UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MÉXICO

PROGRAMA MAESTRÍA Y DOCTORADO EN CIENCIAS AGROPECUARIAS Y RECURSOS NATURALES

PROTECCION INMUNE CONTRA *Trypanosoma cruzi* INDUCIDO POR LA VACUNA TCVAC1 MODELO MURINO DE USANDO EL PROTOCOL DE ELECTROPORACIÓN INTRADÉRMICA

TESIS

QUE PARA OBTENER EL GRADO DE MAESTRO EN CIENCIAS AGROPECUARIAS Y RECURSOS NATURALES

PRESENTA
QFB. WAEL HEGAZY HASSAN MOUSTAFA

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Cerrillo Piedras Blancas, Toluca, México; Febrero de 2015

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IMMUNE PROTECTION AGAINST *Trypanosoma cruzi* INDUCED BY TCVAC1 VACCINE IN A MURINE MODEL USING AN INTRADERMAL/ELECTROPORATION PROTOCOL

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DEDICATION

To my Father Ph.D. Hegazy Hassan Moustafa Hegazy and my Mother B.Ag. Doha Hassan Badr Mohamed for their life, love, care and education, their moral and financial support and for teaching me how to be responsible in my life and everything they have done for me to reach this level of higher education.

To my brother Medical Surgeon Ahmed Hegazy Hassan Moustafa for being a perfect brother, teacher, his enlightenment and guidance in all life aspects.

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To my younger brother Undergraduate St. Nader Hegazy Hassan Moustafa, and my two little angelic sisters Hana Hegazy Hassan Moustafa and Shahd Hegazy Hassan Moustafa for being the happiness of my life.

ABSTRACT

Trypanosoma cruzi, a parasitic protozoan, is the etiologic agent of Chagas disease. Chagas disease is the most common cause of congestive heart failure related deaths among young adults in the endemic areas of South and Central America and Mexico. It has also become an important health issue in the United States and Europe due to large scale migration of Latin Americans over the last few decades. No vaccines are currently available until now. In this study, we tested the vaccine efficacy of two antigen candidates against *T. cruzi* infection and disease in a mouse model. The use of TcVac1 (TcG2, TcG4, *T cruzi* antigen encoding plasmids, interleukin-12 [IL-12] and granulocyte-macrophage colony-stimulating factor [GMCSF] encoding plasmids as genetic adjuvants) anti *T. cruzi* candidate vaccine injected intramuscularly has been previously reported in mice with very encouraging results. Here we evaluated the comparative protection conferred by TcVac1 when administrated intramuscular (IM) versus an intradermal/electroporation (IDE) vaccination protocol. Twelve BALB/c mice per group were vaccinated four times fifteen days apart. Half the animals (n=6) from each treatment were sacrificed two weeks after the last immunization for pre-infection vaccine efficacy evaluation, and the second half (n=6) was sacrificed 60 days post-infection (dpi) with *T.cruzi* Trypomastigotes (Sylvio X10/4 strain). Immune response was assessed through anti-TcG2 and TcG4 *T. cruzi* antigens.TcVac1 induced a strong IgG response (IgG2<u>b</u>>IgG1) that was significantly expanded post-infection, and moved to a nearly balanced IgG2<u>b</u>/IgG1 response in chronic phase. High IgG titers with IgG2 predominance in response to *T. cruzi* infection specific serum antibodies with an Enzyme Linked Immunosorbent Assay (ELISA) and lymphocyte activation against the studied antigens was evaluated through a lymphocyte proliferation assay. We found that IDE induced significantly larger surges of IgG antibodies including subtypes IgG1, IgG2a and IgG2b, during the pre- and post-infection periods for the two antigens used in the experiment. The ratio of antibodies IgG2b/IgG1 was >1 for TcG2 antigen in the pre-

infection period in both administration routes. However for the TcG4 antigen the ratios were opposite for animals belonging to different administration routes<1 for IDE and >1 for IM. During the post infection period for both treatments IgG2b/IgG1 ratio was always <1. Suggesting, as previously reported that a switch from Th1 to Th2 type immune response occurs in vaccinated/infected animals. During the Lymphocyte proliferation assays we observed that both antigens were able to induce lymphocyte proliferation during the pre-infection period. However, we observed that animals from the IDE group induced more proliferation than IM mice group when TcG4 was used to activate the cells, which was also observed during the post-infection phase of the experiment. No animals died due to infection, vaccinated mice appeared to have healthier status than the control animals.

RESUMEN

Trypanosoma cruzi, un protozoario, es el agente etiológico de la enfermedad de Chagas. La enfermedad de Chagas es la causa más común de muertes relacionadas insuficiencia cardiaca congestiva entre los adultos jóvenes en las áreas endémicas de Sudamérica, Centroamérica y México. También se ha convertido en un problema de salud importante en los Estados Unidos y Europa debido a la migración a gran escala de los latinoamericanos en las últimas décadas. Hasta ahora no existen vacunas disponibles. En este estudio, hemos probado la eficacia de la vacuna de dos antígenos contra la infección de *T. cruzi* y la enfermedad en un modelo murino. El uso de TcVac1 (TcG2, TcG4, el antígeno de *T cruzi* que codifica plásmidos, interleucina-12 [IL-12] y factor estimulante de colonias granulocitos-macrófagos [GMCSF] que codifican plásmidos como adyuvantes genéticos) vacuna candidata contra *T. cruzi* inyectada por vía intramuscular ha sido probad anteriormente en ratones con resultados muy alentadores. Aquí se evaluó la protección conferida por la comparativa TcVac1 administrada via intramuscular (IM) contra un protocolo de vacunación electroporación/intradérmica (IDE). Se vacunaron doce ratones BALB/c por grupo, cuatro veces, con quince días de diferencia. La mitad de los animales (n=6) de cada tratamiento, se sacrificaron dos semanas después de la última inmunización para la evaluación de la eficacia de la vacuna en la pre-infección y la segunda mitad (n=6) se sacrificaron 60 días después de la infección (dpi) con *T. cruzi* tripomastigotes (cepa Sylvio X10/4). La respuesta inmune fue evaluada a través de antígenos anti-TcG2 y TcG4 *T. cruzi* .TcVac1 indujo una fuerte respuesta de IgG (IgG2b>IgG1) que se expandió de manera significativa después de la infección, y se mudó a una respuesta equilibrada IgG2b/IgG1 en fase crónica. Se observaron títulos altos de IgG con predominancia de IgG2 en respuesta a los anticuerpos séricos específicos de infección por *T. cruzi* en un ensayo por inmunoabsorción ligado a enzimas (ELISA), la activación de linfocitos frente a los antígenos estudiados se evaluó a través de un ensayo de proliferación de linfocitos. Se encontró que el IDE induce oleadas significativamente mayores de anticuerpos IgG incluyendo subtipos IgG1, IgG2a e IgG2b, durante los períodos pre y post-infección para los dos antígenos utilizados en el experimento. La proporción de anticuerpos IgG2b/IgG1

fue>1 para el antígeno TcG2 en el período previo a la infección en ambas vías de administración. Sin embargo para el antígeno TcG4 la proporción fue opuesta para los animales que fueron vacunados con diferentes vías de administración <1 para IDE y >1 para IM. Durante el período posterior a la infección para ambos tratamientos la proporción de IgG2b/IgG1 fue siempre <1. Estos resultados sugieren que como se informó anteriormente, que un cambio de la respuesta inmune de tipo Th1 a Th2 se produce en los animales vacunados/infectados. Durante los ensayos de proliferación de linfocitos se observó que ambos antígenos fueron capaces de inducir la proliferación de linfocitos durante el período de pre-infección. Sin embargo, se observó que los animales del grupo IDE indujeron una mayor proliferación que en los ratones del grupo IM cuando se utilizó TcG4 para activar las células, lo que también se observó durante la fase posterior a la infección del experimento. No hubo muerte de animales durante la infección, los ratones vacunados parecían más saludables que los animales control.

ACKNOWLEDGMENTS

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1. INTRODUCTION

1. Chagas disease:

Chagas disease is named after Carlos Chagas, the pioneer researcher who first described it in 1909 (Chagas, 1909). Existing only on the American continent, the disease is caused by a flagellate parasite, *Trypanosoma cruzi*, and is mainly transmitted to humans by blood-sucking triatomine bugs. Chagas disease has two successive phases, acute and chronic. The acute phase lasts 6-8 weeks. Once the acute phase abates, most of the infected patients recover an apparent healthy status, where no organ damage can be demonstrated by clinical diagnosis methods. The infection can only be verified by serological or parasitological tests. This form of the chronic phase of Chagas disease is called indeterminate form. Most patients remain in this form of the disease. However, after several years of this phase, 20-35% of the infected individuals will develop irreversible lesions of the autonomous nervous system in the heart, oesophagus, colon, and peripheral nervous system, and it lasts for the rest of the infected individual's life. Chagas disease represents the first cause of cardiac lesions in young, economically productive adults in the endemic countries in Latin America (Coura *et al*., 1983, 1985; Pereira *et al*., 1985).

1.1. Clinical manifestations of the disease:

An important point in the identification of Chagas disease was the observation of an ocular edema known as Romaña's sign. Romaña's sign is a pathognomonic early sign of Chagas disease a one side severe conjunctivitis and swelling of the eye lid, inflammation of the tear gland and swelling of regional lymph glands caused by the entry of *T. cruzi* parasite. The sign has proved of great value in identification the infection in its acute phase.

1.2. First Evidence of American Trypanosomiasis in Latin America:

The first report of American human Trypanosomiasis was discovered Segovia in El Salvador in 1913 (Segovia, 1913), In Panama in 1931, the presence of the disease was proven with the report of 19 human cases in the area of the Panama Canal, De Leon in 1935 highlighted the importance of this disease in this country (León Gómez *et al.*, 1960), In 1949, the first native case of the disease was described in Nicaragua, and as recently as in 1969 Fray Bernadini de Schagen reported that homes were infested by vectors described as "poisonous bloodsucking insects like cockroaches." In Mexico in 1928, Hoffman described the great abundance and domiciliation of *Triatoma dimidiata* in Chiapas and Veracruz. In1938 Bernal Flandes published on transmitter insects and trypanosomatides in Veracruz, and in 1940, Palomo Eroso described two other new cases in Yucatan. It was only in 1972 that the first formal identification of the disease was carried out with reports by Eugenio Palomo and Luis Mazzotti (Symposium: National Academy of Medicine, 1975).

Figure 1: First case of Chagas disease in Bolivia, reported in a girl aged 14 in Capinota, Department of Cochabamba, Bolivia.

1.3. Chagas disease life cycle and invasion mechanism:

Trypanosoma cruzi, the protozoan parasite, has a digenetic life cycle involving both vertebrate and invertebrate hosts within which developmental stages of the parasite arise (Fig. 2). As an obligate intracellular parasite in the vertebrate host, intracellular localization is critical for establishment and maintenance of *T. cruzi* infection. Host cell invasion is accomplished by metacyclic trypomastigotes, which is highly specialized, non-dividing

forms of *T. cruzi* that can penetrate a wide variety of mammalian cell types. Once inside the host cell, trypomastigotes undergo a developmental process that culminates in the formation of replicative amastigotes that proliferate in the host cell cytoplasm for 5−6 days until they occupy most of the cell volume. At this stage, amastigotes division ceases and differentiation to trypomastigotes occurs followed by rupture of the host cell plasma membrane (Costales and Rowland, 2007) releasing trypomastigotes that disseminate infection.
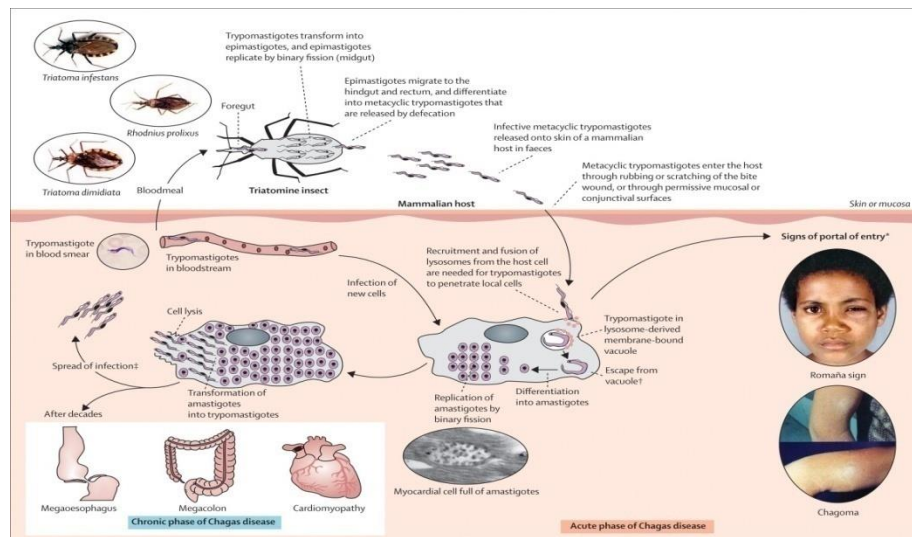
Figure 2: *Trypanosoma cruzi* life cycle. Metacyclic Trypomastigotes arising from Epimastigotes in the reduviid host are transmitted to mammalian host in the feces of the insect vector. Inside the host, Trypomastigotes invade cells and are rapidly targeted to a lysosome-derived vacuole. Within the vacuole, trypomastigotes begin the transformation to amastigotes (2–8 h) after which the vacuole is gradually disrupted and parasites localize to the host cell cytoplasm (8–16 h). Cytosolic amastigotes begin to divide at 24 hrs. post-invasion and continue to divide every 12 hrs for 5–6 days, then differentiate back into trypomastigotes, rupture the host cell, enter the host circulation and disseminate infection.

Cell lysosomes are exploited by *T. cruzi* as the gateway to the host cell cytoplasm, electron micrographs revealed that shortly after invasion, *T. cruzi* trypomastigotes are housed within tight-fitting membrane bound vacuoles that fuse with host cell lysosomes (de Carvalho and de Souza, 1989; de Meirelles Mde *et al*., 1987; Nogueira and Cohn, 1976; Tanowitz *et al*., 1975). As intracellular infection progresses, Trypomastigotes differentiate into Amastigotes which divide in the host cell cytoplasm (Ley *et al*., 1990; Nogueira and Cohn, 1976) the parasitophorous vacuole was rapidly acidified and that acidification was essential for vacuole membrane disruption and release of parasites into the cytoplasm (Ley *et al*., 1990).

These observations coincided with the discovery of a secreted *T. cruzi* lytic activity (TC-TOX) that is released into the lumen of the parasitophorous vacuole where it is optimally active at low pH (Andrews and Whitlow, 1989).

