Sequential combined treatment with allopurinol and benznidazole
in the chronic phase of Trypanosoma cruzi infection: a pilot study


1Instituto Nacional de Parasitologı´ a ‘Dr. Mario Fataла Chaben’, Av. Paseo Colon 568, Buenos Aires (1063), Argentina; 2Chagas’ Disease Section, Hospital Interzonal General de Agudos ‘Eva Pero´ n’, Av. Ricardo Balbı´ n 3170, Provincia de Buenos Aires (1650), Argentina; 3Center for Tropical and Emerging Global Diseases, Coverdell Center for Biomedical Research, University of Georgia, 500 D.W. Brooks Drive, Athens, GA 30602, USA

*Corresponding author. Tel: +(5411) 4331-7732/233; Fax: +(5411) 4331-7142; E-mail: slaucella@yahoo.com
†These authors contributed equally to this work.

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Objectives: Even though the use of combined drugs has been proved to be effective in other chronic infections, assessment of combined treatment of antiparasitic drugs in human Chagas’ disease has not been performed. Herein, a pilot study was conducted to evaluate the tolerance and side effects of a sequential combined treatment of two antiparasitic drugs, allopurinol and benznidazole, in the chronic phase of Trypanosoma cruzi infection.

Patients and methods: Changes in total and T. cruzi-specific T and B cells were monitored during a median follow-up of 36 months. Allopurinol was administered for 3 months (600 mg/day) followed by 30 days of benznidazole (5 mg/kg/day) in 11 T. cruzi-infected subjects.

Results: The combined sequential treatment of allopurinol and benznidazole was well tolerated. The levels of T. cruzi-specific antibodies significantly decreased after sequential combined treatment, as determined by conventional serology and by a multiplex assay using recombinant proteins. The frequency of T. cruzi-specific interferon-γ-producing T cells significantly increased after allopurinol treatment and decreased to background levels following benznidazole administration in a substantial proportion of subjects evaluated. The levels of total naive (CD45RA+CCR7+CD62L+) CD4+ and CD8+ T cells were restored after allopurinol administration and maintained after completion of the combined drug protocol, along with a decrease in T cell activation in total peripheral CD4+ and CD8+ T cells.

Conclusions: This pilot study shows that the combination of allopurinol and benznidazole induces significant modifications in T and B cell responses indicative of a reduction in parasite burden, and sustains the feasibility of administration of two antiparasitic drugs in the chronic phase of Chagas’ disease.

Keywords: Chagas’ disease, trypanosomiasis, chemotherapy, T cells

Introduction

Chagas’ disease, caused by the intracellular protozoan parasite Trypanosoma cruzi, affects around 10 million people from Southern California to South America and in Western Europe.1–3 In 20%–30% of infected individuals, the disease results in heart disease or megaesophagus/megacolon, making Chagas’ disease the most common cause of infectious myocarditis in the world.4

Chemotherapy with the 5-nitro-furan nifurtimox or the 2-nitro-imidazole benznidazole is recommended in both acute and early chronic phases of T. cruzi infection.5,6 Several studies have also demonstrated the benefits of chemotherapy with benznidazole in adults with chronic T. cruzi infection7–10 and, based on this evidence, experts advocate that treatment in adults up to 50 years without advanced heart disease should be generally offered.1 Allopurinol has also been used to treat chronically T. cruzi-infected subjects with variable efficacy.11–15 Gallerano et al.14 found that allopurinol was as efficacious as nitrofurans in eliminating the parasitaemia and rendering patients seronegative in the chronic phase of T. cruzi infection, while adverse reactions were significantly less frequent in subjects treated with allopurinol. In another study, parasitologic- al cure was evident in 44% of the subjects treated with
allopurinol.\textsuperscript{11} Conversely, allopurinol was reported not to be effective in eliminating the persistence of circulating parasites in chronically infected subjects, as evaluated by xenodiagnosis and conventional serological tests.\textsuperscript{15}

Although currently drugs used to treat \textit{T. cruzi} infection have clear efficacy, there is a need for new drugs with higher efficacy and fewer side effects. Even though several potential new drugs for \textit{T. cruzi} infection are under investigation, they are many years away from general clinical use.\textsuperscript{16} One possible strategy to improve the effectiveness of current treatments is to combine existing drugs with different mechanisms of action, as used in other chronic infections, including tuberculosis,\textsuperscript{17} HIV,\textsuperscript{18} malaria,\textsuperscript{19,20} and African trypanosomiasis.\textsuperscript{21} While benznidazole and nifurtimox have similar modes of action, through the generation of nitroanion radicals that cause oxidative damage,\textsuperscript{22} and nitroreduction intermediates that interact with parasite components,\textsuperscript{23,24} respectively, allopurinol is a hypoxanthine analogue that is incorporated into RNA, leading to blockade of de novo synthesis of purine nucleotides.\textsuperscript{25}

Here, we conducted a pilot study to evaluate the tolerance and side effects of a sequential combined treatment of two available drugs, allopurinol and benznidazole, in chronically \textit{T. cruzi}-infected adults. The results of our study show that the sequential combination of allopurinol and benznidazole appears to induce changes in total and \textit{T. cruzi}-specific T and B cell responses indicative of a positive response to therapy, indicating the feasibility of applying a combination of drugs for treatment in the chronic phase of \textit{T. cruzi} infection.

