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Drug-induced cure drives conversion to a stable and protective CD8⁺ T central memory response in chronic Chagas disease

Juan M Bustamante¹, Lisa M Bixby^{1,2}, and Rick L Tarleton^{1,2}

¹Center for Tropical and Emerging Global Diseases, Coverdell Center, 500 D.W. Brooks Drive, University of Georgia, Athens, Georgia 30602, USA.

²Department of Cellular Biology, Coverdell Center, 500 D.W. Brooks Drive, University of Georgia, Athens, Georgia 30602, USA.

Abstract

In this study, we document the development of stable, antigen-independent CD8⁺ T cell memory after drug-induced cure of a chronic infection. By establishing a system for drug cure of chronic *Trypanosoma cruzi* infection, we present the first extensively documented case of total parasite clearance after drug treatment of this infection. Cure resulted in the emergence of a stable, parasite-specific CD8⁺ T cell population with the characteristics of central memory cells, based upon expression of CD62L, CCR7, CD127, CD122, Bcl-2 and a reduced immediate *in vivo* CTL function. CD8⁺ T cells from treated and cured mice also expanded more rapidly and provided greater protection following challenge than those from chronically infected mice. These results show that complete pathogen clearance results in stable, antigen-independent and protective T cell memory, despite the potentially exhausting effects of prior long-term exposure to antigen in this chronic infection.

T cell memory involves the retention, after antigen clearance, of long-lived T cells that have the potential for rapid and highly effective control of re-encountered pathogens. Classically, memory T cells upregulate antiapoptotic molecules that promote their survival^{1–3} and express receptors for the homeostatic cytokines interleukin-7 (IL-7) and IL-15 (refs. 4–7), which allow for their maintenance independently of the presence of antigen. In addition, memory T cells differentially express the homing receptors CD62L and CCR7, which provide the means for trafficking to lymphoid or nonlymphoid tissues and serve as phenotypic markers for classifying this heterogeneous population into T central memory cells (T_{CM} cells; CD62L^{hi}, CCR7^{hi} and lymphoid homing) and T effector memory cells (T_{EM} cells; CD62L^{lo}, CCR7^{lo} and peripheral tissue homing). The maintenance of long-lived T cells in cases in which pathogens, and thus antigens, are not completely cleared is less well defined. In many persistent infections, lymphocytes with the characteristics of T_{CM} cells and T_{EM} cells are clearly present, suggesting their occasional or even rare encounter with antigen, the recruitment of newly generated memory cells from the naive T cell pool, or both^{8–10}. Nevertheless, persistent infections in mice^{2,11} and humans^{12–15} are often characterized by varying degrees of functional impairment of pathogen-specific T cell

Correspondence should be addressed to R.L.T. (Tarleton@cb.uga.edu).

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AUTHOR CONTRIBUTIONS

J.M.B. designed and performed experiments and wrote the manuscript, L.M.B. performed experiments and assisted in writing of the manuscript and R.L.T. designed experiments and wrote the manuscript.

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responses, presumably the result of exhaustion of the T cells under conditions of constant restimulation. In some of these cases, the effects of exhaustion can be reversed by blocking negative regulators of effector T cells, such as IL-10 and PD-1 or PD-L1 (refs. 13,16,17). Substantial evidence supports the conclusion that maintenance of CD8⁺ cells in chronic infections is antigen dependent^{2,18}. But what happens to T cell memory when antigen is removed after an extended chronic infection? To date, no studies have addressed the question of whether or not the T cell exhaustion and antigen-dependence that is potentially engendered by long-term chronic infections could be reversible after antigen clearance. This is a key question in basic immunology, in terms of how T cell responses, and, ultimately, memory, are maintained, if at all, in the presence of persistent antigen. This information is also relevant to the potential for and consequences of curative treatment of many human infections, including those caused by persistent viral and parasitic pathogens.

In this work, we make use of the protozoan pathogen *Trypanosoma cruzi* to evaluate the effect of chronic infection on the immune system's ability to generate stable T cell memory after pathogen clearance. *T. cruzi* is the etiological agent of Chagas disease, a major human health problem in the Americas. The infection is chronic in essentially all of the many mammalian species that the protozoan infects, and approximately 30% of infected humans develop the clinical manifestations of Chagas disease^{19,20}. Although adaptive immune responses control parasite numbers and largely prevent acute-phase mortality, these responses are insufficient to completely clear the infection, leading to parasite persistence in muscle and other tissues. CD8⁺ T cells are an indispensable component of the immune control of this intracellular pathogen²¹⁻²³, and our previous studies have demonstrated that the majority of *T. cruzi*-specific CD8⁺ T cells in chronically infected mice have a T effector (T_{eff}) or T_{EM} phenotype and are dependent on antigen for their maintenance^{24,25} (L.M.B. and R.L.T., unpublished data). In decades-long infections in humans, the degree of severity of Chagas disease has been associated with a decrease in the frequency of *T. cruzi*-specific T cells and changes in the maturational status of the CD8⁺ T cell population^{26,27}.

