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## TcVac1 vaccine delivery by intradermal electroporation enhances vaccine induced immune protection against *Trypanosoma cruzi* infection in mice

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## ABSTRACT

The efforts for the development and testing of vaccines against *Trypanosoma cruzi* infection have increased during the past years. We have designed a TcVac series of vaccines composed of *T. cruzi* derived, GPI-anchored membrane antigens. The TcVac vaccines have been shown to elicit humoral and cellular mediated immune responses and provide significant (but not complete) control of experimental infection in mice and dogs. Herein, we aimed to test two immunization protocols for the delivery of DNA-prime/DNA-boost vaccine (TcVac1) composed of TcG2 and TcG4 antigens in a BALB/c mouse model. Mice were immunized with TcVac1 through intradermal/electroporation (IDE) or intramuscular (IM) routes, challenged with *T. cruzi*, and evaluated during acute phase of infection. The humoral immune response was evaluated through the assessment of anti-TcG2 and anti-TcG4 IgG subtypes by using an ELISA. Cellular immune response was assessed through a lymphocyte proliferation assay. Finally, clinical and morphopathological aspects were evaluated for all experimental animals. Our results demonstrated that when comparing TcVac1 IDE delivery vs IM delivery, the former induced significantly higher level of antigen-specific antibody response (IgG2a + IgG2b > IgG1) and lymphocyte proliferation, which expanded in response to challenge infection. Histological evaluation after challenge infection showed infiltration of inflammatory cells (macrophages and lymphocytes) in the heart and skeletal tissue of all infected mice. However, the largest increase in inflammatory infiltrate was observed in TcVac1\_IDE/Tc mice when compared with TcVac1\_IM/Tc or non-vaccinated/infected mice. The extent of tissue inflammatory infiltrate was directly associated with the control of tissue amastigote nests in vaccinated/infected (vs. non-vaccinated/infected) mice. Our results suggest that IDE delivery improves the protective efficacy of TcVac1 vaccine against *T. cruzi* infection in mice when compared with IM delivery of the vaccine.

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## 1. Introduction

*Trypanosoma cruzi* is the causative agent of devastating Chagas disease in Latin America. Most common route of parasite transmission to humans is through blood-sucking triatomines. Chagas disease is characterized by an acute and a chronic phase. Once the acute parasitemia abates, most of the infected individuals attain a less fulminant chronic infection. Several years after the infection, 20–35% of the infected individuals develop irreversible lesions of the autonomous nervous system of the heart, and/or esophagus, colon, and peripheral nervous system. Chagas disease represents the major cause of cardiac lesions in young, economically productive adults in the endemic countries [1,2]. Vector control programs have not been able to completely prevent parasite transmission [3], the available anti-parasitic drugs are not sufficiently safe or effective, and no vaccines are currently available [4].

The studies of immune responses in resistant and susceptible models of Chagas disease have been valuable in enhancing our knowledge of the protective immunity and designing of the vaccination approaches against *T. cruzi* infection. The sequencing of *T. cruzi* genome and the development of approaches to produce recombinant proteins have made it feasible to produce, deliver, and test the efficacy of a variety of recombinant *T. cruzi* antigens as potential vaccine candidates in experimental models of infection and disease [5–7]. Indeed, many of the recombinant antigens have been tested as prophylactic vaccines. However, recombinant protein based vaccines failed to provide high efficacy in controlling *T. cruzi* infection [8,9]. This is, at least, partially due to the fact that recombinant proteins elicited potent anti-*T. cruzi* antibody response, but failed to stimulate cellular immunity that is required to control the intracellular replicative form of the parasite. Thus, new candidate antigens as well as new delivery approaches are required to design an efficacious vaccine against *T. cruzi* infection.

Our group has performed computational screening of *T. cruzi* sequence databases reported in GenBank and identified genes encoding glycosylphosphatidylinositol (GPI)-anchored proteins as potential vaccine candidates [5]. Through rigorous analysis over a period of several years, we determined that two of the selected candidate antigens, named TcG2 and TcG4, were maximally relevant for the vaccine development. These two antigens are phylogenetically conserved in a variety of *T. cruzi* lineages, expressed in infective and intracellular stages of the parasite's life cycle, and are recognized by immunoglobulins (IgGs) and CD8<sup>+</sup> T cells in multiple *T. cruzi*-infected hosts [10]. Intramuscular co-delivery of these antigens as DNA vaccine (TcVac1) induced higher degree of protection from *T. cruzi* infection than was observed by delivery of single antigens in mice [7]. In dogs, TcVac1 candidate vaccine elicited a significant trypanolytic antibody and Th1 cytokine (IFN- $\gamma$ ) response, a property that has been associated with immune control of *T. cruzi* [11].

Antigen delivery methods can result in varying quality and quantity of immune responses. Physical delivery systems, such as electroporation, micro-injection, gene gun, tattooing, laser and ultrasound have been tested for the delivery of DNA-based vaccines against infectious agents [12]. Electroporation (EP) induces the formation of aqueous pores in lipid bilayers by the application of a brief (microseconds to milliseconds), high-voltage pulse to overcome the barrier of the cell membrane. This transient permeabilized state is used to load cells with a variety of different molecules including ions, drugs, dyes, tracers, antibodies, RNA and DNA [13]. Electroporation of DNA *in vivo* has proved to be an effective method, yielding higher level of the cellular uptake of DNA, increase in the number of DNA-transfected cells, and reduced inter-subject variability. Electroporation of DNA vaccine also pro-

vided increased magnitude of gene expression while requiring less time to reach a maximal immune response when compared to the conventional intramuscular approach of DNA vaccine delivery [14]. Increased efficacy of DNA vaccines delivered by electroporation method vs. other methods, has been shown against a wide range of infectious diseases, such as influenza, HIV, hepatitis C, malaria, and anthrax [15]. Electroporation has also been tested for the delivery of drugs and vaccines against a variety of cancers [16,17].

Herein, we describe the protective efficacy of TcVac1 vaccine applied through an intradermal electroporation (IDE) approach. The TcVac1 vaccine was injected in mice via IDE or intramuscular (IM) approach, and mice were then challenged with *T. cruzi*. We have analyzed the function of vaccine-induced antibody and lymphocyte responses in providing protection from acute parasitemia in mice. We also performed histopathological examination of the heart and skeletal muscle, focusing on the efficacy of vaccination protocol in reducing the tissue injury during the acute *T. cruzi* infection.

