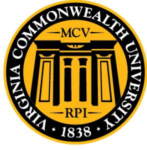

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T-bet-dependent regulation of T cell responses during *Trypanosoma cruzi* infection

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

Dustin Andrew Cobb
B.S., Eastern Mennonite University, 2006

Director: Ronald B. Smeltz, Ph.D.
Assistant Professor, Department of Microbiology and Immunology

Virginia Commonwealth University
Richmond, VA
May, 2012

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List of Abbreviations

+/+	wild-type gene
-/-	homozygous gene deletion
Ab	antibody
Ag	antigen
APC	allophycocyanin
BCG	Bacille Calmette Guerin
C57BL/6	inbred mouse strain
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T lymphocyte
Cy	cyanin
DC	dendritic cell
DNA	deoxyribonucleic acid
Ebi-3	Epstein-Barr virus induced gene 3
ELISA	enzyme-linked immunosorbent assay
Eomes	eomesodermin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
Fc	fragment crystallizable

FITC	fluorescein
GFP	green fluorescence protein
HRP	horseradish peroxidase
ICS	intracellular cytokine staining
IFN	interferon
IL	interleukin
Ig	immunoglobulin
i.p.	intraperitoneal
i.v.	intravenous
LCMV	lymphocytic choriomeningitis virus
LEAF	low endotoxin-azide free
MACS	magnetic activated cell sorting
MHC	major histocompatibility complex
NK	natural killer
NKT	natural killer T
OVA	ovalbumin
PBL	peripheral blood leukocyte
PBS	phosphate-buffered saline
PE	phycoerythrin
PS	phosphatidylserine
p.i.	post infection
RT	room temperature
RV	retrovirus

SD	standard deviation
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box-expressed-in-T-cells
Tbx21	T-box transcription factor 21
T. cruzi	*Trypanosoma cruzi*
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TLR	toll-like receptor
T_{Reg}	T regulatory cell
ts	*trans*-sialidase
TNF	tumor necrosis factor
WT	wild-type

Abstract

T-BET-DEPENDENT REGULATION OF T CELL RESPONSES DURING *TRYPANOSOMA CRUZI* INFECTION

By Dustin A. Cobb, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2012.

Major Director: Ronald B. Smeltz, Ph.D.
Assistant Professor, Department of Microbiology and Immunology

The human pathogen *Trypanosoma cruzi* is an intracellular parasite and the etiological agent of Chagas disease. Protective immune responses to *T. cruzi* are highly dependent on T helper 1 and $CD8^+$ T cells which produce interferon-gamma. A deficiency in these responses has severe consequences on the ability to control infection. Our investigation into the role of the Th1 transcription factor, T-bet, during murine *T. cruzi* infection revealed that T-bet is required for resistance. Contrary to our expectations, T-bet was not required for the development of Th1 immunity during infection, as T-bet-deficient mice still developed interferon-gamma-producing T cells.

However, T-bet was required to suppress the differentiation of Th17 cells and for the expansion of *T. cruzi*-specific $CD8^+$ T cells.

We first sought to determine the cause of reduced numbers of *T. cruzi*-specific $CD8^+$ T cells in infected T-bet-deficient mice. First, we found that impaired migration or survival did not contribute to the low number of *T. cruzi*-specific $CD8^+$ T cells. Secondly, we determined that reduced numbers of $CD8^+$ T cells was not secondary to a defect in antigen-presenting cell activation or priming of $CD8^+$ T cells. A recapitulation of defective expansion in mice with normal T-bet-expressing antigen-presenting cells demonstrated that T-bet expression in T cells was required. Thus, we determined that T-bet regulates the expansion of antigen-specific $CD8^+$ T cells during *T. cruzi* infection in a T cell-intrinsic manner.

Although it was evident T-bet had an integral role in suppressing the development of Th17 cells in response to infection with *T. cruzi*, several issues remained unclear. The first was the apparent lack of a negative regulatory effect of IFN-γ/IFN-γ-signaling on Th17 cells, which contradicted published reports. To clarify the role of IFN-γ, we investigated the effect of IFN-γ- or Stat-1-deficiency during *T. cruzi* infection. Surprisingly, IFN-γ did not have a major role in up-regulating T-bet or for suppressing the development of Th17 responses, whereas Stat-1 was necessary for both. This was unexpected as Stat-1 is an IFN-γ-inducible transcription factor, and its activation leads to T-bet induction. Thus, the T-bet-mediated inhibition of Th17 responses during *T. cruzi* infection is dependent on Stat-1, but not IFN-γ.

The final aim of this project was to identify the cytokines that negatively regulate Th17 differentiation in response to *T. cruzi*. We focused on the IL-12-family cytokines,

IL-12 and IL-27, which are known to regulate T cell responses. Indeed, IL-12-deficient mice infected with *T. cruzi* developed a significant increase in Th17 cells similar to that observed in T-bet-deficient mice. Surprisingly, and in contrast to published results in other models, IL-27-deficient mice did not exhibit an increase in Th17 development. Our results demonstrate that IL-12, but not IL-27, is necessary for optimal T-bet expression and regulation of Th17 responses during *T. cruzi* infection.

General Introduction and Background

Trypanosoma cruzi and Chagas disease

The intracellular protozoa *Trypanosoma cruzi* is a parasite that causes a severe chronic disease in humans called American Trypanosomiasis, or Chagas disease. An estimated 8 to 10 million people are currently infected, with an additional 40 million at high risk of contracting the disease[1,2]. Chagas disease is characterized by severe cardiac and digestive problems in chronically infected people. *T. cruzi* is endemic to countries in Central and South America where it causes significant human health and socioeconomic problems.

The life cycle of *T. cruzi* involves insect vectors of the Reduviidae family and mammalian hosts. When an infected insect bites a human, it defecates on the skin releasing trypomastigotes. The parasites are then scratched or rubbed into the bite wound or eye of the human where they invade host cells. Once invasion of cells occurs, the trypomastigotes differentiate into an amastigote form. During this stage the parasites replicate and transform into infective trypomastigotes, lyse host cells, and invade other cells or enter the bloodstream[3]. The cycle is completed when the insect vector feeds on the blood of an infected host. Strategies to limit human exposure to *T. cruzi* through vector control have had some success in endemic regions, but disease transmission remains a problem. People living in non-endemic countries are also at risk of infection. For example, approximately 700,000 (300,000 in the United States, CDC) infected individuals live abroad, making blood transfusion and organ transplantation a serious threat for disease transmission between humans[4]. Other routes of infection include oral ingestion of contaminated food and beverages and vertical transmission during childbirth.

Chagas disease consists of an acute and a chronic phase. The acute phase of disease begins a short period of time (2-3 weeks) following infection and generally lasts about 2 months. During this time, a large number of parasites can be found circulating in the blood or in the tissues of infected individuals[5]. Despite this high load of parasitemia and parasitism, people remain mostly asymptomatic or may present with non-specific symptoms such as fever and headache. In some instances individuals may develop more specific symptoms including lymphadenopathy, splenomegaly, myalgia, muscles pains, and myocarditis[2]. In rare cases, particularly in the absence of treatment, about 5% may die from heart failure due to acute myocarditis. Infection of immunocompromised persons may also lead to central nervous system involvement and meningoencephalitis which can lead to death[6]. Generally, acute infection is controlled by the host immune response, but the parasites are never fully eliminated. Following the acute phase, infected individuals enter the chronic phase of Chagas disease. During this period, the parasite levels in the blood and tissues are significantly diminished and people are largely free of symptoms. This asymptomatic, or indeterminate phase, can last for years or even the remainder of the patient's life[7]. Unfortunately, approximately 30% of infected people will eventually develop severe medical complications involving the heart and/or digestive organs[2]. One of the most frequent manifestations of Chagas disease is chronic chagasic cardiomyopathy which is characterized by cardiomegaly (enlarged heart), arrhythmias, and congestive heart failure(8). Cardiomyopathy is a leading cause of heart failure in many Latin American countries. The digestive aspects of chronic Chagas disease include megaesophagus and megacolon(9). While most people infected with *T. cruzi* may remain without

symptoms of disease for their entire lives, many will develop these severe and debilitating health complications, which in some cases can be fatal. Therapeutic treatment of infected patients is limited to the use of the drug benznidazole. The drug is primarily effective only for patients who are still in the acute phase of infection, with a therapeutic success rate up to 80%, however, its use causes severe side effects. Treatment of chronically infected patients is much less effective. Additionally, reduced efficacy against certain parasite strains has been observed. Drug studies in *T. cruzi*-infected mice show that combined therapy with interleukin-12 augments the efficacy of benznidazole[10]. This and other studies support the involvement of the immune system in promoting the effectiveness of drug treatment for *T. cruzi* infection.

Protective immunity to *T. cruzi* infection

Successful immune control and subsequent host resistance to *T. cruzi* infection is contingent upon both the innate and adaptive immune responses. Numerous studies of murine *T. cruzi* infection have identified various cell types and molecules spanning the immune system that are critical for control of infection. Initially, infection by *T. cruzi* activates cells of the innate immune system such as macrophages and dendritic cells by engaging specific toll-like receptors (TLRs)[11,12]. *T. cruzi* possesses surface expressed glycolipid molecules such as glycosylphosphatidylinositol (GPI) anchors and glycoinositolphospholipids (GIPLs) that are recognized by TLR-2 and TLR-4, respectively[13,14]. Also, *T. cruzi* DNA that has been liberated from parasites that are destroyed in endolysosomal compartments of phagocytic cells can bind to TLR-9[15]. The engagement of these receptors ultimately results in the production of nitric oxide and

pro-inflammatory cytokines like interleukin (IL)-12 and tumor necrosis factor (TNF)-α. The increased susceptibility of TLR-2, 4, and 9-deficient mice to infection demonstrates the importance of TLR signaling in innate immunity to *T. cruzi* infection[4]. In addition to macrophages and dendritic cells, NK and NKT cells also have important roles in innate resistance to *T. cruzi*[16,17,18]. While innate immunity plays an essential role in initial parasite control, its most important function during *T. cruzi* infection may be in shaping the development of adaptive immune responses.

Adaptive immunity during *T. cruzi* infection is comprised of T and B cell responses. Given the intracellular and extracellular nature of T. cruzi, the development of both cell-mediated and humoral immunity is important. Due to parasites residing intracellularly within host cells, a strong T helper 1 (Th1) mediated response accompanied by cytotoxic T lymphocytes (CTLs) is required for protection. Th1 cells are an integral part of protective immunity to infection because of their ability to secrete interferon-gamma (IFN-γ) which promotes the killing of parasites that have been engulfed by phagocytic cells like macrophages[19]. The need for IFN-γ for protection cannot be overstated as IFN-γ-deficient (*Ifng*$^{-/-}$) mice exhibit severe disease and high mortality rates[20]. Of all mouse knockout strains used in the study of *T. cruzi* infection, *Ifng*$^{-/-}$ mice exhibit the highest degree of susceptibility. Like Th1 cells, CTLs are also a potent source of IFN-γ. CTLs also play an essential role in detecting and killing host cells infected with *T. cruzi* parasites through the perforin/granzyme and Fas/Fas Ligand pathways[21].

B cell immunity and antibody responses are also considered important aspects of protection during infection. *T. cruzi*-specific antibody is involved in opsonizing parasites

for phagocytosis[22], complement-mediated lysis[23], and antibody-dependent cellular cytotoxicity (ADCC)[24]. Antibody effector functions are particularly important because of circulating trypomastigotes in the blood of infected hosts. Furthermore, B cells have been shown to promote $CD4^+$ and $CD8^+$ T cell responses[25] and can engage in cross-priming of $CD8^+$ T cells during *T. cruzi* infection[26].

Thus, engagement of the innate immune system by *T. cruzi* parasites and subsequent generation of appropriate adaptive immune responses is vital to controlling disease. Despite the development of strong and specific adaptive immunity to *T. cruzi*, it is generally not adequate to completely eliminate the parasites from the host, making it possible for them to persist and cause chronic problems. Additionally, inflammatory responses that bring infection under control are also responsible for the severe immunopathology in the heart and digestive tract that are characteristic of Chagas disease. There is a strong consensus that inflammatory T cells are major players in the generation of this immunopathology; however, the basis of what causes these cells to do so remains debatable. The combined influence of parasite persistence, anti-parasite immune responses, and the inability to maintain sufficient immunoregulation may lead to the development of chronic inflammation and pathology of Chagas disease. Nonetheless, this makes understanding the regulatory mechanisms governing inflammatory T cell responses of particular importance for the prevention and control of immunopathology, and especially for vaccine development and protective immunity to *T. cruzi* infection.

T helper cell differentiation

Activation and differentiation of T cells into specialized subsets following encounter with various stimuli is an integral component of appropriate adaptive immune responses. These various subsets of T helper cells have unique effector mechanisms that enable them to direct the appropriate response for a given challenge or infection. A naïve $CD4^+$ T cell can be directed to differentiate into a Th1, Th2, Th17, T regulatory (T_{Reg}), or T follicular helper (T_{FH}) cell depending upon the specific signals, or cytokines, that it receives from its environment. Naïve T cells with a given T cell receptor (TCR) specificity recognize antigen being presented via MHC molecules by antigen presenting cells (APC) (signal 1). This cognate interaction, in addition to co-stimulatory signals from CD40 and CD80 (signal 2), provided by APC, induces T cell activation. The inflammation and the particular cytokine environment (signal 3), which is established by APCs, largely determines the differentiation fate of helper T cells. Cytokines drive T cell differentiation by inducing specific transcription factors which establish the gene expression profiles that are unique to each helper T cell subset.

Th1 cells play an integral role in controlling infection by intracellular pathogens such as *Mycobacterium tuberculosis*, *Leishmania major*, and *Toxoplasma gondii*. The biological effects of Th1 cells are primarily mediated by the cytokine IFN-γ. T-bet, or Tbx21, is a member of the T-box family, and is the lineage-determining transcription factor that governs the Th1 program[27]. T-bet expression is driven by IFN-γ/Stat-1 and IL-12/Stat-4 signaling as well as TCR-mediated signals[28,29]. Induction of T-bet directly activates IFN-γ expression and promotes IL-12 responsiveness via up-regulation of IL-12 receptor β2 (IL-12Rβ2)[28], which results in the amplification of T-bet expression and

further production of IFN-γ. T-bet promotes IFN-γ expression by inducing important epigenetic modifications of the IFN-γ promoter. It accomplishes this by physically recruiting the H3K27-demethylase JMJD3 and the H3K4-methyltransferase Set7/9, which in turn remove the repressive H3K27 modification and adds the permissive H3K4 modification, respectively[30]. The net effect of these epigenetic changes is the establishment of a chromatin state that allows for optimal expression of the IFN-γ gene. The effects of increased IFN-γ production are wide ranging, but include macrophage activation, enhanced antigen presenting cell function, and the induction of cytokines and chemokines involved in the mobilization of monocytes and macrophages to sites of inflammation.

CD4$^+$ T cells may also differentiate into Th2 cells, which are involved in promoting B cell responses and antibody production to protect against extracellular pathogens. Th2 cells develop in response to IL-4/Stat-6 signaling, which promotes expression of the transcription factor GATA-3[31]. The effector cytokines of Th2 cells include IL-4, IL-5, and IL-13. Interestingly, T-bet can inhibit the differentiation of Th2 cells, and thus regulate the balance between Th1 and Th2 development[32].

In contrast, the primary role of T_{Reg} cells is to limit the magnitude of an immune response and thus maintain self-tolerance in order and avoid autoimmunity. T_{Regs} are identified by the expression of the transcription factor Foxp3 and mediate immune suppression by producing the immunosuppressive cytokines IL-10 and TGF-β[33]. T_{FH} cells are another unique subset of CD4$^+$ T cells that secrete IL-21 and IL-4, and are important for promoting B cell activation and germinal center formation in secondary lymphoid tissues[34].

Th17 cells, so named for their ability to produce IL-17A (IL-17), were recently identified in the last decade. Immunity to extracellular bacteria such as *Klebsiella pneumonia*[35] and *Borrelia burgdorferi*[36], as well as fungi like *Pneumocystis carinii*[37] and *Candida albicans*[38], appear to rely on Th17-mediated immune responses. Additionally, Th17 cells have been implicated in a number of autoimmune diseases including multiple sclerosis[39], rheumatoid arthritis[40], and psoriasis[41] and inflammatory bowel disease[42]. The cytokines IL-6, TGF-β, and IL-23 are important factors for the generation of Th17 cells[43]. IL-6 activates Stat-3 expression which induces the Th17-specific transcription factor ROR-γt in developing Th17 cells[44,45]. TGF-β appears to potentiate Th17 development by suppressing Th1 or Th2 differentiation, while IL-23 is critical for establishing and maintaining the Th17 program and the inflammatory characteristics of these cells. Th17 cells produce a multitude of effector cytokines that include IL-17A and F, IL-21, IL-22, and TNF-α[43]. IL-17 secretion induces epithelial cells, fibroblasts, and endothelial cells to produce GM-CSF, IL-6, matrix metalloproteinases, antimicrobial peptides and activates inducible nitric oxide synthase (iNOS)[46]. IL-17 also stimulates the production of the chemokines CXCL1, CXCL2, and CXCL8[47]. The effector responses of Th17 cells thus result in increased granulopoeisis, neutrophil recruitment, and extensive inflammation. Thus, Th17 cells are a potent pro-inflammatory T cell subset that is necessary for some infections, but also can cause severe immune-mediated pathology. This makes understanding the regulation of Th17 cell responses a critical importance.

Transcriptional regulation of T cell responses to *T. cruzi* infection

T-bet is required for the development of IFN-γ-producing Th1 cells that are needed to combat infection by intracellular pathogens. Consistent with this notion, mice that are T-bet-deficient (*Tbx21*$^{-/-}$) have an increased susceptibility to these infections and susceptibility is typically a result of impaired Th1 responses. For example, *Tbx21*$^{-/-}$ mice infected with *L. major* exhibit a diminished Th1 response but an increased Th2 response and show an increase in disease[48]. *M. tuberculosis* and *Salmonella typhimurium* infection of *Tbx21*$^{-/-}$ mice also resulted in increased susceptibility with an accompanying reduction in IFN-γ production, yet in these cases there was no shift to a Th2 phenotype[49,50]. In contrast, there was a greater production of the immunosuppressive cytokine IL-10. These studies demonstrate the necessity for T-bet in providing protective immunity against multiple intracellular pathogens. Furthermore, they show that not only is T-bet important for promoting Th1 immunity, but also for preventing an inappropriate skewing of the helper T cell-mediated immune response.

Studies in the laboratory were initiated to determine the requirement for T-bet in conferring resistance and immunity to *T. cruzi*. The hypothesis was that T-bet would be necessary for resistance given the requirement for a strong Th1 immune response during *T. cruzi* infection. These initial studies yielded several unexpected and interesting findings regarding the role of T-bet in *T. cruzi* resistance, $CD8^+$ T cell responses, and the regulation of helper T cell differentiation during infection. **Importantly, these results challenged the current paradigm at the time regarding the effects of IFN-γ on the regulation of Th17 cells and thus set the stage for the forthcoming studies in this dissertation**.

Following infection with *T. cruzi*, T-bet-deficient mice exhibited increased morbidity based on weight loss compared to infected wild-type C57BL/6 controls (**Figure 1A**). Surprisingly, 100% of infected *Tbx21*$^{-/-}$ mice succumbed to acute infection by approximately four weeks post-infection, whereas all wild-type mice survived (**Figure 1B**). These results clearly demonstrated that T-bet was critically required for resistance to *T. cruzi* infection.

It was hypothesized that defects in T cell activation in mice lacking T-bet would result in an impaired ability to control infection. Thus, T cell activation in infected *Tbx21*$^{-/-}$ mice was assessed. However, a phenotypic analysis of splenic CD4^{+} and CD8^{+} T cells from wild-type and *Tbx21*$^{-/-}$ mice on day 9 p.i. showed that T cell activation was not defective based on CD69 and CD25 expression. Further analysis for the presence of activated effector T cells, which are CD44^{hi}CD62L^{lo}, was performed. This revealed that the numbers of both CD4^{+} and CD8^{+} CD44^{hi}CD62L^{lo} T cells in the spleens of infected mice were comparable between wild-type and *Tbx21*$^{-/-}$ mice (**Figure 2A-B**). Analysis of peripheral blood leukocytes showed that the number of activated CD4^{+} T cells was also comparable (**Figure 2C**). However, the number of activated CD8^{+} CD44^{hi}CD62L^{lo} T cells in the blood of infected *Tbx21*$^{-/-}$ mice was significantly reduced compared to infected wild-type mice (**Figure 2D**). Thus, although *Tbx21*$^{-/-}$ mice showed normal signs of T cell activation during *T. cruzi* infection, they exhibited a striking decrease in the number of effector CD8^{+} T cells in the blood.

CD8^{+} cytotoxic T lymphocytes (CTLs) are an essential component of protective immune responses during infection with intracellular pathogens, including *T. cruzi*. The expansion of *T. cruzi*-specific CTLs is a critical step for controlling parasite replication

and subsequent resistance. T-bet and the related T-box family member Eomesodermin, or Eomes, regulate $CD8^+$ CTL effector mechanisms such as IFN-γ, and expression of perforin and granzyme molecules which are required for cytotoxicity[51,52]. T-bet has also been shown to regulate the expansion of Ag-specific CTLs in several studies[53,54,55]. Therefore, the increased susceptibility of *Tbx21*$^{-/-}$ to *T. cruzi* and the reduced numbers of effector $CD8^+$ T cells in the blood of infected mice could be due to the improper development of *T. cruzi*-specific $CD8^+$ T cell responses. Examination of the immunodominant $CD8^+$ T cell responses to the *T. cruzi*-derived *trans*-sialidase (ts) epitopes Tskb20 and Tskb18 using fluorescent MHC class I tetramers, revealed that infected *Tbx21*$^{-/-}$ mice generated significantly fewer Ag-specific T cells compared to wild-type controls, and this defect was evident in both the spleen (**Figure 3A-B**) and in the blood (**Figure 3C**). These results demonstrated that T-bet regulates the generation of *T. cruzi*-specific $CD8^+$ T cells. However, because T-bet is also expressed in other cell types including APCs, the impaired expansion of Ag-specific $CD8^+$ T cells during infection could be a consequence of defective APC function. Chapter 1 of this dissertation will address this issue and establish in what capacity T-bet is required for the generation of *T. cruzi*-specific $CD8^+$ T cell responses.

In addition to $CD8^+$ T cell-mediated immunity, the development of a strong Th1-immune response is imperative for protection against *T. cruzi* infection. Given the known role of T-bet in Th1 cell differentiation, T cell responses of infected mice were analyzed for IFN-γ production. Recall responses of C57BL/6 and *Tbx21*$^{-/-}$ mice infected with *T. cruzi* were assessed ex vivo by re-stimulation with anti-CD3 antibody or *T. cruzi* lysate. Surprisingly, infected C57BL/6 and *Tbx21*$^{-/-}$ mice produced comparable levels of

IFN-γ (**Figure 4A**). Thus, the increased susceptibility of *Tbx21*$^{-/-}$ mice to *T. cruzi* infection was not a result of impaired Th1 immunity. Surprisingly, infected *Tbx21*$^{-/-}$ mice produced abundant levels of the inflammatory cytokine IL-17 (**Figure 4B**). Importantly, the cellular source of the increased IL-17 was CD4^{+} T cells, as CD4 blockade with anti-CD4 Ab abrogated IL-17 production ex vivo (**Figure 4C**). These results demonstrated that in the absence of T-bet, mice infected with *T. cruzi* display increased Th17 differentiation. CD4^{+} T cells were confirmed to be the source of IL-17 by intracellular cytokine staining. Surprisingly, there were CD4^{+} T cells from infected-*Tbx21*$^{-/-}$ mice that produced either IFN-γ, IL-17, or both cytokines concomitantly, whereas cells from wild-type C57BL/6 mice produced IFN-γ only (**Figure 4D**). These results showed that T-bet inhibits the differentiation of Th17 cells during *T. cruzi* infection. Additional experiments utilizing the adoptive transfer of *Tbx21*$^{-/-}$ T cells into *Rag-2*$^{-/-}$ recipient mice (*Tbx21*$^{+/+}$) established that T-bet regulates Th17 development in a T cell-intrinsic manner (not shown). As a result of the increased Th17 response, *Tbx21*$^{-/-}$ developed a fulminant neutrophilia, a hallmark of Th17-mediated inflammation. Thus, while the results demonstrated that T-bet was dispensable for Th1 development during *T. cruzi* infection, it was required to repress the development of IL-17-producing Th17 cells.

Although *Tbx21*$^{-/-}$ mice infected with *T. cruzi* were capable of mounting a Th1/IFN-γ immune response, it was not sufficient to control acute infection. These results suggest that unregulated Th17 responses and the accompanying inflammation may be detrimental to protective immunity and resistance to *T. cruzi*. This was later supported by the finding that *T. cruzi*-infected mice lacking the bradykinin B2 receptors exhibited increased Th17 development which was associated with increased mortality[56].

However, it is difficult to ascertain whether the exact cause of increased susceptibility of infected *Tbx21*$^{-/-}$ mice is due to the failure to generate sufficient numbers of *T. cruzi*-specific CD8^{+} T cells, or rather a result of the increased pro-inflammatory Th17 response. Alternatively, both of these factors could contribute to increased susceptibility. It could be speculated that the generation of normal CD8^{+} T cell numbers could ameliorate or reduce the exacerbated Th17 response and associated inflammation in infected mice.

At the time of these findings in our laboratory, there were conflicting views on the roles of IFN-γ and T-bet in the negative regulation of Th17 development. Multiple reports cited that IFN-γ inhibited Th17 development[57,58,59], while others showed that T-bet was inhibitory[60,61,62]. However, the confounding issue at the core of these early studies was the presence of the positive feedback loop that exists between IFN-γ and T-bet, making it difficult to delineate the effects of the two on Th17 inhibition. **The novelty of our study was that the CD4^{+} T cell IFN-γ response was intact in infected T-bet-deficient mice, yet it had no effect on inhibiting the development of *T. cruzi*-specific Th17 cells**. This suggested that T-bet regulates Th17 development in an IFN-γ-independent manner. Thus, our findings not only supported the role of T-bet to inhibit the differentiation of Th17 cells, but also argued it could do so independent of IFN-γ.

While these studies were pivotal in establishing the importance of T-bet in antagonizing Th17 differentiation, the particular upstream regulatory cytokines and signaling molecules that lead to the induction of T-bet expression and subsequent inhibition of Th17 development remained to be identified. Given the pathogenic potential of Th17 cells in various disease states, it became imperative to gain a more

adequate understanding of the factors involved in the regulation of this cell type. Specifically, it remained unclear as to which cytokines and signaling pathways favor the induction of T-bet, and thus limit the development of Th17 responses during *T. cruzi* infection.

Figure 1. T-bet is required for resistance to *T. cruzi* infection.

C57BL/6 (dark diamonds) and *Tbx21*$^{-/-}$ mice (open circles) were infected with *T. cruzi* trypomastigotes and observed for A) weight loss and B) survival. Data are representative of five independent experiments, with 4-5 mice per experiment. ***p<0.0001.

Figure 1

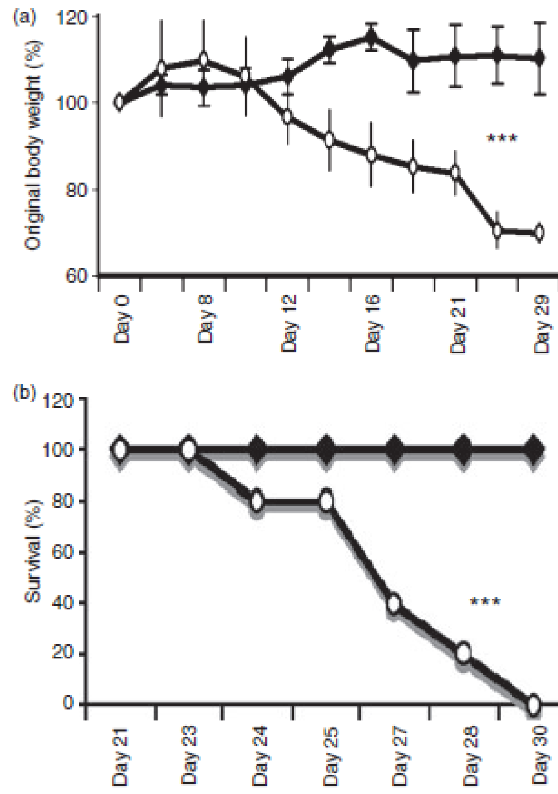


Figure 2. *Tbx21$^{-/-}$* mice infected with *T. cruzi* have reduced numbers of activated $CD8^+$ T cells in the blood.

Spleen and blood from naïve uninfected, infected C57BL/6, and infected *Tbx21$^{-/-}$* mice were collected on day 9 p.i. The total number of $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells (A) and the number of $CD44^{hi}CD62L^{lo}$ $CD8^+$ T cells (B) in the spleen of uninfected and infected mice was determined by flow cytometry. The absolute number of $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells (C) and the absolute number of $CD44^{hi}CD62L^{lo}$ $CD8^+$ T cells (D) in the blood of mice was determined by collecting 50,000 events by flow cytometry and multiplying by the frequency of $CD4^+CD44^{hi}CD62L^{lo}$ cells and $CD8^+CD44^{hi}CD62L^{lo}$ cells, respectively. Results are representative of three independent experiments consisting of three mice per group. ***$p<0.0001$.

Figure 2

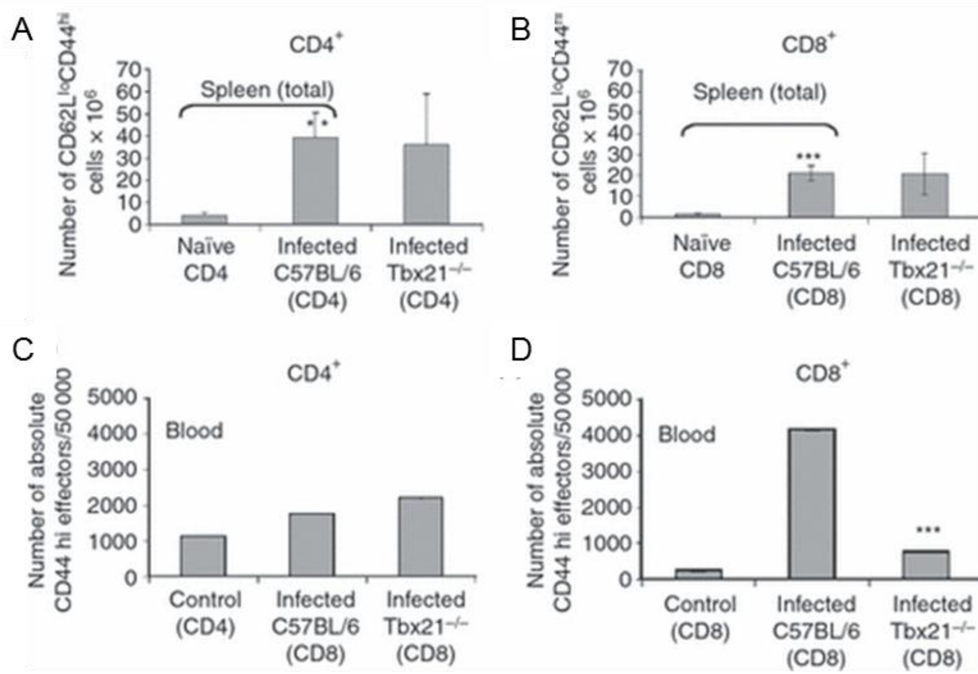


Figure 3. Reduced numbers of *T. cruzi*-specific CD8$^+$ T cells in infected *Tbx21*$^{-/-}$ mice.

