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Diverse Roles For T-Bet In The Immune Response To Toxoplasma Gondii

Abstract

The T-box transcription factor T-bet is most prominently known for its role in production of the cytokine interferon-γ (IFNγ) by effector T cells after infection with Th1-inducing pathogens. Here, we demonstrate additional roles for T-bet during effector T cell responses including an essential function in T cell trafficking to secondary sites of infection during toxoplasmosis. Mice that are deficient in T-bet are unable to survive infection with the intracellular parasite Toxoplasma gondii, and this mortality is caused by uncontrolled parasite replication at secondary sites of infection and is associated with a paucity of T cells at these sites. Additionally, we provide evidence that T-bet is also involved in early events of T cell priming and expansion. Together, the data presented in this thesis provide a better understanding of the diverse roles that T-bet plays in the generation and function of T cell responses during the immune response to T. gondii.

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DIVERSE ROLES FOR T-BET IN THE IMMUNE RESPONSE TO *TOXOPLASMA GONDII*

Gretchen Harms Pritchard

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2016

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DIVERSE ROLES FOR T-BET IN THE IMMUNE RESPONSE TO *TOXOPLASMA GONDII*

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ABSTRACT

DIVERSE ROLES FOR T-BET IN THE IMMUNE RESPONSE TO

TOXOPLASMA GONDII

Gretchen Harms Pritchard

Christopher A. Hunter

The T-box transcription factor T-bet is most prominently known for its role in production of the cytokine interferon-γ (IFNγ) by effector T cells after infection with Th1-inducing pathogens. Here, we demonstrate additional roles for T-bet during effector T cell responses including an essential function in T cell trafficking to secondary sites of infection during toxoplasmosis. Mice that are deficient in T-bet are unable to survive infection with the intracellular parasite *Toxoplasma gondii*, and this mortality is caused by uncontrolled parasite replication at secondary sites of infection and is associated with a paucity of T cells at these sites. Additionally, we provide evidence that T-bet is also involved in early events of T cell priming and expansion. Together, the data presented in this thesis provide a better understanding of the diverse roles that T-bet plays in the generation and function of T cell responses during the immune response to *T. gondii*.

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CHAPTER 1: INTRODUCTION

A: *Toxoplasma gondii* as a model organism to study the immune response

T. gondii **life cycle and biology**

Toxoplasma gondii is an obligate intracellular parasite that can infect any warm-blooded animal (Dubey, 2009). However, sexual reproduction of the parasite and formation of oocysts can only occur in the intestinal epithelium of feline hosts (Elmore et al., 2010). Several million oocysts are shed in the feces and this form is extremely infectious when ingested by an intermediate host (Elmore et al., 2010; Montoya and Liesenfeld, 2004). Within the gastrointestinal tract of the intermediate host, sporulated oocysts differentiate into tachyzoites that actively invade any nucleated cell and rapidly replicate within the parasitophorous vacuole via a unique cellular process called endodyogeny (Montoya and Liesenfeld, 2004). Extensive parasite replication leads to host cell lysis and release of parasites that are able to infect and replicate inside other cells. Repeated cycles of replication allow the parasite to disseminate throughout the host to secondary sites of infection including the central nervous system, skeletal and heart muscle, as well as the placenta (Konradt et al., 2016; Montoya and Liesenfeld, 2004). In current models, pressure from the immune system leads to cellular stress and induces the tachyzoites to transform into a slowly replicating form, the bradyzoite, which forms tissue cysts that persist within long-lived cells that include neurons (Cabral et al., 2016). Consequently, this results in a chronic infection that continues for the lifetime of the host (Montoya and Liesenfeld, 2004).

***T. gondii* infection and global health**

Infection with *T. gondii* has generally been considered asymptomatic, but when parasite replication is not well controlled it can result in clinical toxoplasmosis. Most human infections with *T. gondii* are initiated via *in utero* exposure, consumption of under-cooked meat, ingestion of oocysts from the environment, or transplant of an organ from an infected individual (Dubey, 2009; Montoya and Liesenfeld, 2004). Indeed, infection with *T. gondii* is one of the leading causes of death associated with food-borne illness in the United States (Mead et al., 1999). Approximately 25-30% of people are infected with *T. gondii* worldwide, with variation in prevalence in different regions (Robert-Gangneux and Darde, 2012). The high rate of sero-prevalence worldwide but lack of clinical disease indicates that many immunocompetent adults are asymptomatic. However, approximately 10% of immunocompetent humans infected for the first time develop a self-limiting, non-specific illness that infrequently requires treatment (Montoya and Liesenfeld, 2004). There are rare instances in which "exotic" strains of *T. gondii* can cause lethal disease in immunocompetent patients (Carme et al., 2009). In terms of public health, infection with *T. gondii* can result in severe complications and disease in fetuses and individuals with primary or acquired immune deficiencies in T cell function (Barratt et al., 2010; Dunn et al., 1999). In the Netherlands, infection with *T. gondii* has one of the highest disease burdens from food-borne pathogens, and the cost of illness for toxoplasmosis in this country is estimated to be €55 million per year (Mangen et al., 2015). These findings demonstrate the impact and economic cost of infection with *T. gondii* on human health

and also the importance of the immune system in long-term control of this persistent pathogen.

Immune response to *T. gondii*

In addition to humans, *T. gondii* can naturally infect any warm-blooded vertebrate, including rodents, which makes this mouse-parasite combination an ideal model to study a natural host-pathogen interaction (Subauste, 2012). While the majority of the studies that examine the immune response to *T. gondii* have been performed using strains of the parasite found in the environment, the use of replication-deficient strains have been shown to act as effective vaccines that have also provided insight into the cellular basis of immunity to *T. gondii* (Araujo, 1994; Fox and Bzik, 2002). Live vaccines that are attenuated strains of *T. gondii* are used in veterinary settings, but there are currently no licensed vaccines for human use (Innes et al., 2009; Liu et al., 2012).

Another significant benefit of using *T. gondii* as a model organism is that this parasite is genetically tractable (Kim and Weiss, 2004), which allows the use of genetic approaches to help understand how it evades the host immune response and interacts with host cells (Christian et al., 2014; Koshy et al., 2012; Saeij et al., 2007). Additionally, the same genetic approaches can be used to modify *T. gondii* to aid the study of the host immune response. Such modifications include expression of fluorescent proteins (Christian et al., 2014; John et al., 2009), deletion of the gene carbamoyl phosphate synthetase II (CPS), which renders the parasites unable to salvage uracil and therefore replication-deficient

(Fox and Bzik, 2002), and insertion of model antigens such as ovalbumin, β-galactosidase, and EaRFP, allowing for use of TCR transgenic mice in which the T cells recognize an epitope of the model antigen (e.g. OT-I T cells are specific for the SIINFEKL epitope of ovalbumin) and/or MHC Class I tetramers to track the parasite-specific immune response of the host (John et al., 2009; Jordan et al., 2009; Kwok et al., 2003; Pepper et al., 2008). These approaches will be used in Chapters 2 and 3 of this thesis.

Studies in mice have helped to define the role of multiple immune components in the innate and adaptive response to *T. gondii*, and have provided relevant clinical insights. For example, similar to patients, mice that lack T cells are highly susceptible to *T. gondii* infection (Johnson, 1992; Lindberg and Frenkel, 1977), as are patients and mice that have defects in CD40/CD40L interactions (Leiva et al., 1998; Reichmann et al., 2000). Additionally, treatment of infected humans or mice with anti-TNFα antibodies results in reactivation of latent infection and the development of toxoplasmic encephalitis (TE) (Gazzinelli et al., 1993; Young and McGwire, 2005). There are multiple other examples of clinical relevance (Suzuki, 2002), but together this literature highlights the importance of cell-mediated immunity in controlling infection with *T. gondii*.

***Innate immunity in response to* T. gondii**

The innate immune responses to *T. gondii* are dual-purpose: they suppress early parasite replication and they coordinate the development of adaptive immune responses required

for long-term parasite control. In current models, dendritic cells (DCs) and macrophages recognize parasite-specific products through Toll-like receptors (TLRs) 2, 4, 7, 9, 11 and 12 as well as the NLRP1 inflammasome (Yarovinsky, 2014), which consequently results in the production of IL-12, which induces NK cells to make IFNγ (Gazzinelli et al., 1993). IFNγ signaling leads to an array of antimicrobial mechanisms, in hematopoietic and non-hematopoietic infected cells (Yap and Sher, 1999), that include production of inducible nitric oxide synthase (iNOS) and guanylate-binding proteins (GBPs) (Lieberman and Hunter, 2002; MacMicking, 2012), thus, IFNγ is the major mediator of acute and chronic resistance to *T. gondii* (Suzuki et al., 1988). Furthermore, NK cells have been shown to require expression of the chemokine receptor CCR5 in order to migrate to infected sites within the host and provide protection against this parasite (Khan et al., 2006). While several innate cell types have been shown to produce IL-12 during infection with *T. gondii*, the major sources of IL-12 appear to be $CD8\alpha^+$ DCs and monocyte derived DCs (Goldszmid et al., 2012; Mashayekhi et al., 2011). Additionally, the recruitment of $Gr\text{-}1^+$ monocytes to the site of infection is required to limit local parasite replication (Robben et al., 2005). In addition to IL-12 production, DCs also present antigen to $CD4^+$ and $CD8^+$ T cells, which are required for long-term parasite control (Gazzinelli et al., 1991). DCs are also important for the development of T follicular helper $CD4^+$ T cells (Tfh), which are required for optimal parasite-specific B cell responses necessary for resistance (Goenka et al., 2011). Plasmacytoid dendritic cells (pDCs), have also been implicated in antigen-presentation to $CD4^+$ and $CD8^+$ T cells (Pepper et al., 2008). Recent studies have shown that active invasion of APCs by the

parasite, rather than phagocytosis, is required for optimal T cell activation (Dupont et al., 2014). However, despite the advances in this area, there still remain basic questions about the events that permit successful antigen presentation. For example, it is unclear if the infected cells produce IL-12 and/or present antigen to the T cells, or if there is an intermediate cell that performs this function (Christian et al., 2014; John et al., 2009).

T cell responses to **T. gondii**

As alluded to above, presentation of parasite antigens by APCs in the context of MHC Class I and II leads to signaling through the T cell receptor (TCR) and T cell activation, a process called T cell priming (Lanzavecchia and Sallusto, 2001). When T cells are primed in the presence of IL-12, they differentiate into Th1 cells, characterized by expression of IFNγ (Zhu et al., 2012), which is required for parasite control and host survival (see Figure 1) (Khan et al., 1994; Scharton-Kersten et al., 1996; Suzuki et al., 1988). $CD4^+$ and $CD8^+$ T cells are required for parasite control during a primary infection, but also for protective immune responses after vaccination (Gazzinelli et al., 1991; Parker et al., 1991; Suzuki and Remington, 1988). During toxoplasmosis, both of these cell types have functions in addition to production of IFNγ; T follicular helper $CD4^+$ T cells (Tfh) are required for optimal B cell responses (Glatman Zaretsky et al., 2012), and cytotoxic $CD8^+$ T cells (CTLs) can directly kill infected cells (Gigley et al., 2011).

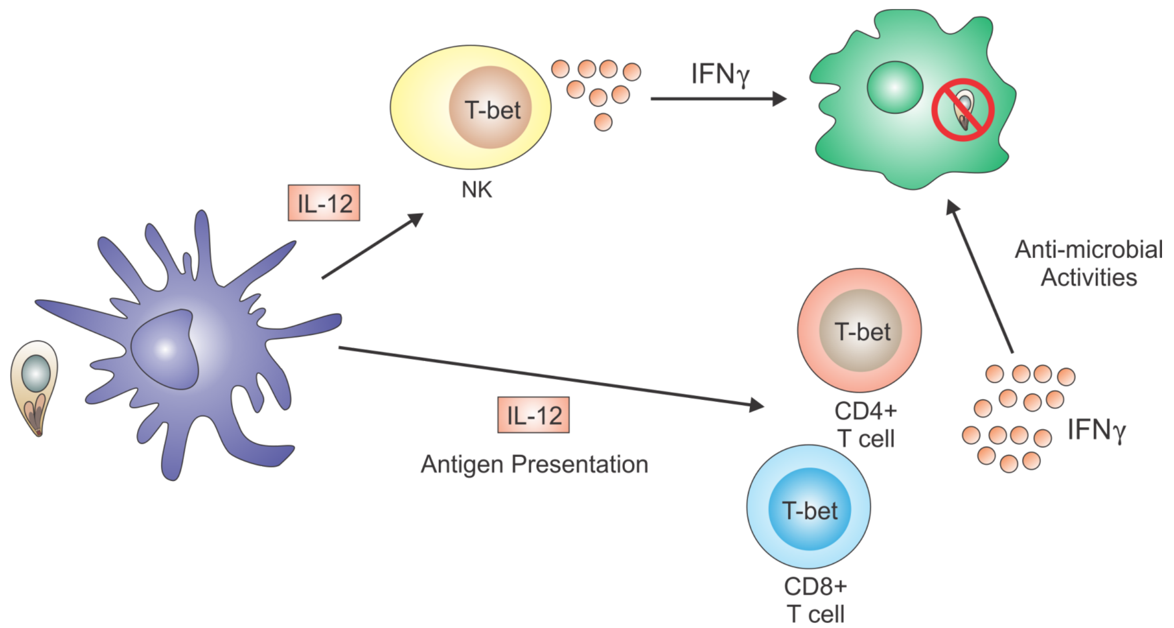

Figure 1. Schematic of NK cell and T cell activation following *T. gondii* challenge.[*]

IL-12 produced after infection induces IFNγ production from NK cells and $CD4^+$ and $CD8^+$ T cells. IFNγ is necessary for parasite killing.

[*] Deb Argento helped prepare this figure.

***B cell responses to* T. gondii**

From a historical perspective, many of the early studies on immunity to *T. gondii* focused on humoral immunity (Sabin and Feldman, 1948); infection with *T. gondii* has been used as a model to study B cell responses and the detection of parasite-specific IgM and IgG is critical for clinical diagnosis (Montoya, 2002). Despite largely intact Th1 responses during the acute phase of infection with *T. gondii*, mice that are deficient in B cells do not survive the chronic phase of infection due to an inability to control parasite replication in the central nervous system (CNS) (Kang et al., 2000). However, transfer of polyclonal anti-*T. gondii* IgG antibody into B cell deficient mice prevented early mortality, suggesting that antibodies from B cells are essential for host survival (Kang et al., 2000). Additional functions of B cells during infection with *T. gondii* include antigen presentation to T cells (Aosai et al., 1994), and interacting with T follicular helper (Tfh) $CD4^+$ T cells in the germinal center (GC) to induce optimal IFNγ production by the Tfh cells, which likely drives antibody class switching (Menard et al., 2007).

Regulation of* T. gondii *induced inflammation

A successful inflammatory response against *T. gondii* limits parasite replication. However, too much inflammation can also be detrimental to the host, and there are multiple examples of situations in which the $CD4^+$ T cell response is pathological (Israelski et al., 1989; Liesenfeld et al., 1996). Over the last 20 years, there have been key studies that have helped to define the regulatory networks that act to limit immune-mediated collateral damage during infection with *T. gondii*. For example, the cytokine

IL-10 is produced by multiple cell types that include APCs, NK cells, effector T cells and regulatory $CD4^+$ T cells (Tregs) and acts to inhibit accessory cell function required for effector T cell responses after infection with *T. gondii* (Gaddi and Yap, 2007). IL-10 deficient mice infected with *T. gondii* appear to control parasite replication but succumb to a systemic inflammatory disease mediated by $CD4^+$ T cells (Gazzinelli et al., 1996; Neyer et al., 1997). IL-27 is another cytokine that acts to regulate inflammatory responses. Initially, due to its structural similarity to IL-12, it was thought to have pro-inflammatory properties associated with the development of Th1 responses. However, mice lacking the IL-27 receptor that are infected with *T. gondii* do not survive the acute phase of infection, despite their control of parasite replication. Rather, these mice succumb to uncontrolled inflammation and pathology mediated by $CD4^+$ T cells (Villarino et al., 2003). Additional regulatory cytokines that act to limit inflammation during infection with *T. gondii* include IL-6 and TGFβ (Buzoni-Gatel et al., 2001; Jebbari et al., 1998). Together, these data demonstrate the principle that the balance between pro- and anti-inflammatory responses is a key component of immune responses.

***Transcriptional regulation of immunity to* T. gondii**

The development and effector function of the varied cell types required for resistance to *T. gondii* are regulated by transcription factors, and many of these transcription factors are essential for this immune response. For example, Batf3 is required for $CD8\alpha^+$ and $CD103^+$ DCs and optimal IL-12 production (Mashayekhi et al., 2011), IRF8 regulates the TLR-dependent DC production of IL-12 (Raetz et al., 2013), and the NFκB family

member c-Rel is important for T cell responses to *T. gondii* (Jordan et al., 2010; Mason et al., 2004). In Chapters 2 and 3, I will explore the underlying reason for susceptibility to *T. gondii* in mice that lack the T-box transcription factor T-bet.

Conclusions from *T. gondii* as a model organism

Not only does *T. gondii* have an impact on global health, but it has also proven to be a valuable model organism used to study the immune response. Thus, it has provided unprecedented insight into the innate components of the immune system contribute to the complex processes required for the development of long-term protective adaptive immunity. Many of the pathways first elucidated using *T. gondii* are broadly applicable to the processes that govern immune response to other pathogens that include intracellular bacteria and viruses. Furthermore, these studies provide insights into basic functions of the immune system which are relevant to conditions such as autoimmunity and cancer. For example, in Chapter 2 I will show that the transcription factor T-bet is essential for control of parasite replication at secondary sites of infection due to its role in T cell trafficking. In Chapter 3 I will describe novel functions of this transcription factor in T cell priming, which are relevant to helping understand the molecular requirements for vaccine-induced immune responses.

B: The role of T-bet in T cell-mediated resistance to infection

A major theme in immunology for the last 50 years has been the study of the functional and phenotypic diversity of T cell subsets and their role in protective or pathological

immune responses. T cells as distinct thymus-derived lymphocytes were first described, albeit controversially, in the 1960s (Miller and Mitchell, 1967; Miller, 1999). However, within a few years, the idea that T cells were a separate population from antibody-producing lymphocytes was more widely accepted (Golstein et al., 1972). These lymphocytes could be further divided into those that helped B cells (now known as $CD4^+$ T cells) and those that were cytotoxic (now known as $CD8^+$ T cells) (Cantor and Boyse, 1975). By the 1980s it was appreciated that different subsets of $CD4^+$ helper T cells predominantly produced either IFNγ (Th1) or the combination of IL-4 and IL-5 (Th2) (Mosmann et al., 1986). The distinct functions of these subsets were highlighted by multiple studies in which $CD4^+$ T cell production of IFNγ was required to activate a variety of cell intrinsic anti-microbial mechanisms central to resistance to intracellular infections while Th2 cells promoted resistance to helminth parasites (Mosmann and Coffman, 1989). Since then, as predicted by Mosmann and Coffman, additional subsets of functionally diverse T cells have been described that include regulatory T cells (Tregs) (Sakaguchi, 2000), anti-fungal Th17 cells (Aggarwal et al., 2003), and T follicular helper cells (Tfh) (Vinuesa et al., 2005).

While the responses described above are associated with resistance to different classes of pathogens, dysregulated T cell activity can lead to an array of inflammatory and autoimmune conditions such as multiple sclerosis, asthma, rheumatoid arthritis, and inflammatory bowel diseases (Fletcher et al., 2010; McInnes and Schett, 2007; Umetsu et al., 2002; Xu et al., 2014). In order to be able to manipulate the immune response to

better manage inflammatory and/or autoimmune conditions, the molecular mechanisms that control T cell differentiation have become widely studied. In the 1990s, the ability of the transcription factors c-maf and GATA3 to direct the generation of Th2 responses was described (Kim et al., 1999; Zheng and Flavell, 1997), and in 2000, the transcription factor T-bet, encoded by the gene *Tbx21*, was identified as a key regulator of Th1 responses (Szabo et al., 2000). Over the next decade, several additional T cell subsets and their specific transcription factors were identified: the transcription factor Foxp3 was shown to be essential for the development and function of Tregs (Fontenot et al., 2003; Hori et al., 2003), Th17 cells were demonstrated to require the transcription factor RORγt (Ivanov et al., 2006), and Bcl-6 was shown to direct the generation of Tfh cells (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009).

The ability to link specific transcription factors with discrete T cell lineages provided a framework to understand the mechanisms that underlie the differentiation of these T cell subsets and reinforced the idea that these transcription factors are "master regulators" of distinct T cell fates. This is a term that was originally used to describe transcription factors that regulate developmental lineages (Chan and Kyba, 2013), but many of these transcription factors associated with different T cell subsets can be co-expressed, often early in the differentiation process, and are not restricted to $CD4^+$ T cells. Indeed, it is now appreciated that the interplay between these transcription factors dictates T cell fate and function. Consequently, it has been proposed that "lineage specifying" is a more

precise term that reflects the complex roles of these transcription factors in T cell differentiation (Oestreich and Weinmann, 2012).

The T-box family of transcription factors

The discovery of the T-box family of transcription factors was based on a series of genetic studies in mice to identify the underlying cause of a defect in tail development; this phenotype was called brachyury, or T for tail (Wattler et al., 1998). Mapping of the locus involved in this defect identified the *T* gene and revealed that the brachyury protein contained an evolutionarily conserved DNA binding motif, termed the T-box (Bollag et al., 1994; Herrmann et al., 1990). In addition to DNA binding, T-box proteins interact with other families of transcription factors, such as homeodomain (encoded by *hox* genes), GATA zinc finger, and LIM domain proteins (Naiche et al., 2005). The T-box genes are present in all metazoans and comprise a large family involved in many developmental processes that include embryonic development of limbs and the CNS (Gibson-Brown et al., 1998; Minguillon and Logan, 2003).

From an immunological perspective, it is worthwhile to consider the major evolutionary events associated with the development of adaptive immunity and how this relates to different T-box transcription factors. Amphioxus, a fish-like aquatic animal, represents the most basal lineage of the chordates and is used as a model organism to study the most recent common ancestor between vertebrates and invertebrates (Bertrand and Escriva,

2011). Amphioxus does not have an adaptive immune system, but does have lymphocyte-like cells (Huang et al., 2007), while lampreys, which are jawless vertebrates and share a more recent common ancestor with higher vertebrates than amphioxus, have a variable lymphocyte receptor (VLR) based adaptive immune system, which is distinct from T and B cell adaptive immunity of higher vertebrates (Boehm et al., 2012). The recombination-activating gene (RAG) and TCR and BCR based immunity first appeared in the gnathostomes, or jawed fish (Flajnik and Kasahara, 2010), and this key event, along with gene duplication, led to novel mechanisms of immunity that adapt to the pathogen, as well as ways to regulate immune responses.

Functional divergence after gene duplication is a major element in the generation of biological complexity (Taylor and Raes, 2004), and there were at least two whole-genome duplication events that occurred during the divergence of the invertebrate and vertebrate lineages (Dehal and Boore, 2005; Okada and Asai, 2008). Indeed, many single amphioxus genes have two or three orthologous vertebrate genes (Ruvinsky et al., 2000). For example, amphioxus has a single gene in the Tbr1 subfamily, *AmphiEomes/Tbr1/Tbx21*, which is involved in specification of the endoderm (Horton and Gibson-Brown, 2002). Lampreys have two paralogs, *tbr1b* and *tbx21*, and gnathostomes have three separate genes in this subfamily, *Tbx21*, *Eomes*, and *Tbr1* (Horton and Gibson-Brown, 2002; Kang and Malhotra, 2015). Many duplicated genes gained new functions, and were important for the development of the adaptive immune system (Flajnik and Kasahara, 2010; Okada and Asai, 2008). Indeed, the diversification

of the Tbr1 subfamily of T-box genes coincides with the emergence of adaptive immunity, and as will be discussed below, the T-box transcription factor T-bet has gained immune functions that appear distinct from its subfamily members Eomes and Tbr1.

Little is known about the T-box genes in immune functions in ancient metazoans, however, in the higher vertebrates, there are three members of the T-box family of transcription factors that are expressed by cells of the immune system: T-bet (*Tbx21*), Eomesodermin (Eomes, *Tbr2*), and *Tbx13*. The immunological functions of T-bet and Eomes have been studied extensively and the goal of this section of my thesis is to highlight the important role that T-bet plays in resistance to diverse intracellular pathogens, and also to examine how the use of infectious disease models has provided novel insights into the function of T-bet in the immune system.

The identification of T-bet and its expression

The description of the Th1 and Th2 $CD4^+$ T cell subsets associated with resistance to different classes of pathogens led to the idea that there might be key environmental signals that directed differentiation into these alternative fates. Indeed, innate cell production of IL-12 that leads to T cell production of IFNγ provides a link between innate and adaptive immunity (Trinchieri, 1995), and both of these cytokines are required for the control of many intracellular pathogens (Trinchieri, 2003). In the search for a Th1-specific transcription factor, Glimcher and colleagues identified a T-box transcription

factor expressed in CD4^{+} T cells that is required for optimal IFNγ production and resistance to the intracellular parasite *Leishmania major* (Szabo et al., 2000; Szabo et al., 2002). Because of its homology to other T-box containing genes, they named this novel protein T-box expressed in T cells (T-bet) (Szabo et al., 2000). While T-bet has been most prominently linked to T cell production of IFN-γ (Szabo et al., 2000; Szabo et al., 2002), it is now recognized that expression of T-bet is not restricted to T cells, but is also present in subsets of innate cells (ILC, NK, NKT) as well as B cells (Klose et al., 2014; Lugo-Villarino et al., 2003; Lugo-Villarino et al., 2005; Szabo et al., 2000; Szabo et al., 2002), although much less is known about its function in these cells.

Molecular basis for effects of T-bet

T-bet inhibits alternative CD4^{+} T cell differentiation fates that include Th2 and Th17 cells (Mathur et al., 2006; Szabo et al., 2000; Szabo et al., 2002). This can be explained by interactions between T-bet and several other proteins that result in the induction or inhibition of key factors in T cell differentiation. For instance, through a tyrosine-kinase mediated-interaction with GATA3, T-bet prevents GATA3 from binding to the IL-5 promoter (Hwang et al., 2005). T-bet also cooperates with Runx3 to activate the IFNγ gene as well as repress the IL4 gene (Djuretic et al., 2007), while its ability to sequester Runx1 prevents Runx1-mediated activation of *Rorct* (Lazarevic et al., 2011), thus limiting production of IL-17. Additionally, T-bet was originally identified as a repressor of IL-2 production (Szabo et al., 2000), and during the late stages of Th1 activation, T-bet recruits Bcl-6 to the IFNγ locus, which limits production of IFNγ (Oestreich et al.,

2011). These latter results imply that T-bet may also regulate Th1 responses through the induction of a negative feedback-loop to limit IFN-γ production. Furthermore, T-bet has been shown to bind to the promoter region of many other immune genes, such as STAT1, CXCR3, and CCL3 which can be regarded as part of the Th1 cassette of genes (Beima et al., 2006; Zhu et al., 2012), suggesting a broader role in the immune response.

<u>Induction of T-bet in naïve T cells</u>

As part of the initial report that identified T-bet, it was recognized that naïve $CD4^+$ and $CD8^+$ T cells do not express T-bet, but stimulation through the TCR leads to T-bet expression (Szabo et al., 2000). Additional studies demonstrated that IFNγ could synergize with TCR signaling to enhance T-bet expression (Afkarian et al., 2002). More recent studies have highlighted that a productive Th1 response is characterized by two distinct waves of T-bet expression (Schulz et al., 2009). Thus, initial signals through the TCR and IFNγR (via STAT1) synergize to induce early synthesis of T-bet (Afkarian et al., 2002; Schulz et al., 2009). IL-12 signaling (via STAT4) then induces the sustained second wave of T-bet expression that stabilizes the Th1 phenotype (Mullen et al., 2001; Schulz et al., 2009). While the ability of IFNγ and IL-12 to reinforce T-bet expression is linked to a commitment to a Th1 phenotype, other cytokines such as Type I IFNs, IL-21 and IL-27 can also promote T-bet expression (Hall et al., 2012; Sutherland et al., 2013; Takeda et al., 2003; Wiesel et al., 2012). The pathogen-dependent cytokine milieu likely induces additional transcription factors and/or binding partners that associate with T-bet, and each distinct environment results in a unique T-bet-dependent transcriptional profile.

