Heart and Vessels (2019) 34:123–133
https://doi.org/10.1007/s00380-018-1228-z

ORIGINAL ARTICLE

Determination of the Th1, Th2, Th17, and Treg cytokine profile in patients with chronic Chagas heart disease and systemic arterial hypertension

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Received: 15 February 2018 / Accepted: 20 July 2018 / Published online: 25 July 2018

Abstract
Chronic Chagas heart disease (CCHD affects about 30% of patients with chronic Chagas disease (CCD). Systemic arterial hypertension (SAH) afflicts about 25% of patients with CCD. The association of CCHD with SAH (CCHD–SAH) predisposes patients to develop chronic heart failure. The role of cytokines in disease progression in patients with CCHD–SAH is unknown. Accordingly, the aim of this study was to evaluate the plasma levels of cytokines expressing the Th1, Th2, Th17 pattern, as well as Treg cytokines, TNF-alpha, IL-1β, IL-8, IL-7 in patients with SAH-CCHD to get insight into the immunomodulation process in patients with this condition. Fifteen patients with CCHD, 22 patients with CCHD–SAH, and 28 controls were studied. All patients underwent history-taking, physical examination, 12-lead resting ECG, chest X-ray, and Doppler-echocardiogram. Ten of 15 (66%) patients with CCHD, and 16 of 22 (73%) patients with CCHD–SAH had decreased left ventricular ejection fraction ($p > 0.05$). Cytokines levels were performed on plasma samples using the ELISA method. Overall, proinflammatory, anti-inflammatory, and regulatory cytokine levels were increased in patients with CCHD–SAH in comparison to patients with CCHD and controls. However, such a difference was higher regarding IL-2, IL-5, IL-17, IL-12, and TNF-alpha cytokine levels, respectively. Cytokine levels were higher in CCHD patients in comparison to controls. Patients with CCHD–SAH have increased plasma levels of pro-inflammatory, anti-inflammatory, and regulatory cytokines in comparison with CCHD patients, thus suggesting a higher level of immunomodulation in patients with CCHD–SAH.

Keywords Chronic heart disease · Chagas disease · Chronic heart failure · Cytokines

Introduction

Discovered more than one century ago, Chagas disease still is a major health problem in South America. In fact, almost six million people are carriers of the disease, and about 70 million people are at risk of acquiring the illness [1]. Chagas disease is no longer confined to South America because international immigration. About 700,000 infected individuals live in non-endemic countries, mainly in USA and Europe [2]. The consequence of this infection disease is an impressive burden on world economy, since more than US 7 billion are spent with the illness annually [3].

Chagas disease is caused by the protozoan *Trypanosoma cruzi*, which is transmitted through humans by the feces of a kissing bug. Many years after initial infection, usually up two decades, patients develop chronic Chagas disease. About 20% of them develop chronic Chagas heart disease (CCHD) [1]. The remaining patients develop either gastrointestinal disease or remain with a positive serology but no detectable disease (indeterminate form). CCHD is clinically manifested by sudden cardiac death [4], chronic heart failure [5], precordial chest pain [6], ventricular dysrhythmias [7], and cardiac thrombosis and thromboembolism [8].

Reinaldo B. Bestetti and Renata Dellalibera-Joviliano takes the responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. Equal contribution of first authors.

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In view of the population aging, systemic arterial hypertension (SAH) affects up to 33% of patients with chronic Chagas disease [9], and SAH is the most frequently comorbidity found in patients with this condition [10]. The clinical characteristics of patients with CCHD and SAH (CCHD–SAH) are similar to those found in patients with isolated CCHD [11, 12]. The presence of SAH predisposes CCHD patients to develop chronic heart failure [13], which can be found in about 31% of patients with CCHD–SAH [13]. Interestingly, in patients with chronic heart failure, outcome is better in CCHD–SAH patients than in those with CCHD alone [14].

Little is known about the pathogenesis of CCHD–SAH. Cytokines are believed to play a central role in the pathogenesis of CCHD. In fact, many studies have shown an increase in the pro-inflammatory cytokine serum levels in patients with CCHD [15, 16]. As far as we know, only one study has determined cytokine serum levels in patients with CCHD–SAH. In that study, no difference was observed in the cytokine serum levels among patients with CCHD normotensive patients, CCHD–SAH patients and controls [17].

Cytokines are proteins produced by different cell types that modulate the inflammatory response, have pleiotropic functions, play critical roles in multiple immune responses, and are mainly produced by T lymphocytes. Particularly, CD4(+) T cells play central roles for adequate protection against pathogens. Besides the classical Th1 and Th2 differentiation model, several subsets of CD4(+) T cells, including Th17, Th9, helper follicular and regulatory T cells (Treg) have recently been determined [18–20].

Accordingly, the purpose of this investigation was to evaluate the plasma levels of cytokines expressing the Th1, Th2, and Th17 pattern, Treg cytokines, as well as TNF-alpha, IL-1 Beta, IL-7, and IL-8 in patients with CCHD–SAH in an attempt to further understand the role of cytokines in the pathogenesis of patients with this condition.

Methods

Patients

Patients usually treated at the São Rafael Clinic, located in Piumhi city, in a region where Chagas disease is endemic, were initially considered for the study. To be included in the study, patients had to have at least two positive serological tests for Chagas disease. Patients with a negative serology, matched by age and sex, served as controls. All patients underwent history taking, physical examination, standard laboratory tests, 12-lead resting ECG, X-Ray chest, and 2-dimensional transthoracic echocardiogram during the work-up period. Patients with abnormal 12-lead ECG and/or abnormal echocardiogram were considered to have CCHD, and received standard treatment for chronic heart failure (CHF) in the case of left ventricular systolic dysfunction, i.e., Betablocker therapy and angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor block (ARB). Those with abnormal SAH on admission (systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg) and a positive serology were diagnosed with CCHD–SAH. Patients with positive serology for Chagas disease, normal systemic arterial pressure upon admission but with a history of previous SAH, and taking antihypertensive medications, were also diagnosed as having CCHD–SAH.

All patients provided a written informed consent before entering the study. The study protocol was approved by the Ethics Committee of the University of Ribeirão Preto, Brazil, and the Brazil Platform Ethics Committee authorized this research (CAAE: 48541715.5.0000.5498).

Laboratory tests

Peripheral venous blood samples were collected from an arm vein by a trained biomedical technician, using trace metal-free tubes to obtain the plasma. The samples were frozen immediately at − 85° before analysis.

Quantitative measurements of cytokines (TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-8, IL-2, IL-7, TGF-beta, IL-17 and IL-23) were performed on plasma samples using the double-ligand/sandwich enzyme-linked immunosorbent assay (ELISA), as previously described [21, 22]. The cytokine concentration was expressed in pg/ml by the kit's standard curve.

