

STABLE CD8+ T CELL MEMORY DURING PERSISTENT INFECTION

by

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(Under the Direction of Rick L. Tarleton)

ABSTRACT

In contrast to antigen-independent, cytokine-driven CD8+ T cell memory arising from acute resolving infections, CD8+ T cell memory during chronic infections is thought to be sustained primarily by the presence of antigen, comprising a population of relatively short-lived T effector memory cells. Consistent with this idea, we have observed that the majority of CD8+ T cells exhibit an effector memory phenotype during experimental chronic infection with *Trypanosoma cruzi*, a protozoan parasite that causes significant morbidity and mortality throughout Latin America. The goal of this study was to explore the possibility that long-term memory is generated in spite of persistent infection by *T. cruzi*. We report that in contrast to what is typically observed during other persistent infections, a stable population of parasite-specific CD8+ T central memory cells capable of antigen-independent survival is maintained in mice despite the presence of persistent antigen.

INDEX WORDS: *Trypanosoma cruzi*, Chagas Disease, chronic infection, memory CD8+ T cell, IL-7R α , IL-15R β , KLRG1, bcl-2, T cell maintenance

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. *Trypanosoma cruzi*

1.1 Transmission

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of American trypanosomiasis, otherwise known as Chagas disease, a major health problem in Latin America. Approximately 16-18 million people in Central and South America are infected with *T. cruzi* and more than 50,000 deaths a year are attributed to Chagas disease. It is estimated that 100 million people are at risk of acquiring infection, with approximately one million new infections occurring each year [1]. *T. cruzi* may be transmitted in several ways, the most common being through blood-feeding Reduviidae insects which infest poorly-constructed houses in rural areas of Latin America [1, 2].

Infection occurs following a blood meal when infective parasite-contaminated feces are deposited onto the skin. Parasites enter the body either through the bite wound or mucus membranes. Transmission also occurs through transfusion of infected blood or organ transplants, ingestion of parasite-contaminated food, and congenitally. While vector-borne transmission is the primary mode of transmission in Latin America, infection via blood transfusion is also a major problem in endemic areas and has become a serious concern in the United States.

The life cycle of *T. cruzi* involves four distinct stages. Transmission to the mammalian host occurs via infective metacyclic trypomastigotes present in the feces of the arthropod vector. Invasive metacyclic trypomastigotes enter a wide variety of phagocytic and non-phagocytic host cells. Differentiation into replicative amastigotes occurs in the host cell cytosol following escape from a parasitophorous vacuole. After several rounds of binary fission, amastigotes released

from the host cell as trypomastigotes infect neighboring host cells or enter the bloodstream and can subsequently be taken up by the vector [3]. In the insect vector midgut, trypomastigotes transform into epimastigotes, replicate, and differentiate into infective metacyclic trypomastigotes in the insect hindgut.

1.2 Infection and disease etiology

Introduction of *T. cruzi* into its mammalian host initiates an acute phase of infection followed by a chronic phase. The acute phase is characterized by widespread tissue parasitism that is controlled, although not cleared, by a robust host immune response [4]. While acutely infected individuals may experience fever, lymphadenopathy and hepatosplenomegaly, the majority of infected individuals remain asymptomatic [5]. Although circulating parasites are often not detected following acute infection, sterile immunity is rarely, if ever, observed, and parasites preferentially persist at low levels in certain tissues during the chronic phase of infection [4, 6]. Decades after initial infection, nearly one-third of chronically infected individuals will develop the clinical manifestations of Chagas disease, including severe cardiomyopathy and/or gastrointestinal pathology, and in some cases sudden death [1].

It is now well-established that tissue damage observed in Chagas disease is the result of *T. cruzi* infection, although different theories regarding the etiology of this disease exist. Some groups support a role for autoimmunity due to the presence of anti-self antibodies and lymphocytes as well as a CD8+ T cell inflammatory infiltrate [7, 8] despite the nearly undetectable parasite levels observed at sites of most severe tissue pathology [9]. However, definitive studies to directly link autoimmunity and the pathology observed are lacking, and strong evidence supports that parasite persistence at a particular site is required for disease in that tissue (reviewed in [10]). In support of this theory, parasite DNA, protein, or whole parasites are detectable at sites of disease but absent from healthy tissues in seropositive cadavers [11] and in murine infection models [12]. Taken together, these data provide strong

evidence that Chagas disease results from the inability of the host immune response to adequately clear parasites with minimal damage to infected tissues [10].

No approved vaccines for *T. cruzi* currently exist and treatment of infected individuals is challenging, particularly in rural areas where access to drugs and proper health care is limited. The principle drug available for the treatment of Chagas disease is N-benzyl-2-nitroimidazole acetamide, benznidazole (BZ), and is generally recommended for clinical use in the acute phase of infection. The efficacy of BZ depends on a variety of factors including the infecting strain, host species, and length of the infection [13]. Treatment with BZ is also limited by toxic side effects, drug resistant parasite strains, and a lack of criteria for determining if and when cure is achieved [13]. For these reasons, a better understanding of the immune response to *T. cruzi* is necessary for the development of treatment options and preventative and therapeutic vaccines.

1.3 Immune response to *T. cruzi*

1.3.1 Humoral and cell-mediated immunity

Both humoral and cellular immune responses are necessary for the control of parasites during *T. cruzi* infection. The extracellular trypomastigote stage of *T. cruzi* is susceptible to antibodies [14, 15] and infection in B cell-deficient mice is lethal [16]. CD4⁺ T cells are also important for control of infection, as mice deficient in MHC class II expression have uncontrolled parasitemia and die during the acute phase of infection [17] while CD4⁺ T cell depletion during the chronic phase results in exacerbation of disease [18]. Furthermore, mice unable to develop a type-1 helper (Th1) CD4⁺ T cell response cannot control *T. cruzi* infection [19]. While it is not known how parasite evasion of the immune response occurs, endogenous CD4⁺ CD25⁺ regulatory T cells [20] and TGF- β (Martin et. al. manuscript submitted) appear to have a limited role in inhibiting protective immune responses at sites of parasite persistence.

1.3.2 CD8+ T cell immunity

Because *T. cruzi* replicates freely in the host cell cytoplasm, *T. cruzi*-derived peptides are presented to CD8+ T cells through the MHC class I presentation pathway of infected cells. Indeed, studies utilizing the model CTL target molecule OVA and transgenic OVA-producing parasites have shown that surface-bound proteins or proteins secreted by *T. cruzi* are processed and presented on MHC class I molecules and generate parasite-specific CTL responses [21]. The importance of CD8+ T cells in control of *T. cruzi* infection has been demonstrated by numerous experiments. Mice depleted of CD8+ T cells with either antibodies [22] or by gene knockout [17, 23-25] have higher parasite burdens and die early in infection. It is not clear how parasites are able to persist in muscle tissue in the presence of *T. cruzi*-specific CD8+ T cell population, although depletion of CD8+ T cells during the chronic phase results in increased parasite loads in muscle, indicating an important role for CD8+ T cells in control of infection at sites of parasite persistence [18].

Several studies have demonstrated *T. cruzi* peptides encoded by members of the *T. cruzi* transsialidase (ts) gene family, are targets of CD8+ T cell responses in both mice and humans [26-29]. The use of MHC class I tetramers has enabled the tracking of CD8+ T cells specific for recently identified immunodominant ts epitopes in our H-2K^b C57BL/6 model of infection [26]. A high proportion of CD8+ T cell responses are focused on the TSKB20 (ANYKFTLV) and TSKB18 (VNYDFTLV) ts epitopes in mice infected with *T. cruzi*, the magnitude and dominance of which depends on the infecting strain. Indeed, the TSKB20-specific response accounts for up to 30% of circulating CD8+ T cells during acute infection with the Brazil strain of parasites, while the TSKB18-specific response is 2-fold lower. While CL strain-infected mice display a similar immunodominance pattern, the TSKB20-specific response is present at a slightly lower frequency and peaks earlier than that observed in Brazil strain infected mice. The kinetics profile of the observed *T. cruzi*-specific CD8+ T cell responses mirrors the parasitemias in these mice, with responses declining and leveling off during the

chronic phase [26]. Thus, as with other intracellular pathogens, it is clear CD8+ T cells are critical to the immune response during *T. cruzi* infection (reviewed in Ref. [30]).

2. Memory CD8+ T cell characteristics

Less clear, however, is our understanding of the development and maintenance of the CD8+ T cell response, particularly during chronic infections. During most infections, initiation of a CD8+ T cell response begins with recognition of antigen followed by activation and clonal expansion. As antigen is eliminated by the immune system, effector cells are deprived of essential survival stimuli and the contraction phase begins. During the contraction phase, the majority of effector CD8+ T cells die by apoptosis while a small subset survives and contributes to the memory pool. In general, memory CD8+ T cells generated during primary infections are different from naïve and effector CD8+ T cells in that they acquire increased resistance to cell death, respond more rapidly and to a greater extent upon re-exposure to antigen, express different cell surface proteins, and provide enhanced protective immunity.

2.1 Classification of memory T cell subsets

Although various memory CD8+ T cell markers have been described, it is clear that the memory population generated during many infections is heterogeneous, with different subsets having distinct phenotypes (reviewed in [31]). The memory CD8+ T cell pool is grouped broadly into two categories based on expression of the lymph node homing molecules CD62L and CCR7 [32]. In general, the CD8+ T cell effector memory subset (T_{EM}) does not express CD62L and CCR7 while the T cell central memory subset (T_{CM}) expresses high levels these lymph node homing molecules [31], although studies have found co-expression of CD62L and CCR7 is not always exclusive [33]. Initial studies demonstrated that T_{EM} cells localized to non-lymphoid tissues while the T_{CM} subset trafficked primarily through secondary lymphoid tissue, although a recent report indicated expression of CD62L and CCR7 were not absolutely required by CD8+ effector and memory CD8+ T cells for entry into inflamed lymph nodes [34].

Several models have been proposed to describe the relationship between these two subsets of cells. Wherry *et al.* proposed a model in which with time and in the absence of antigenic stimulation, TCR-transgenic CD8+ T_{EM} cells differentiate into T_{CM} cells following cleared lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes* (LM) infection [31]. Analyzing the gene expression profiles of antiviral CD8+ T cells over time similarly revealed that memory precursors present during LCMV infection require several weeks following antigen clearance to acquire the hallmark characteristics of memory cells [35]. A noted disadvantage to these studies was the use of non-physiologically high numbers of TCR-transgenic CD8+ T cells for adoptive transfer. Marzo *et. al.* showed that T_{EM} cells generated from infection do not convert to T_{CM} cells and are stably maintained when either a low number of TCR-transgenic cells is transferred or when the precursor population is polyclonal [36]. Additionally, Baron and colleagues have suggested that T_{EM} and T_{CM} cells may represent two distinct lineages utilizing separate TCR repertoires [37]. Studies have also shown that T_{CM} cells convert directly to T_{EM} cells following stimulation or re-exposure to antigen. These findings are based on analyses of memory CCR7+ cells which gained effector functions and lost expression of CCR7 after *in vitro* stimulation [32].

Agreement on the functional abilities of T_{EM} and T_{CM} cells is also inconsistent. It was originally proposed that the T_{EM} subset had immediate effector function but showed reduced proliferative responses to secondary challenge while the T_{CM} subset lacked immediate effector function but proliferated strongly to challenge [32]. More recently, studies have demonstrated both T_{EM} and T_{CM} subsets can produce IFN- γ upon antigen re-stimulation [38, 39] and possess cytolytic activity [39, 40], although the T_{CM} subset displays a greater proliferative capacity following secondary stimulation and provides superior protective immunity [38]. It has also been shown that T_{CM} cells can rapidly convert to T_{EM} cells and localize to non-lymphoid tissues following antigen challenge [38]. A number of factors are likely to dictate which subset is the most protective for each unique infection.

2.2 Maintenance

Much progress has been made in understanding how the memory CD8⁺ T cell pool is maintained for extended periods of time. Studies have suggested memory CD8⁺ T cells do not require MHC-TCR interactions or antigen since they can survive in class I MHC-deficient mice [41, 42]. The common γ chain (γ_c) family of cytokines, including Interleukin 2 (IL-2), -4, -7, -15, and -21, are thought to mediate the homeostasis of most subsets of T cells. At least two of these cytokines, IL-7 and IL-15, are known to be important for the maintenance of CD8⁺ T cell memory following pathogen clearance (reviewed in Ref. [43]) and are discussed in more detail below. Studies of acute resolving infections have demonstrated long-lived memory CD8⁺ T cells are maintained through IL-7 and IL-15 cytokine-driven homeostatic proliferation [43] and thus express the receptors for these cytokines, IL-7R α and IL-15R β , respectively, on their cell surface [40, 44-46]. In addition, memory CD8⁺T cells are capable of up-regulating the expression of anti-apoptotic molecules, most notably in response to IL-7 or IL-15 [44, 47, 48].

