Inducible Nitric Oxide Synthase in Heart Tissue and Nitric Oxide in Serum of *Trypanosoma cruzi*-Infected Rhesus Monkeys: Association with Heart Injury

Cristiano Marcelo Espinola Carvalho, Jaliane Coutinho Silverio, Andrea Alice da Silva, Isabela Resende Pereira, Janice Mery Chicharino Coelho, Constança Carvalho Britto, Otacílio Cruz Moreira, Renato Sergio Marchevsky, Sergio Salles Xavier, Ricardo Tostes Gazzinelli, Maria da Glória Bonecini-Almeida, Joseli Lannes-Vieira

1 Laboratório de Biologia das Interações, Instituto Oswaldo Cruz (IOC/Fiocruz, Rio de Janeiro, Brazil.
2 Serviço de Imunologia, Instituto de Pesquisa Clínica Evandro Chagas (IPCC/Fiocruz, Rio de Janeiro, Brazil.
3 Departamento de Patologia, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil.
4 Serviço de Anatomia Patológica, IPCC/Fiocruz, Rio de Janeiro, Brazil.
5 Laboratório de Biologia Molecular e Doenças Infecciosas, IOC/Fiocruz, Rio de Janeiro, Brazil.
6 Laboratório de Neuroinfluença, BioManguinhos/Fiocruz, Rio de Janeiro, Brazil.
7 Laboratório de Imunoparasitologia, Instituto René Rachou/Fiocruz, Belo Horizonte, Minas Gerais, Brazil.
8 Departamento de Imunologia e Bioquímica, Instituto de Ciências Biológicas (ICB), Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

Abstract

**Background:** The factors contributing to chronic Chagas' heart disease remain unknown. High nitric oxide (NO) levels have been shown to be associated with cardiomyopathy severity in patients. Further, NO produced via inducible nitric oxide synthase (iNOS/NOS2) is proposed to play a role in *Trypanosoma cruzi* control. However, the participation of iNOS/NOS2 and NO in *T. cruzi* control and heart injury has been questioned. Here, using chronically infected rhesus monkeys and iNOS/NOS2-deficient mice, we explored the participation of iNOS/NOS2-derived NO in heart injury in *T. cruzi* infection.

**Methodology:** Rhesus monkeys and C57BL/6 and Nos2−/− mice were infected with the Colombian *T. cruzi* strain. Parasite DNA was detected by polymerase chain reaction, *T. cruzi* antigens and iNOS/NOS2+ cells were immunohistochemically detected in heart sections and NO levels in serum were determined by Griess reagent. Heart injury was assessed by electrocardiogram (ECG), echocardiogram (ECHO), creatine kinase heart isoenzyme (CK-MB) activity levels in serum and conneXin 43 (Cx43) expression in the cardiac tissue.

**Results:** Chronically infected monkeys presented conduction abnormalities, cardiac inflammation and fibrosis, which resembled the spectrum of human chronic chagasic cardiomyopathy (CCC). Importantly, chronic myocarditis was associated with parasite persistence. Moreover, Cx43 loss and increased CK-MB activity levels were primarily correlated with iNOS/NOS2+ cells infiltrating the cardiac tissue and NO levels in serum. Studies in Nos2−/− mice reinforced that the iNOS/NOS2-NO pathway plays a pivotal role in *T. cruzi*-elicited cardiomyocyte injury and in conduction abnormalities that were associated with Cx43 loss in the cardiac tissue.

**Conclusion:** *T. cruzi*-infected rhesus monkeys reproduce features of CCC. Moreover, our data support that in *T. cruzi* infection persistent parasite-triggered iNOS/NOS2 in the cardiac tissue and NO overproduction might contribute to CCC severity, mainly disturbing of the molecular pathway involved in electrical synchrony. These findings open a new avenue for therapeutic tools in Chagas' heart disease.


Editor: Rodrigo Correa-Oliveira, René Rachou Research Center, Brazil

Received: October 4, 2011; Accepted: March 30, 2012; Published: May 8, 2012

Copyright: © 2012 Carvalho et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants from FAPERJ (E-26/111.756/2008 to JLTV, E-26/211.756/2008 to JLTV), the Brazilian Research Council/CNPq (Universal #471518/2006-7 to JLTV,INCT-Parasites/2008 to JLTV, PROEP/OC/CNPq to JLTV) and the Program for Technological Development in Tools for Health [PDTIs-Fiocruz] - Real Time PCR-based platform. JLTV, CCB, and RTG are fellows from CNPq. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: lannes@ioc.fiocruz.br

Introduction

Chagas disease, which is caused by the protozoan parasite *Trypanosoma cruzi*, affects 8–15 million individuals in endemic areas of Latin America and several hundred thousand people in other countries as a result of migration. Although vector transmission has been controlled, there are still more than 50,000 new cases of Chagas disease each year [1,2]. Despite high parasitism, which usually declines at immunity onset, the clinical signs are usually mild in the acute infection. After decades, most of the infected individuals remain in the asymptomatic indeterminate form, and 30% of the patients present arrhythmias and heart failure due to
Author Summary

Chagas disease, a neglected tropical disease caused by the protozoan Trypanosoma cruzi, affects from 8 to 15 million people in the Latin America. Chronic chagasic cardiomyopathy (CCC) is the most frequent manifestation of Chagas disease. Currently, patient management only mitigates CCC symptoms. The pathogenic factors leading to CCC remain unknown; therefore, their comprehension may contribute to develop more efficient therapies. In patients, high nitric oxide (NO) levels have been associated with CCC severity, in T. cruzi-infected mice, NO, mainly produced via inducible nitric oxide synthase (iNOS/NO2), is proposed to work in parasite control. However, both NO and NO2 deficiency in transgenic mice have failed to control parasite infection. In this work, we report that NO and NO2 deficiency in transgenic mice failed to control parasite infection and heart injury in T. cruzi infection. Chronically infected mice presented electrical abnormalities, myocarditis and fibrosis, resembling the spectrum of human CCC. Moreover, cardiomyocyte lesion correlated with iNOS/NO2 deficiency in the cardiac tissue.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cncaeb.org.br/) and the Federal Law 11.794 (October 8, 2008), The Institutional Committee for Animal Ethics of Fiocruz (CEUA/Fiocruz, Licenses 161/03 and 004/09) and the Biosafety National Committee (CQBB/CTNBio, License 105/09) approved all the procedures used in this study.

