DTU I isolates of *Trypanosoma cruzi* induce upregulation of Galectin-3 in murine myocarditis and fibrosis

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SUMMARY

Chagas heart disease is a major public concern since 30% of infected patients develop cardiac alterations. The relationship between *Trypanosoma cruzi* discrete typing units (DTUs) and the biological properties exhibited by the parasite population has yet to be elucidated. In this study, we analysed the expression of α-smooth muscle actin (α-SMA) and galectin-3 (Gal-3) associated with cardiac extracellular matrix (ECM) remodelling a murine chronic cardiomyopathy induced by Tc I genotypes. We found the induction of myocarditis was associated with the upregulation of Col I, α-SMA, Gal-3, IFN-γ and IL-13, as analysed by q-PCR. In myocardial areas of fibrosis, the intensity of myocarditis and significant ECM remodelling correlated with the presence of Col I-, Gal-3- and α-SMA-positive cells. These results are promising for the further efforts to evaluate the relevance of Gal-3 in Chagas heart disease, since this galectin was proposed as a prognosis marker in heart failure patients.

Key words: Chagas disease, galectin-3, fibrosis, smooth muscle actin, DTU.

INTRODUCTION

Chagas disease, which is caused by *Trypanosoma cruzi*, is a major neglected tropical disease, with more than 8 million infected people in the endemic region (WHO, 2002; Rassi *et al.* 2010). The infection can also spread, including in non-endemic countries, mainly by blood transfusion and congenital transmission (WHO, 2002). While the acute phase of the infection is usually asymptomatic, 30% of infected patients develop cardiac alterations and 10% develop megaoesophagus, megacolon, or mixed alterations late during the chronic phase of the infection. In South America, megaviscera and severe cardiomegaly were both observed, but with different rates of incidence between the countries. In Mexico and Central America, the prevalence of megaviscera is rare (Rassi *et al.* 2010). The reasons for the different geographical distributions of these clinical manifestations remain unknown (Campbell *et al.* 2004; Marin-Neto *et al.* 2007; Rassi *et al.* 2010). Chagas heart disease (ChHD) is a major public health concern, and it is estimated that 20 000 patients die

annually in endemic countries (PAHO, 2007). *Trypanosoma cruzi* populations cluster into six discrete typing units (DTU), named Tc I–VI (Zingales *et al.* 2009). The relationship between DTUs and the diverse biological properties, including virulence, invasion and tissue tropism, exhibited by *T. cruzi* strains is still not understood. It was proposed that Tc II, V and VI are most frequently implicated in the pathogenesis of chronic Chagas disease in southern South America, where severe megasyndromes are commonly observed. In contrast, while Tc I was initially considered to be less pathogenic (innocuousness) primarily in Mexico, Central America and northern South America (Di Noia *et al.* 2002; Feliciangeli *et al.* 2002; Miles *et al.* 2003; Campbell *et al.* 2004; Macedo *et al.* 2004; Freitas *et al.* 2005; Carranza *et al.* 2009), in recent years it has been detected in cardiac tissue of patients in the end-stage of ChHD from Brazil and Argentina (Teixeira *et al.* 2006; Burgos *et al.* 2010).

The myocardium invasion by *T. cruzi* destroys cardiac myocytes and induces damage caused by inflammation, which results in the loss of cardiomyocytes, hypertrophy of the surviving myocytes, conduction network damage, chamber dilation, proliferation of cardiac fibroblasts (CFs), and significant extracellular matrix (ECM) remodelling that may result in severe cardiac insufficiency and in the eventual need for a cardiac transplant (WHO, 2002; Marin-Neto *et al.* 2007; Tanowitz *et al.* 2009; Rassi *et al.* 2010). Although the pathogenesis of *T. cruzi*

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infection is not completely understood, its persistence has been postulated to be a co-requisite, or possibly a prerequisite, for the development of ChHD (Tarleton, 2003). It has been demonstrated that during the cardiac ECM remodelling processes, the phenotypic transformation of CFs to myofibroblasts is a key event. Myofibroblasts are highly active cells that express α-smooth muscle actin (α-SMA) and exhibit increased proliferative, migratory and secretory properties. Under normal circumstances, the myofibroblasts are removed from the repaired wound site by apoptosis (Desmouliere *et al*. 1995; Gurtner *et al*. 2008; Shi *et al*. 2011). However, persistence of myofibroblasts can facilitate hypertrophic scarring and fibrosis, which results in myocardial stiffness and impairment of cardiac function (Sun and Weber, 2000; Frangogiannis, 2006).