2.2.1 Interleukin 7 and IL-7R α

IL-7 is a 25 kDa glycoprotein produced by stromal cells, thymic epithelial cells and intestinal epithelial cells. Although IL-7 mRNA transcription is constitutive, IL-7 is generally thought to be limiting *in vivo* and thus plays a crucial role in regulating the size of the T cell pool [49]. IL-7 binds to a heterodimeric receptor composed of the common γ chain and a “private” IL-7R α chain, inducing signals through the Jak1/3/Stat5 pathway [50]. Deletion of IL-7 or its receptor results in a unique phenotype even though several other cytokines also activate the Jak1/3-Stat5 pathway [51]. IL-7 signaling downregulates IL-7R α surface expression and IL-7R α transcription in both CD4⁺ and CD8⁺ T cells but does not significantly affect IL-7R α mRNA stability, indicating regulation of IL-7R α expression occurs at the transcriptional level [52]. Because IL-7 is limiting *in vivo*, IL-7-mediated IL-7R α downregulation thus provides a regulatory mechanism that serves to maximize IL-7 availability [52].

The IL-7R α chain is important for IL-7 signaling in T cells [53]. Although IL-7R α is expressed by naïve and memory CD8 $^{+}$ T cells, it is down-regulated by effector CD8 $^{+}$ T cells following viral and bacterial infections. This observation is in line with the fact that IL-7R α downregulation occurs following TCR stimulation [54-56]. A number of viral infection models have demonstrated ~5-15% of effector CD8 $^{+}$ T cells re-express or retain high levels of IL-7R α and preferentially become memory CD8 $^{+}$ T cells [57]. The effector cells which retained high expression of IL-7R α survived to the memory phase and were functionally superior to IL-7R α ^{lo} cells as assessed by proliferative capacity [58] and IL-2 and IFN- γ production following peptide restimulation [59]. Expression of IL-7R α has therefore been used to identify memory CD8 $^{+}$ T cell precursors following infections in both mice and humans.

The significance of the expression of IL-7R α on a small percentage of effector CD8 $^{+}$ T cells transitioning into the memory phase is not clear, but it has been suggested that expression of IL-7R α provides survival signals during the contraction phase as the antigen load is reduced [44]. Indeed, signaling through IL-7R α has been shown to induce the up-regulation of anti-apoptotic molecules, and IL-7R α signaling during the effector to memory transition has been shown to be dependent on IL-7 [44]. However, a recent report indicated that IL-7R α expression on antiviral CD8 $^{+}$ T cells was not affected in IL-7^{-/-} hosts following vesicular stomatitis virus (VSV) infection and was not required for memory generation [60]. Whether expression of IL-7R α can be used as a memory precursor marker in other situations remains unknown. Lacombe *et al.* found IL-7R α was not an appropriate marker for memory precursors, as the majority of Ag-specific CD8 $^{+}$ T cells expressing IL-7R α during contraction failed to survive following peptide immunization [61].

The effects of IL-7 on T cells have been documented extensively. Mice overexpressing the IL-7 transgene exhibit a massive expansion of T cells in secondary lymphoid tissues, particularly in the CD8 $^{+}$ T cell compartment, with similar effects observed following recombinant

IL-7 administration to wild type (WT) mice [62]. While IL-7 administration preferentially increases the basal proliferation rates of CD8⁺ T cells *in vivo* and has been shown to increase the number of CD8⁺ T cells entering the cell cycle, IL-7 does not have an activating effect on T cells [63]. IL-7 has also been shown to promote the survival of T lymphocytes through the induction of various anti-apoptotic molecules and inhibition of pro-apoptotic molecules [64, 65].

IL-7 is an absolute requirement for naïve CD8⁺ T cell survival and for the homeostatic proliferation of naïve CD8⁺ T cells under lymphopenic conditions, as naïve CD8⁺ T cells fail to undergo homeostatic proliferation in IL-7^{-/-} lymphopenic hosts [57, 66]. It has been suggested that the increased availability of IL-7 during lymphopenic conditions could account for the high proliferation rates observed for naïve CD8⁺ T cells in this situation.

Memory CD8⁺ T survival also depends upon IL-7. Mice overexpressing the IL-7 transgene have ~20-fold higher numbers of memory phenotype CD8⁺ T cells than normal mice [62]. The pro-survival effects of this cytokine are in part due to its ability to up-regulate the anti-apoptotic molecule B-cell lymphoma 2 (bcl-2) in CD8⁺ T cells [44, 47]. Indeed, CD8⁺ T cells from IL-7^{-/-} mice have lower levels of bcl-2 and are less likely to become memory CD8⁺ T cells than their WT counterparts following infection. Furthermore, high concentrations of IL-7 can compensate for the absence of IL-15 in IL-15^{-/-} mice by promoting the survival of memory phenotype CD8⁺ T cells [62, 66].

2.2.2 Interleukin 15 and IL-15R

IL-15 is a 14-15 kDa membrane-associated cytokine produced by many cell types including monocytes, activated dendritic cells, and stromal cells. Although IL-15 mRNA is constitutively expressed in many tissues, IL-15 protein is not easily detected in normal mice and humans [67]. IL-15 binds and signals via a trimeric receptor composed of the γ_c chain, the IL-2/15R β chain, and a “private” α chain that confers high affinity binding to IL-15 [68]. IL-15 can bind to the γ_c and IL-2/15R β chains with intermediate affinity and can transduce signals through

these subunits in the absence of IL-15R α [69]. Although it was originally proposed that memory CD8⁺ T cells expressed IL-15R α in order to receive IL-15 signals, recent studies have indicated that IL-15R α expression on the affected cell type is not required for receiving IL-15-mediated signals [70]. A transpresentation model for IL-15 was proposed demonstrating IL-15R α can bind IL-15 alone and subsequently transpresent this cytokine to opposing cells (reviewed in [71]). Thus, cells expressing the γ c and β chains but lacking the IL-15R α chain are still able to receive IL-15 signals [70, 72].

IL-15 is a pleiotropic cytokine, affecting a wide range of cell types. Although survival of naïve CD8⁺ T cells is unimpaired in IL-15^{-/-} hosts, exogenously added IL-15 enhances their proliferation *in vitro* [73], suggesting IL-15 may contribute to the homeostatic proliferation of naïve cell. IL-15 also up-regulates bcl-2 in naïve CD8⁺ T cells and can rescue these cells from apoptosis [74, 75].

Viruses, polyinosinic-polycytidylic acid (poly I:C) treatment, and LPS are all inducers of type I IFNs and cause the selective stimulation of CD44^{hi} CD8⁺ T cells [48]. Initial studies first demonstrated IL-15 could be induced by type I IFNs and was also capable of stimulating the proliferation of CD44^{hi} CD8⁺ T cells *in vitro* and *in vivo* [76]. Furthermore, IL-15R β is highly expressed on memory phenotype CD8⁺ T cells and is required for IL-15-mediated signaling [77]. IL-15 also enhances T cell survival through the induction of bcl-2 expression and by stabilizing telomere lengths in memory CD8⁺ T cells through the sustained induction of telomerase [78]. The requirement for IL-15 is particularly evident in IL-15^{-/-} mice, which have a severe reduction in the number of memory phenotype (CD44^{hi}) CD8⁺ T cells [78]. The basal proliferation of memory phenotype CD8⁺ T cells is completely blocked in IL-15^{-/-} hosts and thus contributes to the poor survival of memory CD8⁺ T cells in these mice [79].

The impact of IL-15 on the generation and maintenance of antigen-specific CD8⁺ T cell memory has also been demonstrated. IL-15 is required for the primary expansion of antiviral

CD8⁺ T cells during VSV infection [80], but not for the generation and effector function of antigen-specific CD8⁺ T cells during LCMV infection [81], indicating this requirement may depend on the model being utilized. IL-15 is sufficient for driving the expansion of antigen-specific CD8⁺ T cells, resulting in a population of cells displaying efficient effector function [82]. Similarly, primary *L. monocytogenes* (Lm) infection in transgenic mice overexpressing IL-15 results in a substantial increase in Lm-specific memory CD8⁺ T cells that confers a higher level of protection following challenge than observed in their wild type counterparts [83].

Similar to IL-15^{-/-} mice, IL-15R α ^{-/-} deficient mice have decreased numbers of memory phenotype CD8⁺ T cells, but also show reductions in single positive CD4⁺ and CD8⁺ thymocytes and double positive thymocytes [79, 80]. Despite a minimal requirement for IL-15R α in the generation of primary anti-viral CD8⁺ T cell responses [79], antigen-specific CD8⁺ T cell populations decline over time in IL-15R α ^{-/-} mice in part due to decreased CD8⁺ T cell proliferation rates *in vivo* [84]. IL-15R α deficiency also results in a reduction of bcl-2 expression in both naïve CD8⁺ T cells and in the residual memory phenotype CD8⁺ T cells present in IL-15R α ^{-/-} mice [85].

The above studies support models of fully resolved infections showing memory CD8⁺ T cells are maintained through cytokine-driven homeostatic proliferation. Recently however, studies have demonstrated that CD8⁺ T cells generated during some chronic infections may have properties quite distinct from those of “classical” memory cells. This emerging body of evidence is critical to the study of immune responses to infections such as *T. cruzi* in which antigen is not cleared from the host, and is the focus of the remainder of this chapter.

2.2.3 Effects of persistent infection on CD8⁺ T cells

Chronic antigen exposure can impact both the phenotype and function of CD8⁺ T cells. Work in the LCMV model of infection has allowed for the tracking of antigen-specific CD8⁺ T cell populations following either an acute resolving or chronic infection [40, 45, 86]. Analysis of

CD8⁺ T cell subsets based on CD62L and CCR7 expression in this model has revealed that the differentiation pathways of antigen-specific CD8⁺ T cells is altered during persistent infections compared to resolved infections. Wherry *et al.* have demonstrated that the generation of a long-lived T_{CM} population from a T_{EM} population is observed following acute viral infection but fails to occur in the presence of persistent viral antigen [40]. Additionally, removal of T_{EM} cells from an environment containing antigen did not lead to the conversion to a T_{CM} phenotype [40].

Other studies with LCMV have demonstrated that memory CD8⁺ T cells generated during acute infection are capable of self renewal following antigen removal while those generated during chronic infection are not. These “antigen addicted” memory CD8⁺ T cells in persistent infection lack responsiveness to IL-7 and IL-15 and fail to upregulate bcl-2 [40]. The lack of responsiveness to IL-7 and IL-15 is reflected in the phenotype of these cells, as low levels of IL-7R α [40, 45, 87, 88] and IL-15R β [40] are often found on antigen-specific CD8⁺ T cells during persistent infections.

The IL-7R α^{lo} phenotype is also associated with a reduced capacity of antigen-specific CD8⁺ T cells to produce IFN- γ [45] and IL-2 [87] upon peptide restimulation, and IL-7R α downregulation following continuous antigen exposure [44, 55, 89] leads to reduced bcl-2 expression and increased levels of apoptosis by CD8⁺ T cells [57]. The duration and strength of antigen stimulation also likely contributes to differences in function observed for CD8⁺ T cells during various persistent infections. T cells chronically exposed to a high antigen load, such as after infection with certain strains of LCMV, lose their functional abilities gradually; proliferative capacity, IL-2 production, and cytolytic activity are lost first, followed by tumor necrosis factor alpha (TNF- α) and IFN- γ secretion (reviewed in [90]). Antigen-specific CD8⁺ T cells during chronic infections also have poor proliferative recall responses [91], demonstrating the wide range of effects persistent antigen has on T cells.

Reliable phenotypic markers of replicative senescence would be extremely useful for the study of CD8⁺ T cells during persistent infections. The killer cell lectin-like receptor G1 (KLRG1) is one such molecule that has recently been described. KLRG1 is an inhibitory NK cell receptor that binds to cadherins [92]. KLRG1 is expressed by T cells that have undergone a large number of cell divisions and on a small percentage (<10%) of CD8⁺ T cells in naïve mice [93]. Unlike anti-viral CD8⁺ T cells in acute resolving infections, those present during chronic human viral infections are mostly KLRG1⁺, an observation that is consistent with studies showing expression of this molecule is induced by repetitive antigen stimulation and marks CD8⁺ T cells that have impaired proliferative capacities [94]. Although KLRG1⁺ CD8⁺ T cells exhibit replicative senescence, these cells are capable of cytolytic activity and cytokine secretion [94, 95].

2.2.4 Maintenance of CD8⁺ T cells during persistent infections

Although the mechanism for the maintenance of antigen-specific CD8⁺ T cells during persistent infections is not well understood, these cells may be maintained in part through the recruitment of newly-primed CD8⁺ T cells [95]. There has also been at least one report demonstrating antigen contributes directly to this maintenance [88]. Notably, antigen-dependent CD8⁺ T cells found in chronic infections resemble a subset of IL-15-independent memory phenotype CD8⁺ T cells which are found in normal mice and express low levels of IL-15R β [96]. These cells display an activated phenotype; express low levels of IL-7R α , CD62L, and bcl-2; and are dependent on MHC class I interactions. These IL-15R β ^{lo} memory phenotype CD8⁺ T cells also account for the residual CD8⁺ T cell population found in IL-15^{-/-} mice. The significance of this IL-15-independent subset awaits further investigation.