## 2. Materials and methods

### 2.1. Ethics statement

All experimental protocols were conducted under the technical specifications for the production, care and use of laboratory animals of the official Mexican standards (NOM-62-ZOO-1999) [18]. The Laboratory Animal Care Committee at the School of Veterinary Medicine and Zootechnics of the Autonomous Mexico State University (UAEM, Toluca, Mexico) approved all protocols.

### 2.2. Mice and parasites

BALB/c female mice (6–8 weeks old, n = 48) were purchased from CINVESTAV, IPN, Mexico. *T. cruzi* trypomastigotes (Sylvio 10X/4 strain) were cultivated in continuous monolayer of Vero cell line. Vero cells were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and cultured under controlled conditions (37 °C, 5% CO<sub>2</sub>, and saturated humidity) [19]. All media components were purchased from Gibco Laboratories, USA.

### 2.3. TcVac1 vaccine

The cDNAs encoding for TcG2 and TcG4 (Sylvio X10/4 isolate, GenBank: AY727915 and AY727917, respectively) cloned in eukaryotic expression plasmids (pcDNA3.1.TcG2 and pcDNA3.1.TcG4), plasmids encoding IL-12 (pcDNA3.1-msp35 and pcDNA3.1-msp40), and GM-CSF (pcMVI.GM-CSF) have been previously described [5,7]. All plasmids were transformed into *E. coli* DH5- $\alpha$  competent cells, grown in LB-broth containing 100-mg/ml ampicillin, and purified using the GeneJET Plasmid Maxiprep Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to manufacturer's specifications. The vaccine was constituted of 25- $\mu$ g of each plasmid and delivered in 50  $\mu$ l final volume per mouse.

### 2.4. Immunization and challenge infection

Mice were randomly distributed to treatment groups (n = 12 per group) as follows: group I, Only empty pcDNA3.1 plasmid (25  $\mu$ g/mouse) via intradermal electroporation (IDE)  $\pm$  challenge infection; group II, TcVac1 DNA vaccine (25  $\mu$ g each from the 5

DNA plasmids composing the vaccine) via IDE  $\pm$  challenge infection; group III, Only empty pcDNA3.1 plasmid (25  $\mu$ g/mouse) via intramuscular (IM)  $\pm$  challenge infection; and group IV, TcVac1 DNA vaccine (25  $\mu$ g each from the 4 DNA plasmids composing the vaccine/mouse) via IM  $\pm$  challenge infection. Six animals from each group were sacrificed to evaluate the vaccine previous to the infection and the remaining six animals from each group were infected and then evaluated during the acute phase of the disease.

Animals were given DNA vaccine (TcVac1) or placebo (pcDNA3) by intramuscular (IM) injection in the quadriceps muscle or by intradermal electroporation (IDE). The vaccine, or placebo, was delivered four times, at 3 weeks intervals. For IDE, mice were sedated (ketamine/xylazine mix, contains: 87.5 mg/kg Ketamine and 12.5 mg/kg Xylazine, 0.1 ml/20 g mouse wt. IP injected) and shaved at the lower dorsal area, just above the tail base, and TcVac1 DNA vaccine was delivered by intradermal injection. Immediately afterwards, electroporation was performed at the site of injection with 5 pulses at 450 V/cm<sup>2</sup>, 0.050-msec pulse interval and 0.125-msec band width by using a Cyto Pulse Sciences™ PA-4000 PulseAgile® electroporation system. This device consists of 4  $\times$  4 electrode array with 1.5-mm spacing to provide optimal coverage on the DNA injection site [20].

Two weeks after the last immunization, mice were either utilized for assessing the vaccine induced immune responses or challenged with Sylvio 10X/4 strain of *T. cruzi* (10,000 trypomastigotes/mouse, intraperitoneally). Sixty days' later, by the end of the acute phase of infection, mice were sacrificed, and blood was collected for serological evaluation, spleen was collected for lymphocyte activation assays, and heart and skeletal muscle were dissected and prepared for histological analyses.

### 2.5. Blood sample collection

Mice blood was obtained directly from heart at the time of sacrifice [21]. Blood samples were allowed to clot at 4 °C and serum was collected and stored at -20 °C.

### 2.6. Lymphocyte proliferation

Spleens were dissected out in sterile conditions, washed in ice-cold 1X PBS, and meshed using a 40  $\mu$ m Nylon Cell Strainer (Corning, USA). The cells were washed and suspended in 1.5 ml of PBS, gently laid over sterile-filtered Histopaque®-1077 (1.5 ml, density: 1.077 g/mL, Sigma-Aldrich, USA), and centrifuged at 2300 rpm for 30 min at room temperature. Splenocytes were carefully collected, washed twice with PBS, and resuspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich). Splenocytes (2  $\times$  10<sup>5</sup> cells per well) were added to Nunc Maxisorp 96 well tissue culture plates (#M9410) and incubated for 48 h in presence or absence of *T. cruzi* trypomastigotes lysate (TcTL) [22] or recombinant TcG2 or TcG4 antigens (10  $\mu$ g/ml). Splenocytes incubated with phytohemagglutinin (PHA, Thermo Scientific) were used as positive control. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used to measure the number of viable cells. Briefly, cells were incubated with MTS tetrazolium salt (10  $\mu$ l MTS/100  $\mu$ l), and its reduction to colored formazan product was recorded at 490 nm by using an EPOCH microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). Stimulation index (StI) was defined as the ratio of the absorbance signal observed in the negative control (C) and in the stimulated cells (S) and calculated as follows:

$$\text{StI} = (S - C)/C$$

where S and C represents the absorbance values (OD) for stimulated (TcG2, TcG4, TcTL and PHA) and negative control cells (wells containing only splenocytes and RPMI-1640 medium), respectively.

A mice group (n = 6), treated with saline solution, was used to calculate the cutoff value and was represented as follow: mean StI of all negative control groups (saline, pcDNA3.1-IDE and pcDNA3.1-IM)  $\pm$  SD before and after challenge infection.

### 2.7. Serology

Sera samples were obtained two-weeks after the last immunization and 60 days post challenge infection, according to previous reports [10] Briefly: Flat bottom, 96 well, Nunc Maxisorp plates were coated with either *T. cruzi* lysate or recombinant TcG2 or TcG4 proteins (500 ng/well/100  $\mu$ l) diluted in 0.05 M NaCO<sub>3</sub>/NaHCO<sub>3</sub>, pH 9.6 buffer. Protein concentration was standardized in an independent assay to choose the optimal concentration for the assay from 1, 2.5 and 5 ng/well/100  $\mu$ l. Plates were washed twice with PBS-0.05% Tween 20 (PBST), and blocked with (5% non-fat dry milk -PBST). Plates were washed again, and incubated at 37 °C for 2 h each with sera samples (1:50 dilution, 200  $\mu$ l/well) and horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgG2b antibodies (1:5000 dilution in PBST-3% non-fat dry milk (NFDM)). All washing steps were carried out by using an ELx50™ strip washer (BioTech), and all antibodies were purchased from Bethyl Laboratories, Inc. Color was developed by incubation with 100- $\mu$ l/well Sure Blue TMB substrate (Kirkegaard & Perry Labs) at room temperature for 15 min, reaction was stopped with 2 N sulfuric acid, and change in color was monitored at 450 nm using an Epoch microplate reader. Serum from known positive (n = 6) and known negative (n = 6) mice, were used in the ELISA assay for positive and negative controls, respectively. The cut off value was established at the mean value + 2 SD of the negative controls. Positive controls were always above that value.