In this study, we used an experimental mouse model of *T. cruzi* infection to test the effectiveness of benznidazole to clear parasites from infected hosts and to determine the effect of pathogen clearance on chronically stimulated CD8⁺ T cells. Benznidazole (*N*-benzyl-2-nitroimidazole acetamide) is the principal drug available for the treatment of *T. cruzi* infection, although the substantial potential side effects and largely unknown efficacy have limited its use in individuals with chronic infection²⁸. Herein we provide the first evidence, to our knowledge, that appropriate benznidazole treatment results in complete parasite and antigen clearance. Furthermore, we use this treatment protocol to show that cure of this chronic infection results in a marked shift to a stable, *T. cruzi*-specific CD8⁺ T cell population with a T_{CM} cell phenotype that responds more vigorously to and provides enhanced protection against challenge infection as compared to T_{eff} or T_{EM} cells phenotype population present in chronically infected mice. In addition and in contrast to what has been described in other chronic infection models^{12,13,29-31}, substantial exhaustion of pathogen-specific CD8⁺ T cells is not observed despite their long-term exposure to parasite antigens.

RESULTS

Clearance of *T. cruzi* after benznidazole treatment

Infection of C57BL/6 mice with the CL strain of *T. cruzi* results in an acute phase parasitemia that is controlled and that becomes undetectable by approximately 35 d after infection (Fig. 1a). A single 20-d course of benznidazole treatment begun on day 15 post-infection suppressed parasitemia but did not clear the infection, as determined by the reappearance of patent parasitemia after immunosuppression with cyclophosphamide (data not shown). Therefore, a subset of the benznidazole-treated mice (that had not been

cyclophosphamide-suppressed) was submitted to a second round of benznidazole treatment at days 150–170 after infection (Fig. 1a). Blood analysis after cyclophosphamide-induced immuno-suppression 250–260 d post-infection did not reveal parasites in these twice–benznidazole-treated mice but readily showed parasitemias in the chronically infected mice not previously treated with benznidazole. Furthermore, cyclophosphamide-suppressed chronically-infected mice rapidly succumbed to overwhelming parasite loads within 14 d after the beginning of immunosuppressive treatment, whereas mice treated with 2 courses of benznidazole remained healthy and apparently parasite free (data not shown).

To further document the complete clearance of parasites from treated and cured mice, blood from mice immunosuppressed at 265 d after infection was transferred into highly susceptible interferon- γ (IFN- γ)-knockout mice and SCID mice. Previous experiments had shown that both of these immunodeficient strains developed lethal infections after exposure to a single *T. cruzi* parasite (J.M.B., unpublished data). Immunodeficient mice receiving blood from cyclophosphamide-immunosuppressed benznidazole-treated mice did not develop detectable parasitemias and showed no histological evidence of infection or disease (data not shown). In contrast, mice that received blood from cyclophosphamide-immunosuppressed chronically infected (not benznidazole-treated) mice died between 13 and 15 d post–blood transfer with very high parasitemias (data not shown). As previous studies have established that parasites preferentially persist in the skeletal muscle of chronically infected C57BL/6 mice³², we also measured the amount of parasite DNA in skeletal muscle of untreated and benznidazole-treated mice by quantitative PCR. Parasite DNA was undetectable in skeletal muscle from treated and cured mice but was present in untreated mice (Fig. 1b). Cure in mice treated with two courses of benznidazole was also supported by the absence of histological evidence of the infection or disease that is common in chronically infected mice (Fig. 1c). Collectively, these results conclusively show that a two-time 20-d benznidazole treatment regimen effectively cures mice of *T. cruzi* infection. Additional experiments have shown that a single 40-d course of benznidazole treatment during either the acute or chronic phases of infection also provides cure, as assessed with these same criteria (see below).

Emergence of T_{CM} cells after benznidazole treatment

We have previously shown that CD8⁺ T cell responses to *T. cruzi* in C57BL/6 mice are heavily dominated by cells specific for two sets of peptides encoded by the trans-sialidase gene family, the immunodominant TSKB20 and the subdominant TSKB74 epitopes²⁵. CD8⁺ T cells specific for both epitopes were detected in the blood, spleen and lymph nodes of untreated chronic and treated cured mice, although the frequency of *T. cruzi*-specific cells was consistently two- to threefold higher in untreated mice (Fig. 2). *T. cruzi*-specific CD8⁺ T cells from both treated and untreated mice expressed high levels of the surface markers CD44 and CD11a (Fig. 2c), indicating prior antigen experience³³, but did not show evidence of recent activation, as only a minor population expressed either CD25 or CD69 (Fig. 2c). As shown previously^{24,25}, the *T. cruzi*-specific CD8⁺ T cells in long-term persistently infected mice consistently showed a T_{eff} or T_{EM} phenotype (CD62L^{lo}, CD122^{lo} and CD127^{lo}) (Fig. 2c). In contrast, the population of TSKB20-specific CD8⁺ T cells in the cured mice was predominantly CD62L^{hi}, CD122^{hi} and CD127^{hi}, indicative of a shift to a T_{CM} phenotype after antigen clearance (Fig. 2c). The identification of the memory population as classical T_{CM} cells is further supported by the expression of the lymphoid-homing receptor CCR7 on the majority of TSKB20-specific CD8⁺ T cells in treated and cured mice. Cure after benznidazole treatment also resulted in increased expression of the antiapoptotic molecule Bcl-2 in the TSKB20-specific CD8⁺ T cells and a decreased frequency of cells expressing KLRG1, a marker of repeated antigen stimulation^{10,34}. The clear shift in phenotype from a T_{eff} or T_{EM} cell population to a T_{CM} cell population after curative benznidazole treatment indicates that clearance of infection, even after prolonged

