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The sympathetic nervous system affects the susceptibility and course of *Trypanosoma cruzi* infection

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*) is an intracellular parasite that causes Chagas' disease, a major health problem in Latin America. Using a murine model of infection with this parasite, we have previously shown that corticosterone blood levels are markedly elevated during the course of the disease in C57Bl/6 male mice and that this increase is protective for the host by restricting the production of pro-inflammatory cytokines. Since the hypothalamus-pituitary-adrenal (HPA) axis usually operates in a concerted way with the sympathetic nervous system (SNS), we have now studied whether noradrenergic nerves can affect the course of *T. cruzi* infection and the sexual dimorphism observed in the disease. We found a decreased splenic noradrenaline concentration and content, paralleled by a reduction in noradrenergic nerve fibers in the spleen of infected mice, and increased HPA axis activity. These alterations were more marked in males than in females. When the spontaneous loss of noradrenergic nerve fibers was advanced by chemical sympathectomy prior to infection, males died earlier and mortality significantly increased in females. Chemical denervation did not significantly affect the concentration of specific IgM and IgG_{2a} antibodies to *T. cruzi*, and did not worsen myocarditis, but resulted in increased parasitemia and IL-6 and IFN-γ blood levels. The results obtained in this model of parasitic disease provide further indications of the relevance of interactions between the immune system and the SNS for host defense.

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1. Introduction

Trypanosoma cruzi (*T. cruzi)* is an intracellular parasite that causes Chagas' disease, also called American trypanosomiasis, a major health problem in Latinamerican countries, where it currently affects around 10 million people. However, the disease is rapidly extending to Europe, USA, Australia and Japan (Basile et al., 2011; Bonney, 2014; Dutra et al., 2014), becoming a global health problem in this century. In humans, the disease occurs in two phases: an acute stage, manifested shortly after the infection, and a chronic one that may develop over 10 years. Chronic infections result in neurological disorders, damage to the heart muscle, and sometimes dilation of the digestive tract (megacolon and megaesophagus). Left untreated, Chagas' disease can be fatal, in most cases due to the cardiac sequelae. We have developed a murine model of *T. cruzi* infection based on the inoculation of only 200 parasites into C57Bl/6 mice, which leads to a progressive and lethal disease in males, with profound thymic atrophy and loss of $CD4^+CD8^+$ thymocytes, and increased levels of pro-inflammatory cytokines and antibodies against parasite antigens (Roggero et al., 2002; Perez et al., 2005). A strong stimulation of the hypothalamus-pituitary-adrenal (HPA) axis was also detected in infected C57Bl/6 mice, as indicated by a more than 10-fold increase in corticosterone blood levels (Roggero et al., 2006). Interference of glucocorticoid effects, either by administration of a glucocorticoid receptor antagonist or following adrenalectomy, results in increased levels of pro-inflammatory cytokines and in earlier death of infected male mice. These results indicate that neuro-endocrine mechanisms are relevant for the susceptibility and course of the disease caused by *T. cruzi*.

The HPA axis usually operates in a concerted way with the sympathetic nervous system (SNS), and noradrenaline (NA), the main neurotransmitter released by sympathetic nerves, can affect different types of immune cells and immune responses in a selective way (for review del Rey and Besedovsky, 2008). On these bases, we have now studied the possibility that the SNS influences the immune response to *T. cruzi* and the course of the disease induced by this parasite in mice. For this purpose, we have first evaluated NA concentration in the spleen of mice infected with *T. cruzi* and the consequences that interfering with the activity of the SNS have for the course of the disease. Our initial studies were done in males. When we extended our studies to females, a significant proportion of mice survived after inoculation of the same number of parasites that is lethal for males. Thus, we also analyzed whether there were differences in the SNS of

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T. cruzi-infected male and female mice, and studied the possibility that sympathectomy affects the course of the disease and the sexual dimorphism in the susceptibility to infection with the parasite.

2. Materials and methods

2.1. Parasite, mice, and infection

The Tulahuén strain of *T. cruzi* used in this study was maintained by serial passages in Balb/c suckling mice. C57Bl/6 and Balb/c mice were bred at the animal facilities of the Medical Faculty of Rosario, Argentina. Mice were housed individually for one week before experiments were started and kept single-caged throughout the experiments in temperature-, humidity- and light (12 h cycles)-controlled rooms. Two hundred trypomastigotes suspended in 100 µl of physiological saline (0.9% NaCl) were injected subcutaneously (s.c., 50 µl in each flank) when mice were 8–10 week-old. Control mice received the same amount of physiological saline. The studies were approved by the responsible agency (Ethical Committee for Scientific and Technical Research, CEICyT-UAI, Argentina).

2.2. Evaluation of parasitemia

Bloodstream forms of *T. cruzi* were counted under standardized conditions by direct microscopic observation of 5 µl heparinized blood obtained from the tip of the tail. Data are expressed as number of parasites/50 fields in 40× magnification.

2.3. Sympathetic denervation

Groups of mice were sympathetically denervated at birth by intraperitoneal (i.p.) injection of 6-hydroxydopamine hydrochloride (6-OHDA; Sigma-Aldrich; 150 mg/kg dissolved in 0.01% ascorbic acid; 1 injection per day over 5 consecutive days, starting when mice were less than 24 h old). Controls received the vehicle alone.

2.4. Corticosterone determination

Plasma samples for hormone determinations were obtained from the tip of the tail under light ether narcosis between 8 and 10 a.m. before (day 0) and 10, 14 and 17 days post infection (p.i.). Blood samples were also obtained from age- and sex-matched controls subjected to the same experimental conditions. Plasma corticosterone levels were determined by ELISA, using the kits and prescriptions provided by the manufacture (IBL International GmbH, Germany).

2.5. NA and adrenaline (A) determinations

NA, precursor and metabolite determinations in the spleen, and catecholamine concentrations in the adrenal gland and plasma were performed by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (del Rey et al., 2006), using the supernatant of tissue samples homogenized in 0.4 M $HClO_4$. Blood plasma samples underwent a purification step using alumina adsorption prior to HPLC determination. Quantification was done by peak height evaluation using an evaluation software (Chromeleon version 6.08; Dionex, Sunnyvale, CA).

2.6. Spleen cellularity

The spleen was weighted and cell suspensions were prepared from approximately one-third of the organ and counted in a Neubauer chamber at appropriate dilutions.