\section*{Patients and methods}

\subsection*{Selection of study population}
Chronically \textit{T. cruzi}-infected adult volunteers who had lived for decades in areas non-endemic for \textit{T. cruzi} infection in Buenos Aires, Argentina, were recruited at the Chagas' Disease Section of Hospital Interalional General de Agudos "Eva Perón" (Buenos Aires, Argentina). \textit{T. cruzi} infection was determined by indirect immunofluorescence, haemagglutination and ELISA\textsuperscript{26} assays performed at the Instituto Nacional de Parasitología "Dr. Mario Fátala Chaben" (Buenos Aires, Argentina). All the patients included in this study belonged to the G0 group (seropositive individuals with normal findings on electrocardiography, chest X-ray and echocardiography) of the Kuschner grading system.\textsuperscript{27} Patients had not previously received treatment for \textit{T. cruzi} infection at initiation of this study. Fifteen chronically infected subjects (mean age $\pm$ SD 31 $\pm$ 6 years, range 21–42 years) were given the opportunity to participate in this pilot study of a sequential combined treatment with allopurinol and benznidazole. Seventeen untreated subjects (mean age $\pm$ SD 36 $\pm$ 12 years, range 21–58 years), 9 patients receiving benznidazole alone (mean age $\pm$ SD 42 $\pm$ 8 years, range 30–55 years) and 9 untreated controls (mean age $\pm$ SD 42 $\pm$ 5 years, range 22–56 years) were also included for comparison of flow cytometric and serological analyses. \textit{T. cruzi}-infected subjects, infected controls with hypertension, ischaemic heart disease, cancer, HIV infection, syphilis, diabetes, arthritis or serious allergies, and pregnant women were excluded from this study. The protocol was approved by the institutional review boards of the Hospital Interalional General de Agudos "Eva Perón", Buenos Aires, Argentina (Protocol no. 5906).

Signed informed consent was obtained from all individuals before inclusion in the study.

\subsection*{Treatment intervention}
Eligible patients received a combined sequential treatment of allopurinol and benznidazole as follows. Allopurinol was administered at 600 mg/day for 90 consecutive days, indicating a minimum of 2.5 L of fluid intake/day as adjuvant therapy. After 1 week without medication and a normal diet, treatment with benznidazole at a dosage of 5 mg/kg body weight/day was initiated and administered for 30 consecutive days.

\section*{Data collection and follow-up}
Clinical, serological and immunological analyses were performed prior to treatment, at the end of allopurinol administration, and 6 and 12 months following treatment with benznidazole, and at yearly intervals thereafter. The clinical status of enrolled patients was evaluated by electrocardiography and echocardiography. Echocardiography was performed once a year during follow-up. Changes from a lower to a more advanced Kuschner group or cardiac death, and the appearance of new abnormalities on electrocardiography were evaluated during follow-up.

Laboratory tests, including red blood cell and leucocyte counts, haematocrit, erythrocyte sedimentation rate, bilirubin, creatine, aspartate aminotransferase, alanine aminotransferase levels and urine tests, were performed at baseline, during treatment with benznidazole and after completion of treatment. Adverse drug reactions were classified according to their intensity as mild, moderate or severe. Serious adverse drug reactions comprised patient hospitalization, life-threatening conditions and death.

\section*{Monitoring of changes in the levels of \textit{T. cruzi}-specific antibodies}
Immunofluorescence and haemagglutination assays were reported as reactive from sequential half-titre dilutions between 1/32 and 1/756. ELISA tests were considered positive when mean absorbance at 490 nm was higher than the cut-off value of 0.200. The magnitude of serological titre reduction at different timepoints post-treatment with respect to titres at baseline was calculated for each subject. As previously described,\textsuperscript{28} decreases in at least 1 titre dilution (reduction of 50\%) in immunofluorescence and haemagglutination tests and at least a 30\% reduction in the ELISA assay compared with baseline values were considered significant changes, as these variations were not observed in untreated subjects. Conversion to negative serology was confirmed when titres were lower than 1/32 for haemagglutination and immunofluorescence and the mean absorbance at 490 nm in ELISA assays was $<0.200$. Serum specimens were also screened for antibodies reactive to a panel of 34 recombinant \textit{T. cruzi} proteins in a Luminex-based format, as previously described.\textsuperscript{29,30} Serological responses to each individual \textit{T. cruzi} protein were considered to have decreased during the study period if the mean fluorescence intensity (MFI) for at least one recombinant protein decreased $\geq 40\%$ relative to that of pre-treatment sample assessed concurrently.\textsuperscript{28}

\section*{Collection of peripheral blood mononuclear cells (PBMCs) and serum specimens}
Approximately 50 mL of blood was drawn by venipuncture into heparinized tubes (Vacutainer; BD Biosciences). PBMCs were isolated by density gradient centrifugation on Ficoll–Hypaque (Amersham) and were cryopreserved for later analysis. Approximately 10 mL of blood was drawn by venipuncture, allowed to coagulate at room temperature and centrifuged at 2500 rpm for 15 min for serum separation.

