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TGF-β regulates pathology but not tissue CD8$^+$ T cell dysfunction during experimental *Trypanosoma cruzi* infection

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Infection with the protozoan parasite *Trypanosoma cruzi* leads to chronic infection, with parasite persistence primarily in muscle tissue. CD8$^+$ T cells isolated from muscle tissue of *T. cruzi*-infected mice display decreased production of IFN-γ in response to T cell receptor engagement. The expression of TGF-β at the site of CD8$^+$ T cell dysfunction and parasite persistence suggested that this immunoregulatory cytokine might play a role in these processes. Mice expressing a T cell-specific dominant negative TGF-β receptor type II (DNRII) were therefore infected with *T. cruzi*. Infection of DNRII mice resulted in massive CD8$^+$ T cell proliferation, leading to increased numbers but decreased frequencies of antigen-specific CD8$^+$ T cells in the spleen compared to wild-type mice. However, TGF-β unresponsiveness failed to restore effector functions of CD8$^+$ T cells isolated from muscle tissue. Histological examination of skeletal muscle from *T. cruzi*-infected DNRII mice revealed an extensive cellular infiltrate, and DNRII mice displayed higher susceptibility to infection. Overall, while TGF-β does not appear to be responsible for CD8$^+$ T cell unresponsiveness in peripheral tissue in *T. cruzi*-infected mice, these data suggest a role for TGF-β in control of immunopathology in response to *T. cruzi* infection.

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Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease, which is characterized by parasite persistence in muscle tissue, resulting in inflammation and degeneration at the sites of parasite persistence. Approximately 30% of *T. cruzi*-infected individuals develop disease, which typically presents as cardiomyopathy and mega-syndrome. CD8$^+$ T cells [1] and IFN-γ [2, 3] are critical for control of infection in mice. CD8$^+$ T cells isolated from skeletal muscle tissue of C57BL/6 (B6) mice infected with the Brazil strain of *T. cruzi* produce IFN-γ at far lower frequencies than their splenic counterparts [4]. The cause of this apparent lymphocyte dysfunction

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Abbreviations: **B6:** C57BL/6 · **DNRII:** dominant-negative TGF-β type II receptor · **dpi:** days post-infection · **FoxP3:** Forkhead box P3 · **SC:** spleen cells · **TCT:** trypomastigotes

in the muscle of *T. cruzi*-infected mice has not been established; however, neither a lack of costimulation nor the induction of anergy appears to play a role [4]. TGF-β1 (TGF-β) is a major negative regulator of immune responses [5] and is readily detected in muscle tissue of mice infected with *T. cruzi* [6]. The presence of TGF-β at the site of $CD8^+$ T cell dysfunction led us to test the hypothesis that TGF-β inhibits functional responses of tissue-dwelling lymphocytes during experimental *T. cruzi* infection.

TGF-β is the epitome of the multi-functional cytokine, exerting a wide range of effector functions over the life span of an animal. TGF-β plays a critical role in collagen formation and extracellular matrix deposition in addition to its role in down-regulating T cell responses [5]. Consequently, TGF-$\beta^{-/-}$ mice exhibit approximately 50% embryonic lethality and live-born TGF-$\beta^{-/-}$ mice develop multiorgan inflammation, primarily in the heart and lung, and die within 2–3 weeks of birth [7]. The early lethality of TGF-$\beta^{-/-}$ mice makes this an unsuitable model in which to study infectious diseases. The role of TGF-β in regulating T cell responses has recently been studied using mouse strains expressing a dominant negative TGF-β type II receptor (DNRII) under a CD2 promoter, selectively rendering T cells unresponsive to TGF-β [8]. DNRII mice exhibit significant proliferation of $CD8^+$ T cells over time, establishing a role for TGF-β in maintaining $CD8^+$ T cell homeostasis [8].

In the studies described here, the ability of DNRII T cells to respond to *T. cruzi* infection was determined using transfer of DNRII spleen cells (SC) as well as direct infection of DNRII mice. DNRII $CD8^+$ T cells expanded extensively following *T. cruzi* infection, becoming the dominant lymphocyte population during chronic infection. However, a lower frequency of DNRII $CD8^+$ T cells isolated from muscle tissue of chronically infected mice produced IFN-γ than their splenic counterparts, indicating that TGF-β is not responsible for the lower functional responses of muscle-derived $CD8^+$ T cells during experimental *T. cruzi* infection.