Additionally, the functional consequences of the first wave of T-bet expression are unclear. However, in Chapter 3 I will describe a role for this early T-bet in the coordination of events that lead to optimal $CD8^+$ T cell priming. This process is further complicated by the observation that after cessation of TCR signaling, T-bet expression decreases, and is asymmetrically divided between daughter cells (Chang et al., 2011; Schulz et al., 2009).

Many of the studies described above have focused on $CD4^+$ T cells, with the underlying assumption that $CD8^+$ T cells display similar induction mechanisms and kinetics of T-bet expression. However, as previously mentioned, T-bet is expressed in multiple immune cell types of both the innate and adaptive immune system and similar to conventional αβ T cells, T-bet is induced in γδ T cells after TCR signaling (Yin et al., 2002). In B cells, the activation of the BCR synergizes with STAT1-dependent IFNγ signaling to promote T-bet expression (Xu and Zhang, 2005). In contrast to adaptive lymphocytes, NK cells require T-bet for maturation (Gordon et al., 2012; Townsend et al., 2004), but it is dispensable for NK IFN-γ production during infection (Way and Wilson, 2004). ILC1 and ILC3 subsets and invariant NKT cells (iNKTs) require T-bet for development and activity (Klose et al., 2014; Sciume et al., 2012; Silver et al., 2016; Townsend et al., 2004). However, it is unknown what signals induce T-bet expression in these innate cells, but, based on the our understanding of T and B cells, likely candidates would include innate activating receptors and/or cytokine signaling.

The role of T-bet in resistance to intracellular infections

Cell-mediated immunity is critical for the control of intracellular pathogens and, as noted earlier, the IL-12/IFNγ axis is an important pathway required for resistance to many viruses, bacteria, and parasites (Trinchieri, 2003). Consistent with a key role for T-bet in the development of Th1 cells, it is not surprising that this transcription factor is required for IFNγ production during several intracellular infections (see Table 1). For example, in murine models, control of the protozoan parasite *L. major* is dependent on the development of a Th1 immune response dominated by the production of IFNγ, whereas susceptibility is associated with a Th2 response characterized by the secretion of IL-4 and IL-5 (Locksley et al., 1987; Scott et al., 1988). Indeed, while WT C57BL/6 mice can control this infection, the absence of T-bet leads to reduced IFN-γ and increased IL-4 and IL-5, and a progressive disease (Szabo et al., 2002). This study provided the first evidence that T-bet is required for resistance to an intracellular infection and suggested that, in this model, T-bet overrides a default Th2 response in order to induce a Th1 response (Szabo et al., 2002), a conclusion that foreshadowed the complex relationship between T-bet and GATA3 (Hwang et al., 2005).

While Th2 responses are detrimental to host protection during infection with *L. major*, in mice infected with *Mycobacterium tuberculosis* or *Salmonella enterica* serovar *typhimurium*, IFNγ is important for resistance but endogenous Th2 responses do not appear to have a prominent role in disease susceptibility (Cooper et al., 1993; Hess et al., 1996). In this context, T-bet$^{-/-}$ mice challenged with either of these pathogens are more

Pathogen	Relevant source(s) of IFNγ	T-bet$^{-/-}$ phenotype	References
L. major	CD4^{+}	Susceptible	(Szabo et al., 2002)
M. tuberculosis	CD4^{+}	Susceptible	(Sullivan et al., 2005)
S. Typhimurium	CD4^{+}	Susceptible	(Ravindran et al., 2005)
H. simplex virus 2	CD4^{+}	Susceptible	(Svensson et al., 2005)
T. cruzi	CD4^{+}, CD8^{+}, NK	Susceptible	(Cobb et al., 2009)
LCMV-Clone 13	CD8^{+}	Susceptible	(Kao et al., 2011)
LCMV-Armstrong	CD8^{+}	Resistant	(Intlekofer et al., 2008)
L. monocytogenes	CD8^{+}, NK	Resistant	(Way and Wilson, 2004)
P. berghei-ANKA	CD8^{+} (pathological)	Less disease	(Oakley et al., 2013)
T. gondii	CD4^{+}, CD8^{+}, NK	Susceptible	(refer to Chapter 2)

Table 1. T-bet$^{-/-}$ mice and infection

susceptible to infection, associated with reduced CD4$^+$ T cell production of IFNγ, but this is not accompanied by the emergence of a default Th2 response (Ravindran et al., 2005; Sullivan et al., 2005). Paradoxically, T-bet haplo-sufficient mice (with an intermediate level of T-bet expression) challenged with *M. tuberculosis* have an improved ability to control bacterial replication compared to T-bet sufficient mice (Torrado et al., 2015). T-bet has also been shown to be important for NK cell function and CD4$^+$ (but not CD8$^+$) T cell production of IFNγ during the immune response to HSV-2 (Svensson et al., 2005). A common feature in the infection models described above is that CD4$^+$ T cells represent a major source of protective IFNγ, and this helps to explain why T-bet deficiency is associated with increased susceptibility to infection.

While the infection models discussed above link T-bet to the production of IFNγ and pathogen control, in several other infectious settings, T-bet deficiency does not impact global IFNγ production. For example, in T-bet$^{-/-}$ mice infected with *Listeria monocytogenes*, there is reduced production of IFNγ by CD4$^+$ T cells, but the NK and CD8$^+$ T cell populations produce normal amounts of IFNγ (Way and Wilson, 2004). Consequently, serum levels of IFNγ are intact and these mice are able to control bacterial replication (Way and Wilson, 2004). A similar phenotype is apparent with the Armstrong strain of *Lymphocytic Choriomeningitis Virus* (LCMV), which induces an acute viremia in WT mice that is normally cleared via IFNγR-dependent mechanisms (Ou et al., 2001). When T-bet$^{-/-}$ mice are challenged with this strain, the CD8$^+$ T cells produce comparable levels of IFNγ to the WT controls, and there is no significant

difference in viral control or disease severity between these groups (Intlekofer et al., 2008). In contrast, LCMV-Clone 13 usually results in a chronic infection with a low viral load, but T-bet$^{-/-}$ mice infected with this strain have reduced numbers of antigen-specific CD8^{+} T cells and are unable to control viral replication (Kao et al., 2011). Furthermore, T-bet$^{-/-}$ mice do not survive infection with *Trypanosoma cruzi*, and in this model, there is no defect in CD4^{+} and CD8^{+} T cell production of IFN-γ in the absence of T-bet. However, there is an increase in IL-17 production by CD4^{+} T cells, and a decrease in the frequency of CD8^{+} T cells (Cobb et al., 2009; Guo et al., 2009), consistent with a role for T-bet in the suppression of *Rorct* and Th17 responses (Lazarevic et al., 2011; Mathur et al., 2006). Additionally, as I will show in Chapter 2, T-bet$^{-/-}$ mice that are infected with the intracellular parasite *Toxoplasma gondii* have reduced CD4^{+} T cell production of IFNγ, but they have normal NK and CD8^{+} T cell expression and production of IFNγ that allows for control of parasite replication at the initial site of infection. However, as the infection disseminates, T-bet$^{-/-}$ mice are unable to control parasite replication at secondary sites of infection and succumb to parasite burden (Zhu et al., 2012).

These results from diverse infections highlight the context-dependent effects of the pathogen and relevant sources of IFNγ in the determination of the role of T-bet in host protection (See Figure 2). In several of the models described above, despite the absence of T-bet, there is still substantial production of IFNγ, and there are several related explanations to account for this phenotype. One example discussed above is simply that the role of T-bet in host protection is less critical in settings where NK cells and CD8^{+} T

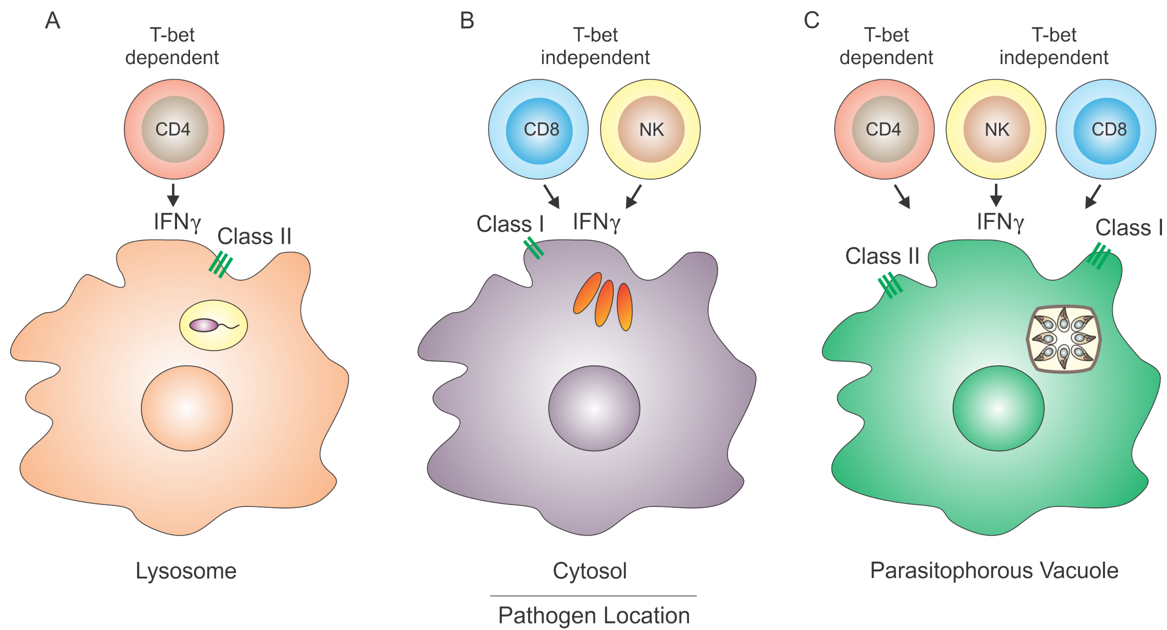

Figure 2. T-bet dependency and pathogen localization.[*] (A) During infection with *Leishmania major*, the parasite is contained within a lysosome, and antigen presentation occurs via MHC-Class II, which activates $CD4^+$ T cells. IFNγ from $CD4^+$ T cells, and resistance to *L. major*, is T-bet dependent. (B) During infection with *Listeria monocytogenes*, the bacteria localize to the cytosol, and antigen presentation occurs via MHC-Class I, which activates $CD8^+$ T cells. NK cells and $CD8^+$ T cells are the major sources of protective IFNγ during infection with *L. monocytogenes*, and these responses are T-bet independent. (C). During infection with *Toxoplasma gondii*, the parasite replicates within a parasitophorous vacuole. Antigen presentation occurs via MHC-Class I and MHC-Class II. NK cells and $CD4^+$ and $CD8^+$ T cells are important sources of IFNγ.

[*] Deb Argento helped prepare this figure.

cells are major sources of IFNγ (i.e. dependent on the pathogen and cytokine environment). A related explanation is that for some situations, additional factors may compensate for T-bet deficiency in the NK cells and $CD8^+$ T cells. For example, a second T-box transcription factor, Eomes (discussed later), is expressed at high levels in $CD8^+$ T cells and NK cells and mediates T-bet independent pathways to IFNγ production (Pearce et al., 2003).

Over the last 20 years, there has been a tendency to describe anti-microbial responses that involve NK and $CD4^+$ and $CD8^+$ T cell production of IFNγ as "Th1-like", perhaps with the implication that the cellular source of IFNγ is not critical. Because intracellular microorganisms can infect distinct host and cellular compartments (e.g. the cytosol, phagosomes, or parasitophorous vacuoles), and have unique mechanisms to evade the host response, the source of protective IFNγ matters. Consequently, these factors likely dictate the relative contributions of NK and $CD4^+$ and $CD8^+$ T cells as relevant sources of IFNγ for diverse infections. Hence, when looking at the variable phenotypes shown in Table 1, T-bet expression and IFNγ produced by $CD4^+$ T cells are essential for resistance to infection with *L. major*, *HSV2*, and *S. typhimurium*. However, T-bet is not required for initial IFNγ-mediated protection in response to *L. monocytogenes, T. gondii* and LCMV, models in which NK or $CD8^+$ T cells are potent sources of innate and adaptive production of IFNγ.

A role for T-bet in T cell trafficking

While IFNγ production is a major component of Th1 responses, there is a cassette of genes that is associated with this response that includes IL-12R expression and the chemokine receptor CXCR3 (Zhu et al., 2012). In that context, T-bet has additional functions relevant to Th1 immune responses, and it is not surprising that T-bet, a "lineage-specifying" transcription factor, can bind to the promoter region of other genes, such as STAT1, IL12Rβ2, CXCR3, and CCL3 (Beima et al., 2006; Jenner et al., 2009; Zhu et al., 2012). Indeed, T-bet promotes T cell expression of the chemokine receptor CXCR3, which binds to CXCL10, an IFNγ-induced chemokine present at sites of Th1 inflammation. CXCR3-dependent T cell trafficking is required for resistance to several intracellular infections that include *T. gondii* and respiratory syncytial virus (RSV) (Cohen et al., 2013; Lindell et al., 2008). Furthermore, T-bet dependent CXCR3 is required on effector T cells for optimal trafficking to the peritoneum (Lord et al., 2005). Conversely, Treg cells that express T-bet and CXCR3 are linked to the regulation of Th1 inflammatory responses (Koch et al., 2009), and, paradoxically, produce IFNγ in inflammatory settings (Hall et al., 2012; Oldenhove et al., 2009). Treg expression of T-bet allows these cells to mirror Th1-like effector cells, while the presence of Foxp3 promotes their suppressive effects. Additionally, T-bet has been linked to $CD8^+$ T cell production of the chemokine CCL3 (Beima et al., 2006; Kao et al., 2011), which promotes optimal T cell priming (Castellino et al., 2006), as well as transendothelial cell migration of Jurkat T cells (Ma and Ma, 2014). Furthermore, during infection with LCMV or *T. gondii*, the absence of T-bet is associated with reduced effector T cell

expression of Ly6C and KLRG1 (Joshi et al., 2007; Shah et al., 2015; Wilson et al., 2008). While expression of these surface proteins is frequently used to identify T cell effector status, Ly6C (which is linked to CD11a) and the ligands for KLRG1 (E-, N-, and R-cadherin) are involved in cellular migration (Cai et al., 2014; Hanninen et al., 1997; Jaakkola et al., 2003). Thus, T-bet expression is associated with multiple proteins involved in T cell trafficking. However, it can be difficult to distinguish whether T-bet directly regulates these molecules or is required for the differentiation of the effector cells that express these markers.

The ability to dissect the impact T-bet has in T cell trafficking during infection has been confounded by the importance of IFNγ and IFNγ-dependent mechanisms of effector T cell trafficking in many of these models. Regardless, two *in vivo* studies using *T. gondii* or *P. berghei*-ANKA illustrate that T-bet is dispensable for global production of IFNγ in these models, but it is essential for effector T cell trafficking and disease progression. In Chapter 2, I will show that after infection with *T. gondii*, parasite-specific $CD4^+$ and $CD8^+$ T cells from T-$bet^{-/-}$ mice express reduced levels of the integrin CD11a as well as CXCR3. Both of these molecules regulate different aspects of T cell migration into inflammatory environments (Cohen et al., 2013; Harris et al., 2012; Smith et al., 2007). Consequently, in the absence of T-bet, mice infected with *T. gondii* have reduced numbers of parasite-specific T cells in tissues such as the lung and brain that represent secondary sites of infection, and this is associated with an increase in parasite burden in these tissues (refer to Chapter 2). In contrast, in a murine model of cerebral malaria the

recruitment of $CD8^+$ T cells to blood vessels of the CNS causes local damage, but T-bet$^{-/-}$ mice have reduced T cell trafficking to the brain and less neuro-pathology (Oakley et al., 2013). Together, these results from two models of parasitic infection highlight the role of T-bet in promoting effector T cell trafficking, but also illustrate that depending on context, this can result in loss of protective immunity or reduced disease. Hence, it may be worthwhile to re-assess previous studies to determine if the absence of T-bet also results in altered distribution or reduced trafficking of effector cells into sites of inflammation, which might contribute to the increased susceptibility of these mice. However, many of the T-bet targets discussed above can affect T cell migration within tissues. Search strategies and migration within tissues are also important facets of T cell behavior (Harris et al., 2012; Krummel et al., 2016), and there remains a major gap in our understanding of whether T-bet affects the behavior of T cells within tissues.

T-bet expression during human infection

There are 40 known polymorphisms of the human *TBX21* gene, one of which has been associated with susceptibility to Type 1 diabetes (Sasaki et al., 2004), while another is associated with HSV-2 incidence (Svensson et al., 2008). How the other 38 known polymorphisms impact human immune responses to infectious disease is unknown. Studies examining the expression patterns of T-bet during disease have also been informative in understanding its role in humans. For example, a recent analysis of human patients infected with *Leishmania viannia* has revealed that T-bet expression in $CD4^+$ T cells correlates with active recurrent disease (Diaz et al., 2010), consistent with the idea

that T-bet is upregulated during effector phases of the immune response. HIV-specific $CD8^+$ T cells from elite controllers (ECs) expressed higher levels of T-bet than HIV-specific $CD8^+$ T cells from either chronically infected progressors (CPs) or highly active antiretroviral therapy (HAART)-suppressed individuals (Hersperger et al., 2011). Furthermore, during chronic infection with HIV, loss of effector function in HIV-specific $CD8^+$ T cells is associated with a decrease in expression of T-bet, which may help explain the decreased functionality associated with HIV progression (Ribeiro-dos-Santos et al., 2012). Additionally, during infection with Hepatitis B and Hepatitis C Viruses, high T-bet expression in antigen-specific $CD8^+$ T cells is associated with spontaneous resolution whereas T-bet deficiency is associated with chronic infection (Kurktschiev et al., 2014). Conversely, dysregulated T-bet expression has been associated with T- and B-cell lymphoproliferative disorders (Dorfman et al., 2003; Dorfman et al., 2004; Dorfman et al., 2005).

Relationship between T-bet and Eomes

As previously mentioned, T-bet belongs to a subfamily of T-box proteins that includes Eomes, which has a critical function in mesoderm development and mammalian trophoblast formation, and consequently, Eomes-deficient mice are embryonically lethal (Russ et al., 2000). In contrast, T-bet$^{-/-}$ mice are viable, which suggests that T-bet has lost developmental functions associated with the other Tbr1 subfamily members. However, both T-bet and Eomes are expressed in cells of the immune system that include NK cells and $CD4^+$ and $CD8^+$ T cells. Because previous studies had shown that $CD8^+$ T cells did

not require T-bet for the production of IFNγ (Szabo et al., 2002), Reiner and colleagues initiated a search for a transcription factor responsible for the T-bet independent production of IFNγ in $CD8^+$ T cells. The use of degenerate primers to amplify T-box sequence DNA in activated $CD8^+$ T cells led to the recognition that Eomes was expressed in these cells and can also promote IFNγ production in $CD8^+$ T cells (Pearce et al., 2003). Mice that are either deficient in T-bet or deficient in Eomes in the T cell compartment are still able to control viral replication after infection with LCMV-Armstrong, and there is no defect in IFNγ production from activated $CD8^+$ T cells (Intlekofer et al., 2008). However, mice with T cells deficient in both of these transcription factors were unable to control viral replication and had increased IL-17 production and disease severity as assessed by weight loss (Intlekofer et al., 2008). These results indicate that there may be some compensatory effects between these two transcription factors.

The studies described above are important because they demonstrate that the balance of these related transcription factors is important for the development and maintenance of productive Th1-like immune responses. For example, during chronic infection with LCMV, T-bet and Eomes are reciprocally expressed in exhausted $CD8^+$ T cells, and T-bet^{hi} precursors generated $Eomes^{hi}$ cells, which display higher expression of inhibitory receptors (Paley et al., 2012). Similarly, during chronic HIV infection, T-bet and Eomes are reciprocally expressed by virus-specific $CD8^+$ T cells, with high Eomes expression associated with expression of the inhibitory receptors PD-1 and CD160 (Buggert et al.,

2014). However, during acute infection with Epstein Barr Virus (EBV) or human cytomegalovirus (HCMV), both T-bet and Eomes are highly expressed in $CD8^+$ T cells specific for EBV or HCMV (Greenough et al., 2015; Hertoghs et al., 2010). Hence, while Eomes can "complement the role of T-bet in governing cellular immunity by providing redundancy and, quite likely, cooperativity in the induction of effector genes of T cells and NK cells" (Pearce et al., 2003), it is now clear that these transcription factors have different mechanisms of induction, unique functions and possibly even antagonistic activities, perhaps mediated through competition for DNA binding sites or transcriptional modifiers. Unfortunately, there are relatively few studies that examine how the absence of Eomes in T cells affects the outcome of infection. Regardless, the finding that Eomes, but not T-bet, is essential during embryonic development suggests that T-bet evolved into a more specialized role in the immune system. Furthermore, while there are examples of compensatory effects between T-bet and Eomes, there are now multiple examples in T-bet-deficient mice in which the presence of Eomes is not sufficient for pathogen control (See Table 1), which is potentially a consequence of which T cell subsets are necessary for pathogen control.

<u>The impact of T-bet on memory responses</u>

Many of the studies described above have focused on how the loss of T-bet affects the magnitude and phenotype of a primary effector response required for resistance to infection. Accordingly, $CD4^+$ and $CD8^+$ short-lived effector T cells (SLECs) express high levels of T-bet, and T-bet is required for the generation of these cells (Intlekofer et al.,

2007; Joshi et al., 2007; Marshall et al., 2011; Shah et al., 2015; Wilson et al., 2008). However, memory precursor cells (MPECs) also express T-bet, albeit at lower levels than effectors (Joshi et al., 2007; Marshall et al., 2011), but relatively few studies have examined if T-bet affects memory formation, or if T-bet contributes to either the maintenance of memory populations or the secondary T cell responses required for pathogen control. In mice infected with LCMV-Armstrong, despite a decrease in effectors, T-bet deficiency results in *enhanced* generation of $CD27^{hi}KLRG1^{lo}$ central memory $CD8^+$ T cells (Intlekofer et al., 2007). These observations are consistent with the idea that during the primary immune response, the magnitude of the effector response is inversely correlated with the generation of memory responses (Darrah et al., 2007; Pearce and Shen, 2007). However, a recent study suggests that a population of $KLRG1^{hi}$T-bet^{hi} $CD8^+$ T cells (typically an effector phenotype) persists more than one month after primary infection to the memory phase, and, despite low proliferative potential, provide optimal protection against *Vaccinia* and *Listeria* challenge (Olson et al., 2013). Furthermore, after epicutaneous challenge with HSV, development of $CD8^+CD103^+$ tissue resident memory cells in the skin requires downregulation of Eomes, but low levels of T-bet are necessary to maintain expression of CD122, the β-chain of the IL-15R, and IL-15 responsiveness in these cells, which promotes memory T cell survival (Mackay et al., 2015). Additionally, a recent report shows that T-bet is highly expressed in effector memory CX_3CR1^+ $CD8^+$ T cells (Bottcher et al., 2015). While this nascent literature seems contradictory, the unique cytokine and transcriptional environment

associated with different models employed in these systems may determine the impact of T-bet in the promotion or inhibition of the generation of effector cells.

Several groups have examined the role of T-bet in vaccine-induced protective immunity upon rechallenge. These types of experiments can be difficult to interpret because it has been challenging to distinguish how the absence of T-bet affects the generation of primary versus secondary responses. Nevertheless, compared to WT controls, HSV-2 vaccinated T-bet$^{-/-}$ mice challenged with a lethal dose of this virus had increased disease severity and decreased survival, which was associated with reduced IFNγ production by $CD4^+$ but not $CD8^+$ T cells (Svensson et al., 2005), and in Chapter 3 I will show that vaccinated T-bet$^{-/-}$ mice do not survive rechallenge with a lethal strain of *T. gondii*. Conversely, memory T-bet deficient GP33-specific $CD8^+$ T cells from LCMV infected mice that were transferred into naïve recipient mice conferred better protection than WT GP33-specific $CD8^+$ T cells against challenge with GP-33 expressing *L. monocytogenes* (Intlekofer et al., 2007).

Conclusion

During the last decade, we have moved from a paradigm in which T-bet was considered the "master regulator" of Th1 responses, to a model in which T-bet and Eomes were functionally redundant in IFNγ induction. Currently, there is a perception that T-bet and Eomes have similar properties, but the literature reviewed indicates that the roles of these transcription factors during infection are distinct and context-dependent. Thus, while both

T-bet and Eomes can promote IFNγ production, these transcription factors are not redundant, as mice deficient in T-bet are susceptible to many pathogens. T-bet and Eomes promote different T cell phenotypes and functions, particularly during chronic viral infection in which T-bet is associated with proliferative potential and Eomes is associated with exhaustion. In these types of situations it can be difficult to distinguish true functional redundancy versus the additive effects of loss of these two genes. There have been many studies examining the role of T-bet during infection, but there is a paucity of studies assessing the function of Eomes. Thus, the contribution of Eomes to the immune response to various bacterial and parasitic pathogens is unclear.

In Chapter 2 I will show that T-bet is required for resistance to *T. gondii*, but surprisingly not due to the role of T-bet in the production of IFNγ. Rather, in this system, the absence of T-bet is associated with reduced T cell trafficking to secondary sites of parasite replication. As discussed earlier, T-bet has previously been linked to proinflammatory T cell trafficking, but, to our knowledge, this is the first report demonstrating that this function of T-bet is required for survival after infection.

Additionally, in Chapter 3, I will provide evidence that T-bet is important in orchestrating the cellular interactions that result in optimal $CD8^+$ T cell priming. T-bet has been known to be expressed early after T cell activation, but the findings presented here indicate that this initial T-bet expression induces upregulation of the integrin CD11a and production of

the chemokine CCL3, both of which are important for the activation and expansion of the effector $CD8^+$ T cell population.

CHAPTER 2: DIVERSE ROLES FOR T-BET IN THE EFFECTOR RESPONSES REQUIRED FOR RESISTANCE TO INFECTION[*]

Abstract

The transcription factor T-bet has been most prominently linked to natural killer (NK) and T cell production of interferon-γ (IFN-γ), a cytokine required for the control of a diverse array of intracellular pathogens. Indeed, in mice challenged with the parasite *Toxoplasma gondii*, NK and T cell responses are characterized by marked increases of T-bet expression. Unexpectedly, T-bet$^{-/-}$ mice infected with *T. gondii* develop a strong NK cell IFN-γ response that controls parasite replication at the challenge site, but display high parasite burdens at secondary sites colonized by *T. gondii* and succumb to infection. The loss of T-bet had a modest effect on T cell production of IFN-γ but did not impact on the generation of parasite-specific T cells. However, the absence of T-bet resulted in lower T cell expression of CD11a, Ly6C, KLRG-1, and CXCR3 and fewer parasite-specific T cells at secondary sites of infection, associated with a defect in parasite control at these sites. Together, these data highlight T-bet independent pathways to IFN-γ production, and reveal a novel role for this transcription factor in coordinating the T cell responses necessary to control this infection in peripheral tissues.

