# Immunological imbalance between IFN-γ and IL-10 levels in the sera of patients with the cardiac form of Chagas disease

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*The immune response is crucial for protection against disease; however, immunological imbalances can lead to heart and digestive tract lesions in chagasic patients. Several studies have evaluated the cellular and humoral immune responses in chagasic patients in an attempt to correlate immunological findings with clinical forms of Chagas disease. Moreover, immunoglobulins and cytokines are important for parasitic control and are involved in lesion genesis. Here, cytokine and IgG isotype production were studied, using total epimastigote antigen on sera of chagasic patients with indeterminate (IND, n = 27) and cardiac (CARD, n = 16) forms of the disease. Samples from normal, uninfected individuals (NI, n = 30) were use as controls. The results showed that sera from both IND and CARD patients contained higher levels of* Trypanosoma cruzi*-specific IgG1 (IgG1) antibodies than sera from NI. No difference in IgG2 production levels was observed between NI, IND and CARD patients, nor was a difference in IL-10 and IFN-γ production detected in the sera of IND, CARD and NI patients. However, IND patients displayed a positive correlation between IL-10 and IFN-γ levels in serum, while CARD patients showed no such correlation, indicating an uncontrolled inflammatory response in CARD patients. These findings support the hypothesis that a lack of efficient regulation between IFN-γ and IL-10 productions in CARD patients may lead to cardiac immunopathology.*

Key words: *Trypanosoma cruzi* - Chagas disease - immunopathogenesis - cytokines - IgG isotype

It is well accepted that the host's immune response plays a key role during *Trypanosoma cruzi* infection, leading to either parasite control during the acute phase or to participation in the pathology development during the chronic phase (Dutra & Gollob 2008). Antibody production and T-cell responses have been studied in patients with Chagas disease (Morgan et al. 1996, Cordeiro et al. 2001) and experimental models (Giordanengo et al. 2000, Guedes et al. 2008). Mononuclear cells from the heart tissue or peripheral blood of patients with the cardiac form of the disease produce higher IFN-γ and TNF-α and lower or absent production of IL-10 and IL-4 compared with asymptomatic individuals (Correa-Oliveira et al. 1999, Ribeirao et al. 2000, Gomes et al. 2003). However, other studies have shown that the mean levels of mRNA expression for IL-5, IL-10, IL-13 and IFN-γ were dramatically increased in peripheral blood mononuclear cells (PBMCs) freshly isolated from chagasic patients, regardless of the clinical form, compared to uninfected individuals (NI) (Dutra et al. 1997). Furthermore, the authors observed no significant differences in cytokine levels between cells from cardiac (CARD) and indeterminate patients (IND) upon stimulation with parasite-derived antigens (epimastigote and trypomastigote) (Dutra et al. 1997).

Similarly, cytokine levels in the sera from CARD and IND patients were analyzed and contradictory results were obtained. Higher TNF-α (Ferreira et al. 2003, Talvani et al. 2004) and NO levels (Perez-Fuentes et al. 2007) were observed in the plasma of chagasic CARD patients with the severe form of the disease, compared to patients with mild cardiopathy and NI. These results suggest a role for systemic NO and TNF-α in dilated cardiomyopathy. In contrast, no difference was observed in cytokine levels in the sera of chagasic patients with different clinical outcomes. IL-2, IFN-γ, and TNF-α production were similar in all groups of chagasic patients with different forms of the disease (Ward et al. 1999). These results were confirmed by other studies, which also showed that, although the levels of TNF-α and NO were higher in sera from chagasic patients, compared to non-chagasic individuals, no difference was observed between asymptomatic and symptomatic chagasic patients (Perez-Fuentes et al. 2008). IFN-γ levels were similar in the sera of asymptomatic individuals and CARD patients that presented the form of the disease (Ribeirao et al. 2000). Thus, the findings of several studies have failed to clearly establish a correlation between the concentration of circulating cytokines and the cardiac form of Chagas disease.