\subsection*{\textit{T. cruzi} lysate}
Protein lysate from \textit{T. cruzi} amastigotes was obtained by four freeze/thaw cycles followed by sonication as previously reported.\textsuperscript{31} Briefly, trypanosomatids of the Brazil strain were cultured overnight in pH 5 Dulbecco’s modified Eagle’s medium (Mediatech) to transform trypanosomatids to
amastigotes. After washing, the parasites were frozen at −20°C and thawed twice. Thereafter, the sample was subjected to two freeze/thaw cycles at −70°C followed by sonication. The supernatant of a 12 000 rpm centrifugation was collected and filter sterilized and the protein concentration was determined.

**Interferon (IFN)-γ and interleukin (IL)-2 enzyme-linked immunosorbent spot (ELISPOT) assays**

The number of *T. cruzi* antigen-responsive 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 Biosciences), as described elsewhere.30,31 Stimulation of PBMCs with 20 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) plus 500 ng/mL ionomycin (Sigma) in medium was used as a positive control for cytokine secretion. Responses to a *T. cruzi*-derived amastigote lysate preparation were considered positive if a minimum of 25 spots/1×10⁶ PBMCs were present/well and if this number was at least twice the value of wells with medium alone.32 To avoid inter-experiment variation, assays were conducted with paired samples from different timepoints. Each timepoint was assessed two or three times.

**Ex vivo cell surface staining for phenotypic analysis**

For counting of naive T cells, 1×10⁶ PBMCs were stained with anti-CD4 (peridin chlorophyll protein (PerCP)) or anti-CD8 (PerCP) in combination with anti-CD45RA (PerCP). As a third color, anti-CD127 (PerCP) was used to identify the naïve T cell population. Staining was performed using a Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. Following staining, cells were washed and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) equipped with FACSDiva software (BD Biosciences). The percentage of CD4+CD45RA+CD127+ cells was determined.

**Figure 1.** Monitoring of *T. cruzi*-specific antibodies by conventional serological tests following sequential combined treatment with allopurinol and benznidazole. *T. cruzi*-specific antibodies, as determined by ELISA (a), haemagglutination (b) and immunofluorescence assays (c), were measured prior to treatment, following allopurinol and at different timepoints after completion of benznidazole administration in the sequential combined treatment. Serological data from 10 untreated subjects with a similar follow-up period (Untreated) are also shown. Each open circle represents the data for single subjects. Broken horizontal lines show the threshold for reactivity for each serological test. ***P = 0.0003 and *P = 0.02 versus untreated. AL, allopurinol; BZ, benznidazole.
with anti-CD45RA (FITC), anti-CCR7 [R-phycocerythrin (PE)] and anti-CD62L [allophycocyanin (APC)]. T cell activation was assessed by staining with anti-CD4 (PerCP) or anti-CD8 (PerCP) in combination with anti-HLA-DR (FITC). Acquisition was performed with a BD FACS Calibur flow cytometer (BD Biosciences) and analysis with FlowJo software (Tree Star). All the antibodies were from BD Pharmingen. To avoid inter-experiment variation, assays were conducted with paired samples from different time points. Each timepoint was assessed two or three times.

**Ex vivo T cell proliferation**

Frozen PBMC samples were thawed, resuspended at $1 \times 10^7$ mL in PBS and stained with 1 μM carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Invitrogen) for 3 min at 37°C. The reaction was quenched with five volumes of ice-cold complete RPMI medium containing 10% fetal calf serum, washed and cultured at 1.5 x 10^6 cells/mL in 24-well flat-bottomed plates in the presence of staphylococcal enterotoxin B (SEB; Sigma, 2 μg/mL), or with medium alone (unstimulated control) for 6 days in 5% CO2. Cells were then harvested, stained with 7-amino-actinomycin D (7-AAD) (BD Pharmingen), and fixed with 1% paraformaldehyde. Dead cells were excluded based on 7-AAD staining. Samples were acquired in a BD FACS Calibur flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star). The proportion of proliferating T cells (CFSElow) was determined by subtracting the percentage of CFSEhigh cells in unstimulated cultures from the percentage of CFSElow cells in SEB-stimulated samples. Typically, <2% CFSEhigh cells were detected in unstimulated cultures. Precursor frequencies were calculated with FlowJo software and represent the percentage of the cells of the original sample that had divided, assuming that no cells died during the culture.

**Statistics**

The Mann–Whitney U-test was applied to compare the percentages of CD4+ or CD8+ naive T cells between T. cruzi-infected subjects and uninfected controls. The Kruskal–Wallis test with pairwise comparison and the Dunn multiple comparison test were applied to compare the percentages of CD4+ or CD8+ T cells expressing different phenotypic markers. Comparisons of the changes in T. cruzi-specific antibodies measured by conventional serological tests were performed using the Mann–Whitney U-test on post-treatment and pre-treatment differences between treated and untreated groups. The proportion of subjects for whom IFN-γ ELISPOT or B cell responses decreased over time following allopurinol or allopurinol-benznidazole treatment was compared using Fisher’s exact test. Correlation analysis between the expression of CD62L on T cells and the proliferation capacity was evaluated using the Spearman correlation test. Differences were considered to be statistically significant at $P < 0.05$.

