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Original papers

Serum cytokines and activation *ex vivo* of CD4+ and CD8+ T cells in chagasic chronic Mexican patients

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ABSTRACT. The clinical manifestations of human Chagas disease are associated with several factors, including immunological alterations, in this regard, many studies propose that tissue damage might be more severe in the absence of immune regulatory mechanisms, other factors are the genetic background of host and parasite. *Trypanosoma cruzi* population is genetically, biochemistry and pathogenic diverse along the Latin-America continent and phylogenetic ally are divided into six intra-species lineages TcI-VI. The TcI lineage has a wide distribution with heterogeneous virulence and pathogenesis within strains. In Mexico, the main circulating lineage is TcI in human infections. We analyzed intracytoplasmic cytokines of unstimulated peripheral T lymphocytes, and the level of cytokines (IL-2, IL-4, IL-12, IL-10, IFN-γ and sIL-2R) in the serum of Mexican chagasic subjects. The population studied consisted of 15 asymptomatic individuals, 17 patients with chronic chagasic cardiopathy (CCC), 20 patients with cardiopathy but negative serology for *T. cruzi*, and 10 healthy subjects. The analysis of CD4+ cells revealed that CCC and asymptomatic patients have higher CD25+ and CD69 activation markers than controls. The Th1 subset (CD4+/IFN- γ +) was higher in CCC than in asymptomatic and control subjects, whereas Th2 subset was markedly high in asymptomatic subjects. Circulating cytokines were below level detection with the exception of IL-2 and sIL-2R. Infection with Mexican *Trypanosoma cruzi* strains in asymptomatic chagasic subjects have a tendency for a Th2 response with higher CD8+/IFN-γ T cells. In contrast, CCC patients have low levels of intracellular IFN- γ and IL-2 cytokines. In both groups circulating serum cytokines are below the detectable level.

Key words: Chagas disease, cytokines, chronic chagasic cardiomyopathy

Introduction

Chagasic infection, caused by the protozoan *Trypanosoma cruzi*, may lead to two stages of the disease that are namely; the acute phase characterized by high parasitemia and parasitism of tissue cells with marked inflammatory infiltration, followed by the chronic stage with a broad spectrum of clinical manifestations, ranging from absence of symptoms to severe heart and or hollow organs involvement [1]. The cardiac chronic stage (CCC) is characterized, histologically, by the presence of mild heart inflammation and parasite persistence at very low concentration, usually detected by PCR in 83% of the cases [2]. The heart inflammatory infiltrate is composed of macrophages (50%), B cells, and T cells (13% and 32%, respectively) and very few NK cells. In addition, immuno-histochemical techniques revealed that IFN-γ+ T cells were present in higher numbers than IL-2+ and IL-4+ T cells. T cells line obtained from endomyocardial biopsies from CCC patients preferentially produce IFN-γ and TNF-α, indicating an inflammatory pattern [3]. In peripheral blood, the immunological alteration has been observed in T lymphocytes from CCC patients. Dutra et al. [4]

found decreased expression of CD28 in many circulating CD4+ and CD8+ in CCC in comparison with non-chagasic subjects, suggesting an anergy state or propensity towards apoptosis.

Many published studies propose that tissue damage might be more severe in the absence of regulatory mechanisms involving both innate and adaptive immune responses. In this context, Vitelli et al. [5] found a high frequency of CD4+ CD25 high T cells in the indeterminate clinical form rather than in CCC patients. In contrast, the increased percentage of activated CD8+HLA-DR+ T cells was associated with severe clinical forms. In this line of research, Gomes et al. [6] found two categories of patients, low and high producers of IFN-γ after in vitro culture of peripheral blood mononuclear cells when stimulated with *T. cruzi* antigens. The CD4+ T cells from CCC patients were high producers of IFN-γ, whereas the indeterminate subjects were classified as low IFN-γ producers, but secreted significantly higher amounts of IL-10.

