Treatment Success in *Trypanosoma cruzi* Infection Is Predicted by Early Changes in Serially Monitored Parasite-Specific T and B Cell Responses

María G. Alvarez¹, Graciela L. Bertocchi¹, Gretchen Cooley², María C. Albareda³, Rodolfo Viotti¹, Damián E. Perez-Mazliah³, Bruno Lococo¹, Melisa Castro Eiro³, Susana A. Laucella¹,³, Rick L. Tarleton²*

¹ Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina, ² Center for Tropical and Emerging Global Diseases, Athens, Georgia, United States of America, ³ Instituto Nacional de Parasitología Dr. Mario Fatale Chaben, Buenos Aires, Argentina

☯ These authors contributed equally to this work.
* tarleton@uga.edu

Abstract

**Background**

Chagas disease is the highest impact parasitic disease in Latin America. We have proposed that changes in *Trypanosoma cruzi*-specific immune responses might serve as surrogate indicators of treatment success. Herein, we addressed in a long-term follow-up study whether cure achieved after treatment can be predicted by changes in non-conventional indexes of anti-parasite serological and T cell activities.

**Methodology/Principal Findings**

*T. cruzi*-specific T cell responses, as measured by interferon-γ ELISPOT and *T. cruzi*-specific antibodies assessed by ELISA, hemagglutination and immunofluorescence tests as well as by a multiplex assay incorporating 14 recombinant *T. cruzi* proteins were measured in 33 patients at 48–150 months post-benznidazole treatment. Cure — as assessed by conventional serological tests — was associated with an early decline in *T. cruzi*-specific IFN-γ-producing T cells and in antibody titers measured by the multiplex serological assay. Changes in the functional status and potential of *T. cruzi*-specific T cells, indicative of reduced antigen stimulation, provided further evidence of parasitological cure following benznidazole treatment. Patients showing a significant reduction in *T. cruzi*-specific antibodies had higher pre-therapy levels of *T. cruzi*-specific IFN-γ-producing T cells compared to those with unaltered humoral responses post-treatment.

**Conclusions/Significance**

Monitoring of appropriate immunological responses can provide earlier and robust measures of treatment success in *T. cruzi* infection.
Author Summary

This study demonstrates that alterations in immunological parameters early after treatment with benznidazole in Chagas disease patients are predictors of treatment efficacy. Cure was associated with an early decline in T. cruzi-specific IFN-γ-producing T cells and in antibody titers and with high basal levels of T. cruzi-specific T cells.

Introduction

Chagas disease is the highest impact parasitic disease in Latin America and the most common cause of infectious myocarditis in the world [1]. The goal of treatment of humans in the chronic phase of Trypanosoma cruzi infection is to prevent the development of heart disease and infection by via blood transfusion, congenital transmission and organ transplants [2]. However, treatment in adult chronic patients is not widely used mainly because of the lack of early metrics of treatment efficacy and the potential adverse effects of these therapeutics [3]. Several studies in adult patients with mild disease symptoms have demonstrated the clinical benefits of treatment with benznidazole [4,5]. However, the results of the recently published BENEFIT clinical trial [6] has raised questions about the benefits of benznidazole treatment in subjects with established cardiomyopathy, thus emphasizing that therapeutic interventions would have greatest benefit when delivered early in the infection.

The current criterion of a positive response to treatment is the complete loss of reactivity in serially performed conventional serological tests (ELISA, hemagglutination and immunofluorescence), as well as the lack of progression to more severe clinical conditions of Chagas disease. The decline in serologic titers using current standard tests is very slow, often requiring > 24 months for antibody titers in conventional tests to begin to fall; complete conversion to negative serology can take more than 10 years [4, 7–11]. Likewise, disease progression also occurs over decades and does not occur in all infected individuals [4, 5]. Consequently, the development of surrogate markers of treatment efficacy is needed for an early assessment of successful treatment and the evaluation of new therapeutic approaches in the chronic phase of T. cruzi infection.

CD4+ and CD8+ T cells derived from patients with chronic T cruzi infection have been shown to produce a variety of cytokines [12–18]. However recent studies using polychromatic flow cytometry revealed that CD4+ and CD8+ T cells with the capacity to produce only one cytokine (i.e. monofunctional T cells) in response to T. cruzi antigens is a common feature in adults with chronic Chagas disease [19–21]. Of note, monofunctional T cells are more prevalent in patients long-standing infections, generally accompanied by advanced cardiomyopathy [20,21], while polyfunctional T cells are often found in children who have shorter term infections [19]. This is consistent with the profile of pathogen-specific T cells in other infections where long-term antigen persistence maintains an active pathogen-specific T cell population but with increasing impairment of T cell function over time. This process known as immune exhaustion has been described for persistent viral, bacterial and protozoan infections [22–27] and is characterized by the loss of IL-2 production, cytokine polyfunctionality, as well as proliferative capacity followed ultimately, by defects in the production of IFN-γ, TNF-α, chemokines and degranulation potential [24]. Several other features of exhausted T cells, such as high expression of inhibitory receptors, a low expression of the IL-7 receptor and high dependence on the presence of antigen for T cell maintenance have been documented in patients with very long-term T. cruzi infections [20, 28–30].
We have proposed that changes in *T. cruzi*-specific IFN-γ-producing T cells [30] and declines in parasite-specific antibodies as measured by the non-conventional multiplex method might serve as surrogate indicators of treatment success, as determined in a 3-5-year post-treatment follow-up study in chronic Chagas disease patients [7, 30]. We hypothesize that treatment decreases parasite load, thus diminishing the antigen necessary to continually activate *T. cruzi*-specific T cells and B cells. In patients successfully cured of the infection, a stable change in T and B cell phenotype and activation, in line with antigen-independent immunological memory, would be expected.

