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Mucin Associated Surface Protein Synthetic Peptide as a Novel Vaccine Candidate Against Chagas Disease

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MUCIN ASSOCIATED SURFACE PROTEIN SYNTHETIC PEPTIDE
AS A NOVEL VACCINE CANDIDATE AGAINST CHAGAS DISEASE

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Carylinda Serna

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Dedication

This work is dedicated to my family, the core of my life.
To the greatest two women in my universe my mother and sister to which I aspire to be every moment. To the two most important men in my life, my dad and my brother, who supply me with endless wisdom and courage. Thank you from the bottom of my heart for all of your sacrifices that you have made to make this possible. I love you eternally.

MUCIN ASSOCIATED SURFACE PROTEIN SYNTHETIC PEPTIDE
AS A NOVEL VACCINE CANDIDATE AGAINST CHAGAS DISEASE

by

CARYLINDA SERNA, B.S

DISSERTATION

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The University of Texas at El Paso
in Partial Fulfillment
of the Requirements
for the Degree of

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Abstract

Trypanosoma cruzi is an intracellular protozoan parasite and the etiological agent for Chagas disease. Chagas is endemic in Latin America affecting 18-20 million people. However, currently worldwide increasing numbers of the disease are being seen due to migration and globalization. This neglected disease causes significant morbidity, mortality, and an economic burden. There are no known vaccines and the only currently available drug is Benznidazole, but its effects are controversial. Nonetheless, a therapeutic or prophylactic vaccine is of urgent need to alleviate this disease. In this study we present an experimental approach using a synthetic peptide-based vaccine against *T. cruzi*. The vaccine was developed based on a mucin-associated surface protein (MASP) (conjugated with keyhole limpet hemocyanin (KLH) [MASP_{syn}]. The study was done using 6-8 week female C3H/HeNHsd mice. 5 groups of mice were used: a placebo group was administered phosphate buffered saline (PBS), adjuvant controls were additionally used, in which, one group was given 0.9% aluminum hydroxide (AlOH), and another KLH. The experimental groups were separated into two groups: one with the MASP_{syn}, and the other with the MASP_{syn} and 0.9% AlOH. The parasitemia and survival of the mice were followed. After challenged with 1x10⁵ trypomastigote Y strain, mice immunized with the MASP_{syn} showed 80% survival followed for 1 year. qPCR showed low parasite burden in heart, liver, and spleen (500-69 times less parasites than the placebo group before being euthanized 20-30 days post infection). The humoral response was analyzed and detection of specific anti-trypomastigote lytic antibody was detected in vaccinated mice, IgG1, IgA and IgM isotypes showed to be important in eliminating the parasite. Moreover, cytokine measurements for IL-4, IL-10, IL-12, IL-17A, and IFN- γ (relevant for resistance against the parasite) also showed to be increased in vaccinated mice. The vaccine also seems to stimulate activation of both CD4⁺ and CD8⁺ T cells. Lastly, depletion of either

CD4⁺ or CD8⁺ showed that CD8⁺ T cells are essential for protection. This neoteric vaccine is able to control survivability by being able to prime both a humoral and cellular immunity.

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Chapter 1: Introduction

1.1 Chagas Disease

Trypanosoma cruzi is a flagellate protozoan that is the causative agent of Chagas' disease. Chagas' disease affects 18-20 million people worldwide and has become a larger concern with growing numbers of immigration. It is more commonly seen in South America, mainly because of the high number of poor communities and the ideal living poor-conditions for the triatomine insect vector [1]. Chagas disease has recently been emerging globally because of the increasing number of immigration [2],[3]. In the USA there is reported to be 300,000 cases and it is speculated to be up to 1 million cases [4].

This parasite is a complex zoonosis as most mammals can serve as hosts and reservoirs for *T. cruzi* infection and, therefore, can help in the distribution of the parasites through migration. Humans are naturally infected with *T. cruzi* with the initial bite of an infected triatomine insect, also known as a “kissing bug”. The insect is the vector for the parasite in which it delivers the infected form, metacyclic trypomastigotes, through its feces [5]. The metacyclic trypomastigotes invade the host cells and transform into amastigotes. In the cell, the amastigotes proliferate via binary fission and eventually differentiate into trypomastigotes [6]. In this stage they are free in the blood and target other cells for invasion and continue the infective cycle. Once released into the blood the trypomastigotes can be transmitted back into the kissing bug through a blood meal in which the parasite transforms into an epimastigote in the insect's gut [6]. The epimastigotes travel to the hind-gut of the insect, and eventually differentiate into infective metacyclic trypomastigotes and the cycle continues (Figure 1.1). The transmission of the parasite is usually from the vector (80%-90%), but it

has known to be transmitted through other methods [7]. Such as contaminated blood (5-20%), organ transplant, and congenital transmission (0.5-8%) [7].

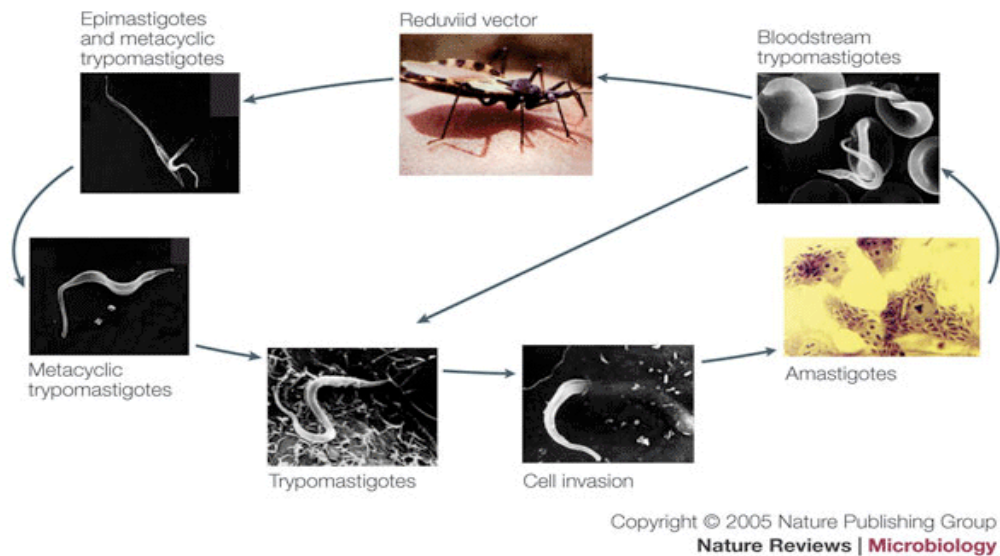


Figure 1.1 Life cycle of *T. cruzi*.

After being infected with *T. cruzi* usually a chagoma forms at site of infection. This can appear anywhere in the body as a form of edema that consist of inflammation due to rupture of parasitized cells [7]. This is a result of the initial acute inflammatory response reaction. Symptoms begin to appear 4 days to 2 weeks after infection [8]. These include spread of edema, chagomas appearing in different parts of the body, but mostly the face. A unique edema to Chagas', forms in the upper and lower eyelid known as Romaña sign. Patients shortly (1-2 months) develop an acute infection [7]. In which symptoms can arise as malaise, chills, high fever, muscles aches, and increase exhaustion [8]. Acute infections are usually asymptomatic and self-limited [4]. The spleen can also become palpable and the liver somewhat enlarged [8]. A non-painful rash can appear in the chest and

stomach area two weeks after infection [8]. Mortality rate is seen mostly in children with a 2-8% of occurrence [7]. 60-70% of patients that survive live an intermediate status that does not develop into the chronic phase of the disease [2]. 30-40% of Chagasic patients go on to develop chronic disease, of which 0-50% are then asymptomatic [2, 7]. Other patients can have sporadic fever and appearances of trypanosomes in the blood. Even though there is a small number of parasites still present in the host during this stage there is an increase amount inflammation in affected tissues [5] [9]. In most cases, symptomatic infection leads to congestive cardiac failure. Patients with Chagas' disease have a higher rate of having a stroke or arrhythmias [10]. During chronic stage parasites usually persist in cardiac and smooth muscle causing inflammation, causing heart and digestive damage [11]. Other results of chronic exposure of *T. cruzi* are megaesophagus and megacolon.

Approximately 22% of patients never seek healthcare and/or have limited access to proper health care [12]. To add to the problem the drug treatment that has been developed is inadequate and no vaccine is available. The only therapeutic agents that have been developed are nifurtimox and benznidazole. However, nifurtimox is no longer available as a therapy due to its high toxicity level [7]. Benznidazole, the only available drug, is extremely toxic, less effective in the chronic stage (especially the late chronic stage), and a growing number of resistant strains are arising [13].

1.2 Host-Parasite interaction

T. cruzi survives in the human hosts mostly as in an intracellular parasite, consequently it is critical to understand the host –parasite interaction. The parasite infects mostly all nucleated cells. Taking advantage of the host's vacuole, dividing in the protected cytoplasm, manipulating the structure and pathways of the host's cell [14]. There are two pathways that the parasite can invade the cell, the lysosome dependent and independent. Either pathway requires the interaction of mucins

on the surface of the protein to interact with the host cell. However, adhesion is not essential for host invasion [15]. The parasite interacts with the extracellular matrix to infect the host cell [15]. The surface of the parasite is covered by trans-sialidases and mucin-type glycoproteins that might help in the attachment of the parasite to the host cell by using glycosylphosphatidylinositol (GPI) anchors [16].

1.3 Mucin-associated surface proteins

About 50% of the *T. cruzi* genome has repeated sequences encoding for retrotransposons and genes for families of surface molecules [17]. These families include trans-sialidases (TS), mucins, surface glycoprotein gp63 proteases, and mucin-associated surface proteins (MASPs) [17]. Mucins are part of the largest super gene family in *T. cruzi*. Since the trypomastigote stage is directly exposed to the immune response it is essential that it have a variety of mucins on the surface. Once in contact with the environment the thick coat of mucins rapidly become negatively charged [18]. This negative charge is a result of the transfer of sialic acid from the host [18]. The trypomastigote stage contains a series of *O*-glycans that contain α -galactosyl residues, which are the main target epitope for antibody production in chagasic patients [19] [18]. All of these surface molecules are known to play key roles in the survival of the parasite.

T. cruzi uses several of these surface proteins to invade host's cells and for parasite protection. In their role of protection they ensure the targeting and invasion of specific cells or tissues [19]. MASPs constitute a large amount of those proteins, in which ~15% of the parasite's genome encodes for it and other mucin-encoding genes [19]. In the genome project for *T. cruzi* were found to MASPs encodes for about >1300 genes [17]. Furthermore, since this family does not have any genes orthologous to other sequenced kinetoplastid, it makes it very specific to *T. cruzi* [20]. Proteomic

analyses of MASP showed that they are highly expressed in trypomastigotes [17, 21]. Genes are present and expressed in low levels in the epimastigote and amastigote stage [17, 21]. MASPs are characterized by a highly conserved N- and C-terminal domains, and a variable and repetitive central region [22]. These domains encode for a presumed signal and a GPI-anchor addition site [22]. Studies from the El-Sayed's [22] group demonstrated that MASP is GPI-anchored and shed into medium culture, contributing to the belief that MASP play an essential role in the immune system as well as the invasion of the host cell. MASPs are believed to have post-translational modifications that contribute to the lack of the proteins interaction with other cells [23]. A conserved region in the family of MASP was used to analyze the expression of MASP at different points of invasion, it was found that there was an increase of expression in trypomastigote and amastigote forms of PAN4 and CL-Brener strains [20]. This supported the already establish notion that MASP is upregulated in the infectious stages [21, 23]. Due to previous confirmed studies that show that mammalian mucins participation in cell-cell interactions, it is contemplated that MASPs could interact in the same way [19]. This leads to the speculation that MASP is might be important for host cell invasion since the other stages do not seem to require it for survival. Also, MASP52, obviously part of the MASP family, was shown to play a role in the process of host invasion, as well as being secreted by metacyclic trypomastigotes [20]. Bartholomeu *et al* (2009) reported that antibodies against a conserved motif of MASPs reacted to the supernatant of parasites, leading to the belief that MASP family members are shed into the culture medium [21]. Hence, it is worth to mention that MASP is important for host cell invasion. Interestingly, according to Murta *et al*, MASP is up regulated in resistant strains of *T. cruzi* compared to those susceptible to benznidazole. MASP expression is known to be up-regulated in the mammalian stages [23].

Trypomastigotes forms of *T. cruzi* secreted vesicles (TcVes), which showed to increase the infection of macrophages (REF). Studies performed by Tricolli et al (2009) showed that mice pre-treated with TcVes presented higher parasitemia and amastigote nests, as well as severe heart pathology [24]. Proteomic characterization of TcVes showed MASP as one of molecules present in it. Furthermore, from the 1,400 MASP encode in *T. cruzi* genome only one was present in TcVes, which we called MASP_{ves} (Nakayasu, unpublished). This study will focus in the characterization of MASP_{ves} as antigen for vaccination against Chagas disease.

