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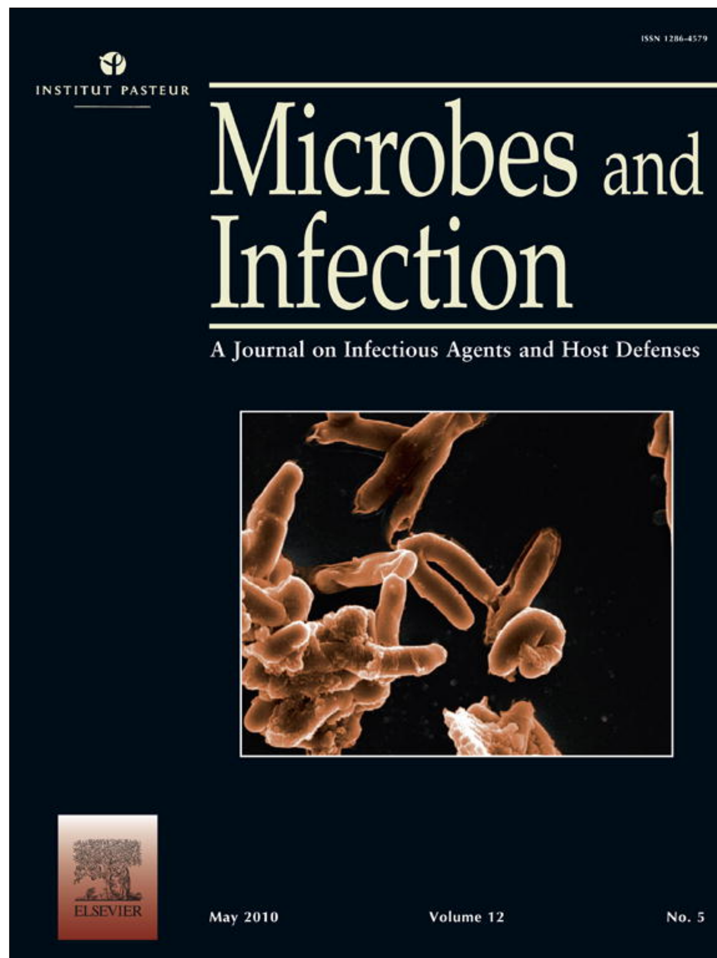

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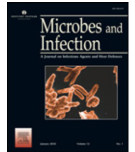

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Original article

Trypanosoma cruzi-specific immune responses in subjects from endemic areas of Chagas disease of Argentina

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Abstract

Trypanosoma cruzi-specific immune responses were evaluated in a total of 88 subjects living in areas endemic of Chagas disease of Argentina by IFN-γ ELISPOT assays and immunoblotting. Positive *T. cruzi* antigen-induced IFN-γ responses were detected in 42% of subjects evaluated (15/26 positive by conventional serology and 22/62 seronegative subjects). Using immunoblotting, *T. cruzi*-specific IgG reactivity was detected in all seropositive subjects and in 11% (7/61) of subjects negative by conventional serology. Measurements of T cell responses and antibodies by immunoblotting, in conjunction with conventional serology, might enhance the capability of detection of exposure to *T. cruzi* in endemic areas.
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Keywords: *Trypanosoma cruzi*; T cell responses; Humoral responses

1. Introduction

Trypanosoma cruzi, the etiologic agent of Chagas disease, is the leading cause of infectious myocarditis [1]. Approximately 30–40% of *T. cruzi* infected individuals may develop cardiac and/or digestive abnormalities many years after the acute phase symptoms have subsided (chronic symptomatic stage), following a sub-clinical period of variable duration (indeterminate stage). In the majority of untreated infected individuals, *T. cruzi*-specific immune responses remain detectable long after the acute infection has subsided, commonly throughout life.