Spleen (A-B) and blood (C) were harvested from infected mice day 9 p.i.. CD8$^+$ T cells were analyzed for staining with Tskb20 tetramers, and the absolute number of Tskb20$^+$ T cells per 50,000 cells (A,C) or the total number of Tskb20$^+$ T cells per spleen (B) determined by flow cytometry. Dot plots shown to right (arrows) are representative of tetramer staining pattern as well as the frequency of Tskb20$^+$ cells. Results shown represent at least five experiments with 3 mice per group. ***p<0.0001.

Figure 3

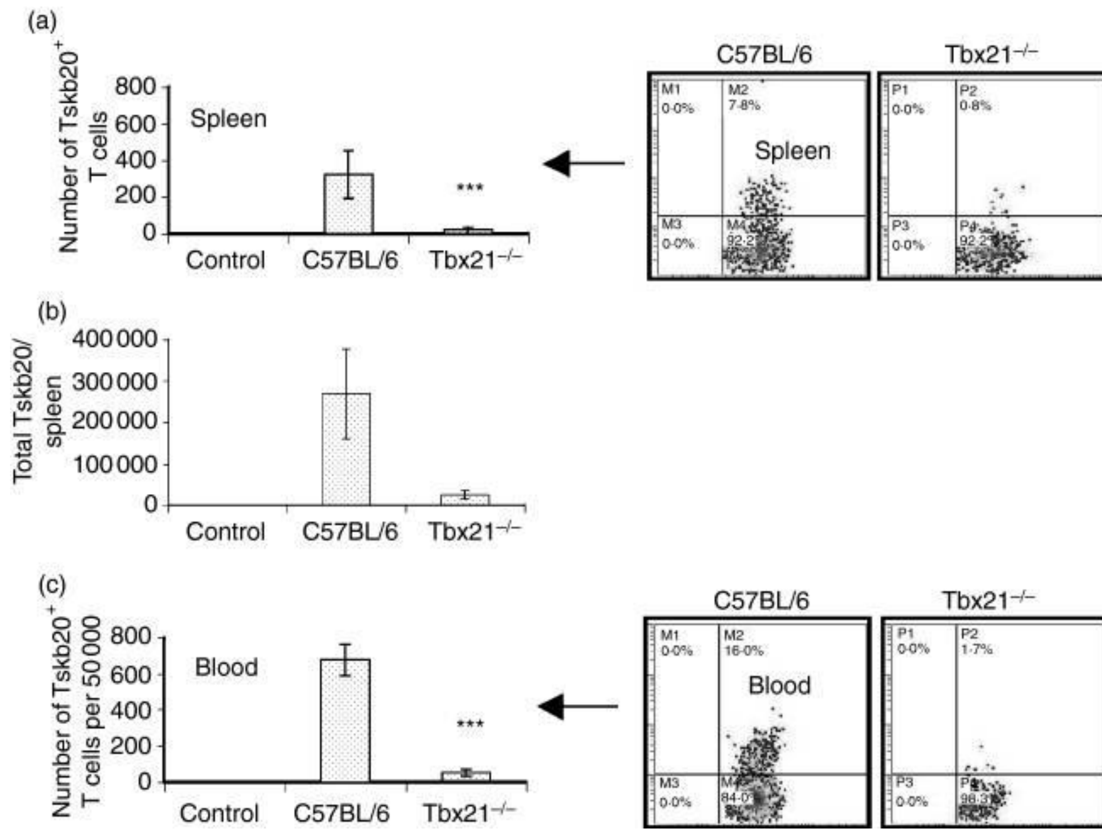
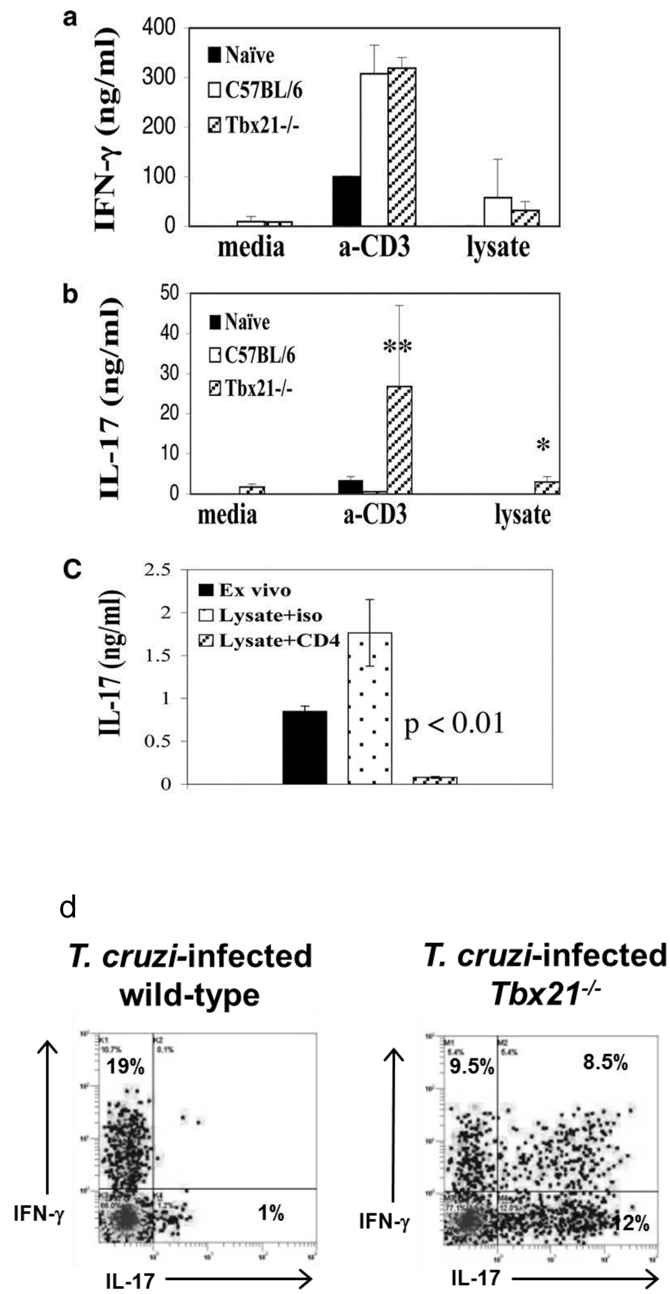


Figure 4. Robust Th17 differentiation in *Tbx21*$^{-/-}$ mice infected with *T. cruzi*. Spleens from infected mice were harvested day 9 p.i. and cultured with anti-CD3 or endotoxin-free *T. cruzi* lysate for 24 hours and supernatants tested for A) IFN-γ and B) IL-17 by ELISA. C) Anti-CD4 or an isotype control antibody was added to cultures to show that the Ag-specific response to *T. cruzi* Ags was mediated by CD4^{+} T cells. D) Spleen cells were stimulated with anti-CD3 in the presence of monensin and cells analyzed for intracellular IFN-γ and IL-17. 10,000 CD4^{+} or CD8^{+} T cells were analyzed by FACS. Numbers in quadrants represent the means of IFN-γ^{+}, IFN-γ^{+}/IL-17^{+}, and IL-17^{+} T cells among five independent experiments with at least two to three mice per group.

Figure 4



Dissertation Objective

Infection of mice genetically lacking the Th1 transcription factor T-bet (Tbx21) with the parasite *T. cruzi* leads to significant morbidity and mortality. Interestingly, these mice developed what normally is a protective Th1/IFN-γ response. However, a robust Th17 response developed simultaneously with this Th1 response. This response correlated with increased Th17-associated inflammation and decreased resistance to infection. The second major observation was that infected T-bet-deficient mice were ineffective at generating normal numbers of *T. cruzi*-specific $CD8^+$ T cells during infection. The purpose of this project was to further investigate these two major findings.

The main objectives of this dissertation project were 1) to characterize the role of T-bet in the generation of *T. cruzi*-specific $CD8^+$ T cells and 2) to further investigate the mechanisms by which T-bet regulates Th17 development during *T. cruzi* infection. The aims of this objective begin with investigating the molecular events and transcription factors involved in regulating Th17 responses during infection. This is followed by an examination of the extrinsic factors known to regulate key aspects of $CD4^+$ T cell differentiation in order to identify the important cytokines involved in the T-bet-dependent regulation of Th17 development during infection with *T. cruzi*.

Chapter 1: Regulation of $CD8^+$ T cell expansion during *Trypanosoma cruzi* infection

Introduction

CD8$^+$ T cells play an unequivocal role in providing protective resistance to *T. cruzi* infection. The most critical function of CD8$^+$ T cells is their ability to detect and kill host cells that are infected with *T. cruzi* parasites. This is accomplished by recognizing parasite-derived antigens displayed by MHC class I molecules in a T cell receptor (TCR)-dependent manner. CD8$^+$ T cells secrete IFN-γ and cytotoxic molecules such as perforin and granzymes which kill infected target cells. Studies in which CD8$^+$ T cells were depleted clearly demonstrate their requirement as these mice fail to survive a non-lethal challenge by *T. cruzi*[63,64,12]. Also, the criticality of IFN-γ has been firmly established in studies where IFN-γ-deficient mice developed accelerated heart pathology following infection[65]. Mice lacking perforin and granzymes are also more susceptible to infection. Thus, CD8$^+$ T cells and IFN-γ are absolutely necessary for resistance and immunity to *T. cruzi*. Efforts by Tarleton and colleagues significantly advanced the understanding of CD8$^+$ T cell immunity to *T. cruzi* infection when they identified immunodominant epitopes derived from the *trans*-sialidase (*Ts*) family of proteins. During acute infection, as much as 30% of the CD8$^+$ T cell response is specific for *T. cruzi Ts* epitope Tskb20[66]. This antigen (Ag)-specific response to *Ts* family is also found at a high frequency in humans and can be detected more than 20 years after initial infection.

The importance of effective CD8$^+$ T cell responses and IFN-γ production is clearly demonstrated in human studies of chronic Chagas disease. Patients with more severe disease had very few *T. cruzi*-specific T cells producing IFN-γ, whereas healthy patients

had a high frequency of responsive $CD4^+$ and $CD8^+$ T cells[67]. Although much is known about the requirement of $CD8^+$ T cell responses for control of *T. cruzi* infection, the exact mechanisms that regulate their generation, expansion, and maintenance are not well characterized. Due to the necessity of $CD8^+$ T cells in immunity to *T. cruzi* infection, many studies have focused on harnessing this response for vaccine development. Unfortunately, a human vaccine that provides protection from *T. cruzi* infection still does not exist. It is imperative that we gain a full understanding of the factors regulating the development and generation of *T. cruzi*-specific CTL immunity to further the advancement of *T. cruzi* vaccine development.

Among the factors involved in promoting $CD8^+$ T cell responses, the T-box family transcription factors, T-bet and Eomes, have a profound influence on the development and effector functions of $CD8^+$ T cells. T-bet was initially characterized for its ability to promote the production of IFN-γ by $CD8^+$ T cells. Additionally, it regulates the generation of cytotoxicity by $CD8^+$ T cells. For example, in both in vitro and in vivo cytotoxicity assays, $CD8^+$ T cells from T-bet-deficient OT-1 TCR transgenic mice do not exhibit the full capacity to kill target cells[51]. Interestingly, their cell killing ability is not always completely lost in the absence of T-bet, indicating the presence of other compensatory factors. Indeed, Eomes can often times provide overlapping functions with T-bet. Like T-bet, Eomes promotes the expression of IFN-γ, perforin, and granzymes by $CD8^+$ T cells[68]. Thus, T-bet and Eomes are critical transcriptional activators of CTL function.

In addition to its ability to confer cytotoxicity, T-bet has been reported to support the activation and expansion of $CD8^+$ T cells. In the absence of T-bet, $CD8^+$ T cells

have impaired proliferative responses to vaccinia virus and LCMV[53,55]. Our initial studies investigating the role of T-bet during infection with *T. cruzi* revealed a profound requirement for T-bet in providing resistance. It was observed that T-bet-deficient mice infected with *T. cruzi* failed to generate sufficient numbers of Tskb20-specific $CD8^+$ T cells[69]. Given the immunodominant nature of this antigen-specific response, it's easy to hypothesize that this defect could have significant consequences on the ability of mice to control *T. cruzi* infection. The primary aim of this study was to establish whether the defective expansion of *T. cruzi*-specific $CD8^+$ T cells was due to a defect that was intrinsic to $CD8^+$ T cells or to the APCs that initiate the activation and subsequent expansion of T cells.

Materials and Methods

Mice and parasite infections

Age and sex-matched C57BL/6, *Tbx21*$^{-/-}$, and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1) mice were obtained from The Jackson Laboratory and were used between six and eight weeks of age. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility under pathogen-free conditions and used in accordance with an Institutional Animal Care and Use Committee-approved protocol. For infections, mice were injected intraperitoneally (i.p.) with $1x10^6$ *T. cruzi* trypomastigotes (CL strain).

Fluorescent antibodies, tetramer staining, and flow cytometry

Peripheral blood leukocytes and splenocytes were harvested from infected mice and pre-incubated with purified anti-CD16/32 (Fc block), then labeled with FITC anti-CD8a (5H10 clone, Biolegend), PE anti-CD11b, PE anti-CD4, and PE anti-B220, followed by allophycocyanin (APC)-coupled MHC class I tetramers consisting of Tskb20 peptides bound to H-2K^b molecules. Tetramers were synthesized by the NIH Tetramer Core Facility and used according to instructions. For detection of tetramer-positive cells, PE-positive cells were first excluded and a gate was set for CD8$^+$ cells. The absolute number of Tskb20$^+$ T cells was calculated by multiplying the frequency of tetramer-positive CD8$^+$ T cells by the total number of CD8$^+$ T cells per 50,000 events. Alternatively, T cells were labeled with FITC anti-CD62L, APC anti-CD44, and PE anti-CD25 fluorescent antibodies and analyzed by FACS to asses T cell activation and

effector cell populations. All flow cytometry analysis was performed on a Beckman Coulter FC500 instrument.

Intracellular T-bet staining

First, cell surface staining with anti-CD4 (FITC) was performed. Cells were then fixed with Foxp3 Fix/Perm (Biolegend) for 20 minutes at room temperature, and then spun down and the supernatant removed. Fixed cells were washed once with cell staining buffer and centrifuged at 250g for 5 minutes and the supernatants were removed. The cell pellets were then washed once with Foxp3 Perm Buffer (Biolegend). Cells were then re-suspended in Foxp3 Perm Buffer and incubated for 15 minutes at room temperature. Following permeabilization, cells were spun down and then re-suspended in 100μl of Foxp3 Perm Buffer and stained with anti-T-bet (PE; Biolegend, clone 4B10) for 30 minutes at 4°C. Cells were washed twice with cell staining buffer and then analyzed by flow cytometry.

Detection of apoptosis

Cells were harvested from blood and spleen of infected mice on day 9 post-infection, and labeled with APC/Cy7 anti-CD8 antibodies as described above. Cells were subsequently re-suspended in Annexin V binding buffer, then stained with PE-anti-Annexin V according to the manufacturer's instructions and analyzed by FACS.

Dendritic cell activation

On days 2, 4, and 6 post-infection, C57BL/6 and *Tbx21*$^{-/-}$ mice were euthanized and spleens removed. Splenocytes were subsequently incubated with purified anti-mouse CD16/CD32 to block Fc receptor binding, followed by incubation with APC anti-CD11c, APC/Cy7 anti-CD8(clone 53-6.7), FITC anti-CD80, PE anti-CD86, PE anti-CD40, FITC anti-H-2K^{b}, and PE anti-I-A^{b} (all from Biolegend). Expression of these markers among CD11c^{+} DC was determined by FACS.

In vivo priming assay

Naïve CD8^{+} T cells were purified from pooled spleen and lymph nodes of OT-1 TCR transgenic mice using CD8^{+} T cell isolation kits and negative selection (Miltenyi Biotec). CD8^{+} T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) at a final concentration of 5μM, and 5 million wild-type OT-1 T cells injected intravenously (i.v.) into C57BL/6 or *Tbx21*$^{-/-}$ mice previously infected with *T. cruzi* (day 2 p.i.). Twenty-four hours post-transfer (day 3 p.i.), infected mice were immunized i.p. with 200 μg of ovalbumin protein emulsified in incomplete Freund's adjuvant. Infected mice were euthanized day 6 p.i., and CFSE dilution was measured by FACS.

Adoptive transfer into Rag-2$^{-/-}$ *recipients*

Thy1.2^{+} T cells were purified from pooled spleens and lymph nodes of naïve C57BL/6 or *Tbx21*$^{-/-}$ mice using Thy1.2^{+} microbeads and positive selection (Miltenyi Biotec). Approximately 20 million T cells were injected i.v. into naïve *Rag-2*$^{-/-}$ recipient mice, which were subsequently infected 24 hours later with 1×10^{6} *T. cruzi* parasites by i.p.

injection. On day 9 p.i., recipient mice were euthanized and the spleens and blood collected for analysis of $CD8^+$ T cell expansion by FACS.

Statistical analysis

One-way analysis of variance (ANOVA), two-tailed tests, and Tukey-Kramer multiple comparsion post-tests were performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software). A p value of less than 0.05 was considered significant.

Results

CD8^{+} T cells in infected Tbx21$^{-/-}$ mice do not accumulate in non-lymphoid tissue.

Chemokines facilitate the trafficking of lymphocytes to sites of ongoing inflammation. The expression of chemokine receptors on activated T cells is important for their appropriate migration to inflamed tissues where they can carry out their effector functions. Th1 and CD8^{+} T cells express the chemokine receptor CXCR3, which binds to its ligands CXCL11 and CXCL10[70]. T-bet controls this migratory potential by directly inducing the expression of CXCR3 in activated T cells[71]. T-bet-deficiency causes CD4^{+} and CD8^{+} T cells to be impaired in their ability to traffic to inflamed tissues in response to chemotactic signals. Thus, we hypothesized that the reduced numbers of *T. cruzi*-specific CD8^{+} T cells in the blood and spleens of infected *Tbx21*$^{-/-}$ mice could be a result of an alteration in T cell trafficking.

On day 9 post-infection, livers were harvested from uninfected, infected C57BL6, and infected *Tbx21*$^{-/-}$ mice. Mononuclear liver cells were prepared and analyzed for the presence of CD8^{+} T cells. The frequency of CD8^{+} cells was increased in the livers of infected C57BL/6 mice (~40%) compared to naïve uninfected mice (~5%) (**Figure 5**). Although infected *Tbx21*$^{-/-}$ mice exhibited an increase in the frequency of CD8^{+} T cells in the liver compared to uninfected mice (~10% vs. ~5%), this frequency was considerably lower than infected C57BL/6 mice (**Figure 5**). Thus, CD8^{+} T cells in *Tbx21*$^{-/-}$ mice infected with *T. cruzi* did not exhibit an abnormal increase in migration to the liver. These results show that the reduced numbers of *T. cruzi*-specific CD8^{+} T cells in *Tbx21*$^{-/-}$ mice was not due to altered trafficking and accumulation in non-lymphoid tissue.

$CD8^+$ T cells from infected $Tbx21^{-/-}$ mice do not exhibit increased apoptosis.

The inability of *$Tbx21^{-/-}$* mice infected with *T. cruzi* to generate sufficient numbers of Ag-specific $CD8^+$ T cells could be due to increased apoptosis. During apoptosis, the phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane of cells to the outer leaflet[72]. This process exposes PS such that it can be readily detected by staining cells with the phospholipid-binding protein Annexin V, which binds PS with high affinity. Thus, the percentage of cells undergoing apoptosis can be measured. To assess the degree of apoptosis in $CD8^+$ T cells during *T. cruzi* infection, cells were analyzed for Annexin V staining by flow cytometry.

Spleens and blood were harvested from uninfected, infected C57BL/6, and infected *$Tbx21^{-/-}$* mice on day 9 p.i. $CD8^+$ T cells in the spleens of infected C57BL/6 mice showed a moderate increase in Annexin V staining compared to uninfected mice (**Figure 6**). $CD8^+$ cells from the spleens of infected *$Tbx21^{-/-}$* showed similar levels of Annexin V staining. The percentage of Annexin V^+ cells in the blood was comparable between all groups. Based upon Annexin V analysis, there was no significant difference in apoptosis between wild-type and *$Tbx21^{-/-}$* $CD8^+$ T cells during infection. Thus, these results demonstrate that the reduced numbers of $CD8^+$ T cells in infected *$Tbx21^{-/-}$* mice is not caused by an increase in apoptosis.

Normal activation of dendritic cells in infected $Tbx21^{-/-}$ mice.

Dendritic cells are essential for inducing $CD8^+$ T cell expansion during infection. Given that T-bet is also expressed in DCs and has been reported to regulate DC function[73], we reasoned that the impaired expansion of $CD8^+$ T cells in *$Tbx21^{-/-}$* mice

could be a result of defective APC functions. Therefore, we sought to determine whether DCs from *Tbx21*$^{-/-}$ mice were adversely affected in their ability to prime CD8^{+} T cell responses during *T. cruzi* infection.

We first hypothesized that the activation of DCs may be impaired in the absence of T-bet, which would then hinder the DC's ability to stimulate optimal CD8^{+} T cell activation and expansion. DC activation is characterized by increased expression of the molecules CD80 (B7.1), CD86 (B7.2), and CD40. Reduced expression of these co-stimulatory molecules could profoundly impact T cell activation and subsequent expansion. To determine if DCs from infected *Tbx21*$^{-/-}$ mice underwent normal activation, C57BL/6 and *Tbx21*$^{-/-}$ mice were infected with *T. cruzi*. On day 6 p.i., splenocytes were stained for the DC-specific marker CD11c and analyzed for signs of activation. CD11c^{+} DCs from wild-type C57BL/6 mice infected with *T. cruzi* exhibited significant up-regulation of CD80, CD86, and CD40 compared to DCs from uninfected mice (**Figure 7A-B**). Importantly, we observed that CD11c^{+} DCs from infected *Tbx21*$^{-/-}$ mice showed similar increases in CD80, CD86, and CD40 expression compared to wild-type DCs (**Figure 7B-C**). The expression of H-2K^{b} (MHC class I) and I-A^{b} (MHC class II) was also similar between wild-type and *Tbx21*$^{-/-}$ DCs (**Figure 7A-C**), which suggests that antigen presentation was normal. These results are in agreement with a report showing that DC maturation is T-bet-independent. Thus, the activation state of DCs from *Tbx21*$^{-/-}$ mice in response to infection was normal and not a cause of reduced numbers of *T. cruzi*-specific CD8^{+} T cells.

Normal antigen presentation functions of dendritic cells in infected Tbx21$^{-/-}$ mice.

In addition to proper activation status, DCs must also be able to effectively process and present antigen to induce T cell activation. It remained plausible that the APC functions of *Tbx21*$^{-/-}$ mice could be leading to the reduction of CD8^{+} T cell numbers. To determine whether APC functions were defective in the absence of T-bet, we utilized an in vivo approach to assess APC function. *Tbx21*$^{-/-}$ APCs were tested for their ability to induce the expansion of ovalbumin (OVA)-specific T cells in vivo during *T. cruzi* infection. First, CD8^{+} T cells from uninfected OT-1 TCR transgenic mice that specifically recognize the OVA protein were labeled with CFSE and then adoptively transferred into either wild-type or *Tbx21*$^{-/-}$ mice infected with *T. cruzi* 2 days prior (**Figure 8A**). One day after transfer of OT-1 cells, infected mice were immunized with OVA protein to assess OVA-specific T cell responses. Infected mice were then rested for three days following immunization to allow for T cell priming, then on day 6 p.i. mice were euthanized and spleens and lymph nodes collected. Dilution of CFSE was used as a means to evaluate cell expansion of transferred OVA-specific CD8^{+} T cells. OT-1 CD8^{+} T cells that were transferred into infected wild-type mice, which have T-bet-expressing APCs, underwent extensive expansion, with most cells reaching 5 to 6 divisions (**Figure 8B**). OT-1 CD8^{+} T cells that were transferred into infected *Tbx21*$^{-/-}$ mice, which have *Tbx21*$^{-/-}$ APCs, expanded just as efficiently as cells that were primed by wild-type APCs (**Figure 9A**). Further, in both cases OT-1 CD8^{+} T cells acquired the ability to produce IFN-γ as determined by intracellular cytokine staining (**Figure 9B**). The results of this experiment showed that T-bet-deficient APCs were just as effective at priming Ag-specific CD8^{+} T cell responses as wild-type APCs during *T. cruzi*

infection. Also, it provides another line of evidence showing that *Tbx21*$^{-/-}$ DCs are not defective at inducing CD8^{+} T cell expansion and thus not the cause of the impaired generation of *T. cruzi*-specific CD8^{+} T cells.

T-bet regulates CD8^{+} T cell expansion in response to T. cruzi infection in a T cell-intrinsic manner.

Based on our findings to this point, which showed that *Tbx21*$^{-/-}$ DCs are normal in their ability to become activated and that they retain normal APC functions during *T. cruzi* infection, we hypothesized that T-bet must regulate the expansion of *T. cruzi*-specific CD8^{+} T cells in a T cell-intrinsic manner. To formally demonstrate this, we sought to determine whether T-bet was specifically required in T cells in order for expansion to occur. Total Thy1.2^{+} T cells (CD4 and CD8 T cells) purified from uninfected C57BL/6 or *Tbx21*$^{-/-}$ donor mice were adoptively transferred into *Rag-2*$^{-/-}$ recipient mice, which have Tbx21$^{+/+}$ wild-type APCs. One day following adoptive transfer of wild-type or *Tbx21*$^{-/-}$ T cells, recipient mice were infected with *T. cruzi*. Spleens and blood were collected on day 9 p.i. and the frequency of CD8^{+} T cells was determined (**Figure 10A**). The percentage of wild-type CD8^{+} T cells was significantly higher than that of the *Tbx21*$^{-/-}$ CD8^{+} T cells in the spleens and blood of the *Rag-2*$^{-/-}$ recipient mice infected with *T. cruzi* (**Figure 10B**). Thus, the expansion of *Tbx21*$^{-/-}$ CD8^{+} T cells during *T. cruzi* infection was impaired despite the presence of wild-type, T-bet-expressing APCs. These results demonstrate that the expansion of CD8^{+} T cells in response to *T. cruzi* infection is regulated by T-bet in a T cell-intrinsic manner.

$CD8^+$ T cell expansion during T. cruzi infection is regulated by T-bet in a $CD8^+$ T cell-intrinsic manner.

Help from $CD4^+$ T cells is an important component for the generation of $CD8^+$ T cell responses[74,75]. $CD8^+$ T cells that do not receive help may not effectively undergo primary expansion or provide adequate secondary protection[76,77,78]. $CD4^+$ T cells help in the priming of $CD8^+$ T cell responses by "licensing" dendritic cells. Engagement of CD40 via CD40 ligand (CD40L)-expressing $CD4^+$ T cells potentiates DCs to become more effective at stimulating naïve $CD8^+$ T cells[79,80,81]. Therefore, we reasoned that the impaired expansion of $CD8^+$ T cells in infected *Tbx21*$^{-/-}$ mice could be a result of defective T-bet-dependent $CD4^+$ T cell help. To address this concern, we used an adoptive transfer approach to determine if the provision of wild-type $CD4^+$ T cells would restore the expansion of T-bet-deficient $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells were purified from wild-type and *Tbx21*$^{-/-}$ mice. T cell subsets were then recombined such that *Rag-2*$^{-/-}$ recipient mice were adoptively transferred with either wild-type $CD4^+$/wild-type $CD8^+$ (positive control for expansion), *Tbx21*$^{-/-}$ $CD4^+$/*Tbx21*$^{-/-}$ $CD8^+$ (negative control for expansion), or wild-type $CD4^+$/*Tbx21*$^{-/-}$ $CD8^+$ T cells. The recipient mice were then infected with *T. cruzi*. Mice were bled on day 10 and 13 p.i. and analyzed for the frequency of $CD8^+$ T cells. As expected, and in accordance with previous results, *Tbx21*$^{-/-}$ $CD8^+$ T cells that were transferred with *Tbx21*$^{-/-}$ $CD4^+$ T cells underwent less expansion than wild-type $CD8^+$ T cells transferred with wild-type $CD4^+$ T cells as demonstrated by the low percentage of cells in the blood. Similar to *Rag-2*$^{-/-}$ mice that received *Tbx21*$^{-/-}$ $CD4^+$ and $CD8^+$ T cells, the percent of *Tbx21*$^{-/-}$ $CD8^+$ T cells in the blood and spleens of mice that received wild-type $CD4^+$ was considerably less than that

observed in the wild-type control group (**Figure 11**). Thus, despite the presence of wild-type CD4$^+$ T cell help, *Tbx21*$^{-/-}$ CD8$^+$ T cells still failed to undergo expansion during *T. cruzi* infection.

CD8$^+$ T cells in IL-12p35-deficient mice have reduced T-bet expression during T. cruzi infection.

In addition to antigen recognition (signal 1) and co-stimulation (signal 2), CD8$^+$ T cells require a third signal to become fully activated and to undergo clonal expansion. The inflammatory cytokines present at the time of CD8$^+$ T cell activation provide signal 3. Multiple cytokines have been shown to effectively act as signal 3 following infection. In particular, the cytokine IL-12 and the type I interferons have been implicated in serving as signal 3 and promoting the expansion of CD8$^+$ T cells following infection with *L. mono* and LCMV, respectively[82,83]. Given the fact that IL-12 significantly mediates resistance to *T. cruzi* and has been shown to promote Ag-specific lymphoproliferation during infection[84,85], we hypothesized that T-bet may be important for mediating the effects of IL-12 in CD8$^+$ T cells during *T. cruzi* infection. To determine if IL-12 was important for the induction of T-bet in CD8$^+$ T cells, IL-12p35-deficient (*IL-12p35*$^{-/-}$) mice were infected with *T. cruzi*. On day 9 p.i. CD8$^+$ T cells were analyzed for T-bet expression. CD8$^+$ T cells from infected C57BL/6 mice significantly up-regulated T-bet expression compared to CD8$^+$ T cells from uninfected mice (**Figure 12A**). In contrast, CD8$^+$ T cells from infected *IL-12p35*$^{-/-}$ mice had reduced T-bet expression compared to infected wild-type mice (**Figure 12B**). Given that T-bet expression was significantly reduced in infected *IL-12p35*$^{-/-}$ mice, we hypothesized that there would be a significant

reduction in the number of $CD8^+$ T cells similar to what we found in infected *Tbx21*$^{-/-}$ mice. Indeed, the frequency of $CD8^+$ T cells in the blood of infected *IL-12p35*$^{-/-}$ mice was reduced compared to infected wild-type mice (**Figure 12C**). These results highlight an important role for IL-12 in promoting T-bet expression and $CD8^+$ T cell expansion during *T. cruzi* infection. Additionally, it suggests that IL-12 is important for the expansion of *T. cruzi*-specific $CD8^+$ T cells.

