***Trypanosoma cruzi* strains cause different myocarditis patterns in infected mice**

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Key words: Myocarditis, T. cruzi, Chagas disease.

**ABSTRACT**

**Aims**. Chagas disease pathology is dependent on the infecting *T. cruzi* strain. However, the relationship between the extent and type of myocarditis caused by different *T. cruzi* strains in the acute and chronic phases of infection has not been studied in detail. To address this, we infected mice with three genetically distant *T. cruzi* strains as well as infected *in vitro* different cell types. **Methods and Results.** Parasitemia was detected in mice infected with the Y and VFRA strains, but not with the Sc43 strain; however, only the Y strain was lethal. When infected with VFRA, mice showed higher inflammation and parasitism in the heart than with Sc43 strain. Y and VFRA caused homogeneous pancarditis with inflammatory infiltrates along the epicardium, whereas Sc43 caused inflammation preferentially in the auricles in association with intracellular parasite localization. We observed intramyocardic perivasculitis in mice infected with the VFRA and Y strains, but not with Sc43, during the acute phase, which suggests that endothelial cells may be involved in heart colonization by these more virulent strains. In *in vitro* infection assays, the Y strain had the highest parasite-cell ratio in epithelial, macrophage and endothelial cell lines, but Y and VFRA strains were higher than Sc43 in cardiomyocytes. **Conclusions.** This study supports parasite variability as a cause for the diverse cardiac outcomes observed in Chagas disease, and suggests that endothelial cells could be involved in heart infection during the acute phase.

## INTRODUCTION

Chagas disease is a neglected tropical disease caused by the intracellular protozoan *Trypanosoma cruzi,* that affects approximately 8 million people in Latin America (Dias, 2006). In humans, infection is characterized by 2 phases: an acute phase, which is generally asymptomatic, and a chronic phase, asymptomatic (indeterminate) or symptomatic (determinate). During the chronic phase, digestive and/or cardiac syndromes may occur and potentially lead to death.

Chagasic cardiomyopathy is the most common cause of disability in infected patients, showing a variable clinical outcome. Myocardial inflammation associated to mononuclear infiltrate is a common finding during the acute phase, although the spatial association between parasites and inflammatory infiltrate is controversial. In this sense, one key point of Chagasic myocarditis that remains to be elucidated is the gateway of parasitical infection in the heart during the acute phase and their involvement in the pathophysiology. The parasite-endothelial cell interactions are among the first to occur during acute *T. cruzi* infection and in recent years the nature of these interactions as well as their consequences have received increased interest. Some authors have proposed that endothelial cells and/or macrophages allow parasite entry into different organs (Epting et al., 2010).

*T. cruzi* genetic variability is increasingly recognized and this parasite has been genetically classified into six discrete typing units (DTUs) – TcI to TcVI (Zingales et al.,

2009), – which have different geographic distributions and may be associated with geographically-restricted clinical profiles (Tibayrenc and Telleria, 2010). Several data indicate that human genetic determinants may impact the clinical outcome of Chagas disease (Florez et al., 2012; Vasconcelos et al., 2012). However, a role for genetic variability among the different parasite strains in the diverse clinical outcomes of Chagas disease cannot be excluded. In this sense, it is possible that differential tropism of *T. cruzi* for the cell types involved in the natural infection could explain the differences observed in virulence in Chagasic patients and in experimental models. It has been reported that parasite strains belonging to 2 different DTUs (TcI and II) have a differential tropism for the heart (Andrade et al., 1999), which correlates with their ability to replicate within cardiomyocytes *in vitro* (Andrade et al., 2010). Nevertheless, the relationship between specific cellular tropism and cardiomyopathy is still an unresolved issue. The present work explores the *in vivo* course of infection with the genetically different *T. cruzi* strains belonging to 3 different DTUs particularly with respect to heart alterations and *in vitro* differences in invasion and replication in epithelial, macrophage, cardiomyocyte and endothelial cell lines.

**METHODS**

*2.1 Cell culture and in vitro infection*

Vero cells were maintained in 25-cm$^2$ Nunc® flask in Dulbecco's modified Minimal Essential Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a controlled atmosphere (5% $CO_2$, 37ºC). The cells were seeded on a coverslip in a 24-well

culture plate at 1,700 cells *per* well twenty-four hours prior to infection. The J774.2 murine macrophage cell line was maintained and seeded under the same conditions at 1 x $10^5$ cells *per* well. The human endothelial-like cell line EAhy926 (Edgell et al., 1983), was a kind gift of Dr. Santiago Lamas, Centro de Biología Molecular, CSIC-UAM. (Madrid, Spain). These cells were maintained in DMEM supplemented with 20% (v/v) FBS and 10% HAT (hypoxanthine-aminoptherine-thymidine) and seeded at 5 x $10^4$ cells *per* well 24h prior to infection. H9c2 cell line, derived from embryonic BD1X rat cardiac tissue (Kimes and Brandt, 1976), was a gift of Dr. Miguel Ángel Íñiguez. Centro de Biología Molecular, CSIC-UAM (Madrid, Spain). H9c2 cells were maintained in DMEM supplemented with 10% v/v of FBS and seeded at 5 x $10^4$ cells *per* well 24h prior to infection.

The Sc43 (TcV) and VFRA (TcVI) strains of *T. cruzi* were obtained from *Triatoma infestan*s specimens in Santa Cruz, Bolivia and Chile, respectively, and were compared with the well-characterized Brazilian Y (TcII) strain, obtained from Dr. John David (Department of medicine, Harvard Medical School, Boston, MA, USA) and previously cloned as was described (Tanuri et al., 1985). Sc43 and VFRA isolates were cloned as described previously (Yeo et al., 2007). Briefly, parasites were mixed with 2.4 ml of 0.9% NaCl (w/v) and 0.6 ml of molten 3% (w/v) low melting point (LMP) agar. The mixture was then poured onto solid blood agar plates, allowed to set and incubated at 28°C for 3–6 weeks. Once colonies were visible they were picked using a sterile pipette tip and inoculated into liquid culture. Colonies were genotyped as was previously described (Lewis et al., 2009). Trypomastigotes were produced by successive passage in Vero cells, and recollected at fifth day post-infection and counted in a Neubauer hemocytometer. The infection was carried out at a parasite-cell ratio of 10:1, in DMEM supplemented with 1%

of FBS in all cases and the infected cells were washed twice 24h post-infection to remove extracellular parasites. Before the experiments, the DTU of the stock used was determined in order to confirm the absence of mislabeling or contamination.

## 2.2 Mouse infection

Young adult (6-8-wk) BALB/c mice were purchased from Charles River Laboratories. Blood trypomastigotes were routinely maintained by infecting IFN-γ receptor deficient mice (129-*Ifngr1$^{tm1Agt}$/J*, The Jackson Laboratory) and isolating them from blood by centrifugation.  Mice were infected intraperitoneally with 5,000 blood-derived trypomastigotes of the Sc43, VFRA and with 2,000 of the Y strain due to their higher virulence. Parasitemia was monitored by the Brener method as described elsewhere (Brener, 1962). Infected mice were sacrificed at 21 and 100 days post-infection (d.p.i). Hearts were washed with heparin 1:100 v/v in PBS, and fixed for 24 h in 4% (v/v) paraformaldehyde (PFA) in PBS, at 4 ºC. The fixed hearts were cut sagittal and processed for paraffin embedding and hematoxylin-eosin or Mason's trichromic staining (Histology Service, Centro Nacional de Biotecnología, Madrid, Spain).