In a previous study we demonstrated that $CD8^+$ T cell responses specific for HLA-A2.1-restricted peptides derived from *T. cruzi* proteins are more frequent among seropositive individuals living in endemic areas of Chagas disease than in chronic Chagas disease patients living in non-endemic areas, suggesting that repeated exposure to *T. cruzi* might boost the level of parasite-specific T cells [2]. Herein, we present a cross-sectional survey conducted to assess *T. cruzi*-specific cellular and humoral immune responses to a wider range of parasite antigens known to activate both $CD4^+$ and $CD8^+$ T cell subsets in subjects living in endemic areas, and thus potentially exposed to infection by *T. cruzi*, irrespective of HLA-haplotype [3].

2. Materials and methods

2.1. Study population

A total of 88 adult subjects (37 males and 51 females) living in endemic areas of Chagas disease of Argentina (Santiago del

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Estero, SE, mean age ± SD = 40.3 ± 16.5 years, $n = 60$, and La Rioja, LR, mean age ± SD = 53.3 ± 15.2 years, $n = 28$) were included in this study. Fifty three additional healthy individuals (19 males and 34 females, mean age ± SD = 40.2 ± 13.4 years) living in Buenos Aires (non-endemic area) were enrolled as unexposed controls. The study protocol was approved by the Ethics Committee of the Instituto Nacional de Parasitología ''Dr. Mario Fatala Chaben'' (INP).

2.2. Conventional serology

The status of *T. cruzi* infection was established by indirect immunofluorescence on formalin-treated *T. cruzi* epimastigotes, indirect hemagglutination assay, and ELISA. All tests were developed at the INP and validated in house using standardized diagnostic procedures [4]. Subjects were considered seropositive for *T. cruzi* infection when at least two of these tests were positive.

2.3. Interferon-gamma ELISPOT assays

The frequency of *T. cruzi*-specific peripheral blood mononuclear cells (PBMC) was estimated in 88 subjects living in endemic areas and 23 unexposed subjects by IFN-γ ELISPOT assays stimulating with a *T. cruzi* amastigote lysate (Brazil strain), as per manufacturer instructions (BD Pharmingen, San Diego, California). Ex-vivo unexpanded PBMC were seeded at 4×10^6 cells/mL and stimulated with 10 μg/mL *T. cruzi* lysate or 10 ng/mL phorbol-12-myristate-acetate (PMA) and 500 ng/mL Ionomycin for 14–16 h at 37 °C in a 5% CO_2 environment; PBMC incubated with complete RPMI were used as non-stimulated controls [2].

T. cruzi-specific cellular immune responses were further evaluated in 18 subjects from endemic areas and 5 unexposed subjects, using *T. cruzi* recombinant proteins immobilized onto beads to stimulate lymphocytes in IFN-γ ELISPOT assays. Recombinant His-tagged protein was expressed in *E. coli* and purified under denaturing conditions using TALON Metal Affinity Resin as previously described [5]. Recombinant proteins acidic ribosomal protein Kn107 (Tc00.1047053508355.250), flagellar calcium-binding protein Kn122 (Tc00.1047053507491.151), conserved hypothetical protein AnoH-G10 (Tc00.1047053-506635.130), microtubule-associated protein homologue AnoL-E02 (Tc00.1047053511633.79), KMP11 Kn56 (Tc00.10470-53510755.89), co-chaperone Grp E AnoL-H11 (Tc00.1047053-507929.20), a pool of *trans*-sialidase protein fragments containing the epitope tskb20, and a pool of *trans*-sialidase protein fragments containing the epitope tskb21 were then coated onto magnetic beads 1.1 μm in diameter (C.V. < 5%) and used to stimulate lymphocytes. Purified denatured protein was coated onto Dynabeads-TALON (Invitrogen) at a ratio of at least 10 μg of protein for every 1 mg beads. Coating was carried out as per the manufacturer's Manual Purification. Pending T cell stimulation, beads were diluted in PBS to a concentration of 3.5 mg/mL and stored at 4 °C. PBMC (4×10^6 cells/ml) were stimulated for 14–16 h with 0.18 μg/mL individual protein Kn122 , pools composed of three different recombinant proteins (0.11 μg/mL/protein; Table 1) or 0.11 μg/mL unbound beads as controls.