Galectin-3 (Gal-3), which is the only galectin with a chimera-type in the family of β-galactoside-binding animal lectins, has several physiological and pathological associated roles (Liu and Rabinovich, 2010). During experimental *T. cruzi* infection, Gal-3 was upregulated in B cells (Acosta-Rodriguez *et al*. 2004) and dendritic cells (Vray *et al*. 2004). Gal-3 binds to a trypomastigote surface mucin (Moody *et al*. 2000; Turner *et al*. 2002) and on human coronary artery smooth muscle cells in a lectin-like manner (Kleshchenko *et al*. 2004). It has been suggested that it plays a role in a trypanosome trapping mechanism, which enables the parasite to accumulate in the basement membrane prior to invasion (Villalta *et al*. 2009). Gal-3 has recently been reported to be involved in heart failure (HF) development in different experimental models. In these systems, its upregulation was associated with macrophages migration, fibroblast activation and the induction of cardiac ECM remodelling in the progression to HF (de Boer *et al*. 2010). In addition, Gal-3 was also upregulated in human hypertrophied hearts and plasma from patients with acute or chronic HF (Lok *et al*. 2010). The role of Gal-3 in the cardiac alterations induced by *T. cruzi* has not been fully investigated (Soares *et al*. 2010).

In order to better understand the mechanisms involved in ChHD pathogenesis, we analysed the expression of Gal-3 associated with cardiac collagen remodelling in a murine chronic cardiomyopathy induced by Tc I strains of *T. cruzi*.

MATERIALS AND METHODS

Parasites

The Ac and Hc strains of *T. cruzi*, which are members of DTU Tc I, were isolated from patients (Risso *et al*. 2004). The remaining isolates (3 Tc V and 1 Tc VI) were also obtained from patients and the DTU were identified as described (Burgos *et al*. 2013).

Mice

Weanling (21 days old) male CF1 mice were provided by the Institute of Medical Microbiology and Parasitology (IMPAM), UBA-CONICET.

Ethics statement

The protocol of this study was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martín (UNSAM), which also approved the protocol development under the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Three R's rule was followed. Therefore the animal number used was reduced to the minimal amount required to obtain statistically valid data.

Experimental design

Groups of five mice were inoculated intraperitoneally with 5×10^4 bloodstream trypomastigotes. Mice were observed daily to determine mortality and morbidity. Cardiac muscle samples were obtained from mice that were euthanased at 3 or 5 (DTU V and VI, respectively) months post-infection (mpi). Animals inoculated with equal volumes of PBS-BSA (mock-infected) were used as negative controls. Circulating parasites were monitored starting at 11 days post-infection (dpi) and parasitaemia was evaluated using a hemacytometer (Risso *et al*. 2004).

Histopathology

Heart samples were removed and split along the longitudinal axis. One-half was fixed in 4% paraformaldehyde, embedded in paraffin, sections stained with hematoxylin-eosin (HE), and examined for the presence of myocarditis according to the parameters based on cell infiltration and necrosis of muscle cells as previously reported (Feldman and McNamara, 2000).

To obtain a myocarditis score for each sample, $5\,\mu$m thick sections were used for HE staining. All fields of each section were analysed. The severity of inflammation was graded as follows: grade $0 =$ no lesions; grade $1 =$ involvement of a single muscle fibre or <5 muscle fibres; grade $2 =$ a lesion involving 5–10 muscle fibres; grade $3 = 10$–20 muscle fibres; and grade $4 =$ diffuse, extensive lesions. When multiple lesions with the same grade were found in a single muscle block or marked fibrosis was observed, 0·5 points were added to the grade. All slides were evaluated independently by researchers who were blinded to the sample status. The picrosirius red (PSR) technique for collagen fibres (Junqueira *et al*. 1979) was carried out as previously described (Gomez *et al*. 1992). Briefly, sections were hydrated, washed, immersed in 0·02% aqueous

phosphomolybdic acid, stained with 0·1% Sirius red F3BA in saturated aqueous picric acid, washed in 0·01 N HCl, stained with hematoxylin, and then dehydrated, mounted and observed using a Nikon E200 microscope.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Gomez *et al*. 2008) with minor modifications. Briefly, after rehydration, the tissue Pro-Bond Plus sections were heated three times for 5 min in a 10 mM citrate buffer in a microwave oven. The sections were then cooled and immersed in 3% H_2O_2 for 15 min, and incubated in 5% normal goat serum in PBS for 20 min at room temperature. The samples were incubated with a primary rat monoclonal anti-Gal-3 antiserum (Clone M3/38; 1 : 50 dilution) or mouse monoclonal anti-α-smooth muscle actin (α-SMA, DAKO; 1 : 50 dilution) for 1 h at room temperature. The secondary antibody was conjugated to peroxide-labelled dextran polymer (DAKO EnVision). Diaminobenzidine (DAB)/hydrogen peroxidase substrate was added for 10–20 min to reach the appropriate intensity and then the slides were rinsed with distilled water. Immunostained sections were counterstained with hematoxylin for 1 min, washed under tap water, rinsed with distilled water, and dehydrated in increasing ethanol concentrations followed by xylene (each treatment, 5 min). They were observed under a Nikon E200 photomicroscope.