1.4. Modes of transmission of Chagas disease:

1.4.1. Transmission through Vectors:

On the basis of country wide cross-sectional surveys, it was estimated that the overall prevalence of human *T. cruzi* infection in the 18 endemic countries has reached 17 million cases.

It was estimated that 100 million people (25% of all the inhabitants of Latin America) were at risk of contracting *T. cruzi* infection (UNDP/WORLDBANK/WHO, 1991). The originally endemic area with vectorial transmission in the human domicile comprised 18 countries with higher *T. cruzi* infection rates in the regions infested by *Triatoma infestans* (Southern Cone countries) and *Rhodnius prolixus* (Andean countries and Central America), which were the triatomine species are adapted to the human domicile.

1.4.2. Transmission through blood transfusion:

The transmission of Chagas disease via blood transfusion is a real threat even for countries where the disease is not transmitted by vector, such as the USA and Canada, where cases of acute Chagas disease have been documented (Grant *et al*., 1989; Kirchhoff *et al*., 1987; Nickerson *et al*., 1989). In 1986, Puebla, México have showed 17.5% positive infected cases with *T. cruzi* from 200 tested samples (Velasco Castrejón and Guzmán Bracho, 1986). The prevalence rates of *T. cruzi* infection in blood varied between 1.3% and 51.0%, these rates were much higher than those of hepatitis or HIV infection. Transmission through transfusion could be prevented if blood is screened by serology and positive units are discarded.

1.4.3. Transmission via oral:

The oral transmission is the fact that several persons are affected simultaneously pointing to the occurrence of a common source outbreak through contaminated food. For the prevention of this transmission is based on surveillance, prevention, and management contaminated food as recommended by a group of experts convened by the PAHO in Brasili (OPS/DPC/CD/CHA, 2006). This route of transmission was established and documented (Shikanai-Yasuda *et al*., 1991; Valente *et al*., 1999; Camandaroba *et al*., 2002; Rodríguez-Morales, 2008). One of the most documented cases is due to the ingestion of açai juice and is concentrated in the States of Pará and Amapá, where the production of this fruit is intensive.

1.4.4. Other ways of transmission:

Transfusional transmission:

which is considered to be the second most important way of spreading the infection in endemic and non endemic areas, this transmission route mainly depends on the presence of the parasite in the blood and the immunological state of the receptor, and it was detected that the parasite is viable at 4°c for a period of time from 18 to 250 days (Schmunis and Cruz, 2005).

Congenital transmission:

The number of cases of congenital Chagas disease has been estimated about 14,385 per year in Latin America, about 66 to 638 per year in the United States, and about 20 to 183 per year in Europe (Pan American Health Organization, 2006), congenital transmission of Chagas disease may occur during any phase of maternal disease. During the first trimester of pregnancy (weeks 1–12), transmission is probably rare. Maternal blood supply becomes continuous and diffuse in the entire placenta only after the 12th week of gestation. Therefore, transmission of blood parasites probably occurs most frequently during the

second and third trimesters of pregnancy (prenatal transmission) and perhaps also closer to delivery and during labor (perinatal transmission) through placental breaches/tears (Carlier and Truyens, 2010, 2012).

Blood banking transmission:

Trypanosoma cruzi transmission by blood transfusion has been much extended in endemic areas due to the lack of controls in blood banks. Due to the persistence of the parasite in the patient, infected people may be responsible for parasite transmission through blood donation throughout their life even when they are asymptomatic and unaware of their infected status. In many of the endemic countries, blood transmission of Chagas disease strongly decreased in the 1990s after the development of the of *T. cruzi*-specific antibodies detection in blood banks (Moncayo, 2003; Schmunis, 2007; Moncayo and Silveira, 2009). Control of *T. cruzi* infection in blood banks was recently extended to non-endemic countries (Wendel, 2010). People who have resided for some months in endemic areas cannot give their blood without serological *T. cruzi* control. The efficiency of blood donor screening programs could be improved by screening only blood donors who were born in Latin America or who have traveled in Latin America for extended periods, using a single enzyme immunoassay (Appleman *et al.*, 1993; O'Brien *et al.*, 2007).

Organ transplantation transmission:

Persons receiving an organ transplant from an infected donor are also at risk for Chagas disease, due to immunosuppressive treatment in the organ receiver, a small number of parasites present in the graft were able to develop quickly. Additionally, an infected patient who is receiving an organ transplant may develop a high parasitemia and clinical signs of Chagas disease when immunosuppressive treatment is implemented before the graft. (Riarte *et al.*, 1999; Altclas *et al.*, 2005; D'Albuquerque *et al.*, 2007; Fore´s *et al.*, 2007; Martin-Davila *et al.*, 2008; Sousa *et al.*, 2008; Kun *et al.*, 2009). The problem also occurred in non-endemic countries and the impacts differed according to the transplanted

organ. Indeed, the consequences of heart transplantation seem much more serious than kidney or liver transplantation from the same donor.

Laboratory accidental transmission:

Investigators working in research or clinical laboratories are at risk of being infected with *Trypanosoma cruzi* through the handling of materials containing viable parasites (e.g., infective trypomastigotes, infective amastigotes and metacyclic trypomastigotes). The most frequent accidental laboratory-acquired contaminations resulted from needle-stick injuries during experimental infection of mice or from transmission by aerosol or droplets of infected materials (*T. cruzi* tissue culture supernatants, triatoma feces, and infected blood) by skin or mucosal contact (Coudert *et al*., 1964; Brener, 1984; Hofflin *et al*., 1987; Herwaldt, 2001). Other sources of laboratory contamination were the spraying of parasites by droplets or the breaking of a tube containing living parasites. In particular, special tubes for cryogenic preservation frequently rupture when they are thawed. Contamination also may occur due to a very low quantity of parasites and the parasite may be also present in dry droplets of infective culture or contaminated blood present on a bench. Epimastigotes culture is often considered to be non-infective; however, old cultures that are enriched in metacyclic trypomastigotes are infectious. Laboratory acquired contamination can be prevented by wearing gloves, a mask, by making cultures in a bio-safe laboratory (L3 type), and by using appropriate facilities for animals.

1.5. Globalization of the transmission:

The increasing mobility of populations and the migration toward non endemic countries have extended the infection to these countries through blood transfusion, organ transplantation and the congenital form among migrants. The risk is related with the country of origin of the migrants and the rate of prevalence in that given country. However, the advances observed in control of the transmission indicate that this potential extension to Europe, the USA, and Canada might be transitory or decreasing. The WHO recently

launched the Global Network for Chagas disease elimination (GNChE) to control this situation. It was also reported the global dispersal of Chagas disease due to migrant South and Central American populations to Europe, North America, Japan, and Australia. Finally, the need for non endemic countries to maintain and develop an awareness of Chagas disease as a consequence of globalization is necessary (Schmunis and Yadon, 2010).

1.6. Vector epidemiology and distribution:

A fully understanding of the epidemiology of Chagas disease across its distribution was proven to be elusive and complex, and remains under intense investigation to the present day. The difficulty in defining the epidemiology of Chagas disease is attributable to several factors. Firstly, Chagas disease is a zoonosis and a variety of widely distributed mammals serve as reservoirs for *T. cruzi*. Moreover, all mammals are susceptible to infection. A further factor that contributes to the complexity of Chagas disease as a zoonosis is the variety of vectors involved, being not simply represented by a range of related species or genera, as is the case for all other insect vectors, associated with any given disease. Triatomine bugs are a subfamily of insects and across this relatively broad taxonomic range there are members from all groups that can harbor *T. cruzi*. Most transmission, however, is attributable to three main genera: *Rhodnius*, *Panstrongylus*, and *Triatoma*, but this diversity still represents two different tribes of the subfamily (*Rhodniiniand* and *Triatomini*). Furthermore, the insects vary in more than ancestry, having a diverse range of vertebrate host and ecological associations. The third factor that complicates Chagas disease epidemiology and accounts for variation in the clinical manifestation of the disease is the sub specific diversity of *T. cruzi* itself. Much work has been conducted over the past 40 years to elucidate the variation of *T. cruzi* across its geographical distribution and associations with hosts and vector species. In addition to vector transmission, a small percentage of cases are attributable to unscreened blood transfusions, congenital transmission, and incidences of oral transmission by contamination of food. In 1990, it was estimated that greater than 80% of transmission was due to vectors

and approximately 16% were due to blood transfusion (Schofield, 1994). The endemic transmission of Chagas disease in humans and wild hosts is restricted to the Americas and corresponds largely with the distribution of triatomine bugs approximately from latitudes 42ºN to 46ºS (i.e., from the mid-USA to Patagonia). The triatomines constitute a subfamily of an otherwise predatory group of bugs, and is relatively small compared to the thousands of predatory reduviids. The Triatominae comprises some 140 species. This diversity is classified into six tribes and 19 genera. Certainly three of the most important vector species, *Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiata*, have distributions across several countries (Abad-Franch *et al*., 2005) (Figure 3).
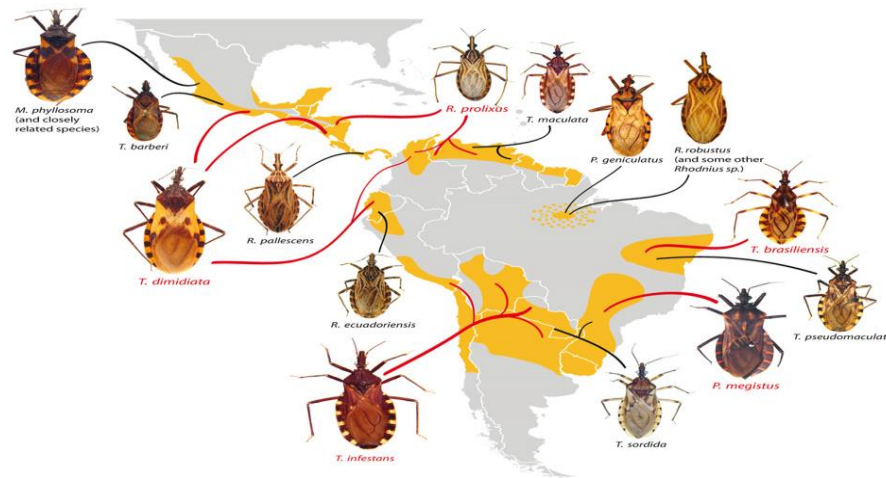
Figure 3: Map of Chagas endemic regions of Central and South America showing respective associations with the 14 most important vector species. The five most important vector species are *T. infestans*, *R. prolixus*, *T. dimidiata*, *P. megistus*, and *T. brasiliensis*. Genera are Triatoma *Rhodnius*, *Panstrongylus* and *Meccus*

Most of the 140 Triatominae species occupy sylvatic ecotopes in association with their respective vertebrate hosts. Examples include: palm crowns, bird nests, possum lodges, rock piles, hollow trees, rodent nests, and bat caves. In most cases triatomine species are adapted to their ecotopes with little tendency to invade human habitations. Therefore, there are only 10-15 species of triatomines that show anthropophilic tendencies and are regularly implicated in disease transmission (Abad-Franch *et al*., 2010; Cohen and Gurtler, 2001; Campbell-Lendrum *et al*., 2007) (Figure 3).

1.7. Chagas disease phases:

Chagas disease is characterized by an acute and a chronic phase of infection. In the acute phase most patients have the unapparent (asymptomatic) form, while in the chronic phase two well-defined forms of disease are distinguished: indeterminate (latent, preclinical) and determinate (clinical), which is subdivided into cardiac, digestive (usually expressed as megaesophagus and/or megacolon), and cardiodigestive forms. Chagas disease is clinically silent in most patients (mainly in the acute phase, but also during the chronic phase), and the diagnosis should be confirmed by the results of laboratory tests. Very often the diagnosis is made fortuitously; for example, when individuals donate blood, during health screening examination, during self-referral testing, and in patients with a strong positive family history or epidemiological antecedents.

1.7.1. Acute phase:

The acute phase of Chagas disease is observed mainly in the first or second decades of life. Clinical manifestations appear around (8-10) days after the penetration of the parasite (Rassi *et al*., 2000). In transfusion transmitted Chagas disease this period may be longer (20-40 days). The acute phase is not clinically recognized in most cases. The experience of those who work in endemic areas is that there is one diagnosed acute case for every 100 chronic patients. Romaña's sign is the most typical sign of portal of entry of the parasite. It is characterized by a painless swelling of one or both eyelids of one eye, the eyelids turn a bluish color, and conjunctival congestion and hypertrophy of satellite lymph nodes (usually preauricular) frequently occur. The edema may spread to half of the face; sometimes dacryoadenitis and diminished conjunctival secretion are observed. Inoculation chagoma is another sign of portal of entry (through the skin), characterized by a maculonodular erythematous lesion, consistent, painless, surrounded by swelling and increased volume of satellite lymph nodes, more often found on open areas and sometimes ulcerated. Fever is a constant sign, frequently accompanied by malaise, asthenia, anorexia, and headache. Fever is usually higher in children, may be continuous or intermittent, and

the temperature may be more elevated during the afternoon. Lymph node enlargement, hepatomegaly, splenomegaly, and subcutaneous edema are the principal systemic signs, together with cardiac and neurologic alterations. Lymph node enlargement is frequent, of slight or moderate intensity, isolated or contiguous, with a smooth surface, painless, hard and non-adherent, and not fistulous. Hepatomegaly and splenomegaly are also frequent, with characteristics similar to that of lymph nodes. Mild to moderate leucocytosis may occur during the acute phase of the disease, with lymphocytosis (atypical lymphocytes), plasmocytosis, and relative neutropenia. Eosinophilia may be observed during the evolution of the disease. The mortality in the acute phase used to be around 5% of all symptomatic cases, often as a consequence of meningoencephalitis or myocarditis. However, nowadays this percentage has decreased as the result of use of specific drugs. Spontaneous cure, although exceptional, may occur as has been described by Zeledón *et al*., (1988) and Francolino *et al*., (2003).

1.7.2. Chronic phase:

The chronic phase begins (2-3) months after the initial infection when the clinical manifestations of the acute phase disappear (if present), and parasitemia falls to undetectable levels. In most cases, the chronic phase presents as an indeterminate form, which may evolve to the cardiac, digestive, or cardiodigestive forms after years or decades. The diagnosis is made by serological tests, such as indirect hemagglutination, indirect immunofluorescence, and ELISA, all of which have high sensitivity and acceptable specificity. The concept of the indeterminate form was not based on histological findings, but on the fact that visceral lesions could not be detected through clinical examination and complementary routine exams in a significant proportion of patients in the chronic phase of Chagas disease. An individual chronically infected with *T. cruzi* remains in the indeterminate form, generally for a period of (10-30) years. There have been few pathological studies focusing on individuals with the indeterminate form. Necropsy studies of patients who died from accidental causes revealed mild myocarditis with scattered small foci of interstitial infiltration by lymphocytes, macrophages, and plasma cells, together with

a limited reduction in the number of cardiac neurons and myenteric plexuses that are insufficient to produce clinical manifestations (Lopes *et al.*, 1981). The cardiac form is the most serious and frequent manifestation of chronic Chagas disease. It develops in 20-30% of individuals and manifests as three major syndromes that may coexist in the same patient: arrhythmic, heart failure, and thromboembolism (systemic and pulmonary) (Rassi *et al.*, 1992, 2000).

