Changes in *Trypanosoma cruzi*-specific immune responses following treatment: surrogate markers of treatment efficacy

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Abstract

**Background**—As many as 20 million people are living with *Trypanosoma cruzi* infection in Latin America, yet few receive any treatment. The major limitation in developing and evaluating potential new drugs for their efficacy is the lack of reliable tests to assess parasite burden and elimination.

**Methods**—Adults volunteers with chronic *T. cruzi* infection were evaluated clinically and stratified according to the Kuschnir classification. Individuals in group 0 and group 1 clinical status were treated with 5 mg benznidazole/kg/day for 30 days. The changes in *T. cruzi*-specific T cell and antibody responses, as well as in clinical status, were measured periodically over the 3-5 year follow-up period and compared to pre-treatment conditions and to an untreated control group.

**Results**—The frequency of peripheral IFN-γ-producing T cells specific for *T. cruzi* declined as early as 12 months after BZ treatment and subsequently became undetectable in a substantial proportion of treated subjects. Additionally decreases in antibody responses to a pool of recombinant *T. cruzi* proteins also declined in many of these same subjects. The shift to negative IFN-γ T cell responses was highly associated with an early increase in IFN-γ-producing T cells with phenotypic features of effector/effector memory cells in a subset of subjects. Benznidazole treatment also resulted in an increase in naïve and early differentiated memory-like CD8+ T cells in a majority of subjects.

**Conclusions**—BZ treatment during chronic Chagas disease has a substantial impact on parasite-specific immune response that is likely to be indicative of treatment efficacy and cure.
Introduction

The most serious long term sequela of chronic *T. cruzi* infection is the development of a persistent inflammatory cardiomyopathy that may lead to congestive heart failure and death. Although therapy with nitroimidazole derivatives is recommended in both acute and early chronic phases of *T. cruzi* infection (1-4) treatment in longer term infections is more controversial, despite the fact that follow-up of individuals treated with benznidazole (BZ) decades after the initial infection demonstrated significant protection from progression of heart pathology due to Chagas disease (5-8).

A defining feature of memory T cells generated following clearance of acute infections is long-term, antigen-independent persistence mediated by homeostatic turnover (9,10). During chronic infections, however, specific antigen has been shown to be essential for maintenance of CD8+ T cells specific for various persisting viruses (11-12) and this repeated antigen stimulation may lead to functional exhaustion or even physical deletion of T cells (13-20). We have shown that individuals chronically infected with *T. cruzi* display a functional profile of IFN-γ only secreting T cells, characteristic of effector/effector memory T cells (T_E/T_EM) (21), and increased frequency of fully differentiated memory CD8+ T cells generally associated with long-term antigen persistence and exhausted T cells (22).

In this study, we sought to gain a clearer understanding of the relationship between parasite persistence and the maintenance of *T. cruzi*-specific T cells during chronic Chagas disease by examining the effect of treatment with BZ on the frequency, function and phenotype of general and *T. cruzi*-specific T cells in chronically-infected subjects treated with BZ 3-5 years previously. We demonstrate that *T. cruzi*-specific T cell responses declined in association with decreases in antibody responses to a pool of recombinant proteins from *T. cruzi* and increases in CD8+ T cells with early differentiated/antigen experienced phenotype in a substantial proportion of treated subjects but not in the untreated group.

Methods

Selection of study population

*T. cruzi*-infected adults volunteers aged 21 to 54 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 ELISA techniques (23) performed at the Instituto Nacional de Parasitología “Dr. Mario Fatala Chaben”. Chronically infected subjects were evaluated clinically and stratified according to the Kuschnir grading system (24). Group 0 individuals with normal electrocardiography, normal chest-X ray and normal echocardiography (G0; n= 67, mean age 38.68 yrs (range= 23-55) and group 1 subjects with normal chest-x and echocardiography ray but abnormalities in the ECG (G1; n= 8, mean age= 43.88 yrs (range= 32-52) were selected for inclusion in the study.

Treatment consisted of 5 mg benznidazole/kg/day for 30 days (5-7). Subjects in G0 clinical group were assigned randomly to the treated and untreated group; the G1 group (8 subjects) were all assigned to the treated group based upon previous studies demonstrating clear evidence of the efficacy of treatment on progression of disease in this subject group (5,7). Clinical, serological and immunological analysis was performed prior to treatment, and at two, six and 12 months post-treatment (PT) and yearly intervals thereafter.

