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Research paper

Development of chronic cardiomyopathy in canine Chagas disease correlates with high IFN-γ, TNF-α, and low IL-10 production during the acute infection phase

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ABSTRACT

When infected with *Trypanosoma cruzi*, Beagle dogs develop symptoms similar to those of Chagas disease in human beings, and could be an important experimental model for a better understanding of the immunopathogenic mechanisms involved in chronic chagasic infection. This study evaluates IL-10, IFN-γ and TNF-α production in the sera, culture supernatant, heart and cervical lymph nodes and their correlation with cardiomegaly, cardiac inflammation and fibrosis in Beagle dogs infected with *T. cruzi*. Pathological analysis showed severe splenomegaly, lymphadenopathy and myocarditis in all infected dogs during the acute phase of the disease, with cardiomegaly, inflammation and fibrosis observed in 83% of the animals infected by *T. cruzi* during the chronic phase. The data indicate that infected animals producing IL-10 in the heart during the chronic phase and showing high IL-10 production in the culture supernatant and serum during the acute phase had lower cardiac alterations (myocarditis, fibrosis and cardiomegaly) than those with high IFN-γ and TNF-α levels. These animals produced low IL-10 levels in the culture supernatant and serum during the acute phase and did not produce IL-10 in the heart during the chronic phase of the disease. Our findings showed that Beagle dogs are a good model for studying the immunopathogenic mechanism of Chagas disease, since they reproduce the clinical and immunological findings described in chagasic patients. The data suggest that the development of the chronic cardiac form of the disease is related to a strong Th1 response during the acute phase of the disease, while the development of the indeterminate form results from a blend of Th1 and Th2 responses soon after infection, suggesting that the acute phase immune response is important for the genesis of chronic cardiac lesions.

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1. Introduction

Chagasic cardiopathy (CC) is a consequence of infection by the *Trypanosoma cruzi* protozoan. This disease is characterized by myocarditis, in which an important lymphomononuclear infiltrate is accompanied by interstitial fibrosis and cardiomyocyte hypertrophy that can lead to dilated cardiomyopathy, end-stage heart failure, and death (Pereira Barretto et al., 1986; Higuchi, 1997). Although it is widely accepted that the inflammatory infiltrate is the ultimate effector of myocardial damage, the exact mechanism through which only a subset of infected individuals develop myocardial inflammation in CC remains poorly understood (Ramasawmy et al., 2006).

The cytokine profile has been shown to constitute an essential key for defining the immunopathological mechanisms involved in CC and controlling immune responses during *T. cruzi* infections (Zhang and Tarleton, 1996; Teixeira et al., 2002). Various cell types and soluble molecules have been shown to participate in both the control and pathogenesis of *T. cruzi* infection. Peripheral blood lymphocytes as well as mononuclear cells infiltrating the heart tissue of patients with CC produce more IFN-γ, TNF-α and IL-6, and less IL-4 and IL-10, compared to those from asymptomatic individuals (Reis et al., 1993; Abel et al., 2001; Gomes et al., 2003). On the other hand, peripheral mononuclear cells from patients with the indeterminate form produce high levels of IFN-γ and TNF-α associated with high IL-10 levels (Bahia-Oliveira et al., 1998; Correa-Oliveira et al., 1999; Ribeirao et al., 2000; Abel et al., 2001; Gomes et al., 2005). However, other studies did not demonstrate a correlation between production of inflammatory and anti-inflammatory cytokines and the clinical signs of Chagas disease (Dutra et al., 1997). Thus, the characterization of the different cytokines present in cardiac tissue, lymphocyte culture or in the serum during the *T. cruzi* infection and their correlation with the intensity of cardiac damage has not been thoroughly elicited. This analysis could be of great value for unraveling the elements involved in the pathogenesis of CC and could be used to reduce morbidity during the chronic phase of the disease, thus enhancing the quality of life for patients.

A major stumbling block for research efforts striving to elucidate the mechanisms of pathogenesis of chronic Chagas' disease is the lack of a suitable animal model. Our group has shown that these animals develop diffuse chronic myocarditis with cardiac, pathologic and electrocardiography changes in the canine model of Chagas' disease that are also found in humans (Lana and Chiari, 1986; Lana et al., 1992; Bahia et al., 2002; Caliari et al., 2002; Guedes et al., 2008). Taking advantage of the great similarity between the pathologies developed by Chagas disease patients and dogs infected with *T. cruzi*, this study explores the role of relationships between pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-10) cytokine levels present in sera, culture supernatant, and heart tissue and the development of the cardiac pathology (heart inflammation, fibrosis and cardiomegaly) using Beagle dogs infected with different *T. cruzi* strains.

2. Materials and methods

2.1. T. cruzi stocks

In this study, three distinct strains were used: the *T. cruzi* Y strain isolated from an acute human case (Silva and Nussenzweig, 1953); the Berenice-78, isolated by xenodiagnosis assay in 1978 from what was considered the first clinical case of the disease in Brazil of a patient with indeterminate form of the disease (Lana and Chiari, 1986); and the ABC strain, which was isolated also by xenodiagnosis from a patient with heart and digestive manifestations from the Minas Gerais state, Brazil (Brener, 1965). We have previously shown that these strains present distinct patterns of virulence and pathogenicity in the canine model (Guedes et al., 2007).