**Results**

**Clinical characteristics, tolerance and adverse drug reactions in chronic Chagas’ disease subjects receiving combined sequential treatment with allopurinol and benznidazole.**

The median time of residence in endemic areas of the 15 subjects included in this pilot study was 19 years (range 1–23 years) and 86% of them were women. Four patients were lost to follow-up (two subjects were lost after the first timepoint post-benznidazole while the other two were followed up to the second timepoint post-benznidazole). Three of the four subjects who were lost to follow-up left voluntarily and the remaining subject moved away from Buenos Aires.

Adverse drug reactions were observed in only one subject after treatment with allopurinol, while seven subjects presented side effects after treatment with benznidazole. The main adverse drug reaction observed was mild (four subjects) to moderate (four subjects) dermatitis. Although no serious adverse drug reaction was observed, treatment discontinuation was required in two cases (suspension rate 13%): one during treatment with allopurinol and one during treatment with benznidazole. Only

**Table 1. Reduction of antibody levels by multiplex assay after combined sequential treatment with allopurinol and benznidazole in the chronic phase of T. cruzi infection**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>No. of proteins altered/total no. of reactive proteins at baseline (%)</th>
<th>ID of proteins altered following treatment</th>
<th>Earliest time of titre decrease following treatment (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP363</td>
<td>6/8 (75)</td>
<td>AnoL-E02, kn107, FaCBP, kn117, kn80, AnoB-A07</td>
<td>7 PBZ</td>
</tr>
<tr>
<td>PP367</td>
<td>3/3 (100)</td>
<td>kn107, FaCBP, FABA4</td>
<td>3 PAL</td>
</tr>
<tr>
<td>PP374</td>
<td>0/11 (0)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PP386</td>
<td>2/8 (25)</td>
<td>FaCBP, kn80</td>
<td>20 PBZ</td>
</tr>
<tr>
<td>PP391</td>
<td>2/7 (29)</td>
<td>FaCBP, kn80</td>
<td>10 PBZ</td>
</tr>
<tr>
<td>PP405</td>
<td>9/11 (82)</td>
<td>AnoL-G10, AnoL-E02, AnoF-F10, kn104, FaCBP, kn117, FABA4, kn80, AnoB-A07</td>
<td>20 PBZ</td>
</tr>
<tr>
<td>PP440</td>
<td>0/5 (0)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PP454</td>
<td>7/8 (88)</td>
<td>AnoL-G10, AnoL-E02, AnoF-F10, FaCBP, kn117, FABA4, AnoB-A07</td>
<td>19 PBZ</td>
</tr>
<tr>
<td>PP465</td>
<td>0/7 (0)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PP486</td>
<td>2/5 (40)</td>
<td>AnoL-G10, FaCBP</td>
<td>6 PBZ</td>
</tr>
<tr>
<td>PP487</td>
<td>1/3 (33)</td>
<td>FaCBP</td>
<td>14 PBZ</td>
</tr>
</tbody>
</table>

PBZ, post-benznidazole administration; PAL, post-allopurinol administration; NA, not applicable; AnoB-A07, oxonemere central apparatus protein, putative; AnoF-F10, TolT3 proteins; AnoL-G10, hypothetical protein, conserved; AnoL-E02, microtubule-associated protein homologue; FABA4, heat shock 70 kDa protein, mitochondrial protein precursor, putative; FaCBP, flagellar calcium binding protein; kn80, poly(A) binding protein; kn104, glycosomal phosphoenolpyruvate carboxykinase, putative; kn107, 605 acidic ribosomal subunit protein, putative; kn117, 69 kDa flagellar bacilli protein, putative.

*a* Number of proteins with decreased antibody levels after treatment out of total reactive proteins at baseline as measured by multiplex assays.28,29

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mild and reversible leucopenia as well as a slight increase in transaminase levels was detected in two subjects. The median time of clinical and serological follow-up in the 11 subjects who received the complete treatment schedule was 36 months (range 9–64). None of the subjects evaluated exhibited progression towards more severe clinical stages of the disease during the follow-up period. No other clinical complications, such as infections or neoplasia, were registered during follow-up.