Spot forming cells were automatically enumerated using an ImmunoSpot analyzer (CTL, Cleveland, Ohio). The mean number of spots in triplicate wells was obtained for each condition. Responses were considered positive if a minimum of 20 spots/10^6 PBMC total were present, and this number was at least twice the value of wells with media alone. The number of specific IFN-γ-secreting T cells was calculated by subtracting the value of the wells containing media alone from the lysate/recombinant protein-stimulated spot count.

2.4. Immunoblotting

T. cruzi Brazil strain epimastigotes cultured in LIT medium at 27 °C were collected during the exponential growth phase, re-suspended in 2% sodium duodecyl sulfate sample buffer and boiled for 5 min at 100 °C. *T. cruzi* lysates (approx. 10^6 epimastigotes per lane) and recombinant proteins Kn122, AnoH-G10, Kn107 and AnoL-E02 (1.5–5 μg per lane) were loaded in 12 to 18% polyacrylamide gel and electrophoresed at 130 V in SDS-PAGE. Proteins were then transferred onto 0.45-μm pore-size nitrocellulose membranes, incubated overnight with human sera diluted 1:50 at 4 °C, followed by incubation with HRP-conjugated anti-human IgG rabbit antibody diluted 1:1000 for 2 h at room temperature, and H_2O_2/4-Cl-1-naphthol.

Table 1
Trypanosoma cruzi recombinant protein-specific cellular immune responses in subjects living in endemic area of Chagas disease as measured by IFN-γ ELISPOT assays.

Conventional serology	*T. cruzi* lysate	*T. cruzi* recombinant proteins			
		Kn 122[a]	Pool 1[b]	Pool 2[c]	Pool 3[d]
Positive	5/6 (353.5 ± 197.55)	4/6 (16.56 ± 6.79)	4/6 (19.56 ± 5.85)	1/6 (15)	1/6 (17)
Negative	4/12 (98.43 ± 52.11)	3/8 (23.33 ± 9.70)	5/12 (30.7 ± 12.15)	3/12 (19.33 ± 5.56)	3/12 (22.75 ± 4.13)

PBMC were stimulated in bulk with *T. cruzi* recombinant proteins and tested for IFN-γ secretion by ELISPOT assays as described in Materials and Methods. The data express the number of responder subjects/total evaluated and the (mean ± SD *T. cruzi* specific IFN-γ-secreting cells/10^6 PBMC).

[a] Kn122 individual recombinant protein.

[b] Includes recombinant protein tskb20, tskb21, AnoH-G10.

[c] Includes recombinant proteins Kn122, Kn107, AnoL-E02.

[d] Includes recombinant proteins AnoL-H11, Kn56.

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2.5. Statistical analysis

Comparisons of the number of spots and antigenic bands between groups were evaluated by Student's *t*-test and analysis of variance (ANOVA) followed by Bonferroni. Comparisons of the frequencies of individuals with positive serology and ELISPOT responses were evaluated with the Fisher's exact test. The relationship between the number of spots and antigenic bands, and reactive conventional serology tests was compared by Spearman's correlation test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Conventional serology

In order to establish the relationship between the prevalence of positive serum reactions for *T. cruzi* and vector transmission status, all subjects were evaluated by conventional serology at enrollment. The results of this evaluation showed a significantly higher seropositive rate in SE, a province with active vector transmission (23/60 subjects, 38%), than in LR, a province under continuous vector surveillance (3/28 subjects, 11%; $P = 0.01$, Fisher exact test). All subjects from Buenos Aires were negative by conventional serology.

3.2. *Trypanosoma cruzi*-specific T-cell responses

ELISPOT analysis revealed measurable numbers of *T. cruzi* lysate-specific IFN-γ producing T cells in 15/26 seropositive subjects (58%; 13 subjects from SE and 2 from LR) and 22/62 seronegative subjects (35%; 15 subjects from SE and 7 from LR), with similar frequencies of IFN-γ secreting T cells in both groups (mean ± SD specific cells = 191.01 ± 222.16 and 114.9 ± 153.39, respectively; Fig. 1).