2.2. Experimental animals and infection

Thirty-two 4-month-old Beagle dogs from the kennels at the Ouro Preto Federal University (UFOP), Minas Gerais State, Brazil were used in this study. All procedures and experimental protocols were conducted in accordance with the directives issued by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Research at this University. Animals were fed with commercial dog food and water *ad libitum*. Prior to the study, the animals were dewormed and vaccinated against several infectious diseases. Twenty-four Beagle dogs were inoculated with 4.0×10^3 bloodstream trypomastigotes per kg of body weight of the Y, Berenice-78 and ABC *T. cruzi* strains. Eight age-matched non-infected dogs were used as controls.

2.3. Macroscopic evaluation

The animals were euthanized by injection with thionenbutal (Abbott, São Paulo, Brazil) 0.5 ml/kg of body weight (0.03 g/ml in 0.8% saline solution), one group at 4 weeks and the other at a 100-week after infection. Necropsies were performed and the heart, spleen, cervical lymph node and animal weights were collected. Cardiomegaly, splenomegaly and lymphadenopathy were evaluated by determining the heart, spleen and cervical lymph node indexes (organ weight/total body weight × 100) and also by inspection for macroscopic alterations. Cardiomegaly, splenomegaly and lymphadenopathy were considered as present in the infected animals when the organ index was significantly higher than that observed in non-infected animals. The digestive systems (esophagus, stomach, caecum and colon) were also macroscopically analyzed for anatomical alterations.

2.4. Histopathology and morphometric analysis

A fragment of approximately 1.0 cm × 1.0 cm × 0.2 cm from the middle of the right atrial wall of each dog was taken for histopathological analyses. Tissue fragments were fixed in 10% buffered formalin solution, dehydrated, cleared and embedded in paraffin. Blocks were cut into 4 μm-thick sections and stained by hematoxylin–eosin

(H&E) for inflammation assessment or Masson's trichromic for fibrosis quantitative evaluation. Thirty fields from each H&E or Masson's trichromic stained section were randomly chosen at a 40× magnification, giving a total of 1.6×10^6 μm^2 analyzed myocardium area. Images were obtained through a JVC TK-1270/RGB micro-camera and the KS300 software built into the Kontron Elektronick/Carl Zeiss image analyzer. The inflammatory process was evaluated by a correlation index between the number of cell nuclei observed in the myocardium muscle from non-infected and infected animals (Maltos et al., 2004). The fibrosis area was quantified using the image segmentation function. All pixels with blue hues in Masson's trichromic section were selected to build a binary image, subsequently calculating the total area occupied by connective tissue in non-infected and *T. cruzi* infected dogs. The cytokine production in serum and supernatant cell cultures were determined by capture ELISA. The blood samples were collected before infection and at 4, 48 and 100 weeks after infection to obtain sera and peripheral blood mononuclear cells (PBMC).

2.5. Peripheral blood mononuclear cells proliferation assay

The PBMC were isolated by Ficoll-Hipaque density gradient centrifugation and washed three times with RPMI (GIBCO, Grand Island, NY, USA). The cells (3.5×10^6) were stimulated with parasite antigens (5×10^6 trypomastigotes/ml) in a final volume of 500 μl. The cells were then incubated for 3 days at 37 °C in a 5% CO_2 atmosphere and supernatant collected for cytokine quantification by ELISA at the third day of culture.

2.6. Cytokine quantification

2.6.1. ELISA

ELISAs for IL-10, IFN-γ and TNF-α were undertaken according to the manufacturers' instructions (R&D Duoset, R&D, Minneapolis, MN). 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.

2.6.2. RT-PCR assay

RNA was isolated from the right atrium and cervical lymph node of Beagle dogs by acidic guanidinium thiocyanate–phenol–chloroform extraction: RNAgentes® Total RNA Isolation System (Promega, US). One microgram of total RNA was treated with RQ1 RNase-Free DNase (Promega, US), before reverse transcribed by the addition of 100 U SuperScript™ II RNase H^- (Invitrogen, US), 10 mM deoxynucleotides (dNTPS) (Invitrogen, US), 0.1 M dithiothreitol—DTT (Invitrogen, US), 1× RNAase H^- reverse transcriptase buffer (Invitrogen, US), 1 μg oligo $(dT)_{12-18}$ primer (Invitrogen, US), 100 U RNaseOUT™ Ribonuclease inhibitor (Invitrogen, US) in a total volume of 20 μl. The reaction proceeded for 1 h at 42 °C. Two microliters of cDNA was used for amplification in a 12-μl PCR reaction containing 2.5 mM dNTPs (Invitrogen, US), a 20-μM concentration of the 3′ and 5′ external primers, 1.5 mM $MgCl_2$; 1× PCR buffer and 0.5 U *Taq* DNA polymerase (Invitrogen, US). PCR conditions were as follows: 94 °C, 5 min (first cycle), 94 °C, 1 min, 57 °C, 1 min, 72 °C, 2 min (*n* cycles), 57 °C, 1 min, 72 °C, 7 min (final cycle). The primers used are shown below. PCR products and molecular weight marker were run on 6% polyacrylamide gel and stained with silver nitrate (Santos et al., 1993). PCR products on silver-stained gels were quantified with a densitometer using a Quantity One program (The Discovery Series 1998, Biorad Laboratories, US). The densitometric values for each cytokine were divided by the average value for the hypoxanthine phosphoribosyltransferase (HPRT) for the same sample. The primer (sense and antisense) sequence from 5′ to 3′, followed by the number of cycles and the expected product size of PCR, shown in brackets. Cytokines: IFN-γ, CGGCCTAACTCTCTGAAACG, CCTCCCTCTTACTGGTGCTG (32, 380 pb); and IL-10, AGCACCCTACTTGAGGACGA, GATGTCTGGGTCGTGGTTCT (40, 249 pb) (Dialab, Brasil). Constitutive gene: HPRT, AAGCTTGCTGGTGAAAAGGA, CAATGGGACTCCAGATGCTT (28, 219 pb) (Dialab, Brasil).