1.4 Immune Response to Infection

The host immune system plays a great role in the infectivity of the parasite. Host resistance is dependent on both the innate and adaptive immune response in a *T. cruzi* infection. Upon initial infection the innate system is able to control parasitism with the involvement of cytokines and activation of macrophages and natural killer cells (NK) [24]. Toll-like receptor (TLR) 2 is activated by the parasite and promotes early-inflammation by dendritic cells [25, 26]. TLR-4 and TLR-9 have also shown to contribute in the activation of host innate immune response leading to the regulation of the infection [27]. The adaptive immune system in response to *T. cruzi* is fairly well understood. It is known that Th1 CD4⁺ cells and CD8⁺ T cells recognize specific antigens from the parasite [28]. Later on, parasitemia is usually controlled by several immune mechanisms such as macrophages, opsonization by antibody, and T cell- mediated cytotoxicity [24]. Since the parasite has an intracellular and extracellular phase the role of CD8⁺ and CD4⁺ T cells is crucial in the moderation of the parasitemia. Abrogation of CD8⁺ and CD4⁺ T cells during the acute phase in model *T. cruzi* infected mice, moderates myocardial inflammation [29], but showed high parasitemia and increase mortality [29, 30]. Both in the acute and chronic phase, cardiac lesions have been characterized by

having predominance for CD8⁺ T cells, fewer CD4⁺ T cells, and basically no B cells [29]. It has been hypothesized that the adaptive immune response occurs after the first round of replication and reinvasion (4-5 days post-infection) [28]. At this time, *T. cruzi* releases proteins that are processed by MHC I [31], and are allowed for detecting from specific-parasite CD8⁺ T cells [28]. CD8⁺ T cells were first analyzed with the identification of different epitopes in the TS gene family [32], surface molecules such as MASP. This analysis showed that 30% of CD8⁺ T cell population in infection is attributed to one TS epitope [32]. In addition, CD8⁺ T cells usually remain high and functional in the chronic stage of the disease [28]. As has been mentioned when infection begins several *T. cruzi* surface proteins are shed into the environment, this is an important role that should stimulate CD4⁺ T cells [22]. CD4⁺ T cells are mainly responsible for controlling parasite replication [33]. Some mucins are able to produce such an effect; however it is important to note that there is a superfamily of surface proteins, SA85-1, that has been shown to possibly inhibit a CD4⁺ response [22].

1.5 Vaccines

The ideal vaccine for Chagas disease would be one that could be used for prophylactic and therapeutic use because of the high number of people that are infected and/or at risk. Experimental vaccines have been produced, but have failed to provide full protection when challenged with parasites [1]. Some potential vaccine candidates have been explored and have shown partial efficiency in protective immunity. Those that have been studied are trypomastigote cDNA expression library [34], paraflagellar rod proteins (PFR) [35], other parasite derived antigens such as amastigote surface protein (ASP)-2, ASP-1 [36], Tc52 [37], TolA-like surface protein [38], as well as a variety of TS [39, 40] some of which have been experimentally used as vaccines and both fail to provide a full protection. Mucosal vaccines have also been explored, among are those containing

proteins such as recombinant cruzipain [41] and gp82 [42]. Besides the fact that these vaccines have not provided full protection these studies have opened a window to understanding the vaccine that needs to be created. The best targets would be genes that are expressed in the trypomastigote stage since this is the stage that is exposed to the host's cells most often [34]. Also, molecules such as TS that are on the surface of the parasite are excellent targets since they are also exposed to the host's cell [39]. Previous studies have demonstrated that only one member of MASP family (MASP_{ves}), is released in vesicles into the culture media by trypomastigotes (Nakayasu and Almeida, personal communication). Based on this data we speculate that MASP_{ves} possibly will aid in the parasite invasion of host cells.

Furthermore, since MASP_{ves} is released in vesicles it is also exposed to the immune system, which is why we decided to explore the possibility to use this molecule as an antigen vaccine candidate. Vaccination has been demonstrated to be a cost-effective approach to illness and death caused by infectious diseases. Here we propose to study an individual MASP_{ves} as a vaccine candidate. Nakayasu *et al* (2012), using cell derived trypomastigotes performed a proteomics-immunoinformatics analysis finding high number MASPs and other surface proteins to have high binding of MHC I and/or II molecules, which make them very valuable as targets for development of vaccines [43]. In further investigation also through proteomic analysis it was shown that a specific MASP is found in vesicles (MASP_{ves}) secreted by trypomastigotes. In addition, our computational analysis of MASP_{ves} indicated the presence of several B-cell, MHC class I and II T-cell epitopes in this molecule in both human and murine. This is a good target since MASP_{ves} epitopes can in principle fit into most known human and murine MHC class I and II alleles, thus suggesting that specific B-cell and T-cell responses toward infected cells and parasites could be triggered.

1.6 Hypothesis

The ideal vaccine against the complex intracellular and extracellular *T. cruzi* is one that can elicit both a humoral (antibody production) and cellular immune response. In this study, we tested the ability of a synthetic peptide-based (conjugated with KLH) vaccine (MASP_{syn}) to provide protection against *T. cruzi*. Based on *in silico* analysis, we hypothesized that: our peptide (MASP_{syn}) will be able to stimulate effectively the humoral and cellular immune response providing protection against Chagas disease in the murine model. Furthermore, the epitopes contained in the vaccine candidate will show similar effect among human infection protection, since theoretically this peptide would be recognized by several MHC I and II haplotypes.

1.7 Specific Aims:

Specific aim 1: To evaluate the humoral response to MASP_{syn}.

Specific aim 2: To validate MASP_{syn} as vaccine candidate.

Specific aim 3: To determine the Th response induced by MASP_{syn}.

Chapter 2: Materials and Methods

2.1 In vitro epimastigote culture

T. cruzi Y strain epimastigotes were cultivated 4 days at 28°C in Liver-infusion tryptone (LIT) supplemented with 10% inactivated fetal bovine serum (Sigma, St. Louis, MO) [44].

2.2 In vitro and in vivo trypomastigote culture

T. cruzi Y and Talahuen strain trypomastigotes were kept in culture by alternating infecting BALB/c mice and monkey kidney epithelial cells (LLC-MK₂, American Type Culture Collection-ATCC, Manassas, VA). After five passages, BALB/c mice were infected with 1×10^4 trypomastigotes. After 3 days bloodstream trypomastigotes were obtained and were used to infect LLC-MK₂. Cells were kept at 37°C in a humid atmosphere supplemented with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% inactivated fetal bovine serum and 5% Antibiotics (ampicillin/ streptomycin) (Sigma, St. Louis, MO) [44].

2.3 Cloning of MASP_{ves}

The complete open reading frame of the mature *T. cruzi* MASP_{ves} (excluding the sequences encoding for the signal peptides for secretion and GPI insertion) was amplified using oligonucleotides S1- *Xho* I sense (5'-GGGATCCGAGCTCGAGG GGGATGATGATGTTTCTGGT) and S2- *Hind* III antisense (5' CAGCCGGATCAAGCT TAGTGTCGCCGGTGGTTGTTTT). Polymerase chain reaction (PCR) was performed using PCR master mix (Promega, Madison, Wisconsin) and 100 ng *T. cruzi* Y strain total genomic DNA [45]. The PCR product was ran in a 0.8% agarose gel and purified using Wizard SV Gel and PCR clean-Up system (Promega, Madison, WI). The amplified fragments were cloned into the pGEM-T vector system I (Promega, Madison, WI) and used to transform

Escherichia coli DH5 α . The clone MASP_{ves}/pGEM was sequenced (Genewiz, Inc, South Plainfield, NJ) and was confirmed using Basic Local Alignment Search Tool (BLAST) and Multiple Sequence Alignment by CLUSTALW (<http://align.genome.jp/>). MASP_{ves}/pGEM was digested using the restriction enzymes *Xho* I and *Hind* III, and then MASP was directionally subcloned into the expression vector pRSET A (Invitrogen, Carlsbad, CA). The MASP_{ves}/pRSET clones were selected by colony screen and the positive clone ORF was confirmed by sequencing (Genewiz, Inc, South Plainfield, NJ). *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) was transformed with MASP_{ves}/pRSET A for expression.

2.4 Expression and Purification of MASP_{ves}

The expression of MASP_{ves} cloned in pRSET A was induced using isopropyl β -D- 1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The expression was induced for four hours. To determine the optimum induction time, aliquots of 1 mL were collected immediately before and each hour after induction. 20 μ L of non-induced and induced samples were resolved in 12% SDS-PAGE. The recombinant MASP_{ves} was purified using B-PER 6XHis Fusion Protein Spin Purification Kit (Thermo Scientific, Rockford, IL).

2.5 Western Blot Analysis of recombinant MASP_{ves}

15- μ L from each elution of the protein purification was electrophoresed on a 12% SDS-PAGE and blotted onto Nitrocellulose membrane (Thermo Scientific, Rockford, Illinois) as described by [46]. Membrane was first was blocked (5% skim milk PBS for 1 hr or overnight), follow by incubation with anti-6XHis (1:5000) antibody (Invitrogen, Carlsbad, CA), washed 3 times with PBS-Tween, and then incubated with anti- mouse antibody IgG (H+L) HRP conjugated antibody

(1:10000) (Invitrogen, Carlsbad, CA). Blots were developed by chemiluminescence following the manufacturer's instructions (ECL kit, Thermo Scientific, Rockford, IL).

2.6 HPLC

To purify MASP_{ves}, to homogeneity, it was subjected to High Performance Liquid Chromatography (HPLC). Solid phase extraction was done of the samples to remove salts before loading them into the HPLC. The DSC-18 (Discovery, Bellefonte, PA) were activated with 100% methanol, then equilibrated with 0.05% trifluoroacetic acid (TFA), after which the sample was loaded, the sample was then washed with 0.05% TFA, and finally eluted (80% Acetonitrile (ACN), 0.05% TFA). The sample was dried using a SpeedVac, then the pellet was resuspended in 0.05% TFA, and centrifuged for 5 minutes at 14,000rpm 4°C. Sample was then loaded onto a Jupiter 5μ C18 300A HPLC column and eluted with a gradient of increasing ACN concentration.

2.7 Protein Purification from Gel extraction

After affinity chromatography purification the recombinant MASP_{ves} was submitted to other step of purification by gel extraction. The MASP_{ves} eluted fractions were resolved in a 12% SDS-PAGE. The gel was placed in freezing KCl (250mM) and shacked at 4°C. The protein was cut out from the gel and placed in 50 mL conical tubes with 10 mL of distilled water with protease inhibitors. The tubes were placed under constant agitation at room temperature for 24 hours. To remove excess SDS the samples were subduced to dialysis against 4 L of PBS at 4°C for 48 hours. The samples were quantified and a western blot was done to detect the protein.

2.8 Selection and synthesis of the synthetic MASP_{ves} peptide (MASP_{syn})

Using adequate computational analyses (ABCpred prediction server for B cell epitope recognition, ProPred I, SYPEITHI, and HLA-BP for MHC I, and TEPITOPE and ProPred for MHC II recognition) we chose a peptide from the protein that seemed to have a high score of epitope recognition in B-cell recognition, MHC I and MHC II in humans and mice. Synthetic MASP_{ves} peptide was synthesized by the company thinkpeptides (thinkpeptides, Bradenton, FL). The peptide was conjugated to KLH.

2.9 Custom Antibody Selection

Two epitopes were selected from MASP_{ves} because of their high immunogenicity. One was used for the production of the synthetic peptide MASP_{syn} and the other was employed to develop a custom antibody (MASP_{cus}). MASP_{cus} was prepared in rabbits on request by Biomatik Research Inc. (Biomatik Research Inc., Wilmington, DE).

2.10 Immunization

C3H/HeNsd (Harlan Laboratory, Indianapolis, IN) female mice (6-8 weeks old) were separated in five groups (4 mice/group). The immunizations were performed via intraperitoneal (i.p.). One control group was immunized with phosphate buffer saline (PBS, placebo). Two control groups for the adjuvants, [0.9% ALOH] (Sigma-Aldrich, St. Louis, MO); and KLH [10 µg/mouse] (Sigma-Aldrich, St. Louis, MO); and two experimental groups. To the experimental groups were administered 20 µg/mouse of MASP_{syn} alone and combined with 0.9% aluminum hydroxide, respectively. A total of 3 immunizations every 10-15 days were administered.

The rationale to use AIOH as adjuvants are: 1) it has been the only approved adjuvant allowed to use in human vaccines by the US federal drug administration (FDA) [47]. 2) It is known to stimulate CD4⁺ T cells, secretion of cytokines, act on macrophages and monocytes, and enhance expression of MHC I [48, 49]. 3) It is also known to stimulate a Th1 –type immune response [50], which has also been shown to help regulate the infection [40, 51].

The procedures described in this study were performed minimizing the distress and pain for the animals following the NIH and ARRIVE guidelines, the animal protocol was approved by The University of Texas at El Paso (Protocol Number: A-2125) Institutional Animal Care and Use Committee (IACUC).

2.11 *Vesicle purification*

Trypomastigotes were collected at different days of culture (first and second day post-infection) and diluted to 1×10^8 parasites/ml with DMEM 12.5 g/L glucose. Trypomastigotes were incubated for 4-6 hours (no longer than 8 hours) at 37°C and 5% CO₂. After, the parasites were centrifuged at 3,000 x g for 7 minutes at 4°C and the supernatant was collected and filtered using 0.45 µM filters (Thermo Scientific, Rockford, IL). The filtrate was collected and centrifuged at 100,000 x g at 4°C for 2 hours. The pellet (S1) was resuspended in 500 µL of PBS and with 1:10 protease inhibitor cocktail (Sigma, St. Louis, MO) was added. The supernatant was collected and centrifuged 100,000 x g at 4°C for 16 hours. After, a second pellet (S2) was resuspended in 500 µL of PBS with 1:10 protease inhibitor cocktail. The vesicle suspension was stored at 4°C until use.