For histological analysis, the micrographs were taken from sections of 8 (Sc43 and VFRA) and 3 (Y) different hearts for acute phase and 6 (Sc43) and 7 (VFRA) for chronic phase (Y strain was not included for this phase) in three independent experiments for each phase. The number of Y strain samples was influenced by the fact of the high mortality observed, which limited the number of available samples. The observation was done with a Leica DMD108 microscope. For a panoramically view of inflammation, one 4x image per heart was taken and for microvasculature morphology analysis five 63x image per heart

were taken at random. Inflammation was classified by a semi-quantitative score, as follows: 0 for the absence of inflammation, 1 for low-level inflammation, 2 for moderate inflammation and 3 for strong inflammation. Quantification was performed in separately in auricles and ventricles.

For the quatification of amastigote nests, sections of three different hearts *per* strain were observed. The whole cardiac section with a 40x objective was monitored and quantification was divided among auricles and ventricles.

2.3 *Ethics statement*

This study was conducted in strict accordance with the recommendations of Spanish legislation and the European Council directive from the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All mice were maintained under pathogen-free conditions in the animal facility at the Centro de Biología Molecular, Universidad Autónoma de Madrid (Madrid, Spain). The animal protocol was approved by the Ethic committee of the Universidad Autónoma de Madrid. Animals had free access to food and water and were handled in compliance with European standards. Mice were euthanized in a $CO_2$ chamber, using a gradually fill of the chamber with $CO_2$, and all efforts were made to minimize suffering.

*2.4 Confocal microscopy*

Ninety-six hours post-infection, the infected cell cultures, placed on coverslips without Poly-L-lysine, were washed three times with PBS and fixed for 10 minutes in (w/v) PFA 4% in PBS pH 7.4. Next, the cells were permeabilized for three minutes in a solution of 0.5% v/v Triton X-100 in PBS. Then, actin fibers were stained with 1µg/ml phalloidin-

TRITC (Sigma-Aldrich) for 20 min, cells were washed twice with PBS. Nuclei were then stained with 1µg/ml DAPI (Merck) for 10 min, washed again once and coverslip were mounted with ProLong Gold® (Invitrogen) and stored at 4ºC until visualization.

The preparations were observed on an AxioObserver LSM 710 confocal microscope, (Zeiss®) with LCI-Plan Neofluar 25x/0.8 Imm Korr DIC M27 and with a Plan-Apochromat 63x/1.40 Oil DIC M27 objectives. Acquisition conditions were determined for each preparation. For parasite-cell quantitation, three images *per* coverslip were taken for three distinct preparations. The experiment was repeated three times for each condition. Images were processed using Fiji software and 63x images were deconvolved using Huygens Professional® software.

### 2.5 *Parasite DNA detection and mRNA analysis by quantitative RT-PCR.*

After blood removal by perfusion, parasite DNA was purified from cardiac tissue with the High Pure PCR Template preparation Kit (Roche), and PCR reactions were performed with 100ng of DNA as described (Calderon et al., 2012; Cuervo et al., 2011). For *T. cruzi* detection, the method described by Peron and collaborators was followed (Piron et al., 2007). Spiking of organs from uninfected animals with known quantities of parasites was used to create bi-dimensional matrices (parasites/mg tissue; total DNA concentration) for two-step regression (unpublished data).

Cardiac RNA was purified with TRIzol reagent (Invitrogen). Quantitative RT-PCR analysis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems) and different genes (*Ifng, Tnf, il4, il10, il13, il12, ptgs2* and *18S rRNA*) were amplified in triplicate using TaqMan MGB probes and the TaqMan Universal PCR Master Mix

(Applied Biosystems) on an ABI PRISM 7900 HT instrument (Applied Biosystems) as previously described (Cuervo et al., 2008). Parasite DNA and gene expression by real-time PCR was quantified by the comparative threshold cycle ($C_T$) method ($RQ= 2^{-\Delta\Delta C_T}$), using normalization by the 18S ribosomal endogenous control levels and reported to uninfected control animals. Graphs were plotted as log RQ when indicated,

## 2.6  Statistical analysis

Data are presented as means ± SEM. Student's t-test for independent samples was used to test for differences between sample means. All analyses were performed using Prism5® (GraphPad Software, Inc., La Jolla, CA, USA).

## RESULTS

We infected BALB/c mice with 3 genetically different *T. cruzi* strains (Sc43, VFRA and Y), belonging to TcV, TcVI and TcII respectively, and monitored the course of infection (Figure 1). Infection by Y strain was lethal during the acute phase; at 30 days post infection (d.p.i.), 100% of the infected mice were dead (Figure 1A). In contrast, the Sc43 and VFRA strains did not cause mortality. Despite this, VFRA produced higher levels of parasitemia than the Y strain with no significant differences in kinetics of parasitemia (Figure 1B). After 45 days of infection, we did not detect circulating parasites in any of the infected mice, and parasites were not detectable in blood at any time point in mice infected with the Sc43 strain. However, parasites were detected at day 21 p.i. in the hearts of mice infected with the Sc43 strain (937 parasites/mg tissue) at a level comparable to that obtained with the VFRA strain (1,840 parasites/mg tissue), albeit at lower levels than the Y

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strain (6,534 parasites/mg tissue, Figure 1C). At 100 d.p.i., parasite DNA was only detected in mice infected with VFRA strain (21 parasites/mg tissue).

Next, we analyzed the pattern of myocardial inflammation during acute infection (Figure 2). Interestingly, the pattern was quite different among the 3 strains. The differences were mainly related with the extension and location of the inflammatory infiltrate a throughout the heart. Focal inflammation of the ventricles was detected in Sc43-infected hearts (Figure 2A, upper, left panel), in contrast with the intense and diffuse inflammatory reaction observed at atria (upper, right panel). For VFRA and Y infected mice, the leukocyte infiltrate was diffuse and more intense at the ventricular epicardium and sub-epicardium. Figure 2B shows a scoring of myocarditis divided by auricles and ventricles, obtained by microscopic observation. Y strain caused the strongest inflammation and Sc43 the lowest one.

In addition, the number of amastigote nests correlated with the intensity of inflammation for the different strains in the different locations (Figure 2C). Thus, in VFRA- and Y-infected mice, we observed more amastigote nests in ventricles than in auricles. As expected, the number of amastigote nests correlated with parasite load observed by quantitative PCR (see Figure 1C).

By analyzing the lesions in the heart in greater detail, we observed perivascular inflammation in myocardial vessels, particularly in mice infected with the VFRA and Y strains (Figure 3). The infiltrate, with a predominance of mononuclear cells, was observed in close apposition to the vessel's adventitia, with slightly rounded endothelial cells and an endothelium occasionally infiltrated with mononuclear cells (Figure 3, white arrows). We

observed a tendency of parasite nests to be located in close apposition to the vessels in mice infected with the Y strain, as illustrated in the central panel in the Y strain's row (black arrows). For Sc43, perivascular infiltrate or intravascular changes were also observed, though to a lesser extent.

During the chronic phase, we observed a moderate inflammatory infiltrate persisted in the hearts of mice infected with VFRA with predominance of mononuclear cells (Figure 4). We still observed perivascular infiltrates (left and center panels) and pericardial fibrosis (right panel). In contrast, no significant pathological alterations were observed in Sc43-infected hearts, which suggests resolution of the inflammation (bottom panels). Though we did not find amastigote nests in mice chronically infected with the VFRA and Sc43strains (data not shown), the higher sensitivity of quantitative, real-time PCR permitted the detection of parasites in mice infected with VFRA but not with Sc43 (Figure 1C). Analysis of the chronic phase with the Y strain could not be performed since BALB/c mice infected with the Y strain died before reaching the chronic phase.

