2018-01-01

Glycoconjugate-Based Vaccines for Chagas Disease

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GLYCOCONJUGATE-BASED VACCINES FOR CHAGAS DISEASE

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GLYCOCONJUGATE-BASED VACCINES FOR CHAGAS DISEASE

by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

May 2018

Abstract

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*. About 6-8 million people are estimated to be infected worldwide. It is a blood borne pathogen and transmitted to humans by the insect vector (*kissing bug*), blood transfusion, organ transplant, contaminated foods and juices, and by congenital contagion. The chemotherapy is partially effective in chronic phase of the disease, and the drugs cause several side effects. There is no vaccine to prevent or treat Chagas disease. The great majority of experimental vaccines have employed whole parasite extracts, purified or recombinant proteins, synthetic peptides, or DNA; however, most of them provide only partial protection. The goal of this project is to evaluate the effect of an αGal-based neoglycoprotein (NGP) vaccine against ChD in the α1,3-galactosyltransferase-knockout (α1,3-GalT-KO) mice. In our first study, mice were vaccinated with NGP Galα(1,3)Galβ(1,4)GlcNAcβ-BSA (3 atom spacer, Dextra) and *T. cruzi*-challenged three consecutive times. This study showed that parasitemia is lower after a second and third challenge; however, they were not protected after immunosuppression. In our second study, NGP Galα(1,3)Galβ(1,4)GlcNAcα-BSA (KM24b) was combined with LMPLA. This study showed that vaccination with KM24b alone gave much lower survival rate as compared to the previous study using Galα(1,3)Galβ(1,4)GlcNAcβ-BSA. The differences between the two NGPs are: (a) the anomeric configuration of the reducing-end GlcNAc residue (α vs. β), (b) and length of the spacer (3-atom linker in the Galα(1,3)Galβ(1,4)GlcNAcβ-BSA vs. 13-atom linker in the KM24b). In our third study, mice were vaccinated with αGal-containing NGPs with linkers with distinct length (3-atom vs. 14-atom-linker) and different carrier proteins (BSA or HSA). This study showed that carrier protein and the linker length are crucial parameters to be considered in the design of NGPs to be tested as experimental vaccines for ChD. In our fourth study, the commercial NGP Galα(1,3)Galβ(1,4)GlcNAc-NH-HSA (3 atom-linker) was combined with LMPLA. This study showed that vaccination with NGP Galα(1,3)Galβ(1,4)GlcNAc-NH-HSA (3 atom-linker) alone gave higher survival rate (100%) and higher protection than vaccination with NGP Galα(1,3)Galβ(1,4)GlcNAc-NH-HSA (3 atom-linker) combined with LMPLA. In conclusion, the use of LMPLA improved the efficacy of KM24b as preventive experimental vaccine against *T. cruzi* challenge; however, did not improve the efficacy of NGP Galα(1,3)Galβ(1,4)GlcNAc-NH-HSA (3 atom-linker).

Keywords: Anti-α-Gal antibodies; Chagas disease; neoglycoproteins; *Trypanosoma cruzi*; vaccine.

Acknowledgements

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List of Abbreviations

anti-α-Gal Abs	anti-α-galactosyl antibodies
ASP-2	amastigote surface protein 2
BZN	benznidazole
ChD	Chagas disease
dpi	day post-infection
dpim	day post-immunization
DTU	discrete typing unit
Galα3LN-3aL-BSA	neoglycoprotein, Galα(1,3)Galβ(1,4)GlcNAcβ linked to bovine serum albumin through a 3-atom linker/spacer
Galα3LN-14aL-BSA	neoglycoprotein, Galα(1,3)Galβ(1,4)GlcNAcβ linked to bovine serum albumin through a 14-atom linker/spacer
Galα3LN-3aL-HSA	neoglycoprotein, Galα(1,3)Galβ(1,4)GlcNAcβ linked to human serum albumin through a 3-atom linker/spacer
Galα3LN-14aL-HSA	neoglycoprotein, Galα(1,3)Galβ(1,4)GlcNAcβ linked to human serum albumin through a 14-atom linker/spacer
GIPL	glycoinositolphospholipid
GPI	glycosylphosphatidylinositol
GPI-AP	GPI-anchored protein
KM24B or KM24b	neoglycoprotein, Galα(1,3)Galβ(1,4)GlcNAcα linked to bovine serum albumin through a 13-atom linker/spacer
LMPLA	liposomal monophosphoryl lipid A
MASP	mucin-associated surface protein
MHC	major histocompatibility complex
NGP	neoglycoprotein
NFX	nifurtimox
Tc24	*Trypanosoma cruzi* flagellar calcium-binding protein of 24 kDa
TCT	**Tissue culture-derived trypomastigote**
TLR	Toll-like receptor
TS	*trans*-sialidase (a.k.a. TS/gp85 glycoprotein)
TSA-1	trypomastigote surface antigen-1

Introduction

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease (ChD). There are 6-8 million people chronically infected, mostly in South and Central America. It is considered to be the most important parasitic infection in Latin America with serious consequences for public health and national economies (WHO, 2018). It is estimated that there are approximately 300,000 currently infected people in the United States, 5,500 in Canada, 80,000 in Europe, 1,500 in Australia, and 3,000 in Japan (dos Santos Virgilio et al, 2014) (**Figure 1**). *T. cruzi* is transmitted to humans by the insect-vector (triatomine, *kissing bug*), blood transfusion, organ transplant, infected mothers during pregnancy, oral infection by tainted foods and juices, and laboratory accidents (WHO, 2017). The acute phase is recognized only in 1-2% of the infected individuals, characterized by mild symptoms that decline after 4-8 weeks. Most of the chronic individuals are asymptomatic. Each year, about 3% of the patients develop lesions in the heart or GI tract (Pinto Dias, 2006). Chronic Chagas heart disease affects about 30% of the patients; and digestive, neurological or mixed alterations affects about 10% of the patients (WHO, 2017).

The *Trypanosoma cruzi* natural life cycle (**Figure 2**) starts in an animal reservoir, usually mammals, such as wild or domestic animals (e.g., opossums, armadillos, and dogs) and humans. A triatomine bug (female or male), also known as *kissing bug*, serves as the vector. When this infective vector bites the mammalian host reservoir, it takes a blood meal. In the vector, the parasite differentiates from infective trypomastigote form or stage to the epimastigote stage and some, spheromastigote stage. Epimastigotes multiply through binary fission in the insect's midgut and, as nutrients become scarce, move onto the rectal cell wall, where they become infective metacyclic trypomastigote forms. When the vector takes a blood meal from a mammal, it defecates to generate space for the large amount of blood entering the midgut. The metacyclic trypomastigotes, present

in the feces, penetrate the mammalian host through the bite wound or exposed oral or ocular mucosal membranes, and cross a network of proteins in the extracellular matrix to invade host cells. Metacyclic trypomastigote infect most nucleated cells and rapidly transforms into amastigote form (Ley et al, 1988). The intracellular amastigote is released from the parasitophorous vacuole 2-3 h after the infection, then multiplying in the cytoplasm and transforming into infective trypomastigote forms after 3-4 days. Trypomastigotes burst out of the cell to reach the extracellular milieu and from there, the blood stream. Blood stream trypomastigotes infect host cells in different tissues and organs or are ingested by a triatomine bug and the life cycle continues (Teixeira et al, 2012).

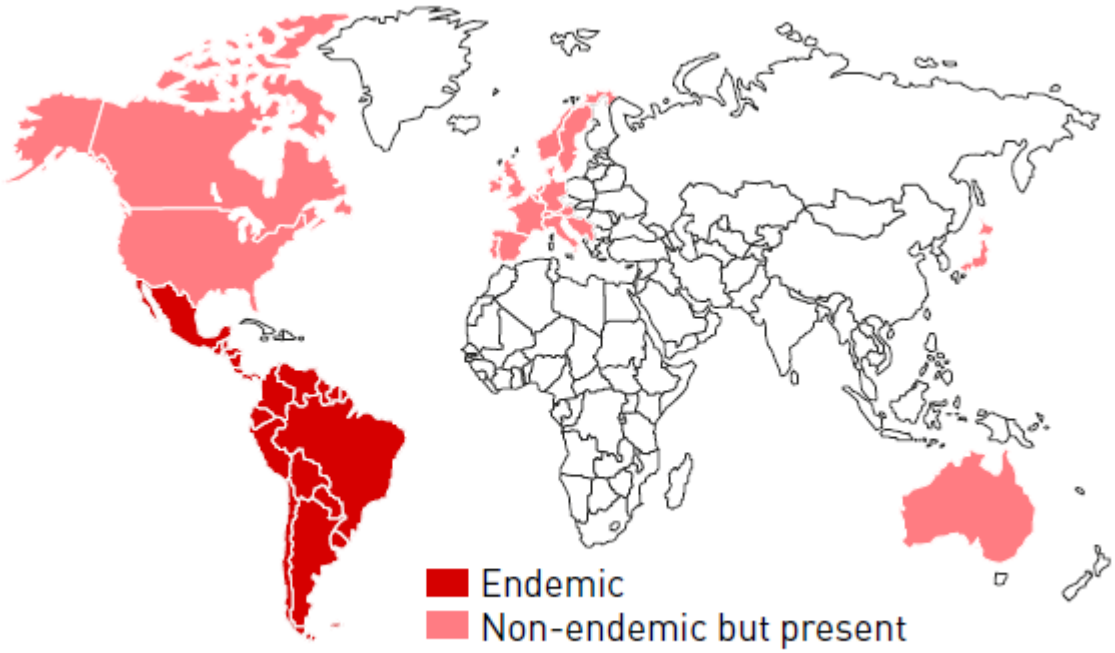

Figure 1. Endemic areas of Chagas disease. Chagas disease is endemic in 21 countries across Latin America, Non-endemic, developed countries are Australia, Canada, Japan, Spain, and the United States. Retrieved on July 14, 2016, from http://www.dndi.org/diseases-projects/chagas.

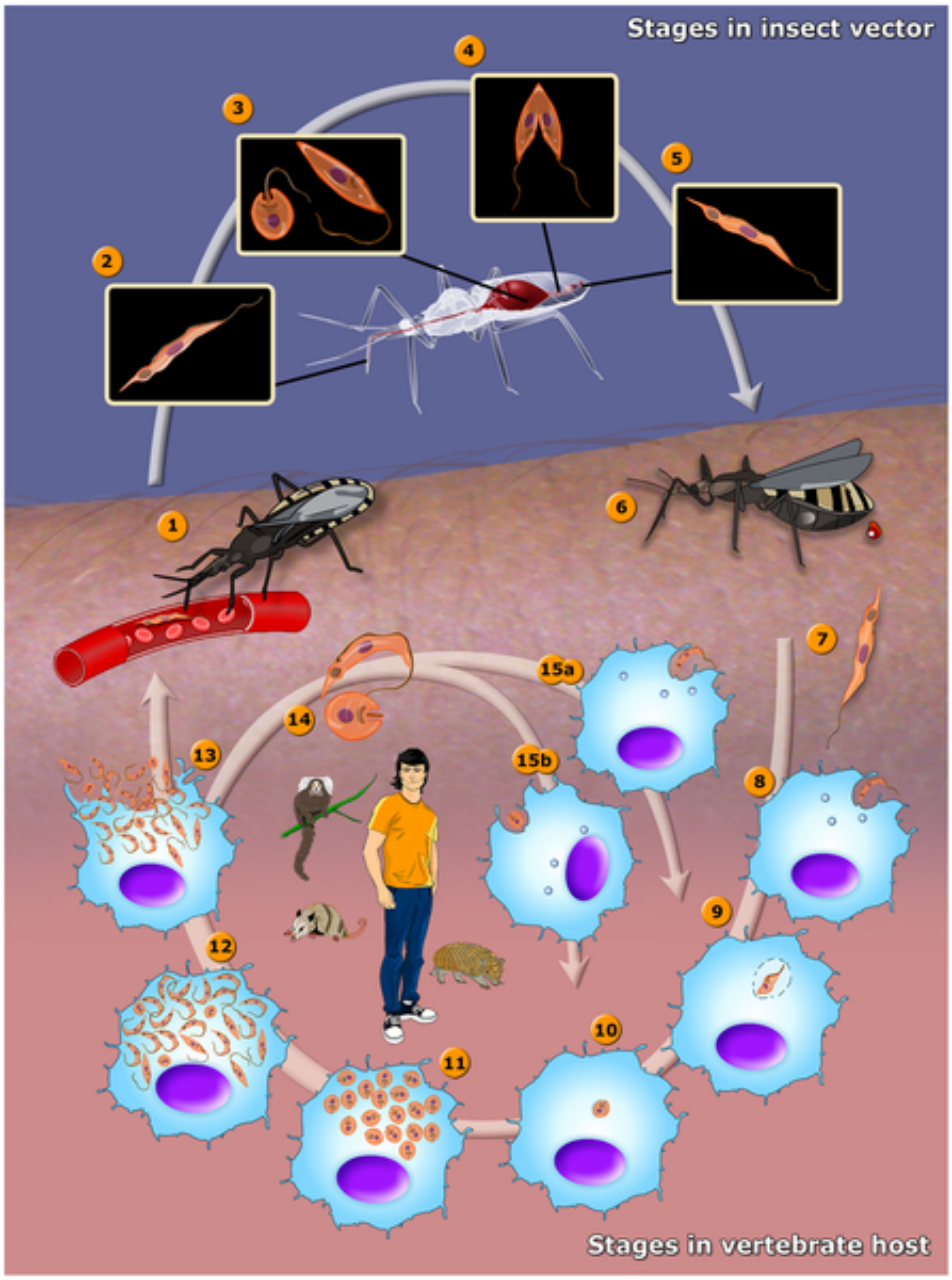

Figure 2. *T. cruzi* natural life cycle. Picture taken from (Teixeira et al, 2012).

ChD can be treated with benznidazole (BZN) or nifurtimox (NFX), which were developed in the 1960s. Both chemotherapeutic drugs are almost 100% effective in curing the disease, if given soon after challenge (Lescure et al, 2010). The standard treatment with BZN is 60 days, whereas with NFX for 90 days, with regular medical visits and tests. Being outdated, these drugs are highly toxic. High rate of adverse effects begins after two weeks of treatment, such as dermatitis with cutaneous eruptions (**Figure 3**), myalgia, arthralgia, lymphadenopathy, polyneuropathy, paresthesia, polyneuritis, and bone marrow disorders. About 9% of patients cease their treatment due to these adverse effects (Urbina, 2015).

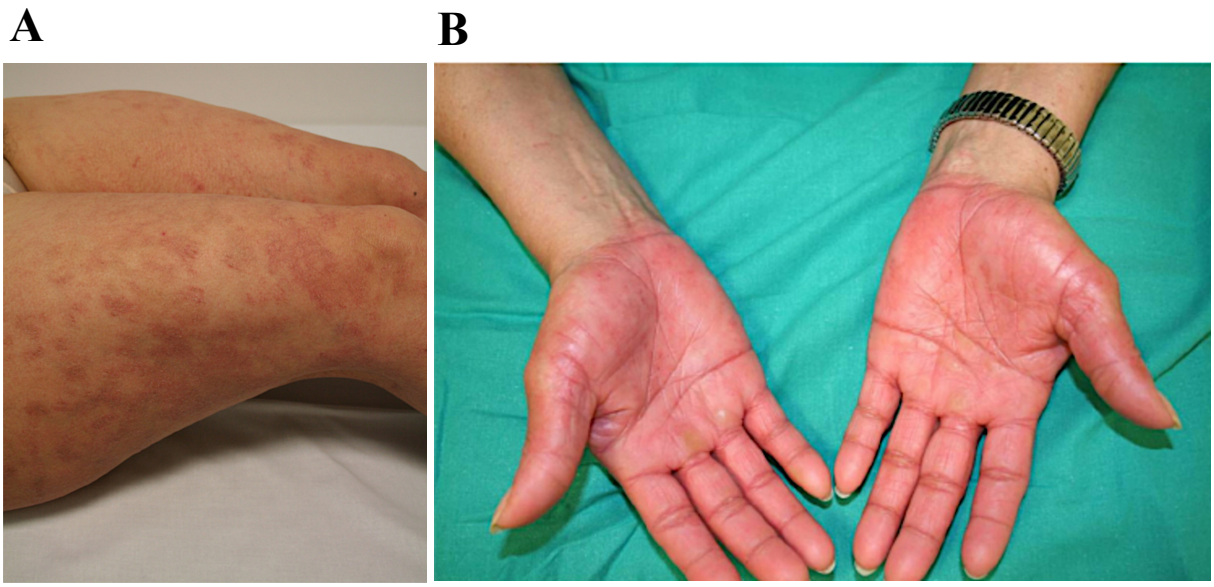

Figure 3. Pruriginous maculopapular rash on limbs (A) and palmar rash (B) of a patient receiving benznidazole. Usually, palmar rash is a concomitant manifestation with micropapular rash and urticaria. Figure taken and modified from (Pinazo et al, 2010).

Currently, there is no preventive or therapeutic human vaccine for ChD. An effective vaccine for ChD could prevent 5.4 million chronic Chagas heart disease, 900,000 megaesophagus and megacolon (Lescure et al, 2010), and about 12,000 deaths annually (Pan American Health Organization). $CD8^+$ T Cell-mediated immunity has been considered as a path for the development

of a human therapeutic vaccine due to its ability to destroy infected cells through the release of perforin, a cytolytic molecule. Newer approaches include the use of *T. cruzi trans*-sialidase (TS), amastigote surface protein 2 (ASP-2), trypomastigote surface antigen-1 (TSA-1), and Tc24 (flagellar calcium-binding protein) as antigens or genes to be delivered through vaccination with DNA plasmid, recombinant adenovirus, or recombinant protein. Results of mouse vaccination with these new approaches provide evidence of high protection; however, perforin-mediated cytotoxic activity may be different across different strains of *T. cruzi* (dos Santos Virgilio et al, 2014). Moreover, it is still not clear how conserved major peptide epitopes form TS, ASP-2, TSA-1 and Tc24 are conserved among the six different parasite genotypes (Discrete Type Units [DTUs] TcI-VI), strains and isolates (Zingales et al, 2012).

T. cruzi is protected by a highly complex coat, which serves as a physical and immunological barrier against host defenses. This is a critical obstacle for the development of an effective vaccine. Glycosylphosphatidylinositol (GPI)-anchored glycoproteins constitute the bulk of this coating. Thousands of genes encode for the mucins, *trans*-sialidases (TSs), and mucin-associated surface proteins (MASPs), which are the major glycoproteins on the parasite surface (Acosta-Serrano et al, 2007; El-Sayed et al, 2005). Proteomics show that hundreds of genes simultaneously and differentially are expressed on infective trypomastigotes. Lytic, protective anti-α-Gal Abs produced during the acute and chronic stages of ChD recognize the αGal residues expressed in the GPI-mucins (Almeida et al, 1994; Almeida et al, 1991; Gazzinelli et al, 1991; Travassos & Almeida, 1993). These lytic anti-α-Gal Abs kill infective trypomastigote controlling the parasitemia in both acute and chronic phases or stages of the disease (Almeida et al, 1994; Almeida et al, 1991; Gazzinelli et al, 1991; Travassos & Almeida, 1993). In contrast to all other mammals, humans and Old-World primates (e.g., chimpanzees, baboons, rhesus monkeys, etc.)

do not express nonreducing, terminal α-Gal residues in their cells or tissues, thus they are not tolerant to these epitopes (Galili, 1993; Galili, 2017; Galili & Swanson, 1991).

Although *T. cruzi* surface glycoproteins contain highly immunogenic epitopes such as α-Gal residues, until recently the development of glycan-based vaccines for ChD have been hampered by technical difficulties in purifying, characterizing, and synthetizing these glycan epitopes, and by the lack of appropriate animal models that resemble or mimic the human immune response. However, recent developments could overcome some these difficulties for the use of glycoconjugates as vaccine candidates for ChD. In our first study, the NGP Galα(1,3)Galβ(1,4)GlcNAcβ covalently linked to bovine serum albumin (BSA) (Galα3LN-3aL-BSA) was used to vaccinate α1,3-galactosyltransferase-knockout (α1,3-GalT-KO) mice, which in contrast to wild-type mice mimic humans in that it does not express terminal αGal epitopes in their cells (Tearle et al, 1996; Thall et al, 1996). These mice showed 100% survival after three consecutive challenges with 10^4, 10^5, and 10^6 of infective trypomastigotes. However, after immunosuppression, we only had 25% survival, indicating therefore that the vaccine did not provide full protection. More recently, the neoglycoprotein Galα(1,3)Galβ(1,4)GlcNAcα-BSA (KM24B) was synthesized and its immunogenicity evaluated in α1,3-GalT-KO mice (Schocker et al, 2016). Immunized mice showed 22-fold higher antibody response to KM24B as compared with pre-immunization levels, whereas mice immunized with BSA alone showed minimal antibody response before and after immunization (Schocker et al, 2016). Thus, KM24B has been proposed as a potential experimental for Chagas disease.

For our second study, we proposed to add an adjuvant to the KM24B (Schocker et al, 2016) to improve the potency of these α-Gal-based vaccine candidate. Adjuvants enhance the efficacy of antigens, enable the use of lower vaccine doses, increase global supply, enable a more rapid

immune response, induce greater magnitude of Ab response and target T cell responses (Reed et al, 2013). Adjuvants are recognized by Toll-like receptors (TLRs) and activate the Th1 response to activate the natural killer (NK) cells to destroy the challenged cells. TLR agonists are potent activators of the innate immune response through activating dendritic cell maturation and inflammatory cytokine secretion by innate immune cells, and they consequently induce an adaptive immunity when co-administered with a foreign antigen (Zhang & Matlashewski, 2008), in this case with the α-containing NGP. We will specifically test liposomal monophosphoryl-lipid A (LMPLA).

LMPLA is a low-toxicity adjuvant derived from the lipopolysaccharide (LPS) of *Salmonella minnesota*. It is being consider as an adjuvant for several human vaccines (Johnson et al, 1956). It has been used in mice and currently undergoing human clinical trials; and experience to date has shown that it is safe, well tolerated, and able to provide a heightened immune response to co-administered antigens (Ulrich & Myers, 1995). LMPLA is recognized by TLR4, located at the cell surface. It leads to signaling through Toll-interleukin 1 receptor domain-containing adapter inducing interferon-beta (TRIF), which has been suggested to be caused by the active suppression, rather than passive loss, of pro-inflammatory activity of this LPS derivative (Mata-Haro et al, 2007). This adjuvant is promising candidate for the development of preventive and therapeutic vaccines against ChD.

Our second study, mice vaccinated with KM24B + LMPLA showed 100% survival and KM24B showed 25% survival after one challenges with 10^4 of infective trypomastigotes. Surprisingly, in our first study we used Galα(1,3)Galβ(1,4)GlcNAcα-BSA from Dextra Laboratories, which provided 100% survival after a third challenge and in our second study Galα(1,3)Galβ(1,4)GlcNAcα-BSA (KM24B) only provided 25% survival after one challenge. For

our third study, we decided to evaluate the difference between these two vaccines considering that both had the same trisaccharide and same carrier protein but different linkers.

For our third study, we investigated whether structural dissimilarities could lead to such considerably diverse immune responses and protection against *T. cruzi*. Mice were vaccinated intraperitoneal (i.p.) with NGP Galα(1,3)Galβ(1,4)GlcNAc-BSA (3 atom linker) a/k/a Galα3LN-3aL-BSA, NGP Galα(1,3)Galβ(1,4)GlcNAc-NH-HSA (3 atom linker) a/k/a Galα3LN-3aL-NH-HSA, NGP Galα(1,3)Galβ(1,4)GlcNAc-BSA (14 atom linker) a/k/a Galα3LN-14aL-BSA, NGP Galα(1,3)Galβ(1,4)GlcNAc-HSA (14 atom linker) a/k/a Galα3LN-14aL-HSA from Dextra; and PBS, BSA and HSA as controls. Only Galα3LN-3aL-NH-HSA showed 100% survival after one challenge with 10^5 of infective trypomastigotes.

For our fourth study, we investigated whether Galα3LN-3aL-NH-HSA incorporating LMPLA (Matyas et al, 2003) could have a different immune responses and protection against *T. cruzi*. Mice were vaccinated with Galα3LN-3aL-NH-HSA from Dextra, Galα3LN-3aL-NH-HSA combined with LMPLA, and HSA + LMPLA (control). Only Galα3LN-3aL-NH-HSA showed 100% survival after one challenge with 10^5 of infective trypomastigotes.

Throughout the years, several attempts have been made to develop carbohydrate-based vaccines against pathogens. One of the first attempts to be carried was through the use of intradermal injections in humans of type-specific polysaccharides from *Pneumococcus,* which showed to induce circulating antibodies against specific each bacterium type (Francis & Tillett, 1930). This was the basis for the first effective vaccine for pneumococcal pneumonia. Therefore, pneumococcal capsular polysaccharides have been proven as effective vaccines against pneumococcal pneumonia providing a long lasting immunity (Heidelberger et al, 1950). Despite this important development, chemotherapeutics and antibiotics took priority, and there was no

interest in developing vaccines based on polysaccharides (Vliegenthart, 2006). In 1983, vaccines based on polysaccharides regained popularity due to the resistance towards antibiotics. However, polysaccharides showed to be poorly immunogenic in children less than 2 years old, in elderly, and in immune-compromised patients (Vliegenthart, 2006). Polysaccharides are known to be independent of T cells; however, some zwitterionic capsular polysaccharides can activate CD4+ T cells. Polysaccharides alone, without conjugation to a carrier-protein, stimulate B-cells to produce IgM antibodies and, thus, no long-term memory protection. On the other hand, glycoproteins are T-cell dependent. It has been reported that carbohydrates conjugated to a protein, induce and enhance immunogenicity to provide a long-lasting protection (Avery & Goebel, 1931).

Glycoconjugates are useful for the development of treatments, vaccines and diagnosis for humans and animals. Glycoconjugates vaccines are a promising strategy to combine the antigenic feature of capsular polysaccharides with the induction of T cell help based on carrier protein derived peptide presentation by MHC-II molecules on antigen presenting cells. Glycoconjugate vaccines promote a protective immune response based on the production of high affinity glycan-specific antibodies as well as the development of B and T cell memory (Johannssen & Lepenies, 2015). There are a number of diseases with no available vaccines to prevent them.