In this study, the evolution of the functional profile of *T. cruzi*-specific T cells and of the humoral immune response to multiple *T. cruzi* antigens, in association with changes in conventional serological tests — an accepted marker of treatment efficacy — was assessed in 33 subjects chronically infected with *T. cruzi* over ~8 years following treatment with benznidazole.

We present evidence that cure — assessed by conventional serological tests — achieved many years after treatment with benznidazole was associated with an early decline in *T. cruzi*-specific IFN-γ-producing T cells, and in antibody titers measured by the multiplex assay. Changes in the activation status and potential of *T. cruzi*-specific T cells, indicative of reduced antigen stimulation, provided additional evidence of parasitological cure following benznidazole treatment. These results further support the case for using immunological markers as indicators of treatment efficacy in *T. cruzi* infection.

**Methods**

**Selection of study population**

*T. cruzi*–infected adult volunteers aged 23–54 years were recruited at the Chagas Disease Section of Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina. *T. cruzi* infection was determined by indirect immunofluorescence assay, hemagglutination, and enzyme-linked immunosorbent techniques [31] performed at the Instituto Nacional de Parasitología Dr. Mario Fatale Chaben, Buenos Aires, Argentina. Chronically infected subjects were evaluated clinically and stratified according to a modified version of Kuschnir grading system [7, 32]. Individuals in group 0 had normal electrocardiograph, normal chest radiograph, and normal echocardiograph findings (n = 27, median age = 39 years, range = 23–54 years), and subjects in group 1 had normal chest radiograph and echocardiograph findings but abnormal electrocardiograph findings (n = 6, median age = 42 years, range, 30–50 years). Treatment consisted of benznidazole, 5 mg/kg per day for 30 days [5–9]. Clinical, serological and immunological analysis was performed prior and after treatment. Patients enrolled in this study did not change the clinical status during the follow-up period. This protocol was approved by the institutional review boards of the Hospital Interzonal General de Agudos Eva Perón, Buenos Aires, Argentina and the University of Georgia, GA, USA. Signed informed consent was obtained from all individuals before inclusion in the study.

**Collection of peripheral blood mononuclear cells (PBMCs) and serum specimens**

PBMCs were isolated by density gradient centrifugation on Ficoll–Hypaque (Amersham) and were cryopreserved in a solution of 20% dimethylsulfoxide in heat-inactivated fetal calf serum for later analysis. Blood to be used for serum analysis was allowed to coagulate at 4°C and centrifuged at 1000 g for 15 min for sera separation.
IFN-γ and interleukin (IL)–2 enzyme-linked immunosorbent spot (ELISPOT) assays

The number of T. cruzi–specific IFN-γ– and IL-2–secreting T cells was determined by ex vivo ELISPOT using a commercial kit (ELISPOT Human IFN-γ or IL-2 ELISPOT Set; BD), as described elsewhere [33]. To avoid inter-experiment variations, assays were conducted with paired samples from different time points assayed in the same experiment. Each time point was assessed 1–3 times.

Monoclonal antibodies

mAb anti-CD3-fluorescein isothiocyanate (FITC), anti-CD134 (FITC), anti-IFN-γ (FITC), anti-CD25 (PE), anti-CD154 (PE), anti-CD3-peridinin chlorophyll protein (PerCP), anti-CD4 (PerCP), anti-CD27-allophycocyanin (APC), anti-TNF-α (APC) and anti-CCR7-phycoerythrin-Cy7 (PE-Cy7) and anti-CD4 (APC-Cy7) were purchased from BD Pharmingen, USA.

Polyfunctionality of peripheral blood mononuclear cells and phenotyping of total T cells

PBMCs isolated from T. cruzi-infected subjects were stimulated with 15 μg/ml T. cruzi amastigote lysate or medium alone in 48-well plates at 37°C in a CO2 incubator for 16–20 h. Ten micrograms of brefeldin A per ml was added to the samples for the last 6 h of incubation. After stimulation, PBMCs were removed from the plates and stained for cell surface markers followed by fixation and permeabilization with cytofix/cytoperm and intracellular staining with a combination of monoclonal antibodies specific for IFN-γ, TNF-α and CD154 (CD40L). In order to confirm that cytokine/co-stimulation expression was derived from T cells, antihuman CD3 was added in polyfunctional staining assays in combination with CD4, IFN-γ and TNF-α or CD4, IFN-γ and CD154, respectively. Typically, 500,000 lymphocytes were acquired on a FACScalibur (Becton Dickinson Immunocytometry Systems, USA) and analyzed using FlowJo software (TreeStar, Inc., USA). Lymphocytes were identified based on their scatter patterns and CD4 expression for the combination of IFN-γ, TNF-α and CD154; and based on scatter patterns as well as CD3 and CD4 expression for the combination of IFN-γ and TNF-α or IFN-γ and CD154. Boolean combination gating was then performed to calculate the frequencies of expression profiles corresponding to the seven possible combinations of functions by using FlowJo. After subtracting the background values, the proportions of the different subsets were expressed as percentages of total cytokine or CD154-positive cells. Responses to the T. cruzi lysate were considered positive, for any particular subset, if the frequency of cytokine/CD154-positive T cells was threefold higher than the frequency in medium alone and above 0.07% of total CD4+ T cells, since the limit of detection was set at 0.01%.