1.8. Diagnosis of Chagas disease:

1.8.1. Acute phase:

Several methods are available to search for parasites when they are present in large numbers. They could be divided into direct tests and concentration methods.

1.8.1.1. Direct method:

The simplest and cheapest direct test is the fresh blood smear. A drop of peripheral blood from the patient is collected from the ear, fingertip, foot, or from a vein through a syringe. Ten μL of blood is immediately deposited on a smear and a cover slip (22×22 mm) covers the drop. The amount of 10 μL is ideal for a preparation (i.e., a very thin smear that allows seeing red blood cells separated from each other). The preparation should be mounted in a microscope with an objective of 40× and ocular of 10× (i.e., 400×). If *T. cruzi* is present, it will be seen as a refringent body with very quick movements, disturbing the quiet red blood cells (Brener, 1984).

1.8.1.2. Concentration method:

The Strout technique is very simple. Blood (3-5 mL) is collected without anticoagulant and left to clot, at room temperature, once the clot is formed (15-60 min) the

blood exudate is transferred with a pipette to a centrifugal tube and spun down at low speed (i.e., 50 g, 500 rpm according to the radius of the centrifuge) for 5 min. This will allow the separation of most of red blood cells. Take all the supernatant and transfer to another centrifuge tube and spin hard (i.e., 500 g, around 2000 rpm) for 10 min. This will clear the suspension, having a clear supernatant. Supernatants are taken and stored for serology tests. The last drop remaining at the bottom of the tube is resuspend and a sample of 10 µL is taken on glass slide and cover slip, with the same methodology as the fresh blood smear (Strout, 1962).

1.8.1.3. Microhematocrit method:

Microhematocrit is very useful for congenital infection, because of the need of low sample quantity needed (100 µL of blood for each test). Collected from the plantar region of the baby's foot, using heparinised glass capillaries Samples are centrifugated and analyzed under microscope for *T. cruzi* parasites. Microhematocrit method has 95% sensitivity (Freilij *et al*., 1983).

1.8.2. Chronic phase:

1.8.2.1. Xenodiagnosis:

This method was firstly applied by Brumpt in 1914, a method of diagnosing a vector-transmitted infection (i.e., Chagas' disease), in which a laboratory-reared, pathogen-free triatomine bug is allowed to suck blood from a patient. The intestinal contents of the insect are then examined for the presence of *T. cruzi*. The advantages of this technique are that it may be performed in the field (it is only necessary to transport bugs with 15 days on fast), does not require sterile handling, and allows for inoculation in animals and humans (Santos *et al*., 1995; Franco *et al*., 2002).

1.8.2.2. Hemoculture:

This method depends on the culture of venous blood in LIT-blood/agar medium, the parasite amastigotes growth is analyzed at 20, 30, and 45 days after inculcation (Chiari *et al*., 1989).

1.8.3. Serological Tests:

1.8.3.1. Indirect Hemagglutination Test (IHA):

This method has several advantages which hold up to now, such as few steps low cost, no equipment, and quick results (1-2 h). The method mainly depends on the specific-antigen adherence on the red blood globules superficies followed by the agglutination resulted from the reaction between homologous antigen and antibody which exists in the inactivated serum sample (Neal and Miles, 1970; Stavitsky and Jarchow, 1954).

1.8.3.2. Indirect Immunofluorescence:

Indirect immunofluorescence is used mainly in research laboratories or diagnostic centers. One of the advantages is that the same conjugate (antihuman IgG) may be used for the diagnosis of several diseases and the ability to use the same equipment (fluorescence microscope). This test is conducted by reacting serum with smear fixed epimastigotes and, after washing, incubating with conjugate. The smears are read in the fluorescence microscope. The key advantage of this test is very high sensitivity. It is quite hard to find a serum from an infected individual which does not react. However, a disadvantage is that this same extreme sensitivity may lead to cross-reactions with several diseases (Luquetti *et al*., 2008; Souza and Amato, 2012).

1.8.3.3. Enzyme Linked Immunosorbent Assay ELISA:

In general terms the mechanism of this assay can be explained as antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. Results are accepted to be positive when the optical density is at least 10% higher than the cutoff (a curve with negative, low-positive, and high-positive controls) (Voller *et al*., 1975).

2. SPECIFIC BACKGROUND

2.1. Vaccines:

Vaccines have an indisputable impact on the control of many important human and veterinary diseases and unquestionably have shaped the health landscape of recent generations. The advantages of a Chagas disease vaccine would be significant not just in terms of public health but also economic and social development (Hotez and Ferris, 2006). Recently, the increasing knowledge about the immune response associated with Chagas disease has been valuable for the design and testing of vaccination approaches, the development of recombinant techniques allowed the production of different immunogens ranging from recombinant proteins to DNA and adenovirus vaccines for experimental *T. cruzi* infection (Garg and Bhatia, 2005; Cazorla *et al*., 2009). Recombinant proteins allowed testing several well defined antigens, but the main immune feature induced by these antigens is the production of specific antibodies. Unfortunately, antibodies are not as effective in controlling *T. cruzi* infection as they are in other infections. Parasites can persist as amastigotes inside host cells, avoiding direct contact with antibodies. Even after release from infected cells, parasites can survive and be readily detected in the bloodstream of chronically infected animals and patients despite the high level of specific antibodies circulating. Vaccination protocols have been successful at decreasing parasitemia, tissue damage, and mortality in mouse models immunized with different *T. cruzi* genes (Rodrigues *et al*., 2009). The main idea for vaccine production was divided in two concepts, the first concept is to produce vaccines against trypomastigotes to block their entry after a bite of an infected triatomine, and this will prevent the initiation or persistence of infection and limit the parasitemia. The second concept is to produce vaccines against intracellular replicative amastigotes would inhibit the propagation of parasites in a host and prevent the parasite from entering the blood.

2.1.1. Vaccine developmental forms:

The earlier vaccine generation against *T. cruzi* was heat killed or subcellular fraction of the parasite, mainly defined in the usage of suboptimal contents of immunogenic proteins of epimastigotes (the divisible stage inside the vector's digestive system), and other infective and intracellular stages of *T. cruzi*. The vaccine was tested in different animal models (i.e., mice, dogs, guinea pigs and monkeys). A loss of protective epitopes during inactivation and fractionation, was believed to be the cause for the limited success met in those attempts (Bhatia and Garg, 2005; Bhatia *et al*., 2009).

The next vaccine generation was testing live vaccines having constituents of *T. cruzi* strains accompanied by pharmacological agents treatments, serial passage in vitro cultures or genetic knockouts with a loss of potentially virulent genes (Bhatia and Garg, 2005; Bhatia *et al*., 2009). These vaccines found to be effective in controlling the infection with a disadvantage the danger of reversion of the attenuated strains to a virulent form and the likelihood of increased virulence

Antigens offered a number of attractive properties as vaccine candidates including those abundantly expressed in the infective and intracellular stages of *T. cruzi* and highly immunogenic in natural infection. Purified recombinant *T. cruzi* proteins were the lately next vaccine candidates' generation, the usage of GP90, GP82, GP56, Cruzipain (Cz), paraflagellar rod (PFRs), TC52, complement-regulatory protein (CRP) and ASP2-purified recombinant proteins showed a significant suitability for inducing antibody responses, but they were not efficient in eliciting the cell-mediated immunity that is essential for controlling the pathogen intracellular stage.

In the last two decades naked DNA vaccines were widely used for controlling *T. cruzi* infection, various plasmid or viral vectors which contain the gene of interest were directly injected to the tested animal, the usage of plasmids facilitate the expression of the recombinant gene inserted directly in the mammals host cells, which followed by the rapid host immune response. Various *T. cruzi* proteins were used as a DNA vaccine (i.e., TSA1, ASP1, ASP2, LYT1, CRP and Cz) Garg and Tarleton (2002). Some of those had a higher

protective immune response in mice models and other was capable of reducing the blocking or decreasing the inflammatory reaction in mice heart tissue and skeletal muscles (Vazquez-Chagoyan *et al.*, 2011).

Recent studies aimed to the importance of adjuvants in enhancing the vaccine efficacy, the usage of adjuvants firstly proposed and applied by (Menezes, 1965), and recently the examination of the cytokines had come to a great expectations cytokines like Interleukin (IL-12), co-stimulatory molecules (e.g. GM-CSF, CD40, HSP70) were used to enhance the Th1 immune response to a defined antigen vaccine, Granulocyte Macrophage Cell Stimulating Factor (GM-CSF) was chosen as a genetic adjuvant because it is a potent cytokine capable of enhancing the antigen-presentation capability of antigen presenting cells, such as dendritic cells. In addition, it facilitates B- and T-cell-mediated immunity (Warren and Weiner, 2000). IL-12 is a key cytokine involved in CD8^{+} T-cell activation and proliferation and in directing the immune responses to type 1 (Pan *et al.*, 1999).

Genome-based vaccines were considered to be the main focus of our study by using the sequence database of *T. cruzi* (El-Sayed *et al.*, 2005). Sophisticated bio-informatics programs are designed to evaluate gene functions on the basis of homologies to genes characterized in other organisms and the presence of motifs predictive of targeting, cellular localization, surface expression and functional characteristics of the gene product. Such programs, allowed to directly proceed from sequence information to antigenic target identification and vaccine design (Bhatia *et al.*, 2004) have employed web based bioinformatics tool coupled with an experimental strategy to Vaccine Development Against *Trypanosoma cruzi* identify the putative genes encoding glycosyl-phosphatidylinositol-anchored (GPI anchor) or secreted proteins in a *T. cruzi*-expressed sequence tag database. Molecular and biochemical characterization of eight of the sequences selected by this approach identified three candidates (e.g. TcG1, TcG2 and TcG4) that were conserved in the genome of *T. cruzi* strains of clinical importance. These were expressed in different developmental stages of the parasite and immunogenic in multiple hosts. The selected candidates were recognized by lytic antibodies and CD8^{+} T cells in infected mice (Bhatia and Garg, 2008). Mice immunized with the selected antigens presented with a trypanolytic

antibody response that was in agreement with the intensity of the surface expression of these proteins in infective and intracellular stages of the parasite (Bhatia *et al*., 2004) and the type 1 cytokine (IFN-γ > IL4) profile. Since then, the protective efficacy of TcG1, TcG2 and TcG4 (individually or in combination with and without IL-12 and GM-CSF cytokine adjuvants) in mice have been examined. Data clearly established that co-delivery of the antigens elicited additive immunity and protection from *T. cruzi* infection, Upon challenge infection, TcVac2-vaccinated mice expanded the antigen-specific IgG2b/IgG1 antibodies (TcG4 > TcG2 > TcG1) and elicited a CD8$^+$-dominant T-cell response (CD8/CD4 ratio > 3) associated with type 1 cytokines (IFN- γ and TNF-α) when compared to controls that elicited a mixed type 1/type 2 cytokine response against *T. cruzi* infection. Importantly, with control of parasite burden, the splenic activation of CD8$^+$ T cells and IFN- γ/TNF- α cytokines that are of pathological importance in chronic disease subsided and IL-4/IL-10 cytokines became dominant in vaccinated mice. Accordingly for the first time, a vaccine showed a successful efficacy in reducing the tissue parasite burden by first priming a polarized type 1 T-cell response and then switching to a type 2 dominance suppressing the evolution of immunopathology and tissue damage that are an outcome of consistent immune activation in chronic Chagasic disease.

2.2. Mice model:

Mice have been more frequently used as experimental models to study Chagas disease for several reasons. They are easily reproduced, of low cost, easy to handle, easy to be experimentally infected, and maintained in experimental conditions. Different strains of mice present distinct patterns of susceptibility to *T. cruzi* infection, chosen according to the virulence of the parasite strain or the objective of the study. Moreover, nowadays the existence of several knockout lineages has facilitated the study of different immunological aspects of *T. cruzi* infection.

The acute phase is easily reproduced in this model (Collier *et al*., 1942; Federici *et al*., 1964) making this species very useful to isolate. Young mice are very susceptible to infection, and parasite strain, inoculum, and route of inoculation influence the development

of the infection. Different aspects of acute disease have been reproduced in mice, including symptoms (anorexia, elevated temperature, weight loss, decrease of general activity, patent parasitemia, general edema, and mortality) and histopathological lesions (diffuse myocarditis, myositis, lymphadenopathy, and congestion with infiltrate of mononuclear cells). The humoral immune response of mice was studied by Peralta *et al*., 1980, Jeng and Kierszembaum (1984), which changed in relation to different parasite and host strains. IgM, IgG, and isotypes IgG1, IgG2a, IgG2b, and IgG3 were observed, with the IgG2a more associated with protection (Andrade *et al*., 1985). Later, it was discovered that IgG1 and IgG2 are the most important IgG isotypes with participation on the phenomenon of lyses mediated by complement, the most important humoral process of protection in chronic chagasic infection (Krettli *et al*., 1984). Several pathological phenomena of *T. cruzi* infection were first studied in murine models, such as cellular damage, inflammation, fibrosis and denervation, which explain the disease evolution and characterize the different clinical forms of the disease.

One of the principal limitations of mouse model is the short life span of this species (approximately 2 years) which probably makes impossible the reproduction of the later clinical forms of the disease, such as intense fibrosis, dilated myocardiopathy, aneurysm, and the digestive clinical forms, specially megaesophagus and megacolon, all of later evolution. However, the recent advances obtained in the study of several aspects of Chagas disease immunology in this animal, as well as the use of murine model for test of new drugs, together with ease of use, fully justify the use of this model.