This protocol was approved by the Institutional Review Boards of the University of Georgia, and the Hospital Interzonal General de Agudos “Eva Perón”. Signed informed consent was obtained from all individuals prior to inclusion in the study.
Collection of PBMC and sera

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficcol-hypaque (Amershan) and were cryopreserved 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 IL-2 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 (BD, San Diego, CA) as previously described (21,25,29). To avoid inter-experiment variations, assays were conducted with paired samples from different time points. Each time point was assessed between one and three times.

Flow cytometric detection of *T. cruzi* antigen-induced intracellular IFN-γ and T cell phenotyping

IFN-γ production was determined after stimulation of PBMC with 15 µg/ml of *T. cruzi* lysate or media alone, for 16-20 h with the addition of 10 µg/ml brefeldin A for the last five hours of incubation as previously described (22,25). The cells were then stained with anti CD4 (PerCP) or (FITC) with the appropriate combination of anti CCR7 (PE), anti KLRG1 (APC), anti CD122 (PE), anti-CD127 (PE) and IFN-γ (APC) or IFN-γ (PE) all from BD-Pharmingen.

Data were acquired on a FACS Calibur cytometer (Becton Dickinson) and analyzed with CellQuest software (Becton & Dickinson). Typically, 500,000 events were collected per sample.

Multiplex serodiagnostic assay

Sera were screened for antibodies reactive to a panel of 14 recombinant *T. cruzi* proteins in a Luminex-based format as previously described (27). Serological responses to each individual *T. cruzi* protein were considered to have decreased during the study period if the mean fluorescence intensity (MFI) decreased by >50% relative to that of the time 0 (pre-treatment) sample assayed concurrently.

Statistical analysis—Comparisons on the frequencies of IFN-γ-producing T cells and on the percentages of CD4+ or CD8+ T cells expressing different phenotypic markers were performed by Mann-Whitney U test on post-treatment/pre-treatment differences between treated and untreated groups. The proportion of subjects for whom IFN-γ ELISPOT or B cell responses decreased over time in the treated and untreated groups was compared by the Fisher's exact test. T cell responses at different time points were compared by Friedman range test. A Spearman correlation test was applied to analyze the association between the frequency of IFN-γ-T cells and serological titers. Differences were considered to be statistically significant at P<0.05.

Results

IFN-γ and IL-2 responses following treatment with BZ

We have monitored IFN-γ ELISPOT responses in 43 chronically *T. cruzi*-infected subjects treated with BZ and a group of 32 untreated individuals followed over a three-five year period. Subjects were grouped according to pre-treatment/baseline IFN-γ ELISPOT responses to an amastigote lysate into those with IFN-γ ELISPOT responses above background levels (responder subjects) and those with negative IFN-γ ELISPOT responses (non-responders; Table 1). There were no significant differences in the magnitude of the baseline IFN-γ ELISPOT responses between treated and untreated subjects either considering responder and
non-responder subjects (p= 0.2) or including responder subjects only (p= 0.10). Within 12 months post-treatment with a 30 day course of BZ, the frequency of IFN- \( \gamma \)-producing T cells specific for \( T. cruzi \) significantly decreased in the treated responder group as compared to the untreated group, who display relatively stable numbers of \( T. cruzi \)-responsive T cells (Figure 1).

To determine the earliest time point at which the decrease in \( T. cruzi \)-responsive T cells could be observed, IFN- \( \gamma \) responses were also measured at two and six months post-treatment in a subset of subjects. Surprisingly, a proportion of BZ-treated (7/19 evaluated) but not untreated (0/7) subjects exhibited an initial increase in IFN- \( \gamma \) responses prior to the decrease at 12 months (Figure 2). This same pattern of transient increase in T cell responses followed by its decay was also observed in three out of seven subjects who were negative for \( T. cruzi \)-induced IFN- \( \gamma \) producing cells prior to treatment (i.e. non-responder subjects PP19, PP21 and PP385; Figure 3). The monitoring of IFN- \( \gamma \) production in a subset of subjects at multiple time points both prior to and after BZ treatment further corroborates the impact of treatment on parasite-specific T cell responses, demonstrating relatively stable responses pre-treatment followed by dramatic decreases at 12-36 months post-treatment in three of four subjects (subjects PP118, PP06 and PP277; Figure 4). The majority of the IFN- \( \gamma \) producing CD4\(^+\) T cells at two or six months post-treatment were CD122 lo, CCR7 lo, KLRG1lo, while a proportion of these CD4\(^+\) IFN- \( \gamma \) cells express CD127 (Figure 5 A and B), consistent with an \( T_E \) or \( T_{EM} \) phenotype. Cumulatively, IFN- \( \gamma \) ELISPOT responses fell below the level of detection (47%) or decreased substantially (25%) in the majority of BZ treated individuals but was unaltered in non-treated subjects (Table 2).