### 2.8. Parasitemia and mortality

Blood samples were collected beginning 7 days post-infection (pi), on alternated days, up to 50 days pi. Fresh blood smears were analyzed by light microscopy at 400 $\times$  final magnification, and parasites were counted in all microscopic fields per slide [23]. Mortality was recorded daily and a survival rate analysis was conducted, results were analyzed statistically by Mantel-Cox test.

### 2.9. Histology

Six mice per group were sacrificed humanly under anesthesia on day 15 after last immunization and the remaining six mice were sacrificed at 60 days post challenge infection. Samples from skeletal muscle and heart were fixed in 10% formaldehyde and embedded in paraffin [24]. Serial sections of five  $\mu$ m thicknesses were prepared from non-consecutive areas, stained with hematoxylin and eosin (H&E), and at least five sections per tissue for each mouse were examined for the presence of acute inflammation and parasite nests (magnification: 400 $\times$  and 100 $\times$ ).

### 2.10. Tissue parasitism and inflammation score

Tissue parasitism was evaluated by counting the amastigotes nests present in 100 microscopic fields in each of the analyzed organs. Likewise, inflammatory infiltrate was visualized in >200 microscopic fields of the tissue sections from heart and skeletal muscle, and scored using the following score system (ordinal method):

Histopathological scoring system key					
Parameters/Score	0	1	2	3	4
Focal lympho-plasmocytes	None (0%)	Rare (1–10%)	Slight (11–25%)	Moderate (26–50%)	Severe ( $\geq 51\%$ )
Diffused lymphoplasmocytes	None (0%)	Slight (1–10%)	Moderate (11–40%)	Coalescing (41–80%)	Diffuse ( $\geq 80\%$ )
Polymorphnuclear Leukocyte/lymphocyte	None (0%)	Rare (1–10%)	Slight (11–20%)	Moderate (21–30%)	Severe ( $\geq 31\%$ )
Necrosis	None (0%)	Rare (1–5%)	Slight (6–10%)	Moderate (11–30%)	Severe ( $\geq 31–70\%$ )
Amastigotes nests	None (0%)	Few (1–2)	Slight (3–5)	Moderate (6–10)	Severe ( $\geq 11$ )

Score criteria was based on previous studies [24,25]. Three pathologists blindly analyzed the samples, and final consensus results were recorded.

### 2.11. Statistical analysis

Female BALB/c mice of 6–8 weeks age were randomly distributed and divided into six groups (n = 12) for homogeneity of variances among each other [26]. Treatments were randomly assigned to the experimental units with six animals per treatment before challenge and six after challenge infection.

One-way analysis of variance (ANOVA) followed by Tukey multiple comparison test were used to assess differences between animal groups in quantitative data; meanwhile, Fisher's Exact test was used for qualitative and semi-quantitative data. All statistical analysis was processed using (SAS<sup>®</sup> University Edition Software) and GraphPad Prism 6 software.

## 3. Results

### 3.1. Effect of route of vaccine delivery on antigen-specific lymphocyte proliferation before and after challenge infection

We monitored the splenic cell response to TcG2, TcG4 and TcTL at two weeks after vaccination and 60 days after challenge infection. The TcVac1\_IDE mice exhibited a significant increase in TcG2- and TcG4- specific splenic lymphocytes' proliferation that was not noted in mice given TcVac1 via IM route. This was

evidenced by TcG2-, TcG4- and TcTL-specific T cell proliferation in TcVac1\_IDE vs. TcVac1\_IM mice (1.32, 1.63, and 1.80 vs. 1.04, 1.13, and 1.47, respectively;  $p < 0.0001$ ) (Table 1-A). All splenocytes, irrespective of the vaccination status, responded to PHA stimulation with a potent increase in T cell proliferation. Splenic cells harvested from control mice (n = 6) injected with empty vector exhibited no stimulation in response to *T. cruzi* antigens; thus, confirming the observed splenic response in vaccinated mice was antigen-specific.

After challenge infection, mice in all groups exhibited a significant level of *T. cruzi*-specific lymphocytes proliferation. Non-Statistically significant effects of TcVac1 (IDE vs. IM) on TcTL-specific lymphocytes' proliferation were observed in challenged mice (Table 1-B). The vaccine-induced TcG2- and TcG4-specific T cell proliferation increased by 3-fold post challenge infection, and was strongest in TcVac1\_IDE mice ( $p < 0.0001$ ). In comparison, TcVac1\_IM mice exhibited similar level of antigen-specific lymphocyte response as was noted in challenged mice injected with pcDNA3 only. Together, these results suggest that IDE (vs. IM) delivery of TcVac1 elicits a stronger, antigen-specific lymphocytes' response in vaccinated and vaccinated/challenged mice.