2.7. Statistical analysis

All the cytokine production and histological data were analyzed by the Student's *t*-test (GraphPad InStat software) for non-infected, *T. cruzi* Y, Berenice-78 and ABC strains in infected animals. Regression analysis was used to compare inflammation and fibrosis, with regression lines compared by covariance analysis (Snedecor and Cochran, 1989). In all cases, differences were considered as significant when $P < 0.05$.

3. Results

3.1. T. cruzi infection induces lymphadenopathy and splenomegaly

All infected dogs showed severe cervical lymphadenopathy and splenomegaly during the acute phase of the disease, regardless of the parasite strain used for infection. Animals infected with ABC strain showed a more intense lymphadenopathy during the acute phase of the disease, compared to Berenice-78 and Y infected animals ($P < 0.05$). In the chronic phase, all the animals showed normalization of cervical lymph node size and persistence of splenomegaly, but to a lesser extent than in the acute phase (Fig. 1).

3.2. Cardiomegaly, inflammation and fibrosis induced by infection are T. cruzi-strain specific

In order to evaluate the influence of the *T. cruzi* strain in the development of heart alterations, a macroscopic and quantitative analysis of the inflammation and fibrosis was carried out in the right atrium of animals infected with different *T. cruzi* strains. During the acute infection phase, half of the animals infected with Y and ABC strains demonstrated mild cardiomegaly and fibrosis, while only 25% of the animals infected with Be-78 strain presented similar alterations. However, no differences were observed for these parameters when comparing all the infected

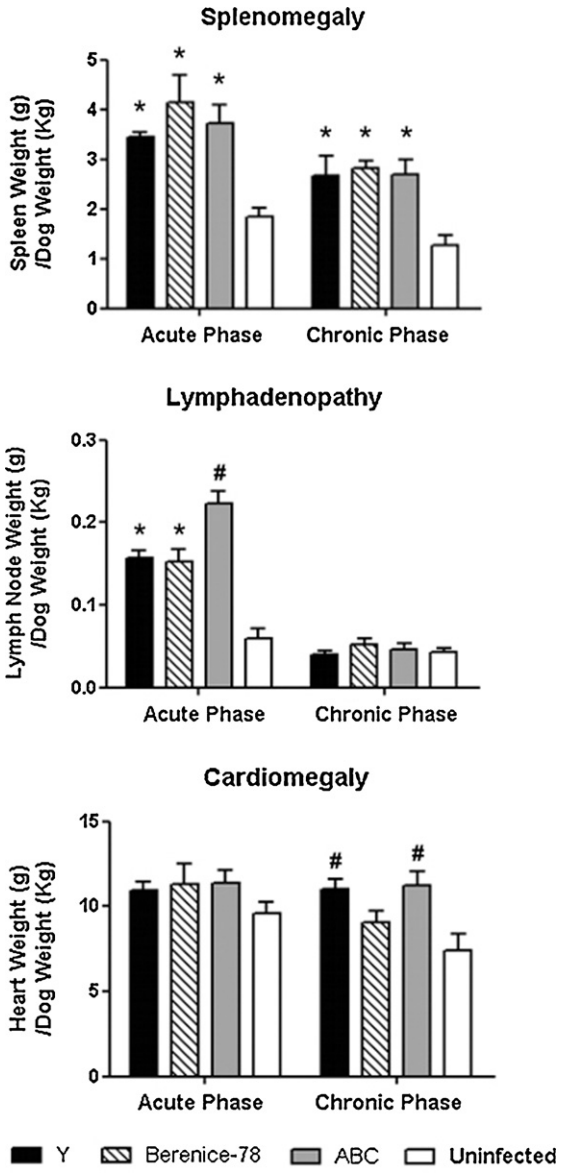

Fig. 1. Splenomegaly (A), lymphadenopathy (B) and cardiomegaly (C) during the acute and chronic phases of infection with 4×10^3 blood trypomastigotes by the intraperitoneal route of Y, Berenice-78 and ABC *T. cruzi* strains in Beagle dogs. #Statistical difference ($P < 0.05$) compared to other groups. *Statistical significance ($P < 0.05$, Student–Newman–Keuls test) when comparing the infected group to non-infected animals.

groups with the control group ($P > 0.05$) (Table 1, Figs. 1 and 2). All the infected dogs showed severe myocarditis during the acute phase of the disease, compared to the non-infected controls ($P < 0.05$). Moreover, the animals infected with the Berenice-78 strain showed discreet hyalin degeneration and parasites, while the animals infected with Y and ABC showed only moderate hyalin degeneration (Fig. 2).

Macro- and microscopic analyses of the dogs' hearts during the chronic phase of the infection showed the presence of cardiomegaly, flaccidity, inflammation and fibrosis in all the animals infected with the Y and ABC strains, in contrast to the dogs infected with the Berenice-78 strain, where only 50% presented these alterations at this stage of the disease (Table 1, Figs. 1 and 2).