In order to gain further insight into the host response resulting from parasite infection we analyzed the inflammatory cytokine expression in hearts at 14 d.p.i., before full damage is induced (inflammation was also evaluated at this point by histological sections with similar trends that the one shown at 21 d.p.i., data not shown). As shown in Figure 5, infection with the Y strain induced the highest expression of inflammatory cytokines (*Ifng*, *Il6* and *Tnf* mRNA) in the heart, whereas the VFRA and Sc43 strains induced lower expression, with VFRA higher than Sc43. Cytokine expression in the acute phase correlates well with the histological evaluation of the inflammation shown in Figure 2B. In the chronic phase (100 d.p.i.), *Il6* mRNA levels significantly decreased regarding to acute

phase and *Ifng* levels decreased in chronically infected animals, but still showed values significantly higher than control. Interestingly, *Tnf* decreased during the chronic phase in mice infected with the VFRA strain (but with values still higher than control animals), while in Sc43 infected mice the values were similar during acute and chronic phase

The above results pointed to clear differences in the cardiac pathophysiology between the strains. A possible explanation could be a differential infective capacity of parasites, in quantitative (virulence) or qualitative (tropism) terms. We thus tested the ability of the studied strains to infect various cell lines derived from different tissues: Vero (epithelial), J774 (macrophage), EAhy926 (endothelial) and H9c2 (cardiomyocyte) cells (Figure 6, Panel A). All parasite strains infected all cell types. Nonetheless, some striking differences appeared. Y strain had the highest amastigote/cell ratio (Panel B) in all cell types, only equaled by VFRA for H9c2 cells. Sc43 showed a higher parasite-cell ratio than VFRA only in Vero cells. VFRA had only a slightly increased parasite-cell ratio in J774 with respect to Sc43 and the same parasite-cell ratio than Y strain in H9c2 cardiomyocytes. EAhy926 cells showed the highest percentage of infection and parasite-cell ratio (Panel C), particularly with the Y Strain (90%) at 96 h post-infection and Vero cells showed the lowest rate for all strains.

The effect on EAhy926 endothelial cells was analyzed in more details. In Figure 7, we present high magnification micrographs of EAhy926 cell infection at 24, 48 and 96 h pi. At 24 h pi, there were no differences in intracellular amastigote number among *T. cruzi* strains, which suggests a similar rate of infection during the early invasion and replication period (non infecting parasites were removed to this point). At 96 h panel demonstrates the cells infected with the Y strain harbored a mixture of intracellular parasites with

amastigote-like (rounded forms) and trypomastigote-like (thin forms) morphologies, unlike the Sc43 and VFRA strains, which exhibited uniform amastigote morphology. This observation suggests an enhanced differentiation from amastigote to trypomastigote for the Y strain, which may be responsible for the observed differences in amastigote numbers show in Figure 6.

## DISCUSION

There is a controversy regarding the role of the genetic variability of *T. cruzi* strains in the clinical evolution and outcome of the disease. In order to clarify this controversial point, we chose to study this question in animal models with cloned parasite strains belonging to 3 different DTUs genetically distant (TcII, TcV and TcVI).

There is little information about the causes involved in the different myocarditis patterns and their evolution observed during the acute and chronic phase. Recent works have linked vasculopathy associated to *T. cruzi* invasion the cardiac inflammation, suggesting that proinflammatory phenotypes of T. cruzi strains may be ascribed, at least in part, to variable expression of TLR2 ligands and cruzipain isoforms during the acute myocarditis (Scharfstein and Andrade, 2011). However, a detailed understanding of the contribution of host and parasite genetics in this process is lacking. To our knowledge, this is the first report of specific cardiac immunopathological patterns associated to genetically distant strains.

A detailed analysis of inflammatory infiltrates in the hearts of infected animals revealed different patterns: Inflammatory infiltrates were more concentrated in the atrial region in Sc43-infected mice during the acute phase, and this finding was related to the

distribution of amastigote nests, although the differences between auricles and ventricles were non-significant, probably due to limitations in the inflammation scoring method caused by the restricted area available (atrial appendage) for amastigote nest and inflammatory infiltrate  scoring. This tendency may be related to differences in virulence observed among the strains, with the most virulent strains (Y and VFRA) causing pancarditis whereas low virulent strains (Sc43) causing only focal/regional inflammation. The Y and VFRA strains induced higher levels of epicardial and sub-epicardial inflammation that was homogenous in the auricles and ventricles. This pattern is consistent with different observations reporting pericardial effusion in acute Chagas disease (Bastos et al., 2010) and may be associated with the involvement of the intra-myocardial vasculature in the establishment of acute myocarditis. Moreover, only VFRA was able to cause cardiac alterations in the chronic phase in mice (Fig 4), a fact that could be related with the inflammation pattern observed during the acute phase and may suggest that progression to chronic phase is also strain-dependent. Therefore, in mice, genetically distinct *T. cruzi* strains show considerable variations in cardiac pathology caused, which parallels the diverse outcome in human Chagas disease.

A recent work reports myocarditis induced in mouse model by a single strain representative of the TcI, TcII, TcV and TcVI DTUs, concluding that a TcVI strain causes moderate myocarditis, whereas a TcV strain that did not cause cardiac inflammation exhibited almost undetectable parasite load (Ragone et al., 2012). This observation is in sharp contrast with the data in humans, where patients infected with TcV strains have been shown to suffer cardiac pathology (Burgos et al., 2010). More recently, some authors have associated mixed infection with different DTUs with severity of inflammation and tissular

tropism (Sales-Campos et al., 2014; Silvina et al., 2014). For our study, we have selected strains with very different biological behavior both *in vitro* and *in vivo*. Indeed, the Y strain causes parasitemia and acute mortality, VFRA causes only parasitemia and Sc43 causes neither parasitemia nor mortality. The Y strain exhibited a higher parasite load in the heart, likely reflecting higher cardiotropism than the VFRA and Sc43 strains, Sc43 being the less infective for the heart. Also, the quantitative differences in parasite load in the heart may not be solely responsible for the highly different cardiomiopathy patterns and suggests that other intrinsic properties of the strains, such as the ability to cause parasitemia (Fig 1) and being available for infecting target cells, are mainly responsible for these differences. Indeed, the differences in the replication of Sc43 and VFRA between auricles and ventricles may explain the differences in the myocarditis patterns observed.

Findings during chronic phase (absence of parasite or cardiac inflammation) would suggest the possibility of spontaneous cure in mice infected with Sc43. There is a previous controversy in the literature about this fact, with suggestions of reactivation of infection in apparently cured mice during the chronic phase (Magalhaes and Andrade, 1994) and another ones that claims cure in elder untreated patients with records of parasitemia during the acute phase (Bertocchi et al., 2013; Francolino et al., 2003). Further studies are necessary in order to evaluate the reactivation due to extra-cardiac parasite homing.

In the case of chronically VFRA infected mice, we can appreciate the presence of inflammatory infiltrate apparently in absence of amastigote nest. Recent articles has highlighted the possibility that  genetic alterations resulting from kDNA integration in the host genome lead to autoimmune-mediated destruction of heart tissue in the absence of T. cruzi parasites (Teixeira et al., 2011). Additionally, parasite antigens were able to induce

autoimmunity and cardiac damage in mice inoculated with heat inactivated parasites (Bonney et al., 2011). Comparing the two strains that can cause chronicity in our infection model (Sc43 vs VFRA), our results suggest that the presence of at least parasite DNA is necessary for causing chronic cardiac disease. Vascular inflammation has been reported in experimental models of Chagas disease (Sunnemark et al., 1998) and acute outbreaks in humans (Alarcon de Noya et al., 2010) and may be associated with different Chagas disease outcomes in patients (Garcia-Alvarez et al., 2011). The observed intramyocardic perivasculitis in mice infected with the VFRA and Y strains, but not Sc43 suggest that endothelial cells may be involved in heart colonization by the most virulent strains. This hypothesis is also in accordance with *in vitro* higher infection in endothelial cell lines by Y strain, as compared to Sc43 and VFRA. Nonetheless, we failed to find intracellular parasites in endothelial cells of cardiac vessels in animals infected with any of the strains, possibly because this invasion process occurs earlier than day 21.Further studies are necessary to solve this question.