Not all chronic infections result in antigen-specific CD8⁺ T cells with a distinctive phenotype. The antigen load and localization likely plays a role in the heterogeneity of the memory CD8⁺ T cell pool observed during different chronic infections. For example, the

majority of antigen-specific CD8+ T cells display a T_{CM} phenotype (IL-7R α ^{hi}, KLRG1-) in the peripheral blood of HCV-infected individuals, while intra-hepatic HCV-specific CD8+ T cells are IL-7R α ^{lo} but remain KLRG1- [55]. Notably, *Salmonella enterica* generates a chronic infection in certain mouse strains resulting in an antigen-specific memory CD8+ T cell population that eventually up-regulates IL-7R α but not CD62L [97]. Because *T. cruzi* establishes a chronic infection, our lab is interested in determining the functional and phenotypic effects of persistent antigen on CD8+ T cells during this infection. The majority of parasite-specific and total CD8+ T cells from mice chronically infected with the Brazil strain of *T. cruzi* show a T_{EM} phenotype (CD44^{hi} and CD62L^{lo}), have cytolytic activity, and are capable of producing IFN- γ upon antigen re-stimulation [98]. However, a more detailed analysis of antigen-specific CD8+ T cell responses is needed in order to gain a better understanding of the maintenance of these cells during chronic *T. cruzi* infection.

Conclusion

T. cruzi establishes a persistent infection in its host, leading in some cases to the development of Chagas disease. Because *T. cruzi* replicates intracellularly, the CD8+ T cell response is especially important for the control of parasite load during infection. While much is known regarding CD8+ T cell responses in acute viral and bacterial models of infection, in-depth knowledge regarding these responses during chronic infections, particularly those caused by complex protozoan pathogens such as *T. cruzi*, is lacking. Antigen-specific memory CD8+ T cells express IL-7R α and IL-15R β , enabling them to receive survival signals from IL-7 and IL-15, two cytokines which are crucial for the maintenance of CD8+ T cell memory following pathogen clearance. However, studies have indicated that CD8+ T cells generated and maintained during persistent infections may not all possess these same qualities. A better understanding of the characteristics of CD8+ T cell responses during chronic infections is crucial not only for the

development of preventative and therapeutic vaccines for *T. cruzi* infection, but for many other debilitating and fatal persistent infections.

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CHAPTER 2

EVIDENCE OF A STABLE CD8⁺ T CELL MEMORY POPULATION IN CHRONIC *TRYPANOSOMA CRUZI* INFECTION¹

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Abstract

Persistent infections caused by intracellular pathogens nearly uniformly result in a population of antigen-specific CD8⁺ T cells with a T effector memory phenotype, with little evidence suggesting that a T central memory population is generated. Using a model of *Trypanosoma cruzi* infection, we demonstrate that in contrast to the T_{EM} phenotype of the majority of *T. cruzi*-specific CD8⁺ T cells, a population of cells displaying hallmark characteristics of T_{CM} cells is also present during long-term persistent infection. This population expressed the T_{CM} marker IL-7R α and was heterogeneous for the expression of three other T_{CM} markers CD62L, IL-15R β and bcl-2. Additionally, the majority of IL-7R α ^{hi} cells were KLRG1⁻, indicating they have not been repetitively activated through TCR stimulation. These IL-7R α ^{hi} cells were better maintained than their IL-7R α ^{lo} counterparts following transfer into naïve mice. This result is consistent with their observed surface expression of IL-7R α and IL-15R β , which confer the ability to self renew in response to IL-7 and IL-15. IL-7R α ^{hi} cells were capable of IFN- γ production upon peptide restimulation and expanded in response to challenge, indicating these cells are functionally responsive upon antigen re-encounter. These results are in contrast to what is typically observed during other persistent infections and indicate that a stable population of parasite-specific CD8⁺ T cells capable of antigen-independent survival is maintained in mice despite the presence of persistent antigen.

Introduction

The study of CD8⁺ T cell memory responses to infections caused by intracellular pathogens is particularly important for understanding defense against secondary infections and persistent infections, where CD8⁺ T cells are chronically exposed to antigen. During chronic infections, repetitive antigenic stimulation can negatively impact the ability of CD8⁺ T cells to remain functionally responsive and to be maintained efficiently as a stable population in the absence of antigen [1]. In contrast, studies in acute resolving models of infection have demonstrated that once established, stable CD8⁺ T cell memory is maintained independently of antigen [2]. The latter type of infection results in a CD8⁺ T cell population that is typically thought of as “classical memory”, while memory in chronic infections is less easily defined. A better understanding of how CD8⁺ T cells are maintained during persistent infections is important for the development of therapeutic vaccines and for the assessment of treatment options.

CD8⁺ T cell memory can be broadly classified into T central memory (T_{CM}) and T effector memory (T_{EM}) subsets. While these subsets were initially defined based on high and low expression of the lymph node homing molecules CD62L and CCR7, respectively [3], a variety of additional markers have subsequently also been used to discriminate between these two subsets of cells. IL-7R α is one such marker that has been used to identify memory CD8⁺ T cell precursors destined to become long-lived T_{CM} cells [4] capable of self renewal and rapid recall to antigen [5]. In these studies, T_{CM} cells are stably maintained through the ability to undergo efficient homeostatic turnover in response to the cytokines IL-7 and IL-15. Therefore, surface expression of IL-7R α and IL-15R β , receptors that confer responsiveness to IL-7 and IL-15, respectively, are hallmark characteristics of T_{CM} cells [6]. In contrast to acute resolving infections, CD8⁺ T cells in chronic infections are maintained primarily as T_{EM} cells [6-10] that are dependent on antigen for survival, generally fail to express IL-7R α and IL-15R β , and show poor

proliferative responses to IL-7 and IL-15 [6-8]. Thus, in the linear differentiation model, T_{CM} cells provide superior protective immunity upon re-exposure to antigen due to their ability to self-renew in the absence of antigen compared to T_{EM} cells [5, 7, 8, 11, 12].

Here we chose to investigate the effects of persistent parasite antigen on the maintenance of CD8⁺ T cells during chronic infection with the protozoan parasite *Trypanosoma cruzi*. *T. cruzi* is the causative agent of Chagas disease and is responsible for more than 50,000 deaths a year in Latin America [13]. Although acute infection is controlled by host immune responses, parasites persist in certain tissues [14, 15]. The use of tools such as MHC class I tetramers has allowed for the tracking and characterization of parasite-specific CD8⁺ T cells during *T. cruzi* infection. This infection therefore provides an opportunity to study the effects of persistent antigen on the parasite-specific CD8⁺ T cell response, and more specifically, whether stable T_{CM} cells are generated during this infection. We report that consistent with the chronic nature of *T. cruzi* infection, the majority of antigen-specific CD8⁺ T cells during chronic infection are classical T_{EM} [16] which are CD62L^{lo} IL-7R α ^{lo} IL-15R β ^{lo} and proliferate poorly to homeostatic cytokines. However, we show that a minor population of IL-7R α ^{hi} parasite-specific CD8⁺ T cells also exists that displays characteristics similar to a T_{CM} population. We show that these IL-7R α ^{hi} antigen-specific CD8⁺ T cells are capable of producing IFN- γ following peptide restimulation, are better maintained in the absence of antigen than their IL-7R α ^{lo} counterparts, and expand following challenge. These data demonstrate that a stable memory CD8⁺ T cell population capable of antigen-independent survival is present even in an environment where antigen persists.

Materials and Methods

Mice, parasites and infections

C57BL/6 (Ly5.2⁺) mice and B6 (Ly5.1⁺) mice were purchased from Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute at Frederick (Frederick, MD). Mice were

maintained at the University of Georgia animal facility in microisolator cages under specific pathogen-free conditions. For the *T. cruzi* infections, tissue culture trypomastigotes (TCT) of the Brazil strain or CL strain of *T. cruzi* were obtained from passage through Vero cells in RPMI containing 10% Fetal Bovine Serum (FBS). Mice were infected either intraperitoneally (i.p.) with 1000 TCT or with 2000 TCT via footpad injection and sacrificed by CO₂ inhalation at different times post infection. In some cases animals were challenged with 10,000 Brazil strain parasites. All animal protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Benznidazole treatment

N-Benzyl-2-nitroimidazole acetamide [benznidazole (BZ); Rochagan, Roche, Rio de Janeiro, Brazil] was used as a trypanocidal drug in the experimental therapy schedules. Mice were treated orally with daily doses of benznidazole of 100 mg/kg body weight for 20 consecutive days from 15 to 35 days post infection (dpi) and from day 150 to 170 dpi. In some cases, BZ was administered from 15-55 dpi or from 30-35 dpi. BZ was prepared by pulverization of one tablet containing 100mg of the active principle, followed by suspension in distilled water. Each mouse received 0.20mL of this suspension, by gavage.

Immunophenotyping

Red blood cells (RBC) in single cell suspensions of spleen cells (SC) were lysed in a hypotonic ammonium chloride solution and washed in staining buffer (2% bovine serum albumin, 0.02% azide in phosphate buffered saline (PAB)). In some cases, mouse peripheral blood was obtained by retroorbital venipuncture, collected in Na citrate solution, and washed in PAB. SC and whole blood were incubated with tetramer-PE and the following labeled Abs: anti-CD62L biotin, anti-CD44 FITC, anti-CD8 APC-Cy7, anti-CD127 APC (eBioscience), anti-CD122 Alexa Fluor 488. Cells were also stained with anti-CD4, anti-CD11b, and anti-B220 Tricolor (Caltag-Invitrogen laboratories) for use as an exclusion channel. Cells were stained for 45 min at 4°C in the dark, washed twice in PAB, and fixed in 2% formaldehyde. For whole blood, RBC were

lysed in a hypotonic ammonium chloride solution after washing twice in PAB. At least 500,000 cells were acquired using a CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star, Inc.). MHC I tetramers were synthesized at the Tetramer Core Facility (Emory University, Atlanta, Georgia, United States). Tetramers used in these studies were TSKB20 (ANYKFTLV/Kb) and TSKB74 (VNYDFTLV/Kb).

Lymphocyte culture

Splenocytes isolated from chronically infected or BZ treated mice were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a final concentration of 1 μ M for 3 min. at room temperature. CFSE labeling was quenched with an equal volume of Horse Serum (HS) and washed three times with RPMI containing 10% FBS. CFSE labeled cells were cultured in RPMI containing 10% FBS for 72 hr at 37°C with IL-7 (15ng/mL), IL-15 (15ng/mL) or media alone. An anti-IL-15 blocking antibody (eBioscience) was added to cell cultures at the following concentrations: 0.78 μ g/ml, 0.078 μ g/ml, or 0.0078 μ g/ml. Following culture, splenocytes were washed twice in PAB and stained with TSKB20 PE, anti-CD8 AF405 and exclusion channel antibodies. In a separate experiment, splenocytes were stained intracellularly for bcl-2 following culture with IL-7 (15ng/mL), IL-15 (15ng/mL), or media alone for 48 hr.

Intracellular cytokine staining

Splenocytes isolated from naïve or chronically infected mice were sorted as described below, cultured with feeder cells from GFP-expressing mice [C57BL/6-Tg(ACTB-EGFP)1Os/J], and stimulated with *T. cruzi* peptides (1 μ M) for 5 hr at 37°C in the presence of Brefeldin A (GolgiPlug, BD Biosciences). *T. cruzi* peptides used in this study were TSKB20 (ANYKFTLV), TSKB18 (ANYDTLV), CRZP5 (PSVRSSVPL), CRZP9 (VPLNKCRL), GFT 16 (AAMSRHPPL), and GFT 17 (RGFDEGNGL). Cells were surface stained with anti-CD8 AF405 and intracellular cytokine staining was performed with anti-IFN- γ APC with a Cytofix/Cytoperm kit (BD

Biosciences) in accordance with the manufacturer's instructions. At least 250,000 cells were acquired on a Cyan flow cytometer and analyzed with FlowJo (Tree star, inc.).

***In vivo* BrdU incorporation assay**

5-bromo-2-deoxy-uridine (BrdU) (Sigma Chemical Co., St. Louis, MO) solution (0.8mg/ml) was made fresh every 2 days and given to mice for 12 days. SC from mice were RBC lysed and washed two times in PBS. Cells were surface stained for 45 min at 4°C in the dark with TSKB20-PE and the following labeled Abs: anti-CD45.2 APC, anti-CD8 AF405 (Invitrogen) and anti-CD127 PE-Cy7 (eBioscience). Cells were permeabilized and stained according to the manufacturer's instructions with a BrdU flow kit (BDbiosciences). Briefly, cells were permeabilized twice and treated with DNase to expose incorporated BrdU. Cells were then stained intracellularly with anti-BrdU FITC (BD Pharmingen), washed twice and fixed in 2% formaldehyde. An exclusion channel was used as described above. At least 2×10^6 were acquired on a Cyan flow cytometer (DakoCytomation) and analyzed with FlowJo (Tree star, inc.).