Figure 5. CD8$^+$ T cells in infected *Tbx21$^{-/-}$* mice do not accumulate in non-lymphoid tissues.

Liver and blood were harvested from naïve uninfected, infected C57BL/6, and *Tbx21$^{-/-}$* mice on day 9 p.i., and the percentage of CD8$^+$ T cells determined by flow cytometry. Results shown are representative of two experiments, with 2-3 mice per group.

Figure 5

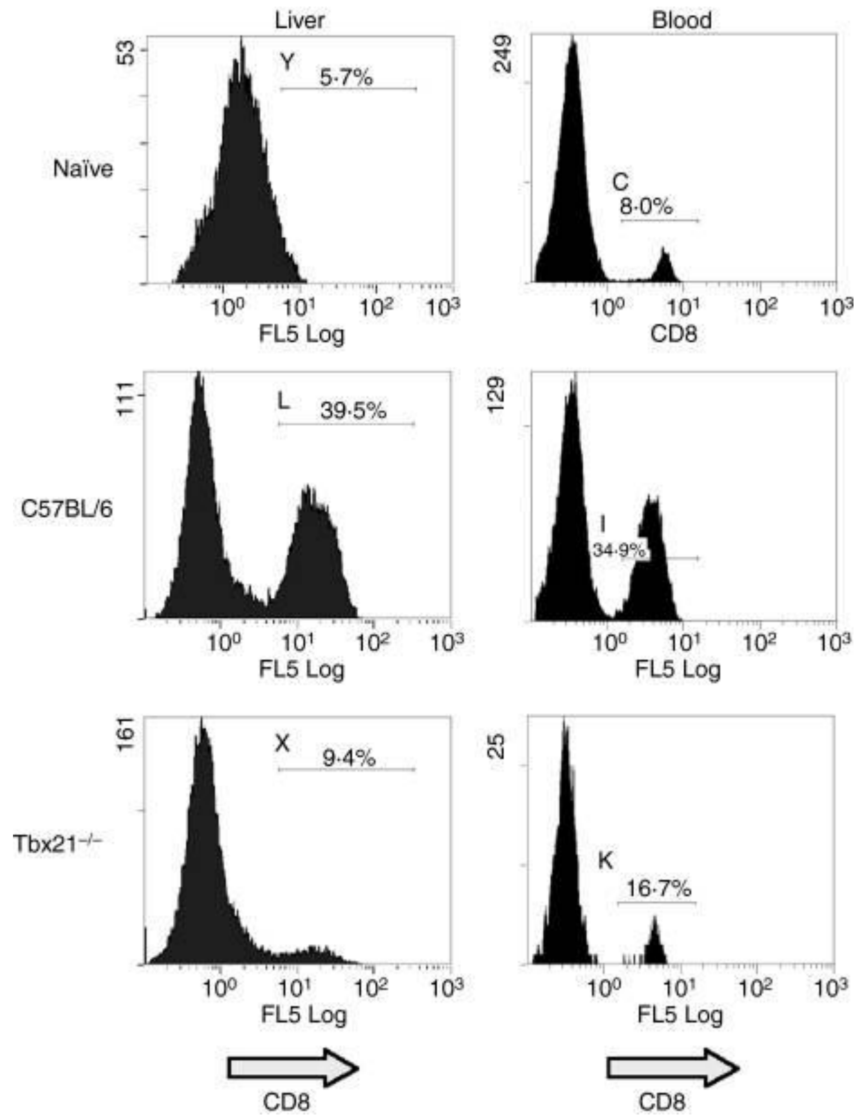


Figure 6. CD8$^+$ T cells from infected *Tbx21$^{-/-}$* mice do not exhibit increased apoptosis.

Spleen and blood were collected from naïve and *T. cruzi*-infected mice on day 9 p.i., and CD8$^+$ T cells were analyzed for Annexin V staining by flow cytometry. Results shown are representative of three independent experiments with 2-3 mice per group.

Figure 6

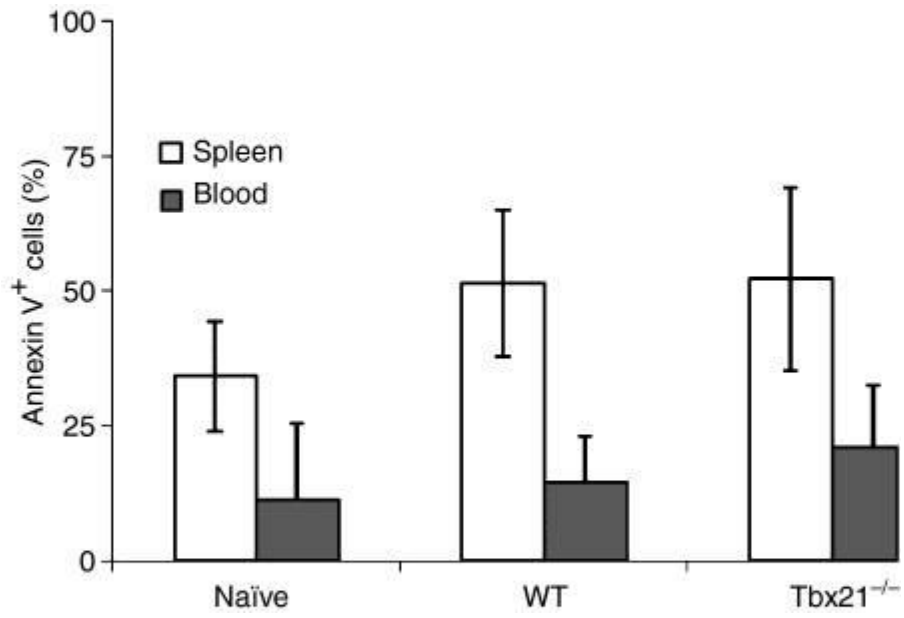


Figure 7. Dendritic cells from infected *Tbx21*$^{-/-}$ mice display normal signs of activation.

Spleen cells were harvested from naïve and infected mice day 6 p.i., and labeled with anti-CD11c, anti-CD80 (B7-1), CD86 (B7-2), CD40, I-A^{b} (MHC class II) and H-2K^{b} (MHC class I). A total of 5,000 CD11c^{+} dendritic cells were collected and analyzed by flow cytometry. Numbers reflect the relative proportion of CD11c^{+} DC expressing the indicated surface marker. Results shown are representative of four independent experiments with 2 mice per group.

Figure 7

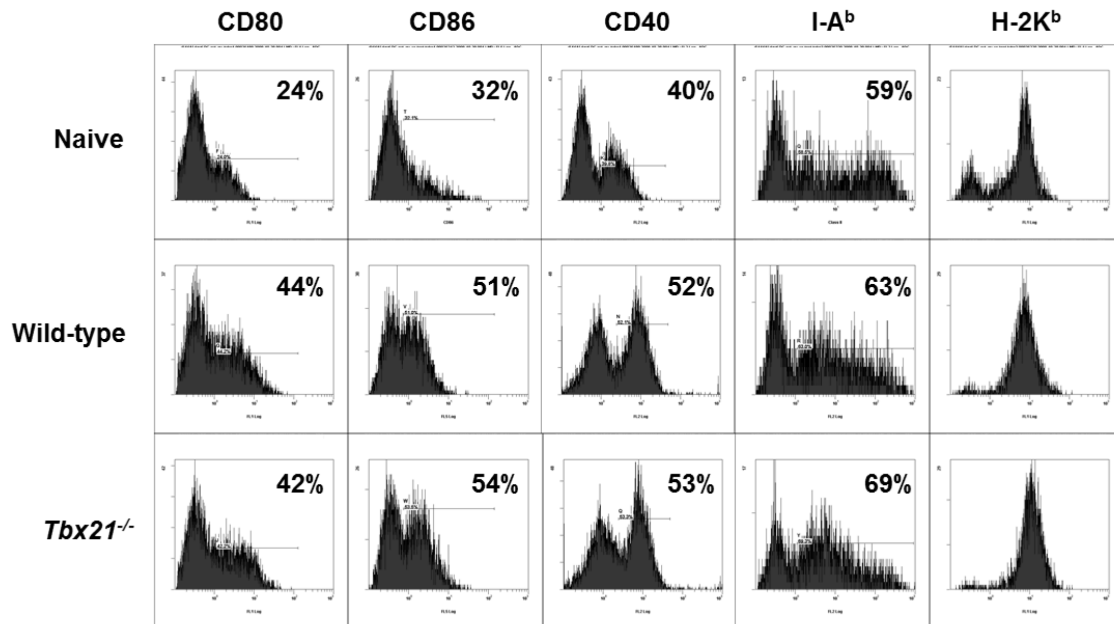
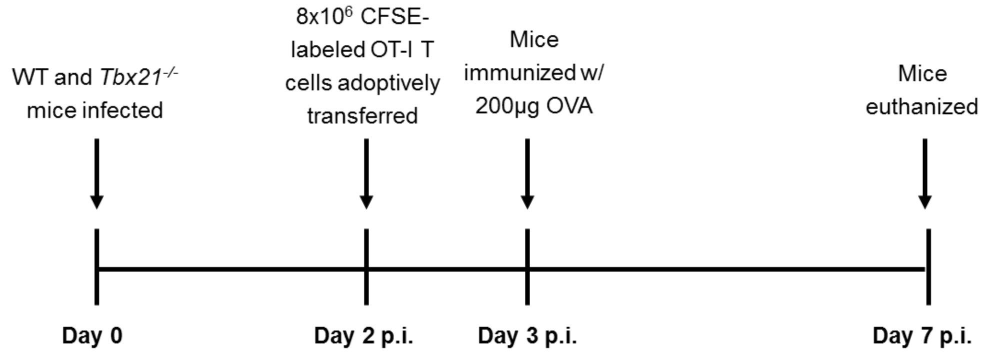


Figure 8. Experimental approach for OT-1 CD8$^+$ T cell priming in *Tbx21$^{-/-}$* mice.

A) CD8$^+$ T cells from naïve OT-1 TCR transgenic mice were purified and labeled with CFSE, and transferred into C57BL/6 or *Tbx21$^{-/-}$* mice previously infected with *T. cruzi* (day 2 p.i.). Twenty-four hours post-transfer, infected mice were immunized with OVA protein emulsified in oil and subsequently euthanized day 6 p.i. On day 7 p.i. mice were euthanized to analyze in vivo CD8$^+$ T cell priming. B) CD8$^+$ gated T cells were analyzed by flow cytometry to assess cell division as measured by CFSE dilution. Representative histograms showing CFSE profiles of OT-1 CD8$^+$ T cells from wild-type and *Tbx21$^{-/-}$* mice are shown.

Figure 8

A



B

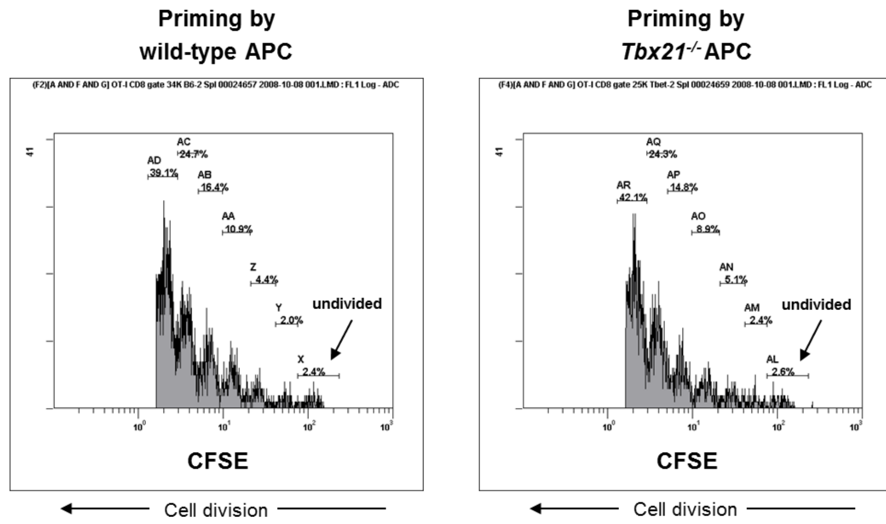
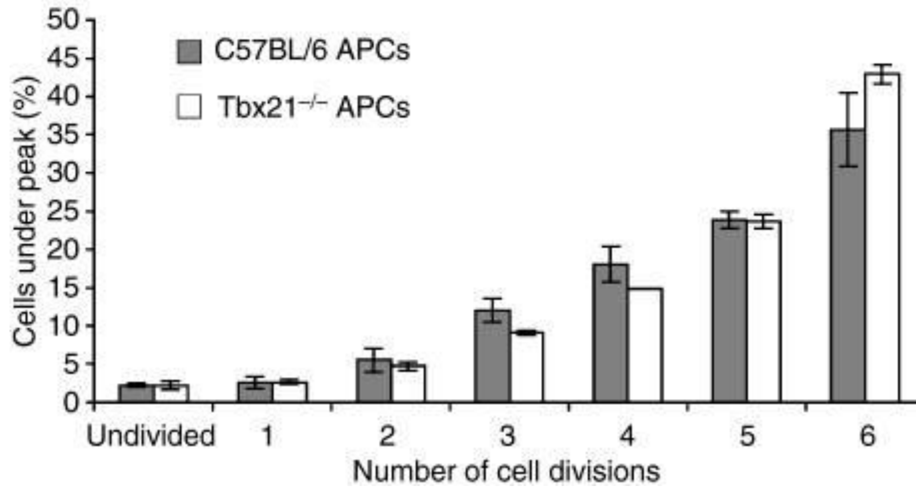


Figure 9. Dendritic cells from infected *Tbx21*$^{-/-}$ mice exhibit normal antigen presentation to CD8^{+} T cells in vivo.

A) Spleens were removed and CFSE^{+} cells were analyzed for CFSE dilution to determine the number of cell divisions induced by OVA. B) CD8^{+}CFSE^{+} cells were analyzed for intracellular IFN-γ expression by ICS. Results from 3 independent experiments are shown, with 2 mice per group.

Figure 9

A



B

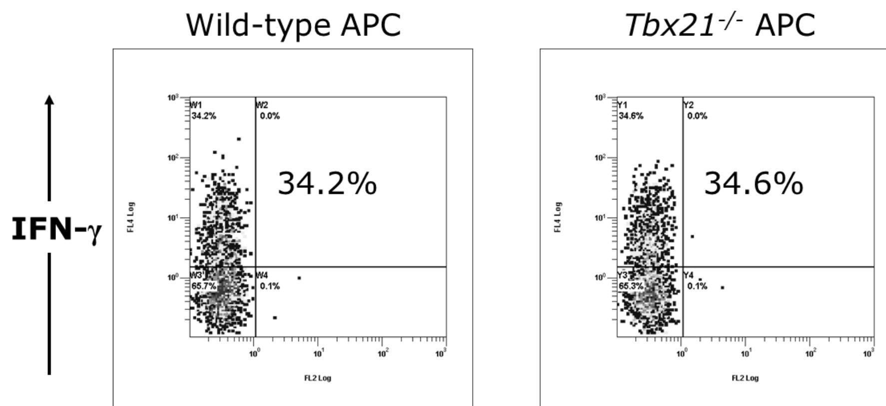
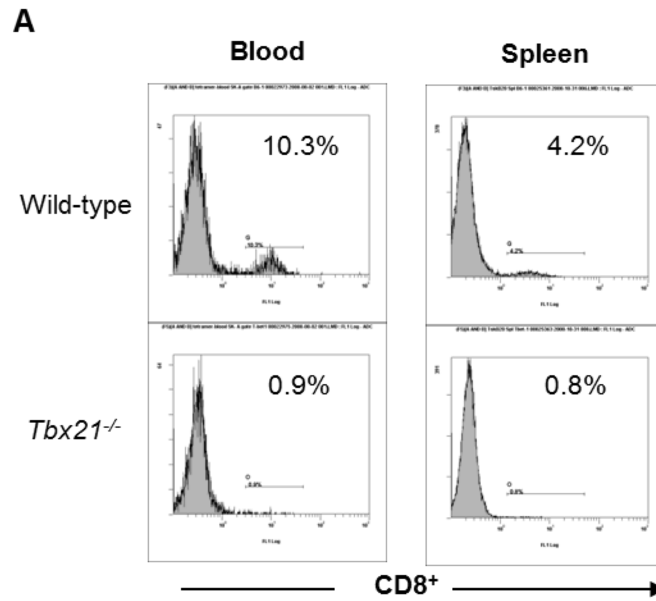


Figure 10. T-bet regulates the expansion of $CD8^+$ T cells during *T. cruzi* infection in a T cell-intrinsic manner.

Thy1.2^+ T cells were purified from pooled spleen and lymph nodes of naïve C57BL/6 or *Tbx21*$^{-/-}$ mice, and approximately 20 million T cells were transferred i.v. into naïve *Rag-2*$^{-/-}$ recipients. *Rag-2*$^{-/-}$ mice were subsequently infected 24 hours post-transfer with *T. cruzi* trypomastigotes and recipient mice were euthanized day 9 p.i. Spleen and blood were collected, and analyzed for the presence of $CD8^+$ T cells. A) Spleen and blood were analyzed by flow cytometry to determine the percentage of $CD8^+$ T cells. Representative histograms show the percentage of $CD8^+$ T cells in *Rag-2*$^{-/-}$ recipient mice. B) Results shown are from five independent experiments, with two mice per group, with each data point representing the percentage of $CD8^+$ T cells from a single mouse.

Figure 10



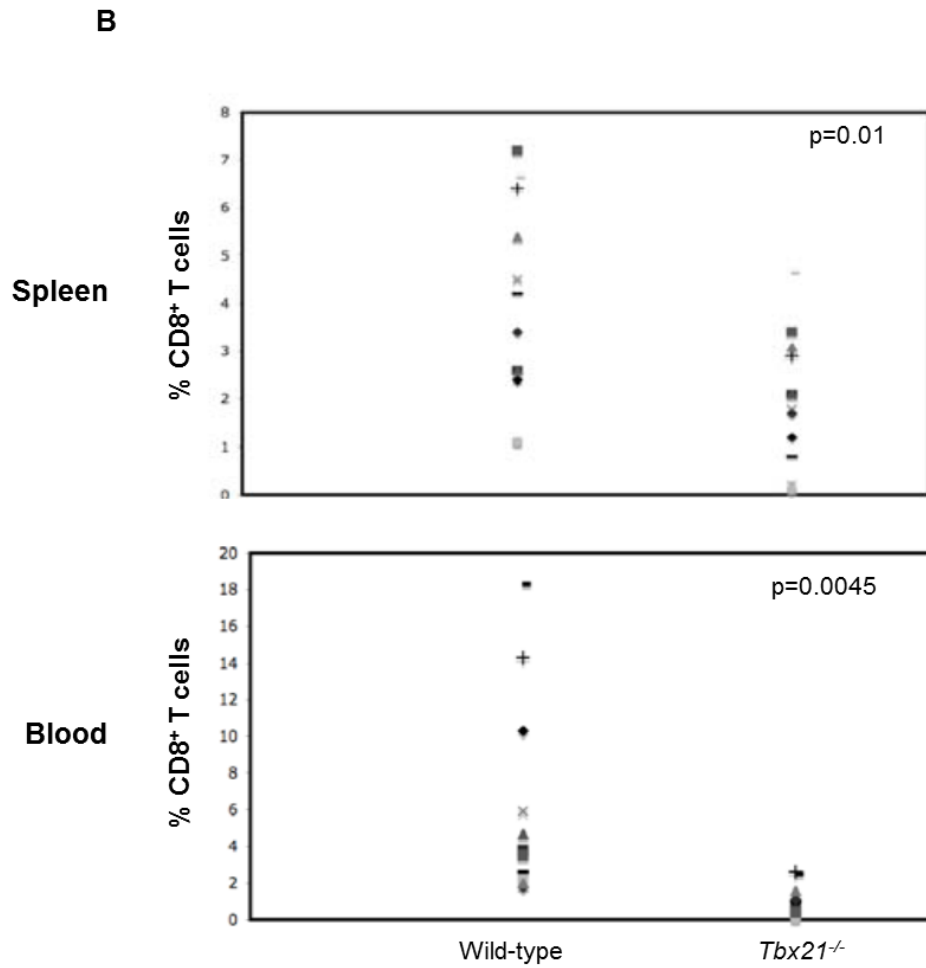


Figure 11. CD8$^+$ T cell expansion during *T. cruzi* infection is regulated by T-bet in a CD8$^+$ T cell-intrinsic manner.

CD4$^+$ and CD8$^+$ T cells were purified from pooled spleen and lymph nodes of naïve C57BL/6 and *Tbx21*$^{-/-}$ mice. Wild-type CD4$^+$/wild-type CD8$^+$, *Tbx21*$^{-/-}$ CD4$^+$/*Tbx21*$^{-/-}$ CD8$^+$, or wild-type CD4$^+$/*Tbx21*$^{-/-}$ CD8$^+$ T cells were combined at the physiological ratio and transferred i.v. into naïve *Rag-2*$^{-/-}$ recipients. Recipient *Rag-2*$^{-/-}$ mice were subsequently infected 24 hours post-transfer with *T. cruzi* trypomastigotes. On day 10 p.i. recipient mice were bled to analyze the percent of CD8$^+$ T cells in the blood. Mice were euthanized on day 13 p.i. and spleen and blood were collected and the percent of CD8$^+$ T cells was analyzed.

Figure 11

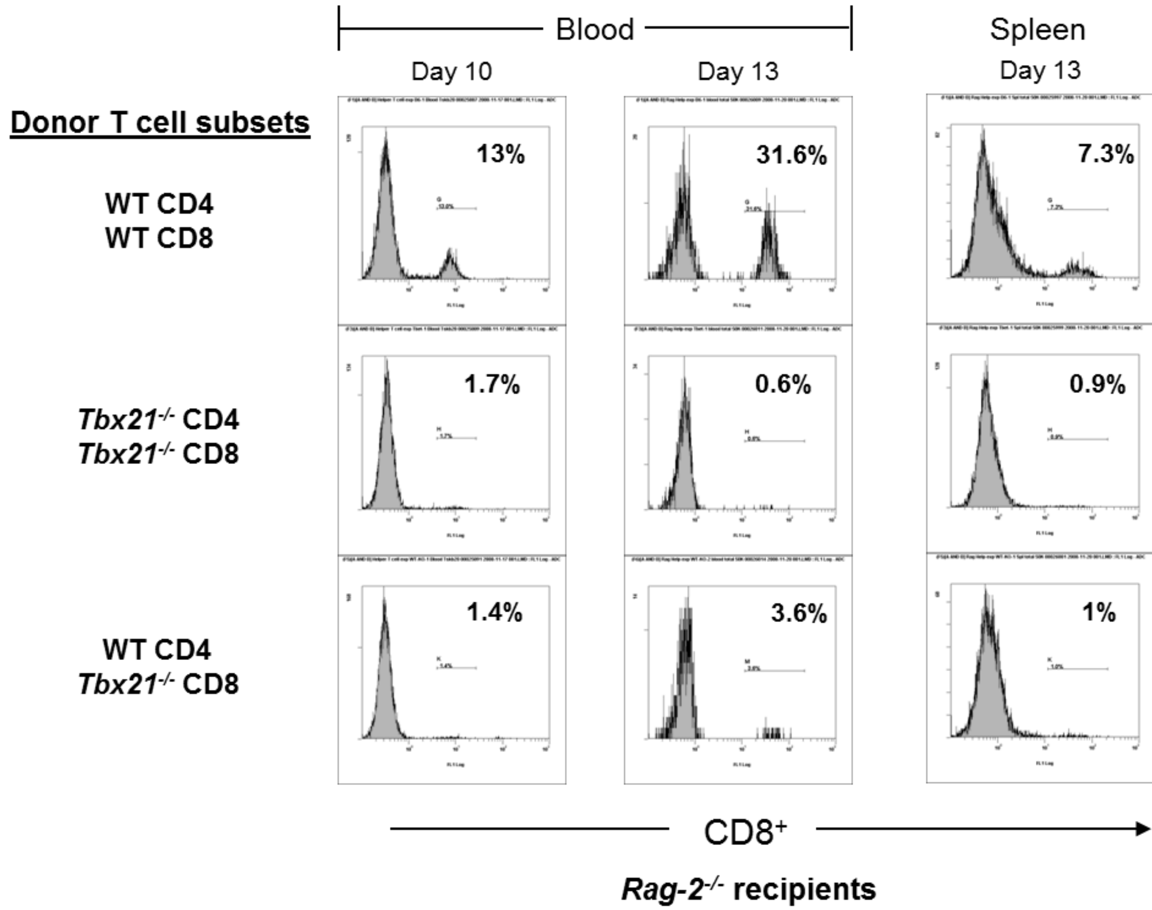
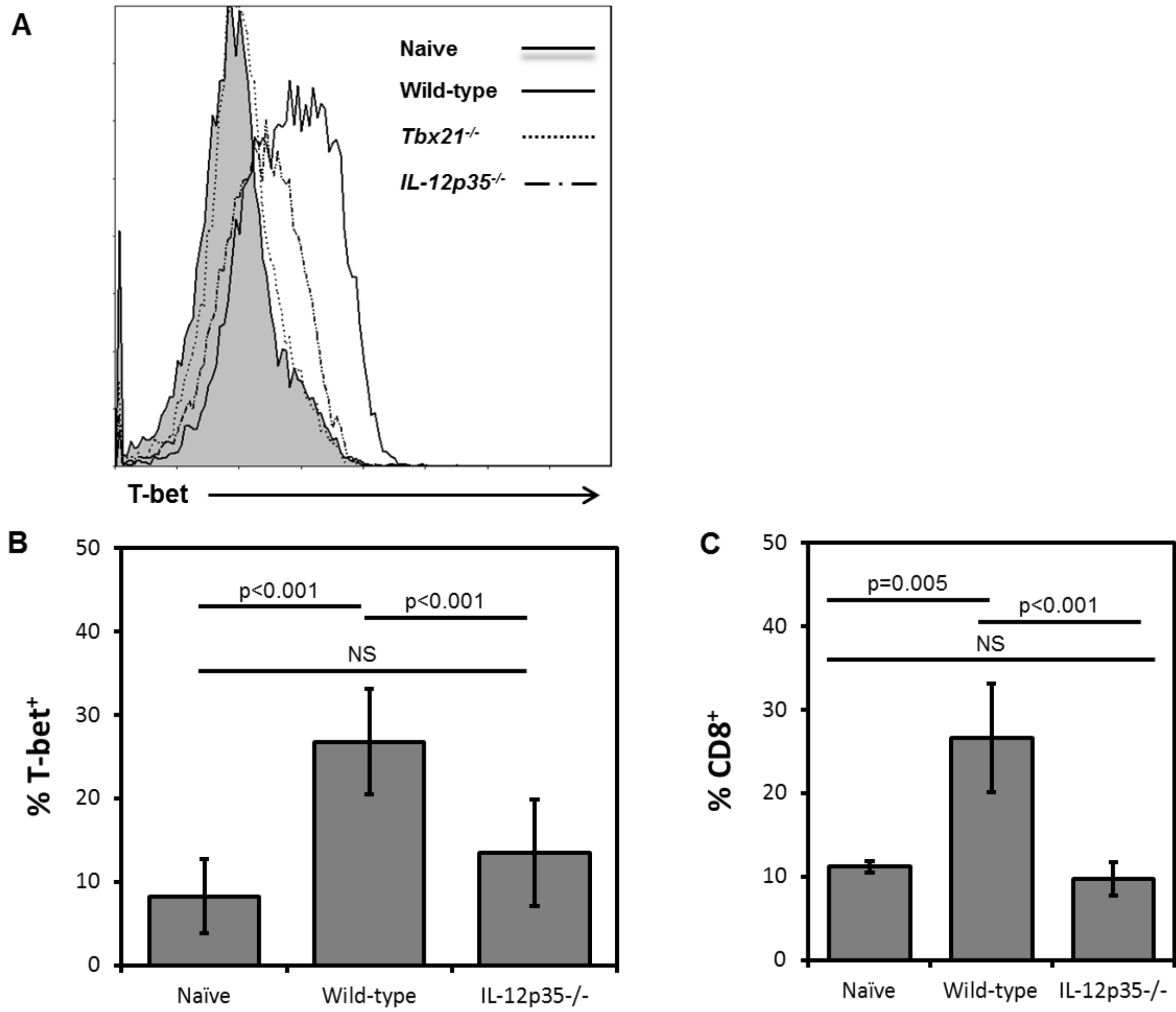


Figure 12. CD8$^+$ T cells in IL-12-deficient mice have reduced T-bet expression during *T. cruzi* infection.

A) Splenocytes from naïve uninfected mice, *T. cruzi*-infected C57BL/6, and *T. cruzi*-*IL-12p35*$^{-/-}$ mice were analyzed by flow cytometry on day 9 p.i. for T-bet expression by gating on CD8$^+$ T cells. Naïve uninfected (shaded), infected C57BL/6 (solid line), infected *Tbx21*$^{-/-}$ (dotted line), and infected *IL-12p35*$^{-/-}$ (dashed line) are represented. Histograms are representative of one individual experiment. B) Percentage of CD8$^+$ T cells expressing T-bet are shown in the bar graph. Results are representative of at least four independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 12



Discussion

CD8$^+$ T cells are a vital component of the adaptive immune response during infection with intracellular pathogens like *T. cruzi*. Their ability to kill *T. cruzi*-infected host cells hinges on the acquisition of cytotoxic effector functions and the production of IFN-γ, all of which require the expression of T-bet. However, the expansion of sufficient numbers of *T. cruzi*-specific CD8$^+$ T cells is equally as important and is critical for controlling infection and providing long-term protection. The expression of T-bet is required for the appropriate expansion of CD8$^+$ T cells that recognize the *T. cruzi* immunodominant epitope Tskb20. The failure to generate this response likely contributes to the increased susceptibility and mortality of *Tbx21*$^{-/-}$ mice infected with *T. cruzi*.