exposure to antigen, can result in development and maintenance of a high-frequency, pathogen-specific T_{CM} cell population. The relative predominance of T_{CM} cells in cured mice was still apparent nearly 300 d post-treatment (460 d after infection; Fig. 2d,e), indicating that this is a stable, long-term memory T cell population that persists in the absence of antigen. Additionally, the substantial change in phenotype of *T. cruzi*-specific CD8⁺ T cells after benznidazole treatment provides additional immunological proof that the benznidazole treatment regimen has rendered these mice parasite free.

Homeostatic maintenance of CD8⁺ T cells in cured mice

T. cruzi-specific CD8⁺ T cells are crucial to the maintenance of tight control of persisting parasites in this chronic infection^{21,23}. To further investigate the CD8⁺ T cell population present in chronic mice and the antigen-independent T_{CM} cells arising in cured mice, we compared the cytokine production and cytolytic activity of these two populations. The *T. cruzi*-specific CD8⁺ T cells from both untreated and cured mice produced IFN- γ (Fig. 3a,b) and tumor necrosis factor- α (TNF- α ; Fig. 3a,c) in response to the TSKB20 peptide, although the frequency of cytokine producing cells was higher in untreated mice, as predicted on the basis of their higher frequency of TSKB20-specific T cells (Fig. 2a). Additionally, *in vivo* cytotoxicity of TSKB20-pulsed target cells was reduced by more than fourfold in treated mice relative to untreated mice at 16 h (Fig. 3d,e) and 4 h (**Supplementary Fig. 1** online) after transfer. T_{CM} cells are reported to have lower rates of proliferation *in vivo* as compared to T_{eff} and T_{EM} cells³. Incorporation of BrdU during days 300–329 post-infection indicated that the majority (>80%) of TSKB20-specific T cells (Fig. 3f) and >40% of all CD8⁺ T cells (Fig. 3g) from untreated mice had divided during this 29-d period, whereas only 30% of the TSKB20-specific (Fig. 3f) and <30% of the total (Fig. 3g) CD8⁺ T cell population from treated mice had divided. Proliferating TSKB20-specific CD8⁺ T cells from treated mice showed a similar turnover rate to that of age-matched naive CD8⁺ T cells (Fig. 3f,g), suggesting that the observed low ‘background’ proliferation of CD8⁺ T cells in treated mice is due to the homeostatic maintenance of these cells. Furthermore, the majority of BrdU⁺ TSKB20-specific and total CD8⁺ T cells in treated mice were CD62L^{hi}CD127^{hi}, whereas BrdU⁺ TSKB20-specific and total CD8⁺ T cells from untreated mice were predominantly CD62L^{lo}CD127^{lo} (Fig. 3h). Finally, in contrast to the long-term maintenance of pathogen-specific CD8⁺ T cells after cure, *T. cruzi*-specific CD4⁺ T cells seem to decline substantially in frequency after cure, as assessed by detection of IFN- γ and TNF- α production in response to a *T. cruzi* antigen preparation and to the nonspecific stimulus antibody to CD3 (**Supplementary Fig. 2** online). Thus, these data document the stable, homeostatic maintenance of pathogen-specific CD8⁺ T_{CM} cells with effector potential, but not CD4⁺ T cell memory, after cure of a chronic infection.

T_{CM} cells in cured mice confer protection to challenge

One of the defining characteristics of T_{CM} cells is their ability to rapidly proliferate upon exposure to a secondary infection³⁵. TSKB20-specific and total CD8⁺ T cells transferred from both chronic and cured mice expanded upon exposure to high-dose parasite challenge (Fig. 4a). However, CD8⁺ T cells from cured donors not only showed a greater post-challenge expansion relative to cells from untreated donors (more than twofold higher), but this expansion was also associated with an inhibition of activation of the endogenous naive parasite-specific CD8⁺ T cells (Fig. 4a). In all cases, the donor TSKB20-specific CD8⁺ T cells in challenged mice had a T_{eff} phenotype, demonstrating T_{CM} cell conversion to a T_{eff} or T_{EM} phenotype after re-exposure to antigen (Fig. 4b). Taken together, these results indicate that CD8⁺ T_{CM} cells present after cured *T. cruzi* infection respond robustly to a rechallenge, outcompete naive parasite-specific T cells and produce a larger pool of T_{eff} cells when compared to CD8⁺ T cells from untreated and chronic mice.