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Introduction

The T-box transcription factor T-bet is expressed in cells of the innate and adaptive immune system (Lugo-Villarino et al., 2003; Lugo-Villarino et al., 2005; Szabo et al., 2000; Szabo et al., 2002), but it is perhaps most prominently linked to the production of IFN-γ in T cells and NK cells (Pearce et al., 2003; Szabo et al., 2000; Szabo et al., 2002). While many studies have focused on the role of T-bet in $CD4^+$ T cell production of IFN-γ, there are studies in which it also impacts on the ability of NK and $CD8^+$ T cells to produce IFN-γ (Intlekofer et al., 2008; Juedes et al., 2004; Sullivan et al., 2003; Townsend et al., 2004). In addition, T-bet has been shown to bind to the promoter region of other genes, suggesting a broader function in the immune response. For example, T-bet controls key checkpoints in NK cell maturation (Gordon et al., 2012) and also inhibits T cell production of IL-2, IL-4, and IL-17, thus repressing other $CD4^+$ T cell differentiation fates that include T helper 2 (Th2) and Th17 cells (Mathur et al., 2006; Szabo et al., 2000; Szabo et al., 2002). Furthermore, T-bet induces expression of the chemokine receptor CXCR3, and *in vitro* studies have identified a role for T-bet in the chemotaxis of $CD4^+$ T cells (Lord et al., 2005). Consistent with a key role for T-bet in the development of Th1 cells, this transcription factor is required for IFN-γ mediated resistance to *Leishmania major*, *Salmonella*, *Mycobacterium tuberculosis*, and HSV-2 (Ravindran et al., 2005; Sullivan et al., 2005; Svensson et al., 2005; Szabo et al., 2002). While production of IFN-γ is also required to limit replication of *Listeria monocytogenes* and LCMV, the IFN-γ produced in the T-bet$^{-/-}$ mice is sufficient for controlling these pathogens (Intlekofer et al., 2008; Way and Wilson, 2004). One explanation for this

phenomenon is that these T-bet independent pathways to IFN-γ production are mediated through a related T-box transcription factor, Eomesodermin (Intlekofer et al., 2005; Pearce et al., 2003).

In current models, challenge of mice with the intracellular parasite *T. gondii* results in the production of interleukin 12 (IL-12) by dendritic cells and macrophages which promotes the activation and expansion of NK cell and T cell populations that express high levels of T-bet and are associated with the secretion of IFN-γ (Gazzinelli et al., 1991; Khan et al., 1994; Lieberman et al., 2004; Sher et al., 2003; Zhu et al., 2012). The production of IFN-γ, which engages numerous antimicrobial mechanisms (MacMicking, 2012; Taylor, 2007), is essential for the local control of *T. gondii* in multiple tissues. The studies presented here demonstrate that when mice deficient in T-bet are challenged with *T. gondii*, they control parasite replication at the site of initial challenge, associated with strong NK cell responses, and can generate parasite-specific T cells that produce IFN-γ. However, these mice do not survive acute infection, and secondary sites of parasite colonization that include the heart, thymus, lung, and brain have high parasite burdens. The increased mortality is associated with alterations in the phenotype of parasite-specific T cell populations that include reduced expression of markers of activation (CD11a), effector status (Ly6C and KLRG-1), and trafficking (CXCR3) as well as a marked reduction in their numbers at these peripheral sites. The use of a mixed bone marrow chimeric approach revealed a cell-intrinsic requirement for T-bet for the development of appropriate parasite-specific effector T cells. Together, these data

highlight a novel role of T-bet in coordinating the $CD4^+$ and $CD8^+$ T cell responses that are essential for the control of infection in peripheral tissues.

Results

Expression of T-bet during the immune response to *Toxoplasma gondii*

As part of studies to understand the role of T-bet during the immune response against *T. gondii,* the populations involved in protective immunity were surveyed for infection-induced alterations in T-bet expression and localization. In naïve WT mice, there is heterogeneous expression of the integrin CD49b (DX5) within the NK cell population, with high expression indicative of NK cell maturity. In uninfected mice, T-bet is not highly expressed in NK cells, but following challenge with *T. gondii*, a population of mature $DX5^{hi}$ NK cells that express high levels of T-bet emerged (Figure 3a). Similarly, based on the use of Ly6C and KLRG-1 as markers of $CD4^+$ and $CD8^+$ T cell effector populations, respectively (Joshi et al., 2007; Marshall et al., 2011; Wilson et al., 2008), naïve mice had few effector cells, and this population expressed low levels of T-bet (Figures 3b and 3c). However, at day 9 post-infection, a subset of $CD4^+$ T cells that expressed T-bet was evident and a portion of these cells also expressed high levels of Ly6C; the number of these $T\text{-}bet^+Ly6C^{hi}CD4^+$ T cells that emerged after infection was significantly increased over naïve mice (Figure 3b). Similarly, at this timepoint, there was an increase in the percentage and number of $CD8^+$ T cells that expressed T-bet and KLRG-1 (Figure 3c).

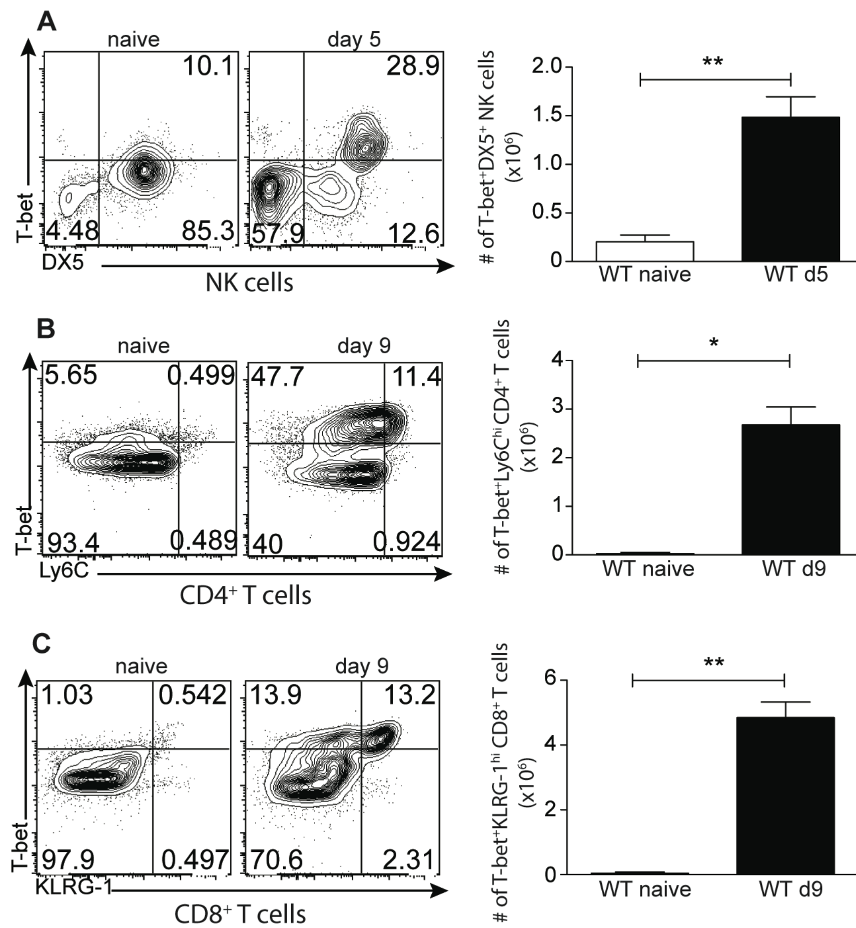

Figure 3. T-bet expression increases in NK and T cells after infection with *Toxoplasma gondii*. WT C57BL/6 mice were infected intraperitoneally (i.p.) with *T. gondii*. (A) After 5 or (B,C) 9 days, splenocytes from infected and uninfected mice were harvested and stained for analysis by flow cytometry. (A) NK cells were identified as $NK1.1^+CD3^-$ live cells. (B) $CD4^+$ T cells were identified as $CD3^+CD4^+CD8a^-$ live cells. (C) $CD8^+$ T cells were identified as $CD3^+CD4^-CD8a^+$ live cells. Data are representative of 3 experiments, n=3 per experiment. * $p<0.05$, ** $p<0.01$

Further analysis revealed that in the $CD4^+$ T cells from infected mice, T-bet expression was associated with expression of the activation markers CD11a, an integrin that, together with CD18, comprises lymphocyte function-associated antigen 1 (LFA-1), CXCR3, a Th1 associated chemokine receptor, and Ly6C (Figure 4a and Figure 5a). More than 95% of $T\text{-}bet^+$ $CD4^+$ T cells expressed at least one of these activation markers, and approximately 20% of these $T\text{-}bet^+$ cells expressed all three activation markers (Figure 4a). In contrast, only 25% of $T\text{-}bet^-$ $CD4^+$ T cells expressed any of these activation markers, and less than 5% of these cells expressed all three activation markers. Expression of CD11a has been used as a marker of activation for the polyclonal antigen-specific T cell population in a variety of infectious settings, including toxoplasmosis (Dupont et al., 2014; McDermott and Varga, 2011; Rai et al., 2009; Srinivasan et al., 2004). Parasite-specific $CD4^+$ T cells were identified using an MHC-II tetramer for AS15 (Grover et al., 2012) and, consistent with the polyclonal ($CD11a^{hi}$) population, these cells expressed T-bet, Ly6C, and CD11a (Figure 5b). Among the $CD8^+$ T cells, T-bet was highly expressed in the infection-induced polyclonal $CD11a^{hi}$ population (Figure 5c), and T-bet expression was closely associated with expression of CXCR3 and KLRG-1 (Figure 4b). More than 95% of T-bet-expressing $CD8^+$ T cells expressed CXCR3, high levels of CD11a, and/or KLRG-1, with nearly 50% expressing all three activation markers. Among the $T\text{-}bet^-$ $CD8^+$ T cell population, the majority (75%) did not express CXCR3, high levels of CD11a, or KLRG-1 and less than 1% of $T\text{-}bet^-$ $CD8^+$ T cells expressed all three of these activation markers (Figure 4b). Moreover, consistent with the polyclonal $CD11a^{hi}$ population, when parasite-specific $CD8^+$ T cells were identified using an MHC-I

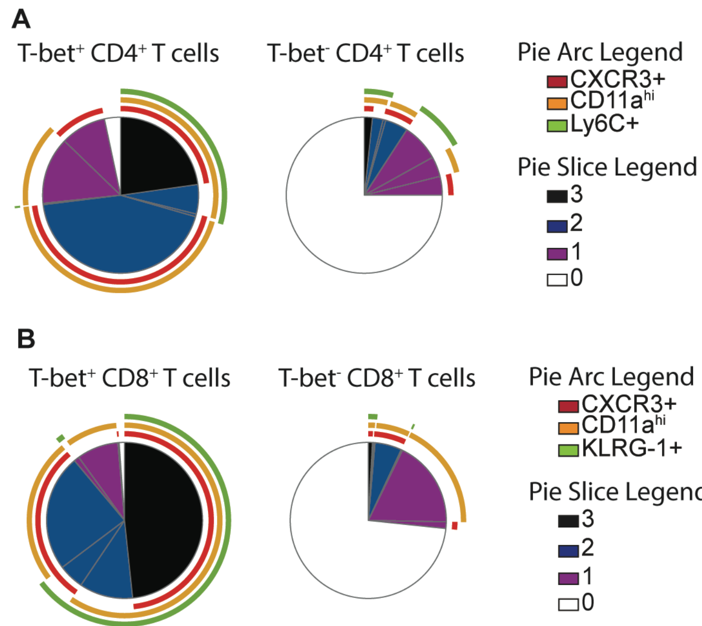

Figure 4. T-bet expression is associated with expression of T cell activation markers. Splenocytes were harvested from *T. gondii* infected mice 9-11 days post-infection and stained for analysis by flow cytometry combined with SPICE. (A) Expression of CXCR3, CD11a, and Ly6C was determined on T-bet$^+$ and T-bet$^-$ CD4$^+$ T cells. (B) Expression of CXCR3, CD11a, and KLRG-1 was determined on T-bet$^+$ and T-bet$^-$ CD8$^+$ T cells.

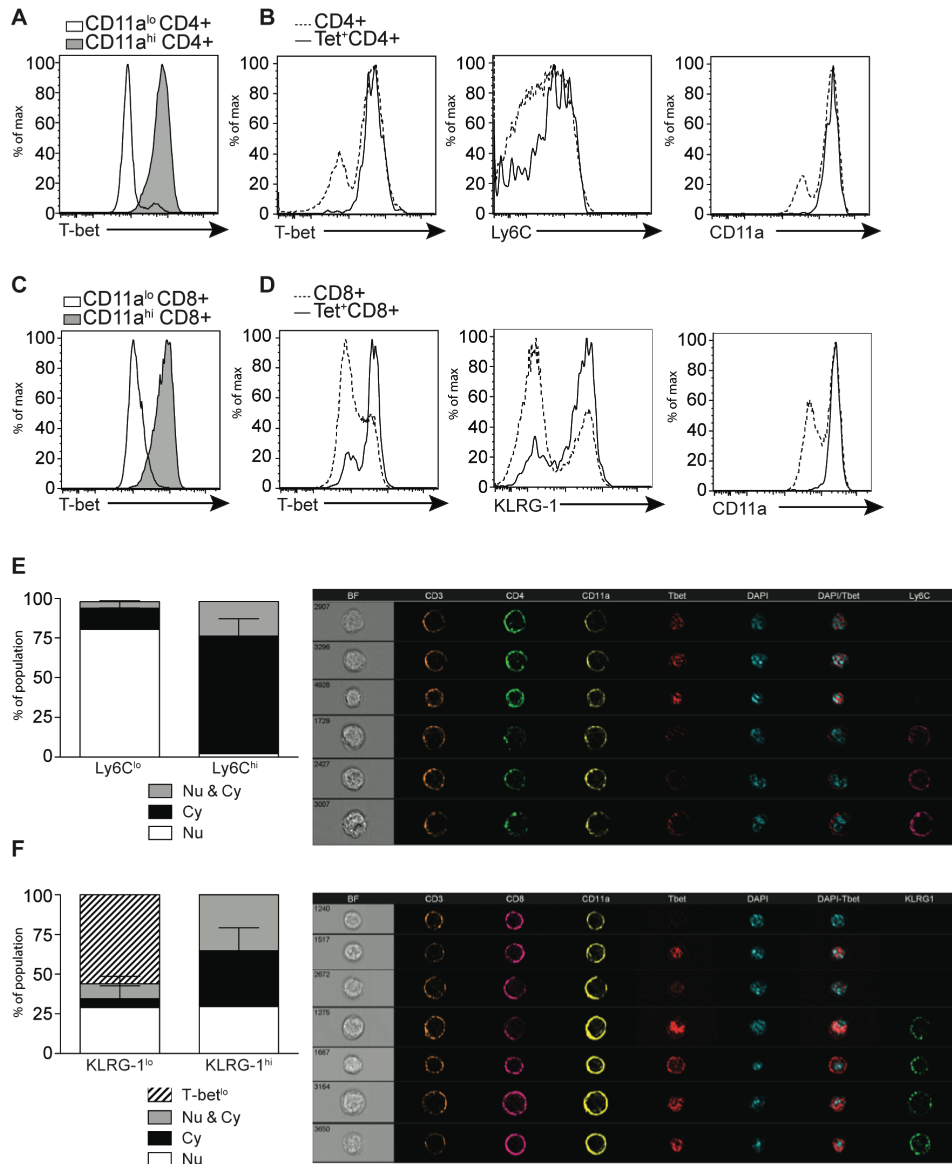

Figure 5. T-bet expressing T cells have a distinct phenotype after infection with *T. gondii*. Splenocytes were harvested from *T. gondii* infected mice 9-11 days post-infection and stained for analysis by flow cytometry and Image Stream. (A) T-bet expression levels were assessed in the polyclonal CD11a^{hi} CD4^{+} T cell population compared to the CD11a^{lo} population. (B) T-bet, Ly6C, and CD11a expression levels were assessed on

total $CD4^+$ T cells (dashed lines) and $tetramer^+CD4^+$ T cells (solid lines). (C) T-bet expression levels were assessed in the polyclonal $CD11a^{hi}$ $CD8^+$ T cell population compared to the $CD11a^{lo}$ population. (D) T-bet, KLRG-1, and CD11a expression levels were assessed on total $CD8^+$ T cells (dashed lines) and $tetramer^+CD8^+$ T cells (solid lines). (E) T-bet localization was assessed within the $Ly6C^{lo}CD11a^{hi}$ and $Ly6C^{hi}CD11a^{hi}$ $CD4^+$ T cell population using the Image Stream. (F) T-bet localization was assessed within the $KLRG\text{-}1^{lo}CD11a^{hi}$ and $KLRG\text{-}1^{hi}CD11a^{hi}$ $CD8^+$ T cell population using the Image Stream. Data are representative of 2-4 experiments, n=3 per experiment.

tetramer for the parasite antigen Tgd057 (Wilson et al., 2010), these cells highly expressed T-bet, KLRG-1, and CD11a (Figure 5d). Thus, following infection with *T. gondii,* T-bet is expressed in activated $CD4^+$ and $CD8^+$ T cells, and this correlates closely with the expression of CD11a, Ly6C and KLRG-1 by parasite-specific effector populations.

Recently, human effector $CD8^+$ T cells have been shown to have heterogeneous levels of T-bet protein that are associated with differential localization of T-bet in the nucleus and cytoplasm (McLane et al., 2013). Since T-bet expression closely correlated with expression of Ly6C and KLRG-1, proteins that aid in defining effector populations in murine T cells, ImageStream analysis was combined with these surface molecules to visualize T-bet localization. Because there were relatively few numbers of cells that bound to the MHC tetramers, the polyclonal $CD11a^{hi}$ $CD4^+$ and $CD8^+$ T cell populations were analyzed. Among the $Ly6C^{lo}CD11a^{hi}CD4^+$ T cell (memory precursor) population, T-bet was present primarily in the nucleus, as quantified by colocalization with DAPI (Figure 5e). In contrast, within the $Ly6C^{hi}CD11a^{hi}CD4^+$ T cell (effector) population, the expression of T-bet in approximately 75% of cells did not colocalize with DAPI, indicating that T-bet was cytoplasmic in these cells (Figure 5e). Analysis of the $CD8^+$ T cells revealed that within the $KLRG\text{-}1^{lo}CD11a^{hi}CD8^+$ T cell (memory precursor) population, a portion of cells expressed low levels of T-bet that was difficult to localize, but in those cells that highly expressed this protein it was present primarily in the nucleus (Figure 5f). However, the majority of $KLRG\text{-}1^{hi}CD11a^{hi}CD8^+$ T cells (effectors) had T-

bet present in the cytoplasm (Figure 5f). Together, these data demonstrate that among the antigen-specific ($CD11a^{hi}$) T cells, T-bet is differentially localized among the effector and memory precursor T cell populations.

T-bet knockout mice are susceptible to infection with *T. gondii*

To directly assess the significance of the infection-induced increases of T-bet in activated NK and T cells, WT and T-bet$^{-/-}$ mice were challenged with ME49, an avirulent strain of *T. gondii*. WT mice survived this challenge, yet mice lacking T-bet succumbed to infection between days 9 and 14 post-infection (Figure 6a). In many instances, susceptibility to *T. gondii* is either a consequence of an inability to control parasite replication (Lieberman et al., 2004; Scharton-Kersten et al., 1996; Suzuki et al., 1988) or the development of T cell-mediated immune pathology (Gazzinelli et al., 1996; Kugler et al., 2013; Liesenfeld et al., 1996; Villarino et al., 2003). In order to determine the cause of the increased mortality seen in T-bet$^{-/-}$ mice, parasite burden and levels of immune-mediated damage were assessed. Analysis of the peritoneal exudate cells (PECs) revealed that there was a small but reproducible increase in parasite burden in the T-bet$^{-/-}$ mice at day 5 (Figure 6b) and day 9 post-infection (data not shown). A more comprehensive analysis of the tissues from infected WT and T-bet$^{-/-}$ mice by quantitative PCR (Figure 6c) and immunohistochemistry (Figures 6d-g) revealed that in WT mice few parasites were detected at peripheral sites of infection such as the thymus, heart, lung, and brain. However, in T-bet$^{-/-}$ mice, these tissues all contained areas of extensive parasite replication. Liver lesions (areas of coagulative necrosis in the liver that are typically

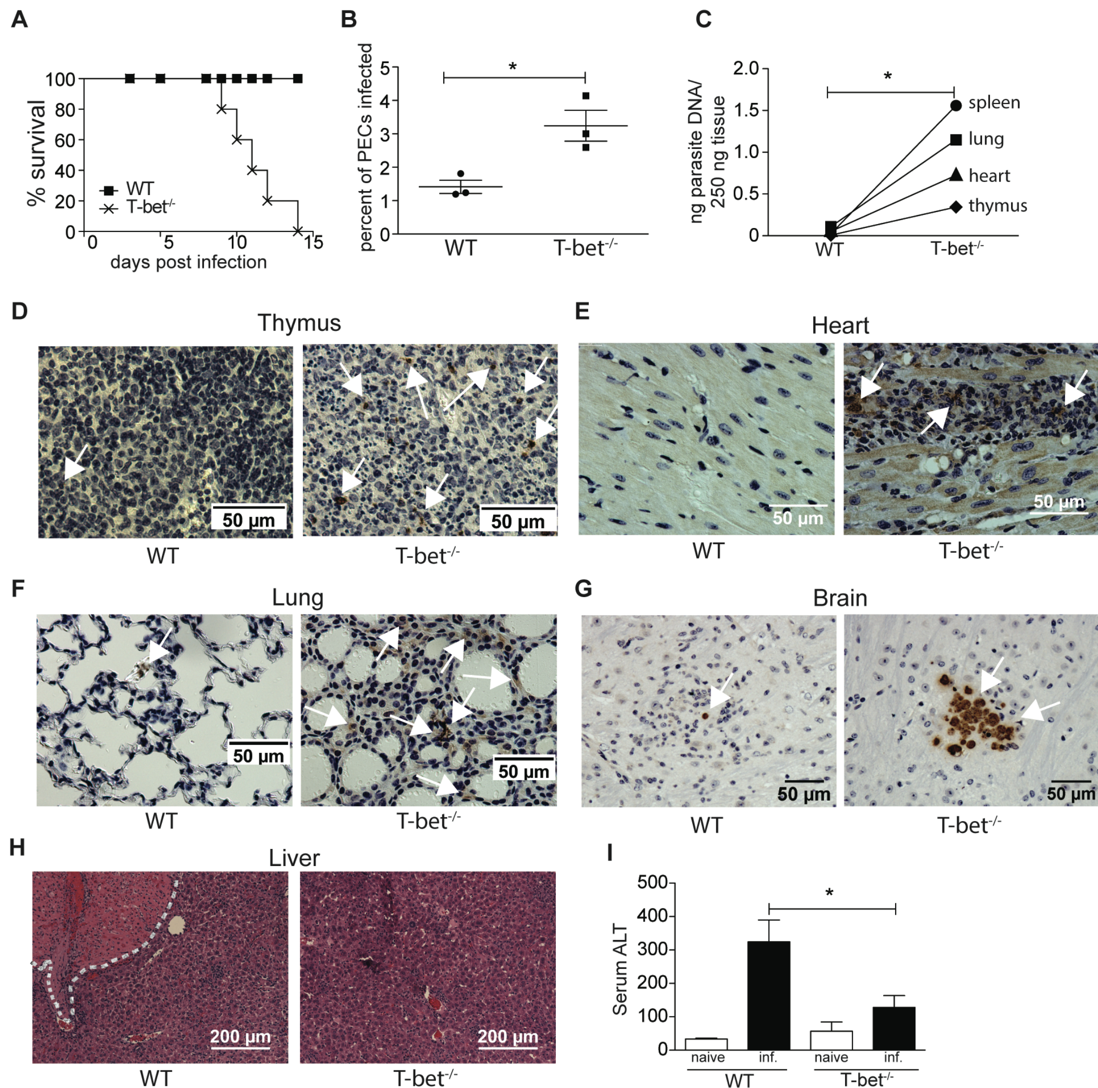

Figure 6. T-bet is necessary for survival and regional parasite control during *T. gondii* infection. (A) WT and T-bet deficient (T-bet$^{-/-}$) mice were infected i.p. with *T. gondii* and were monitored for survival. (B) At day 9 post-infection, peritoneal lavage was performed and peritoneal exudate cells (PECs) were collected for cytospin preparation and analysis of parasite burden. (C) DNA was extracted from spleens (•), lungs (■), hearts (◆), and thymi (▲) from infected WT and T-bet$^{-/-}$ mice and levels of parasite DNA were assessed using qPCR. (D-G) Tissues from WT and T-bet$^{-/-}$ mice were

isolated 9-11 days post infection and fixed in formalin solution, sectioned, and stained for *T. gondii* antigen. (H) Liver samples were harvested from mice 9 days post-infection and stained by H&E. Area of necrosis is outlined with a dashed white line. (I) Serum was harvested from uninfected and *T. gondii* infected WT and T-bet$^{-/-}$ mice and analyzed for ALT levels. Data are representative of 2-3 experiments, n=3-5 per experiment.

associated with $CD4^+$ T cell-mediated immunopathology) were present in the tissues from WT mice but were absent or reduced in those from T-bet$^{-/-}$ mice (Figure 6h). While there was no difference in the frequency of NK cells in the livers from infected WT and T-bet$^{-/-}$ mice, there was a marked reduction in the number of T cells in the livers from the T-bet$^{-/-}$ mice (data not shown). Furthermore, WT mice had higher levels of infection-induced alanine aminotransferase (ALT), which is indicative of liver damage, in the sera than T-bet$^{-/-}$ mice at day 9 post-infection (Figure 6i), consistent with the elevated pathology. Thus, the major cause of susceptibility of the T-bet$^{-/-}$ mice is a failure to control parasite replication at secondary sites of infection.