Multiplex serodiagnostic assay

Serum specimens were screened for antibodies reactive to a panel of 14 recombinant T. cruzi proteins in a Luminex-based format, as previously described [34]. Serological responses to each individual T. cruzi protein were considered to have decreased during the study period if the mean fluorescence intensity in at least one recombinant protein declined by 50% relative to that of the time 0 (pretreatment) sample assessed concurrently.

Statistical analysis

Comparisons on the changes in T. cruzi–specific antibodies after treatment, measured by conventional serological tests, were performed using the Mann-Whitney U test. T cell responses at
different time points were compared by Friedman range test. Comparisons of proportions were performed by use of the \( \chi^2 \) test and Fisher’s exact test. Differences were considered to be statistically significant at \( P < 0.05 \).

**Results**

**Long-term monitoring of T cell responses after treatment with benznidazole in chronic Chagas disease**

We have previously shown in a 3–5 year follow-up study that the frequency of peripheral IFN-\( \gamma \)-producing T cells responsive to *T. cruzi* antigens declined as early as 12 months after treatment with benznidazole and subsequently became undetectable in a proportion of treated subjects [30]. In some cases, these individuals with declining T cell responses experienced rebounds in parasite-specific T cell responses several years after treatment. Additionally, some subjects had undetectable IFN-\( \gamma \)-producing T cells (i.e. below background levels) prior to treatment that became detectable after treatment, whereas the frequencies of IFN-\( \gamma \)-producing T cells did not change relative to pretreatment in a fourth subset of subjects [30]. Herein, we report a 4-12-year follow-up (median 8 years) of humoral and cellular T cell responses in 33 of these subjects. All subjects for which IFN-\( \gamma \)-ELISPOT responses fell below the level of detection between 12–36 months following treatment with benznidazole (\( n = 12 \)) showed a later rebound in IFN-\( \gamma \)-producing T cells (i.e. range 24–72 months post-treatment) [Table 1, Group 1; Fig 1A]. In contrast, in the remaining subjects, T cell responses did not change significantly during long-term follow up (Table 1, Groups 2–4; Fig 1B–1D). Likewise, IFN-\( \gamma \) ELISPOT responses are relatively stable in 6 untreated subjects with a 48–60 month-follow-up (Fig 1E).

**Evolution of *T. cruzi*-specific antibodies in relation to changes in T cell responses in benznidazole-treated subjects**

Monitoring of *T. cruzi*-specific humoral immune responses assessed by the conventional serological tests, as well as by the multiplex assay that examines responses to 14 individual *T. cruzi* proteins [34], was conducted at least yearly following treatment with benznidazole. The levels of *T. cruzi*-specific antibodies measured by conventional serology significantly declined over time in subjects with decreased or rebounding IFN-\( \gamma \)-ELISPOT responses following treatment with benznidazole (Table 2 and Fig 2A and 2B) whereas antibody titers remained relatively stable in the other patient groups (Table 2, Fig 2C and 2D). Of note the seven patients who showed conversion from seropositive to seronegative—the standard metric of infection cure—on at least 2 of the 3 conventional serological tests were patient groups 1 and 2 (Table 2, Fig 2A).
Conversion from seropositive to seronegative was observed on average >5 years post-treatment (24–96 months) and was sustained up to 12 years post treatment (Fig 2B, subject PP31). In concordance with conventional serology, a multiplex assay utilizing recombinant proteins from T. cruzi also revealed a higher rate of declining antibody titers among subjects with decreased or rebounding ELISPOT responses (Table 2, Fig 3A–3D). Seventeen out of nineteen patients with a rebound or a significant decrease in IFN-γ-producing T cells following treatment with benznidazole showed a fall in the levels of antibodies specific for one more
recombinant proteins in comparison to 4 out of 13 in the group of patients in which T cell responses remained unchanged or became detectable after treatment (Table 2, Fig 3A–3D). Notably, the multiplex assay detected declines in antibody levels as early as 2–24 months post-treatment (Fig 3A–3D) while declines in conventional serologic tests were not evident until 24–48 months post-treatment (Fig 2A and 2B). Conversion from seropositive to seronegative by conventional serological tests can take up to 9 years to occur (Fig 2A and 2B). Thus, declines in \( T. cruzi \)-responsive IFN-\( \gamma \)-producing T cells and \( T. cruzi \)-specific multiplex-detected antibodies following benznidazole treatment preceded and were predictive of conversion to negative conventional serology, the accepted standard of treatment success. As previously reported [30], IL-2-producing T cells were low in chronically \( T. cruzi \)–infected subjects and changed in concert with IFN-\( \gamma \) T cell responses after treatment with benznidazole (Fig 2A–2E). Treatment success as measured by declining \( T. cruzi \)-specific antibody responses was not associated either with the age of subject at initiation of treatment or the baseline \( T. cruzi \)-specific antibody titers. However, subjects with declining antibody titers as a group had higher pre-treatment frequencies of IFN-\( \gamma \)- and IL-2 producing T cells as compared to patients who showed no change in humoral responses following treatment (Fig 4).