To support the specificity of the T cell responses for *T. cruzi*, PBMC for a subset of seropositive and seronegative subjects were incubated with recombinant *T. cruzi* proteins and IFN-γ ELISPOT responses measured. The results of this analysis are summarized in Table 1. Three of the five seropositive and three of the four seronegative subjects showing IFN-γ-secreting cells against *T. cruzi* lysate also recognized recombinant antigens in ELISPOT assays. Of the subjects with negative IFN-γ responses against the lysate, 1/1 seropositive and 2/8 seronegative subjects showed specific T cells against the recombinant antigens.

None of the non-endemic seronegative controls responded to *T. cruzi* amastigote lysate or recombinant proteins by IFN-γ ELISPOT assays (Fig. 1).

3.3. Immunoblotting

An in-house immunoblotting assay was used as an experimental tool to further analyze humoral responses in 25 seropositive and 61 seronegative subjects from the endemic area and 30 unexposed subjects. By using this technique, we were able to detect the presence of IgG antibodies specific for *T. cruzi* in all seropositive and in 46 of the 61 seronegative (75%) subjects from endemic area tested (Fig. 2A). The number of bands recognized by seropositive subjects was significantly higher (mean ± SD = 17.48 ± 3.83 bands) than the number of bands recognized by seronegative subjects (mean ± SD = 5.65 ± 3.89 bands; $P < 0.0001$, Student's *t*-test; Fig. 2B). Sera from two unexposed subjects (7%) recognized two bands of *T. cruzi*. Noticeably, seven subjects from the endemic area who were negative by conventional serology

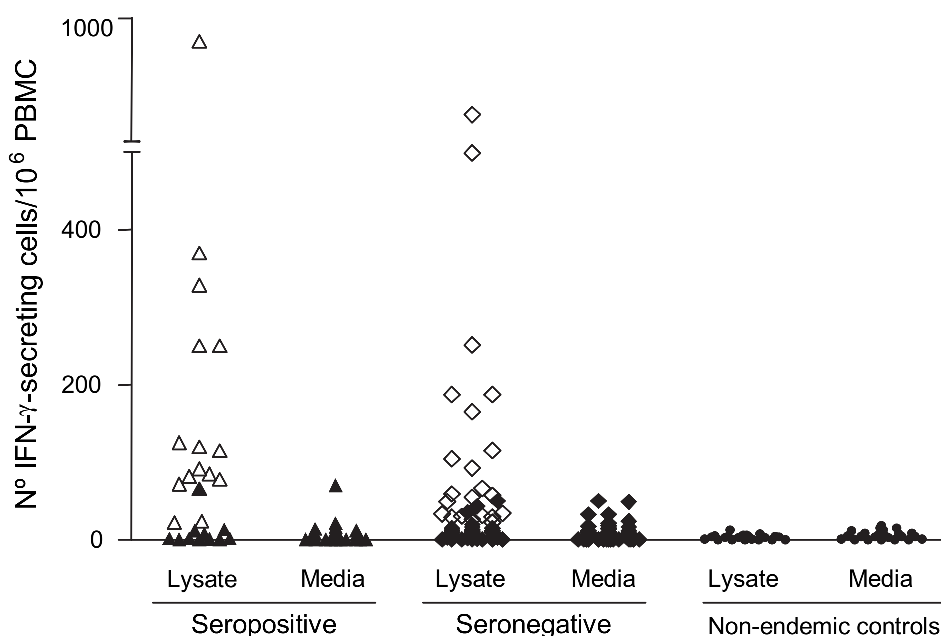

Fig. 1. T cell responses to a *Trypanosoma cruzi* amastigote lysate measured by IFN-γ ELISPOT assays in subjects from endemic areas of Chagas disease of Argentina. Each point represents the mean number of IFN-γ secreting cells per 10^6 PBMC for each subject following in vitro culture with the parasite lysate or media alone during 14–16 h. Open marks indicate positive IFN-γ responses and filled marks non-responders (see Section 2).