Briefly, flat-bottomed 96-well microliter plates were coated with 100 µL/well of specific antibody to one of the above cytokines at a concentration of 1–2 µg/mL of coating buffer and incubated overnight at 4 °C. The plates were washed with appropriate buffer and incubated for 120 min at 37 °C with buffer containing 1% bovine serum to prevent non-specific binding. Standard curves were performed using recombinant human TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-8, IL-2, IL-7, TGF-beta, IL-17 and IL-23. Samples and standards were loaded into wells and incubated overnight at 4 °C. The plates were thoroughly washed and the appropriate biotinylated polyclonal or monoclonal anti-cytokine antibody was added. After 1 h, the plates were washed 5 times with appropriate buffer followed by the addition of avidin-peroxidase diluted 1:5000. The plates were then incubated for 15 min and thoroughly washed again. Finally, the chromogenic substrate O-phenylenediamine (OPD) (0.4 mg OPD plus 0.4 µL of H_2O_2 for 1 mL of substrate buffer) was added, and the reaction was stopped 15 min later with 1 M H_2SO_4. The intensity of the color developed was measured spectrophotometrically at 490 nm using an ELISA plate scanner.

The threshold for quantification of the different cytokines in CCHD and CCHD–SAH patients was determined with the optical density of 492 nm. A standard curve for determination of each cytokine was constructed; only values within the curve sensitivity of 90% were used. The cytokine concentration, expressed in pg/mL, was determined by plotting the optical density obtained against the absorbance curve. The samples were diluted to 1:2 (50 µL of sample + 50 µL of buffer reaction); the concentration seen on the standard curve was multiplied for the dilution factor (×2).

Statistical analysis

Continuous variables are expressed as mean ± standard deviation. The ANOVA test was used for comparison of continuous variables of cytokine plasma levels in controls, CCHD, and CCHD–SAH. The Tukey test was used to compare continuous variables between controls and CCHD patients, and to compare continuous variables between CCHD and CCHD–SAH patients. In this way, the higher difference in each cytokine plasma levels between CCHD and CCHD–SAH was determined. Such a difference was used to establish the cytokines which were more important in the immune process in the CCHD–SAH patients. The *T* test for unpaired sample was used to compare cytokine plasma levels in patients treated and not treated with ACEI/ARB drugs in patients with CCHD and in those with CCHD–SAH. The Chi square test was used to compare the proportion of patients on ACEI and patients not treated with such drugs in patients with CCHD and in those with CCHD–SAH. Statistical comparisons were made using GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA, USA). A p value < 0.05 was considered statistically significant.

Results

Table 1 shows the clinical characteristics of the study population. Overall, 26 of 37 (70%) patients were found to have decreased LVEF. Mean LVEF was 53.5 ± 1.35 in patients with CCHD and 54 ± 1.60 in patients with CCHD–SAH ($p > 0.05$). The proportion of patients on ACEI/ARB was 9 out of 15 (60%) patients in the CCHD group, and 14 out of 22 (68%) patients in the CCHD–SAH group ($p > 0.05$). No statistical difference was observed between the remaining clinical characteristics of both groups. Cytokine concentrations in both groups are given in Table 2.

Table 1 Clinical characteristics of Chagas disease patients enrolled in the study

	CCHD ($n = 15$)	CCHD–SAH ($n = 22$)
Age (years)	62 ± 2	63 ± 2
Male	11 (73%)	9 (41%)
NYHA I/II	14 (93%)	18 (82%)
LAE	4 (60%)	5 (23%)
RAE	1 (7%)	0
LVH	1 (7%)	0
RBBB	5 (33%)	5 (23%)
LBBB	3 (20%)	3 (13%)
LAFB	5 (33%)	8 (36%)
1st degree AV block	3 (20%)	2 (9%)
Advanced AV block	1 (7%)	0
Pathological Q wave	6 (40%)	3 (13%)
LAD (mm)	35 ± 1.3	36 ± 1.5
RVD (mm)	24 ± 1.8	25 ± 1.4
LVDD (mm)	51 ± 1.2	50 ± 1.3
LVSD (mm)	52 ± 1.6	36 ± 1.7
IVST (mm)	11 ± 1.5	14 ± 1.2
LVPW (mm)	11 ± 1.3	10 ± 1.3
LVEF (%)	53.5 ± 1.3	54 ± 1.6
Decreased LVEF	10 (66%)	16 (73%)
Abnormal ECG	13 (87%)	17 (77%)

NYHA New York Heart Association functional class, *LAE* left atrial enlargement, *RAE* right atrial enlargement, *LVH* left ventricular hypertrophy, *RBBB* right bundle branch block, *LBBB* left bundle branch block, *LAFB* left anterior fascicular block, *AV* atrioventricular, *LAD* left atrium diameter, *RVD* right ventricular diameter, *LVDD* left ventricular diastolic diameter, *LVSD* left ventricular systolic diameter, *IVST* intraventricular septum thickening, *LVPW* left ventricular posterior wall, *LVEF* left ventricular ejection fraction

Th1 cytokines

Th1 cytokine plasma levels profile (INF-gamma, and IL-12) was increased in patients with CCHD and in those with SHA-CCHD in comparison to controls (Table 2). In addition, there was a significant difference between groups CCHD and CCHD–SAH with regard to all types of cytokines. However, the higher difference in cytokine plasma levels between CCHD and CCHD–SAH groups was related to IL-12. Table 3 summarizes these findings, whereas Fig. 1 illustrates these features.

Th2 cytokines

Th2 profile cytokine plasma levels (IL-4, IL-10, IL-13, IL-5) were increased in patients with CCHD and in those with SAH-CCHD in comparison to controls. There was also a significant difference between groups CCHD and CCHD–SAH with regard to all types of cytokines

Table 2 Cytokine serum levels in patients with chronic Chagas heart disease alone and in those with systemic arterial hypertension (pg/mL plasma)

	CONTROL ($N=28$)	CCHD ($n=15$)	CCHD–SAH ($n=22$)	p value ANOVA (CCHD × CCHD–SAH)
Th1				
IFN-gamma	60 ± 5.58	101 ± 7.05*	126 ± 8.86*	< 0.01
IL-12	70 ± 8.03	210 ± 9.02*	289 ± 25.0*	< 0.01
Th2				
IL-4	40 ± 6.08	65 ± 5.20*	95 ± 9.56*	< 0.01
IL-10	150 ± 5.88	220 ± 22.27*	276.5 ± 22.30*	< 0.01
IL-13	48 ± 3.78	61 ± 3.30*	91.4 ± 10.34*	< 0.01
IL-5	50 ± 7.53	140 ± 9.65*	207 ± 12.86*	< 0.01
Th17				
IL-17	75 ± 6.54	137 ± 6.5*	190 ± 17.33*	< 0.01
IL-23	50 ± 3.60	140 ± 7.26*	170 ± 9.99*	< 0.01
IL-6	80 ± 11.45	147 ± 9.72*	187.5 ± 9.76*	< 0.01
Treg				
IL-2	22 ± 2.40	43 ± 5.19*	80 ± 9.53*	< 0.01
TGF-beta	30 ± 2.62	55 ± 3.53*	76 ± 9.01*	< 0.01
Other cytokines				
TNF-alfa	230 ± 30.68	412 ± 22.15*	480 ± 18.81*	< 0.01
IL-1 beta	50 ± 4.22	134.5 ± 7.14*	176 ± 8.13*	< 0.01
IL-8	28 ± 2.16	41.75 ± 3.02*	53 ± 3.88*	< 0.01
IL-7	1.85 ± 0.13	2.67 ± 0.09*	2.92 ± 0.22*	< 0.01

control × *CCHD* control × CCHD–SAH), *CCHD* chronic Chagas heart disease patients, *CCHD–SAH* chronic Chagas heart disease patients with systemic arterial hypertension

*$p < 0.01$ for each comparison

(Table 2), but such a difference was higher regarding IL-5 (Table 3). Figure 1 illustrates such findings.