2.7. Tyrosine-hydroxylase staining by immunofluorescence

Spleens were removed 17 days after infection, embedded in Tissue-Tek (Miles Inc., Elkhart, USA) and frozen in liquid nitrogen. Three serial 10 µm-sections from the proximal third of the spleen of 5 mice per group were cut using a freezing microtome, fixed in acetone, and washed three times with PBS. Tissue sections were incubated with sheep anti-tyrosine hydroxylase (Chemicon, USA) overnight at 18 °C, followed by 2 h incubation at 4 °C. After washing with PBS, sections were incubated with donkey anti-sheep-biotin and streptavidin FITC (Dianova, Hamburg, Germany) for 45 min at 25 °C. No binding was detected in the absence of the primary antibody. Following PBS washes, the sections were mounted in 25% glycerol/75% PBS. Ten fields per section were examined with a confocal microscope (Nikon eclipse TE2000-E inverted microscope, D-eclipse C1si, Melville, New York), and representative fields were photographed.

2.8. Evaluation of myocarditis

Evaluation of myocarditis was performed as previously described (Roggero et al., 2002) with slight modifications. Briefly, hearts were removed on day 17 p.i., divided transversally into two parts, and fixed in Bouin-Hollande. Paraffin-embedded 5 µm sections were stained with haematoxylin and eosin. Foci of myocarditis were evaluated by an experienced pathologist blinded to the study groups, and scored as follows: a) small-sized foci, slight infiltration with damage of one or two myocardial fibers (score 1); b) medium-sized foci, aggregated infiltrates compromising three to five muscle fibers (score 2); and c) large-sized foci, heavy accumulation of lymphocytes and macrophages with destruction of more than five muscle fibers (score 3). Results are expressed as total myocarditis score ± SE, calculated by multiplying the number of lesions by the corresponding individual score, and evaluated in whole two slices (each slice obtained from one half of the heart).

2.9. Determination of cytokine levels in serum

Mice were bled by cardiac puncture 17 days p.i. Blood was collected in sterile, endotoxin-free tubes and kept refrigerated until centrifugation. Serum was stored frozen at −20 °C until used. TNFα, IL-1β, IL-6, and IFN-γ concentrations were evaluated by ELISA, using commercially available kits (BD Pharmingen, San Diego, USA). All samples were assayed in duplicate. The limit of quantification (LOQ) of each cytokine was 15.6 pg/ml, except for IL-1β, for which the LOQ was 31.3 pg/ml.

2.10. Determination of T. cruzi-specific IgM and IgG_{2a} antibodies

Specific anti-*T. cruzi* IgM and IgG_{2a} concentrations were measured by ELISA. In brief, microtiter plates (Lockwell Modules, Nalge Nunc International, Naperville, IL, USA) were coated with 20 µg/ml of an epimastigote lysate from the Tulahuén strain, in 0.05 M carbonate–bicarbonate buffer (pH 9.6), blocked with skimmed milk, and incubated for 4 h at 37 °C with a 1:100 dilution of sera from infected

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or control mice. Specific IgM and IgG_{2a} isotypes were detected by incubation with goat anti-mouse IgM (Caltag, Burlingame, California) or goat anti-mouse IgG_{2a} (BD Pharmingen, San Diego, USA) anti-mouse peroxidase-conjugated diluted 1/2000 or 1/4000 respectively. Samples were assessed in duplicate and plates were read at 450 nm in an ELISA reader (Organon Tecknica, Durham, N.C., USA) after 60 min incubation with H_2O_2 and TMB (Sigma Aldrich, USA).

2.11. Statistical analysis

Statistical analysis was done using SPSS version 23. Data that followed a normal distribution (Kolmogorov-Smirnov test), and with homogeneous variances (Levene's test) were analyzed by unpaired *t*-test (two-tailed) when only two groups were compared, or by one-way ANOVA followed by Fischer's LSD for multiple comparisons. For non-normally distributed data, analysis was done using non-parametric tests (Kruskal-Wallis test followed by Mann-Whitney test for comparison between two groups). Survival curves were compared using the Kaplan–Meier's test. Differences were considered statistically significant if $p < 0.05$. Data are presented as mean ± SE.

3. Results

3.1. Sexual dimorphism in the susceptibility of C57Bl/6 mice to infection with T. cruzi

Males and females had a comparable splenomegaly and a significant increase in the total number of nucleated cells 17 days p.i. as compared to non-infected controls (Table 1). When calculated per g spleen, cellularity was decreased in infected mice, but the results were not statistically different from those of the controls of the corresponding sex. Basal corticosterone levels were significantly lower in control male mice than in females, but the plasma levels of the hormone increased to comparable concentrations in infected mice of both genders. Parasitemia was higher in males than in females 21 days after infection. While all male mice died within 25–30 days following infection, 69% of the females survived longer (Table 1).

3.2. Decreased splenic sympathetic innervation following T. cruzi infection

NA concentration was markedly reduced in the spleen 17 days after infection with the parasite (Fig. 1A). When calculated per total spleen, NA content in the whole organ was also significantly reduced (Fig. 1B), indicating that the decreased NA concentration was not just a "dilution" effect due to the splenomegaly induced by the infection. The reduction was proportionally more pronounced in males than in females. Taking the mean of the corresponding controls as 100%, NA concentration was 12.5 ± 2.2% in infected males and 38.9 ± 13.3% in infected females, thus the decrease was about 88% and 61%, respectively. When the NA content in the whole spleen was calculated in a comparable manner, the reduction was about 47% in infected males and 39% in infected females. It is interesting to note that there was a significantly larger variance in the splenic NA concentration (Levene's test, F = 7.830; $p = 0.011$) and content (F = 5.142, $p = 0.035$) in infected females than in infected males (range NA ng/g spleen: infected females: 10–500; infected males 21–69; range NA ng per total

Table 1
Sexual dimorphism in the response to infection with T. cruzi.

