Levels of circulating anti-muscarinic and anti-adrenergic antibodies and their effect on cardiac arrhythmias and dysautonomia in murine models of Chagas disease

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SUMMARY

Antibodies (Ab) recognizing G-protein coupled receptors, such as β_1 and β_2 adrenergic (anti-β_1-AR and anti-β_2-AR, respectively) and muscarinic cholinergic receptors (anti-M_2-CR) may contribute to cardiac damage, however their role in chronic chagasic cardiomyopathy is still controversial. We describe that *Trypanosoma cruzi*-infected C3H/He mice show increased P and QRS wave duration, and PR and QTc intervals, while the most significant ECG alterations in C57BL/6 are prolonged P wave and PR interval. Echocardiogram analyses show right ventricle dilation in infected animals of both mouse lineages. Analyses of heart rate variability (HRV) in chronically infected C3H/He mice show no alteration of the evaluated parameters, while C57BL/6 infected mice display significantly lower values of HRV components, suggesting autonomic dysfunction. The time-course analysis of anti-β_1-AR, anti-β_2-AR and anti-M_2-CR Ab titres in C3H/He infected mice indicate that anti-β_1-AR Ab are detected only in the chronic phase, while anti-β_2-AR and anti-M_2-CR are observed in the acute phase, diminish at 60 dpi and increase again in the chronic phase. Chronically infected C57BL/6 mice presented a significant increase in only anti-M_2-CR Ab titres. Furthermore, anti-β_1-AR, anti-β_2-AR and anti-M_2-CR, exhibit significantly higher prevalence in chronically *T. cruzi*-infected C3H/He mice when compared with C57BL/6. These observations suggest that *T. cruzi* infection leads to host-specific cardiac electric alterations.

Key words: *Trypanosoma cruzi*, muscarinic receptors, adrenergic receptors, chronic Chagas disease.

INTRODUCTION

Chagas disease (CD) is a serious public health problem which currently affects about 7·7 million people, mainly in the poorest endemic rural areas in Latin America (World Health Organization, 2010). It is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted by blood-feeding triatomine bugs. Although the transmission of CD has been controlled in several endemic countries, the prevalence of chronic *T. cruzi* infections in non-endemic areas, such as the USA, Japan and Europe has increased substantially in the past 20 years, mainly due to blood transfusion from infected immigrants (Gascon *et al*. 2007; Bern and Montgomery, 2009; Perez-Molina *et al*. 2012). The disease is clinically divided into acute and chronic phases. The initial acute phase lasts 8–10 weeks and is followed by a chronic phase, which is divided into indeterminate, cardiac, digestive or cardio-digestive forms. The indeterminate form is characterized by reactive serology and/or demonstration of the parasite in the blood and also by the absence of clinical and pathological manifestation of heart and/or digestive disorders. Around 30% of infected individuals progress to disease associated with cardiac and/or digestive disorders (Bilate and Cunha-Neto, 2008). The cardiac form, named chronic chagasic cardiomyopathy (CCC), is the most threatening and frequent manifestation of chronic CD (Rassi *et al*. 2000). CCC is characterized by severe myocarditis, T cell-rich lymphomononuclear infiltrates (Reis *et al*. 1993; Brener and Gazzinelli, 1997), interstitial fibrosis (Rossi, 1991; Prata, 2001), autonomic dysfunction (Ribeiro *et al*. 2001) and cardiomyocyte hypertrophy

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that can lead to dilated cardiomyopathy, end-stage heart failure and sudden death (Prata *et al*. 1986; Ribeiro *et al*. 2001).

Several hypotheses have been proposed to account for the pathogenesis of CCC; among these are parasite persistence in target tissues (Higuchi, 1995) and autoimmune events (Cunha-Neto *et al*. 2006). The parasite persistence theory affirms that tissue parasitism is directly related to tissue damage and would be a prerequisite for the development of CCC. The relatively low number of parasites in the myocardium and the presence of auto-reactive antibodies (Ab) support the autoimmune theory. These Ab can be derived by molecular mimicry between parasite and host antigens or by antigen exposure due to cardiac damage (Cossio *et al*. 1974; Kierszenbaum, 1985; Leon and Engman, 2003; Iwai *et al*. 2005). The emergence of these Ab can be responsible for the destruction of cardiac conduction tissues and cardiac autonomic nerves observed during the chronic state of the disease (Koberle, 1970; Thiers *et al*. 2012).

