, although they can also express IL-17 (Gold and Lewinsohn, 2013; Le Bourhis et al., 2013)

γδ T cells are a unique exception, in that they possess recombined TCRs, but can respond in a rapid, innate-like manner to inflammatory cytokines. Thus, these T cells are technically a component of the adaptive immune response, but are often discussed in the context of early innate responses (Vantourout and Hayday, 2013). Differentiation programming of γδ T cells occurs during thymic development, determining either an IFN-γ, IL-17, or IL-4 producing phenotype (Narayan et al., 2012), but peripheral activation is still required before effector functions can be elicited. The relative contributions of TCR, costimulation and cytokine signals to this activation still seem to be a matter of some debate, and may be partially dependent on the subset, but whatever the mechanism these cells respond far more rapidly than their αβ T cell counterparts (Katsikis et al.; Vantourout and Hayday, 2013).

All of these innate-like T cells (iNKT, MAIT, and γδT), although possessing different TCRs and recognizing different antigen repertoires, share some common features. For one thing, in each cell type the ability to generate or maintain immunological memory is poorly defined, as are the required signals for survival and proliferation (Lanier, 2013). The observation that MAIT cells *in vitro* do not proliferate in response to the conventional common-γ chain cytokine signals, but do proliferate somewhat in response to IL-1 family cytokines, highlights the likelihood of differences in these pathways between conventional T cells and their innate-like counterparts, and the need to elaborate these mechanisms further (Le Bourhis et al., 2013).

For both iNKT and γδT cells functional subsets have now been described analogous to the CD4 T helper subsets (Brennan et al., 2013; Narayan et al., 2012), although unlike CD4 T cells these subsets are pre-determined during development in the thymus. The Th1-like subsets that are of greatest interest to this work respond strongly to IL-12 to produce IFN-γ, and utilize the transcription factor T-bet with some similarities to Th1 (Lazarevic et al., 2013). Although subsets have not yet been defined as such for MAIT cells, and they typically respond to IL-12 to produce IFN-γ in a T-bet-dependent manner, they also express ROR-γt and can express IL-17 and IL-22 under appropriate stimulation. Further, there is now some evidence for an immunoregulatory function of MAIT cells. Thus, whether MAIT cells have functional subsets or are simply functionally promiscuous remains to be determined (Gold and Lewinsohn, 2013; Le Bourhis et al., 2013).

In further similarity, each is described as "innate-like" due to an ability to rapidly respond to innate stimulation- that is, they respond to the inflammatory cytokines that result from innate stimuli. However, for each cell subset the specific requirements of initial priming, and in particular whether this priming can occur without any peripheral TCR stimulation, is still a matter of debate within their respective fields. While earlier literature suggested that these cells respond rapidly because they are able to respond to cytokine alone, other work shows that these cells require TCR interactions (Ribot and Silva-Santos, 2013), and for iNKT at least this requirement can be met by self-antigens under inflammatory

conditions to allow more rapid responses (Brennan et al., 2013; Brigl et al., 2011; Gapin et al., 2013). Herein, we make an argument that conventional αβ T cells can also respond rapidly to inflammatory cytokines in an innate-like manner once they have been primed, suggesting a conservation of these stimulatory mechanisms between conventional and innate-like T cells, and highlighting the need for a better understanding of the activation requirements of non-conventional T cells.

Relevance of innate T cell stimulation

Finally, in considering the mechanisms that might allow T cells to respond in a non-cognate manner, it is important to address the reasons it might be beneficial for a conventional T cell to have retained these functions during evolution. In other words, why might a T cell need to respond to a non-TCR signal? Further, why should a T cell require a TCR signal at all? What is lost and what is gained when a T cell bypasses the strict antigen-specific regulation of the TCR?

The strong inflammatory capacity of the T cell response is mediated in part by a massive capacity for proliferation and cytokine (or other effector) output, but this strength comes at the cost of a severe inflammatory state. Excess inflammation can itself cause cellular damage and can lead to sepsis and shock. For this reason, the strongest responses are regulated to occur only in the direst circumstances. In T cells, one way to localize the effector response is to require direct contact with antigen, thus ensuring the cytokine is only made when and where it is needed. Most of the time this is a logical checkpoint, but what if direct contact with the antigen at the site of infection either cannot occur, or is too limited to facilitate the magnitude required, as discussed earlier?

In fact, many pathogens use this strategy to evade the immune system. *Salmonella*, for example, quickly downregulates many of the antigens that would be recognized by the early T cell response (Lee et al., 2012). The result is large numbers of T cells at the site of early infection primed to respond to antigens that do not exist at the site of infection. Further, while the lymph tissue is designed to streamline movement of APCs and T cells in such a way as to facilitate potential interactions for initial activation, most sites of infection are inflamed and chaotic battlegrounds, wherein finding an APC presenting the

appropriate antigen will, at best, take time that the host may not have (Masopust and Schenkel, 2013; Moreau and Bousso, 2014).

In addition to immune evasion strategies that involve limiting antigens, many strategies of limiting the MHC molecules or their ability to present antigens have been described, with a similar outcome- T cells activated against pathogens that they cannot re-encounter (Bueno et al., 2012; Cheminay et al., 2005; Lapaque et al., 2009). Further, evidence has shown the ability of *Salmonella* to downregulate TCR expression on T cells, which would impair initial priming, but could also prevent cognate secondary restimulation (van der Velden et al., 2008). Many evasion mechanisms of both innate and adaptive immunity have been described for *Salmonella*, as just one example demonstrating an acquired capacity to evade the immune system at just about every turn (Fabrega and Vila, 2013; Jantsch et al., 2011).

Further, while work in the lab reasonably requires limiting variables, infection in nature is content to be as confusing as possible. Thus, it is unlikely that immune responses evolved with a design to combat only one infection at a time, given the selective pressure of many simultaneous infections. In fact, 50 years ago Mackaness described the bystander capacity of macrophages activated in response to one intracellular pathogen to lower the bacterial loads of a second intracellular pathogen (Mackaness, 1964). Bystander responses of innate immune cells during co-infection or polymicrobial infections have become well accepted because the mechanism of cross-reaction in a non-specific response is easily explained. On the other hand, non-cognate T cell responses are often viewed as an abnormal occurrence, a breakdown in the specific regulatory capacities that keep these effector responses in check. However, we now know that a number of T cell types exist with the integral functional capacity to respond in a rapid "innate-like" manner, as detailed earlier. Given our current understanding of the mechanisms that govern these innate-like responses, it seems wholly feasible that not only are T cells capable of responding to antigen-non-specific signals of threats in a crisis, but that this capacity evolved as an important facet of their responsiveness and plays an important physiological role.

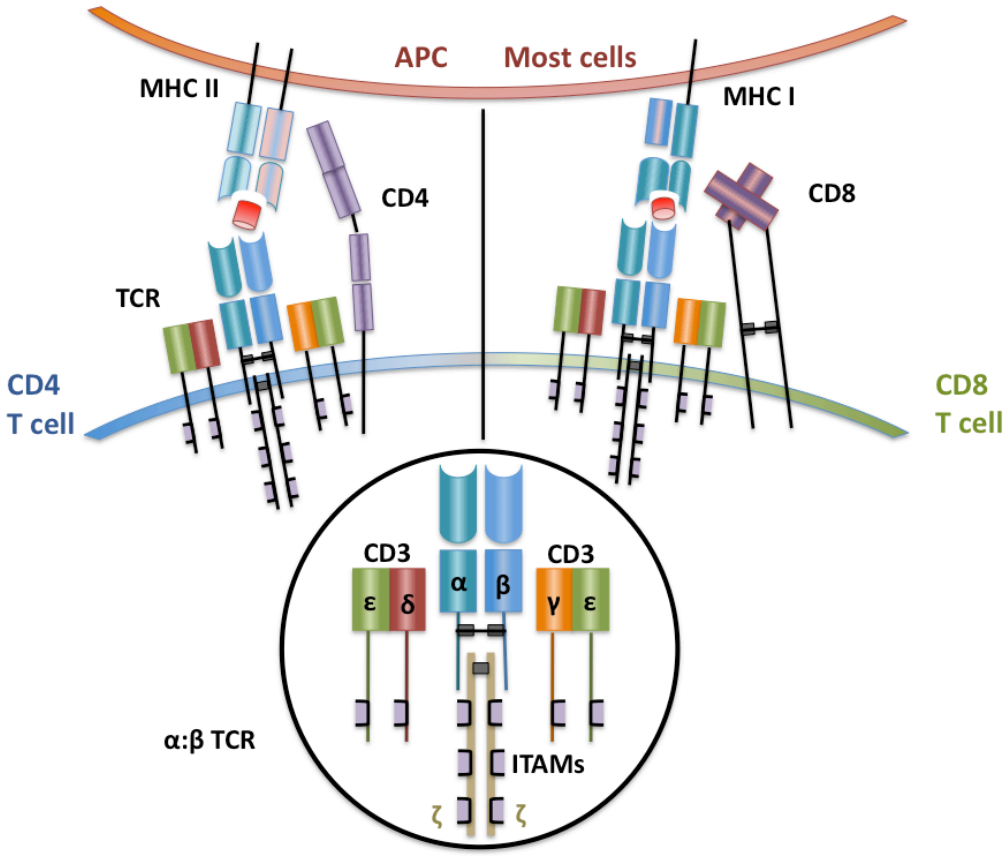

Figure A-2: TCR: MHC interactions on T cells

The T cell receptor (TCR) on CD4 T cells recognizes peptide sequences presented on MHC-II by APCs. They are identified by the cell surface molecule CD4, which interacts with MHC-II to stabilize the TCR: MHC interaction, as shown above. CD8 T cells interact with peptide on MHC-I, which is present on most cells, and this interaction is stabilized by the CD8 cell surface molecule. The inset shows the structure of the α:β TCR, which consists of rearranged α and β peptide recognition chains, a CD3 domain flanking each side, and internal ζ signaling chains. Both the CD3 and ζ chains contain signaling motifs known as ITAMs (immunoreceptor tyrosine-based activation motifs) whose phosphorylation initiates the TCR signaling cascade.

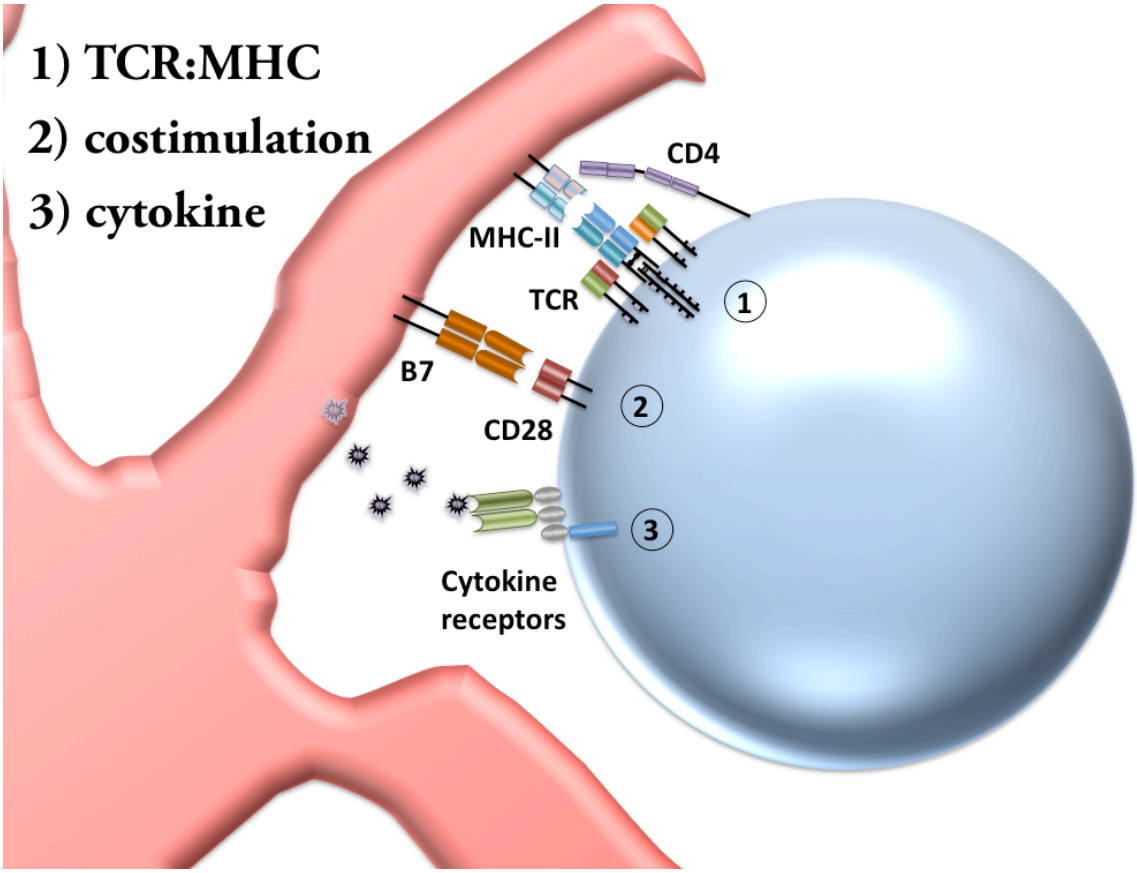

Figure A-3: Priming of CD4 T cells requires 3 signals

Conventional activation of naïve CD4 T cells requires 3 distinct signals. Signal 1: the TCR (T cell receptor) on the T cell must recognize a particular peptide sequence, processed within an APC (antigen presenting cell) and presented by MHC-II (Major Histocompatibility Complex- II) in mice. The CD4 co-receptor shown stabilizes the T cell interaction with MHC-II. Signal 2: activated DC (dendritic cells) upregulate the costimulatory molecules B7.1 and B7.2 (also called CD80 and CD86). CD28 on T cells recognize these costimulatory molecules as a second signal for activation. In the absence of costimulation T cells undergo anergy or death. Signal 3: the cytokine environment instructs the final stage of T cell priming by determining the differentiation pathway undergone by the activated T cell. These differentiation pathways are outlined in Figure A-4.

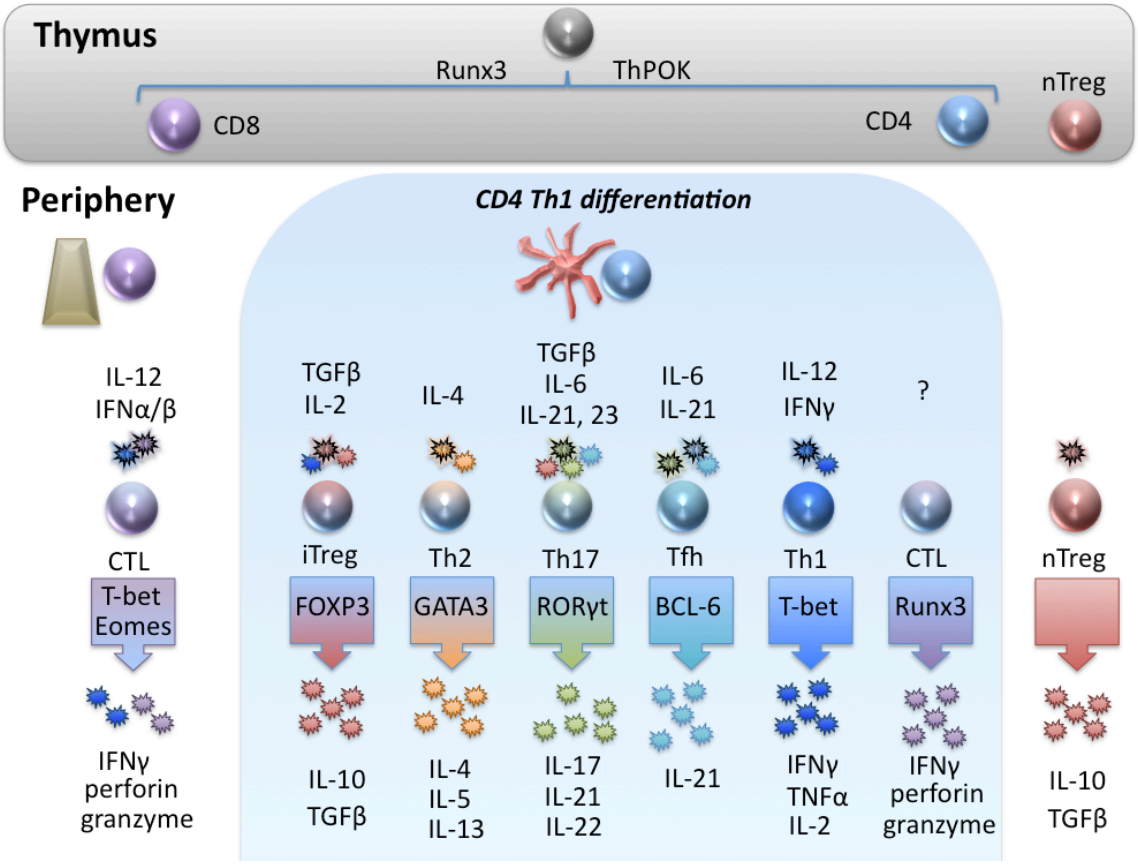

Figure A-4: Development and differentiation of CD4 T cells into helper subsets

T cell lineage is determined in the thymus, where these T cells are referred to as thymocytes. During thymic development these thymocytes receive signals that will either reinforce expression of the transcription factor ThPOK and suppress Runx3, leading to the CD4 lineage, or lose ThPOK expression, allowing upregulation of Runx3 and CD8 lineage determination. Some of these CD4 T cells will upregulate FoxP3 in the thymus to become natural Tregs (nTregs), while most will migrate to secondary lymphoid tissues as naïve CD4 T cells. Naïve CD4 T cells are activated upon interaction with an APC, leading to a TCR:MHC interaction and co-stimulatory signals, as described in Figure A-3. These T cells then need a third cytokine signal to inform differentiation, shown above. The cytokines listed in the top of the blue inset instruct CD4 T cells to differentiate into the cell types listed below them, requiring upregulation of the lineage-defining transcription factors shown in the boxes. The transcription factors then lead to production of the hallmark effector cytokines listed below the arrows, at the bottom of the blue inset. For example, IL-12 serves as a third signal to cause CD4 T cells to differentiate into Th1 cells that upregulate T-bet and secrete IFN-γ, TNF-α, and IL-2, illustrated in dark blue. The cytokines leading to CTL differentiation are listed as a question mark because this cell type is only recently described and these signals are still poorly understood, but the current theory is that TCR signal strength may actually be the driver for differentiation of this subset.

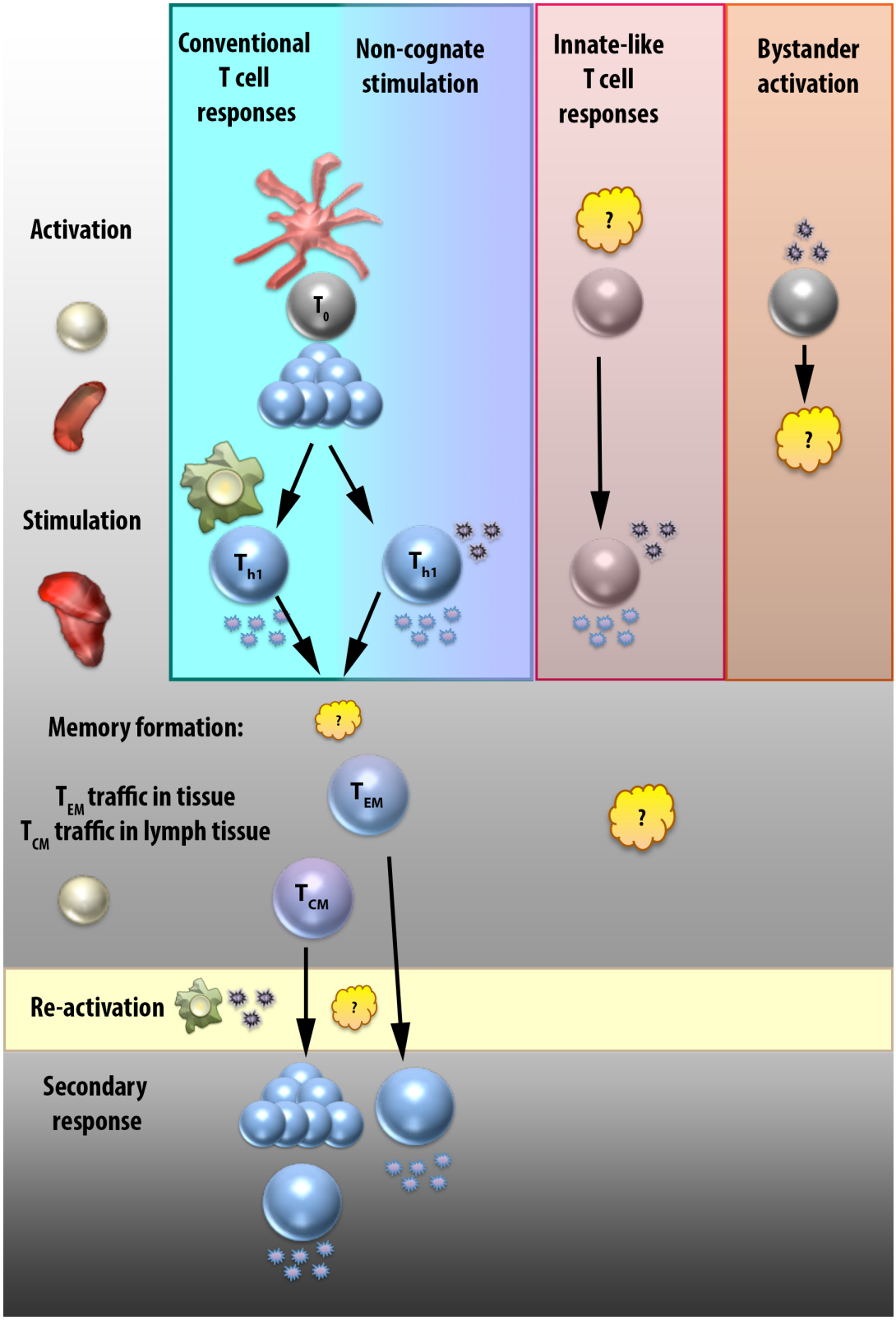
Conventional T cell responses
Non-cognate stimulation
Innate-like T cell responses
Bystander activation
Activation
Stimulation
T0
Th1
Th1
?
?
?
Memory formation:
TEM traffic in tissue
TCM traffic in lymph tissue
TEM
TCM
Re-activation
Secondary response

Figure A-5: Elicitation of CD4 and 'innate-like' T cell effector functions
T cell effector functions can result from interactions with APCs or cytokines at various stages. In the conventional T cell response (first column), naïve T cells are activated by direct interactions with APCs in the secondary lymphoid tissues, proliferate, migrate to sites of infection, and then are stimulated by secondary direct APC contact to produce effector cytokines. Some of these effector T cells will go on to become memory T cells. T central memory (T_{CM}) cells circulate in secondary lymphoid tissues, and upon reactivation will once again proliferate and differentiate into effector cells. T effector memory (T_{EM}) cells can either migrate in the periphery or be resident in tissue, and respond more rapidly than the T_{CM} cells because upon reactivation they can secrete effector cytokines directly. The signals that result in the T_{EM} or T_{CM} fate decision are still unclear, as are the interactions required by each for reactivation, although it is presumed that reactivation occurs after direct interaction with an APC.

Non-cognate stimulation (second column) occurs at sites of infection in T cells that have already undergone conventional activation in the secondary lymphoid tissues. Instead of being stimulated by secondary direct APC contact, these cells receive stimulatory signals from cytokines that induce IFN-γ production. Whether these cells go on to join the memory pool and how this different stimulation signal affects the fate decision of CD4 T cells is unknown. Although the initial priming signals for the different innate-like T cell populations vary and are still unclear in some cases, they include alternative activation mechanisms such as restricted TCRs or constitutive priming. Stimulation of effector responses at sites of infection in innate-like T cells is known to occur rapidly in response to cytokine stimulation, hence the name 'innate-like' T cells. However, it is possible that all T cells have the capacity, once activated, to respond rapidly to cytokine stimulation, and that what really separates these innate-like cell types are their unique priming mechanisms.

Bystander activation is a term that has been used loosely to mean any TCR-independent T cell stimulatory interaction. In the last column, we focus on the idea of bystander activation as a mechanism to prime a CD4 T cell in a TCR-independent manner. While the effect of cytokines on naïve T cells have been studied at length in vitro, there is limited evidence that a naïve CD4 T cell can be activated by cytokine signals alone, and no evidence that TCR-independent activation can produce a fully functional effector T cell.

Chapter 1:
What triggers innate stimulation of Th1 cells?

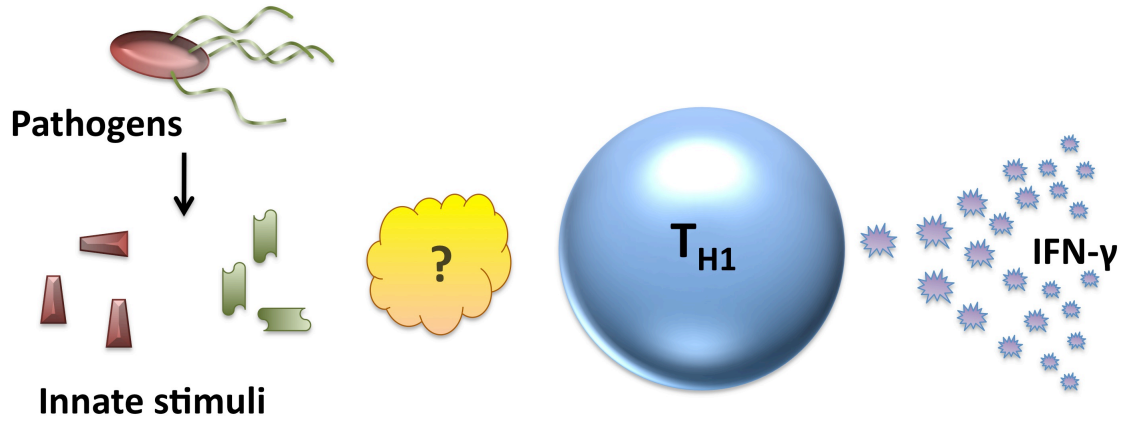

Figure 1-1: Innate ligands cause Th1 cells to produce IFN-γ

Pathogens result in the production of PAMPs and DAMPs. These innate stimuli are known to elicit pro-inflammatory functions from innate cells, like NK cells and DCs. However, activated CD4 T cells can also respond to these stimuli. Large numbers of Th1 cells can produce IFN-γ in response to LPS during *Salmonella* infection. In this chapter we extend that observation to *Chlamydia* infection and other innate ligands, and examine the kinetics of the innate Th1 response over time.