Spleen weight (mg)				Cells × 10^6 per spleen				Corticosterone (μg/dl)				Parasitemia		%Survival after 30 d	
	Control	n	Infected	n	Control	n	Infected	n	Control	n	Infected	n		n	
Females	92 ± 7	5	704 ± 55*	5	183 ± 18	5	805 ± 104*	5	1.5 ± 0.2	7	9.3 ± 1.8*	5	73 ± 23	5	69	16
Males	103 ± 13	5	598 ± 23+	5	260 ± 23	5	1132 ± 303+	5	0.4 ± 0.1#	8	10.0 ± 1.6+	5	403 ± 52§	4	0	7

T. cruzi or the vehicle alone (control) was injected into male and female C57Bl/6 mice as described in Material and Methods. Groups of mice were killed on day 17 p.i. to evaluate spleen weight and cellularity. Corticosterone plasma levels were determined 17 days p.i. and parasitemia 21 days p.i. in other groups of mice. A third group of infected mice were left undisturbed and survival was recorded daily. n: number of animals per group. Results are shown as mean ± SE. Normally distributed data with homogenous variances were analyzed by ANOVA followed by LSD for multiple comparisons or Student's *t* test when only 2 groups were compared. Not normally distributed data were analyzed by non-parametric tests (Kruskal-Wallis followed by Mann-Whitney). Spleen: Kruskal-Wallis $p = 0.002$; Mann-Whitney *vs. control females: $p = 0.008$; + vs. control males: $p = 0.008$. Cellularity: F(3, 16) = 30.126; $p = 8.1381E{-}7$: *vs. control females: $p = 0.000004$; + vs. control males: $p = 0.00001$. Corticosterone: Kruskal-Wallis: $p < 0.0001$; Mann-Whitney: *vs. control females $p = 0.001$; + vs. control males $p < 0.0001$, #vs. control females: $p = 0.004$. Parasitemia: t(7) = −2.804; §vs. infected females $p = 0.026$.

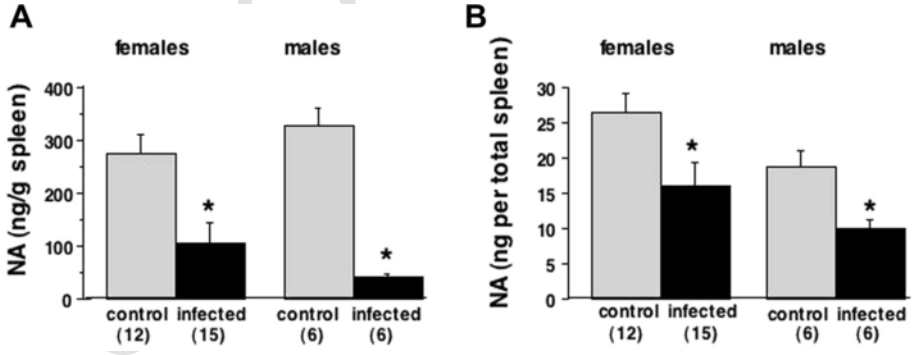

Fig. 1. Decreased splenic NA concentration and content in infected mice. Groups of *T. cruzi*- and vehicle (control)-injected C57Bl/6 male and female mice were killed 17 days after injection. The spleen was collected, weighted, and used for NA determination. The numbers below the bars indicate the number of mice per group. Results are shown as mean ± SE. Data were analyzed by non-parametric tests. ∗ vs. non-infected control of the same sex. NA (ng/g spleen): Kruskal-Wallis: $p < 0.0001$; Mann-Whitney: infected vs. control, for females $p = 0.003$, for males $p = 0.002$. NA (ng per whole spleen): Kruskal-Wallis: $p = 0.004$; Mann-Whitney: infected vs. control, for females $p = 0.014$, for males $p = 0.026$.

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spleen: infected females: 1.5–45.0; infected males: 5.9–14.4). This was not the case in control mice, in which the variances in splenic NA concentration and content was comparable in both genders. The concentrations of dopamine and of the NA metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) in the spleen of control and infected mice were very low, and we did not consider these evaluations reliable.

In another series of experiments, catecholamine concentrations were determined in plasma of male mice 17 days after infection or injection of the vehicle alone. Although NA concentration in the spleen of infected mice was markedly reduced as compared to that of control mice, no significant differences in plasma NA and A concentrations between both groups were detected (results expressed as mean ng/ml plasma ± SE: controls, n = 5, NA: 5.08 ± 0.96; A: 4.40 ± 0.91; infected, n = 6, NA: 5.40 ± 0.73; A: 3.40 ± 0.89).

To study whether the effect of the infection with *T. cruzi* on splenic NA was restricted to the C57Bl/6 strain, we performed comparable experiments in Balb/c male mice, which are less susceptible than C57Bl/6 mice to infection with this parasite (Roggero et al., 2002; Roggero et al., 2006). *T. cruzi* infection also resulted in splenomegaly in Balb/c mice, and the splenic NA concentration and the total NA content in this organ were also decreased in this strain (Fig. 2). As indicated in this figure, the splenic NA concentration in infected Balb/c mice (47.29 ± 4.84 ng/g spleen), although markedly decreased when compared to the corresponding controls, was significantly higher than in infected C57Bl/6 mice (22.22 ± 4.84 ng/g spleen). Significant differences were also observed when results are expressed as NA content in the whole spleen (Balb/c: 23.65 ± 3.59 ng per total spleen; C57Bl/6: 12.00 ± 2.44 ng per total spleen). No comparable decreases were observed in the splenic concentration of tyrosine, the precursor of NA synthesis, and its total content was clearly increased in the spleen of infected mice (Fig. 2). These results also reinforce the idea that the decreased NA concentrations detected in the spleen of infected mice is not just a dilution effect due to the increased spleen weight. To further confirm these indications, we evaluated tyrosine hydroxylase, the rate-limiting enzyme in NA synthesis and a marker of sympathetic nerve fibers, by histological immunofluorescence. As shown in Fig. 3, tyrosine hydroxylase-positive fibers were markedly decreased or even lost 17 days after infection.

3.3. Sympathetic denervation affects survival of T. cruzi-infected mice

We next studied the relevance of the absence of sympathetic nerve fibers for the development the *T. cruzi*-induced disease. To approach this point, sympathetic nerve fibers were destroyed by injection of the neurotoxin 6-OH-DA at birth. As shown in Fig. 4, this treatment resulted in more than 95% depletion in splenic NA concentration in both male and female adult mice. Other groups of mice that had been denervated or treated with the vehicle at birth were infected with the parasite when they were 2 month-old and killed 17 days later. The results of splenic NA concentration and content in these experimental groups are also shown in Fig. 4.

