The TcI and TcII *Trypanosoma cruzi* experimental infections induce distinct immune responses and cardiac fibrosis in dogs

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Trypanosoma cruzi *infection may be caused by different strains with distinct discrete typing units (DTUs) that can result in variable clinical forms of chronic Chagas disease. The present study evaluates the immune response and cardiac lesions in dogs experimentally infected with different* T. cruzi *strains with distinct DTUs, namely, the Colombian (Col) and Y strains of TcI and TcII DTU, respectively. During infection with the Col strain, increased levels of alanine aminotransferase, erythrocytes, haematocrit and haemoglobin were observed. In addition, $CD8^+$ T-lymphocytes isolated from the peripheral blood produced higher levels of interleukin (IL)-4. The latter suggests that during the acute phase, infection with the Col strain may remain unnoticed by circulating mononuclear cells. In the chronic phase, a significant increase in the number of inflammatory cells was detected in the right atrium. Conversely, infection with the Y strain led to leucopoenia, thrombopoenia, inversion of the ratio of $CD4^+/CD8^+$ T-lymphocytes and alterations in monocyte number. The Y strain stimulated the production of interferon-γ by $CD4^+$ and $CD8^+$ T-lymphocytes and IL-4 by $CD8^+$ T-cells. In the chronic phase, significant heart inflammation and fibrosis were observed, demonstrating that strains of different DTUs interact differently with the host.*

Key words: *Trypanosoma cruzi* - strain - dog - immune response - cardiac inflammation

Chagas disease is caused by the flagellate protozoan *Trypanosoma cruzi* and is transmitted by blood-sucking insects of the subfamily Triatominae (Chagas 1909). The *T. cruzi* heterogenic nomenclature is based on grouping the populations into six discrete typing units (DTUs): TcI, TcII, TcIII, TcIV, TcV and TcVI (Zingales et al. 2009).

The parasite exhibits different wild and domestic behaviours. The distribution in South America indicates that TcI is the most abundant and dispersed DTU, as it is found from Central America to the north of South America, and includes chronic cases of cardiomyopathy and severe cases of meningoencephalitis. The DTUs TcIII and TcIV have been predominantly associated with the sylvatic cycle in South America, whereas TcV and TcVI are related to the domestic cycle in southern and central parts of South America. The majority of TcII has been found in South American countries, such as Brazil, Argentina and Chile, where it has been predominately associated with the domestic cycle and with severe chronic forms of Chagas disease, including cardiac and digestive manifestations (Miles et al. 1981, Zingales et al. 1998, 2012, Freitas et al. 2005, Lages-Silva et al. 2006).

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Evaluating organs of the same patient, it was observed that different *T. cruzi* populations can parasitise distinct organs and this pattern might be related to the pathogenesis of chronic forms of the disease (Vago et al. 2000). In Colombian (Col) patients, TcII was detected in the heart tissue along with histological alterations characteristic of chronic chagasic cardiomyopathy (CCC). In contrast, TcI was detected in the muscular layer of oesophageal tissue and was accompanied by lymphocytic infiltrates and interstitial fibrosis (Mantilla et al. 2010).

The mouse was the first and remains the most extensively studied experimental model for Chagas disease. Although murine infection shares some aspects in common with human Chagas disease, such as immunological, pathological and physiological characteristics, there is poor correlation between the chronic alterations observed in mice and in humans (da Costa 1999). In particular, the murine model does not permit accurate and subtle determinations of cardiac dysfunction (Morris et al. 1991). Canine experimental infection with *T. cruzi* shares many of the characteristics of human Chagas disease, including the occurrence of an acute, an indeterminate asymptomatic and a symptomatic chronic phase. Additionally, they share clinical and serological aspects, including patent parasitaemia, parasitism of myocytes, myocardium inflammation, fibrosis and fatty replacement in the cardiac conduction system, with electrocardiographic alteration, evolution to cardiac dysfunction and congestive heart failure. Most importantly, canine infection reproduces diffuse fibrosis, as observed in human CCC (Andrade & Andrade 1980, Andrade et al.

1980, 1984, Laranja & Andrade 1980, Tafuri et al. 1988, Morris et al. 1991, de Lana et al. 1992, Guedes et al. 2002, Diniz et al. 2010, Caldas et al. 2013).

The complex interaction between *T. cruzi* and the host, which determines the pathogenesis and diversity of clinical forms, remains to be elucidated. In this respect, evaluating the influence of different *T. cruzi* DTUs on the course of the disease can provide valuable insight into the host-parasite interaction. For this purpose, we characterised the biochemical, haematological and cardiac histopathological alterations in canine experimental infections with Y (TcII) or Col (TcI) *T. cruzi* strains.

MATERIALS AND METHODS

Ethics statement - Details of the project were submitted to and approved by the Ethical Committee on Animal Research of the Federal University of Ouro Preto (UFOP), Ouro Preto, state of Minas Gerais, Brazil (protocol 2012/14). All procedures in this study were done according to the guidelines set by the Brazilian Animal Experimental College (federal law 11794). Experimental animals were maintained in the kennel at UFOP.

T. cruzi stocks - Trypomastigote forms were obtained from Swiss mice infected with the Y strain (TcII) (Silva & Nussenzweig 1953, Zingales et al. 2009) or the Col strain (TcI) (Federici et al. 1964, Zingales et al. 2009).

