ALESSANDRA TEIXEIRA DE PAULA

AVALIAÇÃO DO POTENCIAL IMUNOGÊNICO E PROTETOR DA VACINA CONTRA DOENÇA DE CHAGAS PELA ESTRATÉGIA "PRIME-BOOST" HETERÓLOGO DNA/rNTPDASE-1

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Gradução em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae.*

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APROVADA: 06 de fevereiro de 2018.

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“ Um pouco de ciência nos afasta de Deus, muito nos aproxima”

Louis Pasteur

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RESUMO

PAULA, Alessandra Teixeira de, D.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Avaliação do potencial imunogênico e protetor da vacina contra doença de Chagas pela estratégia "prime-boost" heterólogo DNA/rNTPDase-1**. Orientador: Leandro Licursi de Oliveira. Coorientadoras: Silvia Almeida Cardoso, Samanta Cristina das Chagas Xavier e Eliziária Cardoso dos Santos.

A doença de Chagas é causada pelo protozoário intracelular obrigatório *Trypanosoma cruzi*, e transmitido a humanos e animais vertebrados através do contato de uma solução de continuidade da pele com fezes de insetos triatomíneos infectados. Atualmente, a doença afeta aproximadamente 16 milhões de pessoas em todo o mundo. Somente duas drogas estão disponíveis no mercado para o tratamento de doentes: Benzonidazol e Nifurtimox, no entanto estas apresentam diversos efeitos colaterais, são contra-indicadas a pacientes imunossuprimidos e são eficientes apenas na fase aguda da doença. Levando-se em consideração a eficácia limitada e toxicidade das drogas disponíveis, o desenvolvimento de uma estratégia de vacinação representa uma alternativa para o controle desta doença. Neste sentido, nosso objetivo foi avaliar a resposta imune induzida pela vacinação utilizando a estratégia vacinal "prime-boost" heterólogo DNA/NTPDase-1 de *T. cruzi*, em camundongos BALB/c. Os resultados indicaram que a imunização com o "prime-boost", induziu produção de níveis semelhantes tanto de IgG1 quanto de IgG2a, e embora o grupo imunizado com três doses da rNTPDase-1 tenha produzido níveis mais elevados de anticorpos precocemente, quando a avaliação foi feita 15 dias após a última imunização, verificamos que esses níveis eram semelhantes para ambos os grupos imunizados, sendo observada produção mista desses isótipos. Também foi observado, para o grupo "prime-boost ", aumento significativo na produção de citocina Th1 (TNF-α) e da quimiocina MCP-1. Avaliando o perfil de células T, verificamos que os animais vacinados seguindo esse mesmo protocolo apresentaram um aumento significativo na frequência de linfócitos T CD8 ($CD3^{+}CD8^{+}$), além de aumentar de forma significativa o percentual de células T CD4 e CD8 com fenótipo de memória ($CD44^{high}CD62L$), quando comparado ao grupo que recebeu três doses da rNTPDase-1 e também o grupo inoculado somente com PBS (controle). Após o desafio, observamos que a imunização tanto com a rNTPDase-1 quando com o "prime-boost" foram eficazes em reduzir a carga parasitária presente no tecido cardíaco dos animais vacinados em comparação ao grupo controle, no entanto a carga parasitária presente no grupo "prime-boost" era inferior quando comparada ao grupo imunizado somente com a rNTPDase-

1. A imunização utilizando esses dois protocolos, também resultou em 100% da sobrevivência quando comparados ao grupo controle, onde 50% dos animais haviam morrido durante o período experimental. Diante dos dados observados, sugerimos que a imunização seguindo a estratégia vacinal “prime-boost” heterólogo DNA/NTPDase-1 de *T. cruzi,* além de induzir polarização da resposta imune para o tipo Th1, também resultou em melhora na proteção. Sendo assim, imunização seguindo a estratégia vacinal “prime-boost” heterólogo DNA/NTPDase-1 de *T. cruzi* pode ser vista como promissora no controle da doença de Chagas.

ABSTRACT

PAULA, Alessandra Teixeira de, D.Sc., Universidade Federal de Viçosa, February, 2018. **Evaluation of the immunogenic and protective potential of the vaccine against Chagas disease by the heterologous prime-boost strategy DNA/rNTPDase-1.** Adviser: Leandro Licursi de Oliveira. Co-advisers: Silvia Almeida Cardoso, Samanta Cristina das Chagas Xavier and Eliziária Cardoso dos Santos.

Chagas disease is caused by the required intracellular protozoan *Trypanosoma cruzi*, transmitted to humans and vertebrates animals through of contacto of a continuity solution skin with feces of infected triatomine insects. The disease currently affects approximately 16 million people worldwide. Only two drugs are commercially available for the treatment of patients, such as Benzonidazole and Nifurtimox, however, they have several side effects, are not indicated in immunosuppressed patients and are efficient only in the acute phase of the disease. Due to the limited efficacy and toxicity of the available drugs, the development of a vaccination strategy represents an alternative for the control of this disease. In this regard, our objective was to study the immune response induced by the vaccination using "prime-boost" heterólogo DNA/NTPDase-1 de *T. cruzi*, in mice BALB/c. Our results indicated that immunization with prime-boost induced production of similar levels of IgG1 and IgG2a, and although the group immunized with rNTPDase-1 produced higher levels of antibodies at the immunization intervals when the evaluation was done 15 days later the last immunization, we check that these levels were similar for both immunized, being observed mixed production of these isotypes. We also observed a significant increase in the production of Th1 cytokine (TNF-α) and chemokine MCP-1 in the prime-boost group, and the T-cell profile showed that the animals vaccinated following the same protocol had a significant increase in the frequency of CD8 T lymphocytes ($CD3^{+}CD8^{+}$), in addition to significantly increasing the percentage of CD4 and CD8 T cells with memory phenotype ($CD44^{high}CD62L$) when compared to the rNTPDase-1 group and also the group inoculated only with PBS (control). After the challenge, we observed that immunization with NTPDase-1 was effective in reducing the level of parasite present in the cardiac tissue of the vaccinated animals compared to the control group, however the parasite level present in the prime-boost group was lower when compared to the group immunized only with rNTPDase-1. Immunization using these two protocols also resulted in 100% survival when compared to the control group, where 50% of the animals died during the experimental period. The observed data, suggest that immunization following "prime-boost" heterólogo

DNA/NTPDase-1 of *T. cruzi* strategy, induced polarization of the Th1-type immune response, also resulted in improved protection. Thus, immunization following the this strategy can be seen as promising in the control of Chagas' disease.

INTRODUÇÃO GERAL

Doença de Chagas

A doença de Chagas, também denominada tripanossomíase americana, é causada pelo protozoário intracelular obrigatório *Trypanosoma cruzi,* e tem como vetores insetos Triatomíneos (Chagas, 1909; Lent e Wygodzinsky, 1979). Descoberta em 1909, a doença de Chagas ainda hoje é um flagelo para humanidade e está associada a impactos econômicos negativos nos países em que está presente. Embora nas últimas três décadas os programas de controle vetorial e triagem de doadores de sangue tenham reduzido de forma significativa casos da doença em todo mundo, alguns desafios permanecem, pois a doença está se tornando um problema em áreas não endêmicas devido as crescentes migrações populacionais (Alpern et al, 2017).

Atualmente, a doença de Chagas representa uma das condições mais comuns de Doenças Tropicais Negligenciadas (DTN), com aproximadamente 16 milhões de pessoas sendo afetadas em todo o mundo: só em 2015 foram 8 mil mortes causadas pelo parasito (Hotez et al, 2012; Lee, 2013; Stanaway e Roth, 2015; Wang Haidonh et al, 2017).

Segundo a Organização Mundial da Saúde, o maior número de portadores da doença de Chagas são encontrados em regiões pobres localizadas na America Latina, onde as condições do meio ambiente favorecem a instalação e proliferação do triatomíneo, sendo elas Argentina, Brasil, México e Bolívia (WHO, 2015). A doença de Chagas se tornou globalizada com milhares de casos notificados no sul da Europa, Austrália e Japão (Gascon et al, 2010). Transmissão autóctone também tem sido observada sendo responsável pelo surgimento de casos da doença no sul dos Estados Unidos (Garcia et al., 2015a; Garcia et al, 2015b; Martinez-Medina et al, 2014).

As principais formas de transmissão da doença incluem o contato de uma solução de continuidade da pele com fezes do vetor infectado, transfusão com sangue contaminado, infecção oral devida á ingestão de alimentos contaminados com triatomíneos ou suas fezes, que recentemente foi reconhecida como causa de pequenos surtos esporádicos em humanos principalmente na região amazônica, e transmissão congenita, que é motivo de preocupação em áreas não endêmicas. A transmissão acidental é pouco frequente e inclui a contaminação durante transplante de órgãos, ingestão de leite materno contaminado com o protozoário, relação sexual e acidentes laboratoriais (Benchimol-Barbosa, 2010; Wendel, 2010; Bern et al,

2011a; Dias, 2011; Diaz-Luján etal, 2012; Shikanai-Yasuda e Carvalho, 2012; Araujo e tal, 2017).