Generation of memory OT-1 cells

SC (50×10^6) from naïve OT-1 mice were transferred i.v. into uninfected congenic Ly5.1 (CD45.1) B6 mice. Recipient mice were infected i.p. with 2×10^6 plaque-forming units of OVA-expressing vaccinia virus 1 day post transfer. SC were harvested >15 dpi and stained as indicated above.

CD8⁺ T cell sorting and adoptive transfers

SC from naïve or chronically *T. cruzi*-infected mice were obtained and washed twice in PBS following RBC lysis. CD8⁺ T cells were negatively selected through magnetic sorting (CD8a⁺ T cell isolation kit, Miltenyi Biotec Inc, Auburn, CA). Purified CD8⁺ T cells were stained with anti-IL-7R α PE and in some experiments stained additionally with anti-CD44 APC. Cells were sorted into IL-7R α^{lo} and IL-7R α^{hi} populations or CD44^{hi} IL-7R α^{hi} and CD44^{hi} IL-7R α^{lo} populations. IL-

7R α ^{hi} and IL-7R α ^{lo} populations were stimulated *in vitro* as described above. 2.3×10^6 CD44^{hi} IL-7R α ^{hi} or CD44^{hi} IL-7R α ^{lo} cells were transferred i.v. into uninfected congenic Ly5.1 (CD45.1) B6 mice. Mice were infected 21 days following transfer with 10,000 Brazil strain *T. cruzi* TCT and fed BrdU in drinking water for 12 days to assess proliferation rates *in vivo*. Mice were sacrificed 12 days post-challenge infection and splenocytes were stained for BrdU and surface markers as described above.

Results

Expression of memory markers on *T. cruzi*-specific and memory OT-1 CD8+ T cells

We have recently shown that *T. cruzi* infection elicits a strong CD8+ T cell response that is highly focused on epitopes encoded by genes belonging to the trans-sialidase (ts) family [17]. To further investigate the heterogeneity of antigen-specific CD8+ T cells from *T. cruzi* chronically-infected mice, we compared the expression of memory markers on *T. cruzi*-specific CD8+ T cells to those found on “classical” memory OT-1 CD8+ T cells (T cell receptor (TCR) transgenic for the OVA peptide SIINFEKL) following an acute resolving vaccinia virus-OVA (VV-OVA) infection. CD8+ T cells from chronically infected mice (>100 dpi) specific for the immunodominant *T. cruzi* epitope TSKB20 displayed very low expression of IL-7R α , IL-15R β , and the anti-apoptotic molecule bcl-2. In stark contrast, analysis of splenocytes > 14 days post vaccinia infection using MHC class I tetramers loaded with SIINFEKL peptide revealed that the majority of *in vivo* generated memory OT-1 cells expressed high levels of IL-7R α , IL-15R β , and bcl-2 (Figure 1). These observations indicate that most memory CD8+ T cells in chronic *T. cruzi* infection have primarily a T_{EM} phenotype as expected based on the persistence of antigen in this model.

A *T. cruzi*-specific T_{CM} population emerges with drug-induced antigen clearance

Although VV-OVA infection provides a model of pathogen clearance for comparison to *T. cruzi* infection, it would be optimal to establish a model that would allow us to investigate *T.*

cruzi-specific memory CD8⁺ T cells in persistent (chronic infection) and cleared (cured infection). We have recently developed and tested a drug treatment protocol whereby *T. cruzi*-infected mice are parasitologically cured following administration of benznidazole (BZ). Cure in BZ-treated mice was documented by clearance of parasites from skeletal muscle as assessed by quantitative PCR, lack of exacerbation of infection following immunosuppression, failure to transfer infection to immunocompromised mice, and failure to detect parasites or pathology by histological examination (Bustamante *et al.*, manuscript in preparation). Interestingly, cure resulted in marked phenotypic changes in both the immunodominant TSKB20-specific and subdominant TSKB74-specific CD8⁺ T cell responses (Figure 2). In contrast to chronically infected mice, the majority of *T. cruzi* specific-CD8⁺ T cells from treated/cured mice expressed CD62L, IL-7R α , IL-15R β and bcl-2, consistent with the development of a T_{CM} population. These results indicate that in the absence of a persistent *T. cruzi* infection, a population of pathogen-specific, long-lived CD8⁺ T cells displaying phenotypic characteristics of a T_{CM} population is found 294 days after the completion of the treatment regimen, implying that the T_{EM} phenotype displayed by the majority of antigen-specific CD8⁺ T cells is due to the persistence of antigen in chronically infected mice. Surprisingly and in contrast to what is reported in other chronic infections, a minority of IL-7R α ^{hi}, IL-15 β ^{hi}, or bcl-2^{hi} TSKB20-specific cells resembling T_{CM} cells was observed in chronic infection (Figures 1 and 2).

Presence of a heterogeneous IL-7R α ^{hi} *T. cruzi*-specific CD8⁺ T cell population during chronic infection

To further examine the phenotypic characteristics of this population of T_{CM}-like cells, we analyzed the longitudinal expression of memory markers on TSKB20-specific CD8⁺ T cells following infection with the Brazil strain of *T. cruzi* (Figure 3A). As previously described [17], the TSKB20-specific response expanded and contracted during acute infection and a population of tetramer⁺ cells was detected at a low frequency during the chronic phase. Expression of IL-

IL-15R β was down-regulated on TSKB20-specific cells at 35 dpi compared to day 14, and was not re-expressed until later in the chronic phase (250 dpi). The frequency of TSKB20-specific cells expressing bcl-2 remained relatively constant throughout the acute phase and increased during the chronic phase. At fourteen days post infection, approximately 8% of TSKB20-specific cells expressed IL-7R α . The percentage of IL-7R α ^{hi} TSKB20-specific cells subsequently decreased until the chronic phase, during which 16% of antigen-specific cells expressed IL-7R α . Thus, these data show that a subset of TSKB20-specific CD8⁺ T cells express the memory markers IL-7R α , IL-15R β , or bcl-2 during chronic infection. Similar frequencies of CD8⁺ T cells from chronically infected mice expressing IL-7R α , IL-15R β or bcl-2 were also observed on T cells specific for the subdominant TSKB74 epitope (Figure 3B), suggesting that the memory phenotype does not vary among different populations within the dominance hierarchy.

A subset of cells from the total CD8⁺ T cell population also expressed IL-7R α , IL-15R β , or bcl-2 in the chronic phase of infection, potentially representing a subset of *T. cruzi*-specific cells of unknown specificity resembling T_{CM} cells. Alternatively, studies have also shown that the expression of such memory markers increases on CD8⁺ T cells in aged mice due to interactions with self-peptides or foreign environmental antigens [18]. To rule out the possibility that this population represented an age-related accumulation of memory phenotype CD8⁺ T cells, we examined expression of memory markers on CD8⁺ T cells from age-matched naïve and chronically *T. cruzi*-infected mice (Figure 3C). Analysis of the CD8⁺ T cell population in these mice revealed a greater percentage of cells from chronically infected mice co-expressed IL-7R α and CD44, a marker of antigen experience. Collectively, these observations show that antigen-specific IL-7R α ^{hi} memory CD8⁺ T cells resembling T_{CM} cells do account for a consistently measurable fraction of the total CD8⁺ T cell response to *T. cruzi* in chronic infection.

We sought to further examine the phenotype of the IL-7R α^{hi} TSKB20-specific CD8+ T cell population by examining the co-expression of memory markers on this population of cells from chronically infected mice 327 dpi (Figure 3D). Approximately 32% and 21% of TSKB20-specific IL-7R α^{hi} cells also expressed CD62L and IL-15R β , respectively. Approximately 40% of IL-7R α^{hi} tetramer+ cells expressed the killer cell lectin-like receptor G1 (KLRG1), a molecule induced by repetitive antigen stimulation that marks CD8+ T cells with impaired proliferative capacities [19]. These results indicate that the *T. cruzi*-specific CD8+ T cell population is heterogeneous for the expression of various memory markers.

The majority of *T. cruzi*-specific CD8+ T cells from chronically infected mice do not proliferate extensively in response to the homeostatic cytokines IL-7 and IL-15

T_{CM} cells are maintained through survival signals provided by the cytokines IL-7 and IL-15 following pathogen clearance; however, this requirement appears to be altered in antigen-specific CD8+ T cells during persistent infections. The observed low levels of IL-7R α and IL-15R β on the majority of antigen-specific CD8+ T cells from chronically *T. cruzi*-infected mice suggest that proliferative responses to IL-7 and IL-15 are likely impaired in this population of cells. In contrast, *T. cruzi*-specific T_{CM} cells from BZ-treated/cured mice could have the potential to proliferate in response to these cytokines based their surface expression of IL-7R α and IL-15R β (Figure 2). To test whether this is the case, splenocytes from infected untreated or infected BZ-treated mice were cultured in the presence of IL-7 and IL-15. Because we have previously observed that endogenous IL-15 in cell cultures contributes to background proliferation in the negative control (media only) conditions, graded doses of an anti-IL-15 antibody were added to block endogenous IL-15 in cultures. Analysis of cells following a 72 hr culture demonstrated endogenous IL-15 contributed to the proliferation of TSKB20-specific cells from both treated/cured and untreated/chronic mice in all culture conditions and could be blocked in a dose-dependent manner with anti-IL-15 blocking antibody (Figure 4A). While the

majority of TSKB20-specific CD8⁺ T cells from untreated/chronic mice showed minimal proliferation in response to IL-7, a greater fraction responded to IL-15. The most extensive proliferative responses to these cytokines, however, were observed in the TSKB20-specific population from treated/cured mice. Interestingly, enhanced proliferation of antigen-specific CD8⁺ T cells from both groups of mice was observed in cultures with IL-7 and the lowest concentrations of anti-IL-15, suggesting IL-7 and IL-15 can act either additively or synergistically to promote the proliferation of *T. cruzi*-specific CD8⁺ T cells. Taken together, these results show that a population of antigen-specific CD8⁺ T cells from chronically infected mice is capable of proliferating in response to IL-7 and IL-15; while a much larger population of antigen-specific CD8⁺ T cells exhibit this ability from BZ treated/cured mice.

Because IL-7 and IL-15 also increase the viability of CD8⁺ T cells through the upregulation of bcl-2, it is possible that the antigen-specific cultured cells may become more resistant to apoptosis but do not proliferate. To address this issue, we determined the level of bcl-2 expressed by *T. cruzi*-specific CD8⁺ T cells from untreated/chronic or BZ-treated/cured mice in the presence of IL-7 or IL-15 (Figure 4B). Compared to CD8⁺ T cells cultured in the absence of cytokines, we observed a small increase in bcl-2 expression in the TSKB20-specific population from chronically infected mice based on median fluorescence intensity following culture with IL-15. These data indicate that parasite-specific CD8⁺ T cells from chronically infected mice may be capable of receiving survival signals from IL-15.

IL-7R α^{hi} T cells are functionally responsive and present in CD8⁺ T cell populations specific for low frequency epitopes

Although TSKB20- and TSKB74-specific CD8⁺ T cells, which make up the bulk of the anti-*T. cruzi* immune response, display predominantly a T_{EM} phenotype, it is possible that *T. cruzi*-specific CD8⁺ T cells of other specificities do not follow the same differentiation program. Immunodominant responses may achieve frequencies due to a greater amount and presentation of cognate antigen throughout the infection, and the abundance of antigen likely

contributes to the maintenance of these cells as a T_{EM} population. On the other hand, low frequency CD8⁺ T cell responses may be specific for scarce or sparsely presented antigens, allowing greater rest from antigen exposure and perhaps a distinct differentiation outcome, such as the development of T_{CM} characteristics. To more rigorously test whether differences in memory phenotype are reflected in immunodominance hierarchy, we examined responses to a number of previously identified non-ts peptides which are present at much lower frequencies than the TSKB20 and 74 responses in chronically infected mice. These peptides are present in proteins belonging to the cruzipain (CRZP) and β -galactofuranosyl transferase (GTF) families of proteins and individually represent < 2% of responding CD8⁺ T cells during chronic infection. Sorted IL-7R α ^{hi} and IL-7R α ^{lo} CD8⁺ T cells from chronically *T. cruzi*-infected mice were stimulated with a pool of ts or non-ts *T. cruzi* peptides and probed for IFN- γ production (Figure 5). Naïve sorted controls did not make IFN- γ in response to any *T. cruzi* peptides. Unexpectedly, the majority of responding CD8⁺ T cells to both pools of peptides was in the IL-7R α ^{lo} population from chronically infected mice. However, we did observe the presence of a small population of responding CD8⁺ T cells to both peptide pools in the IL-7R α ^{hi} sorted population. This indicated that regardless of the epitope specificity and possibly the level of antigen exposure, the majority of *T. cruzi*-specific cells share the IL-7R α ^{lo} phenotype. Importantly, these data show that *T. cruzi* specific IL-7R α ^{hi} T cells are functionally responsive and present in CD8⁺ T cell populations specific for low frequency epitopes as well.