2.12 Isolation of amastigotes

LLC-MK2 cells were infected with 1×10^8 trypomastigotes. After 5 days, the infected monolayers were gently detached by scraping and resuspended in 5 mL of PBS. The suspension containing the infected cells was next transfer to an M-tube (Biotec, CA). This tube has a special stator and rotor that allows for tissue homogenization. It has a pre-inserted mesh that retains larger particles, removing them from the homogenized sample. The tube was then placed in the gentleMACS™ Dissociator (Biotec, CA) for the automated disruption of host cells. The homogenized sample containing the parasites was then passed through a column containing anion exchange preswollen microgranular diethylaminoethyl cellulose (GE Healthcare, NJ). The sialylated trypomastigote forms of the parasites were retained in the resin, while the amastigote forms were recovered [52].

2.13 Lysis of parasites and vesicles

Parasites were cooled on ice for 5 minutes, then centrifuged at 3,500 rpm at 4°C for 10 minutes. The supernatant was removed and the pellet was washed 3 times with PBS (each time centrifuged for 10 minutes at 3,500 rpm). After the washes, 300 µL of lysis buffer (50 mM sodium fluoride, 1 mM sodium ortho-vanadate, 1% triton, 10 mM TRIS, 5mM EDTA, 50 mM sodium chloride, and 30 mM sopdium purophospahte) was added to the pellet. The samples were then nutated for 10 minutes at 4°C, followed by centrifugation at 14,500 rpm for 10 minutes at 4°C. The pellet was kept for immunoprecipitation or directly added to the SDS-PAGE gel. The vesicles were lysed following the above procedure.

2.14 Immunoprecipitation of MASP_{ves} from trypomastigotes

Two microliters of anti- MASP_{syn} was added to the lysed pellets (parasites) and left incubating at 4°C overnight. The next day, 40 µL of protein G-sepharose (Thermo Scientific, Rockford, IL) was added and incubated for 1 hour at 4°C. Subsequent, the samples were centrifuged for 1 minute at 10,000 rpm and washed 3 times with 750 µL of lysis buffer. Aliquot of the samples were then analyzed in SDS-PAGE gel.

2.15 Immunoblotting

IP from epimastigote, amastigote, trypomastigote (Y and Talahuen strain), and vesicles (15 µL of each) were subjected to electrophoresed 12% SDS-PAGE and blotted onto Nitrocellulose membrane (Thermo Scientific, Rockford, IL) as described by [48]. Membrane was first was blocked (5% skim milk PBS overnight), followed by incubation with anti-MASP_{syn} (1:5,000) antibody (obtained from immunized mice, see section 2.10), washed 3 times with PBS-Tween, and then incubated with anti- mouse antibody IgG (H+L) HRP conjugated antibody (2:10,000) (Invitrogen, Carlsbad, CA). Blots were developed by chemiluminescence following the manufacturer's instructions (ECL kit, Thermo Scientific, Rockford, IL). The lysed vesicles were analyzed similarly, as described above.

2.16 Evaluation of humoral immune response

Ten days after the last immunization blood was collected by tail bleeding [53] and serum was separated from blood by centrifugation at 2,000 rpm for 10 min. The serum antibody titers were determined by an ELISA [54]. Human Chagasic and normal serum (kindly donated by Dr. Almeida - UTEP) were also tested for antibody titers against MASP_{syn}.

2.17 Immunoglobulin Isotyping

Immunoglobulin isotyping was examined using Mouse Immunoglobulin Isotyping ELISA Kit (BD Pharmigen, San Jose, CA). The serum from the placebo and MASP_{syn} immunized groups was obtained as described above. The positive control was provided in the kit. The assay was performed following the manufacture guidelines; each sample was done in triplicates and read at an absorbance of 450 nm using VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

2.18 Challenge - Parasitemia and Survival

Ten days after last immunization mice were inoculated via i.p. with 1×10^6 trypomastigote Y strain, the parasitemia and survival were monitored. The parasitemia was evaluated every day for the first 12 days then every third day for a total of 21 days following the Brenner method [55].

2.19 Evaluation of cytokines

Four weeks post-infection the blood was collected by tail-bleeding [55] and pool of serum from each experimental group was obtained as described above. Cytokines were measured using Mouse Inflammatory Cytokines Multi-analyte ELISArray Kit (Qiagen, Valencia, CA).

2.20 Detection of CD4⁺ and CD8⁺ in immunized and acutely infected mice using Flow Cytometry

Eight days post-infection the mice were sacrificed (non-infected and infected). The spleens were harvested and ground through a 0.4 μ M nylon mesh. Cell suspensions were filtered through a second nylon mesh. Afterwards they were centrifuged at 1,200 rpm for 5 minutes to pellet cells. Cells were resuspended in lysis buffer. Cells were centrifuged again at 1,200 rpm for 5 min. To avoid unspecific binding cells were blocked with mouse serum for 15 minutes in 4°C. Cells were

double stained with anti-CD4-FITC, and anti-CD25-biotin or anti-CD69- biotin, the same procedure was performed with anti-CD8-PE. A secondary streptavidin-PC5 antibody was added to anti-CD25/69-biotin stained cells. Cells were left to stain for 1 hour at 4°C wrapped in foil. The cells were then washed with FACS buffer (PBS, 1% fetal calf serum, 0.1% Sodium Azide, and 0.5 mM EDTA) and centrifuged for 5 min at 1200 rpm. The supernatant was tossed and the cells were then resuspended with 300 µL of 1% paraformaldehyde (PFA). Aliquots from cell suspension were taken for individual control stains to verify the presence of CD4⁺ and CD8⁺ cells by staining individually for anti-CD4-FITC, anti-CD8-PE, anti-CD25-biotin and anti-CD69-biotin and subjected to the same treatment as samples. Samples were then analyzed with flow cytometry using Cytomic FC 500 flow cytometer (Beckman Coulter, Miami, FL). For each individual sample, approximately 10,000 events were acquired and analyzed using CXP software (Beckman Coulter, Miami, FL).

2.21 Detection of CD4⁺ and CD8⁺ in mice with chronic stage infection using Flow Cytometry

Immunized mice were chronically infected with 1×10^4 trypomastigotes. The experimental groups were negative control (PBS), AIOH, MASP and MASP/AIOH. In parallel the same group were not infected to be used as control for naïve CD4⁺ and CD8⁺ mice. The survived animals were euthanized nine months after challenge. A boost immunization was given seven days before the animals were sacrificed. The spleens were collected and processed as described above for detection of CD4⁺ and CD8⁺.

2.22 In vivo depletion of immune cell subsets

One week after the last immunization, mice received i.p. injections of 500 µg anti-CD4 (BD Pharmingen, San Jose, CA) or 1000 µg of anti-CD8 (BD Pharmingen, San Jose, CA) mAbs. Unspecific IgG was given to the control groups. Depletion was analyzed by flow and the data were acquired using Cytomic FC 500 flow cytometer (Beckman Coulter, Miami, FL). For each individual sample, approximately 10,000 events were acquired and analyzed using CXP software (Beckman Coulter, Miami, FL). Mice were then challenged with 1×10^5 trypomastigotes 48 hours after last dose of anti-CD4/CD8. Parasitemia and survival were followed as described.

2.23 DNA preparation

In the terminal stage of the disease mice were euthanized and the heart, liver and spleen tissues were collected. This procedure was performed at different end points upon the protection offered by the immunization. DNA was extracted using Wizard SV Genomic DNA Purification System (Promega, Madison, WI) and quantified by Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

2.24 Real-time PCR

Each PCR reaction contained 50 ng genomic DNA, 0.5 µM of *T. cruzi* 195-bp repeat DNA-specific primers TCZ-F 5'-GCTCTTGCCCACAMGGGTGC-3' where M=A or C and TCZ-R 5'-CCAAGCAGCGGATAGTTCAGG-3' primers were obtained from Integrated DNA Technologies (IDT) (Integrated DNA Technologies, Coralview, IA) [56]. In addition, 12.5 µL of IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and RT-PCR-grade water (Life Technologies, Grand Island, NY) was added to a final volume of 25 µL. Independently, reactions containing 50 ng genomic DNA, 0.5

μM of murine-specific tumor necrosis factor-α (TNFα) primers TNF-5241 5' TCCCTCTCATCA-GTTCTATGGCCCA-3' and TNF 5411 5'-CAGCAAGCATCTATGCACTTAGAC- CCC-3', (Integrated DNA Technologies, Coralview, IA) [56], 12.5 μL of IQ SYBR Green Supermix and RT-PCR-grade water were added to a final volume of 25 μL. Negative controls with no DNA added were done. All reactions were performed in triplicate. The reactions were placed onto a 96-well plate (Bio-Rad, Hercules, CA), centrifuged for 2 min at 2000 rpm, and placed in the Eppendorf Mastercycler ep Realplex (Eppendorf, Hauppauge, NY). The reactions were exposed to four phases, denaturation, amplification, melting, and cooling. The denaturation phase the plate is heated to 95°C for 2 min, the amplification was done during 35 cycles for *T. cruzi* primers (45 cycles for TNFα primers) at three steps: 95°C for 15 s, then 65°C for 10 s, 72°C for a 5 s hold, and then 79°C for 20 s, at which the fluorescence intensity is acquired. The melt phase then begins with 95°C for 15 s, then 60°C for 15s, and finally 95°C for 15s hold. Data was collected and analyzed using the software provided in the Eppendorf Mastercycler ep Realplex. Also, the amplification products for each sample were subjected to ethidium bromide, ran through an electrophoresis 1% agarose gel, and finally viewed under UV light.

The standard curve for the qPCR reactions were generated as described by Cummings and Tarleton [56]. Briefly, to 150 mg of normal tissue was added 3×10^6 *T. cruzi* trypomastigotes, and then the DNA was extracted (Wizard SV Genomic DNA Purification System, Promega, Madison, WI). DNA was quantified using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The standard curve obtained was used to determine the parasite load of infected tissues.

2.25 *Trypomastigote Lysis Assay*

Pool of serum was collected from non-infected placebo (complement system active), MASP_{syn} immunized (complement system active), and MASP_{syn} immunized (inactivated complement system, 56°C for 30 min,) mice. Trypomastigote suspensions (200 µL of 1×10^7 parasites/mL, in DMEM) were incubated with 0.8 µL (1:250) of either serum at 37°C for 1 hr. Then propidium iodine (PI) was added (0.5 µg/mL) and cells were incubated for 5 min. at 24°C, followed three washes with PBS. The pellet was re-suspended in 4% paraformaldehyde, incubated at 24°C for 15 minutes, and then washed with PBS. Finally, the pellet was resuspended in 300 µL PBS. The positive and negative controls (live and dead parasites, respectively) were prepared similarly. Briefly, the trypomastigote suspension (200 µL of 1×10^7 parasites/mL in DMEM) was washed three times with PBS and PI was added (0.5 µg/mL), then followed by three washes with PBS, the parasites were then fixed with 4% paraformaldehyde, followed by three PBS washes, and finally resuspended in 300 µL PBS. In the negative control the trypomastigote suspension was treated with hydrogen peroxide (200 µM), then washed with PBS, the pellet was resuspended in PI (0.5 µg/mL), then followed by three washes with PBS, the parasites were then fixed with 4% paraformaldehyde, followed by three PBS washes, and finally resuspended in 300 µL PBS. The samples were evaluated by flow cytometry using Cytomic FC 500 flow cytometer (Beckman Coulter, Miami, FL). The experiment was performed in triplicate in three independent experiments. For each individual sample, approximately 10,000 events were acquired and analyzed using CXP software (Beckman Coulter, Miami, FL). The results were plotted as a percentage of dead parasites.

2.26 *Statistical analysis*

Statistical significance of comparison of mean values was evaluated by a one-tailed ANOVA Student's t-test, two-way ANOVA using GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, CA).

Chapter 3: Results

3.1 *Production of recombinant MASP_{ves}*

The recombinant MASP_{ves} (42.62 kDa) was expressed in *E. coli* (BL21) and purified by affinity chromatography, using B-PER 6XHis Fusion Protein Spin Purification Kit following the manufacture protocol (Thermo Scientific, Rockford, IL). The MASP_{ves} was eluted using 200, 150, and 100 mM, the purity of the protein was verified by SDS PAGE. The Figure 1A showed large contamination with bacterial proteins when used 200 mM imidazole (Figure 1A). We believe that at the concentration of 150 mM imidazole the protein was the purest; nevertheless, other protein bands still were observed in the gel.

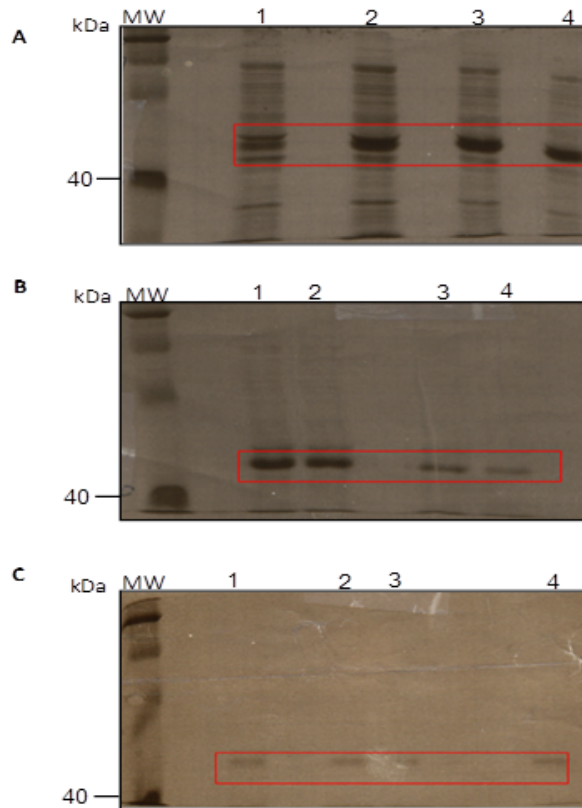


Figure 3.1. SDS-PAGE of the recombinant MASP_{ves} purified by affinity chromatography. A) Elution using 200mM imidazole buffer B) Elution using 200 mM imidazole elution buffer C) Elution using 150 mM imidazole elution buffer.