Several authors have reported the presence of circulating Ab in the sera of animals and patients affected by CCC (de Oliveira *et al*. 1997; Labovsky *et al*. 2007; Hernandez *et al*. 2008) and dilated cardiomyopathy (Jahns *et al*. 1999, 2004; Stork *et al*. 2006; Dandel *et al*. 2012). These Ab are able to interact with the second extracellular loop of G-protein coupled receptors, such as β_1 and β_2 adrenergic (anti-β_1-AR and anti-β_2-AR, respectively) and muscarinic cholinergic receptors of the myocardium (anti-M_2-CR) (Wallukat *et al*. 1995; Elies *et al*. 1996; Jahns *et al*. 1999; Escobar *et al*. 2006) and ultimately lead to receptor activation (Iwata *et al*. 2001; Feldman *et al*. 2005). The presence of such functionally active, receptor-stimulating Ab is associated with a markedly worse prognosis in dilated cardiomyopathy (Schulze *et al*. 2005). The continuous stimulation of the receptors by the Ab could induce desensitization and/or down-regulation of the receptor, explaining the progressive loss of function and consequent autonomic disturbance observed in CD patients (Sterin-Borda and Borda, 2000). In fact, the presence of muscarinic cholinergic receptor activating antibodies in patients' sera have been shown to induce complex cardiac arrhythmias and AV conduction block in isolated rabbit hearts (de Oliveira *et al*. 1997). Ribeiro *et al*. (2007) showed that vagal impairment, evidenced by reduced indexes of heart rate variability (HRV), occurs early in the course of infection, i.e. before the appearance of left ventricle (LV) dysfunction, and that it is correlated to the levels of anti-M_2-CR Ab. Circulating autoantibodies with partial muscarinic cholinergic agonistic activity have also been found in CD patients in the indeterminate form, in the absence of ECG and X-ray alterations (Borda and Sterin-Borda, 1996). Together, these data suggest that the circulating reactive Ab can have a causal role in the cardiac alterations and dysautonomia observed in Chagasic patients.

To investigate more rigorously the relation between the presence of the Ab and the occurrence of cardiac disturbances, and the influence of the host in disease pathogenesis in the present report we: (a) investigated using ELISA the time-course of anti-β_1-AR, anti-β_2-AR and anti-M_2-CR Ab appearance in the sera of C3H/He *T. cruzi*-infected mice in the acute and chronic phases of the disease and (b) determined the levels of reactivity to β_1-AR, β_2-AR and M_2-CR of the serum of chronically infected C3H/He and C57BL/6 mice and their correlation to cardiac function evaluated by histopathological alterations, echocardiography (ECHO), electrocardiography (ECG) and heart rate variability indexes (HRV).

MATERIALS AND METHODS

Animals and parasite infection

All experiments were performed with 5–7-week-old female C3H/He ($H\text{-}2^K$) or C57BL/6 (H-2) mice from our animal facilities (CECAL, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). The animals were maintained under standard conditions and treated according to institutional guidelines regarding ethics of animal usage (CEUA-Fiocruz, protocol #161/03). Mice were infected intraperitoneally with 100 blood trypomastigote forms of the low virulence *T. cruzi* Colombian strain isolated from a Colombian chagasic patient obtained by serial passages from mouse to mouse (Marino *et al*. 2004). Parasitaemia was estimated from 5 μL of tail vein blood and established as a parameter for acute and chronic phases (dos Santos *et al*. 2001). Analyses in the acute phase were done at 30 and 60 days post infection (dpi) and included histopathology and ELISA; while the chronic phase analyses (120–150 dpi) included histopathology, ELISA, ECG, ECHO and HRV indexes (see descriptions below).

Histopathology

Left ventricles (LV) were excised and fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections were stained with haemotoxylin and eosin (H&E) and with picrosirius and evaluated by light microscopy.

Anti-β1-AR, anti-β2-AR and anti-M2-CR Ab detection

ELISA plates were coated with 20 $\mu g\,mL^{-1}$ of synthetic peptides comprising the second extracellular loops of the β_1, β_2 or M_2 cardiac receptors (Ribeiro *et al*. 2010; Daliry *et al*. 2014) in 0·1 M

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Na_2CO_3 or buffer alone for 16 h at 4 °C. After saturation of the wells with PBS/0·1% Tween/2% BSA, mouse sera from control or from infected animals were diluted 1:100 in PBS/0·05% Tween and added to the wells. After incubation for 2 h at room temperature (RT), bound antibodies were detected by a secondary anti-mouse IgG antibody labelled with peroxidase, diluted 1:5000 in PBS/0·1% Tween/2% BSA. Between each step, plates were washed 4× with PBS/0·05% Tween. Afterwards, 100 μL of TMB substrate solution was dispensed into the wells. The plates were covered and incubated for 5 min at RT, in a dark room. The enzyme reaction was stopped by addition of 100 μL stop solution (1 N HCL) to each well. The absorbance was read at 450 nm. ELISA values were expressed as the ratio (R) between the optical densities (OD) determined for each sample and cut-off values. Cut-off was the mean OD of non-infected animals plus 2 standard deviations (S.D.). Positivity was defined as R > 1·2 (Daliry *et al*. 2014).

Electrocardiography

For the analysis of P duration, PR interval, QRS duration and QTc in the infected animals during the chronic phase the following methodology was used: all mice were intraperitoneally injected with diazepam (10 mg kg^{-1}) and the electrodes were carefully placed subcutaneously according to the chosen preferential derivation (DII). ECGs were recorded for at least 2 min using a digital system Power Lab 2/20 that was connected to a bio-amplifier (PanLab Instruments, Barcelona, Spain). Filters were standardized to 0·1–100 Hz and traces were analysed using the Scope software for Windows V3.6.10 (PanLab Instruments, Barcelona, Spain). Heart rate (HR) (in beats per minute, bpm) and duration of P wave, QRS, PR and QT intervals (in milliseconds, ms) were measured. The relationship between the QT interval and the RR interval in the mouse was assessed in all animals. To obtain physiologically relevant values for the heart rate-corrected QT interval (QTc) in units of time (rather than time to a power that is not equal to 1), the observed RR interval (RR0) was first expressed as a unitless multiple of 100 ms, yielding a normalized RR interval, RR100 = RR0/100 ms. Next, the value of the exponent (y) in the relationship $QT0 = QTc \times RR100^{y}$ was assessed, with QT0 indicating the observed QT (in ms) and the unit for QTc being milliseconds. The natural logarithm was computed for each side of this relationship [ln (QT0) = In (QTc) + y ln (RR100)]. Thus, the slope of the linear relationship between the log-transformed QT and RR100 defined the exponent to which the RR interval ratio should be raised to correct QT for HR (Silverio *et al*. 2012).