Experimental animals and infection protocol - Ten four-month-old mongrel dogs were obtained from the kennel at UFOP. The 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. Seven dogs were intraperitoneally inoculated with 2×10^3 blood trypomastigotes/kg of body weight (Guedes et al. 2002) of the Y strain (n = 3) or the Col strain (n = 4). Three age-matched uninfected dogs were used as negative controls (NI). After inoculation, 5 µL of blood was collected daily from the marginal ear vein of the animals to confirm infection (Brener 1962).

Haematological and biochemical analyses - Peripheral blood (5 mL) was collected from the jugular vein of each dog and transferred to tubes containing anticoagulant ethylenediamine tetraacetic acid. Blood samples were stored at room temperature (RT) for up to 12 h prior to processing. The absolute count of lymphocytes in each sample was obtained using a BC-2800 VET auto haematology analyser (Mindray, China) (Aguiar-Soares et al. 2014). The blood was centrifuged and the separated serum was used for biochemical determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-GT, urea and CK-NAC (Diagnostic Labtest SA, Brazil). These were evaluated using the Automatic Biochemical System (CELM SBA-200; CELM, Brazil) following the method described by the manufacturer.

Immunophenotyping and flow cytometry - Immunophenotyping analysis of canine peripheral blood was carried out *via* flow cytometry as follows: fresh whole blood samples were incubated at RT for 30 min in the dark in the presence of 50 µL of fluorochrome-labelled anti-canine cell surface marker monoclonal antibodies (mAbs), Anti-T CD3 (FITC, cat.: MCA1774), Anti-T CD4 (APC, cat.: MCA1038), Anti-T CD8 (Alexa Fluor, cat.: MCA1039647), Anti-B cell (PE, cat.: MCA1781) and Anti-CD14 (Cy5, cat.: MCA1568), all purchased from AbD Serotec (USA). The mAbs were previously diluted in phosphate-buffered saline (PBS) 20% foetal bovine serum (FBS) (PBS 0.15 M, pH 7.2, supplemented with 20% of FBS). After incubation, erythrocytes were lysed by adding 2 mL of lysis solution followed by incubation for 10 min at RT. Canine whole blood leucocytes were then washed twice with 2 mL of PBS and centrifuged at 400 *g* for 10 min at RT. The labelled cells were then fixed at RT with 200 µL of FACS FIX solution (10.0 g/L paraformaldehyde, 10.2 g/L sodium cacodylate and 6.65 g/L sodium chloride, pH 7.2) before analysis on the cytometer. The stained cells were stored at 4-8ºC up to 24 h before cytofluorometric analysis. Each assay included an internal control for autofluorescence, in which the cells were incubated in the presence of PBS 0.5% FBS. Flow cytometric measurements were performed on a FACSCalibur instrument (Becton Dickinson, USA). The Cell-Quest software package was used for data acquisition and analyses were performed with FlowJo software (TreeStar Inc, USA). A total of 20,000 events were acquired for each preparation.

T. cruzi epimastigote antigen preparation - Soluble epimastigote antigen was prepared from stationary phase Y and Col strain *T. cruzi* epimastigotes grown in liver infusion tryptose-medium, similar to the method described by Sathler-Avelar et al. (2006). After the third or fourth in vitro passage, epimastigotes were harvested, washed and resuspended in PBS, pH 7.2-7.4. The suspension was rapidly frozen at -70ºC and thawed at 37ºC three times, with a sonication procedure between each step. The crude lysate was centrifuged (37,000 *g*) for 90 min and the supernatant was collected, dialysed overnight against 15 mM PBS, pH 7.4, sterilised by filtration through a 0.22 µm pore membrane (Millex Filter; Millipore Products Division, USA) and stored at -70ºC until use. The protein content was assayed by the method described by Lowry et al. (1951).

Immunophenotyping of intracellular cytokines and flow cytometry - Peripheral blood samples were collected in sterile tubes containing heparin, as described by Leal et al. (2014), and 1 mL blood aliquots were incubated in the presence of 1 mL RPMI-1640 (GIBCO, USA) in 14 mL polypropylene tubes (Falcon; BD Pharmingen) ("unstimulated culture"). Epimastigote antigens of the Y or Col *T. cruzi* strains were added to the "stimulated culture" at a final concentration of 25 µg/mL. The tubes were incubated for 12 h at 37ºC in 5% CO_2. Brefeldin A (Sigma, USA) was added to each tube at a final concentration of 10 mg/mL and the cultures were submitted to an additional period of 4 h of incubation in a 5% CO_2 incubator at 37ºC. A tube containing phorbol myristate acetate at a final concentration of 25 ng/mL was used as a positive control after 4 h of incubation. At the end of the incubation period, 400 mL aliquots of suspension culture were immunostained with FITC labelled canine mAbs against

CD4 (1:200, mouse IgG2a: clone YKIX322.3) and CD8 (1:100, mouse IgG2a: clone YKIX302.9), all purchased from Serotec (UK). After resuspension of labelled cells in U-bottom 96-well plates, intracellular cytokine staining was done with phycoerythrin-labelled anti-bovine mAbs against interferon (IFN)-γ (clone CC302) and interleukin (IL)-4 (clone CC303) that are cross-reactive with canine cytokines (Serotec). The microtubes were kept at 4ºC until the acquisition of counts on the flow cytometer FACScalibur (Becton Dickinson). Data acquisition and analysis were done with CELLQUEST software (Franklin Lakes, USA) based on 30,000 events/microtube. The cytokine analysis of $CD4^+$ and $CD8^+$ lymphocyte subsets was performed by first establishing a scatter gate on the lymphocyte population using SSC vs. FL1 dot plot distributions. After selecting the region of interest (R1), dot plots were constructed for FSC vs. IFN-γ/FL2 or IL-4/FL2 to determine the percentage of intracellular cytokine. The results were expressed as the index of positive cells, which was calculated as follows: mean percentage of positive cells of "stimulated culture"/mean percentage of positive cells of "unstimulated culture". The cultures were stimulated with antigens corresponding to the strain used to inoculate the animals; thus, dogs infected with the Y strain were stimulated with Y epimastigote antigen, those infected with the Col strain were stimulated with Col epimastigote antigen and the uninfected dogs were stimulated with both Y and Col antigen.