### The cytokine and phenotype profile of rebound populations of \( T. cruzi \)-specific CD4\(^+\) T cells reflects absence of antigen stimulation

Since rebound in \( T. cruzi \)-specific T cells making IFN-\( \gamma \) was associated with declining serological titers, suggestive of a decreased presence of parasite antigen, we hypothesized that these \( T. cruzi \)-responsive T cells re-emerging long-term after treatment would result in enhanced functional capacity of \( T. cruzi \)-specific T cells.

Group 1 subjects exhibited an increase in single CD4\(^+\)CD54\(^+\) and CD4\(^+\)IFN-\( \gamma \)\(^+\) T cells (Fig 5B–5D) coincident with a decrease in single CD4\(^+\)TNF\(^+\) T cells (Fig 5B–5E) following treatment with benznidazole. Some subjects also showed an increase in dual IFN-\( \gamma \)\(^+\)CD154\(^+\) T cells

---

**Table 2. Evolution of \( T. cruzi \)-specific humoral immune responses according to changes in T cell responses during long-term follow-up of benznidazole-treated subjects.**

| Patient group | ELISPOT responses (from Table 1) | ELISA | IHA | IFI | Changes in Serology | No of subjects with seroconversion/total evaluated (%) \(^{A}\) | Multiplex serology (%) \(^{B}\) | Months of follow-up (range) |
|---------------|-----------------------------------|-------|-----|----|-----------------------|---------------------------------|----------------------------|-----------------------------|
| 1             | Rebound                           | 0.0015\(^{C}\) | 0.0052\(^{C}\) | NS | 2/12 (17)             | 11/12 (92)\(^{E}\)                  | 65–150                     |
| 2             | Decreased                         | 0.0205\(^{C}\) | 0.0072\(^{C}\) | NS | 5/8 (63)\(^{D}\)     | 6/7 (86)\(^{F}\) (*)               | 48–132                     |
| 3             | Became detectable                 | NS     | NS  | NS | 0/5                   | 2/5 (40)                         | 80–137                     |
| 4             | Unchanged                         | NS     | NS  | NS | 0/8                   | 2/8 (25)                         | 48–96                      |

\(^{A}\) No. of subjects with negative findings post-treatment for 2 out of 3 or 3 out 3 conventional serological tests.

\(^{B}\) No. of subjects/total evaluated with a 50% decrease in mean fluorescence intensity for > 1 recombinant \( T. cruzi \) protein in the 14-protein multiplex panel.

\(^{C}\) \( P < 0.05 \) compared with group 4, by the Fisher exact test.

\(^{D}\) \( P < 0.01 \) compared with group 4, by the Fisher exact test.

\(^{E}\) \( P < 0.01 \) compared with group 4, by the Fisher exact test.

\(^{F}\) \( P < 0.05 \) compared with group 4, by the Fisher exact test.

(*) No sample available for one patient.

ELISA, enzyme-linked immunosorbent assay; IHA, indirect hemagglutination; IFI, indirect immunofluorescence; NS, no significant change relative to pretreatment values.

---

\[ \text{doi:10.1371/journal.pntd.0004657.t002} \]
Fig 2. Evolution of T. cruzi-specific humoral responses in relation to changes in T cell responses after treatment with benznidazole. T. cruzi-specific humoral responses were measured at different time points after benznidazole treatment by enzyme-linked immunosorbent assay (ELISA), indirect.
hemagglutination (IHA) and indirect immunofluorescence (IFI). Each panel exhibits representative humoral responses for single patients with different kinetics of IFN-γ producing T cells after benznidazole treatment. A) Parasite-specific T cell responses became undetectable after treatment and experienced a rebound thereafter. B) Parasite-specific T cell responses decreased after treatment. C) Undetectable cytokine-producing T cells prior to treatment became detectable after treatment. D) The frequencies of cytokine-producing T cells did not change relative to pretreatment. Time 0 indicates the assay point just prior to benznidazole treatment. Broken lines indicate the cut-off value for ELISA assays; full lines indicate the cut-off value for IHA and IFI assays.