BZ treatment did not alter the previously reported infrequent detection of IL-2 producing T cells responsive to \( T. cruzi \) in these subjects (21). However in individuals with IL-2 producing cells, the frequency of these cells changed in concert with IFN- \( \gamma \) \( T \) cell responses (Figure 4), demonstrating that the alteration in T cell responses generated by therapeutic treatment was not restricted to the production of a single cytokine. Interestingly, three subjects who became negative for both IFN- \( \gamma \) and IL-2 responses by 12-24 months post-treatment showed a rebound in cells producing both cytokines by 36 months post treatment (subjects PP01, PP120 and PP179; Figure 4).

Taken together, these results show that BZ treatment induced substantial changes in \( T. cruzi \)-specific T cell responses in a significant proportion of BZ treated subjects, suggestive of a decrease in antigen load, and possibly, parasite clearance.

**Changes in serologic and clinical status after BZ treatment**

Not surprisingly, given the relatively short follow-up period, the changes in the frequency of \( T. cruzi \)-specific IFN- \( \gamma \)-producing T cells correlated poorly with the titers of anti-\( T. cruzi \) antibodies as measured using conventional (5,7,8) assays (data not shown); in only six of 43 treated subjects did conventional serological tests became negative during the follow-up period and in five out of these six subjects IFN- \( \gamma \)-producing T cells also decreased to below background levels. Additionally, only three subjects exhibited progression in the assessment of disease severity over the follow-up period, two in the BZ-treated group and one in the untreated group. However in sharp contrast to conventional serologic tests, the changes in serologic status determined by a recently developed multiplex serodiagnostic assay (27) showed very strong correlation with both the treatment group and with changes in T cell responses post-treatment (Table 3). Antibody titers to 14 recombinant \( T. cruzi \) proteins were essentially unchanged in untreated subjects (Figure 6A and E) with only two of 30 subjects followed for 12-36 months showing slight alterations over time. Alternatively, over one-half of the treated subjects showed a >50% decrease in reactivity to at least one of the possible 14
target proteins (Figure 6C, D, F). Collectively, 33 of the 43 treated subjects showed significant changes in either or both T cell and B cell responses consistent with a change in infection status (Table 3).

Discussion

This study aimed to tackle the major limitation of treatment in the chronic phase of Chagas disease, the assessment of treatment efficacy, by measuring changes in parasite-specific T cell responses. To our knowledge, this is the first report describing the monitoring of T. cruzi-specific T cell responses after BZ treatment of chronically infected adult subjects and the first to clearly associate alterations in T and B cell responses with proposed treatment efficacy. We found that the frequency of T. cruzi-specific IFN-γ-producing T cells decreased within one year after BZ treatment in the majority of subjects and was below the level of detection in nearly 50% of treated individuals. Similar changes in T cell responses were not evident either prior to treatment in the same individuals or in untreated subjects over the same observation time. Additionally, changes in T cell responses were highly correlated with decreases in serological responses as determined using a multiplex assay, but not as assessed by conventional serology. Together these data argue strongly that BZ treatment has a significant impact on chronic T. cruzi infection in humans.

The most straightforward interpretation of these results is that BZ treatment decreases parasite load, thus diminishing the antigen necessary to activate T. cruzi-specific T cells and B cells. It is also reasonable to hypothesize that the treatment actually cures T. cruzi infection in a substantial number of treated subjects. Similar positive effects of BZ treatment has been well-documented in animal models and in early/acute infections (1-4,29,30) but it has been argued that treatment is ineffective in chronically infected humans (31,32). However the conclusion that drug treatment in chronic Chagas disease is ineffective is based largely on the lack of effective means to assess treatment efficacy rather than actual data demonstrating the lack of efficacy. Indeed, a number of clinical studies with 8-10 year follow-up periods (5,7,8) have shown that drug treatment in the chronic phase of T. cruzi infection significantly decreases the progression of clinical disease. Although these studies are criticized because they are not randomized or controlled, they nonetheless are highly suggestive of cure of the infection in a substantial number of drug treated subjects – something that is rarely observed in the absence of drug treatment.