A doença de Chagas persiste como um problema médico-social grave de difícil abordagem clínica devido á sua característica sistêmica e á variabilidade de manifestações clínicas que se desenvolvem ao longo de sua evolução (Hotez et al, 2008; Biolo et al, 2010). A doença se desenvolve em dois estágios distintos e sucessivos: a fase aguda e a fase crônica. A fase aguda ocorre durante os primeiros meses após a infecção, e é caracterizada por alta carga parasitária na corrente sanguínea do hospedeiro. Normalmente essa fase é assintomática, no entanto manifestações locais podem ser observadas, como por exemplo edema bipalpebral unilateral (sinal de Romaña), que surge quando o parasito penetra na conjuntiva, ou o chagoma de inoculação, que é um edema provocado na pele devido á picada do inseto triatomíneo (Andrade et al, 2011; WHO, 2014).

Uma vez que o paciente entra na fase crônica, ele poderá conviver com a infecção para o resto de sua vida. Esta fase da doença pode ser apresentada como forma indeterminada (assintomática), onde testes sorológicos ou parasitológos podem estar positivos, mas com eletrocardiograma, exames radiológicos do tórax, esôfago e cólon normais. A maioria dos pacientes infectados em áreas endêmicas estão nesta fase da doença (Marin-Neto etal, 2002; Melo et al, 2011). No entanto, as manifestações clínicas na fase crônica podem ser evidentes (forma sintomática), podendo estar relacionadas a distúrbios degenerativos graves em órgãos vitais (formas cardíacas, digestivas e nervosas). A cardiomiopatia é o aspecto clínico mais importante nessa fase da doença devido à sua frequência e gravidade. Aproximadamente 40% dos pacientes que sobrevivem à fase aguda da doença têm uma evolução do quadro para a fase crônica, caracterizado pelo surgimento de cardiomiopatia chagásica (Limon-Flores et al, 2010; Aparicio-Burgos et al, 2015). Esse quadro clínico pode se desenvolver décadas após a infecção inicial e é uma fase altamente incapacitante, levando à insuficiência cardíaca e tendo a morte como consequência (Coura et al, 2010; Rassi et al, 2010; Nunes et al, 2011; Ribeiro et al, 2012).

Transmissão vetorial e ciclo de vida de *Trypanosoma cruzi*

T. cruzi é transmitido a humanos e animais vertebrados por insetos triatomineos que pertencem a um grupo de cerca de 140 espécies, muitas das quais são vetores reais ou potenciais da doença de Chagas e que habitam tanto florestas como áreas secas presentes na América do

Sul e Central, México e o sul Estados Unidos (WHO, 2002; Yamagata e Nakagawa, 2006; Bern et al, 2011b; Costa et al, 2012).

Ao longo do seu ciclo evolutivo, *T. cruzi* sofre alterações morfológicas que possibilitam sua sobrevivência e desenvolvimento no inseto vetor e no hospedeiro vertebrado (Figura 1). Os epimastigotas são as formas encontradas no intestino médio e posterior do inseto vetor e são formas replicativas que se multiplicam por fissão binária. Os tripomastigotas metacíclicos são as formas infectantes para o hospedeiro vertebrado, presentes na ampola retal e consequentemente nas fezes dos triatomíneos (Brener, 1971; Rassi et al, 2009; Pérez-Molina e Molina, 2017).

Em humanos, *T. cruzi* é basicamente observado sob duas formas: amastigotas e tripomastigotas sanguíneos. As amastigotas são formas intracelulares obrigatórias que não possuem flagelo e são responsáveis pela multiplicação que ocorre em vários tipos de células (Tyles e Engman, 2001; De Souza, 2002). Os tripomastigotas sanguíneos possuem um flagelo que se estende ao longo de seu corpo e são formas não-replicativas. São derivados do ciclo intracelular de multiplicação, sendo encontrados na circulação após a ruptura da célula infectada, podendo invadir novas células nucleadas ou serem ingeridas pelo triatomíneo durante um novo repasto sanguíneo. Durante o estágio agudo da doença, todos os tipos de células nucleadas no hospedeiro vertebrado são alvos potenciais de infecção. Com o desenvolvimento da resposta imune, a parasitemia reduz-se a uma concentração subpatente e o número de parasitos nos tecidos diminui substancialmente, sinalizando o fim da fase aguda. No entanto, uma vez que o parasito não é completamente eliminado, a infecção de tecidos específicos no hospedeiro persiste por décadas (Rassi et al, 2010).

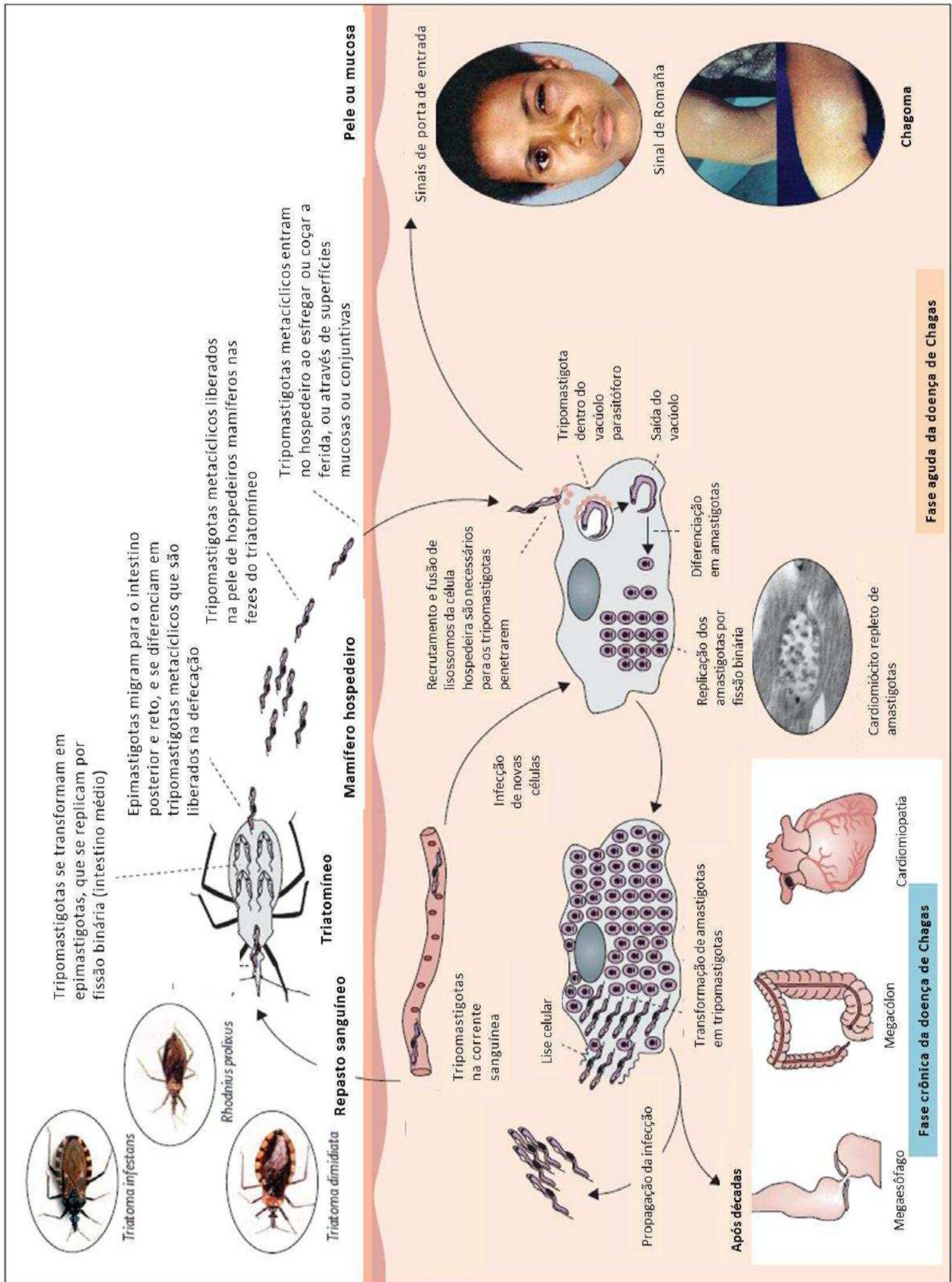

Figura 1: Transmissão vetorial e ciclo evolutivo do *Trypanosoma cruzi* (Rassi, 2010) Adaptado: Mariotini-Moura, 2014a.

Tratamentos disponíveis para a doença de Chagas

A doença de Chagas é uma das doenças tropicais negligenciadas mais importante e um fator relevante neste contexto é o seu tratamento. Na década de 1970 duas drogas foram disponibilizadas no mercado: Benzonidazol e Nifurtimox (Sosa-Estani et al, 2012). Essas drogas são contra-indicadas a pacientes imunossuprimidos, exigem uso prolongado e necessitam de um cuidadoso monitoramento dos usuários devido á elevada toxicidade, além de não garantir a cura após a disseminação e instalação do parasito nos órgãos e tecidos do hospedeiro, pois apresentam eficácia apenas na fase aguda da doença, o que se torna um problema levando-se em consideração que a maioria dos pacientes não recebe um diagnóstico preciso nessa fase (Rassi et al, 2010; Bern, 2015; Grando et al, 2017). Atualmente o Nifurtimox não é mais comercializado no Brasil, e o Benzonidazol é a única droga convencionalmente comercializada nas últimas décadas e clinicamente prescrita para tratamento de paciente chagásico (Muñoz et al, 2011).