Th17 cytokines

Th 17 profile cytokine plasma levels (IL-17, IL-23, IL-6) were increased in patients with CCHD in comparison to controls. The same occurred regarding SHA-CCHD patients in comparison to controls (Table 2). There was also a significant difference between groups CCHD and CCHD–SAH with regard to all types of cytokines. Nevertheless, the difference was more robust regarding IL-17 plasma levels (Table 3). Figure 2 illustrate these features.

Treg cytokines

Treg profile cytokine plasma levels (IL-2/TGF-β) were increased in patients with CCHD in comparison to controls, as well as in SHA-CCHD patients in comparison to controls. There was also a significant difference between groups CCHD and CCHD–SAH with regard to all types of cytokines. Nonetheless, such a difference was higher concerning IL-2. Tables 2 and 3 summarizes these findings, whereas Fig. 2 illustrates these features.

TNF-alfa, IL-1 β, IL-8, IL-7 cytokines

TNF-alfa, IL-1 β, IL-8, IL-7 Th2 profiles cytokine plasma levels were increased in patients with CCHD in comparison to controls. The same occurred regarding SHA-CCHD patients in comparison to controls. There was also a significant difference between groups CCHD and CCHD–SAH with regard to all types of cytokines, but such a difference was higher regarding TNF-alpha. Table 2 and 3 summarizes these findings, whereas Fig. 3 illustrates these features.

Il-4 and IL-17 plasma levels were decreased in patients with CCHD–SAH treated with ACEI/ARB in comparison to those not treated with such drugs (Table 4). Il-10 plasma levels were decreased, but IL-23 and Il-1 beta were increased in patients with CCHD alone treated in comparison to patients not treated with such drugs (Table 4). No difference was observed in the remaining cytokine plasma levels between patients treated and not treated with ACEI/ARB in patients with CCHD and in those with CCHD–SAH (Table 4).

Table 3 Comparison of the cytokine plasma levels among controls, CCHD, and CCHD–SAH patients according to the Tukey test analysis

	Tukey analysis	Difference	*p* value
Th1			
IFN-gamma	Control vs CCHD	41.33	< 0.01
	Control vs CCHD–SAH	65.97	< 0.01
	CCHD vs CCHD–SAH	24.65	< 0.01
IL-12	Control vs CCHD	139.83	< 0.01
	Control vs CCHD–SAH	219.77	< 0.01
	CCHD vs CCHD–SAH	79.94	< 0.01
Th2			
IL-4	Control vs CCHD	24.8929	< 0.01
	Control vs CCHD–SAH	55.2565	< 0.01
	CCHD vs CCHD–SAH	30.3636	< 0.01
IL-10	Control vs CCHD	69.2048	< 0.01
	Control vs CCHD–SAH	125.1623	< 0.01
	CCHD vs CCHD–SAH	55.9576	< 0.01
IL-13	Control vs CCHD	13.1014	< 0.01
	Control vs CCHD–SAH	43.2578	< 0.01
	CCHD vs CCHD–SAH	30.1564	< 0.01
IL-5	Control vs CCHD	89.9667	< 0.01
	Control vs CCHD–SAH	156.6364	< 0.01
	CCHD vs CCHD–SAH	66.6697	< 0.01
Th17			
IL-17	Control vs CCHD	62.0619	< 0.01
	Control vs CCHD–SAH	115.5195	< 0.01
	CCHD vs CCHD–SAH	53.4576	< 0.01
IL-23	Control vs CCHD	89.7571	< 0.01
	Control vs CCHD–SAH	119.4026	< 0.01
	CCHD vs CCHD–SAH	29.6455	< 0.01
IL-6	Control vs CCHD	66.2143	< 0.01
	Control vs CCHD–SAH	106.5325	< 0.01
	CCHD vs CCHD–SAH	40.3182	< 0.01
Treg			
IL-2	Control vs CCHD	21.1333	< 0.01
	Control vs CCHD–SAH	58.5909	< 0.01
	CCHD vs CCHD–SAH	37.4576	< 0.01
TGF-beta	Control vs CCHD	24.6548	< 0.01
	Control vs CCHD–SAH	45.5032	< 0.01
	CCHD vs CCHD–SAH	20.8485	< 0.01
Other cytokines			
TNF-alfa	Control vs CCHD	182.1929	< 0.01
	Control vs CCHD–SAH	250.1201	< 0.01
	CCHD vs CCHD–SAH	67.9273	< 0.01
IL-1beta	Control vs CCHD	83.25	< 0.01
	Control vs CCHD–SAH	125.6591	< 0.01
	CCHD vs CCHD–SAH	42.4091	< 0.01
IL-8	Control vs CCHD	13.6214	< 0.01
	Control vs CCHD–SAH	25.5167	< 0.01
	CCHD vs CCHD–SAH	11.8952	< 0.01
IL-7	Control vs CCHD	0.828	< 0.01
	Control vs CCHD–SAH	1.0729	< 0.01
	CCHD vs CCHD–SAH	0.245	< 0.01

CCHD chronic Chagas heart disease, *CCHD–SAH* chronic Chagas heart disease patients with systemic arterial hypertension

Discussion

The results of this investigation clearly show that patients with CCHD–SAH have a higher inflammatory response, expressed by the Th1, Th2, Th17 and Treg patterns, in comparison with patients with CCHD and with controls. The difference in cytokine plasma levels in patients with CCHD–SAH in comparison to CCHD patients was more pronounced in the plasma levels of the cytokines IL-2, IL-5, IL-12, IL-17, and TNF-alpha. Moreover, cytokine plasma levels were higher in CCHD patients in comparison to controls. It is important to emphasize that, to our knowledge, this is the first complete investigation including cytokines with pro-inflammatory and anti-inflammatory profiles in patients with CCHD–SAH.

Rodrigues-Angulo and associates [17] have been measured cytokines serum levels in 14 patients with CCHD–SAH. They measured the plasma levels of the cytokines IL-17, IFN-gamma, TNF, IL-4, IL-6, IL-2, and IL-10. They failed to show differences in the cytokine serum levels between CCHD–SAH and CCHD patients. Although we do not have a conclusive explanation for such differences, in our study, about three-quarter of CCHD–SAH patients were male, whereas female predominated in the study by Rodrigues-Angulo et al. [17] It is conceivable that such differences can account, at least in part, for the disparity observed in both studies.

Several types of increased cytokines serum levels with the Th1 and the Th17 pattern have been detected in patients with CCHD. Thus, increased serum levels of TNF alpha [23, 24], IFN-gamma [25, 26], IL-2 [27, 28], IL-6 [25, 27, 28], IL-9 [25], and IL-12 [27, 29] have been observed in patients with this condition. Our findings, therefore, confirm the occurrence of such cytokines abnormalities in the plasma of patients with CCHD, and expand the current knowledge by showing that IL-5 and IL-17 may be increased as well. Furthermore, our study does not lend support to the suggestion that the balance between Th1/Th2 cytokines favored the immune response [30] probably because our patients had no or mild chronic heart failure.