Mice receiving CD8⁺ T cells from cured mice also showed a two- to threefold lower tissue parasite burden ($P < 0.01$) relative to recipients of CD8⁺ T cells from naive or chronic donors (Fig. 5a) and showed significantly lower mortality (Fig. 5b). Histological examination of skeletal muscle confirmed the protective capacity of the T_{CM} cells from cured donors, as numerous amastigote nests and a greater degree of inflammation was observed in recipients of naive or chronic CD8⁺ T cells compared to recipients of cured CD8⁺ T cells (Fig. 5c). These results show that the predominantly T_{CM} CD8⁺ cells from treated mice transfer greater protection to recipient mice as compared to the predominantly T_{eff} and T_{EM} CD8⁺ cells from untreated mice.

Cure in the late chronic phase of *T. cruzi* infection

As shown above, two-time benznidazole treatment in the acute (days 15–35) and early chronic (days 150–170) stages of infection cured mice of *T. cruzi* infection and resulted in the development of a potent and protective T_{CM} CD8⁺ T cell response. To exclude the possibility that the (failed) treatment in the acute phase might nevertheless be crucial for the development of stable T cell memory after chronic phase cure, as well as to further explore the question of whether stable T_{CM} responses can be established after more lengthy infections, we treated mice with a single 40-d course of benznidazole initiated at 240 d after infection. Like the two-time treatment protocol, the 40-d benznidazole treatment regimen beginning at 240 d post-infection provided cure, as assessed by the same criteria described above. Mice cured by treatment during the late chronic phase of the infection and examined 135 d post-treatment (415 d post-infection) also retained TSKB20-tetramer⁺ T cells (Fig. 6a) with excellent effector function (for example, IFN- γ and TNF- α production; Fig. 6b) and with the surface phenotype of T_{CM} cells (for example, CD127^{hi} and CCR7^{hi}; Fig. 6c). In comparison to CD8⁺ T cells from chronic but untreated mice, these ‘late cure’ CD8⁺ T cells expanded more robustly and, as with the ‘early cure’ CD8⁺ T cells, outcompeted the endogenous T cells for activation when transferred to naive recipients that were then high-dose challenged with *T. cruzi* (Fig. 6d).

In order to directly compare the effect of early and late cure on the generation of T_{CM} phenotype CD8⁺ T cells, mice were cured by a single 40-d course of treatment beginning on day 15 of infection (early) or on day 180 post-infection (late). The kinetics of development of the T_{CM} cell population, as determined by the expression of CD62L and CCR7, were similar in the early cure and the late cure mice (Fig. 6e,f). In both cases, CD127 expression (Fig. 6g) provided the earliest and most prominent indication of an alteration in the parasite-specific CD8⁺ T cell population, reaching nearly 50% in the TSKB20-specific T cells at day 40 post-treatment. Induction of expression of CD62L and CCR7 was slower, requiring >100 d after completion of treatment to appear on >40% of the TSKB20-specific T cells. Thus, even when treatment is delayed as long as 240 d post-infection, cure can be obtained and a stable population of highly functional CD8⁺ T_{CM} cells can be established, despite the extended period of antigen persistence and exposure.

DISCUSSION

Chronic infections are reported to result in the gradual loss of pathogen-specific CD8⁺ T cell functions such as production of cytokines, killing of infected target cells and proliferation in response to antigen¹⁵. This antigen-induced exhaustion was first extensively studied in persistent lymphocytic choriomeningitis virus (LCMV) infection in mice^{29,36} and has been extended to other model systems and more recently to chronic infections in humans, in particular HIV and hepatitis^{12,14,29,31}, making T cell exhaustion a general paradigm for chronic infections. In most of these well-studied cases of immune exhaustion after persistent infection, comparisons have been made between T cell responses in hosts that rapidly clear the infection and those that do not, often using different pathogen strains to obtain either

persistent or cleared infection. However, because of the limitations of the model systems, what has not been previously investigated are the characteristics and competency of pathogen-specific immune responses after an extended infection that is then completely resolved. Herein we report on just such a system of *T. cruzi* infection in mice. Our results show that long-term exposure to a persistent pathogen does not lead to marked exhaustion of pathogen-specific T cells *per se*, and pathogen cure after an extended infection can result in the development of a stable, antigen-independent, T_{CM} cell population capable of showing effector function and providing protective immunity upon rechallenge.