Levels of TNF-α in the sera of children with acute chagasic infection and patent parasitemia presented higher values than in patients in the indeterminate stage or patients treated with benznidazole; however, a similar pattern for sIL-2R was found in both groups, only those patients in the acute phase and positive parasitemia showed high levels above 2000 U/ml [7]. This finding suggests that patent parasitemia elicits production of mediators of the immune system very efficiently. In this line of evidence, Cuna et al. [8] found higher concentrations of IFN-γ and TNF-α in serum samples from peripheral, placenta, and cord blood of chronically infected mothers with detectable parasitemia than in healthy controls and subjects with undetectable parasitemia.

In contrast to the acute phase, parasitemia is very scarce and cytokines in serum could be expected at very low concentration in the chronic stage. Ward et al. [9] found that TNF-α was present at an undetectable level and IFN-γ did not differ from that of healthy controls.

From the above data, we can see that the immune profile could be different for each stage of the disease. These findings provide support to the search for disease severity biomarkers.

However, the high variation within *T. cruzi* populations introduce another variable in the disease; for example, it can influence the immune response in the mammal host. In this regard, dos Santos et al. [10] have found that genetic diversity of the parasite influences the profile of the humoral immune response.

Trypanosoma cruzi has been genotypically divided into six intra-species lineages (discrete typing unit, DTU), recently renamed TcI-VI [11]. The geographical distribution of the DTUs is complex. TcI is found as far north as the USA; in contrast, TcII, V, and VI predominate in the southern cone countries.

In Mexico, one of the main circulating lineages of *T. cruzi* is TcI [12,13]. Little is known about the immunological alteration that Mexican TcI lineage could induce in infected subjects. Until recently, it was demonstrated that Mexican chagasic patients have higher serum levels of TNF-α than control healthy subjects [14]. On the other hand, using a murine experimental infection model infected with Mexican strains, a poor induction of cytokine at the inoculation site was shown; in contrast, regional lymph node showed cytokine induction, detected from the first day post infection, whereas pro-inflammatory and anti-inflammatory cytokines were demonstrated in serum during the acute and chronic stages of the infection, in addition, *T. cruzi* strain influences the pattern of immune response [15–17].

A common approach to study the immunological status of immune cells is to set an *in vitro* culture and proceed to antigen stimulation. Lorena et al. [18] analyzed the relationship between the production of intracytoplasmic cytokines after *in vitro* stimulation with recombinant antigens, they found that IFN-γ and TNF-α produced by CD8+ T lymphocytes differed between CCC and indeterminate subjects.

In the present work, we analyzed intracytoplasmic cytokines of unstimulated peripheral T lymphocytes (basal state), and the level of cytokines in serum of Mexican chagasic subjects infected with Mexican *T. cruzi* strains.

Materials and Methods

The study included unrelated Mexican individuals serologically positive for *T. cruzi* diagnosed at the Instituto Nacional de Cardiologia I Chávez. The patients were classified according to clinical, electrocardiographic (ECG), and echocardiogram characteristics as asymptomatic or CCC. The asymptomatic population consisted of 15 apparently healthy individuals having a normal ECG, without cardiomegaly. The CCC group included 17 patients with either arrhythmia-related

symptoms, five or more extra systoles per minute, embolic episodes, cardiomegaly or congestive heart failure.

Additionally, one group of 10 not related healthy individuals with neither symptoms nor previous diagnosis of Chagas disease was studied as a control group. All patients with Chagas disease, as well as healthy controls, shared the same environmental and socioeconomic conditions.

Blood samples for cytometry. We obtained 2 ml of blood samples from all patients and healthy controls in heparinized collection tubes. We used 800 µl of each sample divided in 8 assay tubes (100 µl in each one) with 2×10^5 cells each one. Four samples were stained with monoclonal anti-human CD4+, and four with monoclonal anti-human CD8+ antibodies.

We performed three-color flow cytometry analysis of mononuclear cells (MC) surface markers by using different combinations of the following monoclonal antibodies (mAbs) and their matched control isotype: CD4-PerCP, CD8-PerCP, CD25-phycoerythrin (PE), CD69-PE, and mouse IgGγ1-PE, and mouse IgGγ2a fluorescein isothiocyanate (FITC) as isotype control (Beckton Dickinson, Erembodegem, Belgium). We analyzed cells using CD4+ and CD8+ cell gate. We performed data acquisition and analysis using Becton Dickinson fluorescence-activated cell sorter scanner (FACSscan) flow cytometer and CellQuest software (Becton Dickinson).