To study the effect of sympathetic denervation on the survival of infected mice, comparable groups of intact and denervated males and females were left undisturbed with the only exception that a blood sample was obtained from the tip of the tail on day 14 to evaluate parasitemia (see below). Survival was recorded daily (Fig. 5). While 100% of the intact, infected mice were still alive on day 19 p.i., all infected, denervated, males and about half of the infected, denervated females were dead at this time. Thus, sympathetically denervated, infected male and female mice died earlier than the infected intact animals of the corresponding sex. Furthermore, denervated, infected males died earlier than denervated, infected females. These differences were all statistically significant.

3.4. Sympathetic denervation affects parasitemia in T. cruzi- infected female mice

Parasitemia was evaluated 14 days after infection. Denervated, infected females had a significantly higher parasitemia than intact, in-

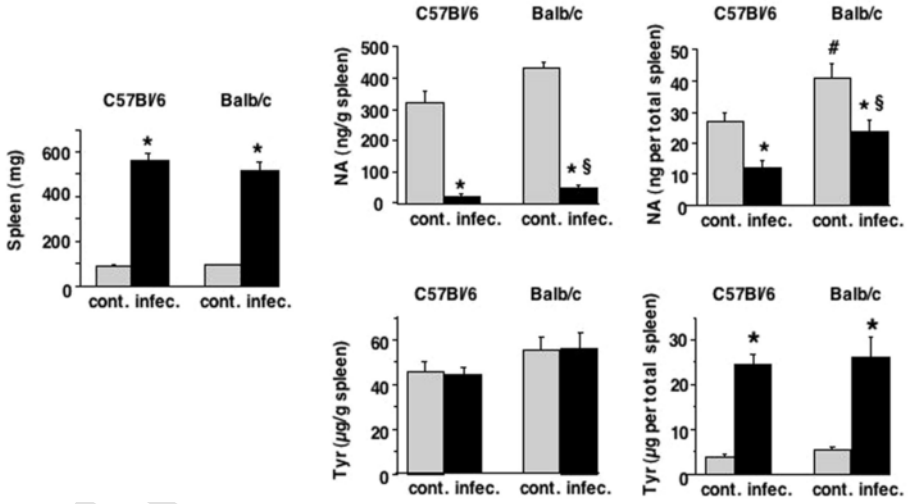

Fig. 2. *T. cruzi* infection results in decreased splenic NA in C57Bl/6 and Balb/c mice. Groups of *T. cruzi* (infec.)- and vehicle (cont.)-injected C57Bl/6 and Balb/c male mice were killed 17 days after injection. The spleen was collected, weighted, and used for the evaluations. Results are shown as mean ± SE of determinations performed in 7–9 mice per group. Data were analyzed by parametric (ANOVA followed by LSD for multiple comparisons) or non-parametric tests (Kruskal-Wallis followed by Mann-Whitney), depending on the distribution of the data. Spleen weight: Kruskal-Wallis: $p < 0.0001$; Mann-Whitney: control vs. infected: C57 $p = 1.748E{-}4$; Balb/c $p = 5.828E{-}4$. NA (ng/g spleen): Kruskal-Wallis: $p < 0.0001$; Mann-Whitney: control vs. infected: C57 $p = 1.748E{-}4$; Balb/c $p = 5.828E{-}4$; C57 infected vs. Balb/c infected: $p = 0.031$. NA (ng/total spleen): $F(3,26) = 12.707$, $p = 0.000026$; LSD: control vs. infected: C57 $p = 0.003$; Balb/c $p = 0.002$; C57 infected vs. Balb/c infected $p = 0.019$; Balb/c control vs. C57 control $p = 0.011$. Tyr (µg/total spleen): $F(3,26) = 72.929$, $p = 8.7001E{-}13$; control vs. infected: C57 $p = 1.890E{-}11$; Balb/c $p = 4.5746E{-}10$. ∗ vs. non infected control of the same strain; # vs. C57 control; §vs. C57 infected.

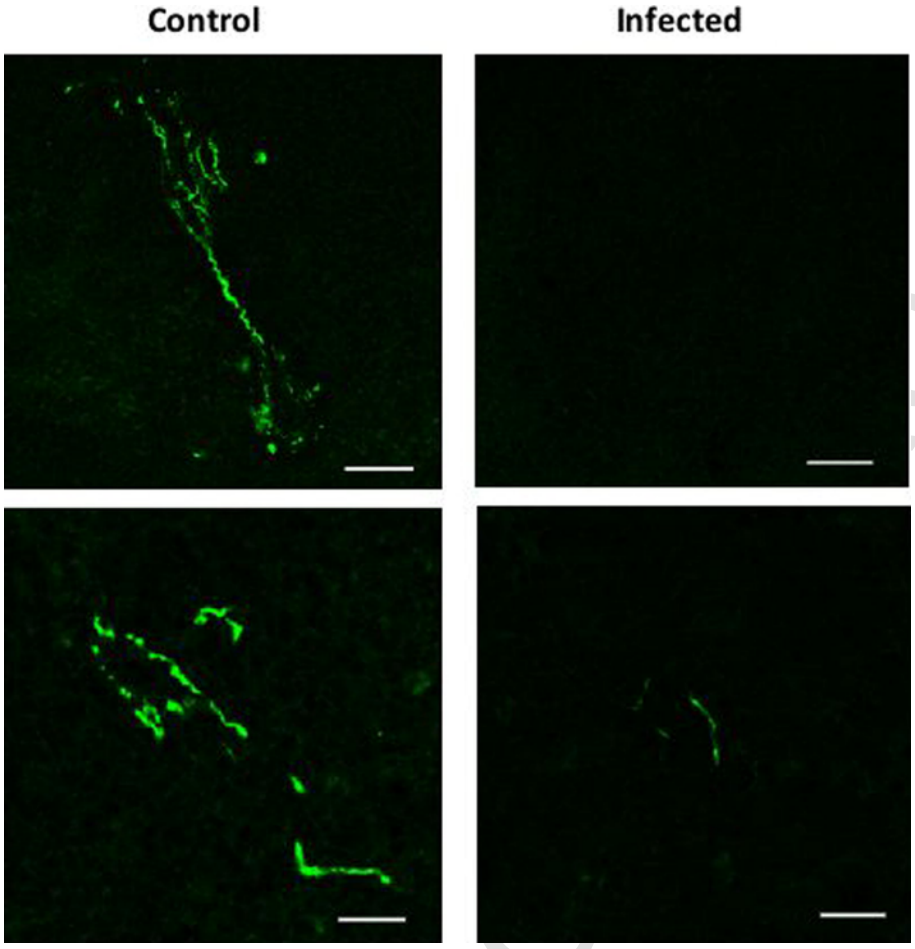

Fig. 3. Tyrosine hydroxylase staining in the spleen of control and infected mice. Nerve fibers containing tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, were detected by fluorescent immunohistochemistry. The photos show representative examples in the spleen of two control and two infected C57Bl/6 male mice 17 days p.i. White bars: top = 50 μm; bottom = 20 μm.

fected mice (infected, n = 11: 10 ± 2 parasites/50 fields; denervated infected, n = 10: 39 ± 10 parasites/50 fields; Mann-Whitney: $p = 0.004$). The same tendency was observed in males, but the difference was not statistically significant (infected, n = 9: 23 ± 8 parasites/50 fields; denervated infected, n = 10: 36 ± 5).