The intensity of inflammatory infiltrate and fibrosis areas in the hearts of infected dogs was also correlated to the parasite strain. Quantitative analyses of inflammation (Fig. 2) in the right atrial wall during the chronic phase showed moderate inflammatory infiltrate in Y and ABC, and discreet inflammation in dogs infected with the Berenice-78 strain. At this stage in the development of the disease, hyaline degeneration was discreet or absent in animals infected with Berenice-78, and moderate in animals infected with Y and ABC strains (data not presented). During the chronic infection phase, two cardiac fibrosis patterns (Fig. 2) were observed in the right atrial wall. Animals infected with Y and ABC showed severe intra-fascicular collagen deposition, while dogs infected with the Berenice-78 strain presented discreet fibrosis. Infected animals did not present any anatomical alterations in the esophagus, stomach, caecum and colon.

3.3. High IFN-γ, TNF-α and low IL-10 production during the acute infection phase correlates to inflammation and fibrosis in the heart during the chronic phase

In order to correlate cardiac alterations with cytokine profiles, the IFN-γ, TNF-α and IL-10 were quantified in the sera and supernatants of PBMC cultures of infected dogs. Cytokine production in the sera of infected dogs was observed only 4 weeks after infection, while high and similar levels of IFN-γ and TNF-α were detected in dogs infected with the three different strains. We also noted high IL-10 levels in the sera of infected animals during the acute phase, compared to the non-infected animals. However, the production observed in the groups of animals infected with the different strains was similar (Fig. 3). Antigenic stimulation of PBMC from animals infected with the Y and ABC strains during the acute infection phase led

Table 1
Cardiomegaly, inflammation and fibrosis in right atrium of infected dogs with Y, Berenice-78 and ABC *T. cruzi* strains and euthanized during acute (4 weeks) and chronic (100 weeks) phase of infection.

T. cruzi strains	Acute phase		Chronic phase	
	Dogs with cardiomegaly/total	Dogs with inflammation/total	Dogs with fibrosis/total	Dogs with cardiomegaly, inflammation and fibrosis/total
Y	2/4	4/4	2/4	4/4
Berenice-78	1/4	4/4	1/4	2/4
ABC	2/4	4/4	2/4	4/4

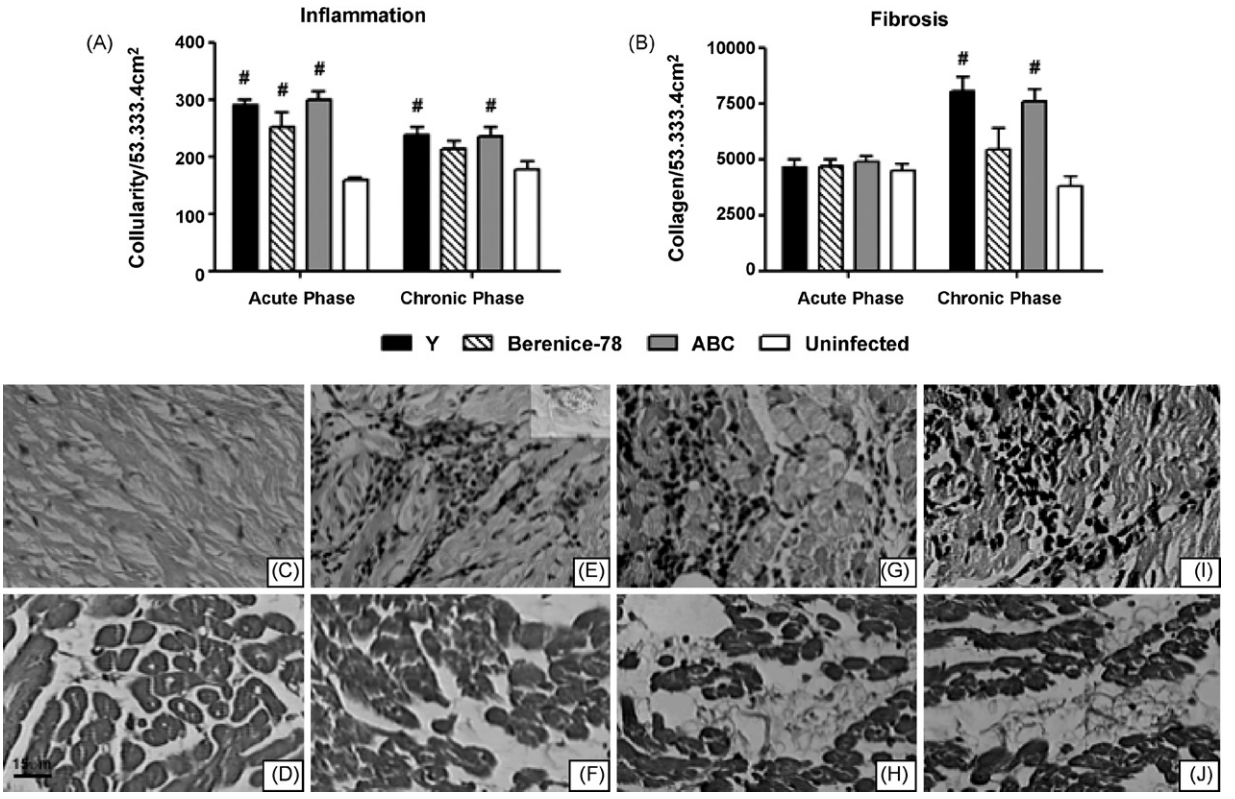

Fig. 2. Dogs infected with Y, Berenice-78 and ABC *T. cruzi* strains showed different patterns of cardiac inflammation and fibrosis. Inflammation (A) and fibrosis (B) in the right atrium during the acute and chronic phases of infection with 4×10^3 blood trypomastigotes of Y, Berenice-78 and ABC *T. cruzi* strains in Beagle dogs. $^{\#}P < 0.05$ indicates a significant difference compared to the control group. Histology of the right atrium of the dogs: non-infected dogs euthanized at the same age as the infected dogs during the acute (C: HE), and chronic (D: Masson trichrome) phases of infection; Berenice-78 *T. cruzi* strain, (E) acute phase (amastigote nest) (F) and chronic phase; Y *T. cruzi* strain (G), acute phase (H) and chronic phase; ABC *T. cruzi* strain acute phase (I) and chronic phase (J).