Animals and infection

Seven male thymus monkeys (Macaca muotata, 26±1.7 years old) chronically infected with T. cruzi (monkeys #42, #46, #69, #90, #95, #99, #105) were individually caged in the non-sterile primate care boxes (Double L Group Ltd., USA) of the Nonhuman Primate Breeding Service (SPCR), of the Laboratory of Animal Breeding Center at Fiocruz (CIFAL/Fiocruz, Rio de Janeiro, Brazil). The monkeys were provided with water ad libitum and fed a commercial chow (Novilab Primates 6030, Novilab, Brazil) that was supplemented daily with fruits, eggs and vegetables. Temperature, humidity and light/dark cycles were standardized. Two noninfected age-matched male monkeys (#81 and #94) were analyzed as controls. Four noninfected male monkeys (L17, L21, M31, M33) from our colony were used as controls for the experiments.

Metacyclic trypanosomes of the Colombian T. cruzi strain were used to infect the monkeys subcutaneously in the arm [15]. These animals were studied during the acute and early chronic infection [15]. In this follow up study, the chronically infected monkeys were analyzed at 16-19 years postinfection (pi) and followed for 18 months. The animals did not receive etiological treatment for T. cruzi. Examinations and procedures were performed under anesthesia with 10 mg/kg ketamine chloride (Vetuset, Fort Dodge, Iowa, USA) intramuscularly according to the Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Blood was obtained by puncture of the femoral vein with appropriate tubes (Vacutainer, Becton & Dickinson, USA). Prior to necropsy, monkeys were sedated with ketamine chloride and euthanized by exsanguination under deep plane of sodium thiopental (Thiopental, Cristália, Belo Horizonte, MG, Brazil). Samples of the heart and all major organs were taken for histological studies and polymerase chain reaction (PCR) for parasite kDNA and genomic DNA detection. For the histopathology study, heart samples taken from five infected monkeys, which were sacrificed at the parasitemia peak (41 days postinfection (dpi), monkeys #57 and #67), when parasitemia was negative (70 dpi, monkey #77, and 76 dpi, monkey #98) and 3 years postinfection (monkey #4) used in a previous work [15] were added to our study.

Five- to seven-week-old female C57BL/6 (H-2b) and inos- deficient (NOS2−/−; B6.129P2-Nos2tm1J/L) mice, resulted of seven backcrossings of the original Nos2-deficient lineage in C57BL/6 mice, were obtained from the animal facilities of Fiocruz and were maintained in specific pathogen-free conditions. The mice were infected intraperitoneally with 100 blood trypomastigotes of the Colombian strain [16].
Electrocardiogram, echocardiogram and radiology

The classic 12-lead human electrocardiogram (ECG) system was used to analyze rhesus monkeys. Tracings were made at 25 mm/s at a voltage of 1 mV standardized to 1 cm (ECG-6, Eacafix, Brazil) [14]. Two-dimensional and M-mode echocardiogram (ECHO) was performed on a regular basis and recorded with a multi-image camera (Ultrasonic Scanner EUB-355, Hitachi, Japan). The ventricular function was assessed in the M-mode; by calculating the ejection fraction, and in the bidimensional mode, by semiquantitatively analyzing the global systolic function [14]. Electrocardiogram, ECHO and radiology (chest, esophagus, colon) were performed at 12-month intervals.

Mice were intraperitoneally tranquilized with diazepam (20 mg/Kg), and transecters were placed under the skin for DII derivation. Electrocardiogram tracings were recorded during two minutes using Power Lab 2/20 (PanLab Instruments, Spain), analyzed with Scope Software for Windows v3.6.10 (PanLab Instruments, USA) [16] and independently analyzed by two investigators.

Histopathology studies

Tissue fragments were fixed in 10% formalin, processed, and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and immunohistochemically (IHS) stained. To evaluate collagen deposits, heart sections were stained with Sirius Red (Croma Gesellschaft, Germany) in a saturated aqueous solution of picric acid and fast green. The proportion of collagen-positive areas was evaluated with the digital morphometric apparatus and analyzed with AnalySIS AUTO Software (Sof Imaging System, USA).

Immunohistochemical staining

A polyclonal anti-NO/NO2 antibody was obtained from Cayman Chemical (#160002, USA) and an anti-connexin 43 (Cx-43) polyclonal antibody was purchased from Sigma (#C6219, USA). In addition, a polyclonal antibody recognizing T. cruzi antigens was produced in rabbits (LBI/IUC-Fiocruz, Brazil). A biotinylated anti-rabbit and peroxidase-streptavidin complex was purchased from Amersham (England). Antibodies and reagents were utilized in compliance with the manufacturers’ instructions. Serial 3-μm sections were subjected to standardized IHS [16]. The material was counterstained with Mayer’s hematoxylin. The T. cruzi, iNOS/NO2- or Cx43-positive areas in 25 fields (12.5 mm²) per section, in 3 sections per heart, were evaluated with a digital morphometric apparatus. For the quantitative studies of Cx43 expression only areas with preserved myocardial cells were analyzed. The images were analyzed with AnalySIS AUTO Software (Sof Imaging System, USA). The areas that expressed the molecule of interest were integrated with the areas that did not express the molecule of interest, and the data were presented as the percentage of the positive area.