2.3. Electroporation system:

Different non-viral approaches have been proposed for drug and gene delivery such as physical and chemical methods. Physical delivery systems are one of the efficient non-viral methods including electroporation, micro-injection, gene gun, tattooing, laser and ultrasound (Bolhassani and Rafati, 2011). Electroporation (EP) is the formation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) high-voltage pulse to overcome the barrier of the cell membrane. This transient, permeabilized

state can be used to load cells with a variety of different molecules including ions, drugs, dyes, tracers, antibodies, oligonucleotides, RNA and DNA (Faurie *et al.*, 2005). Electroporation has proven useful both in vitro. In addition, the data show that electroporation of DNA vaccines in vivo is an effective method to increase cellular uptake of DNA and gene expression in tissue leading to marked improvement in immune responses. Electroporation represents a way of increasing the number of DNA-transfected cells and enhancing the magnitude of gene expression, while reducing intersubject variability and requiring less time to reach a maximal immune response compared to conventional intramuscular injection of the vaccine (Monie *et al.*, 2010). Delivery of DNA vaccines using electroporation has already been tested successfully in a wide range of disease models. Electroporation has been used to enhance immune responses using DNA vaccines directed against infectious diseases such as influenza, HIV, hepatitis C, malaria, anthrax or to treat or prevent the development of tumors including breast cancer, prostate cancer and melanoma (Daemi *et al.*, 2012; Best *et al.*, 2009).

The application of in vivo electroporation to the sites receiving injected plasmid DNA has allowed for dramatic increases in immune responses compared with plasmid DNA injection alone (Widera *et al.*, 2000). Regarding to in vivo EP is predominantly carried out intramuscularly, currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ producing T cells and protective efficacy to infection (Daemi *et al.*, 2012; Brave *et al.*, 2011). Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+Tcell immune responses while limiting invasiveness of delivery (Brave *et al.*, 2011). The investigators have shown that low-voltage electroporation can induce immunity and protect mice effectively (Daemi *et al.*, 2012; Zhou *et al.*, 2008). In addition, intradermal DNA electroporation is one of the most efficient non-viral methods for the delivery of gene into the skin (Lin *et al.*, 2012).

3. JUSTIFICATION

Chagas disease (CD) is one of the most important Neglected Tropical Disease (NTD) in Latin America, this disease also known as the "forgotten" which affects most vulnerable population who has limited public voice. NTDs have an enormous impact on individuals, families in low income countries. CD is endemic in Latin America, but due to the large population mobility, CD has reached many countries (i.e. Japan, Canada; USA, Europe, Australia), CD causes around 10,000 death each year ad it was estimated that 10 million people are infected, and around 75-90 million are at risk of being infected, CD is a global health problem so that the world Bank have classified CD as it is more prevalent than Malaria or Dengue and states that each year 725,000 workdays are lost due to premature deaths, the loss of 1,200 million USD within the seven countries of the southern cone and 5.6 million USD are lost in Brazil due to the absence of workers.

CD has only two treatments of limited access until now (Nifurtimox and Benznidadzol) moreover, they are not perfect because: They are not effective in the chronic phase of the disease, cannot be used for children of age under 10 years old and finally it may cause serious adverse side effects. The vaccine research institutions have not been able to develop a new vaccine with 100% sterile immunity. The budget of NTD organization only give less than 1% for the research of CD, just 20 million out of the 3.063 million USD are spent on Chagas research. More research and development are needed to secure the families who suffer the infection with CD.

With our work in investigating new techniques and ways in improving and enhancing the immune responses to the actual developed vaccines, we are so close to produce a long lasting, low cost and effective vaccine to treat CD, so more investigation is needed to solve the endemic Chagas disease problem.

4. HYPOTHESIS

Electroporated TcVac1-vaccinated BALB/c mice induce higher protective immune response against *T. cruzi* infection with trypomastigotes Sylvio10x/4 strain more than intramuscularly vaccinated mice.

5. OBJECTIVES

Evaluation of the recombinant DNA vaccine efficiency (TcVac1) using the electroporation system, measuring the immune response in electroporated and intramusculary vaccinated BALB/c mice with *T. cruzi* Sylvio 10X/4 strain infection.

1. Specific objectives:

1. Recombinant DNA vaccine, cloning and production.
2. Evaluate the vaccine immune response of the mice before challenging.
3. Production of *T. cruzi* (Sylvio 10X/4) strain in vitro and prepare it for mice challenging.
4. Infecting the vaccinated mice with *T. cruzi* (Sylvio 10X/4).
5. Clinical evaluation for the mice during the experiment.
6. Evaluate the vaccine immune response of the mice after challenging.

6. MATERIAL AND METHODS

The presented work was done in the molecular biology laboratories of CIESA (Centro de Investigaciones y Estudios Avanzados en Salud Animal) of the FMVZ-UAEM (Facultad de Medicina Veterinaria y Zootecnia-perteneciente a la-Universidad Autónoma del Estado de México).

6.1. General strategy

In our investigation 72 BALB/c mice were separated into 12 mice groups (n=6) on two phases according to the following diagram:

a. Phase one (pre-infection):

Table 1: Experimental groups: Vaccination before Challenge infection with *T. cruzi*.

Technique	Group	Mice No.
Electroporation	1-TcVac	6
	2-pcDNA3.1	6
	3-No DNA	6
Intramuscular	4-TcVAC	6
	5-pcDNA3.1	6
	6-No DNA	6

TcVac1; PcDNA3.1.TcG2, PcDNA3.1.TcG4, PcDNA3.MSP35, PcDNA3.MSP45, PcDNA3.GMCSF; Positive Control, pcDNA3.1; No DNA, Saline solution.

b. Phase two (post-infection):

Table 2: Experimental groups: Vaccination after Challenge infection with *T. cruzi*.

Technique	Group	Mice No.
Electroporation	7-TcVac	6
	8-pcDNA3.1	6
	9-No DNA	6
Intramuscular	10-TcVac	6
	11-pcDNA3.1	6
	12-No DNA	6

TcVac1; PcDNA3.1.TcG2, PcDNA3.1.TcG4, PcDNA3.MSP35, PcDNA3.MSP45, PcDNA3.GMCSF; Positive Control, pcDNA3.1; No DNA, Saline solution.

6.2. Immunization:

All the mice groups were vaccinated with antigen encoding plasmids (PcDNA3-TcG2, PcDNA3-TcG4) 25µg of each plasmid/mouse and cytokines encoding plasmids (IL-12) (PcDNA3-msp-35, PcDNA3-msp-40) and (PcMVI-GMCSF, 25µg of each plasmid/mouse) where GMCSF (granulocyte macrophage stimulating factor), one total dose of 125µg DNA in 4 applications with 15 days difference between each application (Shivali Gupta and Nisha Jain Garg, 2010). All the antigen encoding plasmid and cytokines encoding plasmid were constructed donated by Dr. Nisha Garg, UTMB, Texas, USA.

Two weeks after the last immunization, mice were challenged with *T. cruzi* trypomastigotes (Sylvio 10X/4 strain), 10,000 parasites/mouse were intraperitoneally injected with 30µl total volume.

Sixty days post-infection mice were sacrificed which correspond to the acute phase of the infection. Blood sample drops were taken every other day from each mouse to evaluate parasite count (parasitemia) using the "Fresh tail blood drop analysis", blood was collected before mice sacrifice for serological analysis using ELISA technique.

6.3. Animals

Female BALB/c homozygous mice with age between 6-9 weeks, the mice were obtained from the animal facility of CINVESTAV-IPN (Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México). Mice were kept during the experiment in CIESA (Centro de Investigacion y Estudios Avanzados en Salud Animal). All experimental protocols were conducted under the technical specifications for the production, care and use of laboratory animals from the Norma Official Mexicana (NOM-62-ZOO-1999), and the council for international Organizations of Medical Science. Mice were euthanized according to the Norma Oficial Mexicana (NOM-033-Z00-1995). All protocols were approved by the Laboratory Animal Care Committee at the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma del Estado de México (UAEM).

6.4. Parasites

T. Cruzi trypomastigotes (Sylvio 10X/4) strain was cultivated in continuous monolayer phases of C2C12 cell line, in DMEM medium with 10% fetal bovine serum (HyClone, USA), PH 6.8, at 37 ºc, 5% CO_2 and 85% humidity (Chagoyan *et al*., 2011).

6.5. Vaccination

Mice groups were immunized intramuscularly and intradermally then electroporated in only intradermally injected mice groups with 5 pulses at 450 volts, 0.050

msec pulse interval and 0.125 msec band width. In both, antigen encoding plasmids and cytokines encoding plasmids were injected with (25µg of each plasmid/mouse) in total volume of 40µl and 30µl for intramuscularly and intradermally vaccination respectively. The plasmids obtained from Dr. Nisha were produced and cloned by transformation protocol in calcium chloride E. coli (DH5α) competent cells, and were cultivated on LB-Broth plates with 100µg/ml ampicillin (MAX Efficiency® DH5α™ Competent Cells, Cat. No. 18258-012, Invitrogen). Then plasmids were extracted and purified using GeneJET Plasmid Maxiprep Kit (Cat. K0492). The DNA vaccine was prepared as a cocktail of all plasmids and administered as mentioned above.

6.6. Serology

The mice blood were collected from the eye cavity during the experiment or directly from the heart at the time of sacrifice, the blood samples were kept to clot at 4ºc and serum was separated, all serum samples were kept in Eppendorff tubes at -20ºc for analysis.

6.6.1. Lymphocyte proliferation analysis

Lymphocyte proliferation assay was used to determine lymphocyte activation and the cell-mediated immune responses. When B cells encounter their specific antigens, with the help of T cells, B cells are stimulated to undergo proliferation. When T cells are activated by antigen-presenting cells and cytokines, T cells undergo proliferation. The proliferation of B and T cells leads to clonal expansion and the initiation of the specific immune responses, indicating that the immune system has been primed with specific antigens.

Mice were sacrificed and spleens were dissected out and washed in chilled PBS. With the help of sterile forceps, spleen tissue was teased and then mashed in 40 µm Nylon

Cell Strainer, then washed with 3ml of PBS. Cell suspension was collected and gently added to Histopaque®-1077 sterile-filtered, density: 1.077 g/mL (Sigma), then centrifuged at 2300 rpm for 30 min. at room temperature. Splenocytes were gently isolated and washed twice with PBS. Supernatants were discarded and pellet was resuspended with RPMI-1640 medium (Product. No. R0883, Sigma) complemented with 10% Fetal Bovine Serum (FBS). Splenocytes were stained with Trypan blue and counted by hemocytometer. The 96 plates were prepared with antigens which correspond to the vaccine (TcG2, TcG4 proteins 10µg/ml). Reagent grade Phytohaemaglutinin (PHA, Thermo Scientific Remel™) was used to stimulate mitotic division of lymphocytes maintained in cell culture as positive control, and splenocytes with RMPI-10%FBS were used as negative control. MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Sigma) was used as a colorimetric method for determining the number of viable cells in splenocytes proliferation assay. Optical density was read at 495 nm using EPOCH microplate reader.

6.6.2. Specific antibody response through ELISA technique

Serum samples were collected and preserved at -20ºc, then analyzed for IgG and IgG subtypes (IgG1, IgG2a, IgG2b) levels using an Enzyme Linked Immunosorbent Assay. Nunc Maxisorp (96 well) plates were coated with $NaCO_3$/$NaHCO_3$ PH 6.8 coating buffer containing *T. cruzi* antigens (TcG2, TcG4, 5µg/ml protein each) 200µl/well then incubated sequentially over night at 4ºc. Then plates were washed twice with 200µl PBS 1X-0.05% solution, then blocked with 5% NFDM/PBS (non fat dry milk) and incubated for 60 min. at 37ºc.Plates were incubated for 2 hrs with test sera diluted to 1:100 with PBS-0.05%/NFDM, 100µl/well then washed 6 times as mentioned. Plates were incubated at room temperature for 30 min with 100µl of biotin-conjugated goat anti-mouse IgG and IgG subtypes IgG1, IgG2a, IgG2b (1:2000 dilution in PBST-0.5% NFDM), color were developed with 100 µl/well sure blue TMB substrate (3,3´5,5´-tetramethyl benzidine) and the reaction was stopped with100 µl/well 5N sulfuric acid. Optical density was measured at 450 nm using EPOCH microplate reader (Garg *et al*., 2010).

6.7. Parasitemia

Parasitemia was determined by fresh blood smear test every other day starting 2 weeks post infection during the experiment for each mouse until no parasites were detected. Samples were analyzed under 400x magnification power simple microscope, and parasites were counted in all sample fields.

6.8. Statistical analysis

Data are expressed as means with SEM (standard error of the mean), and derived from at least triplicate observations per sample (n=6 animals/group). Results were analyzed for significant differences using one way analysis of variance ANOVA procedures, Bartlett's test for equal variance and Tukey's multiple comparison tests. The level of significance was calculated vector only-versus-immunized TcVac1 and IDE versus IM immunization with. Differences were considered significant at $p<0.05$, $p<0.001$, $p<0.0001$ levels.

7. RESULTS

7.1. Lymphocyte proliferation levels for intradermally electroporated and intramuscularly vaccinated mice groups.

Some differences were found in lymphocyte proliferation between animals from different treatments before and after challenge. As expected, animals treated with saline solution and pcDNA3 had no proliferation neither when intramuscularly injected nor in animals intradermally injected and electroporated, with the exception of PHA stimulated splenocytes which proliferated in an equivalent manner to the splenocytes from all other groups (data not shown). When comparing TcVac1 treatments, splenocytes belonging to IDE animals, displayed higher proliferation values than splenocytes coming from IM vaccinated animals for TcG2 (0.417) or TcG4 (0.504) recombinant proteins (Fig 4A), but they did not proliferated when exposed to pcDNA3 or saline solution (data not shown). After challenge infection (60 dpi) splenocytes proliferation, was evident for all infected animals when exposed to PHA, TcG2 or TcG4 recombinant proteins, but they did not proliferated when exposed to pcDNA3 or saline solution (data not shown). Splenocytes from animals from IDE/TcVac1 group showed higher splenocyte proliferation, than splenocytes from IM/TcVac1 and control groups when exposed to TcG2 (1.109) or TcG4 (1.279) recombinant proteins (Fig. 4B).