Histopathology and morphometric analysis - Uninfected and infected dogs were euthanised 240 days post-infection and a fragment from the middle of the right atrial wall was taken for histopathological analysis. Tissue fragments were fixed in 10% formalin and embedded in paraffin. The blocks were cut into 4-µm-thick sections and stained with haematoxylin and eosin (H&E) for inflammatory analysis and with Masson trichrome for fibrosis quantitation.

Twenty fields stained with H&E and 30 fields stained with Masson trichrome were randomly chosen at 40X magnification, giving a total of 1.5×10^6 μm^2 and 2×10^6 μm^2 myocardium areas analysed, respectively. Images were obtained using a Leica DM 5000 B microscope with a coupled micro camera (model 2.4.0R1; Leica Application Suite; Leica Microsystems, Germany) and processed with Leica Qwin V3 image analyser software (Leica Microsystems).

Inflammation was estimated by subtracting the average number of cells found in the myocardium of control animals from the total number of cells present in the myocardium of each infected animal.

Fibrosis was determined using the image segmentation function. All pixels with blue hues in the Masson trichrome section were selected to build a binary image and fibrosis was subsequently calculated as the total area occupied by connective tissue in the myocardium of each infected animal minus the average area of the myocardium of control animals.

Statistical analysis - Statistical tests were conducted with GraphPad Prism 5 software (Prism Software, USA) using the two-way analysis of variance test, followed by the Bonferroni test, to compare the NI, Y and Col groups. For histopathology, one-way analysis of variance was used, followed by the Tukey test, to compare the NI, Y and Col groups. Differences were considered significant at $p < 0.05$.

RESULTS

T. cruzi infection mainly alters hepatic enzymes - Our results demonstrate alterations in hepatic enzymes compared to values in non-infected dogs. This standard parameter was calculated as the mean value of all animals before infection with and without the value of two-times the standard deviation (SD) (mean ± 2 SD). The increased value of ALT in the Y and Col groups in relation to standard parameters was significant at nine and 30 days after infection (d.a.i.) for the Y group compared with day 0 and the NI group. For the Col group, this increase was statistically significant at 9 d.a.i. compared with day 0 (Fig. 1).

Serum levels of AST, urea, gamma-GT, ALP and CK-NAC were evaluated, but no significant differences were observed (data not shown).

Haematological alterations resulting from T. cruzi infection - When analysing the haematological alterations, it was observed that the erythrocyte and haematocrit levels were increased in the Col group at 30, 90 and 240 d.a.i. compared to day 0. Haemoglobin was increased at 240 d.a.i. compared to day 0 for the NI and Y groups. In the Col group, this increase was observed at 30 and 240 d.a.i. compared to day 0. Platelets were decreased at 9 d.a.i. compared to day 0 in the Y group (Supplementary data, Table I). Infection with the Y strain resulted in leucopoenia at 9 d.a.i. compared to day 0. A reduction in peripheral blood monocytes was observed at nine and 240 d.a.i. with the Y strain compared to day 0, whereas this was observed at 90 d.a.i. for the Col group (Supplementary data, Table II). Y strain infection altered peripheral blood leucocytes in ex vivo analysis; T-lymphocyte $CD4^+$

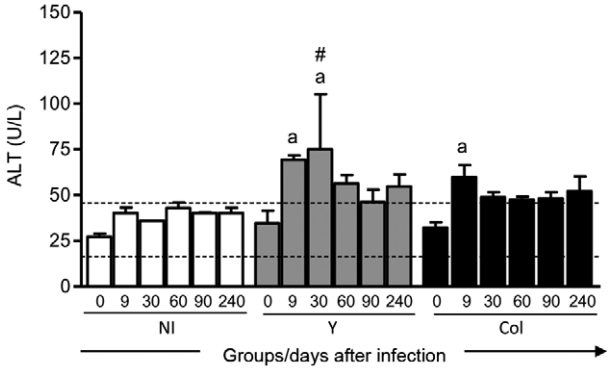

Fig. 1: alanine aminotransferase (ALT) serum level before infection (0) and nine, 30, 60, 90 and 240 days after infection with Y strain or Colombian strain (Col) of *Trypanosoma cruzi*. The results are expressed as mean enzyme level ± standard error. Significant differences at $p < 0.05$ are indicated by letter a for comparisons with day 0. Dotted line indicates mean ± 2 standard deviations for all animals at day 0 as the standard parameter. #: differences between the non-infected group (NI) and infected groups (Y or Col).