For the analysis of the HRV indexes the ECG recordings used were acquired based on the following methodology: recording was carried out in conscious animals by a non-invasive method. Electrodes were positioned in DI derivation and connected by flexible cables to a differential AC amplifier (model 1700, A-M Systems, USA), with signal low-pass filtered at 500 Hz and digitized at 1 kHz by a 16-bit A/D converter (Minidigi 1-D, Axon Instruments, USA) using Axoscope 9.0 software (Axon Instruments, USA). Data were stored in a PC for offline processing.

Transthoracic echocardiography (ECHO)

For analysis of cardiac function, mice were anaesthetized with 1·5% isoflurane in 100% O_2, trichotomized in the precordial region and examined with a Vevo 770 ultrasound apparatus from Visual Sonics (Canada) coupled to a 30 MHz transducer. Left ventricular ejection fractions (LVEF) were determined using Simpson's method, and left and right ventricular area (LV and RV) were obtained in B-mode using a short axis view at the level of the papillary muscles.

HRV indexes

For HRV analyses, stable 60 s segments were extracted from 180s ECGs acquired in the conscious state, and, in order to allow for a more accurate R wave peak detection process, all signals were resampled by cubic spline interpolation at 10 kHz. Baseline drift was subtracted from the 10 kHz ECG signals and, after R wave peak detection, 60 s tachograms were generated, containing all heart period fluctuations within this time segment. In the time domain, the following indexes were obtained: HR, standard deviation of the RR intervals (SDNN) and square root of the mean squared differences of successive RR intervals (RMSSD). For spectral (frequency domain) analysis of HRV, beat-by-beat HR time series were resampled to equal intervals by the spline cubic interpolation method, at 20 Hz, and the linear trend was removed. Power spectrum was obtained using a fast Fourier transform-based method (Welch's periodogram: 512 points, 50% overlap and Hanning window), and high-frequency power (HF: 1–8 Hz) was estimated as the area under the spectrum within this frequency range, being expressed as ln bpm^2.

Statistical analysis

Data are expressed as mean ± S.D. Analysis was performed using GraphPrism (GraphPad, San Diego, CA, USA). Comparison between groups was carried out by analysis of variance (ANOVA) followed by

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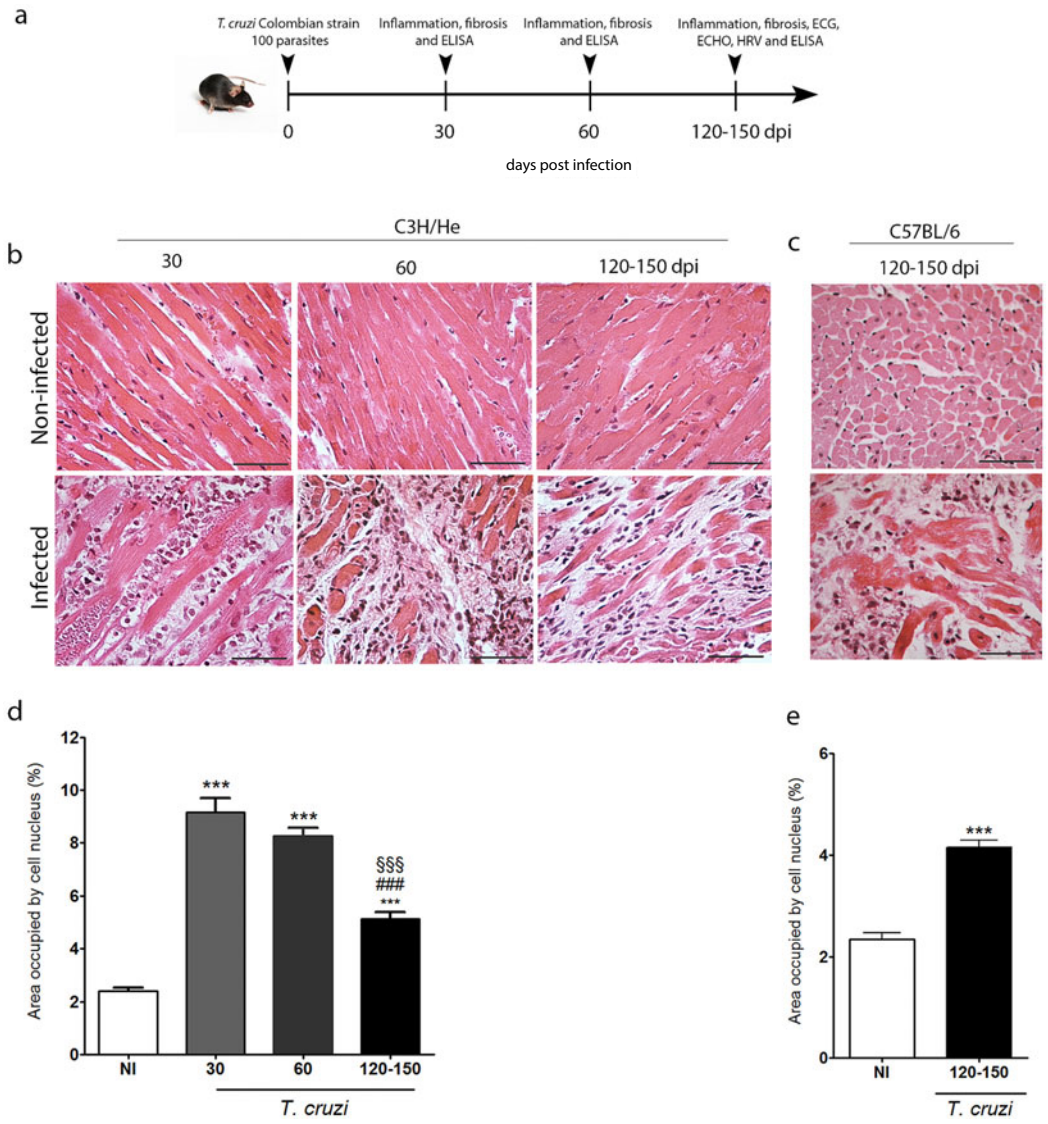