DNA and RNA isolation and RT-PCR

Total DNA or RNA was isolated from half heart samples by mechanical homogenization and Trizol (Invitrogen), as recommended by the manufacturer. The DNA or RNA was resuspended in 100 μL nuclease-free water (Epicenter) and quantified using a spectrophotometer (Nanodrop spectrophotometer ND-1000). Prior to cDNA synthesis, DNase treatment was performed with an RNase free DNase Kit (Qiagen). cDNA was synthesized from 500 ng of total RNA with 15 mM random hexamers and MMLV reverse transcriptase (Promega), according to the manufacturer's instructions.

Real-time PCR

For q-PCR studies, PCR amplification and analysis were performed with a Line-Gene K instrument and software (Bioer). The 5× HOT FIREPol® EvaGreen® qPCR Mix Plus was used for all reactions following the manufacturer's instructions. For PCR efficiency calculations, 10-fold serial dilutions of Standard cDNA samples were used. Cycle threshold (Ct) values were obtained for each individual reaction, and the Ct of the ubiquitously host-expressed β-actin was subtracted to obtain the values for CCL-2, CXCL-10, pro-collagen type I, α-SMA, Gal-3, TGF-β1, IL-10, IL-13 and IFN-γ normalized, respectively (Jaquenod De Giusti *et al*. 2011). Parasite burden was assessed as described previously (Solana *et al*. 2012). Briefly, a sequence of the satellite nuclear repeat (SNR) was amplified and the number of parasites was referred to that of the host cells. Host cell number was assessed by quantifying the single copy murine-specific TNF-α gene. The primer sequences and sizes of the amplified fragments are shown in Table 1.

Statistics

Data were expressed as the mean ± S.E.M. and were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test to determine significant differences between groups. $P < 0·05$ was considered statistically significant.

RESULTS

Human isolates belonging to DTU I were associated with heart disease of different severity

To study the progression of the murine infection and its eventual effect on the heart, we used human isolates of *T. cruzi* belonging to DTU I, V and VI. Their evolution was studied by monitoring circulating parasites, mortality and heart damage during the chronic phase at 3–5 mpi. Results showed that only the two isolated Tc I samples, named Ac and Hc, induced mortality (6%) and quantifiable parasitaemia by hemocytometer. The remaining isolates induced low circulating parasite levels. Ac and Hc trypomastigotes were detected from 11 dpi, but quantified from 14 dpi (Fig. 1A). Both strains induced maximal parasitaemia values between 20–50 dpi and trypomastigotes were undetectable by this method after 70 dpi. Additionally, q-PCR of the SNR gene was used to study the parasitic burden in heart tissue samples of mice inoculated with Ac and Hc strains at 90 dpi, as well as the mock-infected mice. As expected, no amplification was observed in samples from mock-infected mice. In contrast, both Ac- and Hc-inoculated mice exhibited cardiac parasite burden, with higher values in the former ($P < 0·05$; Fig. 1B). For histopathological analysis, myocardial samples from mock-infected mice served as controls for routine HE staining and, as expected, showed no abnormalities (Fig. 1C). The findings we observed in infected animals consisted, to varying degrees of focal myocarditis, featuring a few necrotic myocytes with pyknotic nuclei and basophilic cytoplasm, together with slight mononuclear cell infiltrates within or surrounded by necrotic fibres. The degree of myocarditis ranged from minimum to mild for DTU V and VI (scoring 0·5–1), to moderate to intense