We examined intracellular molecules with FITC-conjugate mAbs anti-human IL-2 and IFN-γ (Becton Dickinson). We first stained MC with both

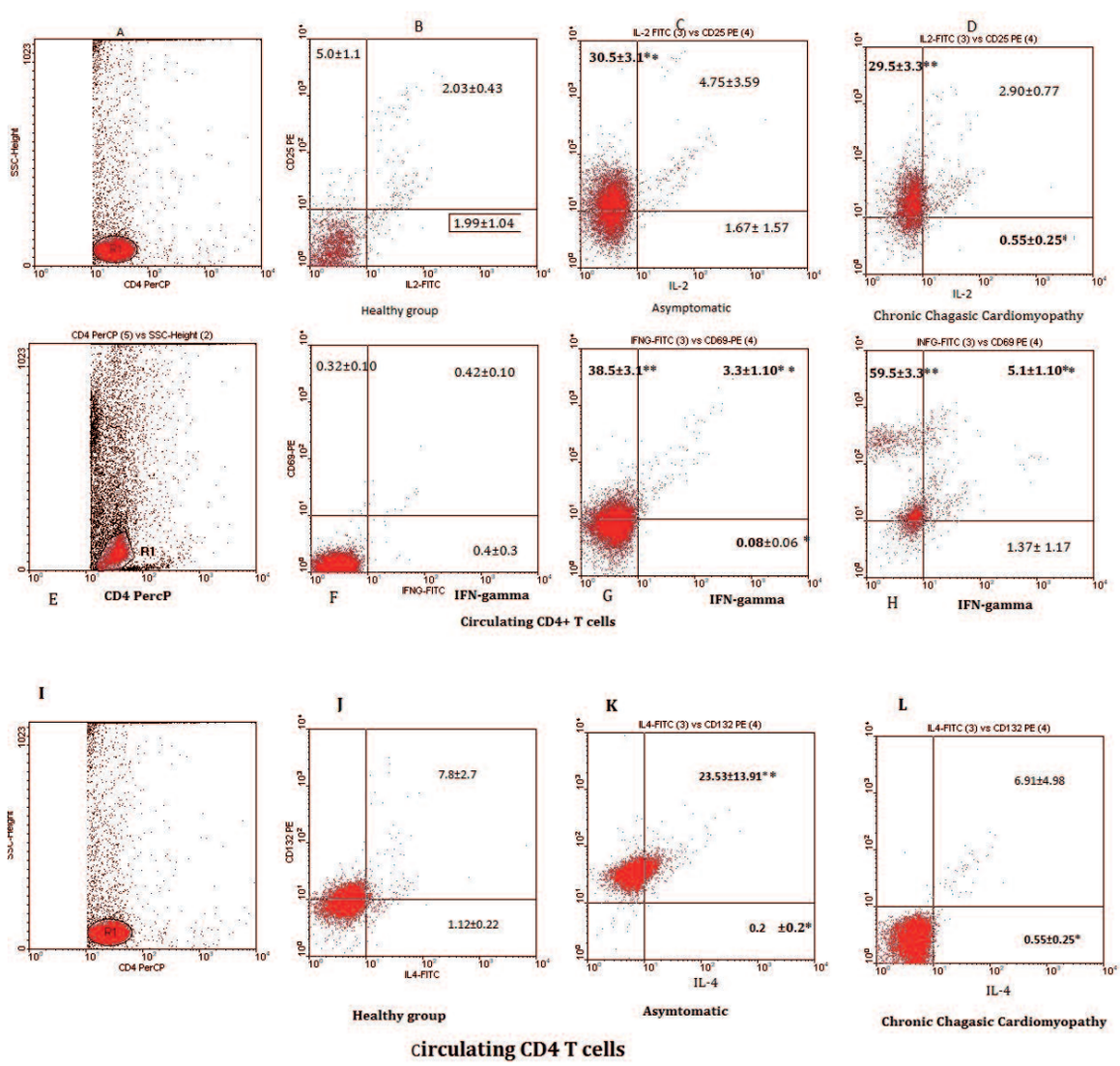

Fig. 1. Circulating $CD4^+$ lymphocytes containing intracellular cytokines, and the surface activation markers in Asymptomatics, Chronic Chagasic Cardiomyopathy and Control Groups