O Benzonidazol tem ação sobre as formas tripomastigotas e amastigotas, inibindo a síntese proteica (DoCampo e Moreno, 1984; Silveira et al, 2000; Urbina e DoCampo, 2003). Apesar da sua eficácia no tratamento da fase aguda da doença, o Benzonidazol possui efeitos adversos que vão desde reações de hipersensibilidade a alterações sanguíneas e polineuropatia periférica. Alguns estudos têm utilizado combinações de drogas, tais como Benzonidazol, Nifurtimox e Alopurinol entre outras, com intuito de minimizar os efeitos adversos causados por estes quimioterápicos (Muñoz et al, 2011). É ainda importante ressaltar que parasitos naturalmente resistentes à quimioterapia já foram relatados em várias regiões da América Latina (Camandaroba et al, 2003).

Tendo em vista a morbidade causada pela doença de Chagas, a toxicidade e ineficiência do tratamento com as drogas disponíveis, é de extrema importância desenvolver métodos alternativos para o controle da doença, que poderiam reduzir de forma significativa a população afetada e os agravos decorrentes da infecção (Beaumeira et al, 2016).

Desenvolvimento de vacinas contra a Doença de Chagas

Entre os principais fatores que dificultam o desenvolvimento de uma vacina contra a doença de Chagas, a diversidade intraespecifica observada em *T. cruzi* pode ser a mais relevante, uma vez que até o momento pelo menos sete linhagens já foram identificadas, e essa

variabilidade genética representa um desafio para o desenvolvimento de uma vacina amplamente eficaz (Marcili et al, 2009; Knight et al, 2014). Apesar dos desafios, nos útimos anos, vários grupos de pesquisa vêm estudando em modelos animais a capacidade imunogênica e protetora de uma variedade de moléculas como candidatas para o desenvolvimento de vacinas contra a doença de Chagas. Nessa linha, os grupos têm buscado tanto o desenvolvimento de vacinas profiláticas que poderiam prevenir novos casos de infecção aguda, como também vacinas terapêuticas para pacientes soro-convertidos ou na fase indeterminada da doença. A avaliação de uma ampla gama de formulações vacinais ao longo dos anos proporcionam prova clínica da vacinação como estratégia preventiva e potencialmente terapêutica contra a doença de Chagas (Quijano-Hernandez e Dumonteil, 2011; Dumonteil et al, 2012). Até o momento, nenhuma vacina contra a doença de Chagas encontra-se em fase de testes clínicos, no entanto, algumas abordagens envolvendo imunização utilizando diferentes antígenos, estratégias e formulações vacinais, vêm demonstrando bons resultados nos testes pré-clínicos entre elas, as indicadas na Tabela 1.

Tabela 1: Candidatos Vacinais

Candidato vacinal	Antígeno	Referência
Organismo Atenuado	*T. cruzi* vivo atenuado	Basombrio et al, 1987 Basombrio et al, 1993 Brandan e Basombrio et al, 2012 Sanchez-Valdez et al, 2015
Capa viva não patogênica	*T. rangeli*	Basso e Marini, 2014 Basso et al, 2014
Microrganismo vetores carreando sequências que codificam antígenos de *T. cruzi*	ASP-2 gp83 Tc52 Cruzipaína, Tc24 e Tc52	Barbosa et al, 2013 Farrow et al, 2014 Matos et al, 2014 Cazorla et al, 2015
Peptídeo	MASP	Serna et al, 2014
Proteína recombinante	TSA-1 Trans-sialidase (Ts) Tc24	Knight et al, 2014 Bontempi et al, 2015 Martinez-Campos et al, 2015

Vacina de DNA	Trans-sialidase TSA-1 e Tc24	Fujimura et al,2001 Quijano-Hernandez et al, 2013
“Prime-boost” heterólogo	DNA-“Prime/proteína-boost” (TcVac2) DNA-“Prime/Adenovirus-boost” (ASP-2+Ts) DNA-“Prime/-T.rangeli-boost” (TvVac4)	Gupta e Garg, 2010 Araújo et al, 2014 Aparício- Burgos et al, 2015

As diferentes estratégias vacinais adotadas, evidenciam a necessidade de induzir resposta imune celular com perfil Th1 e ativação de linfócitos T $CD8^+$ citotóxicos, que desempenham papel crucial no desenvolvimento de imunidade protetora, e consequentemente, no controle da infecção, aumentando a sobrevida dos pacientes e diminuindo possíveis patologias cardíacas (De Alencar et al, 2009; Limon-Flores et al, 2010; Quijano-Hernandez e Dumonteil, 2011; Konduri et al, 2017).

A imunização com DNA se mostrou promissora contra uma variedade de patógenos, sendo capaz de induzir ativação de linfócitos T $CD4^+$ e $CD8^+$, que secretam citocinas e têm função reguladora na produção de anticorpos (Espíndola et al, 2014; Maciel et al, 2015; Chen et al, 2017; Maspi et al, 2017; Wang Lei et al, 2017; Wang Qian et al, 2017; Zheng et al, 2017). Vacinas baseadas na tecnologia do DNA recombinante apresentam diversas vantagens em relação às técnicas clássicas de vacinação, visto que o custo de produção em larga escala é inferior à produção de proteínas recombinantes, peptídeos sintéticos ou ainda frações celulares purificadas. Ainda apresenta a vantagem de ser estável em temperatura ambiente ou após liofilização, o que facilita sua comercialização e distribuição em regiões endêmicas e de difícil acesso, comum em países em desenvolvimento (Waine e McManus, 1995; Robinson, 1997; Glenting e Wessels, 2005). A vacinação com DNA também pode ser a forma de imunização mais indicada para determinados grupos de risco, como por exemplo crianças, indivíduos imunocomprometidos e idosos, cujo sistema imunológico apresenta-se imaturo ou deficiente, e vacinas formuladas com organismos vivos atenuados ou inativados não seriam recomendadas (Whalen, 1996; Siegrist, 1997). Nesse caso, o processo de imunização utilizando vacina de DNA não proporcionaria riscos a saúde desses grupos.

Ecto-Nucleosídeo Trifosfato Difosfohidrolases (E-NTPDases)

Dentro do contexto da busca por novas biomoléculas envolvidas na patogênese da doença de Chagas, assim como fatores de virulência envolvidos no processo de infecção que podem representam potenciais alvos para o desenvolvimento de novas estratégias vacinais, as ecto-enzimas de parasitos se destacam.

Ecto-enzimas são compostas por um grupo de enzimas ecto-localizadas que hidrolisam uma variedade de nucleotídeos di e trifosfatos extracelulares para suas formas difosfatadas ou monofosfatadas. Um importante membro desta família são as ecto-nucleosídeo trifosfato difosfohidrolases (E-NTPDases), que podem tanto ser secretadas quanto estarem localizadas na porção externa de membranas celulares ou organelas (Plesner, 1995; Vasconcelos et al, 1996; Zimmermann, 2000; Coutinho-Silva et al, 2007). Elas têm sido estudadas devido sua importância em infecções causadas por uma variedade de patógenos. Em protozoários a atividade dessas enzimas foi identificada em parasitos do gênero *Toxoplasma* (Nakaar et al, 1998), *Herpetomonas* (Alves-Ferreira et al, 2003), *Leishmania* (Meyer-Fernandes et al, 1997; Berredo-Pinho et al, 2001; Peres-Sampaio et al, 2001), *Entamoeba* (Barros et al, 2000), *Trichomonas* (Jesus et al, 2002), *Crithidia* (Dos Passos et al, 2002), *Plasmodium* (Levano-Garcia et al, 2010) e *Trypanossoma* (Fietto et al, 2004), e estão relacionadas a características de virulência desses parasitos, favorecendo mecanismos de sobrevivência e replicação (Sanson et al, 2008).

Os nucleotídeos extracelulares podem ser liberados das células em virtude de lesão tecidual ou infecção causada por patógenos, e estão envolvidos em diversos processos biólogicos como por exemplo, ativação de receptores purinérgicos presentes em diversos tipos celulares e em muitos tecidos, desencadeando o início da resposta imune (Di Virgilio, 2005; Sansom, 2008). O ATP extracelular é uma molécula com propriedade imuno-moduladora envolvida na modulação de linfócitos, estimulando a secreção de citocinas inflamatórias como a IL-12 e o IFN-γ (Langston et al, 2003). No entanto, a adenosina extracelular acumulada, que é produto da hidrólise do ATP e ADP exógenos pela ação das ecto-enzimas, pode interagir com receptores purinérgicos modulando negativamente múltiplos efeitos das células, como por exemplo suprimindo a produção de citocinas inflamatórias e aumentando a produção de IL-10 por monócitos e macrófagos, permitindo assim, o estabelecimento da infecção pelo parasito (Hasko, 2004). A inosina, produto da quebra da adenosina, também possui efeito

imunomodulador relacionado com a inibição de processos inflamatórios (Jijon et al, 2005; Nemeth et al, 2005).

As plaquetas possuem importante função, pois participam da remoção de parasitos opsonizados da circulação, e um dos fatores de virulência envolvidos nos mecanismos de escape de *T. cruzi* é a inibição da agregação plaquetária dependente de ADP, mediada por purinorreceptores em sítios de injúrias vasculares (De Aguiar et al, 2001; Mizumoto et al, 2002; Sansom, 2008).