Drug-mediated cure in mice after chronic *T. cruzi* infection resulted in a shift in the *T. cruzi*-specific CD8⁺ T cells from a predominantly T_{EM}-like phenotype (CD62L^{lo}, CCR7^{lo}, CD122^{lo}, CD127^{lo} and Bcl-2^{lo}) during persistent infection to a majority T_{CM} cell population (CD62L^{hi}, CCR7^{hi}, CD122^{hi}, CD127^{hi} and Bcl-2^{hi}). This change was observed when cure was achieved early in the acute phase (treatment at day 15–55 post-infection) or delayed for as long as 240 d after initiation of infection. Notably, in both cases the kinetics of the shift to a classical T_{CM} phenotype (CD62L^{hi}, CCR7^{hi}) is similar and also relatively slow. A comparable slow rate of reacquisition of the T_{CM} cell marker CD62L has also been reported in CD8⁺ T cells after secondary exposure to *Listeria monocytogenes*³⁷, suggesting that this slow transition is a common characteristic of repeatedly exposed, but not exhausted, CD8⁺ T cells. The *T. cruzi*-specific CD8⁺ T cells in drug-cured mice had additional hallmarks of T_{CM} cells—they underwent low-level, antigen-independent turnover *in vivo*, allowing for their stable maintenance, and expanded rapidly in response to re-infection. Indeed, the re-infection-induced expansion of T cells transferred from cured mice to naive mice completely abrogated the response of endogenous naive T cells, suggesting a greater ability of the T_{CM} cells to compete for antigen and other resources relative to naive T cells or *T. cruzi*-specific T_{EM} cells. These T_{CM} cells from cured mice also showed increased protective capacity as compared to the predominantly T_{eff} and T_{EM} population from chronically infected mice.

How CD8⁺ T cells in long-term *T. cruzi* infection avoid the degree of exhaustion documented in other chronic disease models is not clear. A major factor is probably antigen load, which is relatively low and extremely low (for example, <1,000 parasites/mouse) in the acute and chronic stages of persistent *T. cruzi* infection, respectively. In contrast, viral load is relatively high in persistent murine LCMV, the classical model of CD8⁺ T cell exhaustion in a persistent infection^{16,38}. The host cell types and tissues where these different pathogens reside could also have an important role in the degree of exhaustion induced. *T. cruzi* is in multiple tissues early in infection but persists primarily in muscle, neuronal tissue and fat during the chronic stage. In persistent LCMV infection, the early and substantial viral presence in splenic dendritic cells is crucial for establishing viral chronicity and T cell exhaustion^{16,39}. The relative absence of CD69 and CD25, markers of recent activation, as well as the lack of expression of PD-1 (M. Collins and R.L.T., unpublished data), a marker of persistent antigen stimulation^{12,13,40}, in circulating CD8⁺ T cells in chronic *T. cruzi* infection provide further support for the conclusion that antigen encounter by circulating CD8⁺ T cells is much less frequent in *T. cruzi* infection than in infections such as LCMV in mice and HIV in humans. A third factor in determining the fate of CD8⁺ T cells in chronic infection may be the length of exposure to persistent antigen. Although we do not see any evidence of immune exhaustion in 2-year-long *T. cruzi* infections in mice, more extensive examination of the full functional potential of T cells from these chronically infected mice might reveal more subtle signs of dysfunction. Indeed, we have observed signs of immune dysfunction that are consistent with exhaustion in humans infected for >20 years^{26,27}, suggesting that very long-term antigen exposure, even at low levels, can ultimately have deleterious effects on T cell function. We propose that the combination of antigen load, the cell and tissue types presenting the antigens and the length of antigen exposure may

collectively govern the rate and extent of antigen-induced immune exhaustion. Chronic LCMV infection in mice may occupy one end of the spectrum, where persistent antigen load is high and in the appropriate cell types to rapidly induce CD8⁺ T cell exhaustion. *T. cruzi* infection may fall near the other end of the spectrum, where low antigen load in nonprofessional antigen-presenting cells does not drive T cells to exhaustion or does so only after a very extended (decades-long) exposure.

Although benznidazole-mediated cure can rescue a stable and protective pathogen-specific CD8⁺ T cell population in chronically infected mice, it does not result in the full retention of pathogen-specific CD4⁺ T cells capable of recall cytokine production. This finding is consistent with other evidence of differences in the memory characteristics of CD8⁺ and CD4⁺ T cells⁴¹ and of the more rapid degradation in CD4⁺ T cell memory in the absence of antigen^{8,42,43}.

A second key finding in this study is the documentation of complete parasite clearance in hosts with established *T. cruzi* infection. Even with the most sensitive methods, it has been difficult to directly detect *T. cruzi* in untreated, chronically infected hosts (including humans), much less to use parasite detection to assess treatment efficacy. Although multiple studies have claimed cure of infection after drug treatment, this assessment is based largely on the suppression of parasitemia very early in the infection or on the use of techniques that frequently do not detect parasites even in the absence of treatment⁴⁴⁻⁴⁶ and not on definitive proof of complete parasite clearance. Furthermore, both benznidazole and nifurtimox are reported to have variable efficacy in humans primarily on the basis of decreases in titers of antibodies to *T. cruzi* several years after treatment²⁸. Although treatment of chronic *T. cruzi* infection in humans has also been associated with slowed progression of clinical disease^{47,48}, the lack of methods to conclusively show that these treatments can completely clear *T. cruzi* infection, along with the considerable potential side effects of these drugs, has drastically limited the use of these compounds in the treatment of established (chronic) adult *T. cruzi* infections.