Previous work from our laboratory examined the ability of LPS to stimulate Th1 cells to produce IFN-γ during *Salmonella* infection (Srinivasan et al., 2007). Further, as discussed above, a number of non-cognate interactions have been shown to amplify T cell responses, although these have typically looked at CD8 T cells (Freeman et al., 2012; Raué et al., 2004). In this chapter, we focus on Th1 cells to examine the kinetics of this response and determine under what circumstances Th1 cells can be innately stimulated. We show that while activation and differentiation of Th1 cells are not altered by administration of innate stimuli, various ligands of extracellular TLRs can elicit strong IFN-γ production from Th1 cells. Further, LPS stimulation is dose-dependent, occurs throughout *Salmonella* infection, and during *Chlamydia* infection as well. These findings illustrate the versatility of the Th1 response to innate stimuli and suggest a broad application for this response pathway.

Results

Th1 cell activation and effector function after LPS stimulation

Innate amplification of Th1 effector responses was primarily explored within our work in the context of a model of systemic salmonellosis, because of the strong CD4 T cell response that occurs during this infection. To establish systemic salmonellosis, an attenuated strain of *Salmonella* (BRD509) was administered i.v. to C57BL/6 mice. At 2 weeks post-infection the CD4 T cell population has expanded and differentiated into a strong Th1 response, thus we administered LPS stimulation at this timepoint i.v. and waited 4 hours to examine the effect of this stimulation on Th1 cell IFN-γ production, as assessed by flow cytometry.

In order to describe this response further, we first showed that during *Salmonella* infection the CD44hi population of both CD4 and CD8 T cells produce IFN-γ in response to 4 hour in vivo LPS stimulation, confirming our previous findings (Figs 1-2a and b, bottom row). In these experiments, it is important to note that LPS stimulation of uninfected mice in contrast did not elicit an IFN-γ response (Fig 1-2a and b, top row). Uninfected control mice did not exhibit IFN-γ production in response to any conditions tested in the context of our work, and as such, although they were included in most of the experiments, they are often not shown in the results. We then go on to demonstrate that the IFN-γ-producing CD4 T cells in these mice contained the transcription factor T-bet, thus confirming that these were Th1 cells (Fig 1-2c).

Next, we characterized the kinetics of this response over time to show that administration of innate stimulation did not alter T cell activation or differentiation of Th1 cells. The first timepoint of these kinetics illustrates the response of naïve (uninfected) mice, and then samples were taken at day 6 (week 1), weeks 2, 3, 4, 6, 8, 12, and 24 post-infection. *Salmonella* infection is typically cleared by around week 8 post-infection, thus the last 2 timepoints should technically represent the memory response post clearance. Throughout the course of infection, LPS stimulation did not impact activation, measured by the percent of CD44hi CD4 or CD8 T cells in the LPS stimulated (gray line) compared to the

unstimulated (black line) mice (Figs 1-3a and b, respectively). Further, this stimulation did not change the ability of CD4 T cells to develop into Th1 cells, since the kinetics of T-bet expression with or without stimuli was not changed (Fig 1-3c).

In contrast, production of IFN-γ throughout the course of infection was remarkably altered by LPS stimulation. Throughout this work, we examine production of IFN-γ by Th1 cells as the relative proportion of T-bet+ CD4+ T cells producing IFN-γ out of the T-bet+ CD4+ T cell total, all of which should be capable of IFN-γ production. In other words, (IFN-γ+ T-bet+ CD4+)/(T-bet+ CD4+ total). Thus, we are comparing the proportion of Th1 cells which become stimulated. A proportion of Th1 cells were producing IFN-γ at any given point throughout the infection in response to the cognate and non-cognate interactions provided by the infection itself, with a maximal response observed at 2 weeks post-infection (Fig 1-4a). However, providing LPS stimulation at any of the timepoints examined increased this Th1 response, demonstrating that innate stimulation of Th1 can occur throughout infection, and in fact continued to be observed at low levels even after infection (weeks 12 and 24 post-infection). A similar response was seen in CD44hi CD8+ T cells, at lower levels (Fig 1-4b).

Innate Th1 stimulation occurs during both *Salmonella* and *Chlamydia* infections

We next wanted to determine whether this innate response of Th1 cells occurs particularly during *Salmonella* infection, or whether this responsiveness could be observed during any infection that is able to induce a strong Th1 cell response. Th1-dominant responses are generated during intracellular infections, so we chose to examine another intracellular pathogen for which a strong Th1 response has been observed. *Chlamydia muridarum* is an intracellular bacteria for which it has been shown that IFN-γ production is required to prevent bacterial dissemination. C57BL/6 mice infected systemically with *Chlamydia* showed differentiation of a Th1 cell population, and upon LPS stimulation these Th1 cells were also able to produce IFN-γ (Fig 1-5a and b). As with *Salmonella* infection, CD8 T cells were also innately stimulated during *Chlamydia* infection (Fig 1-5c), albeit to a lesser extent than was seen for CD4 T cells in either infection. Further, both CD4 and CD8 T cells in *Chlamydia* infected mice that had not

received additional innate stimulation (no stim) produced small amounts of IFN-γ, as seen in *Salmonella* infection.

Extracellular TLR ligands elicit Th1 stimulation

Thus far, we have examined the ability of LPS to stimulate IFN-γ production, however, we hypothesized that this was not an LPS-specific response, but rather a broader response to innate stimuli. To test this, we administered a broad range of TLR ligands during *Salmonella* infection and examined their ability to elicit IFN-γ production. Interestingly, while Th1 cells produced IFN-γ in response to LPS, flagellin, and Pam3CSK4, they did not exhibit significantly increased production of IFN-γ after stimulation with CpG DNA or Imiquimod (Fig 1-6a and b). A similar, but smaller, response pattern was observed in CD8 T cells (Fig 1-6c). Further, the response to LPS administration was dose-dependent in both Th1 and CD8 T cells (Fig 1-7a and b).

Discussion

Together, these data demonstrate that T cells can be stimulated to produce IFN-γ under a broad variety of conditions and throughout infection, suggesting that this pathway has broad implications to the function of T cells. Stimulation occurred during both *Salmonella* and *Chlamydia* infection, supporting the idea that this response pathway may be important in a range of intracellular infections. Future work exploring additional infectious contexts is required to determine whether innate CD4 T cell stimulation occurs as a general rule of T cell function. Further, innate T cell stimulation was not limited to LPS, occurring in response to LPS, flagellin, and Pam3CSK4, although not CpG DNA or Imiquimod. This may be due to the cellular location of the receptors that recognize these ligands. TLR4 (recognizes LPS), TLR5 (flagellin), and the heterodimeric complex of TLR1 and 2 (triacylated lipopeptides, such as Pam3CSK4) are all expressed on the cell surface and can therefore recognize extracellular innate ligands. In contrast, TLR7 (single-stranded RNA, such as Imiquimod) and TLR9 (CpG DNA) are expressed within the cell, recognizing innate ligands in the endosome. This suggests that the reason for the response to some but not other TLR ligands may be explained by the direct

administration of ligands that cannot be internalized on their own to interact with their receptor.

Further, the response to LPS was dose-dependent, with a small level of stimulation observed at doses as low as 0.1 μg per mouse. This supports the assertion that innate stimulation of T cells occurs in response to the lower levels of innate ligands produced during the course of a normal infection without the addition of exogenous TLR ligands. In our system, high doses of ligands were used to observe large and quantifiable effects upon T cell responses. However, in our system mice only have to survive this strong cytokine response for 4 hours, and in fact many did not survive that long with doses at 25 μg per mouse or higher. Thus, to balance the toxicity of the inflammatory response against the anti-bacterial effects of these cytokines, a delicate balance is required. It is likely that a proportion of the small IFN-γ response observed in infected, unstimulated controls is due to the innate stimulation of ligands produced by the infection itself. In chapter 4 we will explore the effect that losing even this small proportion of the response has on the overall ability of T cells to fight *Salmonella* infection.

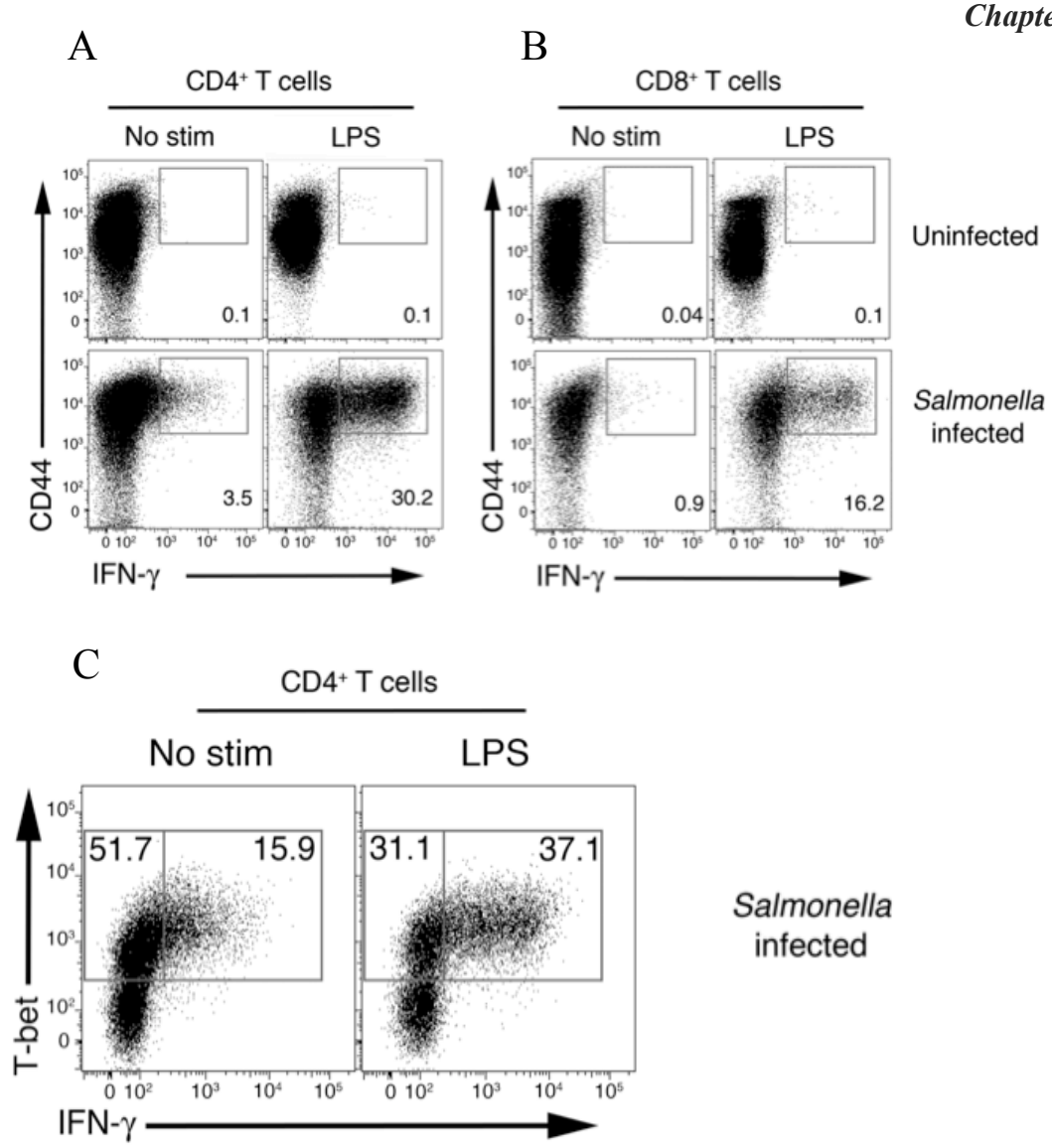

Figure 1-2: LPS stimulates activated T cells to produce IFN-γ during infection

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella* (BRD509) and the proportion of $CD4^+$ and $CD8^+$ T cells in the spleen that express CD44 and IFN-γ (or T-bet on $CD4^+$ T cells) was assessed by flow cytometry. Four hours prior to analysis, mice were injected i.v. with 10μg LPS or received no additional stimulus (no stim). Representative flow cytometry plots showing the production of IFN-γ at day 14 after infection by (A) $CD44^{hi}$ $CD4^+$ and (B) $CD44^{hi}$ $CD8^+$ T cells, or (C) T-bet^+ $CD4^+$ T cells. Numbers show the percentage of IFN-γ positive or negative T cells within the boxed gates. Experiments contained at least 3 mice/group at each time point and representative flow cytometry plots show data that are representative of at least three experiments. Error bars represent mean +/- SEM.

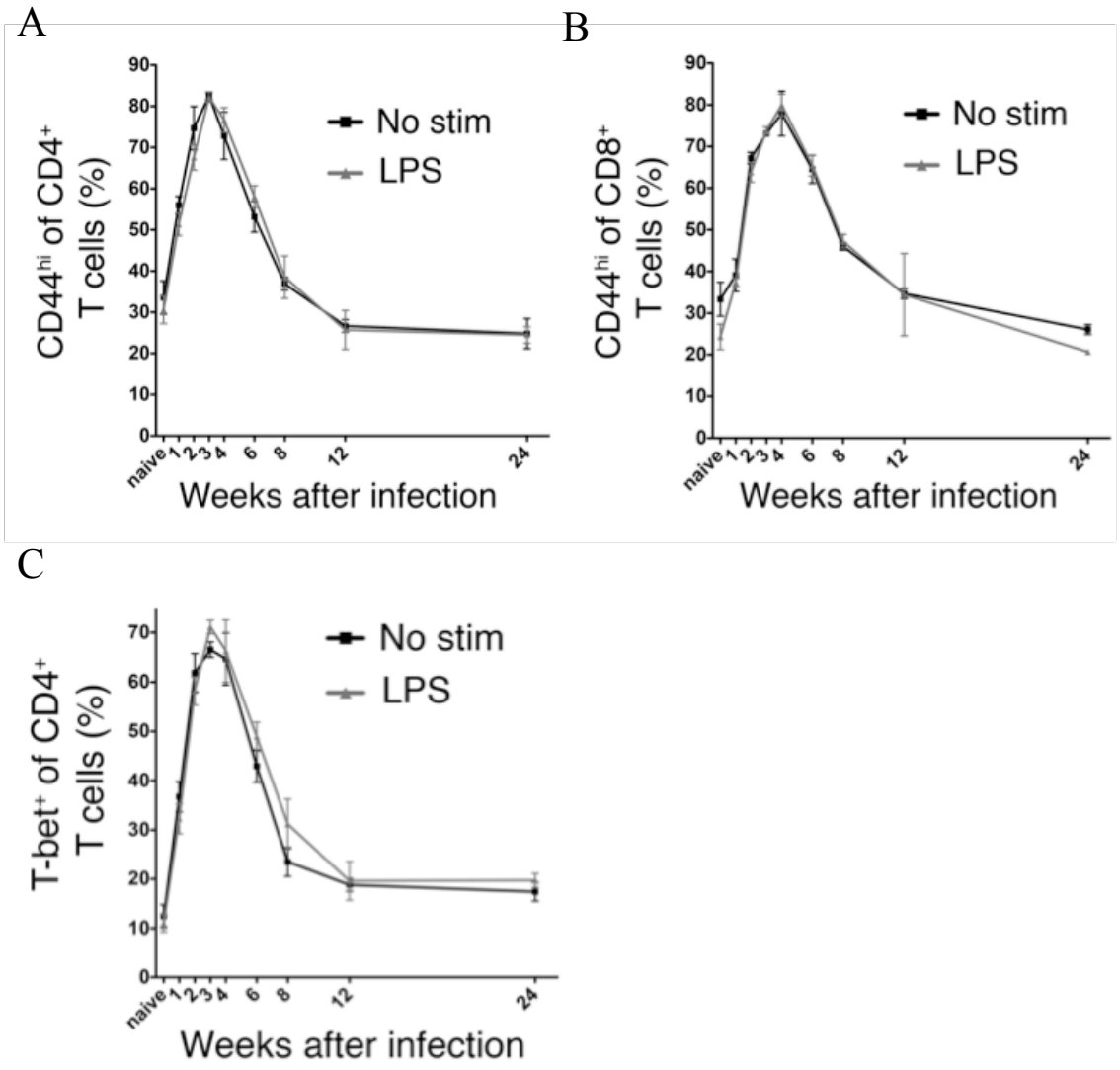

Figure 1-3: Kinetics of activation and Th1 differentiation are not altered by innate stimulation

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella* (BRD509) and at various times later, the proportion of $CD4^+$ and $CD8^+$ T cells in the spleen that express CD44 and IFN-γ or T-bet on $CD4^+$ T cells was assessed by flow cytometry. Four hours prior to analysis, mice were injected i.v. with 10μg LPS or received no additional stimulus (no stim). Graphs show change in the percentage of (A) $CD44^{hi}$ $CD4^+$, (B) $CD44^{hi}$ $CD8^+$, or (C) T-bet^+ $CD4^+$ over time in the spleen. Experiments contained at least 3 mice/group at each time point and show data that are representative of at least three experiments. Error bars represent mean +/- SEM.

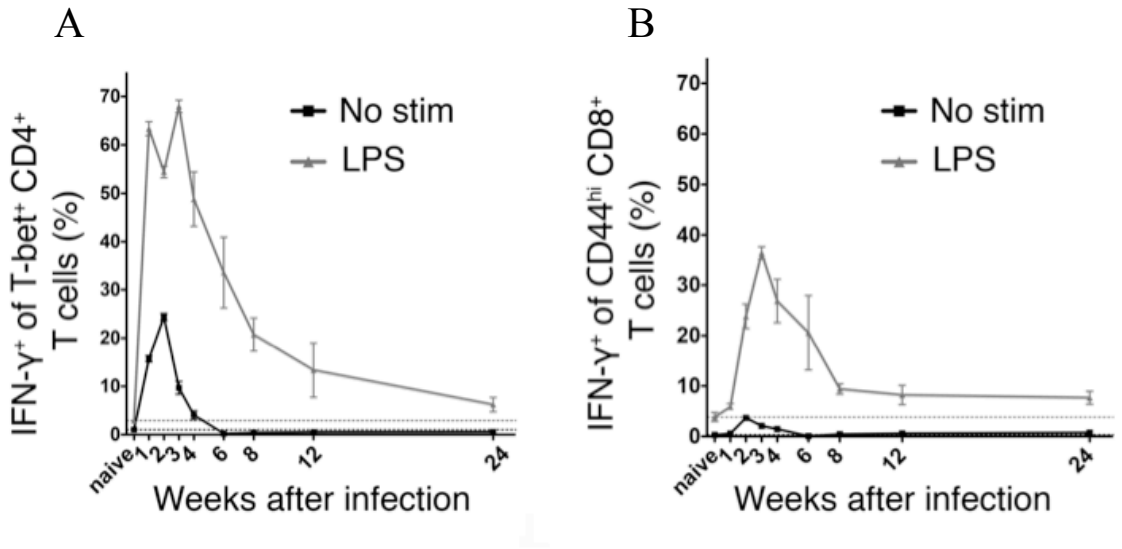

Figure 1-4: Kinetics of IFN-γ production in response to LPS

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella* (BRD509) and at multiple times later splenocytes were assessed for IFN-γ production by flow cytometry. Four hours prior to analysis, mice were injected i.v. with 10μg LPS or received no additional stimulus (no stim). Graphs show change in the percentage of (A) IFN-γ^+ T-bet^+ $CD4^+$ Tcells (percentage of Th1 cells producing IFN-γ) and (B) IFN-γ^+ $CD44^{hi}$ (percentage of activated $CD8^+$ T cells producing IFN-γ) in the spleen. Experiments contained at least 3 mice/group at each time point and data are representative of at least three experiments. Error bars represent mean +/- SEM.

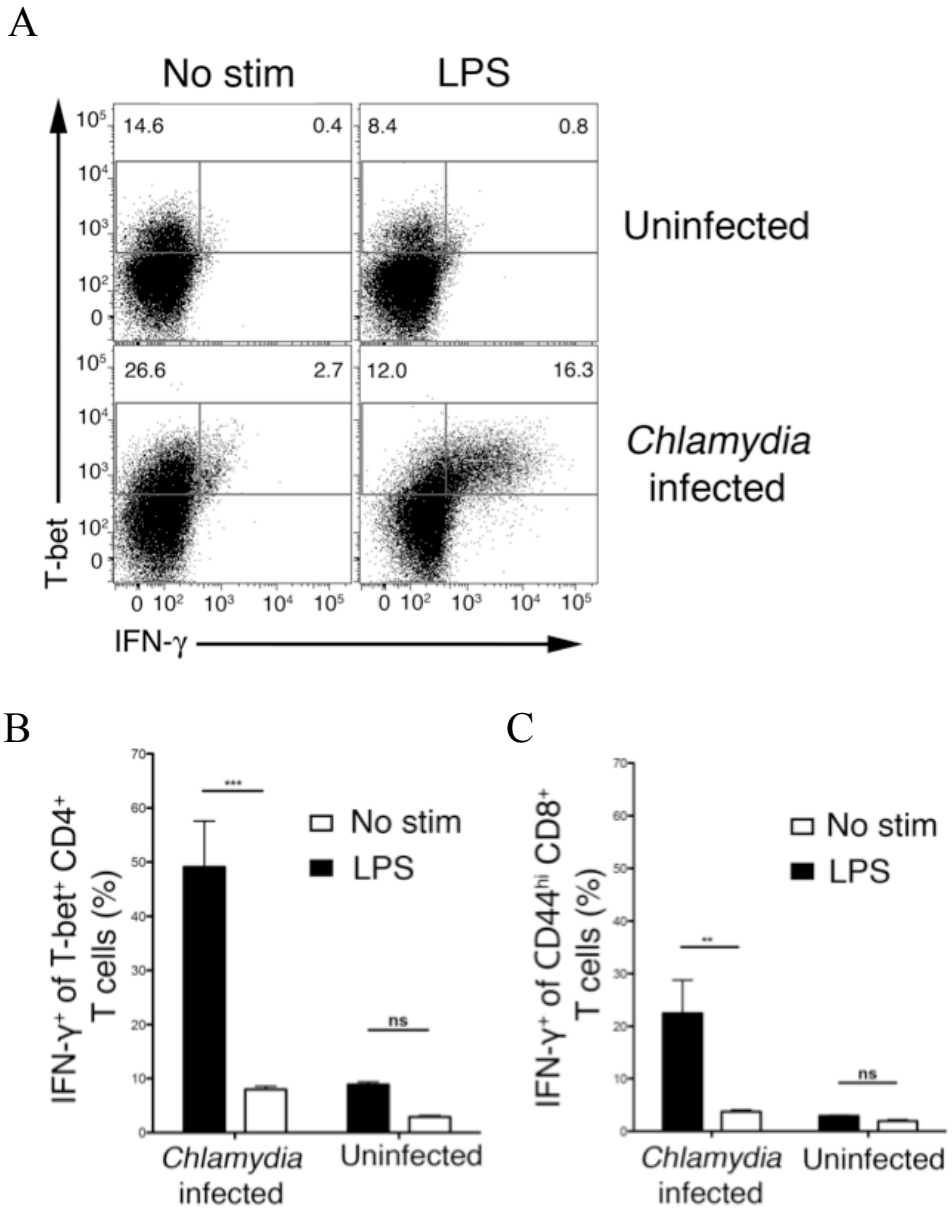

Figure 1-5: T cells also produce IFN-γ after LPS stimulation during *Chlamydia* infection

C57BL/6 mice were infected i.v. with $1x10^7$ *Chlamydia muridarum* Ebs. One week later, infected or uninfected mice were injected i.v. with LPS and spleens harvested four hours later to determine IFN-γ production. Representative (A) flow cytometry plots and bar graphs of combined data showing (B) intracellular T-bet or (C) CD44 and IFN-γ staining in *Chlamydia*-infected mice after gating on $CD4^+$ or $CD8^+$ as indicated. (B, C) Statistical significance was determined by two-way ANOVA with a bonferroni post-test. $P< 0.005$ (***), $p<0.01$ (**), or $p>0.05$ (ns). All experiments contain at least 3 mice/ group and were conducted at least twice. Error bars represent mean +/- SEM.