It is well known that *T. cruzi* can invade a large variety of cells both *in vitro* and *in vivo* (de Souza et al., 2010) and the tissue homing ability of *T. cruzi* has been reported to be strain-specific (Andrade et al.; Tibayrenc and Telleria). However, there is little information regarding the relationship between strains tropism and pathology. Interestingly, our results show that the 3 distinct *T. cruzi* strains have important differences in their abilities to replicate in cells of epithelial (Vero), monocyte (J774), endothelial (EAhy926) and cardiac (H9c2) origin, with Y strain having the highest overall parasite to cell ratio and infection rates in all cell lines studied.

The affinity of *T. cruzi* for endothelial cells has been associated with endothelin and bradykinin receptors (Andrade et al., 2012). Additionally, gp85 – a *T. cruzi* glycoprotein involved in interaction with endothelial cells (Tonelli et al., 2010) – has a greater affinity for cardiac-derived endothelial cells with respect to visceral-derived endothelial cells (Tonelli et al., 2010). It is possible that diverse expression of surface glycoproteins in genetically distant strains may be involved in the differential tropism observed. On the other hand, our results shown in Figure 7 suggest that the differences observed in intracellular parasite-cell ratio in endothelial cells between Sc43, VFRA and Y strains are related to the amastigote differentiation rate to trypomastigote. Experiments to confirm this model with our strains are underway.

In conclusion, our results show that the myocarditis pattern observed in experimental acute infection by *T. cruzi* depends on the strain. Importantly, the myocarditis pattern depends on differential cardiac parasite distribution and the higher parasite invasion rate and replication in endothelial cell *in vitro* would suggest that the interaction between the parasite and the endothelium may play a key role in heart colonization during acute Chagas disease. Moreover, our results indicate that the strains have differing capacities for replication in several cells types, with the more pathogenic *T. cruzi* strains general replicating better in all cell types. This observation may explain the differential pathogenicities of the strains. Our results in mice reflect the great variability in clinical outcomes and syndromes observed in chagasic patients, and offer the possibility of studying the parasite factors involved in different outcomes, including their interactions with different cell types.

**Conflicts of interested:** None declared

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**FIGURE LEGENDS**

**Figure 1. Different outcomes of *in vivo* infection by different *T cruzi* strains.** BALB/c mice were infected with 5,000 trypomastigotes of the *T. cruzi* Sc43 (open box) and VFRA (filled triangle) strains and 2,000 of the Y (open circle) strain. A) Survival. B) Parasitemia. Means +/- SEM  from three independent experiment are shown (n=9) C) Quantification of *T. cruzi* DNA in the heart tissue of infected BALB/c mice. Panel C shows results of real-time PCR with heart samples taken at 21 (acute) and 100 (chronic) d.p.i.. Results are

expressed as the mean values (±SD) for triplicates of pooled DNA from 5 different mice. (*) Statistically significant differences (p< 0.05).

**Figure 2. Heart inflammation by different *T. cruzi* strains in the acute phase.** BALB/c mice were infected with 5,000 trypomastigotes of the Sc43 and VFRA strains and 2,000 of the Y strain and the hearts of the infected mice were analyzed at 21 d.p.i. A) High magnification images of infected and healthy heart (last panel) with representative inflammation for each strain. B) Estimation of inflammation in auricles, ventricles by an arbitrary index: 0 corresponds to absence of inflammation, 1 to low inflammation, 2 to moderate inflammation and 3 to severe inflammation. C) Quantification of the number of amastigote nests divided into auricles and ventricles (three different hearts per infecting strain). Results for inflammation score are expressed as the mean values (±SEM) for independent microscopic fields from 8 different mice for the Sc43 and VFRA strains and 3 different mice for the Y strain (20 fields for each) performed in three independent experiments. * p value <0,05>0,01 ; ** p value <0.01. A capped line shows statistical differences between auricle/ventricle, and a zigzag line shows statistical differences between strains. Scale bars 100 μm

**Figure 3. Perivascular inflammation in intracardiac vessels caused by different *T cruzi* strains during the acute phase.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains and 2,000 of the Y strain, and the hearts of infected mice were analyzed at 21 d.p.i. The upper, middle and bottom rows show perivascular inflammatory infiltrates in the intramyocardial vessels of mice infected with the Sc43, VFRA and Y strains, respectively. White arrows indicate cellular infiltration in endothelial walls and black arrows show amastigote nest. Shown are three representative hearts of 8

(Sc43 and VFRA) and 3 (Y) performed in three independent experiments. Scale bars 100 µm.

**Figure 4. Cardiac inflammation by different *T. cruzi* strains during the chronic phase.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains, and the hearts of infected mice were analyzed at 100 d.p.i. The upper panel shows cardiac infiltration with macrophage-like cells, muscle fibers, disorganization and pericardial fibrosis in VFRA-infected mice. Sc43-infected mice (bottom panel) did not exhibit significant pathological changes. Shown are three representative hearts of 7 (VFRA) and 6 (Sc43) performed in two different experiments. VFRA section are stained with Masson staining and Sc43 with conventional H&E. Scale bars 50 µm.

**Figure 5. Cytokine production in infected hearts by different *T. cruzi* strains during the acute and chronic phases.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains and 2,000 of the Y strain. Fold expression of IFN-γ, IL-6 and TNF-α mRNA in the hearts of infected mice during the acute phase (14 d.p.i.) and chronic phase (100 d.p.i.) over uninfected control mice of the same age. RNA from cardiac tissue at different d.p.i. was used to perform RT-PCR with specific probes and normalized to ribosomal 18S RNA as described in "Materials and Methods". Values are expressed as means ± standard errors (s.e.) from 3 separate experiments (2 mice each). * p value <0.05 and >0.01; ** p value <0.01; *** p value < 0.001.

**Figure 6. Replication of different *T. cruzi* strains in different cell types *in vitro*.** Vero, J774.2, EAhy926 and H9c2 cells were infected with *T. cruzi* Sc43, VFRA and Y strains at a parasite-to-cell ratio of 10:1 in DMEM supplemented with 1% FBS as described in

"Materials and Methods". A) Confocal micrographs of cells harboring intracellular amastigotes at 96 h.p.i. For Vero cells, DAPI staining is shown (white staining) for better visualization of the intracellular amastigotes, and for J774.2, EAhy926 and H9c2, DAPI (green) and phalloidin (red) were used. One representative experiment is shown. B) Quantification of the amastigote-to-nucleus ratio and C) percent infection in the different parasite/cell systems. The number of amastigotes per cell and the percentage of infected cells, respectively, are shown. For parasite-to-cell quantitation, three images per coverslip were taken in three distinct wells per experiment. The experiment was repeated three different times for each condition, and an average of 100 cells was counted in each measurement. Values are expressed as means ± s.e. * p value <0.05 and >0.01; ** p value <0.01; *** p value < 0.001. Scale bars 50 µm.

**Figure 7. Dynamics of replication of different *T cruzi* strains in endothelial cells *in vitro*.** EAhy926 cells were infected with *T. cruzi* Sc43, VFRA and Y strains at a parasite-to-cell ratio of 10:1 in DMEM supplemented with 1% FBS as described in "Materials and Methods". The upper and middle rows show intracellular amastigotes in EAhy926 cells infected with the different *T. cruzi* strains at 24 and 96 h.p.i., respectively. DAPI staining is visualized in green and phalloidin in red. Scale bars 10 µm (confocal microscopy).

**_Trypanosoma cruzi_ strains cause different myocarditis patterns in infected mice**

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Key words: Myocarditis, T. cruzi, Chagas disease.