Maintenance and expansion of IL-7R α ^{hi} CD8⁺ T cells from chronically infected mice

Preservation following pathogen clearance and rapid expansion following secondary antigen exposure are important characteristics of T_{CM} cells. To further examine these characteristics in the IL-7 α ^{hi} CD8⁺ T population from chronically infected mice, purified CD8⁺ T cells from naïve and chronically infected mice were sorted into CD44^{hi} IL-7R α ^{hi} and CD44^{hi} IL-7R α ^{lo} groups. Equal numbers of sorted cells were transferred into naïve mice, and mice were

rested in order to determine the maintenance of these cells in the absence of antigen. Monitoring the peripheral blood of these mice at 20 days revealed the IL-7R α^{hi} donor populations from naïve and chronically infected mice were better maintained at this time than the IL-7R α^{lo} population (Figure 6A), although TSKB20-specific cells were undetectable in the transferred populations at this time. Notably, IL-7R α^{lo} CD8+ T cells from naïve control mice were poorly maintained in naïve mice. Phenotypic analysis revealed the IL-7R α^{hi} transferred cells retained high expression of IL-7R α . Interestingly, approximately 40% of the transferred IL-7R α^{lo} cells from chronically infected mice were IL-7R α^{hi} .

To determine the proliferative capacity of the donor IL-7R α^{hi} cells, mice were infected with *T. cruzi* 21 days post transfer and administered BrdU in drinking water. Examination of spleen cell populations 12 days after infection revealed that transferred CD8+ T cells from both IL-7R α^{hi} and IL-7R α^{lo} groups from chronically infected mice had expanded (Figure 6B) and incorporated similar amounts of BrdU (Figure 6C), although IL-7R α^{hi} donor cells were present at much higher numbers in the spleen than the IL-7R α^{lo} donor cells. This is not surprising considering that IL-7R α^{hi} transferred cells were better maintained in naïve mice and were therefore present in higher numbers prior to challenge. The majority IL-7R α^{hi} and IL-7R α^{lo} donor cells from chronically infected mice also showed a T_{EM} phenotype (IL-7R α^{lo}) following challenge (Figure 6C). Naïve IL-7R α^{hi} donor cells had also expanded following challenge, although far fewer of these cells incorporated BrdU and converted to an IL-7R α^{lo} phenotype compared to chronic donor cells (Figure 6C).

TSKB20-specific cells also expanded in the IL-7R α^{hi} and IL-7R α^{lo} chronic donor populations but were undetectable in the naïve IL-7R α^{hi} population (Figure 6B). Transferred TSKB20-specific CD8+ T cells from chronic IL-7R α^{hi} and IL-7R α^{lo} donors incorporated similar amounts of BrdU compared to each other and to the total transferred CD8+ T cell population

(Figure 6C). These results indicate that IL-7R α^{hi} CD8+ T cells from chronically infected mice are more efficiently maintained in naïve mice than IL-7R α^{lo} cells, however both IL-7R α^{hi} and IL-7R α^{lo} donor cells expand robustly following antigenic challenge.

Discussion

The characteristics of stable CD8+ T cell memory generated during acute resolving infections have been well-documented. However, only recently has work focused on memory responses during chronic infections. CD8+ T cells present in chronic infections are often phenotypically and functionally distinct from CD8+ T cells in acute resolving infections. Functional exhaustion followed by eventual deletion of CD8+ T cells exposed to a high antigen load represents an extreme example of the consequences of persistent antigen exposure [7]. Many factors, including the frequency and duration of antigen encounter as well as the pathogen tropism will dictate the final outcome of antigen-specific CD8+ T cells during various persistent infections.

In this study, we investigated the effects of persistent antigen on memory CD8+ T cell responses during chronic *T. cruzi* infection. We have previously shown that antigen-specific CD8+ T cells are maintained predominantly as CD44 $^{\text{hi}}$ CD62L $^{\text{lo}}$ T $_{\text{EM}}$ cells during chronic *T. cruzi* infection, although here we show that there is also a small population of IL-7R α^{hi} IL-15R β^{hi} , or bcl-2 $^{\text{hi}}$ cells resembling T $_{\text{CM}}$ cells that exist in an environment where antigen persists.

We chose to focus on the expression of the memory marker IL-7R α on antigen-specific CD8+ T cells following *T. cruzi* infection. Expression of IL-7R α confers responsiveness to IL-7, a cytokine crucial to the survival of classical T $_{\text{CM}}$ cells following pathogen clearance. Because IL-7R α is down-regulated on CD8+ T cells following recent TCR stimulation [20, 21], CD8+ T cells generated during many chronic infections generally fail to express IL-7R α [6-8, 22]. Consistent with these findings, the majority of TSKB20-specific CD8+ T cells showed an IL-

7R α ^{lo} phenotype over the entire course of infection. However, a subset of TSKB20- and TSKB74-specific cells did express IL-7R α , IL-15R β , or bcl-2 during chronic infection.

The TSKB20-specific population in chronically infected mice was heterogeneous, as approximately 32% and 20% of IL-7R α ^{hi} tetramer+ cells also expressed CD62L and IL-15R β , respectively. Expression of KLRG1, a receptor marking antigen-specific CD8+ T cells that have experienced repetitive antigen stimulation yet are still capable of immediate effector functions [23], was also examined on the IL-7R α ^{hi} TSKB20-specific population. Interestingly, the majority of IL-7R α ^{hi} cells were KLRG1^{lo}, indicating this population of *T. cruzi*-specific CD8+ T cells has not been recently or repeatedly activated in a way to down-regulate IL-7R α or up-regulate KLRG1. Therefore it is possible that the majority of the IL-7R α ^{hi} subset of cells present in chronically infected mice represent a population of T_{CM} cells that have not recently encountered antigen or that have encountered antigen and failed to become fully activated. This is perhaps not surprising given that *T. cruzi* persists at very low levels in only certain tissues and only occasionally in the circulation during the chronic phase, thus the likelihood of encountering antigen may be lower than what is seen during high antigen load infections. Taken together, these data suggest that parasite-specific cells may have distinct histories of antigen encounter and/or may be at different points along a differentiation pathway leading to the acquisition of a stable T_{CM} phenotype. Priming of newly produced naïve CD8+ T cells during the chronic phase of infection could also account for the heterogeneity of this population [24], although further experiments are needed to determine if this is the case. It will be interesting to determine whether a greater proportion of the *T. cruzi*-specific CD8+ T cell population eventually re-express other memory markers such as CD62L following transfer into naïve hosts. This is certainly a possibility as studies by Kaech *et al.* have demonstrated that acquisition of CD62L expression occurs over time and in the absence of antigenic stimulation [4]. Indeed, we observed that approximately 43% of sorted IL-7R α ^{lo} CD8+ T cells from chronically infected

showed an IL-7R α^{hi} phenotype following transfer into naïve mice, suggesting these cells may be capable of recovering a T_{CM} phenotype, which allows for their long-term survival following removal from antigen. However we cannot rule out the possibility that IL-7R α^{hi} cells present in the IL-7R α^{lo} donor population represent contaminating IL-7R α^{hi} cells from the sort.

Long-lived T_{CM} cells generated during acute resolving infections are maintained through IL-7 and IL-15 cytokine-driven homeostatic proliferation and thus express the receptors for these cytokines, IL-7R α and IL-15R β , respectively, on their cell surface [4, 6, 8, 25]. Wherry and colleagues have recently demonstrated that anti-viral CD8⁺ T cells during chronic infection show poor proliferative responses to IL-7 and IL-15 and survive poorly following removal from antigen, consistent with the low levels of IL-7R α and IL-15R β expressed by these cells [6]. Given these findings, we sought to determine if the phenotypic expression of IL-7R α and IL-15R β (or lack thereof) on *T. cruzi*-specific CD8⁺ T cells was indicative of the function of these cells. Although the majority of TSKB20-specific CD8⁺ T cells from chronically infected mice did not proliferate extensively in response to IL-7 and IL-15 compared to TSKB20-specific cells from BZ treated/cured mice, we observed that a small population of antigen-specific CD8⁺ T cells from chronically infected mice is capable of proliferating in response to IL-7 and IL-15. Additionally, parasite-specific CD8⁺ T cells from chronically-infected mice were capable of up-regulating bcl-2 in the presence of IL-15. Taken together, these results suggest that a population of *T. cruzi*-specific T cells can indeed respond to homeostatic cytokines.

We next sought to further explore the maintenance of IL-7R α^{hi} and IL-7R α^{lo} *T. cruzi*-specific CD8⁺ T cells during chronic infection following removal from antigen. Memory phenotype IL-7R α^{hi} CD8⁺ T cells were better maintained following transfer into naïve hosts than their IL-7R α^{lo} counterparts. Notably, a subset of IL-7R α^{lo} cells showed an IL-7R α^{hi} phenotype following the transfer. This observation may be due to a contamination by IL-7R α^{hi} cells during sorting, although a purity check following the sort showed >95% purity. Challenging these mice

revealed that the IL-7R α^{hi} donor cells from chronically infected mice expanded to the greatest extent following infection, consistent with the observation that IL-7R α^{hi} donor cells were better maintained and thus were present at a higher frequency prior to challenge than IL-7R α^{lo} donor cells. IL-7R α^{lo} donor cells also expanded following challenge infection and incorporated a similar amount of brdU as the IL-7R α^{hi} donor population, indicating both populations from chronically infected mice proliferated at approximately the same rate. TSKB20-specific IL-7R α^{hi} and IL-7R α^{lo} donor cells from chronically infected mice were recovered from mice following challenge and incorporated similar amounts of brdU, indicating that both of these donor populations had expanded at the same rate following challenge infection. Whether the IL-7R α^{lo} TSKB20-specific and total CD8+ T cell population present following challenge represents a residual IL-7R α^{lo} population or a subset of cells that have converted to an IL-7R α^{hi} phenotype remains to be determined. The majority of CD8+ T cells from both IL-7R α^{hi} and IL-7R α^{lo} donor populations also showed an IL-7R α^{lo} phenotype following infection, consistent with a T_{EM} phenotype.

We also noted that CD44 $^{\text{hi}}$ IL-7R α^{hi} donor cells from naïve mice also expand following *T. cruzi* challenge infection. This expansion of CD44 $^{\text{hi}}$ IL-7R α^{hi} donor cells from naïve mice could result from memory CD8+ T cells specific for environmental antigens cross-reacting with *T. cruzi* antigens. It is also possible that IL-7R α^{hi} CD8+ T cells from naïve mice, which have proliferated and upregulated CD44 [26] are responsive to *T. cruzi* antigen or to cytokines produced during *T. cruzi* infection.

Our results indicate that a heterogeneous population of parasite-specific cells is present in *T. cruzi* chronically-infected mice. The T_{CM} marker IL-7R α was notably expressed by a subset of *T. cruzi*-specific cells despite the presence of persistent antigen during infection. This population of CD8+ T cells may represent a subset of parasite-specific cells which have not recently encountered antigen and thus could potentially preserve long-term T cell memory in

situations where antigen is cleared – such as after successful drug treatment. Indeed, we have shown for the first time, a *T. cruzi*-specific CD8⁺ T_{CM} population exists that is capable of providing enhanced protection to infection after mice are cured with the drug benznidazole (Bustamante *et al.*, manuscript in preparation). It will be important to determine if IL-7R α^{hi} CD8⁺ T cells eventually acquire additional phenotypic and functional characteristics of T_{CM} cells, such as expression of CD62L and a greater recall potential upon secondary infection if removed from antigen for a sufficient amount of time. It is also possible that a portion of antigen-specific IL-7R α^{lo} CD8⁺ T cells could acquire T_{CM} characteristics over time in the absence of antigen. These results suggest that long-term T cell memory with T_{CM} characteristics can be maintained even in the face of persistent antigen during *T. cruzi* infection. Retention of such a memory population could have implications for long-term protection in individuals successfully treated with benznidazole and in the development of therapeutic vaccines targeting long-lived memory CD8⁺ T cells.

Figure 1: Expression of memory markers on *T. cruzi*-specific and OT-1 CD8+ cells

OT-1 cells were adoptively transferred into naive C57BL/6 mice and infected with a vaccinia virus expressing OVA. VV-OVA-infected mice were sacrificed > 14 days later. Splenocytes from mice chronically infected (>100 dpi) with the Brazil strain of *T. cruzi* (top panel) and VV-OVA-infected mice (bottom panel) were stained for surface expression of CD8, IL-7R α or IL-15R β and non-CD8 lineage abs (anti-CD4, anti-CD11b, anti-B220), MHC class I tetramers loaded with the OVA peptide SIINFEKL or the *T. cruzi* peptide TSKB20. Cells shown in right panels are gated on CD4⁻ CD11b⁻ B220⁻ CD8⁺ T cells identified in left-most panels. The numbers indicate the percentage of tetramer⁺ CD8⁺ T cells that express each given marker. Data is representative of 2 independent experiments.