Vários trabalhos sugerem uma correlação entre a capacidade de hidrólise de ATP extracelular por parasitos e o seu sucesso em estabelecer o parasitismo (Asai et al, 1995; Nakaar et al, 1998; Barros et al, 2000; Jesus, 2002; Maioli et al, 2004; Tascar et al, 2004; De Almeida Marques-da-Silva et al, 2008), demonstrando, portanto, o envolvimento dessa ecto-nucleotidase nos mecanismos de virulência do parasito. Além disso, também tem sido descrita a participação das NTPDases na via de salvação de purinas, processo importante no metabolismo de muitos parasitos (Robson, 2006; Volonte, 2009).

Até o momento, somente um gene da familia da E-NTPDase foi isolado e sequenciado para o *T. cruzi*, e a proteína deduzida foi denominada NTPDase-1 (Fietto et al, 2004). Nesse mesmo trabalho, foi demonstrado que a NTPDase-1 presente nas formas tripomastigotas de T.*cruzi* hidrolisa preferencialmente ATP em relação ao ADP, e essa taxa de hidrólise é mais elevada nessa fase evolutiva quando comparada com a forma epimastigota. Mais tarde foi demostrado que a inibição parcial da NTPDase-1 de *T.cruzi* e, consequentemente, da sua atividade ecto-nucleotidásica, está diretamente ligada á capacidade de virulência do parasito, assim como sua participação durante o processo de adesão do parasito à célula hospedeira (Santos et al, 2009). Ensaios de imunolocalização utilizando anticorpos policlonais específicos, revelaram que em epimastigotas, a NTPDase-1 está localizada na superfície celular interna e externa do parasito, núcleo, cinetoplasto, flagelo, região de inserção do flagelo e vesiculas intracelulares. A presença da NTPdase-1 na região de inserção do flagelo e vesículas intracelulares em *T. cruzi*, sugere que essa enzima pode ter um papel na aquisição de nutrientes, e sua ampla distribuição dentro do parasito, sugere que ela também pode estar envolvida em outros processos biológicos (Mariotini-Moura et al, 2014b).

Como mencionado, alguns trabalhos evidenciam a relação da NTPDase-1 de *T. cruzi* com o potencial do parasito de subverter e evitar mecanismos de defesa do hospedeiro, mantendo desta forma sua capacidade de virulência e estabelecendo o parasitismo, ficando evidente que a NTPDase-1 é uma importante mólecula envolvida no processo de infecção,

podendo ser utilizada como alvo para o desenvolvimento de novas alternativas para prevenção e controle da doença de Chagas.

Resultados obtidos após imunização com DNA/NTPDase-1 de *T. cruzi*

Nosso grupo havia avaliado previamente a resposta imune induzida pela vacinação com DNA codificando para a NTPDase-1 de *T. cruzi* (dados não publicados). A imunização com três doses de DNA administradas em intervalos de 15 dias, mostrou-se eficaz como intervenção preventiva. Os camundongos foram imunizados com três doses de DNA plasmidial codificando a NTPDase-1 ou com três doses de NTPdase-1 recombinante (rNTPDase-1) emulsificada com saponina.

Os resultados demonstram que a imunização com rNTPDase-1 foi mais eficiente que o DNA na estimulação da resposta específica de IgG total. Ambos os protocolos de imunização testados não alteram o perfil de linfócitos T ($CD4^+$ e $CD8^+$) e B ($CD19^+$) no baço. No entanto, a vacinação com rNTPDase-1 foi capaz de aumentar significativamente a porcentagem de linfócitos T ativados. A avaliação da produção de citocinas mostrou que não houve produção significativa de citocinas Th1 quinze dias após a imunização (IFN-γ e TNF-α), para os grupos imunizados. Entretanto, foram observados níveis séricos de NO significativamente elevados no grupo imunizado com rNTPDase-1. Rredução significativa de citocinas anti-inflamatórias (IL-6 e IL-10) também foram observadas para ambos protocolos vacinais.

A imunização com DNA codificando a NTPDase-1 resultou em níveis significativamente aumentados de IL-17A no tecido cardíaco, associado a uma redução significativa da parasitemia trinta dias após o desafio do *T. cruzi*. A taxa de sobrevivência dos camundongos imunizados, também foi significativamente mais elevadas quando comparadas ao grupo controle

Em conclusão, os dados prévios observados pelo nosso grupo demonstraram que a NTPDase-1 de *T. cruzi* apresentou potencial imunogênico, uma vez que a imunização com rNTPDase-1, foi capaz de estimular o aumento da produção de anticorpos específicos, induzir ativação em células T CD4 e CD8 esplênicas, reduzir os níveis séricos de citocinas anti-inflamatórias e aumentar a produção de NO, enquanto que a imunização com três doses de DNA plasmidial foram responsáveis pelo aumento da produção de IL-17A no tecido cardíaco após o desafio, bem como pela redução significativa da carga parasitária no mesmo órgão

Diante dos resultados obtidos no estudo prévio, no presente trabalho buscamos induzir

melhora da resposta imune tanto celular quanto humoral, pela utilização do protocolo vacinal "prime-boost" heterólogo DNA/NTPDase-1, sendo esta resposta imune capaz de conferir proteção frente a infecção por *T. cruzi.*

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OBJETIVOS

- Objetivo Geral

- Avaliar a resposta imune induzida pela vacinação utilizando a estratégia vacinal "prime-boost" heterólogo DNA/NTPDase-1 de *T. cruzi*, em camundongos BALB/c.

- Objetivos específicos

- Avaliar a capacidade imunogênica do protocolo vacinal, por intermédio da avaliação de produção de anticorpos (IgG1 e IgG2a) específicos contra a rNTPDase-1, bem como verificar os níveis das citocinas TNF-α, INF-γ, IL-4, IL-6, IL-10, IL-12 da quimiocina MCP-1, e das populações de linfócitos presentes no baço dos animais imunizados,

- Verificar a capacidade de proteção da vacina pela avaliação dos níveis de citocinas e carga parasitária presente no tecido cardíaco dos animais desafiados, e a influência da imunização na capacidade de sobrevivência desses animais.

CAPÍTULO 1

Evaluation of the immunogenic and protective capacity of the DNA-prime/rTcNTPDase-1 boost vaccine against *Trypanosoma cruzi*

Abstract

Chagas disease is caused by the protozoan *Trypanosoma cruzi* and represents a serious public health problem, mainly in Latin America. In this work, we evaluated the protection induced after immunization with a vaccine protocol DNA-prime/rNTPDase-1 of *T.cruzi* boost (Prime-boost), followed by challenge with *T. cruzi* in murine model. Evaluation of the production of specific antibodies during the immunization phase showed that both immunization with rNTPDase-1 of *T. cruzi* or prime-boost protocol, induced a significantly IgG1 and IGg2a production 15 days after the last immunization dose when compared to the control group. The Prime-boost protocol led to an increase significant in the production of Th1 cytokine (TNF-α), chemokine MCP-1 and in the frequency of CD8 T cells population ($CD3^+CD8^+$), besides increasing noteworthy the percentage of CD4 and CD8 T cells with memory phenotype ($CD44^{high}CD62L$). Immunization using the proposed vaccination protocol also showed lower parasite load present in cardiac tissue of the animals, resulting in increased survival after challenge. Based on the observed data, it is possible suggest that in the immunized mice following the prime-boost protocol, resulted in polarization of the immune response to the Th1 standard, being possible observe improvement in the induced protection, thus demonstrating that these animals were better prepared to deal with the infection. Thus, the *T. cruzi* vaccine protocol DNA-prime/rNTPDase-1 could be seen as promising strategy in the control of Chagas disease.

Keywords: Prime-boost vaccine, NTPDase-1, *Trypanosoma cruzi*

1. Introduction

Discovered more than 100 years ago by the Brazilian doctor Carlos Chagas, Chagas disease or American Trypanosomiasis is still considered one of the most important neglected tropical diseases in the western hemisphere (1,2).

The disease is caused by the protozoan *Trypanosoma cruzi*, which is transmitted to the vertebrate host by contact with feces of infected hematophagous triatomine insects (3). Currently, the disease affects around 16 million people worldwide with another 75-90 million living in areas at risk of transmission (4), a fact that occurs mainly in Latin America where the parasite is widespread, in 2015 there were 8 thousand deaths due to the disease (5, 6).

Chagas disease manifests itself in two different phases, the acute phase, which is usually asymptomatic and where it is possible to observe parasites circulating in the patients' blood. In some cases, signs indicate the location of the insect vector bite (Romaña sign and chagoma of inoculation). In the chronic phase which may be present indeterminate, specific antibodies can be detected but there are no clinical signs and symptoms. Although most chronically infected patients have the indeterminate form of the disease, about 40% of patients present severe cardiac and gastrointestinal complications that may develop decades after the initial infection, and in most cases they die (7,8).

At present there is no vaccine available for the disease and the pharmacological treatments used are more effective when administered in the acute phase. Besides having numerous side effects, these drugs are contraindicated for immunosuppressed patients, thus presenting limited action (9, 10). It is still important to emphasize that parasites naturally resistant to chemotherapy have already been reported (11). Development of prophylactic or therapeutic vaccines could significantly reduce the number of people affected or serious problems due the infection, especially in endemic areas. To this end, in recent years several research groups have been trying to develop an effective vaccine against Chagas disease. Thus, a number of antigens and vaccine formulations have been investigated as potent candidates for the development of prophylactic and therapeutic vaccines to be used as an additional tool, mainly in endemic areas (12-19).