Fig. 1. Histopathological analysis of the cardiac tissue of *T. cruzi*-infected mice. Panel (a) shows the general scheme of the study; (b) representative photomicrography of left ventricle of *T. cruzi*-infected C3H/He mice showing mononuclear inflammatory cells (Bar = 50 μm); (c) representative photomicrography of left ventricle of *T. cruzi*-infected C57BL/6 mice at the chronic phase showing mononuclear inflammatory cells (scale bar = 50 μm); (d) quantification of the number of mononuclear inflammatory cells of left ventricle of *T. cruzi*-infected C3H/He mice at 30, 60 and 120–150 days post infection (d.p.i.) and (e) quantification of the number of mononuclear cells of left ventricle of *T. cruzi*-infected C57BL/6 mice at 120–150 d.p.i. Data are represented as mean ± S.D. $^{***}P < 0{\cdot}001$ *vs* non-infected (NI) group, $^{\#\#\#}P < 0{\cdot}001$ *vs* 30 dpi, $^{\S\S\S}P < 0{\cdot}001$ *vs* 60 dpi. Abbreviations: Electrocardiography (ECG), echocardiography (ECHO) and heart rate variability analyses (HRV).

Bonferroni's post-test or Student's *t*-test were indicated. Probability values were considered significant when $P < 0{\cdot}05$.

RESULTS

Figure 1a shows the experimental design of the present study. We first evaluated the number of mononuclear cells in the myocardium of *T. cruzi*-infected-C3H/He mice during the course of infection (Fig. 1b and d) and in chronically infected C57BL/6 mice (Fig. 1c and e). During the acute infection (30 dpi), we detected intense mononuclear inflammatory infiltrates in the myocardium of C3H/He mice. These inflammatory infiltrates persisted at 60 dpi and decreased in intensity in the chronic phase of infection, although were still significantly higher than non-infected controls (Fig. 1b and d). A similar increase in inflammatory infiltrates was observed in chronically infected C57BL/6 mice (Fig. 1c and e) when compared with C3H/He mice at the same stage of infection.

The fibrotic area was evaluated by collagen deposition (picrosirius staining), which in *T. cruzi*-infected C3H/He mice showed a significant increase in the acute and chronic phases of infection (Fig. 2a