Results

Increased mortality in *T. cruzi*-infected DNRII mice

In order to determine the effect of TGF-β on T cell responses during experimental *T. cruzi* infection, wild-type (B6) and DNRII mice were infected with 1000 Brazil strain trypomastigotes. B6 mice infected in this way control the infection but have persistent parasitization of skeletal muscle [9]. In contrast, DNRII mice exhibited increased mortality; by the late acute phase of infection (35–50 days post-infection, dpi), 30% of DNRII mice had succumbed to infection (Fig. 1). In total, DNRII mice exhibited 50% mortality (25/50 total mice) by 200 days after infection. To determine whether the increased mortality of DNRII mice was due to increased parasite load, quantitative real-time PCR was performed on skeletal muscle tissue from infected B6 and DNRII mice. Analysis of parasite DNA in muscle failed to detect any differences in tissue parasitism between B6 and DNRII mice (data not shown).

Extensive $CD8^+$ T cell expansion in *T. cruzi*-infected DNRII mice

T. cruzi-infected DNRII mice had approximately twice the number of splenic lymphocytes as infected B6 mice in both the acute (Fig. 2A) and chronic (Fig. 2B) phase of infection. The lymphocytic composition of the spleen varied between infected DNRII and B6 mice as well. Notably, infected DNRII mice exhibited a significant increase in the T:B cell ratio as early as 30 days after infection (Fig. 2C) and becoming much more pronounced in chronically infected mice (Fig. 2D). B cells comprised approximately 55–60% of splenic lymphocytes in infected B6 mice, but less than 20% of splenic lymphocytes in infected DNRII mice (Fig. 2D). The alteration in the T:B cell ratio in chronic DNRII mice was due primarily to $CD8^+$ T cell expansion, as nearly 70% of total splenic lymphocytes in chronically infected DNRII mice were $CD8^+$ compared to approximately 20% in chronically infected B6 mice (Fig. 2D). The inability of T cells to respond to TGF-β in DNRII mice resulted in an increased proportion of $CD8^+$ T cells over time even in the absence of infection (Fig. 2D and [8]); however, *T. cruzi* infection of DNRII mice induced an even greater degree of $CD8^+$ T cell expansion (Fig. 2D).

The effect of TGF-β on priming of T cells was determined by comparing the proliferation of adoptively transferred naive CFSE-labeled B6 and DNRII T cells during acute infection. Both DNRII $CD8^+$ and $CD4^+$ T cells proliferated much more extensively following

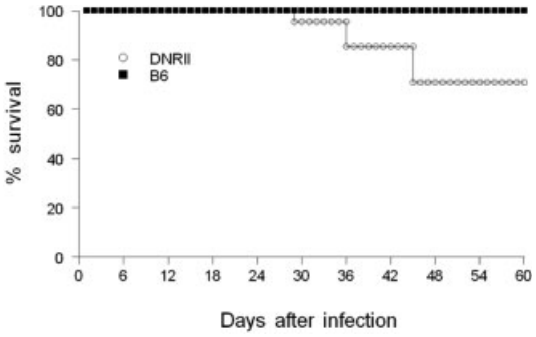

Figure 1. DNRII mice are more susceptible to *T. cruzi* infection. Mortality of mice infected with *T. cruzi* was recorded. Data are representative of greater than 25 mice per group.