A- Electroporation and Intramuscular vaccine evaluation pre-infection

B- Electroporation and Intramuscular vaccine evaluation post-infection
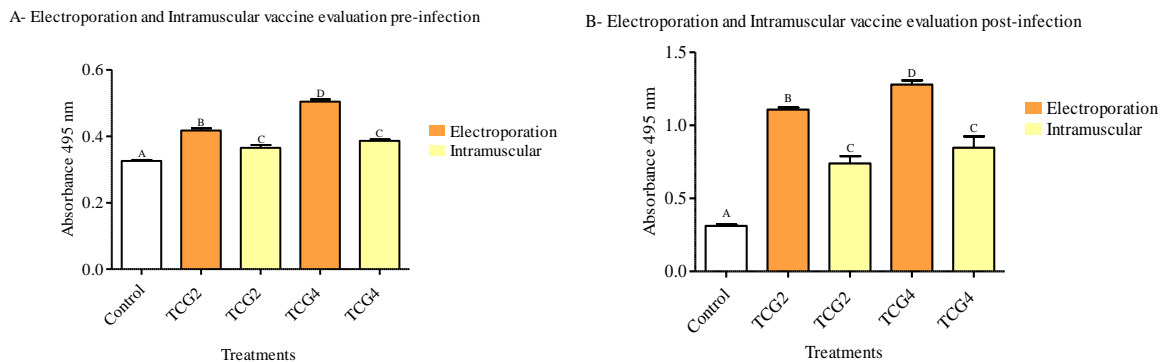
Figure 4: TcG2 and TcG4 recombinant proteins elicit a stronger *in-vitro* proliferation in splenocytes from TcVac1/IDE than from TcVac1/IM immunized animals before

and after challenge infection. (A) IDE mice groups' showed higher significant differences of splenocytes response corresponding to TcG2 and TcG4 antigen encoding plasmids with $p<0.001$ and $p<0.0001$ versus control and $p<0.05$ and $p<0.001$ levels versus IM, while intramuscular mice groups showed a lower significant difference with $p<0.05$ levels for both TcG2 and TcG4 antigen encoding plasmids versus control. (B) IDE mice groups showed a significant high level for TcG2 and Tcg4 antigen encoding plasmids with $p<0.0001$ for both versus control and $p<0.001$ versus both antigen encoding plasmids versus IM mice groups. Finally, IM mice groups shoed a lower significant level for both antigen encoding plasmids with $p<0.001$ levels versus control. Splenocytes proliferations analysis was monitored at 495 nm using EPOCH microplate reader.

7.2. Immunoglobulin G (IgG), IgG subtypes (IgG1, IgG2a,IgG2b) and Immunoglobulin M levels in all mice groups determined by ELISA Assay.

Immunoglobulin G (IgG) and IgG subtypes (IgG1, IgG2a, IgG2b) levels in all treatment groups were assessed to evaluate the immune response for the vaccine before animals were experimentally infected with *T. cruzi* trypomastigotes. Serum samples were analyzed 15 days after the last immunization and substantial levels of recombinant protein-specific antibodies IgG>IgG2a>IgG2b>IgG1 in mice immunized with TcG4- and TcG2-encoding plasmids for both IDE and IM vaccinated groups were detected. It was found that animals from IDE/TcVac1 group had larger surges of all IgG's studied than animals from the IM/TcVac1 group (Fig 5A, 5B, 5C). After challenge, IgG levels from all subtypes increased significantly in all TcVac1 vaccinated animals; however IgG increments were significantly larger for IDE/TcVac1 vaccinated animals than for IM/TcVac1 vaccinated animals (Fig 6A, 6B, 6C).

A- TcG2 pre-infection

B- TcG4 pre-infection

C- TcVAC IDE+IM Pre-infection
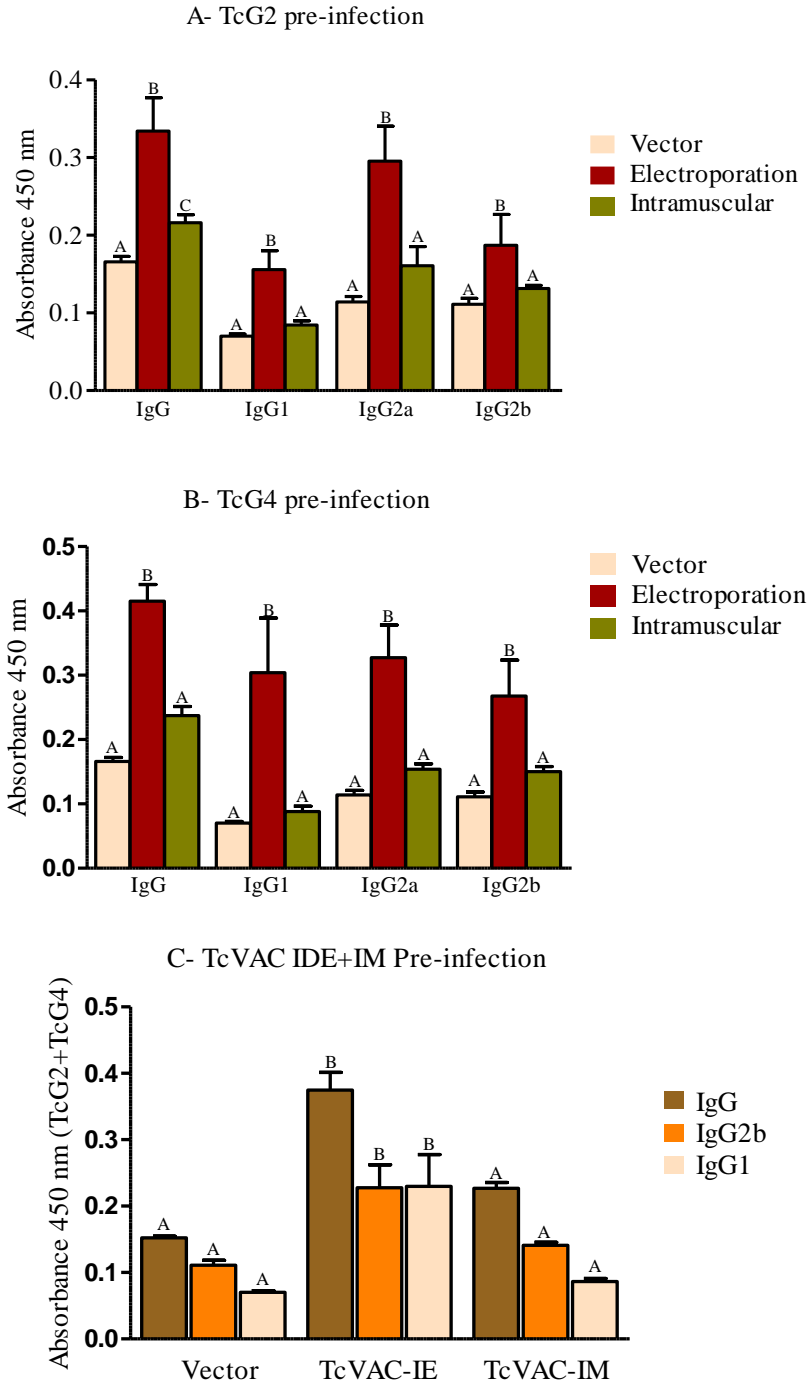
Figure 5: rTcG2 and rTcG4 proteins elicit stronger IgG antibody levels in mice with intradermal electroporation than with Intramuscular injection with TcVac1, before challenge infection. Mice were immunized with 4 vaccine boosts, 15 days after the last

immunization mice sera were collected and analyzed by ELISA assay at (1:100) sera dilution. (A & B) Specific antibody responses were measured corresponding to immunoglobulin G and IgG subtypes (IgG1, IgG2a, IgG2b). A higher antibody response was observed in electroporated mice groups versus intramuscular mice groups (P<0.001 and p<0.0001) in pre- and post-infection periods. IDE versus control showed a high antibody responses with (P<0.001 and p<0.0001) while, IM versus control did not showed a lower response but not statistically significantly different. (C) TcG2 and TcG4 antigen-encoding plasmids Specific antibody response were measured for IgG, IgG2b and IgG1 respectively for IDE mice groups versus IM mice groups showing a significant higher responses of all compared immunoglobulins for IDE mice more than IM groups (P<0.001 and p<0.0001).

A- TcG2 post-infection

B- TcG4 post-infection

Figure 6: rTcG2 and rTcG4 proteins elicit stronger IgG antibody levels in mice with intradermal electroporation than with Intramuscular injection with TcVac1, after challenge infection. Mice were immunized with 4 vaccine boosts, 15 days after the last immunization mice were infected with *T. cruzi* trypomastigotes (Sylvio 10X/4) (10,000/mouse). 60 days post infection sera were collected and analyzed by ELISA assay at (1:100) sera dilution. (A & B) Specific antibody responses were measured corresponding to immunoglobulin G and IgG subtypes (IgG1, IgG2a, IgG2b). A higher antibody response was observed in IDE mice groups versus IM mice groups ($P<0.001$ and $p<0.0001$) in all IgG and IgG subtypes for TcG2 and TcG4 antigen-encoding plasmids. A significant higher response for IDE mice groups versus Control ($P<0.001$ and $p<0.0001$) and lower significant antibody responses ($P<0.05$ and $p<0.001$) for IM mice groups versus control in both TcG2 and TcG4 antigen-encoding plasmids. (C) TcG2 and TcG4 antigen-encoding plasmids Specific antibody response were measured for IgG, IgG2b and IgG1 respectively for IDE versus IM mice groups showing a significant higher responses of all compared immunoglobulins for IDE vaccinated mice ($P<0.001$ and $p<0.0001$).

8. ARTICLE

Immune protection against *Trypanosoma cruzi* induced by TcVac1 vaccine in a murine model using an intradermal/electroporation protocol

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Immune protection against *Trypanosoma cruzi* induced by TcVac1 vaccine in a murine model using an intradermal/electroporation protocol

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Immune protection against *Trypanosoma cruzi* induced by TcVac1 vaccine in a murine model using an intradermal/electroporation protocol

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Abstract

Introduction: *Trypanosoma cruzi*, a parasitic protozoan, is the etiologic agent of Chagas disease. Chagas disease is one of the most common causes of congestive heart failure related deaths among young adults in the endemic areas of South and Central America and Mexico. It has also become an important health issue in the United States and Europe due to large scale migration of Latin Americans over the last few decades. No vaccines are currently available until now. In this study, we tested the vaccine efficacy of two antigen candidates against *T. cruzi* infection and disease in a mouse model. The use of TcVac1 (TcG2, TcG4, *T cruzi* antigen encoding plasmids, interleukin-12 [IL-12] and granulocyte-macrophage colony-stimulating factor [GMCSF] encoding plasmids as genetic adjuvants)

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anti *T. cruzi* candidate vaccine injected intramuscularly has been previously reported in mice with encouraging results. Here we evaluated the comparative protection conferred by TcVac1 when administrated intramuscularly (IM) versus an intradermal/electroporation (IDE) vaccination protocol. Twelve BALB/c mice per group were vaccinated four times fifteen days apart. Six animals (n=6) from each treatment were sacrificed two weeks after the last immunization for pre-infection vaccine efficacy evaluation, after challenge with *T. cruzi* trypomastigotes (Sylvio X10/4 strain), the remaining animals (n=6) were sacrificed 60 days post-infection (dpi). Immune response was assessed through anti-TcG2 and TcG4 *T. cruzi* antigens. Specific serum antibodies with an Enzyme Linked Immunosorbent Assay (ELISA) and lymphocyte activation against the studied antigens was evaluated through a lymphocyte proliferation assay.

Results: We found that IDE induced significantly larger surges of IgG antibodies including subtypes IgG1, IgG2a and IgG2b, during the pre- and post-infection periods for the two antigens used in the experiment. The ratio of antibodies IgG2b/IgG1 was >1 for TcG2 antigen in the pre-infection period in both administration routes. However for the TcG4 antigen the ratios were opposite for animals belonging to different administration routes<1 for IDE and >1 for IM. During the post infection period for both treatments IgG2b/IgG1 ration was always <1. These results suggest, as previously reported, that a switch from Th1 to Th2 type immune response occurs in vaccinated/infected animals. Our splenocyte proliferation assays demonstrated that both antigens were able to induce splenocyte proliferation during the pre-infection period. However, we observed that animals from the IDE group induced more proliferation than IM mice group when TcG4 was used to activate

the cells, which was also observed during the post-infection phase of the experiment. We did not record any animal deaths during the infection however vaccinated mice appeared to have healthier status than the control animals.

Conclusion: Electroporation technique enhances the TcVac1 vaccine uptake in the experimented mice skin tissue leading to high specific immune response in both pre- and post-infection periods compared to the intramuscular technique.

Key words

Trypanosomacruzi; TcVac1; Electroporation; Chagas Disease; Murine model.

Introduction

Trypanosoma cruzi is one of the most devastating parasite infection diseases in Latin America [5]. It is the etiologic agent of Chagas disease and it is mainly transmitted to humans by blood-sucking triatomine bugs. Chagas disease has two successive phases, acute and chronic. Once the acute phase abates, most of the infected patients recover an apparent healthy status in the form of the chronic infection. After several years of the chronic phase, 20-35% of the infected individuals will develop irreversible lesions of the autonomous nervous system in the heart, esophagus, colon, and peripheral nervous system, outlasting the rest of the infected individual's life. Chagas disease represents the first cause of cardiac lesions in young, economically productive adults in the endemic countries of Latin America [1, 2, 3, 4]. It is an important health issue in most of the Latin American countries and due

to human migration; it has become an important health issue in the United States and Europe [5]. Vector control programs have not been able to completely prevent parasite transmission [6], the available anti-parasite drugs are not sufficiently safe or effective [7], and no vaccines are currently available.

Vaccines have an indisputable impact on the control of many important human and veterinary diseases and unquestionably have shaped the health landscape of recent generations. The advantages of a Chagas disease vaccine would be significant, not just in terms of public health but also economic and social development [8]. Recently, the increasing knowledge about the immune response associated with Chagas disease has been valuable for the design and testing of vaccination approaches, the development of recombinant techniques allowed the production of different immunogens ranging from recombinant proteins to DNA and adenovirus vaccines for experimental *T. cruzi* infection [9, 10].

Many, *T. cruzi* derived proteins have been tested as potential vaccine antigens. Unfortunately, antibodies are not as effective in controlling *T. cruzi* infection as they are in other infections. In spite of vaccination protocols with different *T. cruzi* antigens most approaches have been only partially successful at decreasing parasitemia, tissue damage, and mortality in immunized mouse models [11]. The main idea for vaccine production is, on one hand to prevent the initiation or persistence of infection and limit the parasitemia, and on the other hand to fight intracellular replicative amastigotes.