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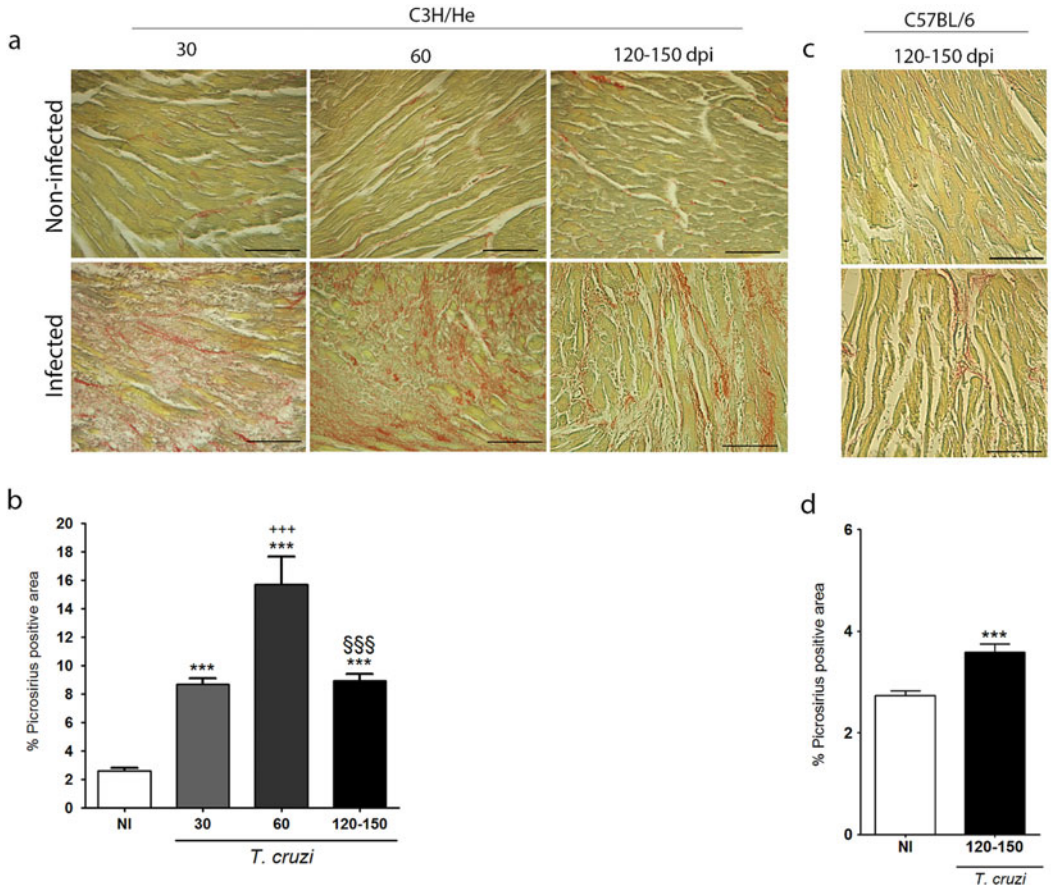

Fig. 2. Histopathological analysis of fibrotic area of cardiac tissue of *T. cruzi*-infected mice stained with picrosirius red: (a) representative photomicrography of left ventricle of *T. cruzi*-infected C3H/He mice showing fibrotic area; (b) quantification of the fibrotic area of the *T. cruzi*-infected C3H/He mice at 30, 60 and 120–150 d.p.i.; (d) representative photomicrography of left ventricle of *T. cruzi*-infected C57BL/6 mice at the chronic phase showing fibrotic area and (e) quantification of the fibrotic area of left ventricle of *T. cruzi*-infected C57BL/6 mice at 120–150 d.p.i. Data are represented as mean ± S.D. $^{***}P<0{\cdot}001$ *vs* non-infected (NI) group, $^{+++}P<0{\cdot}001$ *vs* 30 dpi, $^{§§§}P<0{\cdot}001$ *vs* 60 dpi.

and b). The percentage of picrosirius positive area was higher at 60 dpi in C3H/He infected mice (Fig. 2b). In chronically infected C57BL/6 mice, there was also a significant increase in fibrotic area in comparison with non-infected controls (Fig. 2c and d).

We then analysed the ECG alterations induced by *T. cruzi* infection in the chronic phase in both mouse strains (Fig. 3a–d). *Trypanosoma cruzi*-infected C3H/He mice showed a substantial increase in P wave and QRS duration, and prolonged PR and QTc intervals (Fig. 3a, b, c and –d, respectively). In contrast, C57BL/6 mice only showed a significant increase in P wave duration and PR interval (Fig. 3a and b, respectively).

The analyses of HRV of chronically *T. cruzi*-infected C3H/He mice showed no alteration in any of the evaluated parameters (Fig. 4a–d), while infected C57BL/6 mice showed significantly lower values of many components of HRV: HR (Fig. 4a), SDNN (Fig. 4b), RMSSD (Fig. 4c) and HF (Fig. 4d).

The echocardiogram analyses were performed in chronically infected mice from both lineages (Fig. 5a–c). The LVEF and left ventricular area (LV) were not affected by *T. cruzi*-infection in mice of both lineages (Fig. 5a and b, respectively), but there was a significant enlargement of the right ventricle in chronically infected C3H/He and C57BL/6 mice (Fig. 5c).

We further analysed the anti-β_1-AR, anti-β_2-AR and anti-M_2-CR Ab titres of C3H/He infected mice during the course of infection (Fig. 6a–c, respectively). Significantly higher levels of anti-β_1-AR Ab were detected only in the chronic phase in C3H/He mice (Fig. 6a). However, much higher levels of anti-β_2-AR and anti-M_2-CR Ab were observed in the acute phase (30 dpi), diminishing to non-significant levels at 60 dpi and increasing again in the chronic phase (Fig. 6b and c). Analyses of Ab titres in C57BL/6 mice were performed only in the chronic phase of infection (Fig. 6a–c). There were no differences in anti-β_1-AR and anti-β_2-AR Ab titres in *T. cruzi*-infected C57BL/6