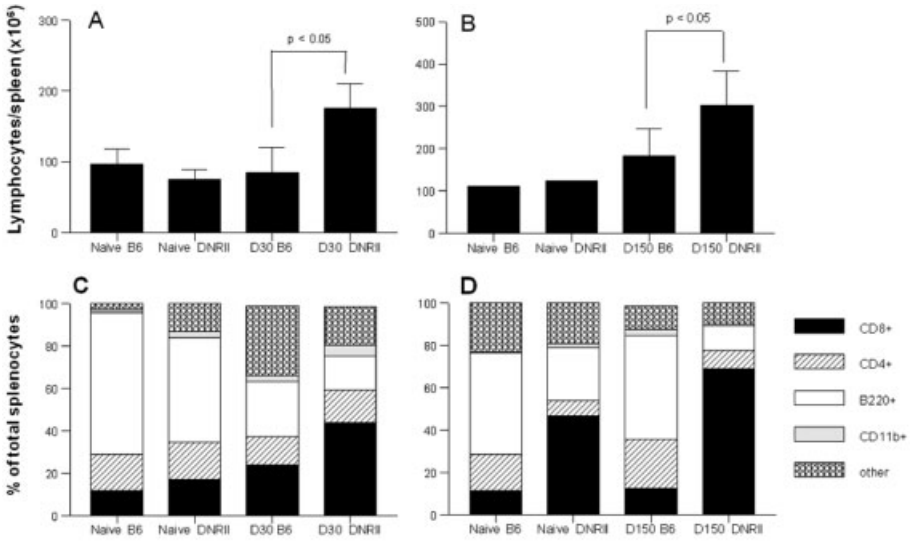

Figure 2. DNRII mice exhibit increased splenic cellularity and CD8$^+$ T cell hyperproliferation. Mice were infected i.p. with *T. cruzi* and examined at 150 days post-infection (dpi). (A and B) Number of non-RBC splenocytes from mice infected for 30 days (A) or 150 days (B); $n = 2$–5 mice/group. Bars represent SD. (C and D) Major leukocyte populations are presented as percent of all live-gated splenocytes for mice infected for 30 days (C) or 150 days (D). Age-matched uninfected mice are shown for each group. For comparison of CD8$^+$ T cell frequencies in infected B6 and infected DNRII, $p = 0.0004$. Data are representative of three to four experiments.

T. cruzi infection of recipient mice than did WT B6 CD8$^+$ T cells. The majority of B6 CD8$^+$ or CD4$^+$ T cells had not divided at 14 dpi (Fig. 3). In contrast, less than 25% of DNRII T cells had not divided at least once at the same time point (Fig. 3), demonstrating that TGF-β is a crucial regulator of T cell expansion following *T. cruzi* infection. Although both CD4$^+$ and CD8$^+$ DNRII T cells proliferated more extensively than WT T cells in response to primary *T. cruzi* infection, CD8$^+$ T cell expansion outpaced that of CD4$^+$ T cells in the absence of TGF-β signaling, resulting in CD8$^+$ T cells becoming the dominant lymphoid population in chronically infected DNRII mice (Fig. 2B), similar to what is observed in uninfected DNRII mice.

Antigen-specific responses in *T. cruzi*-infected DNRII mice

Because CD8$^+$ T cells comprised a higher percentage of lymphocytes in *T. cruzi*-infected DNRII mice compared to either uninfected DNRII or infected B6 mice, we sought to determine whether the higher frequency of CD8$^+$ T cells represented generation of higher frequencies of *T. cruzi*-specific CD8$^+$ T cells in DNRII mice. *T. cruzi* peptide-specific CD8$^+$ T cell responses were examined by IFN-γ ELISPOT assay and staining with MHC I-peptide tetramers using previously described immunodominant *T. cruzi* epitopes [10]. SC from chronically infected DNRII mice had lower frequencies of IFN-γ-producing cells in response to peptide stimulation than did SC from infected B6 mice (Fig. 4A, $p < 0.05$ for each peptide tested). Frequencies of CD8$^+$ T cells specific for the dominant epitope TSKB20 were equivalent in acutely infected B6 and DNRII mice (Fig. 4B, $p > 0.05$ at each time point), but chronically infected DNRII mice contained far lower frequencies of TSKB20-specific CD8$^+$ T cells than infected B6 mice (Fig. 4C, top number, $p = 0.03$). However, taking into account the significant increase in spleen size and frequency of CD8$^+$ T cells in the spleen, an increase in the total number of TSKB20-specific CD8$^+$ T cells was observed in spleens of infected DNRII mice compared to