The interplay of autoimmunity, microvascular abnormalities, and autonomic dysfunction may act in concert to produce CCHD [31]. Myocardial lesions observed in CCHD are similar to those seen in catecholamine cardiomyopathy [32]. In this model, increased levels of TGF-beta have been associated with the production of myocardial fibrosis [33]. TGF-Beta serum levels were more increased in patients with CCHD–SAH than in patients with CCHD in this investigation. Therefore, our study also suggests a potential role of myocardial inflammation-induced fibrosis mediated by cytokines in the pathogenesis of patients with CCHD–SAH.

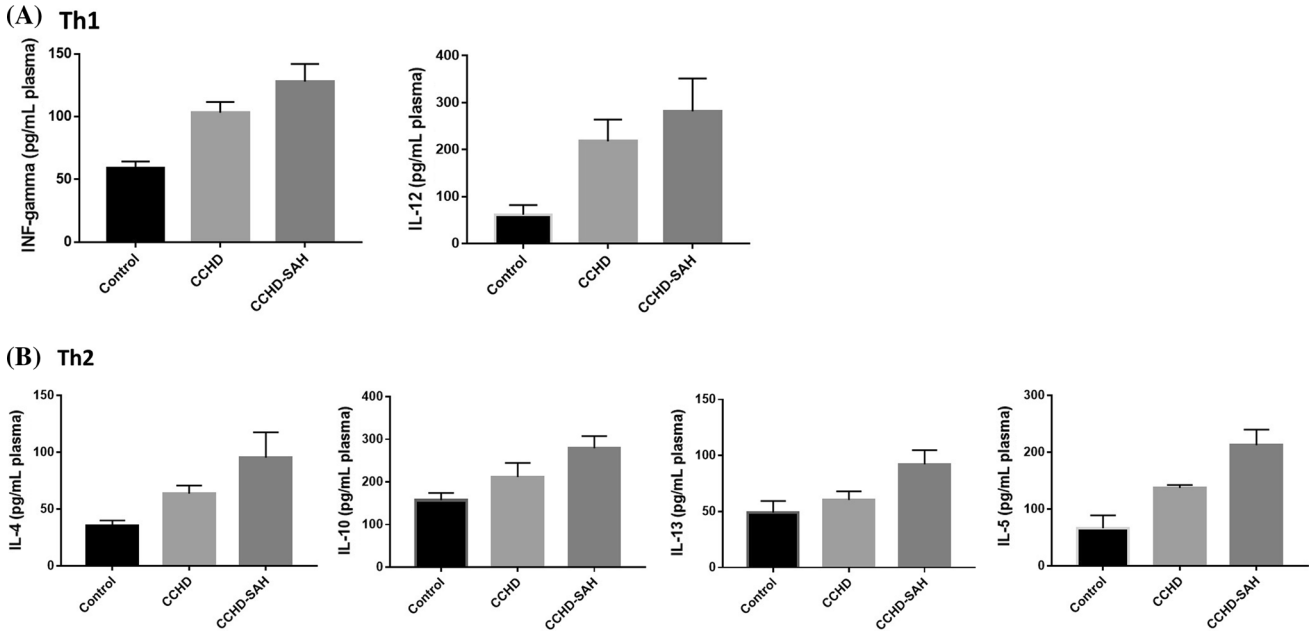

Fig. 1 Determination of Th1 and Th2 cytokine profile levels in subjects with chronic Chagas heart disease (CCHD), chronic Chagas heart disease and systemic arterial hypertension (CCHD–SAH) and controls, expressed as pg/mL plasma. **a** Plasma *Th1 (INF-gama/IL-12 levels:* The data reveal an increase of these cytokines in Chagas' patients compared with control subjects ($p < 0.0001$ for each comparison). A significant difference was also observed between groups CCHD and CCHD–SAH (INF-gama $p < 0.0001$/IL-12 $p = 0.0079$). **b** *Plasma Th2* (*IL-4, IL-10, IL-13, IL-5*) *cytokine profile levels*: The data reveal an increase of these cytokines in the plasma of Chagas patients compared with control subjects ($p < 0.0001$ for each comparison). A significant difference was also observed between groups CCHD and CCHD–SAH (IL-4 $p = 0.0005$/IL-10 $p < 0.0001$/IL-13 $p < 0.0001$/IL-5 $p < 0.0001$)

Another point that has to be addressed is the fact that patients with left ventricular systolic dysfunction were treated with ACEI and Betablockers, drugs that produce beneficial effects in animals infected with *T. cruzi* and in patients with CCHD [34–36], and may antagonize cytokines action, thus partially explaining the balance observed in cytokine measurements.

In fact, IL-4 plasma levels were decreased in patients with CCHD–SAH treated with ACEI/ARB in comparison to patients not treated with such drugs in our study. Despite such a decrease in plasma levels produced by ACEI/ARB, the IL-4 plasma levels continued to be higher than those observed in CCHD patients and controls. Furthermore, IL-4 were not among the cytokines that more contributed to the differential cytokine profile in patients with CCHD–SAH. Therefore, the treatment with ACEI/ARB drugs seems not to have affected the cytokine profile detected in patients with CCHD–SAH. However, a prospective, randomized study addressing this question specifically is necessary to clarify this matter.

IL-10 plasma levels were increased in CCHD patients treated with ACEI/ARB in comparison with those not treated with these drugs. Increased serum levels of IL-10, an anti-inflammatory cytokine, have been observed in patients with CCHD, mainly in those without heart failure [37]. Our findings are in accordance with this study, and suggest a role for IL-10 in the immunomodulation process, thus protecting against the appearance of CHF in patients with this condition. The role of decreased plasma levels of IL-23 are not clear in the context of CCHD, whereas IL-1 beta have been found to be increased in patients with this condition [38]. Therefore, the role of ACEI/ARB drugs appears to contribute to a balance in the pro-inflammatory and anti-inflammatory cytokine in patients with CCHD, which needs to be tested in a formal randomized trial.

The striking feature of this investigation is the more increased plasma cytokine levels observed in patients with CCD-SAH, particularly IL-2, IL-5, IL-12, IL-17, and TNF-alpha, in comparison with patients with CCHD alone. Increased plasma levels of IL-2 have been associated with Chagas disease [39] as well as with the severity of CHF in patients with this condition. [26] Our study, therefore, does not suggest that IL-2 serum levels may be used as a marker of heart function deterioration in patients with CCHD–SAH because patients had only minor left ventricular dysfunction. Further studies are necessary to elucidate such a finding.