The western blot (Figure 3.2) effectively confirms that MASP_{ves} correspond to 42 kDa band in the SDS-PGE (eluted at 150 mM imidazole). The recombinant protein was detected using anti-6XHis antibodies.

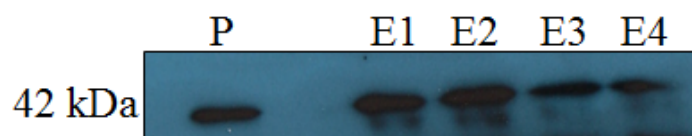


Figure 3.2. Western blot of MASP_{ves} purified by affinity chromatography. The recombinant protein was eluted 4 times using 150 mM imidazole, the fractions collected were resolved in SDS-PAGE 12%, blotted and MASP_{ves} identity was confirmed using anti-6XHis monoclonal antibodies by western blot. P, whole cell extract. E1, elution 1. E2, elution 2. E3, elution 3. E4, elution 4.

Since the purification by affinity chromatography was not sufficient to obtain a pure protein, the elution fractions of the purified proteins were further subjected to HPLC. Fractions were collected 1 mL/min for 80 minutes and MASP_{ves} seemed to have been eluted between the 5th through the 10th minute where was observed a high peak (Figure 3.3)

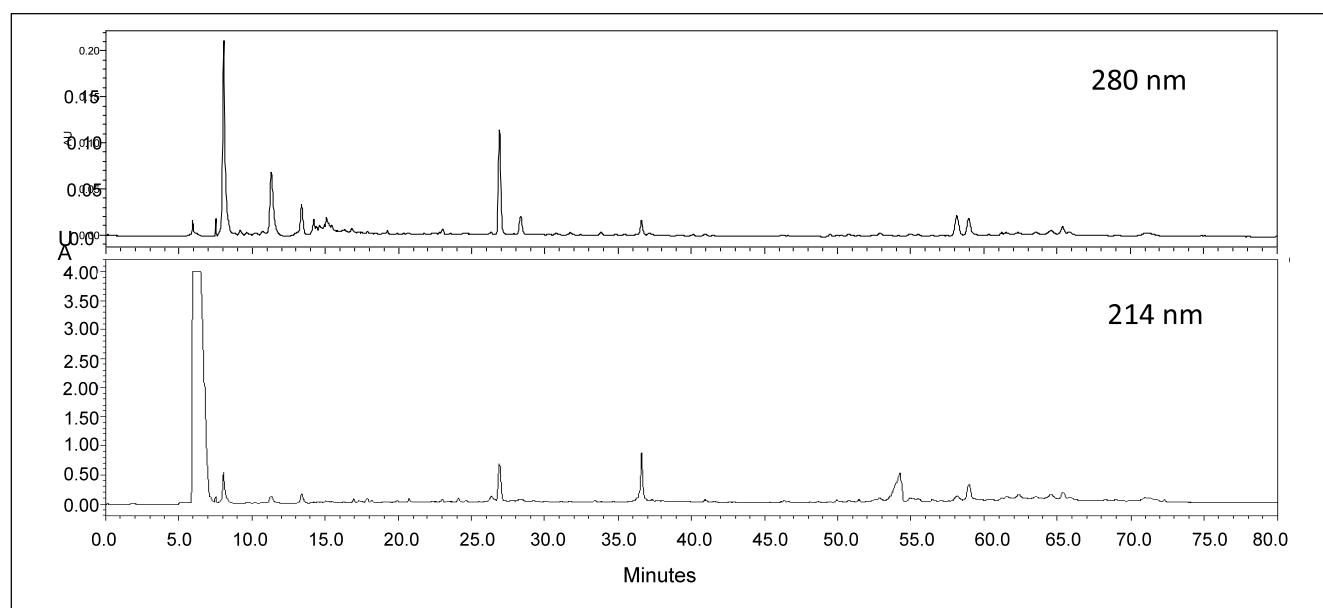


Figure 3.3. HPLC elution profile of MASP_{ves} with increasing acetonitrile gradient and the resulting $A_{280\text{ nm}}$ and $A_{214\text{ nm}}$ readings. Samples were collected from elution peak.

These fractions were then quantified, western blotted, and submitted for mass spectrometry.

The results were not favorable since we were not able to detect the protein using any of the techniques mentioned above. As a consequence, fractions from the first minute to the 15th were subjected to these procedures as well. The protein was not detected in any of these fractions by western blot or mass spectrometry.

Since the protein was not detected, a different approach was taken. The protein was purified straight from the gel and the samples were subjected to dialysis. After, this procedure the protein was not detected by western blot or by quantification. Therefore, we conclude that the recombinant $MA SP_{ves}$ was degraded during the purification process and we decided to produce a synthetic peptide.

3.2 Selection and synthesis of the synthetic $MA SP_{ves}$ peptide ($MA SP_{syn}$)

The selection of the peptide was based on computational analyses that showed the highest number of potential peptide binding to both human and mice MHC I and II as well as its antigenicity prediction.

The amino acid sequence of $MA SP$ contains two signal peptides, one at the N- and other at C-terminal. The N-terminal peptide is responsible for targeting $MA SP_{ves}$ to the endoplasmic reticulum (ER), while the C-terminal is replaced by a GPI-anchor in a post-translation transamidation reaction [57, 58]. The computational analysis for antigenicity, MHC I and II binding peptide were performed with the mature $MA SP_{ves}$.

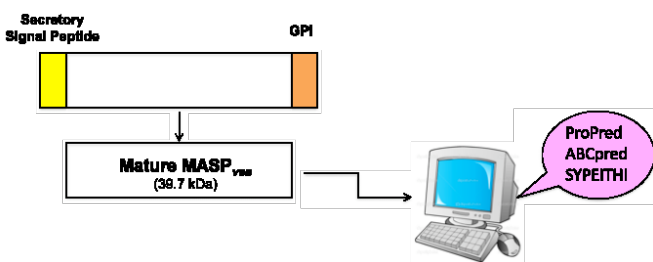


Figure 3.4. Schematic representation of computational analysis strategy.

Table 3.1. MASP_{syn} MHC class-I Binding Peptide Prediction, threshold 5%. (ProPred prediction server). Human alleles (1-15) and mice alleles (16-17). Blue letters represent binding site, red letter represents the putative anchor amino acid. The score represents the probability of its binding to given MHC molecule

	Allele	Predicted Binding Site	Score
1	HLA-A1	DAENPGGEVFNDNKKGLSRV	12.2
2	HLA-A24	DAENPGGEVFNDNKKGLSRV	48.59
3	HLA-B*2702	DAENPGGEVFNDNKKGLSRV	10.66
4	HLA-B*2705	DAENPGGEVFNDNKKGLSRV	32.99
5	HLA-B*3701	DAENPGGEVFNDNKKGLSRV	8.66
6	HLA-B*3801	DAENPGGEVFNDNKKGLSRV	17.46
7	HLA-B*3902	DAENPGGEVFNDNKKGLSRV	12.65
8	HLA-B40	DAENPGGEVFNDNKKGLSRV	32.71
9	HLA-B*4403	DAENPGGEVFNDNKKGLSRV	43.35
10	HLA-B*5101	DAENPGGEVFNDNKKGLSRV	52.16
11	HLA-B*5102	DAENPGGEVFNDNKKGLSRV	34.34
12	HLA-B*5103	DAENPGGEVFNDNKKGLSRV	63.38
13	HLA-B*5201	DAENPGGEVFNDNKKGLSRV	22.95
14	HLA-B*5401	DAENPGGEVFNDNKKGLSRV	65.89
15	HLA-B*51	DAENPGGEVFNDNKKGLSRV	71.01
16	MHC-Kd	DAENPGGEVFNDNKKGLSRV	87.24
17	MHC-Kk	DAENPGGEVFNDNKKGLSRV	28.27
18	MHC-Ld	DAENPGGEVFNDNKKGLSRV	39.14

Table 3.2. MHC II peptide binding prediction. Blue letters represent binding site, red letter represents the putative anchor amino acid.

	Allele	Predicted Binding Site	Score
1	DRB1_0301	DAENPGGEVFNDNKKGLSRV	41.68
2	DRB1_0305	DAENPGGEVFNDNKKGLSRV	21.98
3	DRB1_0306	DAENPGGEVFNDNKKGLSRV	40.91
4	DRB1_0307	DAENPGGEVFNDNKKGLSRV	40.91
5	DRB1_0308	DAENPGGEVFNDNKKGLSRV	40.91
6	DRB1_0309	DAENPGGEVFNDNKKGLSRV	31.16
7	DRB1_0311	DAENPGGEVFNDNKKGLSRV	40.91
8	DRB1_0802	DAENPGGEVFNDNKKGLSRV	12.50
9	DRB1_1101	DAENPGGEVFNDNKKGLSRV	13.25
10	DRB1_1107	DAENPGGEVFNDNKKGLSRV	32.97
11	DRB1_1128	DAENPGGEVFNDNKKGLSRV	17.24
12	DRB1_1307	DAENPGGEVFNDNKKGLSRV	14.71

Table 3.3. Summary of the immunological theoretical activity of MASP_{syn}. MHC I and MHC II predicting binding sites and antigenicity. The ABCPred software predict a 70 % of chances that B cell generate antibodies against this peptide.

MASP _{syn}	Human MHC I alleles	Human MHC II alleles	Mice MHC I H2 ^{Kd, Kk, Ld} haplotypes	B cell
DAENPGGEVFNDNKKGLSRV	15	12	3	+

Synthetic Peptides alone are known to have low antigenicity, for this reason we decided to conjugate MASP_{syn} to keyhole limpet hemocyanin (KLH). KLH works as peptide carrier and stimulator of the immune system, through interaction with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes [59].

3.3 *Immunoblotting*

Using serum from mice immunized with MASP_{syn} a western blot analysis was done for detection of MASP_{ves} at the different stages of the parasite. Immunoprecipitation of the trypomastigote stage two different strains, Y and Talahuen were analyzed. Epimastigote, amastigote, and trypomastigote from Talahuen and Y strain all show to have the presence of MASP_{ves} (Figure 3.5A-B).

Furthermore, vesicles were collected at different time points, first and second day of shedding material from trypomastigotes in culture. Vesicles were then fractionated to collect different sizes (S1>100 nm, S2 <100 nm, and S3: Pellet after acetone precipitation). At the time of this experiment, the pool of serum of α -MASP_{syn} from immunized mice was limited because of the continuous use of the serum for other experiments and to preserve the wellbeing of the mice, consequently, we had a custom antibody made (α -MASP_{cus}). This antibody was produced with a different MASP_{ves} peptide sequence than the synthetic. Using MASP_{cus} we were able to detect vesicles from first day of shedding material, and also in vesicles <100 nm from the second day of shedding. Vesicles from the first day have also been recognized by α -galactosyl antibodies (Almeida, personal communication), which are found at elevated levels in Chagasic patients [60]. On the other hand, this is the first reported marker to detect vesicles at such later shedding (Figure 3.5C).