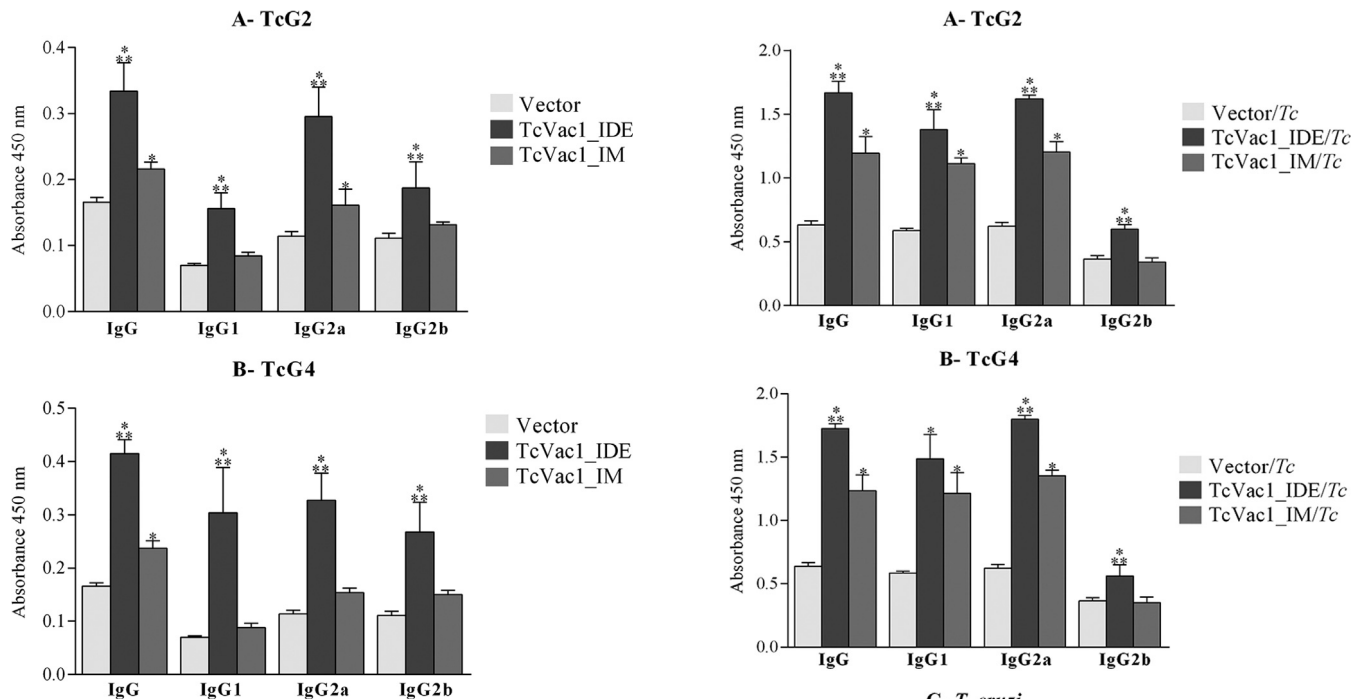
### 3.2. Effect of route of vaccine delivery on antigen-specific antibody response ( $\pm T. cruzi$ )

Immunoglobulin G (IgG) and IgG subtypes (IgG1, IgG2a, IgG2b) titers were evaluated two-weeks after the last vaccine dose and

**Table 1**  
Lymphocyte proliferation stimulation index assay for TcVac1 IDE- and IM-vaccinated mice before and after challenge infection.

A						
Stimulation antigens	Mice treated with (before <i>T. cruzi</i> challenge)				SE	P-value
	pcDNA3.1_IDE	TcVac1_IDE	pcDNA3.1_IM	TcVac1_IM		
TCG2	1.09	1.32 <sup>***</sup>	1.05	1.04	0.027	< 0.0001
TCG4	1.13	1.63 <sup>***</sup>	1.06	1.13	0.031	< 0.0001
TcTL	1.06	1.80 <sup>***</sup>	1.12	1.47 <sup>*</sup>	0.038	< 0.0001
PHA	4.77	4.77 <sup>**</sup>	3.48	3.54	0.162	< 0.0001
B						
Stimulation antigens	Mice treated with (after <i>T. cruzi</i> challenge)				SE	P-value
	pcDNA3.1_IDE	TcVac1_IDE	pcDNA3.1_IM	TcVac1_IM		
TCG2	1.69	3.21 <sup>***</sup>	1.85	2.09	0.139	< 0.0001
TCG4	1.53	3.70 <sup>***</sup>	1.97	2.39	0.199	< 0.0001
TcTL	1.57	2.01	1.84	1.85	0.175	< 0.0001
PHA	4.86 <sup>*</sup>	4.29 <sup>**</sup>	3.80	3.33	0.178	< 0.0001

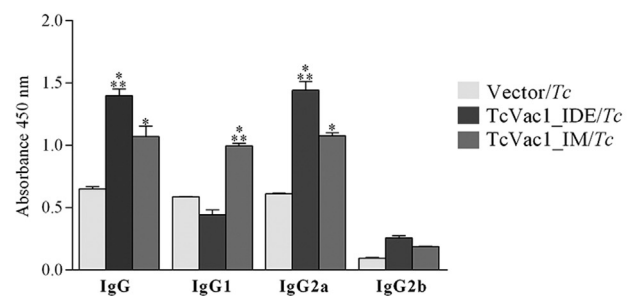
Mice were given empty pcDNA3.1 plasmid, or TcVac1 vaccine by intradermal electroporation (IDE) or intramuscular (IM) injection (1-A). Two weeks after last vaccine dose, half number of mice in each group was challenged with *T. cruzi* (1-B). Splenocytes were incubated with recombinant TcG2 and TcG4 proteins, *T. cruzi* trypomastigotes lysate (TcTL), or phytohemagglutinin (PHA, T cell mitogen, assay positive control) for 72 h. The lymphocytes' proliferation was recorded and data are presented as mean stimulation index value and derived from at least triplicate observations per sample (n = 6 mice per group), fully described in Materials and Methods. Results were analyzed for significant differences by using one-way analysis of variance ANOVA and Tukey's multiple comparison test, and statistical differences are shown at a  $p < 0.0001$ . Data was tested for normal distribution using Normality Test (Kolmogorov-Smirnov) and indicated that data was drawn from a population with a normal distribution ( $P > 0.200$ ). <sup>\*</sup>: Shows statistical differences between TcVac1-IDE or -IM vs pcDNA3.1-IDE or IM, respectively. <sup>\*\*</sup>: Shows statistical differences between TcVac1-IDE vs TcVac1-IM. Samples were considered positive for values  $\geq 1.14 \pm 0.10$ , SE: Mean standard error.



**Fig. 1.** Antigen-specific antibody response in mice vaccinated with TcVac1 via IDE or IM route. Mice were immunized as detailed in Material and Methods. *T. cruzi* specific IgG and subtypes (IgG1, IgG2a, IgG2b) were assessed by ELISA assay by using sera samples at 1:50 dilution. Data are presented as mean value  $\pm$  SD, and derived from triplicate observations by sample per mouse ( $n = 6$  mice per group). Vector was expressed as mean value of pcDNA3.1-IDE and -IM data. Results were analyzed for significant differences by using one-way analysis of variance ANOVA and Tukey's multiple comparison test. \*: show statistical differences between TcVac1-IDE or -IM vs Vector for each IgG or IgG subtype at a  $p < 0.05$ . \*\*: show statistical differences between TcVac1-IDE vs TcVac1-IM for each IgG or IgG subtype at a  $p < 0.05$ .

