Journal of Microbiology, Immunology and Infection 56 (2023) 400—407

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Original Article

# Safety and preservation of cardiac function following therapeutic vaccination against *Trypanosoma cruzi* in rhesus macaques

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Received 5 April 2022; received in revised form 10 June 2022; accepted 20 September 2022
Available online 29 September 2022

**KEYWORDS**
Chagas disease;
DNA vaccine;
Cardiomyopathy;
Non-human primates;
Electrocardiograph

**Abstract** *Background:* Chronic Chagasic cardiomyopathy is responsible for a large disease burden in the Americas, and a therapeutic vaccine would be highly desirable. We tested the safety and efficacy of a therapeutic DNA vaccine encoding antigens TSA-1 and Tc24 for preventing cardiac alterations in experimentally infected macaques. A secondary objective was to evaluate the feasibility of detecting changes in cardiac alterations in these animals.
*Methods:* Naïve rhesus macaques were infected with *Trypanosoma cruzi* and treated with three doses of DNA vaccines.
*Results:* Blood cell counts and chemistry indicated that therapeutic vaccination was safe, as hepatic and renal function appeared unaffected by the vaccination and/or infection with *T. cruzi*. Electrocardiographic (ECG) recordings indicated that no marked arrhythmias developed up to 7 months post-infection. Univariate analysis of ECG parameters found no significant differences in any of these parameters between vaccinated and control macaques. However, linear discriminant analysis revealed that control macaques presented clear alterations in their ECG patterns at 7 months post-infection, indicative of the onset of conduction defects and cardiac alterations, and these changes were prevented in vaccine treated macaques.
*Conclusions:* This is the first evidence that therapeutic vaccination against *T. cruzi* can prevent cardiac alterations in non-human primates, strengthening the rationale for developing a human vaccine against Chagas disease.


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https://doi.org/10.1016/j.jmii.2022.09.003

## Background

Chagas disease, caused by *Trypanosoma cruzi* parasites, is responsible for 0.55 million disability-adjusted life years lost, and 10,600 annual deaths, and an estimated $7.2 billion in annual economic losses.[1] In spite of this major disease burden, it remains a neglected tropical disease that is often underdiagnosed and with substandard access to care for patients.[2]

Following infection with *T. cruzi*, patients present a short acute phase (about 6—8 weeks in duration), characterized by a high parasitemia and non-specific signs and symptoms such as fever, malaise, anorexia, myalgia, or headache. Next, they enter a chronic phase that is initially asymptomatic, with apparent control of the parasite, which can last many years. However, 20—30% of patients will develop cardiac arrhythmias of increasing severity, as long as 20 years or more after the initial infection, associated with dilated cardiomyopathy and eventually leading to cardiac failure.[3] Some patients may also present a digestive form of the disease, with megaesophagus or megacolon. In the US, chronic Chagasic cardiomyopathy is of growing concern.[4,5]

Current options for therapeutic treatment are limited to benznidazole or nifurtimox, which can be effective during the acute phase, but their efficacy becomes very variable during the chronic phase, as they can reduce parasite burden but fail to stop or delay the progression of fibrotic heart disease in symptomatic chronic patients.[6] These observations also suggest that cardiac function should be a key indicator of treatment efficacy. Adverse side effects associated with drug hepato- and nephrotoxicity are also frequently observed and contribute to treatment interruption, which also leads to reduced treatment efficacy.[7]

As an alternative, therapeutic vaccination has been explored to control ongoing *T. cruzi* infections,[8,9] as a vaccine would be very cost effective for Chagas disease control.[10,11] Several studies have provided evidence that therapeutic vaccination of *T. cruzi* infected mice with several vaccine formulations can reduce parasite burden and improve survival, and some studies further evidenced a reduction in fibrosis and cardiac dysfunction.[9] In particular, studies in mouse models have shown that vaccines based on trypomastigote surface antigen (TSA-1) and the flagellar calcium-binding Tc24 parasite antigens formulated as a DNA or recombinant protein vaccines can control *T. cruzi* infection.[12—16] Evaluation of a DNA vaccine encoding these antigens as both a preventative and therapeutic vaccine in dogs also showed a good efficacy at reducing *T. cruzi* parasite burden and cardiac tissue damage, although the reduction in the frequency of cardiac arrhythmias did not reach statistical significance.[17] As a key step to further vaccine development, we tested here the safety and efficacy of a DNA vaccine encoding TSA-1 and Tc24 antigens to prevent cardiac alterations in experimentally infected rhesus macaques. As mentioned above, this outcome is one of the most critical to evaluate vaccine efficacy against *T. cruzi* infection. While cardiac arrhythmias comparable to those reported in humans have been described in Chagasic non-human primates,[18—20] the time course of cardiac disease progression is poorly understood. Thus, a secondary objective of this study was to evaluate the feasibility of detecting cardiac alterations following an experimental *T. cruzi* infection to validate this macaque model for further vaccine and drug studies.