Table 1. Mean proportion (mean±SE) of circulating $CD4^+$ lymphocytes containing intracellular cytokines, and the surface activation markers in Asymptomatics, Chronic Chagasic Cardiomyopathy and Control Groups

	Controls (n=10)	Asymptomatics (n=15)	Chronic Chagasic Cardiomypathy (n=17)
$IL\text{-}2^+$	1.99 ± 1.04	1.67 ± 1.57	0.55 ± 0.25*
$CD25^+$(α-chain)	5.0 ± 1.1	30.5 ± 3.1**	29.5 ± 3.3**
$IL\text{-}2^+/CD25^+$	2.03 ± 0.43	4.75 ± 3.59	2.90 ± 0.77
$IFN\text{-}\gamma^+$	0.4 ± 0.30	0.08 ± 0.06	1.37 ± 1.17**
CD69	0.32 ± 0.10	38.5 ± 3.1**	59.5 ± 3.3**
$IFN\text{-}\gamma^+$/ CD69	0.42 ± 0.09	3.3 ± 1.10**	5.1 ± 1.10**
$IL\text{-}4^+$	1.12 ± 0.22	0.20 ± 0.2*	0.55 ± 0.25*
$CD132^+/IL\text{-}4^+$	7.8 ± 2.7	23.53 ± 13.91**	6.91 ± 4.98
Th1 (all $IFN\text{-}\gamma^+$)	0.82	3.38**	6.47**
Th2 (all $IL\text{-}4^+$)	8.92	25.7**	7.46

*diminished when compares with healthy controls ($p<0.05$)
**increased when compares with healthy controls ($p<0.05$)

anti-CD4-PerCP and CD8-PerCP for 15 minutes at room temperature. We then fixed the cells for 10 minutes at room temperature with the FACS lysis solution (Beckton Dickinson), washed them with 1% PBS-BSA and then permeabilized them for 10 minutes with the FACS permeabilizing solution (Beckton Dickinson), prior to staining with anti-human cytokines in the dark for 30 minutes. Finally, we washed with PBS-BSA 1%, fixed with PBS-BSA 1% and sodium azide at 0.1% and proceeded with the analysis in the FACScan. The data were analyzed by U of Man-Withney and were expressed as a mean proportion ± standard error.

Cytokines in serum. The production of cytokines (IL-2, sIL-2R, IL4, IL-10, IL-12, and IFN-γ) in serum was determined by BD OptEIA test (CA, USA) according to manufacturer's instruction. This is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). Polystyrene microtiter plates (Maxisorp surface, Nunc, USA) were coated with 100 µl per well of capture antibody diluted (1:250) in coating buffer, the plate was incubated overnight at 4°C. After incubation, the plates were washed 3 times with ≥300 µl/well PBS with 0.05% Tween 20, and blocked at room temperature (RT) for 1 hour with ≥200 µl /well assay diluent (PBS with 10% FBS). Plates were then incubated at RT for 2 hours with 100 µl of each standard and serum sample (17 asymptomatic instead of the 15 used for cytometry, 23 CCC instead of the 17 used in cytometry, 20 non-chagasic cardiopathy patients, this group was not included for cytometry analysis, and 20 healthy subjects instead of the 10 used for cytometry), into appropriate wells, followed by incubation at RT for 1 hour with 100 µl of working detector (Detection Antibody + Avidin-HRP reagent, 1:250 dilution). The next step was to add 100 µl of substrate solution (0.1 M citrate buffer, pH 4.5, containing 8 mG o-phenylenediamine and 8 µl H_2O_2) to each well for 30 min at RT in the dark. The reaction was stopped by the addition of 50 µl of 5 N H_2SO_4 and the absorbance value (OD 492 nm) measured with an ELISA plate reader (BIO-RAD Model 550).

The Institutional Ethics and Research Committees approved this study and all patients signed an informed consent.