Innate responses in the absence of T-bet

To better understand the underlying cause of the increased susceptibility of the T-bet$^{-/-}$ mice, the innate response to *T. gondii* was characterized. These studies revealed no significant differences in serum levels of infection-induced IL-12 or IFN-γ in the WT and T-bet$^{-/-}$ mice at days 5 (data not shown) and 9 post-infection (Figures 7a and 7b). Moreover, at day 5 post-infection, a period of enhanced NK cell activity (Hunter et al., 1994; Neyer et al., 1997), there was equivalent expansion of $DX5^+$ NK cells from T-bet$^{-/-}$ and WT mice (Figure 7c) and these cells expressed similar levels of the activation marker CD69 (Figure 7d). Analysis of NK cells from the spleens of infected and uninfected mice showed that there were basal differences in IFN-γ production from WT and T-bet$^{-/-}$ NK cells (data not shown), but after challenge these cells produced similar levels of IFN-γ

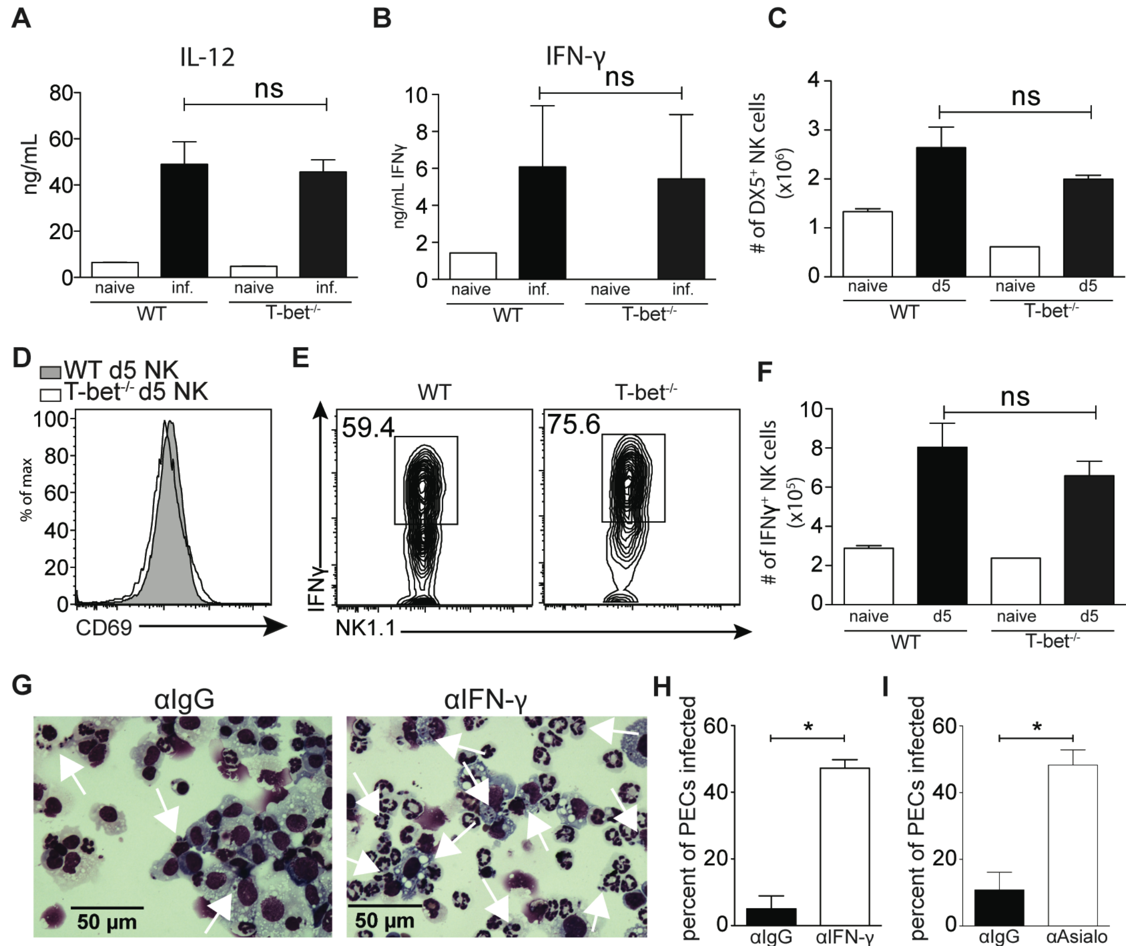

Figure 7. NK and cytokine responses are intact in infected T-bet$^{-/-}$ mice. (A-F) WT and T-bet$^{-/-}$ mice were infected i.p. with *T. gondii*. (A,B) Serum was harvested from day 9 infected and uninfected WT and T-bet$^{-/-}$ mice and assayed by ELISA for (A) IL-12 and (B) IFN-γ. (C) Splenocytes from uninfected and day 5 infected WT and T-bet$^{-/-}$ mice were analyzed by flow cytometry for mature (DX5^{+}) NK cells (NK1.1^{+}CD3^{-} live cells). (D) NK cells (NK1.1^{+}CD3^{-} live cells) from day 5 infected WT and T-bet$^{-/-}$ mice were analyzed by flow cytometry for expression of CD69 (WT CD69 MFI=1246±89.41, T-bet$^{-/-}$ CD69 MFI=1086±24.83). (E,F) Whole splenocytes from uninfected and day 5 infected WT and T-bet$^{-/-}$ mice were restimulated *in vitro* with PMA and ionomycin in the presence

of Brefeldin A and monensin for 4 hours. Cells were then fixed and stained for cytokine production by NK cells (E) and quantified (F). (G,H) T-bet$^{-/-}$ mice were infected i.p. with *T. gondii*. On days 0, 3, and 6, mice were given 0.5mg αIFN-γ or isotype control (αIgG) i.p. On day 7 post-infection peritoneal lavage was performed and PECs were collected for cytospin preparation and analysis of percentage of cells infected. (I) T-bet$^{-/-}$ mice were treated with 50ul αAsialo gm-1 or isotype control on days -1, 3, and 6 of *T. gondii* infection. At day 9 post-infection, peritoneal lavage was performed and peritoneal exudate cells (PECs) were collected for cytospin preparation and analysis of parasite burden. Data are representative of 2-3 experiments, n=3 per experiment. * $p<0.05$

(Figures 7e and 7f). Additionally, when WT and T-bet$^{-/-}$ mice were treated with an IFN-γ blocking antibody, there was a marked increase in the percentage of infected PECs over the isotype control treated mice (Figures 7g and 7h). Furthermore, depletion of NK cells in T-bet$^{-/-}$ during infection with *T.gondii* also resulted in a similar increase in the percentage of infected PECs (Figure 7i). Together, these data establish that T-bet is dispensable for NK cell activation and the early IFN-γ produced in the absence of T-bet contributes to the control of parasite replication at the local site of infection.

Role of T-bet in the T cell response to *T. gondii*

In order to assess the role of T-bet in the generation of the adaptive T cell response to *T. gondii*, WT and T-bet$^{-/-}$ mice were infected with *T. gondii* and responses were analyzed 9-11 days later. Splenocytes from infected and uninfected mice were harvested and stimulated *in vitro* with either anti-CD3 or with soluble *T. gondii* antigen (STAg) and assessed for IFN-γ production (Figures 8a and 8b). Intracellular staining revealed that among WT cells, the majority of CD4^{+} T cells producing IFN-γ were T-bet^{+} (Figure 8c). While infection induced an increase in IFN-γ production by both WT and T-bet$^{-/-}$ CD4^{+} T cells (13-fold increase in the WT and 10-fold increase in the T-bet$^{-/-}$), the percentage of IFN-γ-producing CD4^{+} T cells was reduced in the T-bet$^{-/-}$ mice compared to the WT mice (Figure 8c), but this was not associated with increases in IL-4 (data not shown). Similarly, the majority of WT CD8^{+} T cells producing IFN-γ also expressed T-bet; however, WT and T-bet$^{-/-}$ CD8^{+} T cells produced comparable levels of IFN-γ (Figure 8d).

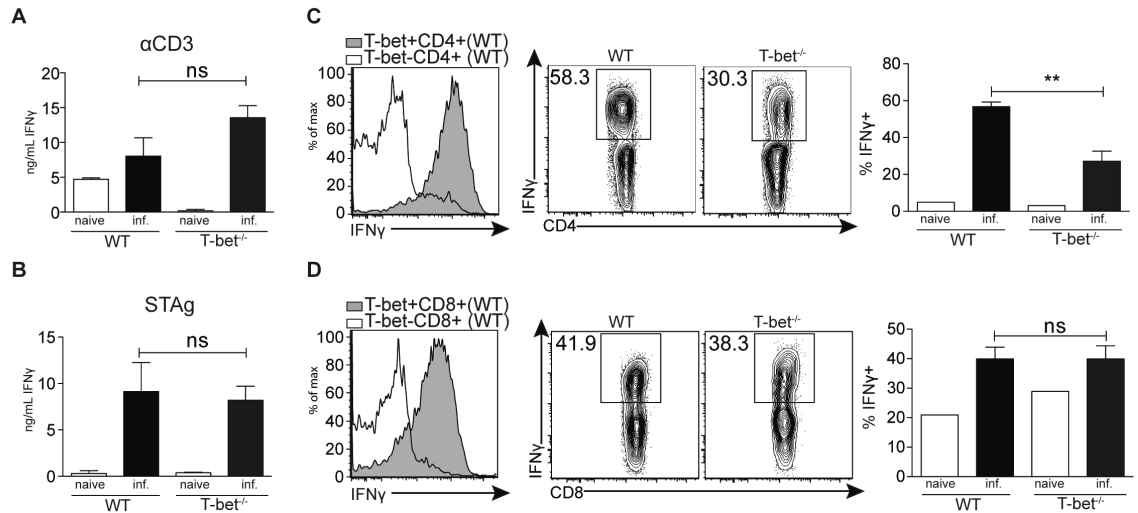

Figure 8. T-bet-deficient T cells produce IFN-γ during infection with *T. gondii*. WT and T-bet$^{-/-}$ mice were infected i.p. with *T. gondii*. Splenocytes were restimulated in vitro with (A) αCD3 or (B) STAg and supernatants were assayed for IFN-γ by ELISA. (C,D) Splenocytes from uninfected and infected WT and T-bet$^{-/-}$ mice were restimulated in vitro stained for IFN-γ production by (C) CD4^{+} T cells (CD3^{+}CD4^{+}CD8a^{-} live cells) and (D) CD8^{+} T cells (CD3^{+}CD4^{-}CD8a^{+} live cells). Data are representative of 3 experiments, n=3-4 per experiment. ** p<0.01

Thus, there is a significant proportion of $CD4^+$ and $CD8^+$ T cells that produce IFN-γ independently of T-bet.

To characterize the impact of T-bet on T cell activation, a panel of markers that included CD25, CD11a, Ly6C, KLRG-1 and CXCR3 was used to compare the T cell populations in WT and T-bet$^{-/-}$ mice. This analysis revealed that following challenge WT $CD4^+$ T cells had high levels of CD11a and this was decreased in the T-bet$^{-/-}$ $CD4^+$ T cells (Figure 9a). Interestingly, although there was no difference in CD25 expression between naïve WT and T-bet$^{-/-}$ mice (data not shown), T-bet$^{-/-}$ $CD4^+$ T cells from infected mice displayed higher levels of CD25 than $CD4^+$ T cells from WT mice (Figure 9a). However, there was no difference in the frequency or numbers of Foxp3 expressing cells between the WT and T-bet$^{-/-}$ $CD4^+$ T cell populations (data not shown). In the $CD8^+$ T cell population, there were $CD11a^{hi}$ and $CD11a^{lo}$ populations of WT $CD8^+$ T cells, and this heterogeneity was less apparent in the T-bet$^{-/-}$ $CD8^+$ T cells (Figure 9b). Moreover, similar to the $CD4^+$ T cells in the T-bet$^{-/-}$ mice, $CD8^+$ T cells expressed higher levels of CD25 than WT $CD8^+$ T cells (Figure 9b). Initially, these data on CD11a expression suggested that in infected T-bet$^{-/-}$ mice there are fewer parasite-specific $CD4^+$ T cells. However, the use of a *T. gondii*-specific AS15 MHC-II tetramer (Grover et al., 2012) revealed that in the spleens of WT and T-bet$^{-/-}$ mice there was an equivalent expansion of the these $CD4^+$ T cells (Figure 9c). Additionally, there were no differences in PSGL-1, CD44 or CD62L expression between the WT and T-bet$^{-/-}$ tetramer$^+$ $CD4^+$ T cells (data not shown). Nonetheless, there were phenotypic differences between the WT and T-bet$^{-/-}$

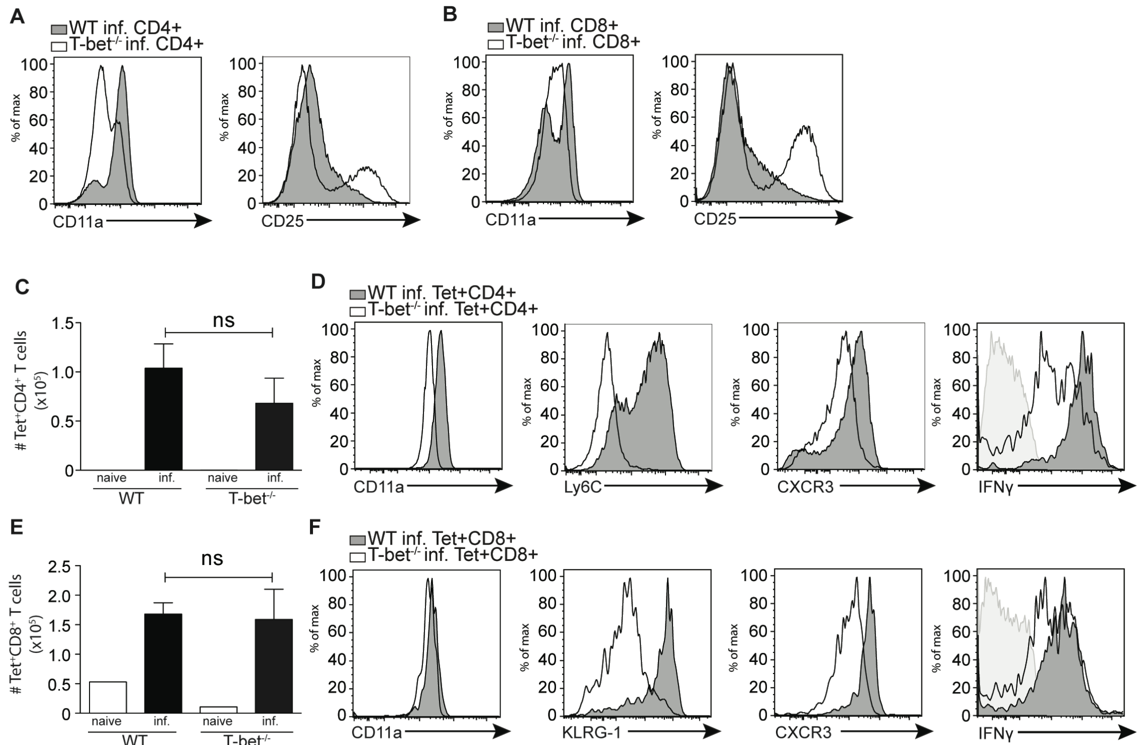

Figure 9. Altered phenotype of parasite-specific T cells in infected T-bet$^{-/-}$ mice. WT and T-bet$^{-/-}$ mice were infected i.p. with *T. gondii*. (A,B) Splenocytes from infected mice were harvested and stained for analysis by flow cytometry for CD11a and CD25 expression levels on (A) CD4^{+} and (B) CD8^{+} T cells. (C,D) Splenocytes from infected or uninfected mice were stained for parasite-specific CD4^{+} T cells using an MHC II tetramer and analyzed for (C) quantification, and (D) activation and effector status markers CD11a, Ly6C, CXCR3, and IFN-γ by flow cytometry. In the IFN-γ flow plots, the IFN-γ FMO is light grey (E,F) Splenocytes from infected or uninfected mice were stained for parasite-specific CD8^{+} T cells using an MHC I tetramer and this population was (E) quantified and analyzed for (F) activation and effector status markers CD11a, KLRG-1, CXCR3, and IFN-γ by flow cytometry. In the IFN-γ flow plots, the IFN-γ FMO is light grey. Data are representative of 3-5 experiments, n=3-4 per experiment.

cells that mirrored the polyclonal population. Thus, the WT AS15-specific $CD4^+$ T cells expressed high levels of the activation markers CD11a, Ly6C, and CXCR3, but these were reduced in parasite-specific $CD4^+$ T cells from the T-bet$^{-/-}$ mice (Figure 9d). In addition, while these T cells did produce IFN-γ, the MFI was reduced compared to WT T cells (Figure 9d).

In naïve WT and T-bet$^{-/-}$ mice, the frequency of splenic Tgd057-specific $CD8^+$ T cells is low and infection with *T. gondii* results in an equivalent expansion of this population (Figure 9e), which expressed similar levels of CD11a (Figure 9f). Additionally, there were no differences in CD44 or CD62L expression between the WT and T-bet$^{-/-}$ Tetramer$^+$ $CD8^+$ T cells (data not shown). However, while the majority of Tgd057-specific WT $CD8^+$ T cells expressed KLRG-1 and CXCR3, the parasite-specific $CD8^+$ T cells from T-bet$^{-/-}$ mice expressed significantly lower levels of these proteins (Figure 9f), but the WT and T-bet$^{-/-}$ parasite-specific $CD8^+$ T cells expressed comparable levels of IFN-γ (Figure 9f). These data indicate that T-bet is not required for the activation and expansion of parasite-specific $CD4^+$ and $CD8^+$ T cells but is required for these cells to acquire an effector phenotype associated with expression of CD11a, CXCR3, Ly6C and KLRG-1.

<u>Role of T-bet in T cell responses in peripheral sites</u>

Given that the T-bet$^{-/-}$ mice had an increased parasite burden in peripheral tissues, the decreased expression of CXCR3 and CD11a by parasite-specific T cells from the spleens

of these mice suggested a defect in their ability to either access distal sites of infection or function within these sites to control parasite replication. Therefore, WT and T-bet$^{-/-}$ mice were infected and the T cell responses were assessed at peripheral sites. In the lungs of WT mice, there was a large population of parasite-specific CD4^{+} T cells characterized by high levels of Ly6C. In the T-bet$^{-/-}$ mice, the numbers of these cells were markedly reduced and those that were present expressed reduced levels of Ly6C (Figure 10a,b). Similarly, there were significantly more parasite-specific CD8^{+} T cells in the lungs of WT mice than in the T-bet$^{-/-}$ mice (Figure 10c), and the WT cells expressed higher levels of KLRG-1 (Figure 10d). Immunohistochemical analysis of brain sections taken at day 9 post-infection showed T cells associated with areas of parasite replication in WT mice (Figure 10e), but in T-bet$^{-/-}$ mice there was a marked absence of T cells despite the increased parasite burden (Figure 10e). Thus, the susceptibility of the T-bet$^{-/-}$ mice is not due to a defect in the numbers of parasite-specific T cells generated, but rather is associated with an altered phenotype and reduced numbers of effector T cells at local sites of parasite replication.

<u>Cell intrinsic versus extrinsic requirement for T-bet</u>

Altered CD4^{+} T cell activity during toxoplasmosis leads to changes in the CD8^{+} T cell response (Jordan et al., 2009; Lutjen et al., 2006), and the studies described above do not distinguish the cell intrinsic versus cell extrinsic effects of T-bet deficiency on the development and function of parasite-specific T cells. In order to start to address this issue, a competitive mixed-bone marrow chimera approach was used in which irradiated

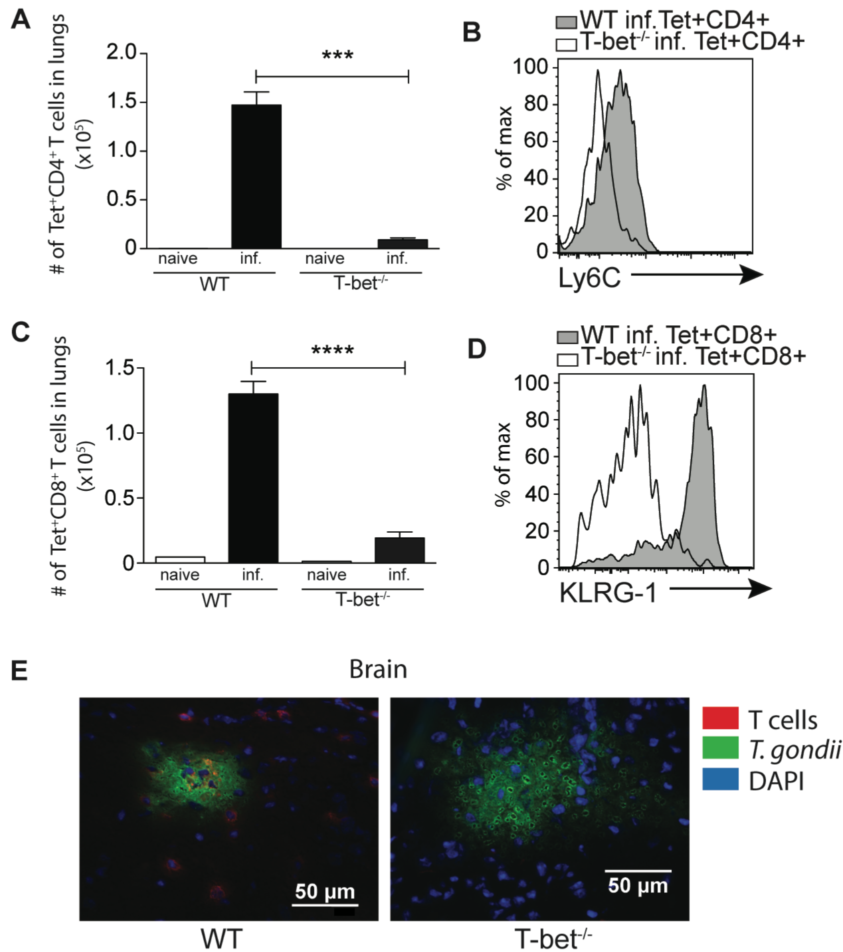

Figure 10. T-bet is required for T cell localization at secondary sites of parasite colonization. WT and T-bet$^{-/-}$ mice were infected i.p. with *T. gondii.* (A,B) Lungs were harvested and cells were isolated and stained for parasite-specific CD4^{+} T cells using an MHC II tetramer and Ly6C expression was assessed on these cells. (C,D) Lung cells were also stained for parasite-specific CD8^{+} T cells using an MHC I tetramer and KLRG-1 expression was assessed on these cells. (E) Brains were harvested from WT and T-bet$^{-/-}$ mice and stained for T cells (Cy7, red), *T. gondii* (FITC, green), and DAPI (blue). Data are representative of 2-3 experiments, n=3 per experiment.

recipient mice received a 1:1 ratio of WT:T-bet$^{-/-}$ bone marrow (Figure 11a). After >8 weeks post-reconstitution, the mice were sacrificed and tissues were examined to determine levels of chimerism. Similar to previous reports (Juedes et al., 2004), the majority of the reconstituted cells were derived from the T-bet$^{-/-}$ donor. At steady state in all four experiments performed, cells from the T-bet$^{-/-}$ donor comprised 65-75% of the total CD4^{+} T cells in the spleen as well as the lung (Figure 11b), and this ratio of WT:T-bet$^{-/-}$ CD4^{+} T cells was maintained for more than 6 months post-reconstitution (data not shown). These observations indicate that T-bet$^{-/-}$ CD4^{+} T cells have a competitive advantage over WT CD4^{+} T cells in this experimental setting. In contrast, the CD8^{+} T cell compartment had equal proportions of WT and T-bet$^{-/-}$ cells in the spleen, and an increased frequency of WT CD8^{+} T cells in the lung (Figure 11c).

When the chimeras were infected with *T. gondii* and analyzed 9-11 days later (Figure 11d), there was a 70-fold expansion of the WT parasite-specific CD4^{+} T cells but only a 6-fold expansion of the T-bet$^{-/-}$ parasite-specific CD4^{+} T cells (data not shown), resulting in no difference in the number of WT and T-bet$^{-/-}$ parasite-specific CD4^{+} T cells in the spleen or lung after infection (Figure 11e). However, the WT parasite-specific CD4^{+} T cells from both the spleen and lung expressed higher levels of Ly6C (Figure 11f) and CD11a (data not shown) than T-bet$^{-/-}$ parasite-specific CD4^{+} T cells after infection. Additionally, there was reduced IFN-γ from the T-bet$^{-/-}$ CD4^{+} T cell compartment compared to the WT CD4^{+} T cells (Figure 11g). In contrast, there were significantly more WT parasite-specific CD8^{+} T cells in both the spleen and lung after challenge (Figure

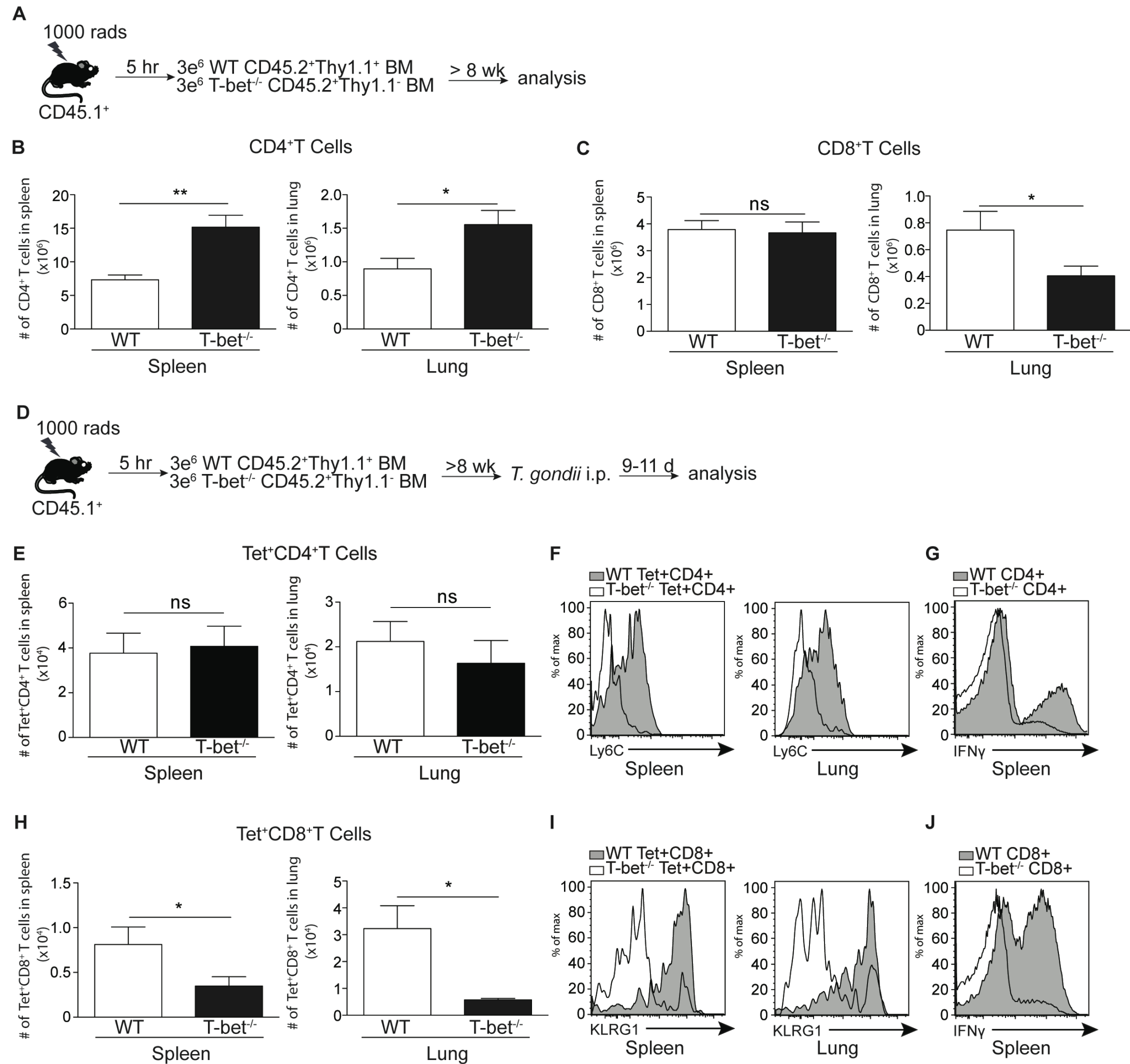

Figure 11. T-bet is intrinsically required for T cell localization at secondary sites of parasite colonization. (A-H) Bone marrow was harvested from WT and T-bet$^{-/-}$ mice, and then transferred via i.v. injection into mice irradiated (1000rads). (B,C) >8 weeks post-transfer, cells from the spleen and lung were analyzed for reconstitution in the (B) CD4^{+} T cell compartment, and the (C) CD8^{+} T cell compartment. (E-J) >8 weeks post-transfer, the mice were infected with *T. gondii* i.p. and 9-11 days post-infection cells from the spleen and lung were (E) analyzed for numbers of parasite-specific (Tet^{+}) CD4^{+} T cell

and replicates were concatenated and (F) assessed for expression of Ly6C on WT and T-$bet^{-/-}$ $Tet^{+}CD4^{+}$ T cells. (G) IFN-γ production was assessed in WT and T-$bet^{-/-}$ $CD4^{+}$ T cells. (H,I) The parasite-specific (Tet^{+}) $CD8^{+}$ T cell compartment was (H) quantified, and concatenated samples were (I) assessed for KLRG-1 expression on WT and T-$bet^{-/-}$ $Tet^{+}CD8^{+}$ T cells. (J) IFN-γ production was assessed in WT and T-$bet^{-/-}$ $CD8^{+}$ T cells. Data are representative of 4 experiments, n=4-5 per experiment.

8h), and these WT parasite-specific $CD8^+$ T cells from the spleen and lung expressed higher levels of KLRG-1 (Figure 11i) and CD11a (data not shown) than the T-bet$^{-/-}$ parasite-specific $CD8^+$ T cells. Surprisingly, there was more IFN-γ production from the WT $CD8^+$ T cells than from the T-bet$^{-/-}$ $CD8^+$ T cells (Figure 11j). These data indicate that following challenge with *T. gondii*, there is a cell-intrinsic requirement for T-bet for the activation and acquisition of effector status of the $CD4^+$ and $CD8^+$ T cell populations, and these effector populations are required for control of the parasite in peripheral tissues.