Table 1. Primers used in q-PCR assays

| Gene product	Primer	Primer sequence (5′–3′)	Amplicon length (bp)
SNR[a]	F	GCAGTCGGCKGATCGTTTTCG	150
	R	TTCAGRGTTGTTTGGTGTCCAGTG	
CCL-2	F	TGCCCTAAGGTCTTCAGCAC	150
	R	AAGGCATCACAGTCCGAGTC	
CXCL-10	F	GGAGTGAAGCCACGCACAC	65
	R	TGATGGAGAGAGGCTCTCTGC	
Pro-collagen type I	F	TTCACCTACAGCACCCTTGTG	66
	R	GATGACTGTCTTGCCCCAAGTT	
α-SMA	F	GCTCTGCCTCTAGCACACAA	150
	R	GCCAGGGCTACAAGTTAAGG	
Galectin-3	F	GACCACTGACGGTGCCCTAT	149
	R	GGGGTTAAAGTGGAAGGCAA	
TGF-β1	F	TGCGCTTGCAGAGATTAAAA	82
	R	AGGTAACGCCAGGAATTGTTGCTA	
IFN-γ	F	CTTGGATATCTGGAGGAACTGGC	234
	R	GCGCTGGACCTGTGGGTTGTTGA	
IL-10	F	CCAGTTTTACCTGGTAGAAGTGATG	324
	R	TGTCTAGGTCCTGGAGTCCAGCAGACTCAA	
IL-13	F	GACCAGACTCCCCTGTGCAA	121
	R	TGGGTCCTGTAGATGGCATTG	
β-actin (cDNA)	F	CGTCATCCATGGCGAACTG	60
	R	GCTTCTTTGCAGCTCCTTCGT	

[a] SNR, satellite nuclear repeat.
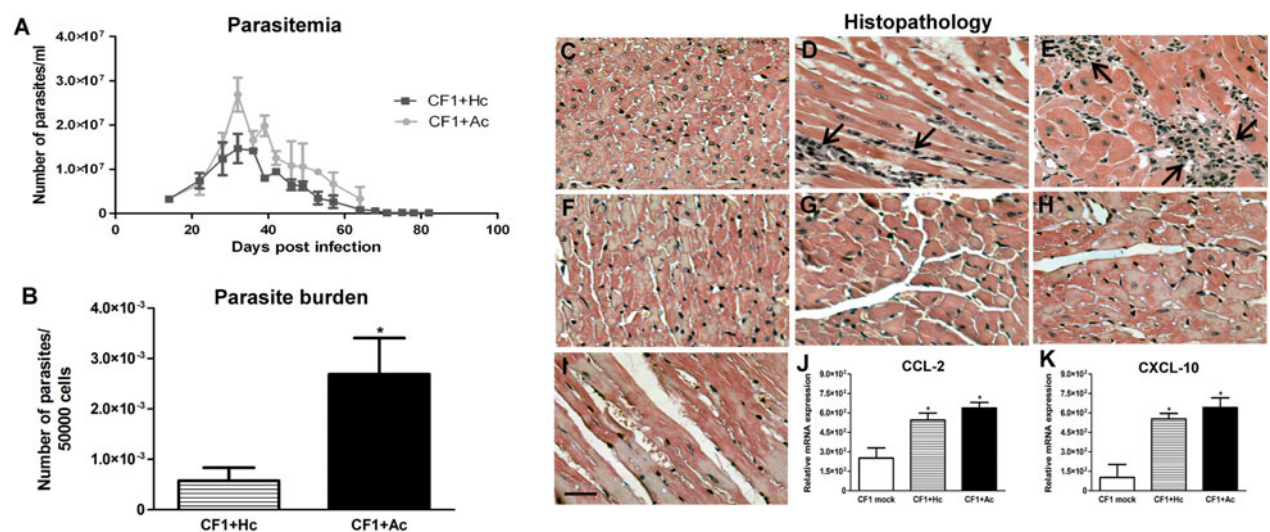
Fig. 1. DTU I isolates induce murine myocarditis to different intensities. (A) Parasitaemia levels were monitored, in *T. cruzi* Hc- and Ac-inoculated CF1 mice, from 14 days post-infection and the number of parasites/mL was calculated. (B) Parasite burden at hearts was quantified by q-PCR of the SNR gene and expressed as number of parasites per number of host cells. Hc- and Ac-infected CF1 mice were euthanased at 3 mpi. Bars represent the S.E.M. for assays performed on a group of five mice. Each organ was analysed independently. mpi, months post-infection. *$P < 0.05$. (C–I) Representative histopathology sections. Samples were harvested and processed for routine staining with HE. (C) Mock-infected heart tissue. (D) *T. cruzi* DTU I (Hc)-infected CF1 mice showed slight myocarditis with minimal mononuclear cell infiltrate (arrows). (E) *T. cruzi* DTU I (Ac)-infected CF1 mice showed a moderate to intermediate myocarditis with predominant mononuclear cell infiltrates localized between necrotic muscle fibres (arrows). (F–H) *T. cruzi* DTU V and (I) *T. cruzi* DTU VI infected-CF1 mice showed normal or minimal myocarditis. Scale bar: 80 μm. (J) Quantitative measurement of CCL-2 mRNA in heart samples from mock-infected or infected animals at 3 mpi. (K) Quantitative measurement of CXCL-10 mRNA in heart samples from mock-infected or infected animals at 3 mpi. Bars represent the S.E.M. of assays from a group of 5 mice. Each organ was analysed in triplicate by q-PCR and normalized to host β-actin expression; *$P < 0.05$ *vs* mock-infected.
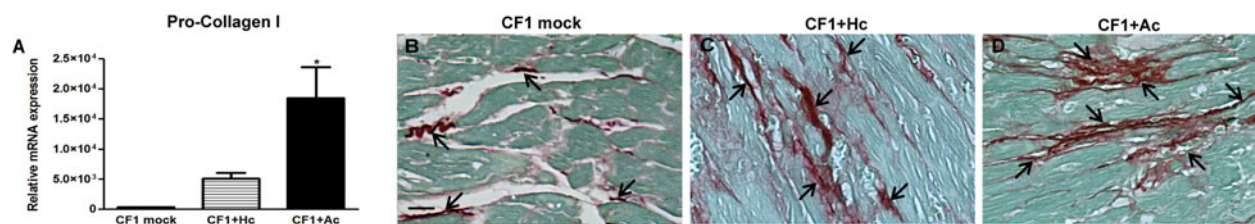
Fig. 2. DTU I isolates induce murine cardiac fibrosis of different intensity. (A) Quantitative measurement of pro-collagen I mRNA in heart samples from mock-infected or infected animals at 3 mpi. Bars represent the S.E.M. of assays from a group of 5 mice. Each organ was analysed in triplicate by q-PCR and normalized to host β-actin expression; *$P < 0.05$; (B–D) PSR staining of representative heart histology sections from *T. cruzi* Hc- and Ac-inoculated CF1 male mice at 3 mpi. As revealed by PSR staining, a collagen matrix made up by fibres was located within the intercellular spaces in; (B) mock-infected; (C) Hc-infected, and; (D) Ac-infected CF1 mice. Scale bar: 80 μm. mpi, months post-infection. Main PSR stains are indicated by arrows.