**Monitoring of T. cruzi-specific antibodies in sera from chronic Chagas’ disease patients treated with allopurinol followed by benznidazole**

Changes in T. cruzi-specific antibodies by conventional serological tests and by a multiplex assay were monitored in the 11 subjects who received complete combined treatment. The levels of T. cruzi-specific antibodies did not vary after treatment with allopurinol, as measured by ELISA, haemagglutination or immunofluorescence assays (Figure 1a–c, left panels). A marked reduction in antibody levels specific for T. cruzi was observed after the sequential combined treatment, as determined by ELISA (Figure 1a, left panel) and haemagglutination (Figure 1b, left panel), but not by immunofluorescence (Figure 1c, left panel) assays. On an individual basis, 3 out of the 11 (27%) treated subjects showed a decrease in haemagglutination titres following treatment with allopurinol while seroconversion by this same assay was detected in one patient. A reduction in antibody levels in at least one serological test was observed in 5 out of the 11 (45%) subjects following the combined sequential allopurinol + benznidazole treatment compared with levels prior to treatment at a mean time of follow-up of 14 months (range 10–18), while 8 out of the 11 (73%) subjects exhibited

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**Figure 2.** IFN-γ ELISPOT responses specific for T. cruzi antigens in chronic Chagas’ disease patients after combined sequential treatment with allopurinol and benznidazole. IFN-γ-producing T cells/1×10⁶ PBMCs upon stimulation with an amastigote lysate preparation were measured at different timepoints after treatment with allopurinol followed by benznidazole. Subjects with positive (a and b) and negative (c) ELISPOT responses at baseline. Each filled triangle represents the data for a single subject from a selected group. Arrowheads indicate the initiation of allopurinol or benznidazole administration. Broken horizontal lines show the threshold for positive ELISPOT responses for each subject as indicated in the Patients and methods section. AL, allopurinol; BZ, benznidazole.
seronegative conversion, as revealed by ELISA (2 patients) (Figure 1a, left panel), haemagglutination (4 patients) (Figure 1b, left panel), or both tests (2 patients) by a median time of follow-up of 17 months (range 9–60) following treatment. T. cruzi-specific antibody levels remained unaltered or even increased in untreated subjects over a 36 month-follow-up period (Figure 1a–c, right panels). Antibody titres, as determined by haemagglutination assays, decreased during follow-up in only two untreated subjects (Figure 1b, right panel).

Treatment with allopurinol followed by benznidazole also decreased the levels of antibodies against a set of T. cruzi-specific recombinant proteins, as determined by a multiplex assay previously reported to be useful for the monitoring of treatment in the chronic phase of the infection. Decreases in MFI for one or several proteins were observed in 8 out 11 (73%) subjects between 12 and 24 months following combined treatment (i.e. between 7 and 20 months after completion of treatment with benznidazole) (Table 1). Moreover, one subject showed decreases in antibody levels by the multiplex technique after completion of the allopurinol schedule. It is of note that decreases in antibody levels specific for a T. cruzi-derived calcium-binding protein (FaCBP) were detected in all the subjects who showed serological changes by multiplex assays.

## Combined sequential treatment with allopurinol followed by benznidazole had a marked impact on the frequency of IFN-γ-producing T cells responsive to T. cruzi antigens

We have previously shown that monitoring of IFN-γ ELISPOT responses specific for T. cruzi antigens could be used as a surrogate biomarker for treatment efficacy in chronic Chagas' disease. Here, IFN-γ and IL-2 ELISPOT responses to a T. cruzi lysate were periodically monitored in 11 chronically T. cruzi-infected subjects given combined sequential treatment over a median follow-up of 36 months after initiation of treatment (range 9–64).

In accordance with our previous findings, two different types of T cell responses were registered at baseline; 7 out of 11 (64%) subjects showed IFN-γ responses greater than background levels (‘responder subjects’) (Figure 2a and b) while negative IFN-γ responses were observed in 4 out of 11 (36%) individuals (‘non-responder subjects’) (Figure 2c). Among responder subjects, IFN-γ-producing T cells fell below the level of detection in four out of seven subjects at the end of allopurinol administration and remained low after benznidazole treatment (i.e. subjects PP374 and PP487; Figure 2a). In the remaining three responder subjects, IFN-γ ELISPOT responses significantly increased (i.e. at least a 3-fold increase) immediately after allopurinol administration and decreased (i.e. at least a 3-fold decrease) at later timepoints following benznidazole treatment (i.e. subjects PP391 and PP454; Figure 2b). Responses to polyclonal stimulation remained unaltered at all timepoints assessed.

All non-responder subjects at baseline also showed a marked increase in the frequencies of T. cruzi antigen-responsive IFN-γ-producing T cells immediately after allopurinol administration, with a significant decrease afterwards (i.e. subject PP465; Figure 2c), even reaching background levels at 24–31 months following sequential combined treatment (i.e. subject PP363; Figure 2c). As previously observed in subjects treated with benznidazole alone, IL-2 ELISPOT responses specific for the T. cruzi lysate varied along with IFN-γ responses during the follow-up period (data not shown). Taken together, these findings show that not only the combination of allopurinol and benznidazole but also allopurinol alone has a large impact on T. cruzi-specific T cell responses, likely indicating a reduction in parasite load.

**Table 2. Correlation of changes in T cell responses to changes in serological values following sequential combined treatment of allopurinol and benznidazole**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Allopurinol-treated patients</th>
<th>Allopurinol + benznidazole-treated patients</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease/change in both ELISPOT responses and serological test values $^b$</td>
<td>2</td>
<td>9</td>
<td>0.0089</td>
</tr>
<tr>
<td>Decrease/change in ELISPOT responses only $^c$</td>
<td>8</td>
<td>9 $^d$</td>
<td>0.0159</td>
</tr>
<tr>
<td>Decrease in serological test only $^d$</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>No decrease/change in ELISPOT or serological test values $^e$</td>
<td>1</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Number exhibiting altered immune profile/total examined</td>
<td>10/11 (91%)</td>
<td>11/11 (100%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, not significant.

