UNIVERSIDADE FEDERAL DE PELOTAS

Programa de Pós-Graduação em Biotecnologia

Tese

Avaliação da resposta imune induzida por *Mycobacterium bovis* BCG recombinante expressando antígenos de *Trypanosoma cruzi* contra doença de Chagas

Guilherme Senna dos Santos

Pelotas, 2024

Guilherme Senna dos Santos

Avaliação da resposta imune induzida por *Mycobacterium bovis* BCG recombinante expressando antígenos de *Trypanosoma cruzi* contra doença de Chagas

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de conhecimento: Biotecnologia).

Orientadora: Drª Sibele Borsuk

Pelotas, 2024

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação da Publicação

S237a Santos, Guilherme Senna dos

Avaliação da resposta imune induzida por *Mycobacterium bovis* BCG recombinante expressando antígenos de *Trypanosoma cruzi* contra doença de Chagas [recurso eletrônico] / Guilherme Senna dos Santos ; Sibele Borsuk, orientadora. — Pelotas, 2024.
214 f.

Tese (Doutorado) — Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, 2024.

1. Vacina recombinante. 2. Tripanossomíase americana. 3. Vacina de subunidade. 4. Vetor bacteriano. 5. Resposta imune. I. Borsuk, Sibele, orient. II. Título.

CDD 616.96

Elaborada por Gabriela Machado Lopes CRB: 10/1842

BANCA EXAMINADORA:

Dr.ª Sibele Borsuk (Orientadora, Universidade Federal de Pelotas, Centro de Desenvolvimento Tecnológico)

Dr.ª Daniela Fernandes Ramos (Universidade Federal do Rio Grande, Faculdade de Medicina)

Dr.ª Fabiana Kömmling Seixas (Universidade Federal de Pelotas, Centro de Desenvolvimento Tecnológico)

Dr. Sérgio Jorge (Universidade Federal de Pelotas, Departamento de Clínicas Veterinárias)

Agradecimentos

Primeiramente, à minha mãe, Adriana Senna, que nunca poupou esforços para que eu chegasse até aqui.

À minha esposa, Larissa Vieira, pelo amor e companheirismo inigualáveis, nos momentos bons e difíceis, sendo o sol que aquece meu coração.

Aos meus amigos, de perto e de longe, que tornam minha vida mais leve e descontraída.

À minha orientadora, Sibele Borsuk, pelos ensinamentos e pela confiança em me tornar parte da equipe do LBIP e em realizar o primeiro trabalho na linha de *Trypanosoma cruzi* do laboratório.

Aos colegas do Laboratório de Biotecnologia Infecto-Parasitária, pelas incontáveis conversas, experimentos e risadas compartilhadas no dia-a-dia em convivência.

À minha amiga, Bárbara Fonseca, pela parceria indispensável dentro e fora do laboratório, este trabalho é teu também.

Aos laboratórios parceiros e seus integrantes, por sempre me receberem bem e me auxiliarem na realização deste trabalho.

Ao Programa de Pós-Graduação em Biotecnologia e à Universidade Federal de Pelotas, pela infraestrutura e condições para realizar este trabalho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001

“A frase mais empolgante de se ouvir na ciência, aquela que anuncia novas descobertas, não é 'Eureka!' mas sim 'Que engraçado...'”