T. cruzi Nucleoside Triphosphate Diphosphohydrolase-1 (TcNTPDase-1) is an ecto-enzyme present on the surface of *T. cruzi* with the ability to hydrolyze extracellular nucleotides. This enzyme was discovered, characterized and had its gene cloned (20). TcNTPDase-1 was then identified as an important molecule involved in the parasite infection process and is also able to modulate the host immune system by interfering with defense mechanisms related to the immune response induced by extracellular ATP (21, 22, 23). Under this approach, our group decided to work with recombinant TcNTPDase-1 (rTcNTPDase-1) evaluating its ability to induce protective immune response using the DNA-prime/rTcNTPDase-1-boost (Prime-boost) vaccine protocol. This one has been increasingly studied as an alternative vaccine especially against intracellular microorganisms (24, 25), in which the efficacy of immunization will depend not only on its ability to induce a specific immune response with Th1 profile, but also on stimulating cytotoxic $CD8^+$ T cells needed to reduce parasitic burden (18, 26), being therefore TcNTPDase-1 used as a potential target for the development of a preventive vaccine against Chagas disease.

2. Materials and methods

2.1 Expression in *Escherichia coli* and purification of rTcNTPDase-1

The plasmid containing the TcNTPDase-1 gene construct was kindly provided by Dra Juliana Lopes Rangel Fietto (20), and used to transform *E. coli* strain BL21 (DE3) for expression of the recombinant protein. The rTcNTPDase-1 was obtained by transfer an aliquot of transformed *E. coli BL21* (DE3) to 1L of sterile LB medium containing 100 µg/ml of ampicillin. Culture was then incubated at 37°C with shaking at 220 rpm until reaching OD 600. The expression of rTcNTPDase-1 was induced with 0.4 mM IPTG for 4 hours at 37°C and shaking at 220 rpm, being protein expressed in inclusion body. The cells were then collected and lysed with lysis buffer. The resulting precipitate containing the inclusion bodies was solubilized with denaturation buffer (150 mM NaCl, 400 mM Tris-HCl, 8 M urea), pH 8.0 and purified. The protein was then dialyzed against PBS to reduce urea concentration and subsequently eluted with 150 mM NaCl, 20 mM Tris-HCl, 300 mM imidazole, pH 8.0. The expression of rTcNTPDas-1 was confirmed by SDS-PAGE and its concentration determined by using the bicinchoninic acid-BCA method (Thermo Scientific, Massachusetts, USA) and then used in the immunizations.

2.2 Construction of recombinant DNA vaccine

For the construction of the DNA vaccine, the cDNA for TcNTPDase-1 was subcloned in the eukaryotic expression vector pVAX (Invitrogen™). The recombinant plasmid and also the empty vector were used to transform competent *E. coli* DH5-α cells by heat shock, which were cultured separately on plates containing LB culture medium and 100 µg/ml of kanamycin and then incubated overnight at 37°C. The purified plasmids were obtained after inoculation of an aliquot of the bacterium into 100 ml of LB culture medium (Sigma-Aldrich, St. Louis, Missouri, USA) containing kanamycin for approximately 16 hours at 37°C and shaking at 220 rpm. Thereafter, the cell culture was centrifuged at 7.000 x *g* for 15 minutes at 4°C to obtain the precipitate. The cells were subjected to alkaline lysis according to the methodology described by Birnboim (1983) (27), the plasmids purified by Miniprep and their concentration determined by NanoDrop apparatus (Thermo Scientific-NanoDrop2000™ GO) at wavelengths 260 and 280 nm and then used in immunizations.

2.3 Animals

The BALB/c mice 4-6 week old females used in this study were obtained from the Centro de Criação de Animais de Laboratório (CECAL/Fiocruz) and kept in the Biotério de Manipulação Animal (IOC/Fiocruz) under controlled conditions during the tests. The experimental protocols were approved by the Comitê de Ética em Uso Animal (CEUA/Fiocruz, protocolo 051/2015) and carried out in accordance with guidelines issued by the Conselho Nacional para o Controle de Experimentação Animal (CONCEA).

2.4 Immunization protocol

Immunization was performed following the DNA-prime/rTcNTPDase-1 of *T.cruzi* boost protocol, i.e. 100 µg intramuscular (im) of the recombinant plasmid encoding TcNTPDase-1 emulsified with PBS, followed by 10 µg of rTcNTPDase-1 emulsified with the adjuvant saponin (0.1 %) applied subcutaneously (sc). It was also evaluated the response induced by the immunization with three doses of 10 µg of rTcNTPDase-1 emulsified with the adjuvant saponin (0.1%) applied by subcutaneous route. Control groups received three intramuscularly doses of 100 µl of PBS. All vaccines were applied with final volume of 100 µL per dose at 15-day intervals (Table 1).

Table 1: Immunization protocols

Day 0	Day 15	Day 30
10 µg rTcNTPDase-1[1] sc	10 µg rTcNTPDase-1[1] sc	10 µg rTcNTPDase-1[1] sc
100 µg pVAX/NTPDase-1[2] im	10 µg rTcNTPDase-1[1] sc	—
CTRL[3]	CTRL[3]	CTRL[3]

[1] vaccine formulated with 10 µg of rTcNTPDase-1 and applied subcutaneously

[2] vaccine formulated with 100 µg of the recombinant plasmid encoding TcNTPDase-1 and applied intramuscularly

[3] Control

2.5 Evaluation of immunogenicity

2.5.1 Antibody response

Six animals per group were immunized following the vaccine protocol (Table 1). Serum samples were collected from the retro venous plexus orbital (0.5 ml) one week after each immunization dose and also at the time of euthanasia, which occurred 15 days after the last immunization, being these samples evaluated by ELISA. The polystyrene microplates (96 wells) were sensitized overnight at 4ºC with 1 µg of purified *T.cruzi* rNTPDase-1. Following the washing and blocking steps with PBS and 1% gelatin, 100 µl of diluted serum (1:40) of all groups were added to the selected wells and the plate was incubated for 45 minutes at 37°C. Following washing steps, rabbit anti-mouse IgG subtypes IgG1 or IgG2a (Sigma, St. Louis, MO, USA) were added to the specific wells and the plate was incubated for 45 minutes at 37°C. The plate was then incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA). There action was revealed using OPD (orthophenylenediamine/Aldrich™) and optical density (OD) was determined using a 490 nm filter from the Thermo Scientific-Multiskan™ GO plate reader.

2.5.2 Cytokines production

Due to the fundamental role of cytokines in the control of *T. cruzi* infection (18, 26) we evaluated their concentration at a systemic level 15 days after the last immunization dose and in the cardiac tissue of challenged animals. Samples were obtained from six animals per group at the time of euthanasia. Cytokine concentrations were determined using the BD Cytometric Bead Array (CBA)/Mouse Th1/Th2 Inflammation Kit (BD Biosciences, New Jersey, USA). The cytokines measured were: Tumor Necrosis Factor (TNF), Interferon-γ (IFN-γ), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10) and monocyte-1 chemotactic Protein (MCP-1). Standard curves were determined from a range of 20 to 5.000 pg/ml according to the manufacturer. Data were collected using the BD FACSVerse cytometer from the Núcleo de Microscopia e Microanálise of the Universidade Federal de Viçosa (UFV) and analyzed in FCAP 3.0 software.

2.5.3 Immunophenotyping

Euthanized animals 15 days after the last immunization dose had the spleens collected aseptically and divested in DMEM medium (Sigma, St. Louis, MO, USA) to obtain whole cells for immunophenotyping analysis. After red cell lysis in ammonium chloride buffer, the sample was washed twice with 10 ml DMEM medium by centrifugation at 250 × g for 10 minutes at 4°C. Cell pellets were resuspended in 1 ml of medium and the total number of viable cells was determined in Neubauer's chamber using 0.2% Trypan Blue vital stain (Vetec, Rio de Janeiro, Brazil). The cell suspension was adjusted to 1×10^6 cells/ml in PBS buffer and incubated for 30 minutes at 4°C. Next, incubation was performed with specific antibodies of interest, anti-CD4-APC, anti-CD8-FITC, anti-CD19-PerCP, anti-CD44-PE, anti-CD62L-APC (Tonbo Biosciences, USA). As the control of labeling, antibodies of the same isotype with unrelated specificity (control isotype) were used. The tubes were incubated for 30 minutes at room temperature in the absence of light for subsequent average acquisition of 10.000 events. Data were collected using the BD FACSVerse of the Núcleo de Microscopia e Microanálise of the Universidade Federal de Viçosa (UFV).

2.6 Protection Assessment

2.6.1 Infection, parasite burden and mortality

Twelve animals per group were challenged on the 15th day after the last immunization (Table1). Groups were inoculated intraperitoneally (ip) with *T. cruzi* Y strain (5.000 trypomastigote forms in 0.1 ml of infected mouse blood). The inoculum was obtained from mice previously infected with metacyclic trypomastigote forms obtained from stationary phase culture in liver infusion tryptose (LIT) medium. The number of parasites in each inoculum was determined according to the method of Toledo *et al* (28). Then the animals were divided into two independent experiments with six animals per group, intended to evaluate the parasitic load on the cardiac tissue and survival curve. To obtain the survival curve the groups of animals infected with *T. cruzi* were observed daily for a period of 30 days. The survival rate was expressed as a percentage of the accumulated deaths within the experimental period. On the other hand, the experimental groups destined to collect samples of infected cardiac tissue for cytokine and qPCR dosing were euthanized two weeks after the challenge date.