## Methods

### Study design

Nine naïve male rhesus macaques (*Macaca mulatta*) were enrolled in the study (Fig. 1). A baseline blood sample was taken, and animals were infected with *T. cruzi* parasites. A group of five randomly selected animals received three doses of the therapeutic vaccine at months 2, 3 and 4 post-infection, and four animals received empty plasmid as control. Monthly blood samples were collected for blood cell counts and chemistry to evaluate vaccine safety. Electrocardiographic recordings were performed at baseline (month 0), and after 3 and 7 months of infection to assess cardiac function. Three animals from each group were euthanized after 7 months of infection and histopathologic tissue damage was evaluated.

### Ethical concerns

Animal housing, care, and research were performed in compliance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council and the guidelines at the TNPRC, an institute fully accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and in accordance with the Animal Welfare Act guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University. All procedures were performed on sedated animals.

### *T. cruzi* infection and DNA vaccination

Nine male rhesus macaques, 6—13 years old, weighting 6—13 kg, were enrolled in the study. Macaques were infected with $3.5 \times 10^5$ *T. cruzi* parasites (Y strain) via subcutaneous injection at month 0. Infection was confirmed by detecting anti-*T. cruzi* antibodies using Stat-Pack rapid test (Chembio) and parasite DNA by PCR in blood samples taken at 1 and 2 months post-infection. DNA vaccines based on the pcDNA3.1 plasmid vector and encoding TSA-1 and Tc24 *T. cruzi* antigens have been described before.[12,17] Vaccinated monkeys received three doses of 500 μg of DNA vaccine (250 μg of each plasmid) with 225 μg of aluminum phosphate as adjuvant, via intramuscular injection, at months 2, 3 and 4 post-infection, while controls received the empty pcDNA3.1 plasmid with aluminum phosphate. While aluminum phosphate favors a Th2 response with recombinant protein vaccines, it favors a Th1 response with DNA vaccines.[21,22]

### Blood cell counts and chemistry

Body weight, blood chemistry and cell counts were measured monthly to assess vaccine safety. EDTA-