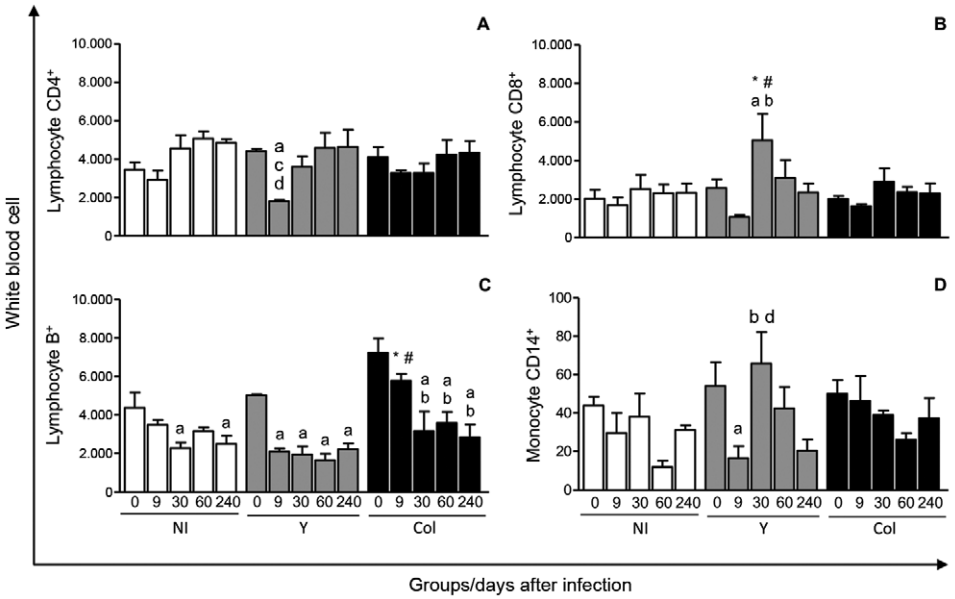

Fig. 2: ex-vivo quantification of $CD4^+$ (A) and $CD8^+$ T lymphocytes (B), B^+ lymphocytes (C) and $CD14^+$ monocytes (D) before infection (0) and nine, 30, 60 and 240 days after infection with Y strain (Y) or Colombian strain (Col) of *Trypanosoma cruzi*. The cell subsets were identified by flow cytometric immunostaining as described in Materials and Methods. Results are expressed as absolute cell counts in scatter plots ± standard error. Significant differences at $p < 0.05$ are indicated by letters a, b, c and d for comparisons with days 0, 9, 60 and 240, respectively. #: differences between the non-infected group (NI) and infected groups (Y or Col); *: differences between Y and Col groups at each time point.

levels were reduced at 9 d.a.i. with the Y strain compared to day 0, 60 and 240 d.a.i. (Fig. 2A). T-lymphocyte $CD8^+$ cells of the Y strain-infected group increased at 30 d.a.i. compared to day 0 and 9 d.a.i. and increased in comparison with the NI and Col groups at the same experimental time points (Fig. 2B). In terms of $CD4^+$ and $CD8^+$ T lymphocyte ratios, a reduction was observed during Y infection at 30 d.a.i. compared to the non-infected group (data not shown). The B^+ lymphocyte frequency dropped dramatically between day 0-9 d.a.i. with the Y strain and remained constant throughout the rest of the experiment, whereas in the case of infection with the Col strain, the decrease in B^+ lymphocytes was progressive from day 0-30 d.a.i. (Fig. 2C). $CD14^+$ monocyte frequencies were highly variable and were drastically lower at 9 d.a.i. compared to day 0 in the Y group, but this percentage was higher at 30 d.a.i. (Fig. 2D).

Each strain favours distinct cytokine profiles - Stimulation of NI groups with Y and Col strain epimastigote antigens did not yield any significant cytokine responses, indicating that the antigen was not able to stimulate the production of IFN-γ or IL-4 by $CD4^+$ and $CD8^+$ T-lymphocytes in NI dogs. In cultures stimulated with the Y antigen, the production o IFN-γ by $CD4^+$ T lymphocytes was higher at 60 and 240 d.a.i. compared to 9 d.a.i. (Fig. 3A) and the production of IFN-γ by $CD8^+$ T lymphocytes was higher at 240 d.a.i. compared to the NI group at this same time point and to that at 9 and 60 d.a.i. (Fig. 3C). IL-4 production by $CD8^+$ T lymphocytes increased in cultures stimulated with the Y strain at 240 d.a.i. compared to 9 and 90 d.a.i. and in comparison with the NI group (Fig. 3D). In cultures stimulated with the Col antigen, IL-4 production was favoured by $CD8^+$ T lymphocytes in comparison with the NI group at 90 d.a.i. (Fig. 3H).

Cardiac inflammation and fibrosis - Infection promoted inflammatory cell migration to the right atrium (Fig. 4). During the chronic phase, a focal inflammatory infiltrate was observed, which consisted predominantly of mononuclear cells with lymphocyte morphology. The infection led to statistically significant increases in the number of cells in groups infected with either the Y or Col strain compared to the NI group. Moreover, the Y strain infection favoured collagen deposition and fibrosis formation was diffuse in the right atrium. This increase of areas occupied by fibrosis and the reduction of heart muscle were statistically significant in the Y group compared to the NI and Col groups (Fig. 4).

DISCUSSION

In Central and South America, different strains of *T. cruzi* with distinct DTUs are present, causing a variety of chronic forms of Chagas disease. Although various studies have tried to elucidate the relationship between *T. cruzi* strains and clinical alterations, there are no straightforward proven associations. This article exposes some aspects of acute and chronic Chagas disease with strains from different DTUs in a canine model to better understand the relationship between strains and clinical manifestations.