2.6.2 qPCR of cardiac tissue

To determine the parasitic load, total genomic DNA was extracted from the cardiac tissue of all mice infected with *T. cruzi* 15 days after the challenge date using the Wizard® Genomic DNA Purification Kit according to Caldas *et al* (29). The extracted DNA was quantified by the NanoDrop apparatus (Thermo Scientific-NanoDrop2000™ GO) at wavelengths of 260 and 280 nm and the concentrations were adjusted to 50 ng/ µl. Real-time quantitative PCR (qPCR) was performed in a final volume of 20 µl containing 50 ng of genomic DNA, 10 µl of master mix SYBR green (Applied Biosystems, Carlsbad, CA, USA) and 0.25 µl *T. cruzi* DNA specific primer or 0.25 µl of specific primer for murine GAPDH. The primers for *T. cruzi* repetitive DNA (TCZ-F,5'-GCTCTTGCCCACAMGGGTGC-3', where M = A or C, and TCZ-R, 5'-CCAAGCAGCGGATAGTTCAGG-3') amplify a 195-bp fragment. The amplification program consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds and 64.3°C for 1 minute with fluorescence acquisition at 64.3°C. Amplification was immediately followed by a melting program with initial denaturation of 15 seconds a 95°C, cooling at 60°C for 1 minute and then a temperature rise of 0.3°C/s from 60°C to 95°C. The mean values of *T. cruzi* DNA quantification were normalized by the data obtained with the GAPDH-specific primers.

3. Statistical analysis

The comparison between the vaccinated and control groups was performed using the One-way ANOVA test, followed by the Tukey test. P values were considered significant com (*) when $p < 0.05$; (**) when $p < 0.01$ ou (***) when $p < 0.005$. All tests were executed on Prism 6.0 software.

4. Results

4.1 Induced immunogenicity by *T. cruzi* NTPDase-1

4.1.1 Production of anti-rNTPDase-1 specific antibodies

Initially, the production of different subtypes of specific antibodies against *T.cruzi* rNTPDase-1 was evaluated in an enzyme immunoassay. We observed that the animals immunized with rNTPDase-1 showed kinetics of total IgG, IgG1 and IgG2a production higher than the animals

of the Prime-boost and control groups, reaching maximum values after the second dose. Even with lower kinetics, we observed that the animals of the Prime-boost group obtained similar detections to the group that received rNTPDase-1 15 days after the last dose, period where total IgG, IgG1 and IgG2a production was higher in the vaccinated groups than in the control group (Fig. 1A, B and C).

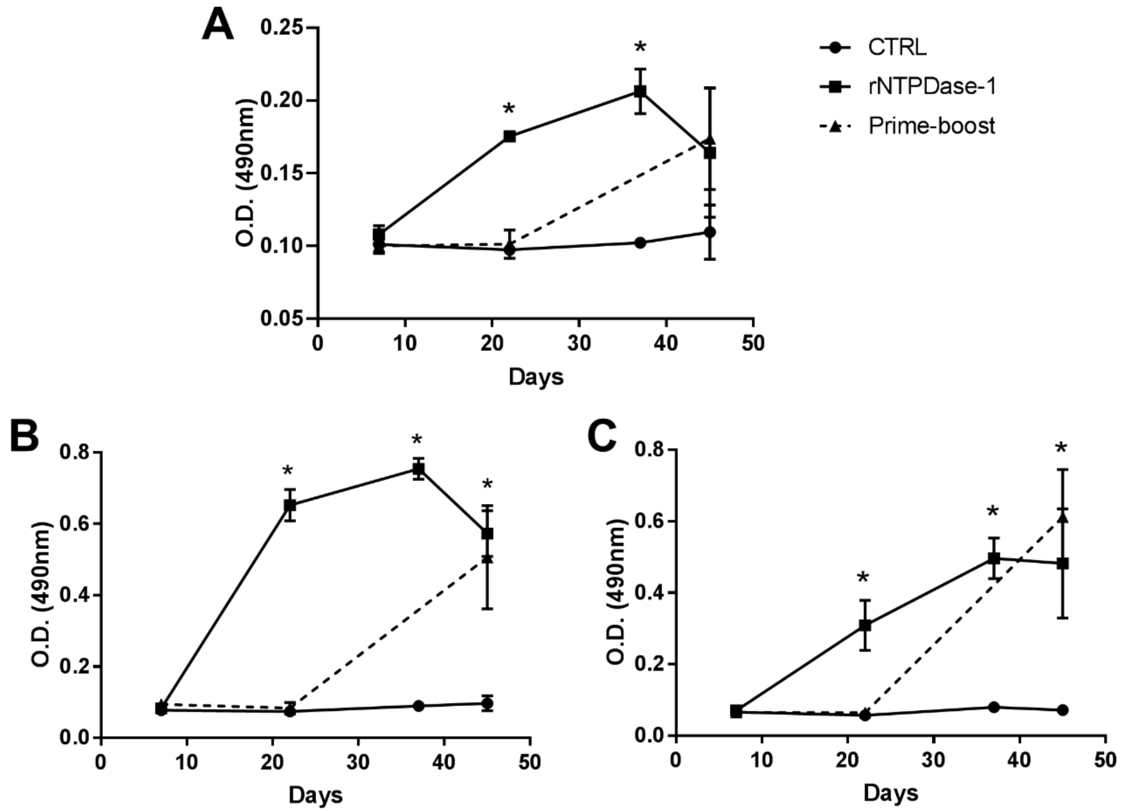

Figure 1: Immunoglobulins total IgG (A), IgG1 (B) and IgG2a (C) present in the serum of mice. Serum samples were collected and evaluated by ELISA. Control group (CTRL), immunized group with three doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1), and the vaccine group with one dose of pVAX/NTPDase-1 and one dose of *T. cruzi* recombinant NTPDase-1 (Prime-boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * $p < 0.05$ versus control in two experiments with similar results.

4.1.2 Evaluation of cytokine production

To characterize the systemic production of cytokines induced after immunization, the serum of the animals was evaluated. We observed that the mice immunized following the Prime-boost protocol were able to produce increased levels of TNF-α and MCP-1, which were statistically significant when compared to the groups evaluated (Fig. 2A and B). No significant difference was observed in IFN-γ (Fig. 2C) and IL-2 (unpublished data) production in the two vaccinated groups. There was a significant reduction of IL-10, both in protein-immunized animals and in the Prime-boost protocol (Fig. 2D). For IL-4 and IL-6 cytokines no alteration was observed (Fig 2E and F).

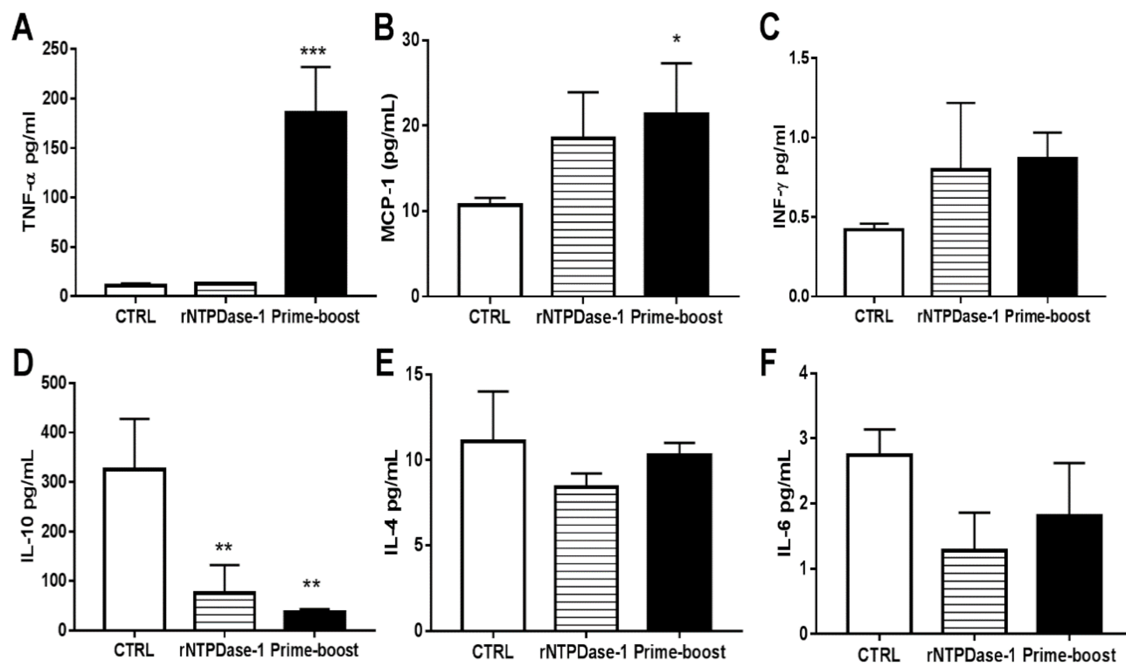

Figure 2: Cellular immune response in mice. TNF(A), INF-γ (B), MCP-1 (C), IL-10 (D), IL-4 (E), IL-6 (F) cytokines levels present in the serum of animals 15 days after the last immunization. Control group (CTRL), immunized group with three doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1), and the vaccine group with one dose of pVAX/NTPDase-1 and one subcutaneously dose of *T.cruzi* recombinant NTPDase-1 (Prime-boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ versus control in two experiments with similar results.