The conclusion that appropriately treated mice were indeed parasite free was reached on the basis of the absence of exacerbation of infection after immunosuppression, the failure to transfer infection to immunocompromised mice through either blood or tissues from the drug-treated mice and the inability to detect parasite DNA by PCR or infection or disease by standard histology in the benznidazole- and cyclophosphamide-treated mice. The change in phenotype of the *T. cruzi*-specific CD8⁺ T cells in drug-treated mice to a stable T_{CM} cell population further supports the conclusions that these mice are not only free of *T. cruzi* but also essentially free of *T. cruzi* antigens.

In addition to establishing that benznidazole treatment can render a host free of *T. cruzi* infection, this work also establishes a system that should have considerable value in the rigorous testing of candidate anti-*T. cruzi* compounds. Perhaps the most useful direct impact of this work on human Chagas disease is the finding that parasitological cure results in the emergence of stable and protective *T. cruzi*-specific T_{CM} CD8⁺ cells that can be detected in the blood. The detection of cells of this phenotype could represent a useful surrogate method for the assessment of treatment efficacy—something that is currently sorely lacking. It will be noteworthy to determine whether parasitological cure of these chronic human infections will result in the rescue of a stable, *T. cruzi*-specific T cell memory pool, as observed here in the treatment of shorter-term infections in mice.

We find the documentation herein of a clear exception to the emerging paradigm that persistent infections invariably drive T cells to exhaustion somewhat comforting. Antigen-driven immune exhaustion would have especially dire consequences for individuals with

parasitic infections, the majority of which are persistent. These results also suggest that tight clinical maintenance of pathogen and antigen load—even if cure cannot be obtained, and as is possible in some infections (for example, HIV)—could potentially delay immune exhaustion and thus maintain the participation of immune effectors in the antipathogen response.

METHODS

Mice and parasites

We purchased C57BL/6 (Ly5.2⁺) (B6), B6.SJL (Ly5.1⁺) and B6.SCID mice from Jackson Laboratory and bred B6.IFN- γ -knockout mice in our animal facility. We obtained tissue culture trypomastigotes of the CL strain of *T. cruzi* from passage through Vero cells. Mice were infected intraperitoneally with 1,000 CL strain tissue culture trypomastigotes and killed by CO₂ inhalation at different time points after infection. All mouse protocols were approved by the University of Georgia Institutional Animal Care and Use Committee.

Benznidazole treatment

We used benznidazole (Rochagan) as a trypanocidal drug in the experimental therapy schedules. We treated mice orally with daily benznidazole doses of 100 mg per kg body weight for 20–40 d as indicated. Some groups received two rounds of treatment (days 15–35 and 150–170 post-infection). We prepared benznidazole by pulverizing one tablet containing 100 mg of the active principle and then suspending it in distilled water. Each mouse received 0.20 ml of this suspension by gavage.

Assessment of treatment efficacy

Mice were immunosuppressed with cyclophosphamide (200mg/kg/d) intraperitoneally at 2–3-d intervals for a total of four doses. After this immunosuppression, we collected blood from the tail vein and quantified the number of parasites with a Neubauer hemocytometer. We monitored survival daily. In some experiments, we obtained blood from naive, chronic and treated immunosuppressed and nonimmunosuppressed mice by retro-orbital venipuncture and collected it in sodium citrate solution. We then transferred the blood into either IFN- γ knockout or SCID mice and quantified the number of parasites as described earlier. We carried out DNA preparation, generation of PCR standards and detection of parasite tissue load by real-time PCR as described previously⁴⁹. We collected skeletal, heart and intestinal tissues at various time points after treatment and fixed them in 10% buffered formalin. Sections (5- μ m) from paraffin-embedded tissues were stained with H&E for histopathological analysis.

T cell phenotyping and function

Single-cell suspensions of spleen cells were prepared, red blood cells lysed and the remaining cells were washed in staining buffer (2% BSA, 0.02% azide in PBS (PAB)). In some cases, we obtained mouse peripheral blood by retro-orbital venipuncture, collected it in sodium citrate solution and washed it in PAB. We then incubated spleen cells or peripheral blood mononuclear cells with tetramer-phycoerythrin (PE) and the following antibodies: biotin-labeled antibody to CD62L, FITC-labeled antibody to CD44, allophycocyanin (APC)-Cy7-labeled antibody to CD8, FITC-labeled antibody to CD11a (all from BD Pharmingen), CD69 PE-Cy5 (eBioscience), APC-labeled antibody to CD127 (eBioscience), Alexa Fluor 488-labeled antibody to CD122 (Caltag-Invitrogen) and APC-labeled antibody to CD25 (Caltag-Invitrogen). We also stained cells with antibodies to CD4, CD11b and B220 (Caltag-Invitrogen) for use as an exclusion channel. We stained cells for 45 min at 4 °C in the dark, washed them twice in PAB and fixed them in 2% formaldehyde.