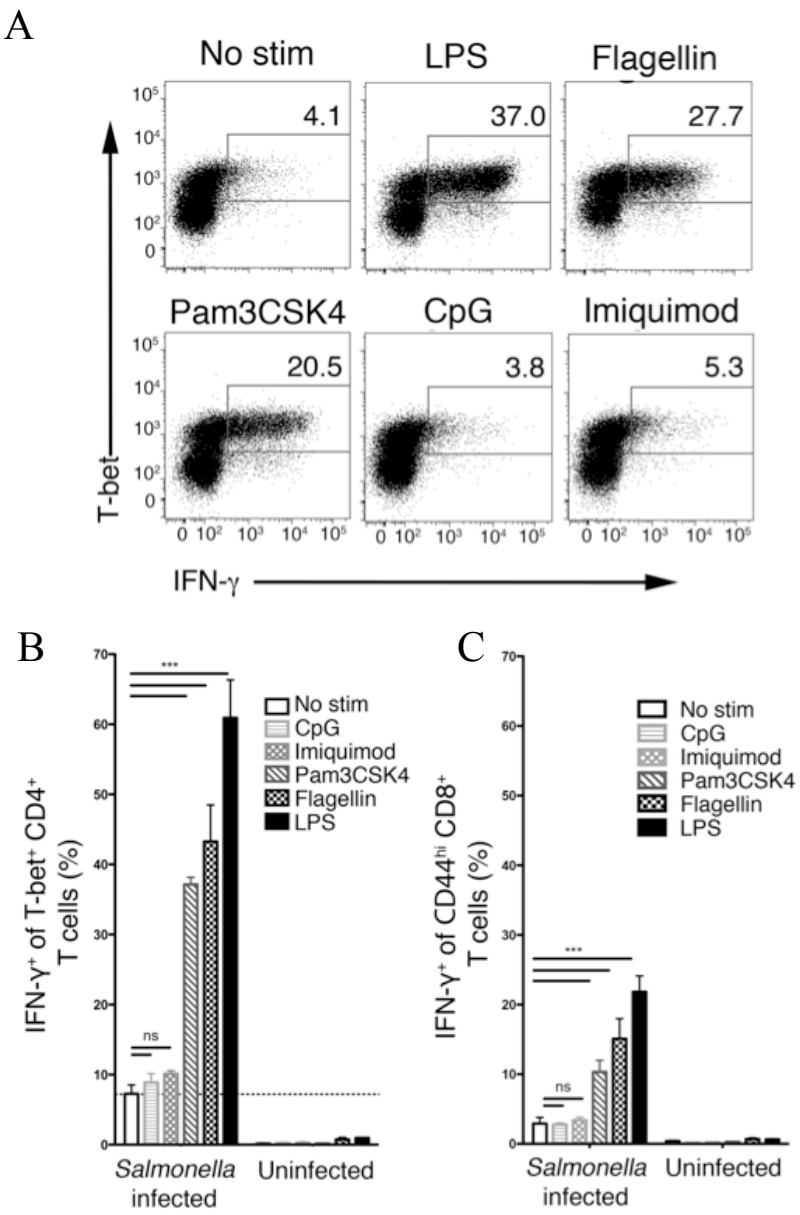

Figure 1-6: Ligands of extracellular TLRs induce T cells to produce IFN-γ

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella*. Two weeks later, infected or uninfected mice were injected i.v. with LPS, flagellin, CpG DNA, Imiquimod, or Pam3CSK4 and spleens harvested four hours later to determine IFN-γ production. Representative (A) flow cytometry plots and bar graphs of combined data showing (B) intracellular T-bet or (C) CD44 and IFN-γ staining in *Salmonella*-infected mice after gating on $CD4^+$ or $CD8^+$. (B, C) Statistical significance was determined by two-way ANOVA with a bonferroni post-test. $P< 0.005$ (***), $p<0.01$ (**), or $p>0.05$ (ns). All experiments contain at least 3 mice/ group and were conducted at least twice. Error bars represent mean +/- SEM.

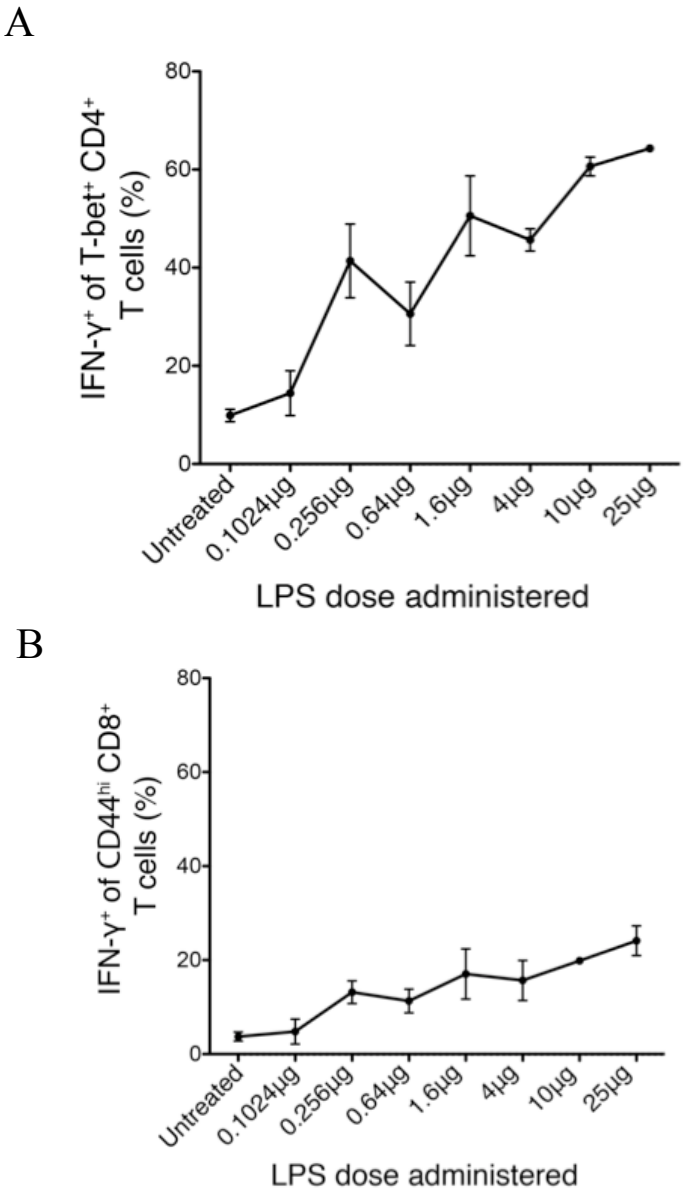

Figure 1-7: T cell response to LPS is dose-dependent

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella*. Two weeks later, infected or uninfected mice were injected i.v. with LPS and spleens harvested four hours later to determine IFN-γ production. Graphs show percentage of IFN-γ^+ cells after i.v. LPS administration at various doses. $CD4^+$ are shown as percentage of total T-bet^+ cells producing IFN-γ and $CD8^+$ as percentage IFN-γ^+ of total $CD44^{hi}$. All experiments contain at least 3 mice/ group and were conducted at least twice. Error bars represent mean +/- SEM.

Chapter 2:
How do T cells recognize innate stimulation?

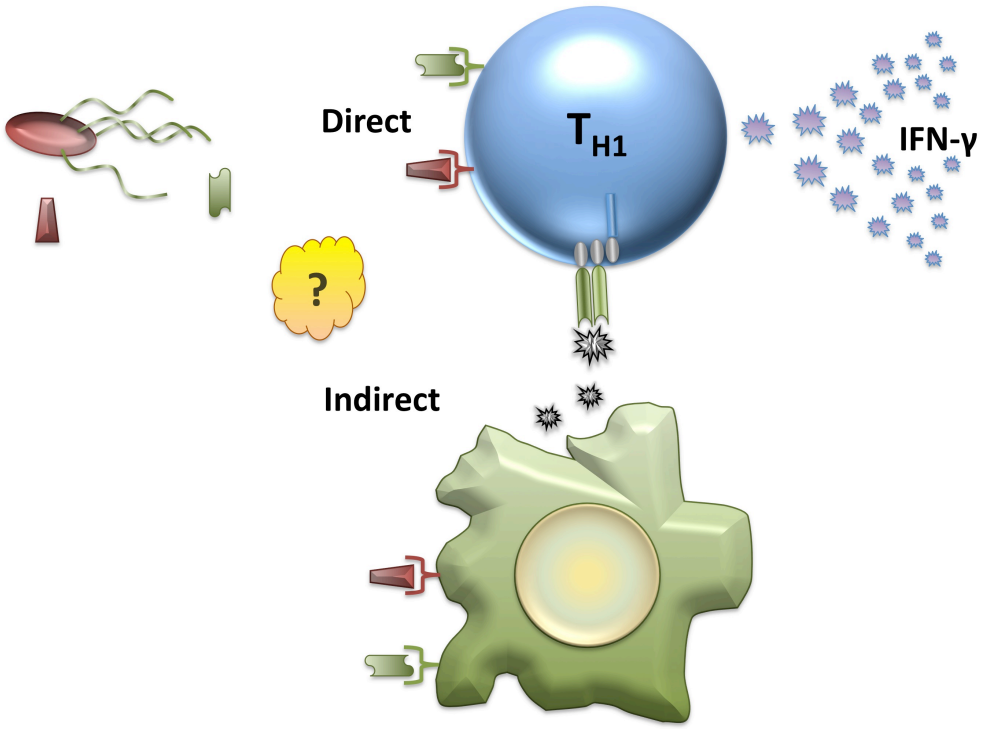

Figure 2-1: Recognition of innate ligands could be directly or indirectly mediated

Th1 cells recognize various innate ligands, resulting in production of IFN-γ as demonstrated in Chapter 1. However, this response could be due to direct PRR signaling or indirect stimulation by PRR expressing innate cells. In this chapter we focus on the T cell intrinsic signals required to mediate the innate Th1 response.

In Chapter 1 we established that Th1 cells can respond to many different innate stimuli in the context of multiple intracellular infections throughout the course of infection. In this chapter we begin to examine the mechanisms for how this response occurs. Recognition of TLR ligands could occur directly by the expression of TLRs on the Th1 cells, or indirectly by TLR recognition on innate cells that produce secondary signals, which then trigger a Th1 response. This chapter focuses on the T cell intrinsic requirements of innate Th1 stimulation, showing that MyD88-dependent IL-18 and IL-33 receptor signaling are necessary for the maximal Th1 response, while TLR4, IL-1R and IFN-γR signaling are not necessary to elicit IFN-γ production after LPS stimulation.

Results

Direct TLR4 recognition is not required for T cell response to LPS

To distinguish between direct and indirect mechanisms of T cell stimulation we first needed a way to compare functional and deficient T cells within an otherwise similar environment. In the case of TLR4 recognition of LPS, for example, it has been proposed that direct TLR4 signaling on the T cell may mediate a variety of effects upon T cell function (Reynolds and Dong, 2013). However, using conventional TLR4-deficient mice it is impossible to separate the potential effects of direct TLR4 signaling on the T cell from the well-established effects of TLR4 signaling on other cell types that could indirectly influence T cell function. To resolve this uncertainty, we utilized a well-established tool for generating mice that contain multiple distinct cell populations: mixed bone marrow chimeras. In a mixed bone marrow chimera (BMC), a host is irradiated and reconstituted with a mix of bone marrow, in our case from both a wild-type and a deficient mouse. Each of these mice generally presents different cell surface markers that allow them to be distinguished from one another by flow cytometry. When the bone marrow cells have expanded to replace the original host cells lost by irradiation, they can be looked at by flow cytometry as distinct populations, as illustrated in Fig 2-2. These plots show 3 separate populations representing the endogenous (host) cells, the transferred wild-type cells, and the transferred deficient cells, as labelled. This enables examination of the cell function based solely on the intrinsic deficiency.

The important point to remember here is that while the T cells being compared are intrinsically different, their environment is not. Although other cell types will also be affected by the same deficiencies, since both the deficient and normal T cells exist within the same environment, these extrinsic deficiencies should impact each population similarly. While this may result in an overall impairment in all T cell responses, by comparing T cell functions within the same host these effects are taken into account. Thus, BMCs are a powerful tool to examine the effect of T-cell specific deficiencies.

We first used this tool to address the question of direct or indirect LPS recognition by making TLR4-deficient BMCs. If direct TLR4 recognition on T cells were required for

LPS to cause innate stimulation, these mice would display defective IFN-γ production in the TLR4-deficient T cells, but not in the wild-type T cells. However, if the effect of LPS on T cells occurs indirectly, with recognition occuring on other cell types that can then influence the T cell response, both deficient and wild-type T cells should be similarly affected by the loss of TLR4 on a portion of the other cells. These mice conclusively demonstrated that direct TLR4 signaling on T cells is not required for LPS stimulation of IFN-γ production, since TLR4-deficient T cells produced IFN-γ at the same rate as wild-type T cells (Fig 2-3). Further, both T cell populations maintained strong IFN-γ production, suggesting that the loss of TLR4 in some of the other cells did not sufficiently lower the overall reponse to LPS. Given that direct LPS recognition by T cells is not required, we next examined what pathways might mediate an indirect response by T cells.

Innate stimulation of T cells requires intrinsic MyD88 signaling

All TLRs contain an intracellular signalling domain known as a TIR (toll/IL-1R) domain that allows them to interact with TIR domain-containing signaling molecules, such as MyD88. However, as the name TIR implies, TLRs are not the only receptors that contain TIR domains. The IL-1R family includes IL-1R, IL-18R, IL-33R, and the recently identified and poorly described IL-36R (Garlanda et al., 2013). To narrow down the possible interactions required by T cells, we decided to begin broadly by looking at the requirement for T cell-intrinsic MyD88. Again using BMCs, wild-type and MyD88-deficient T cells were compared. Th1 cells lacking MyD88 signaling were severely deficient in IFN-γ production compared to wild-type Th1 cells in the same host after LPS stimulation (Fig 2-4a and b). While a small impairment in Th1 differentiation was also observed in MyD88-deficient CD4 T cells, these differences are factored in by displaying the results as IFN-γ production by T-bet+ CD4 T cells, since this calculation excludes cells that have not developed the capacity to produce IFN-γ. Similar results were seen in CD8 T cells from MyD88-deficient hosts (Fig 2-4c).

IL-18R and IL-33R are necessary for maximal Th1 cell response

While the T cell response to innate stimuli requires MyD88 signaling, the role of MyD88 is independent of TLR recognition, as seen earlier in Fig 2-3. As mentioned above, MyD88 mediates intracellular signaling for both the TLR and IL-1R families. In addition, IFN-γR has been shown to be able to use MyD88, although this receptor is usually thought to utilize Jak/STAT signaling. Thus, the requirement for MyD88 must be due to a role for one or more of the following receptors: IL-1R, IL-18R, IL-33R, IL-36R, and IFN-γR. Each of the IL-1 receptors is a heterodimeric complex consisting of a unique recognition chain and a signaling chain, which in the case of IL-1R, IL-33R, and IL-36R are the same chain, IL-1Rap. We tested each of these receptors individually by generating BMCs with mice deficient in the unique recognition chains, with the exception of IL-36R, which is not yet commercially available. Both IL-1R1-deficient and IFN-γR1-deficient T cells showed no impairment as compared to the wild-type T cells in the same mice, for either Th1 cells (Fig 2-5a and b) or CD8 T cells (Fig 2-5c and d).

Additionally, we examined IL-18R1- and IL-1Rl1 (IL-33R)-deficient BMCs. Loss of IL18 signaling in Th1 cells closely mirrored the effects of MyD88 loss, with a small decrease in Th1 cell frequency and a severe defecit in IFN-γ production in response to innate stimulation (Fig 2-6a). CD8 T cells also required IL-18R for stimulation (Fig 2-6c). Perhaps surprisingly, loss of IL-33 signaling also impaired Th1 production of IFN-γ by a smaller, but still highly significant amount (Fig 2-6b). While these results were not reflected in the CD8 T cells (Fig 2-6d), this may simply be due to the smaller percentage of activated CD8 T cells, from which a small change would be difficult to observe.

Discussion

The data in this chapter demonstrate that while the ability of Th1 cells to respond to innate stimuli requires MyD88 signaling, it is not mediated by direct TLR signaling. Innate stimuli interact directly with other cell types, and these cells produce cytokines that allow an indirect stimulation of T cells. Given the requirement for MyD88, a series of BMCs were generated to test receptors known to utilize MyD88 signaling. T cell intrinsic IL-18R signaling was shown to be required for innate Th1 stimulation, which on

its own sufficiently explains the deficiency resulting from loss of MyD88. However, surprisingly an effect was also seen in Th1 cells deficient for IL33R.

IL-33 has primarily been described, within the context of T cell stimulation, as a Th2 associated cytokine. However, some reports describe it as a generally pro-inflammatory cytokine. Prior to being secreted, IL-33 has the unique capacity to act as a transcription factor, with a possible role in NF-κb regulation recently described. Thus, it has been proposed that the release of IL-33 from the cell may serve as a signal of cell damage. Although the impact of IL-18R-deficiency on Th1 cells was certainly more severe than that seen for IL-33R-deficiency, these results suggest that IL-33 may further amplify the Th1 response during infection.

While these results indicate that IL-18 is required for innate stimulation, and that IL-33 may further stimulate this response, they do not establish whether these cytokine signals are sufficient to cause innate stimulation on their own. It is likely that additional inflammatory signals exist that can amplify this innate response. In fact, recently in vitro cytokine stimulation of effector or memory CD8 T cells was examined for 43 different cytokines, either alone or in pairs (Freeman et al., 2012). The results demonstrated a surprising promiscuity, with many cytokine pairs resulting in IFN-γ production. This suggests that innate stimulation of both CD4 and CD8 T cells still requires further exploration in vivo to fully understand how these responses can be induced naturally during infection. Further, while our work has focused on elicitation of IFN-γ from Th1 cells, to fully appreciate the role of innate CD4 T cell stimulation future work will need to examine whether similar stimulation can elicit effector responses from other CD4 T cell subsets.

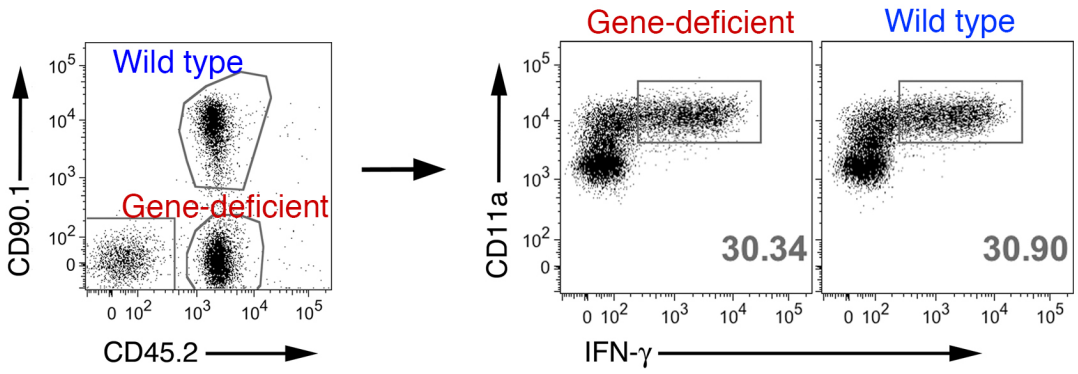

Figure 2-2: Flow cytometry gating strategy used for mixed bone marrow chimeras

CD45.1$^+$ C57BL/6 mice were irradiated and reconstituted with a mixture of bone marrow from wild-type (CD90.1$^+$ CD45.2$^+$) and various gene-deficient (CD90.2$^+$ CD45.2$^+$) mice. After immune reconstitution BM chimeras were infected i.v. with 5x10^5 *Salmonella* and response to LPS determined in the spleen. Gating strategy used to examine donor wild-type and gene-deficient CD4$^+$ T cells in mixed bone marrow chimeras (MBCs). Splenocytes were gated as live, singlets, and CD4$^+$ CD8$^-$, then gated as shown in the left panel for congenic markers. Each gated cell population was assessed for IFN-γ production and either CD11a (TLR4) or T-bet.

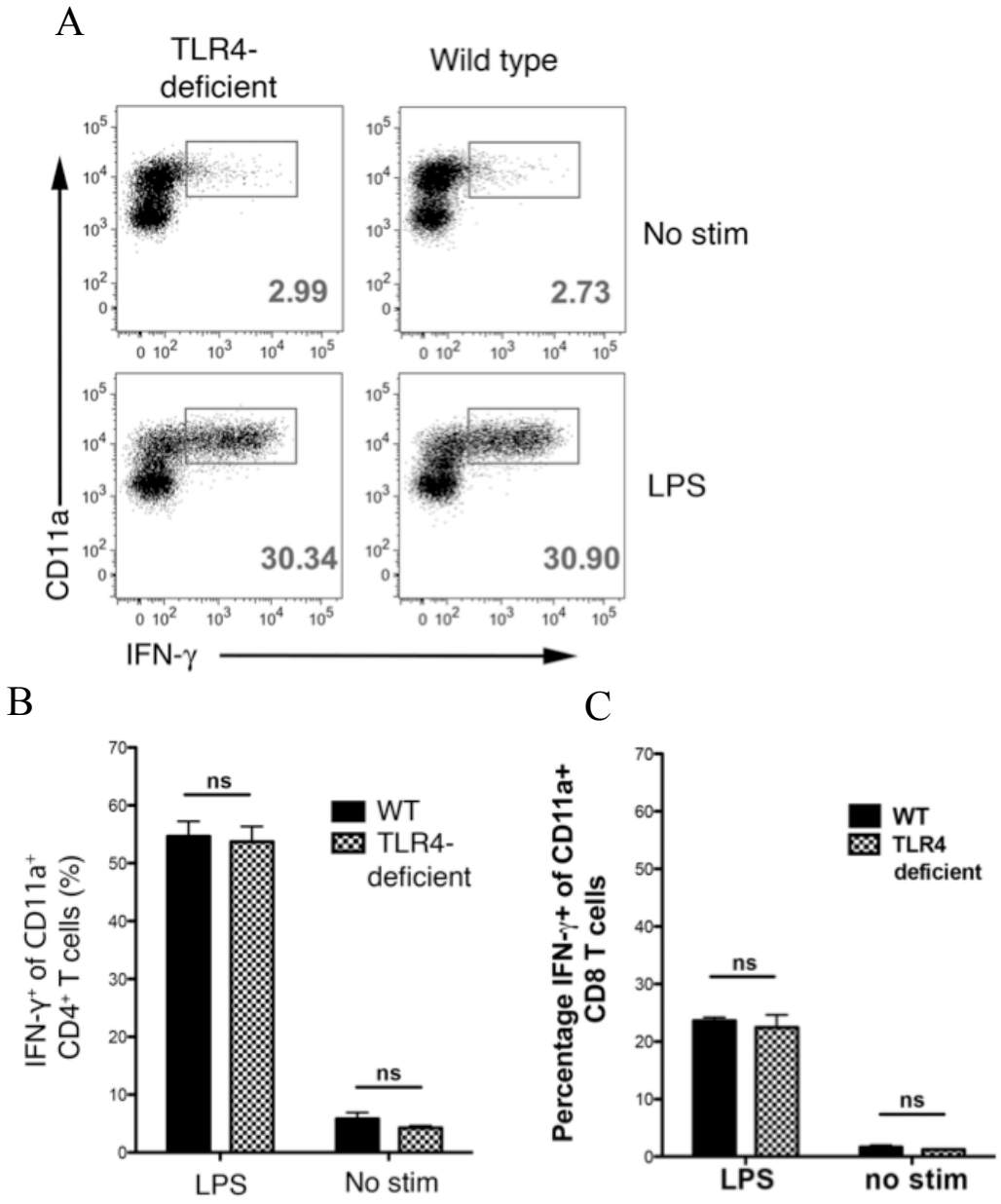

Figure 2-3: TLR4 is not intrinsically required for innate T cell stimulation by LPS

CD45.1$^+$ C57BL/6 mice were irradiated and reconstituted with a mixture of bone marrow from wild-type (CD90.1$^+$ CD45.2$^+$) and TLR4-deficient (CD90.2$^+$ CD45.2$^+$) mice. After immune reconstitution BM chimeras were infected i.v. with 5x10^5 *Salmonella* and response to LPS determined in the spleen. Representative (A) flow cytometry plots and a graph of combined data for (B) CD4$^+$ CD11a^{hi} and (C) CD8$^+$ CD11a^{hi} are shown for TLR4 deficient MBCs. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/- SEM.