Discussion

The experiments presented here reveal that during experimental toxoplasmosis T-bet is not required for the innate NK cell dependent mechanism of resistance nor for the development and expansion of parasite-specific $CD4^+$ and $CD8^+$ T cells. Indeed, the ability of NK and $CD8^+$ T cells to produce IFN-γ appears intact. However, T-bet is required for optimal IFN-γ production by $CD4^+$ T cells. It has been reported that in response to restimulation with αCD3 and αCD28, a significant proportion of T-bet$^{-/-}$ $CD4^+$ T cells from mice infected with *T. gondii* produced IFN-γ, and a smaller subset produced IL-4 (Zhu et al., 2012). In the studies presented here, the parasite-specific T-bet$^{-/-}$ $CD4^+$ T cell restimulated with STAg also produce IFN-γ, but not IL-4, and thus appear to be Th1 cells (Mosmann and Coffman, 1987). Because the loss of $CD4^+$ T cells alone does not lead to acute susceptibility to *T. gondii* (Casciotti et al., 2002; Johnson et al., 1993), this partial defect in $CD4^+$ T cell IFN-γ production is not sufficient to explain the susceptibility of the T-bet$^{-/-}$ mice challenged with *T. gondii*. Rather, the increased

parasite numbers at many secondary sites of parasite dissemination was associated with reduced numbers of parasite-specific effector T cells. However, in the spleen, despite the presence of parasite specific effector T cells, there was still a marked increase in parasite burden. These latter results have to be interpreted with care as the parasite burden detected in the spleen also reflects the numbers of *T. gondii* in the blood, but suggest that the reduced expression of CD11a, CXCR3, Ly6C and KLRG1 may also compromise the function of these parasite-specific T cells. Thus, these data highlight that additional functions of T-bet are required for the ability of effector T cells to access and operate within local sites of infection. To the best of our knowledge, this is the first report that, in the context of infectious disease, highlights this key role for T-bet in coordinating multiple facets of the effector response and localization to peripheral sites and may help to explain the basis for the susceptibility of T-bet$^{-/-}$ mice in other infectious settings (Mayer et al., 2008; Ravindran et al., 2005; Sullivan et al., 2005; Svensson et al., 2005; Szabo et al., 2002).

A common feature of the acute phase of many intracellular pathogens is the presence of an IFN-γ dependent, NK cell mediated mechanism of resistance (Korbel et al., 2004; Lieberman and Hunter, 2002; Lodoen and Lanier, 2006). Because T-bet controls key checkpoints in NK maturation (Gordon et al., 2012) and has been linked to the ability of these cells to produce IFN-γ (Svensson et al., 2005; Szabo et al., 2002), it seemed likely that the NK response during toxoplasmosis would be T-bet dependent. However, others have reported that T-bet is not required for the production of IFN-γ from these cells

(Gordon et al., 2012; Townsend et al., 2004), and following challenge of the T-bet$^{-/-}$ mice with *T. gondii*, NK cell IFN-γ production appeared normal. Similarly, when T-bet$^{-/-}$ mice are infected with *L. monocytogenes*, the early NK cell response is intact and is associated with acute resistance to the bacteria (Way and Wilson, 2004), but the long-term consequences of T-bet deficiency on the T cell responses to this bacterium remain unclear. Similarly, T-bet$^{-/-}$ mice survive for more than 2 weeks after challenge with *Salmonella* (Ravindran et al., 2005), indicating that early IFN-γ production in this model is intact. These results from multiple experimental systems are consistent with a model in which high levels of inflammation associated with many parasitic and bacterial infections provide sufficient signals to overcome the requirement for T-bet in NK cell maturation.

Previous *in vitro* studies have identified a role for T-bet in T cell chemotaxis through its induction of CXCR3 (Lord et al., 2005), while more recent work has implicated T-bet in trafficking of Tregs *in vivo* (Koch et al., 2009). The findings presented here that T-bet is required *in vivo* for the ability of parasite-specific T cells to express CXCR3 and the integrin CD11a (a component of LFA-1) reinforce this notion. CXCR3 and/or its ligands have a key role in the trafficking of effector T cells necessary to control of number of pathogens including *T. gondii*, Respiratory Syncytial Virus, and influenza (Cohen et al., 2013; Fadel et al., 2008; Khan et al., 2000; Lindell et al., 2008). Thus, the reduced number of effector T cells at peripheral sites of infection in the T-bet$^{-/-}$ mice could be a result of defective T cell trafficking to these sites. However, this alteration in trafficking is not always detrimental; during toxoplasmosis, immune-mediated pathology in the liver

is caused by $CD4^+$ effector T cells (Casciotti et al., 2002; Villegas et al., 2000; Wille et al., 2002), and in our studies T-bet$^{-/-}$ mice have reduced levels of liver pathology. Likewise, the loss of T-bet or CXCR3 provides protection against experimental cerebral malaria, associated with a reduced number of T cells in the brains of infected mice (Campanella et al., 2008; Oakley et al., 2013), and T-bet-deficiency confers protection in a murine model of Type 1 diabetes that is associated with reduced islet infiltration by T cells (Juedes et al., 2004). These findings from diverse infectious and autoimmune models highlight that the ability to target T-bet (Peng, 2006) may also influence T cell trafficking and limit inflammation. Interestingly, T-bet was not restricted to the nucleus in highly activated cells. Although the function of T-bet as a cytoplasmic protein remains unknown, differential T-bet localization has been linked to effector status (McLane et al., 2013), activation (Neurath et al., 2002), cell cycle (Chang et al., 2011), and protein stability (Jang et al., 2013). The biological significance of the cytoplasmic localization of T-bet, the mechanisms that underlie this partitioning, and whether localization influences T cell phenotype and/or migration remain unclear but may provide opportunities to target different functions of this transcription factor.

Although the studies presented here highlight the impact of T-bet on expression of CD11a and CXCR3 and their links to T cell trafficking, these molecules are involved in many facets of T cell activation, which may contribute to the defect in effector populations. For example, CD11a is upregulated on T cells after TCR signaling and its expression has been used as a marker of activation for infection-induced polyclonal T cell

populations (McDermott and Varga, 2011; Rai et al., 2009; Srinivasan et al., 2004). Consequently, the reduced levels of CD11a expression on T cells from infected T-bet$^{-/-}$ mice initially suggested that T-bet was required for their ability to generate parasite-specific populations. However, the use of tetramers demonstrated that the generation of parasite-specific CD4^{+} and CD8^{+} T cells was intact in the T-bet$^{-/-}$ mice and implied that upregulation of this integrin was, in part, dependent on T-bet. Thus, because CD11a is important during priming for the generation of antigen-specific effector CD8^{+} T cells (Bose et al., 2013; Gerard et al., 2013), upregulation of CD11a is a potential mechanism by which T-bet influences the phenotype of the effector population. Recent studies have also shown that CXCR3 is important for T cell differentiation and behavior (Groom et al., 2012; Harris et al., 2012), therefore, the combination of decreased CD11a and CXCR3 expression on T-bet$^{-/-}$ T cells could impact the differentiation and function of these populations. Indeed, the T-bet$^{-/-}$ T cells from infected mice display altered effector phenotypes, with a decrease in the Ly6C^{hi} CD4^{+} and KLRG-1^{hi} CD8^{+} effector T cells. While there was a decrease in the numbers of KLRG-1^{hi} effector CD8^{+} T cells in the T-bet$^{-/-}$ mice, it should be noted that the KLRG-1^{lo} CD8^{+} memory precursor T cell population was intact in the absence of T-bet. Similarly, during LCMV infection, memory-precursor CD8^{+} T cells display lower T-bet expression (Intlekofer et al., 2007; Joshi et al., 2007), and T-bet deficiency results in enhanced generation of memory CD8^{+} T cells (Intlekofer et al., 2007), but the ability of this T-bet$^{-/-}$ population to protect against secondary challenge is unclear (Intlekofer et al., 2007; Sullivan et al., 2003). Thus, while the studies described here help to provide a better understanding of the role

of T-bet in resistance to *T. gondii*, they also highlight the need to investigate the role of T-bet in T cell priming and the generation protective memory responses.

Materials and Methods

Mice, infection, and antibody treatment

T-bet deficient (T-bet$^{-/-}$), CBA/CaJ, Thy1.1^{+}C57BL/6 and Swiss Webster mice were purchased from Jackson Laboratory. Wild-type C57BL/6 (WT) mice were purchased from Taconic. CD45.1^{+}C57BL/6 mice were purchased from NCI. All mice were housed in a specific-pathogen free environment at the University of Pennsylvania School of Veterinary Medicine in accordance with federal guidelines and with approval of the Institutional Animal Care and Use Committee. The ME49 strain of *T. gondii* was maintained in Swiss Webster and CBA/CaJ mice and used as a source of tissue cysts for i.p. (10-20 cysts) infections. Soluble *Toxoplasma* Ag (STAg) was prepared from the RH strain of *T. gondii* as previously described (Hauser et al., 1983). For IFN-γ neutralization experiments, mice were treated with 1mg αIFN-γ or isotype control on days 0, 3, and 6 of infection. For depletion of NK cells, mice were treated with 50ul αAsialo gm-1 or isotype control on days -1, 3, and 6 of infection. For bone marrow chimeras, CD45.1^{+} congenic mice were irradiated (1000rads) and i.v. injected with a 1:1 mixture of 6x10^{6} cells isolated from WT (CD45.2^{+}Thy1.1^{+}) and T-bet$^{-/-}$ (CD45.2^{+}Thy1.1^{-}) bone marrow. Mice were given water containing sulfamethoxazol for the first 2 weeks post irradiation. Mice were allowed to reconstitute >8 weeks following irradiation, and were infected with *T. gondii* as described.

Isolation and Analysis of Immune Populations

Single-cell suspensions from the spleens, lymph nodes (LNs), and peritoneal exudate cells (PECs) were prepared as previously described (Wille et al., 2004). Lungs were inflated with a solution of 1mg/ml Collagenase A (Roche) and 100ug/ml DNase (Roche) and then diced and digested in the same solution for 60 minutes at 37°C to obtain a single cell suspension. The resulting cells were then passed through a 70μm filter and used for FACS analysis. Cells were stained for surface markers in FACS buffer (0.5% BSA, 2mM EDTA in PBS), fixed with Foxp3 Fixation buffer (eBioscience), and stained for intracellular markers in Foxp3 Permeabilization buffer (eBioscience). To measure intracellular cytokine production, isolated cells were cultured in cRPMI (1% penicillin/streptomycin, 2 mM L-glutamine, 10% fetal bovine serum, 0.1% beta-mercaptoethanol, 1% nonessential amino acids, and 1 mM sodium pyruvate) in triplicate at 1×10^6 cells/mL in a 96-well U-bottom plate, with PMA and ionomycin for 4 hr with Brefeldin A (Sigma) and monensin (BD). Cells were rinsed, stained for surface markers at 4°C, and fixed with 4% PFA in PBS for 11 min at room temperature. Intracellular cytokines were detected by staining in FACs buffer containing 0.5% saponin (Sigma). Splenocytes were also restimulated at 1×10^5 cells/mL in cRPMI alone (media) or with either αCD3 (1ug/mL) or soluble *T. gondii* antigen (STAg, 12.5ug/mL) and incubated at 37°C for 72 hours. Sera were assayed for ALT, and supernatants and sera were assayed for IL-12p40 and IFN-γ by ELISA.

Flow Cytometry and Imaging

The following antibodies were purchased from BD: CD3 FITC, Ki67 FITC, CD49b (DX5) PE, Ki67 AF700, CD4 Pacific Blue, PSGL-1 BV421, CD8α PE-CF594, CD3 PE-CF594, CD122 Biotin, and Streptavidin PE-TexasRed. The following antibodies were purchased from eBioscience: CD25 FITC, Foxp3 AF488, CD11a FITC, NK1.1 FITC, CD19 FITC, CD4 FITC, KLRG-1 FITC, CD11a PE, Ly6C (clone HK1.4) PerCP-Cy5.5, CD69 PerCP-Cy5.5, NKp46 eFluor660, T-bet eFluor660, CD11c PE-Cy7, KLRG-1 PE-Cy7, IFN-γ PE-Cy7, CD25 APC-eFluor780, CD3 APC-eFluor780, Ly6C (clone HK1.4) APC-eFluor780, Thy1.1 APC-eFluor780, Foxp3 AF700, CD45.2 eFluor450, Ly6C (clone HK1.4) eFluor450, and CD4 eFluor650NC. The following antibodies were purchased from BioLegend: CD8 APC-Cy7, MHCII Pacific Blue, CD11a PerCP-Cy5.5, CXCR3 PE-Cy7, TCRβ AF700, CD3 Pacific Blue, and NK1.1 Pacific Blue. Invitrogen live/dead Aqua stain was used to determine viability. Biotinylated Tgd-057 MHC-I monomers were kindly provided by E. John Wherry (University of Pennsylvania) and tetramerized by incubation with streptavidin-conjugated PE. PE-conjugated AS-15 MHC-II tetramers were kindly provided by Marion Pepper (University of Washington). All samples were run on an LSRFortessa (BD) and analyzed using FlowJo software (Tree Star). Analysis and presentation of distributions was performed using SPICE version 5.35, downloaded from http://exon.niaid.nih.gov/spice/ (Roederer et al., 2011). Images were obtained using the ImageStream (Amnis) and analyzed using IDEAS software (Amnis). To determine T-bet localization, nuclear and cytoplasmic masking functions were made using DAPI staining; these masks were then applied to T-bet expression.

Parasite Detection

For IHC detection of *T. gondii*, tissues were fixed in 10% formalin solution and then paraffin- embedded and sectioned. Sections were deparaffinized, rehydrated, antigen retrieved in 0.01M Sodium Citrate Buffer (PH 6.0) and endogenous peroxidase blocked by 0.3% H2O2 in PBS. After blocking with 2% normal goat serum, the sections were incubated either with anti-*Toxoplasma* antibody or isotype control. The sections were then incubated with Biotinylated goat anti-rabbit IgG (Vector, Burlington, CA), and ABC reagent was applied (Vectastain ABC Kit,Vector Labs). Then DAB substrate (Vector Labs) was used to visualize specific staining according to manufacturer's instructions and slides were counterstained with hematoxylin. For fluorescence microscopy, brains were frozen in OCT, 6 μm sections prepared and stained with antibodies against CD4 and CD8 in Cy3 and against *T. gondii* in Alexa488 as well as DAPI for nuclear staining. To quantify parasite burden in the peritoneal exudate, 100,000 cells were used to prepare cytospins. Cells were methanol fixed and then stained with the Protocol Hema-3 Stain Set, and the ratio of infected cells to total cells in a field of vision was calculated. All images were obtained on a Nikon E600 microscope using a 20X (brain) or 40X (liver, lung, heart, thymus, PECs) objective and NIS Elements Imaging software. For quantitative PCR, DNA was isolated from tissues using the High Pure PCR Template Preparation Kit (Roche) followed by qPCR measuring the abundance of the *T. gondii* gene B1 using the primers 5′-TCTTTAAAGCGTTCGTGGTC-3′ (forward) and 5′-GGAACTGCATCCGTTCATGAG-3′ (reverse).

Statistics

Bar graphs and scatter plots were plotted as means with the standard error of the mean in Prism 5 software (GraphPad). All statistics were performed using an unpaired Student's *t* test, except the bone marrow chimera experiments in which the statistics were performed using a paired Student's *t* test in Prism 5 software.

CHAPTER 3: THE TRANSCRIPTION FACTOR T-BET IS REQUIRED FOR OPTIMAL CD8$^+$ T CELL PRIMING FOLLOWING CPS VACCINATION

Abstract:

The T-box transcription factor T-bet is perhaps most prominently known as a "master regulator" of Th1 differentiation and IFNγ production. However, there are T-bet independent pathways to IFNγ production, and T-bet has been shown to be involved in other facets of T cell function associated with Th1 responses. Here, we show that following vaccination with a replication-deficient strain of *T. gondii*, T-bet is intrinsically required for early CD8$^+$ T cell activation and proliferation for optimal expansion of parasite-specific effector CD8$^+$ T cells. T-bet coordinates these early events of T cell activation by promoting CD8$^+$ T cell production of the chemokine CCL3 and upregulation of the integrin CD11a, both of which influence the early cellular interactions involved in T cell priming.

Introduction

Understanding the transcriptional regulation of polarized T cell responses and their link to disease outcome has been a major theme in immunology. The identification of T-bet, GATA3 and RoRγt as lineage-specifying transcription factors of the development of Th1, Th2 and Th17 type responses have provided key milestones in understanding T cell function and plasticity (Ivanov et al., 2006; Szabo et al., 2000; Zheng and Flavell, 1997). However, there are still many gaps in our knowledge of how these factors impact on their canonical cellular targets (Oestreich and Weinmann, 2012). The transcription factor T-bet has been shown to be essential for protective effector T cell responses to many intracellular pathogens as well as having a pathological role in other diseases (Lazarevic and Glimcher, 2011), which has primarily been linked to the ability of T-bet to promote T cell production of IFNγ. However, there are T-bet independent pathways to IFNγ, mediated in part through the expression of a related T-box transcription factor, Eomesodermin (Pearce et al., 2003). In addition, T-bet has been linked to other effector T cell activities including T cell trafficking (Koch et al., 2009; Lord et al., 2005; Oakley et al., 2013) that highlight a broader role for T-bet in the function of Th1 type responses.

Naïve T cells do not express T-bet, but, as shown in the initial study that identified T-bet, signals through the T cell receptor (TCR) induce expression of this transcription factor (Szabo et al., 2000). Subsequent work has shown that signals from cytokines, such as type I IFNs, IFNγ, IL-12, IL-21, and IL-27, serve to reinforce T-bet expression (Afkarian et al., 2002; Hall et al., 2012; Schulz et al., 2009; Sutherland et al., 2013; Takeda et al., 2003; Wiesel et al., 2012). Many of the studies to understand the function of T-bet *in vivo*

have focused on its role during the effector phase of the immune response (Oakley et al., 2013; Ravindran et al., 2005; Sullivan et al., 2005; Szabo et al., 2002; Way and Wilson, 2004). However, in at least one model, TCR activation leads to an early transient wave of T-bet expression, and a second sustained phase of T-bet expression is influenced by cytokine responsiveness (Schulz et al., 2009). It is unclear if the initial T-bet expression has a role in the co-ordination of T cell activation that is distinct from its function during the effector phase.

Models of infection have been useful in expanding our understanding of the role of T-bet in coordinating the development of Th1 responses (Intlekofer et al., 2007; Oakley et al., 2013; Ravindran et al., 2005; Sullivan et al., 2005; Szabo et al., 2002; Way and Wilson, 2004). However, T-bet has also been shown to impact T cell trafficking (Koch et al., 2009; Lord et al., 2005). Furthermore, our previous studies (refer to Chapter 2) showed that while T-bet is not required for the generation T cell responses to *T. gondii*, its expression is necessary for T cell trafficking to secondary sites of infection. In those studies, T-bet$^{-/-}$ mice are unable to control parasite replication, and consequently, have higher levels of parasite burden than WT controls. Elevated antigen load present in the T-bet$^{-/-}$ mice in the context of a systemic inflammatory response confounds the ability to dissect the role of T-bet in the generation of T cell responses (refer to Chapter 2). Similarly, many of the studies from other laboratories examining the role of T-bet *in vivo* are also complicated by increased pathogen burden (Ravindran et al., 2005; Svensson et al., 2005). Therefore, in an attempt to better understand the basis of the requirement of T-

bet for T cell mediated resistance to *T. gondii*, additional studies were performed using a replication-deficient strain of this parasite, CPS, in order to control for antigen load. Previous studies have shown that CPS induces robust parasite-specific $CD4^+$ and $CD8^+$ T cell responses (Dupont et al., 2014; Jordan et al., 2009; Wilson et al., 2010), and this model has been used by multiple laboratories to understand many aspects of parasite and host biology, including T cell priming and the regulation of effector and memory T cell responses (Dupont et al., 2014; Wilson et al., 2010). The data presented here provide evidence that T-bet has an unanticipated cell-intrinsic essential function in $CD8^+$ T cell priming and the generation and expansion of effector T cells. Mechanistically, this may be explained by the role of T-bet in $CD8^+$ T cell upregulation of the integrin CD11a and production of the chemokine CCL3, both of which play key roles in the differentiation and expansion of activated T cells (Castellino et al., 2006; Gerard et al., 2013). Thus, early T-bet has a critical function in coordinating the generation and optimal expansion of $CD8^+$ T cell effector populations.

Results

T-bet is required for effector T cell expansion and phenotype after immunization

To determine the role of T-bet in the development of T cell responses in a low inflammatory environment with a defined antigen load, WT and T-bet$^{-/-}$ mice were injected intraperitoneally with the CPS parasite and the parasite-specific T cell responses were assessed using Class I and Class II tetramers specific for *T. gondii* antigens at day 10 post-immunization. In the WT mice, immunization induced the expansion of parasite-

specific $CD4^+$ T cells, and this population was not diminished in the absence of T-bet (Figure 12a). However, the phenotype of the parasite specific $CD4^+$ T cells was altered in T-bet deficient cells, specifically levels of Ly6C, which is frequently used to identify effector $CD4^+$ T cells (Marshall et al., 2011), and LFA1 were both reduced in the $CD4^+$ T cells from the T-bet$^{-/-}$ mice (Figure 12b). Similarly, in the WT mice, immunization induced a robust expansion of parasite specific $CD8^+$ T cells, however this was markedly reduced in the T-bet$^{-/-}$ mice (Figure 13a). Further phenotyping of the parasite-specific $CD8^+$ T cells from WT and T-bet$^{-/-}$ mice revealed that WT parasite-specific $CD8^+$ T cells expressed high levels of KLRG1, which is often used to identify effector $CD8^+$ T cells (Joshi et al., 2007; Wilson et al., 2008), and LFA1, and the expression of these surface proteins was reduced in the absence of T-bet (Figure 13b). However, the number of parasite-specific $CD8^+$ T cells that did not express KLRG-1 was increased in the absence of T-bet (Figure 13b).

To understand whether T-bet was intrinsically required in the $CD8^+$ T cells for their optimal expansion, WT and T-bet$^{-/-}$ OT-I T cells were adoptively transferred into WT congenic mice that were then immunized with an Ova-expressing strain of CPS (CPS-Ova). In this system, both the WT and T-bet$^{-/-}$ OT-I T cells were expanded in an environment in which the $CD4^+$ helper T cells were T-bet sufficient, but there was a reduced frequency of T-bet$^{-/-}$ OT-I T cells at day 10 post-immunization (Figure 13c). Furthermore, T-bet$^{-/-}$ OT-I T cells had lower expression of KLRG-1 and LFA1 than WT OT-I T cells (Figure 13d). Together, these studies indicate that in response to CPS, T-bet

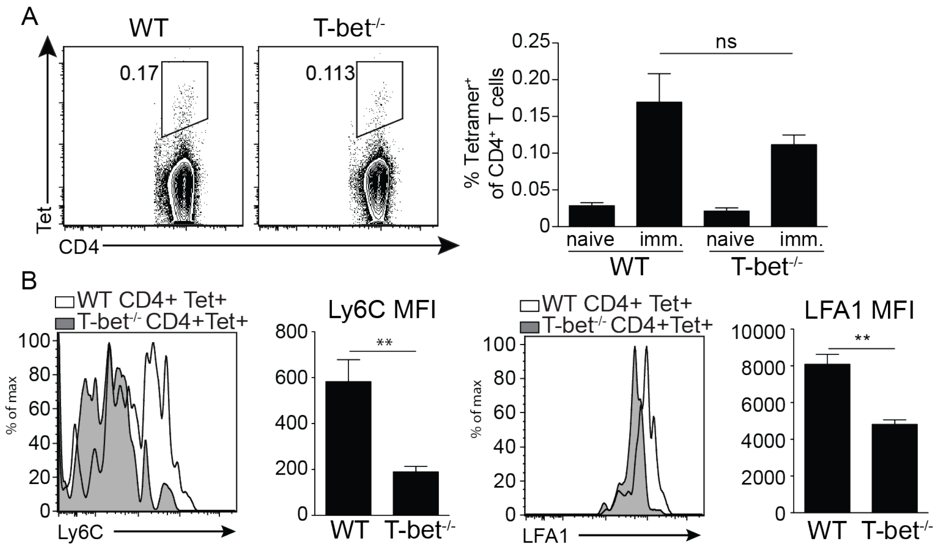

Figure 12. T-bet is required for the phenotype of effector CD4$^+$ T cells following CPS Vaccination. WT and T-bet$^{-/-}$ mice were immunized intraperitoneally (i.p.) with the CPS strain of *T. gondii* and the responses were assessed in the spleen 9-11 days later. (A) Splenocytes were isolated and stained with MHC-II tetramers, CD3, CD4 and a dead cell exclusion dye to detect live parasite specific CD4$^+$ T cells. (B,C) Tetramer$^+$ CD4$^+$ T cells were assessed for expression of (B) Ly6C and (C) LFA-1 (CD11a/CD18). ** $p<0.01$

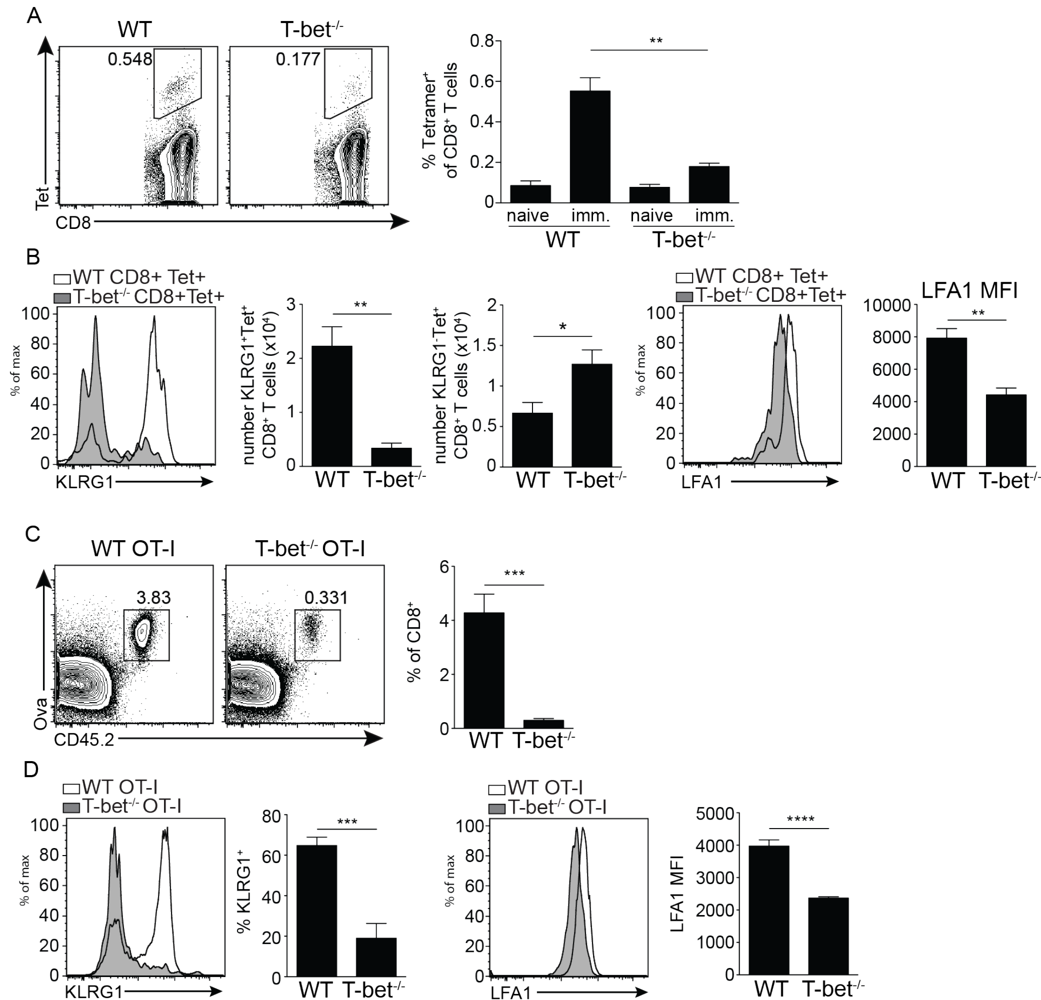

Figure 13. T-bet is required for the expansion and phenotype of effector CD8$^+$ T cells following CPS Vaccination. (A,B) WT and T-bet$^{-/-}$ mice were immunized i.p. with the CPS strain of *T. gondii* and the responses were assessed in the spleen 9-11 days later. (A) Splenocytes were isolated and stained with MHC-I tetramers, CD3, CD8 and a dead cell exclusion dye to detect live parasite specific CD8$^+$ T cells. (B) Tetramer$^+$ CD8$^+$ T cells were assessed for expression of KLRG-1 and LFA-1 (CD11a/CD18). (C,D) WT or T-bet$^{-/-}$ OT-I T cells were transferred into T-bet sufficient congenic hosts and immunized i.p. with CPS-Ova. (C) Splenocytes were assessed for frequency of OT-I T cells,

identified by expression of CD45.2 and binding to MHC-I tetramers loaded with SIINFEKL, at day 9 post-immunization. (D) OT-I T cells were assessed for expression of KLRG-1 and LFA-1. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

is intrinsically required for the expansion and phenotype of parasite-specific $CD8^+$ T cells.