Increased IL-5 serum levels have been observed in patients in the indeterminate form in comparison with patients with Chagas disease heart failure [39]. The higher levels of IL-5, an anti-inflammatory cytokine, observed in

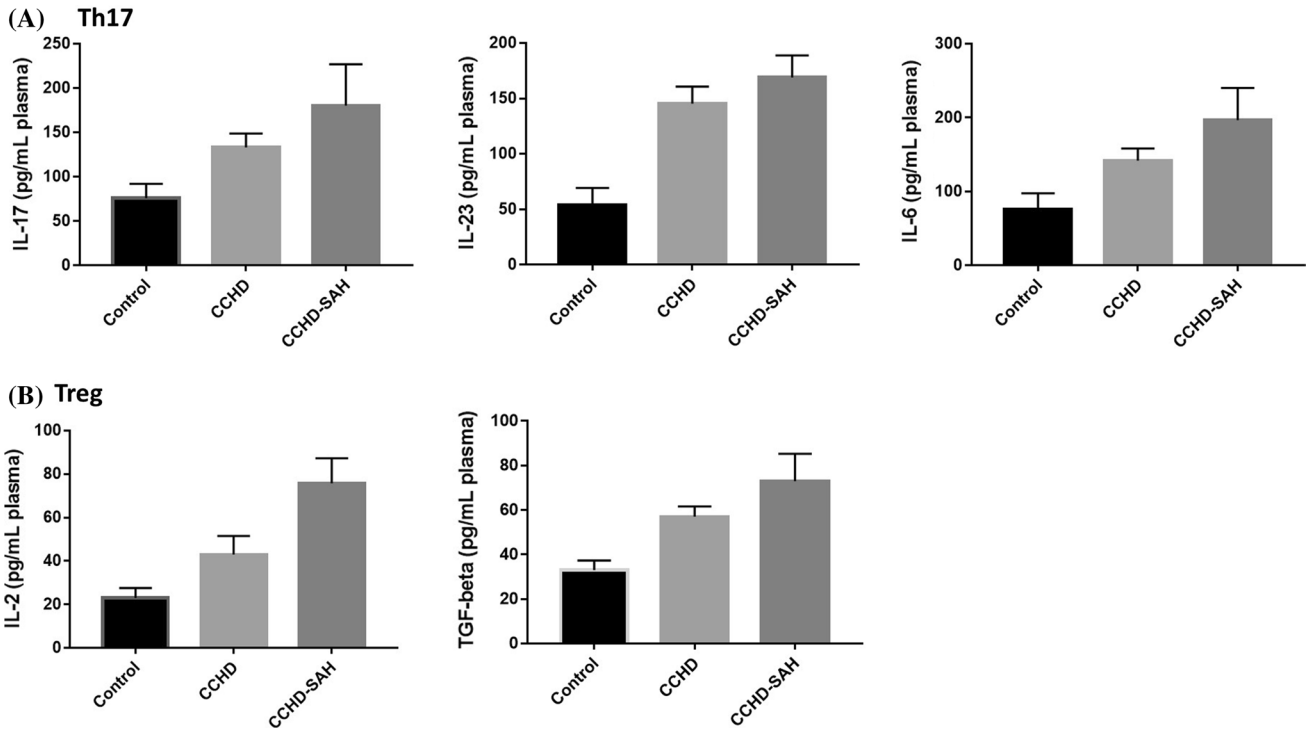

Fig. 2 Determination of Th17 and Treg cytokines plasma levels profile in subjects with chronic Chagas heart disease (CCHD), chronic Chagas heart disease and systemic arterial hypertension (CCHD–SAH) and controls, expressed as pg/mL plasma. **a** *Plasma Th17* (*IL-17, IL-23, IL-6*) *cytokine profile levels:* an increase of these cytokines in the plasma of Chagas patients compared with control subjects is observed ($p<0.0001$ for each comparison). A significant difference was also observed between groups CCHD and CCHD–SAH (IL-17 $p=0.0001$/IL-23 $p<0.005$/IL-6 $p<0.0001$). **b** Plasma *Treg* (*IL-2/TGF-β*) *cytokine levels:* the data show an increase of these cytokines in the plasma of Chagas patients compared with control subjects ($p<0.0001$ for each comparison). A significant difference was also observed between the groups CCHD and CCHD–SAH (IL-17 $p=0.0001$/TGF-β $p=0.00037$)

patients with CCHD–SAH may suggest the contribution of this cytokine for the modulation of immune response in patients with this condition, thus counteracting the pro-inflammatory activity and avoiding the appearance of severe cardiomyopathy.

In patients with CCHD, IL-12 plasma levels have been found to be increased [26, 29], but no correlation with the severity of heart function impairment have been established. We also observed a more increased plasma levels of the cytokine IL-12 in patients with CCHD–SAH in our study. This fact is consistent with the mild left ventricular systolic dysfunction observed in such patients.

Another interesting finding was the more increased levels of the cytokine IL-17 in patients with CCHD–SAH. Some authors have found this cytokine to produce a protective effect against the development of severe cardiomyopathy in patients with CCHD [15, 40], but others have been found an association with the severity of heart disease. [41]. Our finding seems to lend support to the association of plasma levels of Th17 with minor heart disease in patients with CCHD–SAH. The effect of ACEI/ARB treatment on IL-17 plasma levels requires further studies.

TNF-alpha have been correlated with heart failure in patients with CCHD [42–44], and may precede the appearance of heart failure. [44]. Our study shows that more increased levels of TNF-alpha can be found in patients with CCHD–SAH with mild left ventricular dysfunction. Overall, the higher levels of proinflammatory cytokines were counteracted by the higher levels of anti-inflammatory and regulatory cytokines, and this might have protected patients with CCHD–SAH to develop severe cardiomyopathy.

As far as SAH is concerned, previous studies have produced conflicting results regarding cytokine plasma levels in patients with this condition. Matsumori et al. have not found increased serum levels of cytokines in patients with SAH [45]. Conversely, in patients with isolated SAH, Mirhafez et al. have observed an increase in serum levels of IL-2, IL-8, IFN-gamma, and TNF-alpha, and decreased IL-10 serum levels. This cytokine imbalance may have played a role in the pathogenesis of SAH [46]. Kuznetsova et al. found increased levels of IL-18 in patients with SHA, left ventricular hypertrophy, and asymptomatic left ventricular diastolic dysfunction [47].