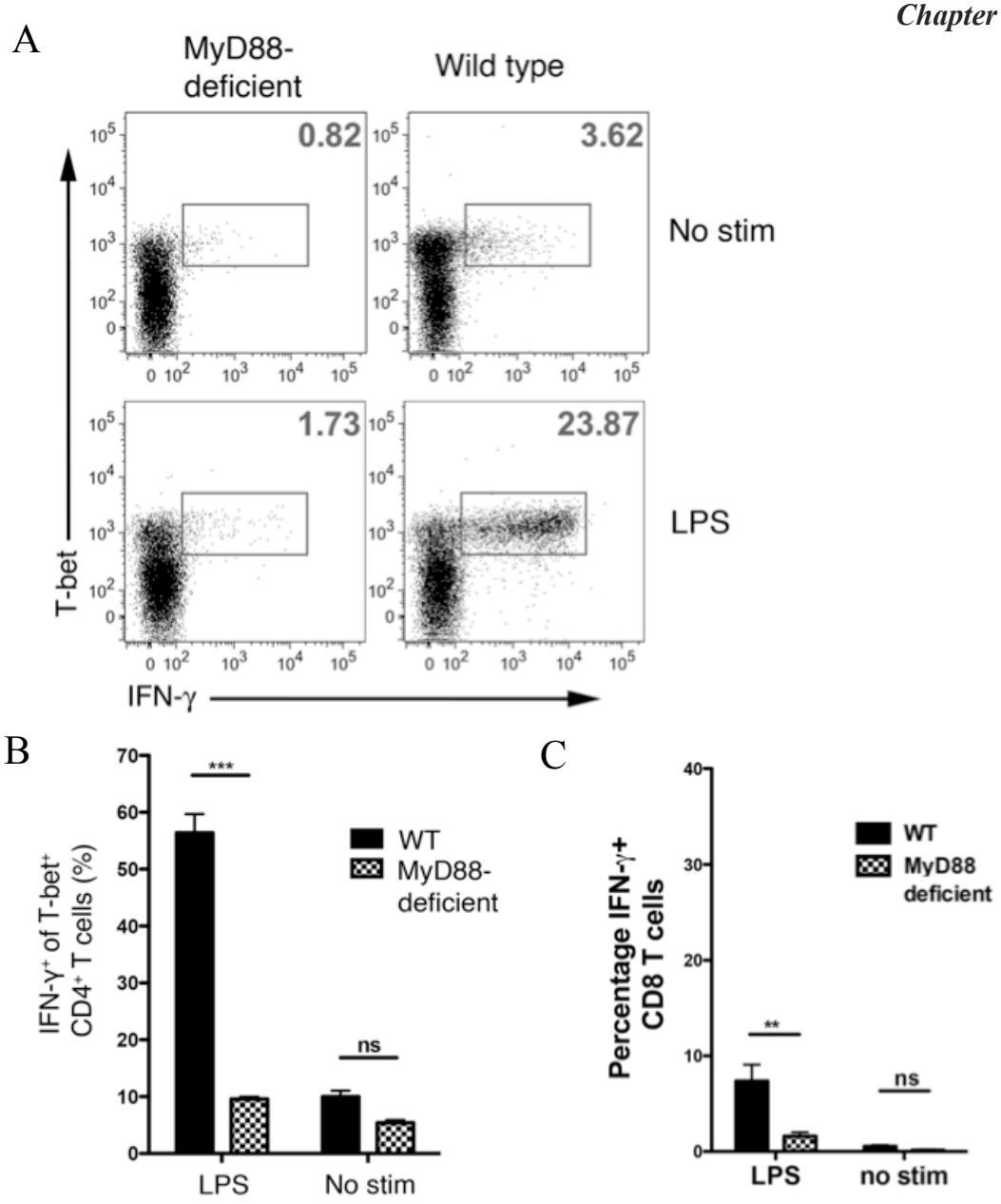

Figure 2-4: T cell response to LPS requires intrinsic MyD88 signaling

CD45.1$^+$ C57BL/6 mice were irradiated and reconstituted with a mixture of bone marrow from wild-type (CD90.1$^+$ CD45.2$^+$) and MyD88-deficient (CD90.2$^+$ CD45.2$^+$) mice. After immune reconstitution BM chimeras were infected i.v. with 5x10^5 *Salmonella* and response to LPS determined in the spleen. Representative (A) flow cytometry plots and a graph of combined data for (B) CD4$^+$ T-bet$^+$ and (C) CD8$^+$ are shown for MyD88-deficient MBCs. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/- SEM.

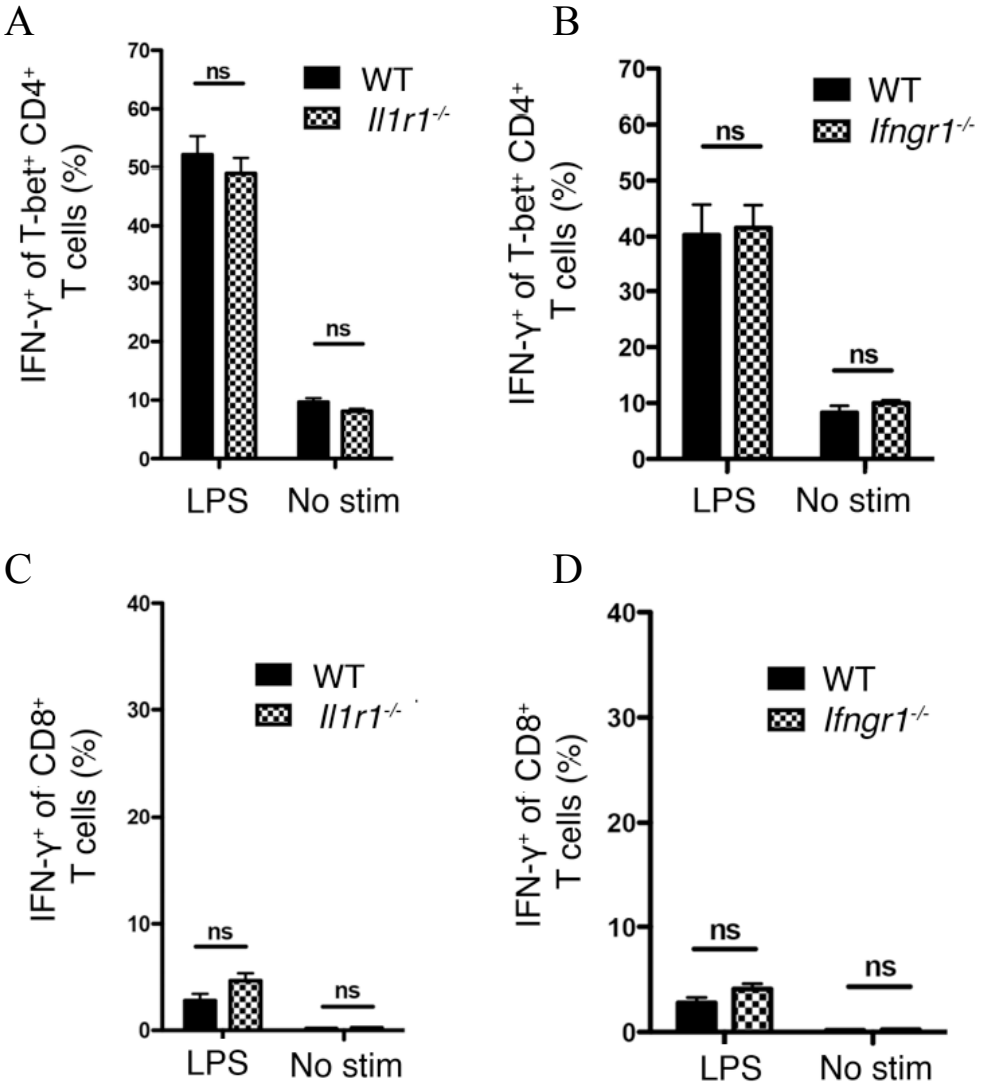

Figure 2-5: T cell response to LPS does not require intrinsic IL1R or IFN-γR signaling

MBCs were generated, infected, and stimulated, as described in Figure 2-2. A graph showing the percentage of IFN-γ^+ (A, B) Th1 cells and (C, D) total $CD8^+$ cells in wild-type versus gene-deficient $CD4^+$ and $CD8^+$ T cells is shown for (A, C) IL-1r1 and (B, D) IFN-γr1 MBCs. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/- SEM.

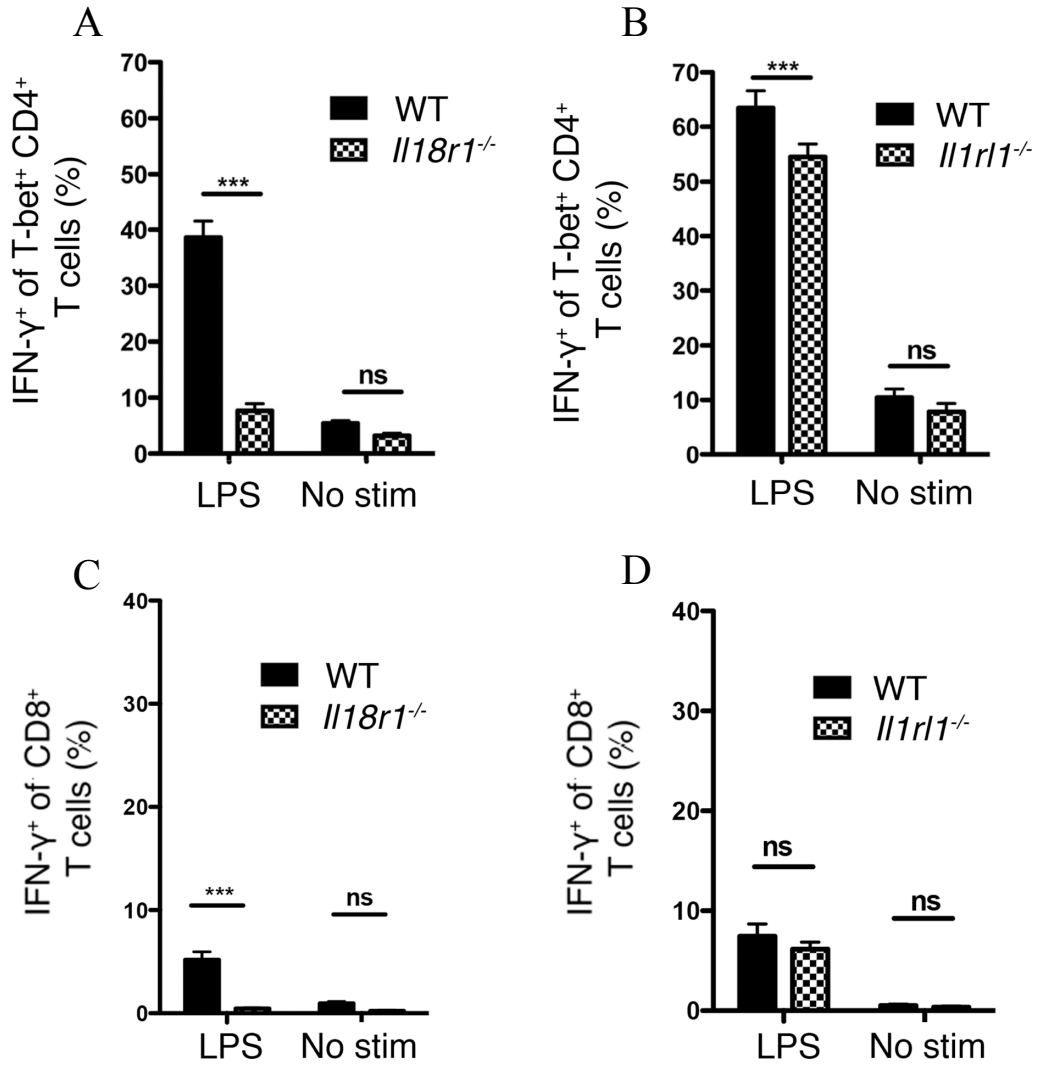

Figure 2-6: Loss of intrinsic IL18R or IL33R signaling impairs innate T cell response

MBCs were generated, infected, and stimulated, as described in Figure 2-2. A graph showing the percentage of IFN-γ^+ (A, B) Th1 cells and (C, D) total $CD8^+$ cells in wild-type versus gene-deficient $CD4^+$ and $CD8^+$ T cells is shown for (A, C) IL-18r1 and (B, D) IL-1rl1 (IL-33R) MBCs. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/- SEM.

Chapter 3:
How do PRRs elicit T cell effector functions?

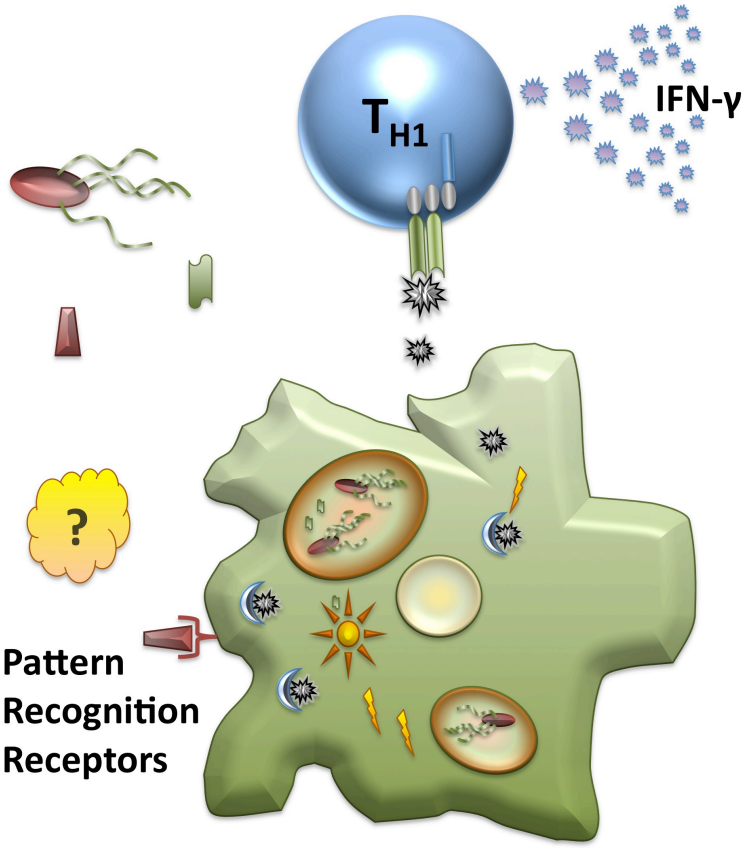

Figure 3-1: Innate T cell stimulation requires multiple PRR pathways

Administration of innate stimuli during infection amplifies the innate response to the infecting pathogen. In the last chapter we showed that innate stimulation of Th1 cells requires IL-18. In this chapter we focus on how innate stimuli lead to IL-18 production.

In Chapter 2 we demonstrate that the recognition of TLR ligands by T cells is indirectly mediated, in part, by IL-18. In this chapter, we next address the question of how IL-18 is induced by innate stimuli. We show that both TLR4 and the inflammasome components NLRP3 and NLRC4 are required for T cells to produce IFN-γ in response to LPS stimulation. However, aflagellate *Salmonella* induced the same response as flagellated *Salmonella*, implying redundant inflammasome recognition of stimuli other than flagellin. Further, the loss of inflammasomes resulted in lower circulating levels of IL-18, most likely due to impaired secretion. These results suggest that both TLR and NLR signals are required to innately stimulate T cells, and offer one possible explanation for the requirement of a concurrent infection for optimal T cell response to innate stimuli.

Results

LPS stimulation increases mRNA and circulating levels of IFN-γ and IL-18

Although our T cell-intrinsic studies in chapter 2 show that IL-18 and IL-33 receptors are required for maximal T cell stimulation by LPS, in order for these cytokines to mediate the indirect effects of LPS administration they would need to increase in response to LPS. RT-QPCR of mRNA extracted from total splenocytes or total hepatocytes showed an increase after LPS stimulation of the transcripts for IFN-γ, IL-18, and IL-33, as well as IL-12, which has been shown to act synergistically with IL-18 (Table 3-1). Further, cytokine ELISAs (performed by Oanh Pham) on sera collected from mice 4 hours after LPS stimulation displayed a dramatic increase in circulating levels of IFN-γ, from around 3000 pg/ml in an infected mouse to nearly 10^6 pg/ml in infected mice after LPS stimulation (Fig 3-2a). This demonstrates that not only are more T cells producing IFN-γ, but that the impact of innate stimulation on overall IFN-γ levels is severe. In support of our earlier data, a greater than 30-fold increase in circulating IL-18 was also observed after LPS stimulation, confirming its availability as a mediator of the T cell response to LPS (Fig 3-2b). Cytokine ELISAs for both IL-12 and IL-33 were attempted on the same sera samples, but all samples were below the limit of detection. For this reason, circulating cytokine levels in the remaining figures focus on IL-18 production.

Ability of TLR ligands to increase circulating IL-18 reflects their T cell stimulating capacity

Given the evidence above that IL-18 production after innate stimulation correlated with both circulating IFN-γ levels and T cell production of IFN-γ observed by FACS, we next asked whether IL-18 is produced after stimulation with innate ligands that do not result in IFN-γ production. In chapter 1 we demonstrate that T cells respond to ligands for extracellular, but not endosomal, TLRs. We hypothesize that this is due to their location, and that this prevents recognition of exogenous ligands by phagosomal receptors. If this is true, than these ligands should also not be able to stimulate IL-18 production. Serum cytokine ELISAs confirmed that while LPS, flagellin, and Pam3CSK4 were able to

stimulate increased circulating IFN-γ levels, Imiquimod was not (Fig 3-3a). Further, as predicted, Imiquimod did not result in IL-18 production, while LPS, flagellin, and Pam3CSK4 all increased IL-18 (Fig 3-3b).

Inflammasome processing of IL-18 is required for innate T cell stimulation

Since IL-18 appears to play a pivotal role in this response pathway, we next wanted to explore the requirements for IL-18 production. IL-18, like IL-1, is first expressed as a pro-peptide requiring caspase-cleavage for maturation and secretion. Caspase maturation is one of the hallmark functions of the NLRC4 inflammasome, which recognizes flagellin. Previous work has shown that during *Salmonella* infection both NLRC4 and NLRP3 are required, but the ligand for NLRP3 during *Salmonella* is unclear (Broz et al., 2010). Therefore, we asked whether recognition of innate stimuli by these inflammasomes could be indirectly involved in the T cell response pathway. Mice deficient for both NLRC4 and NLRP3 produced markedly less IL-18 after stimulation with either LPS or flagellin (Fig 3-4). Interestingly, a small amount of IL-18 was still produced in response to stimulation, despite the loss of both inflammasome pathways. Further, stimulation of either Th1 or CD8 T cells was severely hampered by the loss of these inflammasome molecules, presumably due to the loss of IL-18 production (Fig 3-5a and b).

Recognition of flagellin by the inflammasome is not required for innate T cell stimulation

Recently, it was reported that NLRC4 recognition of flagellin is required for bystander stimulation of CD8 T cells during *Salmonella* infection (Kupz et al., 2012). This data is in agreement with our findings, particularly because they show a similar requirement for IL-18. However, in their model this response occurs only in response to flagellin- no stimulation was observed after administration of LPS. We wanted to determine whether our response pathway is similarly dependent upon flagellin, or whether these are in fact different mechanisms. Although we typically administer LPS, the *Salmonella* itself may provide the necessary flagellin. Thus, to examine the role of NLRC4, we needed to

remove all flagellin from our system. To do this, an aflagellate BRD509 strain was generated in the Baumler lab. Infection with this strain followed by LPS stimulation elicited a similar IFN-γ response from both Th1 and CD8 T cells as in wild-type BRD509, despite the absence of flagellin stimulation, suggesting that the requirement for inflammasome signaling in our model relies on redundant NLR ligands (Fig 3-6a and b).

TLR4 is required for innate stimulation of T cells by LPS

Finally, there is some evidence that LPS can be recognized by NLRP3. We show in chapter 2 that T cell intrinsic TLR4 recognition of LPS is not required to mediate T cell stimulation. However, in light of the requirement for inflammsome signaling, we wanted to confirm that T cell extrinsic TLR4 recognition of LPS does in fact mediate innate stimulation. TLR4-deficient mice were infected with BRD509 and stimulated with LPS as usual, and IFN-γ production by both Th1 and CD8 T cells was examined (Fig 3-7a and b). In the absence of TLR4, no response to LPS was able to occur. Combined with the MBC data from Figure 2-3, these data demonstrate that the T cell response to LPS is mediated indirectly by TLR4.

Discussion

In this chapter, we characterize some of the T cell extrinsic mechanisms that result from innate stimulation and can mediate stimulation of T cells. Upregulation of mRNA transcripts for IFN-γ, IL-18, IL-33 and IL-12 were all observed in splenocytes and hepatocytes after LPS stimulation, although only IFN-γ and IL-18 were found in circulation. It is possible that these cytokines are upregulated after stimulation, but are not able to be secreted within the 4 hour window used in this assay, or that additional post-transcriptional regulation prevents expression or secretion. Further, because IL-33 can also act as a transcription factor, it is possible that IL-33 mRNA is upregulated for this purpose rather than for secretion as a cytokine. All of these explanations would suggest that neither cytokine mediates the effect of LPS upon T cells, although it does not rule out the possibility that the levels induced by the infection itself are sufficient to provide T cell stimulatory effects without requiring LPS mediated amplification. It is also possible that these cytokines are secreted at higher levels after LPS stimulation and do

increase T cell responses, but that they are primarily retained in the tissue rather than circulating in the sera.

Both the production of and the requirement for IL-18 are clear in this pathway, therefore we examined the mechanism leading to IL-18 production after innate stimulation. Not only were all of the ligands of the extracellular TLRs tested able to induce IL-18 production, but the inflammasome components NLRP3 and NLRC4 were also required. Interestingly, a small amount of IL-18 was still seen after stimulation in the absence of either inflammasome, suggesting that either an additional caspase-1 activating inflammasome is stimulated during Salmonella infection, or that caspase-1-independent maturation of IL-18 occurred. In fact, there is evidence for caspase-8 mediated maturation of both IL-18 and IL-1β (Bossaller et al., 2012). The signals that allow for caspase 8 activation are still unclear, but may involve an NLRP3/NLRC4 inflammasome-independent pathway (Broz and Monack, 2013). Together, these findings may explain the low level of seemingly inflammasome-independent IL-18 and IFN-γ production.

The requirement for both TLR and NLR signaling for a strong T cell response to innate stimuli demonstrates a mechanism in which during infection simultaneous stimulation of both the TLR and NLR pathways results in cytokines that are able to amplify the T cell response. While it is remotely possible that the TLR ligands administered were also able to provide NLR stimulation, it is unlikely given the timing and intracytoplasmic location of the NLRs. More likely, the NLR stimulation was provided by the infection itself, either by production of PAMPs or by damage resulting in DAMPs. This theory would also explain why administration of innate ligands alone is not sufficient to result in production of large amounts of IL-18 or IFN-γ in uninfected mice. Further, this dual requirement suggests that a pathway has evolved to allow the innate recognition of pathogens and damage to be communicated to the adaptive T cell response. Clarification of the mechanisms of this response in different models is needed to demonstrate whether this communication is a function inherent in all T cell responses.

		effect of LPS (relative to unstimulated controls)			
Spleen	**gene:**	**IFN-γ**	**IL12**	**IL18**	**IL33**
	naïve	1	1	1	1
	naïve + LPS	8.67	0.99	1.30	1.60
	d14	1	1	1	1
	d14 + LPS	22.02	4.52	2.26	5.35
Liver					
	naïve	1	1	1	1
	naïve + LPS	11.07	1.47	1.96	6.16
	d14	1	1	1	1
	d14 + LPS	27.9	5.36	2.6	16.2

Table 3-1: LPS stimulation leads to increased mRNA expression of IFN-γ, IL-12, IL-18, and IL-33

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella* and two weeks later, infected or uninfected mice were injected i.v. with LPS. Spleens and livers were harvested four hours later and RT-QPCR performed on extracted mRNA. Numbers shown are normalized to gapdh expression and indicate the fold-change increase over unstimulated mice.

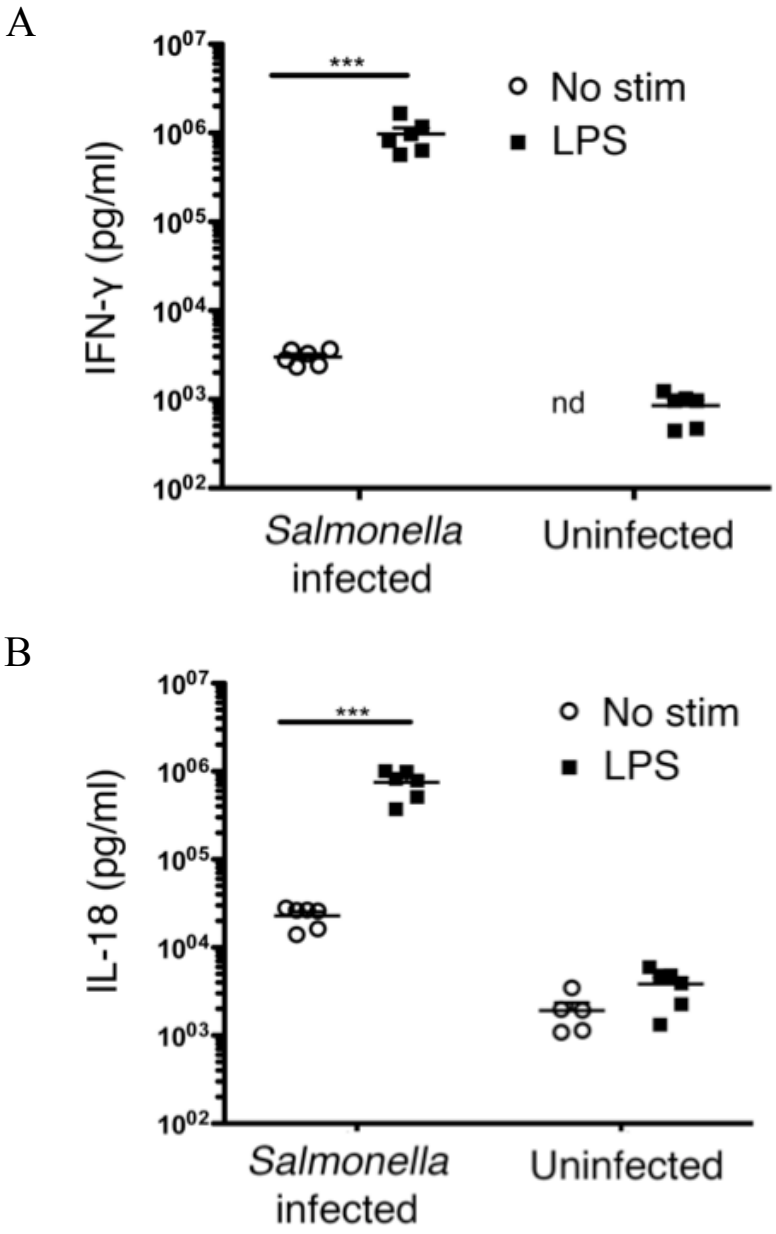

Figure 3-2: LPS stimulation elicits systemic IFN-γ and IL-18 production

C57BL/6 mice were infected i.v. with $5x10^5$ *Salmonella* BRD509 and the splenic response to LPS injection determined 2 weeks later, and four hours after stimulation. Plots show serum cytokine concentrations as determined by ELISA, for (A) IFN-γ and (B) IL-18. Data shown are pooled from 2 experiments. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/- SEM.