Malaria, toxoplasmosis, leishmaniasis, and hook-worm infections (schistosomiasis) are caused by protozoan parasites, affect millions of people worldwide and kill many thousands of people annually. There is no vaccine to prevent to treat these protozoan infections (Hotez et al, 2016). Malaria is caused mainly by *Plasmodium falciparum and P. vivax*. Plasmodial glycosylphosphatidylinositol (GPI) anchors are of major interest due to their capacity to activate Toll-like receptors and induce signal transduction (Gowda, 2007), as well inducing specific antibodies. NH_2CH_2-CH_2PO_4(Manα1-2)6(Manα1-2Manα1-6Manα1-4)Glc$NH_2\alpha$1-6*myo*-inositol-

1,2 cyclic phosphate is a synthetic GPI-anchor-related compound and was conjugated to ovalbumin or keyhole limpet hemocyanin (KLH). Mice vaccinated with these GPI conjugates and showed a significant protection against malarial acidosis, pulmonary edema, cerebral syndrome, and mortality (Schofield et al, 2002). Thus, synthetic GPI-based neoglycoproteins have been proposed as anti-malaria toxin vaccine candidates (Franca et al, 2017; Kamena et al, 2008; Naik et al, 2000; Schofield et al, 2002).

Toxoplasmosis in humans is caused by *Toxoplasma gondii*. GPIs are also of major interest in this parasite, since it abundantly expresses these glycoconjugates on its cell surface, including ($NH_2CH_2CH_2PO_4$)Manα1-2Manα1-6(GalNAcβ1-4)Manα1-4GlcNH2α1-*myo*-inositol-PO_4-lipid and the novel GPI-related compound (NH_2CH_2-CH_2PO_4)Manα1-2Manα1-6(Glcα1-4GalNAcβ1-4)Manα1-4GlcNH_2α1-*myo*-inositol-$P0_4$-lipid (Striepen et al, 1997). Both compounds occur with and without terminal ethanolaminephosphate (EtNP) group. GPIs carrying the Glcα1-4Gal-NAcβ1-4 side chains are immunogenic to humans (Striepen et al, 1997). That study showed that *T. gondii* GPIs are bioactive compounds that enhance production of TNF-α during toxoplasmal pathogenesis; therefore, GPIs could be suitable to prevent infection. In 2012, C-terminal 19-kDa merozoite surface protein-1 (MSP1-19) was conjugated to *Pichia pastoris*-secreted cholera toxin B subunit (PpCTB) inducing high protection against a lethal parasite infection. It is believed that this high protection was due to the antigen linked to the extended branches of the oligosaccharide (Miyata et al, 2012).

Leishmaniasis is caused by various species of *Leishmania*. These parasites have a very complex cell surface of glycoconjugates (McConville & Ferguson, 1993). Several attempts with glycan vaccines have been done searching for protection against leishmaniasis. Purified lipophosphoglycan (LPG) had shown to have a protective effect on infected mice with *Leishmania*

major (McConville et al, 1987; Moll et al, 1989; Russell & Alexander, 1988). Also, a synthetic lipophosphosaccharide, namely the tetrasaccharide cap (Manα1-2Manα1-2(Galβ1-4)Man-O(CH_2)6NH–CO–CH_2-S-CH_2CO-NH), was conjugated to KLH. In another attempt, the tetrasaccharide was linked to a spacer and then conjugated to the immunostimulator tripalmitoyl-S-glycerylcysteine. More recently, the neoglycoprotein Galα(1,6)Galβ-BSA (NGP5B), which is an α-Gal-terminating vaccine candidate based on Type-II GIPL-2 of *L. major* (McConville & Ferguson, 1993), in the presence or not of CpG adjuvant, showed to induce partial but significant protection against *L. major* infection (Iniguez et al, 2017). When incubated with luciferase-expressing *L. major* metacyclic promastigotes, sera from mice vaccinated with NGP5B caused complement-independent lysis in 60% of the parasites, whereas mice vaccinated with NGP5B+CpG caused lower (40%) parasite lysis. However, when complement was active, there was no significant parasite lysis, indicating that active complement inhibits parasite lysis (Iniguez et al, 2017). This α-Gal-based vaccine holds a great potential for prevention of cutaneous leishmaniasis.

The parasitic flatworm of *Schistosoma* is known as blood-flukes and it causes schistosomiasis, which is transmitted by contaminated water with fecal material and urine. Mainly, the urinary tract and intestines are infected. Currently, there is no vaccine. However, Bilhvax3 is a candidate vaccine currently in Phase III clinical trial, in association with praziquantel, as a preventative vaccine for *Schistosoma haematobium* infection (Clinical Trials.gov; https://clinicaltrials.gov/ct2/show/NCT00870649). This vaccine targets the antigen *Sh*28-GST of *Schistosoma haematobium*, which is a 28-kDa recombinant glutathione-S- transferase formulated with the adjuvant Alum (PD-VAC) (Ricciardi & Ndao, 2015). In 2013, a study isolated a glycoprotein extract (AWBE) from whole membrane fraction of adult worms with which mice

were immunized showing IgG response and 43% protection against infection (Sulbaran et al, 2013). In 2014, a study generated Chinese hamster ovary (CHO) Lec8 cell lines expressing poly-LacdiNAc (GalNAcβ1,4GlcNAc, LDN) (L8-GT) and poly-fucosylated LacdiNAc (GalNAcβ1,4(Fucα1-3)GlcNAc; LDNF) (L8-GTFT). Mice were immunized with these cells. L8-GTFT cells induced a sustained booster response generating antibodies to the glycan of *S. mansoni* (Prasanphanich et al, 2014). In a current study, microarray was used to follow anti-glycan IgG and IgM responses in infected macaques, showing that these animals became resistant to reinfection while eliminating existing worms. It is still unknown if anti-glycan antibodies are associated with the elimination of the worm; however, it is known that glycan fucosylation is important to trigger the host immune response (Yang et al, 2017).

Glycoconjugates have been used to developed vaccines, treatments and medical diagnosis. Vaccination against parasitic diseases should remain the ultimate goal in the fight against parasites. An effective vaccine could prevent organ damage and even death. In this current thesis, we have evaluated different formulations and immunization conditions of α-Gal-based glycoconjugate vaccines against experimental Chagas disease. Here, we will present three independent studies evaluating these vaccines.

Hypotheses

Hypothesis 1: We hypothesize that we can improve the efficacy of the αGal-containing vaccine by incorporating the adjuvant liposomal monophosphoryl lipid A in the vaccine formulation.

Hypothesis 2: We hypothesize that the length of the linker between αGal-NGP and the carrier protein (BSA or HSA) might affect the vaccine efficacy.

Specific Aims

Specific Aim 1: Analyze the effect of LMPLA adjuvant in the efficacy of Galα(1,3)Galβ(1,4)GlcNAc covalently linked to BSA or HSA as vaccine against *T. cruzi* infection in the α1,3-GalT-KO mouse model of Chagas disease.

Specific Aim 2: Analyze the effect of anomeric configuration of the GlcNAc (α or β) and the length of the linker between Galα(1,3)Galβ(1,4)GlcNAc and the carrier protein (BSA or HSA) in the effectiveness of the vaccine against *T. cruzi* infection.

Innovation

This project is highly innovative regarding different conceptual and technical aspects:

The epitope/glycotope: Galα(1,3)Galβ(1,4)GlcNAcα was identified by our research group as a conserved epitope in *T. cruzi* strains, and here we validated as effective vaccine target for Chagas disease. **The linker and carrier protein:** We performed experiments to prove the importance for vaccine efficacy of the length of the linker/spacer between the glycan and the carrier protein (BSA or HSA), and the latter itself. **The animal model:** The α1,3-GalT-KO mouse model was validated to mimic the human humoral immune response against α-Gal glycotopes. We have proved that these animals are not tolerant to these glycotopes and, therefore, are able to produce lytic, protective anti-α-Gal antibodies. **The vaccine:** Here, we have proved that an αGal-based neoglycoprotein vaccine, in the presence or not of an adjuvant (MPLA), can effectively protect against experimental *T. cruzi* infection.

First Study:

Evaluation of NGP Galα3LN-3aL-BSA as Vaccine Candidate

First Study: Evaluation of NGP Galα3LN-3aL-BSA as Vaccine Candidate

In our second study, we evaluated the efficacy of NGP Galα(1,3)Galβ(1,4)GlcNAcβ conjugated to BSA (3 atom spacer, catalog number NGP0334, Dextra Laboratories, Reading, UK) as a potential experimental Chagas disease vaccine in the α1,3-GalT-KO mouse model. We also evaluated whether these mice were protected against *T. cruzi* after a second and third challenge, and after immunosuppression.

Materials and Methods

Ethics Statement

Animal procedures were performed according to NIH guidelines and the appropriate protocol (A-201211-1) approved by UTEP's Institutional Animal Care and Use Committee (IACUC). This ethics statement applies to all animal studies performed in this project.

Mice

C57BL/6 α1,3-Galactosyltransferase-knockout (α1,3GalT-KO) mice (Tearle et al, 1996; Thall et al, 1996), were kindly donated by Prof. Peter J. Cowan, St. Vincent's Hospital Melbourne and University of Melbourne, Australia. Animals were bred by Brenda G. Zepeda and maintained under biosafety level 2 (BSL-2), pathogen-free conditions at the Laboratory Animal Resources Center (LARC) at UTEP. Six to eleven-week old female α1,3GalT-KO mice were used for all experiments.

Parasites and Mammalian Host Cells

Tissue culture-derived *T. cruzi* trypomastigotes (TCTs), Y strain, were grown in LLC-MK2 cells at 37°C humidified with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) medium, supplemented with 10% heat-inactivated fetal bovine serum (hi-FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA) and 1% penicillin-streptomycin (Corning, Penicillin Streptomycin Solution, 100X).

Tissue culture-derived *T. cruzi* trypomastigotes (TCTs) CL Brener strain expressing red-shifted luciferase (CL-B_{Luc}) (Lewis et al, 2015), kindly donated by Dr. Jair Siqueira-Neto (University of California, San Diego in La Jolla, California) and Prof. John Kelly (London School of Hygiene & Tropical Medicine in Bloomsbury, London), were grown in LLC-MK2 cells at 37°C, 5%-CO_2 humidified atmosphere, in DMEM medium supplemented with 10% hi-FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Corning, Penicillin Streptomycin Solution, 100X).

Immunizations

Female α1,3-GalT-KO (C57BL/6) (Tearle et al, 1996; Thall et al, 1996) mice at 6 to 10 weeks old were used for immunization against *T. cruzi*. The NGP Galα(1,3)Galβ(1,4)GlcNAcα covalently conjugated to BSA (Galα3LN-3aL-BSA) was purchased from Dextra Laboratories (formerly, V-Labs, Inc). Three groups of α1,3-GalT-KO mice (n=6 per group) were used. The groups were Galα3LN-3aL-BSA, PBS (control), and BSA (control). Immunizations were performed with 100 µl per dose and 4 immunizations at one-week intervals. Prime was given via subcutaneous (s.c.); boost 1 to 3 were given via intraperitoneal (i.p.). Groups were vaccinated with 10 µg Galα3LN-3aL-BSA per mouse; 10 µg per mouse of PBS; and 10 µg BSA per mouse.

Detection of Antibodies by Chemiluminescent Enzyme-linked Immunosorbent Assay (CL-ELISA)

To assess the humoral immune response to the three vaccinated groups, levels of specific anti-α-Gal antibody titers were determined by CL-ELISA (Almeida et al, 1997). MaxiSorp Nunc polystyrene microplates, donkey biotinylated anti-mouse, NeutrAvidin-HRP, and SuperSignal Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. Blood was collected via facial vein of each mouse on the same day, but prior each immunization and one week after last immunization. Serum was separated from every blood collection by centrifugation (2,500 x *g*, 15 min, at room temperature (RT). All serum samples were analyzed by CL-ELISA prior to immunization (naïve) and during the immunizations. Microplates were coated with 125 ng/well of Galα3LN-3aL-BSA, PBS, and BSA in CBC buffer and incubated overnight at 4˚C. Microplates were blocked with 200 μL 1% BSA in PBS or 5% skim milk in PBS for 1 h at 37ºC. Microplates were incubated with 50 μL mouse sera from vaccinated or control group at 1:100 dilution for 1 h at 37ºC. Donkey biotinylated anti-mouse (code number 715-065-150, Lot number 68134, Jackson ImmunoResearch) was used at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP (product number 31030, lot number NL180713, Thermo Fisher Scientific) was used at 1:5000 dilution in 1% BSA/PBS-T. Microplates were washed 3x between steps with PBS-T, except before blocking. The reaction was developed with SuperSignal Pico Chemiluminescent Substrate (product number 37069, Lot number RD232185, Thermo Fisher Scientific) at a 1:1:8 ratio in 50 mM carbonate-bicarbonate CBC buffer, pH 9.6, 0.1% BSA. Luminescence in relative luminescence units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

Parasite Challenge

Mice were inoculated 1 week after boost 3 with $1x10^4$ Y-strain trypomastigotes via intraperitoneal (i.p.), in 100 µL PBS. On 31 dpi, mice were inoculated for the second time with 10^5 Y-strain trypomastigotes. On 94 dpi, mice were inoculated for the third time with 10^6 Y-strain trypomastigotes.

Parasitemia

For first challenge, parasitemia was from 3 to 13 dpi, 17 dpi, 20 dpi, 23 dpi, 26 dpi; for second challenge, from 31 to 50 dpi; and for third challenge, from 94 to 112 dpi. 5 µl of blood was collected from tail vein of each mouse and trypomastigotes were quantified using a light microscope (Leica DMI6000 B), using the following formula:

$$\textbf{Total of parasites in 5 } \mu\textbf{l} = \frac{\left(\frac{\text{Number of parasites in 50 fields}}{50} \times \text{Area of coverslip (22 mm x 22 mm)}\right)}{\text{Area of microscope field } (\pi)(r^2)}$$

Total of parasites in 1 ml = Total of parasites in 5 μl x 200

Immunosuppression

To suppress the host immune system (Bustamante et al, 2014), we used cyclophosphamide (Sigma-Aldrich), diluted with PBS to 40 mg/ml. Challenged mice were immunosuppressed according to their weight with cyclophosphamide/PBS (200 mg/kg/day) intraperitoneally (i.p.) at three-day intervals for a total of three doses on 126, 129, and 132 dpi.

Weight

To detect any sign of toxicity in the animals, post-immunization or post-challenge, weight was measured from 0 to 147 dpi. Weight change was normalized using the mouse weight before challenge.

Results

This study was designed to evaluate whether C57BL/6 α1,3-Gal-KO mice were protected against *T. cruzi* after a second challenge; however, based on survival, we decided to challenge them for a third time and immunosuppress them. This study was designed to evaluate the NGP Galα(1,3)Galβ(1,4)GlcNAcα covalently conjugated to BSA (Galα3LN-3aL-BSA) in C57BL/6 α1,3-GalT-KO mice. For first study, experiment history (**Table 1**) and timelines (**Figure 4**) are provided.

TABLE 1. History of first study.

Procedure/Event	Day Post-Immunization	Day Post-Infection
Collected blood and Prime	0	-28
Collected blood and Boost 1	7	-21
Collected blood and Boost 2	14	-14
Collected blood and Boost 3	21	-7
1st Challenge with Y-strain 10^4 TCTs	28	0
Parasitemia	31- 41	3 -13
Parasitemia	45, 48, 51, 54	17, 20, 23, 26
2nd Challenge with Y-strain 10^5 TCTs	59	31
Parasitemia	61 - 63	33 -35
Parasitemia	65 - 74	37 - 46
Parasitemia	76, 78	48, 50
3rd Challenge with Y-strain 10^6 TCTs	122	94
Parasitemia	125 - 140	97 - 112
1st Immunosuppression	154	126
2nd Immunosuppression	157	129
3rd Immunosuppression	160	132
Endpoint	175	147

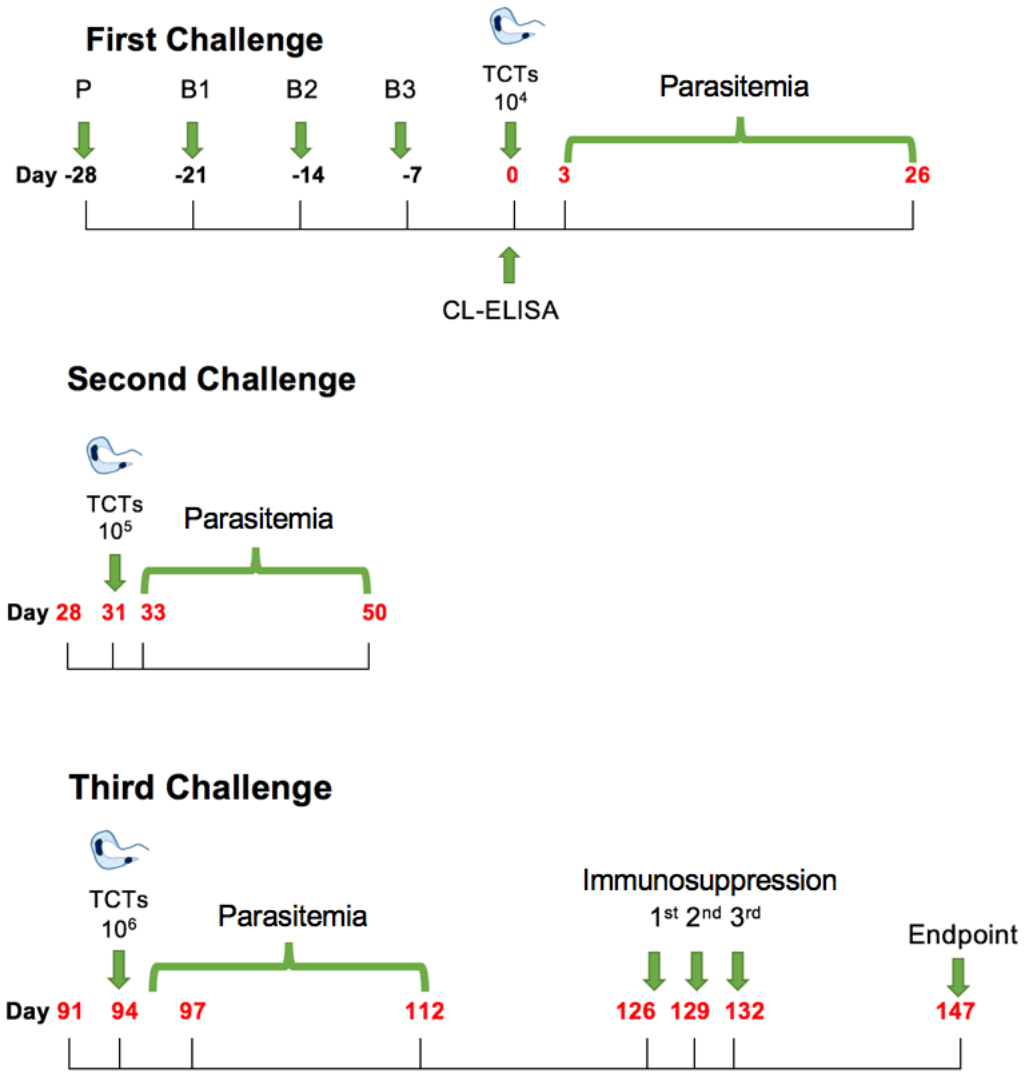

Figure 4. First study's timelines. Four immunizations with Galα3LN-3aL-BSA, BSA, or HSA were given at one-week interval. First challenged day became 0 dpi. Parasitemia after each challenge. Immunosuppression after third challenge on 126 dpi, 129 dpi and 132 dpi. Endpoint on 147 dpi. Vaccinations were prepared and administered by Brenda G. Zepeda (unpublished data).

Female C57BL/6 α1,3-GalT-KO mice groups (n=6) were vaccinated. Prime was performed via s.c. and all three boosts via i.p. with 10 μg of Galα3LN-3aL-BSA per mouse, 10 μg of BSA per mouse, or 10 μg PBS per mouse. Four immunizations at one-week intervals were given.

To determine the production of IgG antibodies levels before challenge, sera was collected via facial vein of each mouse on the same day, but prior each immunization and one week after last immunization. Sera was analyzed with CL-ELISA blocked with PBS-1% BSA (**Figure 5A**). To remove the background provided by BSA, we repeated the CL-ELISA blocking free sites on the microplates with PBS-5% skim milk (Carnation Instant Nonfat Dry Milk, Nestlé) (**Figure 5B**). We were successful removing the BSA background. As expected, mice vaccinated with Galα3LN-3aL-BSA showed high levels of specific anti-α-Gal Abs.

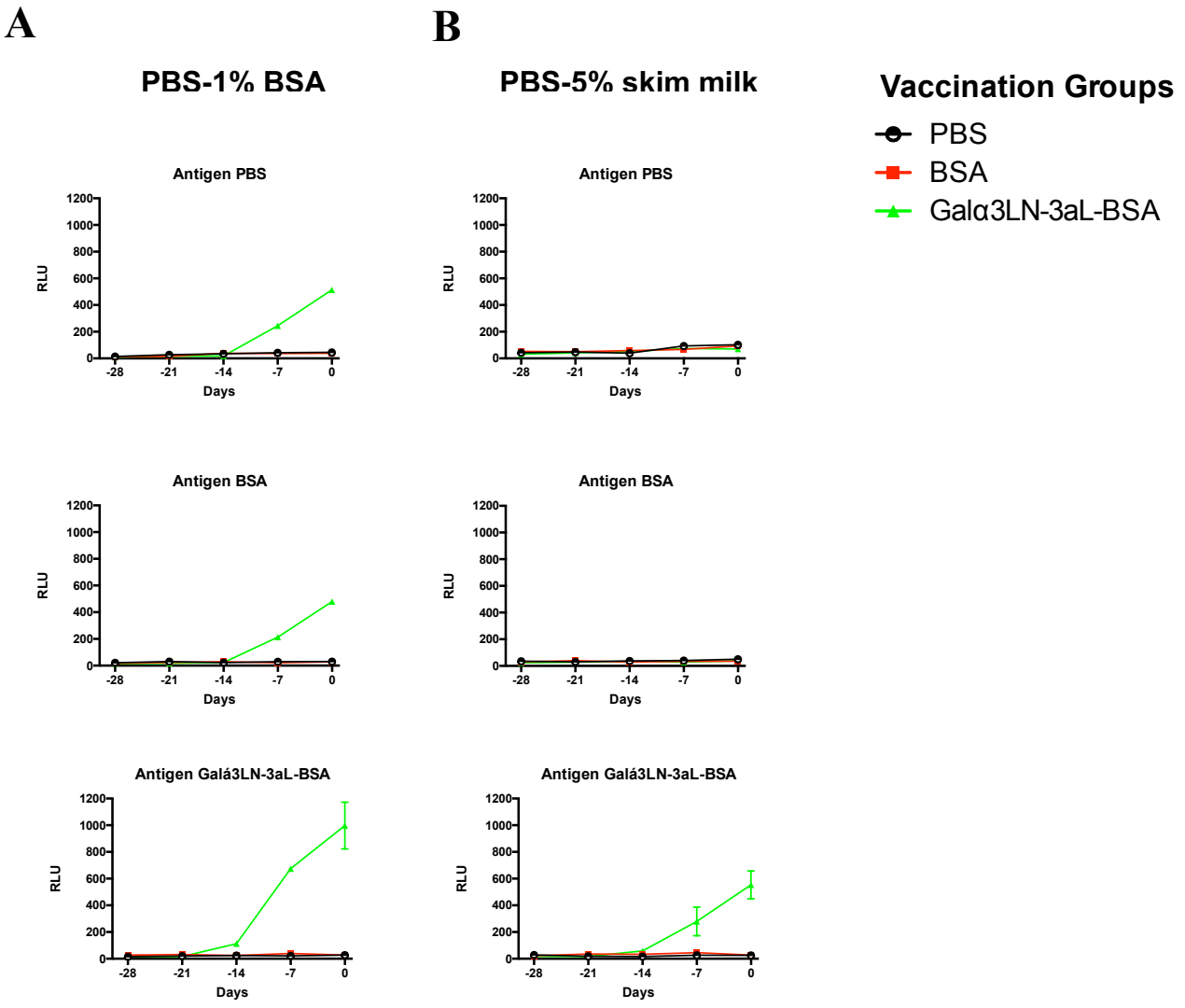

Figure 5. CL-ELISA blocked with PBS-1% BSA (A) and PBS-5% skim milk (B). Levels of anti-α-Gal Abs using Galα3LN-3aL-BSA, BSA, or PBS antigens. Data provided and graphed by Brenda G. Zepeda (unpublished data).

To determine whether these mice were protected against *T. cruzi*, we challenged the animals one week after the last immunization with 10^4 infective trypomastigotes (Y strain). Five microliters of blood via tail vein of each mice was collected individually to quantify parasitemia 3 to 13, 17, 20, 23, and 26 dpi. Animals vaccinated with Galα3LN-3aL-BSA showed lower parasitemia compared to the other groups. However, all animal groups showed 100% survival. On 31 dpi, we performed a second challenge with 10^5 infective TCTs (Y strain). As expected, all groups had lower parasitemia because they had previously encountered the parasites; however, we did not expect to continue having 100% survival for all three groups. Therefore, we did a third challenge on 94 dpi with 10^6 infective trypomastigotes (Y strain). Parasitemia levels continued low (**Figure 6**) and survival continued at 100% for all groups.

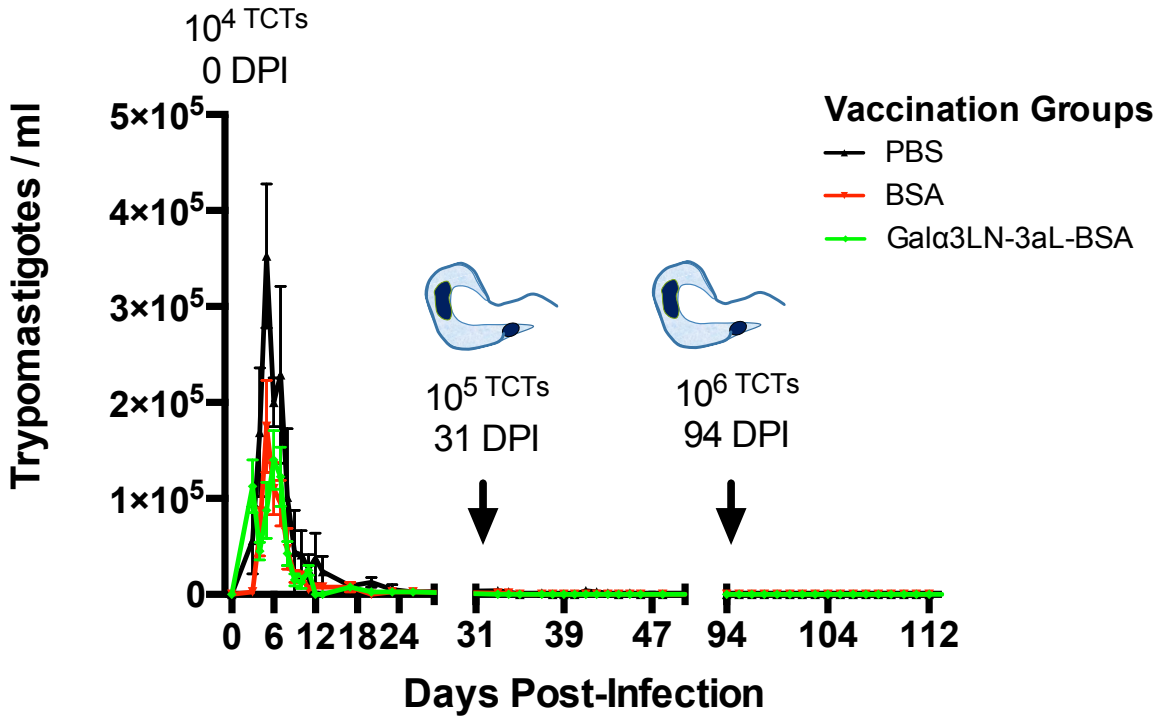

Figure 6. Parasitemia. Quantification of trypomastigotes per milliliter after first, second and third challenge. Data provided and graphed by Brenda G. Zepeda (unpublished data).