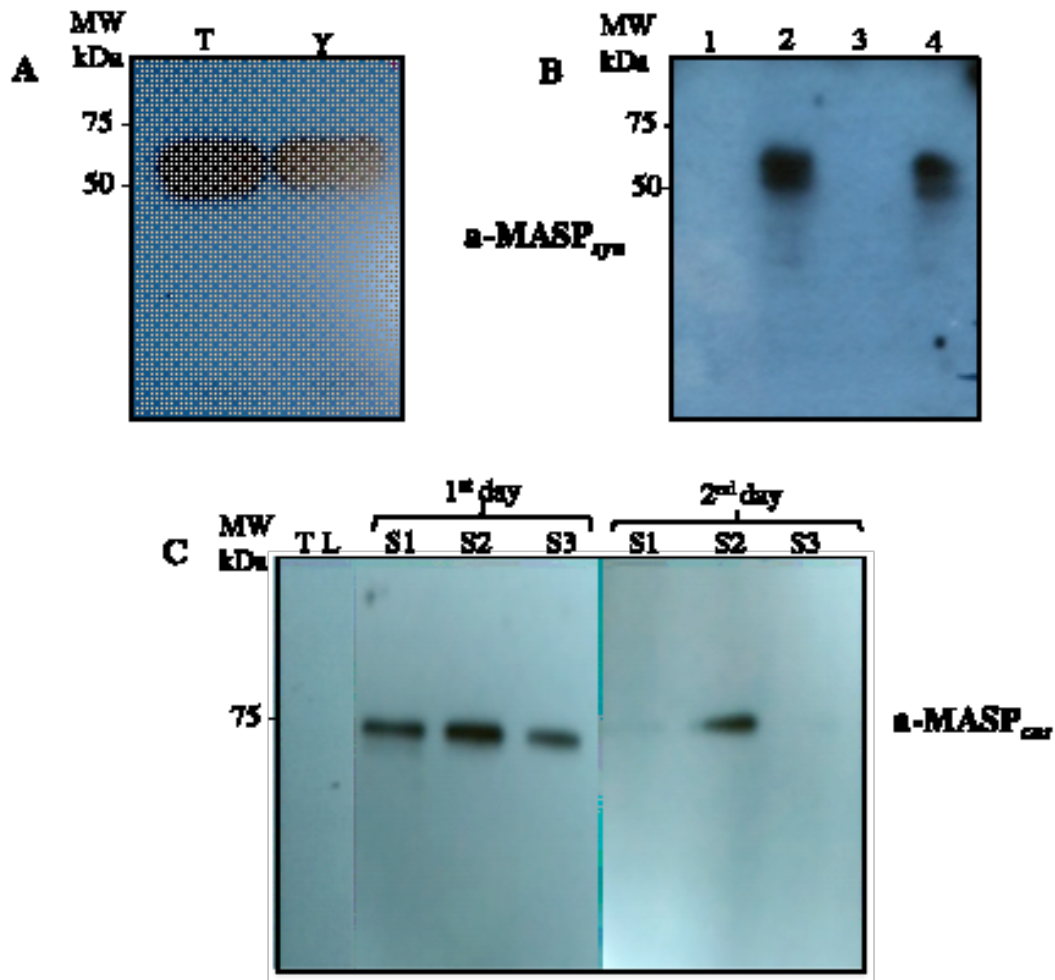


Figure 3.5. Immunoprecipitation and western blot. Detection of MASP_{ves} by antibodies present in sera from mice immunized with MASP_{syn} (A-B). A) Immunoprecipitation of Trypomastigote form of Talahuen and Y strain. B) Lysates of Epimastigote and amastigote stages. C) Vesicles from first and second day shedding using α -MASP_{cus}. Sizes of vesicles S1>100 nm, S2 <100 nm, and S3: Pellet after acetone precipitation.

3.4 Humoral immune response

It has been shown that parasite specific antibody production in *T. cruzi* infected mice aid in the survival.[61]. The purpose of this experiment is to determine capability of MASP_{syn} peptide to elicit a protective humoral immune response.

Pooled sera was collected from all immunized mice groups twelve days after last immunization to determine total IgG antibody levels by ELISA, the dilution of the sera at 1:100 provided specific MASP_{syn} antigen specific antibodies. To determine the production of antibodies against this epitope in the human infection Human Chagasic and non-Chagasic serum were used as positive and negative control. As expected, mice immunized with MASP_{syn} and MASP_{syn}/AIOH both showed antigen-specificity of antibody response as well as the human Chagasic serum (Figures 3.6A and 3.6B), while the non-Chagasic serum did not showed any reactivity. MASP_{syn}/AIOH had a slightly higher detection than the MASP_{syn} group (not significant).

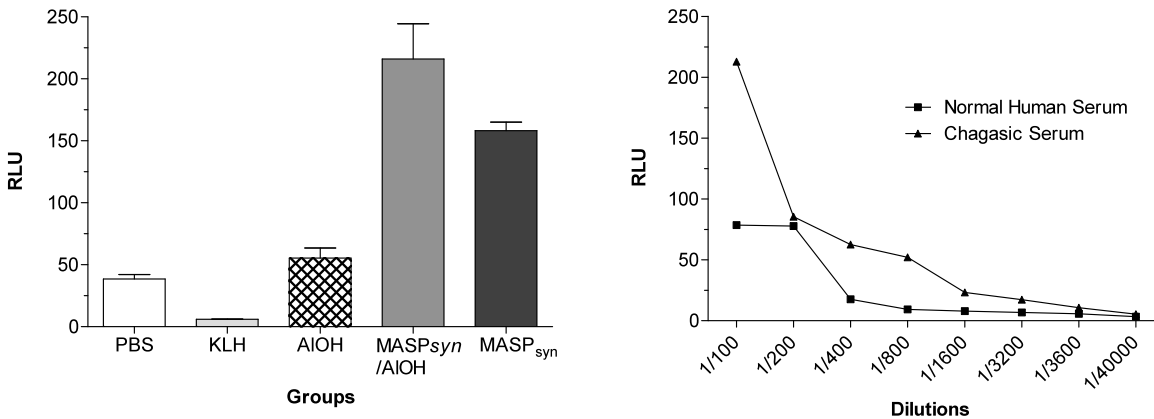


Figure 3.6. Antibody levels from pooled whole serum of non-infected mice (all groups) 10 days after last immunization and pooled human Chagasic serum. A.) Detection of anti- MASP_{syn} IgG levels were done in triplicates per group and by ELISA. Higher levels of antibody titer were detected in MASP_{syn} and MASP_{syn}/AIOH groups. B.) Serum from Chagasic human patients also showed an increase level of antibody titer against MASP_{syn} when compared to normal human serum.

3.5 Immunoglobulin Isotyping

Immunoglobulin (Ig) isotyping was also of interest since the acute phase of the disease is characterized for hypergammaglobulinemia, especially higher titers of IgA, IgM and IgG especially, IgG1, IgG2A, IgG2B isotype antibodies (lytic antibodies) which are considered to have antiparasitic properties [62-64]. In immunized-not infected mice, higher levels of IgA, IgM ($p \leq 0.05$), IgG1 ($p \leq 0.05$), IgG2A were detected (Figure 3.7). The data states that MASP_{syn} as vaccine is stimulating most suitable Igs to kill the parasite.

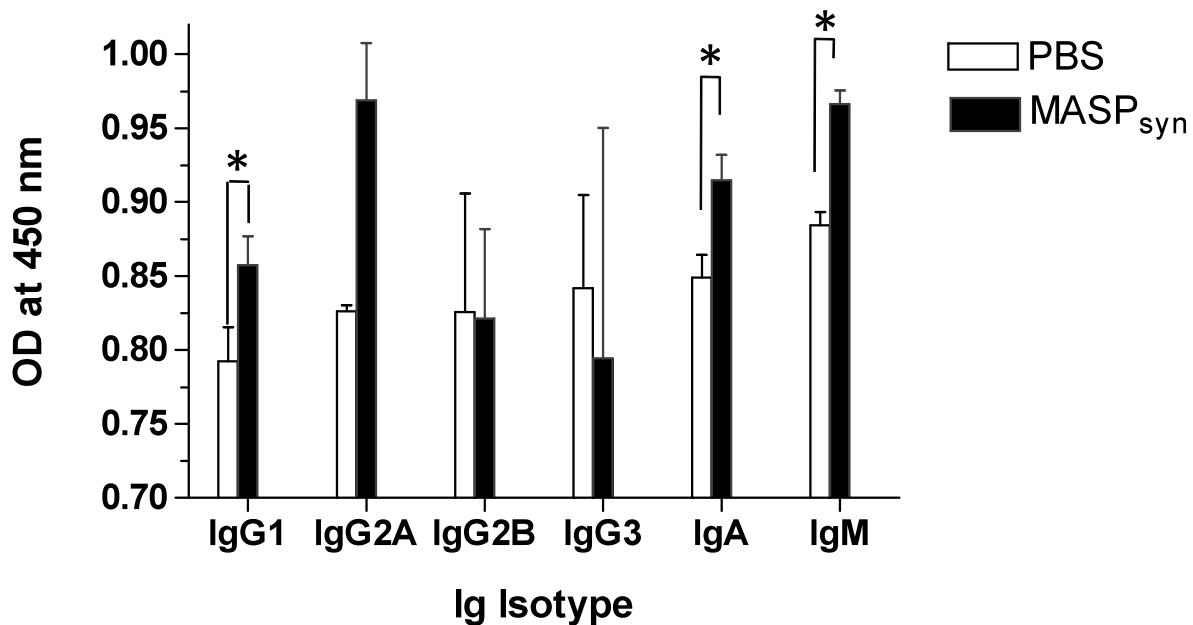


Figure 3.7. Effects of vaccine on production of different immunoglobulins (Ig). A.) Pool serum (n=4/group) from non-infected immunized mice was analyzed by ELISA for Ig isotypes (IgG1, IgG2A, IgG2B, IgG3, IgA, IgM). PBS serum was used as a baseline control, and the positive controls were used from the kit to mark for presence of corresponding Ig. Experiment was done in triplicates per Ig. P-value: $p \leq 0.05 = (*)$.

3.6 Lysis Assay

It has been characterized that Chagasic patients as well as experimental chronic infected mice produces lytic antibodies (IgGs) are able to bind to the membrane surface of trypomastigotes aiding

to control *T. cruzi* infection [65], by lowering the parasitemia. In this regard, the lysis assay was performed to address the possible lytic activity of the anti-MASP_{syn} antibodies produced by immunize mice. In this experiment live trypomastigotes were incubated with α -MASP_{syn} serum, with inactive and active complement, to verify specific MASP-antibody and background lytic activity of the immune system, respectively. Figure 3.8 shows that MASP_{syn} serum is able to lyse the trypomastigotes 3.4 fold higher than the placebo serum (which was taken from mice immunized with PBS and had active complement).

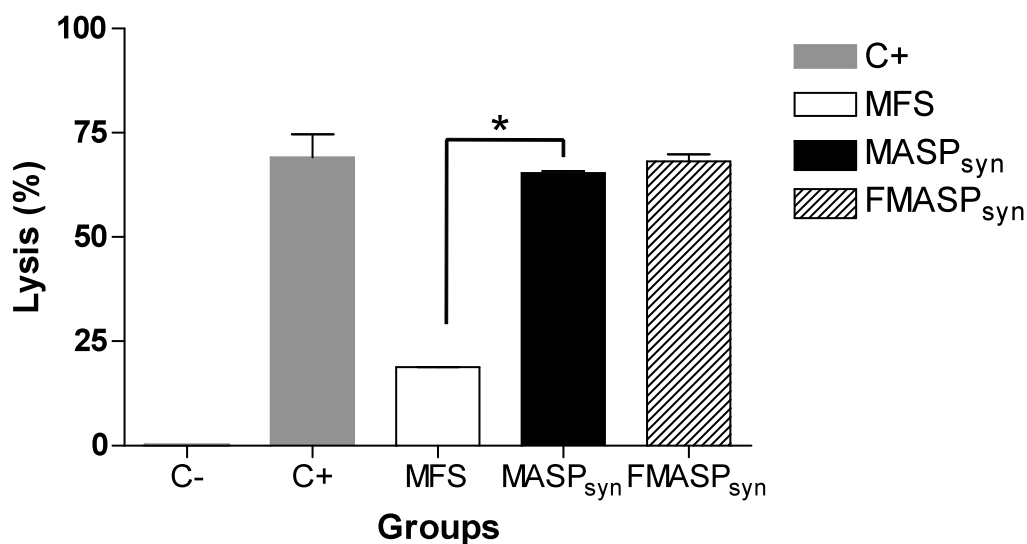


Figure 3.8. Trypomastigote lysis by MASP_{syn} antibody from whole serum immunized mice. A.) Serum from not immunized, not infected mice was collected and was used freshly with natural active complement for the experiment (MFS: Mice fresh serum). Fresh serum from immunized mice and active complement (FMASP_{syn}). Complement system was deactivated in the serum collected from non-infected immunized (MASP_{syn} group) mice (MASP_{syn}). Live trypomastigotes were used as negative control (0% lysis) (C-). A positive control of dead trypomastigotes ($\approx 75\%$ lysis) was also used (C+). The sera from immunized mice (inactivated complement) was able to lyse trypomastigotes more effectively than the placebo group. The data is representative of 3 independent experiments. P-value: $p \leq 0.05 = (*)$.

3.7 *Parasitemia and Survival*

The parasitemia in all groups was followed for 20 days post-infection. Mice immunized with MASP_{syn} alone or with adjuvant did not show any significant decrease in parasitemia during the time monitored (Figure 12A). Survivability was followed as well. Our data shows that immunized mice with solely MASP_{syn} increased survival to 80% for up to a year, when the experiment was ended (Figure 12B). In the case of immunized mice with AIOH and MASP_{syn}/AIOH low survival was observed. In both groups all mice were dead at 15 and 22 days post-infection respectively. Also, in Figure 12B it is shown that mice immunized KLH (control for KLH immunogenicity) are not able to control infection and all die by day 23. This control group was used to address the possible role of KLH (conjugated to the peptide) in the survived mice.