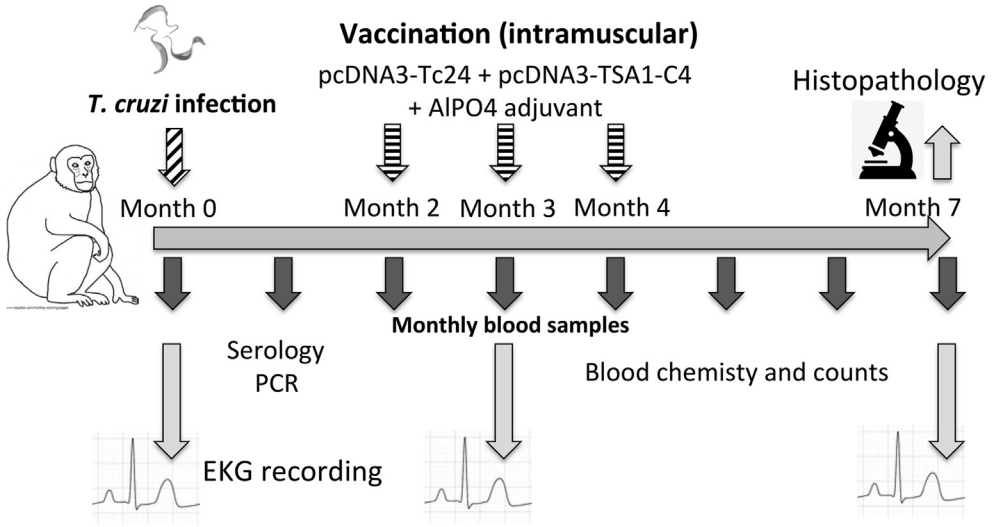


**Figure 1.** Summary of study design.

anticoagulated blood was analyzed using a Sysmex XT2000i for blood cell counts. $CD4^+$ and $CD8^+$ cell counts were measured by flow cytometry with the following fluorochrome-labeled monoclonal antibodies: anti-rhesus monkey CD3-fluorescein isothiocyanate (clone SP34; Pharmingen), anti-human CD4-phycoerythrin (SK3; Becton Dickinson), and anti-human CD8-peridinin chlorophyll protein (SK1; BectonDickinson) as described before.[23] Blood chemistry was performed using the Beckman AU480 chemistry analyzer. Data are presented as mean ± SD.

## Cardiac function and histopathology

Cardiac function was assessed by six lead electrocardiographic (ECG) recordings made at a speed of 25 mm/s and 40 mm/mV using a DRE TrueVET ECG-1 Single Channel recorder on sedated macaques (10 mg/kg ketamine HCl (100 mg/ml, Dechra)). The duration of the RR, QRS, QT and QTc intervals was manually measured (average of 10 beats for each animal) and compared between groups using Student's t test. For multivariate analysis, all ECG variables were integrated into a linear discriminant analysis (LDA), and compared according to the first and second axis. One-way permutation ANOVA (PERMANOVA) was used to assess statistical significance of differences among groups with 10,000 permutations. The confusion matrix of the LDA analysis was also used to evaluate the accuracy of the reclassification of individual macaques among experimental groups based on the similarity/differences in their ECG patterns.

For histopathologic analysis, tissue samples of the heart, colon, stomach, liver, lung, brain, esophagus, mesenteric lymph node, pancreas, spleen, testes and skeletal muscle were collected at the necropsy of three animals per group, embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin.

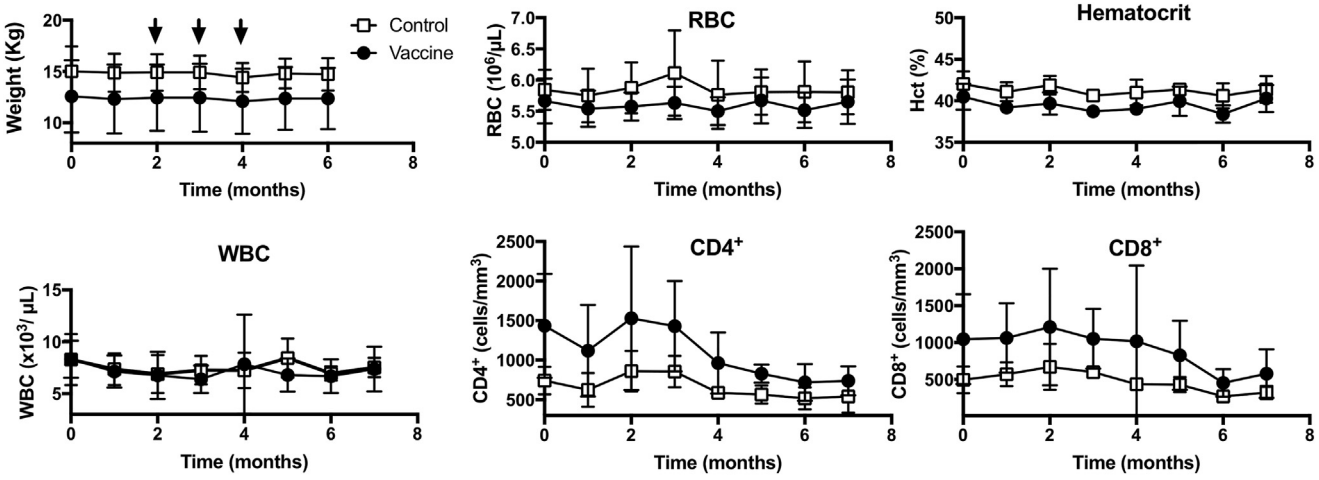


**Figure 2. Body weight and blood cell counts following *T. cruzi* infection and therapeutic vaccination.**
Changes in body weight, red blood cells (RBC), hematocrit, white blood cells (WBC), CD4+ cells and CD8+ cells over time are shown. *T. cruzi* infection occurred at time 0, and therapeutic vaccination at months 2, 3 and 4 post-infection (arrows). Open square: control macaques, closed circles: vaccinated macaques. All data are presented as mean ± SD.