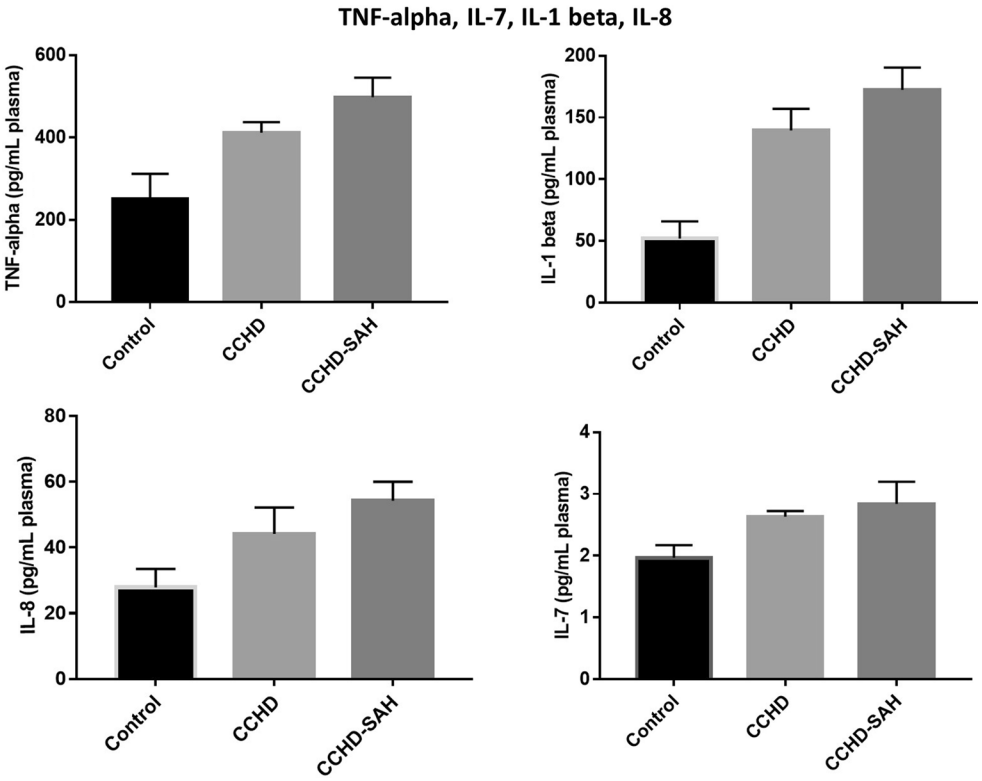

Fig. 3 Determination of TNF-alpha, IL-1beta, IL-8, IL-7 cytokines plasma levels in subjects with chronic Chagas heart disease (CCHD), chronic Chagas heart disease and systemic arterial hypertension (CCHD–SAH) and controls, expressed as pg/mL plasma. The data reveal an increase of these cytokines in the plasma of patients compared with control subjects ($p < 0.0001$ for each comparison). A significant difference was also observed between the groups CCHD and CCHD–SAH (TNF-alfa $p < 0.0001$, IL-1 β $p < 0.0001$, IL-8 $p < 0.001$, IL-7 $p = 0.015$)

Table 4 Cytokines plasma levels in patients with CCHD and CCHD–SAH treated versus not treated with ACEI/ARBs (pg/mL)

	Treated with ace inhibitors		Not treated	
	CCHD ($n=9$)	CCHD–SAH ($n=14$)	CCHD ($n=6$)	CCHD–SAH ($n=8$)
Th1				
IFN-gamma	100 ± 2.17	125 ± 7.98	101 ± 6.79	127 ± 9.99
IL-12	207 ± 8.64	290 ± 28.21	214 ± 8.62	290 ± 19.94
Th2				
IL-4	66 ± 4.95	91 ± 10.42*	62 ± 4.93	101 ± 2.82*
IL-10	233 ± 17.69**	279 ± 23.68	199 ± 9.17**	270 ± 21.47
IL-13	62 ± 3.78	89 ± 11.50	60 ± 2.42	94 ± 6.52
IL-5	143 ± 10.01	207 ± 15.91	136 ± 7.96	206 ± 5.06
Th17				
IL-17	135 ± 5.96	183 ± 17.43$^{\#}$	135 ± 6.85	202 ± 9.38$^{\#}$
IL-23	137 ± 6.38$^{\#\#}$	171 ± 10.14	145 ± 6.36$^{\#\#}$	168 ± 9.95
IL-6	149 ± 11.42	190 ± 9.85	144 ± 6.16	182 ± 8.30
Treg				
IL-2	44 ± 5.94	81 ± 11.34	41 ± 3.16	80 ± 5.61
TGF-beta	54 ± 3.64	76 ± 10.90	56 ± 3.20	75 ± 4.55
Other cytokines				
TNF-alfa	406 ± 19.63	475 ± 19.32	423 ± 23.45	489 ± 15.17
IL-1 beta	130 ± 6.05$^{\&}$	177 ± 9.05	139 ± 6.38$^{\&}$	174 ± 6.95
IL-8	42 ± 2.44	53 ± 3.96	41 ± 3.76	55 ± 3.65
IL-7	2.66 ± 0.05	2.88 ± 0.27	2.7 ± 0.15	2.98 ± 0.11

CCHD = Chronic Chagas heart disease; *CCHD–SAH*: chronic Chagas heart disease and systemic arterial hypertension

*$p = 0.0006$ (CCHD–SAH treated × not treated); ** $p = 0.001$ (CCHD treated × not treated); $^{\#}$ $p = 00.1$ (CCHD–SAH treated × not treated); $^{\#\#}$ $p = 0.04$ (CCHD treated × not treated); $^{\&}$ $p = 0.003$ (CCHD treated × not treated)

In patients with CHF secondary to SAH, cytokine plasma levels are also been increased. Testa et al. studied 80 patients with CHF (30 with hypertensive heart failure, 50 with ischemic heart failure). Increased circulating levels of TNF-alpha, IL-1, and IL-6 were observed in patients with severe CHF, according to the New York Heart Failure Association classification; however, the levels of IL-2 were not increased [48]. Therefore, in our study, the association of two pathologies capable of increasing cytokine plasma levels may account for our results of the higher cytokine plasma levels observed in patients with CCHD–SAH.

In this study, we observed a significant increase in IL-17 in CCHD–SAH patients compared to the control and CCHD groups. IL-17 stimulates the survival and expansion of immature precursors committed to T and B lymphocyte lines. Furthermore, the IL-17 receptor consists of a single binding alpha chain as a component of IL-2 receptors, IL-4 and IL-15. It is also essential for the survival of mature T cells, naive, memory cells such as CD4+ [49]. Thus, these findings may contribute to stimulation of Treg cells via the production of IL-2 and pro-inflammatory cytokines via the Th2 profile through IL-4 observed in patients with CCHD–SAH.

The consequences of increased levels of cytokines are well recognized. Pro-inflammatory cytokines can induce apoptosis and necrosis of the myocardial cells. In addition, left ventricular remodeling can be associated with TNF-alpha activity via matrix metalloproteinases [50]. However, a specific therapy against proinflammatory cytokines action has been proved to be disappointed [51].

The measurement of cytokines in the plasma of patients with CCHD–SAH may have practical consequences in the treatment of patients with this condition. Antagonists of TNF-alpha, for example, may be of benefit for patients with CCHD-SAH because, by decreasing TNF-alpha plasma levels, they not only attenuate inflammatory response, but may also interfere with left ventricular remodeling process. In fact, Sliwa et al. performed a randomized, double-blinded, placebo controlled trial evaluating pentoxifylline in patients with ischemic cardiomyopathy, and noted an improvement in functional status and in the left ventricular ejection fraction [52]. Similar results have been observed in patients with severe CHF secondary to idiopathic dilated cardiomyopathy on ACE inhibitors and carvedilol [53]. Whether a similar beneficial effect could be observed in patients with CCHD–SAH requires further studies.

The relative small number of patients is a limitation of this study. However, no difference was observed in clinical, electrocardiographic, and echocardiographic findings between CCHD–SAH and CCHD alone groups. A similar number of patients ($n = 38$) has been enrolled in the study by Rodrigues-Angulo and colleagues in which cytokine serum levels were measured in Chagas disease patients with and without SAH [17]. Finally, the statistical difference observed in cytokine serum levels among controls, CCHD and CCHD–SAH patients were strong, thus suggesting that he results obtained in our investigation have not occurred by chance alone.

In summary, our study allow us to better understand the inflammatory mechanisms in patients with CCHD–SAH by showing a balance in proinflammatory, anti-inflammatory and regulatory cytokines in patients with this condition.