The EB11 ligand chemokine (ELC)-immunoglobulin chimera was used for detecting CCR7 expression and was a gift from K. Klonowski (University of Georgia). For CCR7 detection, we incubated cells at 37 °C for 1 h, stained them with ELC-immunoglobulin for 45 min and washed them. We then stained cells with Alexa Fluor 488–conjugated goat antibody to human IgG (Invitrogen) for 30 min, washed them and stained them with surface markers as indicated above. For whole blood, we lysed red blood cells in a hypotonic ammonium chloride solution after washing twice in PAB. We acquired at least 500,000 cells with a CyAn flow cytometer (DakoCytomation) and analyzed with FlowJo software (Tree Star). Major histocompatibility complex (MHC) class I tetramers were synthesized at the Tetramer Core Facility (Emory University). The tetramers used in these studies were TSKB20/K^b (ANYKFTLV on H2K^b) and TSKB74/K^b (VNYDFTLV on H2K^b).

We stimulated spleen cells from naive, untreated and chronic or treated and cured mice with *T. cruzi* peptide (5 µM) TSKB20 and processed them for intracellular cytokine staining as previously described²⁴. For measurement of *in vivo* cytotoxicity, we incubated spleen cells from naive mice either with *T. cruzi* peptide TSKB20 or with no peptide for 1 h at 37 °C, labeled them with carboxyfluorescein succinimidyl ester and determined the percentage of specific killing in mice as previous described²⁴.

***in vivo* BrdU incorporation assay**

BrdU (Sigma) solution (0.8 mg/ml) was made fresh every 2 d and given to mice for 29 d. We washed spleen cells from naive, untreated and chronic or treated and cured mice obtained at 329 d post-infection in PAB and incubated them with tetramer-PE and the following antibodies: biotin-labeled antibody to CD62L (BD Pharmingen), APC-Cy7–labeled antibody to CD8 (BD Pharmingen) and APC-labeled antibody to CD127 (eBioscience). An exclusion channel was used as described above. We stained cells for 45 min at 4 °C in the dark. We then fixed and permeabilized them with Cytotfix/Cytoperm buffer (BD Pharmingen), washed them in 1× Perm Wash buffer (BD Pharmingen) and treated them with DNase to expose BrdU epitopes. We then stained them intracellularly with FITC-labeled antibody to BrdU (BD Pharmingen), washed them twice in Perm Wash and fixed them in 2% formaldehyde.

Statistical analysis

We calculated statistical significance with a two-tailed Student's *t*-test.

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Figure 1.

Benznidazole treatment results in parasitological cure in chronic *T. cruzi* infection. **(a)** Evolution of parasitemia after infection with 1×10^3 CL strain *T. cruzi* on day 0 in untreated and benznidazole-treated mice. Benznidazole (BZ) bars below *x* axis indicate period of benznidazole treatment. The arrows denote the times of administration of the immunosuppressant cyclophosphamide (days 250, 253, 258 and 260 after infection). Error bars represent means $\pm$ s.d. **(b)** Quantitative real-time PCR measurement of *T. cruzi* DNA in skeletal muscle of naive, untreated and benznidazole-treated mice determined at 265 d post-infection. The bar represents the mean. **(c)** Histological sections of the skeletal muscle of naive, untreated infected and benznidazole-treated infected mice at 330 d post-infection. Scale bar, 20 μ m. Data are representative of three to five independent experiments with three to six mice per group.

Figure 2.

Chronic phase cure results in conversion of parasite-specific CD8⁺ T cells to a T_{CM} phenotype. **(a,b)** MHC-peptide tetramers of the immunodominant TSKB20/K^b **(a)** or the subdominant TSKB74/K^b **(b)** epitopes were used to detect *T. cruzi*-specific CD8⁺ T cells in the blood, spleen and lymph nodes of chronic (untreated) and treated (cured) mice at 260 d post-infection. Numbers indicate the percentage of tetramer⁺ cells among the CD8⁺ population. **(c)** Expression of activation and memory markers on the TSKB20/K^b splenic T cells from chronic (filled histograms) and cured (open histograms) mice at ~260 d post-infection. Dashed lines in CD44 and CD11a panels show negative control staining patterns of CD8⁺ T cells from naive mice. Data are representative of three to five independent experiments with three mice per group. **(d,e)** The population of TSKB20/K^b cells **(d)** and TSKB20/K^b cells expressing CD62L **(e)** stabilizes within 90 d after benznidazole treatment (treatment completed at 170 d post-infection). Error bars represent means $\pm$ s.d.

Figure 3.