to enhanced production of IFN-γ and TNF-α compared to both the control group and animals infected with Berenice-78 ($P < 0.05$). During this period, high IFN-γ and TNF-α levels were also observed in animals infected with Berenice-78, compared to non-infected animals ($P < 0.05$). During the chronic phase, low cytokine production (IL-10, IFN-γ and TNF-α) was observed and no significant differences were noted between the groups of dogs infected with distinct *T. cruzi* strains. Interestingly, the animals infected with Berenice-78 showed higher IL-10 production than dogs infected with the Y and ABC strains during the acute phase of the disease in PBMC stimulated cultures (Fig. 3). These findings suggest that cytokine production during the acute phase is relevant for clinical manifestations during the chronic phase.

3.4. IL-10 and IFN-γ expression in dogs with inflammation and fibrosis in the heart during the acute and chronic phases of Chagas disease

Cytokine mRNA expression was examined through semi-quantitative RT-PCR in the cervical lymph nodes and hearts of animals infected with Berenice-78, Y and ABC *T. cruzi* strains during the acute and chronic phases of the infection. No differences were observed *in situ* for the IFN-γ and IL-10 mRNA expression in cervical lymph nodes during the acute and chronic phases of Chagas disease, compared to non-infected animals (data not presented). However, we observed higher IFN-γ and IL-10 mRNA expression during the acute phase in the right atrium of infected animals compared to the control group ($P < 0.05$) (Fig. 4). During the chronic phase, the IFN-γ mRNA expression was higher in the heart tissue of infected animals, compared to the control animals ($P < 0.05$). In contrast, higher IL-10 mRNA expression ($P < 0.05$) was observed in the heart tissue of animals infected with the Berenice-78 strain, compared to dogs infected with the Y and ABC strains as well as non-infected animals (Fig. 4).

To analyze the correlation between IFN-γ, TNF-α and IL-10 production and the development of heart pathology, the animals were divided into groups based on the degree of heart damage: (i) cardiac form—defined by the presence of cardiomegaly, inflammation and fibrosis in the heart; (ii) indeterminate form—defined by the absence of these pathological symptoms of the disease. All the dogs infected with the Y and ABC strains, and 50% of those infected with the Berenice-78 strain presented the cardiac form of the disease, while 50% of the animals infected with the Berenice-78 showed the indeterminate form. Analysis of the association between the clinical manifestation and