60 days post-challenge with *T. cruzi*. Vaccination with TcVac1, irrespective of delivery route, elicited an antigen-specific antibody response in mice (Fig. 1). A higher level of IgG as well as IgG subtype response to TcG2 (Fig. 1A) and TcG4 (Fig. 1B) antigens was observed in TcVac1\_IDE (vs. TcVac1\_IM) group ( $p < 0.0001$ ). Mice vaccinated with TcVac1 by IDE route exhibited 35.3%, 45.8%, 45.6% and 29.7% increase in TcG2-specific and 42.9%, 71.0%, 53.0% and 43.9% increase in TcG4-specific IgG, IgG1, IgG2a and IgG2b response, respectively, when compared to that noted in mice vaccinated through IM route (Fig. 1A&B, all,  $p < 0.05$ ). The TcG2-specific IgG2a/b response was dominant in TcVac1\_IDE group. In comparison, IM delivery of TcVac1 elicited low levels of TcG2- and TcG4-specific IgG and IgG2a, and non-significant changes in the IgG2b and IgG1 levels when compared with vector controls (Fig. 1A&B). These results suggest that IDE delivery of TcVac1 elicits stronger, antigen-specific antibody response than was observed with IM delivery of the vaccine.

The antibody response monitored at 60 days post-challenge infection in vaccinated and control mice is presented in Fig. 2. All mice responded to challenge infection with a significant increment in anti-parasite antibody response. The levels of TcG2- and TcG4-specific total IgG and IgG subtypes (IgG1, IgG2a, IgG2b) were significantly higher in vaccinated/infected, vs. non-vaccinated/infected, mice (Fig. 2A&B;  $p < 0.05$ ). The TcVac1\_IDE/Tc mice exhibited 28.5%, 19.3%, 25.7% and 42.9% increase in TcG2-specific and 28.4%, 18.3%, 24.8% and 37.0% increase in TcG4-specific IgG, IgG1, IgG2a and IgG2b responses, respectively, when compared to that observed in TcVac1\_IM/Tc mice (Fig. 2A&B). Likewise, *T. cruzi*-specific antibody response was detected in all mice post-



**Fig. 2.** Antigen-specific antibody response to challenge infection in mice vaccinated with TcVac1 via IDE or IM route. Mice were immunized and infected as detailed in Material and Methods. *T. cruzi* specific IgG and subtypes (IgG1, IgG2a, IgG2b) were assessed by an ELISA assay, using the sera samples at 1:50 dilution. Data were expressed as mean value  $\pm$  SD, and derived from triplicate observations by sample per mouse ( $n = 6$  mice per group). Vector was expressed as mean value of pcDNA3.1-IDE and -IM data. Results were analyzed for significant differences by using one-way analysis of variance ANOVA and Tukey's multiple comparison test. \*: show statistical differences between TcVac1-IDE or -IM vs Vector for each IgG or IgG subtype at a  $p < 0.05$ . \*\*: show statistical differences between TcVac1-IDE vs TcVac1-IM for each IgG or IgG subtype at a  $p < 0.05$ .

challenge infection. The maximal levels of parasite-specific IgGs and IgG subtypes were noted in TcVac1\_IDE/Tc group, while mice in TcVac1\_IM/Tc and pcDNA3/Tc groups exhibited significantly higher levels of IgG1 than was noted in TcVac1\_IDE/Tc mice (Fig. 2C). Together, the results presented in Fig. 2, along with those presented in Fig. 1 suggest that TcVac1 delivery by IDE (vs. IM) route elicits a strong, antigen-specific and parasite-specific antibody response that significantly expanded upon challenge infection. In comparison, non-vaccinated mice responded to challenge infection with low antigen-specific antibody levels.

### 3.3. Parasitemia and mortality in vaccinated/infected mice

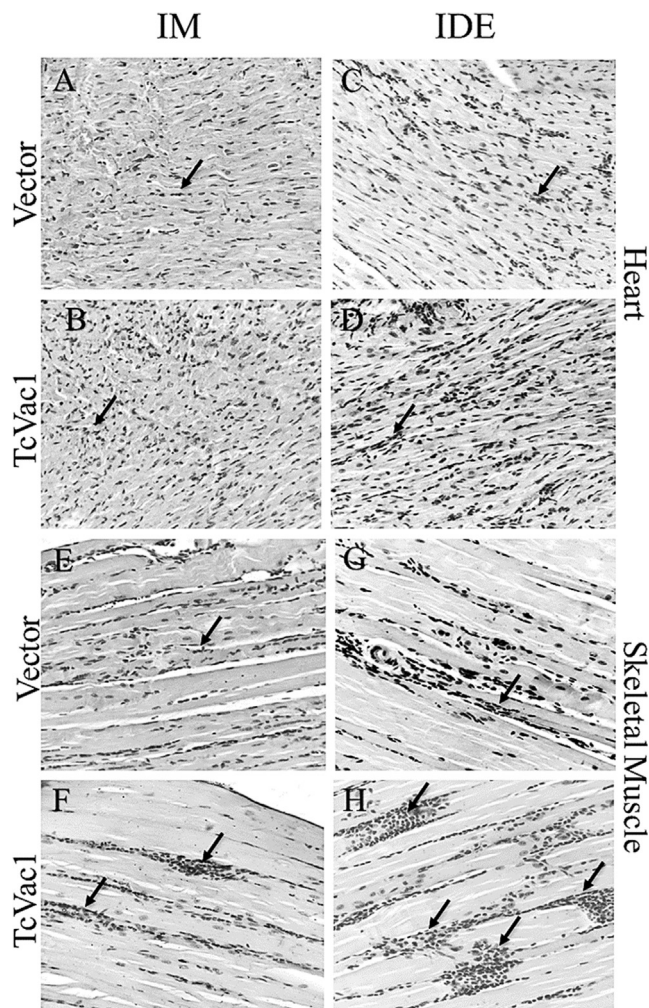
Challenge infection with SylvioX10 isolate of *T. cruzi* did not produce detectable blood parasitemia in any of the mice in vaccinated and non-vaccinated groups. Up to 33.3% (2 of 6) of mice injected with vector only (IM) succumbed to challenge infection during the 60 days of monitoring period. No mortality statistical difference ( $P = 0.0870$ ) was observed among groups (Fig. 5).