Data are numbers of patients unless otherwise indicated.

$^a$Determined by Fisher’s exact test for allopurinol-treated versus allopurinol + benznidazole-treated subjects.

$^b$Decrease after treatment to less than the detection limit or a 3-fold decrease relative to pre-treatment level for responder patients at baseline and a 3-fold increase relative to pre-treatment level followed by a decay to background levels in non-responder subjects at baseline for ELISPOT; and seronegative conversion on at least one conventional serological test or a >40% decrease in MFI for >1 recombinant T. cruzi protein in the 14-protein multiplex panel compared with baseline.

$^c$Decrease after treatment to less than the detection limit or a 3-fold decrease relative to pre-treatment level for responder patients at baseline and a 3-fold increase relative to pre-treatment level followed by a decay to background levels in non-responder subjects at baseline for ELISPOT, while changes in serological tests did not meet the criteria described in b.

$^d$Multiplex test was negative for these two subjects prior to treatment.

$^e$Seronegative conversion on at least one conventional serological test or a >40% decrease in MFI for >1 recombinant T. cruzi protein in the 14-protein multiplex panel, while ELISPOT responses remained unaltered.

$^f$Changes in ELISPOT responses or serological tests did not meet the criteria described in b.
Correlation between T and B cell responses following treatment with allopurinol or combined sequential treatment with allopurinol + benznidazole

Correlation of changes in T and B cell responses specific for *T. cruzi* compared with baseline data was analysed following treatment with allopurinol alone or after a median follow-up of 36 months after combined sequential treatment with allopurinol + benznidazole. Changes in both ELISPOT and serological tests, either by conventional tests (seronegative conversion in at least one test) or by a multiplex assay, were observed in only 2 out of 11 subjects after completion of allopurinol while in 8 subjects ELISPOT responses were significantly modified (Table 2, column headed ‘Allopurinol-treated patients’). No variations were recorded in the remaining allopurinol-treated subjects. Conversely, after completion of the sequential combined treatment, changes in ELISPOT responses were strongly correlated with decreases in the levels of *T. cruzi*-specific 25

\[ P = 0.027 \]

\[ P < 0.01 \]

\[ P < 0.05 \]

\[ P < 0.05 \]

\[ P < 0.05 \]

\[ P < 0.05 \]

**Figure 3.** Combined sequential treatment with allopurinol and benznidazole significantly increases the frequencies of naive CD4+ and CD8+ T cells in chronically *T. cruzi*-infected subjects. PBMCs were isolated by centrifugation on Ficoll-Hypaque and analysed for the expression of CD45RA, CCR7 and CD62L by flow cytometry. (a and b) Frequencies of CD4+(a) and CD8+(b) naive T cells in untreated *T. cruzi*-infected subjects and uninfected controls. (c and d) Percentage ratios of naive CD4+(c) and CD8+(d) T cells following treatment with allopurinol or allopurinol + benznidazole (9–24 or 48–61 months following combined treatment) to those prior to treatment. Percentage ratios in subjects treated with benznidazole alone (6 and 12 months after treatment) and in untreated chronically *T. cruzi*-infected subjects are also shown. Each filled triangle represents an individual subject. Comparisons were performed by Kruskal–Wallis analysis and the Mann–Whitney U-test. Horizontal lines indicate mean values. (e) Representative contour plots of the monitoring of naive CD4+(top panel) and CD8+(bottom panel) T cells from one *T. cruzi*-infected subject following combined treatment with allopurinol and benznidazole. The numbers in the upper right quadrant reflect the proportion of CD4+ or CD8+ T cells co-expressing CD45RA, CCR7 and CD62L. (f) Longitudinal analysis of naive CD4+(broken lines) and CD8+(continuous lines) T cells from a selected group of subjects treated with allopurinol followed by benznidazole and untreated subjects. Plots represent the data for single subjects. Arrowheads indicate the initiation of allopurinol or benznidazole administration. AL, allopurinol; BZ, benznidazole.
antibodies (Table 2, column headed ‘Allopurinol + benznidazole-treated patients’).