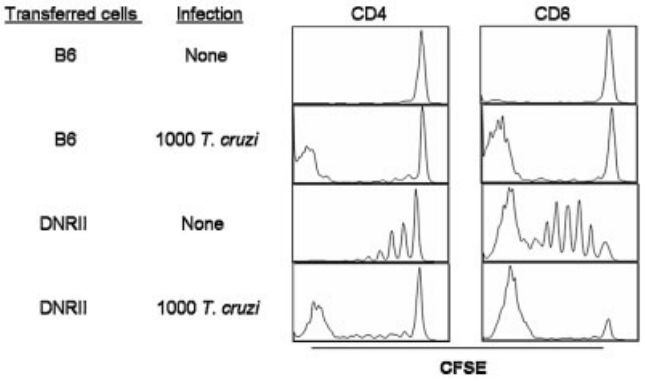

Figure 3. TGF–β inhibits T cell proliferation in response to *T. cruzi* infection. SC from naive B6 or DNRII mice were labeled with CFSE and then transferred to congeneic mice. The next day, recipient mice were infected i.p. with *T. cruzi* and T cell proliferation was measured by CFSE dilution in the transferred CD8$^+$ (right) and CD4$^+$ (left) T cell populations at 14 dpi. Data are representative of two experiments.

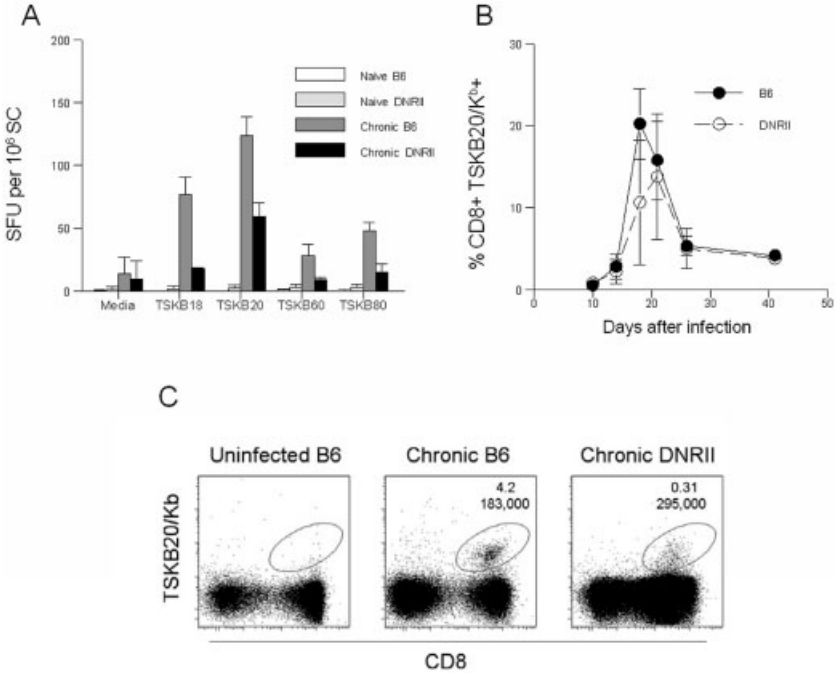

Figure 4. Increased number but decreased frequency of Ag-specific CD8$^+$ T cells in DNRII mice. (A) Splenocytes from chronically infected mice (150 dpi) were stimulated overnight with the respective peptides in anti-IFN-γ coated ELISPOT plates. The number of IFN-γ spot-forming units (SFU) per 100 000 splenocytes is presented. Data are representative of two experiments with three mice per group. (B) Blood from infected mice was obtained and stained with TSKB20/K^b tetramers at the indicated days post-infection. (C) SC from chronically infected mice (150 dpi) were stained with TSKB20/K^b tetramers, and the frequencies of CD8$^+$ TSKB20/K^{b+} (top number) and total TSKB20-specific cells (bottom number) in the spleen were calculated. Data are representative of four chronic DNRII and six chronic B6 mice.

infected B6 mice (Fig. 4C, bottom number $p = 0.05$). Therefore, larger numbers of TSKB20-specific CD8$^+$ T cells were generated following infection of DNRII mice than B6 mice, but TSKB20-specific CD8$^+$ T cells represent a lower proportion of the total CD8$^+$ T cell population.