Results

The analysis of CD4+ cells obtained from blood and immediately processed revealed that CCC and asymptomatic patients have higher CD25+ and CD69 activation markers than controls, but expressed lower IL-4 intracytoplasmic cytokine. The Th1 subset (CD4+/IFN-γ +) was higher in CCC

Table 2. Mean proportion (mean ± SE) of $CD8^+$ lymphocytes containing intracellular cytokines and the surface activation markers in Asymptomatics, Chronic Chagasic Cardiomyopathy and Control Groups

	Controls (n=10)	Asymptomatic (n=15)	Chronic Chagasic Cardiomypathy (n=17)
$IL\text{-}2^+$	1.06 ± 0.41	0.67 ± 0.41	0.35 ± 0.1*
$CD25^+$	0.62 ± 0.11	16.66 ± 2.7**	12.26 ± 2.6**
$IL\text{-}2^+/CD25^+$	0.72 ± 0.11	0.91 ± 0.11	2.42 ± 0.11**
$IFN\gamma^+$	1.09 ± 0.5	2.09 ± 0.87**	0.34 ± 0.09
$CD69^+$	0.04 ± 0.01	6.82 ± 2.4**	5.33 ± 3.4**
$IFN\gamma^+/CD69^+$	0.06 ± 0.01	2.3 ± 1.5**	0.96 ± 0.04

*diminished when compares with healthy controls ($p<0.05$)
**increased when compares with healthy controls ($p<0.05$)

than in asymptomatic and control subjects, whereas Th2 subset was markedly high in asymptomatic subjects (Table 1 and Fig. 1).

Although intracytoplasmic IL-2 in CD4+ T cells was lower in CCC patients, the presence of IL-2+ and its receptor α chain CD25+ in CD4+ T cells was similar among chagasic and healthy subjects, but the presence of IL-4 and its receptor γ chain was higher in asymptomatic subjects than in CCC and healthy individuals (Table 1).

The expression of CD25+/intracytoplasmic IL-2+ cytokine in *ex vivo* CD8+ T cells was higher in CCC patients compared to the control and asytomptomatic subjects, suggesting an activation