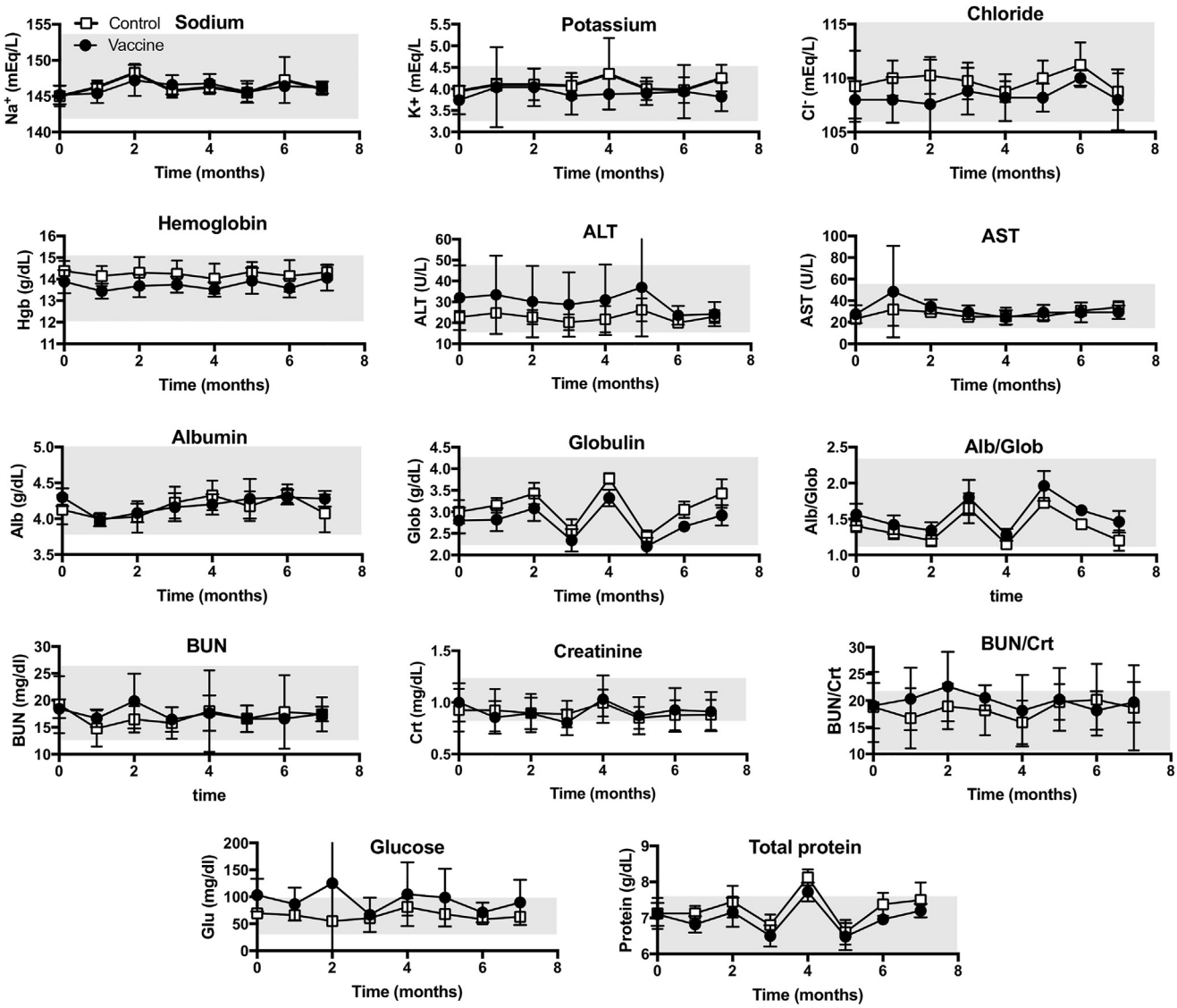


**Figure 3. Blood chemistry following *T. cruzi* infection and therapeutic vaccination.**
*T. cruzi* infection occurred at time 0, and therapeutic vaccination at months 2, 3 and 4 post-infection. Open square: control macaques, closed circles: vaccinated macaques. All data are presented as mean ± SD. Changes in blood chemistry over time are shown for the indicated variables: sodium, potassium, chloride, hemoglobin, ALT, AST, albumin, globulin, albumin/globulin ratio, BUN, creatinine, BUN/creatinine ratio, glucose and total protein. Gray shaded areas indicate normal range.