Acknowledgements Funding was provided by Universidade de Ribeirão Preto.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. World Health Organization (2015) Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Wkly Epidemiol Rec 90:33–43
2. Pinazo MJ, Miranda B, Rodrigues-Villar C, Altclas J, Serra MB, Otero ECG, Almeida EA, Garcia MM, Gascon J, Rodrigues MG, Manito N, Camacho AM, Oppenheimer F, Puente SP, Riarte A, Coronas JS, Lheti MS, Sanz GF, Torrico F, Tendero DT, Ussetti P, Yasuda MA (2011) Recommendations for management of Chagas disease in organ and hematopoietic tissue transplantation programs in nonendemic areas. Transplant Rev (Orlando) 25:91–101
3. Lee BY, Bacon KM, Bottazzi ME, Hotez PJ (2013) Global economic burden of Chagas disease: a computational simulation model. Lancet Infect Dis 13:342–348
4. Bestetti RB, Cardinalli-Neto A (2008) Sudden cardiac death in the contemporary era. Int J Cardiol 131:9–17
5. Bertolino ND, Villafanha DF, Cardinalli-Neto A, Cordeiro JÁ, Arcanjo ML, Theodoropoulos TAD, Bestetti RB (2010) Prognostic impact of Chagas disease in patients awaiting heart transplantation. J Heart Lung Transplant 29:449–453
6. Bestetti RB, Ariolli MT, do Carmo JL, Passos AD, Santos CR, Machado Júnior O, Costa NK, de Oliveira RB (1992) Clinical characteristics of acute myocardial infarction in patients with Chagas disease. Int J Cardiol 35:371–376
7. Bestetti RB, Santos CRF, Machado-Jr O, Ariolli MTM, Carmo JL, Costa NK, Oliveira RB (1990) Clinical profile of patients with Chagas disease before and during sustained ventricular tachycardia. Int J Cardiol 29:39–46
8. Samuel J, Oliveira M, Correa De Araujo RR, Navarro MA, Muccillo G (1983) Cardiac thrombosis and thromboembolism in chronic Chagas heart disease. Am J Cardiol 52:147–151
9. Gurgel CBFM, Miguel A Jr, Mendes CR, Zerbini CO, Carcioni TM (2003) Frequency of arterial hypertension in chronic Chagas disease. A retrospective study. Arq Bras Cardiol 81:541–544
10. Alves RMA, Thomaz RP, Almeida EA, Wanderley JS, Guariento ME (2009) Chagas disease and ageing: the coexistence of other chronic diseases with Chagas disease in elderly patients. Rev Soc Bras Med Trop 42:622–628
11. Pinheiro MB, Bestetti RB, Cordeiro JA (2007) Assessment of QTc interval dispersion in patients with chronic heart failure secondary to Chagas-hypertensive cardiomyopathy. Int J Cardiol 121:93–94

12. Dourado KCC, Bestetti RB, Cardinalli-Neto A, Cordeiro JA (2010) Evaluation of the six-minute walk test in patients with chronic heart failure associated with Chagas disease and systemic arterial hypertension. Rev Soc Bras Med Trop 43:405–408
13. Guariento ME, Ramos MC, Gontijo JAR, Carvalhal SS (1993) Chagas disease and primary arterial hypertension. Arq Bras Cardiol 60:71–75
14. Veiga MF, Rodrigues IF, Cardinalli-Neto A, Otaviano AP, da Rocha BF, Bestetti RB (2011) Clinical course of patients with chronic systolic heart failure due to the association of Chagas disease and systemic arterial hypertension. Int J Cardiol 149:122–124
15. Guedes PM, Gutierrez FR, Silva GK, Dellalibera-Joviliano R, Rodrigues GJ, Bendhack LM, Rassi A Jr, Rassi A, Schmidt A, Maciel BC, Marin-Neto JA, Silva JS (2012) Deficient regulatory T cell activity and low frequency of IL-17-producing T cells correlate with the extent of cardiomyopathy in human Chagas' disease. PLoS Negl Trop Dis 6:e1630
16. de Oliveira AP, Ayo CM, Bestetti RB, Brandão de Mattos CC, Cavasini CE, de Mattos LC (2016) The role of CCR5 in Chagas disease—a systematic review. Infect Genet Evol 45:132–137
17. Rodrigues-Angulo H, Marques J, Mendoza I, Villegas M, Mijares A, Gironès N, Fresno M (2017) Differential cytokine profiling in Chagasic patients according to their arrhythmogenic-status. BMC Infect Dis 17:221
18. Jutel M, Akdis CA (2008) T-cell regulatory mechanisms in specific immunotherapy. Chem Immunol Allergy 94:158–177
19. Kumagai J, Hirahara K, Nakayama T (2016) Pathogenic Th cell subsets in chronic inflammatory diseases. Nihon Rinsho Meneki Gakkai Kaishi 39:114–123
20. Dimeloe S, Burgener AV, Grählert J, Hess C (2017) T-cell metabolism governing activation, proliferation and differentiation: a modular view. Immunology 150:35–44
21. Dellalibera-Joviliano R, Dos Reis ML, Cunha FQ, Donadi EA (2003) Kinins and cytokines in plasma and cerebrospinal fluid of patients with neuropsychiatric lupus. J Rheumatol 30:485–492
22. Dellalibera-Joviliano R, Joviliano EE, Silva JS, Evora PR (2012) Activation of cytokines corroborate with development of inflammation and autoimmunity in thromboangiitis obliterans patients. Clin Exp Immunol 170:28–35
23. Cunha-Neto E, Dzau VJ, Allen PD, Stamattou D, Benvenutti L, Higuchi ML, Koyama NS, Silva JS, Kalil J, Liew CC (2005) Cardiac gene expression profile provides evidence for cytokinopathy as a molecular mechanism in Chagas disease cardiomyopathy. Am J Pathol 167:305–313
24. Lorena VMB, Lorena IMB, Braz SCM, Melo AS, Melo FAD, Melo MGAC, Silva ED, Ferreira AGP, Morais CNL, Costa VMA, Correa-Oliveira R, Gomes YM (2010) Cytokine levels in serious cardiopathy of Chagas disease after in vivo stimulation with recombinant antigens from *Trypanosoma cruzi*. Scand J Immunol 72:529–539
25. Mocelin AO, Issa VS, Bacal F, Guimarães GV, Cunha E, Bocchi EA (2005) The influence of aetiology on inflammatory and neurohumoral activation in patients with severe heart failure: a prospective study comparing Chagas heart disease and idiopathic dilated cardiomyopathy. Eur J Heart Fail 7:869–873
26. Bravo Tobar I, Parra F, Nello Pertez C, Rodríguez-Bonfante CR, Useche F, Bonfante-Cabarcas R (2011) Prevalence of *Trypanosoma cruzi* antibodies and inflammatory markers in uncompensated heart failure. Rev Soc Bras Med Trop 44:691–696
27. Poveda C, Fresno M, Gironés N, Martins-Filho O, Ramirez JD, Rocca JS, Marin-Neto JA, Morilo CA, Rosas F, Guhl F (2014) Cytokine profiling in Chagas disease: towards understanding the association with infecting *Trypanosoma cruzi* discrete typing units (A BENEFIT trial sub-study). PLoS One 9:e91154
28. Herrera RN, Amaya EID, Aguillar RCP, Turoni CJ, Maranon R, Berman SG, Luciardi HL, Coviello A, Bruno MP (2011) Inflammatory and prothrombotic activation with conserved endothelial function in patients with chronic asymptomatic Chagas disease. Clin Appl Thromb Hemost 17:502–507
29. Vitelli-Avelar DM, Sathler-Avelar R, Teixeira-Carvalho A, Dias JCP, Gontijo ED, Farias AM, Elói-Santos SM, Martins-Filho AO (2008) Strategy to assess the overall cytokine profile of circulating leukocytes and its association with distinct clinical forms of human Chagas disease. Scand J Immunol 68:516–525
30. Bocchi EA, Bestetti RB, Scanavacca MI, Cunha-Neto E, Issa VS (2017) Chronic Chagas heart disease management: from etiology to cardiomyopathy treatment. J Am Coll Cardiol 70:1510–1524
31. Bestetti RB (1996) Role of parasites in the pathogenesis of Chagas cardiomyopathy. Lancet 347:913–914
32. Bestetti RB, Ramos CP, Figuerêdo-Silva J, Sales-Neto VN, Oliveira JSM (1987) The ability of the electrocardiogram to detect myocardial lesions in isoproterenol induced rat cardiomyopathy. Cardiovasc Res 21:916–921
33. Chen R, Xue J, Xie M (2012) Puerarin prevents isoprenaline-induced myocardial fibrosis in mice by reduction of myocardial TGF-Beta1 expression. J Nutr Biochem 23:1080–1085
34. Bestetti RB, Sales-Neto VN, Pinto LZ, Soares EG, Muccillo G, Oliveira JSM (1990) Effects of long-term metoprolol administration on the electrocardiogram of rats infected with *Trypanosoma cruzi*. Cardiovasc Res 24:521–527
35. Dobaczewski M, Chen W, Frangogiannis NG (2011) Transforming growth factor (TGF)-B signaling in cardiac remodeling. J Mol Cell Cardiol 51:600–606
36. Bestetti RB, Otaviano AP, Cardinalli-Neto A, da Rocha BF, Theodoropoulos TAD, Cordeiro JA (2011) Effects of beta-blockers on outcome of patients with Chagas cardiomyopathy with chronic heart failure. Int J Cardiol 151:205–208
37. Vasconcelos RH, Azevedo Ede A, Diniz GT, Cavalcanti Mda G, de Oliveira W Jr, de Morais CN, de Gomes YM (2015) Interleukin-10 and tumor necrosis factor alpha serum levels in chronic Chagas disease patients. Parasite Immunol 37:376–379
38. Sousa GR, Gomes JA, Fares RC, Damásio MP, Chaves AT, Ferreira KS, Nunes MC, Medeiros NI, Valente VA, Corrêa-Oliveira R, Rocha MO (2014) Plasma cytokine expression is associated with cardiac morbidity in Chagas disease. PLoS One 9:e87082
39. Gomes JAS, Molica AM, Keesen TSL, Morato MJF, Araujo FF, Fares RCG, Fiuza JÁ, Chaves AT, Pinheiro V, Nunes MCP, Correa-Oliveira R, Rocha MOC (2014) Inflammatory mediators from monocytes down-regulate cellular proliferation and enhance cytokines production in patients with polar clinical forms of Chagas disease. Hum Immunol 75:20–28
40. Magalhães LMD, Villani FNA, Nunes MCP, Gollob KJ, Rocha MOC, Dutra WO (2013) High interleukin-17 expression is correlated with better cardiac function in human Chagas disease. J Infect Dis 207:661–665
41. De Alba-Alvarado M, Salazar-Schetinno PM, Jiménez-Álvarez L, Cabrera-Bravo M, García-Sancho C, Zenteno E, Vazquez-Antona C, Cruz-Lagunas A, Zúniga J, Bucio-Torres MI (2018) Th-17 cytokines are associated with severity of *Trypanosoma cruzi* chronic infection in pediatric patients from endemic areas of Mexico. Acta Trop 178:134–141
42. Talvani A, Rocha MOC, Barcelos LS, Gomes YM, Ribeiro AL, Teixeira MM (2004) Elevated concentrations of CCL2 and tumor necrosis factor-alpha in Chagasic cardiomyopathy. Clin Infect Dis 38:943–950
43. Rocha Rodrigues DB, dos Reis MA, Romano A, Pereira SA, Teixeira Vde P, Tostes S Jr, Rodrigues V Jr (2012) In situ expression of regulatory cytokines by heart inflammatory cells in Chagas disease patients with heart failure. Clin Dev Immunol 2012:361730
44. Ferreira RC, Ianni BM, Abel LCJ, Buck P, Mady C, Kalil J, Cunha-Neto E (2003) Increased plasma levels of tumor necrosis