Parasite-host interactions during the initial events of *T. cruzi* infection reveal that the parasite is viable in the liver and that numerous diffuse cell infiltrates are present in the liver parenchyma, such as macrophages, $CD4^+$ and $CD8^+$ T-lymphocytes and natural killer cells. These cells combine to form focal inflammatory infiltrates in the liver parenchyma and perivascular spaces and are important for clearing parasites and for controlling parasitaemia, as previously observed in mice (Sardinha et al. 2010). We

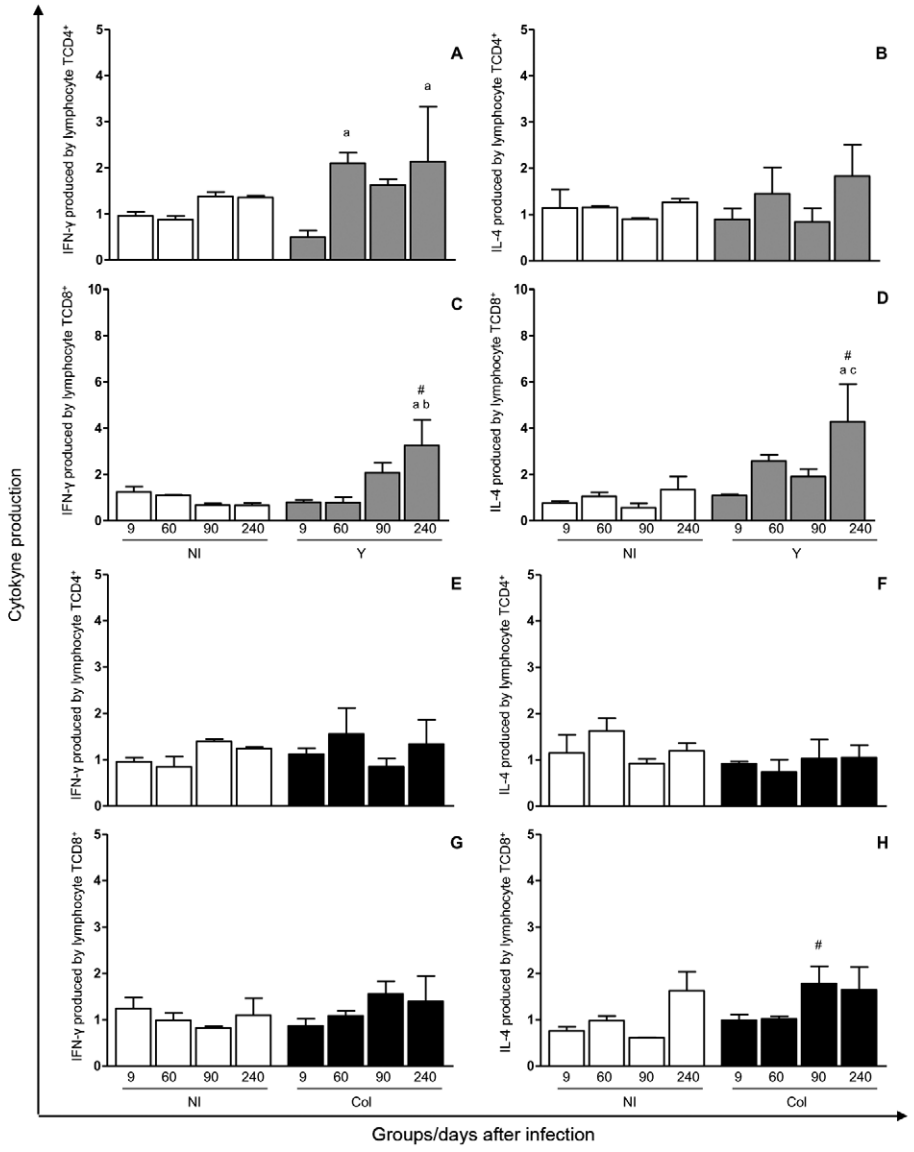

Fig. 3: immunophenotypic profile and cytokine patterns [interferon (INF)-γ and interleukin (IL)-4] of $CD4^+$ and $CD8^+$ T-lymphocyte present in peripheral blood before infection (0), nine, 60, 90 and 240 days after infection with Y strain (Y) or Colombian strain (Col) of *Trypanosoma cruzi*. T-cells subsets were identified by flow cytometric immunostaining as described in Materials and Methods. Data were expressed as the index, i.e., the mean percentage of positive cells of cultures stimulated/mean percentage of positive cells of unstimulated cultures, within gated lymphocytes. A-D: index of cytokine production by $CD4^+$ or $CD8^+$ in culture stimulated with epimastigote antigens of the Y strain; E-H: index of cytokine production by $CD4^+$ or $CD8^+$ in culture stimulated with epimastigote antigens of the Col strain. The results are expressed as index ± standard error. Significant differences at $p < 0.05$ are indicated by letters a, b and c for comparisons with days 9, 60 and 90, respectively. #: differences between the non-infected group (NI) and infected groups (Y or Col).

performed hepatic biochemical analyses and found that serum levels of ALT were significantly increased during the early stages of infection (at 9 d.a.i.) with the Y and Col strains, as observed in coatis (*Nasua nasua*) infected with *Trypanosoma evansi* (Herrera et al. 2002) and in dogs infected with *T. cruzi* (Barr et al. 1991).