T-bet regulates the expression of the chemokine receptor CXCR3 on activated T cells. However, we failed to detect an accumulation of CD8$^+$ T cells in non-lymphoid tissues such as the liver. Since trafficking of CD8$^+$ T cells from *Tbx21*$^{-/-}$ mice did not appear to be altered, this likely did not account for the reduced numbers of Tskb20-specific CD8$^+$ T cells in the spleens and in the circulation of *Tbx21*$^{-/-}$ mice during infection with *T. cruzi*. Impaired survival also did not appear to be the cause of the low frequency of *T. cruzi*-specific T cells as there was no evidence of increased apoptosis. Thus, we reasoned that the defect in the overall numbers of antigen-specific CD8$^+$ T cells originated at the time of priming or expansion. Because DCs express T-bet in addition to T cells, it was necessary to determine if the defective generation of CD8$^+$ T cells was a result of impaired APC function. The activation of DCs in infected *Tbx21*$^{-/-}$

mice proved to be normal, thus ruling this out as a cause of reduced T cell priming. APCs in *Tbx21*$^{-/-}$ mice were also capable of priming and inducing the expansion of OT-1 CD8^{+} T cells in vivo during *T. cruzi* infection, demonstrating that antigen processing and presentation functions were intact. These results showed that the defective CD8^{+} T cell response was not due to a defect intrinsic to APCs as a result of T-bet deficiency. Lastly, because the defect in the expansion of *Tbx21*$^{-/-}$ CD8^{+} T cells was recapitulated in *Rag-2*$^{-/-}$ mice which have wild-type, T-bet-sufficient APCs, we concluded that T-bet regulates CD8^{+} T cell expansion in a T cell-intrinsic manner.

Our finding regarding the T-bet-dependent regulation of Ag-specific CD8^{+} T cell expansion during *T. cruzi* infection has several similarities as well as differences with other studies involving different pathogens and disease models. In agreement with our study, the infection of *Tbx21*$^{-/-}$ mice with vaccinia virus (VV) led to a defective proliferative capacity of VV-specific CD8^{+} T cells[53]. In a similar manner, T-bet was required for the development of sufficient numbers of CD8^{+} T cells to induce autoimmune disease in the rat insulin promoter-lymphocytic choriomeningitis virus (RIP-LCMV) transgenic model of type 1 diabetes[55]. Consistent with findings in our study, the reduced numbers of diabetes-inducing T cells was not due to increased cell death, and this defect was also intrinsic to CD8^{+} T cells. A minor difference between our study and the RIP-LCMV model was that on day 8 of the LCMV response, the number of effector CD8^{+} T cells in *Tbx21*$^{-/-}$ mice was equivalent to wild-type mice and was not significantly lower until day 14. In our model, *Tbx21*$^{-/-}$ mice infected with *T. cruzi* never developed a significant increase in Ag-specific cells. Nevertheless, these studies and ours show that T-bet is important for the proper expansion of Ag-specific CD8^{+} T cells. In contrast,

other infection models of *Tbx21*$^{-/-}$ mice have yielded different results. For instance, absence of T-bet during infection of mice with influenza, Sendai virus, or herpes simplex virus (HSV)-2 did not impair the expansion of CD8^{+} T cells[86,54]. Thus, the requirement for T-bet in the regulation of CD8^{+} T cell expansion may vary depending on pathogen virulence and may be different depending on whether the pathogen is viral or parasitic.

In some cases, the generation of CD8^{+} T cell responses is reliant upon CD4^{+} T cell-mediated help. Depletion of CD4^{+} T cells during infection with *Listeria monocytogenes*[87] or influenza virus[88] adversely affected the expansion of Ag-specific CD8^{+} T cells. However, normal CD8^{+} T cell responses have been observed in other infections that include LCMV[89] and vesicular stomatitis virus[87]. There remains some debate as to whether CD4^{+} T cell help is required for *T. cruzi*-specific CD8^{+} T cell responses. In one instance, CD4 knockout and MHC II knockout mice failed to develop *T. cruzi*-specific CD8^{+} T cell cytotoxicity[90]. In a separate study, MHC class II knockout mice infected with *T. cruzi* generated CD8^{+} T cell responses that included immunodominant Tskb20-specific cells, although the magnitude of the Tskb20 response was consistently reduced in the blood and spleens[91]. Based on these studies, it appears that CD4^{+} T cell help is important for the proper development of CD8^{+} T cell responses during *T. cruzi* infection. Therefore, the failure of CD8^{+} T cells to undergo expansion in infected *Tbx21*$^{-/-}$ mice could be due to defective CD4^{+} T cell help as a result of T-bet deficiency. To address this possibility, wild-type CD4^{+} T cells were transferred with *Tbx21*$^{-/-}$ CD8^{+} T cells into *Rag-2*$^{-/-}$ mice that were then infected with *T. cruzi*. Our preliminary results suggested that even when provided with wild-type CD4^{+}

help, the ability *Tbx21*$^{-/-}$ CD8$^+$ T cells to expand was not restored. Thus, it appears that T-bet regulates expansion in a CD8$^+$ T cell-intrinsic manner.

The activities of T-bet and Eomes provide much of the transcriptional regulation of CD8$^+$ T cell effector functions. Although Eomes can often compensate for the loss of T-bet by providing overlapping functions, it did not appear to replace T-bet in the expansion of *T. cruzi*-specific CD8$^+$ T cells. In addition to T-bet and Eomes, the runt-related transcription factor 3 (Runx3) is involved in the regulation of CD8$^+$ T cell differentiation and CTL programming[92]. Interestingly, Runx3 accomplishes this partly through induction of Eomes. Real-time polymerase chain reaction (PCR) analysis of *Tbx21*$^{-/-}$ CD8$^+$ T cells showed that Runx3 expression was normal during *T. cruzi* infection (data not shown), thus discounting altered Runx3 levels as a cause of defective expansion. Furthermore, because Runx3 levels were not affected by T-bet-deficiency, it would suggest that Eomes expression was also unaffected. However, despite the supposed normal Runx3-induced expression of Eomes, it did not compensate for the loss of T-bet in the regulation of expansion. Thus, T-bet appears to be a critical factor for the expansion of CD8$^+$ T cells during infection, and this role cannot be fulfilled by Runx3 or Eomes.

Signal 3 cytokines, like IL-12, have been shown to be critical for the proper expansion of Ag-specific CD8$^+$ T cells during pathogenic infections[93,83]. We reasoned that T-bet may responsible for mediating the effects of IL-12 for the expansion of CD8$^+$ T cells during *T. cruzi* infection. To determine if there was a link between IL-12 signaling and T-bet expression in CD8$^+$ T cells, IL-12-deficient mice were infected with *T. cruzi*. We found that the induction of T-bet expression in CD8$^+$ T is significantly

dependent upon IL-12, as $CD8^+$ T cells from IL-12-deficient mice failed to up-regulate T-bet. Furthermore, IL-12p35-deficient mice infected with *T. cruzi* had significantly fewer $CD8^+$ T cells in the blood similar to what we observed in infected T-bet-deficient mice. Thus, it appears that IL-12 is important for promoting the expansion of *T. cruzi*-specific $CD8^+$ T cells. The defective expansion of T-bet-deficient $CD8^+$ T cells during *T. cruzi* infection may be a result of their inability to respond to IL-12. This could have important implications for the development of vaccines against *T. cruzi* and other intracellular pathogens that induce $CD8^+$ T cell responses. For instance, the induction of signal 3 cytokines by vaccine adjuvants is an important aspect of promoting successful vaccine-induced $CD8^+$ T cell immunity. Thus, the utilization of adjuvants that enhance IL-12 production and T-bet expression may favor the development of vaccines that target *T. cruzi*-specific T cell responses.

In addition to IL-12, the interferons IFN-α/β and IFN-γ may function as signal 3 during $CD8^+$ T cell activation. Most evidence favors a role for IFN-α/β in promoting the expansion of Ag-specific $CD8^+$ T cells during viral infections including LCMV[94], vaccinia virus, and vesicular stomatitis virus[95,96,97]. Like the type I interferons, IFN-γ has also been shown to promote optimal expansion in vivo[98,99]. The common feature of the IFNs is that both types activate Stat-1, which has been demonstrated to mediate the effects of IFN-signaling during $CD8^+$ T cell expansion[100]. On the other hand, there are reports that suggest that the effects of IFN/Stat-1 signaling may be anti-proliferative[101,102,103,104], thus implicating a role for IFNs in negatively regulating expansion. Stat-1 activation is linked with T-bet expression, thus the effects of IFNs on $CD8^+$ T cell expansion may be mediated by T-bet. Hence, the defective expansion of T-bet-deficient $CD8^+$ T cells

during *T. cruzi* infection could be a result of not receiving T-bet-mediated IFN signals. Conversely, if IFN/Stat-1 signaling is inhibitory towards proliferation, it suggests a possible divergence in the effects of Stat-1 and T-bet during $CD8^+$ T cell expansion. Future experiments are planned to address the effects of IFN-γ and its potential to function as signal 3 for $CD8^+$ T cell expansion in response to *T. cruzi*.

What T-bet specifically does to promote $CD8^+$ T cell expansion has not been completely established. However, it may in part be related to the ability of T-bet to repress the expression of the programmed death-1 (PD-1) inhibitory receptor and other inhibitory receptors[105]. The PD-1 signaling pathway is involved in causing Ag-specific $CD8^+$ T cells to enter a state of exhaustion in which proliferation, cytotoxicity, and survival are compromised. $CD8^+$ T cell exhaustion is a major consequence of chronic infection, particularly during chronic viral infections. In the absence of T-bet, $CD8^+$ T cells exhibited increased expression of PD-1 and other inhibitory receptors during LCMV infection, and this correlated with a reduction in Ag-specific cells by day 9 p.i.[105]. It remains to be determined whether the PD-1 signaling pathway is directly involved in the defective expansion of T-bet-deficient $CD8^+$ T cells during *T. cruzi* infection. Nevertheless, T-bet may play a role in maintaining T cell responses during chronic *T. cruzi* infection by limiting the extent of T cell exhaustion. Indeed, exhaustion of both $CD4^+$ and $CD8^+$ T cells has been observed during *T. cruzi* infection and T cells from infected mice exhibit high levels of expression of PD-1[106]. While blockade or deletion of PD-1 increased lymphocyte proliferative responses and reduced parasitemia levels, it resulted in a heightened inflammatory response and immunopathology, and led to increased mortality[106]. These findings highlight an important regulation of inflammatory

responses during *T. cruzi* infection where T-bet may play an important role in balancing T cell-mediated inflammation and T cell exhaustion.

In conclusion, these results demonstrate that T-bet is a critical factor for promoting the expansion of Ag-specific $CD8^+$ T cells during infection with *T. cruzi*, and strategies that enhance T-bet expression should be used in designing vaccines for the treatment and prevention of *T. cruzi* infection.

Chapter 2: Role of phosphorylation in T-bet-dependent regulation of Th17 development

Introduction

Transcription factor networks govern the differentiation of $CD4^+$ T cells into their effector fates. Importantly, transcription factors involved in establishing $CD4^+$ T cell commitment utilize a variety of mechanisms to promote and/or repress the differentiation of the various T cell subsets. In the case of T-bet, it has been demonstrated that it can act as a direct transcriptional activator of signature Th1 genes. For instance, T-bet directly binds to the promoter of the IFN-γ locus, thereby inducing expression of the IFN-γ gene[107,71]. The gene encoding the chemokine receptor CXCR3 is another target of T-bet[108,71]. In addition to the ability of T-bet to act as a transcriptional activator, it can also influence T cell differentiation by additional, albeit indirect mechanisms. Through its ability to physically interact with other transcription factors, T-bet can indirectly repress the differentiation of other T cell subsets. For example, T-bet can bind to and sequester the Th2-promoting transcription factor GATA-3[109], thus limiting Th2 development and favoring Th1 differentiation. The mechanism underlying this regulation involves the phosphorylation of a particular tyrosine residue within the T-bet protein that allows it to bind to GATA-3. The ability of T-bet to physically interact with other factors to bring about changes in T cell differentiation is also highlighted by its ability to induce expression of Runx3, which complexes with T-bet to further promote Th1 polarization and Th2 inhibition by silencing IL-4 transcription[110]. Additionally, T-bet regulates IL-2 production in T cells by a similar mechanism where it interacts with RelA, preventing it from promoting IL-2 expression[111]. The structure of T-bet and critical

amino acid residues involved in the previously mentioned functions of T-bet are shown in **Figure 13**.

These regulatory mechanisms of T cell function involving physical interactions between transcription factors are not unique to T-bet. For instance, an interesting scenario exists involving the Th17-specific transcription factor ROR-γt, Runx1, and Foxp3, that regulates the balance between Th17 and T_{Reg} cell differentiation. The interaction of Runx1 and ROR-γt promotes IL-17 and Th17 development, whereas the interaction of Runx1 with Foxp3 inhibits Th17 development[112].

We sought to determine the importance of known phosphorylation sites within T-bet that might be important for regulating Th17 differentiation. We hypothesized that T-bet mediates Th17 inhibition by physically interacting with ROR-γt to limit its activity, in similar fashion to how T-bet regulates Th2 differentiation. To address this, we examined the requirement for T-bet residues known to be important in sequestering other factors. This was accomplished by utilizing retrovirus-mediated gene delivery to introduce T-bet or T-bet which has had critical residues mutated into $CD4^+$ T cells.

Materials and Methods

Retroviral plasmids

Retroviral plasmids encoding empty-GFP, full-length T-bet, and single-point mutations in T-bet (Y525F, Y437F, S508A and S498A) were a generous gift of Dr. Eun Hwang and have been described previously[32,111]. Retroviral plasmids were transformed into competent OneShot TOP10 *E. coli*, plated on Luria agar plates containing ampicillin, and incubated at 37°C overnight. Transformants were then picked and large-scale cultures were grown in Luria broth containing ampicillin at 37°C overnight. The following day, bacteria were harvested by centrifugation and plasmid DNA was purified using the PerfectPrep Endo Free Plasmid Maxi Kit (5 Prime). Plasmid DNA sequences were confirmed by sequencing.

Phoenix cell transfection

To generate ecotrophic retroviruses, Phoenix cells which are based on the 293T human embryonic kidney cell line were obtained from ATCC (SD-3444). Phoenix cells were maintained in culture in T25 tissue culture flasks and were split 1:4 approximately every 3 days. Stability of Phoenix cells ability to produce retrovirus particles was routinely monitored by CD8 expression. One day prior to transfection, Phoenix cells were re-suspended in complete RPMI media at a concentration of 0.12×10^6 cells/ml. 0.06×10^6 Phoenix cells (0.5ml) were then seeded in a 24-well tissue culture plate and incubated overnight at 37°C to obtain a monolayer cell density of 80-90% confluency. Twenty-four hours later, media was carefully removed from the cells and replaced with 0.5ml of fresh complete RPMI media 30-60 minutes before transfection. Phoenix cells were

transfected using PolyJet In Vitro DNA Transfection Reagent (SignaGen Laboratories, Cat# SL100688) as follows for each well. First, 0.5μg of plasmid DNA was diluted into 25μl of serum-free DMEM. Next, 1.5μl of PolyJet was diluted into 25μl of serum-free DMEM. The diluted PolyJet was then added immediately to the diluted DNA and vortexed. The PolyJet-DNA mixture was then incubated at room temperature for 15 minutes to allow PolyJet-DNA complexes to form. 50μl of PolyJet-DNA mixture was added drop-wise onto the media in each well. Transfected Pheonix cells were incubated at 37°C for 48 hours. At 48 hours post-transfection, retroviral supernatants were collected and stored at -70°C.

T cell purification and differentiation

$CD4^+$ T cells were purified from spleen and lymph nodes of naïve *Tbx21*$^{-/-}$ mice, or in some cases from the spleens of *T. cruzi*-infected mice on day 9 post-infection by positive selection using $CD4^+$ microbeads (Miltenyi Biotec). For in vitro activation, $0.5x10^6$ $CD4^+$ T cells were cultured with $2x10^6$ irradiated splenocytes and 0.5μg/ml of low-endotoxin/azide-free (LEAF) anti-CD3 (clone 2C11, Biolegend) in 24-well plates. For in vitro differentiation, T cells were cultured under Th17-polarizing conditions (10ng/ml IL-6, 5ng/ml TGF-β, 20ng/ml IL-23, and 0.5μg/ml anti-IFN-γ). For $CD4^+$ T cells from *T. cruzi*-infected mice, cell cultures were supplemented with 20ng/ml IL-23 and 10ng/ml recombinant human IL-7.

Retroviral transduction and cell culture

Purified CD4$^+$ T cells were activated for 24 hours under Th17 conditions then transduced with retrovirus supernatant by centrifugation at 1000g for 1 hour at room temperature in the presence of 10μg/ml of polybrene. Viral supernatants were removed after 24 hours and replaced with fresh media. Cultures were maintained under Th17-polarizing conditions for the duration of the experiment. After 4-5 days of culture, T cells were re-stimulated with plate-bound anti-CD3 (5μg/ml) for 6 hrs with the addition of the golgi inhibitor monensin during the final 4 hrs.

Flow cytometry and intracellular cytokine staining

Following re-stimulation, GFP$^+$ cells were FACS sorted (FACSAria). For intracellular cytokine staining, sorted cells were stained with anti-CD4 antibodies (Biolegend) and fixed with 4% paraformaldehyde. After fixation, cells were permeabilized using Permeabilization Wash Buffer (Biolegend) and subsequently stained with anti-IFN-γ and anti-IL-17 antibodies (Biolegend). For extracellular staining, cells were separately labeled with PE anti-CXCR3 antibodies. Samples were analyzed by flow cytometry using an FC500 instrument (Beckman Coulter). Analysis was performed by gating on CD4$^+$GFP$^+$ events.

Statistical analyses

Data were analyzed using student t test or Mann Whitney *U* test as appropriate. (SigmaPlot 12.0, Systat Software, Inc.). A p value <0.05 was considered significant. Data are represented as means ±SD of experimental groups.

Results

Generation of T-bet-expressing retrovirus by transfection of 293T Phoenix cells.

To investigate the T-bet-dependent mechanism of Th17 repression, retroviral plasmids that encode T-bet and the fluorescent marker GFP, as well as plasmids that encode T-bet with various point mutations were obtained from Dr. Eun Hwang. A transformation step was performed to produce sufficient amounts of plasmid DNA to be used for retrovirus production.

The purified DNA was then used to generate T-bet-expressing retroviruses. This was accomplished by transfecting 293T Phoenix cells with plasmid DNA encoding T-bet. Transfected Phoenix cells then produce retroviruses that express the gene of interest. This system was utilized for the rapid production of retrovirus carrying the T-bet gene. Following transfection, Phoenix cells were incubated for two days to allow retrovirus to be produced and secreted into the culture supernatant. The supernatant was then harvested and used for subsequent T cell experiments. Successful transfection was determined by the percent of GFP-expressing Phoenix cells (**Figure 14**). Thus, Phoenix cells exhibited a high level of transfection efficiency and produced T-bet-expressing RV.

Ectopic T-bet expression inhibits IL-17 production by T cells from T. cruzi-infected $Tbx21^{-/-}$ mice.

We first wished to formally demonstrate that ectopic expression of T-bet could inhibit IL-17 production by Th17 cells from *Tbx21*$^{-/-}$ mice infected with *T. cruzi*. To accomplish this, we first purified $CD4^+$ T cells from infected *Tbx21*$^{-/-}$ mice on day 9 p.i.,

which are IL-17$^+$, IFN-γ^+, and IL-17$^+$IFN-γ^+. Empty-GFP or T-bet-GFP RV was transduced into *Tbx21*$^{-/-}$ CD4$^+$ T cells in vitro and the cells were cultured for four days. To demonstrate that the effects of ectopic T-bet expression were independent of IFN-γ, anti-IFN-γ neutralizing antibody was included in the CD4$^+$ T cell cultures. The experimental approach is diagrammed in **Figure 15A**. CD4$^+$ T cells expressing GFP were then analyzed for IFN-γ and IL-17 to determine the effect of T-bet complementation. As expected, transduction of *Tbx21*$^{-/-}$ CD4$^+$ T cells with Empty RV exhibited a phenotype similar to cells isolated from infected *Tbx21*$^{-/-}$ mice, with approximately 40% of cytokine-producing cells positive for IL-17 only and approximately 20% of cytokine-producing cells positive for IFN-γ only (**Figure 15B**). Importantly, the transduction of Empty RV did not alter the percentages of cytokine-producing cells compared to control cells not transduced with RV (**Figure 15C**). Restoration of T-bet expression by transduction with T-bet RV led to approximately a 30% reduction in the percentage of cells producing only IL-17, while the percentage of cells producing only IFN-γ was increased by approximately 50% (**Figure 15B**). Though there were still double-positive cells producing both cytokines, it remains unclear whether these cells were simply still undergoing transition or whether they are resistance to the effects of ectopic T-bet expression. Nevertheless, ectopic T-bet expression significantly inhibited IL-17 production in *Tbx21*$^{-/-}$ CD4$^+$ T cells from *T. cruzi*-infected mice and did so independently of IFN-γ. These results demonstrate that primed Th17 cells generated in vivo during *T. cruzi* infection exhibit plasticity and undergo conversion into Th1 cells after ectopic expression of T-bet.

T-bet tyrosine residues 437 and 525 are dispensable for the suppression of IL-17 production by Th17 cells.

To further investigate the mechanism by which T-bet inhibits IL-17 production, we turned out attention to critical amino acid residues known to be important in mediating other functions of T-bet. We first focused on tyrosine 525, whose phosphorylation is required for T-bet to bind to GATA-3, and inhibit Th2 differentiation. We hypothesized that this residue could also mediate Th17 inhibition. $CD4^+$ T cells isolated from naïve *Tbx21*$^{-/-}$ mice were cultured in vitro under Th17-polarizing conditions. *Tbx21*$^{-/-}$ $CD4^+$ T cells were transduced with Empty RV, T-bet RV, T-bet Y525F RV, or T-bet Y437F RV. Nearly 100% of cytokine-producing cells remained IL-17^+ when transduced with Empty RV (**Figure 16A-B**). As expected, transduction with wild-type T-bet RV resulted in a significant inhibition of IL-17-producing cells and an increase in IFN-γ producing cells. T-bet Y525F and T-bet Y437F were also capable of significantly inhibiting IL-17 production, as the degree of inhibition of IL-17 was comparable to wild-type T-bet (**Figure 16A-B**). Further, both T-bet mutants were able to promote IFN-γ production. As a control for ensuring that the level of T-bet activity was similar between the mutants and wild-type T-bet, staining for the T-bet-inducible chemokine receptor CXCR3 was performed. CXCR3 expression was up-regulated to comparable levels in all groups, confirming equal activity among wild-type T-bet and the tyrosine mutants (**Figure 16C**). Thus, in contrast to the T-bet-mediated suppression of Th2 development, T-bet tyrosine residues 525 and 437 are dispensable for the inhibition of Th17 development in $CD4^+$ T cells.

T-bet serine residues 498 and 508 are also dispensable for the suppression of IL-17 production by Th17 cells.

We next tested the requirement for T-bet S508 in the regulation of Th17 development. $CD4^+$ T cells isolated from naïve *Tbx21*$^{-/-}$ mice were cultured in vitro under Th17-polarizing conditions. *Tbx21*$^{-/-}$ $CD4^+$ T cells were transduced with Empty RV, T-bet RV, T-bet S508A RV, or T-bet S498A RV. Again, transduction with wild-type T-bet RV significantly inhibited IL-17 production while simultaneously promoting IFN-γ production (**Figure 17A-B**). Surprisingly, neither serine residue 508 or 498 was necessary for control of IL-17 production, as both mutants inhibited Th17 development equally as well as wild-type T-bet and promoted IFN-γ production (**Figure 17A-B**). As before, CXCR3 was up-regulated equally well by wild-type T-bet and the serine mutants, confirming equal activity of the mutants. Thus, serine phosphorylation of T-bet residues 508 and 498 are not required for T-bet to suppress Th17 development.

Figure 13. Schematic diagram of the transcription factor T-bet and critical residues that regulate T-bet functions.

The T-box transcription factor family member, T-bet, is a 530 amino acid protein consisting of a DNA-binding domain that is common to all T-box family members. T-bet also consists of an N-terminal and a C-terminal transcriptional-activation domain. Within the C-terminal domain resides a serine residue at position 508 that is phosphorylated by casein kinase, which allows T-bet to repress IL-12 expression. Also within the C-terminal domain is a tyrosine residue at position 525 that when phosphorylated by Itk, allows T-bet to interact and sequester the Th2-promoting transcription factor GATA-3.

Figure 13

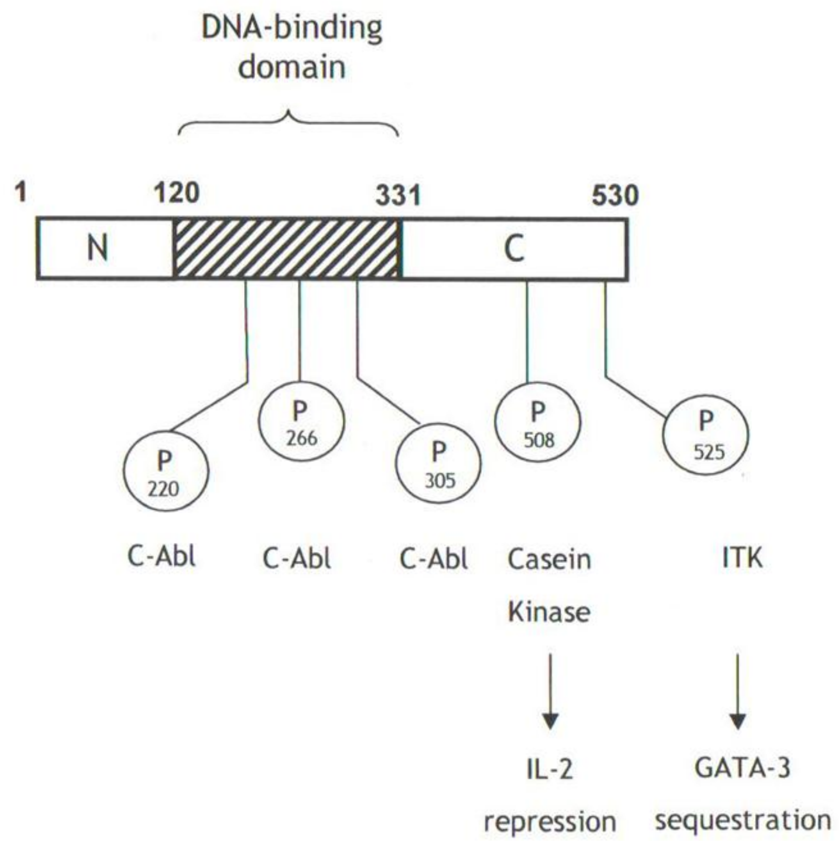


Figure 14. Transfection of 293T cells for retrovirus generation.

Phoenix cells were plated and transfected with retroviral DNA plasmids. After 48 hours, culture supernatants containing retrovirus were harvested and used for subsequent T cell transduction experiments. Phoenix cells GFP expression was analyzed by flow cytometry to determine the percent of cells successfully transfected.

Figure 14

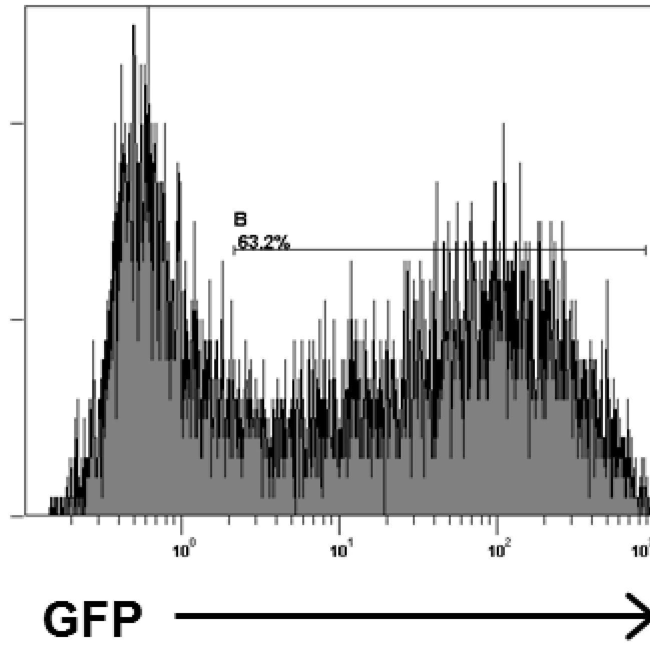


Figure 15. Ectopic T-bet expression inhibits IL-17 production by T cells from *T. cruzi*-infected *Tbx21*$^{-/-}$ mice.

A) CD4^{+} T cells were purified on day 9 post-infection from *Tbx21*$^{-/-}$ mice infected with *T. cruzi* and re-stimulated in vitro with anti-CD3 (0.5μg/ml) for 24 hrs in the presence of irradiated splenocytes as a source of antigen-presenting cells. Cells were cultured with IL-23 and IL-7 to promote the maintenance and survival of Th17 cells. In vivo primed cells were transduced with either Empty RV or T-bet RV on day 10. On day 14, cells were re-stimulated with plate-bound anti-CD3 (5μg/ml) and GFP^{+} cells were sorted by FACS and analyzed for cytokines by intracellular cytokine staining. B) Intracellular staining for IL-17 and IFN-γ on CD4^{+}GFP^{+} gated cells. Representative dot plots are shown for Empty RV (left) and T-bet RV (right). C) Graphical representation of the percentage of cells producing only IL-17 (top) or only IFN-γ (bottom) from the total population of cytokine-producing cells. Experimental groups were performed in duplicate. Results are representative of five independent experiments and are expressed as means ±SD.

Figure 15

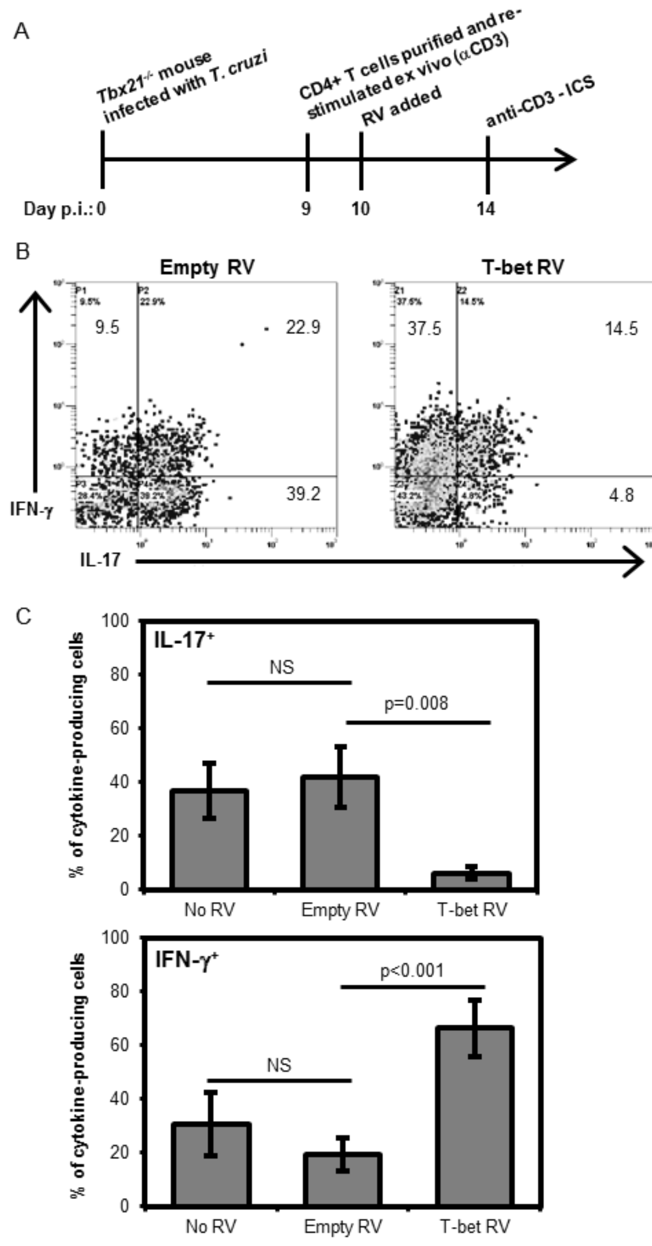


Figure 16. T-bet tyrosine residues 437 and 525 are dispensable for the suppression of IL-17 production in Th17 $CD4^+$ T cells.