for both DTU I Hc and Ac strains (scoring 2 and 2·6, respectively; Fig. 1D–I). CCL-2 and CXCL-10 mRNA expression was evaluated as established molecular markers of myocarditis (Hardison *et al.* 2006; Paiva *et al.* 2009). Both Hc- and Ac-infected mice showed increased significant CCL-2 and CXCL-10 mRNA levels compared with mock-infected mice ($P < 0.05$, Fig. 1J and K).

Higher cardiac parasitic burden and pathology were associated with increased fibrosis

To study if cardiac parasitic burden and pathology correlated with fibrosis, we first analysed the levels of pro-Collagen I mRNA in samples from Hc- and Ac-infected mice. The results showed that expression was higher in samples from the Ac-inoculated animals ($P < 0.05$; Fig. 2A). To confirm this, we also employed the PSR staining of cardiac samples from Ac- and Hc-infected animals, as well as of mock-infected controls. The PSR staining showed normal collagen distribution in samples from mock-infected mice (Fig. 2B). In contrast, we observed an enhancement of the collagen matrix in samples from Hc- and Ac-infected animals, represented by increased numbers of collagen fibres in the intercellular spaces and along the major axis of affected myocytes. The ECM remodelling was less intense in samples from Hc-inoculated mice as compared with Ac-inoculated mice, where, in several areas, the collagen pattern had undergone striking alterations resulting in a highly distorted lattice (Fig. 2C and D).

Tc I-induced cardiac ECM remodelling correlates with high levels of Gal-3 and activated myofibroblasts

The relevance of Gal-3 and myofibroblasts in the ECM remodelling has been described, and their enhanced expression and hyperplasia has been associated with liver (Henderson *et al.* 2006) and renal fibrosis (Henderson *et al.* 2008).

Here, we studied the expression of Gal-3 and α-SMA antigen in hearts from mock-, Hc- or

Ac-infected animals. As expected, the results showed a significant increase in the levels of α-SMA ($P < 0.05$; Fig. 3A) and Gal-3 transcripts in samples from Ac-infected mice as compared with the mock-infected controls ($P < 0.01$; Fig. 3E). By immunohistochemistry staining, α-SMA antigen was only detectable in cells located in the tunica media of vessels in the samples from mock-infected mice. Meanwhile, in samples from infected mice, an additional slight staining was observed in mononuclear cells located in the interstitium (Fig. 3B–D), with staining being higher in fibrotic areas. The Gal-3 antigen was primarily detected in cells located in the interstitium, with it also being higher in fibrotic areas (Fig. 3F–H). Interestingly, both transcripts, as well as protein expression levels, were minimal in mock-infected mice, intermediate in Hc-inoculated mice and maximal in Ac-inoculated (Fig. 3A–H).

mRNA cytokine levels in hearts with interstitial fibrosis triggered by Tc I infection

Using q-PCR, we measured the mRNA levels of several cytokines that had been previously reported to have a role in the development of ChHD in the hearts of Ac-infected mice (Araujo-Jorge *et al.* 2012; Calvet *et al.* 2012; Machado *et al.* 2012; Roffe *et al.* 2012). The IL-13 and IFN-γ mRNA expression levels were significantly elevated in Ac-infected CF1 mice at 3 mpi compared with their expression in mock-infected CF1 mice ($P < 0.01$ and <0·05, respectively). In contrast, there were no significant differences in the expression levels of TGF-β1 and IL-10 at 3 mpi when compared to mock-infected mice (Fig. 4A–D).