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Studies concerning antibody production during the chronic phase of Chagas disease have focused on the analysis of IgG isotypes. Differences in the biological properties of these antibodies may have an important effect on the natural history of Chagas disease. Moreover, the production of different antibody isotypes is controlled by distinct cytokines. In chronic chagasic patients, IgG1 and IgG2 form about 90% of the IgG produced (Watthanakulpanich et al. 2008). It has been demonstrated that Th1 cytokines (IL-12, IFN-γ, TNF-α) are responsible for the production of IgG1 and IgG3 isotypes, while Th2 cytokines (IL-4 and IL-10) stimulate IgG2 production (Briere et al. 1994, Kawano et al. 1994). IgG1 mediates lysis binding to complement C1q protein and macrophage phagocytosis, while IgG2 mediates immunity by non-phagocytic effectors cells (Fanger et al. 1991). Correlation between the different clinical forms of Chagas disease and the levels of IgG isotypes has not been clearly defined. Several authors have tried to demonstrate the correlation between IgG isotypes and the severity of the different clinical forms of Chagas disease both in patient groups (Cerban et al. 1993, Morgan et al. 1996, Michailowsky et al. 2003) and in experimental models (Giordanengo et al. 2000, Guedes et al. 2008). While some studies detected no differences between the levels of these immunoglobulins (IgG) among individuals with different clinical manifestations (Cerban et al. 1993, Michailowsky et al. 2003), others demonstrated higher levels of IgG2 antibodies in the sera of patients with cardiac and digestive manifestations of the disease (Morgan et al. 1996, Cordeiro et al. 2001).

The aim of the present study was to analyze the levels of IL-10, IFN-γ and anti-*T. cruzi* antibody (IgG1 and IgG2 isotypes) production and determine the possible usefulness of these cytokines and antibodies as immunological markers of clinical evolution in the sera of chagasic IND and CARD patients with clinical forms of the disease. Correlation analysis between regulatory and inflammatory cytokine production in CARD and IND patients was performed to improve the current understanding of the immunoregulation involved in pathological development in human Chagas disease. Analysis of the results showed an imbalanced immune response in CARD patients, suggesting that an uncontrolled immune response is associated with cardiac disease.

## PATIENTS AND METHODS

*Patients* - The inclusion of all subjects in the present investigation was approved by two independent Ethical Committees [Universidade Federal de Minas Gerais (087/99), Belo Horizonte, Minas Gerais, and Hospital São Salvador, Goiânia, Goiás, Brazil]. Signed, informed consent forms were obtained from the participants. All patients (n = 43) showed positive serology for Chagas disease, as determined by ELISA and immunofluorescence tests (Camargo 1966). The patients were from endemic areas in Brazil. The data (number, sex, age and clinical form) are summarized in Table. Based on their clinical records, the chagasic patients were divided into two different groups, IND and CARD. Patients presenting asymptomatic *T. cruzi* infection, classified as IND (n = 27), presented no clinical manifestations of the disease other than their positive serology. Patients with cardiac dysfunction (n = 16) presented with dilated cardiomyopathy and were identified via a detailed clinical examination, including electrocardiogram, chest X-ray, Holter and echodopplercardiography. Sera from NI (n = 30) were obtained from seronegative healthy inhab-

TABLE

Age, sex and clinical forms of chronic chagasic patients from states of Minas Gerais, Goiás and Mato Grosso, central region of Brazil

| Patient number | Sex | Age (years) | Clinical form |
|---|---|---|---|
| 1 | F | 27 | IND |
| 2 | M | 51 | IND |
| 3 | M | 45 | CARD |
| 4 | F | 33 | IND |
| 5 | M | 33 | IND |
| 6 | F | 27 | IND |
| 7 | F | 39 | IND |
| 8 | M | 25 | IND |
| 9 | M | 44 | CARD |
| 10 | F | 46 | IND |
| 11 | F | 54 | CARD |
| 12 | F | 49 | CARD |
| 13 | F | 54 | IND |
| 14 | F | 35 | CARD |
| 15 | F | 31 | CARD |
| 16 | F | 37 | IND |
| 17 | F | 26 | IND |
| 18 | F | 44 | IND |
| 19 | F | 43 | IND |
| 20 | F | 33 | IND |
| 21 | F | 20 | IND |
| 22 | M | 30 | IND |
| 23 | M | 42 | IND |
| 24 | F | 22 | IND |
| 25 | M | 30 | IND |
| 26 | M | 38 | IND |
| 27 | F | 35 | IND |
| 28 | F | 53 | IND |
| 29 | F | 33 | CARD |
| 30 | M | 42 | IND |
| 31 | F | 44 | IND |
| 32 | F | 40 | IND |
| 33 | M | 50 | CARD |
| 34 | M | 75 | IND |
| 35 | M | 57 | CARD |
| 36 | M | 45 | CARD |
| 37 | F | 39 | CARD |
| 38 | F | 50 | CARD |
| 39 | F | 59 | CARD |
| 40 | M | 25 | CARD |
| 41 | M | 57 | CARD |
| 42 | M | 76 | IND |
| 43 | F | 47 | CARD |

CARD: cardiac; F: female; IND: indeterminate; M: male.

itants of the same endemic areas. Normal individuals did not differ from Chagas disease patients with regard to age or gender.