DNA extraction and PCR conditions

Two or three different tissue samples per studied organ were processed separately for DNA extraction, and the purified DNA was PCR amplified using T. cruzi-specific kDNA minicircle primers [14] or analyzed by real-time quantitative PCR (qPCR) using primers directed to the nuclear satellite DNA [17]. DNA integrity and the possible presence of PCR inhibitors were checked by amplification of the human b-globin sequence [14]. Samples showing no amplification for b-globin were restested after a new DNA extraction. For the real-time PCR assays heart and spleen samples of one to two cm[3] kept in liquid nitrogen were sectioned using a cryostat in conditions to avoid DNA cross-contamination and the studied area calculated using the AnalySIS AUTO Software (Sof Imaging System, USA). As negative and positive controls, respectively, heart tissues from noninfected C57Bl/6 mice or mice at 50 dpi with the Colotribion T. cruzi strain were used [16].

The qPCR experiments were performed using the ABI Prism 7500 Fast (Applied Biosystems, USA), in a final volume of 20 μL containing 2 μL DNA samples and 10 μL GoTaq qPCR Master Mix (Promega, USA). Primers Cruzi 1 and Cruzi 2 were used for the T. cruzi nuclear satellite target [17]. The cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 58°C for 1 minute. After amplification, the specificity of these primers was confirmed through melting curve analysis of the generated amplicons, revealing a solely melting temperature (Tm) for the amplified fragment. Each sample submitted to qPCR analysis was performed in triplicates and the results were expressed as mean values. Parasitic load quantification was obtained by absolute quantification of T. cruzi DNA, following normalization of the heart and spleen analyzed

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Acute phase</th>
<th>Chronic phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.i.</td>
<td>p.i.</td>
<td>p.i.</td>
</tr>
<tr>
<td></td>
<td>p.i.</td>
<td>p.i.</td>
</tr>
<tr>
<td>42</td>
<td>6-12</td>
<td>AVB, RB88</td>
</tr>
<tr>
<td>64</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>68</td>
<td>4-12</td>
<td>AVB, RB88, LvQRS</td>
</tr>
<tr>
<td>99</td>
<td>20</td>
<td>Normal</td>
</tr>
<tr>
<td>103</td>
<td>6-8</td>
<td>AVB, AVR</td>
</tr>
<tr>
<td>90</td>
<td>4-8</td>
<td>AVB, LVQRS</td>
</tr>
<tr>
<td>95</td>
<td>4-12</td>
<td>AVB, AVR</td>
</tr>
</tbody>
</table>

*IEC patterns were evaluated using the following standard criteria: AVB - atrioventricular block, AVR - Abnormal ventricular repolarization, LBBB - Left bundle branch block - second-degree His bundle, LVQRS - Low voltage QRS, RB88 - Right bundle branch block - first-degree His bundle, RAD - right QRS axis deviation. doi:10.1371/journal.pmed.0091044.e1099

www.plosntds.org 3 May 2012 | Volume 6 | Issue 5 | e1644
areas. The standard curve was generated by a 1:10 serial dilution of DNA extracted from T. cruzi Colombian epimastigotes culture stocks, ranging from 10⁶ to 10 parasites equivalents.

**Total IgG and IgM and specific anti-T. cruzi serology**

The levels of total IgG and IgM were determined by kinetic nephelometry (Beckman Coulter Array 360, Beckman Coulter, USA) in accordance with the manufacturer’s recommendations. Specific anti-T. cruzi IgG antibodies were measured by ELISA (ELISA Chagas, BioManguinhos, Fiocruz, Brazil) in accordance with the manufacturer’s instructions.

**Nitric oxide quantification**

Nitrate and nitrite were determined in plasma samples from noninfected and infected monkeys using Griess reagent and vanadium chloride III with a standard curve of 0.3–100 μM NaNO₂ and NaNO₃ [18].

**Creatine kinase detection**

The activity of the CK-MB isoenzyme was measured in serum with a commercial kit (Labtest, Brazil) according to the manufacturer’s recommendations. The optical density at 340 nm (Microplate Reader Benchmark, Bio-Rad, USA) was recorded every 2 minutes for 15 minutes [19].

**Statistical analysis**

Data are expressed as arithmetic mean + SD. Student’s t test was adopted to analyze the statistical significance of the apparent differences. All statistical tests were performed with SPSS 8.0 software. Differences were considered statistically significant at p<0.05.

**Results**

**Spectrum of clinical forms of chronic Chagas disease is reproduced in Trypanosoma cruzi-infected rhesus monkeys**

The general characteristics of the studied monkeys are shown in Table S1. Electrical conduction abnormalities found in acute and chronically T. cruzi-infected monkeys are summarized in Table 1. Interestingly, the mild electrical abnormalities that we detected during the acute T. cruzi infection disappeared at the 4th month postinfection [15]. In this follow up study, during the chronic infection (18-25 ypi), three monkeys (#12, #64 and #60) showed transient electrical alterations, and three animals (#90, #95 and #103) presented significant electrical conduction abnormalities. The ECG patterns were normal in the 4 analyses of one monkey (#99). In infected monkey #90, multifocal ventricular extrasystoles were observed at 16 ypi, atrial extrasystoles were observed at 17 ypi, an incomplete left bundle branch block (LBBB) was observed at 20 ypi, and T-wave inversion was observed in all 4 analyses. In monkey #103, first-degree anterograventricular (AV) conduction disturbance and T-wave inversion were seen at 17 ypi. In monkey #95, LBBB was seen in 4 exams, and the T-wave inversion was more accentuated and right QRS axis deviation at 20 ypi. Representative ECG registers of chronically T. cruzi-infected are shown in Figure S1. As shown in Table S2, only monkey #95 showed ECHO abnormality with asynchronous interventricular septum motility and a decreased left ventricular ejection fraction (72.4% and 33.2% at 16 ypi and 17 ypi, respectively). Altogether, monkeys #12, #64, #68 and #99 were considered non-cardiopathic and monkeys #90, #93 and #103 were considered cardiopathic. All T. cruzi-infected monkeys showed normal radiological exams of the chest, esophagus and colon at the end-point of this study (data not shown). Therefore, monkeys #12, #64, #68 and #99 presented the indeterminate form of the chronic Chagas disease.