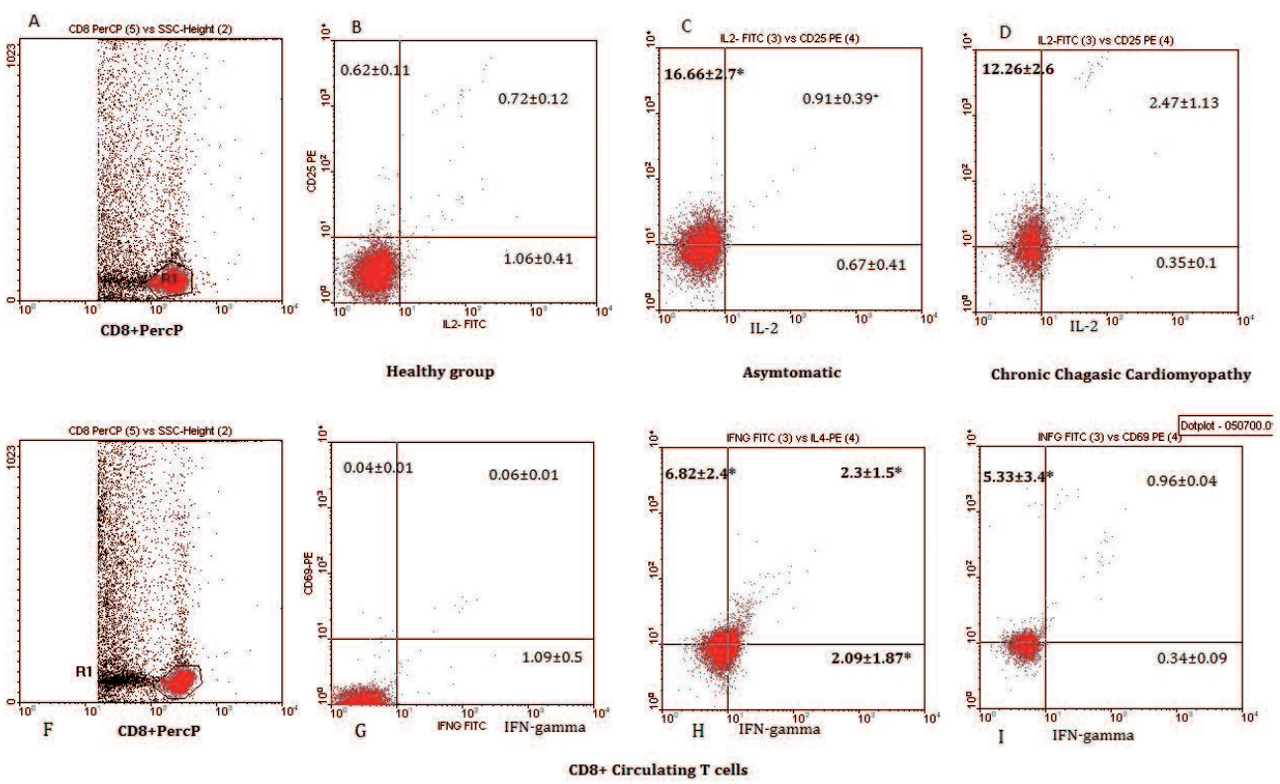

Fig. 2. Circulating $CD8^+$ lymphocytes containing intracellular cytokines and the surface activation markers in Asymptomatics, Chronic Chagasic Cardiomyopathy and Control Groups

state; however; asymptomatic subjects expressed higher intracytoplasmic IFN- γ in CD8+ T cells than the CCC and control group Individuals, suggesting activity state (Table 2 and Fig. 2).

The surface activation markers such as CD69+ and CD25+ in CD8+ T cells were higher in CCC and asymptomatic chagasic subjects than in the control group (Table 2).

It is well-known the compartmentalization of the immune system, we determined concentrations of different pro-inflammatory cytokines (IFN-γ and IL-12) in serum from healthy subjects and chagasic patients (CCC and asymptomatic). Both IFN-γ and IL-12 were recorded at limits below the sensitivity of the technique and identical to the healthy group (Fig. 3).

The concentration of IL-2, in chagasic subjects, was very low in serum, only in a few cases did the concentration reach 25 pg/ml, but even the healthy control group had similar behavior (Fig. 3). Similar results were observed for IL-4, a cytokine preferentially produced by Th2, in general, its concentration was at undetectable levels in all groups tested (Fig. 3).

The anti-inflammatory cytokines IL-10 and TGF-β could be recorded only in few asymptomatic subjects and, exceptionally, in CCC patients and control groups (Fig. 3).

Finally, levels of sIL-2R, as a homeostatic marker for T cell activation, was determined in chagasic serum and controls subjects. We did not find any differences between them. Both groups had a similar concentration of sIL-2R, in general terms, the media concentration was at 1000 pg/ml (Fig. 3).

Discussion

The relevance of both CD4+ and CD8+ T cell compartments in both the control and the pathology of the Chagas disease has been demonstrated in humans and in experimental models, supporting the notion that tissue damage might be more severe in