**Discussion**

One of the primary drawbacks in treatment of chronic *T. cruzi* infections is the difficulty of assessing treatment efficacy [4, 35, 36], principally in the short term. In this study, we investigated if the early, post-treatment changes in *T. cruzi*-specific T cell and antibody responses, previously reported by our group [30], are predictors of treatment efficacy. To answer this question we compared these non-conventional immune assessments with the conversion from parasitological cure.
Fig 4. Levels of pre-therapy IFN-γ- or IL-2 secreting T cells in relation to the evolution to *T. cruzi*-specific humoral responses in Chagas disease patients treated with benznidazole. Treated subjects were grouped as those with stable or declining *T. cruzi*-specific antibodies post-treatment as measured by conventional serological tests and multiplex assays. Each dot represents the mean IFN-γ (A) and IL-2 (B) spot number of triplicate wells for each patient sample assessed. Spot counts with media alone were subtracted from *T. cruzi*-antigen stimulated spot numbers. Horizontal lines depict median values. Comparisons between groups were performed using the Mann-Whitney U test. P < 0.05 was considered as statistically significant.

doi:10.1371/journal.pntd.0004657.g004
positive to negative conventional serology — the accepted standard of cure — in a longitudinal over ~8-year post-benznidazole treatment follow-up study. Our study revealed that cure—as determined by seronegative conversion by conventional serology—was strongly correlated with an early decline in both T. cruzi-specific T cells and in the levels of antibodies specific for a panel of T. cruzi antigens. Significant declines in IFN-γ-producing T cells and multiplex-monitored antibody responses post-treatment also preceded detection of reductions in anti-T. cruzi antibodies detectable by conventional serological tests. In contrast, subjects exhibiting stable T cell responses post-treatment were generally associated with unaltered conventional
and multiplex-assessed humoral responses. Thus, this work identifies dependable and early markers of treatment efficacy in Chagas disease.

These results support and extend our previous studies [7] indicating the superiority of assaying responses to >10 recombinant proteins using a multiplex format over conventional serologic tests. Other studies have also demonstrated that the use of recombinant proteins as antigens can often detect changes in parasite-specific antibodies earlier than the complex *T. cruzi* antigen preparations normally used in many conventional tests [37, 38]. However, in 15 out of the 33 patients evaluated in this study slight or no changes in *T. cruzi*-specific humoral and cellular T cell responses were observed, suggesting a failure of treatment and confirming previous studies showing that benznidazole treatment is not uniformly successful curing *T. cruzi* infection [4, 6].

Some subjects with declining or negative anti-*T. cruzi* antibody levels and T cell responses experienced rebounds in T cell responses, prompting the question of whether these T cell reflected renewed antigen stimulation, and thus persistence of *T. cruzi* infection. However rebounding IFN-γ-producing T cells were associated with decreasing serological titers by both conventional and multiplex assays and two of the seven subjects who converted to negative conventional serology—the accepted standard of cure—exhibited this rebound in T cell responses. Therefore, it seems likely that these parasite-specific T cells in rebound responses are maintained in the absence of or very low levels of antigen, a characteristic of TCM. Such responses are evident in mice cured of *T. cruzi* infection by benznidazole treatment [8, 39, 40]. Herein, benznidazole treatment resulted in a different functional quality of CD4⁺ T cells with a prominent decline in single producers of TNF-α and an increase in either monofunctional or polyfunctional CD4⁺ T cells expressing CD154 after treatment. Several studies have shown that constant antigen stimulation during chronic infections might skew T cell responses to single TNF-α-producing T cells [41] and low CD154 expression [42, 43] which are restored after suppression of antigen load [41, 44].

Other studies have also shown that therapy with benznidazole in the chronic phase of the infection resulted in a shift toward a type-1 T cell profile profile [45–47]. Collectively, these findings further support that parasite persistence in chronic *T. cruzi* infection induces significant alterations in T cell function.

An interesting observation that deserves further investigation is that subjects who showed the greatest decrease in *T. cruzi*-specific antibodies following treatment also had on average higher baseline levels of IFN-γ-producing T cells compared with subjects with modest or no changes in humoral responses. Studies in the experimental models have suggested that the quality of the anti-*T. cruzi* immune response plays a role in the efficacy of benznidazole treatment [48–51]. Studies in larger patient groups and in experimental models are needed to confirm these findings.

This study validates the ability of appropriate and sensitive immunological tests to provide early evidence of treatment efficacy in chronic Chagas disease. Providing tools to not only monitor but to more rapidly predict treatment success or failure will facilitate the development of new and better therapeutic options in Chagas disease.

**Acknowledgments**

We thank the staff and patients of the Hospital Eva Peron who provided blood samples and the Diagnostic Department of the Instituto Nacional de Parasitologia Dr. Mario Fatala Chaben for serological tests. We are grateful to Claudia Nose for technical assistance with figures.

**Author Contributions**

Conceived and designed the experiments: RLT SAL RV. Performed the experiments: MGA GLB GC MCA DEPM MCE. Analyzed the data: MGA GLB SAL MCE. Contributed reagents/materials/analysis tools: BL. Wrote the paper: SAL RLT.
References

1. Feldman AM, McNamara D. Myocarditis. N Engl J Med. 2000; 343: 1388–1398. PMID: 11070105

2. Bern C, Montgomery SP, Herwaldt BL, Rassi A Jr, Marin-Neto JA, Dantas RO, et al. Evaluation and treatment of Chagas disease in the United States: a systematic review. JAMA. 2007; 298: 2171–2181. PMID: 1800201

3. Viotti R, Vigliano C, Lococo B, Alvarez MG, Petti M, Bertocchi G, et al. Side effects of benznidazole as treatment in chronic Chagas disease: fears and realities. Expert Rev Anti Infect Ther. 2009; 7: 157–163. doi: 10.1586/14787210.7.2.157 PMID: 19254164

4. Viotti R, Vigliano C, Lococo B, Bertocchi G, Petti M, Alvarez MG, et al. Long-term cardiac outcomes of treating chronic Chagas disease with benznidazole versus no treatment: a nonrandomized trial. Ann Intern Med. 2006; 144: 724–734. PMID: 16702588

5. Fabbro DL, Streger ML, Arias ED, Bizzai ML, del Barco M, Amicone NA. Trypanocide treatment among adults with chronic Chagas disease living in Santa Fe city (Argentina), over a mean follow-up of 21 years: parasitological, serological and clinical evolution. Rev Soc Bras Med Trop. 2007; 40: 1–10.

6. Morrillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A Jr, Rosas F, et al. BENEFIT Investigators. Randomized Trial of Benznidazole for Chagasic Cardiomyopathy. N Engl J Med. 2015; 373: 1295–1306. doi: 10.1056/NEJMoa1507574 PMID: 26323937

7. Viotti R, Vigliano C, Alvarez MG, Lococo B, Petti M, Bertocchi G, et al. Impact of aetiological treatment on conventional and multiplex serology in chronic Chagas disease. PLoS Negl Trop Dis. 2011; 5: e1314. doi: 10.1371/journal.pntd.0001314 PMID: 21909451

8. Fernandes CD, Tiecher FM, Balbinot MM, Liarte DB, Scholl D, Steindel M, et al. Efficacy of benznidazole treatment for asymptomatic chagasic patients from state of Rio Grande do Sul evaluated during a three years follow-up. Mem Inst Oswaldo Cruz. 2009; 104: 27–32. PMID: 19274372

9. Sánchez Negrette O, Sánchez Valdés FJ, Lacunza CD, García Bustos MF, Mora MC, Uncos AD, et al. Serological evaluation of specific-antibody levels in patients treated for chronic Chagas’ disease. Clin Vaccine Immunol. 2008; 15: 297–302. PMID: 18057194

10. Bertocchi GL, Vigliano CA, Lococo BG, Petti MA, Viotti RJ. Clinical characteristics and outcome of 107 adult patients with chronic Chagas disease and parasitological cure criteria. Trans R Soc Trop Med Hyg. 2013; 107: 372–376. doi: 10.1093/trstmh/trt029 PMID: 23612468

11. Machado-de-Assis GF, Diniz GA, Montoya RA, Dias JC, Coura JR, Machado-Coelho GL, et al. A serological, parasitological and clinical evaluation of untreated Chagas disease patients and those treated with benznidazole before and thirteen years after intervention. Mem Inst Oswaldo Cruz. 2013; 108: 873–880. doi: 10.1590/1757-2271201222024037109

12. Albareda MC, Laecolla SA, Alvarez MG, Armenti AH, Bertocchi G, Tarlefon RL, et al. Trypanosoma cruzi modulates the profile of memory CD8+ T cells in chronic Chagas’ disease patients. Int Immunol. 2006; 18: 465–471. PMID: 16431876

13. Alvarez MG, Postan M, Weatherly DB, Albareda MC, Sidney J, Sette A, et al. HLA class I-T cell epitopes from trans-sialidase proteins reveal functionally distinct subsets of CD8+ T cells in chronic Chagas disease. PLoS Negl Trop Dis. 2008; 2: e288. doi: 10.1371/journal.pntd.0000288 PMID: 18846233

14. Cuellar A, Rojas F, Bolaños N, Diez H, Del Carmen Thomas M, Rosas F, et al. Natural CD4(+) T-cell responses against Trypanosoma cruzi KMP-11 protein in chronic chagasic patients. Immunol Cell Biol. 2009; 87: 149–153. doi: 10.1038/icb.2008.76 PMID: 18957935

15. de Araújo FF, Corrêa-Oliveira R, Rocha MO, Chaves AT, Fiuza JA, Fares RC, et al. FOXp3+CD25(high) CD4+ regulatory T cells from indeterminate patients with Chagas disease can suppress the effector cells and cytokines and reveal altered correlations with disease severity. Immunobiology. 2012; 217: 768–777. doi: 10.1016/j.imbio.2012.04.008 PMID: 22672991

16. Lasso P, Mesa D, Cuéllar A, Guzmán F, Bolaños N, Rosas F, et al. Frequency of specific CD8+ T cells for a promiscuous epitope derived from Trypanosoma cruzi KMP-11 protein in chagasic patients. Parasite Immunol. 2010; 32: 494–502. doi: 10.1111/j.1365-3024.2010.01206.x PMID: 20591120

17. Lorena VM, Lorena IM, Braz SC, Melo AS, Melo MF, Melo MG, et al. Cytokine levels in serious cardiopathy of Chagas disease after in vitro stimulation with recombinant antigens from Trypanosoma cruzi. Scand J Immunol. 2010; 72: 529–539. doi: 10.1111/j.1365-3083.2010.02462.x PMID: 21044127

18. Fiuza JA, Fujiwara RT, Gomes JA, Rocha MO, Chaves AT, de Araújo FF, et al. Profile of central and effector memory T cells in the progression of chronic human chagasic disease. PLoS Negl Trop Dis. 2009; 3: e512. doi: 10.1371/journal.pntd.0000512 PMID: 19742301