DISCUSSION

In the present study, we analysed different *T. cruzi* isolates obtained from Argentine patients and genotyped as DTU I, V or VI. Only those belonging to
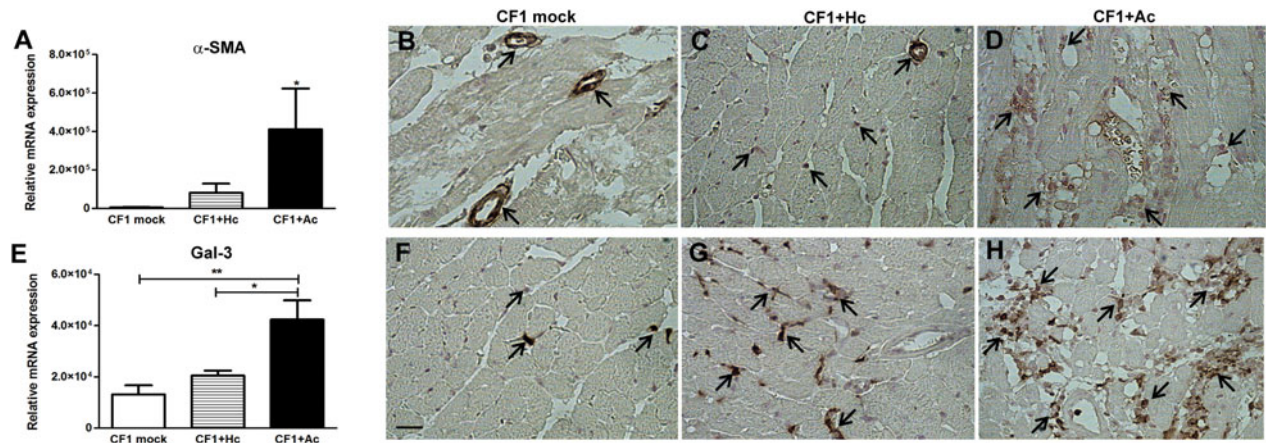
Fig. 3. Gal-3 and α-SMA expression in *T. cruzi* Hc- and Ac-inoculated CF1 male mice at 3 mpi. Quantitative measurements of (A) α-SMA and (E) Gal-3 mRNA expression in heart samples from CF1 mock-infected (CF1 mock) and Hc- and Ac-infected (CF1 + Hc and CF1 + Ac, respectively) animals at 3 mpi. Bars represent the S.E.M. of assays from a group of 5 mice. Each organ was analysed in triplicate by q-PCR and normalized to host β-actin expression; *$P < 0.05$, **$P < 0.01$. Immunohistochemistry of heart sections, from (B and F) mock-,-; (C and G) Hc-, and (D and H) Ac-infected CF1 mice, with antiserum specific for α-smooth muscle actin (α-SMA) or galectin-3 (Gal-3) at 3 mpi. Scale bar: 80 μm. mpi, months post-infection. Positive foci are indicated by arrows.
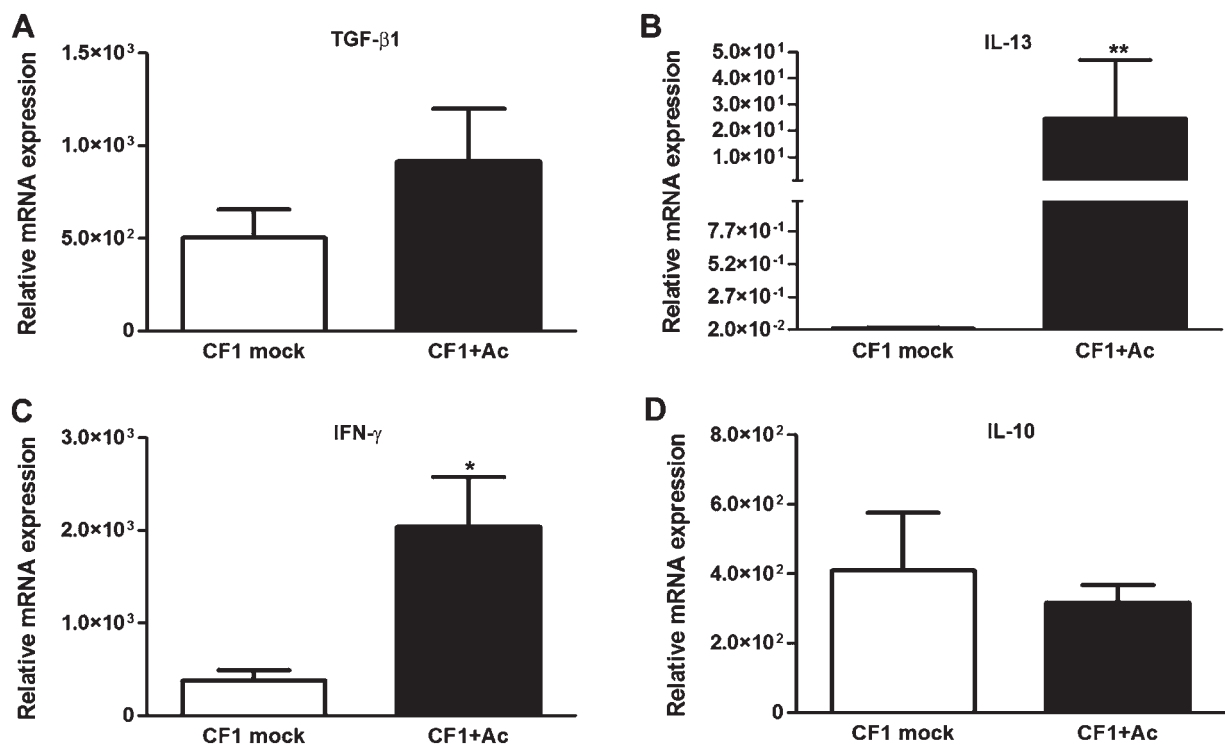