**ABSTRACT**

**Aims**. Chagas disease pathology is dependent on the infecting *T. cruzi* strain. However, the relationship between the extent and type of myocarditis caused by different *T. cruzi* strains in the acute and chronic phases of infection has not been studied in detail. To address this, we infected mice with three genetically distant *T. cruzi* strains as well as infected *in vitro* different cell types. **Methods and Results.** Parasitemia was detected in mice infected with the Y and VFRA strains, but not with the Sc43 strain; however, only the Y strain was lethal. When infected with VFRA, mice showed higher inflammation and parasitism in the heart than with Sc43 strain. Y and VFRA caused homogeneous pancarditis with inflammatory infiltrates along the epicardium, whereas Sc43 caused inflammation preferentially in the auricles in association with intracellular parasite localization. We observed intramyocardic perivasculitis in mice infected with the VFRA and Y strains, but not with Sc43, during the acute phase, which suggests that endothelial cells may be involved in heart colonization by these more virulent strains. In *in vitro* infection assays, the Y strain had the highest parasite-cell ratio in epithelial, macrophage and endothelial cell lines, but Y and VFRA strains were higher than Sc43 in cardiomyocytes. **Conclusions.** This study supports parasite variability as a cause for the diverse cardiac outcomes observed in Chagas disease, and suggests that endothelial cells could be involved in heart infection during the acute phase.

**INTRODUCTION**

Chagas disease  is a neglected tropical disease caused by the intracellular protozoan *Trypanosoma cruzi,* that affects approximately 8 million people in Latin America (Dias, 2006). In humans, infection is characterized by 2 phases: an acute phase, which is generally asymptomatic, and a chronic phase, asymptomatic (indeterminate) or symptomatic (determinate). During the chronic phase, digestive and/or cardiac syndromes may occur and potentially lead to death.

Chagasic cardiomyopathy is the most common cause of disability in infected patients, showing a variable clinical outcome. Myocardial inflammation associated to mononuclear infiltrate is a common finding during the acute phase, although the spatial association between parasites and inflammatory infiltrate is controversial. In this sense, one key point of Chagasic myocarditis that remains to be elucidated is the gateway of parasitical infection in the heart during the acute phase and their involvement in the pathophysiology. The parasite-endothelial cell interactions are among the first to occur during acute *T. cruzi* infection and in recent years the nature of these interactions as well as their consequences have received increased interest. Some authors have proposed that endothelial cells and/or macrophages allow parasite entry into different organs (Epting et al., 2010).

*T. cruzi* genetic variability is increasingly recognized and this parasite has been genetically classified into six discrete typing units (DTUs) – TcI to TcVI (Zingales et al.,

2009), – which have different geographic distributions and may be associated with geographically-restricted clinical profiles (Tibayrenc and Telleria, 2010). Several data indicate that human genetic determinants may impact the clinical outcome of Chagas disease (Florez et al., 2012; Vasconcelos et al., 2012). However, a role for genetic variability among the different parasite strains in the diverse clinical outcomes of Chagas disease cannot be excluded. In this sense, it is possible that differential tropism of *T. cruzi* for the cell types involved in the natural infection could explain the differences observed in virulence in Chagasic patients and in experimental models. It has been reported that parasite strains belonging to 2 different DTUs (TcI and II) have a differential tropism for the heart (Andrade et al., 1999), which correlates with their ability to replicate within cardiomyocytes *in vitro* (Andrade et al., 2010). Nevertheless, the relationship between specific cellular tropism and cardiomyopathy is still an unresolved issue. The present work explores the *in vivo* course of infection with the genetically different *T. cruzi* strains belonging to 3 different DTUs particularly with respect to heart alterations and *in vitro* differences in invasion and replication in epithelial, macrophage, cardiomyocyte and endothelial cell lines.

**METHODS**

*2.1 Cell culture and in vitro infection*

Vero cells were maintained in 25-cm$^2$ Nunc® flask in Dulbecco's modified Minimal Essential Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a controlled atmosphere (5% $CO_2$, 37ºC). The cells were seeded on a coverslip in a 24-well

culture plate at 1,700 cells *per* well twenty-four hours prior to infection. The J774.2 murine macrophage cell line was maintained and seeded under the same conditions at $1 \times 10^5$ cells *per* well. The human endothelial-like cell line EAhy926 (Edgell et al., 1983), was a kind gift of Dr. Santiago Lamas, Centro de Biología Molecular, CSIC-UAM. (Madrid, Spain). These cells were maintained in DMEM supplemented with 20% (v/v) FBS and 10% HAT (hypoxanthine-aminoptherine-thymidine) and seeded at $5 \times 10^4$ cells *per* well 24h prior to infection. H9c2 cell line, derived from embryonic BD1X rat cardiac tissue (Kimes and Brandt, 1976), was a gift of Dr. Miguel Ángel Íñiguez. Centro de Biología Molecular, CSIC-UAM (Madrid, Spain). H9c2 cells were maintained in DMEM supplemented with 10% v/v of FBS and seeded at $5 \times 10^4$ cells *per* well 24h prior to infection.

The Sc43 (TcV) and VFRA (TcVI) strains of *T. cruzi* were obtained from *Triatoma infestan*s specimens in Santa Cruz, Bolivia and Chile, respectively, and were compared with the well-characterized Brazilian Y (TcII) strain, obtained from Dr. John David (Department of medicine, Harvard Medical School, Boston, MA, USA) and previously cloned as was described (Tanuri et al., 1985). Sc43 and VFRA isolates were cloned as described previously (Yeo et al., 2007). Briefly, parasites were mixed with 2.4 ml of 0.9% NaCl (w/v) and 0.6 ml of molten 3% (w/v) low melting point (LMP) agar. The mixture was then poured onto solid blood agar plates, allowed to set and incubated at 28°C for 3–6 weeks. Once colonies were visible they were picked using a sterile pipette tip and inoculated into liquid culture. Colonies were genotyped as was previously described (Lewis et al., 2009). Trypomastigotes were produced by successive passage in Vero cells, and recollected at fifth day post-infection and counted in a Neubauer hemocytometer. The infection was carried out at a parasite-cell ratio of 10:1, in DMEM supplemented with 1%

of FBS in all cases and the infected cells were washed twice 24h post-infection to remove extracellular parasites. Before the experiments, the DTU of the stock used was determined in order to confirm the absence of mislabeling or contamination.

### 2.2 Mouse infection

Young adult (6-8-wk) BALB/c mice were purchased from Charles River Laboratories. Blood trypomastigotes were routinely maintained by infecting IFN-γ receptor deficient mice (129-*Ifngr1$^{tm1Agt}$/J*, The Jackson Laboratory) and isolating them from blood by centrifugation. Mice were infected intraperitoneally with 5,000 blood-derived trypomastigotes of the Sc43, VFRA and with 2,000 of the Y strain due to their higher virulence. Parasitemia was monitored by the Brener method as described elsewhere (Brener, 1962). Infected mice were sacrificed at 21 and 100 days post-infection (d.p.i). Hearts were washed with heparin 1:100 v/v in PBS, and fixed for 24 h in 4% (v/v) paraformaldehyde (PFA) in PBS, at 4 ºC. The fixed hearts were cut sagittal and processed for paraffin embedding and hematoxylin-eosin or Mason's trichromic staining (Histology Service, Centro Nacional de Biotecnología, Madrid, Spain).