Our results demonstrate that haematological parameters were minimally altered in dogs infected with the Y strain, whereas erythrocyte, haemoglobin and haematocrit numbers were increased during infection with the Col strain. This strain, which belongs to the TcI DTU, apparently has the capacity to alter haematological parameters in the chronic phase. In rats, *T. cruzi* infection resulted in increased plasma viscosity and haematocrit numbers, with morphological changes in red blood cells (Berra et al. 2005). Contradictory results were obtained by other authors during experimental canine infection with *T. cruzi*, as a decrease in erythrocyte frequency was observed in Beagle dogs infected with the Y, ABC or Be-78 strain (Guedes et al. 2012). However, no significant haematological changes were observed during out-bred canine infection with Be-62 and Be-78 strains (de Lana et al. 1992). Rhesus monkeys experimentally infected with metacyclic trypomastigotes of the Col strain did

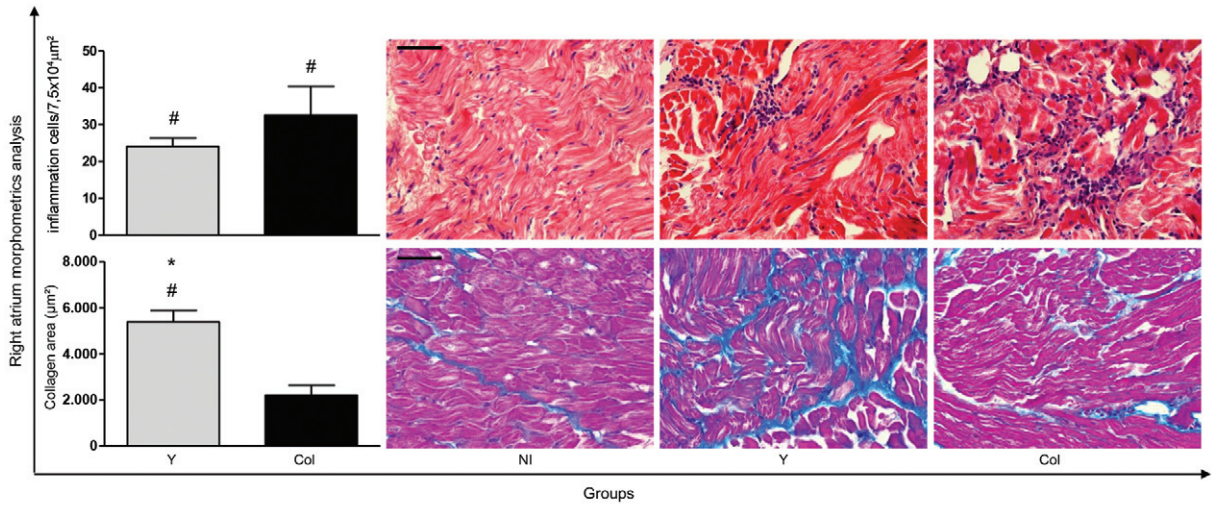

Fig. 4: morphometric analysis and photomicrographs of the heart showing the number of inflammation cells (haematoxylin and eosin) and fibrosis area (Masson trichrome) at 240 days after infection with Y strain (Y) or Colombian strain (Col) of *Trypanosoma cruzi*. The results are expressed as mean number of cells or collagen area ± standard error. Significant differences at $p < 0.05$ are indicated by the symbols # for comparison between the non-infected group (NI) and infected groups (Y or Col) and * for comparison differences between Y and Col groups at each time point. Bar = 50 µm.

not show any statistically significant difference in erythrocyte numbers or haemoglobin levels during the acute phase (Bonecini-Almeida et al. 1990). Such variability in experimental data suggests that both the experimental model and the *T. cruzi* strain may influence the impact of *T. cruzi* infection on haematological characteristics.

Haemoglobin levels were significantly increased at 240 d.a.i. in all groups. This may be because an increase in haemoglobin levels normally occurs during the developmental stages in young dogs (Comazzi et al. 2004). However, anaemia was reported in dogs infected with the Sylvio X10/4 *T. cruzi* strain (Quijano-Hernández et al. 2012) and in Beagle dogs infected with the *T. cruzi* Y strain (Guedes et al. 2012).

The parasitaemia curve for dogs inoculated with Y or Col *T. cruzi* strains presented different pre-patent and patent periods. During Y infection, the patent period was between 11-21 d.a.i., the parasitaemia peak occurred at 15 d.a.i. (data not shown) and hepatic alterations occurred at 9 and 30 d.a.i.. The haematological alterations occurred at different times, with a decrease in platelets, leucocytes and monocytes at 9 d.a.i., whereas the increase in erythrocytes, haemoglobin, haematocrit and platelets occurred at 60 d.a.i (later than the parasitaemia peak). The animals infected with the Col strain presented higher parasitaemia levels and the patent period occurred between 21-38 d.a.i., which was later than during Y infection. Furthermore, the parasitaemia peak occurred at 33 d.a.i. (data not shown). The increase in ALT occurred at 9 d.a.i., the significant increase in erythrocyte and haematocrit levels was detected at 30, 90 and 180 d.a.i. and the haemoglobin increase was detected at 30, 60, 90 and 180 d.a.i. In conclusion, there was no clear correlation between the occurrence of the parasitaemia peak and that of the hepatic and haematological alterations.

We observed thrombocytopoenia during Y strain infection. This reduction in platelet count is characteristic of the acute phase in humans (Shikanai-Yasuda et al. 1990), in mice infected with *T. cruzi* (Cardoso & Brener 1980, Marcondes et al. 2000) and in dogs experimentally infected with *T. evansi* (La Rue et al. 1997). In the present study, we found that dogs presented leucocytopoenia during the early stages of Y strain infection, as described in the murine model (Marcondes et al. 2000). By evaluating other white blood cell parameters, we observed that infected animals presented monocytopoenia, similar to naturally infected dogs and dogs seropositive for *T. cruzi* (Cruz-Chan et al. 2009).