Sections were scored by a veterinary pathologist blinded to the experimental group of each animal, based on a 5 level scale: 0: normal tissue, 1: minimal inflammation, 2: mild inflammation, 3: moderate inflammation, and 4: severe inflammation.

## Results

### Vaccine safety

Nine naïve male rhesus macaques (*M. mulatta*) were infected with *T. cruzi*. A group of five randomly selected animals received three doses of the therapeutic DNA vaccine encoding TSA1 and Tc24 antigens at month 2, 3 and 4 post-infection, and four animals received empty plasmid as control (Fig. 1). Monitoring of body weight during *T. cruzi* infection and after therapeutic vaccination indicated that there was no change during the 7 months of follow up in both the control and vaccine group (Fig. 2). Monthly blood samples were collected for blood cell counts and chemistry to further evaluate vaccine safety. Blood cell counts remained rather constant over the study period (Fig. 2), although some minor fluctuations in $CD4^+$ and $CD8^+$ cell counts could be observed in both groups, particularly following infection. No changes were detected in any of the blood chemistry parameters measured (Fig. 3), which mostly were within their expected range, indicating a normal hepatic and renal function in both groups of animals. The only notable observation was that one animal in the vaccine group was diabetic from the onset of the experiment, but glucose levels were not further affected by *T. cruzi* infection nor by therapeutic vaccination (Data not shown).