Unfortunately there is no ethical way to determine with 100% certainty the efficacy of drug treatment in chronic human T. cruzi infection. We have used immunosuppression with cyclophosphamide to demonstrate that BZ can achieve parasitological cure of mice with chronic T. cruzi infections (28). However, because direct detection of T. cruzi or its productions or constituents (e.g. DNA, proteins) is unreliable in chronically infected hosts, including humans (33-35) these same measures are not informative with respect to the determining of the effectiveness of any treatment. Thus assessment of treatment efficacy has generally relied upon measurement of disease progression and conventional serological responses – both of which require up to a decade post-treatment to effectively evaluate. Indeed in this study we could not correlate changes in conventional serology or clinical status with the other immunological changes noted at earlier time points post-treatment. However previous studies by our group using the identical treatment protocol and similar patient populations found a significant decrease in progression toward disease in BZ-treated subjects relative to untreated subjects at eight-ten years post-treatment (5,7). In contrast to the results herein, a recent study from Fernandez et. al. (36) reported a very high failure rate of treatment – based upon positive PCR-based detection of T. cruzi both prior to and after treatment. However, all subjects became hemaculture negative after treatment and showed consistently falling serological titers over
the 3 year follow-up period, both indicative of a decreasing parasite load in these subjects rather than complete treatment failure.

We attribute the decline in *T. cruzi*-specific T cell responses post-BZ treatment to decreased parasite antigen needed to drive antigen-dependent effector T cells. Interestingly, the reduction in *T. cruzi*-specific T cells was often preceded by a rise in T_E/T_EM IFN-γ-producing cells early after treatment, perhaps due to the release of parasite antigens as a consequence of drug-induced parasite death. Although this response was not documented in all subjects, this may have been the result of infrequent sampling.

A subset of subjects also showed a rebound in parasite-specific T cell responses very late (>24 months) post-treatment. Unfortunately, the frequency of these cells has not been sufficiently high to allow for the collection of reliable phenotyping data. However, this rebound was associated with falling serological titers by the multiplex assay and thus it seems likely these parasite-specific T cells are maintained in the absence of antigen – a characteristic of T_CM, as shown in mice that are cured of *T. cruzi* infection (28). Ultimately a metric such as a high percentage of T_CM phenotype cells among the parasite-specific T cell population (when they can be measured) may be the best surrogate for assessing treatment efficacy in individual patients.

One of the drawbacks to the assessment of *T. cruzi*-specific T cell responses as an indicator of treatment efficacy in chronically infected subjects is the fact that these responses were below the level of detection in nearly 1/4 of these individuals. This result is consistent with previous studies showing low and often undetectable parasite specific T cell responses (21,25) along with a differentiated status of the overall CD8+ T cell pool (24) in individuals with decades long infections with *T. cruzi*, indicating a more general dysfunction of the immune system during chronic infections as a consequence of persistent activation (13,14,18,37).

Based upon the substantial changes in immunological responses as well as the arrest in disease progression reported in previous studies (5,7), we conclude that the protocol for BZ treatment used in these subjects is effective in altering the course of infection and likely achieves parasitological cure in up to 3/4 of subjects with decades long infections. Importantly, this estimate is in line with the 76-83 percent reduction in the relative risk of progression in BZ-treated subjects reported in several studies (6,38,39) including those using a similar patient group and an identical treatment protocol to that used in the current study (5,7).

Nevertheless, BZ treatment is not uniformly effective, as immunological changes are not observed in all treated individuals and progression to more severe disease continues post treatment in a small proportion of cases (5,7). This result is not surprising – various *T. cruzi* isolates are differentially susceptible to the actions of BZ and even among inbred mice infected with the identical dose and strain of *T. cruzi*, not all animals cure when treated with a shorter course (<40 days) of BZ (28).