**Trypanosoma cruzi-infected rhesus monkeys presented histopathological alterations compatible with chronic chagasic cardiomyopathy**

In the acute infection, intense heart parasitism was associated with pronounced inflammatory infiltrates at 41 dpi (monkeys #37 and #67). This effect subsided at 76dpi (monkey #93), which coincided with parasitemia control. Inflammation was focal or absent at 3 ypi (monkey #45) [15]. In the chronic infection, none of the analyzed organs showed macroscopic alterations. In addition, no ventricular conduction system alteration was observed in the analyzed areas of the fibers composing the bundle of His in chronically T. cruzi-infected rhesus monkeys (data not shown). The main histopathological alterations found in the cardiac tissue are represented in the Figure S2. In monkeys with electrical abnormalities (#90, #95 and #103) and decreased left ventricular ejection fraction (#95), histopathological alterations were heterogeneous with areas with normal aspect (Figure S2E), but mostly areas with a mild or intense multifocal myocarditis (more pronounced in the left ventricle), with lymphocytes, macrophages, and an apparent lack of parasites, was associated with the disrupted spaces between cardiomyocytes and hyperplasty of the myocardial fibers (Figure S2D, Figure S2F). Further, monkeys with the indeterminate form of Chagas disease presented focal myocarditis (Figure S2A, Figure S2B) or complete absence of cardiac inflammation or myocardial fiber injury (Figure S2C). Conversely, noninfected controls (Figure S2G) consistently did not have inflammation or myocardial fiber injury.

Compared with noninfected controls (Figure 1A, Figure 1G), myocardial fibrosis with collagen deposits was detected in the early (41 dpi) acute infection (Figure 1B, Figure 1G). Fibrosis was residual in the early (3 ypi) chronic infection Figure 1C, Figure 1G) and characterized the indeterminate form of the disease. In the late (20 ypi) chronic infection, the monkey that had transient alterations in clinical score (#64) presented intense fibrosis (Figure 1D, Figure 1G), while in the monkey with normal clinical score (#90) heart histological aspects and collagen deposits resembling those of noninfected controls (Figure 1E, Figure 1G). In the cardiopathic monkeys (#90, #95 and #103), fibrosis (Figure 1F, Figure 1G) was related to the severity of electrical abnormalities. Collagen deposits varying from slight to severe were...
Figure 2. Persistence of *T. cruzi* in chronically infected rhesus monkeys. The persistence of *T. cruzi* parasite and antigens was evaluated by immunohistochemistry, PCR and antibody response. (A) Photomicrographs of section of myocardium of left ventricle of monkey #95 (20 ypl), immunohistochemistry for *T. cruzi* antigens (black arrows and insert) associated (dotted square) or not associated (black arrow) with focal inflammation. Inflammatory infiltrates lacking parasite antigens (white arrow heads). B–C. PCR for *T. cruzi* kDNA (330 bp) in blood of noninfected controls (NI) and *T. cruzi*-infected rhesus monkeys at (B) 16–20 ypl and (C) 20–23 ypl. D) PCR for *T. cruzi* kDNA (330 bp) in fragments of the left ventricle (LV) of the heart of noninfected controls and *T. cruzi*-infected rhesus monkeys at 20–23 ypl. Negative (−) and positive (+) controls were heart fragments of noninfected and *T. cruzi*-infected C3H/Bl/6 mice, respectively. E) Real-time qPCR for the *T. cruzi* satellite DNA sequences Cruz1/Cruz2 in...
frequently associated with inflammatory foci (Figure 1H, K). In areas of severe inflammation (Figure 1H), fibrosis was abundant (Figure 1I), and there was substitution of cardiomycocytes by mesenchymal cells (Figure 1J) and dense bundles of interstitial matrix that extended from the subepicardium into the subjacent myocardium (Figure 1K). Therefore, in six out of seven T. cruzi-infected thymus monkeys, clinical and histological alterations were compatible with chronic Chagas disease. Interestingly, three of these monkeys reproduced major aspects of CCC in patients [2, 19].