*Conventional serology (ELISA)* - Peripheral blood was collected and maintained at 4°C for 30 min to promote clot formation. Serum samples were stored at -80°C prior to cytokine quantification. ELISA tests were performed according to the methods of Voller et al. (1976). Briefly, ELISA plates were sensitized with *T. cruzi* antigen prepared by alkaline extraction of the Y strain, obtained by exponential growth in LIT medium. The sera were added to the plates and antibody binding was detected using peroxidase-labeled anti-human IgG. Total IgG or IgG1 and IgG2 isotypes conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, USA) were used to determine antibody levels. The plates were read in a spectrophotometer using a 490 nm filter (BIO-RAD, 3550). The cut-off was determined using the mean absorbance of 10 NI individuals plus two standard deviations.

*Cytokine quantification (ELISA)* - Cytokine levels in the sera were determined by ELISA. The ELISA sets were IL-10 and IFN-γ (R & D Duoset, R & D, Minneapolis, MN). Procedures were performed according to the manufacturer's instructions. The reaction was detected by peroxidase-conjugated streptavidin followed by a substrate mixture containing hydrogen peroxide and ABTS (Sigma Aldrich, St. Louis, MO) as a chromogen.

*Statistical analyses* - Regression analysis was used to compare antibody and cytokine levels; regression lines were compared by analysis of covariance. In all cases, differences were considered statistically significant when $p < 0.05$. All analyses were performed using the PRISM 3.0 software (GraphPad, San Diego, CA).

## RESULTS

In order to correlate clinical findings with the immunological response observed during chronic Chagas disease, and to identify potential immunological pathogenesis markers, circulating cytokine (IL-10 and IFN-γ) and IgG isotype (total IgG, IgG1 and IgG2) levels were determined in IND and CARD chagasic patients.

Higher production of total IgG was observed in IND (1.26 ± 0.17) and CARD (1.23 ± 0.13) patients compared to NI (0.18 ± 0.04, $p < 0.05$) patients (Fig. 1A). An analysis of the results showed that sera from both IND (0.86 ± 0.23) and CARD (0.88 ± 0.22) patients presented with higher levels of *T. cruzi*-specific IgG1 antibodies than NI (0.14 ± 0.03, $p < 0.05$) patients. However, no difference was observed between IND and CARD patients in terms of IgG1 production (Fig. 1B). No difference was observed in IgG2 levels between IND (0.34 ± 0.14), CARD (0.32 ± 0.16) and NI (0.23 ± 0.09) individuals (Fig. 1C).

Statistically similar levels of IL-10 were detected in the sera of IND patients (9.48 ± 3.09), CARD chagasic patients (13.68 ± 2.35) and NI individuals (8.13 ± 3.82) (Fig. 2). Patients with IND (59.44 ± 18.63) and CARD (80.12 ± 22.32) forms of Chagas disease, as well as NI individuals (53.83 ± 17.41) presented similar IFN-γ levels (Fig. 2).

In an attempt to clarify the immunoregulatory mechanisms involved in the chronic cardiac pathology of Chagas disease, our group investigated whether a correlation exists between cytokine and IgG isotype production, and between regulatory (IL-10) and inflammatory (IFN-γ) cytokine levels in CARD and IND patients. No correlation was observed between cytokine (IL-10 and IFN-γ) and IgG isotype (IgG1 and IgG2) production (data not shown); however, a positive correlation ($R = 0.82$, $p < 0.0001$) (Fig. 3A) was observed between IFN-γ and IL-10 levels in the sera of IND patients. Interestingly, an analysis of CARD patients showed no correlation ($R = 0.11$, $p = 0.69$) between regulatory and inflammatory cytokine production (Fig. 3B).

## DISCUSSION

Distinct cytokine profiles exert important influences in IgG production, and antibodies are important to control parasite infection and immunopathological processes (Tibbetts et al. 1994). The current results, using total epimastigote antigen, showed high levels of IgG1 in chagasic patients compared to non-chagasic individuals and similar IgG1 levels in with the IND versus the CARD clinical forms. These results are in agreement with data that demonstrated high IgG1 concentrations against epimastigote antigen in sera from all groups of chagasic pa-