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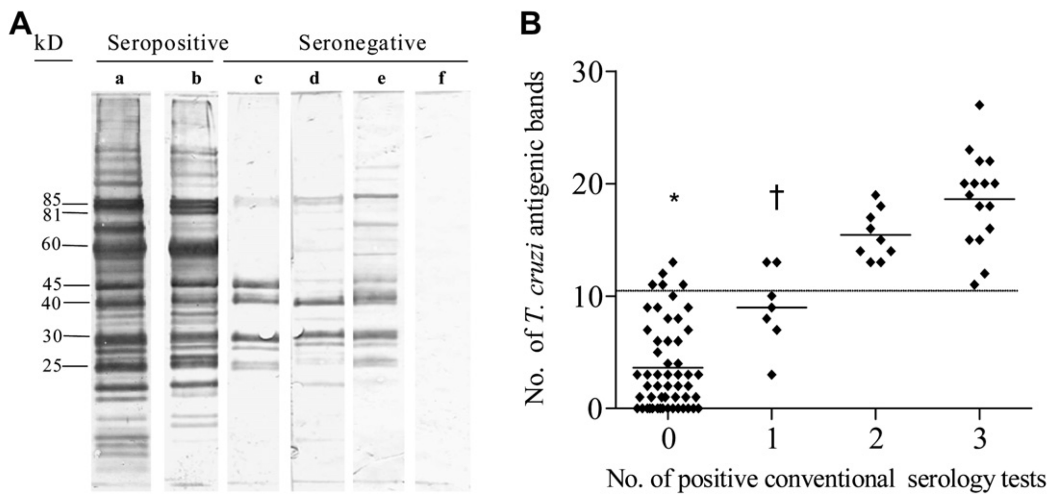

Fig. 2. Western blot analysis of *Trypanosoma cruzi* epimastigotes. A. Sera from subjects of SE (a, c, d) and LR (b, e), with positive (a, b) and negative (c–e) conventional serology, and a non-endemic control (f). B. Each point represents the number of bands recognized by each individual from endemic area tested. Horizontal lines indicate the mean values for seronegative (0 and 1 positive conventional serology tests) and seropositive individuals (2 or 3 positive serodiagnosis tests). The dotted line indicates the mean minus 2SD number of bands recognized by seropositive subjects. *0 vs. 1 positive tests, $P < 0.01$, 0 vs. 2 and 3 positive tests, $P < 0.001$; †1 vs. 2 and 3 positive tests, $P < 0.01$ (ANOVA followed by Bonferroni test). Correlation coefficient between the number of positive serodiagnostic tests and the number of bands, $r = 0.81$; Spearman test.

(11%, four subjects from SE and three from LR) recognized ≥11 bands and thus, their level of serum reactivity was within the range of subjects positive by conventional serology. Additionally, four of these subjects had *T. cruzi*-specific T cells in IFN-γ ELISPOT assays, supporting the likelihood of prior contact with the parasite.

To investigate whether individuals showing IFN-γ responses to recombinant *T. cruzi* proteins also show serological reactivity against the same antigens, recombinant proteins Kn122, Kn107, AnoH-G10 and AnoL-E02 were used as antigens in immunoblotting assays. All subjects who had IFN-γ secreting cells against the recombinant proteins (four seropositive and five seronegative by conventional serology) recognized at least one of the possible target proteins (Fig. 3). Sera from five non-endemic controls tested with immunoblotting bound weakly to recombinant protein Kn107, but not to recombinant proteins Kn122, AnoH-G10 and AnoL-E02, indicating the potentiality of the later as targets for serodiagnosis of human Chagas disease.