3.5. Sympathectomy does not affect specific IgM and IgG_{2a} antibodies against T. cruzi, catecholamine concentration in the adrenal glands, and corticosterone plasma levels in infected mice

Sympathetic denervation did not affect the concentration of IgM and IgG_{2a} specific antibodies against *T. cruzi* antigens evaluated in females 17 days p.i. (results are given as mean ± SE of the optical density measured. IgM: infected, n = 8: 0.410 ± 0.045; denervated infected, n = 7: 0.339 ± 0.030. IgG_{2a}: infected 0.230 ± 0.053; denervated infected: 0.199 ± 0.036). Neither were statistically significant differences between control, infected, denervated, and denervated infected female mice after injection of *T. cruzi* or vehicle in the concentration of NA and A in the adrenal gland. (Results are given as mean μg catecholamine/g adrenal ± SE: control (n = 4) NA: 442 ± 62, A: 834 ± 59; denervated (n = 3) NA: 556 ± 51; A: 899 ± 99; infected (n = 8): NA: 408 ± 53; A: 934 ± 111; denervated infected (n = 7): NA: 540 ± 57; A: 967 ± 112).

We have previously reported that corticosterone levels are increased in plasma of *T. cruzi*-infected C57Bl/6 male mice (Roggero et al., 2006). As shown in Table 1, such an increase was also observed in infected females. In a separate series of experiments, we evaluated corticosterone blood levels in adult female mice that received vehicle or 6-OH-DA at birth and that were infected or not when they were 2 month-old. Blood samples were obtained immediately before infection or injection of the corresponding medium, and 10, 14 and 17 days later. No statistically significant difference in corticosterone blood levels between non-infected, adult mice that received either vehicle or 6-OH-DA at birth were detected at any of the time points studied. Corticosterone blood levels on day 17 p.i. were significantly different from those on day 0 in intact infected and in denervated infected females, but there were no statistically significant differences between both groups (Supplementary Fig. 1).

3.6. Sympathetic denervation does not aggravate myocarditis in infected mice

Myocarditis was evaluated in the heart of the same mice in which NA concentration was quantified in the spleen (Fig. 4). Sympathetic denervation did not result in an increase in the number and degree of inflammatory foci in infected males (results are given as mean myocarditis score ± SE: infected, n = 6: 22.00 ± 4.67; denervated infected, n = 6: 25.33 ± 4.68); and this score was even decreased in infected females (infected n = 4: 31.50 ± 7.76; denervated infected, n = 5: 10.20 ± 1.63; t(7): 3.410, $p = 0.011$).

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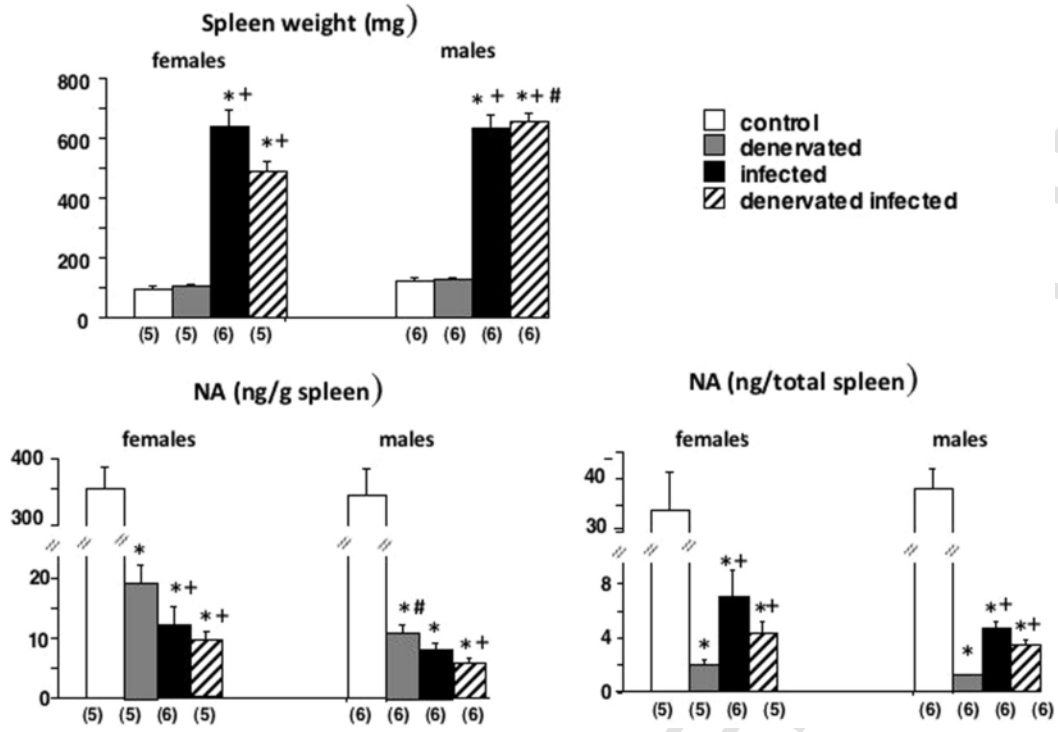