Parasite persistence in chronically Trypanosoma cruzi-infected monkeys

Exhaustive microscopic examinations of paraffin heart tissue sections failed to detect T. cruzi pseudocysts or isolated amastigotes in all of the chronically infected monkeys. Immunohistochemistry experiments were performed to detect T. cruzi antigens, and extramyocardial antigens were seen as red amorphous spots (Figure 2A) in six out of seven infected animals (the exception was monkey #99). T. cruzi antigens were commonly surrounded by mononuclear cell infiltrates (Figure 2A, black arrows); however, inflammatory cells were also seen associated to apparently noninfected fibers (Figure 2A, white arrow heads). In addition, blood samples and fragments of spleen and cardiac tissue were submitted to conventional PCR amplification targeting T. cruzi kDNA minicircles [11]. In the 1st analysis (16-19 ypi) of blood, all T. cruzi-infected animals showed a PCR signal at 330 bp for kDNA, whereas the noninfected animals (#81 and #94) did not show a signal (Figure 2B). In the 4th analysis (20-25 ypi), a PCR signal at 330 bp was detected in the blood of six infected rhesus monkeys (Figure 2G). Furthermore, T. cruzi kDNA was identified in the cardiac septum, left (Figure 2D) and right (Figure 2E) ventricles, left and right atria, and aorta of all infected monkeys except monkey #99. All of the infected monkeys were parasitologically positive for T. cruzi at 16-19 ypi [14]. At 20 ypi, however, attempts to identify T. cruzi kDNA in the blood and heart of monkey #99 were consistently negative (Figure 2C, Figure 2D). Considering that low parasitemia could contribute to these results, we performed additional studies using two or three tissue samples per analyzed monkey. Real time qPCR for the conserved repetitive nuclear satellite DNA sequences revealed low parasitemia in heart samples of all T. cruzi-infected rhesus monkeys except monkey #103. Conversely, real time qPCR revealed low parasitemia in spleen samples of all T. cruzi-infected monkeys (Figure 2E, Figure 2F). Heart and spleen samples of noninfected controls were repeatedly negative (Figure 2E). The b-globin sequence was detected as a control for DNA integrity and PCR inhibition in all analyzed samples (data not shown). Taken together, these results support parasite persistence in indeterminate (#81, #64, #68, #99 and cardiopathic (#90, #95, #103) chronically T. cruzi-infected rhesus monkeys. Because antibody detection is a criterion for persistent infection [20], we analyzed the kinetics of the anti-T. cruzi antibody in a group of infected monkeys that were cardiopathic (#90, #95 and #103) and a monkey that was indeterminate (#99) at 20 ypi. Figure 2G shows that all monkeys were seronegative prior to infection. The anti-T. cruzi antibody was only detected in one monkey (#103) when parasitemia was positive (18 dpi). When parasitemia was controlled (59 dpi) [15] all of the infected monkeys presented anti-T. cruzi antibodies. At the end-point of our study, anti-T. cruzi antibodies were detected in cardiopathic monkeys (#90, #95, #103), but not in indeterminate monkey #99. Importantly, there were no alterations in the levels of total IgM (Figure 3A) and IgG (Figure 3B) in serum of T. cruzi-infected monkeys in comparison with noninfected controls at the end-point of this study (20-25 ypi).

iNOS/NOS2* cells in the cardiac tissue and high NO levels in serum are associated with cardiomyopathy in chronically Trypanosoma cruzi-infected monkeys

Because parasite antigens were scarce in the cardiac tissues of monkeys with the indeterminate and cardiac forms of Chagas disease, we evaluated the cardiac tissue for the expression of iNOS/NOS2, which is an enzyme potentially involved in an important parasitic control pathway [9, 11]. Analysis of serial heart tissue sections revealed that most of the iNOS/NOS2 cells infiltrating the myocardial interstitium of infected animals (Figure 3A-D) were CD68+ macrophages (data not shown). There was a significant increase in the number of iNOS/NOS2* cells in the myocardium of monkey #92 that presented high parasitemia detected by qPCR for genomic DNA (Figure 3A-D, Figure 3E), whereas iNOS/NOS2* cells were scarce or absent in noninfected animals (Figure 3E).

In chronic Chagas disease, severity of CCC correlated with high NO levels in serum [6]. Because an increased number of iNOS/NOS2* cells in the cardiac tissue of infected monkeys was related to the severity of electrical abnormalities, we decided to study NO levels in the serum of infected monkeys. A high NO concentration was only detected in the monkey (#95) with severe CCC (Figure 3G). In this monkey, parasitic control was achieved at 2 months postinfection, at which point antibodies were detected (Figure 2G), in absence of significant NO concentration in serum (Figure 3G). A high NO concentration was only detected after 6 months of infection, and the NO level was persistently elevated at 16 and 20 ypi (Figure 3G). In the case of noninfected monkeys #81 and #94 the low numbers of iNOS/NOS2* cells in the cardiac tissue was parallel to low NO levels in serum (Figure 3G, Figure 3G). In the cardiopathic T. cruzi-infected monkey #95 the high number of iNOS/NOS2* cells in the cardiac tissue paralleled the high NO concentration in serum (Figure 3E, Figure 3F). However, there was no significant correlation 2 (p = 0.0866) between the number of iNOS/NOS2* cells in the cardiac tissue and NO concentration in serum of T. cruzi-infected rhesus monkeys.

Cardiac tissue injury is severe in Trypanosoma cruzi-infected monkeys: overexpressing iNOS/NOS2 and NO

Overexpression of iNOS/NOS2 and NO has been shown to be associated with heart injury in noninfectious conditions [8]. To test the possible consequences of iNOS/NOS2 overexpression in the heart tissue of T. cruzi-infected monkeys, we analyzed the expression of Cx43. Connexin 43 is the major gap junction protein in the heart, and Cx43 is primarily responsible for the electrical synchrony of cardiomyocytes [21]. Connexin 43 distribution was homogeneous in the intercalated disks of
Figure 4. Cardiomyocyte injury in *T. cruzi*-infected rhesus monkeys. Cardiomyocyte damage was assessed by immunohistochemical detection of Cx43 in the myocardium of the left ventricle and CK-MB activity levels in the serum of noninfected and chronically *T. cruzi*-infected rhesus monkeys. (A) Photomicrograph of myocardium section of the left ventricle of the noninfected monkey #94 showing normal pattern of Cx43 expression in intercalated discs. (B) Photomicrograph of myocardium section of the left ventricle of the *T. cruzi*-infected monkey #64 (23 yr) showing normal aspect. (C) Photomicrograph of left ventricle section of the *T. cruzi*-infected monkey #99 (20 yr) showing normal Cx43 pattern. (D)
related to the 12-lead ECG QRS scoring and the severity of Chagas' heart disease [23]. Fibrosis, which is one of the most important features of CCC [19], shows a progressive evolution in T. cruzi-infected mice [24]. Fibrosis is directed by inflammatory processes that provoke chemokine-driven accumulation of mesenchymal cells [25]. In the present studies, collagen deposition occurred early in the acute infection (11–76 dpi) concomitantly with inflammation. Interestingly, inflammation resolution and collagen degradation can occur without specific treatment. This finding was seen in monkey #45, which presented the indeterminate form of Chagas disease at 3 ypi. Monkey #99 did not exhibit cardiac inflammation or fibrosis; however, we could not prove whether fibrosis was established and later remodeled. The predominance of type III, pro-III, and pro-IV collagens in the heart tissue of individuals with chronic T. cruzi infection [24] may favor reversibility of fibrosis because these collagens have a high turnover. In T. cruzi infection, extracellular matrix deposition in the cardiac tissue can be remedied if inflammation subside, which has been observed after exogenous chemotherapy with benzimidazole in the chronic infection [21] and after modulation of inflammation with a partial CG-chemokine receptor antagonist in the acute and chronic infection [26,27]. These data suggest paralleling and inflammation as triggers of fibrogenic factors in CCC, which reinforces the idea that therapeutic strategies targeting parasites and inflammation, possibly combined, may be beneficial in remodelling fibrosis and restoring heart function in Chagas disease.