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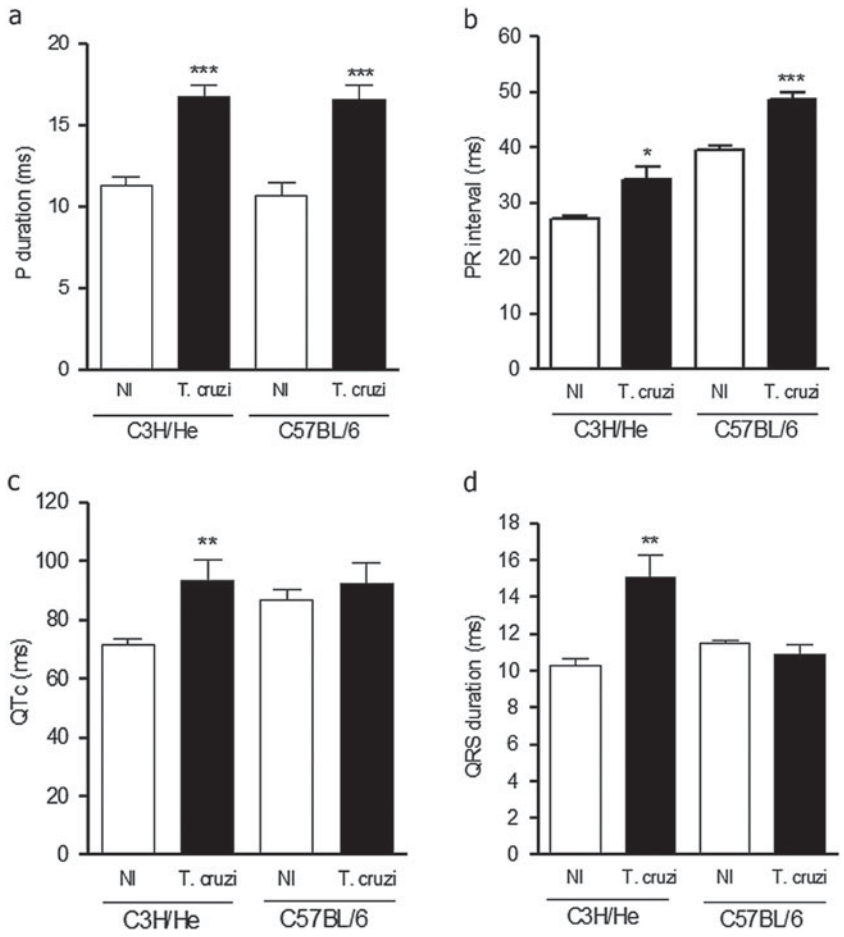

Fig. 3. Electrocardiographic analyses of *T. cruzi*-infected C3H/He and C57BL/6 mice: (a) P duration (ms); (b) PR interval (ms); (c) QTc (ms) and (d) QRS duration (ms). ***$P<0{\cdot}001$ *vs* non-infected (NI) group, **$P<0{\cdot}01$ *vs* NI group and *$P<0{\cdot}05$ *vs* NI group.

mice when compared with the non-infected group (Fig. 6a and b, respectively), but anti-M_2-CR Ab titres were higher in *T. cruzi*-infected C57BL/6 mice when compared with their respective non-infected controls (Fig. 6c). Comparing Ab titres in *T. cruzi*-infected mice of both mouse lineages in the chronic phase all three Ab evaluated, namely anti-β_1-AR, anti-β_2-AR and anti-M_2-CR, presented significantly higher prevalence in infected C3H/He mice when compared with C57BL/6 (Fig. 6a–c).

DISCUSSION

The physiopathology of Chagas disease, particularly with regard to chagasic cardiomyopathy, is very complex and not completely understood. It is well accepted that the balance between parasite invasiveness and the host immune response plays a major role in the development and evolution of the acute and chronic manifestations of CD. Additionally the presence of auto-reactive antibodies that recognize cardiac epitopes adds even more complexity to the disease. However, the exact contribution of each of the components of the disease – parasite invasiveness, inflammatory responses and autoimmunity – is difficult to evaluate, especially in human patients. Trying to help understand this issue, we infected two different lineages of mice – C3H/He and C57BL/6 – with the same *T. cruzi* strain and the same number of parasites, and evaluated heart morphology and function, HRV indexes and their relation to anti-muscarinic and anti-adrenergic Ab levels.

During the chronic phase, cardiac inflammatory infiltrates, fibrotic area deposition and echocardiogram parameters in both lineages presented similar patterns in response to parasite infection. ECG analyses showed prolonged P wave and PR interval in infected mice of both lineages, which is a common finding in experimental infection and in human patients with CD (Williams-Blangero *et al*. 2007; Eickhoff *et al*. 2010). Interestingly, QRS and corrected QT (QTc) intervals significantly increased only in infected C3H/He mice. The differences in ECG abnormalities between the mouse lineages infected with the same *T. cruzi* strain shown here, suggest that the degree of electrical cardiac dysfunction is dependent not only on the *T. cruzi* strain