T-bet is required for protection against lethal rechallenge

Although the effector $CD8^+$ T cells appeared deficient in the T-bet$^{-/-}$ mice, the memory precursor population was intact and perhaps even expanded compared to WT mice. Indeed, 30 days post-immunization, there were equal numbers of parasite specific $CD4^+$ and $CD8^+$ T cells between WT and T-bet$^{-/-}$ mice (Figure 14a). Therefore, it was unclear if T-bet$^{-/-}$ mice would be protected from rechallenge with a lethal strain of *T. gondii* (RH). To assess the functionality of these cells, WT and T-bet$^{-/-}$ mice were immunized with CPS and rechallenged 30 days later with RH. Consistent with previous reports, WT mice were largely protected from this lethal strain and were able to control parasite replication, as assessed by frequency of infected cells in the peritoneum 5 days post-challenge (Figure 14b). However, in two experiments, T-bet$^{-/-}$ mice were not able to control parasite replication and displayed a significant increase in the frequency of infected cells in the peritoneum (Figure 14b). These data indicate that while T-bet is not required for the presence of parasite-specific $CD8^+$ T cells with a memory-precursor phenotype, T-bet is necessary for memory cell function.

Analysis of CPS-induced T cell priming

To better understand at what stage of the T cell response to CPS that T-bet is required, WT mice were immunized in the peritoneum with CPS and the T cell responses were

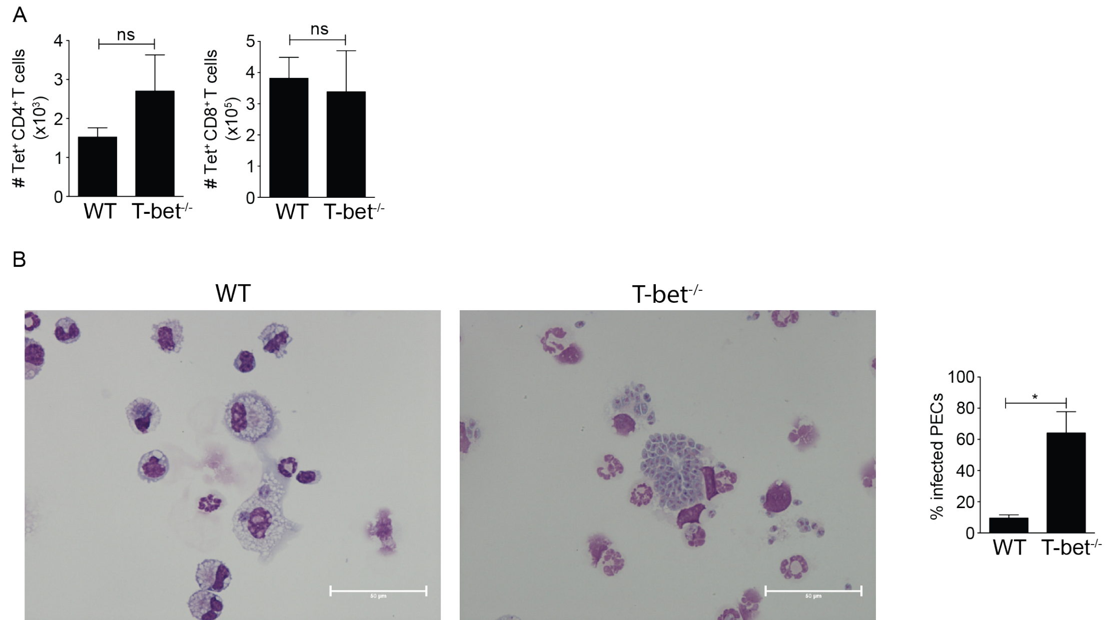

Figure 14. T-bet is required for protective immunity against lethal rechallenge. WT and T-bet$^{-/-}$ mice were immunized i.p. with CPS. (A) Thirty days post-immunization, the numbers Tetramer^{+} CD4^{+} and CD8^{+} T cells were assessed in the spleen. (B) Thirty days post-immunization, WT and T-bet$^{-/-}$ mice were rechallenged with a lethal strain of *T. gondii*, RH. Parasite burdens were assessed in the peritoneal exudate cells (PECs) 5 days following rechallenge. *$p<0.05$

assessed in the omentum, a fatty tissue that connects the stomach, spleen, colon, and pancreas. The omentum is considered the site of T cell priming after peritoneal injection of antigens and/or pathogens, and can function as a secondary lymphoid organ that supports the generation of B and T cell responses in the absence of lymphoid tissue-inducer cells (Carlow et al., 2009; Rangel-Moreno et al., 2009). CD69 is rapidly upregulated after T cell engagement (Testi et al., 1989) and is frequently used to identify cells that have recently been activated. In addition to upregulation of CD69 by TCR activation, type I IFNs are also potent inducers of CD69 (Feng et al., 2005; Shiow et al., 2006). Across multiple experiments, approximately 10-20% of $CD4^+$ T cells and 5-10% of $CD8^+$ T cells from naïve mice displayed basal levels of CD69 expression (Figures 15a and 15b). However, by 48 hours post-immunization, two distinct populations of activated ($CD69^+Ki67^+$ and $CD69^+Ki67^-$) $CD4^+$ and $CD8^+$ T cells could be detected and T-bet expression was assessed in these populations. Compared to the $CD69^-Ki67^-$ population, T-bet was upregulated in each of the activated populations, and was most highly expressed in the cells that were $CD69^+Ki67^+$ (Figures 15a and 15b). Because T-bet localization in the nucleus and cytoplasm can vary with activation state (Chang et al., 2011; McLane et al., 2013), $CD8^+$ T cells isolated from the omenta of mice two days after CPS immunization were analyzed using the Amnis ImageStream. Interestingly, T-bet was localized to both the nucleus and cytoplasm in each of the T-bet expressing populations, however, the $CD69^+Ki67^+$ $CD8^+$ T cell population had the highest frequency of cells with exclusively nuclear T-bet (Figure 15c). This observation suggests that T-bet translocation to the nucleus correlates with entry into cell cycle.

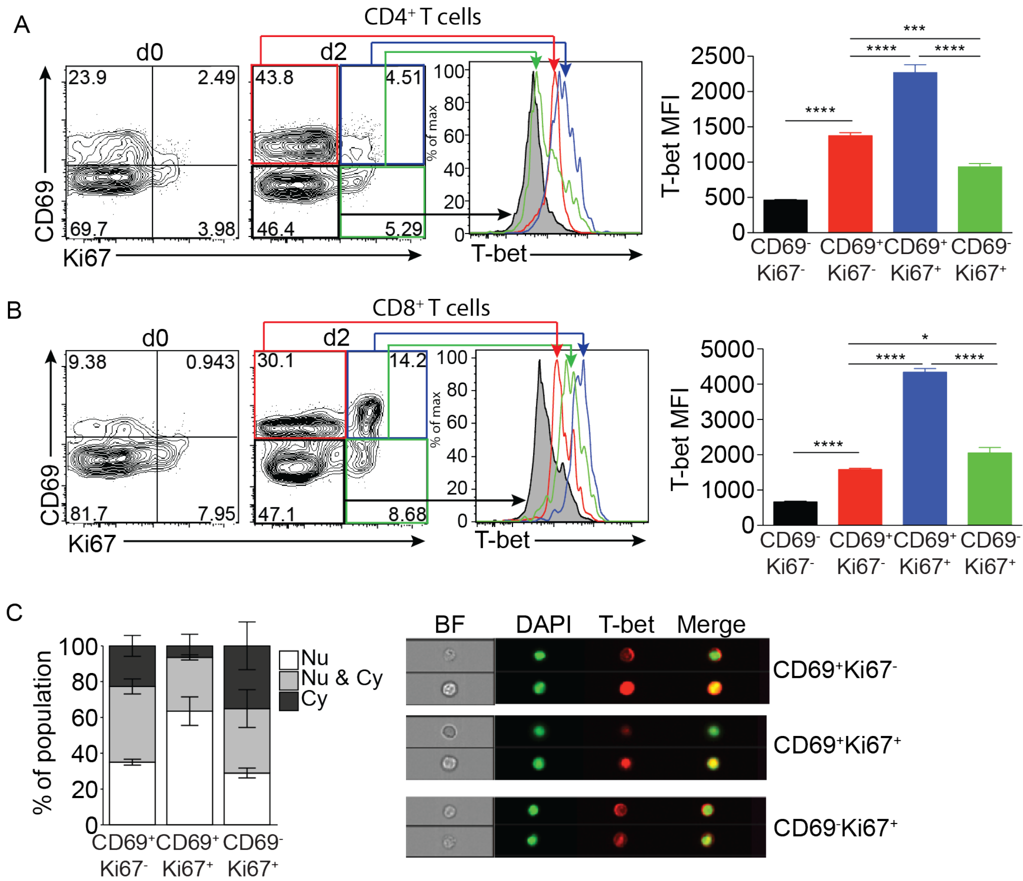

Figure 15. T-bet is expressed early after T cell activation. WT mice were immunized i.p. with CPS and 2 days later the T cell responses were assessed in the omenta. (A) CD4$^+$ T cells from naïve and day 2 immunized mice were analyzed for CD69 and Ki67 expression, and T-bet was assessed within these populations. (B) CD8$^+$ T cells from naïve and day 2 immunized mice were analyzed for CD69 and Ki67 expression, and T-bet (B) expression and (C) localization was assessed within these populations. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$

Kinetics of CD8$^+$ T cell activation

To distinguish between antigen-induced and bystander activation of the CD8$^+$ T cells, purified CD69$^-$ OT-I T cells (which recognize Ova) were adoptively transferred into congenic hosts that were then immunized with CPS that did not express Ova or CPS-Ova. CPS administration led to a modest increase in CD69 expression, but most of the OT-I T cells remained CD69$^-$. CPS-Ova, however, induced a robust upregulation of CD69 (Figure 16a). These data indicate that while there is some antigen-independent CD69 expression on the OT-I T cells, it is largely upregulated in response to TCR stimulation. To better understand the kinetics of CD69 and Ki67 upregulation and their association with proliferation, OT-I T cells were labeled with CFSE and adoptively transferred into congenic hosts that were subsequently immunized with CPS-Ova. At day 2 post-immunization, the OT-I T cells from the omentum were isolated and phenotyped. CD69$^+$Ki67$^-$ cells did not display any dilution of CFSE (Figure 16b), similar to OT-I T cells from mice that were not given CPS-Ova (data not shown), indicating that they had not undergone cell division. However, the CD69$^+$Ki67$^+$ and CD69$^-$Ki67$^+$ populations did display diluted CFSE, indicating that these cells had undergone cell division (Figure 16b). Furthermore, the CD69$^-$Ki67$^+$ population had diluted CFSE more than the CD69$^+$Ki67$^+$ population (Figure 16b), suggesting that CD69 is downregulated as activated cells continue to divide. Together, these data are consistent with the idea that CD69 is upregulated proximal to TCR activation and show that the use of CPS immunization provides a tractable experimental system to examine the role of early T-bet expression on T cell priming and expansion.

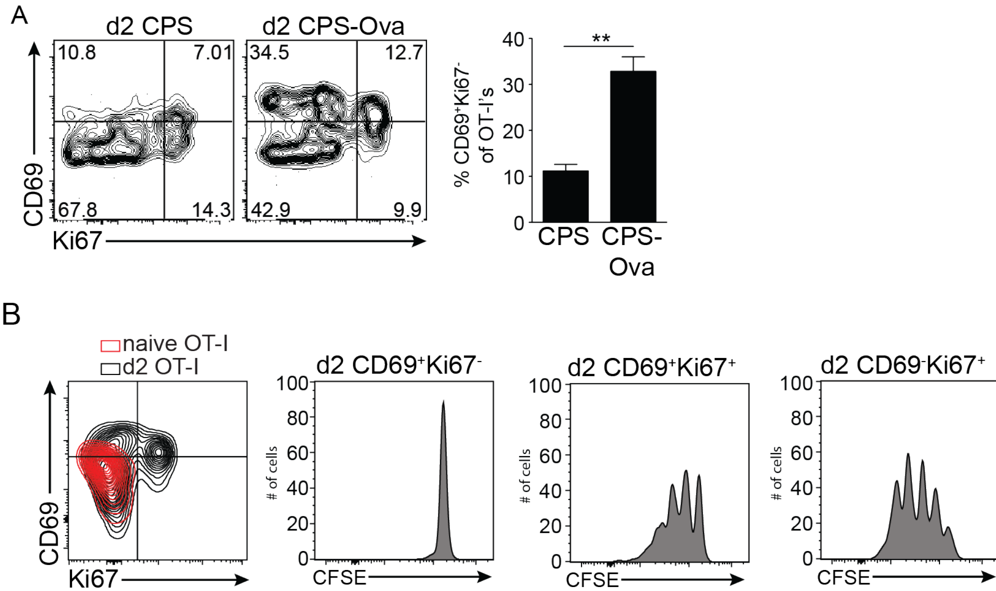

Figure 16. Induction and kinetics of CD69 expression following CPS challenge.* (A) $CD69^-$ WT OT-I T cells were purified and transferred into congenic hosts which were subsequently immunized with CPS-Ova or CPS. Two days later, expression of CD69 and Ki67 was assessed on the OT-I T cells. (B) WT OT-I T cells were purified, labeled with CFSE, and transferred into congenic hosts which were subsequently immunized with CPS-Ova. Two days later, expression of CD69 and Ki67 and dilution of CFSE was assessed. **$p<0.01$

* John Johnson helped prepare this figure.

T-bet dependent effector T cell expansion

To understand if early T-bet expression impacted T cell activation, WT and T-bet$^{-/-}$ mice were immunized with CPS and the CD4^{+} and CD8^{+} T cell responses in the omenta were analyzed 1-3 days later. Both WT and T-bet KO CD4^{+} upregulated CD69 expression by 1 day after immunization, and absence of T-bet did not result in a significant difference of CD69 expression on these cells (Figure 17). However, by day 2, CD69 was decreased in the T-bet KO CD4^{+} T cells compared to WT CD4^{+} T cells, and this difference was maintained at day 3. Furthermore, fewer T-bet KO CD4^{+} T cells upregulated Ki67 beginning at day 2, indicating differences in cell cycle entry (Figure 17).

Similar to the CD4^{+} T cells, both WT and T-bet KO CD8^{+} T cells upregulated CD69 by 1 day post-immunization (Figure 18a). However, at 2 days post-immunization, WT CD8^{+} T cells continued to upregulate CD69 whereas T-bet KO CD8^{+} T cells had reduced CD69 compared to day 1. Additionally, while WT CD8^{+} T cell upregulation of Ki67 was apparent beginning at day 2 post-immunization, and was sustained at day 3, this was markedly reduced in the T-bet$^{-/-}$ mice on day 2 and day 3 (Figure 18a). These results indicate that initial T cell activation is T-bet independent, but T-bet is necessary for the maintenance of the activated phenotype and for entry into cell cycle, and that this phenotype is most apparent in the CD8^{+} T cell population.

Because the effect of T-bet deficiency on the initial T cell activation and long term expansion is most striking in the CD8^{+} T cells, we focused on role of T-bet in the CD8^{+} T

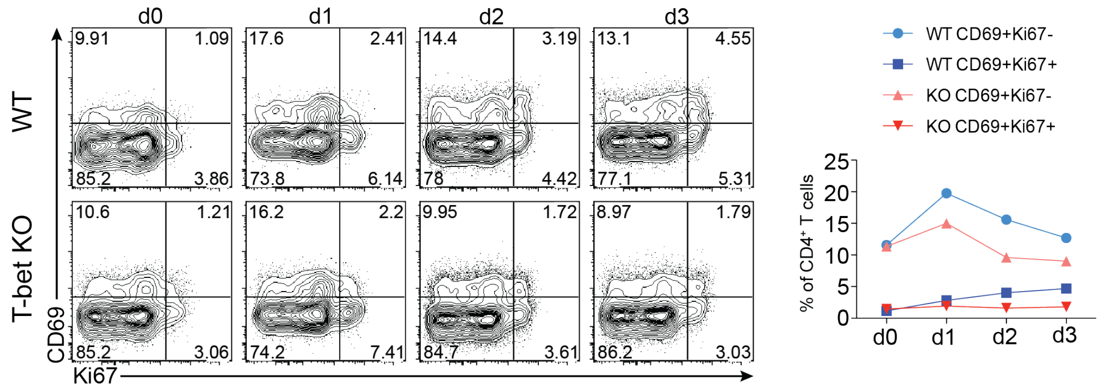

Figure 17. Early activation of CD4$^+$ T cells is largely T-bet independent. WT and T-bet$^{-/-}$ mice were immunized i.p. with CPS and the CD4$^+$ T cell responses were assessed in the omenta 1-3 days later.

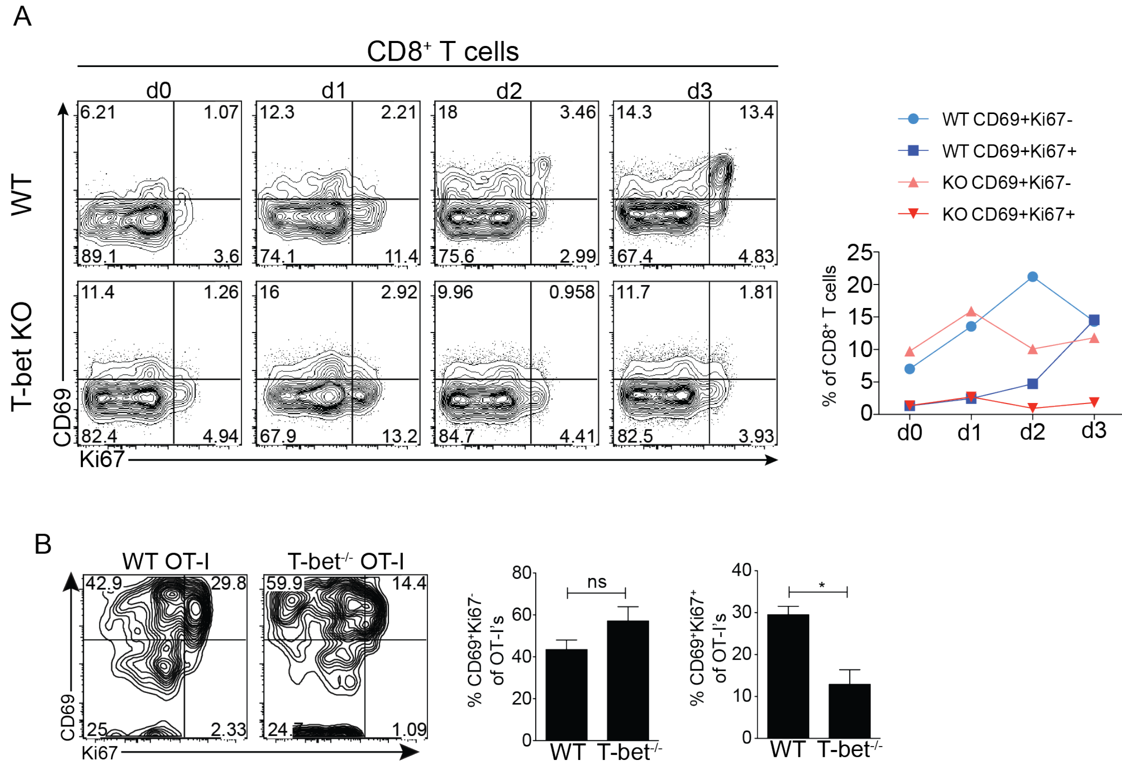

Figure 18. Early activation of CD8^{+} T cells is largely T-bet dependent. (A) WT and T-bet$^{-/-}$ mice were immunized i.p. with CPS and the CD8^{+} T cell responses were assessed in the omenta 1-3 days later. (B) WT and T-bet$^{-/-}$ OT-I T cells were transferred into T-bet sufficient congenic hosts which were subsequently immunized i.p. with CPS-Ova. Two days later, CD69 and Ki67 expression was assessed on the OT-I T cells isolated from the omenta. *p<0.05

cell response to CPS. While the studies shown in Figure 13 demonstrate that T-bet is required in a cell-intrinsic fashion for the expansion of parasite-specific $CD8^+$ T cells at day 10, similar studies were performed to determine if T-bet is intrinsically required for initial $CD8^+$ T cell activation and proliferation at day 2. Thus, WT and T-$bet^{-/-}$ OT-I T cells were adoptively transferred into congenic T-bet sufficient hosts, which were then immunized with CPS-Ova. At day 2 post-immunization, cells were isolated from the omenta and the OT-I T cells were phenotypically assessed. Both WT and T-$bet^{-/-}$ OT-I T cells displayed high levels of CD69 expression (Figure 18b). Between the WT and T-$bet^{-/-}$ OT-I T cells, the frequency of cells expressing CD69 but not Ki67 was the same (Figure 18b), which is consistent with the idea that T-bet is not required for initial T cell activation and upregulation of CD69. However, in the absence of T-bet, there was a reduction in the frequency of $CD69^+Ki67^+$ OT-I T cells (Figure 13c). These results demonstrate that in the $CD8^+$ T cell compartment, the absence of T-bet results in reduced entry into cell cycle during priming. There are several possible explanations for this phenomenon and the following sections will address the possible roles of CD11a and CCL3 that may help explain how T-bet impacts early T cell activation.

T-bet is required for optimal CD11a upregulation

The integrin CD11a, which together with CD18 comprises LFA-1, has a key role in T cell biology and the co-ordination of T cell interactions during T cell priming (Gerard et al., 2013). While CD11a is constitutively expressed on WT and T-$bet^{-/-}$ $CD8^+$ T cells, T-bet appears to be required to promote the upregulation of the integrin CD11a on activated

$CD8^+$ T cells (refer to Chapter 2). Furthermore, after *in vitro* activation, T-bet$^{-/-}$ T cells display lower adherence to ICAM than WT T cells (Figure 19a). To determine whether CD11a was important for the early activated phenotype of $CD8^+$ T cells, WT mice were treated with either an isotype control or a CD11a blocking antibody and then immunized with CPS. At day 2 post-immunization, the frequency of $CD69^+Ki67^-$ $CD8^+$ T cells was equal between the control and the treatment groups (Figure 19b). However, blockade of CD11a resulted in a significant reduction in the frequency of cells that expressed both CD69 and Ki67 (Figure 19b). Furthermore, blocking CD11a at the time of immunization resulted in a reduction in parasite-specific $CD8^+$ T cells at ten days post-immunization (Figure 19c), and this population expressed reduced KLRG1 at this time-point (Figure 19d). Together, these results demonstrate that CD11a blockade does not impact initial upregulation of CD69, but it does affect entry into cell cycle and expansion and effector phenotype of $CD8^+$ T cells. While these data need to be interpreted with care, the results with CD11a-blockade phenocopy the T-bet$^{-/-}$ mice in response to CPS (i.e. there is a reduced frequency of parasite-specific $CD8^+$ T cells and an altered phenotype).

T-bet promotes CCL3 production

CCL3, and its receptor CCR5, facilitate the ability of a $CD8^+$ T cell to find and interact with antigen bearing DCs during priming (Castellino et al., 2006). T-bet has been shown to bind to the promoter region of CCL3 in activated T cells (Beima et al., 2006; Jenner et al., 2009). Although the biological significance of this observation is unclear, the absence of T-bet during infection with LCMV results in fewer CCL3-producing $CD8^+$ T cells

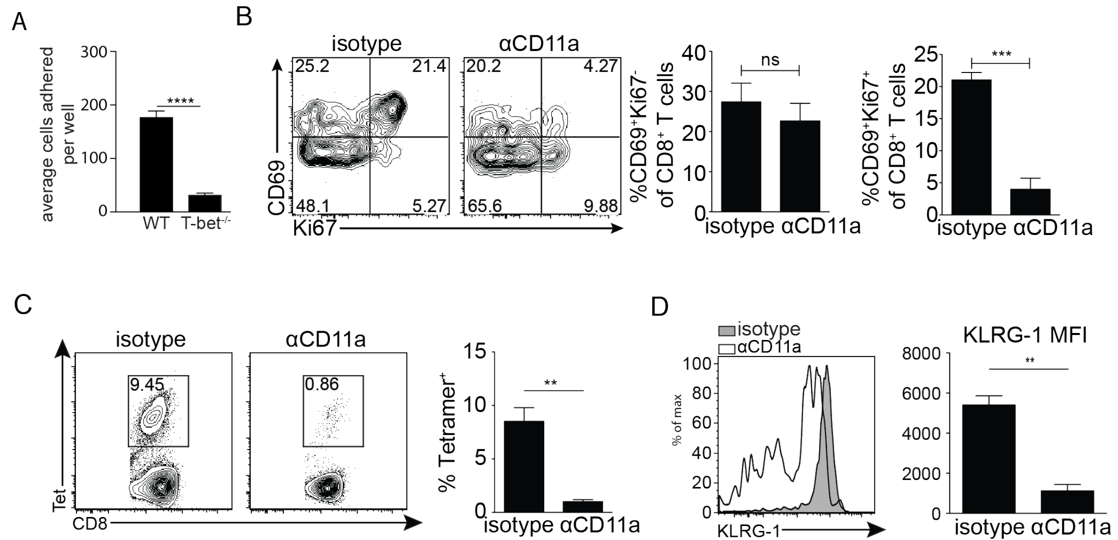

Figure 19. CD11a blockade phenocopies T-bet deficiency in early T cell activation. (A) WT and T-bet$^{-/-}$ T cells were activated *in vitro* and adherence to ICAM-1 (coated on a slide) was assessed. (B) WT mice were treated with CD11a-blocking antibody or isotype control and immunized i.p. with CPS. Expression of CD69 and Ki67 was assessed on the CD8$^+$ T cells two days after immunization. (C,D) WT mice were treated with CD11a-blocking antibody or isotype control and immunized i.p. with CPS-Ova. Nine days after immunization (C) SIINFEKL-loaded MHC-I tetramers were used to identify parasite-specific CD8$^+$ T cells, and (D) KLRG1 expression was assessed on the parasite-specific CD8$^+$ T cells. **p<0.01, ***p<0.001, ****p<0.0001

(Kao et al., 2011). To determine whether CCL3 production was associated with T-bet expression, naïve WT $CD8^+$ T cells were activated *in vitro* with various concentrations of αCD3 antibody. In the absence of TCR activation, there was minimal expression of T-bet or production of CCL3 (Figure 20a). Stimulation with an increasing concentration of αCD3 (1-10ug/ml) resulted in a dose-dependent co-expression T-bet and CCL3 (Figure 20a). To assess whether T-bet expression is required for CCL3 production, WT and T-bet$^{-/-}$ $CD8^+$ T cells were activated *in vitro* and CCL3 production was measured by flow cytometry and ELISA. In these experiments, T-bet$^{-/-}$ $CD8^+$ T cells produced less CCL3 than WT $CD8^+$ T cells (Figure 20b). It should be noted that neither WT nor T-bet$^{-/-}$ $CD4^+$ T cells produced appreciable levels of CCL3 in either the *in vitro* or *in vivo* assays (data not shown).