Observations of chronic myocarditis and fibrosis in the apparent absence of T. cruzi have suggested that autoimmunity is a central mechanism for CCC pathogenesis [3]. In a classic study, however, amastigotes forms of T. cruzi were detected inside myocardial cells in all analyzed CCC patients [19]. Furthermore, a PCR signal for T. cruzi kDNA has been observed in the hearts of CCC patients, but it was not detected in seronegative nonCCC patients [28]. In indeterminate and cardiopathic T. cruzi-infected monkeys, parasites remain, kDNA of T. cruzi, and nuclear satellite DNA persisted in the heart and spleen. Although in some tissues samples parasite kDNA was not detected in repeated analyses, the study of different fragments of heart and spleen and the use of more sensitive assay resulted in detection of parasitic persistence in all chronically T. cruzi-infected rheas monkeys. Considering the short life span of parasitic DNA in host tissues [29], PCR signals in the heart and spleen of infected monkeys constitute real proof of T. cruzi persistence. The presence of anti-T. cruzi antibodies in cardiopathic monkeys coincided with parasitic persistence in the blood, heart and spleen. In monkey #99, however, anti-T. cruzi antibodies were restricted to the acute infection and coincided with parasitic control. Detection of a specific immune response in chronically infected individuals reflects continuous antigens stimulation by persistent parasites. The consistent absence of detection of specific antibodies [15], T. cruzi kDNA in peripheral blood and heart in monkey #99, which had no electrical abnormalities, cardiac inflammation or fibrosis at 20 ypi in the absence of treatment, led us to consider the possibility of spontaneous cure in this monkey [19]. However, the absence of different fragments of spleen and heart revealed the presence of T. cruzi genomic DNA in both tissues of monkey #99, supporting the persistence of parasite in these tissues in this monkey with the indeterminate form of Chagas disease. Therefore, the low parasitemia restricted to focal areas in different organs may explain difficulties to reveal parasite and antibodies presence in this monkey.

Although there was no correlation between the quantity of antigens and the intensity of myocarditis, the detection of T. cruzi DNA in the cardiac tissue of infected monkeys supports the idea that persisting parasites trigger detrimental inflammation that can act on cardiomyocytes. Heart inflammation is a major factor that contributes to an increased risk of death in CCC compared with other heart conditions [30]. The components of inflammatory infiltrates contributing to this picture remain unclear. In noninfectious human diseases and murine models of cardiac pathologies, iNOS/SID6 and NO were shown to be both protective and detrimental for heart physiology, and these effects may be dependent on NO concentration [31]. Therefore, the increased number of iNOS/NOS2 cells in the heart tissue of T. cruzi-infected cardiopathic monkeys led us to search for cardiomycocyte lesion. Cxcccused #45 was severely depleted in cardiopathic monkey #99, which presented a high number of iNOS/NOS2 cells infiltrating the cardiac tissue. T. cruzi infection of human cardiomycocytes in vitro [32] and mice in vivo [27,33] has also been shown to cause Cx3c loss. This effect was hampered in infected T. cruzi-infected mice, which supports that TNF/TNF1 signaling is involved in Cx43 loss [33]. Tumor necrosis factor, particularly associated with INIFs, is an iNOS/SID6 inducer that results in NO production [78]. In this context, we detected IFNc in the serum of monkey #95 (150 pg/ml) and TNP in the serum of monkey #103 (28 pg/ml) and high IFNc and TNP levels in PMA-stimulated peripheral blood cells of monkeys #103 and #95, but not in noninfected controls or infected monkeys with the indeterminate form of Chagas disease (our unpublished data). Although consistent with the idea that the cardiopathic monkeys #103 and #95 overproduce inflammatory cytokines that may stimulate NO production, the low number of reactive and responsive animals hampered any definitive conclusion. Thus, the mechanism by which high iNOS/NOS2 is induced in cardiac tissue following T. cruzi infection remains unknown. The present study was the first to show that in CCC the numbers of iNOS/NOS2 cells in cardiac tissue are associated with Cx3c loss and, more clearly, with increased CK-MB activity levels in serum, markers of cardiomycocyte injury [21,22]. Interestingly, iNOS/NOS2 upregulation in an inflammatory milieu results in increased NO levels in the cardiac tissue in autoimmune myocarditis [34]. In addition, NO is induced in macrophages and cardiomyocytes by T. cruzi and inflammatory cytokines and chemokines, which require the NO/L-arginine pathway [35]. The severity of CCC is associated with high NO levels in chagasic patients [6]. Nitric oxide has also been shown to be involved in heart denervation in acute T. cruzi infection in rats [31]. Therefore, persistent T. cruzi in the cardiac tissue might sustain continuous iNOS/NOS2 expression and a large supply of NO in a tissue that normally experiences low and tightly controlled levels of this mediator [23,36]. In consequence, the increased expression of iNOS/NOS2 and supply of NO could lead to cardiomyocyte lesion and heart injury. To test
this idea, we adopted a Nos2−/− murine model. Initially, T. cruzi infection of C57BL/6 mice (the Nos2−/− genetic background) led to increased systemic NO production and, particularly, iNOS/NOS2+ cells in the cardiac tissue, which reproduced our findings in chronically infected monkeys. In addition, iNOS/NOS2+ cells have previously been detected in the cardiac tissue of acutely infected mice [9] and dogs [37]. Therefore, the present data show that independent of the host and the phase of infection, T. cruzi
**Table 2.** Electrocardiographic parameters of C57BL/6 and Nos2⁺/⁻ mice infected with the Colombian T. cruzi strain.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Heart rate (bpm)</th>
<th>PR interval (ms)</th>
<th>P-wave duration (ms)</th>
<th>QRS duration (ms)</th>
<th>QTc (ms)</th>
<th>Cardiac conduction (% of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 NIl</td>
<td>539 ± 28.1</td>
<td>35.8 ± 2.1</td>
<td>123.7 ± 1.4</td>
<td>11.1 ± 0.4</td>
<td>163.2 ± 2.2</td>
<td>Brad (0%) AVBl (0%) AVB2 (0%)</td>
</tr>
<tr>
<td>T. cruzi</td>
<td>441 ± 35.8**</td>
<td>41.1 ± 0.4**</td>
<td>125.5 ± 0.9</td>
<td>18.6 ± 1.5***</td>
<td>32.2 ± 2.3***</td>
<td>Brad (43%) AVBl (57%) AVB2 (43%)</td>
</tr>
<tr>
<td>Nos2⁻/⁻ NIl</td>
<td>400 ± 38.12</td>
<td>36.5 ± 4.0</td>
<td>140.1 ± 1.0</td>
<td>11.1 ± 1.1</td>
<td>20.7 ± 1.3**</td>
<td>Brad (60%) AVBl (20%) AVB2 (80%)</td>
</tr>
<tr>
<td>T. cruzi</td>
<td>423 ± 81.9</td>
<td>39.5 ± 4.3</td>
<td>140.2 ± 2.3</td>
<td>13.9 ± 2.4**</td>
<td>28.3 ± 9.8</td>
<td>Brad (57%) AVBl (43%) AVB2 (28%)</td>
</tr>
</tbody>
</table>