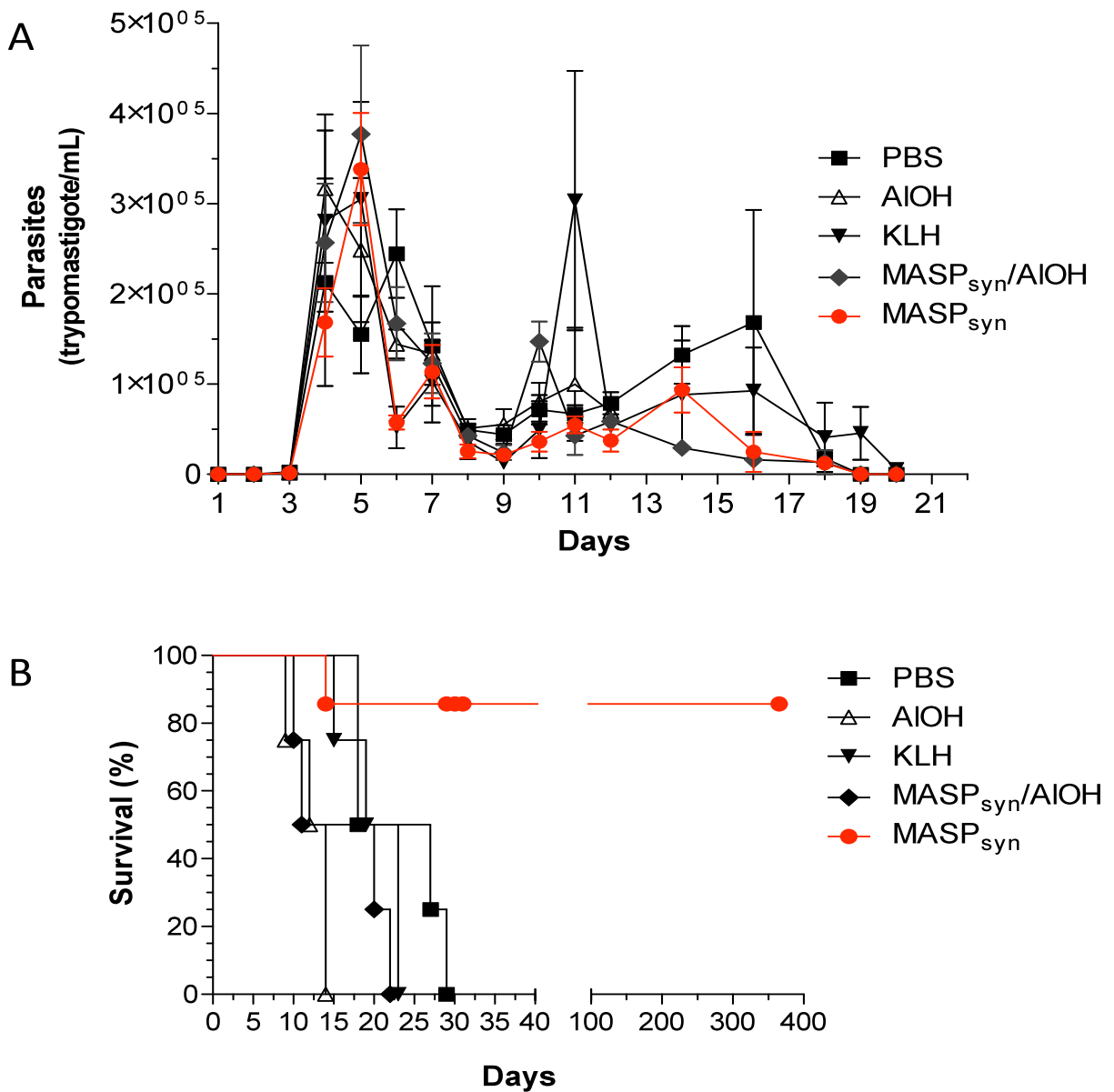


Figure 3.9. Parasitemia (A) and survival (B) of mice infected with 1×10^6 *Trypanosoma cruzi* Y strain trypomastigotes. A.) Parasitemia levels are shown as trypomastigotes/ml of the mice infected. Each point corresponds to the mean parasitemia level in blood of mice groups. Blood parasitemia was followed in all groups (n=4/group). From the 3rd to the 20th day post-infection, 5 μ L of blood was taken from the tail and the number of trypomastigotes was counted. B) Survival was monitored daily in all groups (n=4). Each point is representative of one mouse. Three independent studies were done with similar results.

3.8 *Real-time PCR*

In order to test if the vaccine produces sterile protection to the immunized mice qPCR was performed. The heart, liver and spleen were removed from all animals at the humane endpoint (see Figure 3.9). DNA was extracted and a RT-PCR analysis, with proper controls, was done to determine the parasite burden in the selected organs. $MASP_{syn}$ and $MASP_{syn}/AIOH$ groups both showed lower amount of parasites in all organs. More exceptionally, mice that had been immunized with $MASP_{syn}$ and survived past a year had statistically significant lower burdens in all organs when compared to PBS (placebo control) (Figure 10).

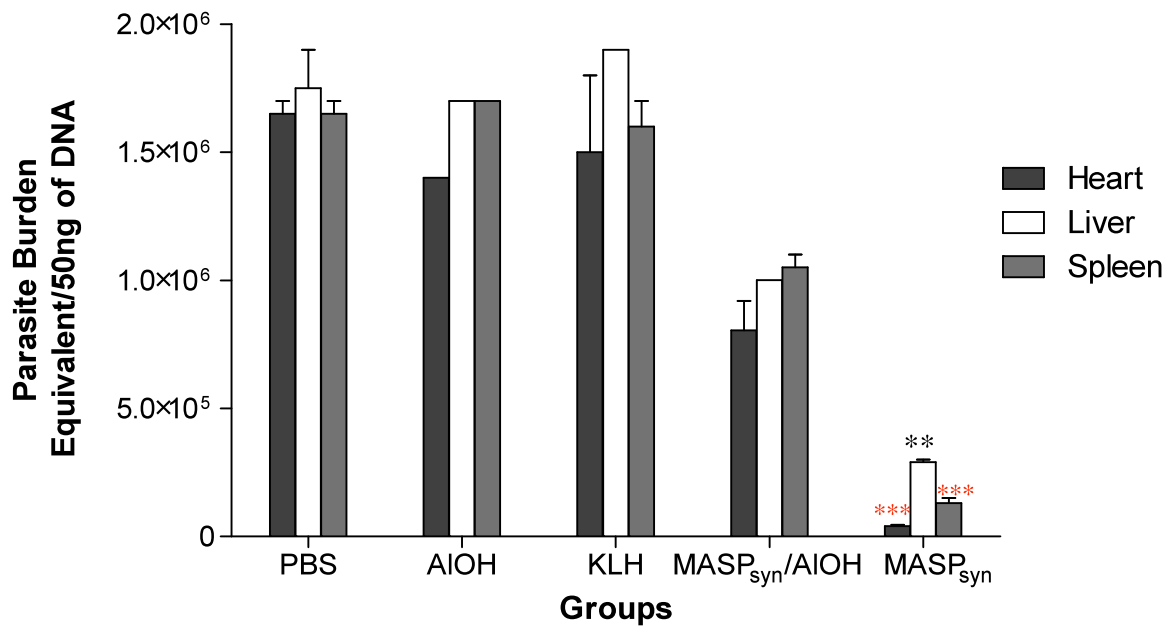


Figure 3.10. Parasite burden in heart, liver, and spleen of immunized mice infected with 1×10^6 *Trypanosoma cruzi* Y strain trypomastigotes. Each bar represents corresponding organs of one mouse, done in triplicates. Data are a comparison of parasite (trypomastigote form) burden of 50 ng of DNA isolated from infected corresponding organ. Mice immunized with $MASP_{syn}$ had significantly lower parasite burden in all organs (heart: P value of $p \leq 0.001$ (***), liver $p \leq 0.01$ (**), spleen $p \leq 0.001$ (***)).

3.9 Evaluation of cytokines

Stimulation of cytokines play a crucial role in resistance of Chagas disease. Therefore, we analyzed the cytokine profile of mice immunized with MASP_{syn}. Four weeks after the last immunization, serum was collected and the cytokines measured were IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFN γ , and TNF α (not all shown) via ELISA. Circulating cytokines that were seen to have a significant increase in either MASP_{syn} or MASP_{syn}/AIOH groups when compared to the PBS group were IL-4, IL-10, IFN γ , IL-12, and IL-17A (Figure 8A-E). Expectantly, the vaccine induced cytokines that are known to play an important role in the response to a *T. cruzi* infection [66-73].

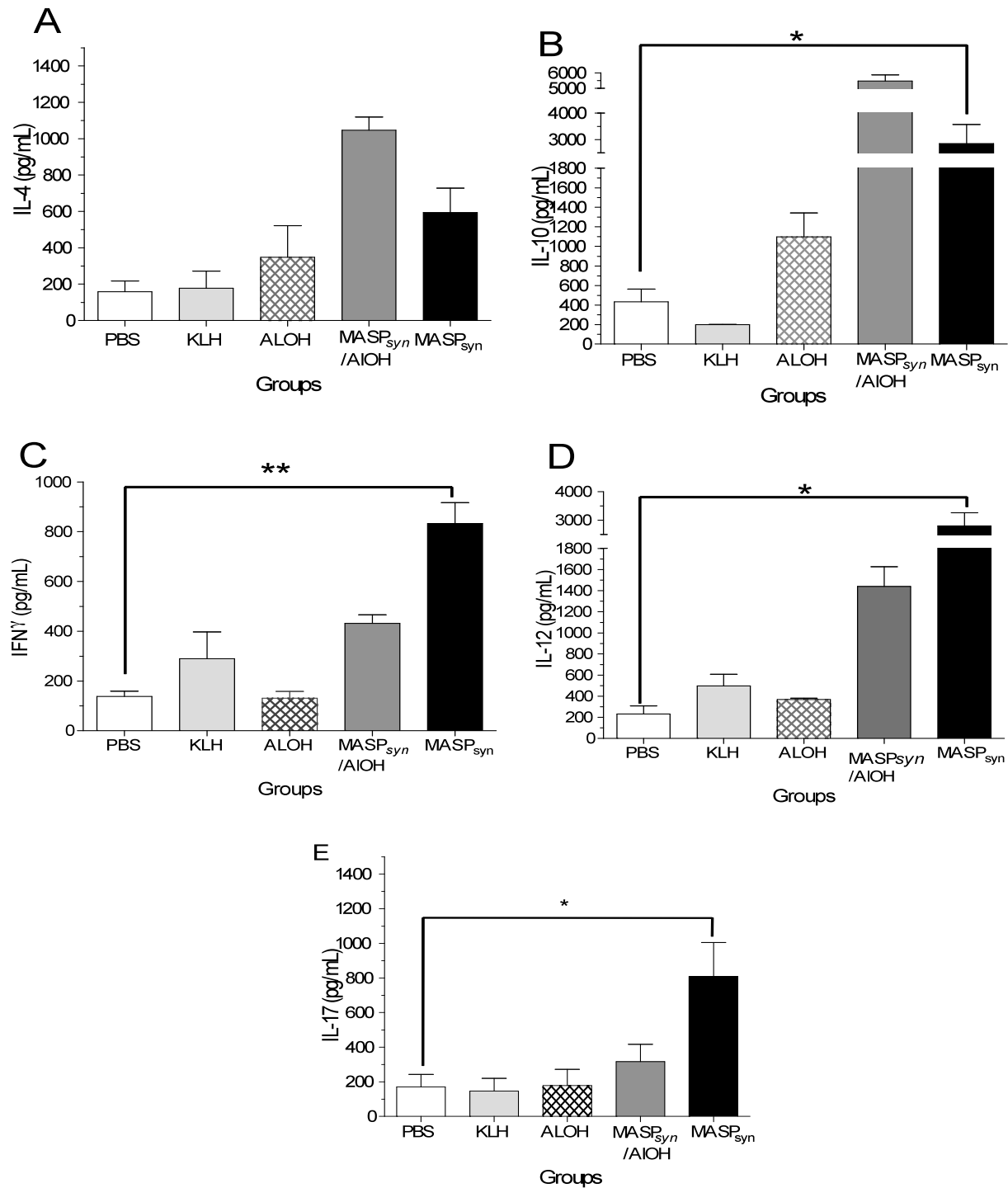


Figure 3.11. Pool serum cytokine levels of non-infected, immunized mice 4 weeks post-last immunization. Cytokines were assayed in triplicate by ELISA. A.) IL-4, Difference in PBS and MASPSyn/AIOH is statistically significant with a P-value of $p \leq 0.05$ (*). B.) IL-10, Difference in

production level in PBS and MASP is statistically significant with a P-value of $p \leq 0.05$ (*). C.) IFN γ Difference in production level in PBS and MASP is statistically significant with a P-value of $**= p \leq 0.01$. D.) IL-12 Difference in production level in PBS and MASP_{syn} is statistically significant with a P-value of $p \leq 0.05$ (*). E.) IL-17, Difference in production level in PBS and MASP_{syn} is statistically significant with a P-value of $p \leq 0.05$ (*). Each bar represents the mean (n=4/group) for the different groups.

3.10 Detection of CD4⁺ and CD8⁺ in immunized mice using Flow Cytometry

In order to evaluate the role of the vaccine in the cellular immune response, we analyzed the activation of CD4⁺ and/or CD8⁺ T-cells. T-cells were isolated from spleens of non-infected and acutely infected mice of all groups after 8 days of infection. Activation was determined by using two different known activation markers, CD25 and CD69 [74]. Mice immunized with AIOH at the acute level of infection, had an overwhelming CD4⁺ and CD8⁺ T cell activation (Figure 9). Clearly AIOH is not able to stimulate the adequate immune response in fact it seems to over stimulate the response possibly leading to early death in infected mice. When looking at mice immunized with either MASP_{syn} or MASP_{syn}/AIOH, in both infected and non-infected mice, the activation of CD4⁺ and CD8⁺ seems to have a homeostatic effect, when monitored with either marker. Upon infection immunized mice are also able to provide a higher activation when compared to the placebo group.