Fig. 4. NA concentration in the spleen of denervated and infected mice. Groups of mice were sympathetically denervated at birth by injection of 6-OH-DA. Control mice received the vehicle (ascorbic acid) alone. Other groups of mice that had been denervated or treated with the vehicle at birth were infected with the parasite when they were 2 month-old and killed 17 days p.i. The spleen was removed, weighted and used for NA determinations. The numbers below the bars indicate the number of mice per group. Results are shown as mean ± SE. Data were analyzed by parametric or non-parametric tests, as indicated. Spleen (mg): Kruskal-Wallis $p = 0.001$; Mann-Whitney: infected vs. control: $p = 0.004$ (females) and 0.002 (males); denervated infected vs. control: $p = 0.008$ (females) and 0.002 (males); denervated vs. infected $p = 0.004$ (females) and 0.002 (males); denervated infected vs. denervated: $p = 0.008$ (females) and 0.002 (males); infected denervated: males vs. females: $p = 0.009$. NA (ng/g spleen): F (7, 37) = 118.08 $p = 2.4487E{-}23$, denervated vs. control: $p = 3.2403E{-}15$ (females) and $1.9119E{-}18$ (males); infected vs. control: $p = 5.913E{-}18$ (females) and $1.3763E{-}19$ (males); denervated infected vs. control: $p = 3.7705E{-}18$ (females) and $7.3499E{-}21$ (males); infected vs. denervated: $p = 0.016$ (females); denervated infected vs. denervated: $p = 0.004$ (females) and 0.005 (males); denervated females vs. denervated: males $p = 0.023$. NA (ng/total spleen): F(7, 37) = 54.418 $p = 1.4796E{-}17$, denervated vs. control: $p = 7.8327E{-}14$ (females) and $2.7378E{-}17$ (males); infected vs. control: $p = 2.5068E{-}8$ (females) and $4.7741E{-}11$ (males); denervated infected vs. control: $p = 5.4107E{-}10$ (females) and $1.1596E{-}12$ (males); denervated infected vs. denervated: $p = 0.003$ (females) and 0.000072 (males); infected vs. denervated $p = 0.000013$ (females) and 0.000001 (males).∗ vs. control of the corresponding sex; + vs. denervated of the corresponding sex; # vs. female of the corresponding group.

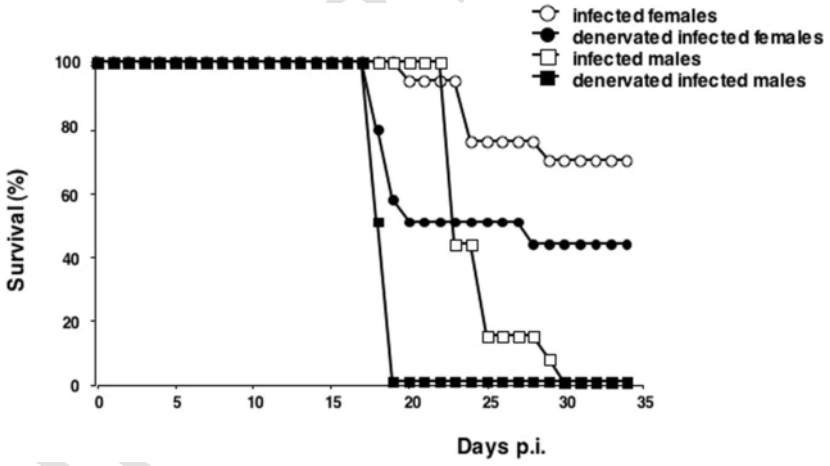

Fig. 5. Survival of intact and denervated infected mice. Groups of mice were treated as described in legend to Fig. 4 (ascorbic acid: 16 females and 7 males; 6-OH-DA: 14 females and 8 males), infected with *T. cruzi* when they were 2 month-old, and left undisturbed throughout. Survival was recorded daily. Survival curves were compared using Kaplan-Meier's test followed by Generalized Wilcoxon (Breslow). The following comparisons resulted in statistically significant differences: a) infected: males vs. females (Chi-square: 9.178, $p = 0.002$); b) females: infected vs. infected denervated (Chi-square: 4.596, $p = 0.032$); c) males: infected vs. infected denervated (Chi-square: 12.327, $p < 0.0001$); d) infected denervated: females vs. males (Chi-square: 5.565, $p = 0.018$).

3.7. Cytokine blood levels in sympathetically denervated T. cruzi-infected mice

Due to the early mortality of denervated, infected male mice and because we were interested in establishing the relevance of the SNS for the course of the disease to chronicity as far as it is possible in this model, we mainly concentrated on the evaluation of some selected immune parameters in infected female mice. TNFα, IL-1β, IL-6, and IFN-γ concentrations were below the limit of quantification in serum of control and denervated females. The increased levels of TNFα and IL-1β observed after infection were not significantly affected by prior sympathetic denervation (Fig. 6). However, the concentration of IL-6 and IFN-γ, which can also exert pro-inflammatory effects, was clearly enhanced in denervated, infected mice when compared to those of intact, infected females (Fig. 6).

4. Discussion

We show here that there is a sexual dimorphism in the response of mice to infection with *T. cruzi*, as indicated by a higher parasitemia and a shorter survival time in C57Bl/6 males than in females. Using this model, our studies provide the first demonstration that the sympathetic innervation of a lymphoid organ, the spleen, is markedly altered during a protozoan parasitic disease, without parallel changes in the concentration of catecholamines in plasma. It is known since a long time that the spleen has a rich sympathetic innervation (for review Felten, 1993) with a particular distribution of nerve fibers that can make close contact with lymphoid cells located in the parenchyma. It is also well established that the SNS can exert relevant regulatory effects on the functioning of the immune system (for review Elenkov et al., 2000; Sanders, 2012). Here we report that the concentration of NA, the main neurotransmitter synthesized and released by sympathetic nerves, is so profoundly decreased in the spleen of mice infected with *T. cruzi* that it is closed to the concentration found in pharmacologically denervated, non-infected mice. The reduction of NA levels was observed not only in the concentration of the neurotransmitter but also in its total content in the spleen. Conversely, no significant differences between control and infected mice in the splenic concentration of tyrosine were detected, and its content in the whole organ was significantly increased. These facts indicate that the decreased splenic NA concentration in infected mice does not merely reflect the splenomegaly that characterizes the disease. This was further confirmed by immunohistochemical studies, which showed that fibers containing tyrosine hydroxylase, the rate-limiting enzyme in NA synthesis, were very scarce in the spleen of infected mice. The mechanism that leads to decrease NA concentration in the spleen during immune responses is still not clarified. In a model of viral infection, a direct effect of the infective agent on nerve fibers and a neurotoxic oxidative effect caused by increased NA re-uptake have been discarded (Kelley et al., 2003). Other possibilities are that: 1) a markedly increased production of some immune-derived mediators could destroy sympathetic fibers (Kessler et al., 1993; Gutierrez et al., 2008; Li et al., 2012) or stimulate the local degradation of tyrosine hydroxylase (Shi and Habecker, 2012); 2) there is an increased NA utilization or an increased expression of catecholamine degrading enzymes (Bloemker et al., 2016), without the possibility that the rate-limiting enzyme in NA synthesis could compensate for this effect. Although our immunohistochemical studies cannot ascertain if sympathetic nerve fibers were destroyed during infection with the parasite, they clearly show that tyrosine hydroxylase was markedly decreased. Independently of the mechanism(s) involved, the findings reported here show that splenic lymphoid cells are less exposed to the modulatory effects of NA during a parasitic infection.