To determine whether CCL3 was produced during T cell priming after immunization with *T. gondii*, WT mice were immunized and CCL3-producing $CD8^+$ T cells were quantified by incubating the cells with Golgi-inhibitors followed by intracellular staining for CCL3. One day following immunization, there was no significant increase in the number of CCL3-producing $CD8^+$ T cells in the omentum, however, by day 2 post-immunization, there was a significant increase in these cells, which did not further increase at day 3 post-immunization (Figure 20c). Furthermore, at day 2 post-immunization, the majority of the CCL3-producing $CD8^+$ T cells were $CD69^+$ (Figure 20d). To determine if T-bet was necessary for *in vivo* CCL3 production, WT and T-bet$^{-/-}$ mice were immunized and CCL3-producing $CD8^+$ T cells were assessed in the omentum.

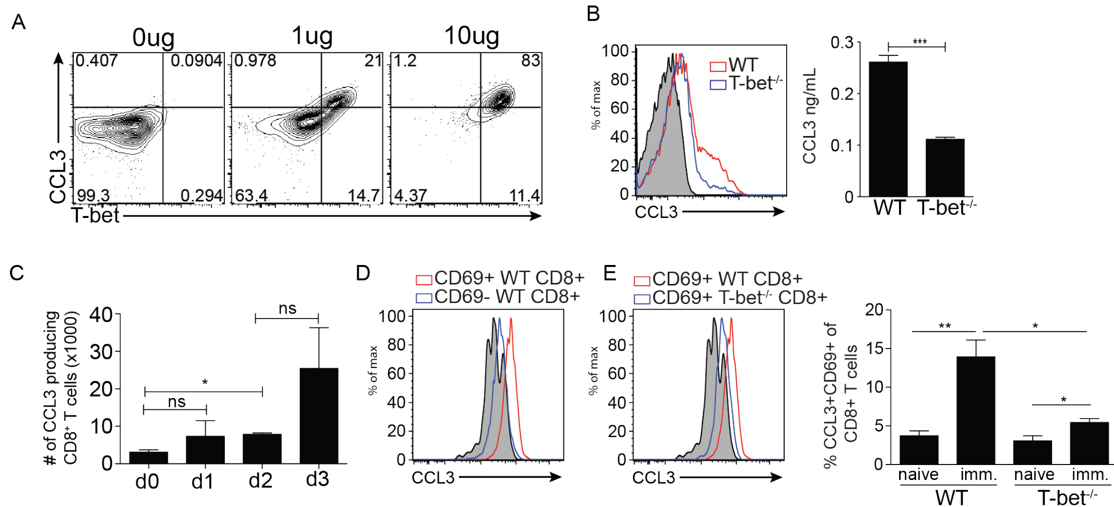

Figure 20. A role for T-bet in CCL3 production. (A) WT CD8$^+$ T cells were activated *in vitro* with the shown concentrations of αCD3/ml and 1ug/ml αCD28. Forty hours after activation, the cells were incubated with Golgi inhibitors for 4 hours, then fixed and stained for CCL3. (B) WT and T-bet$^{-/-}$ CD8$^+$ T cells were activated *in vitro* with 1ug/ml αCD3 and 1ug/ml αCD28. Forty hours after activation, the cells were incubated with Golgi inhibitors for 4 hours, then fixed and stained for CCL3. (C) WT mice were immunized i.p. with CPS and 1-3 days later cells from the omenta were incubated with PMA, ionomysin, and Golgi inhibitors and then stained for CCL3. (D) WT mice were immunized i.p. with CPS and 2 days later cells from the omenta were incubated with PMA, ionomysin, and Golgi inhibitors and then stained for CD69 and CCL3. (E) WT and T-bet$^{-/-}$ mice were immunized i.p. with CPS and 2 days later cells from the omenta were incubated with PMA, ionomysin, and Golgi inhibitors and then stained for CD69 and CCL3. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Indeed, there was a reduction in the CCL3 producing $CD69^+CD8^+$ T cells in the absence of T-bet (Figure 20e). Together, these data demonstrate that following CPS challenge, CCL3 is produced during T cell priming, and that T-bet is required for optimal production of this chemokine.

To determine whether CCL3 was necessary for early T cell activation after CPS immunization, WT and $CCL3^{-/-}$ mice were immunized with CPS and $CD8^+$ T cell responses were assessed in the omentum at day 2. Similar to what was seen with the T-$bet^{-/-}$ animals, $CCL3^{-/-}$ mice had a reduction of the $CD69^+Ki67^+$ $CD8^+$ T cell population, but unlike the T-$bet^{-/-}$ mice, they also had a reduction in the $CD69^+Ki67^-$ $CD8^+$ T cell population (Figure 21a). These results indicate that CCL3 production is required for optimal $CD8^+$ T cell activation, and the decrease in activation is more severe in the $CCL3^{-/-}$ mice than the T-$bet^{-/-}$ mice. Additionally, $CCL3^{-/-}$ mice had reduced numbers of parasite-specific $CD8^+$ T cells at day 10 post-immunization (Figure 21b), which was comparable to what was seen in the absence of T-bet. Similar results were obtained at day 2 and day 10 post-immunization when using a CCL3-blocking antibody (data not shown). These findings are consistent with the idea that T-bet coordinates the events of early T cell activation in part through its promotion of CCL3 production. However, CCL3 is not completely absent in the T-$bet^{-/-}$ mice, and this residual CCL3 production may explain why the T-$bet^{-/-}$ mice have increased T cell activation compared to the $CCL3^{-/-}$ mice.

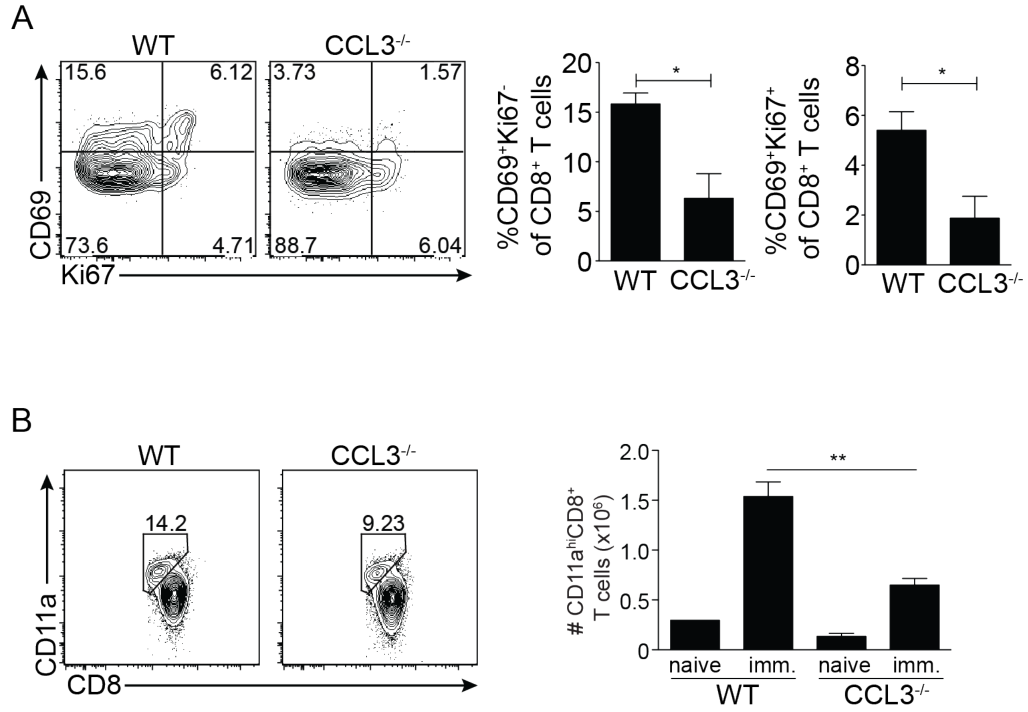

Figure 21. CCL3 is required for early activation and expansion of CD8+ T cells. (A,B) WT and CCL3-/- mice were immunized i.p. with CPS and T cell responses were assessed (A) two and (B) ten days later. *p<0.05, ***p<0.001

TCR-induced histone upregulation is T-bet–dependent

The data described above point to a role for T-bet following T cell activation. Therefore, in an effort to gain additional insight into the effects of early T-bet expression, a microarray was performed on WT and T-bet$^{-/-}$ CD8^{+} T cells activated *in vitro*. In these preliminary experiments, purified naïve (CD62L^{hi}CD44^{lo}) WT and T-bet$^{-/-}$ CD8^{+} T cells were stimulated *in vitro* with αCD3 and αCD28. To control for the effects of IFNγ and IL-12 signaling on T-bet expression, neutralizing antibodies against these cytokines were included in the cultures. After 40 hours in culture, the cells were removed and microarray analysis was performed on the transcripts isolated from the cells. Gene set enrichment analysis (GSEA) of the stimulated WT and T-bet$^{-/-}$ cells identified the Reactome RNA Pol. I Promoter Opening gene set as being enriched in the WT cells compared to the T-bet$^{-/-}$ cells; there were 17 genes identified in this gene set that were part of the core enrichment, and all 17 of these were histones (data not shown). Therefore, an a priori analysis was performed to assess expression of histones within these samples. Among WT CD8^{+} T cells, activation induced a robust upregulation of the majority of histones (Figure 22a). However, consistent with the GSEA results, there was lower expression of histones among stimulated T-bet$^{-/-}$ CD8^{+} T cells compared to stimulated WT CD8^{+} T cells (Figure 22b). To validate these findings, purified naïve WT and T-bet$^{-/-}$ CD8^{+} T cells were stimulated *in vitro* and qPCR was performed assessing the expression of a subset of the histones identified on the array. Consistent with the results from the array, there was no difference in expression of *hist1h4j* between activated WT and T-bet$^{-/-}$ CD8^{+} T cells, but *hist1h2af* and *hist2h2be* were decreased in activated T-bet$^{-/-}$ CD8^{+} T cells

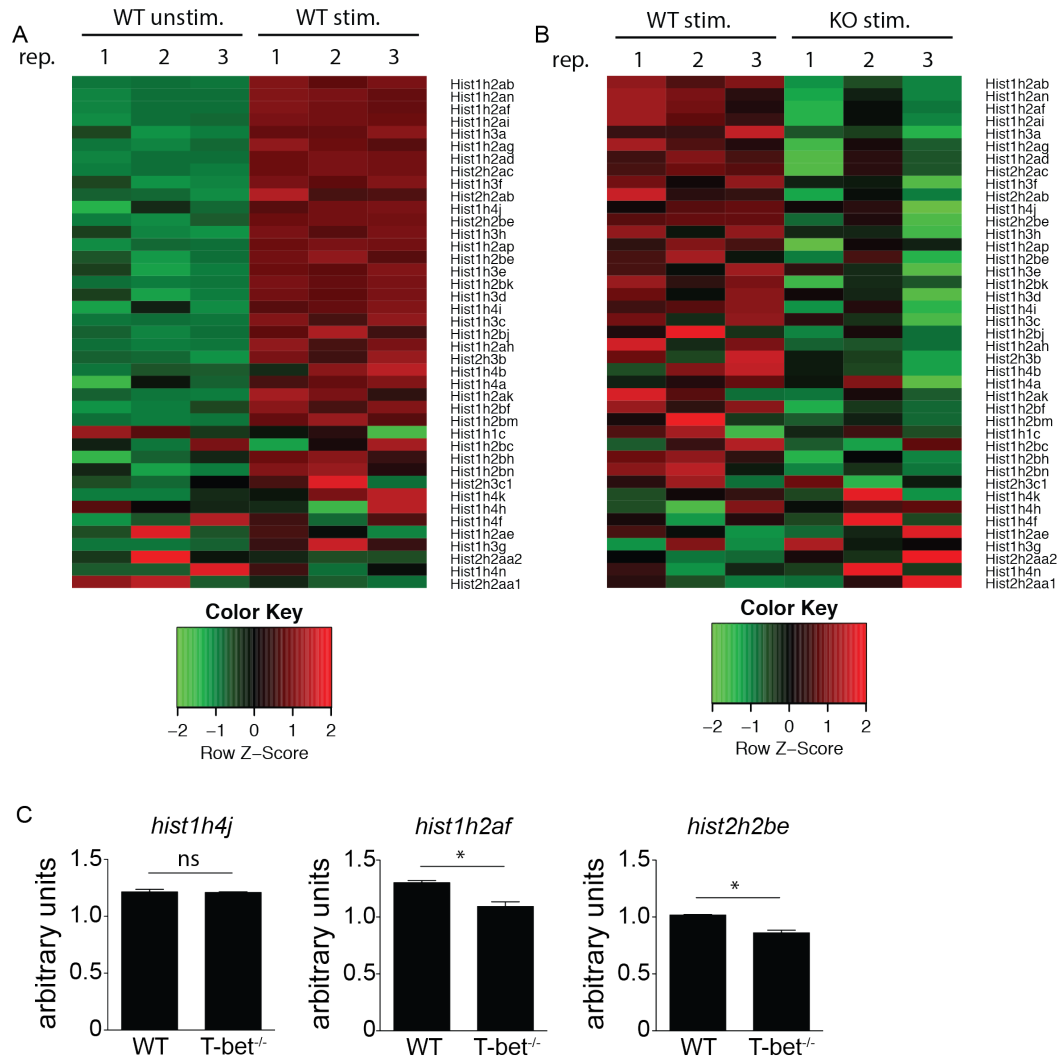

Figure 22. T-bet dependent histone expression. (A) Naïve ($CD44^{lo}CD62L^{hi}$) WT $CD8^+$ T cells were sorted and activated *in vitro* with αCD3 and αCD28 in neutral conditions (i.e. in media with αIFNγ, αIL-12 and αIL-4). Forty hours after activation, cells were harvested and prepared for micro-array analysis. (B) Naïve ($CD44^{lo}CD62L^{hi}$) WT and T-bet$^{-/-}$ $CD8^+$ T cells were sorted and activated *in vitro* with αCD3 and αCD28 in neutral conditions (i.e. in media with αIFNγ, αIL-12 and αIL-4). Forty hours after activation, cells were harvested and prepared for micro-array analysis. (C) Naïve WT and T-bet$^{-/-}$

CD8$^+$ T cells were sorted and activated *in vitro* with αCD3 and αCD28 in neutral conditions (i.e. in media with αIFNγ, αIL-12 and αIL-4). Forty hours after activation, cells were harvested and prepared for qPCR. *$p<0.05$

compared to activated WT $CD8^+$ T cells (Figure 22c). Taken together, these preliminary data indicate that optimal TCR-induced histone upregulation is reduced in the absence of T-bet.

Discussion

A role for T-bet early after T cell activation

While T-bet has primarily been associated with IFNγ production during effector T cell responses, it has been known to be expressed in T cells shortly after activation through the TCR (Chang et al., 2011; Schulz et al., 2009), although its biological significance at this early phase was unclear. The data presented here demonstrate that the initial T-bet expression after TCR activation promotes production of CCL3 and upregulation of CD11a. Thus, T-bet coordinates events involved in T cell priming and expansion. Furthermore, preliminary experiments presented here suggest that one potential effect that T-bet may exert at this early timepoint is the induction of histone upregulation. Whether the reduction in histone mRNA is a cause or a consequence of the reduced proliferation in $T\text{-bet}^{-/-}$ $CD8^+$ T cells is unclear. However, published CHIP-Seq datasets show that T-bet binds to histone promoters in activated $CD4^+$ T cells (Golnaz Vahedi, personal communication), which would suggest that T-bet is directly influencing histone transcription.

Because T-bet is necessary for the early activation of $CD8^+$ T cells, targeting T-bet during vaccination may be useful in generating protective immune responses. Several

successful vaccines are characterized by the induction of strong cell-mediated immunity in addition to potent antibody responses, and a current challenge in vaccine design is understanding the requirements to generate these responses (Salerno-Goncalves and Sztein, 2006). Thus, increasing T-bet expression early may help to increase the antigen-specific $CD8^+$ T cell population, potentially resulting in better protection, but its long-term impact on memory may be detrimental.

Bystander activation

Many of the studies presented in this chapter have focused on early T cell activation, and we have used CD69 as a surrogate marker. CD69 is upregulated proximal to TCR engagement (Testi et al., 1989). However, CD69 upregulation can also be induced by cytokines, in particular type I IFNs (Feng et al., 2005; Shiow et al., 2006). Therefore, it was important to know whether CD69 was upregulated in a TCR-dependent or independent manner in our studies. The data assessing CD69 upregulation on OT-I T cells suggested that while there was basal CD69 expression when Ova was not present during immunization, the $CD69^+$ OT-I T cells did not proliferate in this setting. Rather, CD69 was expressed at much higher levels and the OT-I T cells proliferated when Ova was present. Thus, it appears that cytokines enhance CD69 expression, an observation that is similar to what is reported in the literature regarding T-bet expression (Schulz et al., 2009).

T-bet and CCL3 production

While both T-bet and CCL3 were required for the expansion of parasite specific $CD8^+$ T cells, the phenotype was more severe at day 2 in the $CCL3^{-/-}$ mice (i.e. CCL3 appeared to be required for CD69 upregulation whereas T-bet was not required for its initial upregulation). Here, we propose that one mechanism by which T-bet impacts the events of T cell priming is through promotion of CCL3 production by $CD8^+$ T cells. However, CCL3 is reduced but not absent in T-$bet^{-/-}$ mice. Thus, the residual CCL3 present in the absence of T-bet likely contributes to coordinating the events of T cell activation. Furthermore, supplementary data from the initial report on the role of CCL3 during $CD8^+$ T cell priming demonstrate that increased expression of the receptor, CCR5, was not dependent on TCR engagement (Castellino et al., 2006), suggesting that it is activated, at least partially, in a bystander manner.

Unexpected role for T-bet in $CD8^+$ T cell priming

Since its discovery in 2000 (Szabo et al., 2000), the function of T-bet as a "master regulator" of $CD4^+$ Th1 responses has been largely attributed to its role in production of IFNγ, but $CD8^+$ T cells responses were thought to be T-bet independent (Szabo et al., 2002). Instead, $CD8^+$ T cells were largely dependent on a related transcription factor, Eomes, for IFNγ production (Pearce et al., 2003). Therefore, we were surprised that T-bet appeared to have a key role in $CD8^+$ T cell priming, but not $CD4^+$ T cell priming. This difference in requirement for T-bet may be rooted in what T-bet is doing at this stage of activation. We propose a model in which T-bet enhances $CD8^+$ T cell activation by

production of CCL3 and by promoting upregulation of CD11a. The original report from the Germain laboratory demonstrated that $CD4^{+}$ T cell help is optimized when $CD8^{+}$ T cells respond to CCL3, and while both $CD4^{+}$ and $CD8^{+}$ T cells express CCR5, it is more highly expressed on $CD8^{+}$ T cells (Castellino et al., 2006). Therefore, it is plausible that T-bet dependent CCL3 is more important for $CD8^{+}$ T cell priming. Additionally, studies from the Krummel laboratory have demonstrated a role for CD11a in synaptic $CD8^{+}$ T cell: $CD8^{+}$ T cell interactions during a second phase of T cell priming (Gerard et al., 2013). Because T-bet appears to be important for CD11a upregulation, it could be that T-bet is required in $CD8^{+}$ T cells in order to enhance these secondary interactions. *In vivo* imaging studies will be very informative to understand whether cellular interactions (e.g. T-DC and/or T-T) are impacted by T-bet expression.

Materials and Methods

Mice and immunization

T-bet deficient (T-bet$^{-/-}$) and CCL3 deficient (CCL3$^{-/-}$) mice were purchased from Jackson Laboratory. Wild-type C57BL/6 (WT) mice were purchased from Taconic. CD45.1^{+}C57BL/6 mice were purchased from Charles River. All mice were housed in a specific-pathogen free environment at the University of Pennsylvania School of Veterinary Medicine in accordance with federal guidelines and with approval of the Institutional Animal Care and Use Committee. CPS-Ova parasites and were derived from the RHΔ*cpsII* (CPS) clone, which was provided as a generous gift by Dr. David Bzik (Fox and Bzik, 2002). Parasites were cultured and maintained by serial passage on human foreskin fibroblast cells in the presence of parasite culture media (Dulbecco modified Eagle medium [DMEM] [Invitrogen, Carlsbad, CA], 20% medium M199 [Invitrogen], 10% fetal bovine serum [FBS] [Serum Source International, Charlotte, NC], 1% penicillin-streptomycin [Invitrogen], 25 μg/ml gentamycin [Gibco]) which was supplemented with uracil [Sigma-Aldrich](final concentration of 0.2 mM uracil). Tachyzoites of each strain were prepared for infection by serial needle passage and filtered through a 5-μm-pore-size filter. Mice were infected i.p. with 10^5 live parasites.

Antibody treatment

For antibody treatment experiments, mice were treated with 50ug polyclonal αCCL3 (R&D), 150 ug αCD11a (M17/4, BioXcell), or isotype control (BioXcell) at the time of immunization.

Cell culture and tissue harvesting

Splenocytes were obtained by grinding spleens over a 70 µm filter (VWR) and washing them in complete media. Red blood cells were then lysed by incubating for 5 minutes at room temperature in 5 ml of lysis buffer [0.864% ammonium chloride (Sigma-Aldrich) diluted in sterile de-ionized H_2O)], followed by washing with complete media. Cells from the omentum were obtained by incubating omenta for 1hr in 0.2U/mL liberase TL (Roche) diluted in RPMI at 37°C, followed by grinding over a 70 µm filter (VWR) and washing in complete media.

Flow cytometry

The following antibodies were purchased from BD: Ki67 AF700, CD4 Pacific Blue, CD8α PE-CF594, CD3 PE-CF594, CD4 BV650. The following antibodies were purchased from eBioscience: CCR5 PE, CCL3 PerCP-eFluor710, KLRG-1 FITC, Ly6C (clone HK1.4) PerCP-Cy5.5, CD69 eFluor450, CD69 FITC, CD69 PE-Cy7, T-bet eFluor660, CD45.2 APC-eFluor780, CD3 APC-eFluor780. The following antibodies were purchased from BioLegend: LFA-1 PerCP-Cy5.5, CD3 Pacific Blue, CD3 BV785. Invitrogen live/dead Aqua stain was used to determine viability. PE conjugated Tgd-057 MHC-I tetramers and PE-conjugated AS-15 MHC-II tetramers were provided by NIH Tetramer Core. All samples were run on an LSRFortessa (BD) or Canto (BD) and analyzed using FlowJo software (Tree Star). Images were obtained using the ImageStream (Amnis) and analyzed using IDEAS software (Amnis). To determine T-bet

localization, nuclear and cytoplasmic masking functions were made using DAPI staining; these masks were then applied to T-bet expression.

Parasite detection

To quantify parasite burden in the peritoneal exudate, 100,000 cells were used to prepare cytospins. Cells were methanol fixed and then stained with the Protocol Hema-3 Stain Set, and the ratio of infected cells to total cells in a field of vision was calculated. All images were obtained on a Nikon E600 microscope using a 20X (brain) or 40X (liver, lung, heart, thymus, PECs) objective and NIS Elements Imaging software.

Microarray and analysis

Total RNA was isolated and a cDNA library was made using the TargetAmp Pico labeling kit. Samples were analyzed using Illumina bead arrays. Data analyses were carried out using the statistical computing environment, R (v3.1), the Bioconductor suite of packages for R, and RStudio (v0.98). The total dataset was be analyzed using Gene Set Enrichment Analysis (GSEA) and the Broad Institute's Molecular Signatures Database (MSigDB, v4.0).

Statistical analysis

Statistical analyses were performed using PRISM software (Graphpad Software). Significance was calculated using an unpaired two-tailed Student's *t*-test except when otherwise noted.

CHAPTER 4: DISCUSSION

Preface

In this Thesis Chapter, I will describe some of the toolsets that have facilitated my experiments to define the role of T-bet in the immune response to *T. gondii*. Next, I will discuss the role of T-bet in T cell priming and T cell trafficking, and why the necessity for T-bet differs between infection and immunization. I will then present the implications for these findings on the generation of $CD8^+$ T cell memory. Finally, I will discuss the relationship between T-bet and Eomes and future studies that may help define how their relationship impacts on the immune system.

Parasite and Host Toolset to study the T cell response to *T. gondii*

The genetic tractability of *T. gondii* has enabled the development of transgenic parasites that have been fundamental to the studies presented in Chapter 3. For example, deletion of the CPS gene renders the parasites incapable of replicating *in vivo*, and these parasites induce a robust T cell response without killing the host (Fox and Bzik, 2002; Jordan et al., 2009; Wilson et al., 2008). This strain of *T. gondii* induces low levels of inflammation, and this reductionist approach has been important because it has allowed several groups to study T cell priming with a defined antigen load. Additional useful modifications of *T. gondii* include the insertion of fluorescent proteins, which can be used with *in vivo* imaging experiments and with flow cytometry to identify cells that have been infected (John et al., 2010). Originally, in the absence of ways to identify endogenous parasite-specific T cells, the expression of model antigens by *T. gondii* facilitated the identification of antigen-presenting cells and antigen-specific lymphocytes (Gubbels et al., 2005; Jordan et al., 2009; Jordan et al., 2009; Pepper et al., 2008; Tait et al., 2010).

Frequently used in parallel, transgenic mice have been equally beneficial to studying the host immune response. For example, in order to understand the intrinsic versus extrinsic requirements for T-bet in the $CD8^+$ T cells, several of the experiments in this thesis used the combination of Ovalbumin secreting parasites and transgenic mice that had $CD8^+$ T cells specific for the SIINFEKL peptide of Ovalbumin, commonly referred to as OT-I T cells. Confoundingly, while the OT-I transgene should be inherited as a whole, some of our mice experienced spontaneous partial loss of this transgene. Because the loss was only partial, the mice still expressed high levels of the alpha or beta chain of the TCR,

obfuscating the true genotype of these mice. Communications with the Jackson Laboratories and collaborators (Drs. Kedl, Hogquist, and Jameson) revealed that this occurs in approximately 0.1% of pups. We published these findings, with a recommendation to screen for both chains of the TCR transgene, in a recent commentary in *Nature Immunology* (Pritchard et al., 2016). While this experience was frustrating and delayed several planned experiments, we were able to highlight this issue to the immunology community, hopefully helping others avoid our experience.

While the TCR transgenic mice have been useful, there are several experimental concerns about these approaches. For example, many of the transgenic TCRs commonly used by immunologists have a high avidity for their antigen, and their biology may not be reflective of the affinity and activation requirements of endogenous TCRs. The examination of the requirements for the generation and function of optimal T cell were enhanced by the ability to detect and characterize "natural" parasite-specific T cells. Several markers of activation (e.g. CD11a and CD44) have been used to help identify parasite specific T cells, but both MHC-I and MHC-II tetramers for *T. gondii* epitopes are now available that identify endogenous parasite-specific $CD4^+$ and $CD8^+$ T cells (Grover et al., 2012; Wilson et al., 2010), and these tetramers were used throughout this thesis. In our studies, the combination of CD11a with MHC-I and MHC-II tetramers helped to uncover a role for T-bet in CD11a upregulation. Initially, while only using high expression of CD11a to identify parasite-specific T cells, it appeared that the loss of T-bet resulted in a failure to generate parasite-specific $CD4^+$ T cells. However, use of the

tetramers showed that the frequencies of parasite-specific T cells were equal in the secondary lymphoid organs between infected WT and T-bet$^{-/-}$ mice, but the expression of CD11a on these cells was reduced in the absence of T-bet (refer to Chapter 2). The technologies to manipulate host and the parasite have advanced the field, and new tools continue to be developed. For example, mice with transgenic TCRs that have defined high and low affinities to endogenous *T. gondii* antigens were generated using epigenetic reprogramming via somatic cell nuclear transfer (Kirak et al., 2010), and these mice could be useful in studying how (or if) TCR affinity impacts levels of T-bet expression in the setting of a natural infection.