### 3.4. Clinical and pathological (macroscopic and microscopic) abnormalities in vaccinated and non-vaccinated mice post-challenge infection

Immunization with TcVac1 produced no apparent presentation of physical, or clinical macroscopic abnormalities. All mice injected with pcDNA3.1 vector or TcVac1 by IDE or IM route exhibited no inflammatory, degenerative, necrotic or proliferative injuries, thus, suggesting that vaccine alone is non-toxic and safe to deliver. In response to challenge infection with *T. cruzi*, 60–70% of mice that were injected with empty vector only by IM or IDE route displayed low levels of physical activity and nest building behavior, decreased interaction with cage mates, hirsute bristling hair, and poor general appearance. In comparison, mice vaccinated with TcVac1 (IDE or IM route) had a healthy appearance with no observable abnormalities in socialization, physical activity, or food and water uptake. Macroscopic evaluation of the heart showed that control mice injected with vector only (IDE or IM route) developed bi-ventricular dilation (7 out of 10 mice) and epicardial hemorrhage (1 out of 10 mice) post challenge infection (Fig. 4). In comparison, mice immunized with TcVac1 (IDE or IM route) exhibited no morphological changes of the heart post-challenge infection.

Histological analysis of tissue sections was performed at sixty days post-challenge infection when mice exhibited the end of the acute infection phase. The histological evaluation of heart tissue from representative mice in each group is shown in Fig. 3. We noted mild, diffused inflammatory infiltrate, primarily constituted by mononuclear lymphocytes, in the atria and ventricles of mice that were injected with empty vector via IDE or IM route after challenge infection. In comparison, vaccinated/infected mice (TcVac1\_IDE/Tc and TcVac1\_IM/Tc) exhibited a potent increase in inflammatory infiltrate constituted of lymphocytes and polymorphonuclear leucocytes in the myocardium. The myocardial infiltration of inflammatory infiltrate was maximally noted in TcVac1\_IDE/Tc mice. Likewise, cardiomyocytes necrosis was observed more frequently in TcVac1\_IDE/Tc and TcVac1\_IM/Tc mice, while mice from the control groups presented infrequent and moderate number of necrotic cells. The increase in myocardial inflammatory infiltrate was associated with a decline in tissue parasite burden in vaccinated mice. We observed the largest number of amastigotes nests in the atria and ventricles of mice in control groups. The amastigotes nests' counts were also significantly higher in the left and right ventricle walls of acutely infected mice in control groups than was observed in vaccinated mice. Overall, we observed 5–8 foci of pseudocysts/microscopic field (mf) in the cardiac tissue of mice in control groups and 0–2 foci of pseudocysts/mf in the heart tissue of vaccinated mice. Minimal tissue parasite burden was observed in TcVac1\_IDE/Tc group.

Similar to the heart tissue, skeletal muscle of mice in control groups exhibited mild inflammation characterized by mononuclear inflammatory infiltrate (macrophages and lymphocytes) and moderate level of myocytes' necrosis. In comparison, vaccinated mice (TcVac1\_IDE/Tc and TcVac1\_IM/Tc) showed stark increase in skeletal muscle infiltration of inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and macrophages) and moderate level of muscle fibers' necrosis post challenge infection. Generally, skeletal muscle amastigotes nests were larger when compared to those detected in the myocardial tissue. The abundance of amastigotes nests in skeletal tissue of control groups was observed as follows: pcDNA3.1\_IDE/Tc (4 foci of pseudocysts/mf); pcDNA3.1\_IM/Tc (3 foci of pseudocysts/mf). In comparison, vaccinated mice showed a significantly lower number of parasitic pseudocysts ( $p < 0.01$ ), and fewer amastigotes nests were observed in skeletal tissue of TcVac1\_IDE/Tc mice (0–1 foci of pseudocysts/mf) than in the TcVac1\_IM/Tc mice (2 foci of pseudocysts/mf). Further, parasitic nests

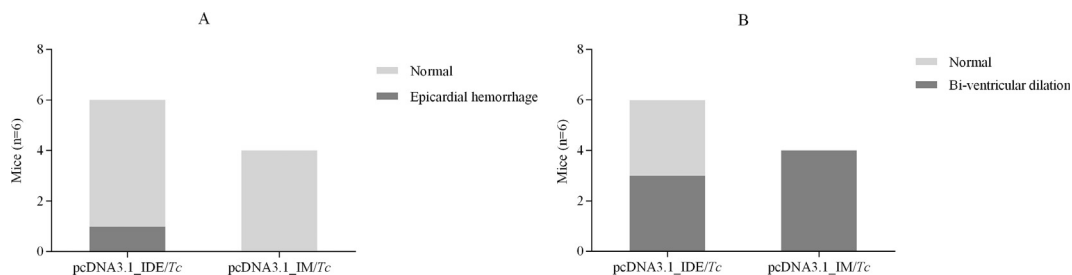


**Fig. 3.** Histological visualization of acute phase lymphocyte infiltration in TcVac1 vaccinated/infected mice. Mice were given empty pcDNA3.1 vector or TcVac1 via intramuscular (IM) injection or intradermal electroporation (IDE), challenged with *T. cruzi*, and harvested at 60 days post-infection. Shown are representative images of the heart and skeletal muscle sections stained with H&E (magnification 400 $\times$ ). Arrows show lymphocyte infiltration.

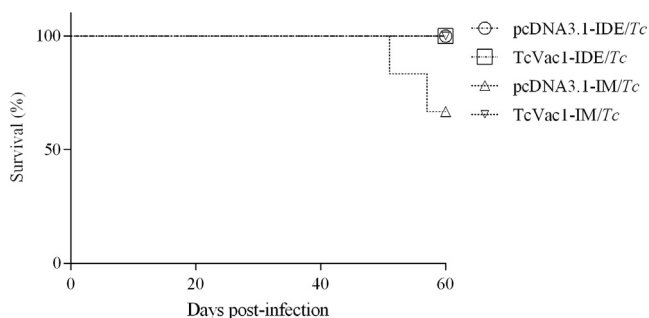
were frequently presented adjacent to the inflammatory infiltrate in the myocardial and skeletal tissue of the vaccinated/infected mice (Fig. 3 and Table 2). Together, these results suggest that the TcVac1 induced innate and adaptive immune cells responded to challenge infection with vigorous proliferation and tissue migration, and provided a significant control of tissue parasite burden. A higher degree of destruction of infected myocardial and skeletal myocyte cells was also noted in vaccinated, vs. non-vaccinated, mice post challenge infection.