*ECG parameters were evaluated at 40 dpi, using the following standard criteria: I. heart rate (monitored by beats per minute (bpm)), and II. the variation of the P wave and QRS complex (ms) and T wave (ms). III. Brad, first-degree atrioventricular block; AVBl, second-degree atrioventricular block.

1. These data represent two independent experiments, with 5–7 mice/group.

2. *p < 0.05;

3. **p < 0.01;

4. ***p < 0.001;

5. No significant differences were found between the values for C57BL/6 and Nos2⁻/⁻ noninfected mice of each group.

6. No significant differences were found between the values for Nos2⁻/⁻ noninfected and T. cruzi-infected control groups of each group.

7. No significant differences were found between the values for Nos2⁻/⁻ noninfected and T. cruzi-infected control groups of each group.

8. doi:10.1371/journal.pntd.0001044.r002

**Supporting Information**

**Figure S1** Representing electrocardiographic registers of chronically T. cruzi-infected rhesus monkeys. Rhesus monkeys were infected with metacystic trypomastigotes of the Colombian T. cruzi strain and analyzed at 20–23 years post-infection (ypi). (A) ECG registers of monkey # 61 (20 ypi) showing normal pattern of electrical activity. (A) ECG registers of monkey # 99 (20 ypi) showing normal pattern. (C) ECG registers of monkey # 103 (20 ypi) demonstrating first degree right bundle branch block (RBB1, arrow). (D) ECG registers of monkey # 90 (20 ypi) showing first degree left bundle branch block (LBBB1, arrow). (E) ECG registers of monkey # 95 (20 ypi) showing second degree right bundle branch block (RBB2, arrow) and right QRS axis deviation (RAD, arrow head). (11F)

**Figure S2** Histological alterations in the myocardium of T. cruzi-infected rhesus monkeys. Sections of the myocardium of the left ventricle of noninfected and T. cruzi-infected rhesus monkeys were stained with H&E and analyzed under light microscope. Photomicrographs of heart tissue sections showing: (A) Monkey # 42 (20 ypi), with multifocal infiltrates of mononuclear inflammatory cells. (B) Monkey # 61 (25 ypi), intense focal mononuclear inflammation. (C) Monkey # 69 (20 ypi), normal aspect of the myocardium. (D) Monkey # 103 (20 ypi), multifocal