A role for T-bet in optimal CD8$^+$ T cell priming

Several groups have shown T-bet induction after TCR activation (Chang et al., 2011; Schulz et al., 2009). These results have led to a model in which T-bet is expressed after cognate interactions between the TCR and peptide:MHC. In Chapter 3 I provided evidence for a novel function of early T-bet expression during CD8$^+$ T cell priming, but T-bet appeared less critical for CD4$^+$ T cell priming. Interestingly, these results were foreshadowed by work from the Glimcher laboratory in which T-bet is required for the acquisition of a CD8$^+$ T effector phenotype (CD62L^{lo}Ly6C^{hi}) when stimulated with peptide-loaded APCs (Sullivan et al., 2003). In this system, T cell activation requires physical interactions between T cells and APCs and the formation of an immunological synapse, of which CD11a/CD18 and ICAM-1 are essential components of the peripheral supramolecular activation cluster (pSMAC) (Friedl et al., 2005). However, when

stimulated with αCD3/αCD28, which does not require cellular contacts, T-bet is not required for the development of $CD8^+$ T cells that express an effector phenotype (Sullivan et al., 2003). One possible interpretation of these data is that T-bet is necessary for optimal cellular interactions during T cell priming and the data presented in Chapter 3 suggest a model in which T-bet influences these cellular interactions via promoting upregulation of the integrin CD11a and the chemokine CCL3 (Figure 23). While it is unclear which cell type(s) respond to these proteins via interactions between CD11a and ICAM-I or signaling through CCR5 (the receptor for CCL3), an obvious candidate would be dendritic cell-T cell interactions. However, recent studies from the Krummel laboratory have demonstrated that a secondary phase of $CD8^+$ T cell priming is characterized by synaptic interactions between $CD8^+$ T cells (Gerard et al., 2013). These studies showed that blocking CD11a during the "critical differentiation period" shifted the balance of $CD8^+$ T cells, with a reduction in SLECs ($KLRG1^{hi}IL7R^{lo}$) but a slight increase in the frequency of MPECs ($KLRG1^{lo}IL7R^{hi}$). This is a phenotype that is similar to the parasite-specific $CD8^+$ T cells from CPS-treated mice deficient in T-bet, or mice that were treated with CD11a-blocking antibody at the time of immunization (refer to Chapter 3).

One of the main findings described in Chapter 3 was that CCL3 was required for the early activation and expansion of the $CD8^+$ T cells in response to CPS. However, blocking CCL3 did not alter the phenotype of the effector $CD8^+$ T cells after CPS immunization. Work from the Luster laboratory has demonstrated an essential function of

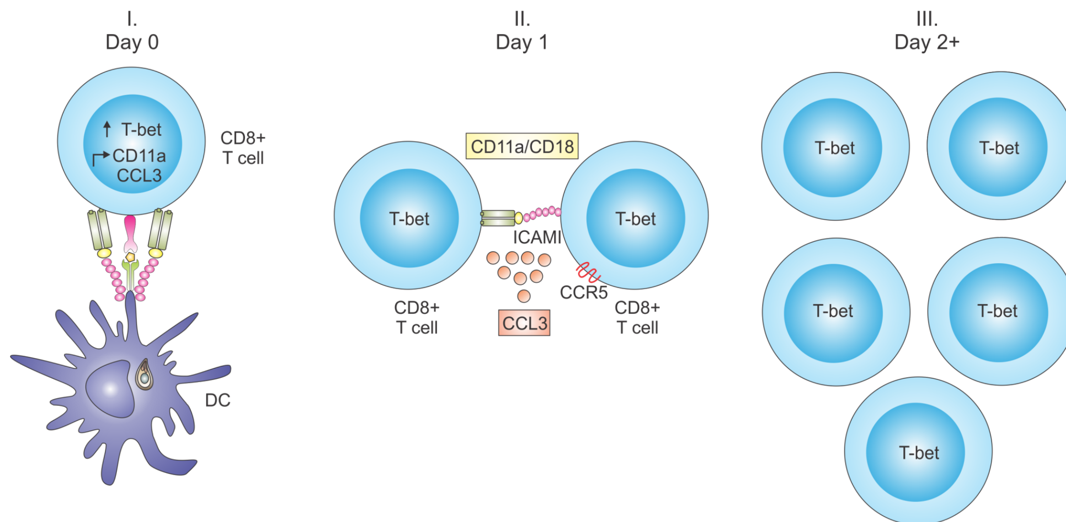

Figure 23. A role for T-bet during CD8$^+$ T cell priming.[*] Naïve T cells have basal levels of CD11a expression, but do not express T-bet. (I) TCR signaling promotes T-bet expression, which induces the upregulation of CD11a and CCL3. CD11a/CD18 interactions with ICAM-1 on APCs are an essential part of the immune synapse. (II) After cessation of TCR signaling, a second phase of CD8$^+$ T cell priming occurs in which CD11a-dependent synaptic interactions between CD8$^+$ T cells enhance activation. CD8$^+$ T cells also express the receptor for CCL3, CCR5, and this chemokine may also impact these interactions. (III) T-bet promotes the expansion of activated CD8$^+$ T cells.

[*] Deb Argento helped prepare this figure.

the receptor for CCL3, CCR5, on NK cells for migration and control of *T. gondii* replication, but suggested that CCR5 was dispensable for the T cell responses during infection (Khan et al., 2006). However, experiments from the Germain laboratory have shown that CCL3 is important for early T cell activation and expansion. *In vivo* imaging experiments demonstrated that CCL3-signaling increases the migratory speed of $CD8^+$ T cells and enhances the frequency of cellular interactions between $CD8^+$ T cells and antigen-bearing APCs (Castellino et al., 2006). Future studies examining the role of T-bet in the cellular interactions involved in T cell priming should include live imaging of these interactions to determine if the absence of T-bet alters T cell migration and/or the cellular interactions (i.e. T-DC, T-T, and/or T-NK) during T cell priming. For example, in the Hunter laboratory, we have WT and T-bet$^{-/-}$ OT-I T cells that express different fluorescent proteins and CPS-Ova parasites. With these tools, we can transfer WT and T-bet$^{-/-}$ OT-I T cells into the same immunized mouse and image cellular interactions in the omentum. This experimental system will permit analysis of a large number of parasite-specific $CD8^+$ T cells during the priming process.

T-bet and T cell trafficking

We have known that T cells are required for long-term resistance to *T. gondii* for almost 30 years (Gazzinelli et al., 1991; Parker et al., 1991; Suzuki and Remington, 1988). However, effective control is not just dependent on the ability to expand the parasite-specific T cells and generate effector responses, rather recently activated parasite-specific T cells also must traffic from secondary lymphoid organs into the infected tissues in order

to mediate local control. Indeed, several chemokines and chemokine receptors (e.g CCL19/CCL21 and CCR7, and CXCL10 and CXCR3) are implicated in these events (Cohen et al., 2013; Harris et al., 2012; Noor et al., 2010; Norose et al., 2011; Ploix et al., 2011). In Chapter 2, I demonstrated that in the absence of T-bet, parasite specific $CD4^+$ and $CD8^+$ T cells were generated but were absent from secondary sites of infection, a finding that suggests a role for T-bet in the coordination of optimal T cell trafficking during inflammation. In this system, infected T-bet$^{-/-}$ mice had intact circulating levels of IFNγ, yet the mice still succumbed to infection associated with the inability of the $CD4^+$ and $CD8^+$ T cells to access to secondary sites of infection. While the major function of T-bet has been considered to be the induction of IFNγ in effector $CD4^+$ T cells, previous reports implicated T-bet in effector $CD4^+$ T cell and regulatory $CD4^+$ T cell trafficking (Koch et al., 2009; Lord et al., 2005). Our studies provide a life and death example that its role in effector T cell trafficking should also be considered a major function of T-bet. As discussed in Chapter 1, T-bet regulates a cassette of genes associated with Th1 responses, not just IFNγ, and therefore it might be expected that T-bet has diverse roles in these effector populations. Based on the literature and our own studies, several of these potential T-bet targets involved in T cell migration include PSGL-1, CCL3, CXCR3, and CD11a. Furthermore, preliminary data from a micro-array on *in vitro* activated $CD8^+$ T cells identified several additional potential targets of T-bet involved in T cell trafficking (including CD7, CD53, and CD96), but to date these have not been validated (data not shown). As shown in Chapter 2, expression of CD11a and CXCR3 are reduced on parasite-specific $CD4^+$ and $CD8^+$ T cells. In preliminary data that I have generated with

Nathan Roy in the Burkhardt laboratory, *in vitro* activated T-bet$^{-/-}$ T cells have reduced adherence to endothelial cell monolayers. Since CD11a/CD18-dependent adhesion to endothelial cells is required for effector T cell entry into inflamed tissues (Shulman et al., 2011), our data may help explain why T-bet is required for optimal T cell trafficking into inflamed tissues (see Figure 24). Additionally, during toxoplasmic encephalitis, CXCR3 has been shown to be important for T cell migration into and within tissues (Harris et al., 2012), and the reduced CXCR3 expression on the parasite specific CD4^{+} and CD8^{+} T cells from T-bet$^{-/-}$ mice suggests that the few T cells that do make it into the inflamed tissues likely have altered migration or localization (see Figure 24).

In many of the infection models previously used to study the role of T-bet, the major source of protective IFNγ is either CD4^{+} T cells or CD8^{+} T cells, but not both, and this dichotomy generally aligns with susceptibility of T-bet$^{-/-}$ to infection (i.e. T-bet$^{-/-}$ mice die from infections in which CD4^{+} T cells are important for IFNγ and they survive when infected with pathogens that require IFNγ from NK and/or CD8^{+} T cells). Perhaps the essential role of T-bet in T cell trafficking during infection was masked by the absence of IFNγ in the settings in which the T-bet$^{-/-}$ mice do not survive infection. However, during infection with *T. gondii*, NK cells and CD4^{+} and CD8^{+} T cells are all major sources of protective IFNγ, and global IFNγ production was largely intact in the absence of T-bet. Instead, the reduced T cell trafficking (discussed earlier) was associated with reduced control of parasite replication at secondary sites of infection. Thus, this system enabled the differentiation of distinct functions of T-bet, and it would be informative to re-assess

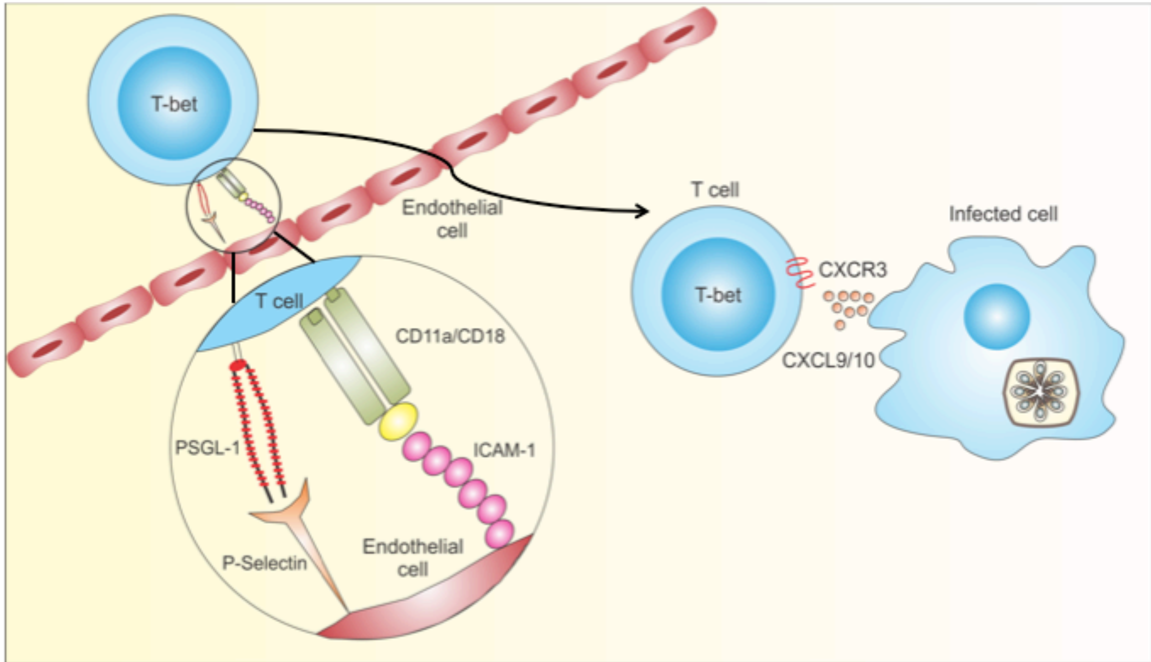

Figure 24. T-bet is required for T cell trafficking.* T-bet promotes upregulation of the integrin CD11a, which binds to ICAM-1 on endothelial cells, facilitating extravasation into inflamed tissues. T-bet also promotes PSGL-1-mediated binding to P-selectin on endothelial cells. Within tissues, T-bet dependent CXCR3 impacts migration and behavior in response to the chemokines CXCL9 and CXCL10.

* Deb Argento helped prepare this figure.

other infectious disease settings and other models of inflammation to determine if T-bet impacts on T cell trafficking in these systems. For instance, during infection with *L. major*, parasites are sequestered to the site of infection. $CD4^+$ T cells are the major source of IFNγ that limits disease in this system, and presumably, in order for these cells to exert their protective effects, they must be present at the site of infection. While mice deficient in T-bet infected with *L. major* have reduced IFNγ and increased disease (Szabo et al., 2002), it is unclear if T-bet deficient $CD4^+$ T cells are able to traffic to the site of infection, thus exacerbating the lesion created by reduced IFNγ. In contrast, infection with a related parasite, *Leishmania braziliensis*, induces the development of both $CD4^+$ and $CD8^+$ T cell responses. In this model, the $CD4^+$ T cells produce IFNγ, but the cytotoxic activity of $CD8^+$ T cells can cause pathology (Scott and Novais, 2016). Hence, T-bet deficiency may result in reduced $CD8^+$ T cell trafficking and consequently increased protection during infection with *L. braziliensis*. A similar precedent has been observed in mice infected with *P. berghei* and in this setting, T-bet deficiency reduces immunopathology (Oakley et al., 2013).

Differences in the role of T-bet between infection and immunization

As shown in Chapters 2 and 3, T-bet is required for the presence of parasite-specific specific $CD4^+$ and $CD8^+$ T cells that express an effector phenotype (i.e. high expression levels of Ly6C and KLRG1, respectively) after injection with replicating (infection) and non-replicating (immunization) strains of *T. gondii.* However, during infection, parasite-specific T cells are present in secondary lymphoid tissues at similar frequencies between

WT and T-bet$^{-/-}$ mice, but after immunization, T-bet is required for the expansion of the parasite-specific CD8^{+} T cells. These results seem contradictory, but there are several important differences between these model systems that may explain this discrepancy. First, in the immunization model, because the parasite cannot replicate, the antigen load is likely equivalent between the WT and T-bet$^{-/-}$ mice, and declines with time. Conversely, with the replication-sufficient parasite, antigen presentation peaks and then persists (and presumably is reflective of parasite burden) and results in the continuous production of effector T cells (Chu et al., 2016). Similar to studies have demonstrated that immunization with heat-killed *L. monocytogenes* results in lower CD8^{+} T cell activation and proliferation than live bacteria (Lauvau et al., 2001). During infection with *T. gondii*, T-bet is required to control parasite replication, and in the infected T-bet$^{-/-}$ mice infected, there is an increased antigen load compared to WT mice. Thus, increasing antigen burden, and presumably increased opportunities for T cell priming, may help to explain why the frequencies of parasite-specific T cells appear normal in the secondary lymphoid organs.

An alternative explanation regarding the difference in the requirement of T-bet between infection and immunization is the relative levels of inflammation induced by these two strains of *T. gondii*. The replication competent strains of *T. gondii* elicit a much higher inflammatory response than the replication-deficient strains, and IL-12 promotes effector responses (Pearce and Shen, 2007). These differences in inflammation may help to overcome the requirement for T-bet in the generation and expansion of parasite-specific

$CD8^+$ T cells. Nevertheless, during infection or immunization with *T. gondii*, T-bet is required for the "effector" phenotype of both $CD4^+$ and $CD8^+$ T cells. Perhaps T-bet has evolved to enhance and optimize early responses so that they are more efficient and expand faster, in effect better combatting pathogens before they become uncontrollable.

Memory responses and T-bet as a target for vaccine design

As previously discussed, T-bet is required for $CD4^+$ and $CD8^+$ T cells with an effector phenotype (i.e. high expression levels of Ly6C and KLRG1, respectively), both during immunization and infection. However, the $KLRG1^{lo}$ $CD8^+$ memory-precursor T cell population appears to be intact. Collaborations with Ross Kedl's laboratory at the University of Colorado have demonstrated that T-bet is also required for the early $CD8^+$ T cell activation in an adjuvant vaccine setting. It is notable that IL-27 promotes T-bet expression (Takeda et al., 2003), and IL-27 signaling intrinsic to T cells dictates the magnitude of the T cell response to an adjuvant vaccine (Pennock et al., 2014). These concepts could be beneficial in vaccine design; strategies to enhance T-bet expression at the time of vaccination could yield a higher frequency of T cells specific for the antigen, thus providing better protection against future infection. Conversely, *inhibition* of T-bet could prove advantageous when treating immune-mediated inflammatory conditions, but potential additional uses could include suppression of T-bet during T cell priming. At first, this approach may seem counter-intuitive because of the reduction in effector T cells in the absence of T-bet. However, while there are fewer total parasite-specific cells generated in the absence of T-bet, the memory-precursor population is intact (refer to

Chapter 3), which may be beneficial to the establishment of a protective memory population. As previously mentioned, many successful vaccines are characterized by the induction of strong cell-mediated immunity as well as antibody responses, and a current challenge in vaccine design is understanding the requirements to generate reliable cell mediated immunity that is dependent on memory $CD4^+$ and $CD8^+$ T cells (Salerno-Goncalves and Sztein, 2006). Thus, strategies to modulate T-bet may aid in rational vaccine design.

Regardless, whether there are distinct requirements for T-bet during priming, effector responses, memory formation and secondary expansion or the function of memory effectors remains unclear (see Figure 25). Our data suggest that T-bet is not necessary for the development of memory precursors after immunization, but whether there are inherent defects in MPECs from T-bet$^{-/-}$ mice or if T-bet is simply required for the secondary expansion and effector function after rechallenge is not known. One potential method to address the question of temporal requirement of T-bet would be timed deletion of T-bet using a ER-Cre-T-bet$^{flox/flox}$ system, which would allow for T-bet to be deleted only after administration of tamoxifen. Indeed, in preliminary data that I have generated using the CPS strain of *T. gondii*, deletion of T-bet by injection of tamoxifen into ER-Cre-T-bet$^{flox/flox}$ mice starting at day 4 after immunization (i.e. after T cell priming), but before the peak of the effector response, results in an intermediate phenotype compared to WT mice or mice lacking T-bet at the time of immunization. This intermediate phenotype is characterized by a partial reduction of parasite-specific $CD8^+$ T cells, but

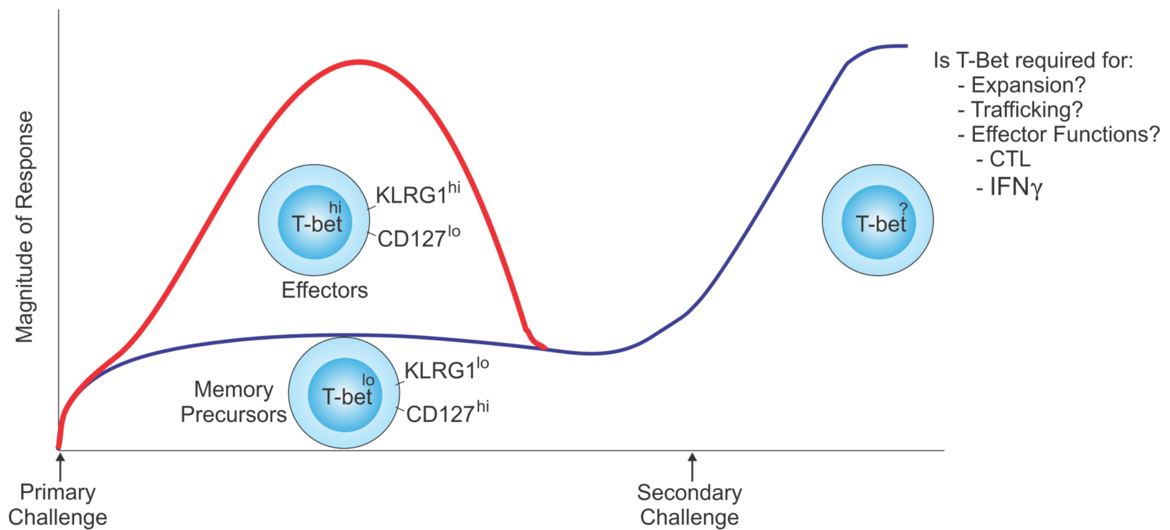

Figure 25. Differential roles for T-bet during primary effector responses, the generation of memory, and secondary challenge.* T-bet is highly expressed in effector $CD8^+$ T cells after primary challenge, and this population is reduced in the absence of T-bet. T-bet is expressed at low levels in memory precursor cells, however their generation is T-bet independent. The role of T-bet during rechallenge is unclear.

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* Deb Argento helped prepare this figure.

these cells express high levels of KLRG1. These data imply that early T-bet expression is sufficient for the effector phenotype of these cells, but that T-bet is also required for the maintenance or survival of the parasite-specific $CD8^+$ T cells. Experiments in which T-bet was absent during priming but then introduced during the effector phase would be ideal to thoroughly dissect and understand the role of T-bet at this early time point. Additional useful experiments using this system include deletion of T-bet after the development of memory responses, but before secondary challenge.

Studies using persistent murine cytomegalovirus (MCMV) have suggested that memory cells are derived early during infection, and short-lived effector cells are continuously generated from this population (Snyder et al., 2008). More recent studies from the Robey laboratory have demonstrated a similar phenotype using *T. gondii*, but in this setting, persistent antigen induces the continuous generation of SLECs from a novel memory-effector hybrid intermediate cell that expresses both CXCR3 and KLRG1. Interestingly, the *T. gondii* studies also implied that there was no contraction phase of the dominant effector T cell response (Chu et al., 2016). These studies have raised new questions such as how best to target this population of intermediate cells during vaccination. Additionally, T-bet is expressed in this novel intermediate population, but it is not known whether T-bet is required for its generation and/or maintenance, although the studies presented in Chapter 2, as well as previously published data (Joshi et al., 2007; Lord et al., 2005), suggest that T-bet is required for this population (i.e. T-bet is necessary for expression of both KLRG-1 and CXCR3).

Potential avenues of future investigation

One of the unexpected elements of my studies was the initial observation that T-bet localization within the cell is not restricted to the nucleus. While this is not a topic that is generally discussed in reviews, we are not the first group to note this difference. Thus, cytosolic T-bet has been observed in T cells isolated from the lamina propria of patients with Crohn's disease (Neurath et al., 2002), in $CD8^+$ T cells that are dividing (Chang et al., 2011), in $T\text{-bet}^{lo}$ $CD8^+$ T cells from human donors (McLane et al., 2013), in parasite-specific effector T cells from mice infected with *T. gondii* (refer to Chapter 2), and in activated $CD8^+$ T cells following priming (refer to Chapter 3). A portion of activated $CD8^+$ T cells from human donors also displayed cytoplasmic Eomes localization (McLane et al., 2013). The biological significance of the cytoplasmic localization of T-bet, or how the balance of nuclear/cytoplasmic T-bet affects T cell function is unclear. One potential mechanism underlying this partitioning was identified in studies in which mutation of the T-bet lysine residue at 313 resulted in decreased ubiquitination and increased cytoplasmic T-bet localization (Jang et al., 2013). Perhaps excess T-bet is stored in the cytoplasm and can quickly translocate to the nucleus when needed for effector responses upon rechallenge. Alternatively, sequestration of T-bet to the cytoplasm could represent a regulatory process to limit T-bet induced immunopathology. Through its interaction with Bcl-6, T-bet is able limit production of IFNγ (Oestreich et al., 2011), and localization of T-bet to the nucleus may be another mechanism of inhibition of inflammatory responses by T-bet. It is also possible that T-bet could have a

distinct function by interacting with cytosolic proteins, independent of its DNA binding ability.

Nevertheless, T-bet is primarily known for its transcriptional effects mediated through its ability to bind DNA. T-bet was first identified as a transactivator of the IFNγ gene in Th1 and NK cells (Szabo et al., 2000), but $CD8^+$ T cells are still able to produce IFNγ in the absence of T-bet (Szabo et al., 2002). We now know that Eomes is able to induce IFNγ production in $CD8^+$ T cells (Pearce et al., 2003), but these linked transcription factors have many additional functions that relate broadly to Th1-like responses. In addition to its role in IFNγ production, T-bet exerts many effects during Th1 immune responses that include inhibition of alternative T cell fates and impact on trafficking of effector and regulatory T cells (discussed earlier). However, T-bet may impact immune responses by additional mechanisms. For example, T-bet has been implicated in cellular metabolism (Stolarczyk et al., 2013). Although its precise role in the process remains unclear, T-bet expression is upregulated after TCR-engagement, and T-bet, along with mTOR complex 1 (mTORC1), is asymmetrically divided between daughter cells (Chang et al., 2011; Pollizzi et al., 2016), and recent studies highlight that the metabolic profiles differ between the daughter cells (Pollizzi et al., 2016). T-bet is also expressed in a variety of cells in the immune system, including ILCs, NK cells, and B cells, and it may have many diverse functions in these populations. Additional studies on the role of T-bet in these cells in the context of infection would provide insights into lineage specific effects of T-bet.

At the start of these studies we had an expectation that, based on the role of T-bet in $CD4^+$ and $CD8^+$ T cell effector function (i.e. IFNγ production), T-bet might be important for $CD4^+$ T cell priming but not the $CD8^+$ T cell response. Therefore, it was surprising that we observed a key role for T-bet in $CD8^+$ T cell priming. In Chapter 3, I presented data in which the $CD4^+$ T cell responses in T-bet$^{-/-}$ mice were largely intact after immunization with CPS, but the $CD8^+$ T cell responses were impaired. In Chapter 1 I highlighted the evolutionary background of T-bet and Eomes, two related T-box transcription factors, which diverged around the development of adaptive immunity. Interestingly, while expressed in cells of the immune system, Eomes also has a key role during embryonic development. Preliminary data that I have generated have shown that mice with a T cell specific deletion of Eomes have intact T cell responses following CPS vaccination with *T. gondii*. Conversely, T-bet appears to be integral to immune responses but has a negligible effect, if any, during embryonic development (although it is involved in the development and maturation of NK cells). It is unclear why the requirement for these transcription factors is diverse during embryonic development versus the generation of effective immune responses. However, as discussed in Chapter 1, T-box transcription factors can interact with other transcription factors and these combinations result in different effects. Therefore, it is likely that T-bet and Eomes have distinct binding partners resulting in diverse transcriptional profiles in different cell types and developmental processes.

Several newer assays have become available that can help us to understand how these transcription factors mediate their distinct effects. For example, a technique that may prove useful in understanding how T-bet (or Eomes) impacts epigenetic programming of newly activated $CD8^+$ T cells T cells is mass spectrometry. Preliminary data from a microarray identified histones a potential T-bet target, and with assistance from the Garcia laboratory, I generated preliminary data on *in vitro* activated WT and T-$bet^{-/-}$ $CD8^+$ T cells assessing post-translational modifications (PTMs) of histones. These data suggested that there may be differences of PTMs on the histones H3K27 and H4K20 (data not shown). These preliminary data are consistent with studies from the Weinmann laboratory that have shown that T-bet is able to recruit the H3K27-demethylase Jmjd3 to the *Ifng* and *Cxcr3* promoters to remove the repressive H3K27me3 mark, thus impacting chromatin accessibility and transcription (Miller and Weinmann, 2010). Furthermore, the Assay for Transposase-Accessible Chromatin with high throughput sequencing (ATAC-seq) can be used to determine how T-bet and Eomes impact chromatin accessibility in activated T cells. Indeed, these technologies and others can be used to better understand the molecular events of T-bet and Eomes interactions (e.g. with other proteins or DNA binding) and give clues to additional functions of these transcription factors.

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