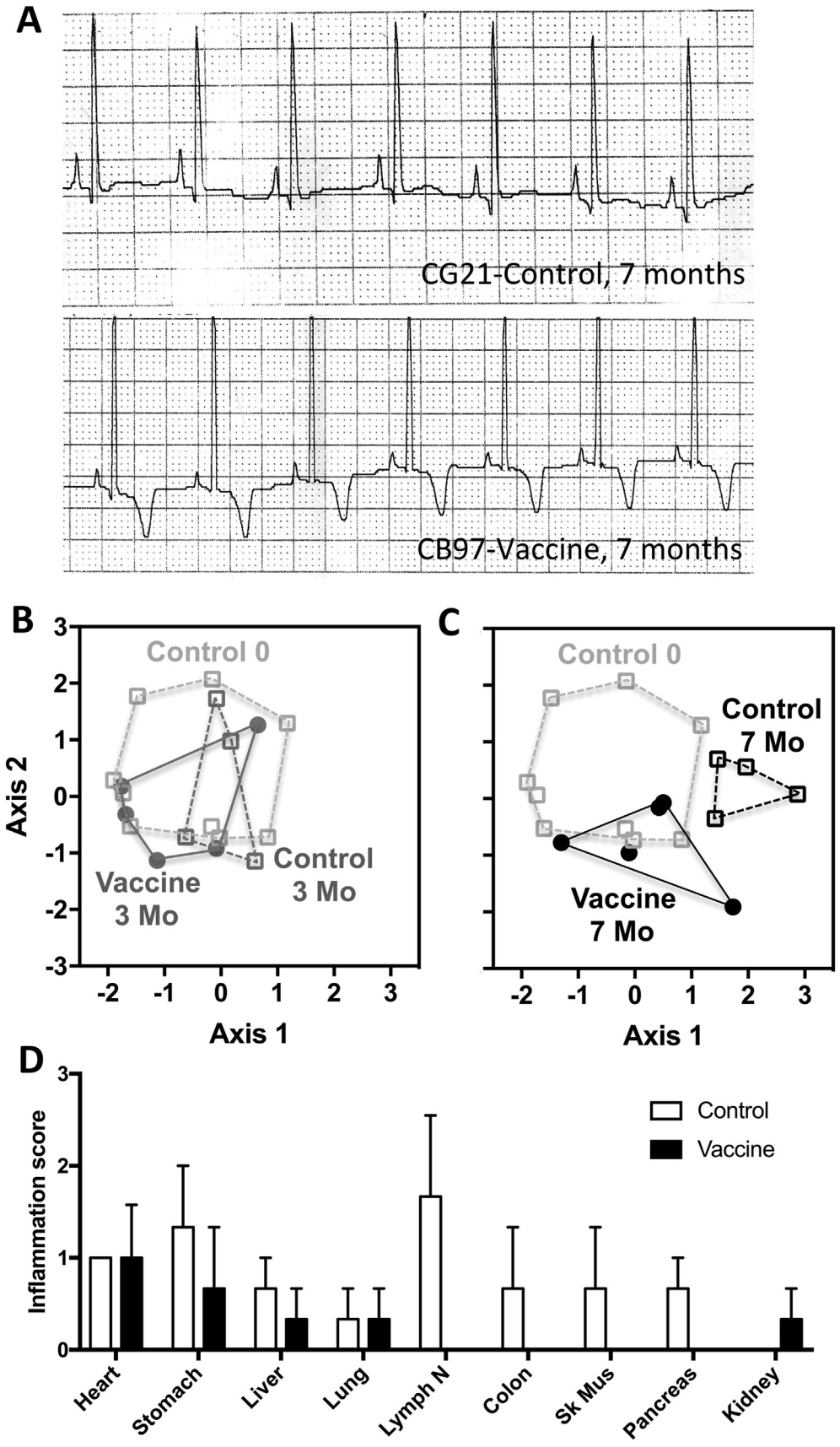


**Figure 4. Cardiac function and tissue inflammation following therapeutic vaccination in *T. cruzi* infected macaques.** **(A)** ECG recording from a control (CG21) and vaccinated (CB97) macaque after 7 months of *T. cruzi* infection. **(B and C)** LDA analysis

### Cardiac function and tissue damage

Monitoring of cardiac function was performed by electrocardiographic (ECG) recordings, and no notable cardiac arrhythmias were detected during the study period in any of the groups (Fig. 4A). Measurement of time intervals for the RR, QRS, QT and QTc intervals were also within the expected range and were not significantly different between the vaccinated and control group for all time points (Student's t test, $P > 0.05$ for all parameters). Linear discriminant analysis (LDA) of ECG parameters further confirmed that control macaques at 3 months post-infection presented no alterations in ECG parameters compared to the baseline ECG before infection (Fig. 4B, PERMANOVA $P = 0.62$). However, at 7 months post-infection, control macaques presented clear alterations in their ECG patterns, indicative of the onset of conduction defects and cardiac dysfunction (Fig. 4C, PERMANOVA $P = 0.029$ with uninfected baseline EKG and $P = 0.058$ with 3 months ECG, respectively). On the other hand, the ECG from vaccinated macaques remained unaltered at both 3 and 7 months post-infection and not different from the ECG at baseline before infection (Fig. 4B and C, PERMANOVA $P = 0.92$ for 7 months ECG vs baseline ECG and $P = 0.49$ vs 3 months ECG, respectively). The confusion matrix of the LDA analysis also confirmed that the ECGs from vaccinated macaques were indistinguishable from uninfected control ECGs at baseline with a low group classification accuracy among these (7/23, 30% correctly classified), and only the ECGs from the control macaques at 7 months post-infection allowed to classify these in a separate group (4/4, 100% correctly classified).

Following necropsy of three animals per group, histopathologic analysis indicated some minimal to mild inflammation in several tissues including the heart, colon, stomach, liver, lung, mesenteric lymph node, pancreas, and skeletal muscle after 7 months of *T. cruzi* infection, with a few instances of moderate inflammation in the heart, stomach and mesenteric lymph node (Fig. 4D). On the other hand, no histologic alterations were observed in the brain, esophagus, spleen and testes. Comparable levels of inflammation were observed between control and vaccinated macaques in the heart, stomach, liver and lung. However, inflammation was observed in the mesenteric lymph node, colon, skeletal muscle and pancreas of control macaques, but not in vaccinated animals. Some inflammation was observed in the kidney of one vaccinated animal but not in the control group. Taken together, these results suggest a more diffuse infection in control animals compared to vaccinated macaques, and confirm the safety profile of the therapeutic vaccination.