## 4. Discussion

In this study, we compared intradermal electroporation approach and the conventional intramuscular administration technique to determine the differential efficiency of these two protocols for the administration of TcVac1 vaccine in a murine model. Candidate vaccines against Chagas disease have mainly been tested by intramuscular immunization route. Several investigators have reported that permeabilizing the target cells through electroporation (EP) led to enhanced uptake of naked recombinant plasmid and a superior expression of the gene of interest, and thereby an improved immunization efficacy [12–14,16,17,20,27–29]. Other



**Fig. 4.** Macroscopic evaluation of the heart for control mice (non-vaccinated) groups. Mice were vaccinated via IDE ( $n = 6$ ) or IM ( $n = 4$ ) with pcDNA3.1 empty plasmid and evaluated after challenge infection, and postmortem macroscopic examination was established for the identification of heart abnormalities (epicardial hemorrhage and bi-ventricular dilation). Data were recorded as case incidence of the heart abnormality for each mouse and recorded in a contingency table. Data were analyzed for statistical differences using Chi-square test. (A) Shows the incidence of mice with epicardial hemorrhage for each control mice groups ( $P = 0.389$ ). (B) shows the incidence of mice with bi-ventricular dilation for each control mice groups ( $P = 0.091$ ). pcDNA3.1\_IDE/Tc: Control mice group vaccinated with empty plasmid via intradermal electroporation. pcDNA3.1\_IM/Tc: Control mice group vaccinated with empty plasmid via intramuscular injection.



**Fig. 5.** Survival percentage of mice groups after *T. cruzi* infection. Mice were monitored for 60 days for any sign of disease and survival. Survival data were analyzed statistically through Mantel-Cox test, survival curves did not show any significant differences ( $P = 0.0870$ ).

authors have shown that electroporation, after intradermal or intramuscular injection of DNA vaccines provide an efficient system to activate antigen presenting cells (APCs), mainly dendritic cells, since they are directly transfected at the site of DNA administration. The expressed antigen is presented in association of the major histocompatibility complex (MHC) class 1 molecules [20]. This can subsequently stimulate the synthesis of antigen-specific cytotoxic T lymphocytes in conjunction with T-helper cells. Skin tissues, especially epidermis and dermis, are crowded with various types of APCs including dendritic cells, in contrast with the muscle tissue. Moreover, intradermal vaccine route specifically targets

dermal DCs and macrophages that, when activated, will carry vaccine components to draining lymph by passive diffusion or cell transport of antigens. Afterwards, presentation of the antigens to immature  $CD4^+$  and  $CD8^+$  T cells, leads to their activation, as well as, the activation of specific B cells within the germinal center. Finally, the generation of a pool of specific memory T and B cells in the secondary lymphoid organs (draining lymph nodes and bone marrow) and in the periphery (skin and mucosa) is also noted by IDE delivery of candidate antigens [30]. Other studies compared the effect of plasmid vaccination via intradermal (ID) only vs intradermal followed by electroporation (EP), they proved that EP + ID showed better and higher antibody responses than ID only [31]. Therefore, we hypothesized that intradermal electroporation would enhance the effectiveness of the DNA based vaccine against Chagas disease.

It is important to note that both types of immune responses, humoral and cellular, are necessary to control the infection. This is evidenced by the findings that mice deficient in  $CD8^+$  T cell and/or B cell function were susceptible to *T. cruzi* infection and exhibited high parasitemia and mortality [32]. Thus, a candidate vaccine capable of eliciting both B and T cell responses and Th1 cytokines is considered to offer the best protection from *T. cruzi* infection. Our group has identified two vaccine candidates, named TcG2 and TcG4, that when co-delivered as DNA-prime/DNA-boost vaccine (TcVac1), elicited protective immunity and resistance against *T. cruzi* infection in mice and dogs [5,10,11]. Along the use of parasite antigens, plasmid DNA GM-CSF adjuvant use

**Table 2**

Histo-pathological abnormalities during the acute phase of *T. cruzi* infection and disease development (day 60 post-infection) in mice vaccinated with TcVac1.

Parameters	IDE/Tc		IM/Tc	
	pcDNA3.1	TcVac1	pcDNA3.1	TcVac1
<b>Myocardial Tissue</b>				
Focal lympho-plasmocytes	1.8	3.2 <sup>*</sup>	1.5	2.2
Diffused lympho-plasmocytes	2.5	2.3	2.2	2
Polymorphnuclear Leukocyte/lymphocyte	1	2.8 <sup>*</sup>	0.8	1.8 <sup>*</sup>
Necrosis	1.2	2.5 <sup>*</sup>	1	2.2 <sup>*</sup>
Amastigotes nests	2.5 <sup>*</sup>	1	2.3 <sup>*</sup>	1.2
<b>Skeletal Muscle</b>				
Focal lympho-plasmocytes	1	2.2 <sup>*</sup>	0.8	1.8 <sup>*</sup>
Diffused lympho-plasmocytes	1.3	2.8 <sup>*</sup>	1.2	1.8
Polymorphnuclear Leukocyte/lymphocyte	0.8	2.8 <sup>*</sup>	0.7	2 <sup>*</sup>
Necrosis	1.5	2.5 <sup>*</sup>	1.7	2.2
Amastigotes nests	2 <sup>*</sup>	0.8	2.3 <sup>*</sup>	1.3

Mice were injected with empty pcDNA3.1 plasmid, or TcVac1 via intradermal electroporation (IDE) or intramuscular (IM) route and challenged with *T. cruzi* (Tc) as described in Materials and Methods. Tissue sections were examined for inflammatory infiltrate, necrosis, and parasite nests as described by Barbabosa-Pliego and Slauson [24,45]. Semi-quantitative scoring of histopathological lesions is presented and explained in material and methods section. Data represents the average score of the evaluation of 100 microscopic fields/treatment/tissue and were analyzed for statistical differences using as mean score of mice per group ( $n = 6$ ). <sup>\*</sup> indicates statistical differences of vaccinated groups (TcVac1-IDE or -IM) vs control groups (pcDNA3.1-IDE or IM) respectively.

enhances the antigen-presenting capability of dendritic cells and facilitates B- and T-cell-mediated immunity and plasmid DNA IL-12 is a key cytokine involved in CD8<sup>+</sup>-T-cell activation and proliferation reported to enhance antiparasitic effects of vaccines [33]. However, when these adjuvant plasmids were tested without parasite specific antigens to prevent *T. cruzi* infection in mice they did not show any specific-antibody responses [10,34,35]. Therefore, the anti-*T. cruzi* specific immune response observed in the present report can be assumed to rely on the TcG2 and TcG4 parasite antigens used in the candidate vaccine. The immune activation provided by the GM-CSF and IL-12 plasmids were not analyzed in the present report.