4. Discussion

In the present study, we demonstrate that a substantial proportion of individuals living in endemic areas of Chagas disease of Argentina who were negative for *T. cruzi* by

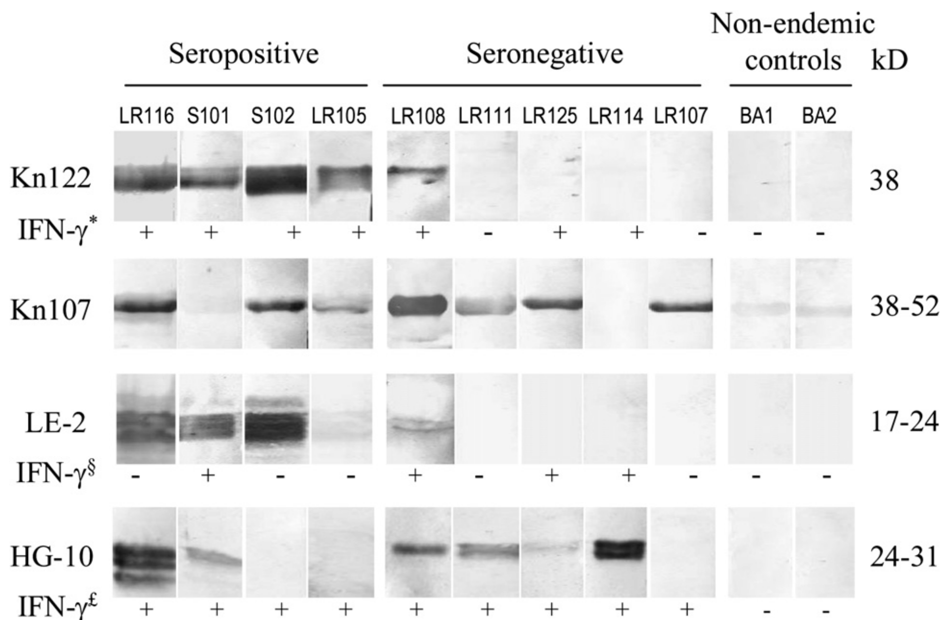

Fig. 3. Serum reactivity to recombinant *Trypanosoma cruzi* proteins by Western blot. Different patterns of antigen recognition were obtained in subjects from endemic areas of Chagas disease with positive and negative conventional serology. *, § and £ indicate positive (+) and negative (−) IFN-γ responses to protein Kn122, Pool 2 and Pool 1 in ELISPOT assays, respectively.

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conventional antibody testing, nevertheless had *T. cruzi*-specific T cells capable of producing IFN-γ in ELISPOT assays. A similar pattern of immune responses was described in individuals exposed to viral infections but who remained without detectable viremia or seroconversion [6–8]. Exposure to low-dose viruses inducing virus-specific cellular immunity, sub-clinical spontaneously resolving infections, and the maintenance of cellular responses by cross-reacting environmental antigens were all postulated to account for this type of responses. It is possible that healthy individuals exposed to *T. cruzi* in endemic areas of Chagas disease may have had prior, resolved *T. cruzi* infections with subsequent parasite clearance and partial loss of antibodies but with the retention of a strong T cell memory. Occasional spontaneous seronegative conversion described in chronic *T. cruzi* infections supports this hypothesis [9]. Alternatively, repetitive exposure to *T. cruzi* in endemic areas may provide a persistent source of antigens that maintain memory T cell responses, possibly resulting in protective immunity to healthy individuals. It can be argued that some of the immune responses elicited by *T. cruzi* antigens in vitro could have been induced by other protozoan parasites circulating in Argentina [10,11]. However, the reactivity to recombinant *T. cruzi* antigens found in this study strongly argue against other microorganisms as the target for these responses.

One implication of this work is that definitions of exposure and infection solely on the basis of the presence of antibodies against *T. cruzi* detectable by conventional serology tests at a single point in time may significantly underestimate the true rate of exposure to the parasite in endemic areas. The lack of gold standard techniques for accurately determining *T. cruzi* infection indicates that a number of assays, such as serology, T cell assays [2,12], immunoblotting, etc, will be required to have a more complete picture of the infection status.

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