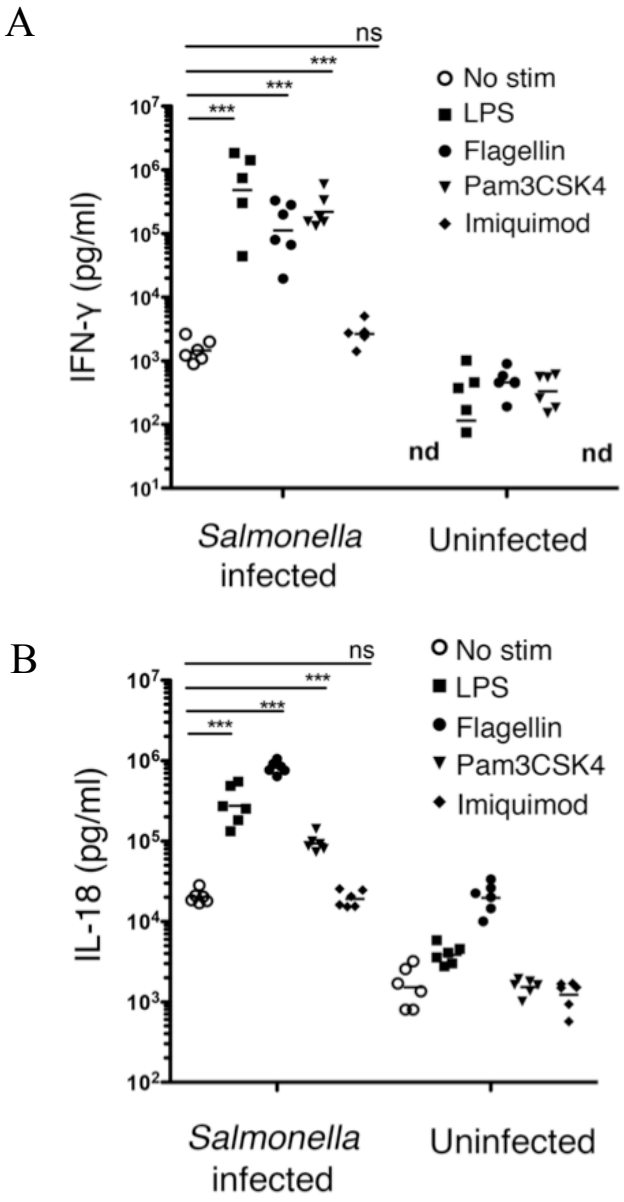

Figure 3-3: Extracellular TLR ligands elicit increased systemic IFN-γ and IL-18

C57BL/6 mice were infected i.v. with $5x10^5$ BRD509 and the splenic response to LPS, flagellin, Pam3CSK4, and Imiquimod injection determined 2 weeks later, and four hours after stimulation. Plots show serum cytokine concentrations as determined by ELISA, for (A) IFN-γ and (B) IL-18. Data shown are pooled from 2 experiments. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/- SEM.

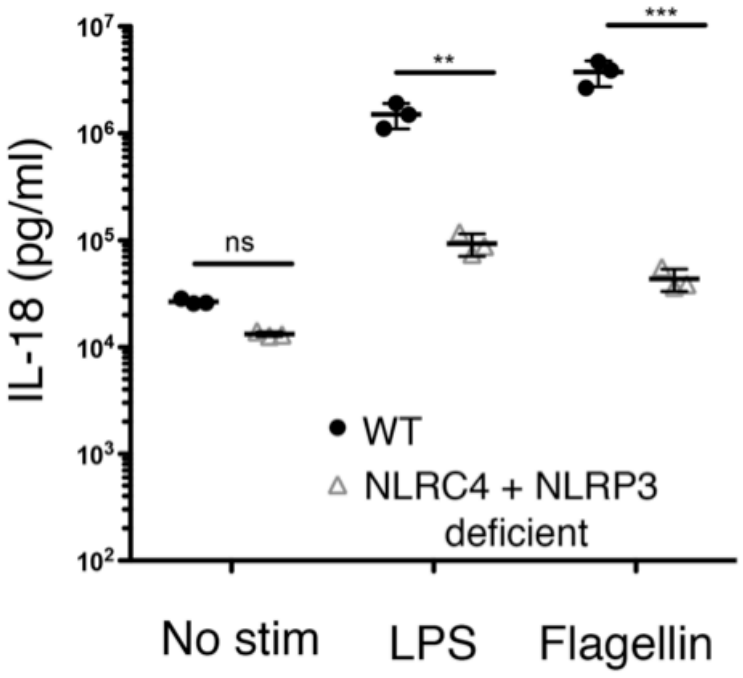

Figure 3-4: Impaired inflammasome signaling results in decreased systemic IL-18 after innate stimulation

Wild-type or mice deficient in NLRP3 and NLRC4 were infected i.v. with $5x10^5$ BRD509 and the splenic response to LPS or flagellin injection was determined 2 weeks later, and four hours after stimulation. Plots show serum cytokine concentrations as determined by ELISA for IL-18. Data shown are pooled from 2 experiments. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/- SEM.

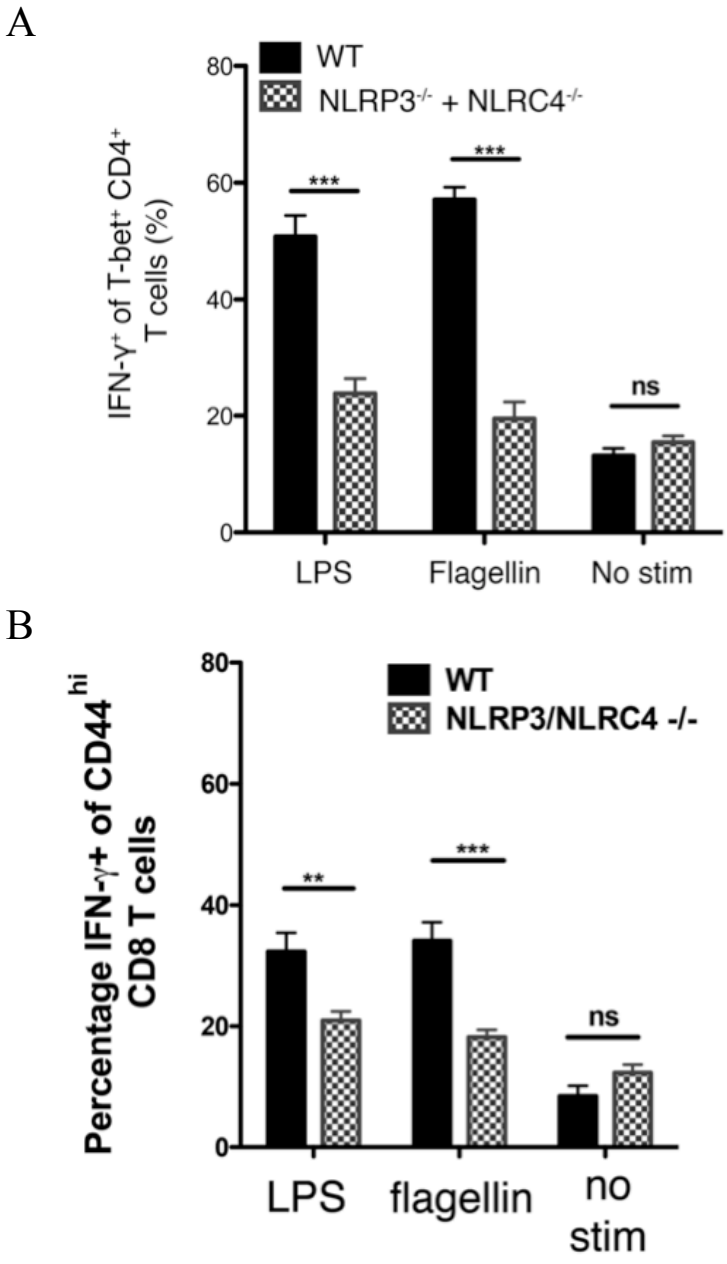

Figure 3-5: Innate stimulation of T cells requires inflammasome signaling

Wild-type or mice deficient in NLRP3 and NLRC4 were infected i.v. with $5x10^5$ BRD509 and the response to stimulation determined 2 weeks later, and four hours after stimulation. Plots show IFN-γ production by (A) Th1 cells or (B) $CD8^+$ $CD44^{hi}$ T cells in *Salmonella*-infected wild-type, or mice lacking NLRP3 and NLRC4, after administration of LPS or flagellin. Data is pooled from 2 experiments with more than 3 mice per group. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/- SEM.

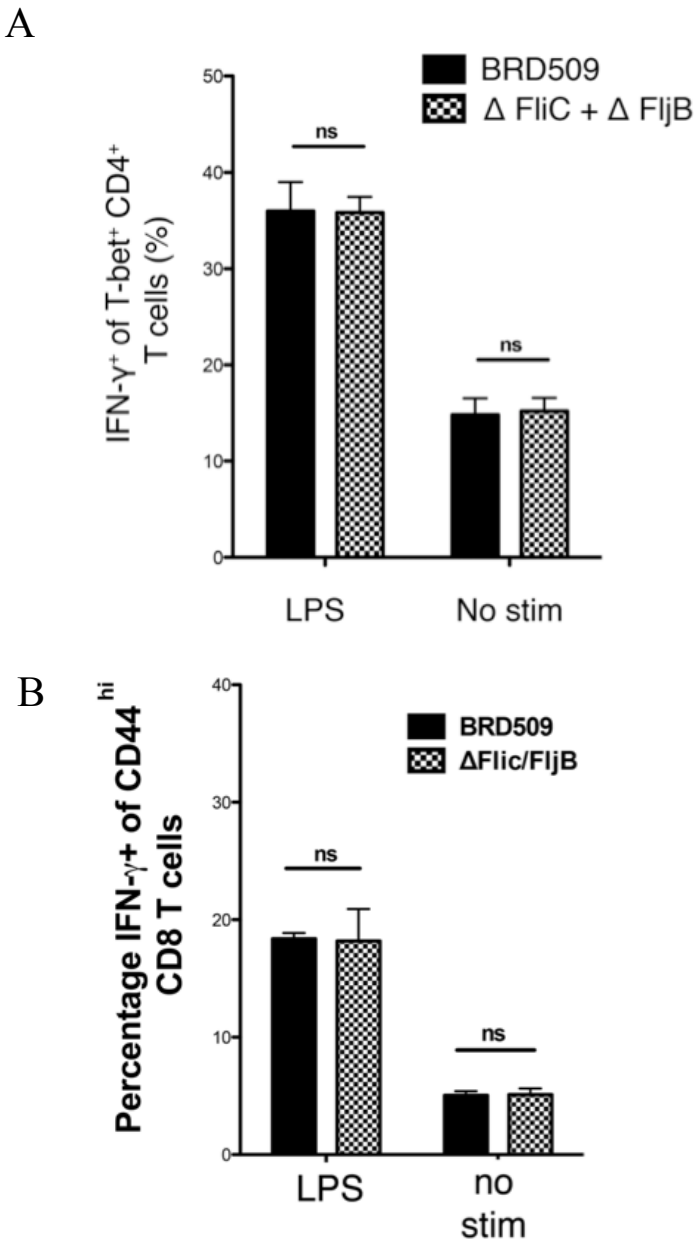

Figure 3-6: Innate T cell stimulation occurs independent of flagella

C57BL/6 were infected i.v. with $5x10^5$ *Salmonella* (BRD509 or flagellin-deficient BRD509) and the splenic response to LPS was determined 2 weeks later, and four hours after stimulation. IFN-γ production by (A) Th1 cells or (B) $CD8^+$ $CD44^{hi}$ T cells after LPS stimulation of mice infected with BRD509 (flagellin-expressing) or a flagellin deficient mutant of BRD509 (ΔfliC ΔfljB). Data is representative of at least 2 experiments with at least 3 mice per group. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/- SEM.

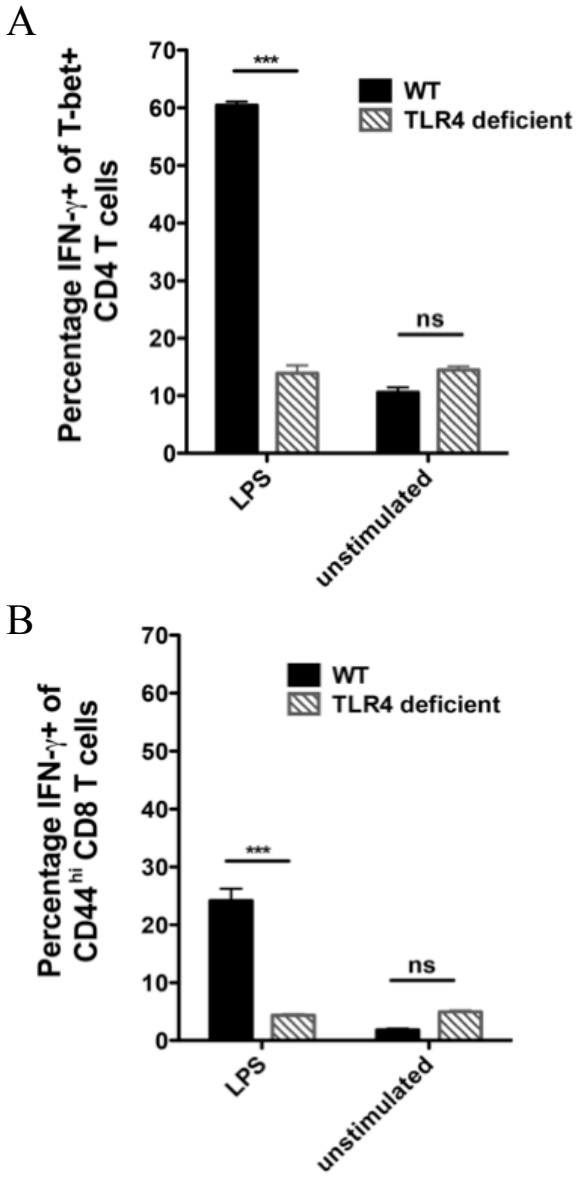

Figure 3-7: TLR4 is extrinsically required for innate T cell stimulation by LPS

TLR4 is required for response to LPS. Wild-type (C57BL/6) or TLR4-deficient mice were infected with BRD509 iv 14 days prior to stimulation with 10μg LPS i.v. 4 hours after stimulation mice were euthanized and spleens were removed and prepared for flow cytometry. Graphs indicate (A) the % of total T-bet^{+} CD4 T cells that produced IFN-γ or (B) the % of total CD44^{hi} CD8 T cells that produced IFN-γ. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. $P < 0.005$ (***) or $p > 0.05$ (ns).

Chapter 4:
Does innate stimulation of Th1 cells impact bacterial clearance?

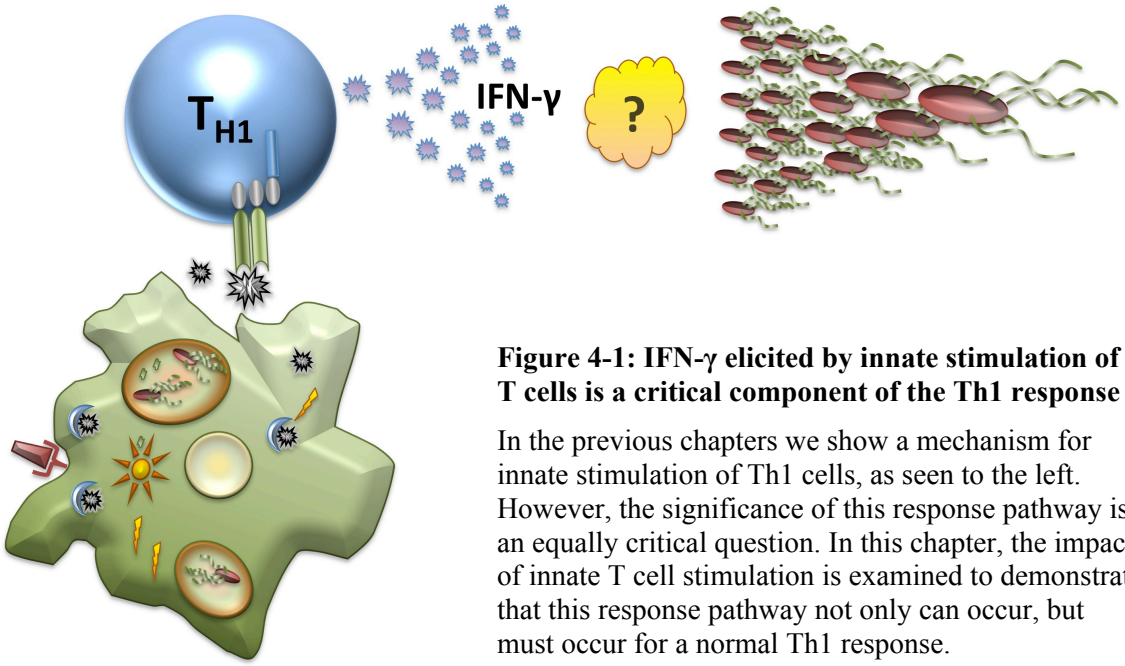

Figure 4-1: IFN-γ elicited by innate stimulation of T cells is a critical component of the Th1 response

In the previous chapters we show a mechanism for innate stimulation of Th1 cells, as seen to the left. However, the significance of this response pathway is an equally critical question. In this chapter, the impact of innate T cell stimulation is examined to demonstrate that this response pathway not only can occur, but must occur for a normal Th1 response.

The previous chapters have demonstrated that during infection various innate stimuli are able to trigger both TLR and NLR signaling pathways that result in pro-inflammatory cytokine secretion. This includes IL-18, which is required by Th1 cells to produce IFN-γ in response to innate stimuli. However, the fact that T cells can respond to high dose TLR ligand administration does not necessarily mean that this response is a major component of the response to infection. In this chapter, we use the information that IL-18 signaling is required for innate T cell response to explore the impact of the loss of this response on infection outcome. We show that T cell-intrinsic MyD88 deficiency impairs bacterial clearance, suggesting that this response is a critical component of an efficient Th1 response.

Results

CD4 T cells are required for *Salmonella* clearance and are a major source of IFN-γ after innate stimulation

Before examining the effect of innate T cell responses, we first wanted to demonstrate the requirement of total CD4 T cell responses within our system. Previous work has shown that both CD4 T cells and IFN-γ are required during *Salmonella* infections. However, these studies were done with other strains of mice or bacteria than those used for our experiments. Thus, the extent of the deficiency that could be expected in our mice was unclear. Here, we show that loss of CD4 T cells from day 7 to day 30 of infection resulted in a greater than 50-fold increase in the splenic bacterial loads (Fig 4-2a). Depletion of CD8 T cells also led to increased bacterial burden, although to a lesser extent than loss of CD4. Bacterial loads in the liver were similarly increased in both groups (Fig 4-2b). Although loss of CD8 T cells impaired clearance, they did not appear to significantly compensate during CD4 depletion, since depletion of both CD4 and CD8 did not result in significantly higher bacterial loads than those seen in the CD4 depletions alone. These results confirm in our model that CD4 T cells play a major role in bacterial clearance. Further, they set an upper threshold for our later experiments, since the role of innate stimulation in CD4 T cells would not be expected to exceed the requirement for CD4 T cells.

While CD4 T cells are required, they have many functions. It is possible that their contribution to clearance is not through production of IFN-γ. Many cells are known to produce IFN-γ during *Salmonella* infection, and any of these has the potential to be influenced by innate stimulation, directly or indirectly. It is plausible that while CD4 T cells can produce IFN-γ, other cells contribute equally or more to IFN-γ levels, and therefore the production of IFN-γ by CD4 is not the function for which they are required. Thus, we next wanted to examine the sources of IFN-γ production after innate stimulation, to determine if CD4 T cells were a major contributor to IFN-γ levels. Flow cytometry gated on CD4+ or CD8+ splenocytes showed the high frequency of CD4+ T cells within the total live cell population, and the high frequency of those total CD4 T

cells that were producing IFN-γ at 3 different timepoints throughout infection (Fig 4-3a). The bar graph shows the relative frequency of each cell subset that produced IFN-γ after innate stimulation (Fig 4-3b), as gated in a.

Mice with MyD88-deficient T cells show defects in survival, clearance, and resolution of *Salmonella* infection

Given that CD4 T cells are required during Salmonella infection and are a major source of IFN-γ production after innate stimulation, we next wanted to find a way to ask what impact the innate production of IFN-γ by CD4 T cells has on the course of infection. To do this, we used the information gained in chapter 2 to design mice whose T cells lack an innate response, but are still capable of cognate activation and stimulation. Since we showed that MyD88 signaling was critical to innate stimulation of IFN-γ production, but did not prevent Th1 differentiation or IFN-γ production by other means, we generated mice with a T cell-specific deficiency in MyD88 using the cre-lox mouse system. Lck is a kinase involved in TCR signaling, thus by crossing Lck promoter driven cre-recombinase expressing mice to mice whose MyD88 gene is flanked by loxP sites, we were able to selectively knock-out MyD88 from T cells (called Lck-MyD88$^{f/f}$ for short). Thus, T cells are present, but unable to respond to innate cytokine stimulation. This provides a model for exploring the effect of innate T cell stimulation.

During early *Salmonella* infection, the innate immune response plays a critical role in bacterial control. The requirement for CD4 T cells has typically begun to be observed around week 3, when the innate immune response fades and the T cell response must take over (Ravindran et al., 2005). Thus, we examined the role of innate T cell stimulation at later timepoints during infection, when T cell production of IFN-γ is most critical. At 5 weeks post-infection, bacterial loads in wild-type mice should be declining. However, in the Lck-MyD88$^{f/f}$ mice we saw deaths beginning at 3 weeks, with 1 in 6 mice dying by the 5 week timepoint (Fig 4-4). Among the survivors, bacterial loads in the spleen were significantly higher in Lck-MyD88$^{f/f}$ mice than in either wild-type or the littermate (Lck-MyD88$^{f/w}$) controls (Fig 4-5a). Higher bacterial loads were also seen in the livers of these mice (Fig 4-5b).

Further, by 8 weeks post-infection mice in our model should be resolving *Salmonella* infection, showing low or no detectable bacterial loads. While we show 1 wild-type mouse with no detectable bacteria and most with bacteria no longer detectable in the draining lymph nodes, only 1 Lck-MyD88$^{f/f}$ mouse had no detectable bacteria in the draining lymph nodes and none had resolved infection (Fig 4-6a and b). Overall, bacterial loads in both spleen and mesenteric lymph nodes were significantly higher in the Lck-MyD88$^{f/f}$ mice than in wild-type control mice. Together, these data demonstrate that innate stimulation of T cells can improve survival, bacterial clearance, and resolution of infection.

Discussion

In this chapter, we begin to explore the function of innate T cell stimulation. First, we showed that CD4 T cells are required for Salmonella clearance and that they composed a substantial proportion of the IFN-γ producing cells after innate stimulation. Then, we used the data from chapter 2 to design a system in which the T cell-specific response to innate stimuli is absent. In this model, we demonstrated that the innate T cell response improved survival, bacterial clearance and ultimately resolution of *Salmonella* infection. While we focused on the role of T cell stimulation during Salmonella infection here, we have established a model in which innate T cell responses can be explored further in a variety of contexts.

Work in other labs has examined the role of MyD88 in T cells using other model systems. For example, it was recently shown that MyD88 is required in CD4 T cells for the resolution of Chlamydia infection (Frazer et al., 2013). This was done in a mixed BMC model, wherein mice were reconstituted with a mixture of CD4-deficient and MyD88-deficient bone marrow. However, studying infection outcomes in these mice presents problems, both as a result of the partial deficiencies in other cell types, and the effects of radiation on the non-hematopoietic compartment.

Ultimately, the selective deficiency generated in cre-lox mice provides the best model currently available for interrogating innate T cell responses, with limited manipulation of the host or cells in question. Future work in these mice can further explore the

significance of innate T cell stimulation under various contexts, including additional infections. Although not currently available, this system could also be further refined in the future to allow for selective loss of innate T cell signaling using an inducible cre system. Tools such as these may be necessary to answer critical questions such as how innate T cell stimulation impacts fate decisions in order to separate the effects of innate stimulation in priming, stimulation, and re-activation.

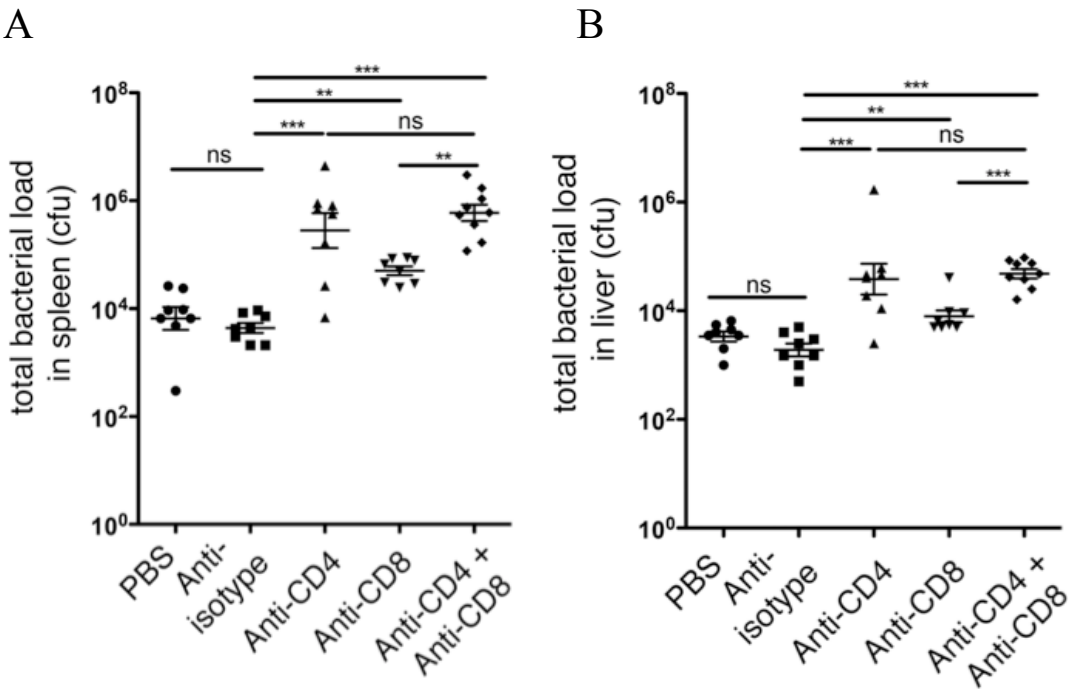

Figure 4-2: Depletion of CD4 T cells severely impairs bacterial clearance during *Salmonella* infection

T cell-depleted mice were infected i.v. with $5x10^5$ *Salmonella* BRD509. Mice given PBS, anti-CD4, anti-CD8, both, or an isotype control antibody were sacrificed at day 30 post-infection and bacterial loads in the (A) spleen and (B) liver were determined. Statistical significance was determined on log transformed data by one-way ANOVA. Error bars represent mean +/- SEM.