19. Albareda MC, De Rissio AM, Tomas G, Serjan A, Alvarez MG, Viotti R, et al. Polyfunctional T cell responses in children in early stages of chronic Trypanosoma cruzi infection contrast with monofunctional responses of long-term infected adults. PLoS Negl Trop Dis. 2013; 7: e2575. doi: 10.1371/journal.pntd.0002575 PMID: 24349591
20. Lasso P, Mateus J, Pavia P, Rosas F, Roa N, Thomas MC, et al. Inhibitory Receptor Expression on CD8+ T Cells Is Linked to Functional Responses against Trypanosoma cruzi Antigens in Chronic Chagasic Patients. J Immunol. 2015; 195: 3748–3758. doi: 10.1049/jimmunol.1500459 PMID: 26385520

21. Mateus J, Lasso P, Pavia P, Rosas F, Roa N, Valencia-Hernández CA, González JM, Puerta CJ, Cuéllar A. Low frequency of circulating CD8+ T stem cell memory cells in chronic chagasic patients with severe forms of the disease. PLoS Negl Trop Dis. 2015; 9: e3432. doi: 10.1371/journal.pntd.0003432 PMID: 25569149

22. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who’s who of T-cell differentiation: human memory T-cell subsets. Eur J Immunol. 2013; 43: 2797–2809. doi: 10.1002/eji.201343751 PMID: 24258910

23. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. Cell. 2009; 138: 30–50. doi: 10.1016/j.cell.2009.06.036 PMID: 19596234

24. Kahan SM, Wherry EJ, Zajac AJ. T cell exhaustion during persistent viral infections. Virology. 2015; 479–480: 180–193. doi: 10.1016/j.virol.2014.12.033 PMID: 25620767

25. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interrupts. Semin Immunol. 2014; 26: 559–577. doi: 10.1016/j.smim.2014.09.003 PMID: 25311810

26. Gigley JP, Bhadra R, Moretto MM, Khan IA. T cell exhaustion in protozoan disease. Trends Parasitol. 2012; 28: 377–384. doi: 10.1016/j.pt.2012.07.001 PMID: 22832368

27. Rodrigues Vasco, Anabela Cordeiro-da-Silva, Mireille Laforge, Ouaisi Ali, Akharid Khadija, Silvestre Ricardo, Je ro’me Estaquier. Impairment of T Cell Function in Parasitic Infections. PLOS Neglected Tropical Diseases. 2014; 8: e2567. doi: 10.1371/journal.pntd.0002567 PMID: 24551250

28. Argüello RJ, Albareda MC, Alvarez MG, Bertocchi G, Armenti AH, Vigliano C, et al. Inhibitory receptors are expressed by Trypanosoma cruzi-specific effector T cells and in hearts of subjects with chronic Chagas disease. PLoS One. 2012; 7: e35966. doi: 10.1371/journal.pone.0035966 PMID: 22574131

29. Albareda MC, Perez-Mazliah D, Natale MA, Castro-Eiro M, Alvarez MG, Viotti R, et al. Perturbed T cell IL-7 receptor signaling in chronic Chagas disease. J Immunol. 2015; 194: 3883–3889. doi: 10.4049/jimmunol.1402202 PMID: 25769928

30. Laucella SA, Mazliah DP, Bertocchi G, Alvarez MG, Cooley G, Viotti R, et al. Changes in Trypanosoma cruzi-specific immune responses after treatment: surrogate markers of treatment efficacy. Clin Infect Dis. 2009; 49:1675–1684. doi: 10.1086/648072 PMID: 19877967

31. World Health Organization. Control of Chagas disease. World Health Organ Tech Rep Ser. 2012; 975: 1–116.

32. Kuschnir E, Sgammini H, Castro R, Eavequoz C, Ledesma R, Brunetto J. Evaluation of cardiac function by radioisotopic angiography, in patients with chronic Chagas cardiopathy. Arq Bras Cardiol. 1985; 45: 249–256. PMID: 3835868

33. Laucella SA, Postan M, Martin D, Hubby Fralish B, Albareda MC, Alvarez MG, et al. Frequency of interferon-gamma-producing T cells specific for Trypanosoma cruzi inversely correlates with disease severity in chronic human Chagas disease. J Infect Dis. 2004; 189: 909–918. PMID: 1516/j.infdis/jit420 PMID: 23945371

34. Pinazo MJ, Thomas MC, Bua J, Perrone A, Schijman AG, Viotti R, et al. Biological markers for evaluation of effective serodiagnostics for Trypanosoma cruzi infection. PLoS Negl Trop Dis. 2008; 2: e316. doi: 10.1371/journal.pntd.0000316 PMID: 18841200

35. Sosa-Estani S, Viotti R, Segura EL. Therapy, diagnosis and prognosis of chronic Chagas disease: insight gained in Argentina. Mem Inst Oswaldo Cruz. 2009; 104 (Suppl. I): 167–180.