Limited role of TGF-β in depressed muscle lymphocyte responses

The well-documented regulatory activities of TGF-β and the previous documentation of TGF-β expression in muscle from *T. cruzi*-infected mice prompted us to examine if this cytokine was responsible for the decreased responsiveness of muscle-derived CD8$^+$ T cells. To this end, splenic and muscle-derived CD8$^+$ T cells were assessed for the ability to elaborate IFN-γ in response to polyclonal stimulation. Muscle CD8$^+$ T cells from DNRII mice produced little IFN-γ upon anti-CD3 stimulation (Fig. 5), demonstrating that TGF-β plays a minor role in CD8$^+$ T cell dysfunction in the muscle. In contrast, splenic CD8$^+$ T cells from chronically infected B6 or DNRII mice produced IFN-γ at similar frequencies in response to anti-CD3 stimulation (Fig. 5). CD8$^+$ DNRII T cells are therefore unimpaired in their overall ability to respond to polyclonal stimulation, but TGF-β unresponsiveness does not restore the functional capacity of muscle CD8$^+$ T cells from *T. cruzi*-infected mice.

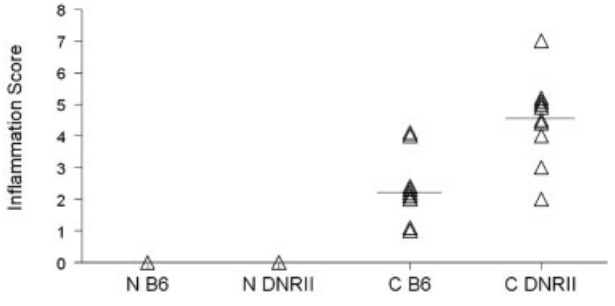

Figure 5. TGF-β responsiveness does not alter CD8$^+$ T-cell derived IFN-γ production following polyclonal stimulation. SC and muscle-derived lymphocytes from mice infected for at least 60 days were stimulated with anti-CD3 overnight and assessed for intracellular IFN-γ accumulation. Each data point represents the percent of CD8$^+$ T cells producing IFN-γ from a single mouse; bars represent the median. Less than 1% of CD8$^+$ T cells produced IFN-γ when unstimulated. N, naïve; C, chronically infected.

T. cruzi-infected DNRII mice display more severe tissue inflammation than B6 mice

Inflammatory foci are observed at the sites of parasites persistence in infected humans and in murine models of *T. cruzi* infection [11, 12]. To determine whether T cell unresponsiveness to TGF-β enhanced tissue inflammation following *T. cruzi* infection, chronically infected DNRII and B6 mice were examined for lymphocytic infiltrate at the site of parasite persistence. Inflammation in muscle of infected DNRII mice was much more pronounced than in infected B6 mice (Fig. 6A and B). Inflammation in DNRII mice was directly related to the infection, as aged uninfected DNRII mice did not exhibit any tissue inflammation (Fig. 6A).