The role of $CD4^+$ T-lymphocytes in controlling the parasite is not clear. The resistance to *T. cruzi* infection may be favoured by $CD4^+$ cells, which can increase the production of IFN-γ, a cytokine that upregulates nitric oxide in macrophages, thus controlling parasite replication and promoting the synthesis of immunoglobulin isotypes (Brener & Gazzinelli 1997). Our identification of peripheral blood mononuclear cells in ex vivo analyses showed a reduction in $CD4^+$ T lymphocytes and an increase in $CD8^+$ T lymphocytes during infection with the Y strain. These same features were noted in dogs experimentally infected with the Be-78 strain (Carneiro et al. 2007). Importantly, Y and Be-78 are TcII strains, indicating that this cellular response may be related to DTU classification. Moreover, we observed an inversion of the ratio of $CD4^+/CD8^+$ T lymphocytes during Y strain infection, where the $CD8^+$ T lymphocyte frequency was increased in the peripheral blood. This has been observed in studies of *T. cruzi* experimental infection, in which this ratio was considered an intrinsic immunological feature of infection (Carneiro et al. 2007).

The quantification of $CD14^+$ cells showed significant reductions at 9 d.a.i. and sequential increases at 30 d.a.i during Y strain infection. Similarly, the lower level of monocytes during the acute phase of *T. cruzi* infection was the most relevant phenotypic alteration during experimental canine infection (Carneiro et al. 2007). Again, this feature depends on the *T. cruzi* strain.

In *T. cruzi* infection, B-cell apoptosis (Zuñiga et al. 2000) or polyclonal activation (Minoprio et al. 1989, Montes et al. 2002) is required for the establishment of parasitic infection. Moreover, *T. cruzi* infection reduces the levels of $CD21^+$ lymphocytes (Carneiro et al. 2007), as was also described in the present study during infection with either the Y or Col strain.

Moreover, peripheral blood mononuclear cell cultures of patients with chronic Chagas disease in ex vivo analyses indicated cytokine profiles with high expression of IL-5, IL-10, IL-13 and IFN-γ compared to those without Chagas disease. However, when mononuclear cells of Chagas disease patients were incubated with *T. cruzi* antigens, IFN-γ expression was increased and IL-10 levels were reduced (Dutra et al. 1997). Another study comparing the indeterminate and cardiac forms observed high levels of IFN-γ and IL-10, respectively, but was unable to demonstrate a correlation between a particular cytokine expression profile and clinical manifestations (de Melo et al. 2012).

This contradictory pattern suggests that the role of IFN-γ in tissue lesion development is unclear. We postulate that the increased production of IFN-γ may have an impact on tissue inflammation, particularly in the case of infection with the Y strain, as observed with heart inflammatory infiltrates. However, this same strain favours IL-4 production, which can help control the tissue lesion. Similar observations were made during murine infection with Be-78 (TcII), which favours early production of IFN-γ and coincides with cardiac inflammation (Vieira et al. 2012). Petray et al. (1993) studied the influence of anti-IFN-γ and anti-IL-4 treatment on the course of experimental murine infection with two reticulotropic strains and the Col strain, a myotropic strain. They found that during infection with the Col strain, anti-IFN-γ and anti-IL-4 treatment did not influence the course of infection. In contrast, during infection with the reticulotropic strains, anti-IFN-γ treatment increased host susceptibility to the parasite and anti-IL-4 treatment increased resistance. We speculate that this TcI strain was silent in that it did not activate cytokine expression by T lymphocytes during the acute phase.

Histopathological analyses of the myocardium of NI animals or animals infected with the Y or Col strain indicated a correlation between the *T. cruzi* strain and the intensity of inflammation and fibrosis. Therefore, infection with both strains caused significant increases in inflammation, similar to that observed with canine experimental *T. cruzi* chronic infection (Guedes et al. 2009, Diniz et al. 2010). In addition, fibrosis was increased in dogs infected with the Y strain compared to NI animals and animals infected with the Col strain. The fibrosis area was characterised as intrafascicular collagen deposition (Guedes et al. 2009, Diniz et al. 2010), which caused disorganisation and isolation of the cardiomyocytes and likely contributed to the electrocardiographic alterations observed in canine models and in human CCC (Caliari et al. 2002). These anatomohistopathologic features of canine experimental infection by *T. cruzi* were described by de Lana et al. (1992), who thus considered the dog to be a suitable model for studying the acute and chronic phases of Chagas disease.

In summary, the Y strain triggers a more drastic immune response during the acute phase of infection in dogs in comparison with the Col strain. In the chronic phase, inflammation in the heart was balanced by tissue rearrangement and fibrosis, whereas Col infection at the chronic phase showed characteristics of inflammation. Sales-Campos et al. (2014) noted that in mixed infection with TcI and TcII, parasitism in the acute phase was similar in composition to that of the inoculum, whereas in the chronic phase, TcI was prevalent.

We hypothesise that the Col strain (TcI) can escape the host's acute immune response, remain unnoticed by peripheral blood mononuclear cells and hence parasitise target organs faster. For the Y strain (TcII), the specific immune response begins at the acute phase. We think that this helps in controlling the myocardial lesion in the early chronic phase. We conclude that different DTU strains interact differently with the host, which is possibly related to variable expression of cell surface molecules depending on the DTU. More studies are necessary to further elucidate the parasite-host interaction.