**Treatment with allopurinol promotes a sustained improvement in the frequency of naive T cells along with a decrease in T cell activation in total peripheral CD4+ and CD8+ T cells**

The *T. cruzi*-specific and overall T cell compartments in chronically *T. cruzi*-infected subjects display several features compatible with a process of immune exhaustion, including a parasite-specific functional profile of T cells secreting IFN-γ only, characteristic of effector/effector memory T cells, decreased frequencies of total naive CD45RA+CD27+CD28+CD4+CD8+ T cells and increased levels of activated T cells.33–35

We confirmed that naive CD4+ (Figure 3a) and CD8+ (Figure 3b) T cells are diminished in subjects with very long-term *T. cruzi* infection, by measuring the expression of CD45RA, CD62L and CCR7 in a similar group of untreated *T. cruzi*-infected subjects and uninfected controls. In 10 out of 14 individuals who at least completed allopurinol treatment, the frequencies of total peripheral CD4+ (Figure 3c (AL; AL+BZ)) and CD8+ (Figure 3d (AL; AL+BZ)) naive T cells significantly increased (i.e. at least a 3-fold increase) after treatment with allopurinol compared with the levels prior to treatment, reaching levels comparable to those in uninfected subjects. A mean 5- and 9-fold increase in naive T cells was observed for CD4+ and CD8+...
Figure 4. Expression of HLA-DR in total peripheral T cells from chronically T. cruzi-infected subjects after sequential combined treatment with allopurinol and benznidazole. The plots show the percentages of CD4+(a) and CD8+(b) T cells expressing HLA-DR following treatment with allopurinol or allopurinol +benznidazole with respect to values prior to treatment, as well as in untreated chronically T. cruzi-infected subjects.
T cells, respectively. The frequency of naive T cells generally declined following treatment with benznidazole, but remained higher than was observed prior to treatment (median follow-up of 36 months, range 9–61). Conversely, no major alterations in naive T cell levels were found during follow-up either in nine patients treated with benznidazole alone [Figure 3f (B2)] or in untreated subjects [Figure 3f (Untreated)] over a similar follow-up period.

The elevations in the levels of naive T cells was paralleled by a decrease in the number of CD4+ (Figure 4a, c and d) and CD8+ (Figure 4b–d) T cells expressing the activation marker HLA-DR, measured either after allopurinol or after the completion of the combined treatment, while the frequencies of HLA-DR+ T cells in untreated subjects were maintained or even increased over time (Figure 4a and b). CD62L expression, the hallmark of naive T cells, in total peripheral CD4+ and CD8+ T cells was correlated with the proliferation capacity of these T cell populations in response to SE, establishing a link between T cell phenotype and function (Figure 5a–c). Moreover, the frequency of cells that divided was higher in PBMCs derived from subjects treated with the combination of allopurinol and benznidazole compared with the untreated group (Figure 5d). Altogether, these findings document a decline in T cell activation after combined treatment with allopurinol and benznidazole.

**Discussion**

The present study constitutes the first report on the use of a combination of drugs for the aetiological treatment of human Chagas’ disease. In this pilot study, the sequential combination of allopurinol and benznidazole was chosen based on the different mechanisms of action and previous use of these drugs during the chronic phase of T. cruzi infection. This combined treatment was well tolerated, demonstrating the feasibility of the application of a combination of drugs in the chronic phase of T. cruzi infection. The good tolerance to allopurinol is in agreement with previous studies, and serious adverse reactions, like Stevens–Johnson syndrome, that are infrequent in everyday practice, were not observed in this pilot study.

Treatment in the chronic phase of T. cruzi infection has been largely postponed mainly because of the lack of early metrics of treatment efficacy. The main criterion of a positive response to treatment has been complete seronegative conversion by conventional serological tests, something that requires long-term follow-up. More recently, other likely indicators of treatment success have been proposed, comprising decreases in parasite-specific IFN-γ-producing T cells as well as partial seronegative conversion or significant decreases in antibody levels specific for T. cruzi. By applying the same tools we had previously reported to assess treatment efficacy, we observed that T. cruzi-specific T and B cell responses are modified following the administration of allopurinol as well as after the completion of this combined sequential treatment, indicating a putative reduction in parasite burden.

The pool of T. cruzi-specific T cells in very long-term chronically infected subjects is mainly composed of single IFN-γ-producing effector/effector memory T cells with low expression of CCR7 (a lymph node homing molecule), and IL-7 receptor (involved in homeostatic proliferation and memory T cell maintenance). As shown in other chronic infections, these responses are dependent on antigen for maintenance, and thus the disappearance of these cells is indicative of antigen withdrawal.

In agreement with this notion, studies conducted in HIV+ subjects undergoing antiretroviral therapy have demonstrated that the decrease in the frequency of HIV-specific CD8+ T cell response during therapy is paralleled by a decrease in viral load.

A novel finding of the current study is that, following treatment with allopurinol, T. cruzi-specific IFN-γ ELISPOT responses either initially increased and then decreased, or became negative in a substantial proportion of patients. These changes are similar to those observed in our previous study in which parasite-specific T cell responses were monitored after treatment with benznidazole, as a single drug, in chronically infected subjects. The early rise in IFN-γ-producing T cells following allopurinol administration observed here and in our previous study with benznidazole-treated patients might have been due to the trypanocidal action of the drugs, resulting in parasite release and inducing a boost in T cell responses. Moreover, early variations in T cell responses after treatment with benznidazole were correlated with decreases in parasite-specific B cell responses in a long-term follow-up. In the present study, the levels of T. cruzi-specific antibodies did not vary in most patients after allopurinol, but decreased considerably along with changes in T cell responses over a longer follow-up period subsequent to the sequential administration of benznidazole. These findings further support the idea that changes in parasite-specific T cell responses appeared to be a suitable early indicator of positive response to treatment.