A) Naïve *Tbx21*$^{-/-}$ $CD4^+$ T cells were cultured in vitro with anti-CD3 (0.5μg/ml) and irradiated splenocytes under Th17-polarizing conditions (IL-6, TGF-β, IL-23, and anti-IFN-γ). Activated T cells were transduced with Empty RV, T-bet RV, T-bet Y437F, or T-bet Y525F RV and cultured for an additional 4 days. Intracellular staining for IL-17 and IFN-γ was performed on $CD4^+GFP^+$ gated cells. B) Bar graphs represent the percentage of cells producing IL-17 (top) or IFN-γ (bottom) from the total population of cytokine-producing cells. C) FACS analysis of $CD4^+GFP^+$ cells for the expression of CXCR3 confirms equal activity among T-bet tyrosine mutants. Error bars represent means ±SD. All experimental groups were performed in triplicate. Results are representative of three independent experiments.

Figure 16

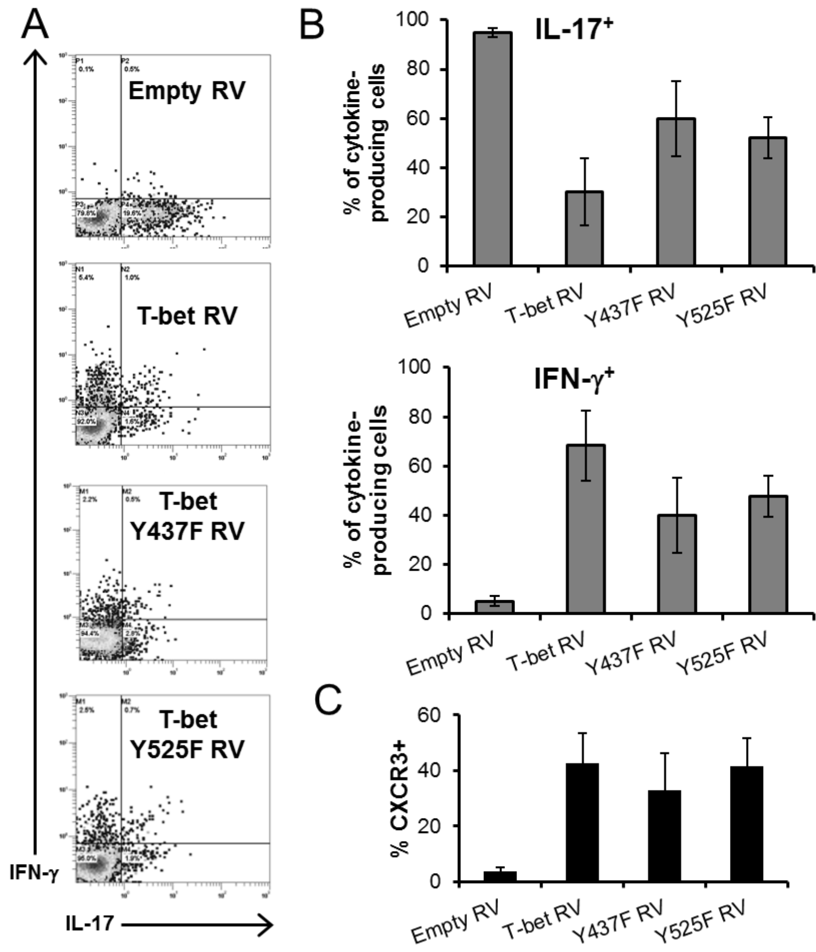
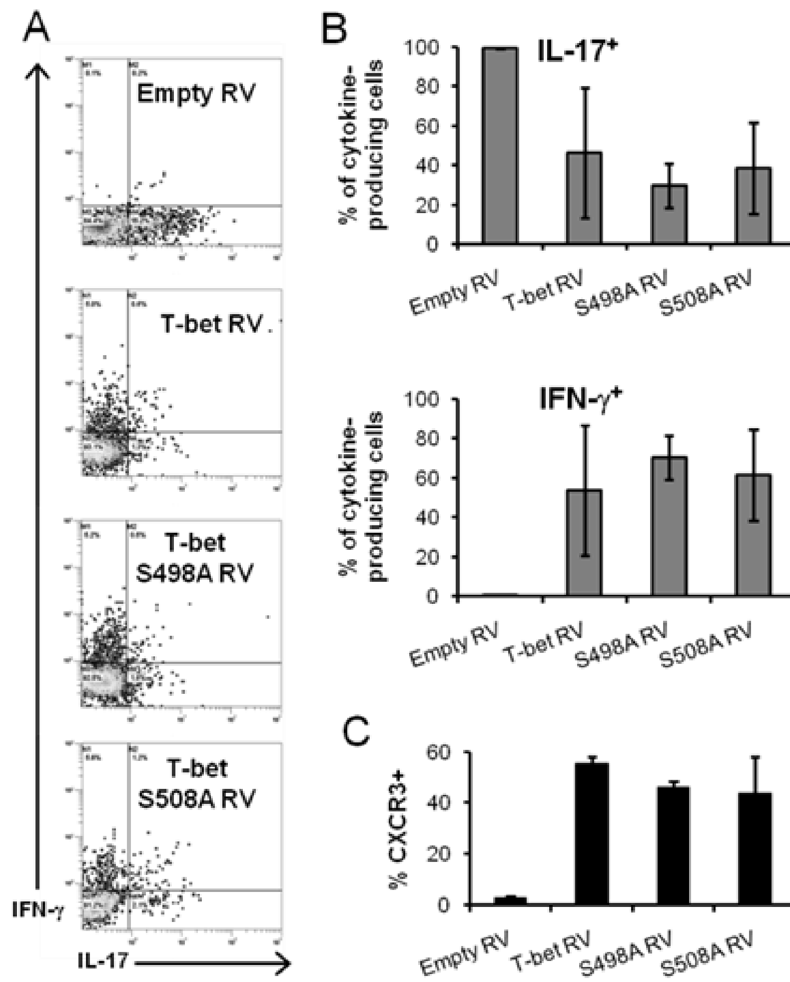


Figure 17. T-bet serine residues 498 and 508 are dispensable for the suppression of IL-17 production in Th17 $CD4^+$ T cells.

A) Naïve *Tbx21*$^{-/-}$ $CD4^+$ T cells were cultured in vitro with anti-CD3 (0.5μg/ml) and irradiated splenocytes under Th17-polarizing conditions (IL-6, TGF-β, IL-23, and anti-IFN-γ). Activated cells were transduced with Empty RV, T-bet RV, T-bet S498A, or T-bet S508A RV and cultured for an additional 4 days. Intracellular staining for IL-17 and IFN-γ was performed on $CD4^+GFP^+$ gated cells. B) Bar graphs represent the percentage of cells producing IL-17 (top) or IFN-γ (bottom) from the total population of cytokine-producing cells. C) FACS analysis of $CD4^+GFP^+$ cells for the expression of CXCR3 confirms equal activity among T-bet serine mutants. Error bars represent means ±SD. All experimental groups were performed in triplicate. Results are representative of at least two independent experiments.

Figure 17



Discussion

Transcription factors ultimately control the differentiation fate of effector T helper cells. Various mechanisms are utilized by transcription factors to not only drive the expression of particular genes that are unique to a given subset, but also to antagonize the development of other subsets. This coordinated activity is critical for inducing and preserving the type of T cell response that is appropriate for a given pathogen. Furthermore, the proper balance in transcription factor networks is necessary to prevent T cell responses that could be become detrimental to the host. Our previous studies highlighted a critical role for T-bet in repressing the development of pro-inflammatory Th17 responses during *T. cruzi* infection. Here, we focused on increasing our understanding of the molecular events that are involved in the T-bet-dependent suppression of Th17 development.

Using retrovirus-mediated gene delivery, we ectopically expressed T-bet in T cells from *Tbx21*$^{-/-}$ mice infected with *T. cruzi*. The introduction of T-bet significantly reduced the numbers of cells producing IL-17 and led to an increase in those producing IFN-γ. Thus, expression of T-bet was necessary and sufficient for inhibition of IL-17 expression. This observation definitively shows that T-bet is important for regulating *T. cruzi*-specific Th17 development. Further, it shows that Th17 cells from *Tbx21*$^{-/-}$ mice do not exhibit a stable phenotype and can readily undergo further differentiation into Th1 cells when T-bet expression is restored. Importantly, the inhibitory effects of T-bet on IL-17 production occurred independently of IFN-γ, as IFN-γ was neutralized during these experiments.

We next sought to further elucidate the molecular mechanism by which T-bet suppresses Th17 differentiation and IL-17 production. We reasoned that T-bet could function in a manner similar to how it regulates other T cell fates such as Th2 inhibition and IL-2 repression. We first examined whether phosphorylation of tyrosine 525 was involved in inhibiting IL-17 production. The reasoning for this experiment was that tyrosine phosphorylation at this site by Itk allows T-bet to interact with GATA-3 and sequester it from promoting a Th2 phenotype[32]. Thus, our hypothesis was that phosphorylation of this tyrosine residue may also be required for T-bet to physically interfere with a factor that would normally promote Th17 development. However, mutation of this residue had no impact on T-bet's ability to suppress the differentiation of $CD4^+$ T cells into Th17 cells. Thus, the mechanism by which T-bet suppresses Th17 differentiation is not the same as it is for suppression of Th2 responses. We then examined the requirement for the serine residue 508, a residue that when phosphorylated by casein kinase I and glycogen synthase kinase-3, allows T-bet to bind to RelA and prevent it from inducing IL-2 production[111]. However, T-bet was still capable of inhibiting Th17 cell differentiation when this serine phosphorylation site was mutated. Therefore, in contrast to their roles in regulating Th2 differentiation and IL-2 production, tyrosine 525 and serine 508 are dispensable for the T-bet-dependent inhibition of Th17 differentiation.

Interestingly, these results suggest that T-bet may utilize specific mechanisms to accomplish its diverse functions, instead of allowing one particular mechanism to mediate multiple functions. Although these residues were not important for the negative regulation of Th17 differentiation, it did not eliminate the possibility of other

phosphorylation-mediated events being important for this regulation. Additionally, we could not rule out the possibility that T-bet-dependent regulation of Th17 responses may involve direct interactions with DNA, as opposed to a particular protein-protein interaction with another transcription factor.

However, during the time of this study, a report identified that T-bet suppresses Th17 cell development by preventing the expression of the Th17-promoting transcription factor ROR-γt[113]. Specifically, it was found that T-bet physically associates with the transcription factor Runx1, thereby preventing Runx1-induced expression of ROR-γt, and subsequent Th17 differentiation. This interaction between T-bet and Runx1 required T-bet tyrosine residue 304, however it is unclear as to whether phosphorylation of this residue is required. Even with the identification of this mechanism, the T cell extrinsic factors and the signaling molecules that are involved in T-bet-mediated inhibition of Th17 responses are yet to be definitively established, particularly during *T. cruzi* infection. However, with an improved understanding of the pathways and mechanisms that regulate Th17 differentiation, it will open possibilities for the targeting of specific molecules for therapeutic purposes in treating many inflammatory diseases and autoimmunity.

Chapter 3: Effects of IFN-γ and Stat-1 signaling on Th17 responses during *Trypanosoma cruzi* infection

Introduction

IFN-γ and Stat-1 are important factors for promoting Th1 responses and thus necessary for immunity to *T. cruzi* infection. The effects of IFN-γ are mediated primarily through Stat-1 which resides upstream of T-bet[28,114]. Phosphorylation and subsequent activation of Stat-1 by IFN-γ causes it to translocate to the nucleus, where it induces T-bet expression (**Figure 18**). Since T-bet can be up-regulated in a Stat-1-dependent fashion in response to IFN-γ[114], it seems likely that IFN-γ would be involved in the regulation of Th17 responses through its effect on T-bet.

Indeed, previous studies reported that IFN-γ was required for the negative regulation of Th17 differentiation, whereas other studies supported a T-bet-dependent suppression of Th17 responses. In a study investigating the inhibition of IL-17 production, it was observed that IL-4 or IFN-γ alone had no effect, whereas together they suppressed IL-17 expression in vitro[59]. However, in the same study, immunization of *Tbx21*$^{-/-}$ mice did lead to an increase in Th17 cells[59]. Elsewhere, IFN-γ was shown to suppress Th17 development in vitro under Th17-polarizing conditions[58]. However, IFN-γ did not suppress IL-17 production by already committed Th17 cells. Lastly, infection of *Ifng*$^{-/-}$ mice with *Mycobacterium bovis* Bacille Calmette Guerin (BCG) led to an increase in Th17 cells[57]. However, the interpretation of some of these early studies was complicated because of the positive feedback loop that exists between IFN-γ and T-bet, thus the effects of IFN-γ and T-bet could not be separated (**Figure 18**).

Our previously published studies suggested that IFN-γ had no effect on the regulation of Th17 development during *T. cruzi* infection, as it did not inhibit IL-17

production by CD4$^+$ T cells. This observation contrasted with those reports which claimed that IFN-γ was inhibitory towards the development of Th17 cells. Although our observations suggested that T-bet was the critical factor required for this inhibition, it remained possible that IFN-γ could have other direct or indirect effects that were not evident in our previous studies. Therefore, the role of IFN-γ in regulating Th17 responses during *T. cruzi* infection required further clarification.

Given that T-bet is a Stat-1-inducible transcription factor, it is easy to hypothesize that its role in the development of Th1 and Th17 responses would resemble those of T-bet. However, the effects of Stat-1 during infection are not that predictable, as Stat-1 has been shown to have contrasting roles during Th1 development. For instance, *Stat-1$^{-/-}$* mice infected with LCMV actually had increased IFN-γ/Th1 responses[115]. In contrast, others propose that Stat-1 may negatively regulate Th1 responses through inhibition of Stat-4 activation[116]. Along these lines, the requirement for Stat-1 during infection with *T. gondii* was not for the development of protective Th1 responses, but rather for the development of macrophage effector functions[117]. Although in this study, T-bet expression was reduced in the absence of Stat-1 signaling. Conversely, Stat-1 was necessary for the induction of Th1-mediated immunity against *L. major*[118]. Therefore, the role of Stat-1 in the regulation of T cell responses to infection can be complicated. Surprisingly, the effects of Stat-1 on immunity to *T. cruzi* are largely unresolved as no studies to our knowledge have focused on its role during infection. The goal of this study was to determine the effects of IFN-γ and Stat-1 signaling on the development of Th1 and Th17 cells during infection with *T. cruzi*.

Materials and Methods

Mice and parasite infections

Age and sex-matched C57BL/6, *Tbx21*$^{-/-}$, and *Ifng*$^{-/-}$ mice were obtained from The Jackson Laboratory and were used between six and eight weeks of age. *Stat-1*$^{-/-}$ mice were generously provided by Dr. Andrew Larner (Virginia Commonwealth University). Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility under pathogen-free conditions and used in accordance with an Institutional Animal Care and Use Committee-approved protocol. For infections, mice were injected intraperitoneally with 1×10^6 *T. cruzi* trypomastigotes (CL strain).

Ex vivo cell culture and recall responses to T. cruzi

2×10^6 splenocytes from *T. cruzi*-infected mice were cultured in complete RPMI 1640 (10% FBS, 10mM Hepes, 1 IU/ml penicillin and 100μg/ml streptomycin, 2mM L-glutamine, 1×10^{-5} M 2-β-mercaptoethanol). Splenocytes were re-stimulated with 0.5μg/ml of LEAF-purified anti-CD3 (clone 145-2C11, Biolegend) or 10-20μg/ml *T. cruzi* lysate for antigen-specific responses for 72 hours. $CD4^+$ T cells were purified from *T. cruzi*-infected mice using positive selection by MACS microbeads (Miltenyi). 2×10^5 $CD4^+$ T cells were cultured with plate-bound LEAF-purified anti-CD3 (5μg/ml). For the neutralization of IFN-γ and IL-12, LEAF-purified anti-IFN-γ (clone XMG1.2, Biolegend) and anti-IL-12 (clone C17.8, Biolegend) antibodies were administered to ex vivo cell cultures at a concentration of 5μg/ml. In some experiments, LEAF-purified anti-CD4

(Biolegend) was used to confirm that antigen-specific responses were mediated by $CD4^+$ T cells. Murine recombinant IL-23 was used at 10ng/ml. For stimulation of T cells with recombinant IFN-γ, a concentration of 20ng/ml was used. Cell-free culture supernatants were collected after 72 hours and IL-17 was measured using Biolegend's ELISA MAX Standard Set according to manufacturer recommendations.

Fluorescent antibodies, intracellular cytokine staining, and flow cytometry

For intracellular cytokine staining, spleen cells were re-stimulated on plate-bound anti-CD3 (5μg/ml) for 6 hours. Monensin was added for the final 4 hours of re-stimulation to induce intracellular cytokine accumulation. Cells were then stained extracellularly with APC/Cy7 anti-CD4 (clone GK1.5) and fixed with 4% paraformaldehyde. After fixation, cells were permeabilized using Permeabilization Wash Buffer (Biolegend) and subsequently stained with APC anti-IFN-γ (clone XMG1.2) and PE anti-IL-17 (clone TC11-18H10.1) antibodies. For phenotypic T cell analysis, splenocytes were incubated with anti-CD16/32 (Fc block), then stained with anti-CD4 and PE anti-CXCR3 (clone CXCR3-173) or PE anti-IFN-γR (clone MOB-47) fluorescent antibodies. Cells were analyzed by flow cytometry using an FC500 instrument (Beckman Coulter). All fluorescent antibodies were obtained from Biolegend.

Statistical analyses

One-way ANOVA, two-tailed tests, as well as Tukey-Kramer multiple comparison post-tests were performed using GraphPad InStat, Version 3.0a. A p value of <0.05 was considered significant.

Results

Stat-1 is required to inhibit Th17 responses during T. cruzi infection.

Since Stat-1 is upstream of T-bet, we hypothesized that its expression would be necessary for proper T-bet induction, and ultimately regulation of Th17 development during *T. cruzi* infection. To determine if Stat-1 was required to inhibit Th17 responses during infection, Stat-1-deficient (*Stat-1*$^{-/-}$) mice were infected with *T. cruzi* and cytokine responses were analyzed. On day 9 p.i., C57BL/6, *Tbx21*$^{-/-}$, and *Stat-1*$^{-/-}$ mice were euthanized and splenocytes harvested for analysis. Intracellular cytokine staining revealed that $CD4^+$ T cells from *Stat-1*$^{-/-}$ mice produced IFN-γ, IL-17, or both IFN-γ and IL-17 concomitantly, similar to T cells from *Tbx21*$^{-/-}$ mice (**Figure 19A-B**). Additionally, these results show that Th1 development during *T. cruzi* infection is Stat-1-indepentent. Ex vivo re-stimulation of splenocytes from infected mice with anti-CD3 resulted in a significant increase in IL-17 production by cells from *Stat-1*$^{-/-}$ mice that was comparable to that observed in cells from *Tbx21*$^{-/-}$ mice (**Figure 19C**). Importantly, re-stimulation with *T. cruzi* lysate also resulted in an increase in IL-17 production, confirming that Th17 cells generated in *Stat-1*$^{-/-}$ mice were *T. cruzi*-specific (**Figure 19D**). To determine that Stat-1 inhibition of Th17 development was indeed T cell-intrinsic, *Stat-1*$^{-/-}$ $CD4^+$ T cells were adoptively transferred into *Rag-2*$^{-/-}$ mice, which have normal Stat-1 functions in all other cellular components, and recipient mice were subsequently infected. Following infection, there was a similar increase in Th17 development of *Stat-1*$^{-/-}$ T cells, demonstrating that Stat-1 inhibits Th17 responses to *T. cruzi* in a T cell-intrinsic manner.

Thus, in the absence of Stat-1, Th17 responses are increased, demonstrating a critical role for Stat-1 in inhibiting the generation of Th17 cells during infection with *T. cruzi*.

IFN-γ is mostly dispensable for T-bet-dependent regulation of Th17 responses during T. cruzi infection.

Based on our findings to this point, we demonstrated that the regulation of Th17 development occurs in a Stat-1- and T-bet-dependent manner. IFN-γ signaling activates Stat-1 and has been proposed to negatively regulate Th17 development. However, in our studies, the presence of IFN-γ in *Tbx21*$^{-/-}$ mice infected with *T. cruzi* did not appear to affect Th17 responses. We wished to address this issue regarding the precise role of IFN-γ in limiting Th17 differentiation during *T. cruzi* infection. Thus, we wanted to formally exclude a role for IFN-γ in controlling Th17 differentiation.

IFN-γ-deficient (*Ifng*$^{-/-}$) mice were infected with *T. cruzi* and on day 9 p.i. were analyzed for the generation of Th17 cells. Intracellular cytokine staining revealed a population of CD4^{+} T cells producing IL-17 in *Ifng*$^{-/-}$ mice. However, the percentage of IL-17-producing T cells from *Ifng*$^{-/-}$ mice was significantly lower than that observed in infected *Tbx21*$^{-/-}$ mice (**Figure 20A-B**). Ex vivo re-stimulation of *Ifng*$^{-/-}$ splenocytes revealed an increase in IL-17 production compared to wild-type cells, however the levels of IL-17 were significantly less relative to cells from infected *Tbx21*$^{-/-}$ and *Stat-1*$^{-/-}$ mice (**Figure 20D-E**). Therefore, in surprising contrast to the requirement for Stat-1, IFN-γ was mostly dispensable for regulating Th17 differentiation during infection.

CXCR3 expression correlates with the relative levels of T-bet activity in Stat-1$^{-/-}$ and Ifng$^{-/-}$ mice.

T-bet is a transcriptional activator of CXCR3 in T cells(108), thus analysis of CXCR3 expression provides a reliable means of assessing the level of T-bet activity in vivo. Using this approach, we sought to correlate the relative levels of T-bet activity in *Stat-1*$^{-/-}$ and *Ifng*$^{-/-}$ mice infected with *T. cruzi*, to the degree of Th17 development. CD4^{+} T cells from *T. cruzi*-infected mice were analyzed for surface expression of CXCR3 by flow cytometry. Analysis of naïve uninfected mice showed approximately 20% of CD4^{+} T cells expressing CXCR3 (**Figure 21A**). Upon infection with *T. cruzi*, CXCR3 expression was significantly increased, as 65% of CD4^{+} T cells in wild-type mice expressed CXCR3 (**Figure 21B**). As expected, CD4^{+} T cells from infected *Tbx21*$^{-/-}$ mice had failed to up-regulate CXCR3 (**Figure 21C**). Interestingly, CXCR3 expression by T cells from infected *Stat-1*$^{-/-}$ mice mirrored that of *Tbx21*$^{-/-}$ T cells, with only 25% of cells being positive for CXCR3 (**Figure 21D**). In contrast to *Stat-1*$^{-/-}$ cells, approximately 48% of *Ifng*$^{-/-}$ CD4^{+} T cells expressed CXCR3, which was comparable to wild-type levels (**Figure 21E**). As shown in **Figure 21F**, the mean levels of CXCR3 expression were significantly reduced in *Stat-1*$^{-/-}$ and *Tbx21*$^{-/-}$ mice, whereas there was no significant difference in *Ifng*$^{-/-}$ mice, compared to wild-type mice. These results show that during *T. cruzi* infection, Stat-1 is necessary for optimal induction of T-bet, whereas IFN-γ is dispensable for T-bet expression. Importantly, the results reinforce the conclusions regarding the roles of Stat-1 and IFN-γ in regulating *T. cruzi*-specific Th17 generation.

$CD4^+$ T cells from infected $Tbx21^{-/-}$ mice are not defective in the expression of the IFN-γ receptor.

Next, we wanted to understand why IFN-γ had no apparent regulatory effect on IL-17 production. Although T-bet induction and T-bet-dependent control of Th17 responses were mostly IFN-γ-independent (**Figures 20-21**), there remained the possibility that IFN-γ could still exert some effects on Th17 cells and IL-17 production. The previous observation showing concomitant production of IFN-γ and IL-17 in infected *$Tbx21^{-/-}$* mice suggested that IFN-γ had no regulatory effect on IL-17 (**Figure 4** and **Figure 19A**). However, in this case there was the potential that IFN-γ could be exerting effects despite the absence of T-bet, which would not be evident until IFN-γ was neutralized. To test this, $CD4^+$ T cells from infected *$Tbx21^{-/-}$* mice were purified and re-stimulated with anti-CD3 in the presence of anti-IFN-γ. The hypothesis was that if IFN-γ exerted any influence on IL-17, its neutralization would cause an increase in IL-17 levels. However, neutralization of IFN-γ had no effect on IL-17 production (**Figure 22A**). Likewise, when exogenous recombinant IFN-γ was added, there was no inhibitory effect on IL-17 production (**Figure 22A**).

IFN-γ signals by binding to the IFN-γ receptor (IFN-γR) on the cell surface (**Figure 18**). Thus, one explanation for the lack of an effect of IFN-γ could be the loss of the IFN-γ receptor. To determine that $CD4^+$ T cells from *$Tbx21^{-/-}$* mice were not defective in the expression of the IFN-γR, and thereby rendered unable to respond to IFN-γ stimulation, cell surface staining for the signaling chain of the receptor (IFN-γRβ) was performed. Following infection with *T. cruzi*, $CD4^+$ T cells from *$Tbx21^{-/-}$* mice

exhibited significant up-regulation of IFN-γRβ, compared to cells from uninfected mice, and did so equally as well as cells from infected wild-type mice (**Figure 22B**). Thus, the expression of the IFN-γR was not defective in *Tbx21*$^{-/-}$ T cells, confirming that they are capable of responding to IFN-γ stimulation. Although CD4^{+} T cells from *T. cruzi*-infected *Tbx21*$^{-/-}$ mice can respond to IFN-γ, it does not appear to have any direct inhibitory effects on IL-17 production.

IFN-γ can act on APCs to limit Th17 responses in an IL-12-dependent manner.

In addition to T cells, IFN-γ can have wide-ranging effects on other cell types during an immune response. For example, APC functions such as IL-12 production can be augmented by IFN-γ[119]. While no direct effect of IFN-γ was observed on T cells, it could potentially function indirectly to regulate Th17 responses during *T. cruzi* infection via modulation of APC function. Since APC-derived IL-12 is critical for promoting/stabilizing Th1 responses, we hypothesized that it could also serve to limit Th17 responses.

To test this possibility splenocytes from infected *Tbx21*$^{-/-}$ mice were cultured with *T. cruzi* lysate in the presence of anti-IFN-γ, anti-IL-12, or both anti-IFN-γ and anti-IL-12 together to determine if these cytokines had any regulatory effect on IL-17 production. Neutralization of IFN-γ resulted in a minor increase in IL-17, but this effect was not statistically significant. Surprisingly, the neutralization of IL-12 led to a significant increase in IL-17 production (**Figure 23**). Interestingly, the neutralization of both IFN-γ and IL-12 led to an even further increase in the levels of IL-17 (**Figure 23**). These results suggest that IFN-γ may indirectly limit *T. cruzi*-specific Th17 responses, possibly

by promoting APC-derived IL-12 production, which in turn regulates IL-17 production by T cells. Additionally, it demonstrates that IL-12 can have inhibitory effects on IL-17 production by T cells in a T-bet-independent manner.

IL-23 can enhance T. cruzi-specific Th17 responses in T-bet-independent manner.

In contrast to potential inhibitory effects of IL-12 on the Th17 program, the IL-12-family cytokine member IL-23 is important for promoting Th17 cell differentiation, growth, and stability of the Th17 phenotype. Since IL-12 had the ability to limit the production of IL-17 by T cells from *Tbx21*$^{-/-}$ mice infected with *T. cruzi*, we wanted to determine if IL-23 exerted a similar influence in the absence of T-bet. Splenocytes from naïve uninfected mice cultured with *T. cruzi* lysate did not produce any measurable levels of IL-17, whereas addition of recombinant IL-23 yielded a minor increase in IL-17. This increase in IL-17 production is mostly likely due to the presence of endogenous memory T cells responding to IL-23, as these cells were derived from uninfected mice and had not previously been exposed to *T. cruzi*. As expected, cells from infected C57BL/6 mice produced little IL-17 in response to *T. cruzi* lysate, and this response was increased to a level comparable to that observed with cells from uninfected mice with the addition of IL-23. Lastly, the addition of IL-23 to cells from infected *Tbx21*$^{-/-}$ mice cultured with *T. cruzi* lysate resulted in a significant increase in IL-17 production (**Figure 24**). Thus, unlike IL-12 which limited *T. cruzi*-specific IL-17 production by *Tbx21*$^{-/-}$ T cells, IL-23 enhanced its production. These results show that IL-12 and IL-23 are can regulate Th17 responses in vivo during *T. cruzi* infection in a T-bet-independent

manner, and suggest an even broader role for IL-12 family cytokines in normal wild-type mice.

Figure 18. T-bet is an IFN-γ/Stat-1-inducible transcription factor in T cells.

The IFN-γ receptor consists of the α binding chain and the β signaling chain. Binding of IFN-γ results in receptor chain dimerization and Stat-1 activation. Activated Stat-1 induces T-bet expression, which then in turn drives IFN-γ expression. Secreted IFN-γ can then act in a positive-feedback manner to augment and stabilize T-bet expression.