Then we decided to immunosuppress the mice to reduce their immune system on 126, 129, and 132 dpi. After immunosuppression, their weight decreased significantly (**Figure 7**). Unexpectedly, challenged animals vaccinated with BSA (control) showed 87.5% survival; PBS (control) showed 75% survival; and Galα3LN-3aL-BSA showed 25% survival (**Figure 8**).

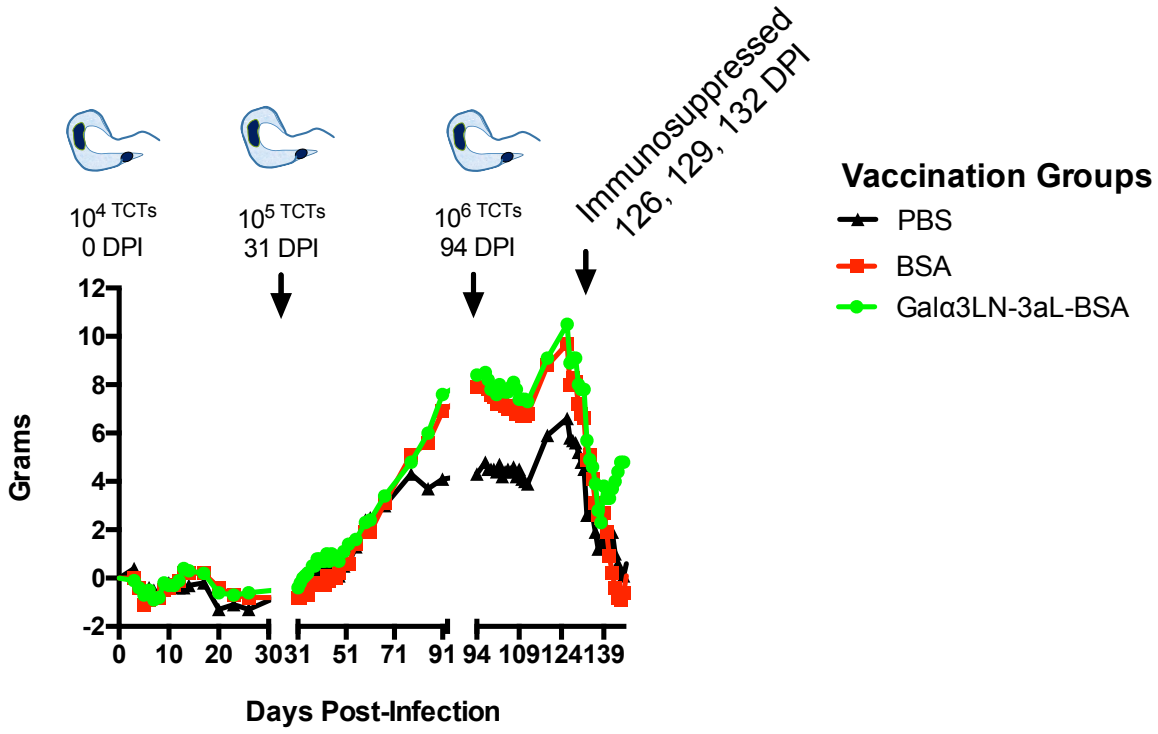

Figure 7. Animal weight post-infection. Animal weight was measured at 0 to 147 dpi. Data provided and graphed by Brenda G. Zepeda (unpublished data).

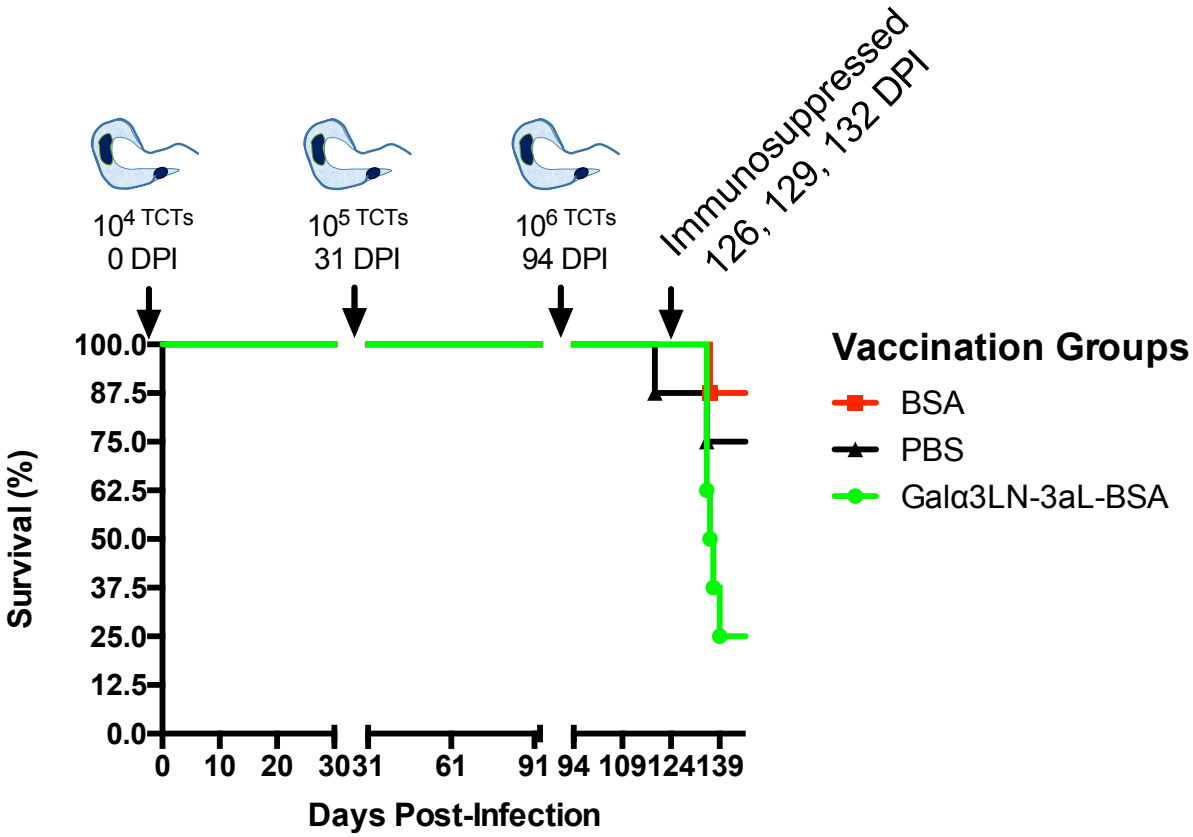

Figure 8. Survival of mice challenged with *T. cruzi* TCTs (Y strain). 87.5% BSA (control), 75% PBS (control); and 25% Galα3LN-3aL-BSA. Data provided and graphed by Brenda G. Zepeda (unpublished data).

This study showed that vaccination with Galα3LN-3aL-BSA from Dextra (formerly, V-Labs, Inc) had lower parasitemia after the first challenge; and after a second and third challenge, parasitemia levels were low for all three groups. Therefore, this study shows recognition of the parasite by the immune system after a sequence of challenges. After immunosuppression, Galα3LN-3aL-BSA showed the lowest survival: therefore, mice vaccinated with Galα3LN-3aL-BSA were not protected from *T. cruzi*.

Second Study:

Evaluation of KM24b as Vaccine Candidate

Second Study: Evaluation of KM24b as Vaccine Candidate

In our second study, we evaluated the efficacy of the synthetic NGP Galα(1,3)Galβ(1,4)GlcNAcα conjugated to BSA (KM24b) (Schocker et al, 2016) as a potential experimental Chagas disease vaccine in the α1,3-GalT-KO mouse model. We also evaluated the adjuvant LMPLA for the improvement of vaccine efficacy.

Materials and Methods

LMPLA Adjuvant

LMPLA was prepared as described (Matyas et al, 2003). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'rac-glycerol) (sodium salt) (DMPG), and cholesterol (plant-derived) were purchased from Avanti Polar Lipids, Inc. Lipid A, monophosphoryl from *Salmonella enterica* serotype minnesota Re 595 (Re mutant) was purchased from Sigma-Aldrich (St. Louis, MO). DMPC was dissolved in freshly distilled chloroform ($CHCl_3$) (LC-MS grade, Thermo Fisher Scientific), which was kindly purified and provided by Dr. Katja Michael, Chemistry Department, University of Texas at El Paso (UTEP), giving a stock concentration of 180 mM. DMPG was dissolved in pure chloroform, giving a stock concentration of 20 mM. Cholesterol was also dissolved in pure chloroform, giving a stock concentration of 150 mM. Lipid A was dissolved in chloroform:methanol (9:1, v/v), giving a stock concentration of 1 mg/ml.

Immunizations

Female α1,3-GalT-KO (C57BL/6) (Tearle et al, 1996; Thall et al, 1996) mice at 6 to 10 weeks old were used for immunization against *T. cruzi*. The synthetic glycan Galα(1,3)Galβ(1,4)GlcNAcα covalently conjugated to BSA (KM24b) was provided by Dr. Katja Michael's group (Chemistry Dept., UTEP). Three groups of α1,3-GalT-KO mice (n=4 per group) were used. The groups were KM24b alone, KM24b + LMPLA, and BSA + LMPLA (control). Immunizations were performed with 100 μl per dose and 4 immunizations at one-week intervals were given via subcutaneous (s.c.). Groups were vaccinated with 10 μg LMPLA and 0.01% BSA per mouse (BSA + LMPLA); 20 μg per mouse of KM24b; and 20 μg KM24b and 10 μg LMPLA per mouse (KM24b + LMPLA).

Detection of Antibodies by Chemiluminescent Enzyme-linked Immunosorbent Assay (CL-ELISA)

To assess the humoral immune response to the three vaccinated groups, levels of specific anti-α-Gal antibody titers were determined by CL-ELISA (Almeida et al, 1997). MaxiSorp Nunc polystyrene microplates, donkey biotinylated anti-mouse, NeutrAvidin-HRP, and SuperSignal Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. Blood was collected via facial vein of each mouse the day prior the first immunization, before the third immunization, 2 weeks after the last immunization and at endpoint. Serum was separated from every blood collection by centrifugation (2,500 x *g*, 15 min, at room temperature (RT)). All serum samples were analyzed by CL-ELISA prior to immunization (naïve), during the immunizations, and at the experimental endpoint. Microplates were coated with 125 ng/well of KM24b + LMPLA, KM24b or BSA + LMPLA, in CBC buffer and incubated overnight at 4˚C. Microplates were blocked with 200 μL 1% BSA in PBS or 5% skim milk in PBS for 1 h at 37ºC. Microplates were

incubated with 50 μL mouse sera from vaccinated or control group at 1:100 dilution for 1 h at 37ºC. Donkey biotinylated anti-mouse (code number 715-065-150, Lot number 68134, Jackson ImmunoResearch) was used at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP (product number 31030, lot number NL180713, Thermo Fisher Scientific) was used at 1:5000 dilution in 1% BSA/PBS-T. Microplates were washed 3x between steps with PBS-T, except before blocking. The reaction was developed with SuperSignal Pico Chemiluminescent Substrate (product number 37069, Lot number RD232185, Thermo Fisher Scientific) at a 1:1:8 ratio in 50 mM carbonate-bicarbonate CBC buffer, pH 9.6, 0.1% BSA. Luminescence in relative luminescence units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

Parasite Challenge

Mice were inoculated with $1x10^4$ Y-strain trypomastigotes via intraperitoneal (i.p.), in 100 μL PBS.

Parasitemia

From 3 to 17 dpi, 5 μl of blood was collected daily from tail vein of each mouse and trypomastigotes were quantified using a light microscope (Leica DMI6000 B), using the following formula:

$$\textbf{Total of parasites in 5 } \mu\textbf{l} = \frac{\left(\frac{\text{Number of parasites in 50 fields}}{50} \times \text{Area of coverslip (22 mm x 22 mm)}\right)}{\text{Area of microscope field } (\pi)(r^2)}$$

Total of parasites in 1 ml = Total of parasites in 5 μl x 200

Weight

To detect any sign of toxicity in the animals, post-immunization or post-challenge, animal weight was measured from 0 to 28 dpi. Weight change was normalized using the mouse weight before challenge.

Results

Previous studies showed that C57BL/6 α1,3-GalT-KO mice vaccinated with the NGP Galα3LN-3aL-BSA (from Dextra Laboratories, formerly V-Labs, Inc.) had a 40-fold increase in the levels of specific anti-α-Gal antibodies when compared to pre-immunization titers (Schocker et al, 2016). Chronic ChD patients produce high levels of protective anti-α-Gal Abs (Almeida et al, 1997; Almeida et al, 1994; Almeida et al, 1991; Milani & Travassos, 1988). This study was designed to evaluate the synthetic NGP KM24b (**Figure 9**), with or without LMPLA as adjuvant, in C57BL/6 α1,3-GalT-KO mice. For second study, the experimental history (**Table 2**) and experimental timeline (**Figure 10**) are provided.

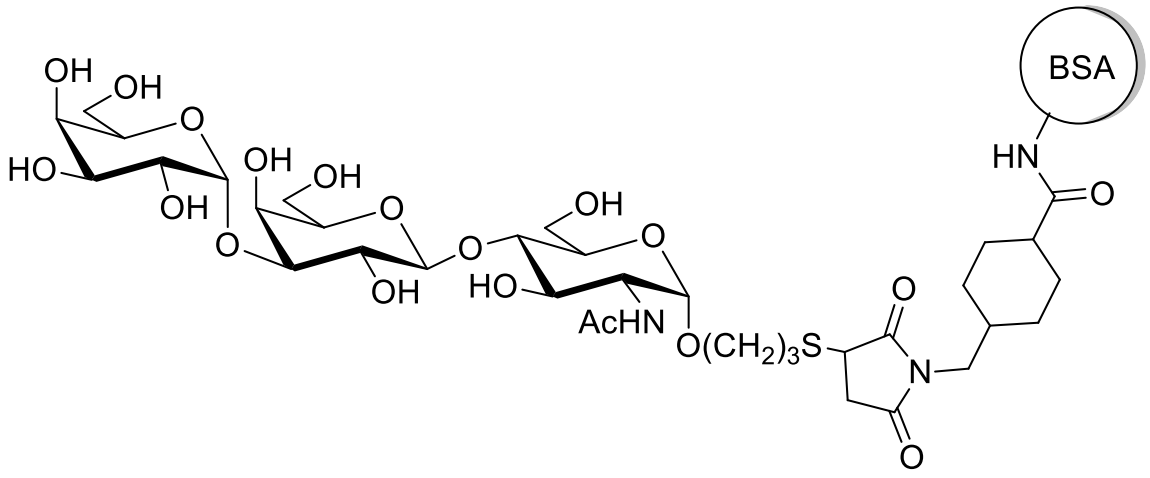

Figure 9. Structure of neoglycoprotein KM24b. Galα(1,3)Galβ(1,4)GlcNAcα conjugated to BSA was synthesized by Dr. Katja Michael and her group (Chemistry Dept., UTEP).

TABLE 2. History of second study.

Procedure/Event	Day Post-Immunization	Day Post-Infection (dpi)
Blood collection	-1	-38
Prime immunization	0	-37
Boost 1	7	-30
Blood collection	13	-24
Boost 2	14	-23
Boost 3	21	-16
Blood collection	34	-3
Challenge with 10^4 trypomastigotes	37	0
Parasitemia measurement	40 - 54	3 to 17
BSA+MPLA #4 dies	46	9
KM24b mouse #3 dies	48	11
KM24b mouse #1 dies	52	15
KM24b mouse #2 dies	57	20
Blood collection	69	32
Euthanasia → organs harvested	69	32

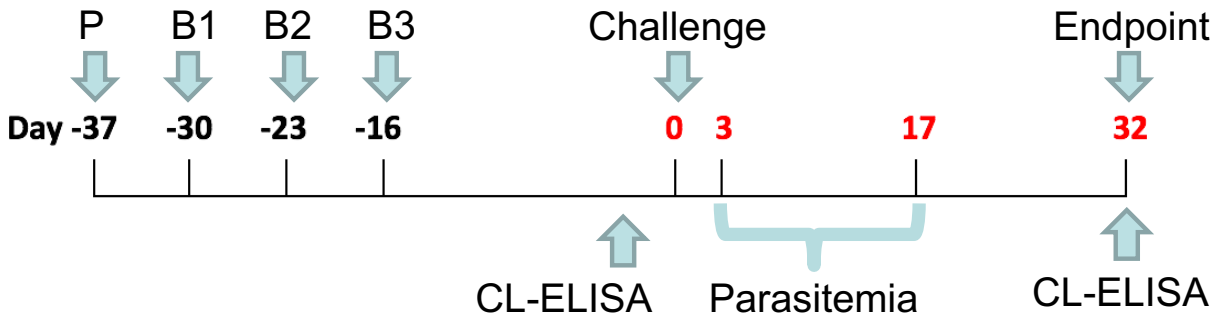

Figure 10. Second study's timeline. Four immunizations with KM24b alone, KM24b + LMPLA or BSA + LMPLA were given at one-week interval. Challenged day became 0 dpi. Parasitemia was measured at 3 to 17 dpi. Endpoint at 32 dpi. CL-ELISA was performed before and after challenge.

Female C57BL/6 α1,3-GalT-KO mice groups (n=4) were vaccinated via s.c. with 20 μg per mouse of KM24b; and 20 μg KM24b and 10 μg LMPLA per mouse (KM24b + LMPLA); and 0.01% BSA and 10 μg LMPLA per mouse (BSA + LMPLA). Four immunizations at one-week intervals were given.

To determine the production of IgG antibodies levels before challenge, serum samples were collected one day before prime, one day before boost 3, and 2 weeks after boost 3. Serum was analyzed by CL-ELISA blocked with PBS-1% BSA (**Figure 11A**). To remove the background given by BSA, we successfully repeated the CL-ELISA blocking with PBS-5% skim milk (Nestle Carnation Instant Nonfat Dry Milk) (**Figure 11B**). As expected, mice vaccinated with KM24b alone or KM24b + LMPLA showed high levels of specific anti-α-Gal Abs. However, the B cell-mediated immune response was earlier and stronger with KM24b + LMPLA vaccinated mice.

To determine whether these mice were protected against *T. cruzi,* we challenged the animals two weeks after the last immunization with 10^4 infective trypomastigotes (Y strain). Five microliters of blood via tail vein of each mice was collected individually to quantify parasitemia 3-17 dpi. Unexpectedly, the group vaccinated with KM24b showed higher levels of parasitemia, whereas the group vaccinated with KM24b + LMPLA showed lower levels of parasitemia at the 4 dpi (**Figure 12**). After challenge, mice vaccinated with KM24b + LMPLA showed higher weight (**Figure 13**).

To determine the production of IgG antibodies levels after challenge, we used sera collected one day before prime immunization, one day before boost immunization 3, and 2 weeks after boost 3, and endpoint (32 dpi). All sera were analyzed by CL-ELISA blocked with PBS-1% BSA (**Figure 11A**) and with PBS-5% skim milk (**Figure 11B**). At the endpoint of the experiment,

anti-α-Gal Abs levels were low for BSA + LMPLA and KM24b groups and present for KM24b + LMPLA group.

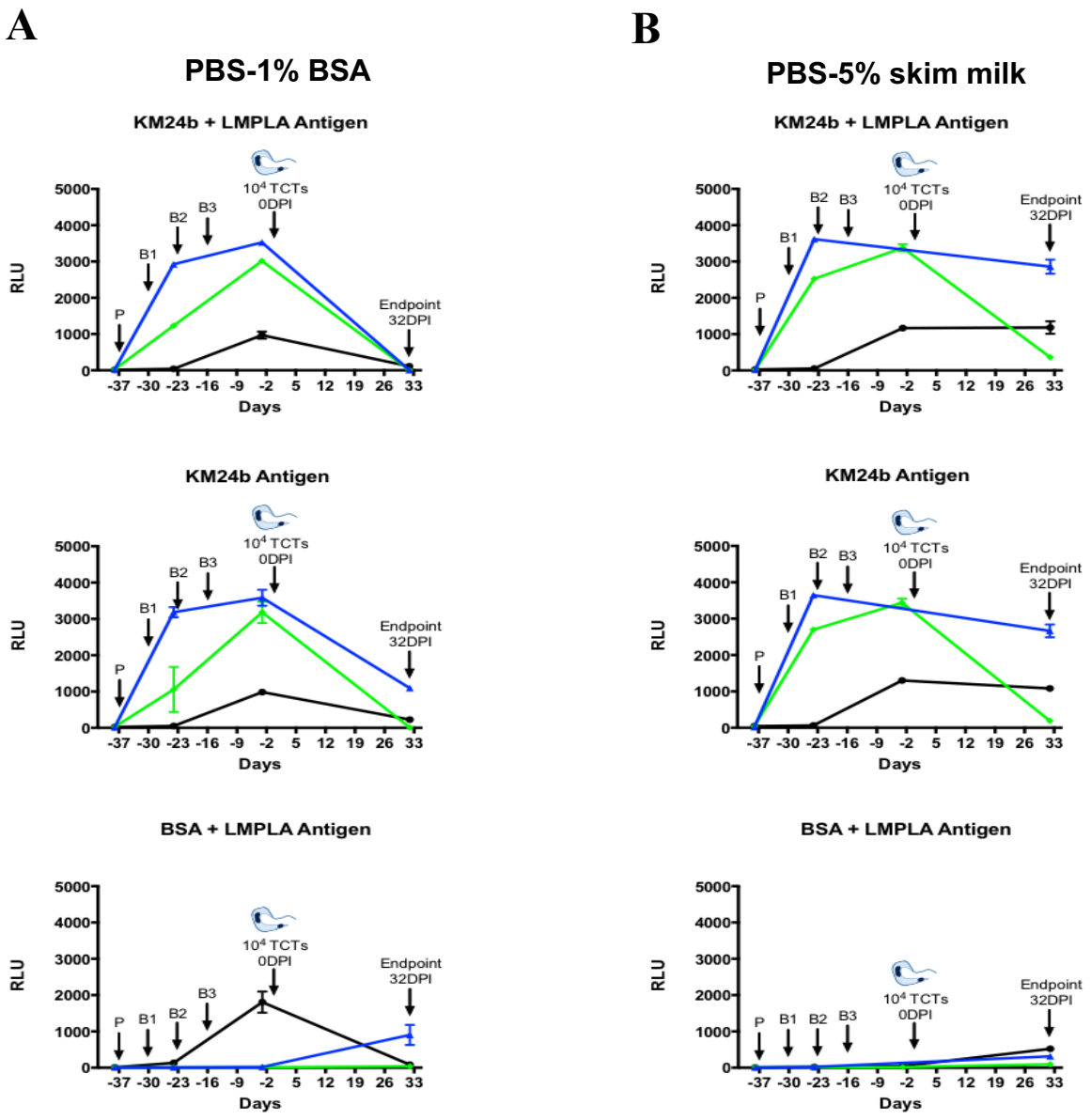

Figure 11. Levels of specific antibodies as measured by CL-ELISA. Levels of anti-α-Gal Abs using BSA+MPLA, KM24b, and MPLA+KM24b as antigens on the CL-ELISA plate.

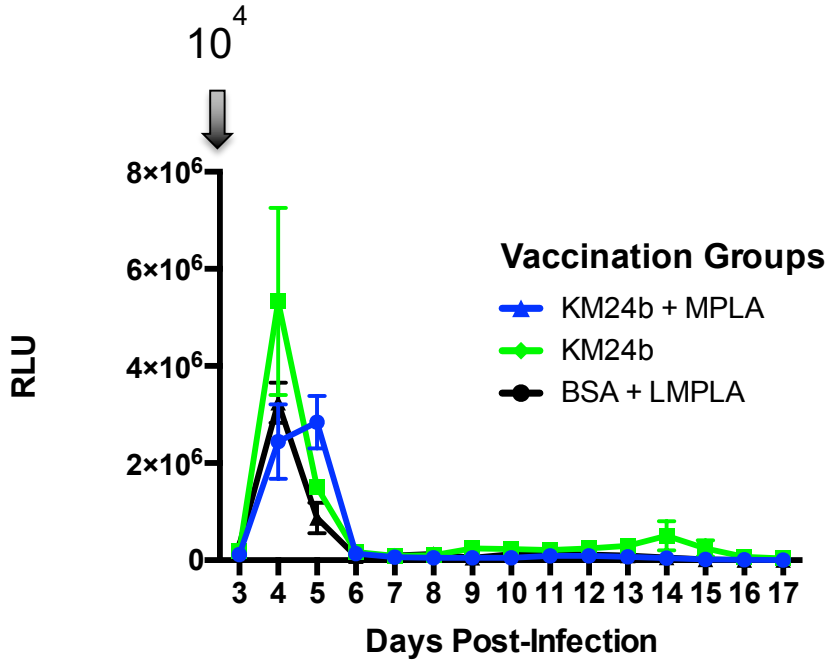

Figure 12. Parasitemia. Quantification of trypomastigotes per milliliter during 3 to 17 dpi.

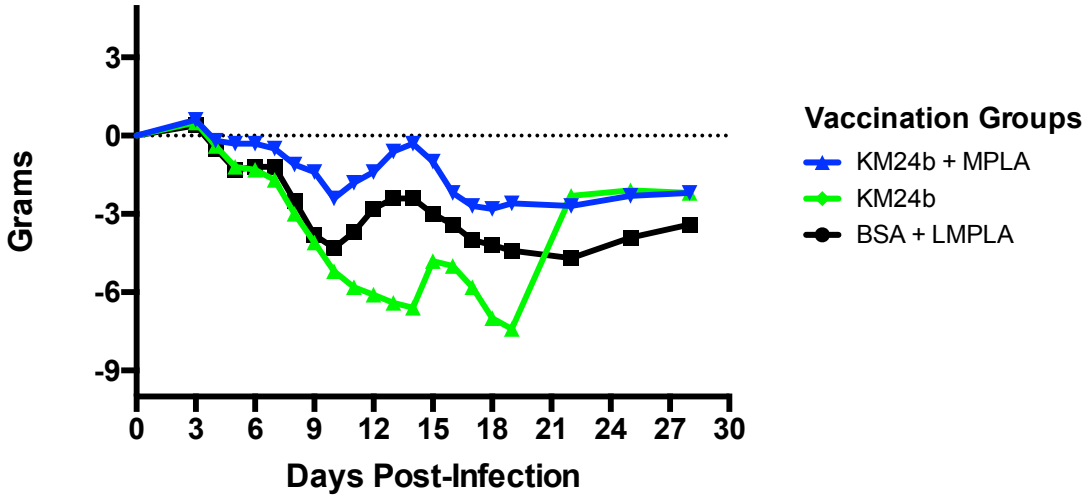

Figure 13: Animal weight post-infection. The weight of mice was measured 0 to 28 dpi. The weight is represented as variation on weight (grams) following infection with parasites.