For histological analysis, the micrographs were taken from sections of 8 (Sc43 and VFRA) and 3 (Y) different hearts for acute phase and 6 (Sc43) and 7 (VFRA) for chronic phase (Y strain was not included for this phase) in three independent experiments for each phase. The number of Y strain samples was influenced by the fact of the high mortality observed, which limited the number of available samples. The observation was done with a Leica DMD108 microscope. For a panoramically view of inflammation, one 4x image per heart was taken and for microvasculature morphology analysis five 63x image per heart

were taken at random. Inflammation was classified by a semi-quantitative score, as follows: 0 for the absence of inflammation, 1 for low-level inflammation, 2 for moderate inflammation and 3 for strong inflammation. Quantification was performed in separately in auricles and ventricles.

For the quatification of amastigote nests, sections of three different hearts *per* strain were observed. The whole cardiac section with a 40x objective was monitored and quantification was divided among auricles and ventricles.

2.3 *Ethics statement*

This study was conducted in strict accordance with the recommendations of Spanish legislation and the European Council directive from the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All mice were maintained under pathogen-free conditions in the animal facility at the Centro de Biología Molecular, Universidad Autónoma de Madrid (Madrid, Spain). The animal protocol was approved by the Ethic committee of the Universidad Autónoma de Madrid. Animals had free access to food and water and were handled in compliance with European standards. Mice were euthanized in a $CO_2$ chamber, using a gradually fill of the chamber with $CO_2$, and all efforts were made to minimize suffering.

*2.4 Confocal microscopy*

Ninety-six hours post-infection, the infected cell cultures, placed on coverslips without Poly-L-lysine, were washed three times with PBS and fixed for 10 minutes in (w/v) PFA 4% in PBS pH 7.4. Next, the cells were permeabilized for three minutes in a solution of 0.5% v/v Triton X-100 in PBS. Then, actin fibers were stained with 1µg/ml phalloidin-

TRITC (Sigma-Aldrich) for 20 min, cells were washed twice with PBS. Nuclei were then stained with 1µg/ml DAPI (Merck) for 10 min, washed again once and coverslip were mounted with ProLong Gold® (Invitrogen) and stored at 4ºC until visualization.

The preparations were observed on an AxioObserver LSM 710 confocal microscope, (Zeiss®) with LCI-Plan Neofluar 25x/0.8 Imm Korr DIC M27 and with a Plan-Apochromat 63x/1.40 Oil DIC M27 objectives. Acquisition conditions were determined for each preparation. For parasite-cell quantitation, three images *per* coverslip were taken for three distinct preparations. The experiment was repeated three times for each condition. Images were processed using Fiji software and 63x images were deconvolved using Huygens Professional® software.

### 2.5  *Parasite DNA detection and mRNA analysis by quantitative RT-PCR.*

After blood removal by perfusion, parasite DNA was purified from cardiac tissue with the High Pure PCR Template preparation Kit (Roche), and PCR reactions were performed with 100ng of DNA as described (Calderon et al., 2012; Cuervo et al., 2011). For *T. cruzi* detection, the method described by Peron and collaborators was followed (Piron et al., 2007). Spiking of organs from uninfected animals with known quantities of parasites was used to create bi-dimensional matrices (parasites/mg tissue; total DNA concentration) for two-step regression (unpublished data).

Cardiac RNA was purified with TRIzol reagent (Invitrogen). Quantitative RT-PCR analysis was performed using the High Capacity cDNA Archive Kit (Applied Biosystems) and different genes (*Ifng, Tnf, il4, il10, il13, il12, ptgs2* and *18S rRNA*) were amplified in triplicate using TaqMan MGB probes and the TaqMan Universal PCR Master Mix

(Applied Biosystems) on an ABI PRISM 7900 HT instrument (Applied Biosystems) as previously described (Cuervo et al., 2008). Parasite DNA and gene expression by real-time PCR was quantified by the comparative threshold cycle ($C_T$) method (RQ= $2^{-\Delta\Delta C_T}$), using normalization by the 18S ribosomal endogenous control levels and reported to uninfected control animals. Graphs were plotted as log RQ when indicated,

*2.6 Statistical analysis*

Data are presented as means ± SEM. Student's t-test for independent samples was used to test for differences between sample means. All analyses were performed using Prism5® (GraphPad Software, Inc., La Jolla, CA, USA).

**RESULTS**

We infected BALB/c mice with 3 genetically different *T. cruzi* strains (Sc43, VFRA and Y), belonging to TcV, TcVI and TcII respectively, and monitored the course of infection (Figure 1). Infection by Y strain was lethal during the acute phase; at 30 days post infection (d.p.i.), 100% of the infected mice were dead (Figure 1A). In contrast, the Sc43 and VFRA strains did not cause mortality. Despite this, VFRA produced higher levels of parasitemia than the Y strain with no significant differences in kinetics of parasitemia (Figure 1B). After 45 days of infection, we did not detect circulating parasites in any of the infected mice, and parasites were not detectable in blood at any time point in mice infected with the Sc43 strain. However, parasites were detected at day 21 p.i. in the hearts of mice infected with the Sc43 strain (937 parasites/mg tissue) at a level comparable to that obtained with the VFRA strain (1,840 parasites/mg tissue), albeit at lower levels than the Y

strain (6,534 parasites/mg tissue, Figure 1C). At 100 d.p.i., parasite DNA was only detected in mice infected with VFRA strain (21 parasites/mg tissue).

Next, we analyzed the pattern of myocardial inflammation during acute infection (Figure 2). Interestingly, the pattern was quite different among the 3 strains. The differences were mainly related with the extension and location of the inflammatory infiltrate a throughout the heart. Focal inflammation of the ventricles was detected in Sc43-infected hearts (Figure 2A, upper, left panel), in contrast with the intense and diffuse inflammatory reaction observed at atria (upper, right panel). For VFRA and Y infected mice, the leukocyte infiltrate was diffuse and more intense at the ventricular epicardium and sub-epicardium. Figure 2B shows a scoring of myocarditis divided by auricles and ventricles, obtained by microscopic observation. Y strain caused the strongest inflammation and Sc43 the lowest one.

In addition, the number of amastigote nests correlated with the intensity of inflammation for the different strains in the different locations (Figure 2C). Thus, in VFRA- and Y-infected mice, we observed more amastigote nests in ventricles than in auricles. As expected, the number of amastigote nests correlated with parasite load observed by quantitative PCR (see Figure 1C).

By analyzing the lesions in the heart in greater detail, we observed perivascular inflammation in myocardial vessels, particularly in mice infected with the VFRA and Y strains (Figure 3). The infiltrate, with a predominance of mononuclear cells, was observed in close apposition to the vessel's adventitia, with slightly rounded endothelial cells and an endothelium occasionally infiltrated with mononuclear cells (Figure 3, white arrows). We

observed a tendency of parasite nests to be located in close apposition to the vessels in mice infected with the Y strain, as illustrated in the central panel in the Y strain's row (black arrows). For Sc43, perivascular infiltrate or intravascular changes were also observed, though to a lesser extent.

During the chronic phase, we observed a moderate inflammatory infiltrate persisted in the hearts of mice infected with VFRA with predominance of mononuclear cells (Figure 4). We still observed perivascular infiltrates (left and center panels) and pericardial fibrosis (right panel). In contrast, no significant pathological alterations were observed in Sc43-infected hearts, which suggests resolution of the inflammation (bottom panels). Though we did not find amastigote nests in mice chronically infected with the VFRA and Sc43strains (data not shown), the higher sensitivity of quantitative, real-time PCR permitted the detection of parasites in mice infected with VFRA but not with Sc43 (Figure 1C). Analysis of the chronic phase with the Y strain could not be performed since BALB/c mice infected with the Y strain died before reaching the chronic phase.