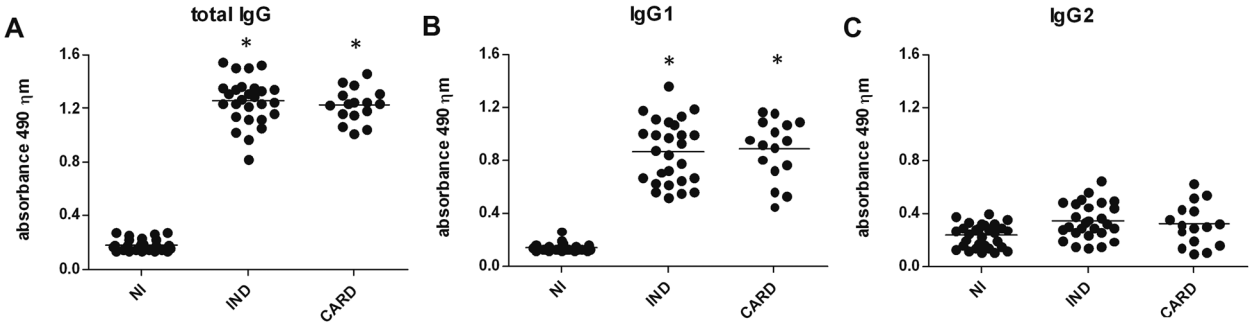


Fig. 1: *Trypanosoma cruzi* specific total-IgG (A), IgG1 (B) and IgG2 (C) antibodies in the sera of indeterminate (IND) and cardiac (CARD) patients with chronic Chagas disease and noninfected individuals (NI). Asterisks indicate statistic significance for comparisons between NI and chagasic patients (IND and CARD) ($p < 0.05$). Each data point represents the mean absorbance of duplicate wells.

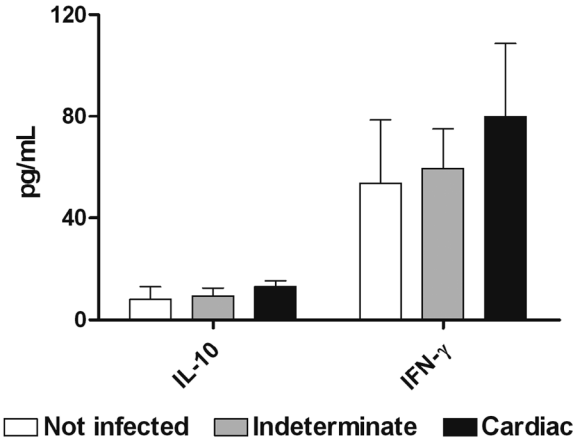


Fig. 2: IL-10 and IFN-γ quantification by ELISA in the sera of indeterminate and cardiac patients with chronic Chagas disease and non infected individuals. Results are mean ± standard error medium.

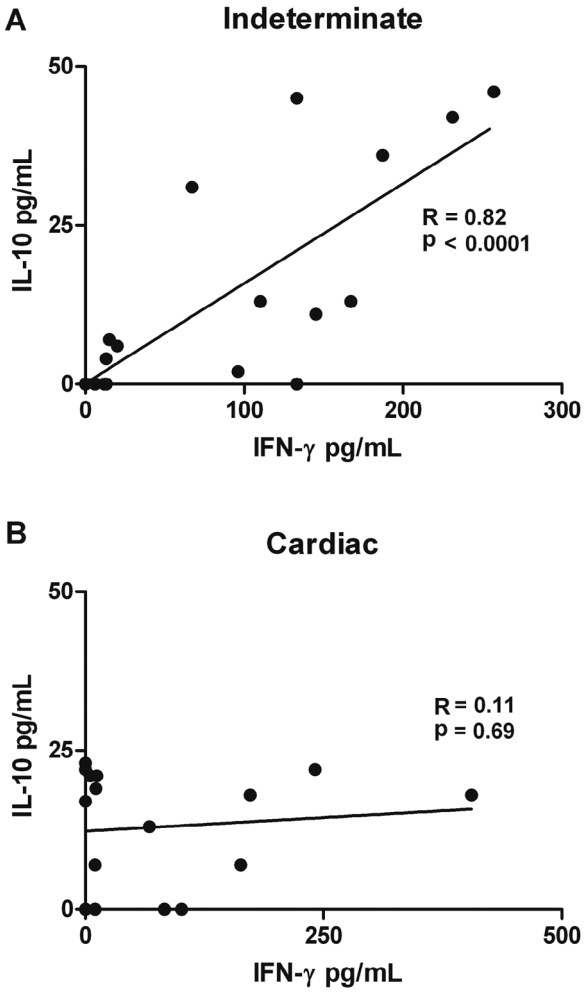


Fig. 3: correlation between IFN-γ and IL-10 levels in the sera of chagasic patients that presented the indeterminate (A) and cardiac (B) forms of the disease. Positive correlation was considered significant when the $p < 0.05$.