Isaac Asimov

Resumo

SANTOS, Guilherme Senna dos. **Avaliação da resposta imune induzida por Mycobacterium *bovis* BCG recombinante expressando antígenos de *Trypanosoma cruzi* contra doença de Chagas.** 2024. 214f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A doença de Chagas (DC) é uma doença parasitária disseminada globalmente, causada pela infecção do protozoário flagelado *Trypanosoma cruzi*. Os medicamentos disponíveis, como o benznidazol, atuam principalmente na fase aguda da infecção, onde o diagnóstico quase é raro, e apresentam uma infinidade de efeitos adversos que culminam na descontinuação do tratamento pela maioria dos pacientes. Consequentemente, a prevenção parece ser a melhor estratégia para controle da DC. Entre vários métodos profiláticos em desenvolvimento, o *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) tem sido recentemente empregado como vetor para entrega de antígenos de *T. cruzi* com resultados positivos no estímulo da resposta imune e proteção contra a infecção. Seguindo essa perspectiva, este estudo teve como objetivo caracterizar a resposta imune desencadeada por BCG recombinante (rBCG) expressando a proteína de ligação ao cálcio de 24 kDa (Tc24) e proteína de superfície de amastigota 2 (ASP-2) de *T. cruzi*. Para tal, cinco grupos de camundongos fêmeas BALB/c (n = 10) foram vacinados com solução salina 0,9% (Grupo A), BCG Pasteur não transformada (Grupo B), rBCG/pUS2000/asp-2 (Grupo C), rBCG/pUS977/asp-2 (Grupo D) ou rBCG/pUS977/tc24 (Grupo E). Embora nenhuma resposta humoral significativa tenha sido detectada com qualquer uma das formulações através de análise com ELISA indireto, as respostas celulares, avaliadas pela expressão de citocinas de esplenócitos cultivados e estimulados por proteínas, foram estatisticamente maiores para todas as formulações vacinais quando comparadas com os níveis basais (Grupo A) e BCG não transformado (Grupo B). O Grupo D obteve melhores resultados para interferon γ e interleucina 10, enquanto as interleucinas 4 e 17 foram fortemente estimuladas pela vacinação com o Grupo C e Grupo E, com todas as formulações reprimindo a interleucina 6. Embora sejam necessárias análises adicionais para avaliar a eficácia total das construções, os resultados aqui apresentados exibem o potencial das vacinas vetorizadas BCG em induzir respostas imunes mistas Th1/Th2/Th17.

Palavras-chave: Vacina recombinante; tripanossomíase americana; vacina de subunidade; vetor bacteriano; resposta imune.

Abstract

SANTOS, Guilherme Senna dos. **Avaliação da resposta imune induzida por *Mycobacterium bovis* BCG recombinante expressando antígenos de *Trypanosoma cruzi* contra doença de Chagas.** 2024. 214f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Chagas disease is a globally spread parasitic illness caused by the infection of the flagellated protozoan *Trypanosoma cruzi*. Although drugs, like benznidazole, have been employed over the past years, with some degree of efficacy, it is not without counter effects. Available drugs work mostly on the acute phase of infection, where diagnosis is hardly ever made, and present a plethora of negative side effects that ends in discontinuation of treatment by most patients. Consequently, prevention seems to be a better strategy to deal with this disease in particular. Among several prophylactic methods in development, *Mycobaterium bovis* Bacillus Calmette-Guérin (BCG) has recently been employed as vector for delivering *T. cruzi* antigens with positive results on immune response stimulus and protection against the infection. Following this perspective, this study aimed to characterize the immune response elicited by recombinant BCG (rBCG) expressing the calcium-binding 24 kDa protein (Tc24) and amastigote surface protein 2 (ASP-2) of *T. cruzi*. To accomplish this, five groups of BALB/c female mice (n = 10) were vaccinated with 0.9% saline solution (Group A), non-transformed BCG Pasteur (Group B), rBCG/pUS2000/asp-2 (Group C), rBCG/pUS977/asp-2 (Group D) or rBCG/pUS977/tc24 (Group E). Even though no significant humoral response was detected with any of the formulations through analysis with indirect ELISA of animal sera, cellular responses, assessed by cytokine expression from cultured and protein stimulated splenocytes, were statistically higher for all vaccinal formulations when compared with basal levels (Group A) and non-transformed BCG (Group B). Group D achieved better results for interferon γ and interleukin 10, while interleukins 4 and 17 where greatly stimulated by vaccination with Group C and Group E, with all formulations repressing interleukin 6. Even though further analyses are needed to evaluate the full efficacy of the constructions, the here presented results exhibit the potential of BCG vectored vaccines in eliciting Th1/Th2/Th17 mixed immune responses.