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TABLE I

Number of erythrocytes and different subtypes of cells in peripheral blood of uninfected (NI) dogs or infected with 2×10^3 blood trypomastigotes/kg of the Y strain or the Colombian (Col) strain

Groups	Day	Erythrocytes (10^{12}/L)	Haemoglobin (g/dL)	Haematocrit (%)	Platelets (10^9/L)
Normal values for dogs		5.08-6.58	12.20-15.55	32.10-39.59	129.50-379.49
NI	0	5.94 ± 0.14	14.03 ± 0.60	35.8 ± 1.20	262.7 ± 40.82
	9	6.08 ± 0.47	15.27 ± 2.06	38.7 ± 3.76	191.7 ± 125.0
	30	6.51 ± 0.43	16.53 ± 1.60	40.8 ± 4.74	219.7 ± 76.25
	60	6.64 ± 0.13	16.63 ± 0.86	38.6 ± 0.62	206.3 ± 38.89
	90	6.78 ± 0.22	16.63 ± 0.30	41.8 ± 1.05	249 ± 48.77
	240	6.91 ± 0.07	17.53 ± 0.11[a]	42.5 ± 1.56	195.3 ± 11.85
Y	0	6.13 ± 0.38	14.63 ± 0.63	37.7 ± 1.90	290.3 ± 96.42
	9	5.91 ± 0.56	14.17 ± 1.01	35.97 ± 2.13	90.3 ± 54.72[a]
	30	6.27 ± 0.69	15.37 ± 1.98	38.83 ± 4.89	217.3 ± 33.65
	60	7.06 ± 0.99	16.83 ± 2.96	43.93 ± 7.53	249.3 ± 58.97
	90	6.44 ± 0.24	15.37 ± 0.47	37.37 ± 1.44	233.0 ± 60.01
	240	7.16 ± 0.40	17.63 ± 0.25[a]	43.27 ± 1.82	277.3 ± 76.23
Col	0	5.66 ± 0.35	13.20 ± 0.62	34.5 ± 1.13	221.5 ± 40.07
	9	5.69 ± 0.41	13.55 ± 0.47	35.55 ± 1.43	245.3 ± 10.37
	30	6.76 ± 0.54[a]	16.68 ± 1.28[a]	41.65 ± 3.15[a]	147.5 ± 52.55
	60	6.53 ± 0.45	16.33 ± 0.37[a]	38.08 ± 2.83	155.8 ± 35.13
	90	7.29 ± 0.62[a]	17.4 ± 1.54[a]	43.55 ± 4.96[a]	174.3 ± 56.23
	240	7.42 ± 1.0[a]	18.18 ± 2.76[a]	43.95 ± 6.43[a]	146.8 ± 25.58

a: significant differences at $p < 0.05$ are indicated for comparisons with day 0. Normal values for dogs indicate the standard parameter [the value of all animals at T0 (mean ± 2 standard deviations)].

TABLE II

Number of leukocytes and different cell subtypes in peripheral blood of uninfected (NI) dogs or dogs infected with 2×10^3 blood trypomastigotes/kg of the Y strain or the Colombian (Col) strain

Groups	Day	Leukocyte (mm^3)	Lymphocyte (mm^3)	Neutrophil (mm^3)	Moncyte (mm^3)
Normal values for dogs		8100.3-20,499.6	2231.6-8778.7	99.4-15,056.3	290.7-913.0
NI	0	11,600 ± 2425	5413 ± 1711	5180 ± 918	496.7 ± 56.86
	9	9633 ± 1762	4202 ± 1548	5178 ± 647	301.7 ± 178.2
	30	11,767 ± 3650	5216 ± 2420	5792 ± 1349	489.7 ± 230.9
	60	13,000 ± 1652	5313 ± 1568	7021 ± 522	211.5 ± 113.8
	90	13,267 ± 1380	4410 ± 1599	7705 ± 359	529 ± 140.8
	240	11,467 ± 2325	3893 ± 774	6589 ± 1451	567.3 ± 216.3
Y	0	14,800 ± 888	5116 ± 1941	7932 ± 2308	790.3 ± 108.8
	9	6300 ± 916[a]	2224 ± 973	3647 ± 15.2	231.7 ± 55.43[a]
	30	14,700 ± 3158	6652 ± 2354	6519 ± 714	735 ± 157.9
	60	13,033 ± 305	5344 ± 1181	6911 ± 1171	475 ± 189.3
	90	13,533 ± 2470	4550 ± 1404	8119 ± 1375	465 ± 282.5
	240	11,167 ± 2281	3802 ± 713	6811 ± 1390	359.7 ± 238.3[a]
Col	0	15,950 ± 3635	5891 ± 1819	9132 ± 5352	541.5 ± 104.3
	9	12,975 ± 1072	4895 ± 1012	7110 ± 1532	487.5 ± 181.1
	30	11,650 ± 4085	5567 ± 2284	5231 ± 1922	545.3 ± 139.7
	60	13,550 ± 4047	5970 ± 927	6990 ± 3654	306.5 ± 115.2
	90	12,350 ± 1702	3937 ± 1208	7730 ± 2876	232.8 ± 141.1[a]
	240	11,875 ± 3595	3213 ± 869	7824 ± 2853	429.8 ± 163.5

a: significant differences at $p < 0.05$ are indicated for comparisons with day 0. Normal values for dogs indicate the standard parameter [the value of all animals at T0 (mean ± 2 standard deviations)].