Several investigators have shown the potential utility of *T. cruzi* surface antigens as vaccine candidates in mice [12, 13]. Our group has performed computational screening of

T. cruzi sequence databases reported in GenBank, and identified genes encoding glycosylphosphatidylinositol (GPI)-anchored proteins TcG2 and TcG4 as potential vaccine candidates. These antigens were chosen after an unbiased computational/bioinformatics screening of the *T. cruzi* genome sequence database that led to the identification of 11 potential candidates [14]. Through rigorous analysis over a period of several years, we determined that three candidates (TcG2, TcG4) were maximally relevant for vaccine development. These two candidates are phylogenetically conserved in clinically important *T. cruzi* strains, expressed in infective and intracellular stages of the parasite [15], and recognized by immunoglobulins and CD8+T cells in multiple *T. cruzi*-infected hosts [16]. When individually delivered as a DNA-prime/DNA-boost vaccine along with adjuvants (IL-12- and GM-CSF-encoding plasmids) in mice, these antigens elicited a significant trypanolytic antibody and Th1 cytokine (IFN-γ) response, a property that has been associated with immune control of *T. cruzi* [15]. Co-delivery of these antigens as DNA vaccine (TcVac1) induced additive immunity and higher degree of protection from *T. cruzi* infection than was observed with single vaccine candidates in mice [9]. Furthermore, these same antigens used as a DNA prime/MVA (modified virus Ankara; an attenuated vaccinia virus) based expression vector boost vaccine (TcVac3)elicited a type-1 effector T cell immunity that improved *T. cruzi* control during the acute phase of infection, and subsequently, predominance of anti-inflammatory responses prevented chronic inflammation and myocarditis in chagasic mice. Also DNA prime/protein boost vaccine (TcVac2) elicited an improved protection response over DNA prime/DNA boost (TcVac1) vaccine [17].

Since antigen delivery protocol results in varying protection successes, we decided to test other DNA delivery systems. Physical delivery systems such as electroporation, microinjection, gene gun, tattooing, laser and ultrasound have been proven as efficient delivery methods [18]. Electroporation (EP) is the formation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) high-voltage pulse to overcome the barrier of the cell membrane. This transient, permeabilized state can be used to load cells with a variety of different molecules including ions, drugs, dyes, tracers, antibodies, RNA and DNA [19]. Electroporation has proven useful both *in-vitro* and *in-vivo* protocols. In addition, the data show that electroporation of DNA vaccines in vivo is an effective method to increase cellular uptake of DNA and gene expression in tissue leading to marked improvement in immune responses. Electroporation represents a way of increasing the number of DNA-transfected cells and enhancing the magnitude of gene expression, while reducing intersubject variability and requiring less time to reach a maximal immune response compared to conventional intramuscular injection of the vaccine [20]. Delivery of DNA vaccines using electroporation has already been tested successfully in a wide range of infectious diseases such as influenza, HIV, hepatitis C, malaria, anthrax or to treat or prevent the development of tumors including breast cancer, prostate cancer and melanoma [21, 22].The application of in vivo electroporation to the sites receiving injected plasmid DNA has allowed for dramatic increments in immune responses compared with plasmid DNA injection alone [23].

Regarding to in vivo EP is predominantly carried out intramuscularly, currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive

immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ producing T cells and protective efficacy to infection [21, 24]. Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+Tcell immune responses while limiting invasiveness of delivery [24]. Researchers have shown that low-voltage electroporation can induce immunity and protect mice effectively [21, 25]. In addition, intradermal DNA electroporation is one of the most efficient non-viral methods for the delivery of gene into the skin [26].

Here we describe the protective efficacy of TcG2 and TcG4 vaccine candidates co-delivered with genetic adjuvants (IL-12 and GM-CSF expression plasmids) through an intradermal electroporation approach. We discuss the function of vaccine-induced antibody and splenocyte responses against *T. cruzi* in providing protection from acute parasitemia in chagasic mice.

Material and Methods

Parasites

T. Cruzi trypomastigotes (Sylvio 10X/4 strain) were cultivated in continuous monolayer phases of C2C12 cell line, in DMEM medium with 10% fetal bovine serum (HyClone, USA), PH 6.8, at 37 ℃, 5% CO2 and 85% humidity (Vázquez-chagoyán *et al*., 2011).

Mice

BALB/c female mice (n=72, 6 to 8 week old) were obtained from CINVESTAV, IPN, Mexico, animal facility and divided into twelve treatment groups (n=6), according to table 1. All experimental protocols were conducted under the technical specifications for the production, care and use of laboratory animals from the Norma Official Mexicana (NOM-62-ZOO-1999) [29], and the council for international Organizations of Medical Science. Mice were euthanized according to the Norma Oficial Mexicana (NOM-033-Z00-1995) [30]. All protocols were approved by the Laboratory Animal Care Committee at the Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma del Estado de México (UAEM).

TcVac1 vaccine preparation

TcG2 and TcG4 cDNAs (SylvioX10/4 isolate, Genbank: AY727915 and AY727917, respectively) were cloned in eukaryotic expression plasmid pCDNA3.1 [31]. Plasmids encoding IL-12 (pcDNA3.msp35 and pcDNA3.msp40) and GM-CSF (pCMVI.GM-CSF) have been previously described [32]. Plasmids were transformed into E. coli (DH5-α) competent cells, grown in L-broth containing 100-mg/ml ampicillin, and purified using the GeneJET Plasmid Maxiprep Kit [33].

Table 1. Treatment groups

   a. Pre-infection phase:

      Table 1:

   b. Infection phase

Table 2:

Immunization:

Animals were treated either with DNA or placebo, either by intramuscular (IM) injection or by intradermal/electroporation (IDE). Intradermally injected mice were electroporated with 5 pulses at 450 volts, 0.050 msec pulse interval and 0.125 msec band width.

Mice from the TcVac1 groups, IDE (n=6) and IM (n=6) were vaccinated with antigen and cytokine encoding plasmids (PcDNA3-TcG2, PcDNA3-TcG4; PcDNA3-msp-35, PcDNA3-msp-40 [IL-12] and pcMVI-GM-CSF), (25µg of each plasmid/mouse) in 4 doses with 15 days apart [27], or with pcDNA3.1 empty plasmid (n=6, 25µg) or saline solution (n=6) with the same frequency as animals receiving TcVac1. Two weeks after the last immunization animals were sacrificed for vaccine assessment through antibody response and lymphocyte proliferation assay.

The remaining 6 groups of mice received the same treatments as the pre-infection groups, with the exception that animals received an intra-peritoneal challenge infection with (10,000 parasites/mouse) of *T. cruzi* trypomastigotes (Sylvio 10X/4 strain) in a 30µl total volume, two weeks after the last immunization.

Sixty days post-infection, during the acute phase of the infection, mice were sacrificed and blood was collected for immunological evaluation.

Serology

Mice blood was collected from the eye cavity during the experiment or directly from the heart at the time of sacrifice. Blood samples were kept to clot at 4ºc and serum was separated, all serum samples were stored at -20ºc until analyzed.

Lymphocyte proliferation analysis

Lymphocyte proliferation assay was used to determine lymphocyte activation and the cell-mediated immune responses. When B cells encounter their specific antigens, with the help of T cells, B cells are stimulated to undergo proliferation. When T cells are activated by antigen-presenting cells and cytokines, T cells undergo proliferation. The proliferation of B and T cells leads to clonal expansion and the initiation of the specific immune responses, indicating that the immune system has been primed with specific antigens.

Mice were sacrificed and spleens were dissected out and washed in chilled PBS. With the help of sterile forceps, spleen tissue was teased and then mashed in 40 µm Nylon Cell Strainer, then washed with 3ml of PBS. Cell suspension was collected and gently added to Histopaque®-1077 sterile-filtered, density: 1.077 g/mL (Sigma), then centrifuged at 2300 rpm for 30 min. at room temperature. Splenocytes were gently isolated and washed twice with PBS. Supernatants were discarded and pellet was resuspended with RPMI-1640 medium (Product. No. R0883, Sigma) complemented with 10% Fetal Bovine Serum (FBS). Splenocytes were stained with Trypan blue and counted by hemocytometer. The 96 plates

were prepared with antigens which correspond to the vaccine (TcG2, TcG4 proteins 10μg/ml). Reagent grade Phytohaemaglutinin (PHA, Thermo Scientific Remel™) was used to stimulate mitotic division of lymphocytes maintained in cell culture as positive control, and splenocytes with RMPI-10%FBS were used as negative control. MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Sigma) was used as a colorimetric method for determining the number of viable cells in splenocytes proliferation assay. Optical density was read at 495 nm using EPOCH microplate reader.

Specific antibody response through ELISA technique

Serum samples were collected and preserved at -20ºc, then analyzed for IgG and IgG subtypes (IgG1, IgG2a, IgG2b) levels using an Enzyme Linked Immunosorbent Assay. Nunc Maxisorp (96 well) plates were coated with $NaCO_3/NaHCO_3$ PH 6.8 coating buffer containing *T. cruzi* antigens (TcG2, TcG4, 5μg/ml protein each) 200μl/well then incubated sequentially over night at 4ºc. Then plates were washed twice with 200μl PBS 1X-0.05% solution, then blocked with 5% NFDM/PBS (non fat dry milk) and incubated for 60 min. at 37ºc.Plates were incubated for 2 hrs with test sera diluted to 1:100 with PBS-0.05%/NFDM, 100μl/well then washed 6 times as mentioned. Plates were incubated at room temperature for 30 min with 100μl of biotin-conjugated goat anti-mouse IgG and IgG subtypes IgG1, IgG2a, IgG2b (1:2000 dilution in PBST-0.5% NFDM), color were developed with 100 μl/well sure blue TMB substrate (3,3´5,5´-tetramethyl benzidine) and the reaction was stopped with100 μl/well 5N sulfuric acid. Optical density was measured at 450 nm using EPOCH microplate reader. [27]

Parasitemia

Parasitemia was determined by fresh blood smear test every other day starting 2 weeks post infection during the experiment for each mouse until no parasites were detected. Samples were analyzed under 400x magnification power simple microscope, and parasites were counted in all sample fields.

8. Statistical analysis

Data are expressed as means with SEM (standard error of the mean), and derived from at least triplicate observations per sample (n=6 animals/group). Results were analyzed for significant differences using one way analysis of variance ANOVA procedures, Bartlett's test for equal variance and Tukey's multiple comparison tests. The level of significance was calculated vector only-versus-immunized TcVac1 and IDE versus IM immunization with. Differences were considered significant at $p < 0.05$, $p < 0.001$, $p < 0.0001$ levels.

Results

Lymphocyte proliferation assay:

Some differences were found in lymphocyte proliferation between animals from different treatments before and after challenge. As expected, animals treated with saline solution and pcDNA3 had no proliferation neither when intramuscularly injected nor in animals intradermally injected and electroporated, with the exception of PHA stimulated splenocytes which proliferated in an equivalent manner to the splenocytes from all other

groups (data not shown). When comparing TcVac1 treatments, splenocytes belonging to IDE animals, displayed higher proliferation values than splenocytes coming from IM vaccinated animals for TcG2 (0.417) or TcG4 (0.504) recombinant proteins (Fig 1A), but they did not proliferated when exposed to pcDNA3 or saline solution (data not shown). After challenge infection (60 dpi) splenocytes proliferation, was evident for all infected animals when exposed to PHA, TcG2 or TcG4 recombinant proteins, but they did not proliferated when exposed to pcDNA3 or saline solution (data not shown). Splenocytes from animals from IDE/TcVac1 group showed higher splenocyte proliferation, than splenocytes from IM/TcVac1 and control groups when exposed to TcG2 (1.109) or TcG4 (1.279) recombinant proteins (Fig. 1B).

Serological evaluation:

Immunoglobulin G (IgG) and IgG subtypes (IgG1, IgG2a, IgG2b) levels in all treatment groups were assessed to evaluate the immune response for the vaccine before animals were experimentally infected with *T. cruzi* trypomastigotes. Serum samples were analyzed 15 days after the last immunization and substantial levels of recombinant protein-specific antibodies IgG>IgG2a>IgG2b>IgG1 in mice immunized with TcG4- and TcG2-encoding plasmids for both IDE and IM vaccinated groups were detected. It was found that animals from IDE/TcVac1 group had larger surges of all IgG's studied than animals from the IM/TcVac1 group (Fig 2A, 2B, 2C). After challenge, IgG levels from all subtypes increased significantly in all TcVac1 vaccinated animals; however IgG increments were

significantly larger for IDE/TcVac1 vaccinated animals than for IM/TcVac1 vaccinated animals (Fig 3A, 3B, 3C).

Discussion

DNA vaccination consists of the administration of a construct engineered to produce an antigen of interest designed to elicit immunity against pathogens or even cancer cells exposed to its action. Eventually, the encoded immunogen will be responsible for the generation of a pool of antigen-specific T cells, some of which will remain as memory cells for long term protection. This technology has been used for a wide range of applications, from laboratory tools to licensed veterinary vaccines [35]. Also relevant to the treatment of human disease, the increasing number of clinical trials predicts the tremendous therapeutic potential for this approach against cancer. Moreover, DNA vaccines present several advantages over other vaccination strategies, among which are the relatively higher stability of DNA that avoids cold chain disruption issues, as well as the low price and the ease in producing good manufacturing practices (GMP) by the use of vaccination grade DNA. [34, 35]

The effectiveness of *T. cruzi* genes as genetic vaccines for the elicitation of parasite-specific immune responses has recently been shown in a number of laboratories with several different *T. cruzi* strains [9, 15, 16, 17, 27, 28, 31, 33, 36, 37, 38, 39, 40, 41, and 42]. The antigenic potential of TcG2 and TcG4 was shown by studies of our group which

demonstrated that the encoded antigens elicited antibodies that bound to the parasite surface and exhibited trypanolytic and agglutination activities [16, 17].

Manipulation of antigen presentation and processing pathways is an important aspect in DNA vaccine technology. Several approaches have been studied in DNA vaccine designs, including: co-delivery of immune-modulatory molecules, diverse routes of administration or prime-boost regimens. Among the different DNA vaccine administration strategies reported, the use of naked plasmid DNA injected with intramuscularly (IM), the subcutaneously (SC), intradermally (ID) or intranasally approaches [43] and the epidermal transfection with either coated or naked DNA administered through gene gun [44]. Pros and cons have been reported for alternative vaccine strategies. For example: Viral mediated gene transfer by genetically modified viruses (lentiviruses, adenoviruses, adeno-associated viruses, and retroviruses) has high transfection efficiency and stability. However, disadvantages of these methods may be the production cost, putative toxic side effects, limited transgene size that can be used and the potential insertional mutagenesis that may be induced by these viral vectors, (reviewed by Fioretti *et al* [45]). Intramuscular or intradermal injection of plasmid predominantly leads to transfection of myocytes or keratinocytes. However, these type of cells lack expression of major histocompatibility complex (MHC) class II and for that reason they do not prime T lymphocytes directly. It is likely that immune priming occurs through dendritic cells (DCs) present at the site of DNA injection or that are attracted in response to inflammatory or chemotactic signals following vaccination. Dendritic cells are thought to be responsible for antigen presentation following direct transfection of plasmid DNA, (reviewed by Fioretti *et al* [45]).

TcG2 and TcG4 antigens have been tested experimentally in a series of combinations of TcVac anti-*T. cruzi* candidate antigens in order to test differential efficacies of the vaccine preparations. Vaccines strategies tested included pcDNA-prime/pcDNA-boost in mice and dogs (TcVac1) [17, 46], pcDNA-prime/recombinant protein boost (TcVac2) [17], and pcDNA-prime/MVA boost (TcVac3) [33].