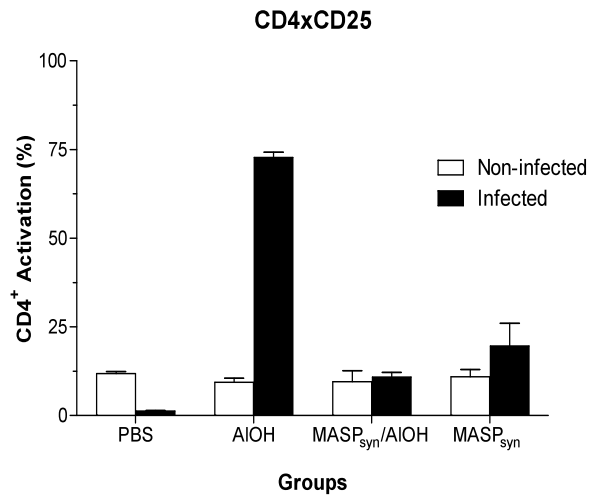
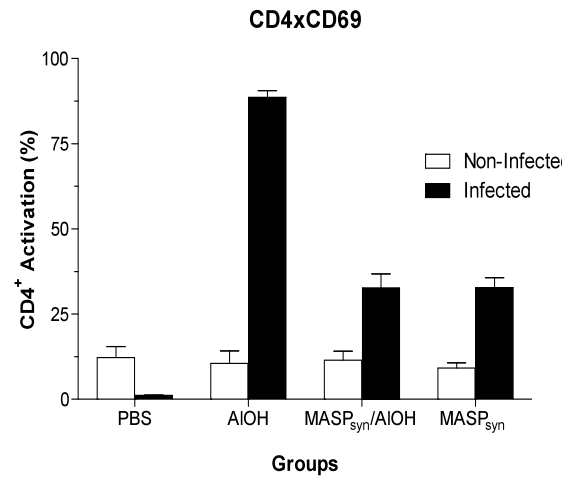
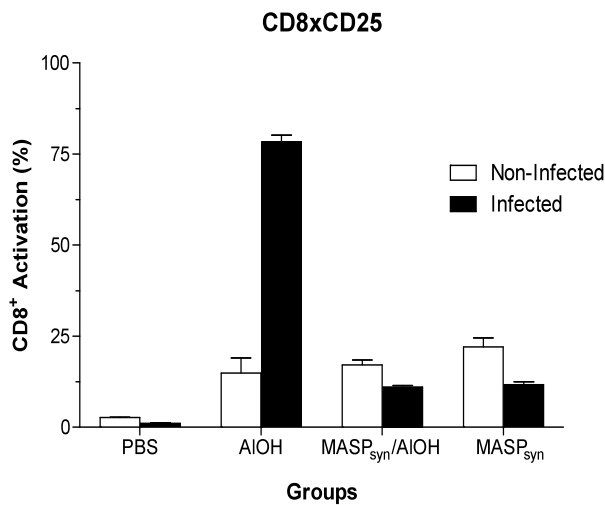
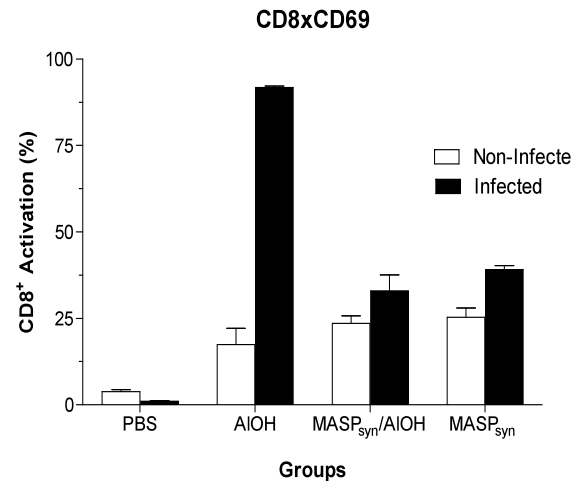
A**B****C****D**

Figure 3.12. CD4⁺ and CD8⁺ activation in acute phase and non-infected mice. Cells were stained for A.) CD4⁺ and CD25 markers. B.) CD4⁺ and CD69 marker. C.) CD8⁺ and CD25 markers. D.) CD8⁺ and CD69 markers. Samples were done in triplicates and analyzed using flow cytometry. Immunized mice show to have a better regulatory system of activation when compared to the two control groups (PBS and AIOH).

3.11 *Detection of CD4⁺ and CD8⁺ in mice with chronic stage infection using Flow Cytometry*

The immunized mice that were challenged with 1×10^4 trypomastigotes develop a chronic disease and some of them survive until nine months when the experiment was terminated. In order to analyze the production of MASP_{syn}-specific CD4⁺ and C8⁺ T cell a boost immunization was administered seven days before euthanize the mice. The spleen was collected just after the animals were sacrificed and the specific T cell production measured using as CD25 and CD69 as markers for CD4⁺ and C8⁺ T (Figure 3.12). It is interesting that mice in the AIOH group died early on therefore, we did not have a chronic model for this group. We were able to compare the non-infected AIOH immunized to all the other groups, since the animals were kept alive. Mice that were immunized with MASP_{syn} and not infected are usually used for collection of serum for other experiments, so there weren't animals available that were immunized, not infected at this age. The overall T lymphocyte level was low as expected in mice at this age. Interestingly, it was an overall slight activation of CD4⁺/CD8⁺ T cells in the mice challenged immunized with MASP_{syn} or MASP_{syn}/AIOH (Figure 3.12), so we can assume that the vaccine was able to stimulate the population of T. cells that had "memory".

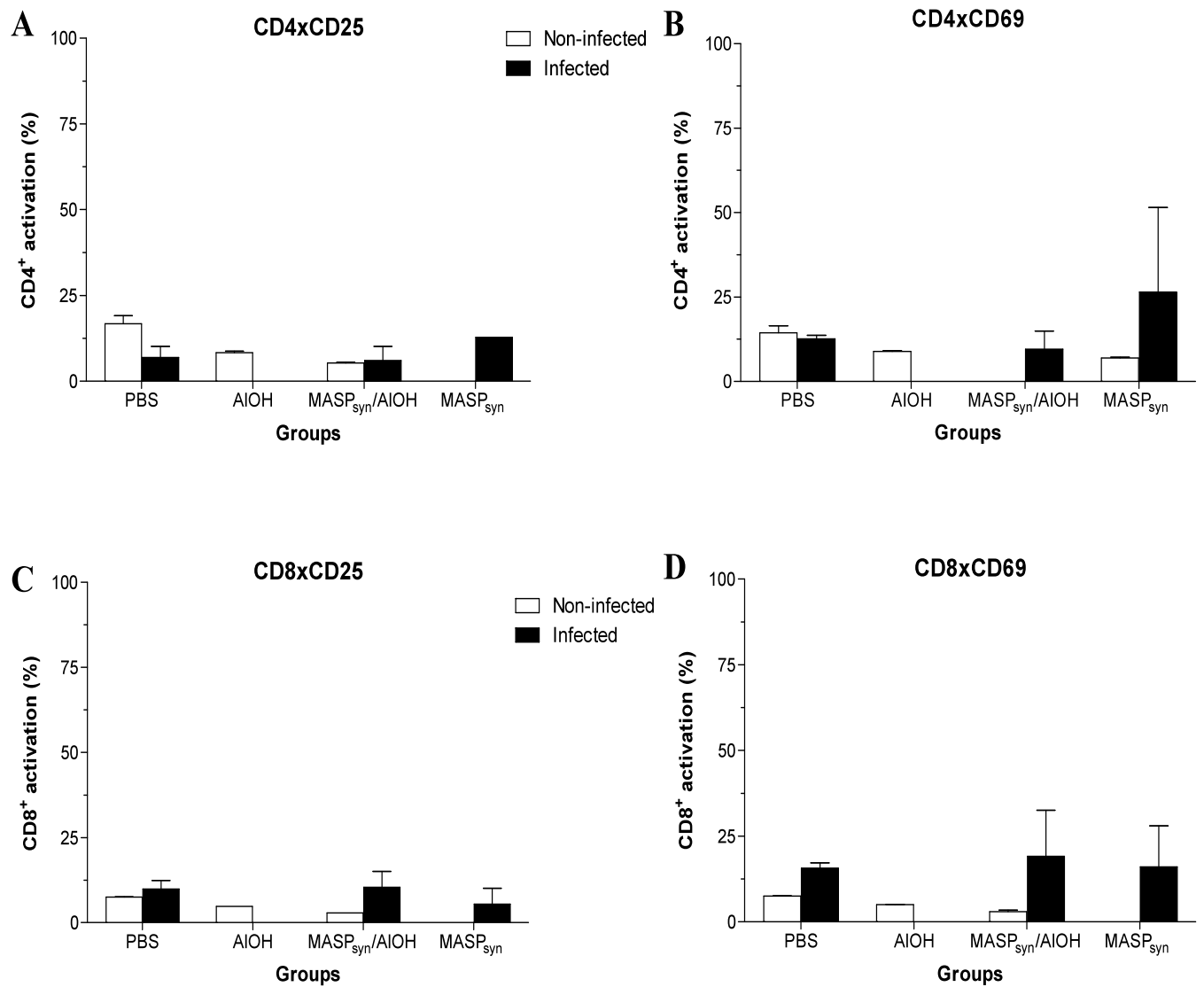


Figure 3.13. CD4⁺ and CD8⁺ activation in chronic phase and non-infected mice. Cells were stained for A.) CD4⁺ and CD25 markers. B.) CD4⁺ and CD69 marker. C.) CD8⁺ and CD25 markers. D.) CD8⁺ and CD69 markers.

3.12 *In vivo depletion of immune cell subsets*

Parasitemia and survival of mice infected, immunized with PBS, KLH or MASP_{syn}, and depleted of CD4⁺ or CD8⁺ were followed to show the role of T cells in the protection by the vaccine. Mice in KLH and PBS groups that were depleted with either T cell type showed higher mortality, and uncontrollable parasitemia. Mice immunized with MASP_{syn} and treated with α -CD8 did not show difference in survivability, but rather a prolonged life. Parasitemia in this group seemed to be controlled until day 15, with a late peak of parasites (17th day) and no detected parasitemia at day 19. Mice immunized with MASP_{syn} and treated with α -CD4 showed increase survival and lower parasitemia when compared to the other groups.

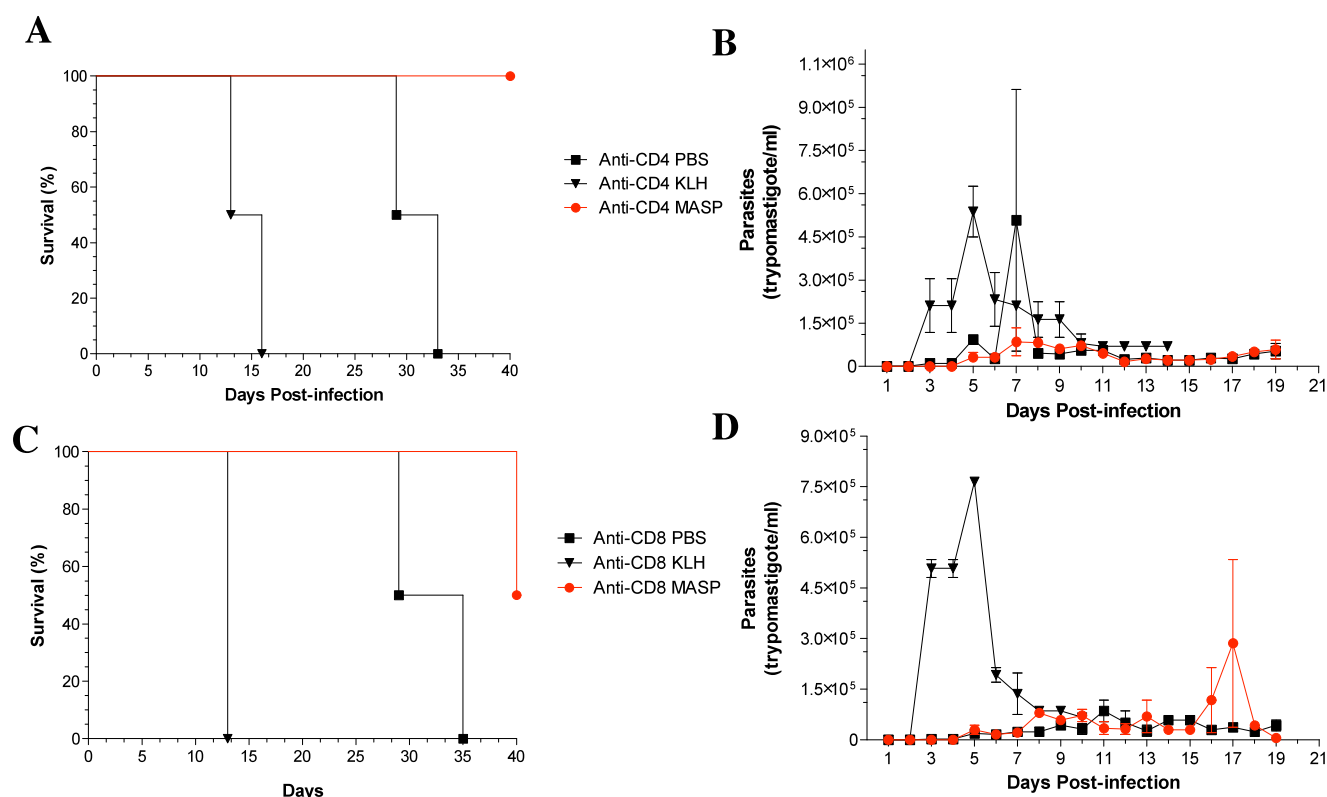


Figure 3.14. Survival and parasitemia of mice depleted of CD4⁺ or CD8⁺ T cells. A.) Survival of mice treated with α -CD4. B.) Parasitemia of mice treated with α -CD4. C.) Survival of mice treated with α -CD8. D.) Parasitemia of mice treated with α -CD8. Immunized mice with vaccine seem to have better response to infection of *T. cruzi*.