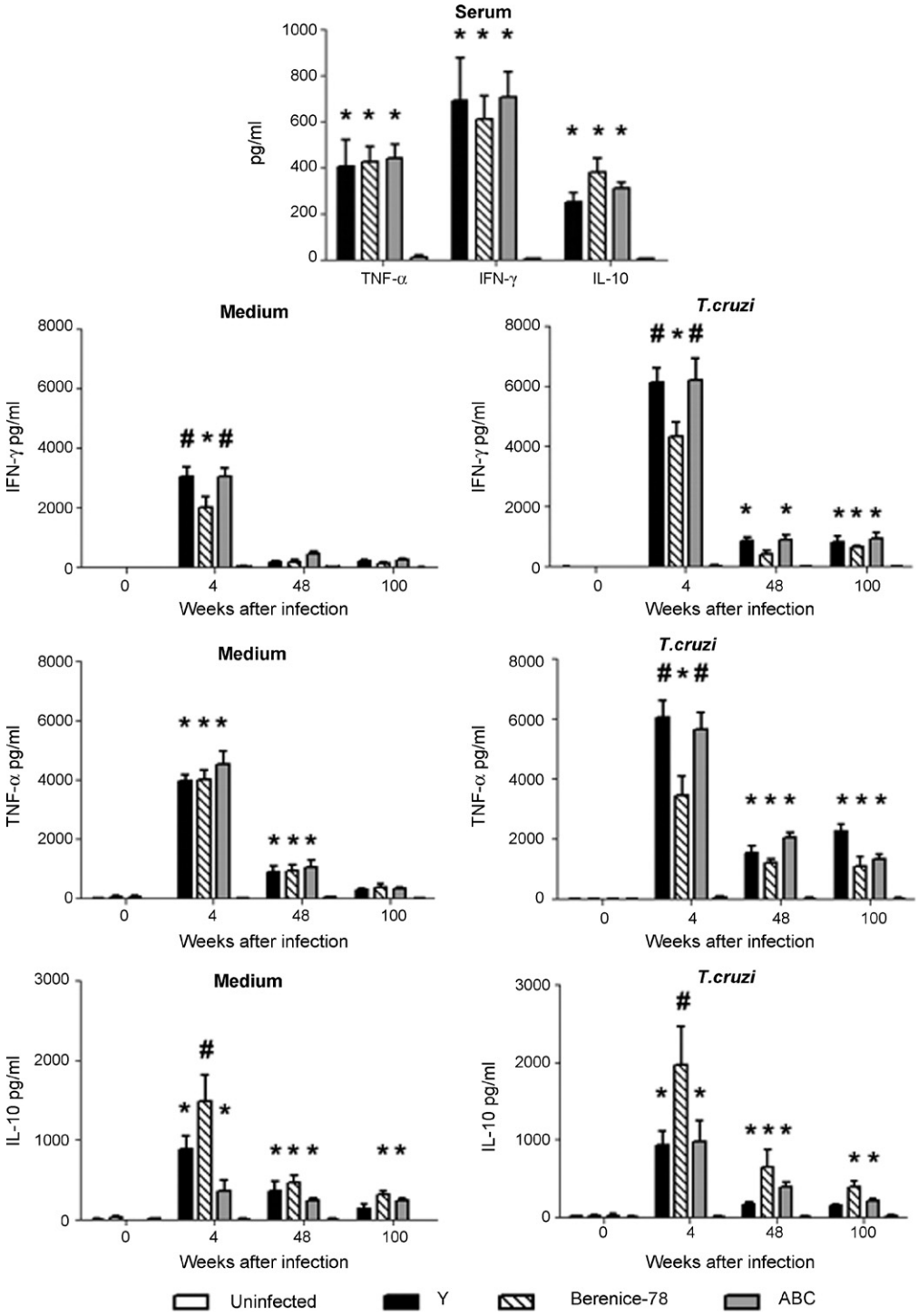

Fig. 3. IL-10, IFN-γ and TNF-α quantification by ELISA in sera (4 weeks after infection) and culture supernatant of PBMC in Beagle dogs infected with 4×10^3 blood trypomastigotes/kg of *T. cruzi* Y, Berenice-78 and ABC strains, which were followed from the acute to the chronic infection phases. The results shown (mean ± S.D.) of four animals per group. #Statistical difference ($P < 0.05$) compared to other groups. *Statistical significance ($P < 0.05$, Student–Newman–Keuls test) when comparing the infected group to non-infected animals.

cytokine production demonstrated a higher IFN-γ and TNF-α and lower IL-10 production in the supernatant culture of dogs with the cardiac form during the acute phase of the disease. Furthermore, while no differences were observed in IFN-γ production in the heart during the acute phase in animals with the cardiac or indeterminate form of the disease, animals with the indeterminate form of the disease presented higher levels of IL-10 mRNA

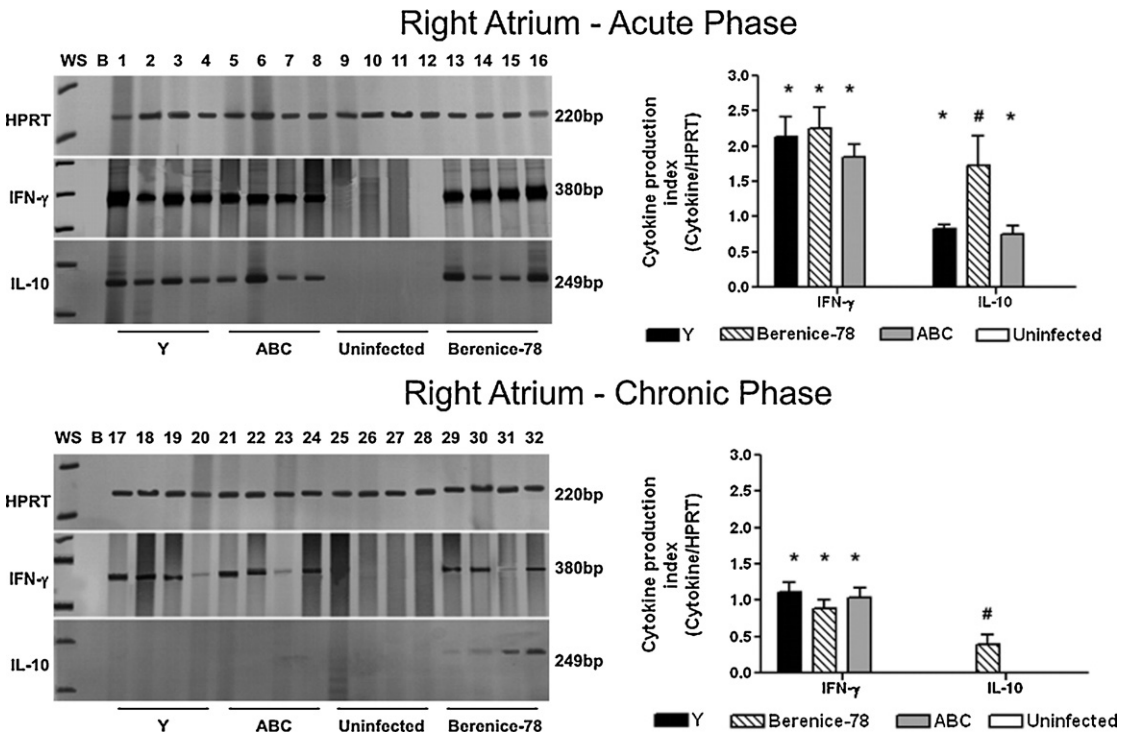

Fig. 4. Cytokine production in the right atrium of Beagle dogs infected with Y, ABC and Berenice-78 *T. cruzi* strains by RT-PCR during the (A) acute and (B) chronic phases. WS: molecular weight standard—100 bp DNA ladder; (B) blank; 1-32: product of RT-PCR obtained from each dog. Semi-quantitative analysis of IFN-γ and IL-10 mRNA expression in the right atrium during the acute (C) and chronic (D) phases of infection with Y, Berenice-78 and ABC *T. cruzi* strains in Beagle dogs. $^{*}P < 0.05$ indicates a significant difference between the infected and non-infected groups, $^{\#}P < 0.05$ indicates a significant difference between the infected group and other groups.

expression *in situ* in the heart during the chronic phase than dogs with the cardiac form of the disease ($P < 0.05$) (Fig. 5).