TcVac1 vaccine elicited a strong Th1-type antibody response dominated by IgG2b/IgG1 isotypes, which were maintained after a challenge infection. Vaccinated animals controlled 50-90% of the acute phase tissue parasite burden. Lack of splenocyte proliferation of vaccinated/infected animals in vitro suggests that protection from challenge infection was not provided by an active T-cell response. TcVac2 vaccine also induced a strong IgG2b>IgG1 antibody response and a moderate level of lymphocyte proliferation in mice. After Challenge infection IgG2b>IgG1 antibody response expanded and elicited a substantial CD8+ T cell response, which is associated with type 1 cytokines (IFN-γ and TNF-α) that resulted in control of acute parasite burden. During chronic phase antibody response persisted, as well as CD8+ T cells, however IL-4/IL-10 cytokines became dominant in vaccinated mice. Tissue parasitism, inflammation and fibrosis in heart and skeletal muscle of TcVac2-vaccinated chronic mice were undetectable by histological techniques, as compared to non vaccinated/infected animals which exhibited persistent parasite burden and immunopathology in the myocardium. TcVac3 vaccine also elicited a strong IgG2b>IgG1 antibody response and a robust *T. cruzi*-specific CD8+ T cell response with Type-1 cytokine (IFN-γ + TNF-α> IL-4 + IL-10) and cytolytic effector phenotype. TcVac3 induced significantly effector T cells upon challenge infection and provided >92%

control of *T. cruzi*. In chronic phase of infection mice exhibited a significant decline (up to 70%) in IFN-γ and CD8+ T cells a predominance of immune-regulatory IL-10/CD4+ T cell, and presented undetectable tissue parasitism, inflammatory infiltrate, and myocardium fibrosis. As a whole the experience with TcVac series vaccines suggests that a protective vaccine elicits a strong antibody response IgG2b>IgG1 and a robust *T. cruzi*-specific CD8+ T cell response with Type-1 cytokine (IFN-γ + TNF-α > IL-4 + IL-10) and cytolytic effector phenotype that even if it does not produce a sterile immunity, it does control parasitemia and tissue parasite burden. Then during the infection Th type 1 is switched for a Th type 2 immune response which is associated to a healing inflammation [27].

The TcVac vaccine series are designed for prime/boost doses. They all start with a DNA prime dose and finish with either, DNA, recombinant protein or viral vector boost. This difference may explain the dissimilarities found among treatments, and mainly with TcVac1.

TcVac2 and TcVac3 induce a more effective immune response, however, production and purification of recombinant proteins or viral vectors is quite more expensive than plasmid DNA production and purification. Therefore we decided to test a more efficient way of administration of a DNA-prime/DNA-boost vaccine approach which we found in intradermal electroporation (IDE) vaccine administration, since it has been shown to be very effective in transfecting in-vivo cells with DNA vaccines and induce a high immune response.

IDE promotes Th1 and Th2 type immune responses because on one hand it reversibly permeabilizes the target cells enhancing the uptake and expression of the gene of interest

[53, 54], and since the site of IDE contains many antigen-presenting cells (APCs), mainly dendritic cells, that can be directly transfected during the vaccination process, and therefore the expressed antigen is presented in association with major histocompatibility complex (MHC) class I molecules, which will subsequently stimulate the synthesis of a pool of antigen-specific cytotoxic T lymphocytes(CTL). Furthermore, epitope display mediated by MHC class 1 is prone to induce both a T helper type 1 (Th1) response in conjunction with the generation CTLs [47, 48], the most potent cytolytic effector cells. On the other hand, the tissue damage caused by the application of IDE causes inflammation and recruits antigen presenting cells (DCs, macrophages) and lymphocytes to the injection site inducing significant immune responses, including antibody production as well as T-cells. Furthermore, IDE can be repeatedly used in a prime/boost vaccine delivery strategy, since it does not induce undesirable immune responses against the delivery system (reviewed by Fioretti *et al* [45]).

Here, we tested TcVac1 vaccine that includes pcDNA3.1-TcG2 and pcDNA3.1-TcG4 mammalian cell expression plasmids, but, in order to maximize its protective capacity, we did not eliminate the adjuvant cytokine genes that can help enhance the response to the antigen-encoding plasmid(s). GM-CSF enhances the antigen-presenting capability of dendritic cells and facilitates B- and T-cell-mediated immunity [49, 50], and IL-12 enhance $CD8^+$-T-cell activation and proliferation, and in promoting type 1 cytokine production [50, 51, 52]. Co-administration of cytokine genes with *T. cruzi* antigen encoding plasmids have

been associated to an increment in the level of antigen-specific CTL activity and humoral immune responses and provided substantially better protection from *T. Cruzi* [27].

In this experiment we expected that the TcVac1 vaccine, delivered through IDE, should induce a stronger immune response than TcVac1 administered intramuscularly. We would expect an immune response more closely related to the responses found for TcVac2 and TcVac3 immunization protocols.

We found that splenocytes coming from IDE /vaccinated animals proliferated more than the splenocytes derived from IM/vaccinated animals. O.D. (optical density) readings for TcG2 or TcG4 recombinant protein stimulated splenocytes were 0.42, 0.50 for TcG2 and 0.38, 0.39 for TcG4 from IDE and IM vaccinated animals, respectively. During the acute phase of infection (60 dpi) the splenocytes proliferated even further for all vaccinated groups, while the differences kept favoring splenocytes coming from IDE vaccinated mice with O.D. values of 1.2, 1.25 for TcG2 and 0.75, 0.08 for TcG4 from IDE and IM vaccinated animals, respectively. This study demonstrates that animals vaccinated with TcVac1 by IDE develop stronger cell immunity than animals vaccinated with IM vaccine administration. These results are not surprising since it has been previously reported that IDE facilitates keratinocyte and dendritic cell transfection, and the proteins product of plasmid expression, are directed to the MHC type I antigen presentation that conducts to a Th1 Type immune response, mentioned above. Further studies should be conducted to better characterize the cell mediated immune response elicited by TcVac1 vaccine when administered by IDE.

Antibodies levels were evaluated in an ELISA assay where plaques were sensitized with TcG2 and TcG4 recombinant proteins, independently. Antibody titers for these proteins can be observed in figures 2 and 3 Unexpectedly, IM administrated TcVac1 induced very low immunoglobulin levels during the vaccination period. In comparison IDE administration of the vaccine induced relatively high levels of all IgG subtypes. High levels of immunoglobulin G production were expected, since the IDE is associated to some tissue damage that attracts antigen-presenting cells (dendritic cells and macrophages) to the injection site that promote antibody production as previously described. For both treatments (IDE and IM) the ratio IgG2b/IgG1 was >1 which suggests a Th type 1 skewed immune response, which is in accordance with the observations made by Bhatia *et al*. (2008) and Gupta and Garg, 2010; 2013 for the TcVac vaccine series [17, 27, 33].

Antibody production after experimental infection (60 dpi) displayed significant increments in all infected animals in anti-TcG2 and TcG4 IgG antibodies. However, the largest antibodies surges for all IgG subtypes was produced from the IDE/TcVac1 animal groups, indicating that antigen presenting cells were more active for IDE than for IM vaccinated animals. The ratio IgG2b/IgG1for both vaccine treatments switched to values <1 as compared to vaccinated animals, indicating a switch from a Th1 to a Th2 cell type immune response as was previously reported by Gupta *et al*. (2010, 2013) for the TcVac vaccine series [27, 33].

Conclusion

Further investigation is required to complete the study of the full protective immune response of TcVac1 IDE protocol, however, if the immune protective response of TcVac1 IDE matches the TcVac2 and TcVac3 vaccination protocols it would be a more practical approach as a clinical vaccine, since production and purification of plasmid DNA can be done at a fraction of a cost of what recombinant proteins or viral vectors are produces and with less effort [45]. Additionally the cold chain needed for proteins and viral vector based vaccines to prevent degradation or inactivation, is not required for plasmid DNA. Therefore, these results are encouraging to perform complementary experiments to fully test the protective capacity of TcVac1 IDE vaccination protocols.

List of abbreviations

IM   Intramuscular

IDE   Intradermal electroporation

Dpi   Days post infection

ELISA   Enzyme Linked Immunosorbent Assay

GPI   Glycosylphosphatidylinositol

EP   Electroporation

IL-12   Interleukin-12

GM-CSF   Granulocyte Macrophage Colony Stimulating Factor

PBMCs   Peripheral Blood Mononuclear Cells

FBS	Fetal Bovine Serum
PHA	Phytoheamaglutinin
NFDM	Non Fat Dry Milk
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween20
TMB	TetramethyleBenzidine
SEM	Standard Error of the Mean
GMP	Good Manufacturing Practices
SC	Subcutaneously
ID	Intradermally
DCs	Dentritic Cells
APCs	Antigen Presenting Cells
MHC	Major Histocompatibility Complex
CTL	Cytotoxic T Lymphocytes
OD	Optical Density

Competing interests

The authors have declared that no competing interests exist.

Authors' contributions

Conceived and designed the experiments: WHHM, JCVC. Performed the experiments: WHHM. Analyzed the data: WHHM, JCVC. Contributed reagents/materials/ analysis tools: JCVC. Wrote the paper: WHHM, JCVC.

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Attachments

Tables

Table 1: Experimental groups: Vaccination before Challenge infection with *T. cruzi*.

Technique	Group	Mice No.
Electroporation	1-TcVac	6
	2-pcDNA3.1	6
	3-No DNA	6
Intramuscular	4-TcVAC	6
	5-pcDNA3.1	6
	6-No DNA	6

TcVac1; PcDNA3.1.TcG2, PcDNA3.1.TcG4, PcDNA3.MSP35, PcDNA3.MSP45, PcDNA3.GMCSF; Positive Control, pcDNA3.1; No DNA, Saline solution.

Table 2: Experimental groups: Vaccination after Challenge infection with *T. cruzi*.

Technique	Group	Mice No.
Electroporation	7-TcVac	6
	8-pcDNA3.1	6
	9-No DNA	6
Intramuscular	10-TcVac	6
	11-pcDNA3.1	6
	12-No DNA	6

TcVac1; PcDNA3.1.TcG2, PcDNA3.1.TcG4, PcDNA3.MSP35, PcDNA3.MSP45, PcDNA3.GMCSF; Positive Control, pcDNA3.1; No DNA, Saline solution.

Figures

Figure 1: TcG2 and TcG4 recombinant proteins elicit a stronger *in-vitro* proliferation in splenocytes from TcVac1/IDE than from TcVac1/IM immunized animals before and after challenge infection.

(A) IDE mice groups' showed higher significant differences of splenocytes response corresponding to TcG2 and TcG4 antigen encoding plasmids with $p<0.001$ and $p<0.0001$ versus control and $p<0.05$ and $p<0.001$ levels versus IM, while intramuscular mice groups showed a lower significant difference with $p<0.05$ levels for both TcG2 and TcG4 antigen encoding plasmids versus control. (B) IDE mice groups showed a significant high level for TcG2 and Tcg4 antigen encoding plasmids with $p<0.0001$ for both versus control and $p<0.001$ versus both antigen encoding plasmids versus IM mice groups. Finally, IM mice groups shoed a lower significant level for both antigen encoding plasmids with $p<0.001$ levels versus control. Splenocytes proliferations analysis was monitored at 495 nm using EPOCH microplate reader.

Figure 2: rTcG2 and rTcG4 proteins elicit stronger IgG antibody levels in mice with intradermal electroporation than with Intramuscular injection with TcVac1, before challenge infection.

A- TcG2 pre-infection

B- TcG4 pre-infection

C- TcVAC IDE+IM Pre-infection

Mice were immunized with 4 vaccine boosts, 15 days after the last immunization mice sera were collected and analyzed by ELISA assay at (1:100) sera dilution. (A & B) Specific

antibody responses were measured corresponding to immunoglobulin G and IgG subtypes (IgG1, IgG2a, IgG2b). A higher antibody response was observed in electroporated mice groups versus intramuscular mice groups (P<0.001 and p<0.0001) in pre- and post-infection periods. IDE versus control showed a high antibody responses with (P<0.001 and p<0.0001)while, IM versus control did not showed a lower response but not statistically significantly different. (C) TcG2 and TcG4 antigen-encoding plasmids Specific antibody response were measured for IgG, IgG2b and IgG1 respectively for IDE mice groups versus IM mice groups showing a significant higher responses of all compared immunoglobulins for IDE mice more than IM groups(P<0.001 and p<0.0001).
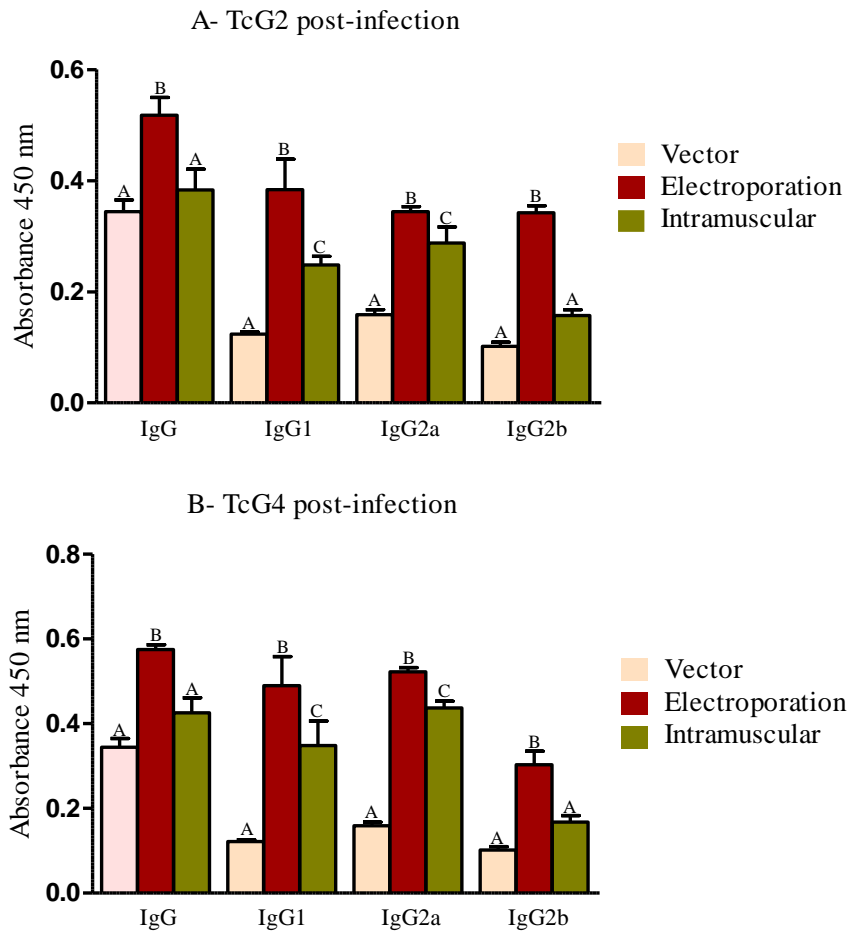
Figure 3: rTcG2 and rTcG4 proteins elicit stronger IgG antibody levels in mice with intradermal electroporation than with Intramuscular injection with TcVac1, after challenge infection.
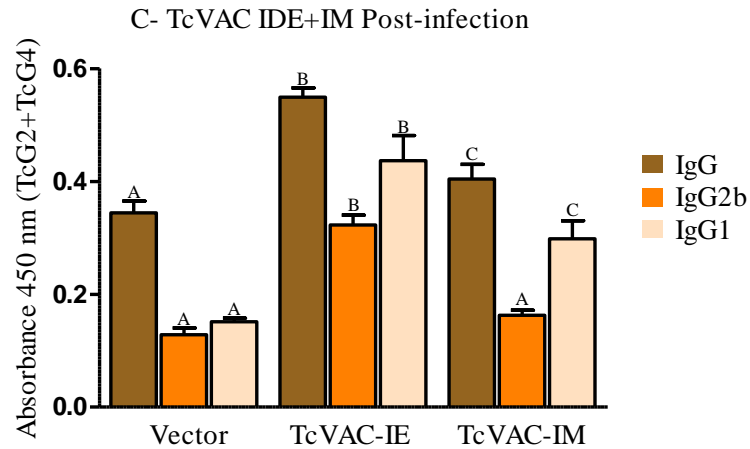
C- TcVAC IDE+IM Post-infection