It will be both important and interesting to continue to follow the subjects in this study to determine if the observed early changes in immunological parameters we report here correlate with long-term disease progression. However in the interim, we suggest that changes in *T. cruzi*-specific T cell responses and in antibody responses to individual parasite proteins can be used as early and effective predictors of the effectiveness of drug treatment in human Chagas disease. Furthermore, these results strongly challenge the contention that drug therapy is ineffective in chronic Chagas disease and provide additional support for the wider use of existing drugs, despite their imperfections, to treat the millions of individuals who are infected and who are likely to develop clinical disease (40,41). As importantly, we believe the tools
developed and assessed in this study will help to remove a major barrier to the development and testing of improved drugs for the treatment of *T. cruzi* infection.

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**References**


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Figure 1.
Effect of treatment with BZ on T. cruzi-specific T cell responses in chronic infection. IFN-γ-producing T cells specific for a parasite lysate/1×10^6 PBMC were determined by ELISPOT at baseline and 12 months after with BZ. Each line represents an individual subject. (*) P = 0.041 Mann-Whitney U test on PT/pre-treatment differences between treated and untreated groups as described in the Materials and Methods.
Monitoring of IFN-γ ELISPOT responses in chronic Chagas disease subjects with positive ELISPOT responses at baseline. IFN-γ-producing T cells were measured at different time points following BZ treatment or enrolment (for untreated subjects). Plots represent the data for single subjects from a selected group. IFN-γ-secreting T cells significantly increased at 2-6 months post treatment and decreased thereafter as determined by Friedman range test (P=0.012).

Figure 2.
Monitoring of \textit{T. cruzi}-specific IFN-\(\gamma\) ELISPOT responses in subjects with negative ELISPOT responses at baseline. IFN-\(\gamma\)-producing T cells were measured at different time points following BZ treatment or enrollment (for untreated subjects). Plots show representative data for single subjects from a selected group. IFN-\(\gamma\)-secreting T cells significantly increased at 2-6 months post treatment and decreased thereafter as determined by Friedman range test (P=0.012). The horizontal line indicates the threshold for positive/negative response.
Figure 4.
Monitoring of IFN-$\gamma$ and IL-2 ELISPOT responses in chronic Chagas disease subjects following treatment with BZ. IFN-$\gamma$ (solid line) and IL-2 (broken line)-producing T cells were measured at different time points of follow-up in BZ-treated and untreated subjects. Time “0” indicates assay point just prior to BZ treatment. Plots show representative data for single subjects whose IFN-$\gamma$ ELISPOT responses became negative during follow-up (PP06), decreased > 3 fold, showed a rebound (PP01, PP120, PP179) or remained stable (PP96, PP100 and RD31). The criteria for determining “positive responses are defined in Materials and Methods.
Figure 5.
Phenotype of *T. cruzi*-specific CD4+ T cells early after treatment with BZ. A. Distribution of CD122, CD127, CCR7 and KLRG1 expression on CD4+ IFN-γ+ T cells after stimulation with a *T. cruzi* lysate. A gate was set on total CD4+ T cells with side-scatter and forward-scatter light for lymphocytes. The percentage of CD4+ IFN-γ+ T cells with low (upper left quadrant) or high (upper right quadrant) expression for each marker is indicated. B. Mean ±SE of cumulative data on the proportion of *T. cruzi*-specific CD4+ IFN-γ+ T cells within the different phenotypic cell populations 6 months following treatment with BZ in 7 *T. cruzi*-infected subjects. The percentage of CD4+ IFN-γ+ T cells expressing each marker is shown. (*) P < 0.0001 vs CD122, CCR7 and KLRG-1; (#) P < 0.0001 vs CCR7 and CD122.
Figure 6.
Multiplex analysis of anti-\( T. cruzi \) antibodies before and after treatment. Sera obtained at the indicated time points were screened using a bead array-based multiplex serologic assay against 14 recombinant \( T. cruzi \) proteins (Antigens 1-14) as well as a negative control protein (green fluorescent protein, GFP) and an \( T. cruzi \) amastigote lysate, as previously described in detail (28). Subject not treated (A) or treated (B) with BZ (at time \( t = 0 \)) did not exhibit a consistent change in antibody levels over 36 and 31 months, respectively. Different patterns of alterations in antibody responses was observed in treated subjects, including changes in antibodies specific for all (C), or just some (D), of the \( T. cruzi \) proteins. Subject PP118 had a stable pattern of antibodies for 12 months prior to treatment (E) but showed a steady decline in responses to most proteins apparent within 12 months following treatment (F). * no data – insufficient numbers of beads recovered for accurate measurement.
Table 1

Characteristics of study population according to IFN-γ ELISPOT responses prior to treatment with BZ.