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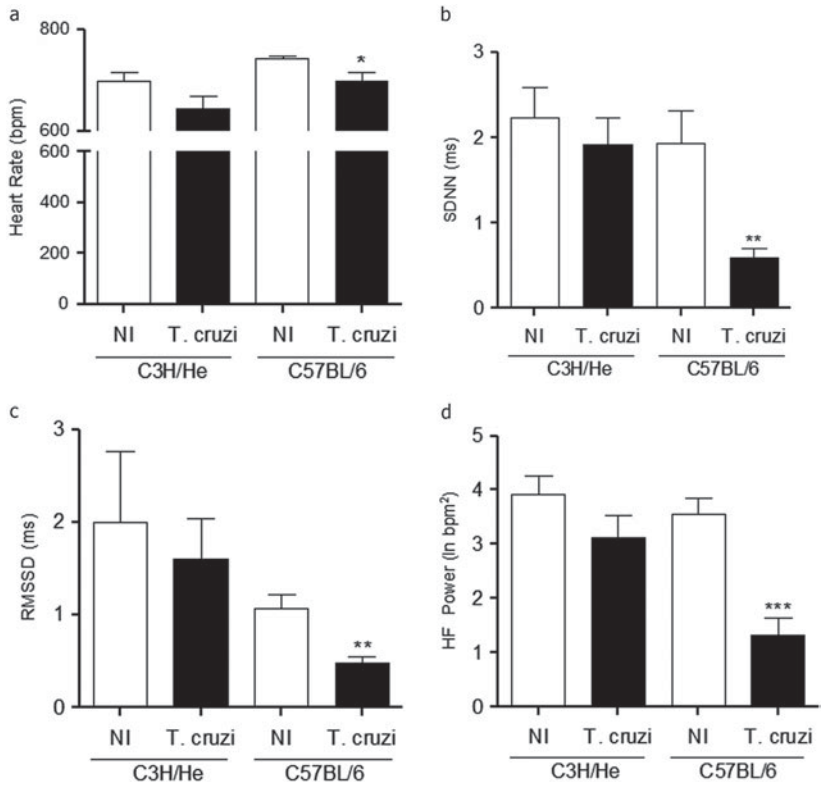

Fig. 4. Heart rate variability (HRV) analysis of *T. cruzi*-infected C3H/He and C57BL/6 mice: (a) Heart rate (bpm); (b) standard deviation of NN intervals (SDNN) (ms); (c) root mean square of successive differences (RMSSD) (ms) and (d) HF power (ln bpm^2). ***$P<0{\cdot}001$ *vs* non-infected (NI) group, **$P<0{\cdot}01$ *vs* NI group and *$P<0{\cdot}05$ *vs* NI group.

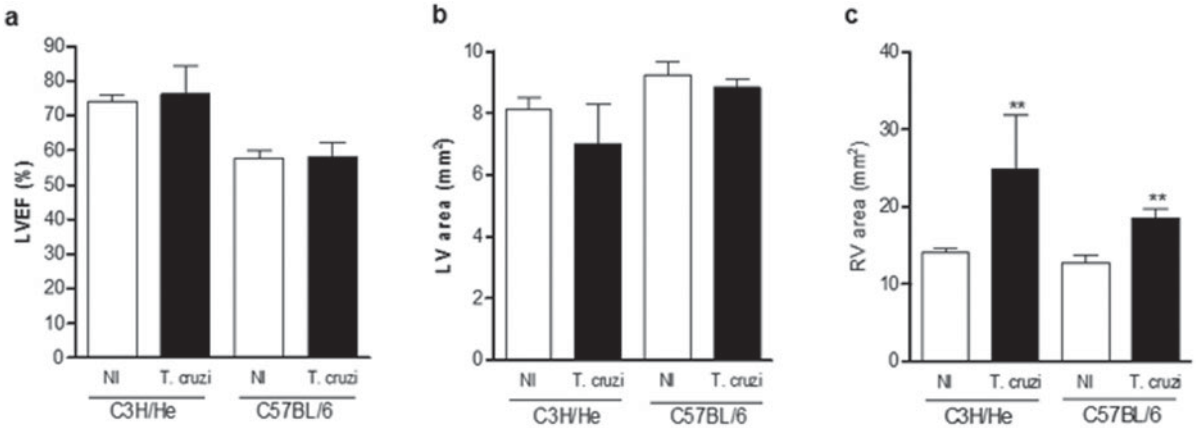

Fig. 5. Cardiac function assessed by echocardiogram of *T. cruzi*-infected C3H/He and C57BL/6 mice: (a) Percentage of left ventricle ejection fraction (% LVEF); (b) left ventricle area (mm^2) and (c) right ventricle area (mm^2). **$P<0{\cdot}01$ *vs* non-infected (NI) group.

(Eickhoff *et al*. 2010; Daliry *et al*. 2014) but also on the host genetic background.

We next detected the presence and titres of anti-β_1-AR, β_2-AR and M_2-CR Ab in the sera of the infected animals. We found that anti-β_1, anti-β_2 and anti-M_2 Ab titres were higher in chronically infected C3H/He mice than in C57BL/6 mice and non-infected controls, while only anti-M_2 titres were significantly high in infected C57BL/6 animals when compared with non-infected controls. Since the number of ECG alterations was higher in infected C3H/He mice, our findings suggest that the degree of ECG abnormalities could be related to the presence of circulating levels of those Ab. This hypothesis is reinforced by our previous study with *T. cruzi*-infected dogs (Daliry *et al*. 2014). The correlation between Ab presence and ECG alterations can be attributed to the arrhythmic effect of those Ab in CCC, as previously shown (Iwata *et al*. 2001; Jahns *et al*. 2004; Medei *et al*. 2008). Escobar *et al*. (2006)