In order to determine if vaccination via IDE or IM route elicited similar (or different) levels of cellular immune responses, we evaluated the lymphocyte proliferation reaction post immunization and challenge infection. While both IDE and IM modes of vaccine delivery elicited antigen-specific lymphocytes' proliferation (TcG4 > TcG2), the extent of antigen-specific lymphocytes' proliferation was higher in the TcVac1\_IDE than in TcVac1\_IM group ( $p < 0.0001$ ). Importantly, the vaccine induced T cells exhibited a better expansion and rapid effector function in controlling the tissue parasite burden in mice immunized via the IDE route. A better protection from challenge infection was associated with increased frequency of TcG2- and TcG4-specific lymphocytes' proliferation in TcVac1\_IDE vs. TcVac1\_IM group. Our data allow us to surmise that IDE delivery of TcVac1 enhances the generation of antigen-specific memory T cells that are capable of responding to pathogen exposure, and thus provided a better vaccine efficiency.

Our finding of higher levels of humoral response post-immunization and post-challenge infection also favored IDE over IM vaccination system (Fig. 1). The TcVac1\_IDE mice exhibited higher levels of the TcG2- and TcG4-specific IgG2a/b response post-vaccination a significant increase in antigen- and parasite-specific IgG2a/b and IgG1 levels post-challenge infection. Both IgG1 and IgG2 antibody subtypes are proposed to be involved in the elimination of blood forms of parasites and in a reduction of animal mortality rate during the acute phase of infection [36]. The IgG2a/b generally constitute prevalent immune response against carbohydrate/polysaccharide antigens, and IgG1s are predominantly considered to recognize proteins and polypeptide antigens bound to the Fc receptor of phagocytic cells and participate in activation of the complement cascade via binding to C1 complex. Whether the TcG2 and TcG4 antigens are post-translationally modified, or whether the IDE delivery of TcVac1 enhances the post-translational modification of TcG2 and TcG4 leading to increase in IgG2a/b response is not known. However, our data suggest that IDE route of TcVac1 delivery is more effective in eliciting a predominant IgG2a/b response over IgG1 for both pre- and post-infection phases, suggesting a skewed Th1 immune profile in experimental mice.

Studies conducted in canine, bovine, primates and humans, indicated that IM vaccination with naked DNA yields low expression level of the recombinant antigen, which in turn induces a sub-optimal immune response. This is explained by the fact that muscle tissue is not an efficient site for antigen presentation, because of a lack of appropriate quantities of DCs, macrophages and lymphocytes. Therefore, larger amounts of DNA vaccine dose delivery by IM route was required to establish a strong immune response in mice and larger species (e.g. dogs, pigs, humans). Comparatively, IDE delivery of the DNA vaccine requires smaller amounts of the plasmid DNA to achieve strong immune responses, and thus offers significant savings in time, cost and effort for vaccine production and delivery [11,37–39]. Thus, IDE delivery of naked plasmids could favorably compete with other vaccine protocols that include efficient antigen expression vectors, such as Adenovirus or Salmonella [40,41].

Blood parasitemia was not detected during the acute experimental phase in any mice from any group, which indicates that the strain *Sylvio* X10/4 of *T. cruzi* has a strong tissue tropism. This strain behavior was previously reported by Marinho et al., 2004, who described the absence of a patent acute phase as well as parasitemia in the weeks following the infection, when using *Sylvio* X10/4 *T. cruzi* parasite strain, with various mouse strains [42]. Additionally, they reported the presence of parasite nests, which were totally ignored by the immune system in mice myocardial tissue. In the present study also, we observed mild, diffused tissue infiltration of inflammatory infiltrate that was not localized close to parasite nests in non-vaccinated/infected animals. In contrast, tissue inflammatory infiltrate was strongly associated with the parasite nests, and contributed to control of size and number of tissue parasite nests in the vaccinated (TcVac1\_IM/Tc and TcVac1\_IDE/Tc) mice. Thus, vaccinated/challenged mice exhibited an ability to control the parasite-mediated tissue destruction that was apparent by the macroscopic and microscopic observation of apparently healthier tissue.

The major pathological manifestations for chagasic cardiomyopathy in mice are cardiomegaly with hypertrophy of cardiac tissue, dilation of the heart chambers and aneurism mostly in the left ventricle associated with strong inflammation, necrotic cardiomyocytes and fibrosis [7]. The IDE vaccination protocol altered the histopathological developments in the myocardium of acutely infected mice through induction of focal, parasite-specific inflammation; moreover, vaccinated mice produced more antibodies and displayed a stronger cellular immune response, which were capable of reducing the number of amastigotes nests in mice heart tissue (Table 2). However, our observations of cardiomyocytes necrosis in TcVac1\_IDE/Tc mice (Fig. 3) suggest that inflammatory responses, if persistent, can also be injurious to the host. This interpretation is consistent with studies in IL-4 deficient mice, which were able to develop a strong Th1 type immune response and control acute parasitic burden but also developed exacerbated inflammation in the myocardial tissue [43]. Future studies will be required to determine if TcVac1\_IDE induced inflammatory responses subside with control of parasite and do not constitute a risk factor in causing consistent, long-term tissue damage in the host. Indeed, mice vaccinated with TcG2 and TcG4 antigens by DNA-prime/protein boost approach exhibited a predominance of type 1 inflammatory response capable of controlling circulating and tissue parasites in acute phase, and then switched to immunomodulatory, tissue-healing type 2 response thus providing protection to the heart [44]. Thus, we believe that IDE would offer an enhanced protective efficacy of TcVac1 against acute infection and chronic Chagas disease.

## 5. Conclusion

Both protocols of TcVac1 vaccination (IDE and IM), protected against an acute *T. cruzi* infection, enhanced survival rate, milder macroscopic heart pathologies and decreased tissue (heart and skeletal muscle) parasite burden than was noted in non-vaccinated/infected mice. The IDE delivery offered a more efficient vaccination system than the IM route. This was evidenced by induction of higher levels of antigen-specific, Th1 type cellular and humoral immune responses that expanded in response to challenge infection and provided a stronger infiltration of inflammatory cells capable of controlling the parasite nests in the heart and skeletal tissues in mice vaccinated with TcVac1 by IDE route than that observed in mice vaccinated via IM route. We conclude that IDE delivery enhances protective efficacy of TcVac1 vaccine in mice.



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## Author contributions

JVCV and WHHM conceived and designed the experiments; WHHM performed the experiments; JAZE, LOG, ETB, JEAB, BRS and NJG substantially contributed to conception and design and acquisition of data; NJG contributed to vaccine design and production, JVCV, ETB, HAD, BRS, JMECO and ROP contributed reagents/materials/analysis tools; ROP, JEAB, ABP and WHHM performed anatomopathological and histopathological analysis; WHHM, JVCV, and NJG prepared the paper; All authors read and approved the manuscript to be published.

## Conflict of interest

The authors declare no conflict of interest.

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