Figure 1

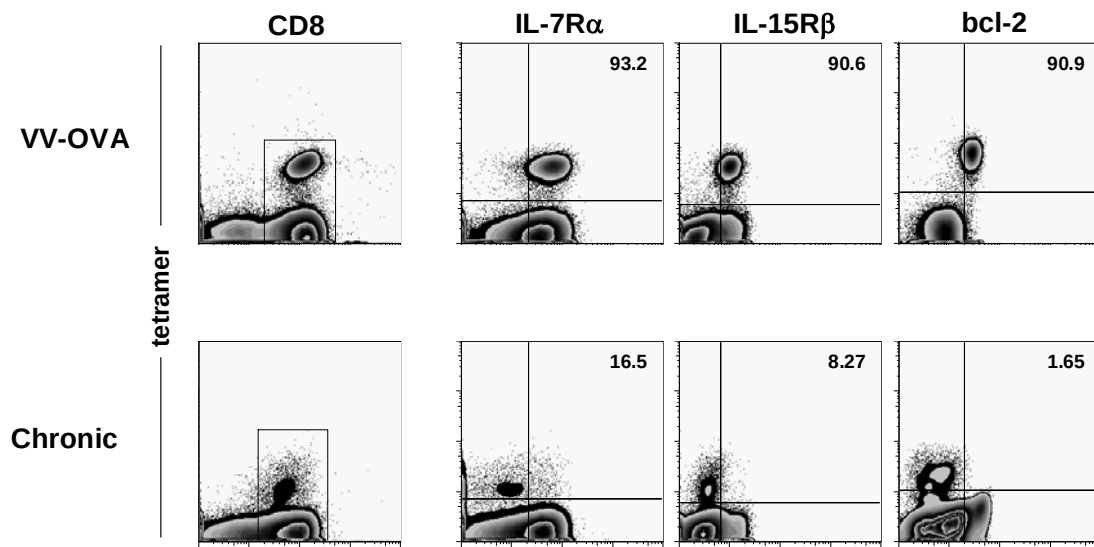


Figure 2: Expression of memory markers on TSKB20-specific CD8⁺ T cells from BZ treated or untreated C57BL/6 mice. Splenocytes were isolated from mice 349 dpi with the Brazil strain of *T. cruzi* at. Mice were left untreated or were treated with BZ from day 15-55 following infection. Splenocytes were stained with MHC class I tetramers bearing the TSKB20 peptide or the TSKB74 peptide and for surface expression of CD62L, IL-7R α , IL-15R β , or bcl-2 and non-CD8 lineage abs (anti-CD4, anti-CD11b, anti-B220). Cells shown are gated on CD4⁻ CD11b⁻ B220⁻ CD8⁺ T cells. Red histograms indicate TSKB20-specific or TSKB74-specific CD8⁺ T cells from untreated/chronic mice and blue histograms indicate TSKB20-specific or TSKB74-specific CD8⁺ T cells from treated/cured mice.

Figure 2

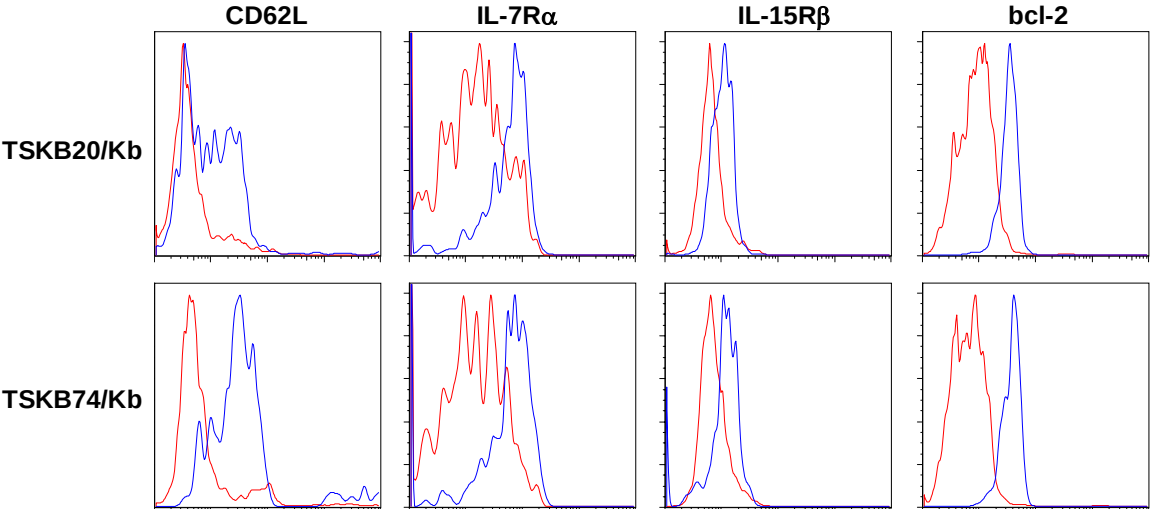
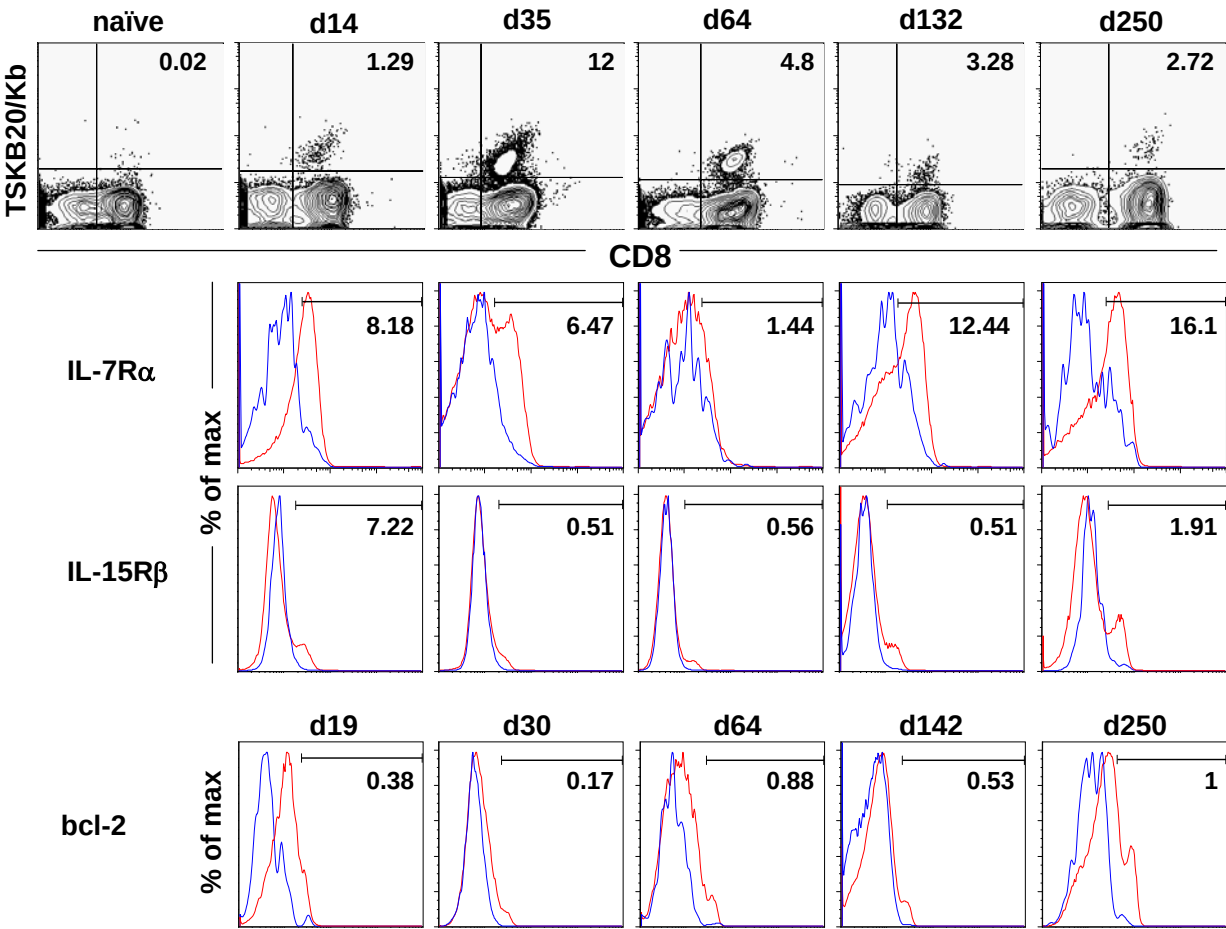


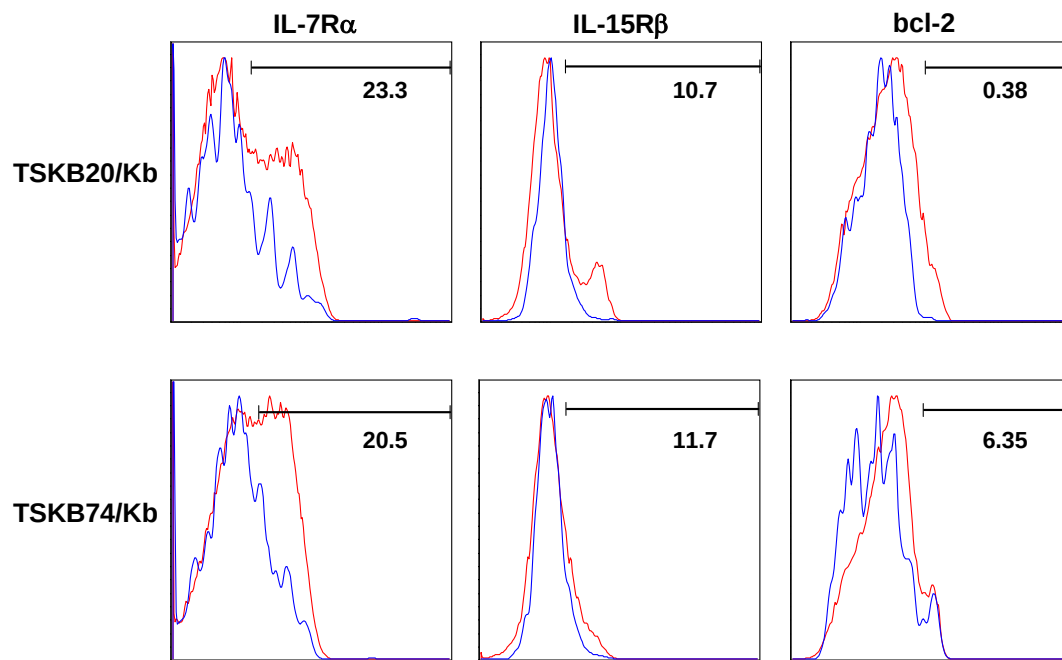
Figure 3: Emergence of a parasite-specific IL-7R α^{hi} population during chronic *T. cruzi* infection. Splenocytes from naïve or Brazil strain-infected mice were isolated and stained with the TSKB20/Kb or TSKB74/Kb tetramer and for surface expression of IL-7R α , IL-15R β , and bcl-2 and non-CD8 lineage abs (anti-CD4, anti-CD11b, anti-B220) at various times following infection. A) Cells shown in top panel are gated on CD4 $^{-}$ CD11b $^{-}$ B220 $^{-}$ T cells. Numbers indicate the percentage of CD8 $^{+}$ T cells that are TSKB20 tetramer $^{+}$. In lower panels, blue histograms indicate the TSKB20-specific population and red histograms represent the total CD8 $^{+}$ T cell population. Numbers indicate the percentage of tetramer $^{+}$ CD8 $^{+}$ T cells that express each given marker. Results are the average of n=3 or n=5 mice per group. Blue histograms indicate the TSKB20-specific population and red histograms represent the total CD8 $^{+}$ T cell population. Numbers indicate the percentage of TSKB20 tetramer $^{+}$ CD8 $^{+}$ T cells that express each given marker. B) Expression of memory markers on TSKB20-specific or TSKB74-specific CD8 $^{+}$ T cells from mice 327 dpi. Blue histograms indicate the tetramer $^{+}$ population while red histograms indicate the total CD8 $^{+}$ T cell population. Results are an average of n=2 mice per group. C) Splenocytes from age-matched (430 days old) naïve mice and chronically infected mice (400 dpi) with the Brazil strain of parasites were stained for surface expression of CD8, IL-7R α , and CD44. Histograms shown are gated on IL-7R α^{hi} CD8 $^{+}$ T cells. Numbers indicate the percent of IL-7R α^{hi} CD44 $^{+}$ CD8 $^{+}$ T cells. Results are an average of n=3 mice per group (naïve) and n=4 mice per group (chronic). D) Co-expression of memory markers on TSKB20-specific CD8 $^{+}$ T cells from mice 327 dpi. Histograms are gated on IL-7R α^{hi} TSKB20/Kb $^{+}$ cells. Numbers indicate the percentage of IL-7R α^{hi} cells that express each of the given memory markers. Results are an average of n=2 mice per group.