36. Pinazo MJ, Thomas MC, Bua J, Perrone A, Schijman AG, Viotti RJ, et al. Biological markers for evaluating therapeutic efficacy in Chagas disease, a systematic review. Expert Rev Anti Infect Ther. 2012; 10: 479–496. doi: 10.1586/14787210.2012.491689 PMID: 22462125

37. Fabbro D, Velazquez E, Bizal ML, Denner S, Olivera V, Arias E, et al. Evaluation of the ELISA-F29 test as an early marker of therapeutic efficacy in adults with chronic Chagas disease. PLoS Negl Trop Dis. 2008; 2: e316. doi: 10.1371/journal.pntd.0000316 PMID: 18841200

38. Bustamante JM, Bixby LM, Tarleton RL. Drug-induced cure drives conversion to a stable and protective CD8+ T central memory response in chronic Chagas disease Nat Med. 2008; 14: 542–550. doi: 10.1038/nm1744 PMID: 18425131

39. Argüello RJ, Albareda MC, Alvarez MG, Bertocchi G, Armenti AH, Vigliano C, et al. Inhibitory receptors are expressed by Trypanosoma cruzi-specific effector T cells and in hearts of subjects with chronic Chagas disease. PLoS One. 2012; 7: e35966. doi: 10.1371/journal.pone.0035966 PMID: 22574131

40. Behar SM, Carpenter SM, Booty MG, Barber DL, Jayaraman P. Orchestration of pulmonary T cell immunity during Mycobacterium tuberculosis infection: immunity interrupts. Semin Immunol. 2014; 26: 559–577. doi: 10.1016/j.smim.2014.09.003 PMID: 25311810

41. World Health Organization. Control of Chagas disease. World Health Organ Tech Rep Ser. 2012; 975: 1–116.
41. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O’rie T, et al. Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. J Immunol. 2011; 187: 2222–2232. doi:10.4049/jimmunol.1101122 PMID: 21775682

42. Subauste CS, Wessendarp M, Smulian AG, Frame PT. Role of CD40 ligand signaling in defective type 1 cytokine response in human immunodeficiency virus infection. J Infect Dis. 2001; 183: 1722–1731. PMID: 11372024

43. Vanham G, Penne L, Devalck J, Kestens L, Colebunders R., Bosmans, K E. et al. Decreased CD40 ligand induction in CD4 T cells and dysregulated IL-12 production during HIV infection. Clin. Exp. Immunol. 1999; 117: 335–342. PMID: 10444266

44. Nyakeriga AM, Ying J, Shire NJ, Fichtenbaum CJ, Chougnet CA. Highly active antiretroviral therapy in patients infected with human immunodeficiency virus increases CD40 ligand expression and IL-12 production in cells ex vivo. Viral Immunol. 2011; 24: 281–289. doi: 10.1089/vim.2010.0142 PMID: 21830900

45. Sathier-Avelar R, Vitelli-Avelar DM, Massara RL, de Lana M, Pinto Dias JC, Teixeira-Carvalho A, et al. Etiological treatment during early chronic indeterminate Chagas disease incites an activated status on innate and adaptive immunity associated with a type 1-modulated cytokine pattern. Microbes Infect. 2008; 10: 103–113. doi: 10.1016/j.micinf.2007.10.009 PMID: 18248755

46. Sathier-Avelar R, Vitelli-Avelar DM, Eloi-Santos SM, Gontijo ED, Teixeira-Carvalho A, Martins-Filho AO. Blood leukocytes from benznidazole-treated indeterminate chagas disease patients display an overall type-1-modulated cytokine profile upon short-term in vitro stimulation with Trypanosoma cruzi antigens. BMC Infect Dis. 2012; 12: 123. doi: 10.1186/1471-2253-12-123 PMID: 22825224

47. Bahia-Oliveira LM, Gomes JA, Cançado JR, Ferrari TC, Lemos EM, Luz ZM, et al. Immunological and clinical evaluation of chagasic patients subjected to chemotherapy during the acute phase of Trypanosoma cruzi infection 14–30 years ago. J Infect Dis. 2000; 182: 634–638. PMID: 10915103

48. Ferraz ML, Gazzinelli RT, Alves RO, Urbina JA, Romana AJ. Absence of CD4+ T lymphocytes, CD8+ T lymphocytes, or B lymphocytes has different effects on the efficacy of posaconazole and benznida- zole in treatment of experimental acute Trypanosoma cruzi infection. Antimicrob Agents Chemother. 2009; 53: 174–179. doi: 10.1128/AAC.00779-08 PMID: 19001113

49. Michailowsky V, Murta SM, Carvalho-Oliveira L, Pereira ME, Ferreira LR, Brener Z, et al. Interleukin-12 Enhances In Vivo Parasiticidal Effect of Benznidazole during Acute Experimental Infection with a Naturally Drug-Resistant Strain of Trypanosoma cruzi. Antimicrob Agents Chemother. 1998; 42: 2549–2556. PMID: 9756574

50. Romana AJ, Alves RO, Murta SM, Silva JS, Ropert C, Gazzinelli RT. Experimental chemotherapy against Trypanosoma cruzi infection: essential role of endogenous interferon-gamma in mediating parasitologic cure. J Infect Dis. 2002; 186: 823–828. PMID: 12198617

51. Toledo MJO, Machado GBN, Pereira MES, Brener Z. Results of treatment in mice immunosuppressed inoculated with different Trypanosoma cruzi strains. Mem Inst Oswaldo Cruz. 1991; 86: 237