Keywords: Recombinant vaccine; American trypanosomiasis; subunit vaccine; bacterial vector; immune response.

Lista de Abreviaturas

ºC – Graus Celsius

% – Porcentagem

µg – Micrograma

µm – Micrômetro

µL – Microlitro

ANOVA – Analysis of variance (Análise de variância)

ASP-2 – Amastigote surface protein 2 (Proteína de superfície de amastigota 2)

BALB/c – Linhagem isogênica de camundongos albinos

BCA - Bicinchoninic acid (Ácido bicincrônico)

BCG - Bacilo Calmette-Guérin

CFU – Colony-forming unit (Unidade formadora de colônia)

BHI – Brain Heart Infusion (Infusão cérebro coração)

BSA – Bovine serum albumin (Albumina sérica bovina)

CD – Chagas disease (Doença de Chagas)

CD4 – Cluster of differentiation 4 (Grupamento de diferenciação 4)

CD8 – Cluster of differentiation 8 (Grupamento de diferenciação 8)

CEEA – Comitê de Ética em Experimentação Animal

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

CONCEA – Conselho Nacional para o Controle da Experimentação Animal

CO_2 – Dióxido de carbono

DC – Doença de Chagas

DMEM – Dulbecco's Modified Eagle's Medium (Meio de Eagle Modificado por Dulbecco)

DNA – Deoxyribonucleic acid (Ácido desoxirribonucleico)

ELISA – Enzyme-Linked Immunosorbent Assay (Ensaio Imunoadsorvente Ligado à Enzima)

FBS – Fetal bovine serum (Soro fetal bovino)

Fig – Figura

g – Grama

GPDH – Glicerol-3-phosphate dehidrogenase (glicerol-3-fosfato desidrogenase)

h – Hora

HCl – Ácido clorídrico

HRP – Horseradish peroxidase (Peroxidase de rábano silvestre)
IFN-γ – Interferon gama
IgG – Imunoglobulina G
IgG1 – Isótipo 1 da imunoglobulina G
IgG2a – Isótipo 2a da imunoglobulina G
IgM – Imunoglobulina M
IL-4 – Interleucina-4
IL-6 – Interleucina-6
IL-10 – Interleucina-10
IL-17 – Interleucina-17
INPI – Instituto Nacional da Propriedade Industrial
IPTG – Isopropyl β-D-1-thiogalactopyranoside (Isopropil-beta-D-1-tiogalactopiranosídeo)
kDa - Quilodaltons
kg – Quilogramas
L - Litro
LB – Luria-Bertani
M – Molar
Mb – Megabase
mL – Mililitro
mM – Milimolar
mRNA – Messenger RNA (RNA mensageiro)
NCBI – National Center for Biotechnology Information (Centro Nacional de Informação em Biotecnologia)
ng - Nanograma
OPD - o-phenylenediamine dihydrochloride (Dihidrocloreto de orto-fenilenodiamina)
PBS – Phosphate-buffered saline (Tampão fosfato-salino)
PCR – Polymerase Chain Reaction (Reação em Cadeia da Polimerase)
PFR-3 – Paraflagellar rod protein 3 (Proteína
PBS-T – Phosphate Buffered Saline with Tween 20 (Tampão fosfato-salino adicionado de Tween 20)
r - Recombinante
RNA – Ribonucleic acid (Ácido ribonucleico)

r.p.m – Rotações por minuto

s.c. – Via subcutânea

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio)

Tc24 – *Trypanosoma cruzi* Calcium-binding 24 kDa protein (Proteína de ligação ao cálcio de 24 kDa de *Trypanosoma cruzi*)

Tc52 – *Trypanosoma cruzi* thiol transferase

Th1 – Linfócito T helper 1 (Linfócito T auxiliar 1)

Th2 – Linfócito T helper 2 (Linfócito T auxiliar 2)

Th17 – Linfócito T helper 17 (Linfócito T auxiliar 17)

TM – Tripomastigotas metacíclicos

TNF-α – Tumor necrosis factor α (Fator de necrose tumoral α)