infection enhances NO in serum and iNOS/NOS2⁺/⁻ cells in the cardiac tissue. The present data also refine the result showing that iNOS/NOS2 is not required for T. cruzi control [12], but support previous findings that iNOS/NOS2 is essential for T. cruzi control in the cardiac tissue [9,11]. Paradoxically, NO might be detrimental in T. cruzi infection because it depresses lymphocyte functions, which could promote parasite survival [7]. Although this idea demands further experimental support, if this is the case this detrimental effect of NO on the immune response explains parasite persistence in chronically infected individuals, including patients [6] and our experimental models. Interestingly, iNOS/NOS2⁻/⁻ mice showed no NO production in T. cruzi-infected mice, which showed that iNOS/NOS2 is essential for NO overproduction. In addition, this result corroborated studies showing that there is no compensation mechanism increasing other ROS isofoms in Chagas disease [12]. Furthermore, iNOS/ NOS2 and locally produced NO are not involved in myocarditis formation, which suggests that the nature rather than the intensity of heart inflammation is determinant of the Chagas’ heart disease outcome [27]. Furthermore, T. cruzi infection of Nos2⁻/⁻ mice demonstrated a role for iNOS/NOS2 in myocardial cell lesion and connectivity loss, which supported our findings in chronically infected monkeys. Interestingly, recent proposals suggest that for Chagas disease to be considered a myoneuropathy [38]. Therefore, iNOS/NOS2⁻/⁻ derived NO may be a direct or indirect critical trigger of the molecular pathway leading to myocardial cell connectivity loss. Further, T. cruzi may directly lead to myocardial cell lesion, as revealed by increased CK-MB activity levels in serum and Gx45 loss, in infected mice. In this context, absence of iNOS/NOS2 led to significant decrease in CK-MB activity levels in serum, placing iNOS/NOS2-derived NO as important myocarditisogenic factor, but did not completely abolish myocardial cell lesion that persisted in presence of high cardiac tissue parasitism in acutely T. cruzi-infected Nos2⁻/⁻ mice. In chronically infected rhesus monkeys, focal persistence of T. cruzi (revealed by detection of antigen spots and of low amounts of parasite DNA) may contribute to maintain iNOS/NOS2 induction and local NO production leading to myocardial cell lesion. A previous study showed that iNOS/NOS2⁻/⁻ derived NO was associated with right ventricular dilation and systolic dysfunction in acute murine T. cruzi-infected myocarditis [9]. Although iNOS/NOS2 deficiency led to bradyarrhythmia in infected controls, T. cruzi-infected Nos2⁻/⁻ mice presented lower frequency of AVB1 and AVB2 than T. cruzi-infected C57BL/6 mice. More importantly, the higher QRS scores detected in C57BL/6 patients [2,23], which were reproduced in infected monkeys and C57BL/6 mice, were significantly decreased in T. cruzi-infected Nos2⁻/⁻ mice, which implicated iNOS/NOS2 and NO in QRS score increases independent of the host. Therefore, the present data support that the iNOS/NOS2 pathway participates in T. cruzi-induced myocardial cell lesion and heart injury and suggest that this pathway should be explored as a therapeutic target in C57BL/6. Considering observations in chronic Chagas disease [6], it is a plausible proposal as the pivotal role of NO in T. cruzi control was restricted to acute infection [39]. Further studies are needed to determine whether inhibition of iNOS/NOS2 will be therapeutically useful in chronic Chagas disease, a condition of vast overproduction of NO.
infiltrates of mononuclear inflammatory cells. (E) Monkey #90 (20 ypi), normal aspect of the myocardium. (F) Monkey #95 (20 ypi), multifocal infiltrates of mononuclear inflammatory cells. (G) Noninfected monkey #94, normal aspect. H&E. Bar = 100 μm. (TIF)

Figure S3 Total IgM and IgG levels in noninfected and chronically T. cruzi-infected rhesus monkeys. The levels of total immunoglobulin of the IgM and IgG classes were determined by nephelometry in the serum of noninfected and T. cruzi-infected rhesus monkeys at the end point (20–25 years post-infection, ypi). Total (A) IgM and (B) IgG levels in the serum. (TIF)

Figure S4 Representative electrocardiographic registers of T. cruzi-infected C57BL/6 and Nss2/ mice. The mice were infected with 100 blood trypomastigotes of the Colombian T. cruzi strain and analyzed at 40 dpi. (A) ECG registers of noninfected control C57BL/6 mice showing normal pattern of electrical activity. (B) ECG registers of T. cruzi-infected C57BL/6 mice showing first- and second-degree atrioventricular block (AVB1, AVB2, arrows). (C) ECG registers of noninfected control Nss2/ mice showing presence of sinus bradycardia. (D) ECG registers of T. cruzi-infected Nss2/ mice showing first-degree atrioventricular block (AVB1, arrows). (TIF)

Table S1 General characterization of Trypanosoma cruzi infection of rhesus monkeys. Rhesus monkeys were infected with metacyclic trypomastigotes from the Colombian T. cruzi strain and analyzed at 20–25 years post-infection (ypi). The original cohort identification used in the present study and the experimental number used in a previous publication that describes the analysis of the T. cruzi-infected monkeys during the acute infection, are provided. (DOC)

Table S2 Echocardiographic patterns detected in Trypanosoma cruzi infected rhesus monkeys during chronic infection. Rhesus monkeys were infected with metacyclic trypomastigotes from the Colombian T. cruzi strain and analyzed at 20–25 years post-infection (ypi). The echocardiographic registers were analyzed and the main findings were asynchronous interventricular septal motion, and decreased left ventricular ejection fraction detected in monkey #95. (DOC)

Acknowledgments

The authors would like to thank Dr. Antonio de Mota Marinho and the staff of the New England Primate Breeding Service SCPRIME, CECAL/ Flórida, Rio de Janeiro, Brazil for their assistance with the daily care and procedures with the monkeys. We would also like to thank Dr. Zeilão Andrade for the discussion of the initial histological findings, Dr. Antonio Carlos Camargo de Carvalho for the revision of the ECG registers of the studied mice, Dr. Beltrina Santos responsible for husbandry and breeding the Nss2/ mice, Mrs. Dazielia R. Martins for technical assistance performing the serology.

Author Contributions

Conceived and designed the experiments: CMGC AAS MBG-B ABB OCM RSM YTG JLA. Performed the experiments: CMGC AAS MBG-B ABB OCM RSM JMC MCG NMB JLA. Analyzed the data: CMGC AAS IRP OCM RSM JMCC SSX CCB MBG-B JLA. Contributed reagents/materials/analysis tools: CMGC AAS OCM RSM JMCC SSX CCB MBG-B JLA. Wrote the paper: CMGC JAS OCM IRP RSM MBG-B JLA.

References