Animals vaccinated with KM24b + LMPLA showed 100% survival, whereas those vaccinated with KM24b and BSA + LMPLA showed 25% and 75% survival, respectively (**Figure 14**). The adjuvant LMPLA significantly increased anti-α-Gal Ab levels and the efficacy of KM24b vaccine in terms of survival. Moreover, the number of vaccinations could be eventually reduced, which is an important factor for eventual vaccination of humans.

This study showed that vaccination with KM24b alone gave much lower survival rate as compared to the previous study using Galα3LN-3aL-BSA from Dextra Laboratories (formerly, V-Labs, Inc). The main differences between the two NGPs are: (a) the anomeric configuration of the reducing-end GlcNAc residue (α vs. β), and (b) Dextra's Galα3LN-3aL-BSA has a 3-atom linker, whereas KM24b has a 13-atom linker (**Table 3**).

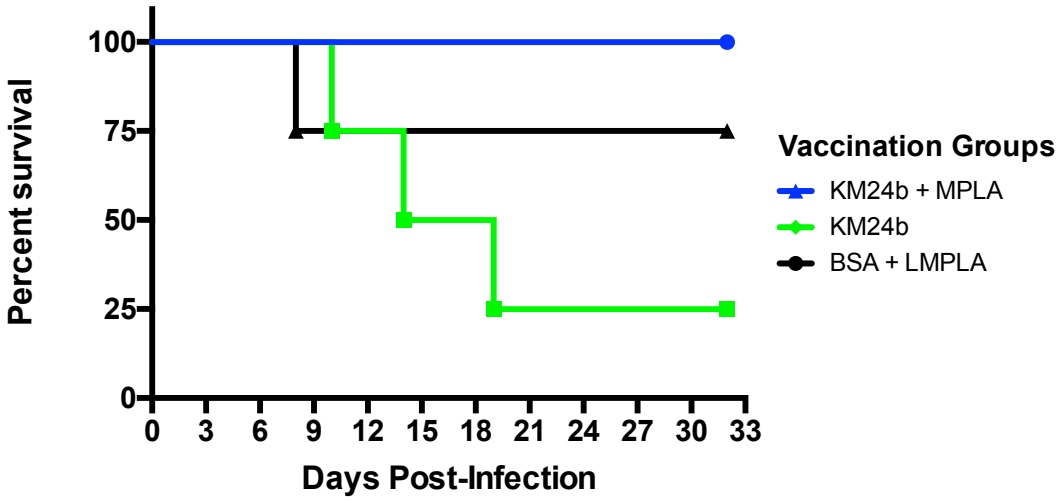

Figure 14. Survival of mice challenged with *T. cruzi* TCTs (Y strain). Survival rates: BSA+MPLA, 75; KM24b+MPLA, 100%; KM24b, 25%.

TABLE 3. Differences between first study with Galα(1,3)Galβ(1,4)GlcNAcβ-BSA from Dextra Labs and second study with KM24b.

Parameter	First Study	Second Study
Antigen	Commercial (Dextra Labs/V-Labs)	Synthesized by Michael Lab
	Galα1,3Galβ1,4GlcNAc**β**-BSA	Galα1,3Galβ1,4GlcNAc**α**-BSA
	3-atom spacer	13-atom spacer
	10 μg/mouse	20 μg/mouse
Challenge	3 challenges	1 challenge
	Challenged 1 week after B3	Challenged 2 weeks after B3
Immunosuppression	yes	no

Third Study:

Evaluation of the Influence of Linker and Carrier Protein in the Neoglycoprotein Vaccine

Third Study: Evaluation of the Influence of Linker and Carrier Protein in Neoglycoprotein Vaccines

In our third study, we evaluated the effect of the linker length and carrier protein nature in different neoglycoproteins used for immunization of α1,3-GalT-KO mice.

Materials and Methods

NGPs and Other Reagents for Immunization

NGPs Galα3LN-3aL-BSA (catalog number NGP0334), Galα3LN-3aL-NH-HSA (catalog number NGP2334), Galα3LN-14aL-BSA (catalog number NGP1334), and Galα3LN-14aL-HSA (catalog number 3334) were purchased from Dextra Laboratories (formerly, V-Labs, Inc., Reading, UK). Bovine serum albumin (BSA, Fraction V) and human serum albumin (HSA) were purchased from Thermo Fisher Scientific. HyClone Phosphate Buffered Saline 1X (PBS) was purchased from GE Healthcare Life Sciences.

Immunizations

Female α1,3GalT-KO (C5B/6) mice at 6 to 10 weeks old were used for immunization. Seven groups of α1,3GalT-KO mice (n=6 per group) were used. The groups were Galα3LN-3aL-BSA, Galα3LN-3aL-NH-HSA, Galα3LN-14aL-BSA, Galα3LN-14aL-HSA, HSA (control), BSA (control), and PBS (control). All NGPs were first diluted in sterile deionized water to a 1.0 mg/mL solution. Immunizations were performed with 100 µl per dose and 4 immunizations at one-week

intervals were given via subcutaneous (s.c.). Groups were vaccinated with 20 μg of each NGP, HSA, BSA, or PBS.

Detection of Antibodies by Chemiluminescent Enzyme-linked Immunosorbent Assay (CL-ELISA)

To assess the humoral immune response for the seven groups, levels of specific anti-α-Gal antibody titers were determined by CL-ELISA, as described (Almeida et al, 1997). MaxiSorp Nunc polystyrene microplates, donkey biotinylated anti-mouse, NeutrAvidin-HRP, and SuperSignal Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. Blood was collected via facial vein of each mouse 3 days after each immunization, weekly after boost 3, at 33 dpi and 40 dpi (endpoint). Serum was separated from every blood collection by centrifugation (2,500 x *g*, 15 min, RT). Serum samples collected 3 days after each immunization and weekly after boost 3 were used and analyzed by CL-ELISA before challenge; and all serum samples, including sera collected at 33 dpi and 40 dpi (endpoint) were analyzed by CL-ELISA. Microplates were coated with 125 ng/well of PBS, BSA, HSA, Galα3LN-3aL-BSA, Galα3LN-3aL-NH-HSA, Galα3LN-14aL-BSA, and Galα3LN-14aL-HSA in CBC buffer and incubated overnight at 4˚C. Microplates were blocked with 200 μL PBS-1% BSA, PBS-5% skim milk, or ELISA Ultrablock (BUF033, BioRad) for 1 h at 37ºC. Microplates were incubated with 50 μL mouse serum from vaccinated or control group at 1:100 dilution for 1 h at 37ºC. Donkey biotinylated anti-mouse (code number 715-065-150, Lot number 68134, Jackson ImmunoResearch) was used at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP (product number 31030, lot number NL180713, Thermo Fisher Scientific) was used at 1:5000 dilution in 1% BSA/PBS-T. Microplates were washed 3x between steps with PBS-T, except before blocking. The reaction was developed with SuperSignal Pico Chemiluminescent Substrate (product number

37069, Lot number RD232185, Thermo Fisher Scientific) a 1:1:8 ratio in CBC buffer/0.1%BSA. Luminescence in relative luminescence units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

Challenge

Female α1,3GalT-KO (C57BI/6) mice (n=3 per group) were challenged with $1x10^5$ red-shifted luciferase expressing CL-Brener clone ($CL\text{-}Brener_{Luc}$) trypomastigotes (Lewis et al, 2015), via intraperitoneal (i.p.), 3 weeks post-boost 3. Parasite inoculation was considered 0 dpi.

Parasite Load

Parasite Load was measured at 3 to 20 dpi, and 23, 26, and 29 dpi using an *in vivo* imaging system Lumina III *In Vivo* Imaging System (IVIS) (PerkinElmer). Mice were injected intraperitoneally (i.p.) with 100 µl of 15 mg/mL D-luciferin (Gold Biotechnology) and anesthetized with 2.5% gaseous isoflurane in oxygen. Images were acquired 15 min after luciferin injection using the IVIS. Luminescence was expressed in radiance (photons/second/cm^2/steradian).

Weight

To detect any sign of toxicity, weight was measured from 0 to 20, 23, 26, 29, 33, and 40 dpi from every mouse before imaging. Weight change was normalized using the mouse weight before challenge.

Immunoglobulin Isotyping

To detect specific levels of IgG antibodies (IgG1, IgG2a, IgG2b, IgG3 and IgE) for the seven

groups (Almeida et al, 1997). Goat polyclonal Ab to IgG1, IgG2a, IgG2b, and IgG3; and rat monoclonal antibody (mAb) to IgE were purchased from Abcam (Cambridge, MA). Serum samples collected after boost 3 and at 40 dpi (endpoint) were analyzed. Isotyping was carried out by CL-ELISA with some modifications. The NGPs Galα3LN-3aL-BSA, Galα3LN-3aL-NH-HSA, Galα3LN-14aL-BSA, Galα3LN-14aL-HSA, or PBS/BSA/HSA in CBC buffer were incubated on the microplate well overnight at 4˚C. Microplates were blocked with 200 μL PBS-5% skim milk. Donkey biotinylated anti-mouse conjugated to HRP was used at 1:2000 dilution in PBS-1% BSA with 0.05% Tween 20 (PBS-1% BSA-T). Microplates were washed 3x between steps with PBS-T, except before blocking. The reaction was developed with SuperSignal Pico Chemiluminescent Substrate (product number 37069, Lot number RD232185, Thermo Fisher Scientific) at a 1:1:8 ratio in 50 mM carbonate-bicarbonate CBC buffer, pH 9.6, 0.1% BSA. Luminescence in relative luminescence units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

Results

The first study showed that C57BL/6 α1,3-GalT-KO mice vaccinated with KM24b alone gave much lower survival rate as compared to the previous study using Galα3LN-3aL-BSA from Dextra/V-Labs. The main differences between the two NGPs are: (a) the anomeric configuration of the reducing-end GlcNAc residue (α vs. β), (b) and Dextra's Galα3LN-3aL-BSA has a 3-atom linker, whereas KM24b has a 13-atom linker (**Table 3**). This study was designed to evaluate whether these structural dissimilarities could lead to such considerably different immune responses and protection against *T. cruzi* in C57BL/6 α1,3-GalT-KO mice, since they do not express the terminal α-Gal terminal in their cells. They mimic humans, in contrast to wild-type mice (Thall et al, 1996). For the third study, timeline (**Figure 15**), experimental design (**Figure 16**), and experiment history (**Table 4**) are provided.

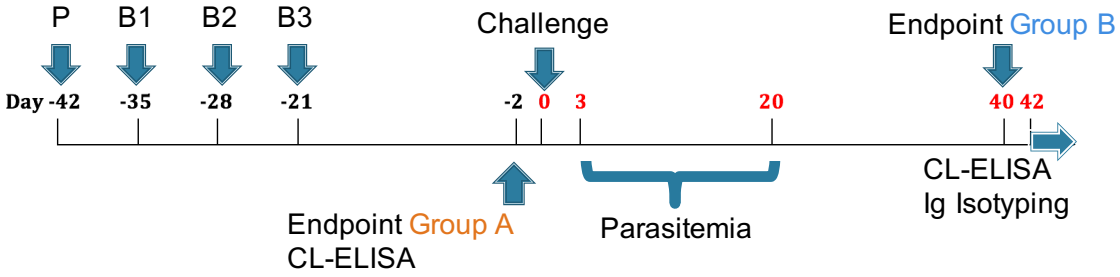

Figure 15. Third study's timeline. Four immunizations with Galα3LN-3aL-NH-HSA, Galα3LN-14aL-HSA, Galα3LN-3aL-BSA, Galα3LN-14aL-BSA, HSA, BSA or PBS were given at one-week interval. Challenged day became 0 dpi. Parasitemia during 3 dpi to 20 dpi. Endpoint for vaccinated mice, but not challenged was on -2 dpi. Endpoint for vaccinated and challenged mice was on 40 dpi. CL-ELISA performed before and after challenge. Isotyping was perform after 40 dpi.

TABLE 4. History of Third study.

Procedure/Event	Day Post-Immunization (dpim)	Day Post Infection (dpi)
Prime immunization	0	-42
Blood collection	3	-39
Boost 1	7	-35
Blood collection	10	-32
Boost 2	14	-28
Blood collection	17	-25
Boost 3	21	-21
Blood collection	24	-18
Blood collection	31	-11
Blood collection	38	-4
Group A final blood collection	40	-2
Group A euthanized	40	-2
Challenge Group B with 10^5 CL Brener$_{Luc}$	42	0
Parasitemia (IVIS)	45 - 62	3 - 20
14-BSA group mouse #4 dies	49	7
PBS group mice # 1,2,3 die	53	11
BSA group #2 dies	53	11
14-HSA group #3 dies	53	11
3-BSA group #2 dies	54	12
BSA group mouse #1 dies	56	14
HSA group mouse #2 dies	60	18
Group B blood collection	75	33
Group B Euthanasia	82	40
Group B final blood collection	82	40

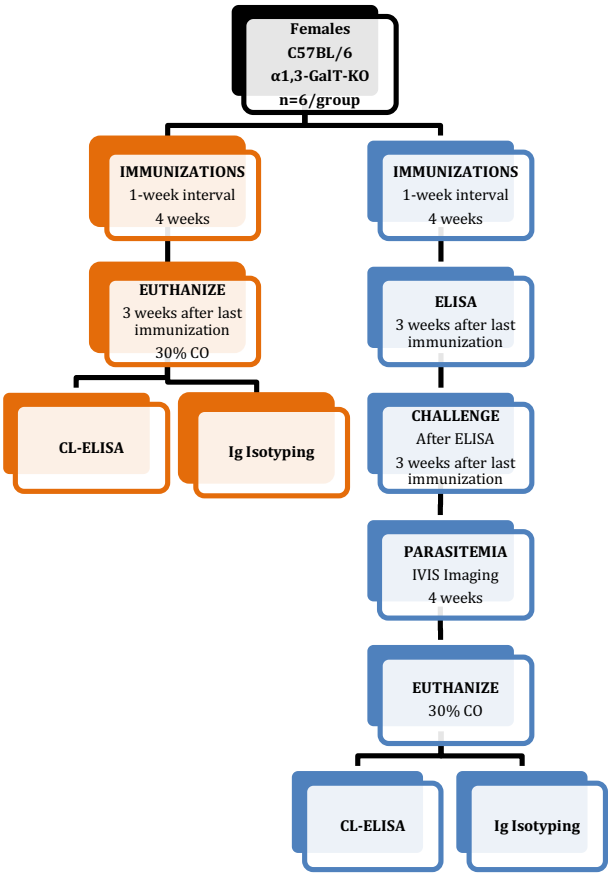

Figure 16. Experimental Design for third study. All 6 mice per group were vaccinated. Before challenge, 3 mice per group were euthanized.

Female C57BL/6 α1,3-GalT-KO mice groups (n=6) were vaccinated via s.c. with 20 μg of Galα3LN-3aL-NH-HSA per mouse, 20 μg of Galα3LN-14aL-HSA per mouse, 20 μg of Galα3LN-3aL-BSA per mouse, 20 μg of Galα3LN-14aL-BSA per mouse, 20 μg of HSA per mouse, 20 μg of BSA per mouse, or 1X PBS per mouse. Four immunizations at one-week intervals were given. Mice (n=3) per group were euthanized before parasite challenge.

To determine the production of IgG antibodies levels before challenge, serum was collected 3 days after each immunization and weekly after boost 3. Sera were analyzed by CL-ELISA

blocked with PBS-1% BSA (**Figure 17**). To remove the background given by HSA or BSA, we repeated the CL-ELISA blocking with PBS-5% skim milk. We were successful removing the BSA background (**Figure 18**). To remove the HSA background, we repeated the CL-ELISA blocking with BioRad ELISA Ultrablock, a fish extract-based blocker. Even though we were unable to completely remove the HSA background, we were able to reduce it (**Figure 19**). As expected, both groups of mice vaccinated with Galα3LN-14aL-BSA or Galα3LN-14aL-HSA showed high levels of specific anti-α-Gal Abs.

The remaining 3 mice per group were challenged 3 weeks after boost 3 to determine whether they were protected against *T. cruzi*. Parasite challenge was done with 10^5 CL-Brener$_{Luc}$ infective trypomastigotes. Parasitemia was measured at 3 to 20 dpi, and 23, 26, and 29 dpi using the IVIS bioimager. Images of mice vaccinated with Galα3LN-3aL-NH-HSA showed lower radiance compared to the other groups (**Figure 20**), and consequently, lower parasitemia (**Figure 21**). At endpoint, we harvested organs and collected serum for future experiments.

To determine the production of IgG antibodies levels after challenge, sera were also collected at 33 and 40 dpi (endpoint). All sera were analyzed by CL-ELISA blocked with PBS-1%BSA (**Figure 17**), PBS-5% skim milk (**Figure 18**), or BioRad ELISA Ultrablock (**Figure 19**). At the endpoint of the experiment, anti-α-Gal Abs levels were low for all groups.

To determine the production of subclasses of IgG (IgG1, IgG2a, IgG2b, and IgG3) and IgE antibodies levels, immunoglobulin isotyping was performed using serum samples collected after immunizations (P, and B1-B3) and at endpoint (**Figure 22**). Levels of IgG1 and IgG2b were higher after vaccination and levels decreased after challenge. Levels of IgE were minimally detectable.

Animals vaccinated with Galα3LN-3aL-NH-HSA showed higher weight post-infection (**Figure 23**), lower parasitemia, and 100% survival. Mice vaccinated with Galα3LN-3aL-BSA,

Galα3LN-14aL-BSA, Galα3LN-14aL-HSA and HSA (control) showed 66% survival. On the other hand, BSA group (control) showed only 33% survival, whereas and PBS group (control) had 0% survival (**Figure 24**). These NGPs produce high levels of IgG1 and IgG2b after vaccination, and no detectable levels of IgE; therefore, these NGPs do not cause allergic reactions. Blocking a CL-ELISA with skim milk removed the BSA background, whereas BioRad ELISA Ultrablock (fish extract) reduced HSA background. In this study, we concluded that the carrier protein and the linker length are crucial parameters to be considered in the design of NGPs to be tested as experimental vaccines for ChD.

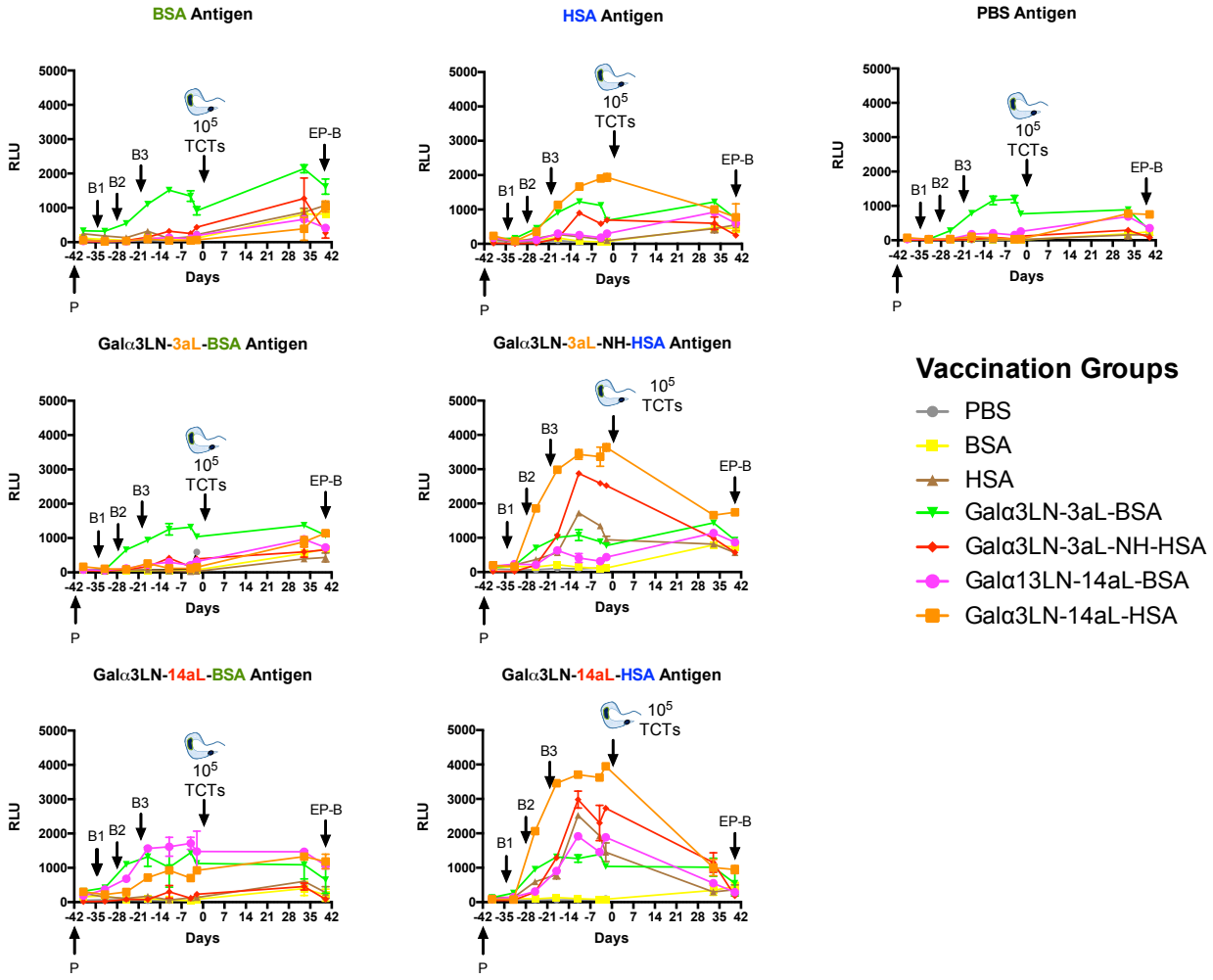

Figure 17. CL-ELISA blocked with PBS-1%BSA. Levels of anti-α-Gal Abs using NGPs with different linkers and carrier proteins, HSA, BSA and PBS antigens.

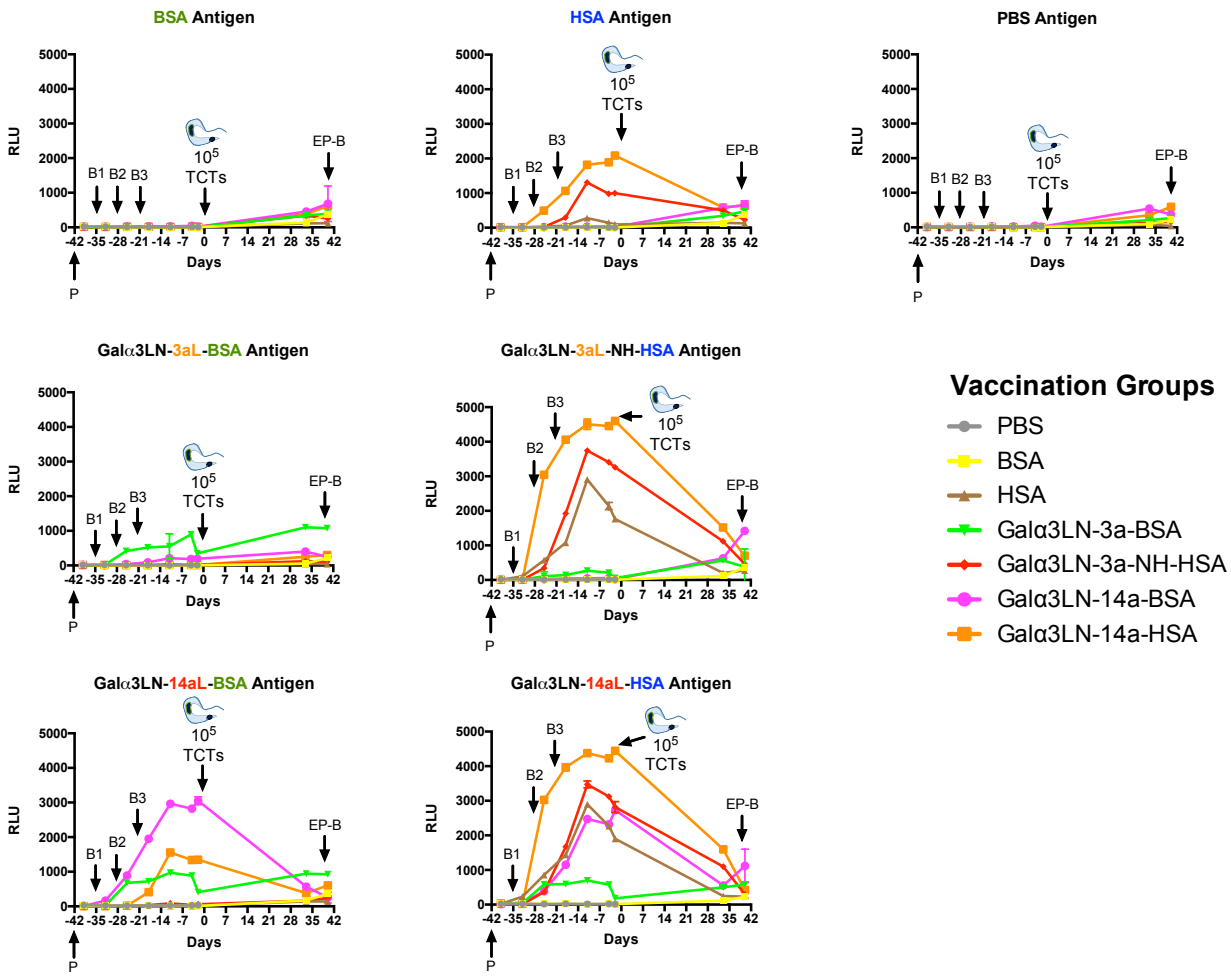

Figure 18. CL-ELISA blocked with PBS-5% skim milk. Levels of anti-α-Gal Abs using NGPs with different linkers and carrier proteins, HSA, BSA and PBS antigens.