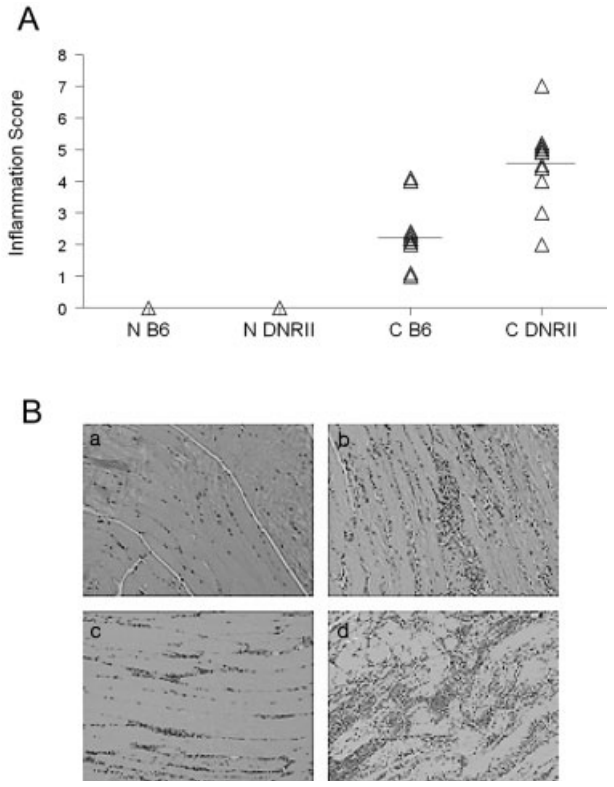

Figure 6. Lack of T cell responsiveness to TGF-β signaling results in increased tissue pathology. (A) *T. cruzi*-infected DNRII mice exhibit increased tissue inflammation compared to wild-type mice. Skeletal muscle from chronically (150 dpi) infected B6 and DNRII mice were fixed in 10% formalin, sectioned and stained with H&E, and analyzed for tissue infiltrating lymphocytes. Data points represent individual mice from multiple experiments. (B) Representative histological sections from: acutely infected B6 (a), acutely infected DNRII (b), chronically infected B6 (c), and chronically infected DNRII (d) mice. N, naïve; C, chronically infected.

Discussion

The studies reported here demonstrate that while TGF-β does not contribute to $CD8^+$ T cell unresponsiveness in muscle tissue of *T. cruzi*-infected mice, TGF-β regulates $CD8^+$ T cell expansion and protects against immunopathology following experimental *T. cruzi* infection. Immune-mediated clearance of *T. cruzi* is typically incomplete and parasites persist indefinitely in muscle tissue [9]. Muscle-derived $CD8^+$ T cells from mice with chronic *T. cruzi* infection fail to produce significant levels of IFN-γ upon polyclonal stimulation, suggesting either an inherent dysfunction in muscle-dwelling lymphocytes or the presence of a suppressive factor in muscle [4]. The expression of the immunoregulatory cytokine TGF-β in muscle of *T. cruzi* infected mice [13] suggested a link with the lack of functional response of muscle-derived $CD8^+$ T cells. Recent evidence has shown that TGF-β treatment of memory $CD8^+$ T cells derived from secondary mixed lymphocyte cultures induces an inert state that, like the tissue-derived $CD8^+$ T cells from *T. cruzi*-infected mice, is unable to be rescued by IL-2 treatment and is therefore not classically anergic [14]. However, the data presented here demonstrate that $CD8^+$ T cells isolated from muscle tissue of DNRII mice produced IFN-γ at lower frequencies than splenic $CD8^+$ T cells from the same mice. TGF-β production therefore cannot completely account for the lower responsiveness of muscle-derived $CD8^+$ T cells.

$CD8^+$ T cell-specific blockade of TGF-β signaling has rarely been studied in models of infectious disease. Instead, the effects of TGF-β on pathogen-specific $CD8^+$ T cell function have typically been studied *in vitro* or by treatment of mice with TGF-β. While TGF-β has a number of T cell inhibitory activities, TGF-β has a protective effect in mice infected with *Listeria monocytogenes* [15], *Candida albicans* [16], or *Cryptococcus neoformans* [17]. In the latter case, TGF-β-mediated protection was associated with increased production of lysozyme in macrophages [17]. However, early production of TGF-β negatively associates with disease outcome in some models of protozoan infection [18, 19], and treatment of *T. cruzi*-infected mice with TGF-β results in increased mortality and blocks the protective effects of IFN-γ treatment [20]. We chose to use DNRII mice in these studies to isolate the effects of TGF-β on T cells and avoid the potential complication of interfering with TGF-β-mediated entry of *T. cruzi* into host cells [21] or other non-T cell-mediated effects of universal TGF-β blockade.

One source of TGF-β is regulatory T cells (Tregs), and while the exact relationship between Tregs and TGF-β is still unclear [22, 23], there is a large body of data demonstrating that Tregs exert their regulatory effects at least in part through TGF-β [24–28]. Our recent studies failed to define a role for regulatory T cells in regulating $CD8^+$ T cell function in muscle of *T. cruzi*-infected mice as determined by depletion (or functional inactivation [29]) of Tregs using PC61 anti-CD25 Ab [30]. The lack of observed effects of TGF-β on muscle $CD8^+$ T cell dysfunction lends further support to the conclusion that Tregs are of little consequence in the poor responsiveness of muscle $CD8^+$ T cells from *T. cruzi*-infected mice to TCR engagement. TGF-β is also critical for the development of the newly described T_H17 subset of effector $CD4^+$ T cells [31, 32]. Since mice lacking CD4-specific TGF- β signaling do not develop T_H17 cells [33], our results suggest that T_H17 T cells are also not contributing to inflammation in the mouse model of *T. cruzi* infection used in the current studies.