In order to gain further insight into the host response resulting from parasite infection we analyzed the inflammatory cytokine expression in hearts at 14 d.p.i., before full damage is induced (inflammation was also evaluated at this point by histological sections with similar trends that the one shown at 21 d.p.i., data not shown). As shown in Figure 5, infection with the Y strain induced the highest expression of inflammatory cytokines (*Ifng*, *Il6* and *Tnf* mRNA) in the heart, whereas the VFRA and Sc43 strains induced lower expression, with VFRA higher than Sc43. Cytokine expression in the acute phase correlates well with the histological evaluation of the inflammation shown in Figure 2B. In the chronic phase (100 d.p.i.), *Il6* mRNA levels significantly decreased regarding to acute

phase and *Ifng* levels decreased in chronically infected animals, but still showed values significantly higher than control. Interestingly, *Tnf* decreased during the chronic phase in mice infected with the VFRA strain (but with values still higher than control animals), while in Sc43 infected mice the values were similar during acute and chronic phase

The above results pointed to clear differences in the cardiac pathophysiology between the strains. A possible explanation could be a differential infective capacity of parasites, in quantitative (virulence) or qualitative (tropism) terms. We thus tested the ability of the studied strains to infect various cell lines derived from different tissues: Vero (epithelial), J774 (macrophage), EAhy926 (endothelial) and H9c2 (cardiomyocyte) cells (Figure 6, Panel A). All parasite strains infected all cell types. Nonetheless, some striking differences appeared. Y strain had the highest amastigote/cell ratio (Panel B) in all cell types, only equaled by VFRA for H9c2 cells. Sc43 showed a higher parasite-cell ratio than VFRA only in Vero cells. VFRA had only a slightly increased parasite-cell ratio in J774 with respect to Sc43 and the same parasite-cell ratio than Y strain in H9c2 cardiomyocytes. EAhy926 cells showed the highest percentage of infection and parasite-cell ratio (Panel C), particularly with the Y Strain (90%) at 96 h post-infection and Vero cells showed the lowest rate for all strains.

The effect on EAhy926 endothelial cells was analyzed in more details. In Figure 7, we present high magnification micrographs of EAhy926 cell infection at 24, 48 and 96 h pi. At 24 h pi, there were no differences in intracellular amastigote number among *T. cruzi* strains, which suggests a similar rate of infection during the early invasion and replication period (non infecting parasites were removed to this point). At 96 h panel demonstrates the cells infected with the Y strain harbored a mixture of intracellular parasites with

amastigote-like (rounded forms) and trypomastigote-like (thin forms) morphologies, unlike the Sc43 and VFRA strains, which exhibited uniform amastigote morphology. This observation suggests an enhanced differentiation from amastigote to trypomastigote for the Y strain, which may be responsible for the observed differences in amastigote numbers show in Figure 6.

## DISCUSION

There is a controversy regarding the role of the genetic variability of *T. cruzi* strains in the clinical evolution and outcome of the disease. In order to clarify this controversial point, we chose to study this question in animal models with cloned parasite strains belonging to 3 different DTUs genetically distant (TcII, TcV and TcVI).

There is little information about the causes involved in the different myocarditis patterns and their evolution observed during the acute and chronic phase. Recent works have linked vasculopathy associated to *T. cruzi* invasion the cardiac inflammation, suggesting that proinflammatory phenotypes of T. cruzi strains may be ascribed, at least in part, to variable expression of TLR2 ligands and cruzipain isoforms during the acute myocarditis (Scharfstein and Andrade, 2011). However, a detailed understanding of the contribution of host and parasite genetics in this process is lacking. To our knowledge, this is the first report of specific cardiac immunopathological patterns associated to genetically distant strains.

A detailed analysis of inflammatory infiltrates in the hearts of infected animals revealed different patterns: Inflammatory infiltrates were more concentrated in the atrial region in Sc43-infected mice during the acute phase, and this finding was related to the

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distribution of amastigote nests, although the differences between auricles and ventricles were non-significant, probably due to limitations in the inflammation scoring method caused by the restricted area available (atrial appendage) for amastigote nest and inflammatory infiltrate scoring. This tendency may be related to differences in virulence observed among the strains, with the most virulent strains (Y and VFRA) causing pancarditis whereas low virulent strains (Sc43) causing only focal/regional inflammation. The Y and VFRA strains induced higher levels of epicardial and sub-epicardial inflammation that was homogenous in the auricles and ventricles. This pattern is consistent with different observations reporting pericardial effusion in acute Chagas disease (Bastos et al., 2010) and may be associated with the involvement of the intra-myocardial vasculature in the establishment of acute myocarditis. Moreover, only VFRA was able to cause cardiac alterations in the chronic phase in mice (Fig 4), a fact that could be related with the inflammation pattern observed during the acute phase and may suggest that progression to chronic phase is also strain-dependent. Therefore, in mice, genetically distinct *T. cruzi* strains show considerable variations in cardiac pathology caused, which parallels the diverse outcome in human Chagas disease.

A recent work reports myocarditis induced in mouse model by a single strain representative of the TcI, TcII, TcV and TcVI DTUs, concluding that a TcVI strain causes moderate myocarditis, whereas a TcV strain that did not cause cardiac inflammation exhibited almost undetectable parasite load (Ragone et al., 2012). This observation is in sharp contrast with the data in humans, where patients infected with TcV strains have been shown to suffer cardiac pathology (Burgos et al., 2010). More recently, some authors have associated mixed infection with different DTUs with severity of inflammation and tissular

tropism (Sales-Campos et al., 2014; Silvina et al., 2014). For our study, we have selected strains with very different biological behavior both *in vitro* and *in vivo*. Indeed, the Y strain causes parasitemia and acute mortality, VFRA causes only parasitemia and Sc43 causes neither parasitemia nor mortality. The Y strain exhibited a higher parasite load in the heart, likely reflecting higher cardiotropism than the VFRA and Sc43 strains, Sc43 being the less infective for the heart. Also, the quantitative differences in parasite load in the heart may not be solely responsible for the highly different cardiomiopathy patterns and suggests that other intrinsic properties of the strains, such as the ability to cause parasitemia (Fig 1) and being available for infecting target cells, are mainly responsible for these differences. Indeed, the differences in the replication of Sc43 and VFRA between auricles and ventricles may explain the differences in the myocarditis patterns observed.

Findings during chronic phase (absence of parasite or cardiac inflammation) would suggest the possibility of spontaneous cure in mice infected with Sc43. There is a previous controversy in the literature about this fact, with suggestions of reactivation of infection in apparently cured mice during the chronic phase (Magalhaes and Andrade, 1994) and another ones that claims cure in elder untreated patients with records of parasitemia during the acute phase (Bertocchi et al., 2013; Francolino et al., 2003). Further studies are necessary in order to evaluate the reactivation due to extra-cardiac parasite homing.

In the case of chronically VFRA infected mice, we can appreciate the presence of inflammatory infiltrate apparently in absence of amastigote nest. Recent articles has highlighted the possibility that  genetic alterations resulting from kDNA integration in the host genome lead to autoimmune-mediated destruction of heart tissue in the absence of T. cruzi parasites (Teixeira et al., 2011). Additionally, parasite antigens were able to induce

autoimmunity and cardiac damage in mice inoculated with heat inactivated parasites (Bonney et al., 2011). Comparing the two strains that can cause chronicity in our infection model (Sc43 vs VFRA), our results suggest that the presence of at least parasite DNA is necessary for causing chronic cardiac disease. Vascular inflammation has been reported in experimental models of Chagas disease (Sunnemark et al., 1998) and acute outbreaks in humans (Alarcon de Noya et al., 2010) and may be associated with different Chagas disease outcomes in patients (Garcia-Alvarez et al., 2011). The observed intramyocardic perivasculitis in mice infected with the VFRA and Y strains, but not Sc43 suggest that endothelial cells may be involved in heart colonization by the most virulent strains. This hypothesis is also in accordance with *in vitro* higher infection in endothelial cell lines by Y strain, as compared to Sc43 and VFRA. Nonetheless, we failed to find intracellular parasites in endothelial cells of cardiac vessels in animals infected with any of the strains, possibly because this invasion process occurs earlier than day 21.Further studies are necessary to solve this question.