Figure 18

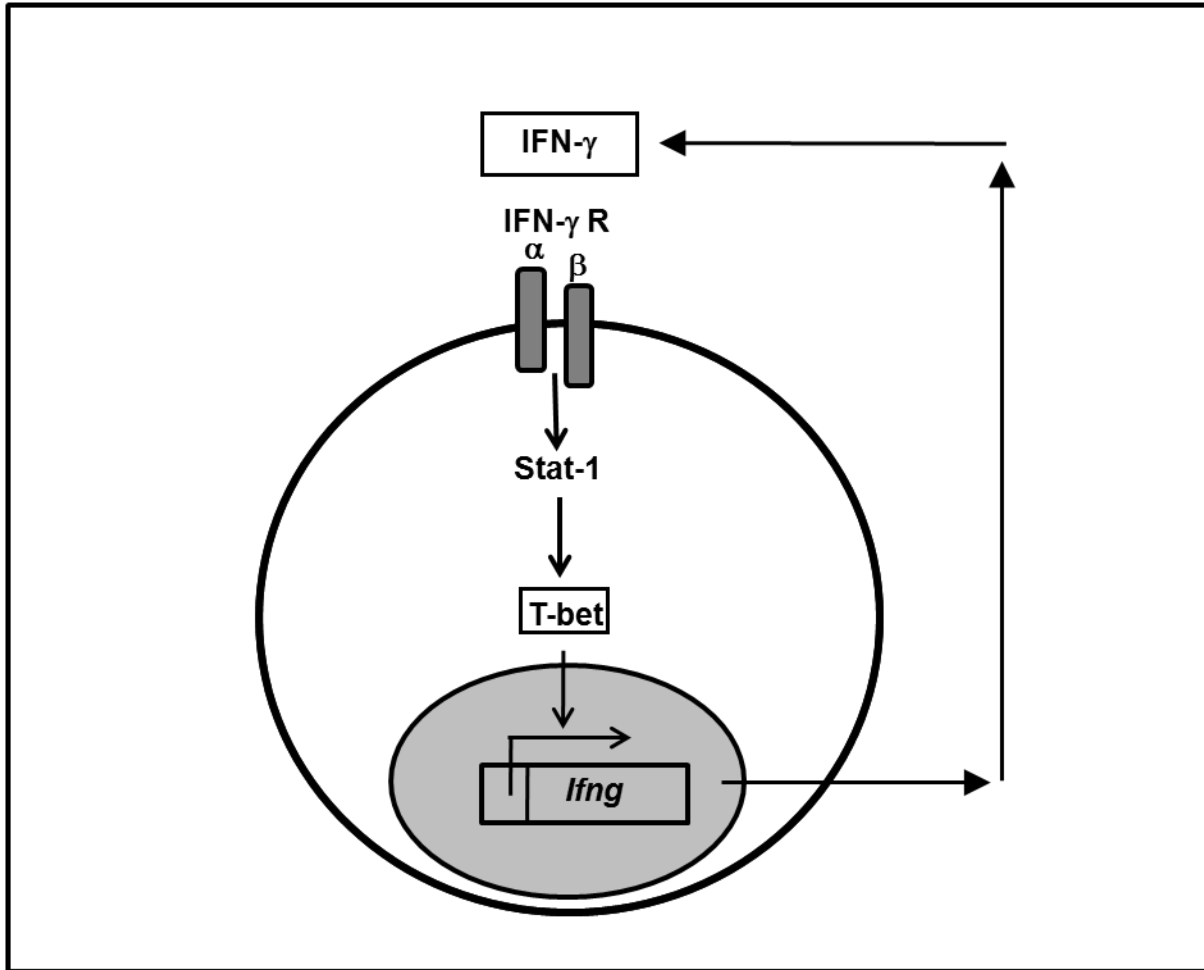


Figure 19. Stat-1 is required to inhibit Th17 development during *T. cruzi* infection. Spleens from *Tbx21*$^{-/-}$ (A and C) and *Stat-1*$^{-/-}$ (B and D) mice infected with *T. cruzi* were harvested at day 9 p.i. and analyzed for recall responses. (A and B) Spleen cells were stimulated with anti-CD3 in the presence of monensin and cells analyzed for intracellular IFN-γ and IL-17 by FACS. Alternatively, cells were stimulated with anti-CD3 (C) or endotoxin-free *T. cruzi* lysate (D) and supernatants harvested and tested for IL-17 protein by ELISA. (A and B) Numbers represent the percentage of IFN-γ^{+}, IFN-γ^{+}/IL-17^{+}, and IL-17^{+} T cells and are representative of three to five independent experiments with two mice per group.

Figure 19

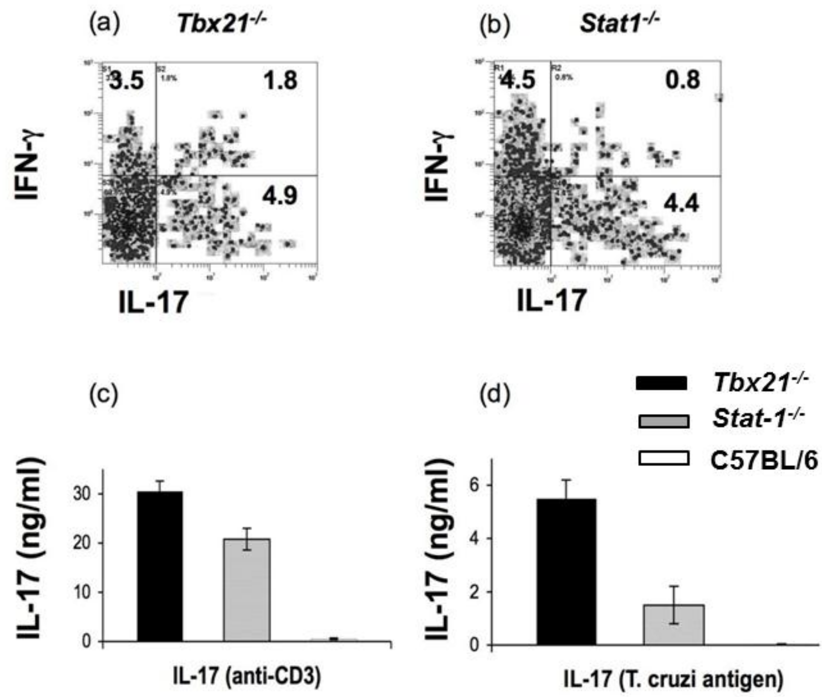


Figure 20. IFN-γ is mostly dispensable for T-bet-dependent regulation of Th17 responses to infection.

Spleens from *Tbx21*$^{-/-}$ (A), *Ifng*$^{-/-}$ (B), and WT C57BL/6 (C) mice infected with *T. cruzi* were harvested at day 9 p.i. and analyzed for recall responses. (A–C) Spleen cells were stimulated with anti-CD3 in the presence of monensin and cells analyzed for intracellular IFN-γ and IL-17 by FACS. Alternatively, cells from *Tbx21*$^{-/-}$ or *Ifng*$^{-/-}$ mice were stimulated with anti-CD3 (D) or endotoxin-free *T. cruzi* lysate (E) and supernatants harvested and tested for IL-17 protein by ELISA. (A and B) Numbers represent percentage of IL-17^{+} T cells and are representative of multiple experiments with two mice per group.

Figure 20

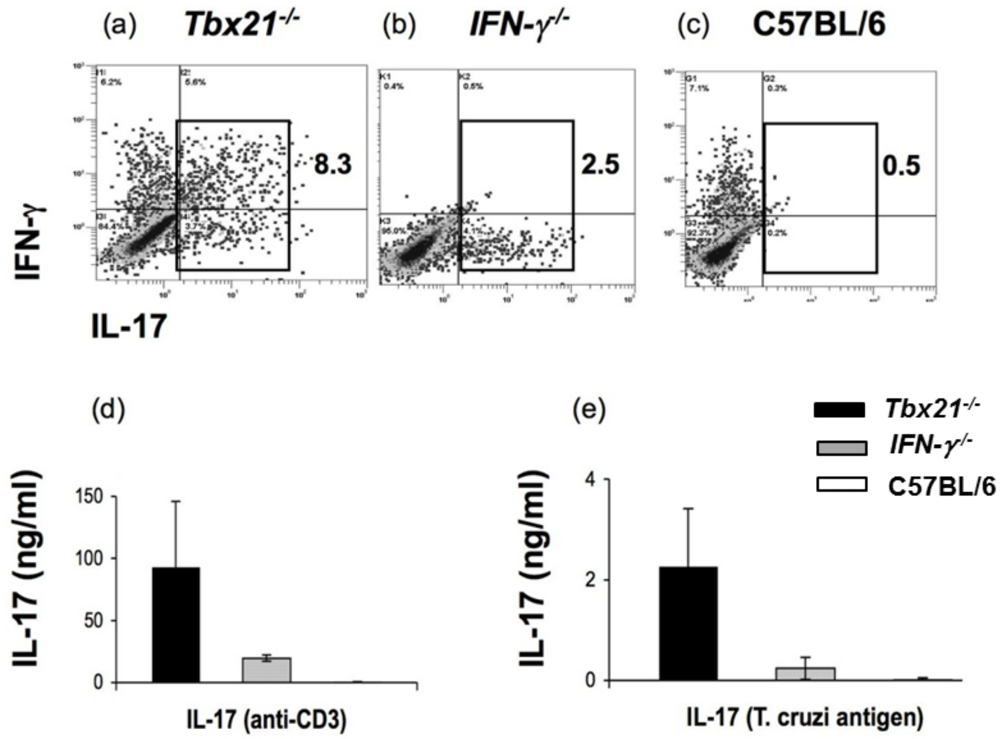


Figure 21. CXCR3 expression correlates with the relative levels of T-bet activity in *Stat-1*$^{-/-}$ and *Ifng*$^{-/-}$ mice.

Spleen cells from WT naive (A) or *T. cruzi*-infected WT (B), *Tbx21*$^{-/-}$ (C), *Stat-1*$^{-/-}$ (D), or *Ifng*$^{-/-}$ (E) mice were harvested at day 9 p.i. and analyzed for surface expression of CXCR3 by FACS. Histogram plots show the relative percentages of $CD4^+$ T cells that express CXCR3 and are representative of one experiment among multiple experiments performed. (F) The average numbers of $CD4^+$ T cells that express CXCR3 are shown. *$p<0.0262$ comparing WT versus *Stat-1*$^{-/-}$ $CXCR3^+$ cells; ***$p<0.0025$ comparing WT versus *Tbx21*$^{-/-}$ cells. Differences between *Stat-1*$^{-/-}$ and *Tbx21*$^{-/-}$ were not significant.

Figure 21

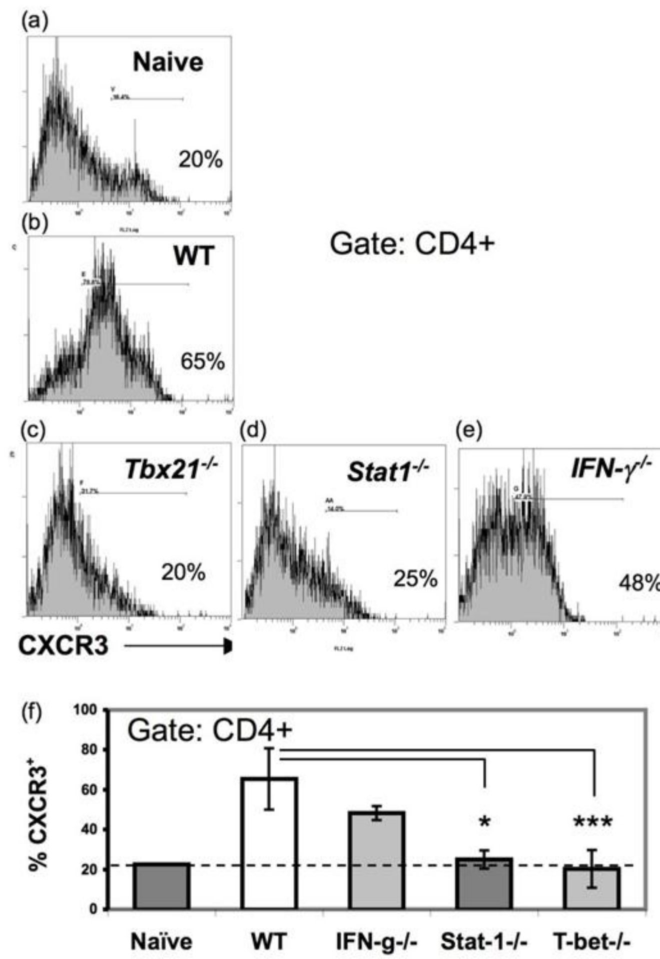
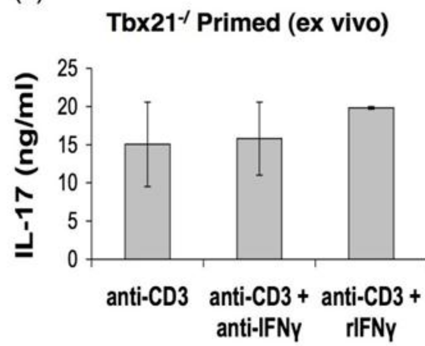


Figure 22. The lack of a direct effect of IFN-γ on IL-17 production is not secondary to defective IFN-γRβ expression.

A) $CD4^+$ T cells were purified from the spleens of *Tbx21*$^{-/-}$ mice, infected previously with *T. cruzi* (day 9 p.i.) and stimulated with anti-CD3 in the presence or absence of neutralizing antibody to IFN-γ or rIFN-γ. Supernatants were harvested and tested for IL-17 protein by ELISA. Results shown reflect the average of three independent experiments. B) Cells from naive WT, infected WT, and infected *Tbx21*$^{-/-}$ mice were stained with PE anti-IFN-γRβ and analyzed by flow cytometry. Results shown are gated on $CD4^+$ T cells; no significant differences were observed between $CD4^+$ T cells from infected WT and *Tbx21*$^{-/-}$ mice.

Figure 22

(a)



(b)

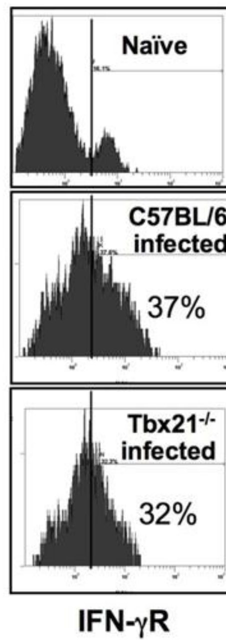


Figure 23. IFN-γ can limit Th17 responses in an IL-12-dependent manner.

Spleen cells from *Tbx21*$^{-/-}$ mice infected with *T. cruzi* (day 9 p.i.) were restimulated ex vivo with endotoxin-free *T. cruzi* lysate in the presence of anti-IFN-γ, anti-IL-12, or anti-IFN-γ and IL-12 neutralizing antibodies. Supernatants were harvested after 72 hours of stimulation and tested for IL-17 protein by ELISA. *$p<0.04$; ***$p<0.005$.

Figure 23

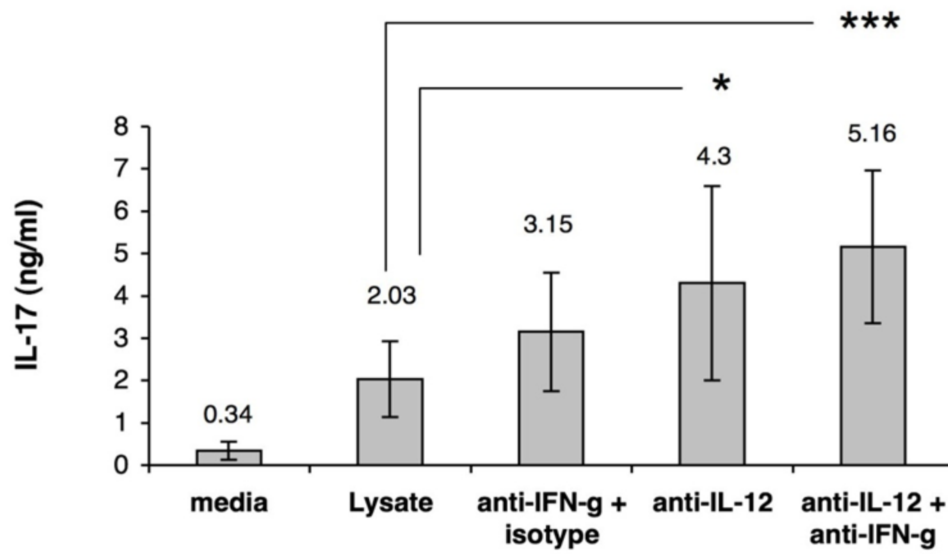
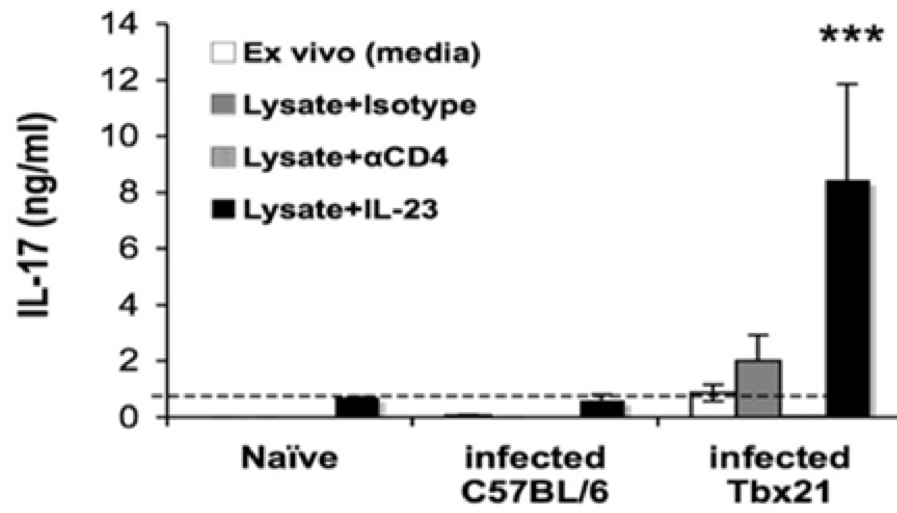


Figure 24. IL-23 enhances *T. cruzi*-specific Th17 responses independent of T-bet. Spleen cells from naive wild-type, *T. cruzi*-infected wild-type, and *T. cruzi*-infected *Tbx21*$^{-/-}$ mice were harvested at day 9 p.i. and cultured with *T. cruzi* lysate and an isotype control or anti-CD4 antibody (to demonstrate the antigen-specific nature of the cytokine response) or in the presence of rIL-23. Supernatants were harvested after 72 h and tested for IL-17 protein by ELISA. ***$p<0.002$ for IL-17 production by cells from infected *Tbx21*$^{-/-}$ mice compared with infected WT or naive WT.

Figure 24



Discussion

We previously demonstrated that T-bet is critical for inhibiting Th17 cell differentiation during infection with *T. cruzi,* while IFN-γ appeared to be ineffective at doing so[120]. The purpose of this study was to definitively determine the role of IFN-γ and Stat-1 in regulating the development of Th17 responses to *T. cruzi* infection. We found that Stat-1 was necessary for controlling Th17 development as infected *Stat-1*$^{-/-}$ mice developed Th17 responses similar to those observed in infected *Tbx21*$^{-/-}$ mice. In contrast to Stat-1, that IFN-γ was mostly dispensable. Infection of *Ifng*$^{-/-}$ mice resulted in only a modest increase in Th17 development relative to infected *Stat-1*$^{-/-}$ or *Tbx21*$^{-/-}$ mice. In regards to T-bet levels, its expression in *Stat-1*$^{-/-}$ mice appeared to be impaired, while T-bet expression in *Ifng*$^{-/-}$ mice was normal, based upon T-bet-dependent CXCR3 expression. These results suggest that Stat-1, not IFN-γ, is critical for T-bet induction and its subsequent inhibition of Th17 responses to *T. cruzi*.

Surprisingly, we found as many IFN-γ-producing CD4^{+} T cells in infected Stat-1-deficient mice as in wild-type or T-bet-deficient mice, indicating that the development of Th1 responses to *T. cruzi* infection is also Stat-1-independent. These results are supported by previous findings that show unimpaired Th1 development in the absence of Stat-1 during *Toxoplasma* and LCMV infection[117,115]. However, a separate study using *L. major* infection showed that Stat-1 expression in APCs rather than T cells was necessary for Th1 responses, as APCs from infected Stat-1-deficient mice exhibited impaired antigen presentation and IL-12 production[121]. Here, our results show that mice

infected with *T. cruzi* generate Th1 cells even though Stat-1 is absent in both non-T cells and T cells.

Although IFN-γ was mostly dispensable for inhibiting the development of Th17 cells during *T. cruzi* infection, we sought to definitively address its exact role. As our initial data from infected T-bet-deficient mice suggested, IFN-γ had no direct effect on regulating T cell IL-17 production as neutralization of IFN-γ or exogenous treatment of $CD4^+$ T cells from $Tbx21^{-/-}$ mice failed to alter the levels IL-17. However, neutralization of both IFN-γ and IL-12 in splenocyte cultures from infected $Tbx21^{-/-}$ mice resulted in an increase in *T. cruzi*-specific IL-17 production. Thus, the importance of IFN-γ appears to be in its ability to regulate Th17 responses indirectly by acting in concert with APC-derived IL-12 and/or by modulating other APC functions. This finding supports results reported in the *Mycobacterium bovis* BCG study, in which the effects of IFN-γ on Th17 responses were due the ability of IFN-γ to augment IL-12 production by APC[57]. IFN-γ may further limit IL-17 production by suppressing APC production of IL-23, which is needed for Th17 maintenance. Regulation of IL-23 production could be important as addition of recombinant IL-23 led to an increase in *T. cruzi*-specific IL-17 levels. These results reveal a minor role for IFN-γ in limiting Th17 responses during *T. cruzi* infection through its effects on APCs.

Importantly, these results show that IL-12 can have inhibitory effects on *T. cruzi*-specific Th17 responses and these effects occur independently of T-bet. This finding is supported by a study in which IL-12 could suppress Th17 responses and associated inflammation in an allergic lung inflammation model[122]. Similar to our study, the effects of IL-12 on Th17-mediated inflammation occurred in a T-bet-independent manner.

However, the suppression of lung Th17 responses by IL-12 was IFN-γ-independent, whereas in our study the greatest suppression of IL-17 was observed when both IL-12 and IFN-γ were active.

Since IL-12 activates Stat-4 to promote Th1 development, the observed inhibitory effects of IL-12 on IL-17 production by Th17 cells may be mediated by Stat-4 but independently of T-bet. Thus, the regulation of Th17 cell development during *T. cruzi* infection may be mediated by both an IL-12/Stat-4 mechanism and a T-bet-dependent mechanism. The concerted effort of these two pathways may lead to the full suppression of Th17 development. Alternatively, there remains the possibility that these pathways may be interconnected and IL-12 may also influence Th17 development via T-bet as IL-12/Stat4 signaling has been shown to be required for full T-bet-driven Th1 differentiation.

Finally, an intriguing issue raised by this study was in regards to the role of Stat-1-signaling in suppressing Th17 development during *T. cruzi* infection. Since IFN-γ-deficiency did not recapitulate the degree of Th17 development that was observed in *Stat-1*$^{-/-}$ and *Tbx21*$^{-/-}$ mice, it was indicative of the potential involvement of another cytokine other than IFN-γ capable of activating Stat-1 signaling.

Chapter 4: Role of IL-12-family cytokines during infection with *Trypanosoma cruzi*

Introduction

Although the exact functions of IL-17 during infection with *T. cruzi* have not been extensively studied or fully characterized, it does appear to have an important role during acute resistance to *T. cruzi*. Neutralization of IL-17 in mice infected with *T. cruzi* resulted in increased myocarditis and mortality[123]. Similarly, infection of IL-17A-deficient mice resulted in increased parasitemia and organ failure[124]. However, the effects of IL-17 on the inflammatory responses of infected mice in these studies varied. However, these results could be due to differences in mouse strains used and experimental protocols, for example neutralization versus gene deletion of IL-17. Nevertheless, the absence of IL-17 led to a decrease in resistance to *T. cruzi* infection, suggesting it does play an important role in providing protection.

Though IL-17 may be necessary for acute resistance, it is clear that chronic IL-17 production during immune responses to *T. cruzi* is associated with increased susceptibility and mortality as we and others have shown[56,120]. To circumvent the pathological consequences of Th17-associated inflammation during *T. cruzi* infection, appropriate and timely regulation of the Th17 response is critical. APC-derived cytokines generally possess a strong potential to influence developing and ongoing T cell responses. We previously demonstrated an important role for Stat-1 and T-bet in limiting Th17 cell responses to *T. cruzi*[120,125], yet the cytokine signal that utilize these transcription factors have not been determined. We believe that cytokines produced by APCs are important for regulating Th17 responses during *T. cruzi* infection.

The IL-12-family of heterodimeric cytokines, which include IL-12, IL-27, and IL-23, are produced by APCs and are highly influential in regulating aspects of T helper

cell development, differentiation, and maintenance[126,127]. The cytokines in this family are grouped according to their shared usage of cytokine and receptor subunits. IL-12 is composed of p35 and p40 and is important for Th1 responses. IL-27 consists of Ebi-3 and p28 and also affects Th1 development, but is known for possessing anti-inflammatory properties as well. Lastly, IL-23 is formed by p19 and p40 and is recognized for its role in promoting and stabilizing Th17 differentiation. Thus, the IL-12 family cytokines may also be important for influencing Th17 responses to *T. cruzi*.

IL-12 has a central role in mediating resistance to *T. cruzi*[20]. Infection with *T. cruzi* elicits a potent IL-12 response, particularly by macrophages[84]. The absence of IL-12 during *T. cruzi* infection results in a significant impairment in the ability of mice to mount an adequate Th1 response[128]. Thus, *T. cruzi*-infected mice that lack IL-12 are severely impaired in their ability to control parasitemia and do not survive acute infection. Accumulating evidence suggests that IL-12 is also important for antagonizing IL-17 production by Th17 cells. These reports showed that Th17 cells possess a considerable amount of plasticity and a tendency to differentiate into Th1 cells. Specifically, they showed that Th17 cells were amenable to the effects of IL-12 and could undergo conversion into Th1 cells[129,130]. Yet, it is unclear whether IL-12 has any regulatory effects on the initial development of Th17 cells in vivo during infection. Furthermore, our previous findings revealed a T-bet-independent role for IL-12 in limiting Th17 responses during *T. cruzi* infection (**Chapter 3**). However, we had not yet addressed whether IL-12 could have regulatory effects in which T-bet is required. Thus, we wished to further investigate the role of IL-12 in regulating Th17 responses to *T. cruzi*.

Like IL-12, IL-27 can promote Th1 responses, and is important for promoting Th1 development during *L. major* infection. Conversely, IL-27 can also serve as a negative regulator of T cell responses, including Th1 and Th17 development. For instance, IL-27 production is important during *T. cruzi* infection, as mice that lack the IL-27-receptor subunit, WSX-1, exhibit exacerbated Th1 responses and display increased susceptibility[131]. However, in this study, IL-17 responses were not investigated. Data originating from a separate study showed that *WSX-1*$^{-/-}$ mice had increased levels of systemic IL-17 as well as increased IL-17 production by liver mononuclear cells (LMNCs) during *T. cruzi* infection[132], although the effects of this on resistance were not investigated in this instance. Therefore, it is unclear if IL-27 signaling regulates CD4^{+} T cell-derived IL-17 in response to *T. cruzi*. It is possible that increased Th17 responses could have contributed to the increased susceptibility *WSX-1*$^{-/-}$ mice. To lend further support to this possibility, infection of *WSX-1*$^{-/-}$ mice with another protozoan parasite, *Toxoplasma gondii*, leads to increased development of Th17 responses in the central nervous system[133] Likewise, multiple in vitro studies have demonstrated a role for IL-27 in suppressing Th17 differentiation and this can occur in a Stat-1-dependent manner. Importantly, Stat-1 was important for inhibiting the development of Th17 cells during *T. cruzi* infection, while IFN-γ had only a minor role which appeared to be indirect via its effects on APCs[125]. Based on these lines of evidence, we hypothesized that IL-27 was the required Stat-1-activating cytokine responsible for inducing Stat-1 and T-bet expression, leading to the negative regulation of Th17 responses during *T. cruzi* infection.

Therefore, the primary goal of this study was to determine the extrinsic factors, or cytokines, involved in the T-bet-dependent regulation of Th17 development during infection with *T. cruzi*. We hypothesized that both IL-12 and IL-27 would provide regulatory effects on Th17 differentiation. Additionally, we felt that the relative contributions of IL-12 and IL-27 would be different, thus, we sought to determine if there was a hierarchy between IL-12 and IL-27 in this regulation.

Materials and Methods

Mice and parasite infections

Age and sex-matched C57BL/6, *Tbx21*$^{-/-}$, *IL12p35*$^{-/-}$, *IL12p40*$^{-/-}$, and *Ebi-3*$^{-/-}$ mice were obtained from The Jackson Laboratory and were used between six and eight weeks of age. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility under pathogen-free conditions and used in accordance with an Institutional Animal Care and Use Committee-approved protocol. For infections, mice were injected intraperitoneally with $1x10^6$ *T. cruzi* trypomastigotes (CL strain).

Ex vivo cell culture

$2x10^6$ splenocytes from *T. cruzi*-infected mice were cultured in complete RPMI 1640 (10% FBS, 10mM Hepes, 1 IU/ml penicillin and 100μg/ml streptomycin, 2mM L-glutamine, $1x10^{-5}$ M 2-β-mercaptoethanol). Splenocytes were re-stimulated with 0.5μg/ml of LEAF-purified anti-CD3 (clone 145-2C11, Biolegend) or 10μg/ml *T. cruzi* lysate for antigen-specific responses for 72 hours. For the neutralization of IFN-γ, LEAF-purified anti-IFN-γ (clone XMG1.2, Biolegend) antibodies were administered to ex vivo cell cultures at a concentration of 5μg/ml.

Recombinant cytokines

For Th17 differentiation, cells were stimulated in the presence of recombinant IL-6 (20ng/ml) and TGF-β (10ng/ml). For ex vivo culture experiments, recombinant mouse

IL-12 (p70), IL-27 (100ng/ml), and IL-23 (20ng/ml) were used. All cytokines were purchased from Biolegend.

Fluorescent antibodies and flow cytometry

Peripheral blood leukocytes and splenocytes were incubated with anti-CD16/32, and then stained with anti-Gr-1 (PE) fluorescent antibodies. Likewise, splenocytes were stained with anti-CD4 (FITC) and anti-CXCR3 (PE) antibodies. For intracellular cytokine staining, cells were stained with anti-CD4 (APC/Cy7) and fixed with 4% paraformaldehyde. After fixation, cells were permeabilized using Permeabilization Wash Buffer (Biolegend) and subsequently stained with anti-IFN-γ (APC) and anti-IL-17 (PE) antibodies. All fluorescent antibodies were obtained from Biolegend. Cells were analyzed by flow cytometry using an FC500 instrument (Beckman Coulter).

Intracellular T-bet staining

First, cell surface staining with anti-CD4 (FITC) was performed. Cells were then fixed with Foxp3 Fix/Perm (Biolegend) for 20 minutes at room temperature, and then spun down and the supernatant removed. Fixed cells were washed once with cell staining buffer and centrifuged at 250g for 5 minutes and the supernatants were removed. The cell pellets were then washed once with Foxp3 Perm Buffer (Biolegend). Cells were then re-suspended in Foxp3 Perm Buffer and incubated for 15 minutes at room temperature. Following permeabilization, cells were spun down and then re-suspended in 100μl of Foxp3 Perm Buffer and stained with anti-T-bet (PE; Biolegend, clone 4B10)

for 30 minutes at 4°C. Cells were washed twice with cell staining buffer and then analyzed by flow cytometry.

Measurement of cytokines

Cell culture supernatants were collected after 72 hours and analyzed for the presence of IFN-γ, IL-17, or IL-10 using Biolegend's ELISA MAX Standard Sets according to manufacturer recommendations.