Given the results that neither TGF- β nor Treg cells appear to regulate $CD8^+$ T cell function in the muscle of *T. cruzi*-infected mice, the question remains as to why these muscle-homing T cells exhibit depressed effector

function. One possibility is that there is in fact no active regulation of T cell responses occurring in the muscle and instead the relative inability to stimulate these tissue-homing T cells is one sign of their prior encounter with antigen in the muscle tissue. In support of this hypothesis, a substantial frequency of muscle-derived $CD8^+$ T cells expresses the activation markers CD69 and PD-1 (Collins and Tarleton, unpublished data). We currently favor the hypothesis that *T. cruzi*-specific $CD8^+$ T cells are activated upon entering the infected muscle tissue, exert effector function, and then lose their ability to further respond and/or undergo apoptosis. The muscle environment itself may also facilitate the regulation of effector $CD8^+$ T cells [4] in this environment where over-exuberant inflammation may be life threatening.

Previous studies have suggested that TGF-β directly acts on $CD8^+$ T cells to preferentially inhibit clonal expansion of Ag-specific $CD8^+$ T cells rather than polyclonal or third party responses [14]. $CD8^+$ T cells recognizing high affinity peptides in a pro-inflammatory environment are particularly susceptible to the effects of TGF-β [34]. However, in the current studies $CD8^+$ T cells comprised 80% of the splenic lymphocyte compartment in *T. cruzi*-infected DNRII mice with no selective expansion of the dominant TSKB20-specific $CD8^+$ T cell population. These data reflect the preferential inhibition of homeostatic $CD8^+$ T cell proliferation by TGF-β in aged DNRII mice [8]. While expansion of very low frequency $CD8^+$ T cells recognizing yet unidentified *T.cruzi* peptides in infected DNRII mice may also occur, it seems unlikely that this would result in $CD8^+$ T cells becoming the majority lymphocyte population in these mice. Instead, the data presented here raise the possibility that TGF-β does not selectively inhibit clonal expansion, but may help focus infection-induced $CD8^+$ T cell expansion by controlling third party activation. This third party control may be especially critical during infections such as *T. cruzi* in which development of Ag-specific $CD8^+$ T cell responses occur over weeks rather than hours or days. The increased mortality and tissue pathology of *T. cruzi*-infected DNRII mice further suggests that TGF-β-mediated focusing of the T cell response on Ag-specific T cells correlates with more efficient control of infection.

Materials and methods

Mice and parasites

DNRII heterozygous mice were obtained from the National Cancer Institute (Bethesda, MD) and bred in the University of Georgia animal facility in microisolator cages under specific pathogen-free conditions. All DNRII $^+/^+$ and DNRII$^{-/-}$ used in experiments were littermates. B6.SJL mice were obtained from Jackson Labs (Bar Harbor, ME). Tissue culture trypomastigotes (TCT) of the Brazil strain of *T. cruzi* were obtained from passage through Vero cells. Mice were infected intraperitoneally with 1000 Brazil strain TCT and were sacrificed by CO_2 inhalation. All animal studies were conducted in compliance with the University of Georgia Animal Care and Use Committee. No human subjects were used in these studies.

In vitro stimulation of lymphocytes

Single-cell suspensions of splenocytes from uninfected or *T. cruzi*-infected mice were generated as previously described [35]. Muscle-derived lymphocytes were obtained by teasing tissues apart and vigorously pushing through a 40-μm nylon mesh screen, followed by extensive washing with RPMI-10. Lymphocytes were cultured 16–20 h at 2×10^6/mL in 48-well plates with 5 μM of a *T. cruzi* peptide mix. Brefeldin A (BFA, BD PharMingen, San Diego, CA) was added for the final 4 h of culture to block protein secretion.