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>Treated IFN-γ ELISPOT</th>
<th>Untreated IFN-γ ELISPOT</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Responder</td>
<td>Non-responder</td>
</tr>
<tr>
<td>G0</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>G1</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>11</td>
</tr>
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</table>

a G0, seropositive individuals with normal findings on ECGs and chest radiographs; G1, seropositive patients with normal chest radiographs but abnormal findings on ECGs according to Kuschnir classification (40).

b IFN-γ ELISPOT responses above background levels at initiation of study.

c IFN-γ ELISPOT responses below background levels at initiation of study.
Table 2
Cumulative decreases in IFN-γ T cell responses after treatment with BZ.

<table>
<thead>
<tr>
<th>Months post enrollment</th>
<th>BZ treated</th>
<th>Not Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative IFN-γ ELISPOT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;3 fold-decrease&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>5/26</td>
<td>4/26</td>
</tr>
<tr>
<td>24</td>
<td>8/32</td>
<td>7/32</td>
</tr>
<tr>
<td>36</td>
<td>15/32</td>
<td>8/32</td>
</tr>
</tbody>
</table>

<sup>a</sup>IFN-γ ELISPOT responses were below detectable levels at one or more points after BZ treatment.

<sup>b</sup>IFN-γ ELISPOT responses decreased by >3-fold relative to pre-treatment levels.

<sup>c</sup><i>P</i> = 0.04, vs. not treated (Fisher’s exact test).

<sup>d</sup><i>P</i> = 0.0001, vs. not treated (Fisher’s exact test).

<sup>e</sup><i>P</i> = 0.00001, vs. not treated (Fisher’s exact test).
### Table 3

Correlation of decreases in T cell responses to changes in serological responses following BZ treatment.

<table>
<thead>
<tr>
<th></th>
<th>BZ-treated</th>
<th>Non-treated</th>
<th>Stats $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease in both ELISPOT responses and serology $^b$</td>
<td>21</td>
<td>0</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Decrease in ELISPOT responses only $^c$</td>
<td>6</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Decrease in serology only $^d, e$</td>
<td>6</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>No decrease in ELISPOT or serology $^f, g$</td>
<td>7</td>
<td>30</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>Insufficient data/complex pattern $^h$</td>
<td>3</td>
<td>0</td>
<td>ns</td>
</tr>
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<table>
<thead>
<tr>
<th></th>
<th>33/43 (74%)</th>
<th>2/32 (6%)</th>
<th>$&lt; 0.0001$</th>
</tr>
</thead>
</table>

$^a$ Fisher’s exact test analysis between BZ-treated and non-treated groups was performed. ns indicates non-significant differences.

$^b$ Decrease at one or more points post treatment to below detection limit or >3-fold relative to pretreatment level for ELISPOT and >50% decrease in mean florescence intensity for 1 or more recombinant T. cruzi protein in the 14 protein multiplex panel.

$^c$ Decrease to below detection limit or >3-fold relative to pretreatment level but changes in serology response do not meet 50% decrease cutoff.

$^d$ >50% decrease in mean florescence intensity for 1 or more recombinant T. cruzi protein in the 14 protein multiplex panel but changes in ELISPOT responses do not drop below level of detection or decrease by >3-fold.

$^e$ Includes 2 subjects for whom changes in ELISPOT responses were not determined at months 2 and 6 post treatment. and/or who had undetectable ELISPOT responses at all time points.

$^f$ Changes in ELISPOT responses do not drop below level of detection or decrease by >3-fold and decreases in serology response do not meet 50% cutoffs.

$^g$ Includes 4 subjects for whom changes in ELISPOT responses were not determined at months 2 and 6 post treatment and/or who had undetectable ELISPOT responses at all time points.

$^h$ Insufficient serological samples and/or follow-up period was <24 months; ELISPOT responses not measured at 2 and 6 months post-treatment and or not evident throughout.