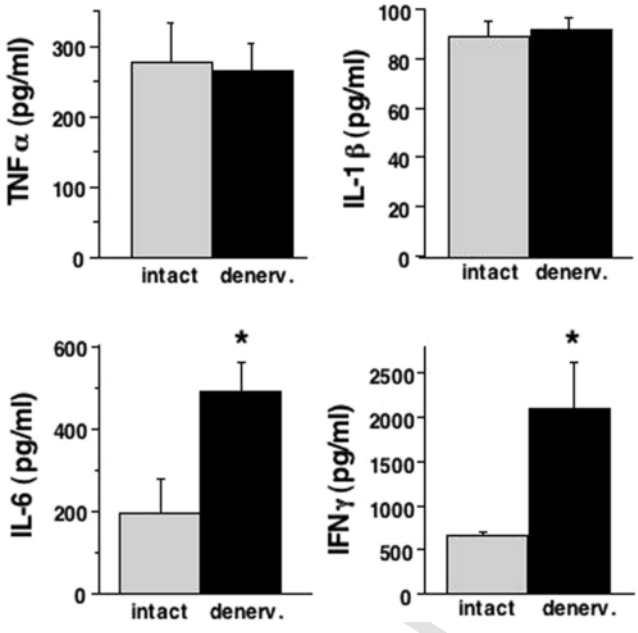

Fig. 6. Cytokine concentration in plasma of intact and denervated infected female mice. Groups of mice were sympathetically denervated at birth by injection of 6-OH-DA. Control mice received the vehicle (ascorbic acid) alone. Other groups of mice that had been denervated or treated with the vehicle at birth were infected with *T. cruzi* when they were 2 month-old. Cytokine concentrations in serum were determined on day 17 p.i. in 5–8 intact, infected and 5–7 denervated, infected females. Cytokine concentrations in serum of non-infected mice were below the limit of quantification, thus these groups are not included in the figure. Results are shown as mean ± SE. Data were analyzed by parametric or non-parametric tests, depending on the distribution and homogeneity of the variances. IL-6: $t(9) = 2.751$, $p = 0.022$; IFN-γ: Mann-Whitney: $p = 0.026$. ∗ vs. infected, non-denervated.

The decrease in NA concentration and content was observed in mice of both genders. However, the depletion was proportionally more marked in males than in females, suggesting that this difference is linked to the sexual dimorphism in response to *T. cruzi* infection. As mentioned, the variance in splenic NA concentration and content measured on day 17 in infected animals was larger in females than in males, and the decrease in a proportion of females fell within the range of those of the males. Although this is difficult to prove, these results make it tempting to speculate that those would be the females that would have died earlier.

We have previously reported that C57Bl/6 and Balb/c male mice differ in the susceptibility to infection with the parasite, as evaluated by an increased survival time and decreased mortality, and by an earlier increase in corticosterone blood levels in Balb/c mice as compared to C57Bl/6 animals (Roggero et al., 2002; Roggero et al., 2006). However, both strains are markedly affected by impeding the effect of endogenous glucocorticoids, since adrenalectomy or treatment with a glucocorticoid antagonist accelerates death in C57Bl/6 mice and results in 100% mortality in the less susceptible Balb/c strain (Roggero et al., 2006). Here we show that the decrease in splenic NA concentration is also observed in infected Balb/c male mice, although, as mentioned, it was still higher than in infected C57Bl/6 male mice.

Long ago, we reported that the immune response to innocuous antigens can alter SNS activity in the spleen (Besedovsky et al., 1979), as a consequence of an immunologically triggered sympa-

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thetic reflex mechanism (for review del Rey and Besedovsky, 2008). *T. cruzi* infection elicits a prolonged immune cell activation that, as shown here, is paralleled by a decreased splenic NA content. We propose that the above-mentioned immune-mediated sympathetic reflex that is triggered in the spleen during acute immune stimulation also operates during prolonged immune responses, as observed during viral infections (Kelley et al., 2003; Bloemker et al., 2016), autoimmune/lymphoproliferative diseases (del Rey et al., 2006), and adjuvant-induced arthritis (Lorton et al., 2005). It is noteworthy that, despite the different nature of the antigens used and of the pathologies so far investigated, a decrease in the NA content in the spleen is observed in all these processes.

To explore the relevance of the SNS for the course of *T. cruzi* infection, we advanced the "spontaneous" denervation observed in infected mice by inducing a permanent destruction of noradrenergic nerve fibers following injection of the neurotoxin 6-OH-DA at birth. As shown here, this treatment resulted in more than 95% depletion in splenic NA concentration in both adult males and female mice. When males were infected, denervated animals died before mice that were treated with the vehicle at birth. In females, which as mentioned, resist better the infection, the death of denervated mice was not only advanced but lethality was significantly increased. These results show that, despite the sexual dimorphism, sympathectomy can increase the susceptibility to the disease induced by *T. cruzi*.

Parasitemia was significantly higher in denervated than in intact female mice, which can be taken as an indication that the activity of noradrenergic nerves play a favorable role in the clearance of the parasite from the blood. The same tendency, although not statistically significant, was observed in denervated males. Although females show a smaller parasitic load and also survive longer than infected males, survival times in *T. cruzi*-infected mice do not necessarily correlate with parasitemia. For example, the differential susceptibility to acute *T. cruzi* infection in Balb/c and C57Bl/6 male mice is not invariably linked to a distinct parasite load but to a different cytokine pattern (Roggero et al., 2002). Also, we have previously shown that blockade of glucocorticoid receptors in *T. cruzi*-infected mice increases TNFα plasma levels, accelerates death in C57Bl/6 mice, and increases lethality to 100% in Balb/c mice without concomitantly affecting parasitemia (Roggero et al., 2006). In fact, as these previous results indicate, survival seems to be rather linked to a different cytokine pattern than to an increased parasitemia.