4. Discussion

These findings suggest that the indeterminate form of experimental Chagas disease in dogs correlates to a balance between inflammatory (IFN-γ and TNF-α) and regulatory (IL-10) cytokine profile production. The chronic cardiac form of the disease was associated with high IFN-γ and TNF-α and low IL-10 production during the acute phase. Moreover, the hearts of animals presenting the cardiac form of the disease showed high IFN-γ and low or nil IL-10 production during the chronic phase. The similarities between the immunopathogenical findings and those reported for patients suggest that this dog is a good model for physiopathogenesis studies of Chagas disease.

Initially, we evaluated the utility of the Beagle as an animal model for human Chagas disease. It was observed that *T. cruzi* infection in Beagle dogs with Y, Berenice-78 and ABC strains resulted in severe splenomegaly and lymphadenopathy during the acute phase of the disease. During the chronic phase, mild splenomegaly and severe cardiomegaly was observed in most of the animals, reinforcing previous studies by our group and other researchers that have already found cardiomegaly, inflammation and fibrosis during experimental Chagas disease in mongrel dogs (Lana et al., 1992; Bahia et al., 2002; Caliari et al., 2002). The presence of clinical signs as splenomegaly, lymphadenopathy and cardiomegaly, was also described during acute and chronic phase of Chagas disease in patients (Salgado et al., 1962; Dias, 1989). The dogs used in this study presented parasitemia, antibody production (IgA, IgM and IgG) (Guedes et al., 2007, 2008) and electrocardiograph alterations (data not presented) similar to those noted in human chagasic patients (Elizari and Chiale, 1993). Moreover, we observed diffuse chronic myocarditis, fibrosis and cytokine production in these Beagle dogs that was very similar to that found in chronic chagasic patients (Higuchi, 1995; Gomes et al., 2003). During the chronic phase, Beagle dogs thus reproduce the clinical indications, pathological and electrocardiographic changes and immune responses seen in humans, demonstrating that they are good experimental models for Chagas disease.

The next step in this study was to evaluate whether different *T. cruzi* strains showing distinct patterns of virulence, *in vitro* infectivity and pathogenicity in mice and mongrel dogs (Lana et al., 1992; Veloso et al., 2001; Guedes et al., 2007), were associated with varying degrees of cardiac pathology. Our findings demonstrated that the Y and ABC strains were very pathogenic for dogs, while animals infected with the Berenice-78 strain presented less severe cardiac damage.

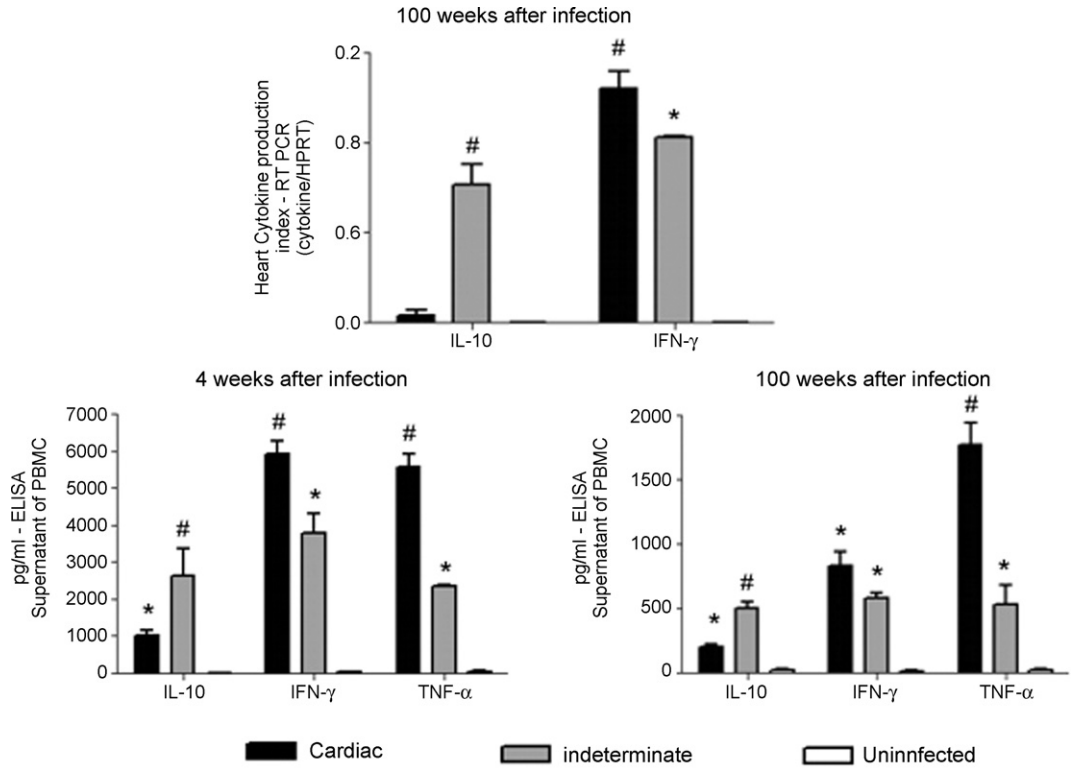

Fig. 5. Semi-quantitative analysis of IFN-γ and IL-10 mRNA in the right atrium during the chronic infection phase with Y, Berenice-78 and ABC *T. cruzi* strains in Beagle dogs. The animals were divided into groups according to clinical manifestations: non-infected control, indeterminate and cardiac. $^{*}P < 0.001$ indicates a significant difference between the infected and non-infected groups, $^{\#}P < 0.001$ indicates a significant difference between the infected group and other groups.