Chapter 4: Discussion

The original goal of this study was to evaluate MASP_{ves} protein as a candidate against Chagas disease. After an unsuccessful attempt to produce a recombinant MASP_{ves} vaccine, our alternate approach was to synthetically develop a peptide. Furthermore, we evaluated the efficacy of a synthetic peptide (MASP_{syn}) based vaccine. As previously discussed the optimal vaccine for Chagas disease is that is able to stimulate both a humoral and cell mediated immune response. We therefore assayed the parasitemia, survivability, and the humoral and cell mediated immune response to evaluate the success of the vaccine. Although there was no sterile protection against the infection, there were several relevant results that showed that our vaccine is a good candidate and it could be optimized for the development of a prophylactic vaccine.

The humoral response plays a key role in controlling parasitic infection [61, 75]. Antibodies have shown to have importance in both the chronic and acute stage of the disease. Serum transferred from chronically infected mice to naïve mice showed reduced parasitemia and prolonged survival after challenge [76, 77]. In addition, in the acute phase of the disease B cells also play a valuable role in recruiting CD4⁺ and CD8⁺ T cells and maintaining memory and effector T cells [78]. We evaluated the humoral response by antibody production of immunized mice, here we analyzed the antibody Ig isotyping stimulation and evaluated lytic activity of α -MASP_{syn}. The levels of antibody production against the synthetic peptide were first evaluated in serum from human Chagasic patients as a positive control, since ideally this vaccine would be for human use. This study suggests that MASP_{syn} induce immunity stimulation in a natural infection. As expected, immunized mice with MASP_{syn} produced antibodies against the peptide as well as MASP_{syn}/AIOH. Increasing levels of antibody production in MASP_{syn}/AIOH can be explained by the ability of AIOH to aid production of antibodies through CD4⁺

T cells [48]. We speculate that early death in mice immunized with AIOH was a result of an over inflammation response from both this adjuvant and the parasite challenge.

Recently it was suggested that the MASP family and other surface molecules may contribute to hypergammaglobulinemia seen in the acute stages of the disease [79]. Our further examination of the Ig isotype of the antibodies revealed appealing results. Our data established that immunized mice have a higher amount of IgG1, IgG2A, IgA, and IgM. We found that IgA, IgM and IgG showed significant high titers in the MASP_{syn} immunized animals, which after challenge have 80 % survival. These results are supported by the literature described the anti-parasitic effects of these Igs in the murine model of Chagas disease [62-64]. Interestingly, when antigens from different life stages of the parasite were tested for immunogenicity in serum from infected individuals, increase levels of IgA, IgM and IgG levels are detected [80]. Also, IgG1 and IgG2 have been shown to attribute to the elimination of the parasite and decrease of mortality [81-83]. In addition, IgG1 and IgG3 have been demonstrated to help in the clearance of other parasites such as *Schistosoma mansoni* and *Plasmodium falciparum* [84-86].

In the acute phase of the disease antibodies are produced against surface molecules of the trypomastigote form [87]. The protective antibodies have the capacity to agglutinate trypomastigotes, lyse them in a complement-mediated path, help in phagocytosis/opsonization, and mediate antibody-dependent cellular cytotoxicity [88-92]. We showed that MASP_{syn} produce lytic antibodies that help in the direct killing of trypomastigotes.

Regarding the T-cell specific immune response, the cytokine profile (IL-4, IL-10, IL-12, IL-17A, and IFN- γ) relevant in the control infection was analyzed. Cytokine IL-4 was shown to be stimulated at higher levels in MASP_{syn} and in MASP_{syn}/AIOH serum. This is interesting since it has

been published that IL-4 is a stimulating factor for the differentiation and proliferation of B cells [93], supporting the higher antibody production as well in these two groups.

IL-4 is not a major determinant of susceptibility to a *T. cruzi* infection, but IL-4 and IL-10 in association have been demonstrated to control myocarditis [66]. It has also been shown that if IL-4 is more dominant than IL-10 parasitemia is enhanced [67]. This is interesting since our vaccine is able to stimulate more dominance IL-10 than IL-4. IL-10 during infection can actually increase parasitemia, however, it is essential for survival. Mice that are depleted of IL-10 show lower parasitemia, but higher mortality [70]. IL-10 is able to prevent inflammatory damage and thus mortality as well [68, 69]. In our case the MASP_{syn} is increased, but still had less production than MASP_{syn}/AIOH, showing some homeostasis. IL-12 is also important for controlling infection, IL-12 KO mice showed higher parasitemia and mortality [67, 94]. IL-12 and IFN- γ are crucial to lower parasitemia, mortality and lower inflammation, respectively [70, 71]. IFN- γ has been identified as a resistant player in the infection of *T. cruzi* [95]. Also, the addition of recombinant IFN- γ to mice increases resistance of *T. cruzi* and neutralizing it causes susceptibility [95]. In our case, both IL-12 and IFN- γ are produced at high levels when compare to the control. IL-17A levels of our immunized mice showed statistically significant increase levels when compared to the placebo immunized mice. This is relevant since IL-17 helps regulate extracellular and intracellular pathogens [96]. Moreover, IL-17 has been shown to play a role in regulating parasite-induced myocarditis and aid in parasitemia and survivability [97, 98]. More specifically IL-17A was recently demonstrated to play a vital role in resistance of a *T. cruzi* infection [73]. Additionally, IL-17 was shown to be produced during acute phase of infection, is able to control cardiac inflammation by modulating a Th1 response [97]. After closely analyzing and associating cytokine production we speculate that this vaccine is activating a Th1, Th9, and Th17 response. Th1 response, through production of IFN- γ , has been linked to *T.*

cruzi [99]. Also, Th1 response that produces IL-12 and IFN- γ are important for protection against a variety of intracellular pathogens [100]. Furthermore, IFN- γ is the main cytokine that stimulates isotype switching to the IgG1 and IgG3 subclasses eliciting a Th1 response [101]. Therefore, our results seem to corroborate all this cascade of events supporting activation of Th 1 results. Th9 in *T. cruzi* infection has not been explored, but it is known to play roles in pro-inflammatory and function in many autoimmune diseases and allergic inflammation [102]. Also, Th9 cells secrete high amounts of IL-10 and to differentiate in the presence of TGF- β and IL-4 [102]. As mentioned briefly above IL-10 and IL-4 both play essential roles in infection. Th9 has also been shown to contribute to the differentiation of Th17 by the secretion of IL-9 [102]. Lastly, Th17 cells, through the production of IL-17 family, are known to be critical for the clearance of extracellular pathogens [103]. Hence, Th1, Th9 and Th17 responses all seem to reflect the cytokine profile that we see with the vaccine.

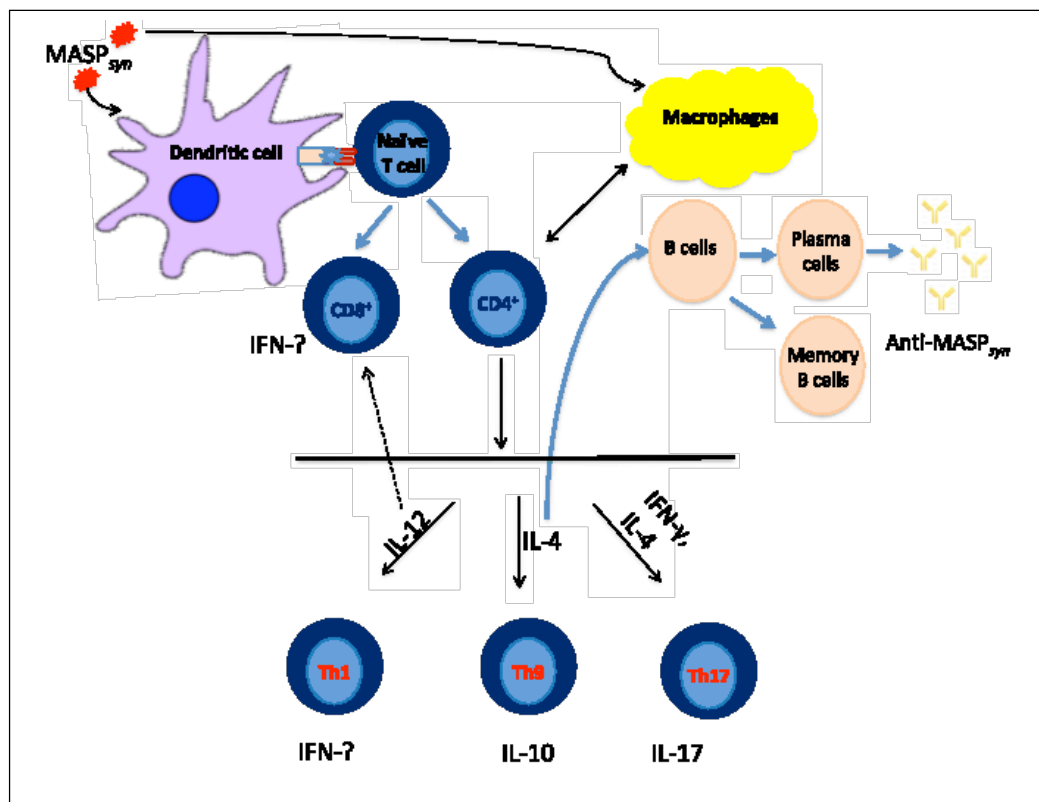


Figure 4.1. Model of the T and B cell activation by MASP_{syn} in immunized mice

Cellular immune response was also analyzed by determining the percentage of activation of CD4⁺ and CD8⁺ T cells in both non-infected, immunized mice and in acute and chronic phase immunized mice. Chronic infected mice showed a slight activation of both T lymphocytes upon boost, which shows that memory was created. In the acute stage of infection mice immunized (non-infected or infected), had a greater activation response from both cells. The activations seem to reflect a homeostatic effect between the cells, probably contributing to the increase survivability and controlling of the disease. Mice with the chronic stage of the disease, although showing the expected overall low lymphocyte number because of their age [104-107], also showed a slight increase in activation within immunized-infected mice. Furthermore, we also abolished either CD4⁺ or CD8⁺ in immunized mice and then challenged. We concluded that the vaccine is probably protecting through more on stimulation of CD8⁺ T cells, since mice with the depletion of CD8⁺ died and had greater parasitemia, developing at a later time. This was also confirmed by the mice with depleted CD4⁺ in which the animals were able to prolong death or survive and had lower parasitemia. This is encouraging since CD8⁺ T lymphocytes have been established to be important of protective immunity against this parasite [108, 109].

It is important to highlight that one year after the challenge (with 1×10^6 parasites) the mice immunized with MASP_{syn} alone showed 80% survival, more than any other infected mouse in the experimental groups. Early in the infection MASP_{syn} mice showed high parasitemia, however from the 8th – 18th day post infection lower parasitemia was maintained, and no parasites were detected in blood after. Most noticeable the vaccine was able to significantly reduce the level of parasites in heart, liver, spleen, probably attributing to the survival of the mice.

In conclusion, surface proteins, such as MASPs, are intriguing epitopes to study for vaccination purposes because of their visibility to host cells. In this study we have provided evidence

of a synthetic based vaccine based, MASP_{syn}, is able to effectively control *T. cruzi* infection, prolonging survival, and possibly reducing progression of the disease. This might be able to be an alternate treatment to the present drug therapy used to treat Chagas disease. The vaccine has shown to also elicit an ideal immune stimulation, therefore, we foresee that efforts in the optimization of this vaccine candidate, through improving delivery system (e.g. virus particles approach), immunizations protocols, adjuvant and carrier molecules, will provide a full protective vaccine against Chagas disease.

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Vita

Carylinda Serna earned her Bachelor of Science from The University of Texas at El Paso (UTEP). She was accepted into the Pathobiology PhD program at UTEP in 2008.

In 2009 she was awarded the HHMI mentor fellowship, in which she mentored numerous undergraduates. Carylinda is also part of distinguish scientific communities such as Society for Advancement of Chicanos and Native Americans in Science (SACNAS) and American Society for Microbiologist (ASM), both nationally and locally.

As a graduate student she was trained in molecular and immunology methods directed to studying parasites. Her dissertation entitled “Mucin Associated Surface Protein Synthetic Peptide as a Novel Vaccine Candidate Against Chagas Disease” was supervised by Dr. Rosa Maldonado. This work has been presented in a variety of conferences; such as, Rio Grande Branch of the ASM (RGASM) (2009-2011), in which she was awarded Best Graduate Poster, UTEP SACNAS Research Expo (2009), wherein she received third place for Graduate Poster, SACNAS National Meeting (2011), Kinetoplastid Molecular Cell Biology Meeting (2012), Symposium on Infectious Disease and Health Disparities in a Changing World (2011), and Gordon Research Conference (2009 & 2013). Carylinda has also been privileged to be invited and speak to early college students about careers in science. As well as had an invitation to speak about her research in a meeting for the Association for Professionals in Infection Control and Epidemiology.

During her process in achieving her doctoral she had the opportunity to interact with undergraduates as an instructor and with laboratory training. As a graduate student she also mentored entering and senior students conducting similar research. As a teaching assistant she taught laboratories for General Biology, General Microbiology, Anatomy and Physiology, Prokaryotic

Molecular Genetics (PMG), and Medical Parasitology. She has been fortunate to give lectures in PMG and Medical Parasitology.

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