Figure 3

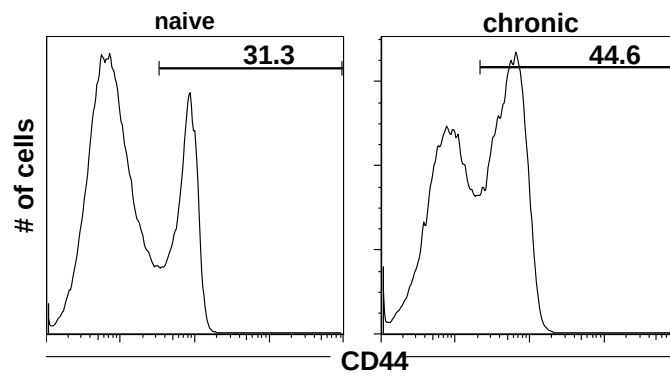
A.



B.



C.



D.

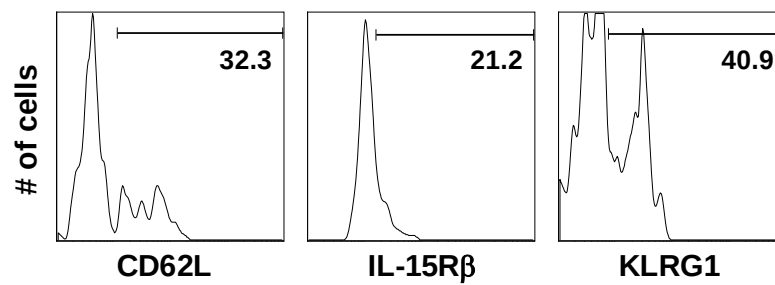
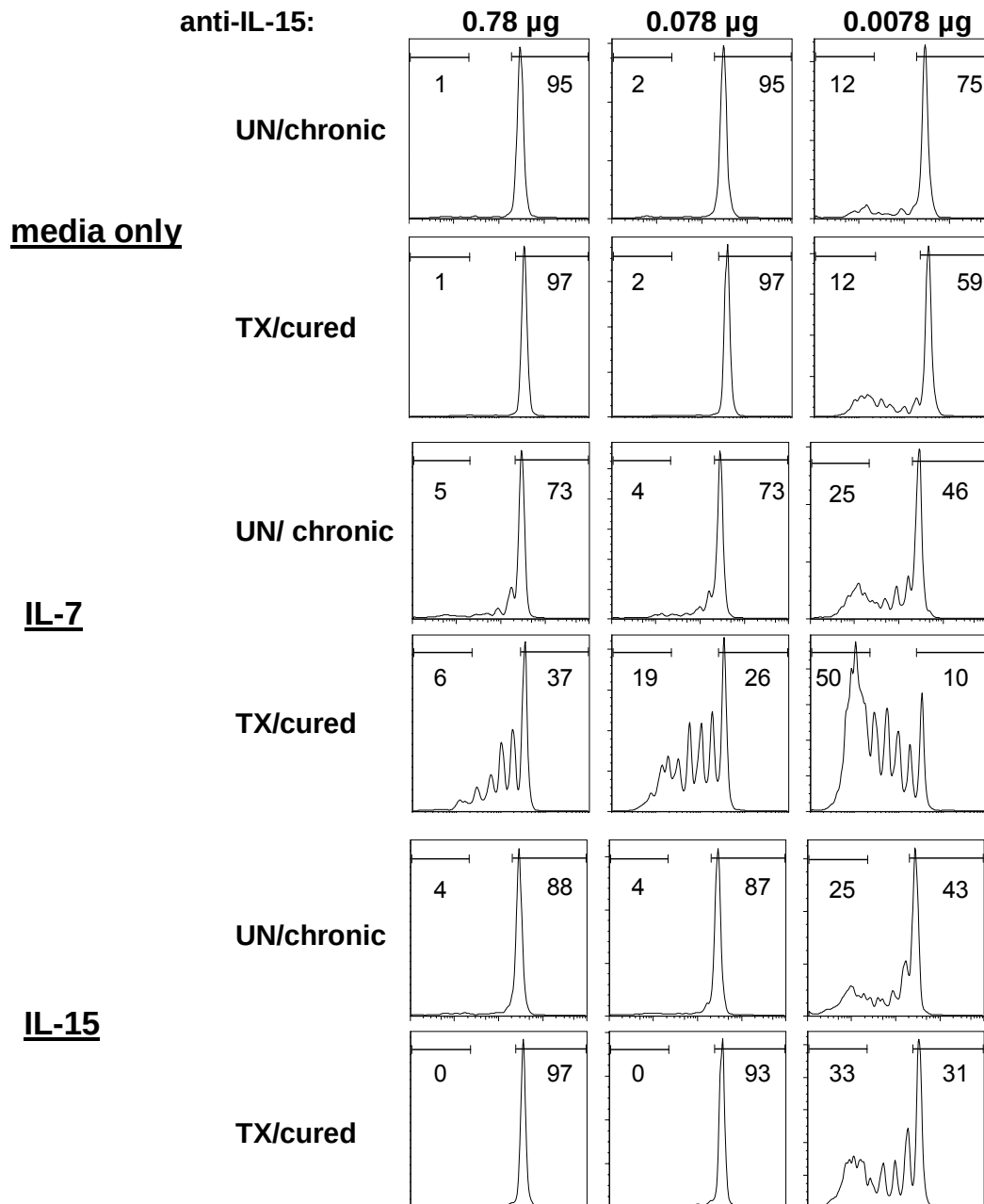


Figure 4: TSKB20 tetramer+ CD8+ T cells from chronically infected mice respond suboptimally to the cytokines IL-7 and IL-15. A) Splenocytes from BZ treated (TX 15-55 dpi) or untreated mice 154 dpi with the CL strain of parasites were labeled with CFSE and cultured in the presence of 15 ng/mL IL-7 or IL-15 or with media alone for 72 hr. An anti-IL-15 blocking antibody was added to cultures at the indicated concentrations. A concentration of 0.78 μ g/ml was used to block 100% of 50ng/ml IL-15 activity. Cells were stained with the TSKB20/Kb tetramer and for surface expression of CD8 and non-CD8 lineage abs (anti-CD4, anti-CD11b, anti-B220). Proliferation of CD4⁻ CD11b⁻ B220⁻ TSKB20-specific CD8⁺ T cells was assessed by CFSE dilution. Numbers indicate the percentage of TSKB20-specific cells in the undivided parent population. B) Splenocytes from Brazil strain infected BZ treated (TX 30-35 dpi) or untreated mice 100 dpi were cultured in the presence of 15ng/mL IL-7 or IL-15 or with media alone for 48 hr. Cells were stained with the TSKB20/Kb tetramer and for surface expression of CD8, non-CD8 lineage abs (anti-CD4, anti-CD11b, anti-B220) and intracellularly for bcl-2 expression. Plots are gated on CD4⁻ CD11b⁻ B220⁻ cells. Numbers indicate the median fluorescence intensity (MFI) of bcl-2 in the TSKB20-specific CD8⁺ T cell population. Data are an average of n=2 mice per group.

Figure 4
A.



B.

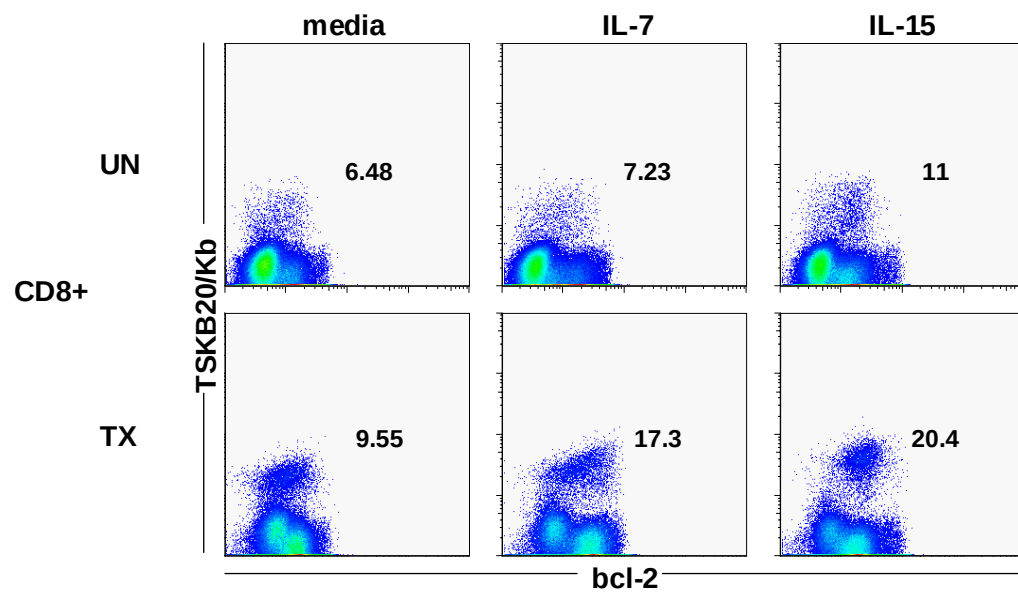


Figure 5: IL-7R α^{hi} *T. cruzi*-specific CD8+ T cells produce IFN- γ following *in vitro* peptide restimulation. Splenocytes from n=3 mice per group were pooled and sorted into CD8+ IL-7R α^{hi} and CD8+ IL-7R α^{lo} groups. IFN- γ production by purified IL-7R α^{hi} and IL-7R α^{lo} CD8+ T cells was assessed following a 5 hr incubation with a pool of ts peptides (TSKB20, TSKB74) or a pool of non-ts peptides (CRZP5 and CRZP9, GFT16 and GFT17) in the presence of GFP+ feeder cells. Splenocyte cultures from naïve (top panel) and mice chronically infected with the Brazil strain of parasites (bottom panel) were stained with anti-CD8 and anti-IFN- γ . Numbers indicate the percent of CD8+ IFN- γ producing cells for each condition. All plots are gated on GFP- cells.

Figure 5

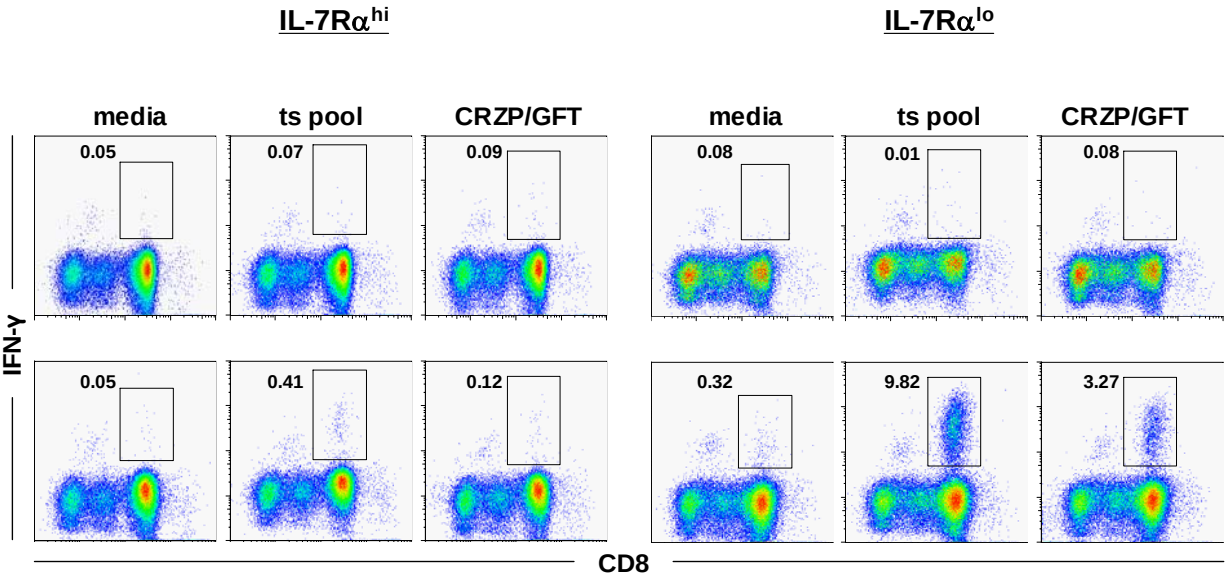
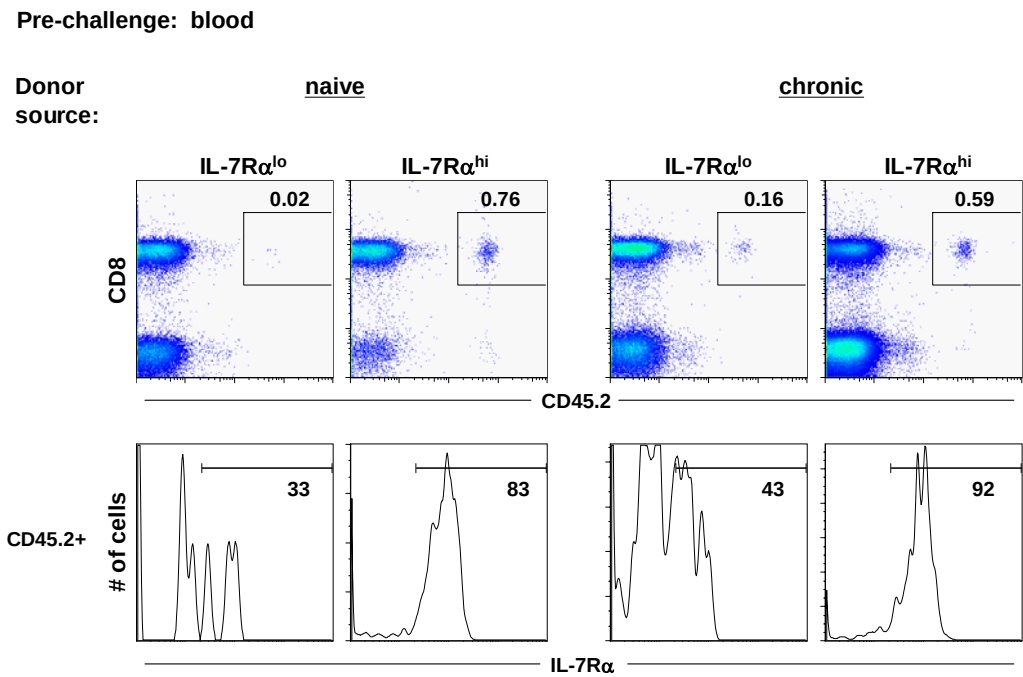