4.1.3 Immunophenotyping of splenocytes

To determine if immunization was able to stimulate subpopulations of cells present in the spleen, the presence of $CD3^{+}/CD4^{+}$ and $CD3^{+}/CD8^{+}$ T lymphocytes with activated phenotype ($CD44^{+}$) and of memory ($CD44^{+}/CD62L^{high}$) as well as B lymphocytes ($CD19^{+}$) was evaluated 15 days after the last immunization. The data indicated there was no change in the total $CD4^{+}$ T cell frequency (Fig. 3A), differently, a significant increase of total $CD8^{+}$ T was observed in the Prime-boost group in relation to the other two groups (Fig. 3D). In relation to the activated cells, a significant increase of this population was detected in the immunized group with protein in relation to the Prime-boost group (Fig. 3B and 3E). Significantly higher percentage of $CD4^{+}$ and $CD8^{+}$ T cells with memory phenotype ($CD44^{+}$/CD62L) was observed when compared to the control group (Fig. 3C and 3F). The evaluation of $CD19^{+}$ expression in B cells indicated there was no significant statistical difference in the frequency of this population in the immunized and control groups (Fig 4).

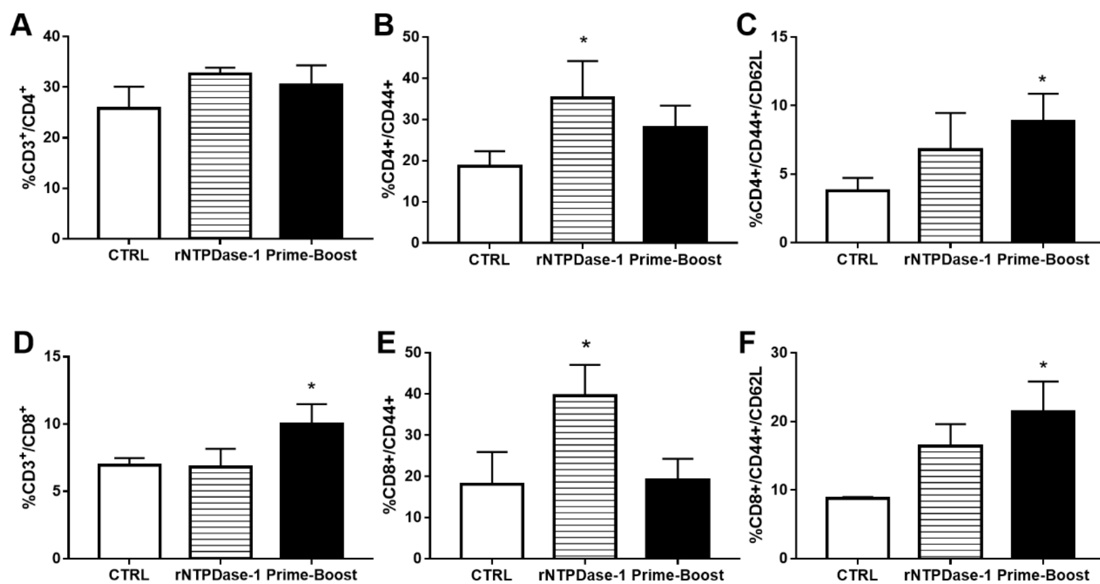

Figure 3: Immunophenotyping of cells present in the spleen after immunization. Percentage of total $CD4^{+}$ and $CD8^{+}$, $CD44^{high}$ (activated T cells) and $CD44^{high}CD62L$ (memory T cells) cells. Control group (CTRL), immunized group with three doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1), and the vaccine group with one dose of pVAX/NTPDase-1 and one subcutaneously dose of *T.cruzi* recombinant NTPDase-1 (Prime-

boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * p <0.05 versus control, in two experiments with similar results.

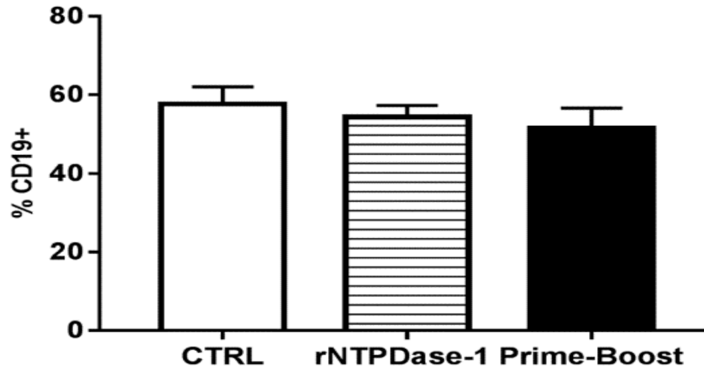

Figure 4: B cells present in the spleen of immunized mice. Percentage of B lymphocytes ($CD19^+$). Control group (CTRL), immunized group with three doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1), and the vaccine group with one dose of pVAX/NTPDase-1 and one subcutaneously dose of *T.cruzi* recombinant NTPDase-1 (Prime-boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * p <0.05 versus control in two experiments with similar results.

4.2 Protection induced after immunization

4.2.1 Cytokine dosing in infected cardiac tissue

In order to evaluate the protection induced by the different vaccine protocols, challenge with lethal dose of *T. cruzi* Y strain (5.000 trypomastigote forms) was performed. Initially, the production of cytokines in the cardiac tissue was evaluated since this is the main organ infected in the vertebrate host. Evaluation of the cytokine profile demonstrated that although a greater trend in IL-10 production was observed in the immunized groups, no significant difference was noted (Fig. 5A). The same applies to the group immunized with rNTPDase-1 when we evaluated the production of TNF-α (Fig. 5B) and INF-γ (Fig. 5C). Regarding IL-2 (unpublished data), IL-4 (Fig. 5D) and IL-6 (Fig. 5E) similar profile was observed for both cytokines in the evaluated groups, without therefore presenting significant difference.

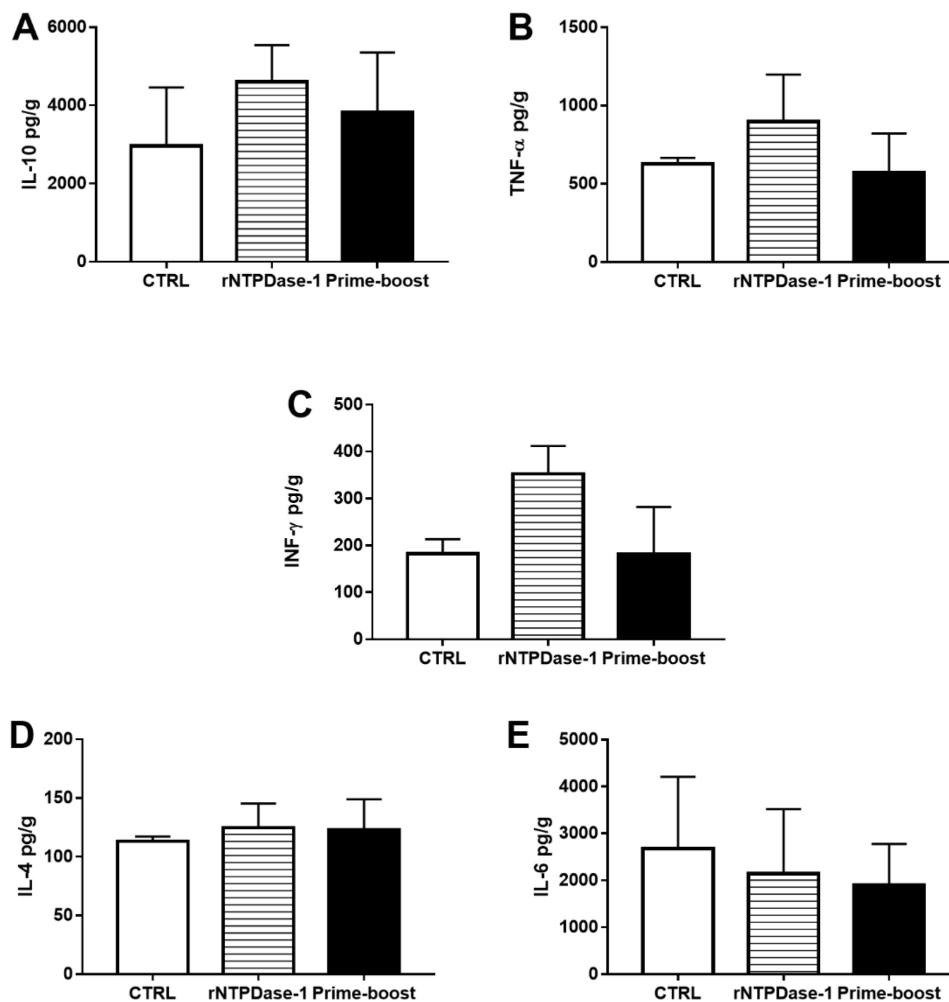

Figure 5: Production of cytokine in infected cardiac tissue. Cytokines TNF (A), INF-γ (B), IL-10 (C), IL-4 (D) and IL-6 (E) was measured by flow cytometry. Control group (CTRL), immunized group with three doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1), and the vaccine group with one dose of pVAX/NTPDase-1 and one subcutaneously dose of *T.cruzi* recombinant NTPDase-1 (Prime-boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * $p < 0.05$ versus control in two experiments with similar results.