The presence of inflammation and fibrosis during the acute and chronic phases of Chagas disease have been described in mongrel dogs (Tafuri, 1970, 1987; Lana et al., 1992; Bahia et al., 2002; Caliari et al., 2002) and chagasic patients (Higuchi, 1995; Higuchi et al., 1997; Rassi et al., 2000). However, the inflammation and fibrosis observed in infected mongrel dogs is highly variable (Lana et al., 1992; Bahia et al., 2002; Caliari et al., 2002). Beagle dogs are genetically homogeneous, making possible to obtain more homogeneous and reproducible outcomes. A key point not studied in the dog model and not yet fully understood in human disease is the correlation between cytokine production and the clinical manifestation of Chagas disease.

In the Beagle model, the presence of dogs with and without cardiac damage provided a great opportunity to study cytokine production during the acute and chronic phases of the disease and its ability to predict the risk of developing the cardiac form of Chagas disease. We thus quantified the IL-10, TNF-α and IFN-γ production in the serum and supernatant from *T. cruzi* stimulated lymphocyte cultures. Furthermore, the *in situ* production of IFN-γ and IL-10 in heart and cervical lymph node of animals presenting cardiac and indeterminate clinical forms of the disease was also evaluated.

The findings indicated that cardiac chronic inflammation and fibrosis were influenced by immune responses to the parasite. Animals presenting cardiac damage produced high IFN-γ, TNF-α and low IL-10 levels by peripheral mononuclear blood cells during the acute phase of the disease, regardless of the *T. cruzi* strain used in the infection. Additionally, we also observed high IFN-γ and low or absent IL-10 mRNA levels during the chronic phase in the right atrium of infected animals presenting heart inflammation and fibrosis. Interestingly, significant IL-10 mRNA levels were detected only in animals infected with the Berenice-78 strain that did not present cardiac damage. Thus, the indeterminate form of Chagas disease in Beagle dogs was associated with high IFN-γ, TNF-α and IL-10 production, while the cardiac form of the disease is associated with high IFN-γ, TNF-α and low IL-10 production. To our knowledge, this is the first description of cytokine production in canine Chagas with long-term infection, suggesting that cytokine production during the acute phase of the disease is a determining factor for cardiac lesions. Due to ethical constraints, the evaluation of this association between acute and chronic infection is not possible in chagasic patients and has not been demonstrated in mice. Our findings showed that intra-fascicular inflammatory foci continue to develop slowly and progressively over the years, being modulated by ineffective and unbalanced immune responses that lead to chronic fibrotic myocarditis.

The pro-inflammatory cytokines IL-12, IFN-γ and TNF-α (Th1 response) work together, activating macrophages that kill parasites through producing nitric oxide and its derived nitrogen free radicals. In addition, they also stimulate the differentiation and proliferation of Th1-

biased CD4 T cells, which orchestrate a CD8 T cells response that causes tissue destruction and fibrosis (Machado et al., 2000). As expected, this inflammatory response must be regulated, mainly through the action of anti-inflammatory (Th2) cytokines IL-4, IL-10 and TGF-β (Silva et al., 1992; Holscher et al., 1998).

Experimental studies using mice have shown that *T. cruzi* infection in resistant animals induces low IL-10 and IL-4 production associated with high IFN-γ and TNF-α production in the supernatant of splenocyte cultures (Eksi et al., 1996). It was suggested that high IFN-γ levels associated with low IL-4 and IL-10 levels are an important cause of fibrosis (Waghabi et al., 2002). However, similar levels of IFN-γ e IL-10 mRNA were observed in *T. cruzi* infected mice that showed different degrees of cardiac lesions (Powell et al., 1998; Teixeira et al., 2002).

Studies using PBMC and human heart biopsies showed that cardiac manifestations are associated with high Th1 (IFN-γ) response, while patients with the indeterminate form are divided evenly between Th1 and Th2 responses (Reis et al., 1997; Bahia-Oliveira et al., 1998; Correa-Oliveira et al., 1999; Abel et al., 2001; Gomes et al., 2003, 2005; Souza et al., 2004). Although Dutra et al. (1997) showed similar levels of anti- and pro-inflammatory cytokine mRNA in the PBMC of chagasic patients with the cardiac and indeterminate forms, the pro-inflammatory cytokines (TNF-α and IFN-γ) are detected in lesions (Reis et al., 1993; Cunha-Neto et al., 1998).

This paper shows that Beagle dogs are good models for studying immunopathogenical mechanisms involving *T. cruzi* infections, through reproducing the clinical signs and immune responses of the acute and chronic phases that are very similar to those noted in human beings. Moreover, the natural course of the disease could be monitored, evaluating immune responses and cardiac analyses. The data indicate that the indeterminate clinical form in these animals is associated mainly with high IL-10 production during the acute phase of the disease, while the cardiac form is associated with high IFN-γ, TNF-α and low or nil IL-10 production, indirectly strengthening the hypothesis that while the type 1-dependent response is important for limiting parasite replication during the acute phase, it may also be involved in the development of severe chronic heart disease in human beings.

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