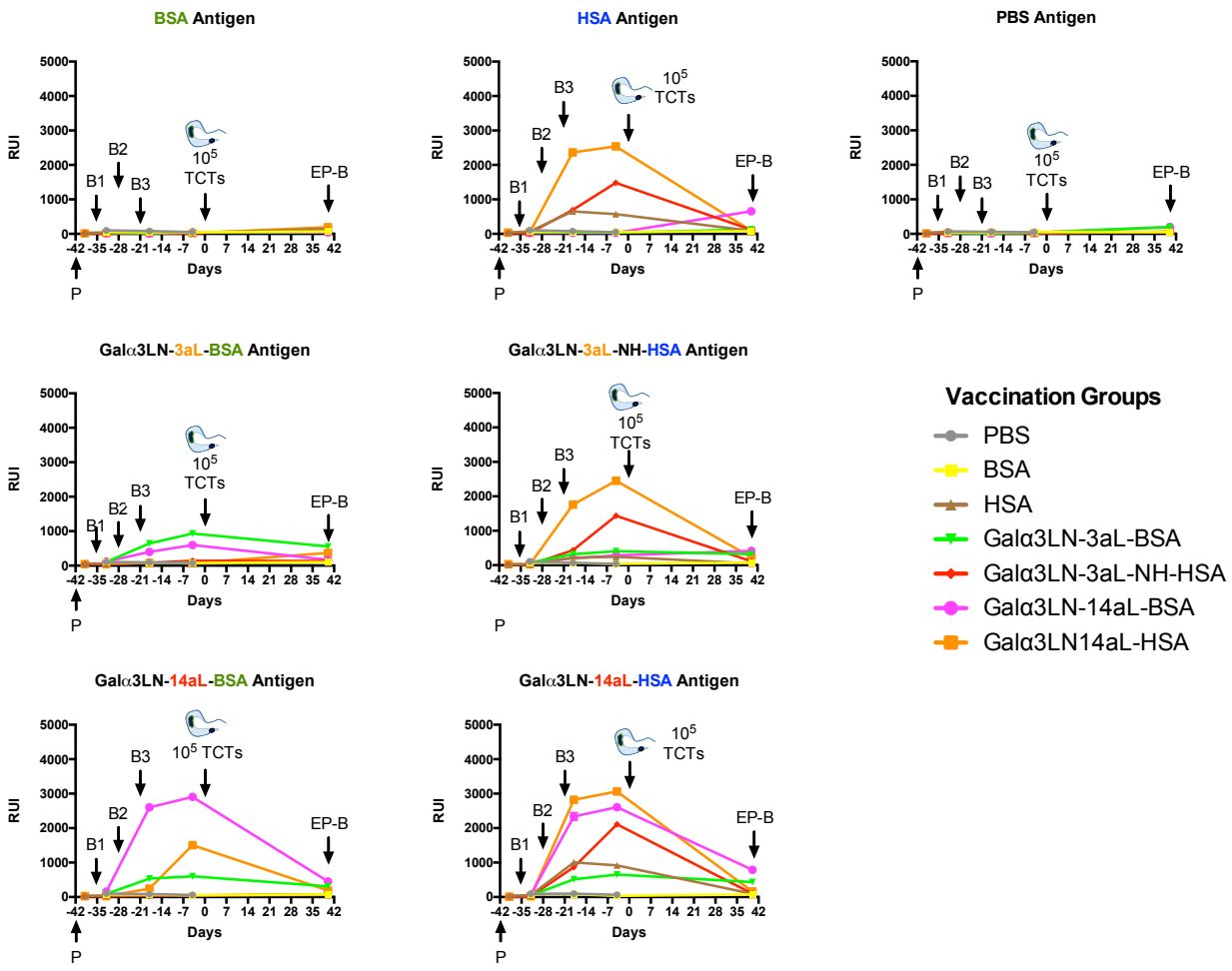

Figure 19. CL-ELISA blocked with BioRad ELISA Ultrablock (fish extract). Levels of anti-α-Gal Abs using NGPs with different linkers and carrier proteins, HSA, BSA and PBS antigens.

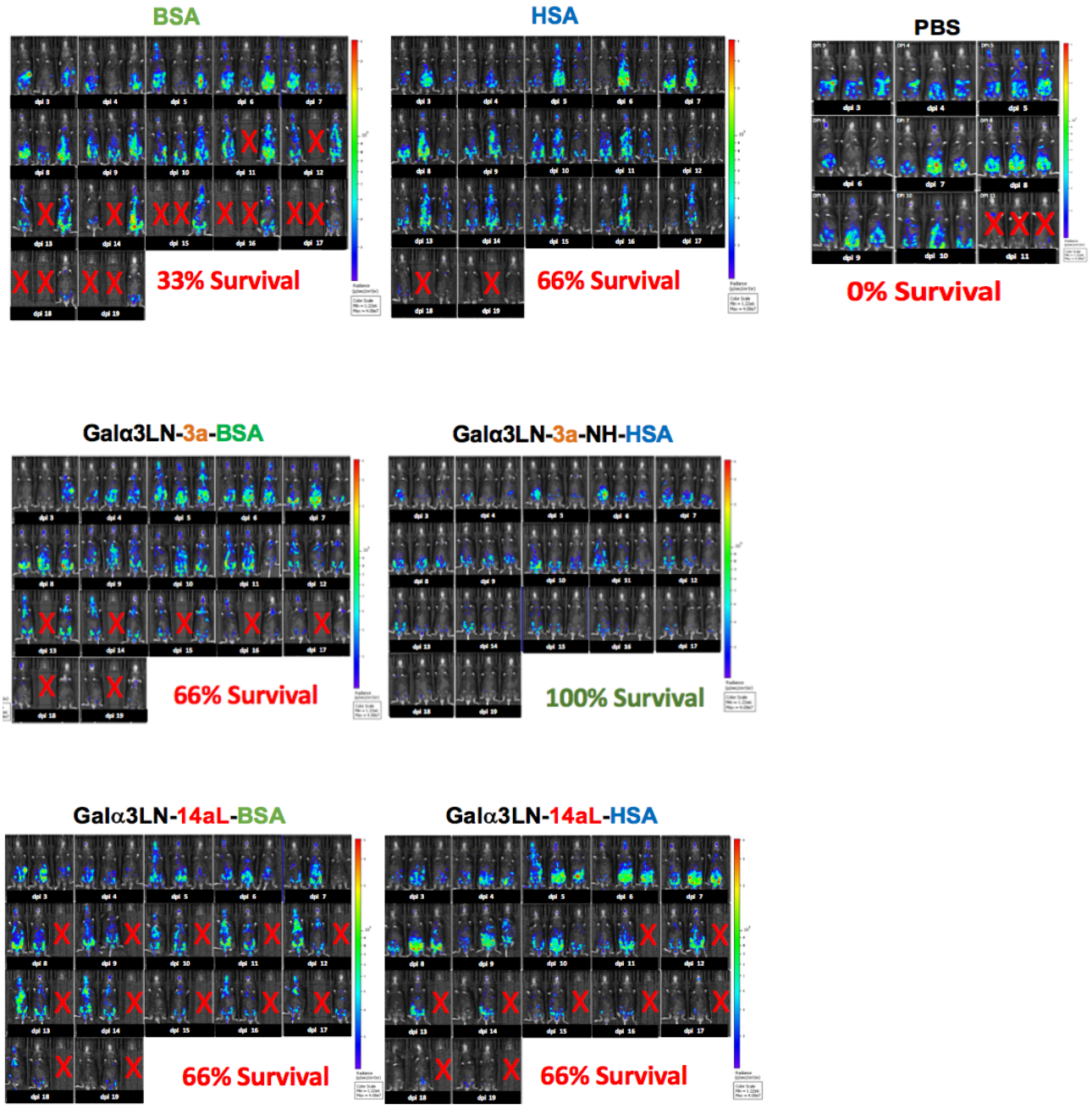

Figure 20. IVIS images. Three out of six mice per group were challenged with $1x10^5$ TCTs CL Brener strain expressing red-shifted luciferase (CL-Brener$_{Luc}$) parasite and analyzed using an in vivo imaging system (IVIS). Images provided by Brenda G. Zepeda, Susana Portillo and Igor Estevao da Silva (unpublished data).

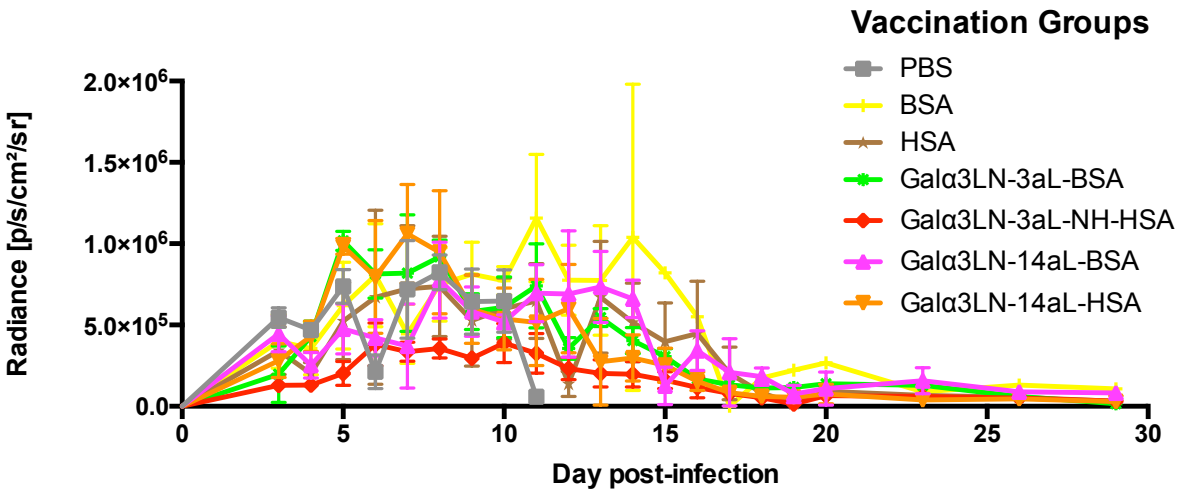

Figure 21. Parasite load. Radiance of trypomastigotes during 3 to 20, 23, 26, and 29 dpi using an in vivo imaging system (IVIS). Data provided and graphed by Brenda G. Zepeda (unpublished data).

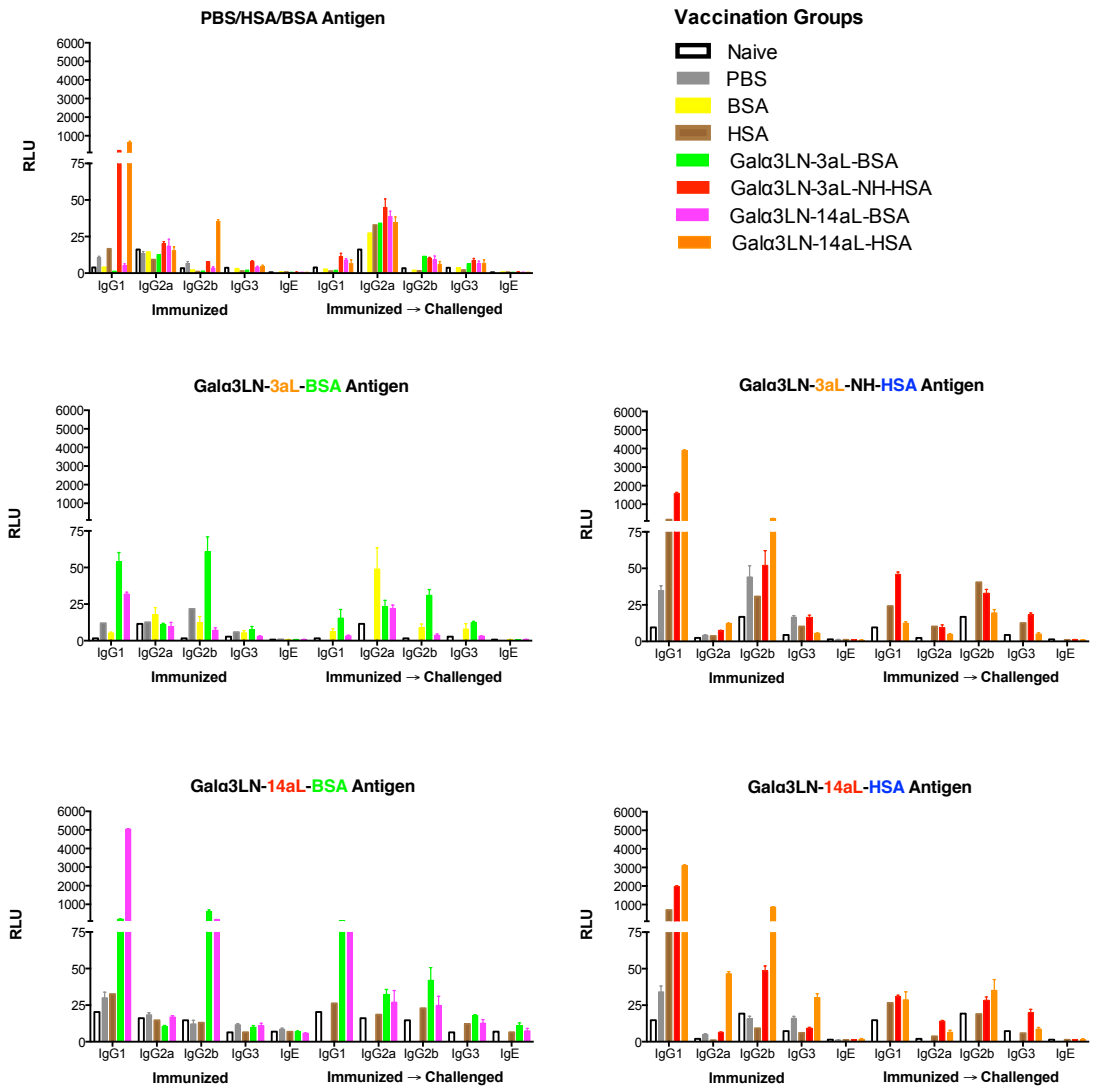

Figure 22. Isotyping. Levels of IgG subclasses and IgE after vaccination and at endpoint (40 dpi) of animals vaccinated with NGPs with different linkers and carrier proteins and parasite challenged. Data provided and graphed by Brenda G. Zepeda (unpublished data).

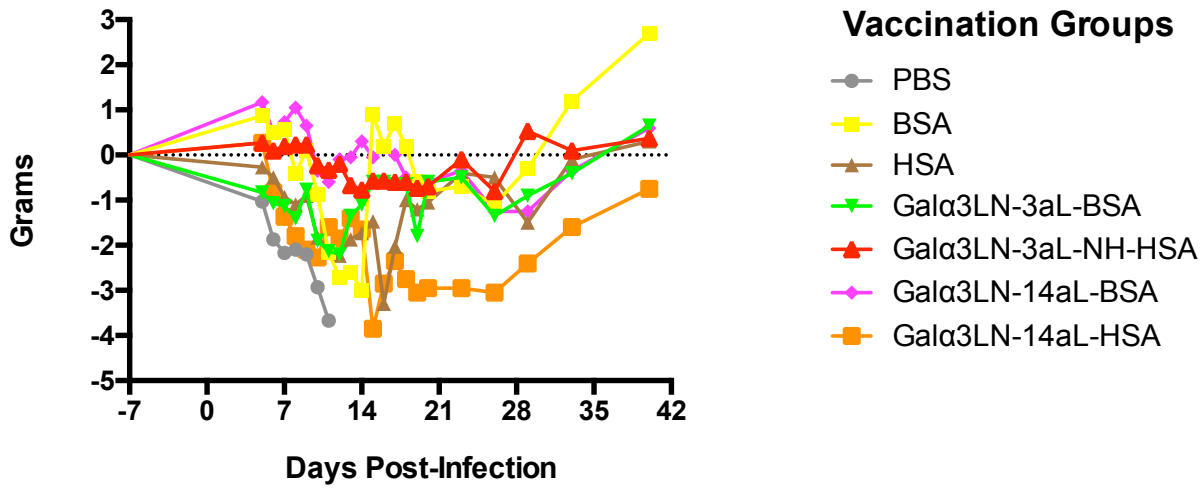

Figure 23. Animal weight post-infection. From -7 to 40 dpi weight of mice was measured. Data provided and graphed by Brenda G. Zepeda (unpublished data).

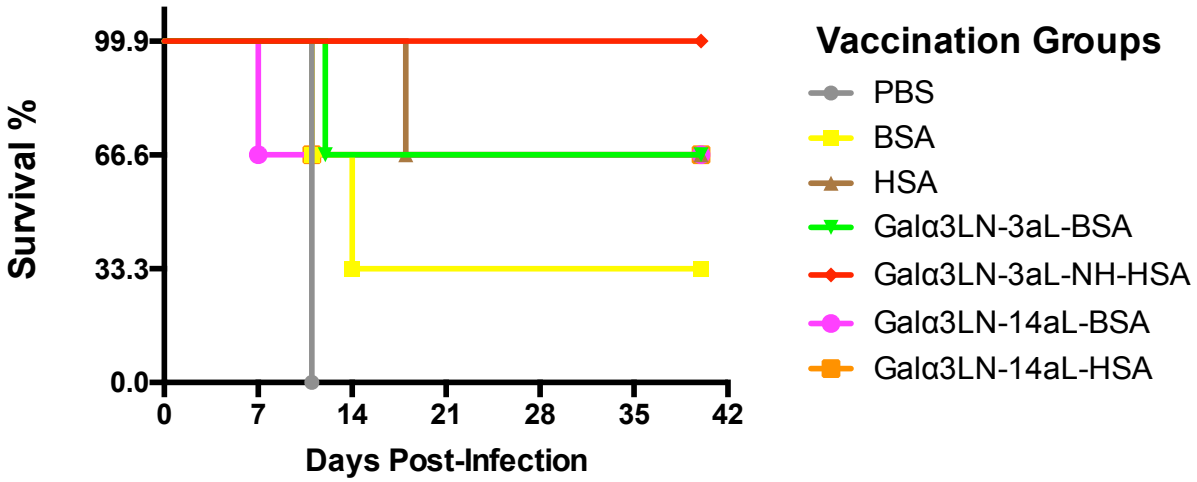

Figure 24. Survival of mice challenged with *T. cruzi* TCTs (CL-Brener$_{LUC}$ strain). Survival rate per group: Galα3LN-3aL-NH-HSA, 100%; Galα3LN-3aL-BSA, 66%; Galα3LN-14aL-BSA, Galα3LN-14aL-HSA and HSA (control), 66%; BSA (control), 33%; and PBS (control), 0%. Data provided and graphed by Brenda G. Zepeda (unpublished data).

Fourth Study:

Evaluation of Galα3LN-3aL-NH-HSA

with or without Liposomal Monophosphoryl Lipid A

Fourth Study: Evaluation and Validation of Galα3LN-3aL-NH-HSA With or Without Liposomal Monophosphoryl Lipid A

In our third study, we evaluated the effect of the adjuvant LMPLA in the efficacy of Galα3LN-3aL-NH-HSA antigen as a vaccine for experimental Chagas disease in α1,3-GalT-KO mice.

MATERIALS AND METHODS

NGP and HSA

The NGP Galα3LN-3aL-NH-HSA (catalog number NGP2334) used in this study was purchased from Dextra Laboratories. It was diluted in sterile deionized water to a 1.0 mg/mL solution. Recombinant HSA was purchased form Thermo Fisher Scientific.

LMPLA Adjuvant

LMPLA was prepared as described (Matyas et al, 2003). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'rac-glycerol) (sodium salt) (DMPG), and cholesterol (plant-derived) were purchased from Avanti Polar Lipids, Inc. Lipid A, monophosphoryl from *Salmonella enterica* serotype minnesota Re 595 (Re mutant) was purchased from Sigma-Aldrich (St. Louis, MO). DMPC was dissolved in freshly distilled chloroform ($CHCl_3$) (LC-MS grade, Thermo Fisher Scientific), which was kindly purified and provided by Dr. Katja Michael, Chemistry Department, University of Texas at El Paso (UTEP), giving a stock concentration of 180 mM. DMPG was dissolved in pure chloroform, giving a stock concentration of 20 mM. Cholesterol was also dissolved in pure chloroform, giving a stock concentration of 150

mM. Lipid A was dissolved in chloroform:methanol (9:1, v/v), giving a stock concentration of 1 mg/ml.

Immunizations

Female α1,3GalT-KO (C57BI/6) mice at 6 to 11 weeks old were used for immunization against *T. cruzi*. Three groups of α1,3GalT-KO mice (n=9 per group) were used. The groups were Galα3LN-3aL-NH-HSA + LMPLA, Galα3LN-3aL-NH-HSA alone, and HSA + LMPLA (control). Immunizations were performed with 100 µl per dose and 4 immunizations at one-week intervals were given via intraperitoneal (i.p.). Prime and boost 1 vaccines did not contain LMPLA; therefore, Galα3LN-3aL-NH-HSA + LMPLA group only received Galα3LN-3aL-NH-HSA vaccine, and HSA + LMPLA group only received HSA vaccine. Boost 2 and boost 3 vaccines contained LMPLA; therefore, Galα3LN-3aL-NH-HSA + LMPLA group received Galα3LN-3aL-NH-HSA + LMPLA vaccine, and HSA + LMPLA group received HSA + LMPLA vaccine. Groups were vaccinated with 20 µg of Galα3LN-3aL-NH-HSA, 10 µg of LMPLA and/or 20 µg of HSA per mouse.

CL-ELISA

To assess the humoral immune response for the three vaccinated groups, levels of specific anti-α-Gal antibody titers were determined by CL-ELISA (Almeida et al, 1997). MaxiSorp Nunc polystyrene microplates, donkey biotinylated anti-mouse, NeutrAvidin-HRP, and SuperSignal Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. 2-mercaptoethanol-reduced maleimide-HSA (2-ME) was provided by Dr. Katja Michael, Chemistry Dept., UTEP. Cellastim S recombinant human serum albumin (rHSA) was purchased from InVitria

(Junction City, KS). HSA and LMPLA were obtained as previously described. Blood was collected via facial vein of each mouse 3 days prior to prime, three days after each immunization, post-immunizations, post-infection, and endpoints. Serum was separated from every blood collection by centrifugation (2,500 x *g*, 15 min, RT). Serum samples collected 3 days prior to prime, three days after each immunization, post-immunizations, and post-challenge were analyzed by CL-ELISA. Microplates were coated with 125 ng/well of recombinant-HSA, HSA, HSA + LMPLA, HSA + LMPLA, or 2-ME in CBC buffer, and incubated overnight at 4˚C. Microplates were blocked with 200 µL 5% skim milk for 1 h at 37ºC. Microplates were incubated with 50 µL mouse sera from vaccinated or control group at 1:100 dilution for 1 h at 37ºC. Donkey biotinylated anti-mouse (Thermo Fisher Scientific) was used at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP (Thermo Fisher Scientific) was used at 1:5000 dilution in 1% BSA/PBS-T. Microplates were washed 3x between steps with PBS-T, except before blocking. The reaction was developed with SuperSignal Pico Chemiluminescent Substrate (Thermo Fisher Scientific) at a 1:1:8 ratio in CBC Buffer/0.1%BSA. Luminescence in relative luminescence units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

Challenge

Six out nine female α1,3GalT-KO (C57BI/6) mice per group were challenged. We inoculated each mouse with $1x10^5$ TCTs of CL-Brener$_{Luc}$ clone (in 100 µL PBS) (i.p.), 3 weeks after boost 3.

Parasite Load

Parasite Load was measured between 4 and 21 dpi and 32 dpi (endpoint). Mice were injected intraperitoneally (i.p.) with 100 µl 15 mg/mL D-luciferin (Gold Biotechnology, St. Louis, MO)

and anesthetized with 2.5% gaseous isoflurane in oxygen. Images were acquired 10 min after luciferin injection using IVIS Lumina III *In Vivo* Imaging System (PerkinElmer, Waltham, MA).

Weight

To detect any sign of toxicity, animal weight was measured from 3 to 21 dpi, every three days (24, 27 and 30 dpi), and endpoint (32 dpi) in every mouse before imaging. Weight change was normalized using the mouse weight before challenge.

Harvest of organs

At experimental endpoint, mice were humanely euthanized by 30% CO_2 overdose. Heart, liver, lung, skeletal muscle, stomach, intestine, spleen, and colon were harvested at endpoint (32 dpi) of challenged mice and washed with PBS. The heart was divided in half (2 x 1/2); one half (1/2) was divided half (2 x 1/4). Spleen was divided in half (2 x 1/2). ½ of the heart was assigned for histopathology; ¼ of heart, for metabolomics; ¼ of heart and ½ of spleen for qPCR; and ½ of spleen for T cell response.

Immunoglobulin Isotyping

The specific levels of IgM, IgG (IgG1, IgG2a, IgG2b, and IgG3) and IgE antibodies for Galα3LN-3aL-NH-HSA + LMPLA group and Galα3LN-3aL-NH-HSA group were measured. Goat polyclonal Ab to IgG1, IgG2a, IgG2b, and IgG3, and rat mAb to IgE were purchased from Abcam. Serum samples collected 3 days prior to prime, three days after each immunization, post-immunizations, and endpoint were used and analyzed. Isotyping was analyzed by CL-ELISA with some modifications. Only Galα3LN-3aL-NH-HSA was incubated overnight at 4˚C. Microplates

were blocked with 200 µL PBS-5% skim milk. Donkey biotinylated anti-mouse was not used; instead, we used the antibodies at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP was not necessary. These antibodies already contain HRP.

Quantitative Real-Time PCR (qPCR)

Harvested tissue was initially weighted. For every 30-50 mg of tissue, except heart, we used 200 μl of PBS to blend the tissue with gentleMACS™ M Tubes by gentleMACS Dissociator (Miltenyi Biotec). Heart was about or less than 30 mg, so we added 200 μl of Tissue Lysis Buffer (TLB) and smashed it with a sterile blender stick. To extract Genomic DNA from tissue, we used all the blended heart and 120 μl of the other blended tissue. High Pure PCR Template Preparation Kit (Roche Molecular Systems, Indianapolis, IN) was used to extract DNA. We followed the manufacture's protocol with some modifications. When we added 40 μl proteinase K, and 5 μl internal amplification control (IAC). gDNA samples were placed in ice and measured protein via Nanodrop (Thermo Fisher Scientific). Based on the protein concentration by Nanodrop, we calculated 20 ng of sample in 50 μl Elution Buffer. Samples were stored at -20 ºC until use. Parasite load was measured by absolute quantification based on a standard gDNA curve ranging from 0.5 to 10^5 *T. cruzi* parasite equivalents/mL. A standard curve was produced by extracting gDNA from a 30 to 50 mg tissue fragment, spiked with 10^5 *T. cruzi* trypomastigotes (Duffy et al, 2009). Also, gDNA was extracted from 30 to 50 mg of tissue fragment from uninfected mice (negative control gDNA). Subsequently, spiked gDNA was 10-fold serially diluted in the negative control gDNA. Amplification of 120 bp-fragment from the kinetoplast DNA of *T. cruzi* was performed using 100 nM of forward primer, 100 nM of reverse primer, and 50 nM of TaqMan probe; a total of 100 ng of gDNA was added to a reaction in a final volume of 20 µL. The internal amplification control

(IAC) was amplified using 100 nM of forward primer, 100 nM of reverse primer, and 50 nM of TaqMan probe. PCR conditions consisted of 50°C for 2 min, 94°C for 10 min, followed by 45 cycles at 94°C for 15 sec and 55°C for 1 min. Samples were run in duplicate in Step One Plus Real Time PCR System (Applied Biosystems).