Statistical analyses

Data were analyzed using student t test or Mann Whitney *U* test as appropriate. (SigmaPlot 12.0, Systat Software, Inc.). A p value <0.05 was considered significant. Data are represented as means ±SD of experimental groups.

Results

Differential effects of IL-12 family cytokines IL-12, IL-27, and IL-23 on IL-17 production by T cells from wild-type mice infected with T. cruzi.

The IL-12-family cytokines, which include IL-12, IL-27, and IL-23, are produced by APCs and are essential for regulating $CD4^+$ T helper cell differentiation. First, we wanted to determine what effect these cytokines had on IL-17 production by T cells that were differentiated in vivo in response to infection with *T. cruzi*. Splenocytes from wild-type *T. cruzi*-infected mice were re-stimulated on day 9 p.i. with anti-CD3 in the presence of IL-12, IL-27, or IL-23. The addition of IL-12 resulted in approximately a 60% decrease in IL-17 production as determined by ELISA (**Figure 25A**). Contrary to our expectation, IL-27 had no inhibitory effect on IL-17 production by primed T cells recovered from *T. cruzi*-infected mice. This is in contrast to the effects of IL-27 on naïve $CD4^+$ T cells in vitro, as IL-27 was capable of suppressing TGF-β+IL-6-mediated Th17 differentiation (**Figure 25B**). While IL-12 exhibited suppressive effects on the ability of T cells to produce IL-17, IL-23 enhanced IL-17 production by approximately 6-fold (**Figure 25A**). It has yet to be determined whether the increase in IL-17 in response to IL-23 stimulation is a consequence of increased expansion of Th17 cells or a direct stimulation of IL-17 production on a per cell basis. These results demonstrate that T cells primed during *T. cruzi* infection are responsive to both IL-12 and IL-23, and that these cytokines have the ability to differentially regulate IL-17 production.

Increased development of Th17 responses to T. cruzi infection in the absence of IL-12p35.

Because IL-12 had a potent inhibitory effect on IL-17 production upon re-stimulation of $CD4^+$ T cells from *T. cruzi*-infected mice, we next sought to determine the role of IL-12 in regulating the development of Th17 responses to *T. cruzi* infection in vivo. To do this, C57BL/6, IL-12p35-deficient (*IL-12p35*$^{-/-}$), IL-12p40-deficient (*IL-12p40*$^{-/-}$), and *Tbx21*$^{-/-}$ mice were infected with *T. cruzi*. On day 9 p.i., infected mice were euthanized and T cell responses were analyzed by culturing splenocytes with *T. cruzi* lysate to measure antigen-specific recall responses. T cells from infected C57BL/6 mice had a Th1/IFN-γ response that predominated over that of IL-17 (**Figure 26**). As expected, infection of *IL-12p35*$^{-/-}$ mice with *T. cruzi* resulted in impaired Th1 development. Importantly, there was a significant increase in *T. cruzi*-specific IL-17 production (**Figure 26**). The addition of anti-CD4 neutralizing antibody significantly reduced *T. cruzi*-specific IL-17 production, confirming that the IL-17 is derived from $CD4^+$ T cells (not shown). The increase in IL-17 in infected *IL-12p35*$^{-/-}$ mice was not evident in *IL-12p40*$^{-/-}$ mice, however, which lack both IL-12 and IL-23 (p40+p19 subunits). This result supports the reported role of IL-23 in the generation of Th17 cells[134] and the notion that the Th17 development that occurs in *IL-12p35*$^{-/-}$ mice infected with *T. cruzi* is mediated at least in part by IL-23.

To evaluate the consequences of increased Th17 development and IL-17 production in *IL-12p35*$^{-/-}$ mice infected with *T. cruzi*, peripheral blood was analyzed for Th17-associated inflammation. On day 9 p.i., blood was collected and cells were stained for the neutrophil marker Gr-1. We observed a significant increase in the

percentage of Gr-1^{+} cells in the blood of infected *IL-12p35$^{-/-}$* mice compared to infected wild-type mice (**Figure 27A-B**), however there was no difference in spleens (**Figure 27C**). These results demonstrate that IL-12 regulates the development of *T. cruzi*-specific Th17 responses to *T. cruzi* infection in vivo.

Increased Th17 responses in IL-12p35$^{-/-}$ mice are associated with a significant reduction in the expression and activity of T-bet.

We previously demonstrated that T cell-specific expression of T-bet is critical for regulating Th17 responses to *T. cruzi*. Since *IL-12p35$^{-/-}$* mice developed a significant Th17 response to *T. cruzi*, we next wanted to determine how IL-12 deficiency correlated with the levels of T-bet expression in CD4^{+} T cells recovered from infected mice. Thus, CD4^{+} T cells were analyzed for T-bet expression by intracellular flow cytometry. As expected, there was a significant increase in the percentage of CD4^{+} T cells that expressed T-bet in C57BL/6 mice infected with *T. cruzi* compared to naïve uninfected control mice (**Figure 28A**). However, the percentage of CD4^{+} T cells from infected *IL-12p35$^{-/-}$* mice that expressed T-bet was significantly lower than in infected wild-type mice (**Figure 28A**). Additionally, T-bet-dependent CXCR3 expression was also significantly reduced in *IL-12p35$^{-/-}$* CD4^{+} T cells compared to wild-type CD4^{+} T cells (**Figure 28B**). Therefore, under conditions of IL-12 deficiency, CD4^{+} T cells from *T. cruzi*-infected mice have reduced levels of T-bet expression and activity, and this reduction is associated with a significant increase in Th17 responses.

IL-27 is dispensable for regulating Th17 responses to T. cruzi infection.

As shown in **Figure 25A**, we observed that IL-27 had no observable effect on IL-17 production upon re-stimulation of T cells ex vivo. However, it is possible that the effects of IL-27 are limited to the initial differentiation of T cells. For example, IL-27 has been shown to regulate Th17 responses during *Toxoplasma gondii* infection[133]. To determine the importance of IL-27 in regulating Th17 development during *T. cruzi* infection, we infected *Ebi-3*$^{-/-}$ mice with *T. cruzi*. Ebi-3, together with the subunit p28, forms the heterodimeric cytokine IL-27. On day 9 p.i., splenocytes from C57BL/6, *Ebi-3*$^{-/-}$, and *Tbx21*$^{-/-}$ mice were cultured ex vivo with *T. cruzi* lysate and cytokine responses were analyzed. Contrary to our expectations, *Ebi-3*$^{-/-}$ mice did not exhibit any significant increase in *T. cruzi*-specific IL-17 production (**Figure 29A**). Because of this unexpected result, we considered the possibility that re-stimulation with *T. cruzi* lysate masked any potential inhibitory effects of IL-27. Instead, splenocytes from infected *Ebi-3*$^{-/-}$ mice were re-stimulated using anti-CD3. However, even with anti-CD3 re-stimulation there was no significant increase in IL-17 production (**Figure 29B**). Finally, as an additional confirmation for the detection of IL-17-producing T cells, we performed intracellular cytokine staining of T cells recovered from infected *Ebi-3*$^{-/-}$ mice. However, we again failed to detect any increase in IL-17-producing T cells in infected *Ebi-3*$^{-/-}$ mice (**Figure 30**). Interestingly, T cell-derived IFN-γ production in infected *Ebi-3*$^{-/-}$ mice was comparable to that of C57BL/6 and *Tbx21*$^{-/-}$ mice (**Figure 29C**). This was unexpected and in contrast to systemic IFN-γ production, as serum IFN-γ levels in infected *Ebi-3*$^{-/-}$ mice were significantly elevated relative to infected wild type mice (not shown). The increase in serum IFN-γ in *T. cruzi*-infected IL-27-deficient mice is consistent with

previous reports[131]. Consistent with the lack of an increase in Th17 development, infected *Ebi-3*$^{-/-}$ mice also did not show any signs of increased neutrophilia compared to wild-type mice (**Figure 31A-C**), further confirming the lack of any significant increase in IL-17 production and Th17-associated inflammation. Thus, IL-27 is not required for regulating the Th17 response during *T. cruzi* infection.

Normal induction of T-bet and T-bet-dependent CXCR3 expression in IL-27-deficient mice infected with T. cruzi.

Since IL-27 was not required for regulating Th17 responses to *T. cruzi*, we hypothesized that in *Ebi-3*$^{-/-}$ mice, other cytokines such as IL-12 were sufficient for induction of T-bet and T-bet-dependent regulation of Th17 responses to *T. cruzi*. Therefore, T-bet expression in $CD4^+$ T cells recovered from *Ebi-3*$^{-/-}$ mice was analyzed on day 9 p.i. Indeed, there was a significant increase in the percentage of $CD4^+$ T cells that expressed T-bet in both C57BL/6 and *Ebi-3*$^{-/-}$ mice infected with *T. cruzi* (**Figure 32A**). Furthermore, T-bet-dependent induction of CXCR3 was normal in $CD4^+$ T cells obtained from infected *Ebi-3*$^{-/-}$ mice (**Figure 32B**). These results demonstrate that during *T. cruzi* infection, IL-27 is dispensable for induction of T-bet and the regulation of Th17 development.

Reduced IL-10 production in infected IL-27-deficient mice does not affect IL-17 production.

The immunoregulatory cytokine IL-10 is known to possess suppressive properties. It has been demonstrated that T cells derived from IL-27R-deficient mice

produce less IL-10 and more IL-17(135). In contrast, another study in which *Ebi-3*$^{-/-}$ mice were infected with *L. major* showed that IL-10 production by T cells was actually increased[136]. Therefore, it was possible that during infection with *T. cruzi*, *Ebi-3*$^{-/-}$ mice may have altered IL-10 production. We hypothesized that if IL-10 production was increased during infection of *Ebi-3*$^{-/-}$ mice with *T. cruzi*, this IL-10 could be potentially suppressing Th17 development.

To determine if IL-10 production was altered by Ebi-3 deficiency during *T. cruzi* infection, splenocytes from infected *Ebi-3*$^{-/-}$ mice were cultured with *T. cruzi* lysate or anti-CD3 and IL-10 production was measured. Interestingly, splenocytes from *Ebi-3*$^{-/-}$ mice actually produced significantly less IL-10 than cells from infected wild-type mice (**Figure 33**). This eliminated the possibility that an increase in IL-10 production was the reason for the lack of an increase in Th17 responses in infected *Ebi-3*$^{-/-}$ mice. Furthermore, IL-10 does not appear to have any suppressive activity on Th17 responses to *T. cruzi* infection, as the decrease we observed did not result in an increase in IL-17 production.

Stability of IL-17-producing T cells from T. cruzi-infected mice.

The propensity of Th17 cells to undergo a conversion in their cytokine expression profile has been demonstrated previously. For example, transfer of in vitro-polarized Th17 cells into lymphopenic recipients results in their conversion into Th1 cells[137]. Furthermore, Th17 cells generated in vivo could be converted into Th1/Th17 cells ex vivo in a manner that was dependent upon both IL-12 and IFN-γ[138]. Therefore, we wanted to address the potential plasticity of Th17 cells generated in response to *T. cruzi*

infection. Splenocytes were harvested from *T. cruzi*-infected *IL-12p35*$^{-/-}$ mice on day 9 p.i. and subsequently re-stimulated ex vivo in the presence of cytokines. The addition of IL-12 led to a significant reduction in IL-17 production (**Figure 34A**). To determine if IFN-γ was necessary for IL-12-dependent inhibition of IL-17 production by T cells from *T. cruzi*-infected mice, we neutralized IFN-γ during re-stimulation. Surprisingly, and in contrast to the above mentioned study, neutralization of IFN-γ had no effect on the ability of IL-12 to inhibit IL-17 (**Figure 34A**), demonstrating that the effects of IL-12 are independent of IFN-γ. To test these results using *T. cruzi*-specific stimulation, splenocytes from infected *IL-12p35*$^{-/-}$ mice were re-stimulated with *T. cruzi* lysate. Similar to results obtained with polyclonal stimulation, IL-12 suppressed *T. cruzi*-specific IL-17 production in an IFN-γ independent manner (**Figure 34B**). Similarly, to determine if IL-27 could compensate for IL-12 deficiency and promote the conversion of Th17 cells, T cells from infected *IL-12p35*$^{-/-}$ mice were cultured as above in the presence of IL-27. We observed that addition of IL-27 had no effect on the cytokine-producing potential of T cells stimulated with either anti-CD3 or *T. cruzi* lysate (**Figure 34A-B**). Despite the lack of a significant effect of IL-27 on IL-17, we reasoned that IL-27 may influence IL-12-dependent inhibition of IL-17 by synergizing with IL-12 to suppress the production of IL-17. For example, IL-27 has been shown to enhance the responsiveness of T cells to IL-12 by causing an increase in the expression of IL-12-receptor β2. However, when T cells were cultured in the presence of both IL-12 and IL-27 there was no additional IL-17 suppression (**Figure 34A-B**). IL-17-producing T cells from IL-12p35-deficient mice were amenable to suppression by IL-12. To determine if these cells converted into Th1 cells following stimulation with IL-12, we looked for an

increase in IFN-γ levels. As expected, following stimulation with *T. cruzi* lysate only, splenocytes from IL-12p35-deficient mice produced much less IFN-γ. However, after addition of IL-12 there was a substantial increase in IFN-γ production, which resembled that of wild-type cells (**Figure 35**). Thus, IL-12 can restore the ability of T cells from infected IL-12p35-deficient mice to produce IFN-γ. We next tested the ability of IL-27 to compensate for the lack of IL-12 in restoring IFN-γ production. In contrast to the effects of IL-12, IL-27 could not promote IFN-γ production by T cells from infected IL-12p35-deficient mice (**Figure 35**). Furthermore, it did not synergize with IL-12 in restoring IFN-γ, as the two cytokines together had no additional effects. In conclusion, despite our prediction of a hierarchy and potential synergy in the abilities of IL-12 family cytokines to regulate Th17 responses to *T. cruzi*, we found IL-12, but not IL-27, to be critical for regulating the development and conversion from Th17 cells into Th1 cells.

Figure 25. Effects of IL-12-family cytokines IL-12, IL-27, and IL-23 on IL-17 production by T cells from wild-type mice infected with *T. cruzi*.

A) Splenocytes from wild-type mice infected with *T. cruzi* were harvested on day 9 p.i. Cells were stimulated with anti-CD3 antibody and cultured in the presence of recombinant IL-12, IL-27, or IL-23. Culture supernatants were collected after 72 hours and tested for IL-17 by ELISA. B) Naïve $CD4^+$ T cells were cultured under Th17-skewing conditions (IL-6, TGF-β, anti-IFN-γ) with or without IL-27. Data are shown as a percentage of IL-17 production with anti-CD3 stimulation only. Results are representative of at least three independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 25

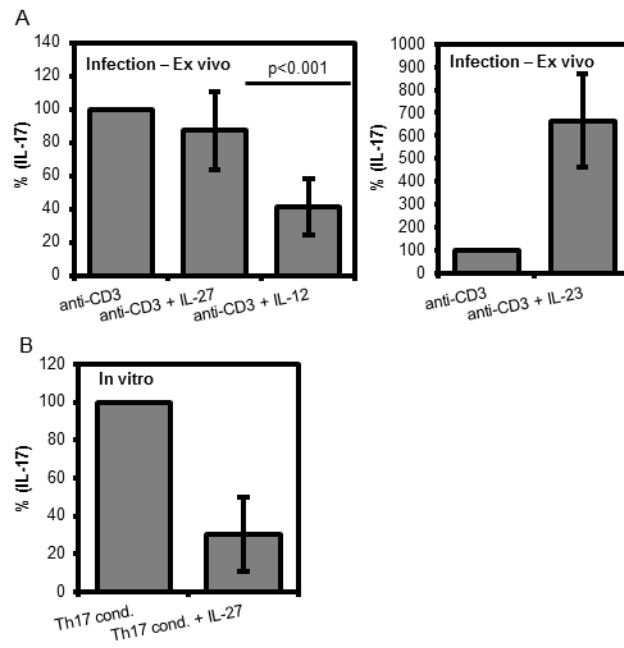


Figure 26. IL-12p35-deficient mice infected with *T. cruzi* exhibit increased Th17 development.

Splenocytes from C57BL/6, *IL-12p35*$^{-/-}$, *IL-12p40*$^{-/-}$, and *Tbx21*$^{-/-}$ mice infected with *T. cruzi* were harvested on day 9 p.i. Cells were re-stimulated with *T. cruzi* lysate. Culture supernatants were collected after 72 hours and tested for IL-17 and IFN-γ by ELISA. Results are representative of at least five independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 26

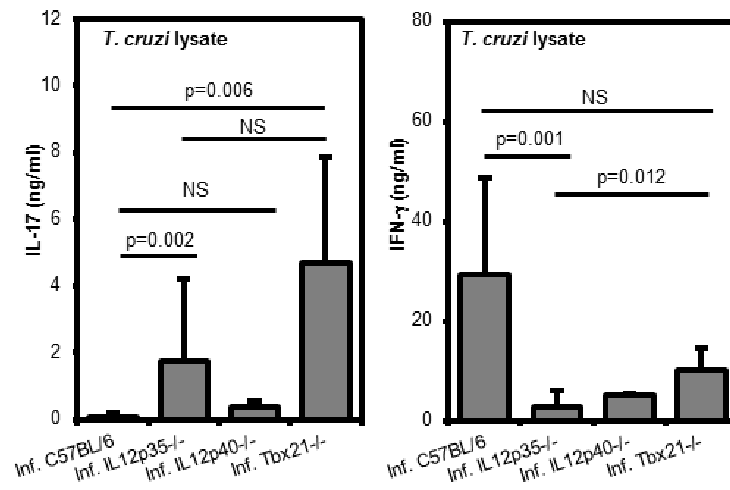
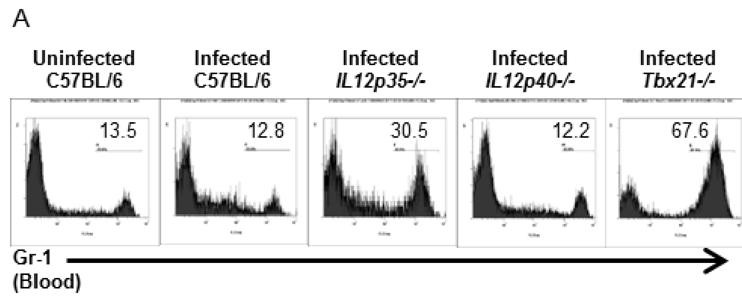


Figure 27. IL-12p35-deficient mice infected with *T. cruzi* development increased neutrophilia.

A) Neutrophilia was determined by analyzing peripheral blood leukocytes on day 9 p.i. for Gr-1 expression by flow cytometry. Histograms from one representative experiment are shown. B) The average percent of PBL that are Gr-1$^+$. C) The average percent of spleen cells that are Gr-1$^+$. Results are representative of at least five independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 27



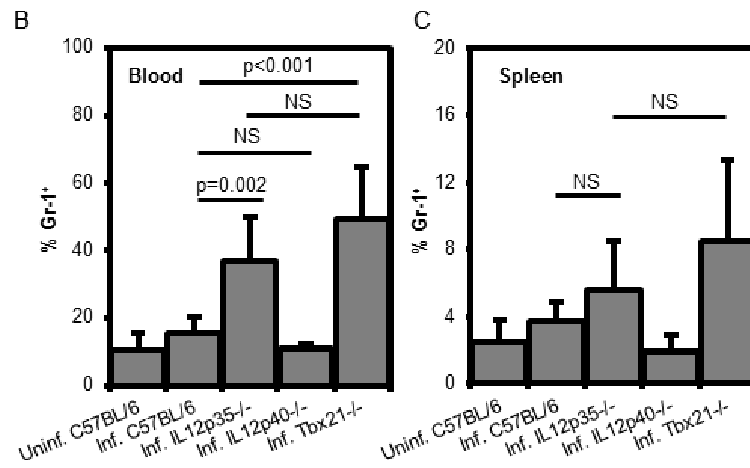
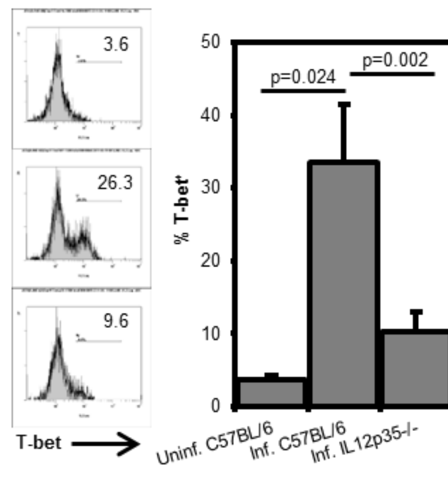


Figure 28. Increased Th17 responses in *IL-12p35$^{-/-}$* mice are associated with a significant reduction in the expression and activity of T-bet.

A) Splenocytes from naïve uninfected mice or *T. cruzi*-infected C57BL/6 and *IL-12p35$^{-/-}$* mice were analyzed by FACS on day 9 p.i. for T-bet expression by gating on $CD4^+$ T cells. Percentage of $CD4^+$ T cells expressing T-bet are shown in the bar graph. B) T-bet activity was determined by analyzing splenocytes for CXCR3 expression. Percentage of $CD4^+$ T cells expressing CXCR3 are shown in the bar graph. Histograms are representative of one individual experiment. Results are representative of at least five independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 28



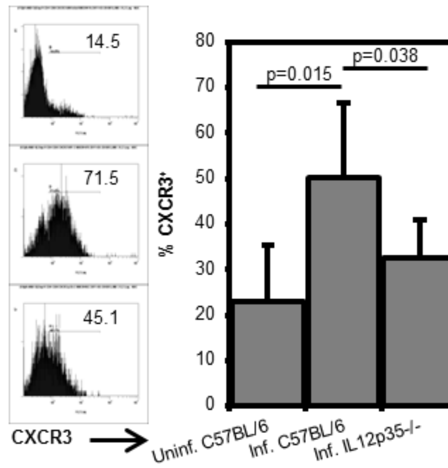


Figure 29. IL-27 is dispensable for regulating Th17 responses to *T. cruzi* infection. Splenocytes from C57BL/6, *Ebi-3*$^{-/-}$, and *Tbx21*$^{-/-}$ mice infected with *T. cruzi* were harvested on day 9 p.i. Cells were re-stimulated with *T. cruzi* lysate or anti-CD3. Culture supernatants were collected after 72 hours and tested for A-B) IL-17 and C) IFN-γ by ELISA. Results are representative of three independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 29

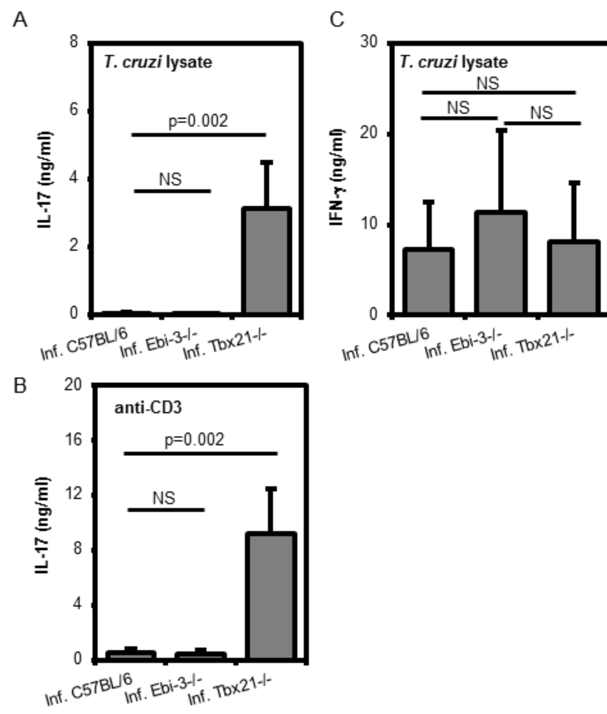


Figure 30. CD4$^+$ T cells from IL-27-deficient mice infected with *T. cruzi* do not produce IL-17.

Splenocytes from infected C57BL/6 and *Ebi-3*$^{-/-}$ mice were re-stimulated on day 9 p.i. with anti-CD3 and stained for intracellular IL-17 and IFN-γ. Cells are gated on CD4$^+$ events. Results are representative of three independent experiments consisting of two mice per group.

Figure 30

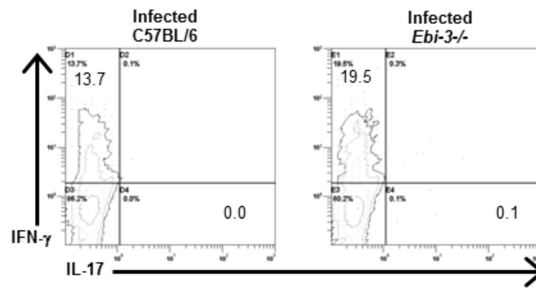


Figure 31. IL-27-deficient mice infected with *T. cruzi* do not exhibit signs of increased Th17 development.

A) Neutrophilia was determined by analyzing peripheral blood leukocytes on day 9 p.i. for Gr-1 expression by flow cytometry. Histograms from one representative experiment are shown. B) The average percent of PBL that are Gr-1^+. C) The average percent of spleen cells that are Gr-1^+. Results are representative of three independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 31

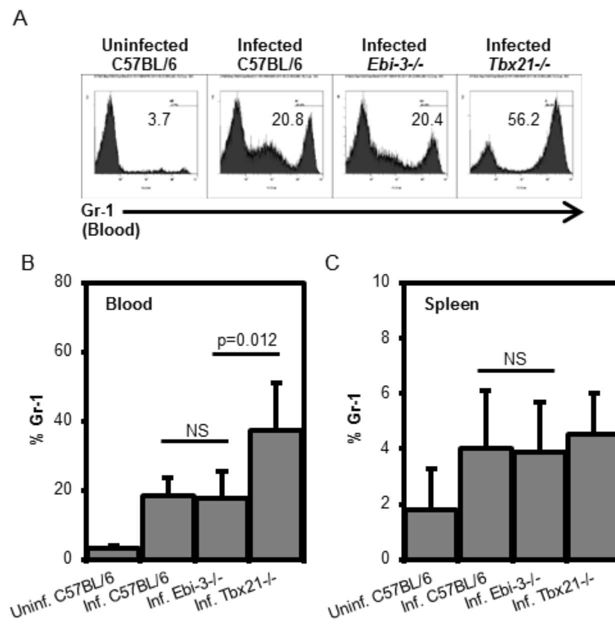


Figure 32. Normal induction of T-bet and T-bet-dependent CXCR3 expression in IL-27-deficient mice infected with *T. cruzi*.

A) Splenocytes from naïve uninfected mice or *T. cruzi*-infected C57BL/6, *IL-12p35*$^{-/-}$, and *Ebi-3*$^{-/-}$ mice were analyzed by flow cytometry on day 9 p.i. for T-bet expression by gating on $CD4^+$ T cells. Percentage of $CD4^+$ T cells expressing T-bet are shown in the bar graph. B) T-bet activity was determined by analyzing splenocytes for CXCR3 expression. Percentage of $CD4^+$ T cells expressing CXCR3 are shown in the bar graph. Histograms are representative of one individual experiment. Results are representative of three independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 32

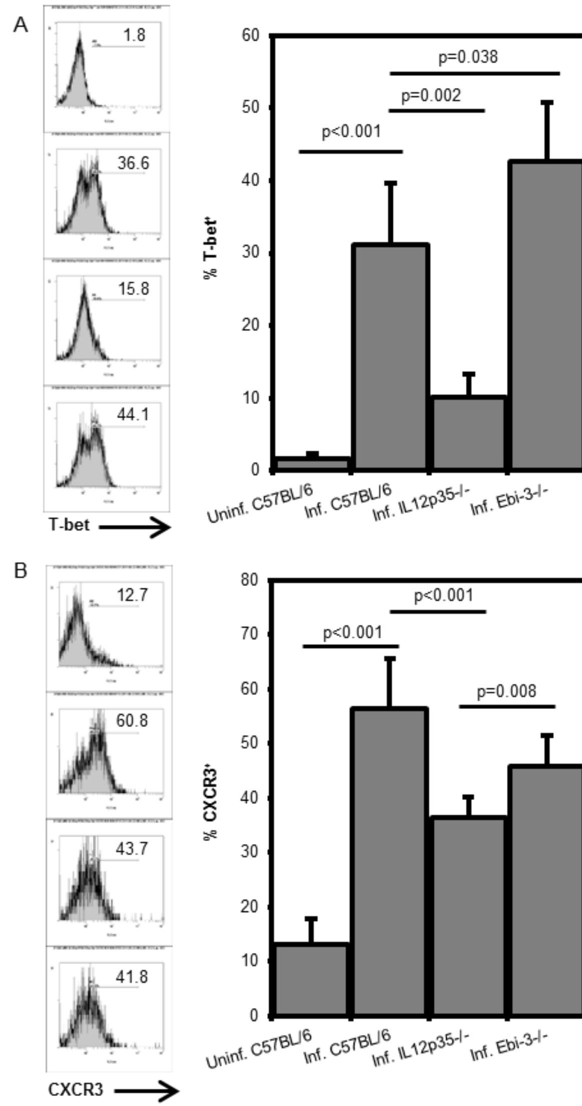


Figure 33. Reduced IL-10 production in infected IL-27-deficient mice does not result in increased IL-17.

Splenocytes from C57BL/6 and *Ebi-3*$^{-/-}$ mice infected with *T. cruzi* were harvested on day 9 p.i. Cells were re-stimulated with *T. cruzi* lysate or anti-CD3. Culture supernatants were collected after 72 hours and tested for IL-10 by ELISA.

Figure 33

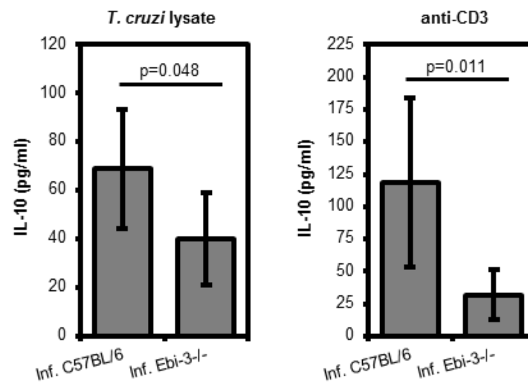
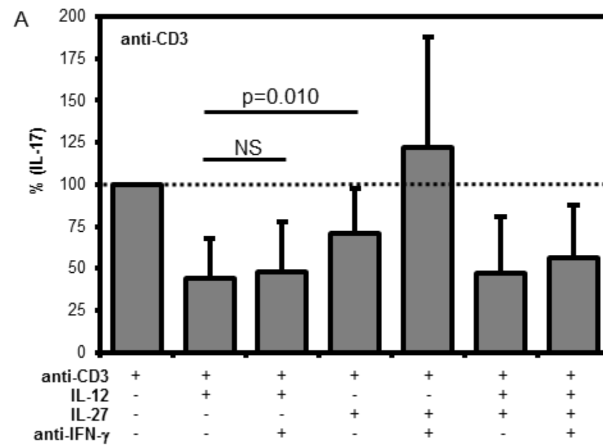


Figure 34. Stability of IL-17-producing T cells from *T. cruzi*-infected mice.

