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Th-17 cytokines are associated with severity of *Trypanosoma cruzi* chronic infection in pediatric patients from endemic areas of Mexico

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ABSTRACT

In Chagas disease the clinical, acute and chronic manifestations are the result of the interaction between the parasite and the host factors. The balance between inflammatory and anti-inflammatory immune responses is essential for the increase or resolution of the manifestations in individuals infected with *T. cruzi*. To identify if children with chronic Chagas disease and heart injury is related with non-regulated Th1, Th2 and Th17 responses. We included 31 children with *T. cruzi* confirmed chronic infection from endemic areas of Mexico. Subsequently, they were separated according to their ECHO and ECG results into three groups according to the severity of cardiac involvement. Circulating Th1, Th2 and Th17 cytokine profiles were performed by Luminex assays and the results were analyzed by bivariate and multivariable analysis. Patients were classified in asymptomatic chronic (group 1, N = 12); individuals with IRBBB in ECG and incipient lesions in ECHO (Group 2, N = 8) and Patients with severe chronic symptomatic disease (Group 3, N = 11). The analysis of immune mediators revealed that patients with severe cardiac manifestations had significant higher levels ($p < 0.05$) of Th17 related cytokines including IL-17 and IL-6 as well as IFN-γ and IL-2. Also patients with severe cardiomyopathy exhibit increased levels of IL-13 ($p < 0.05$) after multivariate analysis. High levels of Th17 related cytokines including IL-17, IFN-γ, IL-6 and IL-2 and pro-fibrotic factors such as IL-13 could be associated to the severity of cardiac involvement in children with chronic *T. cruzi* infection. These cytokines could be useful as indicators for the early identification of cardiac damage associated to the *T. cruzi* infection.

1. Introduction

Trypanosoma cruzi is the etiologic agent of Chagas disease, which is a complex zoonosis that implies interaction of humans and other vertebrates with multiple species of the triatomine transmitter. The World Health Organization (2013) estimates that Chagas disease affects seven to eight million people, with a record of 11,000 deaths in 2008 worldwide. Two phases of Chagas disease have been well clinically characterized; the acute and chronic disease. During the acute phase, 5% of patients present clinical symptoms such as fever and adenitis, 70% remain asymptomatic and 30% may progress to symptomatic chronic phase (Moncayo, 1999). During this phase of the disease, elevated levels of interferon gamma (IFN-γ) and tumor necrosis factor (TNF-α) induce macrophage activation as well as NK and T cell $CD8^+$ infiltrates at the site of infection (Campos et al., 2004; Cardoni, 1997; Golden and Tarleton, 1991).

During the chronic phase of Chagas disease, asymptomatic carriers can be detected, but frequently, Th1 responses have been associated with the progress of symptomatic cardiac damage (Cunha-Neto et al., 2005). In this regard, patients that develop chagasic cardiomyopathy accompanied by a strong Th1-type immune response may be the result of persistence of the parasite and chronic stimulation of inflammatory responses resulting in tissue damage (Guedes et al., 2016; Gutierrez et al., 2009).

Some studies have reported that individuals under the age of 18 usually develop the chronic phase of Chagas disease in a very short period, some of them two years after the acute phase (Salazar-Schettino et al., 2009) suggesting differential clinical outcomes related with age of patients. Thus, it is important to perform studies that analyze the levels of pro- and anti-inflammatory cytokines in early and late stages

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of the infection in order to determine the possible role of the non-regulated inflammatory responses in the clinical progression and outcome of the *T. cruzi* infection. Previous studies have highlighted the role of Th17 responses in the protection of *T. cruzi* infection (Cai et al., 2016), and the correlation of pro-inflammatory mediators in the pathogenesis of cardiac lesions and risk of poor outcomes in both experimental animals and adults in dilated myocardiopathies (Nindl et al., 2012). However, few studies about the immune mediators' profiles in children with and without cardiac involvement have been reported (Moretti et al., 2002).

The main goal of this study was to identify the Th17 cytokine profiles in pediatric patients with confirmed *T. cruzi* chronic infection with different levels of cardiomyopathy and determine if Th17 responses are related with severity of Chagas disease in young patients from an endemic area of Mexico.