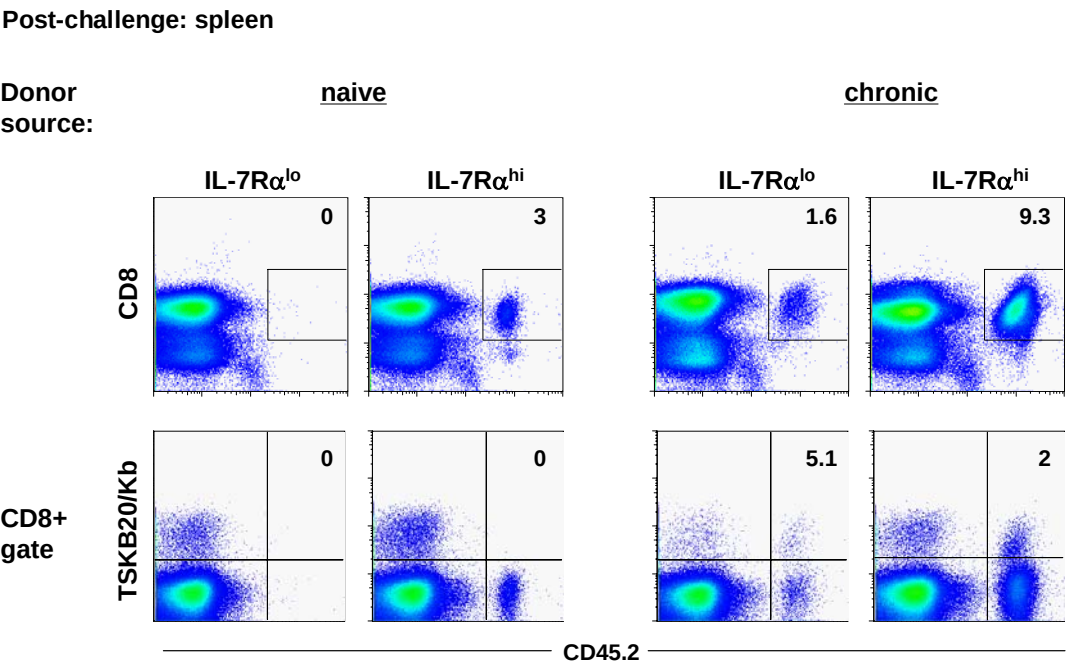
Figure 6: IL-7R α^{hi} CD8+ T cells are better maintained following removal from antigen than IL-7R α^{lo} CD8+ T cells. Equal numbers of purified IL-7R α^{hi} and IL-7R α^{lo} CD8+ CD44 $^{\text{hi}}$ CD45.2+ T cells from mice chronically infected with the Brazil strain of parasites or from naïve aged-matched mice were transferred into naïve CD45.1+ recipients. A) Mouse peripheral blood was stained with the TSKB20/Kb tetramer and for surface expression of CD45.2, CD8, IL-7R α , and non-CD8 lineage abs (anti-CD4, anti-CD11b, and anti-B220) 20 days following transfer. Top panels are gated on and numbers represent the percent of transferred CD8+ T cells of the total CD8+ T cell population. Bottom panels are gated on CD45.2+ CD8+ T cells and numbers indicate the percent of cells that are IL-7R α^{hi} . B) Mice receiving transfers were challenged with 10,000 Brazil strain parasites 21 days post transfer. Splenocytes from mice 12 days post challenge were stained with the TSKB20/Kb tetramer and for expression of CD8, IL-7R α , CD45.2, and BrdU. The top panels are gated on CD4 $^-$ CD11b $^-$ B220 $^-$ cells and bottom panels are gated on the total CD8+ T cell population. C) Analysis of donor populations 21 days post challenge in spleen. Histograms are gated on CD45.2+ CD8+ donor cells. Numbers in the top panels indicate the percentage of cells that have incorporated BrdU and numbers in bottom panels indicate the percentage of cells that express IL-7R α . Red histograms show the total CD8+ T cell donor population and blue histograms show the TSKB20-specific donor population. The top and bottom numbers in each overlay represent the total CD8+ T cell donor population and the TSKB20-specific donor population, respectively. Results are an average of n=3 mice per group.

Figure 6

A.



B.

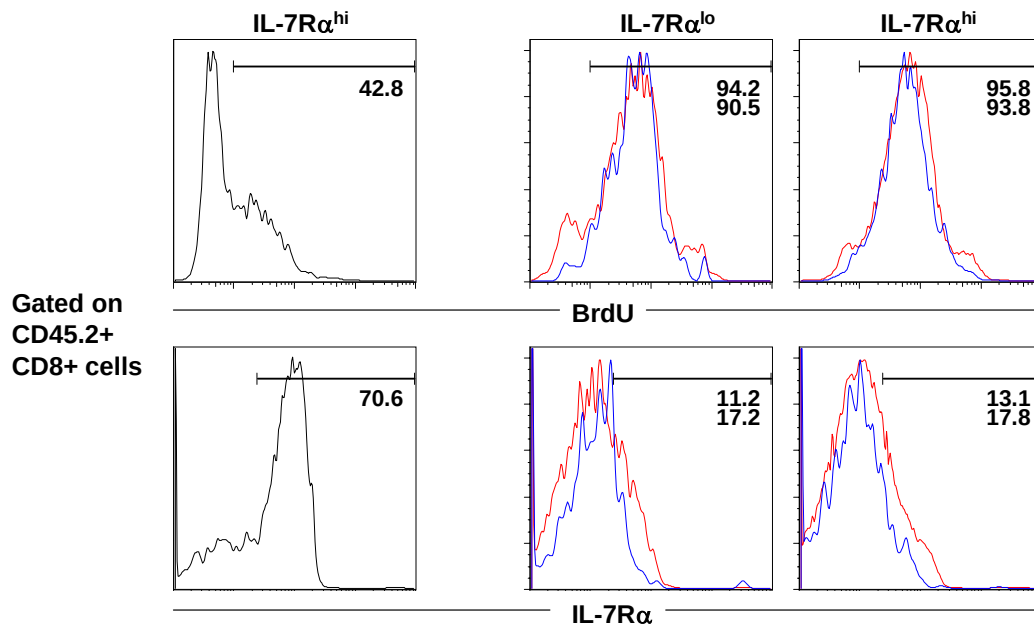


C.

Donor
source:

naive

chronic



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CHAPTER 3 CONCLUSION

The protozoan parasite *Trypanosoma cruzi* infects a variety of mammalian hosts and is the causative agent of Chagas disease. Both humoral and cell-mediated immune responses are induced following infection with *T. cruzi*. Although these responses are capable of controlling acute infection, they are insufficient to clear parasites from certain tissues thus resulting in a chronic infection. Consequently, a long-term relationship is established between the parasite and the host immune response. A precise understanding of the effects of persistent antigen on responding immune cells during *T. cruzi* infection is needed for the evaluation of treatment options and for the development of therapeutic vaccines for Chagas disease.

Because *T. cruzi* replicates intracellularly, CD8⁺ T cells are particularly important for controlling infection. Models of fully resolved infections have demonstrated that memory CD8⁺ T cells are maintained independently of MHC-TCR interactions through IL-7 and IL-15 cytokine-driven homeostatic proliferation and an up-regulation of anti-apoptotic molecules [1, 2]. Therefore, surface expression of IL-7R α and IL-15R β , receptors that confer responsiveness to IL-7 and IL-15, respectively, is a critical quality of memory CD8⁺ T cells following pathogen clearance [3]. In many of these studies, antigen-specific CD8⁺ T cells are maintained as T_{CM} cells and are the superior subset because they are long-lived and provide superior protection over T_{EM} cells [4-8]. Therefore, IL-7R α and IL-15R β are excellent markers for T_{CM} cells. In contrast, CD8⁺ T cells in chronic infections may have characteristics quite distinct from those of T_{EM} cells. Indeed, predominantly T_{EM} cells are generated during chronic infections, which do not express IL-7R α and IL-15R β and lack responsiveness to IL-7 and IL-15, thus compromising their survival following antigen removal [3]. Additionally, depending on the

amount of antigen present and the frequency with which T cells encounter this antigen, persistent infections can result in the eventual exhaustion of the pathogen-specific T cell response. In this situation, T_{EM} cells are not capable of self-renewal and are functionally impaired, making them inferior to T_{CM}.

The study of the maintenance of *T. cruzi*-specific CD8+ T cell memory has significant implications for long-term protection in individuals successfully treated with benznidazole. In this context, it is important to determine 1) if memory is maintained following cure of *T. cruzi* infection and 2) what are the subsets that are responsible for preserving long-term memory in the absence of infection. In this study, I investigated the phenotype and function of CD8+ T cells during chronic *T. cruzi* infection, with particular focus on the T_{CM} subset, which may be responsible for stable, long-term memory in this infection. IL-7R α was found to be expressed on a minority of *T. cruzi*-specific CD8+ T cells. These IL-7R α ^{hi} CD8+ T cells are capable of producing IFN- γ in response to *T. cruzi* peptides, are present in CD8+ T cell populations with distinct *T. cruzi* antigen specificities and frequencies, and are stably maintained when transferred to an antigen-free environment. However this IL-7R α ^{hi} subset is heterogeneous, with only a fraction of these cells expressing other markers characteristic of T_{CM} cells, including IL-15R β , bcl-2, or CD62L. The phenotypic heterogeneity of this population suggests that these cells have distinct histories of antigen encounter and/or may be at different points along a differentiation pathway leading to the acquisition of a stable T_{CM} phenotype. In support of this hypothesis, IL-7R α ^{lo} CD8+ T cells from chronically infected mice re-express IL-7R α following removal from antigen, suggesting that these cells may also be capable of attaining characteristics similar to long-lived T_{CM} memory cells. Interestingly, IL-7R α ^{hi} and IL-7R α ^{lo} donor CD8+ T cells rested in naïve mice for 20 days were capable of rapid expansion in response to challenge infection, indicating their recall potential was not impaired. Whether the IL-7R α ^{lo} donor population present following challenge represents a residual IL-7R α ^{lo} population or a

subset of cells that converted to an IL-7R α^{hi} phenotype prior to challenge remains to be determined.

Thus, these results suggest that IL-7R α^{hi} CD8⁺ T cells from chronically infected mice likely represent a population of cells that have not recently encountered antigen and consequently may be in the process of developing characteristics similar to those observed for CD8⁺ T_{CM} cells in models of acute resolving infections. This population may therefore provide a pool of stable memory cells that is more amenable to manipulation and that would be preserved in situations where *T. cruzi* is cleared – such as following successful drug treatment. Further experiments are needed to determine if this IL-7R α^{hi} population is capable of acquiring additional T_{CM} characteristics, such as the re-expression of CD62L and a more rapid recall to antigen compared to T_{EM} cells.

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