Surface staining of splenocytes

Single-cell suspensions of SC were washed in staining buffer (2% BSA, 0.02% azide in PBS, PAB) incubated for 15 min in FcBlock (anti-CD16/CD32, PharMingen), then incubated with the appropriate labeled antibodies: anti-CD8 FITC, anti-B220 PE, and anti-CD4 PerCP, and anti-CD11b APC (all PharMingen). Cells were stained for 30 min at 4°C in the dark, washed twice in PAB, fixed in 2% formaldehyde, acquired on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using FlowJo (Tree Star, Ashland OR).

Intracellular cytokine staining

In vitro-stimulated splenocytes were assayed for intracellular IFN-γ levels using the Cytofix/Cytoperm intracellular staining kit (PharMingen). Cells were washed in PAB, incubated for 15 min on ice in FcBlock (anti-CD16/32, PharMingen), and stained for surface expression of CD8 using anti-CD8 PE (PharMingen). The cells were fixed and permeabilized using Cytofix/Cytoperm (PharMingen) on ice for 15 min, then washed in PermWash (PharMingen). The cells were then stained with anti-IFN-γ APC (PharMingen) for 20 min on ice. Cells were washed and fixed in 2% formaldehyde for >20 min at 4°C, then washed and resuspended in PAB for flow cytometric analysis.

MHC I tetramer staining

MHC I tetramers were synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA). TSKB20 (ANYKFTLV/K^b) tetramers in PAB were added to mouse splenocytes, incubated 15 min at 37°C, then co-stained with labeled anti-CD8 antibodies, as well as anti-CD4, anti-CD11b, and anti-B220 for use as a dump channel for 30 min an 4 C. In some experiments, mice were infected then serially bled from the retro-orbital cavity, and whole blood was stained with MHC I-peptide tetramers and antibodies. RBC were lysed with 0.83% ammonium chloride solution.

CFSE labeling and adoptive transfer

Single cell suspensions of naive B6 or DNRII SC were washed twice in PBS then incubated for 3 min in 5 μM CFSE. CFSE was quenched with serum, followed by two washes in 10% serum. 2×10^7 cells were transferred to naïve B6.SJL mice, some of which were infected with 1000 Brazil TCT 24 h later. Fourteen days post-infection, mice were sacrificed and cell division analyzed by examining CFSE dilution in the $CD45.1^{neg}CD8^+$ population.

ELISPOT

ELISPOT assays to measure secretion of IFN-γ from peptide-stimulated mouse splenocytes were performed as per manufacturer's instructions (BD PharMingen). Briefly, M200 plates were coated with anti-mouse IFN-γ. After blocking, SC were incubated overnight with 1 μM of *T. cruzi*-derived peptides [10]. Secreted IFN-γ was detected using a secondary biotinylated anti-IFN-γ Ab, followed by SAv-HRP and colorimetric detection. Spots were counted using an Immunospot analyzer (CTL, Cleveland, OH).

Histology

Skeletal muscle was obtained from *T. cruzi*-infected mice and controls, fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were obtained and stained with hematoxylin-eosin. A single-blind microscopic evaluation of the tissue sections was performed on pre-coded slides. The presence and number of parasitized cells per section were recorded. Inflammation was evaluated qualitatively according to the presence or absence of myocyte necrosis and polymorphonuclear leukocytes in the infiltrates (active or chronic inflammation, respectively, [36]), and semi-quantitatively on low-power microscopic examination according to distribution (focal, confluent or diffuse, [37]) and extent of inflammatory cells (1^+ for a single inflammatory foci, 2^+ multiple, non-confluent foci of inflammatory infiltrate, 3^+ for confluent inflammation, and 4^+ for diffuse inflammation extended through the section [12]). Scores from two sections were summed to give the inflammatory score. Images of muscle tissue were taken on an Olympus BX40F-3 camera (Olympus, Center Valley, PA) using a 10X objective.

Statistical analysis

Student's *t*-test (two-tailed, type 2) was used in analysis of comparison of means. Kaplan-Meier survival curves were used to analyze mortality. The p values <0.05 were considered statistically significant.

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