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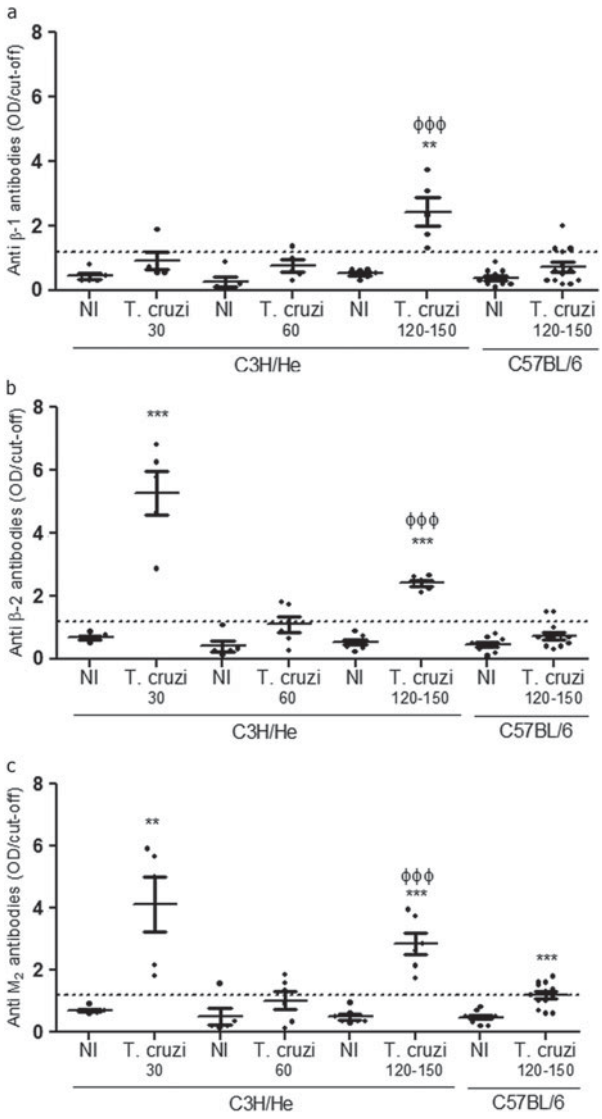

Fig. 6. Autoantibodies titres evaluated by ELISA during the course of infection of *T. cruzi*-infected C3H/He and C57BL/6 mice: (a) anti-β_1-AR antibodies titres of C3H/He and C57BL/6 non-infected and infected mice; (b) anti-β_2-AR autoantibodies titres of C3H/He and C57BL/6 non-infected and infected mice and (c) anti-M_2-CR autoantibodies titres of C3H/He and C57BL/6 non-infected and infected mice. $^{***}P<0{\cdot}001$ *vs* non-infected (NI) group, $^{**}P<0{\cdot}01$ *vs* NI group and $^{\phi\phi\phi}P<0{\cdot}001$ *vs* C57BL/6 infected group.

found that anti-β_2-AR reactivity induced conduction blocks in isolated heart mouse preparations, suggesting that these antibodies could be responsible for ventricular arrhythmias (Escobar *et al*. 2006).

It is interesting to note that the same *T. cruzi* strain induced production of distinct levels of circulating antibodies recognizing the β_1-AR, β_2-AR and M_2-CR in the two mouse lineages. This indicates that there is not only a *T. cruzi* strain-specific modulation of antibody titres – observed previously in infected dogs (Daliry *et al*. 2014) – but also a mouse lineage-specific modulation of Ab production.

Although presenting positivity for anti-β_1, anti-β_2 and anti-M_2 in the chronic phase, *T. cruzi*-infected C3H/He animals did not show any HRV index alteration. Chronically infected C57BL/6 mice, which displayed only anti-M_2-CR Ab, presented a decrease in all HRV indexes analysed, suggestive of dysautonomia. Several reports have shown the presence of circulating anti-M_2 Ab concomitant with autonomic dysfunction, suggesting that those Ab could have a causal effect on clinical manifestations of CD (Goin *et al*. 1994, 1999; Talvani *et al*. 2006; Ribeiro *et al*. 2007). In fact, autonomic disorders have been described before the occurrence of LV dysfunction in patients and even in the indeterminate phase of CD, suggesting that it appears early during the infection, before any cardiac structural and functional alteration can be detected (Ribeiro *et al*. 2007). In agreement with that, we detected anti-M_2 Ab in the serum of C3H/He *T. cruzi*-infected mice at 30 dpi, and similarly in a canine model of CD infected with three different *T. cruzi* strains (Daliry *et al*. 2014).

Another interesting finding of the present report is that the presence of anti-β_1 and anti-β_2-CR Ab, concomitant with anti-M_2 Ab, did not alter HRV indexes, suggesting that the presence of both anti-adrenergic and anti-cholinergic Ab may balance each other's effects, resulting in no autonomic dysfunction.

There are two possible explanations for the effect of anti-M_2 Ab on lowering HRV indexes: (1) impairment of vagal-mediated autonomic modulation of the heart and (2) enhanced parasympathetic modulation of the sinus node. Since we found a significant HR reduction in *T. cruzi*-C57BL/6 infected mice, this could only result from parasympathetic hyperstimulation. However, in chagasic patients Ribeiro *et al*. (2007) found that reduced HRV was not accompanied by reduced HR and that anti-M_2-CR Ab titres did not correlate with basal HR. Based on these findings they stated that vagal enhancement remains a theoretical hypothesis that still needs demonstration. Additional experiments are necessary to establish if these differences are due to species-specific effects.

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