Histopathology

To test for cardiac inflammation and myocyte necrosis of the heart of mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA, Galα3LN-3aL-NH-HSA alone, or HSA + LMPLA, histopathology was performed. At endpoint, the heart was harvested and cut in two-halves. One-half was used for histopathology. Paraformaldehyde 4% was purchased from Alfa Cesar, sucrose was purchased from Sigma-Aldrich, Disposable Basle Molds 15x15x5mm and Tissue-Tek O.C.T. (optimum cutting temperature) were purchased from Fisher Healthcare, and Hexane was purchased from Fisher Scientific. One-half of heart from each mouse was placed in 1 mL. of 4% paraformaldehyde at 4°C for 24 h. Hearts were removed from 4% paraformaldehyde and placed in 1 mL of 15% sucrose at 4°C for 3 days or until the hearts were floating. Hearts were removed from 15% sucrose and placed in 30% Sucrose at 4°C for 1 week. Hearts were removed from 30% sucrose and placed in labeled disposable molds, covered with Tissue-Tek O.C.T., submerged in cold hexane for 10 seconds, submerged in liquid nitrogen for 12 seconds, placed in dry ice, left at -80°C for 1 h, wrapped in foil, and stored at -80°C until they were shipped. Histopathology was performed by Research Histology, Pathology & Imaging Core from the Virginia Harris Cockrell Cancer Research Center at The University of Texas MD Anderson Cancer Center.

α-Galactosidase Treatment

To test the specificity of the IgG antibodies elicited in mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA or Galα3LN-3aL-NH-HSA alone, we pre-treated the antigen on the microplate with α-galactosidase. Chagas anti-α-Gal antibodies (Ch anti-α-Gal Abs) were purified by Dr. Almeida's group, as previously described (Almeida et al, 1991). Chagas human serum pool (ChHSP) from patients with chronic ChD and normal human serum pool (NHSP), used as positive and negative controls, respectively, were from Dr. Joaquim Gascon (ISGlobal, Barcelona), and were prepared as previously described (Ashmus et al, 2013; Schocker et al, 2016). MaxiSorp Nunc polystyrene microplate, donkey biotinylated anti-mouse, NeutrAvidin-HRP, and SuperSignal Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. Green coffee bean α-galactosidase in ammonium sulfate suspension was purchased from Sigma-Aldrich. Briefly, microplate was coated with 125 ng/well of Galα3LN-3aL-NH-HSA in CBC buffer and incubated overnight at 4˚C. The microplate was blocked with 200 μL 5% skim milk-PBS for 1 h at 37˚C, and washed three times with 200 μL PBS-T. To prepare α-galactosidase treatment, 200 μL of green coffee bean α-galactosidase were centrifuged at 10,000 x g for 10 min at 4°C to remove the excess ammonium sulfate. The supernatant was discarded and the pellet containing the enzyme was gently re-dissolved in ice-cold 100 mM potassium phosphate buffer (pH 6.5). 50 μL of the enzyme solution (0.002 units/μL) were added to each well followed by 24 h incubation at 37°C. The microplate was washed twice with 200 μL PBS-T. The microplate was incubated with 50 µL mouse sera from vaccinated groups at 17 days post boost 3 (-4 dpi) at 1:100 dilution for 1 h at 37ºC. Donkey biotinylated anti-mouse was used at 1:2000 dilution in 1% BSA/PBS with 0.05% Tween 20 (1% BSA/PBS-T). NeutrAvidin-HRP was used at 1:5000 dilution in 1% BSA/PBS-T. Microplate was washed 3x between steps with PBS-T, except before blocking. The reaction was

developed with SuperSignal Pico Chemiluminescent Substrate at a 1:1:8 ratio in CBC Buffer/0.1% BSA. Luminescence in relative luminescence Units (RLU) was measured by Luminoskan luminometer (Thermo Fisher Scientific).

CD4+ and CD8+ T cell analysis by flow cytometry

We analyzed T cell response for vaccinated + challenged mice at endpoint by flow cytometry. For 100 mL of ACK lysis buffer (ammonium-chloride-potassium), we used 100 ml deionized water, 0.832 g NH_4Cl (ammonium chloride purchased from Sigma-Aldrich), 0.1g KHCO3 (potassium bicarbonate, Thermo Fisher Scientific), and 3.72 mg Na_2-EDTA (ethylene diamine tetra acetic acid, Thermo Fisher Scientific), pH 7.2-7.4, sterilized using Nalgene 0.2-μm filter; and stored at 4°C for use. Culture media was prepared freshly using Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin solution (Thermo Fisher Scientific). Complete medium was prepared freshly using culture medium and added 0.5 mM 2-mercaptoethanol (2-ME) (Sigma-Aldrich). Sodium azide (NaN_3) was purchased from Sigma-Aldrich. Paraformaldehyde 4% in PBS was purchased from Thermo Fisher Scientific. At endpoint (32 dpi), spleen was harvest from challenged mice (n=6 per group, depending of survival). Half of the spleen was used to collect splenocytes. Splenocytes were placed in 10 ml of ACK red blood cells lysis solution and rinsed with 10 mL of culture medium. Centrifuged at 1300 x g for 5 min at 4°C to pellet the cells. Supernatant was discarded, 3 mL of culture medium were added, 3 cryo tubes were used to store 1 mL of splenocytes in culture medium and stored at -80°C until use. Then, splenocytes were cultured in 12-well flat-bottom plates purchased from Thermo Fisher Scientific and stimulated in vitro with 20 μg/ml of Galα3LN-3aL-NH-HSA antigen at 37°C, in 5% CO_2 atmosphere, for 24 h. Fc-gamma receptor (FcγR) was blocked with 10% heat-

inactivated naïve α1,3GalT-KO mouse serum and cells were stained with fluorochrome-conjugated antibodies PE-Cy7-labeled anti-CD3e, PE-labeled anti-CD4, FITC-labeled anti-CD8, APC-labeled anti-CD44, and Alexa Fluor 700-labeled anti-CD69 (all conjugates were purchased from BD Bioscience), along with the appropriate isotype controls (BD Bioscience) for 30 min at 4°C. Cells were washed with PBS-1% BSA-0.09% sodium azide (NaN_3) and fixed with 1% paraformaldehyde. A total of 10,000 events were acquired using a Flow Cytometer Gallios (Beckman Coulter) and analyzed by Kaluza Software (Beckman Coulter). Gates were set for cells, followed by lymphocytes (CD3e-Pe-Cy7 labeling) using forward and side scatter properties, and the frequencies and percentages of activated CD4+ and CD8+ T cells were obtained on CD3+ T cells.

Quantification of Cytokines and Chemokines

Mouse Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay was purchased from EMD Millipore was used to quantify 32 analytes for mouse serum. Cytokines quantified were IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, and LIF. Chemokines quantified were CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, G-CSF, M-CSF, GMCSF, and VEGF. Sera (at 1:2 dilution) from all vaccination and control groups were analyzed at endpoint (32 dpi), following the manufacturer's protocol.

Lytic Antibody Assay

Lytic anti-α-Gal Ab assay (Almeida et al, 1994; Almeida et al, 1991; Pereira-Chioccola et al, 2000) was used to evaluate the ability of induced IgM and IgG anti-α-Gal Abs to kill the parasites. LLC-

MK2 cells were infected with 10^6 parasites/mL of TCT CL-Brener$_{Luc}$. At the 4th day after infection, parasites were incubated for 2 h 37˚C and 5% CO_2 with DMEM/0.2%BSA. We let the parasites swim for 1 h, pellet was accumulated at the bottom, and resuspended in 1 mL of DMEM/0.2%BSA for counting, 20 μL of sera from 10 days after boost 3 of mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA or Galα3LN-3aL-NH-HSA alone was added to 20 μL of parasites in a 1.5 mL Eppendorf tube (at antiserum dilutions of 1:2 and 1:20), and incubated for 30 min at 37˚C. After incubation, 10 μL of solution was loaded to a hemocytometer. All 16 squares of each quadrant were used to count live and dead parasites. The number of parasites for the 4 quadrants were counted, divided by 4, and multiplied by 10^4 to get the average of live and dead parasites.

Statistical Analysis

For parasitemia and weight, we examined longitudinal associations between changes in weight and parasitemia among groups using generalized nonlinear mixed-effects models. In the mixed models, the treatment group was included as a fixed effect and subjects were modeled as a random effect. We assumed the subject-specific random intercept and slope, to account for inter-subject variability of outcome and the subject-specific change in outcome associated with a unit-change in time. To find a functional relationship between time and outcome, time was re-expressed as a polynomial degree greater than one (e.g., quadratic and cubic) and we finally fitted parametric cubic curves assuming random intercept and random slope for subjects; cubic polynomials showed a significant improvement over the linear or quadratic models for both weight and parasitemia outcomes (p-values < 0.001). For models predicting weight data, we included additional assumption for the repeated measures of the normalized weight using a first-order autoregressive covariance structure (AR(1)).

For qPCR, α-galactosidase treatment, cytokines and chemokines profile, and T-cell response, we presented the data as average of either duplicate or triplicate determinations with their corresponding standard error of the mean (S.E.M.). Student t-test, One-way ANOVA, or Two-way ANOVA were employed in the statistical analysis. Graph Pad Prism 6 Software (GraphPad Software, Inc., La Jolla, CA) was used to graph and for statistical analysis.

Results

The fourth study showed that the carrier protein and the linker length are crucial parameters to be considered in the design of NGPs to be tested as experimental vaccines for ChD. C57BL/6 α1,3-GalT-KO mice vaccinated with Galα3LN-3aL-NH-HSA had much lower parasitemia and higher survival (100%) than other groups. This third study was designed to evaluate whether LMPLA can improve the potency of Galα3LN-3aL-NH-HSA. Timeline (**Figure 25**), experimental design (**Figure 26**), and experimental history (**Table 5**) are provided. Female C57BL/6 α1,3-GalT-KO mice groups (n=9) were vaccinated via s.c. with 20 μg of Galα3LN-3aL-NH-HSA per mouse, 10 μg of LMPLA per mouse, or 20 μg of HSA per mouse (control). Four immunizations at one-week intervals were given. Mice (n=3 per group) were euthanized before challenge.

To determine the production of IgG antibodies levels before challenge, serum samples were collected before prime, 3 days after each immunization and weekly after boost 3, but before challenge. All serum samples collected before challenge were analyzed by CL-ELISA. By then, we knew that blocking with PBS-1% BSA did not remove HSA background, and PBS-5% skim milk reduced HSA background. Therefore, we evaluated whether immobilizing with recombinant HSA vs. HSA provided, with or without LMPLA, and immobilizing with 2-ME-treated HSA would reduce the background (**Figure 27**). Immobilizing with rHSA, with or without LMPLA, showed lower levels of specific anti-α-Gal antibody titers. As expected, mice vaccinated with Galα3LN-3aL-NH-HSA or Galα3LN-3aL-NH-HSA + LMPLA showed high levels of specific anti-α-Gal Abs, and B cell-mediated immune response was earlier compared to control group.

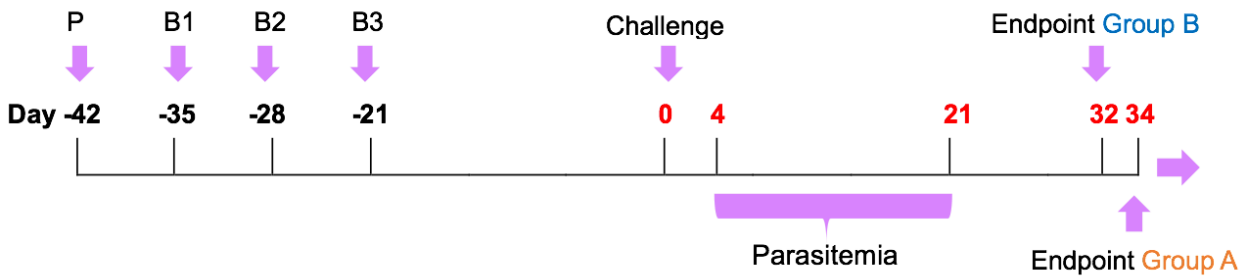

Figure 25. Fourth study's timeline. Four immunizations with Galα3LN-3aL-NH-HSA + LMPLA, Galα3LN-3aL-NH-HSA alone, or HSA + LMPLA were given at one-week interval. Challenged day became 0 dpi. Parasitemia at 4 to 21 dpi. Endpoint for vaccinated mice, but not challenged was on 34 dpi. Endpoint for vaccinated and challenged mice was on 32 dpi. CL-ELISA was performed before and after challenge. Isotyping, qPCR, α-Galactosidase treatment, and cytokines and chemokine profile, T cell response, lytic Assay were performed after 34 dpi. Vaccinations were prepared and administered by Brenda G. Zepeda (unpublished data).

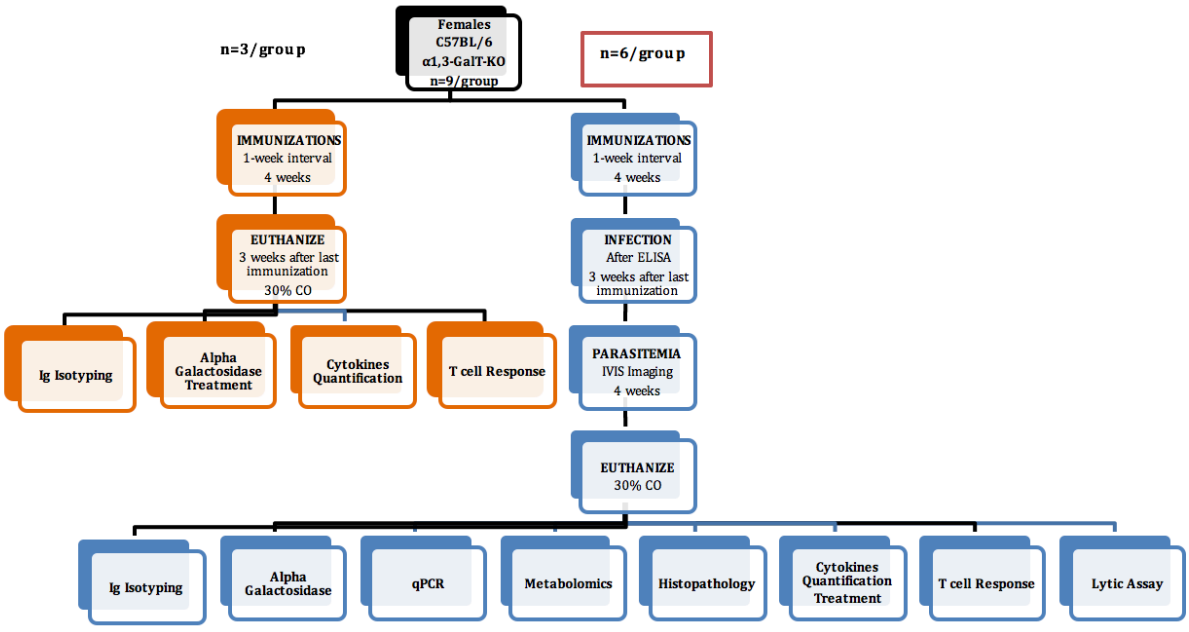

Figure 26. Experimental design for fourth study. All 9 mice per group were vaccinated; however, 6 mice were challenged.

Table 5. History of fourth study.

Procedure/Event	Day Post-Immunization	Day Post-Infection
Blood collection	Day -3	-45
Prime immunization	Day 0	-42
Blood collection	Day 3	-39
Boost 1	Day 7	-35
Blood collection	Day 10	-32
Boost 2	Day 14	-28
Blood collection	Day 17	-25
Boost 3	Day 21	-21
Blood collection	Day 24	-18
Blood collection	Day 31	-11
Blood collection	Day 38	-4
Challenge Group B with 10^5 CL Brener$_{Luc}$ TCTs	Day 42	DPI 0
Parasitemia (IVIS)	Day 45-63	DPI 3-21
Blood collection (Group B)	Day 60	DPI 18
Blood collection (Group A)	Day 66	
Endpoint (Group B)	Day 74	DPI 32
Endpoint (Group A)	Day 76	

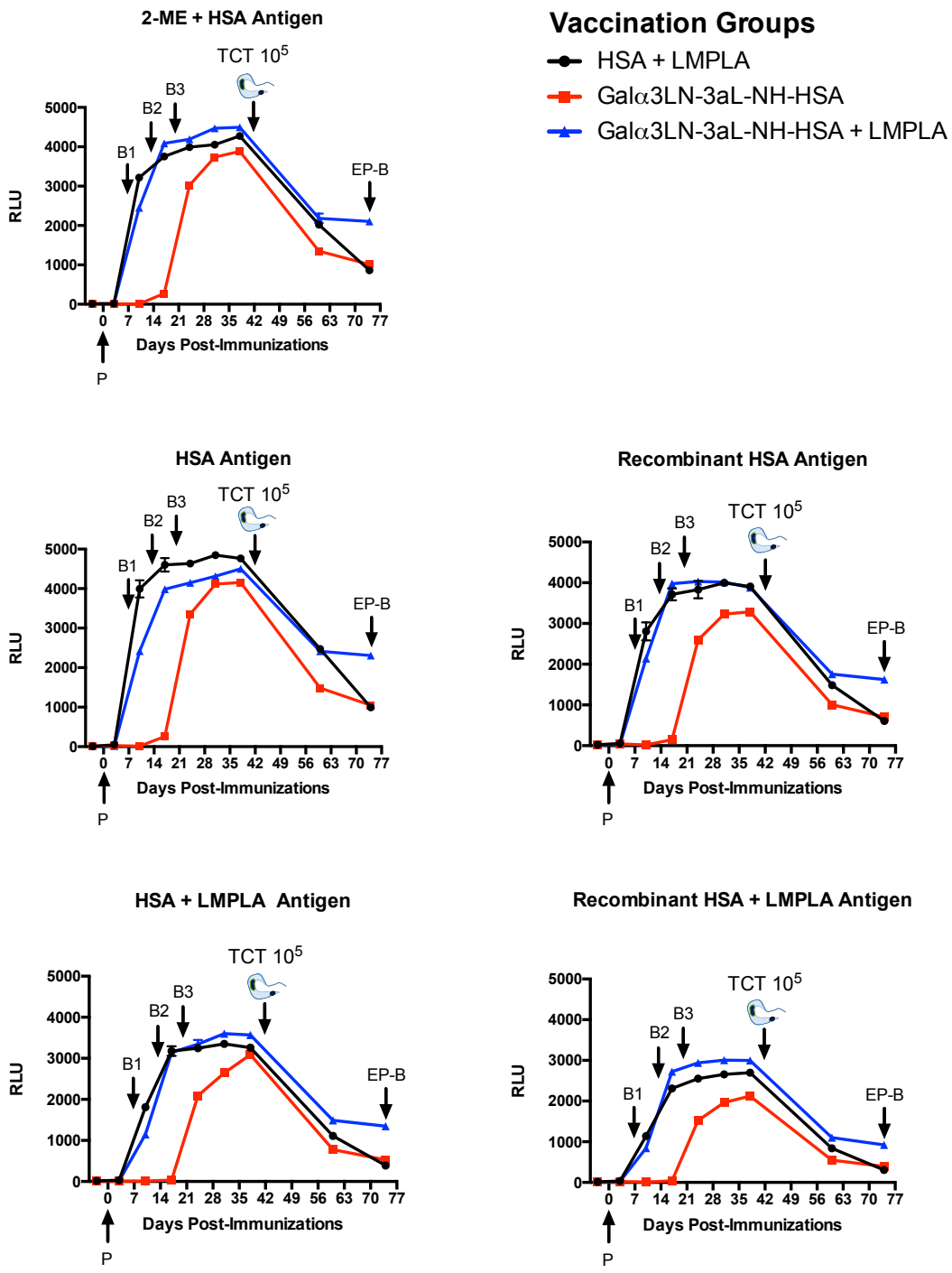

Figure 27. CL-ELISA blocked with PBS-5% skim milk. Levels of anti-α-Gal Abs using 2-ME + HSA, HSA, recombinant HSA, HSA + LMPLA, and recombinant HSA + LMPLA antigens. Data provided by Brenda G. Zepeda and Dr. Eva A. Iniguez, and graphed by Brenda G. Zepeda (unpublished data).

The remaining mice (n=6) per group were challenged 3 weeks after boost 3 to determine whether these mice were protected against *T. cruzi*. Challenge was carried out with 10^5 CL-Brener$_{Luc}$ TCTs. Parasitemia was measured from 4 to 21 dpi and 32 dpi (endpoint) using an in vivo imaging system (IVIS). Images of mice vaccinated with HSA + LMPLA control group showed much higher radiance compared to the other vaccinated groups (**Figure 28**); and therefore, higher parasitemia (**Figure 29**). Challenged animals vaccinated with HSA + LMPLA showed lower weight post-infection (**Figures 30 and 31**). At endpoint, heart, liver, lung, skeletal muscle, stomach, intestine, spleen, and colon were harvested of challenged mice and washed with PBS; and serum was also collected for future experiments. At endpoint, mice vaccinated with Galα3LN-3aL-NH-HSA group showed 100% survival, whereas Galα3LN-3aL-NH-HSA + LMPLA group showed 83% survival, and the HSA + LMPLA (control) group had only 33% survival (**Figure 32**).

All sera collected before challenge and at endpoint were analyzed by CL-ELISA using the same procedure mentioned above. Sera from endpoint showed decreased levels of anti-α-Gal Abs for all groups; however, mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA had higher levels compared to the other groups (**Figure 27**).

To determine the production of IgM, subclasses of IgG, and IgE antibody levels, immunoglobulin isotyping was performed using serum collected during and after vaccinations and at endpoint (**Figure 33**). Galα3LN-3aL-NH-HSA group had constant increment levels of IgM from prime immunization to endpoint. Galα3LN-3aL-NH-HSA + LMPLA group had constant increments from prime to B2, decreased at B3, and constant increments from B3 to endpoint. This suggests that Galα3LN-3aL-NH-HSA + LMPLA group could have received 3 instead of 4 immunizations. Levels of IgG1 and IgG2b were higher after vaccination and levels decreased after challenge; however, B cell-mediated immune response was earlier with mice vaccinated with

Galα3LN-3aL-NH-HSA + LMPLA. Levels of IgE were minimally detectable; therefore, these vaccines do not cause allergic or autoimmune reactions.

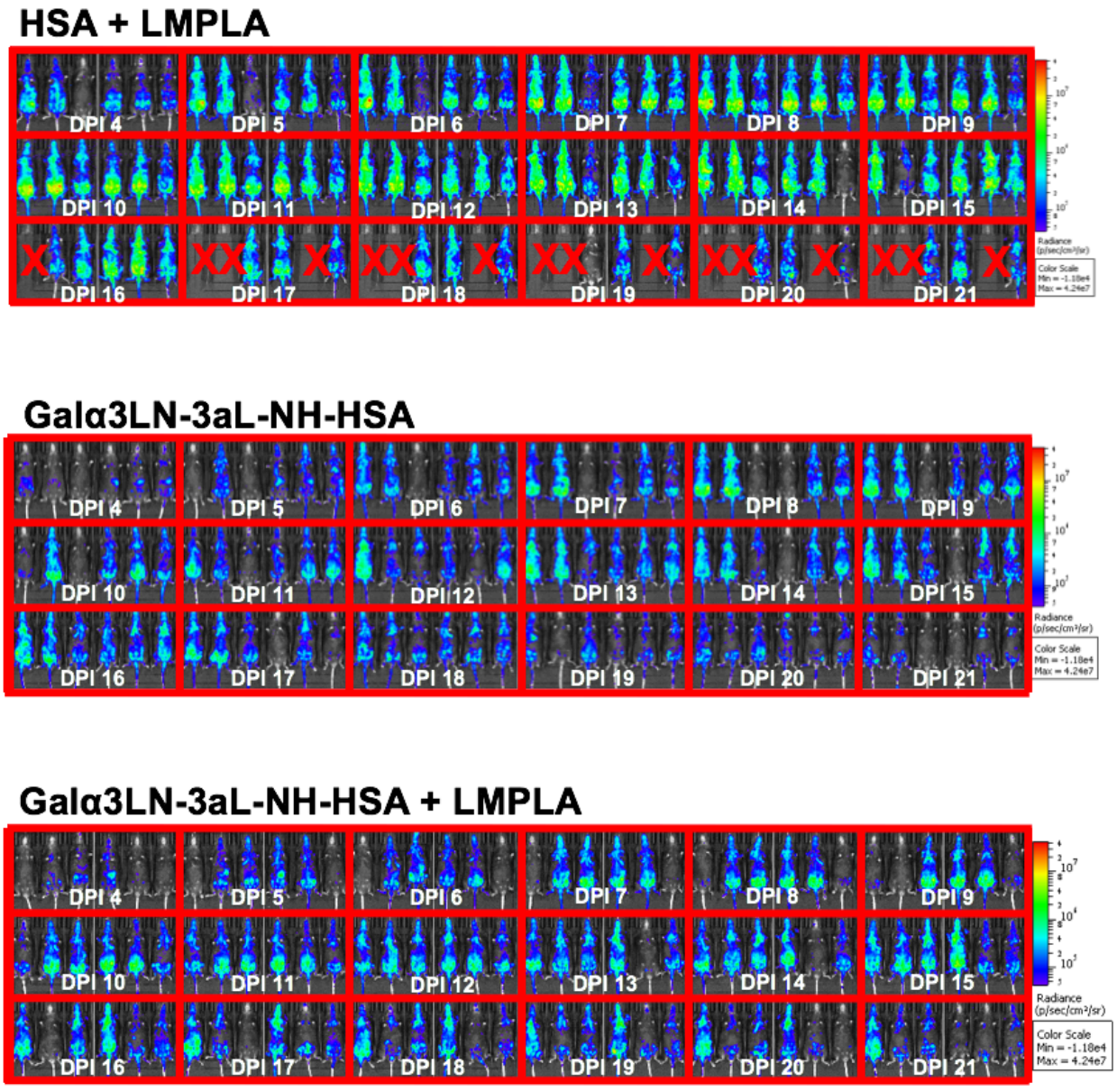

Figure 28. IVIS images. Six out of nine mice per group were challenged with 10^5 TCTs CL Brener strain expressing red-shifted luciferase (CL-Brener$_{Luc}$) parasite and analyzed using an in vivo imaging system (IVIS). Images provided by Brenda G. Zepeda (unpublished data).