## Discussion

While several studies have shown the efficacy of therapeutic vaccination to control *T. cruzi* infection in mouse models, the translational value of these results for a human vaccine is unknown. Indeed, studies have found more variable results when vaccines have been tested in dogs, and while several vaccine formulations were able to significantly reduce parasite burden, vaccine efficacy to prevent or delay cardiac dysfunction has been more difficult to establish.[17,24–26] This outcome is nonetheless critical as evidenced by the BENEFIT clinical trial, which showed that benznidazole drug treatment can significantly reduce parasite burden, but has a more questionable effect on cardiac dysfunction.[6] Thus, we evaluated here the efficacy of the therapeutic vaccination with a DNA vaccine encoding TSA-1 and Tc24 antigens to prevent cardiac dysfunction in experimentally infected rhesus macaques.

Analysis of blood counts and chemistry indicated that therapeutic vaccination with this DNA vaccine was safe, as hepatic and renal function appeared unaffected by the vaccination and/or infection with *T. cruzi*, and all blood parameters remained constant during *T. cruzi* infection in both the vaccinated and unvaccinated groups. Body weight was also unaffected by *T. cruzi* infection and vaccination. These results contrast with a previous report documenting changes in blood cell counts and body weight loss following experimental *T. cruzi* infection.[20] but another study also found no alterations in blood chemistry induced by infection.[19] These discrepancies may reflect differences in parasite strain, route, infective dose or follow-up times.

Monitoring of cardiac function through ECG indicated that no marked arrhythmias developed during the 7 months of the study period. Again, this may indicate differences in parasite infection parameters and/or follow-up time compared with previous studies.[18–20] Nonetheless, while univariate analysis of ECG parameters was unable to identify significant differences in any of these parameters, multivariate analysis had a greater power to identify minimal changes. These changes may be indicative of the onset of small conduction defects, which may eventually lead to arrhythmias and heart failure. These results indicate that cardiac alterations can be detected by ECG in experimentally infected macaques after a few months, highlighting the feasibility of using this infection model for the short-term evaluation of vaccines or novel drug treatments.

Our results also provide the first evidence that therapeutic vaccination against *T. cruzi* can prevent ECG alterations in non-human primates, at least in the short-term as these macaques can be considered in the early chronic phase. The reduction in inflammatory response from multiple tissues following therapeutic vaccination also suggests

of ECG parameters. Open squares: control macaques, filled circles: vaccinated macaques. Control 0: Baseline (uninfected and not vaccinated), (**B**) 3 mo: 3 months post-infection (and 1 month after first vaccine dose), (**C**) 7 mo: 7 months post-infection (and 3 months after last vaccine dose). At 7 months post-infection, control macaques presented significant alterations in their ECG patterns (PERMANOVA $P = 0.029$ with baseline ECG and $P = 0.058$ with 3 months ECG, respectively). ECG parameters from vaccinated macaques were not different from pre-infection baseline (PERMANOVA $P = 0.92$ for 7 months ECG vs baseline ECG and $P = 0.49$ vs 3 months ECG, respectively). **(D)** Tissue inflammation at 7 months post-infection. Mean ± SEM of inflammation scores from 3 macaques per group for the indicated tissues.

an improved control of the infection, with no exacerbated inflammation. While longer-term follow-up is still needed to better characterize vaccine efficacy, and potentially detect additional clinical differences in cardiac dysfunction, including systolic dysfunction, these results expand previous observations in mice and dogs, and greatly strengthen the rationale for further development of a human vaccine against Chagas disease. These findings also indicate the feasibility of detecting ECG alterations following an experimental *T. cruzi* infection in a relatively short time, validating this macaque model for further vaccine and drug studies. The parasite antigens TSA-1 and Tc24 encoded by this vaccine are thus good candidates for further development, which supports ongoing efforts for product development as a recombinant protein vaccine.[9,27]

## Financial support

This work was funded by a Gorgas Memorial Institute Award from the American Society for Tropical Medicine and Hygiene and a TNPRC Pilot Research Program Grant. This work was supported by the National Center for Research Resources and the Office of Research Infrastructure Programs (ORIP) of the NIH through grant P51 OD011104 to the Tulane National Primate Research Center.

## Declaration of competing interest

The authors have no conflict of interest.

## Acknowledgments

We thank the Division of Veterinary Medicine for expert animal care and Theresa Glissman for administrative support. We also thank Dr. Peter Didier, Professor of Pathology and Laboratory Medicine, Chief of Diagnostic Pathology Services, TNPRC, for necropsies and histopathologic analyses, and Kelly Goff for expert technical assistance.

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