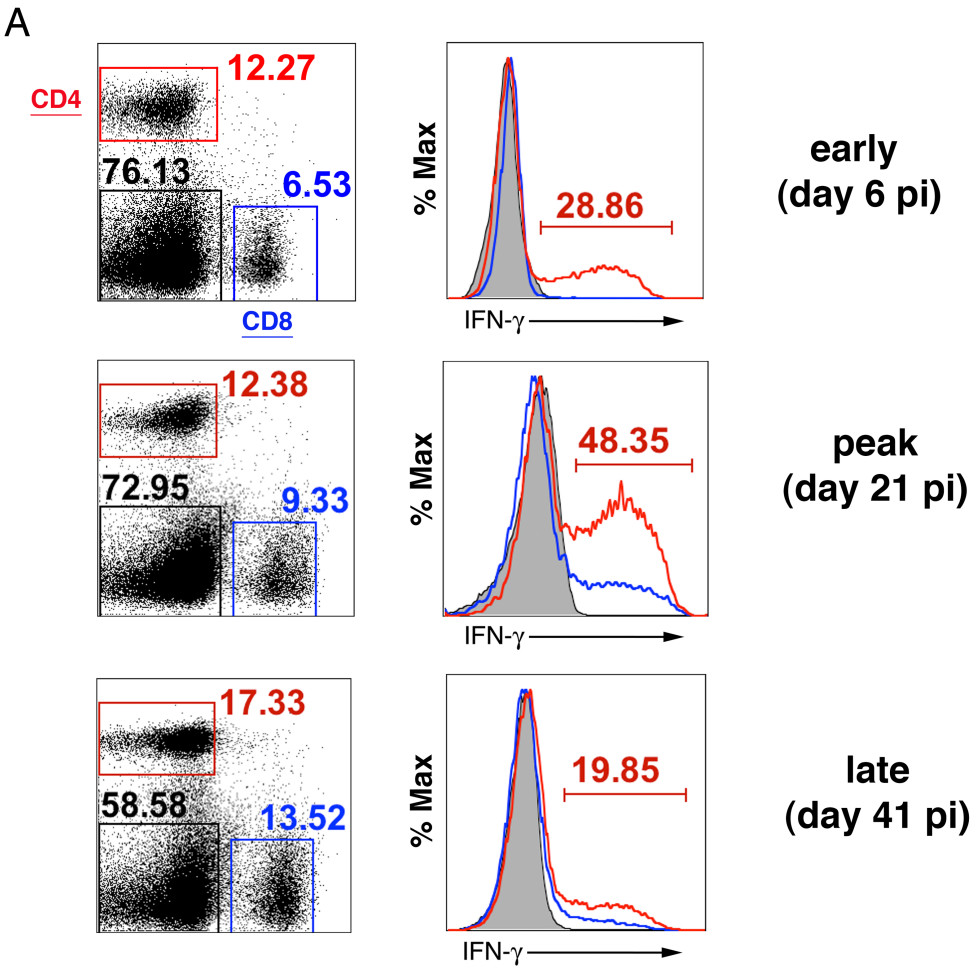
A
CD4
12.27
76.13
6.53
CD8
% Max
28.86
IFN-γ
early
(day 6 pi)
12.38
72.95
9.33
48.35
peak
(day 21 pi)
17.33
58.58
13.52
19.85
late
(day 41 pi)
B
Percent of T cell subset producing IFNγ
60
40
20
0
CD4+
CD8+
CD4- /CD8-
day 6
day 21
day 41
Days post-infection

Figure 4-3: $CD4^+$ T cells are the primary IFN-γ^+ population after LPS stimulation

CD4 T cells are the major producers of IFN-γ in the spleen throughout systemic *Salmonella* infection. C57BL/6 mice were infected with $5x10^5$ BRD509 i.v. then stimulated with 10μg LPS at various timepoints post-infection, as indicated. After 4 hours, mice were euthanized and splenocytes prepared for flow cytometry. IFN-γ-producing populations were examined after a broad live gate and singlet gating here at days 6, 21, and 41 post-infection, representing the various stages of the T cell response. (A) Samples were gated on $CD4^+$/ $CD8^-$, $CD8^+$/ $CD4^-$, or $CD4^-$/ $CD8^-$ populations as shown on the left, then for IFN-γ-production as shown in the histogram on the right. The CD4 are shown in red, the CD8 in blue, and the $CD4^-/CD8^-$ are filled in gray. The red gate and %IFN-γ^+ displayed on the histogram is %IFN-γ^+ of $CD4^+$, but the same gate was used for each population. (B) %IFN-γ^+ CD4, CD8, or $CD4^-$/ $CD8^-$ cells as gated in (A) were compiled for infected, LPS-stimulated mice at indicated timepoints.

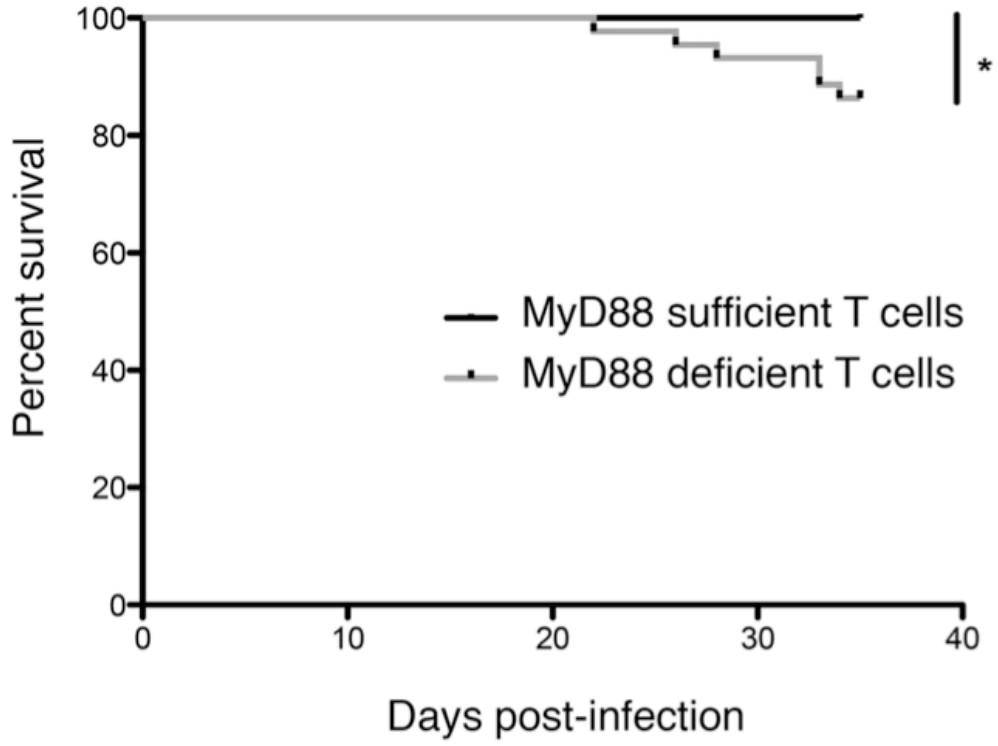

Figure 4-4: *Lck-cre x Myd88*$^{flox/flox}$ mice display impaired survival during *Salmonella* infection

Wild-type, *Lck-cre x Myd88*$^{flox/flox}$ homozygote and *Lck-cre x Myd88*$^{flox/WT}$ heterozygote mice were infected i.v. with $5x10^5$ *Salmonella* BRD509 and monitored for 5 weeks post-infection. Survival curve shows combined data from at least 3 experiments and contains at least 10 mice per group. Wild-type and littermate control *Lck-cre x Myd88*$^{flox/WT}$ mice are grouped together as 'MyD88 sufficient T cells'. *Lck-cre x Myd88*$^{flox/flox}$ mice are shown as 'MyD88 deficient T cells'. Statistical significance was observed by the Log-rank (Mantel-Cox) test.

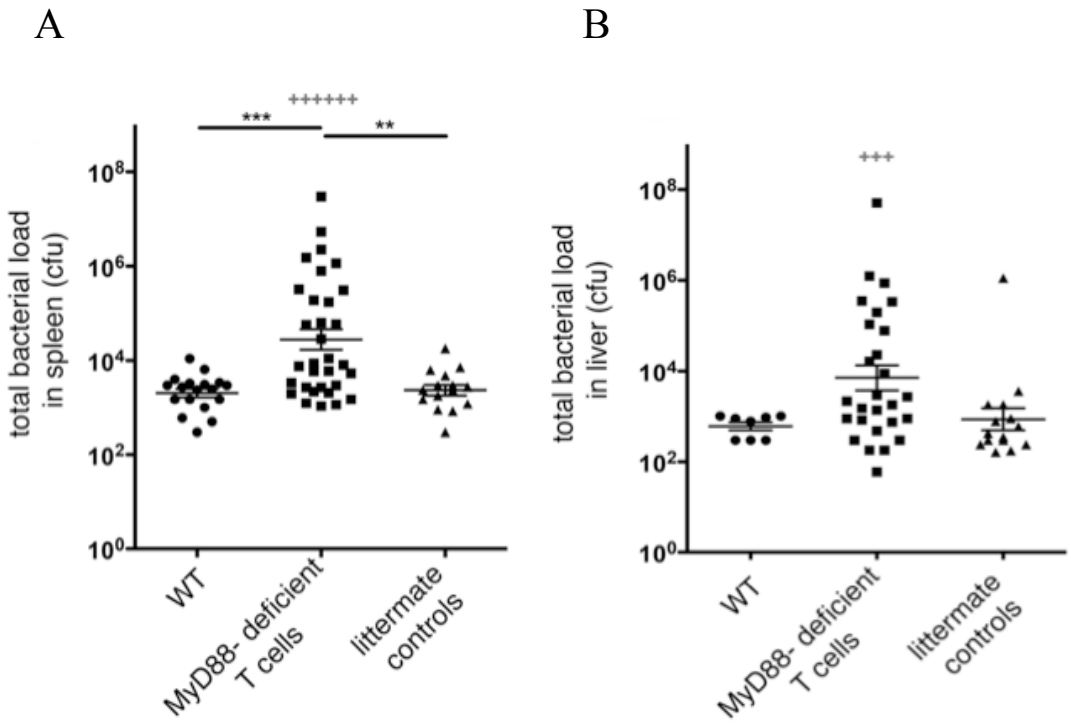

Figure 4-5: Mice lacking MyD88 signalling in T cells show impaired *Salmonella* clearance

Wild-type, *Lck-cre x Myd88*$^{flox/flox}$ homozygote, and *Lck-cre x Myd88*$^{flox/WT}$ heterozygote mice were infected i.v. with $5x10^5$ *Salmonella* BRD509. Bacterial burdens in the (A) spleen and (B) liver were determined five weeks after infection. Gray (+) symbols show the number of mice that became moribund prior to assessment of bacterial burdens at the 5 week time point, also shown in the survival curve in Figure 4-3. Graphs represent pooled data from multiple experiments. Statistical significance was determined on log transformed data by one-way ANOVA. Error bars represent mean +/- SEM.

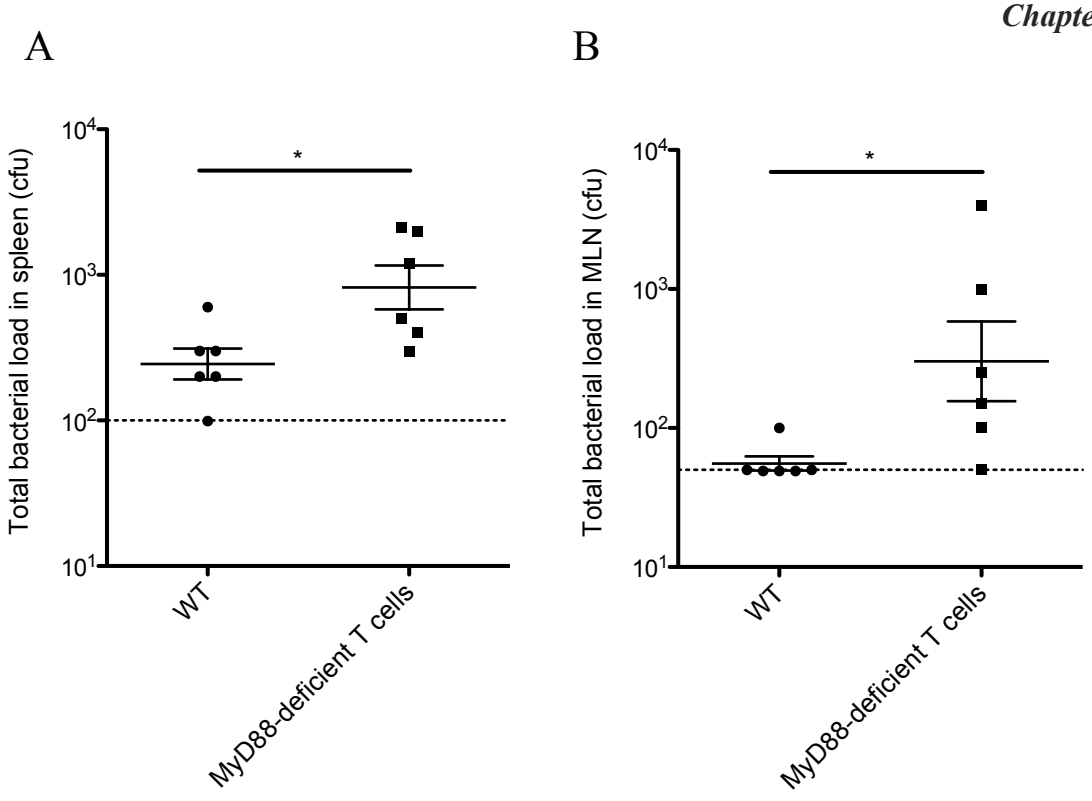

Figure 4-6: Resolution of *Salmonella* infection is impaired in mice with MyD88-deficient T cells

Wild-type and *Lck-cre x Myd88*$^{flox/flox}$ homozygous mice were infected i.v. with 5x10^5 *Salmonella* BRD509. Bacterial burdens in the (A) spleen and (B) mesenteric lymph nodes (MLN) were determined eight weeks after infection. Dotted lines represent the limit of detection, and samples with no detected bacteria fall just below the line. Data is representative of one experiment. Statistical significance was determined on log transformed data by an unpaired, two-tailed T test. $P < 0.05$ (*). Error bars represent mean +/- SEM.

Conclusion

Overview

During an infection, T cells are activated by an interaction of TCRs and MHCs that allow a very powerful response to be limited to a pre-determined set of non-self antigens. This is an important control mechanism that limits severe immunopathology. However, once this checkpoint is passed in the secondary lymphoid tissues during initial T cell priming, the requirement of TCR:MHC interactions for every instance of IFN-γ elicitation by a T cell becomes dangerously redundant. When a T cell can "see" a war going on around it, in the form of inflammatory signals, why should it wait for further instructions to act? Our results have demonstrated that Th1 respond to IL-18 that is produced in response to combined TLR and NLR signaling. This suggests a mechanism for the non-cognate stimulation of effector Th1 responses. We further showed that in the absence of this response clearance of *Salmonella* was impaired, establishing that this pathway contributes to the normal resolution of infection.

The information provided here, as well as considerable work in other mechanisms (Katsikis et al.), allows us to reconsider the conventional division of innate and adaptive immunity in favor of a model in which innate and adaptive immune responses communicate and coordinate their responses according to the needs of the circumstances (Fig B-1). Developing our understanding of these interactions and overlapping pathways further could significantly improve our ability to understand, and ultimately to modify, the immune responses required for vaccination and immunotherapeutics, whether stimulating the clearance of a granuloma, or suppressing the T cell response in autoimmunity.

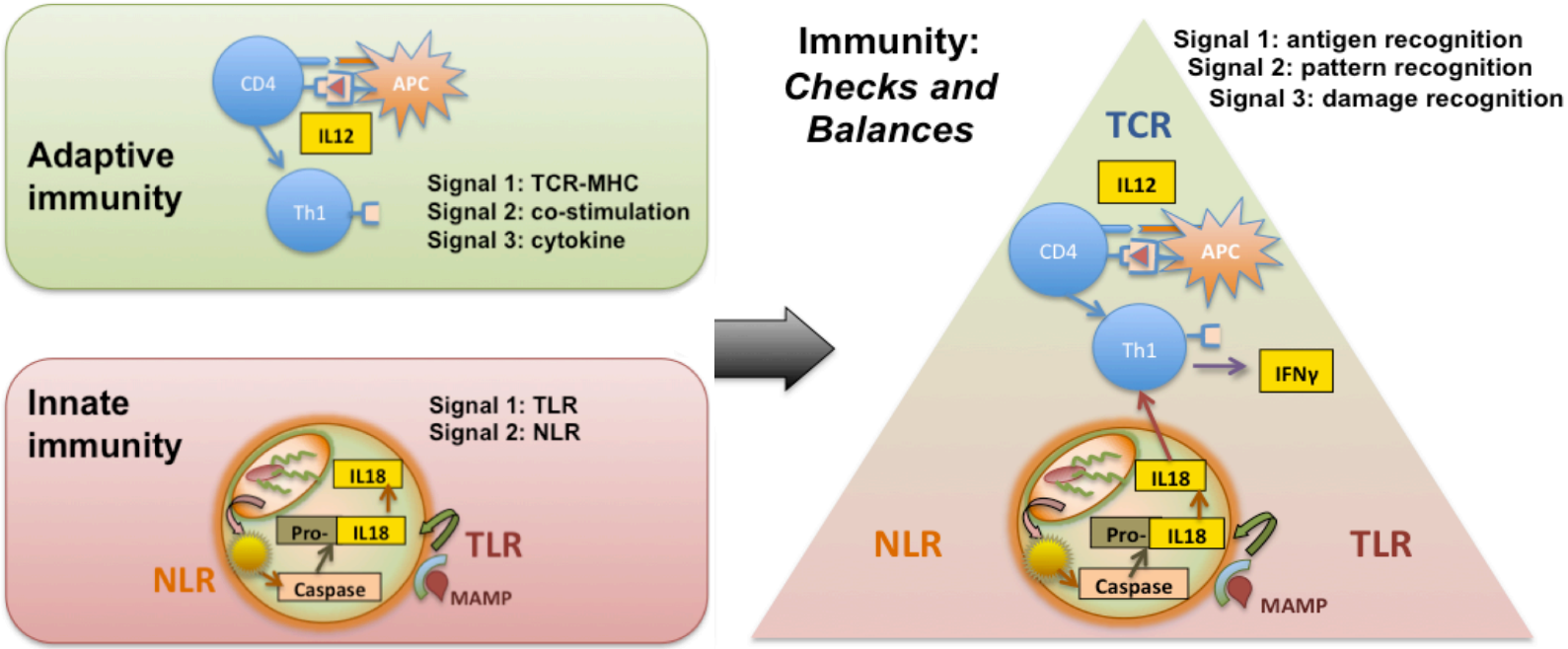

Figure B-1: Checks and balances of T cell responses
The conventional view of immunity is divided into innate and adaptive responses (left panels). Each is associated with particular cell types and signals. T cells recognize specific antigen through the TCR:MHC complex. Cells like DCs and macrophages have PRRs to recognize conserved microbial ligands and damage. However, these responses do not occur in isolation. Our work suggests that the strongest immune response occurs when all of these signals come together. Logically, this helps to limit immunpathology to situations with clearly demonstrated severity.

Mechanisms of innate stimulation of T cells

The signals eliciting production of IFN-γ in various cell types have been studied extensively, and yet the complexity of the regulation of this cytokine continues to unfold and constantly highlights how much we have left to learn. For example, while T-bet is considered to be a master transcriptional regulator of IFN-γ production, in NK cells and CD8 T cells Eomes is able to make up for the loss of T-bet, while in CD4 T cells T-bet signalling is required (Lazarevic et al., 2013). Further, it was shown that the promoter region of IFN-γ that is utilized after TCR signaling is different than the promoter region required for IFN-γ production in response to IL-12 and IL-18 (Yang et al., 1999). In addition to transcriptional regulation, extensive post-transcriptional regulation of IFN-γ mRNA has been shown to take place, and varies between naïve, effector and memory T cell populations.

An activated Th1 cell is primed to produce IFN-γ rapidly, meaning that the IFN-γ gene locus is modified to be open for faster transcription, IFN-γ mRNA has been transcribed and is ready for rapid translation, and IFN-γ protein has been translated and awaits the signals necessary for post-transcriptional modifications and secretion. Despite all of this preparation, Th1 do not constitutively secrete IFN-γ. When they receive antigen-specific TCR signals, the signaling cascade downstream of the TCR allows rapid release of the prepared IFN-γ transcripts and protein from the regulatory mechanisms that otherwise keep this production in check. However, other signaling pathways can also stimulate IFN-γ production in activated Th1, including IL-12 and IL-18 in combination, as mentioned above.

While some evidence has suggested an ability for innate ligands to interact directly with CD4 T cells to stimulate their proliferation or function, it is also likely that innate ligands can stimulate T cells through a second messenger that would allow amplification of the signal. Many cells are capable of responding to TLR or NLR ligands to produce inflammatory cytokines, including those cytokines known to stimulate IFN-γ production from Th1 cells. Thus, it is easy to imagine a mechanism whereby Th1 cells respond indirectly to innate stimuli by responding to inflammation. In fact, this has been shown to

occur during viral infections in response to TLR ligands, and during *Salmonella* infection in response to NLR ligands, both in CD8 T cells (Kupz et al., 2013; Raué et al., 2004).

Here, we define a mechanism for innate Th1 stimulation that relies on the convergence of both TLR and NLR signaling pathways to elicit IL-18 production, which can then be recognized by activated Th1 to result in IFN-γ secretion (Fig B-2). Further, while previous work has typically focused on very small populations of responding T cells, we showed that Th1 cells in an infectious model of strong Th1 activation are highly susceptible to innate stimulation, with a large proportion of the Th1 capable of secreting IFN-γ in response to LPS stimulation. Finally, we demonstrated that mice whose T cells lack the capacity to be innately stimulated suffer a reduced capacity to clear *Salmonella* infection. Together, this suggests a pathway which not only can occur, but must occur for normal immune function. We discuss this distinction further in the next section.

Checks and balances: the significance of adaptive cells with innate functions

The strict rules governing conventional T cell activation as outlined in the introduction are an important checkpoint to prevent unnecessary inflammation and host damage. However, under certain circumstances a rapid response to clear and present danger is required. While these rapid responses are initiated by the conventional innate immune response, the innate response is not always sufficient in strength or duration to combat severe infection. Under these circumstances, the development of the adaptive immune response is critical. However, the specificity and strength of these responses typically comes at a cost. Strong adaptive immune responses require time and abundant specific antigen, and during severe infection neither may be available. For this reason, it is easy to imagine that mechanisms might need to be in place that would allow a less cautious approach to a strong inflammatory response when a crisis occurs. It is equally easy to imagine how this might backfire.

Conventional literature on the subject of innate T cell responses has tended to focus on it more as a phenomenon than a function, although the CD8 T cell literature certainly contains exceptions (Berg et al., 2003; Berg and Forman, 2006). Th1 cells responding to TCR-independent signals were not typically thought of as an alternative response

pathway, but as 'bystanders' of the actual response going on around them. The term bystander itself connotes an effect which is unintentional or unplanned. Given the magnitude of these responses, and the similarity to other described 'innate-like' pathways, it seems unlikely that this stimulation is merely coincidental. More likely, this response is a programmed component of the normal T cell effector function. When cognate interactions are not sufficient, as detailed in the introduction, a non-cognate response could amplify critical effector functions. In a crisis, as recognized by the presence of microbes and damage that lead to specific inflammatory signals, like IL-18, the ability to "shoot first, and ask questions later" may allow Th1 cells at sites of infection to engage in the battle surrounding them earlier and more effectively (Fig B-3).

For every answer, new questions

While this and other recent work have established the foundation of a mechanism and function for an innate T cell stimulation response pathway, our current knowledge is really only the beginning. The ability to elicit effector functions from T cells without specific antigen could have broad clinical implications, but before we can begin to address these applications we need a thorough understanding mechanistically and functionally of how this response differs from the conventional T cell responses described. We'll discuss some specific examples of these questions in three parts: first looking at some mechanistic questions that remain to be resolved, then addressing some key functional questions, before finally asking how understanding of this pathway can be applied to clinical problems.