In the following studies, we evaluated the effect of denervation on corticosterone levels, NA and A concentration in the adrenals, myocarditis, and specific anti-*T. cruzi* antibodies and cytokine levels in serum of infected animals. The stimulation of the HPA axis that occurs during *T. cruzi* infection was not affected by neonatal denervation, indicating that the increased mortality in sympathetically denervated animals is dissociable from the protective endocrine response during this infection (Roggero et al., 2006). There were also no significant differences in the concentration of catecholamines in the adrenals of infected and infected denervated mice. Since sympathetic denervation did not worsen the myocarditis caused by the parasite in males, and it even reduced it in females, the results suggest that increased inflammation and damage of the heart muscle was not the main cause for the accelerated death of infected denervated mice.

Since lytic antibodies are important in parasite elimination during the acute phase of *T. cruzi* infection (Gazzinelli et al., 1991; Antas et al., 1999; Dutra and Gollob, 2008), we evaluated IgM and IgG_{2a} antibodies specific to *T. cruzi* antigens. It is interesting to remark that, as opposed to infections caused by other types of parasites, IgE is not the main immunoglobulin class affected during infection with trypanosomes (for review Rogerio and Anibal, 2012), and its concentration is not altered in patients with Chagas' disease (Geller et al., 1978). Conversely, IgM and IgG_2 concentrations are increased in patients with different clinical manifestations of this disease (Morgan et al., 1996). Furthermore, all mouse strains so far tested are capable of producing IgM antibodies to different stocks of the parasite (Kipnis et al., 1987), but resistant strains produce more IgM than those with a susceptible background. Conversely, susceptible animals produce higher levels of anti-parasite IgG_{2a} antibodies compared to more resistant congenic strains (Powell and Wassom, 1993), and this is the main isotype secreted during experimental Chagas' disease (Spinella et al., 1992). We had therefore chosen to evaluate IgM and IgG_{2a} levels because they could indicate whether potential effects of the SNS on the humoral response to *T. cruzi* could have a protective or aggravating role on the development of the disease, and, eventually, to distinguish if such effect would derive from affecting mainly Th1 (IgG2a) or Th2 (IgM) cell activity. However, the results obtained showed that the specific immune response to the parasite mediated by these immunoglobulins is not affected by denervation in the model of infection that we have used and at the time evaluated.

A strong inflammatory response is triggered during *T. cruzi* infection with the production of inflammatory cytokines that activate cells to eliminate the parasite. While these cytokines are necessary to control the infection (Roggero et al., 2009), if produced in large amounts, they can lead to hypercatabolic conditions and cachexia, which can kill infected mice and aggravate the disease in animals that survive the early phase of the infection (for review Dutra and Gollob, 2008). This evidence indicates that a subtle balance in cytokine production is necessary for an efficient defense against *T. cruzi* infection. The two main pro-inflammatory cytokines that have been involved in this response are IFN-γ and TNFα (for review Dutra and Gollob, 2008; Dutra et al., 2014). The role of both cytokines is however still under debate, and they can be protective or damaging depending on the phase of the infection and on the different clinical forms of Chagas' disease (Rodriguez-Angulo et al., 2013; Rodrigues Pinto Ferreira et al., 2014). Confirming previous results from us and from other groups (Roggero et al., 2006; Perez et al., 2007; Dutra et al., 2014), IFN-γ, IL-6, IL-1β, and TNFα levels were markedly increased in infected mice, as it has been shown in animals infected with all *T. cruzi* clones tested so far. We have not found a significant effect of sympathectomy on circulating TNFα and IL-1β levels in this model. This is interesting in view of the marked effect of glucocorticoid removal on the levels of these cytokines that we have previously reported in this same model (Roggero et al., 2006; Perez et al., 2007). In contrast, sympathectomy resulted in a more than 2-fold increase in IL-6 levels and a more than 4-fold increase in the concentration of IFN-γ, a cytokine that seems to play a protective role during the acute phase but that has been involved in cardiac damage during the chronic phase of the disease (Rodrigues Pinto Ferreira et al., 2014). Thus, since no effect of denervation on the levels of IgM and IgG_{2a} specific antibodies has been detected and this manipulation does not increase myocarditis either, the most likely interpretation at present is that an increase in the production of products released by activated immune cells, including IL-6 and IFN-γ, is a major contributor to the acceleration and increased mortality observed in infected denervated mice.

Our results do not exclude that other mechanisms affected by sympathectomy may have contributed to aggravate the disease. For example, cytotoxic T cells can modulate immune resistance and disease progression during *T. cruzi* infection (Tarleton et al., 1996), and there is evidence that NA and β1-adrenergic agonists can stimulate the generation and action of these cells (Hatfield et al., 1986). Also processes related to the control of blood flow and cell mobilization may play a role. As we have previously reported (Rogausch et al.,

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1997), the increase in regional blood flow that correlates with splenomegaly following immune stimulation is due to reduction of the vascular adrenergic tonus, and NA can also simulate the mobilization of cells from immune organs to the blood (Lang et al., 2003; Rogausch et al., 2003). Thus, an increased blood flow due to sympathectomy could favor *T. cruzi* dissemination and interfere with the cellular uptake of this intra-cellular parasite. In agreement with this possibility is that NA can modulate macrophage chemotaxis and phagocytosis (Ortega et al., 2000; Garcia et al., 2003), and that catecholamines can act as chemoattractant for microorganisms (Bansal et al., 2007). The absence of these effects in sympathectomised mice might reduce the probability of immune cells to encounter and phagocyte circulating parasites.

In conclusion, we have detected that the SNS can favor survival to infection with *T. cruzi* by mechanisms that most likely involve the control of excessive production of certain pro-inflammatory cytokines, although other processes related to immune cell mobilization and restriction of parasite spreading and homing may contribute to this effect. The decreased sympathetic activity that naturally occurs during infection with the parasite may interfere with these potentially protective effects. As a whole, these results further emphasize the relevance of interactions between the immune system and the SNS for immunoregulation and host defense. Furthermore, they provide a clear example that the outcome of complex pathologies, such as those caused by parasites, depend on the fine balanced response of different bodily systems.

Conflict of interest statement

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.07.163.

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