CD8⁺ T cells from treated and cured mice undergo homeostatic proliferation and maintain the ability to carry out effector functions. **(a–c)** Spleen cells from naive, untreated and chronic or treated and cured *T. cruzi*-infected mice (330 d post-infection and 160 d after treatment end) were cultured overnight with or without TSKB20 peptide before assessment of cytokine production by intracellular cytokine staining. Graphs show IFN- γ and TNF- α production (numbers represent the percentage of CD8⁺ T cells producing the respective cytokine) in representative mice **(a)** and the average of three mice for IFN- γ **(b)** and TNF- α **(c)**. Data are representative of two independent experiments with three mice per group. **(d,e)** *In vivo* cytotoxic T lymphocyte activity on TSKB20-pulsed targets 16 h after transfer to naive, untreated and chronic or treated and cured *T. cruzi*-infected mice (330 d post-infection). Data are from representative individual mice **(d)**; numbers above the peaks are percentage specific lysis calculated as described in the Methods) and cumulative data from six mice per group **(e)**. **(f,g)** The plots show the percentage of TSKB20/K^b+ CD8⁺ T cells incorporating BrdU in untreated and chronic (filled histogram) or treated and cured (open histogram) mice **(f)** and the percentage of total CD8⁺ T cells **(g)** incorporating BrdU over a 29-d period (days 300–329 post-infection). **(h)** Expression of CD62L and CD127 in BrdU⁺ cells gated on TSKB20/K^b+ cells (top) and total CD8⁺ cells (bottom) from treated and cured (open histogram) or untreated and chronic (filled histogram) mice. Data are representative of three independent experiments with a total of six mice per group.

Figure 4.

T_{CM} cells in treated and cured mice show strong antigen-induced proliferation after infection. $CD8^{+}$ splenic T cells (3×10^6) from naive, untreated and chronic (460 d post-infection) or treated and cured (460 d post-infection and 290 d after treatment completion) B6 ($CD45.2^{+}$) mice were transferred to uninfected congenic $CD45.2^{-}$ B6 SJL mice, which were then challenged with 1×10^7 CL strain *T. cruzi* trypomastigotes. **(a)** Maintenance before challenge (left) and accumulation 8 d after challenge (right) of donor ($CD45.2^{+}$) and recipient ($CD45.2^{-}$) TSKB20/ K^{b+} cells in the spleens of mice that received $CD8^{+}$ T cells from above-mentioned donors. Donor cells from treated and cured mice respond robustly to challenge and inhibit the activation and expansion of the endogenous naive population of TSKB20/ K^{b} -tetramer⁺ cells. Numbers indicate the percentage of the transferred $CD8^{+}$ population ($CD45.2^{+}$) that are positive for TSKB20/ K^{b} (upper right quadrant) or are TSKB20/ K^{b} negative (upper left quadrant) and the percentage of endogenous $CD8^{+}$ T cells ($CD45.2^{-}$) that are positive for TSKB20/ K^{b} (lower right quadrant). **(b)** Conversion to a T_{eff} phenotype in donor TSKB20/ K^{b+} T cells from treated and cured mice on the basis of loss of expression of CD62L and CD127 at 8 d after challenge. Data are representative of three independent experiments with three mice per group. Numbers indicate the percentage of TSKB20/ K^{b} cells among the $CD8^{+}$ population transferred ($CD45.2^{+}$) that stain positively for CD62L or CD127.

Figure 5.

CD8⁺ T cells from treated and cured mice provide greater protection from challenge infection. CD8⁺ splenic T cells (3×10^6) from naive, untreated and chronic or treated and cured mice were transferred and the recipient mice were infected as described in the legend to Figure 4. Recipient mice were killed 8 d after challenge. **(a)** *T. cruzi* DNA in skeletal muscle of mice that received CD8⁺ T cells from naive, untreated and chronic, or treated and cured mice as determined by quantitative real-time PCR. Bars represent the means. **(b)** Survival rate after challenge of mice that received CD8⁺ T cells from naive, untreated and chronic or treated and cured mice. **(c)** Histological sections of the skeletal muscle in CD8⁺ T cell recipients at 8 d after challenge showing amastigote nests (arrows) and cellular infiltrates. Scale bars, 20 μ m. Data are representative of three independent experiments with three mice per group.

Figure 6.

Benznidazole treatment in the late chronic phase of *T. cruzi* infection provides cure and conversion of *T. cruzi*-specific CD8⁺ T cells to a T_{CM} phenotype. (a) Frequency of TSKB20/K^b⁺ T cells in the spleens of untreated and chronic or treated and cured mice 415 d post-infection (treatment given from 240 to 280 d post-infection). (b) Intracellular cytokine staining for IFN-γ and TNF-α production by CD8⁺ T cells with or without stimulation with TSKB20 peptide in representative mice. Numbers represent the percentage of CD8⁺ T cells producing IFN-γ or TNF-α. (c) CCR7 and CD127 expression in CD8⁺TSKB20/K^b⁺ T cells from untreated and chronic (filled histograms) or treated and cured (open histograms) mice. Data are representative of three independent experiments with three mice per group. (d) Maintenance before challenge (left) and accumulation 8 d after challenge (right) of donor (CD45.2⁺) and recipient (CD45.2⁻) TSKB20/K^b⁺ cells in the spleens of mice that received CD8⁺ T cells from naive, untreated and chronic or chronically treated and cured donors. Donor cells from treated and cured mice respond robustly to challenge and inhibit the activation and expansion of the endogenous naive CD8⁺ T cells. (e–g) Percentages of CD8⁺TSKB20/K^b⁺ T cells expressing CD62L (e) CCR7 (f) or CD127 (g) at the indicated time points after completion of benznidazole treatment. Data are representative of three independent experiments with three mice per group. Error bars represent means ± s.d.