As mentioned earlier, one key question surrounding innate T cell stimulation is the impact these signals have on the cell fate decision, as compared to conventional TCR stimulation. While some work to address CD8 T cell fate decisions has been done, there is currently a limited understanding of CD4 T cell fate decisions in general, whether by TCR stimulation or otherwise. Thus, learning how and when an individual T cell decides between apoptosis and memory, short or long-term memory, central or effector memory, and what other options might be available, is still a critical question of basic T cell biology. How innate stimulation may factor into these decisions will be an important

piece of this puzzle. Further, the extent to which there is plasticity in these decisions remains to be answered, as do the requirements for reactivation, as detailed in the introductory figure A-5. Current work in CD8 T cells suggests that memory CD8 T cells can be reactivated by innate stimuli alone, but the significant decrease in the response kinetics we observed in chapter 1 after *Salmonella* clearance raises questions about the response of memory CD4 T cells. It is possible that at least an initial TCR signal is required to reactivate these memory cells, after which they may again be restimulated by innate signals. It is also possible that in the absence of infection there are simply insufficient NLR stimuli available to partner with the TLR signals.

NLR stimulation is itself an area that requires further exploration, both in this mechanism of T cell stimulation, and in general. While it is well established that *Salmonella* infection triggers NLRC4 and NLRP3 inflammasome activation (Broz et al., 2010), it is less clear how an endosomal pathogen is recognized by a cytoplasmic receptor. It has been proposed that microbial ligands may enter the cytosol alongside the effector molecules injected by the Type-III secretion system (T3SS), and indeed some NLRs recognize components of the T3SS needle complex itself (Broz and Monack, 2013; Sun et al., 2007). However, more studies are needed to determine whether the requirement for NLRC4 and NLRP3 in innate T cell activation is T3SS-dependent, or whether there is an alternative mechanism of inflammasome activation involved.

Another key point mentioned earlier is the involvement of other CD4 T cell subsets. While our work has focused on elicitation of IFN-γ from Th1 cells, there are a myriad of possibilities for the role of this same innate stimulation pathway in other CD4 T cells. It is certainly possible that this mechanism uniquely affects Th1 cells, among the CD4 T cells. However, a far more interesting scenario would involve different innate stimulation mechanisms within different CD4 subsets. For example, given the role of IL-1 in the Th17 response, a similar role for TLR and NLR driven IL-1β production in the innate stimulation of Th17 cells could be hypothesized. Further, while IL-33 is not a caspase matured cytokine, some believe that its release from the cell is itself a danger signal because of its role in the nucleus. Since IL-33 is known to act upon Th2 cells, this

cytokine could also mediate stimulation in this subset. In this manner, one could imagine a set of cytokine signals capable of mediating the innate stimulation of each CD4 T cell subset. Within the Th1 subset itself it is possible that additional signals are present that can act alongside the IL-18 and IL-33 responses we observed here. As mentioned earlier, in CD8 T cells a diverse mix of cytokine signals were shown to elicit some level of response from effector or memory CD8 T cells in vitro (Freeman et al., 2012). A similarly comprehensive look at cytokine stimulation in different CD4 T cell subsets could prove useful as a first step in defining the potential applicability of this response pathway across CD4 T cell subsets.

Finally, although we previously showed that activated CD4 T cells could be transferred into MHC-II-deficient recipients and still be stimulated to produce IFN-γ after LPS administration (Srinivasan et al., 2007), the exact contributions and timing of TCR and cytokine signals remains to be elaborated upon. TCR engagement alone has been shown to be sufficient for stimulation (McLachlan et al., 2009), and stimulation by cytokines in the absence of MHC-II: TCR engagement was sufficient, but whether these interactions can act synergistically, whether the timing of these interactions is a factor, and whether any of these considerations influence functionality, epigenetic programming, apoptosis, or memory fate decisions, are all questions that remain to be considered.

In addition to questions about the mechanisms controlling these innate T cell responses, many questions are raised by the existence of this response pathway as to what functions it serves. Addressing many of these questions would require a deeper understanding of the mechanistic questions outlined above, but here we briefly consider some of the functional questions that can now be asked with our current understanding of how innate Th1 stimulation occurs. First, while numerous groups have looked at non-cognate restimulation of memory CD8 T cells, whether non-cognate restimulation of CD4 T cells is an important component of the rapid secondary immune response is unknown. Using our Lck-MyD88$^{f/f}$ mice we can potentially address the role of this response upon re-challenge with virulent *Salmonella*. Given our limited understanding at this time of the effects of innate stimulation on memory formation, any efforts to interrogate the

secondary response will come with caveats. The inducible cre system offers one possible solution to this problem.

Further, while we have interrogated this response in *Salmonella*, the functional relevance of this pathway in other infection models remains to be proven. Within the context of innate Th1 responses, any intracellular infection requiring IFN-γ could be hypothesized to require this innate pathway. If the mechanism is expanded to other T cell subsets, than it could be presumed that the immune response to a wide variety of infections might require this pathway. Moreover, the ability to elicit non-cognate effector functions makes exploration of a role in co-infection and polymicrobial infection models seem promising. Infection with a first Th1-activating infection (A) could conceivably protect against a second (B), as the already activated A-specific Th1 cells respond to the early innate recognition of B ligands.

Finally, while we have focused on Th1 stimulation in a model for which increased IFN-γ production has been shown to be beneficial, the potential misuse of these same pathways needs to be explored. In particular, during highly inflammatory infections, to what extent does the innate T cell response contribute to immunopathology. Further, could innate T cell stimulation be responsible for some of the T cell-mediated autoimmune conditions described, and if so, how do the responses in these patients differ from responses in healthy patients? It is important to understand the functions of these responses, both positive and negative, before we can really begin to appreciate the applications of this knowledge.

Ultimately, we ask all of these questions for a better understanding of how this information can be applied practically. How can our knowledge be used to improve or suppress the system, and when do we do each? The simplest practical application for these findings relates to a common lab technique. In assays of T cell function it is common to add antigen, either to a culture or in vivo, to assess the effector response to stimulation. When the antigen is unknown, or a polyclonal response is desired, often this antigen stimulation is administered in the form of a heat-killed or otherwise inactivated microbe. It is important to realize that these methods would include not only T cell

antigens, but also a great deal of MAMPs. In some cases this distinction could prove irrelevant, or even useful, but in other cases the innately stimulated T cells could inexplicably confound the results.

For example, currently to test whether a person is infected with tuberculosis (TB) their PBMCs (peripheral blood mononuclear cells) are stimulated with TB antigens and IFN-γ production is measured. Recently, it was shown that addition of TLR ligands improves the sensitivity of this assay (Gaur et al., 2012). Given the non-cognate nature of the T cell stimulation by these ligands, this improved assay may also result in higher false positive rates. Individuals with other current (known or unknown) infections would likely have activated T cells capable of innate stimulation. Thus, theoretically it might be possible to detect anything from a cold to an asymptomatic chlamydia infection by the addition of TLR ligands to PBMCs. The potential for the innate T cell response to confound results needs to be considered and tested in such cases.

As a better understanding of these pathways develops it might be feasible to modulate the innate T cell response to our benefit. With a better understanding of the mechanism for development and restimulation of memory T cells, and the function of these cells in the secondary response, it could be possible some day to tailor vaccine design around the development of stronger innate T cell responses. Further, potential immunotherapeutics could be designed to improve or suppress particular T cell functions. For example, a more targeted treatment to clear mycobacterial granulomas with less toxicity than directly administered IFN-γ. Conversely, monoclonal antibodies against IL-18 could prove useful for autoimmune conditions in which Th1 cells are being activated by non-cognate stimuli.

Overall, we are just beginning to understand the significance of this response pathway. As more and more details unfold as to how these T cells are stimulated innately and what purposes this innate stimulation serves, more opportunities to alter these responses will become apparent.

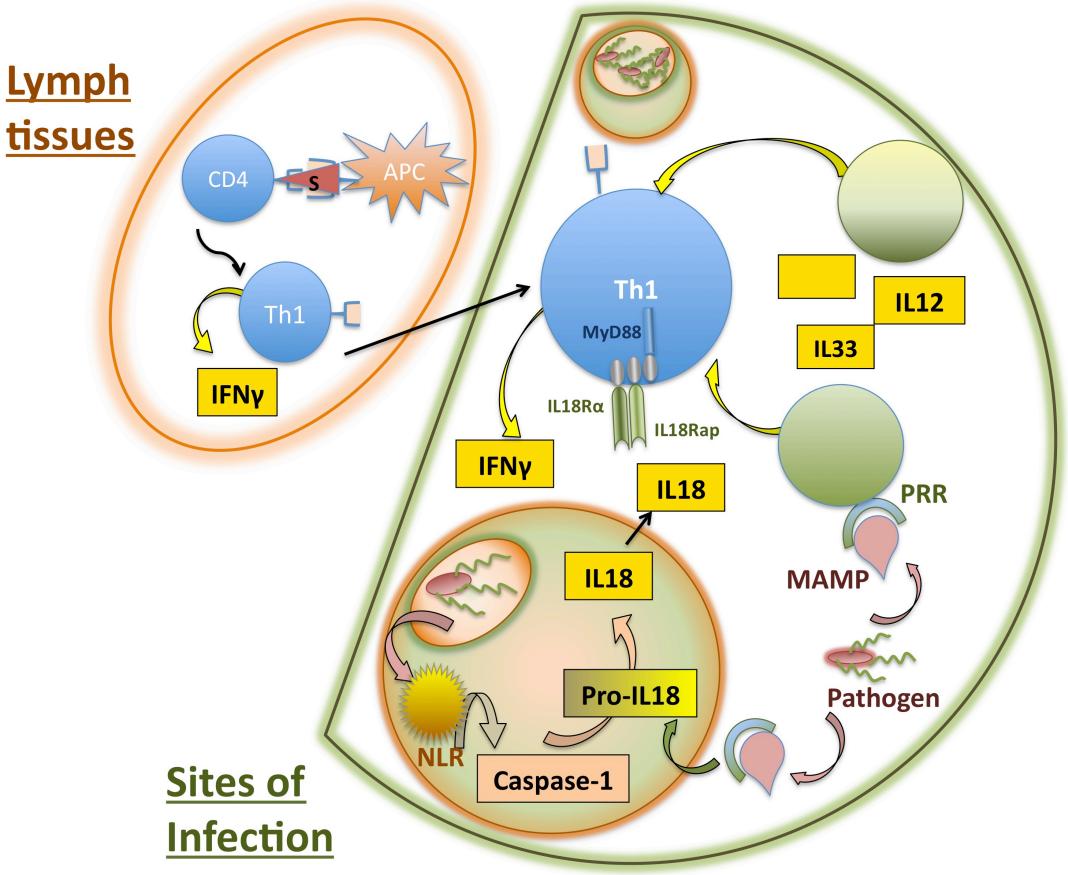

Figure B-2: Mechanism of Th1 response to innate stimuli

CD4 T cells are activated by direct interaction with APCs in the secondary lymph tissues. These effector cells migrate to sites of infection, where they require additional stimulation to provoke effector cytokine secretion. Although conventional wisdom describes this signal as an additional direct interaction with an APC, the data here supports an alternative mechanism of Th1 cell stimulation. We propose that signals from TLR recognition of conserved microbial ligands and NLR recognition of intracellular infection or cell damage converge, resulting in the secretion of IL-18. Stimulation of Th1 after administration of the TLR ligand LPS required Th1-intrinsic expression of the IL-18R. Together, these data suggest a model wherein non-cognate stimulation of Th1 cells can occur in the presence of both TLR and NLR signals, bypassing an adaptive checkpoint under critical inflammation.


T
1
2
3
Adaptive
Th1
Cooperate
INNATE
1
2
Hope

Figure B-3: Optimal Th1 responses require both innate and adaptive immune signals

This illustration highlights the ability of Th1 cells to communicate and cooperate with both the adaptive and innate arms of the immune system for optimal function. In the center, a blue Th1 cell recently emigrated from a lymph node in which conventional T cell activation signals were received encounters inflammatory signals (yellow) generated in response to TLR (pink) and inflammasome signaling (yellow, orange, and green) by an innate cell (orange) at the site of infection. These inflammatory signals allow the Th1 cell to rapidly recognize and respond to an ongoing crisis. The Th1 cell secretes its own signals (blue), such as IFN-γ, to activate other cells in the vicinity. A nearby macrophage (yellow) is warned to 'prepare for battle'.

Materials and Methods

Mice

C57BL/6, B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ, B6.PL-Thy1^{a}/CyJ, TLR4-deficient, and IL-18R-deficient mice were purchased from The Jackson Laboratory or NCI at 6-8 weeks of age. MyD88-, IL-1R1-, and IFN-γR-deficient C57BL/6 mice were obtained from Dr. Jenkins (University of Minnesota) and Dr. Way (University of Cincinnati), and bred in our animal facility. IL-33R-deficient C57BL/6 mice were kindly provided by Dr. Bryce (Northwestern University). Mice deficient in NLRC4 and NLRP3 were maintained at Stanford University (Broz et al., 2010). T cell specific Myd88 deficient mice were generated as illustrated in Figure C-1 by crossing *Lck-cre* mice to *Myd88*$^{loxP/loxP}$ flanked mice purchased from Jackson labs. Mice were genotyped by PCR according to protocols provided by The Jackson Laboratory. All animal procedures were approved by UC Davis IACUC (#16612) and University of Minnesota IACUC (#0801A25442).

Bacterial strains, culture and infection

Bacterial strains used for this work are listed in Table C-1. Aflagellate *Salmonella enterica* serovar Typhimurium strain BRD509 was modified by Sean-Paul Nuccio as detailed below to generate aflagellate BRD509. Unless otherwise indicated, all mice were given 5x10^{5} cfu bacteria intravenously (i.v.). Prior to infection, all *Salmonella* was grown overnight at 37°C in static Luria-Bertani (Becton Dickinson) broth culture. Concentration of overnight culture was determined by spectrophotometry at OD_{600} and bacteria were diluted in sterile 1X PBS (phosphate buffered saline, Gibco) to the appropriate concentration for administration in 200µl total volume. All samples used for infection were titrated and plated on MacConkey agar (Becton Dickinson) to confirm administered bacterial dose.

Chlamydia muridarum strain Nigg II was purchased from ATCC and was cultured and quantitated by Linxi Li within HeLa 229 cells grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum. Elementary bodies (EBs) were purified by discontinuous density gradient centrifugation and stored at -80°C, as previously described (Scidmore, 2005). Purified EBs were enumerated for

inclusion-forming units by infecting HeLa 229 cells and counting inclusions stained with anti-*Chlamydia* MOMP. A fresh aliquot was thawed for each infection, in which $1x10^7$ EBs were administered i.v. in 1X sterile PBS (Gibco).

Generation of aflagellate strain SPN529

Bacteriophage P22 (*HT105/1 int-201*) was utilized for transduction. Transductants were purified from phage P22 on EBU agar and confirmed to be P22-sensitive by cross-streaking colonies against P22-H5. A flagellin-deficient mutant of BRD509 was generated by transducing the *fliC5050*::Mu*d*J insertion of SPN286 into BRD509 and selecting for kanamycin-resistant transductants, yielding SPN527. The *fljB5001*::Mu*d*Cm of SPN287 was transduced into SPN527 by selecting for chloramphenicol-resistant mutants, yielding SPN529. SPN529 was confirmed to be non-motile relative to BRD509 by stabbing in motility agar and incubating at 37^oC for 24 hours.

Quantification of bacterial loads

Mice were euthanized and the indicated organs collected in HBSS (Hank's balanced salt solution) or PBS (both Gibco) on ice, homogenized, and reconstituted in a known volume of HBSS or PBS. Samples were mixed thoroughly and serial 1:10 dilutions were plated on MacConkey agar (Becton Dickinson) plates, incubated overnight at 37^oC, and enumerated the following day to calculate the number of colony forming units (cfu) in the total organ.

Flow cytometry

For flow cytometry, cells were prepared as a single cell suspension of between $1x10^6$ and $8x10^6$ cells/ml and stained with various antibodies from eBioscience, Becton Dickinson (BD), or Tonbo Biosciences, for 30 minutes to 1 hour in Fc block on ice. Intracellular cytokine or transcription factor staining was performed using the Foxp3 intra-nuclear staining kit from eBioscience, as recommended by the manufacturer. Stained cells were analyzed using a FACS Canto, Aria or Fortessa (BD) using appropriate compensation controls and flow cytometry data was analyzed using FlowJo software (TreeStar).

Bone marrow chimeras

Mixed bone marrow chimeras were generated as illustrated in Figure C-2 by first irradiating 45.1+ congenic mice (B6.SJL) (1000 rads irradiation by cesium source). The following day, bone marrow was isolated from congenic CD90.1+ (B6.PL) and genetically-deficient mouse femurs and tibias, cells counted by hematocytometer, and then the bone marrow cells were combined at a 1:1 ratio before being administered ($4x10^6$ total cells) in 200µl 1X PBS (Gibco). After bone marrow transfer, chimeras were maintained on antibiotics for at least 4 weeks and blood collected at 4 weeks post-transfer for flow cytometric analysis of immune reconstitution. Chimeras were infected 6-8 weeks after administration of bone marrow, and at least 2 weeks after discontinuation of antibiotic treatment.

In vivo administration of TLR ligands

Ultrapure lipopolysaccharide (LPS) from *E. coli* strain EH100Rα (Alexis, TLRgrade®), purified bacterial flagellin, endotoxin-free Pam3CSK4, CpG DNA (ODN1585), and Imiquimod (all Invivogen) were diluted in 1X PBS (UltraPure PBS, Gibco) and administered i.v. to mice. Spleens and livers were harvested from infected or uninfected mice 4 hours or less after administration of MAMPs. Flagellin was purified from an LPS-deficient X4700 strain of *S.* Typhimurium using a modified acid-shock protocol and passed multiple times through Detoxi-Gel columns (Thermo Scientific) to remove residual endotoxin, as previously described (Salazar-Gonzalez et al., 2007).

RNA preparation, RT-QPCR, and microarray

RNA was extracted from cryopreserved spleens and livers according to the manufacturer's instructions using TRIzol reagent (Ambion) and quantified using a Nanodrop spectrophotometer (Thermo Scientific). RNA was DNase-treated using a DNA-free kit (Ambion), re-quantified using the Nanodrop, diluted to a stock concentration in sterile RNAse-free water (Gibco), and stored at -80°C for future use in RT-QPCR or microarray.

For RT-QPCR, a SensiFAST SYBR Hi-ROX One-Step RT-QPCR kit (Bioline) was used for both cDNA synthesis and QPCR reactions using 100ng total RNA per reaction. RT-QPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 96 well plate and triplicate data was analyzed in Microsoft Excel by the comparative Ct method (Applied Biosystems) using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the control. Data is expressed as fold change of stimulated over unstimulated ΔCt. Primer sequences and their references are listed in Table C-2.

Serum Cytokine ELISAs

Sera was prepared by collecting blood from retro-orbitally exsanguinated, anesthetized mice that had been infected or stimulated as indicated, then blood was incubated on ice to allow clotting, centrifuged, and serum harvested and stored at -20°C. Cytokine ELISAs for IFN-γ, IL-12 and IL-33 were performed by Oanh Pham according to the instructions provided by the Ready-Set-Go kit (eBioscience) and concentrations determined using the protein standard provided. For the IL-18 ELISA, the same standard protocol was followed using capture and detection antibodies (Medical and Biological Labs, Co) as recommended by the manufacturer with rmIL-18 as a standard (R&D). After substrate was added, plates were read at 450nm using a microplate reader (Spectra Max M2, Molecular Devices, Inc) and analyzed in Microsoft Excel.

Antibody depletion of CD4+ and CD8+ T cells

Loss of $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$ T cells was examined in vivo by depletion of cells with indicated rat anti-mouse monoclonal antibodies (BioXCell). Antibodies were administered intraperitoneally twice per week starting at day 7 and depletions were maintained until euthanization. The initial doses (days 7 and 10) contained 200 μg of antibody per mouse, all subsequent doses contained 300 μg per mouse. Maintenance of depletion was monitored by flow cytometry on blood collected once per week from the lateral tail vein.

Statistical Analyses

All statistical analyses were performed as described in the figure legends using Prism version 5 (Graphpad). All error bars are displayed as mean +/- SEM. P< 0.005 (***), p< 0.01 (**), p<0.05 (*) or p>0.05 (ns).

Designation	Relevant genotype	Source
S. Typhimurium		
BRD509	SL1344 Δ*aroA* Δ*aroD*	(Strugnell et al., 1992)
SPN286	IR715 *fliC5050*::Mu*d*J	(Winter et al., 2009)
SPN287	IR715 *fljB5001*::Mu*d*Cm	(Winter et al., 2009)
SPN527	SL1344 Δ*aroA* Δ*aroD fliC5050*::Mu*d*J	(O'Donnell et al., 2014)
SPN529	SL1344 Δ*aroA* Δ*aroD fliC5050*::Mu*d*J *fljB5001*::Mu*d*Cm	(O'Donnell et al., 2014)
C. muridarum		
Nigg II		ATCC

Table C-1: Bacterial strains used in this work

Primer	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')	Reference
IL12 p35	AAATGAAGCTCTGCATCCTGC	TCACCCTGTTGATGGTCACG	(Liu et al., 2004)
IL18	CAGGCCTGACATCTTCTGCAA	CTGACATGGCAGCCATTGT	(Abu Elhija et al., 2008)
IL33	GGTGTGGATGGGAAGAAGCTG	GAGGACTTTTTGTGAAGGACG	(Talabot-Ayer et al., 2012)
IFN-γ	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG	(Godinez et al., 2008)
Gapdh	ACGGCCGCATCTTCTTGTGCA	AATGGCAGCCCTGGTGACCA	(Talabot-Ayer et al., 2012)

Table C-2: Primers used for RT-QPCR

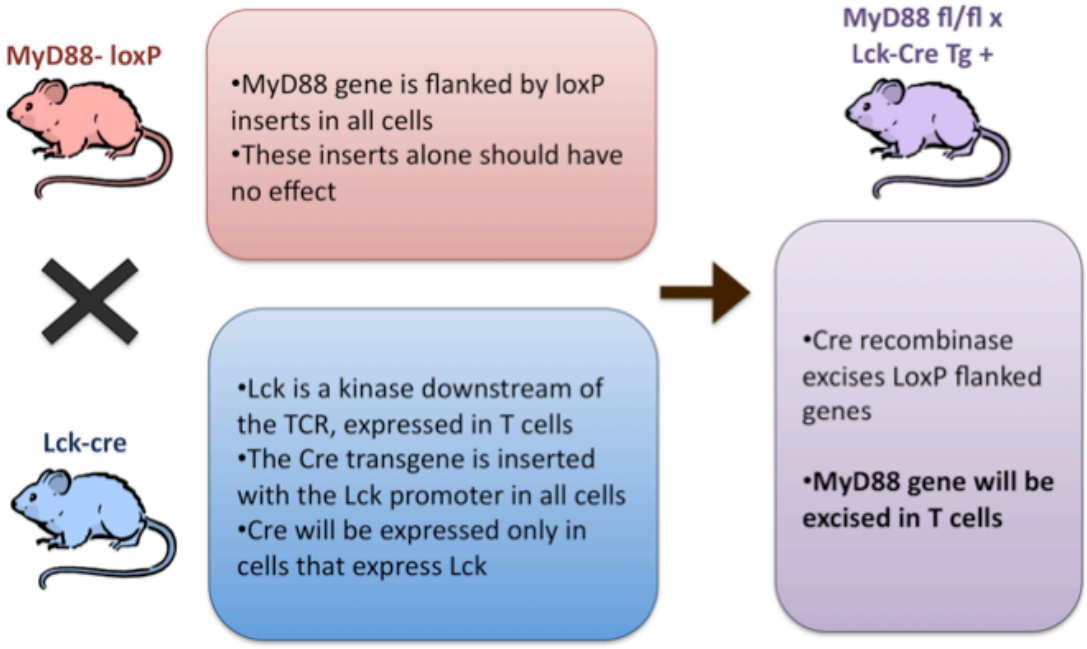

Figure C-1: A conceptual schematic for the generation of Lck-MyD88$^{f/f}$ mice

MyD88-loxP mice are bred to Lck-cre mice to yield F1 offspring that are heterozygous for both the cre recombinase gene and MyD88-loxP (MyD88$^{f/w}$). These mice are then crossed a second time to MyD88-loxP mice (cre negative), resulting in offspring that are either heterozygous (cre +) or negative for Lck-cre, and MyD88$^{f/f}$ or MyD88$^{f/w}$. Cre + mice are shown as Lck-MyD88$^{f/f}$ (MyD88-deficient T cells) or Lck-MyD88$^{f/w}$ (littermate controls) in the text.

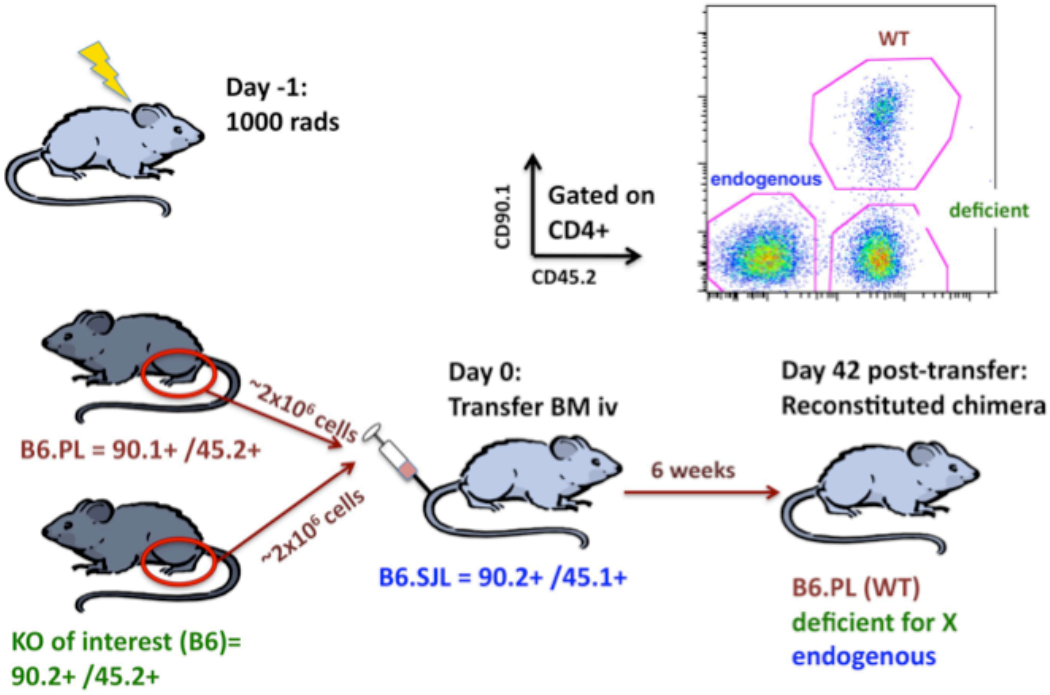

Figure C-2: Making mixed bone marrow chimeras

The diagram shows how mixed bone marrow chimeras are created, as detailed in the methods, and the gating strategy used during flow cytometry to analyze the populations of cells coming from separate mice. Briefly, a congenically marked recipient mouse is irradiated, then the following day bone marrow is isolated from 2 different congenically marked donors and mixed in equal proportion before i.v. administration to the irradiated recipient. After six weeks, the donor marrow has reconstituted the irradiated recipient as a chimera of all three populations, as seen in the flow cytometry gating.