We have shown that humans with decades-long infection with T. cruzi lack polyclonal CD4+ and CD8+ T cell responses to T. cruzi proteins/peptides and that the whole T cell population in these subjects shows signs of immune exhaustion consistent with the persistence of infection in these individuals, comprising diminished levels of naive T cells and an increased frequency of fully differentiated memory CD8+ T cells. Treatment with allopurinol resulted in a reconstitution of naive CD4+ and CD8+ T cells that was maintained after treatment with benznidazole, along with an improvement in the proliferative capacity of T cells and a decline in the general status of T cell activation. The reconstitution of the naive pool did not affect the frequency of total memory T cells, as evidenced by the expression of CD45RA (D. E. Perez-Mazilah, unpublished results). Conversely, patients who received benznidazole as a single drug did not show an improvement in naive T cells in a similar short-term
follow-up period. Altogether, these effects show the impact of this sequential combined treatment not only on parasite-specific T cells but also on the whole T cell compartment. Immune reconstitution of the naive T cell pool has also been shown to occur in HIV+ subjects after successful suppression of viral load by highly active antiretroviral therapy.46,47

Several anti-inflammatory actions of allopurinol have been reported, including the ability to decrease the production of tumour necrosis factor-α by human mononuclear cells,48 down-regulate the expression of intercellular adhesion molecule 1 (ICAM-1 or CD54) and P2X7 receptor on human monocytes/macrophages,49 block the induction of monocyte chemotactic factor-1 and IL-6 production in rat vascular smooth muscle cells50 and free radical scavenging and antioxidant activities.51 Although the action of allopurinol on T cells is less known, lymphocyte blastogenesis induced by phytohaemagglutinin was significantly suppressed by allopurinol.52 We have recently observed that allopurinol attenuates CD62L down-regulation and CD69 up-regulation induced upon T cell activation, and significantly reduces the levels of spontaneous and induced intracellular reactive oxygen species in T cells in vitro (D. E. Perez-Mazliah, unpublished results).

Thus, we cannot rule out the possibility that the immunological improvement reflected in the total T cell population is due to a synergistic action between the trypanocidal and anti-inflammatory activities of allopurinol in the sequential combined protocol applied here.

Benznidazole used in combination with heterocyclic analogues or with ketoconazole has been reported to enhance the efficacy of chemotherapy in experimental T. cruzi infection.53,54 A synergistic activity was also claimed when ketoconazole was used in combination with terbinafine55 or posaconazole with amiodarone56 in T. cruzi-infected mice. Other drug combinations were intended to enhance the immune response against T. cruzi.57,58 In human chronic infection, nifurtimox was added to betamethasone with the aim of combining the respective antiparasitic and anti-inflammatory actions of these drugs.59

A number of studies documented the trypanolytic activity of allopurinol in mice60–62 and in vitro infections,63,64 but the efficacy of allopurinol treatment in the human chronic phase of T. cruzi infection varied among different studies.13–15 Gallerano et al.14 reported that allopurinol was as effective as benznidazole in eliminating parasitaemia and inducing seronegative conversion in chronically T. cruzi-infected subjects, whereas Rassi...
et al. demonstrated that allopurinol was not effective in clearing T. cruzi infection. Allopurinol was also shown to be safe and effective in treating Chagas’ disease reactivation after heart transplantation. Moreover, the association of allopurinol with clomipramine was effective for treatment in the acute phase of T. cruzi infection in mice. Although we cannot ascertain whether the sequential combined treatment of allopurinol and benznidazole is more effective than the current use of benznidazole alone, it is of note that the 11 patients who received complete combined treatment showed changes in either T or B cell responses during follow-up. The trypanocidal activity of allopurinol is reflected in decreases in T. cruzi-specific IFN-γ-producing T cells, which depend on antigen persistence for their maintenance, preceded in many cases by an early increase. On the other hand, the restoration of total naive T cells would be achieved by a diminution in the general activation status of the host immune system induced by a decrease in parasite load as well as the anti-inflammatory action of allopurinol.

In conclusion, this pilot study shows the feasibility of administration of a combined sequential treatment of allopurinol and benznidazole, which resulted in immunological changes indicative of a beneficial outcome of therapy in human chronic Chagas’ disease.

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Transparency declarations
None to declare.

Author contributions
D. E. P.-M. performed ELISPOT, flow cytometric and proliferation assays, and contributed to the analysis and interpretation of data as well as writing. M. G. A. made a substantial contribution to the study design, analysis of conventional serology during follow-up and management of patient appointments for blood sampling, G. C. performed multiplex assays and the corresponding analysis of data. B. E. L. contributed to study design and performed echocardiographic evaluation. G. B. contributed to patient recruitment and treatment. M. P. contributed to patient recruitment and treatment. M. C. A. performed ELISPOT assays and analysis of corresponding data. A. H. A. performed electrocardiographic evaluation. R. L. T. contributed to multiplex analysis, writing and financial support. S. A. L. contributed to study design, analysis and interpretation of data, writing and financial support. R. V. was responsible for the conception and design of the study, interpretation of data and writing.

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