2. Materials and Methods

2.1. Patients

Here we recruited 31 children between 6 and 17 years old with confirmed T. cruzi chronic infection from endemic states of Queretaro, San Luis Potosi and Veracruz located in rural areas of Mexico. Simultaneously, as a control group, we recruited seronegative T. cruzi individuals from the same endemic regions. Mean age of T. cruzi cases was 13.6 ± 3.5 years and patients were classified into three groups according to the clinical criteria of New York Heart Association, (The Criteria Committee of the New York Heart Association, 1994). All patients were considered chronically infected with T. cruzi in different stages of the disease and acute infection was discarded by parasitological methods. The route of infection in all patients was the confirmed repeatedly exposure to the vector. The infection was confirmed by ELISA and indirect immunofluorescence previously described methods (Salazar-Schettino et al., 2009; Bucio et al., 1999). Chronic T cruzi patients were classified in three groups; Group 1 (N = 12): "Chronic Asymptomatic Individuals": Individuals with no alterations in the electrocardiography (ECG) or echocardiography (ECHO); Group 2 (N = 8): "Symptomatic Chronic Incipient": individuals with ECG IRBBB (incomplete block of the right bundle of His) normal ECHO or incipient lesions and Group 3 (N = 11): "Symptomatic Chronic Severe": individuals with RBBB (complete block of the right bundle of His), or IRBBB, with sinus bradycardia and abnormal ECHO with injuries considered incipient and abnormal. Mild hypertrophy of interventricular septum and posterior wall were considered incipient lesions. Abnormal ECHO was characterized by hypertrophy of interventricular septum and posterior wall, ejection fraction of left ventricle ejection fraction (LVEF) < 57%, pulmonary artery pressure (PABP) > 35 mmHg (Salazar-Schettino et al., 2016). Patients with other cardiovascular diseases including congenital cardiac conditions, those with negative serologic results, chronic inflammatory conditions or pregnant were not included in the study. Also, gastrointestinal manifestations of Chagas disease were discarded in all cases.

Simultaneously, we recruited a group of 5 healthy controls from the same endemic areas from patients with no clinical and laboratory data of *T. cruzi* infection with a mean age of 10 ± 6 years.

The Institutional Review Board (IRB) and Biosafety Committees of the School of Medicine of the National University of Mexico (UNAM) and from the National Institute for Respiratory Diseases reviewed and approved this study. At the start of the study, a signed informed consent was obtained from the parents or legal responsible of the children with ages below 18 years old.

2.2. T. cruzi serology testing

Serological confirmation was done by using two serological tests, as recommended by WHO/PAHO guidelines, indirect ELISA techniques and indirect immunofluorescence (IIF). Cases with doubtful or discordant results were confirmed using immunoblotting as described previously (World Health Organization, 2005; Salazar-Schettino et al., 2009). The ELISA assay was performed with an antigen from *T. cruzi* that was previously characterized and validated in our laboratory (Bucio et al., 1999).

2.3. Electrocardiographic (ECG) and Echocardiography (ECHO) studies

Study groups of seropositive individuals to *T. cruzi* were characterized based on electrocardiographic study with 12 leads (conventional ECG), a two-dimensional echocardiography and a conventional Doppler echocardiography (ECHO). These radiologic studies were performed by well-trained physicians at the National Institute of Cardiology "Dr. Ignacio Chavez".

2.4. Cytokine and chemokine measurements

Levels of cytokines, chemokines and growth factors in blood serum, were assessed by using Luminex assay (IL-1β, IFN-γ, TNF-α, IL-2, IL-6, IL-17, IL-8, MIP-1α and anti-inflammatory cytokines IL1-RA, IL-4, IL-10 and IL-13) and, plates were read in a Luminex platform, Bio-Plex Multiplex 200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Standard curves were generated with a dynamic range between 5 and 20,000 pg/ml. Results, were analyzed using the Bio-Plex v 6.0 software (Bio-Rad Laboratories Inc).

2.5. Statistical analysis

Differences were evaluated by one-way ANOVA and Sidak's test with correction for multiple comparisons. A multiple correspondence analysis (MCA) was used to assess: 1) the patter of association of clinical symptoms, demographic characteristics and ECG and ECHO features of *T. cruzi* infected patients at different stages of the disease; and 2) the profile of association of the different groups of patients according to the degree of severity of cardiomyopathy (Group 1-3) and the level of concentration (low, medium and high) of pro- and anti-inflammatory cytokines. In this regard it is important to mention that MCA is a reliable multivariate statistical tool to analyze patterns of association between levels of categorical variables. This analysis simplifies complex sets of categorical variables, thus allowing a detailed description of the data, which can be displayed in a simplified graphical output that shows clouds of points that represent the categories of the variables. The pattern of association between variables was interpreted in terms of the relative position of their points along the displayed dimensions (Dim), which show the percentage of explained variation. Dim 1, first principal component; Dim 2, second principal component, arises from the combination of the associated categories of all the variables (demographic characteristics of patients, clinical symptoms and cardiac function) allowing discrimination of the formed clouds of data. The closer the points are, the more associated (corresponded). To compare differences in the levels of immune mediators among groups we also used the Pearson chi2 tests for categorical variables and median, percentiles 25-75% and test of Equality of populations (Kruskal-Wallis test) for continuous variables. Multivariate analysis was done adjusting for a logistic ordinal model using the statistical package STATA version 9. p values < 0.05 were considered as significant.

3. Results

Clinical and demographic characteristics of studied individuals are summarized in Table 1.

Significant differences in gender, age, irregular heart beats per minute and tachycardia were not detected. Dyspnea was more frequent in patients with severe disease (72.7%) compared to chronic asymptomatic (58%) and chronic incipient patients, however this difference