References

Abu Elhija, M., Lunenfeld, E., and Huleihel, M. (2008). LPS increases the expression levels of IL-18, ICE and IL-18 R in mouse testes. Am J Reprod Immunol *60*, 361-371.

Bangs, S.C., Baban, D., Cattan, H.J., Li, C.K., McMichael, A.J., and Xu, X.N. (2009). Human CD4+ memory T cells are preferential targets for bystander activation and apoptosis. J Immunol *182*, 1962-1971.

Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T.F., Mann, M., and Bumann, D. (2006). Robust *Salmonella* metabolism limits possibilities for new antimicrobials. Nature *440*, 303-307.

Berg, R.E., Crossley, E., Murray, S., and Forman, J. (2003). Memory CD8+ T cells provide innate immune protection against Listeria monocytogenes in the absence of cognate antigen. J Exp Med *198*, 1583-1593.

Berg, R.E., and Forman, J. (2006). The role of CD8 T cells in innate immunity and in antigen non-specific protection. Curr Opin Immunol *18*, 338-343.

Beuneu, H., Garcia, Z., and Bousso, P. (2006). Cutting edge: cognate CD4 help promotes recruitment of antigen-specific CD8 T cells around dendritic cells. J Immunol *177*, 1406-1410.

Bossaller, L., Chiang, P.I., Schmidt-Lauber, C., Ganesan, S., Kaiser, W.J., Rathinam, V.A., Mocarski, E.S., Subramanian, D., Green, D.R., Silverman, N.*, et al.* (2012). Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. J Immunol *189*, 5508-5512.

Bou Ghanem, E.N., and D'Orazio, S.E. (2011). Human CD8+ T cells display a differential ability to undergo cytokine-driven bystander activation. Cell Immunol *272*, 79-86.

Brennan, P.J., Brigl, M., and Brenner, M.B. (2013). Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol *13*, 101-117.

Brigl, M., Tatituri, R.V., Watts, G.F., Bhowruth, V., Leadbetter, E.A., Barton, N., Cohen, N.R., Hsu, F.F., Besra, G.S., and Brenner, M.B. (2011). Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. J Exp Med *208*, 1163-1177.

Broz, P., and Monack, D.M. (2013). Newly described pattern recognition receptors team up against intracellular pathogens. Nat Rev Immunol *13*, 551-565.

Broz, P., Newton, K., Lamkanfi, M., Mariathasan, S., Dixit, V.M., and Monack, D.M. (2010). Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against *Salmonella*. J Exp Med *207*, 1745-1755.

Bueno, S.M., Riquelme, S., Riedel, C.A., and Kalergis, A.M. (2012). Mechanisms used by virulent Salmonella to impair dendritic cell function and evade adaptive immunity. Immunology *137*, 28-36.

Castellino, F., Huang, A.Y., Altan-Bonnet, G., Stoll, S., Scheinecker, C., and Germain, R.N. (2006). Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature *440*, 890-895.

Celli, S., Garcia, Z., and Bousso, P. (2005). CD4 T cells integrate signals delivered during successive DC encounters in vivo. J Exp Med *202*, 1271-1278.

Chakir, H., Lam, D.K., Lemay, A.M., and Webb, J.R. (2003). "Bystander polarization" of CD4+ T cells: activation with high-dose IL-2 renders naive T cells responsive to IL-12 and/or IL-18 in the absence of TCR ligation. Eur J Immunol *33*, 1788-1798.

Chapman, T.J., Castrucci, M.R., Padrick, R.C., Bradley, L.M., and Topham, D.J. (2005). Antigen-specific and non-specific CD4+ T cell recruitment and proliferation during influenza infection. Virology *340*, 296-306.

Cheminay, C., Mohlenbrink, A., and Hensel, M. (2005). Intracellular *Salmonella* Inhibit Antigen Presentation by Dendritic Cells. J Immunol *174*, 2892-2899.

Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol *13*, 227-242.

Cheroutre, H., and Husain, M.M. (2013). CD4 CTL: living up to the challenge. Semin Immunol *25*, 273-281.

Crump, J., and Mintz, E. (2010). Global Trends in Typhoid and Paratyphoid Fever. Clin Infect Dis *50*, 241-246.

Cui, W., Joshi, N.S., Jiang, A., and Kaech, S.M. (2009). Effects of Signal 3 during CD8 T cell priming: Bystander production of IL-12 enhances effector T cell expansion but promotes terminal differentiation. Vaccine *27*, 2177-2187.

Curtsinger, J.M., Lins, D.C., and Mescher, M.F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J Exp Med *197*, 1141-1151.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K., and Mescher, M.F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol *162*, 3256-3262.

de Jong, R., Altare, F., Haagen, I.A., Elferink, D.G., Boer, T., van Breda Vriesman, P.J., Kabel, P.J., Draaisma, J.M., van Dissel, J.T., Kroon, F.P.*, et al.* (1998). Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. Science *280*, 1435-1438.

Di Genova, G., Savelyeva, N., Suchacki, A., Thirdborough, S.M., and Stevenson, F.K. (2010). Bystander stimulation of activated CD4+ T cells of unrelated specificity following a booster vaccination with tetanus toxoid. Eur J Immunol *40*, 976-985.

Dillon, S., Agrawal, A., Van Dyke, T., Landreth, G., McCauley, L., Koh, A., Maliszewski, C., Akira, S., and Pulendran, B. (2004). A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J Immunol *172*, 4733-4743.

Dunne, A., Ross, P.J., Pospisilova, E., Masin, J., Meaney, A., Sutton, C.E., Iwakura, Y., Tschopp, J., Sebo, P., and Mills, K.H. (2010). Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against Bordetella pertussis. J Immunol *185*, 1711-1719.

Dustin, M.L. (2008). T-cell activation through immunological synapses and kinapses. Immunol Rev *221*, 77-89.

Egen, J.G., Rothfuchs, A.G., Feng, C.G., Horwitz, M.A., Sher, A., and Germain, R.N. (2011). Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. Immunity *34*, 807-819.

Ezekowitz, R.A., Dinauer, M.C., Jaffe, H.S., Orkin, S.H., and Newburger, P.E. (1988). Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. N Engl J Med *319*, 146-151.

Fabrega, A., and Vila, J. (2013). Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin Microbiol Rev *26*, 308-341.

Franchi, L. (2011). Role of inflammasomes in salmonella infection. Front Microbiol *2*, 8.

Fraser, A., Paul, M., Goldberg, E., Acosta, C.J., and Leibovici, L. (2007). Typhoid fever vaccines: systematic review and meta-analysis of randomised controlled trials. Vaccine *25*, 7848-7857.

Frazer, L.C., Sullivan, J.E., Zurenski, M.A., Mintus, M., Tomasak, T.E., Prantner, D., Nagarajan, U.M., and Darville, T. (2013). CD4+ T cell expression of MyD88 is essential for normal resolution of Chlamydia muridarum genital tract infection. J Immunol *191*, 4269-4279.

Freeman, B.E., Hammarlund, E., Raue, H.-P., and Slifka, M.K. (2012). Regulation of innate CD8+ T-cell activation mediated by cytokines. Proceedings of the National Academy of Sciences *109*, 9971-9976.

Gapin, L., Godfrey, D.I., and Rossjohn, J. (2013). Natural Killer T cell obsession with self-antigens. Curr Opin Immunol *25*, 168-173.

Garlanda, C., Dinarello, C.A., and Mantovani, A. (2013). The interleukin-1 family: back to the future. Immunity *39*, 1003-1018.

Gaur, R.L., Suhosk, M.M., and Banaei, N. (2012). In vitro immunomodulation of a whole blood IFN-gamma release assay enhances T cell responses in subjects with latent tuberculosis infection. PLoS One *7*, e48027.

Geginat, J., Paroni, M., Facciotti, F., Gruarin, P., Kastirr, I., Caprioli, F., Pagani, M., and Abrignani, S. (2013). The CD4-centered universe of human T cell subsets. Semin Immunol *25*, 252-262.

Godinez, I., Haneda, T., Raffatellu, M., George, M.D., Paixao, T.A., Rolan, H.G., Santos, R.L., Dandekar, S., Tsolis, R.M., and Baumler, A.J. (2008). T Cells Help To Amplify Inflammatory Responses Induced by *Salmonella enterica* Serotype Typhimurium in the Intestinal Mucosa. Infect Immun *76*, 2008-2017.

Gold, M.C., and Lewinsohn, D.M. (2013). Co-dependents: MR1-restricted MAIT cells and their antimicrobial function. Nat Rev Microbiol *11*, 14-19.

Gottschalk, R.A., Corse, E., and Allison, J.P. (2010). TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. J Exp Med *207*, 1701-1711.

Griffin, A.J., and McSorley, S.J. (2011). Development of protective immunity to Salmonella, a mucosal pathogen with a systemic agenda. Mucosal Immunol *4*, 371-382.

Haring, J.S., Badovinac, V.P., and Harty, J.T. (2006). Inflaming the CD8+ T cell response. Immunity *25*, 19-29.

Haring, J.S., and Harty, J.T. (2006). Aberrant contraction of antigen-specific CD4 T cells after infection in the absence of gamma interferon or its receptor. Infect Immun *74*, 6252-6263.

Holland, S.M., Eisenstein, E.M., Kuhns, D.B., Turner, M.L., Fleisher, T.A., Strober, W., and Gallin, J.I. (1994). Treatment of refractory disseminated nontuberculous mycobacterial infection with interferon gamma. A preliminary report. N Engl J Med *330*, 1348-1355.

Honda, T., Egen, J.G., Lammermann, T., Kastenmuller, W., Torabi-Parizi, P., and Germain, R.N. (2014). Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues. Immunity *40*, 235-247.

Huse, M., Lillemeier, B.F., Kuhns, M.S., Chen, D.S., and Davis, M.M. (2006). T cells use two directionally distinct pathways for cytokine secretion. Nat Immunol *7*, 247-255.

Jantsch, J., Chikkaballi, D., and Hensel, M. (2011). Cellular aspects of immunity to intracellular Salmonella enterica. Immunol Rev *240*, 185-195.

Jouanguy, E., Doffinger, R., Dupuis, S., Pallier, A., Altare, F., and Casanova, J.L. (1999). IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. Curr Opin Immunol *11*, 346-351.

Kamath, A.T., Sheasby, C.E., and Tough, D.F. (2005). Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. J Immunol *174*, 767-776.

Katsikis, P.D., Schoenberger, S.P., Pulendran, B., and Aegean Conferences. Crossroads between innate and adaptive immunity IV.

Klose, C.S., Flach, M., Mohle, L., Rogell, L., Hoyler, T., Ebert, K., Fabiunke, C., Pfeifer, D., Sexl, V., Fonseca-Pereira, D*., et al.* (2014). Differentiation of Type 1 ILCs from a Common Progenitor to All Helper-like Innate Lymphoid Cell Lineages. Cell *157*, 340-356.

Kupz, A., Guarda, G., Gebhardt, T., Sander, L.E., Short, K.R., Diavatopoulos, D.A., Wijburg, O.L., Cao, H., Waithman, J.C., Chen, W*., et al.* (2012). NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. Nat Immunol *13*, 162-169.

Kupz, A., Scott, T.A., Belz, G.T., Andrews, D.M., Greyer, M., Lew, A.M., Brooks, A.G., Smyth, M.J., Curtiss, R., 3rd, Bedoui, S., and Strugnell, R.A. (2013). Contribution of Thy1+ NK cells to protective IFN-gamma production during Salmonella typhimurium infections. Proc Natl Acad Sci U S A *110*, 2252-2257.

Lanier, L.L. (2013). Shades of grey--the blurring view of innate and adaptive immunity. Nat Rev Immunol *13*, 73-74.

Lapaque, N., Hutchinson, J.L., Jones, D.C., Meresse, S., Holden, D.W., Trowsdale, J., and Kelly, A.P. (2009). Salmonella regulates polyubiquitination and surface expression of MHC class II antigens. Proc Natl Acad Sci U S A *106*, 14052-14057.

Latz, E., Xiao, T.S., and Stutz, A. (2013). Activation and regulation of the inflammasomes. Nat Rev Immunol *13*, 397-411.

Lazarevic, V., Glimcher, L.H., and Lord, G.M. (2013). T-bet: a bridge between innate and adaptive immunity. Nat Rev Immunol *13*, 777-789.

Le Bourhis, L., Mburu, Y.K., and Lantz, O. (2013). MAIT cells, surveyors of a new class of antigen: development and functions. Curr Opin Immunol *25*, 174-180.

Lee, S.J., McLachlan, J.B., Kurtz, J.R., Fan, D., Winter, S.E., Baumler, A.J., Jenkins, M.K., and McSorley, S.J. (2012). Temporal expression of bacterial proteins instructs host CD4 T cell expansion and Th17 development. PLoS Pathog *8*, e1002499.

Lertmemongkolchai, G., Cai, G., Hunter, C.A., and Bancroft, G.J. (2001). Bystander activation of CD8+ T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens. J Immunol *166*, 1097-1105.

Lewinsohn, D.A., Gold, M.C., and Lewinsohn, D.M. (2011). Views of immunology: effector T cells. Immunol Rev *240*, 25-39.

Liu, J., Guan, X., Tamura, T., Ozato, K., and Ma, X. (2004). Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. J Biol Chem *279*, 55609-55617.

Mackaness, G. (1964). The Immunological Basis of Acquired Cellular Resistance. J Exp Med *120*, 105-120.

Malherbe, L., Mark, L., Fazilleau, N., McHeyzer-Williams, L.J., and McHeyzer-Williams, M.G. (2008). Vaccine adjuvants alter TCR-based selection thresholds. Immunity *28*, 698-709.

Mandl, J.N., Liou, R., Klauschen, F., Vrisekoop, N., Monteiro, J.P., Yates, A.J., Huang, A.Y., and Germain, R.N. (2012). Quantification of lymph node transit times reveals differences in antigen surveillance strategies of naive CD4+ and CD8+ T cells. Proc Natl Acad Sci U S A *109*, 18036-18041.

Manicassamy, S., and Pulendran, B. (2009). Modulation of adaptive immunity with Toll-like receptors. Semin Immunol *21*, 185-193.

Marciano, B.E., Wesley, R., De Carlo, E.S., Anderson, V.L., Barnhart, L.A., Darnell, D., Malech, H.L., Gallin, J.I., and Holland, S.M. (2004). Long-term interferon-gamma therapy for patients with chronic granulomatous disease. Clin Infect Dis *39*, 692-699.

Masopust, D., and Schenkel, J.M. (2013). The integration of T cell migration, differentiation and function. Nat Rev Immunol *13*, 309-320.

McLachlan, J.B., Catron, D.M., Moon, J.J., and Jenkins, M.K. (2009). Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin. Immunity *30*, 277-288.

McNally, J.M., and Welsh, R.M. (2002). Bystander T cell activation and attrition. Curr Top Microbiol Immunol *263*, 29-41.

Mescher, M.F., Curtsinger, J.M., Agarwal, P., Casey, K.A., Gerner, M., Hammerbeck, C.D., Popescu, F., and Xiao, Z. (2006). Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev *211*, 81-92.

Miskov-Zivanov, N., Turner, M.S., Kane, L.P., Morel, P.A., and Faeder, J.R. (2013). The duration of T cell stimulation is a critical determinant of cell fate and plasticity. Sci Signal *6*, ra97.

Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med *208*, 1279-1289.

Moreau, H.D., and Bousso, P. (2014). Visualizing how T cells collect activation signals in vivo. Curr Opin Immunol *26C*, 56-62.

Muller, A.J., Filipe-Santos, O., Eberl, G., Aebischer, T., Spath, G.F., and Bousso, P. (2012). CD4+ T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation. Immunity *37*, 147-157.

Murray, P.J., and Wynn, T.A. (2011). Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol *11*, 723-737.

Narayan, K., Sylvia, K.E., Malhotra, N., Yin, C.C., Martens, G., Vallerskog, T., Kornfeld, H., Xiong, N., Cohen, N.R., Brenner, M.B.*, et al.* (2012). Intrathymic programming of effector fates in three molecularly distinct gammadelta T cell subtypes. Nat Immunol *13*, 511-518.

Neefjes, J., Jongsma, M.L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol *11*, 823-836.

Nyirenda, T.S., Seeley, A.E., Mandala, W.L., Drayson, M.T., and MacLennan, C.A. (2010). Early interferon-gamma production in human lymphocyte subsets in response to nontyphoidal Salmonella demonstrates inherent capacity in innate cells. PLoS One *5*, e13667.

O'Donnell, H., Pham, O.H., Li, L.X., Atif, S.M., Lee, S.J., Ravesloot, M.M., Stolfi, J.L., Nuccio, S.P., Broz, P., Monack, D.M.*, et al.* (2014). Toll-like Receptor and Inflammasome Signals Converge to Amplify the Innate Bactericidal Capacity of T Helper 1 Cells. Immunity *40*, 213-224.

Obst, R., van Santen, H., Mathis, D., and Benoist, C. (2005). Antigen persistence is required throughout the expansion phase of a CD4+ T cell response. J Exp Med *201*, 1555-1565.

Pakkanen, S.H., Kantele, J.M., and Kantele, A. (2012). Cross-reactive gut-directed immune response against Salmonella enterica serovar Paratyphi A and B in typhoid fever and after oral Ty21a typhoid vaccination. Vaccine *30*, 6047-6053.

Pepper, M., and Jenkins, M.K. (2011). Origins of CD4(+) effector and central memory T cells. Nat Immunol *12*, 467-471.

Pham, N.L., Badovinac, V.P., and Harty, J.T. (2011). Differential role of "Signal 3" inflammatory cytokines in regulating CD8 T cell expansion and differentiation in vivo. Front Immunol *2*, 4.

Pien, G.C., Nguyen, K.B., Malmgaard, L., Satoskar, A.R., and Biron, C.A. (2002). A Unique Mechanism for Innate Cytokine Promotion of T Cell Responses to Viral Infections. The Journal of Immunology *169*, 5827-5837.

Pluddemann, A., Mukhopadhyay, S., and Gordon, S. (2011). Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. Immunol Rev *240*, 11-24.

Plumlee, C.R., Sheridan, B.S., Cicek, B.B., and Lefrancois, L. (2013). Environmental cues dictate the fate of individual CD8+ T cells responding to infection. Immunity *39*, 347-356.

Raué, H.-P., Brien, J.D., Hammarlund, E., and Slifka, M.K. (2004). Activation of Virus-Specific CD8+ T Cells by Lipopolysaccharide-Induced IL-12 and IL-18. The Journal of Immunology *173*, 6873-6881.

Ravindran, R., Foley, J., Stoklasek, T., Glimcher, L.H., and McSorley, S.J. (2005). Expression of T-bet by CD4 T Cells Is Essential for Resistance to *Salmonella* Infection. J Immunol *175*, 4603-4610.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat Rev Immunol *6*, 476-483.

Reynolds, J.M., and Dong, C. (2013). Toll-like receptor regulation of effector T lymphocyte function. Trends Immunol *34*, 511-519.

Ribot, J.C., and Silva-Santos, B. (2013). Differentiation and activation of gammadelta T Lymphocytes: Focus on CD27 and CD28 costimulatory receptors. Adv Exp Med Biol *785*, 95-105.

Ritter, M., Gross, O., Kays, S., Ruland, J., Nimmerjahn, F., Saijo, S., Tschopp, J., Layland, L.E., and Prazeres da Costa, C. (2010). Schistosoma mansoni triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. Proc Natl Acad Sci U S A *107*, 20459-20464.

Salazar-Gonzalez, R.M., Srinivasan, A., Griffin, A., Muralimohan, G., Ertelt, J.M., Ravindran, R., Vella, A.T., and McSorley, S.J. (2007). *Salmonella* flagellin induces bystander activation of splenic dendritic cells and hinders bacterial replication in vivo. J Immunol *179*, 6169-6175.

Sallusto, F., and Monticelli, S. (2013). The many faces of CD4 T cells: roles in immunity and disease. Semin Immunol *25*, 249-251.

Schulz, E.G., Mariani, L., Radbruch, A., and Hofer, T. (2009). Sequential polarization and imprinting of type 1 T helper lymphocytes by interferon-gamma and interleukin-12. Immunity *30*, 673-683.

Scidmore, M.A. (2005). Cultivation and Laboratory Maintenance of Chlamydia trachomatis. Curr Protoc Microbiol *Chapter 11*, Unit 11A 11.

Simpson, T.R., Quezada, S.A., and Allison, J.P. (2010). Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Curr Opin Immunol *22*, 326-332.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N., Mebius, R.E.*, et al.* (2013). Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol *13*, 145-149.

Srinivasan, A., Salazar-Gonzalez, R.M., Jarcho, M., Sandau, M.M., Lefrancois, L., and McSorley, S.J. (2007). Innate Immune Activation of CD4 T Cells in *Salmonella*-Infected Mice Is Dependent on IL-18. J Immunol *178*.

Srivastava, S., and Ernst, J.D. (2013). Cutting edge: Direct recognition of infected cells by CD4 T cells is required for control of intracellular Mycobacterium tuberculosis in vivo. J Immunol *191*, 1016-1020.

Strugnell, R., Dougan, G., Chatfield, S., Charles, I., Fairweather, N., Tite, J., Li, J.L., Beesley, J., and Roberts, M. (1992). Characterization of a Salmonella typhimurium aro vaccine strain expressing the P.69 antigen of Bordetella pertussis. Infect Immun *60*, 3994-4002.

Sun, Y.H., Rolan, H.G., and Tsolis, R.M. (2007). Injection of flagellin into the host cell cytosol by Salmonella enterica serotype Typhimurium. J Biol Chem *282*, 33897-33901.

Talabot-Ayer, D., Calo, N., Vigne, S., Lamacchia, C., Gabay, C., and Palmer, G. (2012). The mouse interleukin (Il)33 gene is expressed in a cell type- and stimulus-dependent manner from two alternative promoters. J Leukoc Biol *91*, 119-125.

Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., and Lotze, M.T. (2012). PAMPs and DAMPs: signal 0s that spur autophagy and immunity. Immunol Rev *249*, 158-175.

Thi, E.P., Lambertz, U., and Reiner, N.E. (2012). Sleeping with the enemy: how intracellular pathogens cope with a macrophage lifestyle. PLoS Pathog *8*, e1002551.

Tough, D.F., Borrow, P., and Sprent, J. (1996). Induction of bystander T cell proliferation by viruses and type I interferon in vivo. Science *272*, 1947-1950.

Tough, D.F., Sun, S., and Sprent, J. (1997). T cell stimulation in vivo by lipopolysaccharide (LPS). J Exp Med *185*, 2089-2094.

Trunk, G., and Oxenius, A. (2012). Innate instruction of CD4+ T cell immunity in respiratory bacterial infection. J Immunol *189*, 616-628.

Turner, M.S., Kane, L.P., and Morel, P.A. (2009). Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. J Immunol *183*, 4895-4903.

van der Velden, A.W., Dougherty, J.T., and Starnbach, M.N. (2008). Down-modulation of TCR expression by Salmonella enterica serovar Typhimurium. J Immunol *180*, 5569-5574.

van Stipdonk, M.J., Lemmens, E.E., and Schoenberger, S.P. (2001). Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat Immunol *2*, 423-429.

Vantourout, P., and Hayday, A. (2013). Six-of-the-best: unique contributions of gammadelta T cells to immunology. Nat Rev Immunol *13*, 88-100.

Walker, J.A., Barlow, J.L., and McKenzie, A.N. (2013). Innate lymphoid cells--how did we miss them? Nat Rev Immunol *13*, 75-87.

Winter, S.E., Thiennimitr, P., Nuccio, S.P., Haneda, T., Winter, M.G., Wilson, R.P., Russell, J.M., Henry, T., Tran, Q.T., Lawhon, S.D.*, et al.* (2009). Contribution of flagellin pattern recognition to intestinal inflammation during *Salmonella enterica* serotype typhimurium infection. Infect Immun *77*, 1904-1916.

Yang, J., Murphy, T.L., Ouyang, W., and Murphy, K.M. (1999). Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. Eur J Immunol *29*, 548-555.

Yarke, C.A., Dalheimer, S.L., Zhang, N., Catron, D.M., Jenkins, M.K., and Mueller, D.L. (2008). Proliferating CD4+ T cells undergo immediate growth arrest upon cessation of TCR signaling in vivo. J Immunol *180*, 156-162.

Zehn, D., Roepke, S., Weakly, K., Bevan, M.J., and Prlic, M. (2014). Inflammation and TCR signal strength determine the breadth of the T cell response in a bim-dependent manner. J Immunol *192*, 200-205.