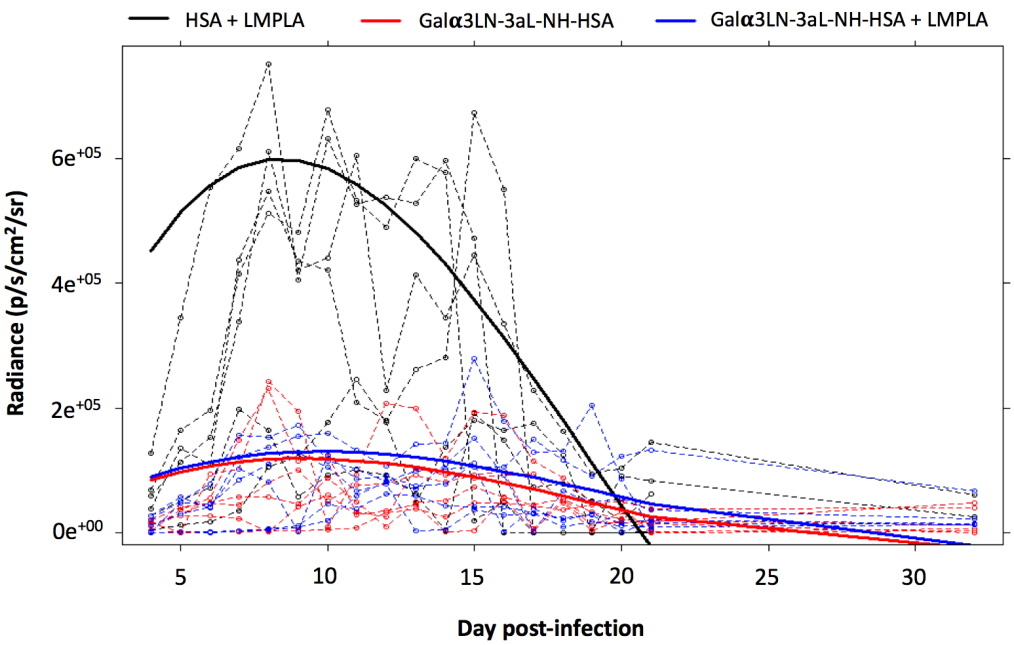

Figure 29. Fitted trend of parasite load using longitudinal mixed-effect models. Radiance of trypomastigotes at 4 to 21 dpi and 32 dpi using an in vivo imaging system (IVIS). Raw data provided by Brenda G. Zepeda and analyzed and graphed by Dr. Soyoung Jeon.

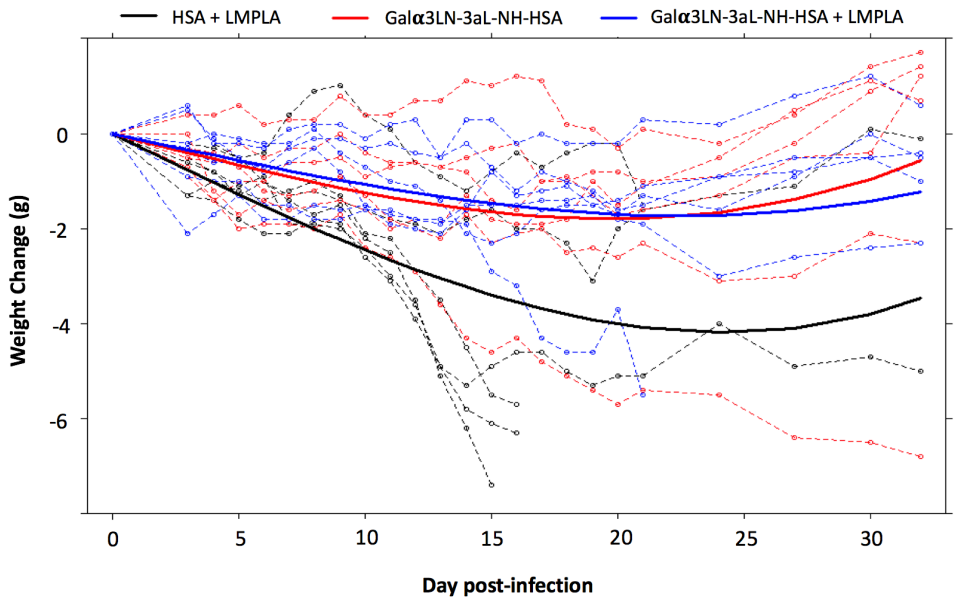

Figure 30. Fitted trend of normalized weight using longitudinal mixed-effect models. 0 dpi to 32 dpi weight of survival mice was measured. Raw data provided by Brenda G. Zepeda and analyzed and graphed by Dr. Soyoung Jeon.

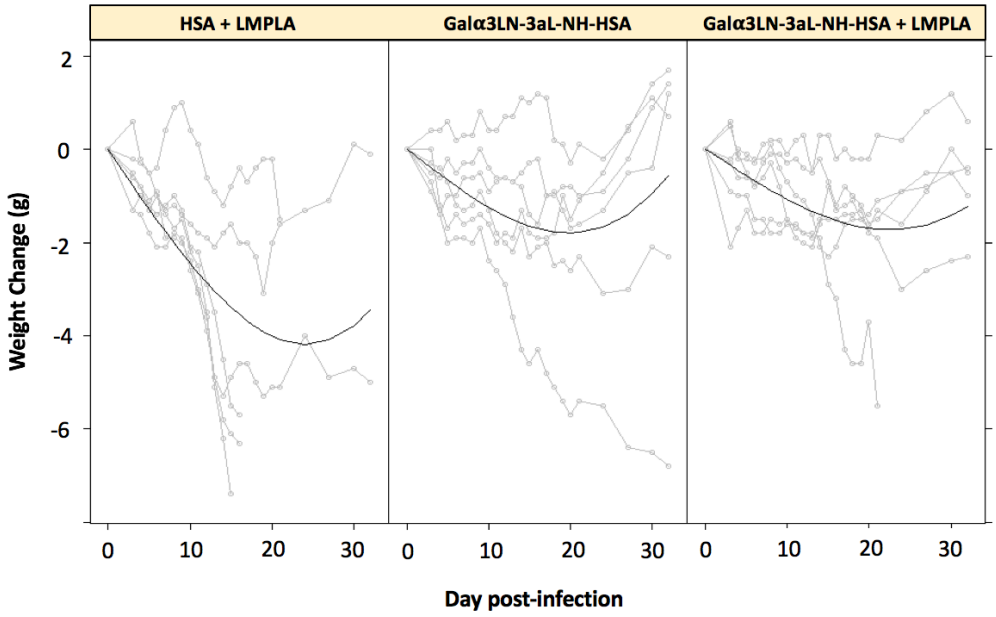

Figure 31. Fitted trend of normalized weight using longitudinal mixed-effect models (by vaccination group). Animal weight at 0 dpi to 32 dpi was measured. Raw data provided by Brenda G. Zepeda and analyzed and graphed by Dr. Soyoung Jeon.

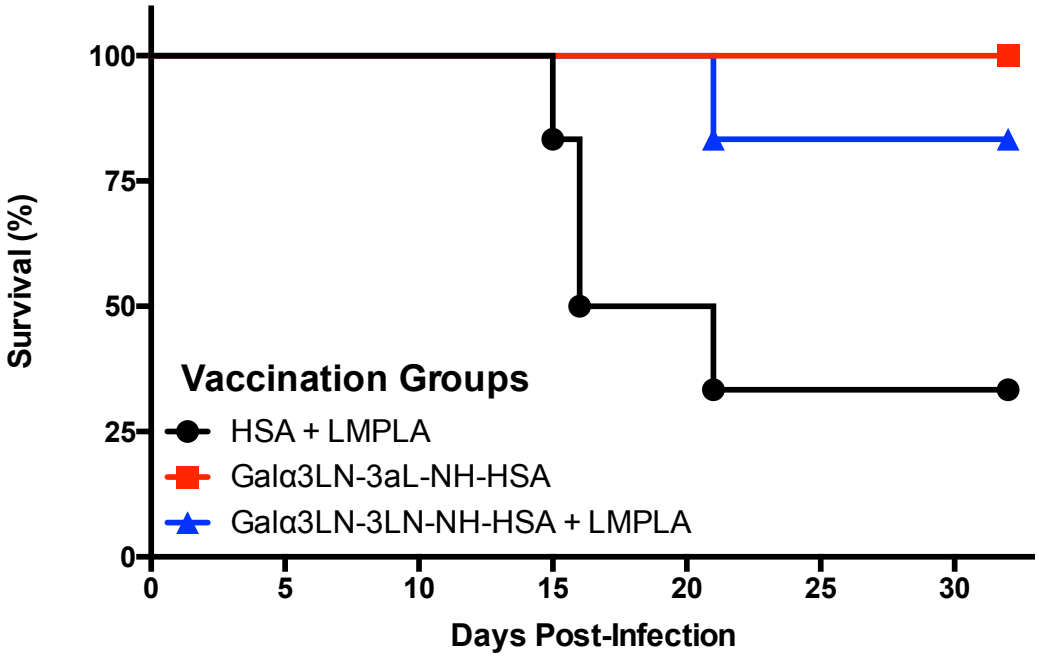

Figure 32. Survival of mice challenged with *T. cruzi* TCTs (CL-Brener$_{LUC}$ strain). Data provided and graphed by Brenda G. Zepeda.

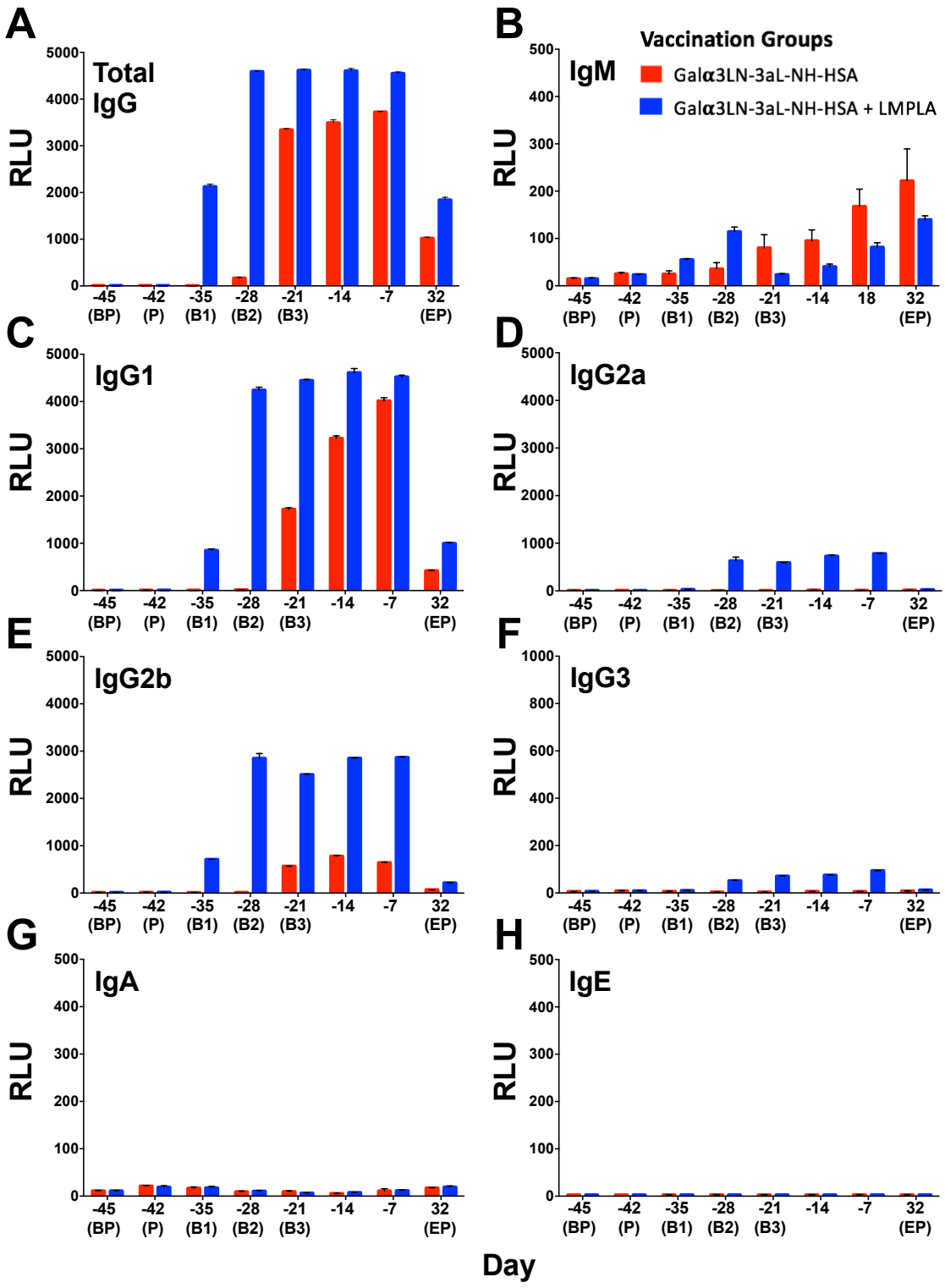

Figure 33. Isotyping. We measured the levels of IgG subclasses and IgE during and after vaccination and at endpoint (32 dpi) of animals vaccinated with Galα3LN-3aL-NH-HSA alone and Galα3LN-3aL-NH-HSA + LMPLA and parasite challenged. Data provided by Brenda G. Zepeda and Dr. Eva A. Iniguez, and graphed by Dr. Eva A. Iniguez (unpublished data).

To evaluate parasite load in the tissues harvested at endpoint, Real-Time Polymerase Chain Reaction (qPCR) was performed (**Figure 34**) using ¼ heart; ½ spleen; and whole liver, lung, skeletal muscle, stomach, intestine, and colon. Because every tissue has a different weight, the equivalent of parasite load 100 ng of tissue was calculated and graphed. Groups vaccinated with Galα3LN-3aL-NH-HSA and Galα3LN-3aL-NH-HSA + LMPLA showed >90% decrease of parasite load in all the harvested organs compared to HSA + LMPLA group (control). However, the two vaccine formulations did not provide 100% sterile protection. Thus, further modifications of vaccine formulation should be carried out in the future to try to achieve sterile protection.

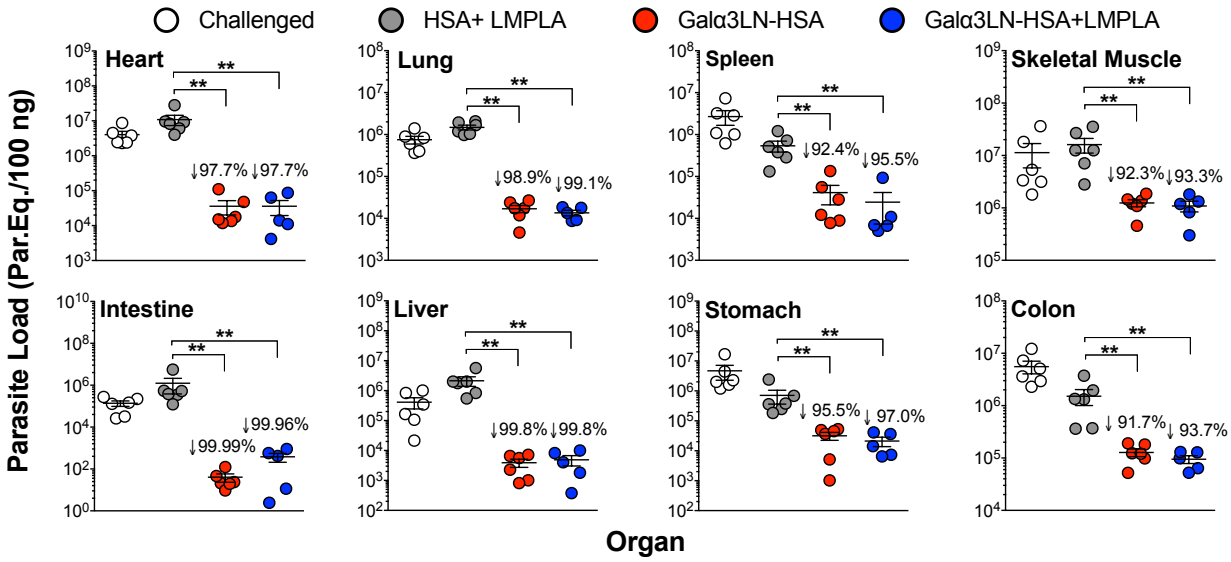

Figure 34. Quantitative real-time polymerase chain reaction (qPCR). Parasite load was significantly decreased in groups vaccinated with Galα3LN-3aL-NH-HSA alone and Galα3LN-3aL-NH-HSA + LMPLA compared to group vaccinated with HSA + LMPLA (control). Data provided by Brenda G. Zepeda and Susana Portillo and graphed by Susana Portillo (unpublished data).

To test for cardiac inflammation and myocyte necrosis of the heart of all mice, heart preparation was done at our facility and histopathology was performed (**Figure 35**) by Research Histology, Pathology & Imaging Core from The Virginia Harris Cockrell Cancer Research Center at The University of Texas MD Anderson Cancer Center. Histopathology shows significant reduction of cardiac inflammation in mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA and Galα3LN-3aL-NH-HSA alone, reduction of 82% and 69%, respectively. It also shows significant reduction of myocyte necrosis (~80%).

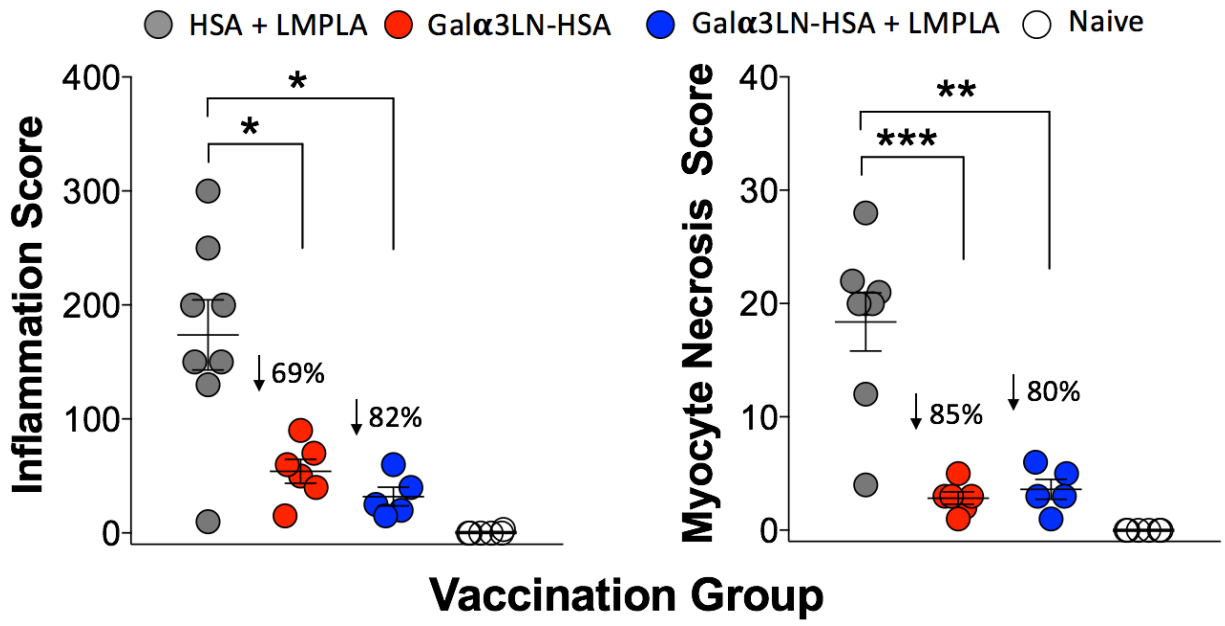

Figure 35. Histopathology of the heart. Heart of all mice were sectioned, placed in glass slide and analyzed for cardiac inflammation and myocyte necrosis.

To test the specificity of the IgG antibodies elicited in mice vaccinated with Galα3LN-3aL-NH-HSA + LMPLA and Galα3LN-3aL-NH-HSA alone, the antigen immobilized on the microplate was pre-treated with α-galactosidase to remove the terminal α-Gal residue (**Figure 36**). Purified Ch anti-α-Gal Abs and ChHSP were used as controls. Treatment with α-galactosidase significantly decreased (~70%) the IgG binding to the immobilized antigen, thus indicating that the majority of the reactivity was directed to the terminal α-Gal residue. The binding of the controls Ch anti-α-Gal Abs and ChHSP, decreased 85% and 58%, respectively.

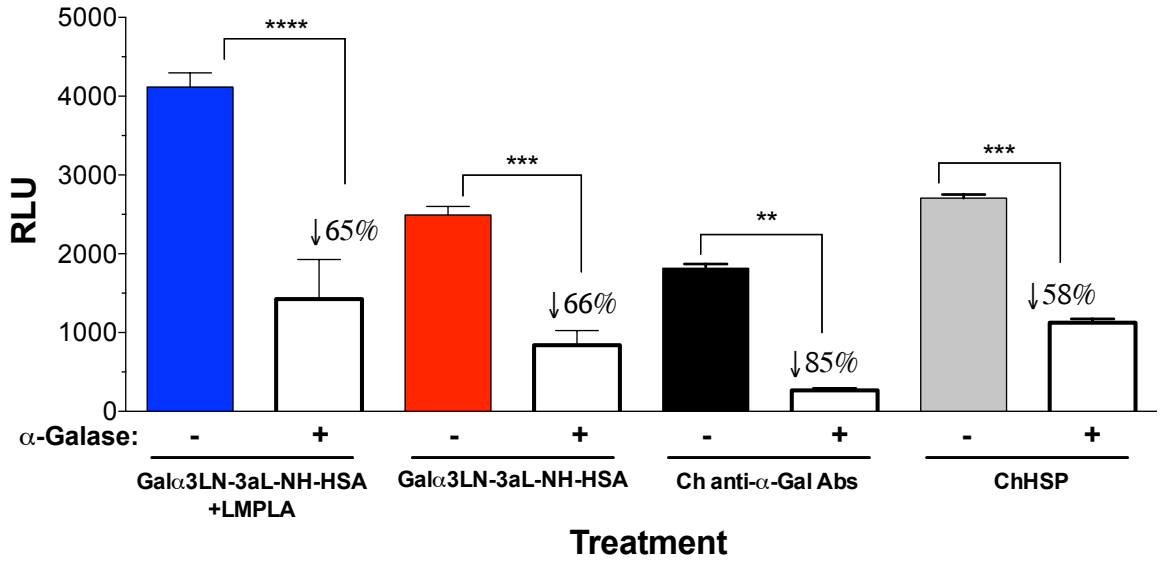

Figure 36. α-Galactosidase treatment. Galα3LN-3aL-NH-HSA + LMPLA, Galα3LN-3aL-NH-HSA alone, Ch anti-α-Gal, and ChHSP were treated with the enzyme α-Galactosidase to hydrolyze the α-Galactosyl terminal of the antigens, and to show specificity to the terminal. Data provided and graphed by Dr. Eva A. Iniguez and Susana Portillo (unpublished data).

To analyze $CD4^+$ and $CD8^+$ T cell response and $CD45^+$ B cell response for only vaccinated mice and for vaccinated + challenged mice at endpoint, we analyzed splenocytes by flow cytometry (**Figure 37**). Splenocytes were collected from ½ of the harvested spleen of each survival mouse. We added a naïve group to compare our results. T helper cell (T_h cell) a/k/a $CD4^+$ T cell releases cytokines during the adaptive immune response; helps with the maturation of B cells to plasma cells and memory B cells; and activation of cytotoxic T cells and microphages (Gutcher & Becher, 2007). Levels of $CD4^+$ T cells were higher for mice vaccinated with Galα3LN-3aL-NH-HSA alone and Galα3LN-3aL-NH-HSA + LMPLA compared to naïve; however, once mice were challenged, Galα3LN-3aL-NH-HSA + LMPLA showed higher levels of $CD4^+$ T cells with or without antigen stimulation with the parasite. $CD44^+$ T cells are central and effector memory T cells found in the lymph nodes and peripheral circulation (Willinger et al, 2005). Levels of $CD4^+$ $CD44^+$ T cells were higher from vaccinated mice compared to naïve after antigen stimulation with parasite. Cytotoxic $CD8^+$ T cells destroy infected cells by intracellular pathogens. Levels of CD8+ T cells were higher for mice only vaccinated and for mice vaccinated + challenged compared to naïve. $CD8^+$ $CD44^+$ T cells decreased for all groups. B220 (CD45R) is found in all developmental stages from pro-B cells through mature B cells. Levels of $B220/CD45R^+$ was higher with mice vaccinated with Galα3LN-3aL-NH-HSA. This experiment shows activation of $CD4^+$ and $CD8^+$ T cells after vaccination and after challenge. However, only memory $CD4^+$ T cells ($CD4^+$ $CD44^+$) were present after challenge.

To identify the inflammatory proteins realized by the cells after they encounter vaccination with Galα3LN-3aL-NH-HSA + LMPLA and Galα3LN-3aL-NH-HSA, and after they encounter the parasite, we quantified of cytokines and chemokines using 32 analytes in a magnetic bead panel (**Figures 38 and 39**). Galα3LN-3aL-NH-HSA + LMPLA showed higher levels of cytokines (IFN-

γ, TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, and LIF) compared to Galα3LN-3aL-NH-HSA alone. Galα3LN-3aL-NH-HSA + LMPLA showed higher levels of some chemokines (CCL2, CCL3, CXCL1, CXCL2, CXCL5, CXCL10, GM-CSF, and VEGF) compared to Galα3LN-3aL-NH-HSA alone. Galα3LN-3aL-NH-HSA alone showed higher levels of some chemokines (CCL4, CCL5, CXCL9, and M-CSF) compared to Galα3LN-3aL-NH-HSA + LMPLA. About the same level of CCL11 and G-CSF chemokines was observed. Most of the observed differences in specific cytokines and chemokines are statistically significant. Proinflammatory cytokines tend to make the disease worse by causing fever, inflammation, tissue destruction, and even shock and death (Dinarello, 2000). We can assume that Galα3LN-3aL-NH-HSA + LMPLA produced more of these proinflammatory cytokines and chemokines than Galα3LN-3aL-NH-HSA alone and, therefore, did not provide 100% mouse survival.

To evaluate the ability of induced IgM and IgG anti-α-Gal Abs to kill TCT CL-Brener$_{Luc}$, we performed a lytic antibodies assay (**Figure 40**). Parasites were incubated with the sera of vaccinated mice at 10 days post boost 3, normal mouse serum (NMS, negative control), at antiserum dilutions of 1:2 and 1:20 1:20. Ch anti-α-Gal antibodies (positive control) was used at 20 μg/mL. We also used for controls live (incubated with medium alone) and dead (killed by 4% paraformaldehyde) parasites. Following 2 h of incubation, 94% parasites were alive with the negative medium alone (negative control 1); 96% parasites were alive with NMS (negative control 2); 100% paraformaldehyde-treated parasites were dead (positive control 1); 65% parasites were dead with Ch anti-α-Gal Abs (positive control 2); 42% and 27% parasites were dead with 1:2 and 1:20 dilution of antisera against Galα3LN-3aL-NH-HSA; and 40% and 19% parasites were dead with 1:2 and 1:20 dilution, respectively, with antisera against Galα3LN-3aL-NH-HSA + LMPLA.