Fig. 4. mRNA cytokine levels in Ac-infected CF1 mice at 3 mpi. Quantitative measurement of (A) TGF-β1, (B) IL-13, (C) IFN-γ and (D) IL-10 mRNA expression in cardiac samples from mock-infected (CF1 mock) or infected (CF1 + Ac) mice. Organs were collected at 3 mpi. Bars represent the S.E.M. of assays performed on samples from a group of 5 mice. Each organ was analysed in triplicate by q-PCR and normalized to host β-actin expression; *$P < 0.05$ and **$P < 0.01$ *vs* controls. mpi, months post-infection.

Tc I progressed towards a chronic infection in mice and had a significant impact on cardiac tissue.

Previously, the Tc I genotype was proposed to be more benign and was not associated with ChHD in South American patients (Feliciangeli *et al.* 2002; Miles *et al.* 2003; Campbell *et al.* 2004). However, in recent years, the persistence of Tc I strains in human cardiac tissue has been correlated with ChHD (Burgos *et al.* 2010; Ramirez *et al.* 2010). Although both Tc I genotypes, analysed here, induced the

persistence of the parasites in cardiac tissue that is associated with chronic myocarditis and significant cardiac ECM remodelling in mice, they differed in the parasite burden and the intensity of the murine ChHD that was induced. These differences may be related to the significant genetic diversity observed in this DTU, which is what prompted the proposal for the subdivision of DTU I (Guhl and Ramirez, 2011).

Taken together, our results suggest that during the host-parasite interplay, in addition to the genetic background of the parasite, other properties are relevant to the outcome of heart disease.

We found that relatively small levels of *T. cruzi* DNA, detected by q-PCR, persisted in diseased heart muscle, as has been reported in previous studies (Brandariz *et al*. 1995). Moreover, the levels of *T. cruzi* burden in CF1 mice correlated with the degree of ECM remodelling induced. These findings are in agreement with the key role of parasite persistence in tissues during the acute phase, and the development of cardiopathy late during infection (Tarleton, 2001, 2003). In this regard, *T. cruzi* persistence may result in cytolysis, which leads to the release of host antigens and large quantities of inflammatory mediators, which can enhance bystander activation (Bonney and Engman, 2008).

In humans, dilation of the left ventricle and cardiac fibrosis are hallmarks of idiopathic dilated cardiomyopathy and ChHD. Enhanced fibrosis is characterized by a disproportionate accumulation of fibrillar collagen, leading to stiffening of the ventricles and impaired diastolic filling (Marin-Neto *et al*. 2007; Rassi *et al*. 2010; Machado *et al*. 2012). The PSR staining was sensitive enough to monitor how collagen fibres accumulated. It could be speculated that such modifications must inevitably affect cardiac function.