Mice were immunized with 4 vaccine boosts, 15 days after the last immunization mice were infected with *T. cruzi* trypomastigotes (Sylvio 10x/4) (10,000/mouse). 60 days post infection sera were collected and analyzed by ELISA assay at (1:100) sera dilution. (A & B) Specific antibody responses were measured corresponding to immunoglobulin G and IgG subtypes (IgG1, IgG2a, IgG2b). A higher antibody response was observed in IDE mice groups versus IM mice groups ($P < 0.001$ and $p < 0.0001$) in all IgG and IgG subtypes for TcG2 and TcG4 antigen-encoding plasmids. A significant higher response for IDE mice groups versus Control ($P < 0.001$ and $p < 0.0001$) and lower significant antibody responses ($P < 0.05$ and $p < 0.001$) for IM mice groups versus control in both TcG2 and TcG4 antigen-encoding plasmids. (C) TcG2 and TcG4 antigen-encoding plasmids Specific antibody response were measured for IgG, IgG2b and IgG1 respectively for IDE versus IM mice groups showing a significant higher responses of all compared immunoglobulins for IDE vaccinated mice($P < 0.001$ and $p < 0.0001$).

9. DISCUSSION

DNA vaccination consists of the administration of a construct engineered to produce an antigen of interest designed to elicit immunity against pathogens or even cancer cells exposed to its action. Eventually, the encoded immunogen will be responsible for the generation of a pool of antigen-specific T cells, some of which will remain as memory cells for long term protection. This technology has been used for a wide range of applications, from laboratory tools to licensed veterinary vaccines (Kutzler, 2008). Also relevant to the treatment of human disease, the increasing number of clinical trials predicts the tremendous therapeutic potential for this approach against cancer. Moreover, DNA vaccines present several advantages over other vaccination strategies, among which are the relatively higher stability of DNA that avoids cold chain disruption issues, as well as the low price and the ease in producing good manufacturing practices (GMP) by the use of vaccination grade DNA. (Senovilla, 2013; Kutzler, 2008)

The effectiveness of *T. cruzi* genes as genetic vaccines for the elicitation of parasite-specific immune responses has recently been shown in a number of laboratories with several different *T. cruzi* strains (Garg *et al*., 1997, 2005; Bhatia *et al*., 2004, 2008; Gupta *et al*., 2010; Vázquez-chagoyán *et al*., 2011; Rodrigues, 2011; Sepulveda, 2000; Tesoro-Cruz, 2008). The antigenic potential of TcG2 and TcG4 was shown by studies of our group which demonstrated that the encoded antigens elicited antibodies that bound to the parasite surface and exhibited trypanolytic and agglutination activities (Bolhassani, 2011; Bhatia *et al*., 2008).

Manipulation of antigen presentation and processing pathways is an important aspect in DNA vaccine technology. Several approaches have been studied in DNA vaccine designs, including: co-delivery of immune-modulatory molecules, diverse routes of administration or prime-boost regimens. Among the different DNA vaccine administration strategies reported, the use of naked plasmid DNA injected with intramuscularly (IM), the subcutaneously (SC), intradermally (ID) or intranasally approaches (Lai, 2009) and the epidermal transfection with either coated or naked DNA administered through gene gun (Fioretti *et al*., 2010). Pros and cons have been reported for alternative vaccine strategies.

For example: Viral mediated gene transfer by genetically modified viruses (lentiviruses, adenoviruses, adeno-associated viruses, and retroviruses) has high transfection efficiency and stability. However, disadvantages of these methods may be the production cost, putative toxic side effects, limited transgene size that can be used and the potential insertional mutagenesis that may be induced by these viral vectors, (reviewed by Fioretti *et al.*, 2010). Intramuscular or intradermal injection of plasmid predominantly leads to transfection of myocytes or keratinocytes. However, these type of cells lack expression of major histocompatibility complex (MHC) class II and for that reason they do not prime T lymphocytes directly. It is likely that immune priming occurs through dendritic cells (DCs) present at the site of DNA injection or that are attracted in response to inflammatory or chemotactic signals following vaccination. Dendritic cells are thought to be responsible for antigen presentation following direct transfection of plasmid DNA, (reviewed by Fioretti *et al.*, 2010).

TcG2 and TcG4 antigens have been tested experimentally in a series of combinations of TcVac anti-*T. cruzi* candidate antigens in order to test differential efficacies of the vaccine preparations. Vaccines strategies tested included pcDNA-prime/pcDNA-boost in mice and dogs (TcVac1) (Aparicio-Burgos, 2011; Gupta and Garg, 2013), pcDNA-prime/recombinant protein boost (TcVac2) (Aparicio-Burgos, 2011), and pcDNA-prime/MVA boost (TcVac3) (Gupta and Garg 2013).

TcVac1 vaccine elicited a strong Th1-type antibody response dominated by IgG2b/IgG1 isotypes, which were maintained after a challenge infection. Vaccinated animals controlled 50-90% of the acute phase tissue parasite burden. Lack of splenocyte proliferation of vaccinated/infected animals in vitro suggests that protection from challenge infection was not provided by an active T-cell response. TcVac2 vaccine also induced a strong IgG2b>IgG1 antibody response and a moderate level of lymphocyte proliferation in mice. After Challenge infection IgG2b>IgG1 antibody response expanded and elicited a substantial CD8+ T cell response, which is associated with type 1 cytokines (IFN-γ and TNF-α) that resulted in control of acute parasite burden. During chronic phase antibody response persisted, as well as CD8+ T cells, however IL-4/IL-10 cytokines became

dominant in vaccinated mice. Tissue parasitism, inflammation and fibrosis in heart and skeletal muscle of TcVac2-vaccinated chronic mice were undetectable by histological techniques, as compared to non vaccinated/infected animals which exhibited persistent parasite burden and immunopathology in the myocardium. TcVac3 vaccine also elicited a strong IgG2b>IgG1 antibody response and a robust *T. cruzi*-specific CD8+ T cell response with Type-1 cytokine (IFN-γ + TNF-α> IL-4 + IL-10) and cytolytic effector phenotype. TcVac3 induced significantly effector T cells upon challenge infection and provided >92% control of *T. cruzi*. In chronic phase of infection mice exhibited a significant decline (up to 70%) in IFN-γ and CD8+ T cells a predominance of immune-regulatory IL-10/CD4+ T cell, and presented undetectable tissue parasitism, inflammatory infiltrate, and myocardium fibrosis. As a whole the experience with TcVac series vaccines suggests that a protective vaccine elicits a strong antibody response IgG2b>IgG1 and a robust *T. cruzi*-specific CD8+ T cell response with Type-1 cytokine (IFN-γ + TNF-α > IL-4 + IL-10) and cytolytic effector phenotype that even if it does not produce a sterile immunity, it does control parasitemia and tissue parasite burden. Then during the infection Th type 1 is switched for a Th type 2 immune response which is associated to a healing inflammation (Gupta and Garg, 2010).

The TcVac vaccine series are designed for prime/boost doses. They all start with a DNA prime dose and finish with either, DNA, recombinant protein or viral vector boost. This difference may explain the dissimilarities found among treatments, and mainly with TcVac1.

TcVac2 and TcVac3 induce a more effective immune response, however, production and purification of recombinant proteins or viral vectors is quite more expensive than plasmid DNA production and purification. Therefore we decided to test a more efficient way of administration of a DNA-prime/DNA-boost vaccine approach which we found in intradermal electroporation (IDE) vaccine administration, since it has been shown to be very effective in transfecting in-vivo cells with DNA vaccines and induce a high immune response.

IDE promotes Th1 and Th2 type immune responses because on one hand it reversibly permeabilizes the target cells enhancing the uptake and expression of the gene of interest (André, 2008, 2010), and since the site of IDE contains many antigen-presenting cells (APCs), mainly dendritic cells, that can be directly transfected during the vaccination process, and therefore the expressed antigen is presented in association with major histocompatibility complex (MHC) class I molecules, which will subsequently stimulate the synthesis of a pool of antigen-specific cytotoxic T lymphocytes(CTL). Furthermore, epitope display mediated by MHC class 1 is prone to induce both a T helper type 1 (Th1) response in conjunction with the generation CTLs (Dobaño, 2007; Wang, 2012), the most potent cytolytic effector cells. On the other hand, the tissue damage caused by the application of IDE causes inflammation and recruits antigen presenting cells (DCs, macrophages) and lymphocytes to the injection site inducing significant immune responses, including antibody production as well as T-cells. Furthermore, IDE can be repeatedly used in a prime/boost vaccine delivery strategy, since it does not induce undesirable immune responses against the delivery system (reviewed by Fioretti *et al.*, 2010).

Here, we tested TcVac1 vaccine that includes pcDNA3.1-TcG2 and pcDNA3.1-TcG4 mammalian cell expression plasmids, but, in order to maximize its protective capacity, we did not eliminate the adjuvant cytokine genes that can help enhance the response to the antigen-encoding plasmid(s). GM-CSF enhances the antigen-presenting capability of dendritic cells and facilitates B- and T-cell-mediated immunity (Warren, 2000; Lin, 1995), and IL-12 enhance CD8^{+}-T-cell activation and proliferation, and in promoting type 1 cytokine production (Lin, 1995; Pan, 1999; Cox, 1997). Co-administration of cytokine genes with *T. cruzi* antigen encoding plasmids have been associated to an increment in the level of antigen-specific CTL activity and humoral immune responses and provided substantially better protection from *T. Cruzi* (Gupta and Garg, 2010).

In this experiment we expected that the TcVac1 vaccine, delivered through IDE, should induce a stronger immune response than TcVac1 administered intramuscularly. We

would expect an immune response more closely related to the responses found for TcVac2 and TcVac3 immunization protocols.

We found that splenocytes coming from IDE /vaccinated animals proliferated more than the splenocytes derived from IM/vaccinated animals. O.D. (optical density) readings for TcG2 or TcG4 recombinant protein stimulated splenocytes were 0.42, 0.50 for TcG2 and 0.38, 0.39 for TcG4 from IDE and IM vaccinated animals, respectively. During the acute phase of infection (60 dpi) the splenocytes proliferated even further for all vaccinated groups, while the differences kept favouring splenocytes coming from IDE vaccinated mice with O.D. values of 1.2, 1.25 for TcG2 and 0.75, 0.08 for TcG4 from IDE and IM vaccinated animals, respectively. This study demonstrates that animals vaccinated with TcVac1 by IDE develop stronger cell immunity than animals vaccinated with IM vaccine administration. These results are not surprising since it has been previously reported that IDE facilitates keratinocyte and dendritic cell transfection, and the proteins product of plasmid expression, are directed to the MHC type I antigen presentation that conducts to a Th1 Type immune response, mentioned above. Further studies should be conducted to better characterize the cell mediated immune response elicited by TcVac1 vaccine when administered by IDE.

Antibodies levels were evaluated in an ELISA assay where plaques were sensitized with TcG2 and TcG4 recombinant proteins, independently. Antibody titers for these proteins can be observed in figures 2 and 3 Unexpectedly, IM administrated TcVac1 induced very low immunoglobulin levels during the vaccination period. In comparison IDE administration of the vaccine induced relatively high levels of all IgG subtypes. High levels of immunoglobulin G production were expected, since the IDE is associated to some tissue damage that attracts antigen-presenting cells (dendritic cells and macrophages) to the injection site that promote antibody production as previously described. For both treatments (IDE and IM) the ratio IgG2b/IgG1 was >1 which suggests a Th type 1 skewed immune response, which is in accordance with the observations made by Bhatia *et al*. (2008) and Gupta and Garg, 2010; 2013 for the TcVac vaccine series.

Antibody production after experimental infection (60 dpi) displayed significant increments in all infected animals in anti-TcG2 and TcG4 IgG antibodies. However, the largest antibodies surges for all IgG subtypes was produced from the IDE/TcVac1 animal groups, indicating that antigen presenting cells were more active for IDE than for IM vaccinated animals. The ratio IgG2b/IgG1for both vaccine treatments switched to values <1 as compared to vaccinated animals, indicating a switch from a Th1 to a Th2 cell type immune response as was previously reported by Gupta *et al*. (2010, 2013) for the TcVac vaccine series.

10. CONCLUSION

Further investigation is required to complete the study of the full protective immune response of TcVac1 IDE protocol, however, if the immune protective response of TcVac1 IDE matches the TcVac2 and TcVac3 vaccination protocols it would be a more practical approach as a clinical vaccine, since production and purification of plasmid DNA can be done at a fraction of a cost of what recombinant proteins or viral vectors are produces and with less effort (Fioretti *et al*., 2010). Additionally the cold chain needed for proteins and viral vector based vaccines to prevent degradation or inactivation, is not required for plasmid DNA. Therefore, these results are encouraging to perform complementary experiments to fully test the protective capacity of TcVac1 IDE vaccination protocols.

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