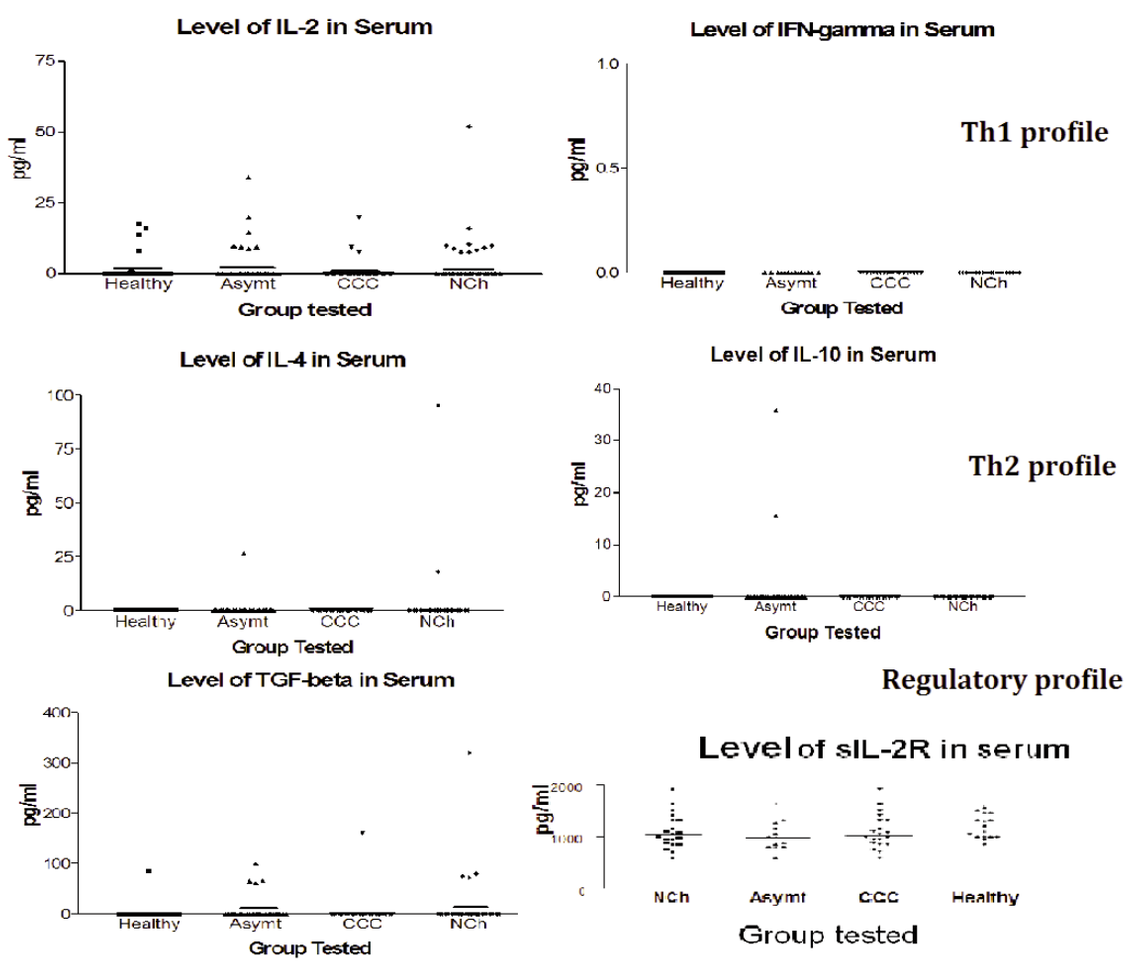

Fig. 3. Serum cytokine concentration (IL-2, sIL-2R, IL4, IL-10, IL-12, and IFN- γ) determined by BD OptEIA test in Asymptomatics, Chronic Chagasic Cardiomyopathy, Cardiopathy non-chagasic and Control Groups

the absence of regulatory mechanisms involving both innate and adaptive immune responses.

In this line of research, there are two categories of patients, low and high producers of IFN-γ after *in vitro* culture of peripheral blood mononuclear cells when stimulated by *T. cruzi* antigens. The CD4+ T cells from CCC patients were high producers of IFN-γ, whereas the indeterminate subjects were classified as low IFN-γ producers, but secreted significantly higher amounts of IL-10 [6]. However, the frequency of individuals with positive IFN-γ-producing CD8+ T cells in ELISPOT revealed a very high frequency of responders among patients with mild clinical symptoms as compared to those with the most severe form [19]. Complementary findings by others have found that individuals with the more severe clinical disease have significantly lower frequencies of *T. cruzi*-specific IFN-γ-producing CD8+ and CD4+ T cells after antigen stimulation than subjects in the asymptomatic stage [20].

In our work, we found that *ex vivo* analysis both CD4+ and CD8+ T cells presented a higher state of activation in chagasic subjects (determined by using CD69 and CD25 markers) compared with control. It is known that the early activation CD69 marker is induced after 8-h stimulation, whereas the late activation CD25 marker enhances its expression after 48-h stimulation [21]. These data may suggest that a fraction of circulating T cells in chagasic subjects was recently activated.

In spite of this state of activation, the presence of intracytoplasmic cytokines, such as IL-2 in CD4+ T cells, was statistically lower in CCC than in asymptomatic or control group individuals. However, there was no difference between CD4+ T cells expressing both CD25+ and IL-2+ in chagasic subjects and the control group (Table 1). In addition, we found in peripheral circulating CD8+ T cells, higher expression of intracytoplasmic cytokine IFN-γ in asymptomatic subjects than in the control group; even CCC presented lower expression of this cytokine than control and asymptomatic subjects (Table 2). Moreover, both chagasic groups expressed lower IL-4 intracytoplasmic cytokine in CD8+ T cells than the control healthy subjects.