Although there have been no previous reports of α-SMA-positive cells in *T. cruzi*-induced ChHD, the presence of myofibroblasts in ChHD was described in an electron microscopy study (Andrade and Grimaud, 1986). As was expected for cells that are considered as the main source of collagen, our study shows that myofibroblasts were colocalized with accumulated collagen, further indicating a relevant role for these cells in the development of ChHD fibrosis. In addition, myofibroblasts-myocyte coupling has the potential to promote reentry-induced arrhythmias (Zlochiver *et al*. 2008), which are frequently observed in ChHD patients. Importantly, myofibroblasts are also a significant source of proinflammatory cytokines, including TNF-α and IL-1β, which have a known deleterious effect on the myocardium (Kubota *et al*. 1997; Cain *et al*. 1999; Haudek *et al*. 2007).

The enhanced expression of Gal-3 that we observed in Tc I-infected animals agrees with previous reports of its upregulated expression following *T. cruzi* infection (Soares *et al*. 2010). Our findings

confirm and extend these results since they were obtained using different mice and *T. cruzi* strains. The parasitological treatment of patients in the indeterminate clinical phase is now recommended to avoid or attenuate cardiac alterations. Therefore, the availability of a biomarker to identify those patients that will progress to ChHD is urgently needed. As such, the results presented here are encouraging for further efforts to evaluate the relevance of Gal-3 in ChHD, since this galectin was proposed as a prognosis marker in HF patients not of chagasic aetiology.

The immunohistochemistry studies showed that the enhanced expression of Gal-3 was primarily located in the cytoplasm of interstitial cells, most likely macrophages (Sato and Hughes, 1994) and/or fibrocytes (Pilling *et al*. 2009). It has been shown that Gal-3 is required for differentiation of cultured hepatic stellate cells into myofibroblasts and that disruption of the *galectin-3* gene blocks myofibroblast differentiation and pro-Collagen I expression, reducing hepatic fibrosis significantly (Henderson *et al*. 2006).

TGF-β1 has an assigned important role in *T. cruzi*-induced myocarditis, induction of myofibroblasts differentiation, and ECM remodelling (Araujo-Jorge *et al*. 2012). However, the TGF-β1 values obtained in our study suggest the possible involvement of other factors, such as the alteration in the ECM components, hyperoxia, and mechanical tension, as have been proposed for other models (Frangogiannis, 2008). Moreover, in a recent *in situ* analysis of cytokines expression, the values for IFN-γ, but not TGF-β1, in hearts from ChHD, showed a correlation with HF and/or fibrosis intensity (Rocha Rodrigues *et al*. 2012). These results are in agreement with the TGF-β1 and IFN-γ expression levels in murine hearts following Ac infection. The upregulation of IFN-γ was also observed using microarrays and immunopathological studies on human cardiomyopathy samples (Bahia-Oliveira *et al*. 1998; Cunha-Neto *et al*. 1998, 2005). It was proposed that the regulatory networks established during the chronic phase in humans are critical for the clinical progression (Dutra *et al*. 2009). The shift towards a proinflammatory pathway was related to the development of cardiomyopathy (Dutra *et al*. 2009). This is in agreement with high IFN-γ expression levels and the unmodified expression of IL-10 observed in our model.

A profibrotic role of IL-13 has been observed in different pathologies. The induction/activation of TGF-β1 through a MMP-9-dependent mechanism has been proposed (Zhu *et al*. 1999; Lee *et al*. 2001). However, the induction of fibrosis by IL-13 in a TGF-β1 independent manner was also described for hepatic fibrosis induced by *Schistosoma mansoni* infection (Kaviratne *et al*. 2004). Our results show fibrosis development in a mixture of cytokines, with

IL-13 upregulated and TGF-β1 maintained at normal levels. Although IL-13 has recently been reported to be elevated after acute *T. cruzi*-induced myocarditis (Roffe *et al*. 2012), to the best of our knowledge, this is the first report of significant upregulation of its mRNA levels in ChHD.

In conclusion, our study shows that Tc I genotypes induce murine ChHD in association with Col I, α-SMA, Gal-3, IFN-γ and IL-13 upregulation. The significant ECM remodelling was associated with Col I-, Gal-3- and α-SMA-positive cells in the myocardial areas of the fibrosis. The Gal-3 upregulation encourages further efforts to evaluate the relevance of Gal-3 in ChHD, since this galectin was proposed as prognosis marker in HF patients. A biomarker is urgently needed to identify patients that evolve to cardiomyopathy and give them anti-parasite treatment. Eventually, the present results may also justify studies using Gal-3 inhibitors to prevent or ameliorate cardiac fibrosis in chagasic patients.

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