factor-alpha in asymptomatics/indeterminate and Chagas disease cardiomyopathy patients. Mem Inst Oswaldo Cruz 98:407–411
45. Matsumori A, Yamada Y, Suzuki H, Matoba Y, Sasayama S (1994) Increased circulating cytokines in patients with myocarditis and cardiomyopathy. Br Heart J 72:561–566
46. Mirhafez SR, Mohebati M, Disfani MF, Karimian MS, Avan A, Eslami S, Pasdar A, Rooki H, Esmaeli H, Ferns GA, Mobarhan MG (2014) An imbalance in serum concentrations of inflammatory and anti-inflammatory cytokines in hypertension. J Am Soc Hypertens 8:614–623
47. Kuznetsova T, Haddad F, Knez J, Hasson YR, Sung J, Cauwenbergs N, Thijs L, Karakikes I, Maecker H, Mahaffey KW, Wu JC, Staessen JA (2015) Cytokines profile in hypertensive patients with left ventricular remodeling and dysfunction. J Am Soc Hypertens 9:975–984
48. Testa M, Yeh M, Lee P, Fanelli R, Loperfido F, Berman JW, LeJemtel TH (1996) Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. J Am Coll Cardiol 28:964–971
49. Schluns KS, Lefrançois L (2003) Cytokine control of memory T-cell development and survival. Nat Rev Immunol 3(4):269–279
50. Bautista-López NL, Morillo CA, López-Jaramillo P, Quiroz R, Luengas C, Silva SY, Galipeau J, Lalu MM, Schulz R (2013) Matrix metalloproteinases 2 and 9 as diagnostic markers in the progression to Chagas cardiomyopathy. Am Heart J 165:558–566
51. Mann DL, McMurray JJV, Packer M, Swedberg K, Borer JS, Colucci WS, Dijan J, Drexler H, Feldman A, Kober L, Krum H, Liu P, Nieminen M, Tavazzi L, van Veldheisen DJ, Waldenstrom A, Warren M, Westheim A, Zannad F, Fleming T (2004) Targeted anticytokine therapy in patients with chronic heart failure. Circulation 109:1594–1602
52. Sliwa K, Woodwiss A, Kone VN, Candy G, Badenhorst D, Norton G, Zambakides C, Petgers F, Essop R (2004) Therapy of ischemic cardiomyopathy with the immunomodulating agent pentoxifylline. Circulation 109:750–755
53. Skudicky D, Bergemann A, Sliwa K, Candy G, Sareli P (2001) Beneficial effects of pentoxifylline in patients with idiopathic dilated cardiomyopathy treated with angiotensin-converting enzyme inhibitors and carvedilol. Circulation 103:1083–1088