This experiment showed that after 2 h of incubation, both vaccine formulations induced complement-independent lysis in a dose-dependent manner, as previously observed for human Ch anti-α-Gal Abs (Almeida et al, 1991; Pereira-Chioccola et al, 2000)

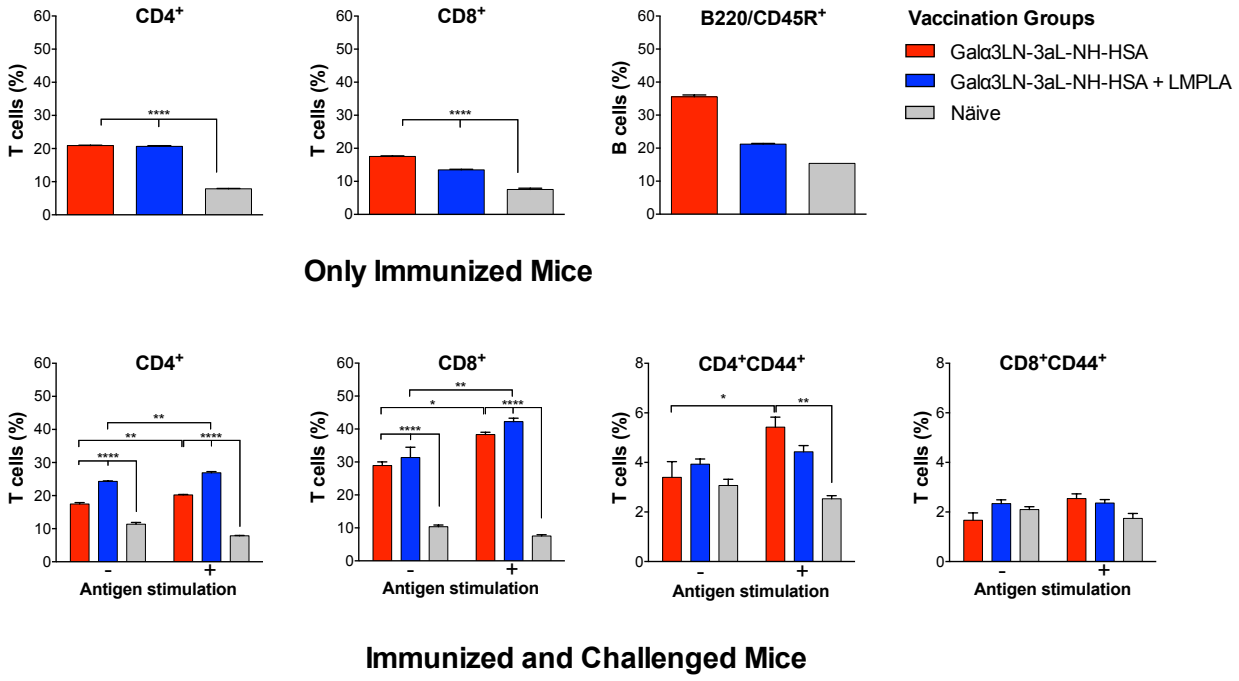

Figure 37. CD4+ and CD8+ T cell response. T cell response and B cell marker were analyzed by flow cytometry for only vaccinated mice and for vaccinated + challenged mice at endpoint. Data provided and graphed by Dr. Eva A. Iniguez and Susana Portillo (unpublished data).

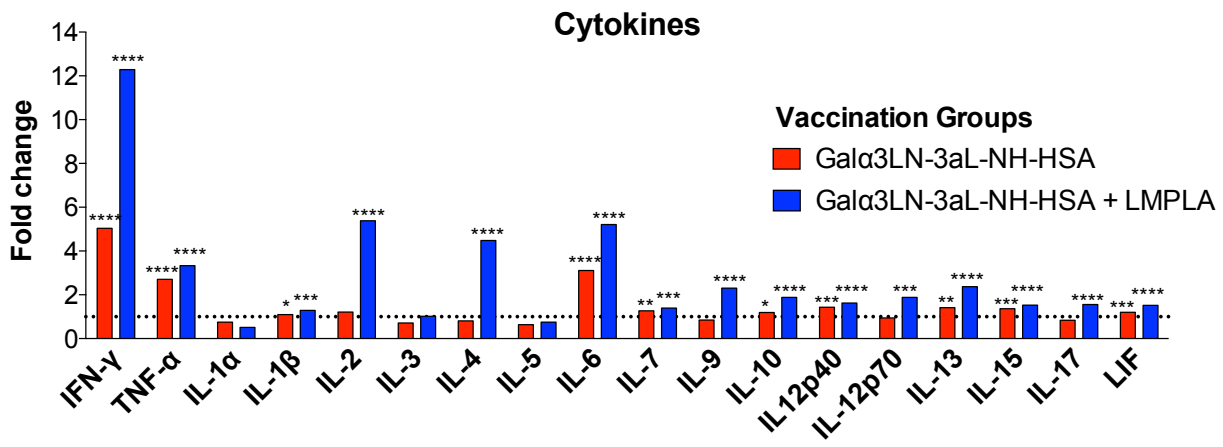

Figure 38. Quantification of cytokines. Galα3LN-3aL-NH-HSA + LMPLA showed higher levels of cytokines compared to Galα3LN-3aL-NH-HSA alone. Fold change was calculated by the ratio of [cytokine concentration of the vaccinated group] / [cytokine concentration of the naïve animal]. Data provided and graphed by Dr. Eva A. Iniguez and Susana Portillo (unpublished data).

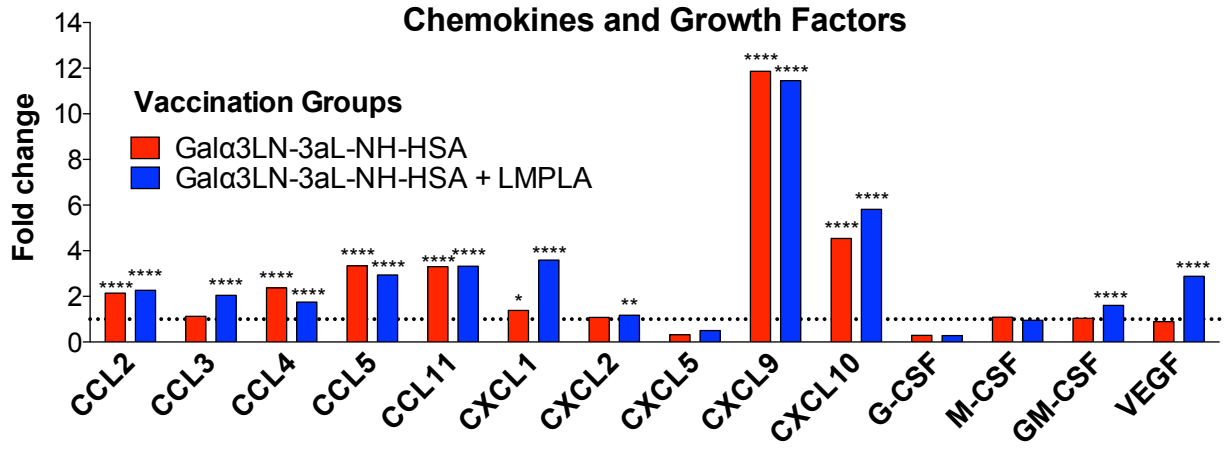

Figure 39. Quantification of chemokines and growth factors. Fold change was calculated by the ratio of [cytokine concentration of the vaccinated group] / [cytokine concentration of the naïve animal]. Data provided and graphed by Dr. Eva A. Iniguez and Susana Portillo.

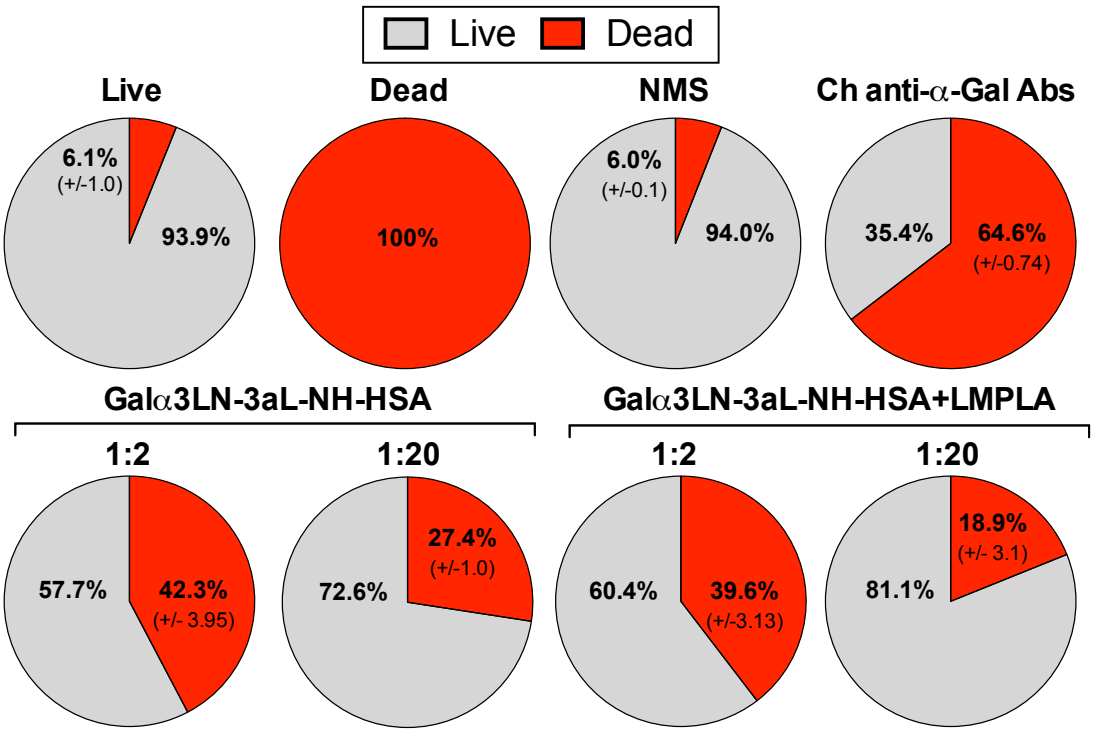

Figure 40. Lytic antibody assay. CL-Brener$_{Luc}$ TCTs were incubated with sera of immunized mice from 10 days after boost 3. Data provided and graphed by Dr. Eva A. Iniguez and Susana Portillo (unpublished data).

Metabolomics and histopathology are experiments that will be carried out soon to assess metabolites and inflammation in the heart tissue. This study shows that vaccination with Galα3LN-3aL-NH-HSA alone and Galα3LN-3aL-NH-HSA + LMPLA had much lower parasitemia, higher weight post-infection, > 90% reduction of parasite loan in tissues; ~70% of the IgG antibodies produced to either vaccine formulation are specific to the nonreducing, terminal αGal residue; high levels of IgG1 and IgG2b after vaccination, and no detectable levels of IgE; activation of $CD4^+$, $CD8^+$, and B220; induction of memory $CD4^+$ T but not $CD8^+$ T cells; and production of complement-independent trypanolytic antibodies. Finally, the Galα3LN-3aL-NH-HSA vaccine alone, without adjuvant, elicited a profile of proinflammatory and regulatory cytokines and chemokines conducive to protection against *T. cruzi* challenge, and thus 100% survival rate.

Discussion

Nowadays, there is no preventive or therapeutic human vaccine for this Chagas disease. Benznidazole and nifurtimox are outdated and partially effective chemotherapeutic drugs and cause high rate of adverse effects. *T. cruzi* is protected by a highly complex carbohydrate-rich surface coat, composed of glycoproteins and glycolipids, which serves as a barrier to host immunological defenses. Newer experimental vaccine approaches use *T. cruzi trans*-sialidase (TS), amastigote surface protein 2 (ASP-2), trypomastigote surface antigen-1 (TSA-1), and Tc24 (flagellar calcium-binding protein) as antigens or genes to be delivered through vaccination with DNA plasmid, recombinant adenovirus, or recombinant protein. These vaccines show high protection to the mice; however, perforin-mediated cytotoxic activity may be different across different strains of *T. cruzi* (dos Santos Virgilio et al, 2014), and it is not clear how conserved these epitopes form TS, ASP-2, TSA-1 and Tc24 are among the six different parasite genotypes (Discrete Type Units [DTUs] TcI-VI), strains and isolates (Zingales et al, 2012).

Trans-sialidase (TS) is a protein that transfers sialic acid from the host cell to a receptor on the parasite surface allowing the parasite to escape from being lysed. Therefore, the parasite can invade mammalian host cells. Amastigote, a noninfective form of *T. cruzi*, expresses surface proteins such as amastigote surface protein 2 (ASP-2). This protein is a member of the TS superfamily of surface proteins of *T. cruzi* (Vasconcelos et al, 2012), and it has been used to target $CD8^+$ cytotoxic T cells (Low et al, 1998). In a study, a plasmid DNA (pIgSPC1.9) and a human replication-defective adenovirus type 5 expressing ASP-2 (AdASP-2) of *T. cruzi* were used. A group of mice received a prime immunization with pIgSPC1.9 followed by a booster with AdASP-2. Other groups of mice received one or two immunizations of AdASP-2. Mice immunized with one or two immunizations of AdASP-2 showed lower parasitemia compared with the control

group; however, it was much lower for mice vaccinated with pIgSPC1.9 (prime) and AdASP-2 (boost). All three groups that received AdASP-2, survived longer (over 220 dpi) compared to the control groups. However, pIgSPC1.9 (prime) and AdASP-2 (boost) group showed higher survival rate (80%) (de Alencar et al, 2009). In another study, a chimeric gene encoding ubiquitin fused to ASP-2 (pUB-ASP-2) was constructed. Mice immunized with pUB-ASP-2 showed lower parasitemia, longer survival and activation of $CD8^+$ T cells compared to mice immunized with pASP-2 alone (Chou et al, 2010).

T. cruzi 24kDa antigen (Tc24) is a flagellar calcium-binding protein that neutralizes the parasite to evade the immune the immune system. In a recent study, Tc24 was combined with the adjuvant monophosphoryl-lipid A (MPLA) showed increased levels of IgG2a and IFNγ and decreased levels of parasitemia after challenge. Also, in this study, protein aggregation was observed due to intermolecular disulfide bond formation. Therefore, they replaced cysteine codons with serine codons resulting Tc24-C4. Mice were immunized with Tc24 or Tc24-C4 in combination with the adjuvant E6020, which is another TLR4 agonist. Results show similar levels of IgG2a and IFNγ; therefore, intermolecular disulfide bonds did not cause any significant effect on the molecule. Mice vaccinated with both vaccines showed increased survival and decrease of parasite load in cardiac tissue (Biter et al, 2018).

T. cruzi trypomastigote surface antigen 1 (TSA1) has been used to induce CD^+8 T cells. Studies were performed using TSA1 in combination with Tc24, which is a flagellar calcium-binding protein that neutralizes the parasite to evade the immune the immune system. Mice vaccinated with Tc24-TSA 1 DNA vaccine during the acute phase of the disease showing about 75% reduction of parasitemia, about 60% reduction of parasite load in cardiac tissue, and reduction of cardiac pathology at endpoint compared to unvaccinated mice (Dumonteil et al, 2004; Limon-

Flores et al, 2010; Sanchez-Burgos et al, 2007). In other studies, mice have been vaccinated with the same combination, Tc24-TSA 1 DNA vaccine, during the chronic phase of the disease showing 80% survival and reduction of cardiac pathology at 180 days post-infection (Dumonteil et al, 2004; Pereira et al, 2015; Sanchez-Burgos et al, 2007). In a study, *trans*-sialidase-based immunogen (TSf) has been used to immunize mice. After infection, mice showed TS-specific antibody response, significant delayed-type hypersensitivity (DTH) reactivity, production of INF-γ, low parasitemia, and 90% survival (Prochetto et al, 2017).

Despite their high abundance on the parasite cell surface, very few experimental vaccines are direct against parasite on glycoconjugates, such as mucins, MASPs, and TS/gp85 glycoproteins. A major and immunodominant epitope on *T. cruzi* trypomastigotes is the terminal α-Gal, which is found on *O*-glycans of tGPI-mucins (Almeida et al, 1994). Specific anti-α-Gal antibodies are highly abundant in chronic ChD patients and they kill infective trypomastigote forms, thus controlling the parasitemia in chronic phase of the disease. In contrast to all other mammals, humans and Old-World nonhuman primates do not express terminal α-Gal residues (Galili, 2017; Galili & Swanson, 1991). For this reason, α-Gal-containing epitopes have been proposed as potential vaccine candidates for Chagas disease (Schocker et al, 2016) and other kinetoplastid infections such as leishmaniasis (Iniguez et al, 2017). However, glycan-based vaccines are hampered by technical difficulties in structurally characterizing and chemically synthesizing glycans, and lack of appropriate animal model that can mimic humans in their immune response to parasite-derived glycans. To this end, α1,3-galactosyltransferase-knockout (α1,3-Gal-KO) mouse model, in contrast to wild-type mice, do not express terminal α-Gal epitopes on their cells and tissues (Tearle et al, 1996; Thall et al, 1996) and, therefore, represent a perfect experimental model for the evaluation of α-Gal-based vaccines in the context of ChD. In this

current study, we used this mouse model to evaluate the immune response to synthetic α-Gal-based neoglycoprotein vaccines, in the presence or absence of an adjuvant, MPLA, which has been approved for use in humans.

Here, in our first study, we evaluated a commercial NGP Galα(1,3)Galβ(1,4)GlcNAcβ (Dextra Labs) conjugated to BSA via a 3-atom linker (Galα3LN-3aL-BSA). α1,3-GalT-KO mice were vaccinated and showed 100% survival after three consecutive parasite challenges with 10^4, 10^5, and 10^6 infective trypomastigotes, respectively. However, after immunosuppression, we only had 25% survival with Galα3LN-3aL-BSA.

In our second study we evaluated an NGP with a 13-atom linker between Galα(1,3)Galβ(1,4)GlcNAcα and the BSA moiety (KM24b) (Schocker et al, 2016). We compared the efficacy of the KM24b vaccine candidate, in the presence or absence of LMPLA as adjuvant. Mice were vaccinated subcutaneously with KM24b alone, KM24b + LMPLA, and BSA + LMPLA (control). Mice vaccinated with KM24b + LMPLA showed 100% survival, whereas animals vaccinated with BSA + LMPLA had a 75% survival. Surprisingly, the group immunized with KM24b alone showed a much lower (25%) survival rate. By CL-ELISA, KM24b + LMPLA showed higher levels of specific anti-α-Gal antibodies, and a rapid and strong B cell-mediated immune response, which was expected using an adjuvant. The main differences between the Dextra's Galα(1,3)Galβ(1,4)GlcNAcβ-BSA, used in the previous study, and KM24b used for the first study were: **(a)** the anomeric configuration of the reducing-end GlcNAc residue (α vs. β), and **(b)** and length of the spacer (3-atom spacer in the Dextra's Galα(1,3)Galβ(1,4)GlcNAcβ-BSA vs. 13-atom spacer in the KM24b). We then proposed to vaccinate α1,3-GalT-KO mice with αGal-containing NGPs with different anomeric configurations in the GlcNAc residue, linkers with different lengths, and distinct carrier proteins.

Therefore, in our third study, because Dextra's Galα3LN-3aL-BSA has a 3-atom linker whereas KM24b a 13-atom linker, we vaccinated α1,3-GalT-KO mice with different linker lengths to evaluate whether the linker had any influence in the vaccine candidate. We also evaluated whether the carrier protein (BSA vs. HSA) could affect the vaccine efficacy. Thus, Galα3LN-3aL-BSA, Galα3LN-3aL-NH-HSA, Galα3LN-14aL-BSA, and Galα3LN-14aL-HSA were evaluated as vaccine candidates. PBS, BSA and HSA were used as placebo controls. Mice vaccinated with Galα3LN-3aL-NH-HSA showed 100% survival, whereas animals vaccinated with Galα3LN-3aL-BSA, Galα3LN-14aL-BSA, Galα3LN-14aL-HSA, and HSA (control) showed 66% survival. The other two controls showed much lower survival rate, with BSA (control) with a survival rate of 33% and PBS with 0% survival. CL-ELISA (Almeida et al, 1997) was performed using the serum collected during and after immunizations and endpoint. Both groups of mice vaccinated with Galα3LN-14aL-BSA and Galα3LN-14aL-HSA showed high levels of specific anti-α-Gal Abs. Immunoglobulin isotyping was also performed and showed that, following vaccination, all NGPs induced high levels of IgG1 and IgG2b, which usually have lytic properties (Stefani et al, 1983), inducing antibody dependent cell-cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (InvivoGen, 2011). We noticed that IgG1 and IgG2b levels decreased after infection. On the other hand, we could only detect very low level of IgE, which may cause allergic reactions and autoimmunity (Hostoffer & Joseph, 2018) and it is not desirable in any vaccine candidate. This study shows that the carrier protein and the linker length are crucial parameters to be considered in the design of NGPs to be tested as experimental vaccines for ChD.

Based on the results of our third study, for our fourth study, we investigated whether an adjuvant such as LMPLA (Matyas et al, 2003) could influence the immune response elicited by Galα3LN-3aL-NH-HSA, increasing the protection against *T. cruzi*. Thus, α1,3-GalT-KO mice

were vaccinated intraperitoneal (i.p.) with Galα3LN-3aL-NH-HSA (Dextra), Galα3LN-3aL-NH-HSA combined with LMPLA, and HSA + LMPLA (control). Mice were then challenged (i.p.) with 10^5 TCTs (CL-Brener$_{Luc}$ strain). Mice vaccinated with Galα3LN-3aL-NH-HSA showed 100% survival, whereas immunized with Galα3LN-3aL-NH-HSA plus LMPLA showed 83.3% survival. On the other hand, the group vaccinated with HSA + LMPLA (control) showed only 33.3% survival rate. Animals vaccinated with Galα3LN-3aL-NH-HSA showed increment levels of IgM, which is the is the first antibody in the response to an initial exposure of an antigen (Alberts, 2002). Galα3LN-3aL-NH-HSA and Galα3LN-3aL-NH-HSA + LMPLA showed high levels of IgG1 and IgG2b; and minimal detectable levels of IgE. Significant parasite load reduction (>90%) in harvested organs (heart, spleen, liver, intestine, skeletal muscle, colon, was detected by real-time polymerase chain reaction (qPCR) in animals immunized with Galα3LN-3aL-NH-HSA and Galα3LN-3aL-NH-HSA + LMPLA. αGalactosidase is an enzyme that hydrolyses the terminal alpha-galactosyl from NGPs (Calhoun et al, 1985). Treatment with αGalactosidase was performed and showed that about 70% of IgG antibodies are specific to the αGal residue of the Galα3LN-3aL-NH-HSA and Galα3LN-3aL-NH-HSA + LMPLA antigens.

Quantification of cytokines (IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, and LIF) and chemokines (CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, G-CSF, M-CSF, GM-CSF, and VEGF) showed that Galα3LN-3aL-NH-HSA + LMPLA produced more than enough cytokines and chemokines and therefore did not provide 100% mice survival. T and B cell response was analyzed by flow cytometry and showed activation of $CD4^+$ and $CD8^+$ T cells after vaccination and after challenge. However, only memory $CD4^+$ T cells ($CD4^+$ $CD44^+$) were present after challenge. It also showed activation of B cells after vaccination. Lytic assay showed that after 2 h

of incubation, parasites died faster with Galα3LN-3aL-NH-HSA alone and when serum is less diluted.

Together, these three studies showed that vaccination with Galα3LN-3aL-NH-HSA alone results in lower parasitemia; high and early B cell response; high levels of IgG1 and IgG2b and no detectable levels of IgE; high weight after infection; 100% survival; more than 90% reduction of parasite load in organs; produced IgG antibodies are specific to the αGal residue of the antigens; enough T cell, cytokine and chemokine response; and high lytic antibodies. Therefore, LMPLA was not able to improve the efficacy of Galα3LN-3aL-NH-HSA. We had hypothesized that we can improve the efficacy of the αGal-containing vaccine by incorporating an adjuvant. LMPLA improved the efficacy of KM24b (14-atom linker), but not Galα3LN-NH-HSA (3-atom linker).

We also hypothesized that the length of the linker between the αGal-glycan and the carrier protein (BSA or HSA) might affect the vaccine efficacy. On this regard, 3aL-HSA alone was recognized as the most effective vaccine candidate. Therefore, we incorporated LMPLA to 3aL-HSA, but we were not successful. The NGP 3aL-HSA reduced more than 90% of parasite load in tissue; however, did not provide sterile protection.

For future projects, Dr. Almeida's group could use different lengths of the linker between αGal-NGP and the carrier-protein with LMPLA, the number of immunizations could be reduced, and different adjuvants could be incorporated to the candidate vaccine. Moreover, the vaccine and adjuvant dose could be reduced or increased. We also believe that monitoring of the animals could be carried out for a longer time.

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Curriculum Vita

From 1999 to 2002, I attended Ysleta High School and graduated as one of the Top 10% students. From 2002 to 2004, I attended El Paso Community College (EPCC) and was in the Honors Program. From 2004 to 2008, I served the U.S. Army as Sergeant E-5, Emergency Medical Technician-Basic, Heath Care Specialist, Gunner, Driver, or Instructor/Writer. As Instructor/Writer, I provided Combat Medic Advance Skills Training, Emergency Medical Technician-Refresher, Annual Combat Medic Skills-Validation Test, Medical Proficiency Training, and Basic Life Saver classes for military and civilian personnel. During my military service, I was awarded with 13 medals, including Purple Heart. From 2007 to 2008, I returned to EPCC and obtained my Associates of Arts. From 2008 to 2010, I attended The University of Texas at El Paso (UTEP) and obtained my first Bachelor's Degree in Microbiology. From 2009 to 2010, I worked at Pharmacy of the U.S. Department of Veterans Affairs. From 2010 to 2014, I worked at The Minton Law Firm as a Certified Paralegal. In 2015, I returned to UTEP, volunteered at Dr. Igor C. Almeida's Laboratory. obtained my second Bachelor's Degree in Forensic Science and was in the Dean's List for my outstanding grades. From 2015 to 2018, I attended UTEP for a Master's Program, continued doing research at Dr. Almeida's Laboratory, worked as a Teaching and a Research Assistant (TA/RA). As a TA, I provided Topics in Study of Life I Laboratory, Human Anatomy and Physiology II Laboratory, and Molecular Cell Biology Laboratory. In 2016, I obtained the Outstanding Graduate Student Poster Presentation from American Society for Microbiology Rio Grande Branch. In 2018, I obtained my Master's Degree in Biological Sciences and awarded with Outstanding College of Science Thesis.

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