The regulation of IL-17 production by T cells from *T. cruzi*-infected *IL-12p35*$^{-/-}$ mice was determined by culturing splenocytes in the presence of IL-12 or IL-27 with and without anti-IFN-γ. Culture supernatants were collected after 72 hours and tested for IL-17 by ELISA. Data are shown as a percentage of IL-17 production with A) anti-CD3 stimulation only or B) *T. cruzi* lysate stimulation only. Results are representative of at least five independent experiments consisting of two mice per group and are expressed as means ±SD.

Figure 34



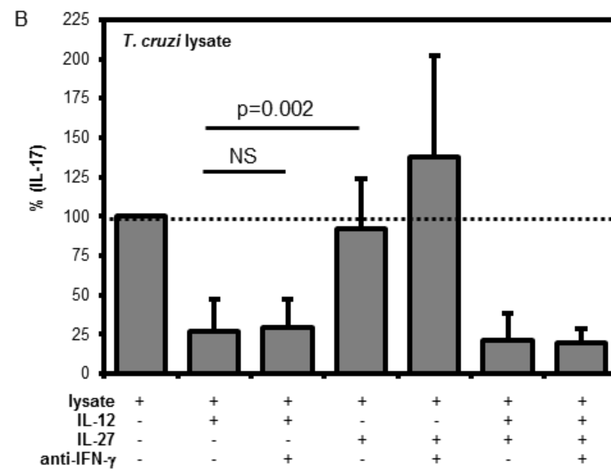
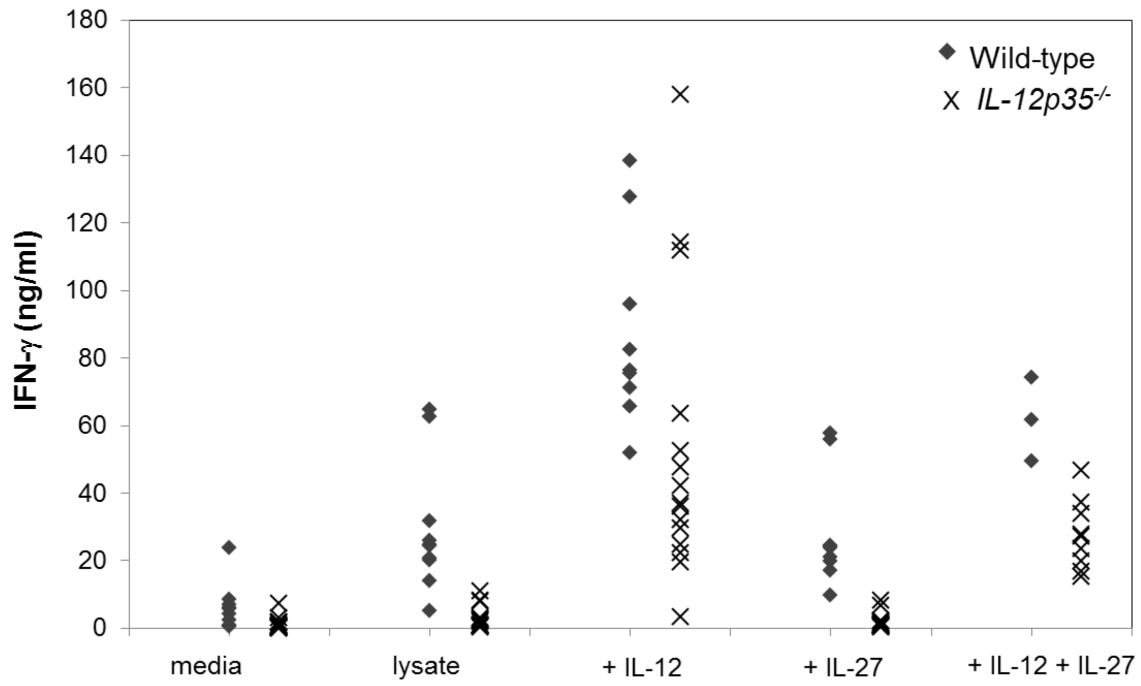


Figure 35. Exposure of T cells from infected IL-12p35-deficient mice to IL-12, but not IL-27, restores IFN-γ production.

Th1 conversion of T cells from *T. cruzi*-infected *IL-12p35*$^{-/-}$ mice was determined by culturing splenocytes with *T. cruzi* lysate in the presence of IL-12 or IL-27. Culture supernatants were collected after 72 hours and tested for IFN-γ by ELISA. Each data point represents an ex vivo splenocyte culture from one mouse. Results are from at least five independent experiments.

Figure 35



Discussion

Understanding the factors that influence $CD4^+$ T cell responses is important for the study of infectious diseases, autoimmunity, and cancer. The role of IL-12 in Th1 immune responses has been well characterized for some time and the role of IL-27 has been recently examined. However, the regulation of pro-inflammatory Th17 responses by these two cytokines is only now being appreciated. Here, we sought to determine the importance of IL-12 and IL-27 in the T-bet-dependent regulation of Th17 responses to *T. cruzi* infection.

First, we found that IL-12, but not IL-27, could suppress IL-17 production by T cells from wild-type mice infected with *T. cruzi*. This result differed from the ability of IL-27 to inhibit the differentiation of naïve T cells into IL-17-producing cells under Th17-polarizing conditions in vitro. Since, these results represented the effects of IL-12 and IL-27 on T cells differentiated in vivo during infection, we wanted to determine if IL-12 had inhibitory effects on the initial development of Th17 cells in vivo in response to *T. cruzi* infection.

To address these issues, IL-12-deficient and IL-27-deficient mice were infected with *T. cruzi* to definitively determine if these cytokines played a role in regulating Th17 development. Infection of IL-12-deficient mice resulted in a significant increase in *T. cruzi*-specific Th17 responses and the mobilization of neutrophils in the blood. The increase in Th17 development in the absence of IL-12 corresponded with a significant decrease in T-bet expression and activity. **These data demonstrate the importance of IL-12 for optimal T-bet induction and subsequent T-bet-dependent inhibition of *T. cruzi*-specific Th17 responses**. Lastly, these results are in agreement with others

and they provide additional evidence for IL-12 as an essential regulator of Th17 cell responses.

Despite the importance of Stat-4 in IL-12 signaling, we do not have evidence that Stat-4 is required for regulating Th17 responses. Experiments to determine whether Stat-4-deficiency results in increased Th17 development during *T. cruzi* infection are being planned. However, the availability of Stat-4-deficient mice is currently restricted to the Balb/c background. The disadvantage of utilizing mice on the Balb/c background is the immunodominant Th2 phenotype in these mice, which could in turn suppress any increase in Th17 development that may occur in the absence of Stat-4. Furthermore, an additional consideration to take into account regarding Stat-4-deficient mice is the use of Stat-4 in IL-23 signaling. Although IL-23 predominantly signals via Stat-3, there does appear to be some Stat-4 utilization in IL-23-driven Th17 development[139]. Thus, it may be difficult to parse the effects of Stat-4 on IL-12/IL-23 responses and Th17 development.

Contrary to our expectations, IL-27 was not involved in suppressing Th17 development during *T. cruzi* infection. Ex vivo re-stimulation of splenocytes from IL-27-deficient mice failed to show any *T. cruzi*-specific increases in IL-17 production. Stimulation with anti-CD3 also did not yield any significant IL-17 production. Furthermore, $CD4^+$ T cells producing IL-17 were not detected using intracellular cytokine staining. Not surprisingly, given that there was no apparent Th17 response, infected *Ebi-3*$^{-/-}$ mice did not display any Th17-associated neutrophilia. Also, in stark contrast to IL-12 deficiency, there was no impairment in T-bet induction in IL-27-

deficient mice. These results demonstrate that in the absence of IL-27, T-bet expression and function are normal, which are required to suppress Th17 development.

Multiple reports show that IL-27 can affect the production of the immunosuppressive cytokine IL-10[135,140,136,141]. However, the lack of a significant Th17 response was not due to inhibition by IL-10, as we did not observe increased levels of IL-10 in *T. cruzi*-infected IL-27-deficient mice. In fact, we found that IL-10 production was actually diminished in the absence of IL-27, arguing against a major role for IL-10 in regulating Th17 responses to *T. cruzi*.

The fact that IL-27 was dispensable for inhibiting *T. cruzi*-specific Th17 development was very surprising based upon existing reports that support IL-27 as a negative regulator of Th17 responses during infection with *Leishmania major*, *Toxoplasma gondii*[142], and *Listeria monocytogenes*[143]. Enhanced Th17 responses in IL-27-receptor-deficient mice infected with *T. gondii* and *L. major* were associated with severe pathology and decreased resistance[142,143]. Interestingly, increased Th17 responses in IL-27-deficient mice actually conferred enhanced resistance to *L. monocytogenes* infection[143], and this protection was independent of any changes in Th1 development. Similarly, the effects of IL-27 on Th1 responses in these studies varied from study to study. However, as the absence of IL-27 signaling caused a reduction in Th1 development during *L. major* infection[136], but had no effect during *T. gondii* infection[142]. In regards to *T. cruzi* infection, IL-27R-deficient mice actually exhibit enhanced Th1 responses, but display severe immunopathology and increased mortality.

On the other hand, there are reports in addition to ours, which show that the lack of IL-27 signaling does not lead to increased Th17 development. For instance, in a

model of helminth infection in which mice are infected with *Schistosoma mansoni* in combination with schistosome egg antigen (SEA) immunization, mice develop severe immunopathology characterized by high levels of IL-17[144]. However, IL-27R-deficient mice infected with schistosomes and immunized with SEA do not exhibit any changes in the levels of IL-17, demonstrating that IL-27 does not regulate schistosome/egg-induced Th17 responses. Additionally, IL-27 did not have a significant role in inhibiting Th17 development during proteoglycan-induced arthritis (PGIA)[145]. While the effects of IL-27 on Th1 responses seem very disparate in terms of whether it promotes or suppresses IFN-γ production, evidence suggests that IL-27 either inhibits Th17 responses, or has no effect at all.

Based on these collective studies, it appears that the pro- and anti-inflammatory properties of IL-27 signaling appear to be dependent on the context of the immune response. The requirement for IL-27 in suppressing Th17 cell responses is likely determined by the cytokine milieu that is established in response to a given infection. For example, different levels of IL-12 induced by various pathogens may dictate the necessity for IL-27 in regulating Th17 responses. Alternatively, varying responsiveness of T cells to IL-12 may exist due to differential requirements for IL-27 in inducing IL-12Rβ2. Within the context of *T. cruzi* infection, we believe that the levels of IL-12 induced by infection may eliminate the need for IL-27 for inducing T-bet expression.

In regards to the factors that promote Th17 responses during *T. cruzi* infection, we believe the IL-12 family member IL-23 to be important because IL-12p40-deficient mice did not exhibit increased Th17 development. Since both IL-12 and IL-23 are composed of the p40 subunit, p40-deficient mice lack the ability to produce either

cytokine. Thus, although IL-12 was absent, this was not sufficient to cause an increase in Th17 development in mice also lacking IL-23, highlighting the necessity for IL-23 in promoting *T. cruzi*-induced Th17 responses. However, definitive proof that IL-23 is necessary for Th17 development during *T. cruzi* infection would require an analysis of infected IL-23p19-deficient mice, which specifically lack IL-23 only.

A well-established feature of Th17 cells is their plasticity and tendency to undergo conversion into Th1 cells. This instability has been shown to be true for both Th17 cells differentiated in vitro as well as for Th17 cells that develop in vivo. Evidence exists for T cells that produce both IL-17 and IFN-γ, and this is consistent with an intermediate phenotype between Th17 and Th1 cells which may represent cells in transition. Thus, we wished to gain insight into the potential plasticity of Th17 cells that were generated in vivo in response to *T. cruzi*. To extend our analysis of the regulatory effects of IL-12-family cytokines on Th17 cells, we wanted to determine if IL-12 and IL-27 had any influence on Th17 cell plasticity. Treatment with IL-12 ex vivo significantly inhibited IL-17 production by T cells from *T. cruzi*-infected *IL-12p35*$^{-/-}$ mice. The decrease in IL-17 levels was simultaneous with an increase in IFN-γ production, demonstrating a shift in the T cell phenotype from Th17 to Th1 in response to IL-12. This conversion also occurred independently of any IFN-γ-mediated effects on Th17 cells, supporting our previous finding that IFN-γ is mostly dispensable for inhibiting Th17 responses. Thus, the importance of IL-12 during infection with *T. cruzi* may be to not only promote the development of Th1 responses, but to first promote conversion of Th17 cells that may develop early during infection into fully-differentiated Th1 cells. The role of IL-12 in driving the conversion of Th17 cells into Th1 cells stems from its ability to

induce significant epigenetic changes to gene loci associated with the Th17 lineage. For example, IL-12 stimulation was linked to repression of the gene encoding RORγt and resulted in repressive histone modifications of the *Il17* locus[146]. Importantly, these epigenetic modifications were Stat4 and T-bet-dependent. The existence of $CD4^+$ T cells that produce both IFN-γ and IL-17 has been demonstrated in various diseases and experimental models including ours[147,148,149,120]. The dual cytokine-producing phenotype of these cells may be a result of the plasticity of Th17 cells. It is possible that cells producing both IFN-γ and IL-17 are representative of Th17 cells undergoing conversion into Th1 cells. T cells concomitantly producing IFN-γ and IL-17 may be advantageous early during immune responses due to their combined effector functions and also co-expression of chemokine receptors unique to Th1 and Th17 cells. However, this may confer upon them an increased pro-inflammatory potential that could contribute to autoimmunity. This highlights the importance for Th17 cells to undergo full conversion into Th1 cells.

In regards to the IL-12-driven conversion of Th17 cells into Th1 cells by T cells from *T. cruzi*-infected mice, we found this to be independent of IFN-γ. This is in contrast to a report suggesting that both IL-12 and IFN-γ were necessary, where IFN-γ was needed to up-regulate IL-12Rβ2 expression[138]. The reason for this difference may be related to the origin of the cells. In the study where both IL-12 and IFN-γ were required for conversion, Th17 cells were isolated from naïve mice, whereas Th17 cells in our study originated from mice undergoing an active immune response to *T. cruzi* infection. Thus, Th17 cells from *T. cruzi*-infected mice may already be poised to respond to IL-12 stimulation without exposure to IFN-γ. Alternatively, even though *IL-12p35*$^{-/-}$ mice have

diminished IFN-γ production during *T. cruzi* infection, Th17 cells in our study may have previously been exposed to low levels of IFN-γ in vivo, negating this requirement for IL-12-induced conversion ex vivo. Regardless, IL-12 plays a dominant role in the stability of Th17 cells.

In contrast to the potent ability of IL-12 to inhibit IL-17 production by T cells from *T. cruzi*-infected *IL-12p35*$^{-/-}$ mice, IL-27 had minimal impact on IL-17. This notion supported by the results obtained with *Ebi-3*$^{-/-}$ mice infected with *T. cruzi*. Furthermore, although IL-27 has been shown to increase IL-12 responsiveness, it did not augment the suppressive effects of IL-12 on IL-17 production, as there was no further inhibition of IL-17. These results show that IL-27 does not affect the stability of Th17 cells generated during *T. cruzi* infection.

From our findings in Chapter 3, it was evident that Stat-1 plays an important role in controlling Th17 responses. We originally hypothesized that IL-27 was the Stat-1-inducing cytokine responsible for limiting Th17 responses to *T. cruzi* in a Stat-1/T-bet-dependent manner. However, as these results demonstrate, IL-27 is not necessary for regulating Th17 responses during *T. cruzi* infection. This leaves the issue regarding Stat-1-inducing cytokines in the T-bet-dependent regulation of Th17 development unresolved, as neither IFN-γ nor IL-27 play a significant suppressive role during *T. cruzi* infection. However, it is clear that in addition to IL-12 signaling, there is a Stat-1 dependent pathway for regulating Th17 development.

It is plausible that the type I interferons, IFN-α/β, may be involved in the Stat-1-dependent regulation of Th17 cells, as these cytokines are known to induce Stat-1 activation, and have been implicated in Th17 suppression. For example, IFN-α was

capable of inhibiting the differentiation of both murine and human naïve T cells into Th17 cells in vitro[150,151]. Additionally, IFN-α treatment suppressed IL-17 in colonic tissue of patients with ulcerative colitis[151], an autoimmune disease in which Th17 cells have been shown to contribute to immunopathology. Along these lines, type I IFNs have been reported to have a protective role in EAE and multiple sclerosis, a disease that involves Th17 cells. *IFN-α$^{-/-}$* and IFN-α-receptor-deficient mice developed more severe EAE[152]. Treatment of MS patients with IFN-α/β ameliorated disease by extending remission and reducing relapse. Thus, it is possible that type I IFNs might also regulate Th17 responses to parasitic infection. However, we have not examined the role of IFN-α/β in regulating Th17 responses to *T. cruzi* infection.

Although the effects of IL-12 or Stat-1 deficiency on Th17 responses never approximated those of T-bet deficiency, these differences were not statistically significant. Thus, in the absence of IL-12, there may be a degree of Stat-1-mediated Th17 inhibition occurring, and vice versa. However, this may require a more direct comparative analysis of the requirements for Stat-1, IL-12p35, and T-bet, as these experiments were originally performed separately. Nonetheless, since these two pathways do not appear to significantly compensate for one another in inhibiting Th17 responses, they may both need to be intact to achieve full suppression. It is logical to hypothesize that in the absence of both IL-12 and Stat-1, the degree of Th17 development would more closely resemble, or may even exceed that observed in T-bet-deficient mice. For example, treatment of infected Stat-1-deficient mice with anti-IL-12p35 may increase the extent of Th17 development.

In conclusion, these studies demonstrate that IL-12 is essential for limiting the development of *T. cruzi*-specific Th17 responses and has the ability to extinguish IL-17 production by previously committed Th17 cells. Importantly, we ruled out a significant role for IL-27 in regulating Th17 responses to *T. cruzi*, which is in contrast to other pathogens. These results have important implications in understanding how pro-inflammatory T cell responses are regulated, as these are associated with both protective immune responses to *T. cruzi* as well as those that contribute to the pathogenesis of Chagas disease. Thus, this improved understanding should benefit the development of more effective treatments targeting T cell responses and potentially the development of vaccines for protective T cell-mediated immunity to *T. cruzi* infection.

Dissertation Conclusion

This research project was initiated to elucidate the factors important for regulating $CD4^+$ and $CD8^+$ T cell responses, especially Th1 and Th17 development, during infection with the intracellular parasite *T. cruzi*. Initial studies examining the role of the transcription factor T-bet during *T. cruzi* infection lead to two major findings. First, they showed that mice lacking T-bet exhibited a significant quantitative defect in the number of antigen-specific $CD8^+$ T cells that recognize *T. cruzi*. This observation presented us with the challenge of examining the potential underlying causes of this defect. Secondly, the finding that T-bet negatively regulates the development of pro-inflammatory Th17 responses to *T. cruzi* prompted the investigation into how T-bet accomplishes this and what signals are required for T-bet-dependent regulation (**Figure 36**). This aspect of the project was particularly interesting as it challenged current paradigms regarding the inhibition of Th17 development. The work involved in this project culminated with the identification of the important cytokines involved in promoting the necessary and appropriate T cell responses to *T. cruzi* infection.

The major goal of this research project was to determine how Th17 cells that are generated in response to *T. cruzi* infection are regulated. The impetus behind these studies was based on our initial finding that T-bet is required to inhibit the differentiation of Th17 cells during *T. cruzi* infection. The need to further characterize this regulation was fueled by the fact that Th17 responses to *T. cruzi* were developed in T-bet-deficient mice despite the presence of IFN-γ, which at the time was believed to be the primary factor responsible for suppressing Th17 development. Furthermore, Th17 cell-mediated responses are often associated with severe immunopathology and autoimmune disorders, making it a priority to understand the regulation of these cells. Thus, we

elected to investigate the regulation of Th17 cells, specifically to identify the molecular aspects and the cytokines that lead to their regulation.

The first step was to identify critical amino acid residues responsible for T-bet-dependent regulation of Th17 differentiation. Retroviral-gene delivery was used to demonstrate that restoration of T-bet by ectopic expression in T-bet-deficient T cells was sufficient to suppress IL-17 production by Th17 cells derived from *T. cruzi*-infected mice. Using this same strategy, we sought to identify which amino acid residue was important for repressing Th17 development. Specific serine and tyrosine residues previously shown to be functionally important in repressing IL-2 expression and Th2 inhibition were tested for their involvement in Th17 inhibition. Surprisingly, these residues were not necessary for the ability of T-bet to regulate Th17 differentiation, suggesting a distinct mechanism for Th17 suppression. Although we were unsuccessful in implicating one of these residues in Th17 regulation, these results provided evidence suggesting that T-bet utilizes different amino acid residues to bring about distinct functional outcomes.

Next, we extended our analysis to IFN-γ and Stat-1 signaling to definitively clarify the role IFN-γ in suppressing Th17 development. Initially, reports were conflicting on whether IFN-γ or T-bet was more important in the regulation of Th17 cells. Our early findings favored a T-bet-dependent regulation and provided strong evidence that IFN-γ alone was insufficient to suppress Th17 responses to *T. cruzi* infection. However, we wished to carry out a more direct examination of the role of IFN-γ. Infection of IFN-γ-deficient mice did not lead to significant Th17 responses, supporting the claim that IFN-γ does not play a major role in suppressing Th17 development. In contrast, Stat-1, which

mediates IFN-γ signals and activates T-bet, was needed as there was significant Th17 development in Stat-1-deficient mice during *T. cruzi* infection. These results suggested that other Stat-1-inducible cytokines were potentially involved. Additionally, in the absence of Stat-1 signaling but not IFN-γ, T-bet activity was diminished based on T-bet-mediated CXCR3 induction. These and other results further diminished a significant role for IFN-γ in directly regulating Th17 development. However, in concert with IL-12, we found that IFN-γ could moderately limit *T. cruzi*-specific IL-17 production, albeit in a T-bet-independent manner. Thus, we concluded that IFN-γ was mostly dispensable for T-bet-dependent control of Th17 development, whereas Stat-1 was critical.

The next step in examining T-bet-dependent regulation of Th17 cell responses to *T. cruzi* infection was to determine the specific cytokines that are involved. Several lines of evidence suggested the IL-12 family cytokines IL-12, IL-27, and IL-23 were involved. Briefly, IL-12 and IL-27 were previously reported to inhibit Th17 responses in other disease models, whereas IL-23 was shown to promote Th17 development. Thus, we tested their involvement in regulating Th17 responses to *T. cruzi* infection. As expected, IL-23 strikingly promoted IL-17 production from Th17 cells from *T. cruzi*-infected mice. We found that IL-12 was critical for the optimal induction of T-bet and the inhibition of *T. cruzi*-specific Th17 development (**Figure 36**). IL-12 was also effective at driving the conversion of Th17 cells differentiated in vivo during *T. cruzi* infection, into IFN-γ-producing Th1 cells. Given that we observed a critical role for Stat-1 in inhibiting Th17 development, we hypothesized that the Stat-1-inducible cytokine IL-27 would also be important for suppressing Th17 responses to *T. cruzi*. Surprisingly, IL-27 was expendable for regulating Th17 development during *T. cruzi* infection, which is in

contrast to its inhibitory effects on Th17 differentiation of naïve T cells in vitro, as well as during other parasitic infections including *T. gondii* and *L. major*.

CD8$^+$ T cell-mediated immune control of *T. cruzi* infection is crucial for host resistance and survival. The appropriate expansion of CD8$^+$ T cells that recognize *T. cruzi*-derived antigens is an essential step in achieving this protection. Therefore, determining how this response develops and the factors that regulate it are important for understanding the immune mechanisms responsible for generating protective immunity. We observed that T-bet was required in order for infected mice to generate normal numbers of *T. cruzi*-specific CD8$^+$ T cells. Through an extensive examination of APC functions, which showed that T-bet-deficiency did not adversely affect the ability of APCs to become activated and induce CD8$^+$ T cell responses, we determined that this was not the cause of impaired CD8$^+$ T cell expansion. These data suggested that the inability of CD8$^+$ T cells to expand was due to a T cell-intrinsic function of T-bet. Indeed, this proved to be the case when the defect in expansion was recapitulated when T-bet-deficient CD8$^+$ T cells failed to expand in the presence of normal APCs in vivo. Further experiments investigating the potential cues necessary for the development of CD8$^+$ T cell responses to *T. cruzi* revealed that IL-12 was important for inducing maximal expression of T-bet. Previous studies established that T-bet was involved in various aspects of CD8$^+$ T cell biology. However, these primarily focused on the role of T-bet in promoting the effector functions and cytotoxicity of CD8$^+$ T cells, such as the production of IFN-γ, granzymes, and perforin molecules. In this study, we identified an essential CD8$^+$ T cell-intrinsic role for T-bet in promoting the generation of CD8$^+$ T cells that fight

T. cruzi infection (**Figure 36**). To the best of our knowledge, this was the first study that examined the role of T-bet in $CD8^+$ T cell responses during *T. cruzi* infection.

This research project has brought to light new information regarding the regulation of T cell responses to the intracellular pathogen *T. cruzi*. These findings have important implications for the study of protective immunity to *T. cruzi*, as T cells are an essential component of resistance. First, we identified a major $CD8^+$ T cell-intrinsic role for T-bet in promoting $CD8^+$ T cell responses to *T. cruzi*. Secondly, through examination of factors regulating pro-inflammatory Th17 development, we identified key regulators of the Th17 response to *T. cruzi* infection. It was demonstrated that Stat-1 and IL-12, which function in a T-bet-dependent fashion, are necessary for the appropriate control of Th17 responses to *T. cruzi*. Additionally, we found that IFN-γ played only a minor role in suppressing Th17 development, and these effects were mostly dependent on APC-derived IL-12. Along these lines, our results demonstrate that IL-12 exerts both T-bet-dependent and T-bet-independent effects on IL-17 production by Th17 cells during *T. cruzi* infection. Lastly, we have shown that the IL-12-family member IL-27 was not important for inhibiting Th17 responses to *T. cruzi* infection.

Numerous studies have demonstrated the vital role of T cells in mediating resistance to *T. cruzi*. Parasites reside and replicate in host cells, making $CD8^+$ T cells an important avenue for vaccine-induced immunity to *T. cruzi* infection because of their ability to detect and kill infected cells. Our demonstration of the importance of T-bet in generating *T. cruzi*-specific $CD8^+$ T cell responses is one of the first to investigate transcription factor involvement in generating $CD8^+$ T cell immunity to *T. cruzi*.

Moreover, it will be important for moving forward with targeting $CD8^+$ T cell responses for therapeutic or preventative purposes in which an understanding of these aspects may be necessary.

Less than a decade ago, T cells capable of producing IL-17 were identified, which were soon defined as a third T helper cell lineage, and labeled Th17 cells. Since then, extensive work has been focused on their roles in infectious diseases and autoimmunity, as well as on the regulation of their development. While the pro-inflammatory potential of Th17 cells has been indisputable since their discovery, determining the factors involved in their regulation has been more difficult. The hope is that by gaining a better understanding of the regulatory mechanisms governing Th17 responses, it will lead to the advent of improved therapeutics for many inflammatory diseases. These studies have contributed to the understanding of the regulation of Th17 responses, specifically, as they pertain to parasite immunity and *T. cruzi* infection. Hopefully the new insights gained from this project as well as the collective research involving Th17 immunity will benefit the treatment of inflammatory diseases and improve the current understanding of T cell immunity to infectious disease.

Despite significant advances made by our research in understanding how T cell responses to *T. cruzi* are shaped and regulated, there remains much more to learn. Employing new strategies for investigating immunity to *T. cruzi* will be important moving forward. We believe that one such approach to meet these needs is the humanized mouse model, in which immunodeficient mice are reconstituted with human hematopoietic stem cells, which allow the formation of a functional human immune system. This approach allows for the study of human immune responses in vivo. The

use of humanized mice has recently taken a significant step forward with the advent of mice that lack the IL-2 receptor γ chain (*IL-2r*$\gamma^{-/-}$), which makes successful engraftment of human cells more likely. Future studies using the humanized mouse model hold a tremendous amount of potential for advancing the knowledge of human immunity to *T. cruzi*. Currently, studies of human immune responses to *T. cruzi* are limited to in vitro experimentation and investigations using patients already infected with the parasite. The humanized mouse model will provide a means of investigating primary immune responses of naïve human T and B cells to *T. cruzi* infection. The use of this model will provide a novel translational approach for studying *T. cruzi* immunity and developing vaccines intended to prevent *T. cruzi* infection. Additionally, it will allow for a unique opportunity to validate of our previous findings using murine *T. cruzi* infection, possibly including those from this project. The development of this approach is currently underway and we are in the process of establishing humanized mice.

In conclusion, this work has advanced our knowledge of the regulation of T cell responses during *T. cruzi* infection (**Figure 36**). One of the exciting aspects of this project was that many of the strides that were made during the course of this research paralleled the ongoing works of others and the evolution of the notions surrounding T cell responses to infection and Th17 biology. However, this project did more than just corroborate studies by others, but it also challenged previous paradigms and contributed important new findings to the field. At the onset of these studies, the field of Th17 cell research was relatively new, and there was much that was not known regarding how the development of Th17 cells was regulated. Since then, the views on Th17 regulation have evolved significantly and much of the work presented here

contributed to and helped shape the current understanding of how Th17 immune responses are regulated. As is the case for many scientific endeavors, each step of this project served to answer critical questions, but at each point, they also presented new avenues of investigation.

Figure 36. Model for T-bet-dependent regulation of T cell responses during *T. cruzi* infection.

Antigen presenting cells (APCs) encounter *T. cruzi* parasites and respond by producing pro-inflammatory cytokines such as IL-12, IL-6, and IL-23. The balance of IL-12, IL-6, and IL-23 determines the differentiation of $CD4^+$ T helper cells. Early during infection, IL-6 and IL-23 may drive the development of IL-17-producing Th17 cells. IL-17 promotes the recruitment of neutrophils to sites of infection where they may be critical for controlling early parasitism. High levels of IL-12 favor the transition of Th17 cells into Th1/Th17 and Th1 cells. Stat-4 and Stat-1 promote T-bet expression which in turn supresses IL-17 production and the Th17 program. Maximal T-bet expression drives the full differentiation of T cells into Th1 cells. IL-12 is important for promoting T-bet expression in $CD8^+$ T cells. Activation of T-bet is necessary for optimal Tskb20-specific $CD8^+$ T cell expansion. The T-bet-dependent regulation of T cell responses during *T. cruzi* infection promotes resistance to infection.

Figure 36

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Vita

Dustin Andrew Cobb was born on February 25, 1984, in Staunton, Virginia and is an American citizen. He graduated in 2002 from Wilson Memorial High School in Fishersville, Virginia. He received a Bachelor of Science degree in Biology in 2006 from Eastern Mennonite University in Harrisonburg, Virginia. He joined the Department of Microbiology and Immunology as a graduate student in 2007 and later entered the Ph.D. program in 2008.

Publications:

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