tients by ELISA, regardless of the clinical form (Cerban et al. 1993). High IgG1 and similar IgG3 production in patients presenting different clinical forms of the disease were described using different antigenic preparations, such as paraflagellar rod protein (Michailowsky et al. 2003). Cerban et al. (1993) observed no significant differences regarding IgG isotype production in chronic chagasic patients with different clinical forms. However, a different antigenic recognition pattern by IgG1 among different clinical groups by immunoblotting using acidic antigenic fraction (AAF) separated from *T. cruzi* cytosol was observed. It seems that some relationship exists between the pattern of IgG1 reactivity with AAF and the degree of heart damage. The distinct production of IgG isotypes identified via ELISA was described for chagasic patients with different clinical forms of the disease. Analysis of literature data shows that when using epimastigote antigenic preparation, CARD patients produced elevated levels of IgG2 compared with the IND and NI groups (Morgan et al. 1996). It is possible that the preferential induction of different subsets of T helper cells by specific antigen or host genetic background may be crucial to the pattern of antibody response and, subsequently, in the immunopathology of Chagas disease. Patients with different clinical forms of Chagas disease have the ability to produce similar antibody levels; therefore, given the clinical and immunological differences among the groups, it is reasonable to hypothesize that these antibodies may recognize different antigenic targets.

Cytokine profiling has been demonstrated as fundamental to defining the immunopathological mechanisms involved in chronic chagasic cardiomyopathy and controlling the immune response during *T. cruzi* infection (Zhang & Tarleton 1996). The proinflammatory cytokines IL-12, IFN-γ and TNF-α (Th1 response) act in concert to activate macrophages to kill the parasites through the production of nitric oxide and NO-derived nitrogen free radicals. In addition, they also stimulate the differentiation and proliferation of Th1-biased CD4 T cells, which may orchestrate a CD8 T cell response that causes tissue destruction and fibrosis (Higuchi et al. 2003). As expected, this inflammatory response needs to be regulated, and this occurs mainly via the action of the anti-inflammatory cytokines IL-4, IL-10 and TGF-β (Silva et al. 1992, Holscher et al. 1998). Thus, cytokine expression may be correlated with clinical manifestations of disease.

A few studies concerning cytokine sera levels have been performed in individuals with different clinical forms of Chagas disease and contradictory results were obtained. It is possible that the contradictions are due to the procedures used for sample collection, which can influence the outcome of laboratory measurements. This study showed that serum cytokine levels in patients with the CARD chronic form of the disease did not differ from those of IND patients. Thus, the present results are in agreement with the finding of Ward et al. (1999) that similar levels of IFN-γ and TNF-α can be found in the sera of chronic chagasic patients (both IND and CARD form) and NI patients. Interestingly, although these authors used different collection procedures than

those used by our group, similar results were obtained. This implies that other factors, possibly related to the patient classification criteria, could also be involved, as suggested by Dutra et al. (2005). Similar high levels of TNF-α were detected in asymptomatic individuals and patients with digestive and CARD clinical forms, respectively (Perez-Fuentes et al. 2007). However, Ferreira et al. (2003) showed that patients with significant left ventricular (LV) dysfunction (LV ejection fraction ≤ 50%) showed higher levels of TNF-α, compared to chagasic patients without LV dysfunction. It has been suggested that chronic TNF-α production prior to heart failure may play a role in chronic Chagas disease cardiomyopathy progression (Ferreira et al. 2003). A similar level of cytokine production in sera of acute chagasic children, compared to NI, has been described previously (Moretti et al. 2002). Although the present results showed no differences between IL-10 and IFN-γ levels in CARD and IND patients, the IND patients displayed a positive correlation between inflammatory and regulatory cytokine production, indicating a controlled immune response. These findings are supported by the higher production of IFN-γ and TNF-α associated with high IL-10 levels described in IND patients, while CARD patients showed an unregulated Th1 response (Bahia-Oliveira et al. 1998, Correa-Oliveira et al. 1999, Ribeirao et al. 2000, Abel et al. 2001, Gomes et al. 2005). A lack of co-regulation between inflammatory and anti-inflammatory cytokines associated with severe disease was also demonstrated in human patients with leishmaniasis (Gaze et al. 2006), strengthening this hypothesis.

In conclusion, these findings suggest that no correlation exists between cytokine (IL-10 and IFN-γ) and IgG isotype (IgG1 and IgG2) levels in the sera of chagasic patients with IND and CARD clinical forms of Chagas disease. However, analysis of the results reinforces the fact that more efficient regulation of IFN-γ and IL-10 production occurs in IND patients, compared to CARD patients, suggesting that the maintenance of a balanced immune response is critical for arresting pathology establishment.

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