4.2.2 Parasitic load present on cardiac tissue after challenge

The parasitic load present on the cardiac tissue after the challenge was determined by qPCR. Immunization using both vaccine protocols showed a reduction in the presence of *T. cruzi* in comparison to the control group. However, the Prime-boost protocol was more efficient, it was able to significantly reduce the parasitic load compared to the protocol with rNTPDase-1 (Fig 6).

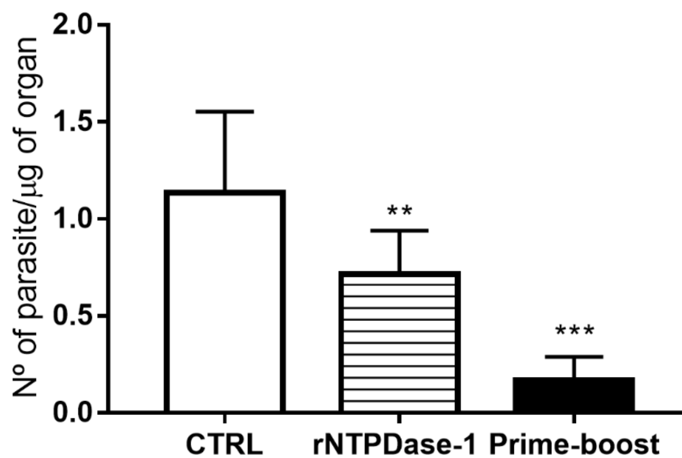

Figure 6: *T. cruzi* DNA present in cardiac tissue of infected mice. Control group (CTRL), immunized group with three ip doses of *T. cruzi* recombinant NTPDase-1 (rTcNTPDase-1) and vaccine group with one im dose of pVAX/NTPDase-1 and one ip dose of *T. cruzi* recombinant NTPDase-1 (Prime-boost). The results represent the mean ± SD of six mice per group obtained using the ANOVA test, ** $p < 0.05$; *** $p < 0.001$ versus control in two experiments with similar results.

4.2.3 Survival rate of infected mice

Monitoring of the survival rate of the animals after challenge with lethal dose of *T. cruzi* Y strain occurred for a period of 30 days. In Figure 7 it is possible to observe there was no death in the two immunized groups and that in 20 days, 50% of the control animals had already died.

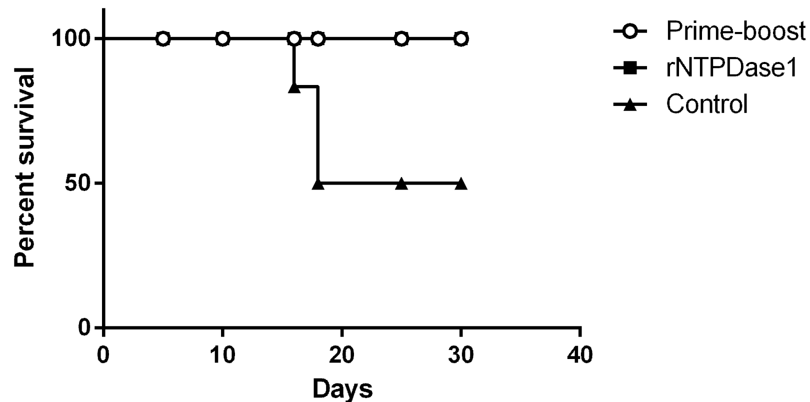

Figure 7: Survival rates of infected mice. Mice were challenged with 5.000 trypomastigote forms 15 days after the last immunization. The survival rate is shown for control group (CTRL) infected mice, immunized with rTcNTPDase-1 and infected mice (rTcNTPDase-1), immunized with Prime-boost and infected (Prime-boost) mice. The results represent the mean ± SD of six mice per group obtained using the ANOVA test, * p <0.05 versus control in two experiments with similar results.

5. Discussion

Our group had previously verified that *T. cruzi* NTPDase-1 has been shown to be a good vaccine candidate being capable of conferring protection against *T. cruzi* infection (unpublished data). In the present work, we proposed to evaluate the improvement in the profile of the induced immune response using DNA-prime/rNTPDase-1 of *T. cruzi* boost vaccination protocol.

Our results showed that immunization with three doses of *T.cruzi* rNTPDase-1 emulsified with saponin stimulated the production of specific antibodies with faster kinetics compared to the Prime-boost protocol. However, the analysis of specific antibodies 15 days after the last dose demonstrated that the two vaccine protocols resulted in similar levels. It is well known that recombinant proteins are more effective in promoting antibody response since they can directly stimulate memory B cells, and the immune response probably has been improved due to the action of saponin which induces the expression of accessory molecules that meliorate the antigen-specific immune response (13, 31).

Analysis of the IgG1 and IgG2a isotypes demonstrated there was a mixed antibody response (Th1/Th2). These data are in agreement with Salgado-Jimènez *et al.* (32) who

evaluated the ability of the trans-sialidase protein of *T. cruzi* to induce protection in immunized BALB/c mice. The authors found that serum antibody levels in mice immunized with the recombinant protein were higher when compared to the group immunized with DNA encoding the corresponding recombinant protein. In the same work after analysis of the cytokine profile, it was observed that both the immunization with the recombinant protein and the DNA resulted in mixed T cell response.

Immunization using three doses of *T.cruzi* rNTPDase-1 resulted in a significant increase in T lymphocytes with activated phenotype ($CD44^+$) when compared to the control group. Unlike the group immunized with recombinant protein the Prime-boost protocol, in addition to leading to a significant increase in the total $CD8^+$ T cell population, also induced a significant increase in T-lymphocytes with memory phenotype ($CD44^{high}$CD62L) when compared to rNTPDase-1 and control groups. This fact is very important as it reflects the success of the immunization, since one of the objectives is to generate cells with the ability to readily proliferate and to differentiate into effector cells capable of eliminating the microorganism in a future contact.

Studies have reported the role of Th1 cytokines in the control of *T. cruzi* infection (13), although recent studies also have demonstrated the involvement of Th17 response cytokines, through the activation of $CD8^+$ T cells and increased microbicidal capacity of macrophages (33,34). This work, no significant production of the proinflammatory cytokine IFN-γ in the vaccinated groups was observed, however the use of the Prime-boost protocol induced Th1 cytokine (TNF-α) and the chemokine MCP-1 production that were statistically significant in comparison to the control group. This fact is very important, since TNF-α is an important mediator in the resistance to infection by intracellular microorganisms in the initial stages of infection, inducing the production of chemokines, like MCP-1 that attracts monocytes/macrophages to the site of infection resulting in the elimination of the parasite (35,36). The decrease in IL-10 production observed in vaccinated groups indicate that although the immunization process induced production of proinflammatory cytokines, especially when the Prime-boost protocol was used, however this response at the immunization stage was not uncontrolled since IL-10 acts as an important regulatory cytokine in several stages during the development of the immune response (37), thus suggesting the immunomodulatory capacity of the vaccine.

T. cruzi DNA was present in the cardiac tissue of all animals evaluated and although no changes were observed in cytokine production levels after challenge, it was still possible to

notice improvement in the protection induced in the groups immunized, since a significant reduction of the parasitic load was verified in relation to the control group. On the other hand, the Prime-boost group had a lower number of parasites present in the cardiac tissue when compared to the group receiving three doses of rNTPDase-1. Interestingly, the survival assessment showed that no animal belonging to the immunized groups died, which did not occur with the control group, where there was 50% death within time mice were monitored.

T. cruzi infection is a real challenge for the host immune system since it requires the involvement of cellular and humoral immune responses to avoid replication of amastigote forms in tissues and to remove circulating trypomastigotes (38). Immunization following the Prime-boost protocol may be more effective in inducing activation of the immune response because it induces a T cell response that can be enhanced by the corresponding recombinant protein. Activation of the immune response using this protocol is often improved over that achieved applying the repeated dose protocol (13,39), reinforcing the idea that this type of vaccine protocol is ideal for achieving the immune response necessary to control the infection caused by *T. cruzi*.

The results obtained this work demonstrate that immunization using the Prime-boost protocol based on *T. cruzi* NTPDase-1 was able to specifically stimulate the immune system. The low production of the anti-inflammatory cytokine IL-4 in conjunction with significant production of proinflammatory cytokine TNF-α and the chemokine MCP-1, suggest that vaccination following this protocol resulted in polarization of the immune response to the Th1 standard, being able to protect the mice against the challenge with lethal doses of *T. cruzi.* We also suggest that the observed cellular immune response can still be improved, if an additional dose of DNA is administered. Therefore, the proposed vaccination protocol can be seen as a promising strategy for the control of Chagas disease.

Acknowledgements

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PERSPECTIVA

Avaliar a resposta imune induzida, após imunização seguindo o protocolo "Prime-boost", administrando no entanto, uma dose a mais de DNA codificando para a NTPDase-1 de *T.cruzi*, objetivando induzir melhora na resposta imune celular.