The above findings might support published data suggesting that the effector T cell pool in chronic *T. cruzi* infection includes a high proportion of newly recruited T cells but a low frequency of long-term memory cells [22].

Our *ex vivo* analysis of circulating T cells without antigen stimulation resembles those that have used antigen stimulation, this feature could be explained according to the model proposed by Lanzavecchia and Sallusto [23]. They proposed that protective memory is mediated by effector memory T cells, which migrate to inflamed peripheral tissues and display their functions, whereas reactive memory cell development is mediated by central memory T cells, which home T cell areas of secondary lymphoid organs where they proliferate and differentiate into effector cells. Thus, the circulating T cells found in this study could belong to effector memory T cells ready to homing inflamed tissue. In addition, naďve T cells that have not encountered antigens do not express activation markers such as CD25 and CD69.

In summary, our data indicate that asymptomatic chagasic subjects have a mixed Th1/Th2 response with tendency to Th2 but with higher CD8+/IFN-γ T cells than CCC patients, whereas in CCC the CD4+/IL-2+ T cells and CD4+/IL-4+ T cells are fewer than in asymptomatic subjects. It is clear that the immune response to *T. cruzi* infection is complex and compartmentalization of the immune system may occur in distinct tissues as recently reviewed [24].

In this context, we investigated the cytokine levels in serum of CCC, asymptomatic subjects, and control groups. Among the cytokines determined, IL-4, IL-10, IL-12, and IFN-γ, all were at the undetectable levels in all groups. This result may suggest that the amount of T cells or cell producers of these cytokines are few, in spite of detecting a small fraction of T cells in the peripheral circulation by cytometry. In addition, it is known that the half-life of cytokines in plasma is very short, in the range of minutes [25]. Similar data was recently reported where IL-2, IL-6, IL-10, TNF-α, IL-4 and IFN-γ are under 10 pg/ml with an average around 2 pg/ml [26].

There are few studies and with contradictory results about the cytokine levels in the serum of CCC and asymptomatic patients [9,14,27–28]. Only a few researchers have demonstrated higher levels of TNF-α, IL-17, IFN-γ, IL-4, IL-6, IL-10 and NO in CCC compared with asymptomatic subjects or healthy control individuals. Even contradictory results have been found in healthy control groups. A partial explanation for these contradictory results could be the fact that obesity and gender influence cytokine level, basically for IL-6, IL-10, and TNF-α [29]. In addition to *T. cruzi*

strain involved in the infection.

On the other hand, if we consider that the presence of cytokines in serum depends on the degree of activation of immune cells in the secondary lymphoid tissues or inflamed tissues; then it is reasonable to detect cytokines in serum when antigen load is high as it occurs in the acute phase of Chagas disease [7]. Interestingly, it is not always possible to detect cytokines even in the acute stage of illnesses. For example, in patients with coronary artery disease, the levels of IL-1, IL-10, and IFN-γ were not detected [30], the same occurs in helminthic diseases, such as schistosomosis, where IFN-γ, IL-2, and IL-13 were negative in 70% of infected people [31].

In our work, only IL-2, TGF-β, and sIL-2R were at detectable levels but without differences in respect to the controls.

In summary, these cytokines in serum are not good markers to distinguish between chagasic infected people and healthy subjects; however, they are good markers to assess the activation intensity of the immune system.

Finally, there are some reports that have pointed out the importance of parasite genetics on the immune system in animal models using Mexican *T. cruzi* strains [15,17]. Our data is more similar to that found in CCC patients from South America, where undetectable levels of serum cytokines were reported [9,26].

In conclusion, Mexican chagasic patients infected with Mexican *T. cruzi* strains show CD4+ and CD8+ T lymphocytes in an activation state, but CCC presents a Th1 tendency whereas asymptomatic subjects towards Th2 profile. However, serum soluble cytokines are below 10 ng/ml or at undetectable levels.

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