It is well known that *T. cruzi* can invade a large variety of cells both *in vitro* and *in vivo* (de Souza et al., 2010) and the tissue homing ability of *T. cruzi* has been reported to be strain-specific (Andrade et al.; Tibayrenc and Telleria). However, there is little information regarding the relationship between strains tropism and pathology. Interestingly, our results show that the 3 distinct *T. cruzi* strains have important differences in their abilities to replicate in cells of epithelial (Vero), monocyte (J774), endothelial (EAhy926) and cardiac (H9c2) origin, with Y strain having the highest overall parasite to cell ratio and infection rates in all cell lines studied.

The affinity of *T. cruzi* for endothelial cells has been associated with endothelin and bradykinin receptors (Andrade et al., 2012). Additionally, gp85 – a *T. cruzi* glycoprotein involved in interaction with endothelial cells (Tonelli et al., 2010) – has a greater affinity for cardiac-derived endothelial cells with respect to visceral-derived endothelial cells (Tonelli et al., 2010). It is possible that diverse expression of surface glycoproteins in genetically distant strains may be involved in the differential tropism observed. On the other hand, our results shown in Figure 7 suggest that the differences observed in intracellular parasite-cell ratio in endothelial cells between Sc43, VFRA and Y strains are related to the amastigote differentiation rate to trypomastigote. Experiments to confirm this model with our strains are underway.

In conclusion, our results show that the myocarditis pattern observed in experimental acute infection by *T. cruzi* depends on the strain. Importantly, the myocarditis pattern depends on differential cardiac parasite distribution and the higher parasite invasion rate and replication in endothelial cell *in vitro* would suggest that the interaction between the parasite and the endothelium may play a key role in heart colonization during acute Chagas disease. Moreover, our results indicate that the strains have differing capacities for replication in several cells types, with the more pathogenic *T. cruzi* strains general replicating better in all cell types. This observation may explain the differential pathogenicities of the strains. Our results in mice reflect the great variability in clinical outcomes and syndromes observed in chagasic patients, and offer the possibility of studying the parasite factors involved in different outcomes, including their interactions with different cell types.

**Conflicts of interested:** None declared

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 **FIGURE LEGENDS**

**Figure 1. Different outcomes of *in vivo* infection by different *T cruzi* strains.** BALB/c mice were infected with 5,000 trypomastigotes of the *T. cruzi* Sc43 (open box) and VFRA (filled triangle) strains and 2,000 of the Y (open circle) strain. A) Survival. B) Parasitemia. Means +/- SEM  from three independent experiment are shown (n=9) C) Quantification of *T. cruzi* DNA in the heart tissue of infected BALB/c mice. Panel C shows results of real-time PCR with heart samples taken at 21 (acute) and 100 (chronic) d.p.i.. Results are

expressed as the mean values (±SD) for triplicates of pooled DNA from 5 different mice. (*) Statistically significant differences (p< 0.05).

**Figure 2. Heart inflammation by different *T. cruzi* strains in the acute phase.** BALB/c mice were infected with 5,000 trypomastigotes of the Sc43 and VFRA strains and 2,000 of the Y strain and the hearts of the infected mice were analyzed at 21 d.p.i. A) High magnification images of infected and healthy heart (last panel) with representative inflammation for each strain. B) Estimation of inflammation in auricles, ventricles by an arbitrary index: 0 corresponds to absence of inflammation, 1 to low inflammation, 2 to moderate inflammation and 3 to severe inflammation. C) Quantification of the number of amastigote nests divided into auricles and ventricles (three different hearts per infecting strain). Results for inflammation score are expressed as the mean values (±SEM) for independent microscopic fields from 8 different mice for the Sc43 and VFRA strains and 3 different mice for the Y strain (20 fields for each) performed in three independent experiments. * p value <0,05>0,01 ; ** p value <0.01. A capped line shows statistical differences between auricle/ventricle, and a zigzag line shows statistical differences between strains. Scale bars 100 μm

**Figure 3. Perivascular inflammation in intracardiac vessels caused by different *T cruzi* strains during the acute phase.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains and 2,000 of the Y strain, and the hearts of infected mice were analyzed at 21 d.p.i. The upper, middle and bottom rows show perivascular inflammatory infiltrates in the intramyocardial vessels of mice infected with the Sc43, VFRA and Y strains, respectively. White arrows indicate cellular infiltration in endothelial walls and black arrows show amastigote nest. Shown are three representative hearts of 8

(Sc43 and VFRA) and 3 (Y) performed in three independent experiments. Scale bars 100 µm.

**Figure 4. Cardiac inflammation by different *T. cruzi* strains during the chronic phase.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains, and the hearts of infected mice were analyzed at 100 d.p.i. The upper panel shows cardiac infiltration with macrophage-like cells, muscle fibers, disorganization and pericardial fibrosis in VFRA-infected mice. Sc43-infected mice (bottom panel) did not exhibit significant pathological changes. Shown are three representative hearts of 7 (VFRA) and 6 (Sc43) performed in two different experiments. VFRA section are stained with Masson staining and Sc43 with conventional H&E. Scale bars 50 µm.

**Figure 5. Cytokine production in infected hearts by different *T. cruzi* strains during the acute and chronic phases.** BALB/c mice were infected with 5,000 trypomastigotes of *T. cruzi* Sc43 and VFRA strains and 2,000 of the Y strain. Fold expression of IFN-γ, IL-6 and TNF-α mRNA in the hearts of infected mice during the acute phase (14 d.p.i.) and chronic phase (100 d.p.i.) over uninfected control mice of the same age. RNA from cardiac tissue at different d.p.i. was used to perform RT-PCR with specific probes and normalized to ribosomal 18S RNA as described in "Materials and Methods". Values are expressed as means ± standard errors (s.e.) from 3 separate experiments (2 mice each). * p value <0.05 and >0.01; ** p value <0.01; *** p value < 0.001.

**Figure 6. Replication of different *T. cruzi* strains in different cell types *in vitro*.** Vero, J774.2, EAhy926 and H9c2 cells were infected with *T. cruzi* Sc43, VFRA and Y strains at a parasite-to-cell ratio of 10:1 in DMEM supplemented with 1% FBS as described in

"Materials and Methods". A) Confocal micrographs of cells harboring intracellular amastigotes at 96 h.p.i. For Vero cells, DAPI staining is shown (white staining) for better visualization of the intracellular amastigotes, and for J774.2, EAhy926 and H9c2, DAPI (green) and phalloidin (red) were used. One representative experiment is shown. B) Quantification of the amastigote-to-nucleus ratio and C) percent infection in the different parasite/cell systems. The number of amastigotes per cell and the percentage of infected cells, respectively, are shown. For parasite-to-cell quantitation, three images per coverslip were taken in three distinct wells per experiment. The experiment was repeated three different times for each condition, and an average of 100 cells was counted in each measurement. Values are expressed as means ± s.e. * p value <0.05 and >0.01; ** p value <0.01; *** p value < 0.001. Scale bars 50 μm.

**Figure 7. Dynamics of replication of different *T cruzi* strains in endothelial cells *in vitro*.** EAhy926 cells were infected with *T. cruzi* Sc43, VFRA and Y strains at a parasite-to-cell ratio of 10:1 in DMEM supplemented with 1% FBS as described in "Materials and Methods". The upper and middle rows show intracellular amastigotes in EAhy926 cells infected with the different *T. cruzi* strains at 24 and 96 h.p.i., respectively. DAPI staining is visualized in green and phalloidin in red. Scale bars 10 μm (confocal microscopy).