TSA-1 – Trypomastigote surface antigen 1 (Antígeno de superfície de tripomastigota 1)

UFC – Unidade formadora de colônia

UFPel – Universidade Federal de Pelotas

x – Vezes

Sumário

1. Introdução 11
2. Hipótese e Objetivos 18
2.1 Hipóteses 18
2.2 Objetivo geral 18
2.3 Objetivos específicos 18
3. Resultados 19
3.1) Manuscrito 1 19
3.2) Manuscrito 2 51
3.3) Patente 1 125
3.4) Manuscrito 3 144
3.5) Patente 2 173
3.6) Patente 3 191
4. Conclusões 208

1. Introdução

Descrita pela primeira vez há mais de um século por Carlos Chagas e assim adquirindo sua denominação, a doença de Chagas (DC) é uma zoonose causada pela infecção do protozoário *Trypanosoma cruzi* em seres humanos, geralmente transmitida pela picada e deposição das fezes de insetos da subfamília Triatominae (Centers for Disease Control and Prevention of the U.S. 2021). A doença é endêmica em 15 países da América Latina, mas, devido à migração populacional, casos foram identificados em outros países das Américas, no Pacífico Ocidental e na Europa, com aproximadamente 8 a 10 milhões de pessoas infectadas globalmente (Antinori et al. 2017). Estima-se que cerca de 75 milhões de pessoas que vivem em áreas endêmicas estejam em risco de infecção (World Health Organization 2024).

A DC se distingue clinicamente em duas fases: a fase aguda, que marca o início da infecção e na qual podem surgir os primeiros sintomas, e a fase crônica, que pode se prolongar por anos ou décadas. A forma cardíaca é a manifestação mais grave e comum da doença de Chagas crônica, ocorrendo em 20% a 30% dos indivíduos infectados e frequentemente levando à insuficiência cardíaca, tromboembolismo e morte súbita (Rassi, Rassi, e Marin-Neto 2010). Existe também uma fase indeterminada, caracterizada pela presença do protozoário no organismo hospedeiro sem que haja sinais clínicos evidentes (Guarner 2019).

As manifestações clínicas da DC resultam do ciclo reprodutivo do *Trypanosoma cruzi* no interior do hospedeiro definitivo (Macaluso et al. 2023). O entendimento desse ciclo é fundamental para o combate efetivo à infecção, uma vez que o parasita é capaz de se diferenciar em três formas distintas, de acordo com o ambiente em que se encontra, adotando diferentes mecanismos de escape conforme a forma assumida (Freire-de-Lima et al. 2015).

Conforme mencionado anteriormente, o ciclo de vida do parasita depende de um vetor de transmissão, o *Triatoma infestans*, inseto da subfamília Triatominae, popularmente conhecido como "barbeiro". Quando infectado, esse vetor libera tripomastigotas metacíclicos (TM) em suas fezes próximo ao local da picada, ao se alimentar do sangue do hospedeiro. No interior do novo

hospedeiro, os TM invadem as células próximas ao local de inoculação, onde se diferenciam em amastigotas intracelulares. Estes se multiplicam por fissão binária e se diferenciam em tripomastigotas, que são então liberados na corrente sanguínea. Os tripomastigotas infectam células de vários tecidos e se transformam em amastigotas intracelulares em novos locais de infecção. Manifestações clínicas podem resultar desse ciclo infeccioso. Os tripomastigotas presentes no sangue não se replicam; a replicação recomeça apenas quando os parasitas entram em outra célula ou são ingeridos por outro vetor. O "barbeiro" é infectado ao se alimentar de sangue humano ou animal contendo parasitas circulantes. No intestino médio do vetor, os tripomastigotas ingeridos se transformam em epimastigotas, que posteriormente se diferenciam em TM infectantes no intestino posterior (Centers for Disease Control and Prevention of the U.S. 2021).

Os medicamentos benznidazol e nifurtimox têm sido utilizados no tratamento contra *T. cruzi* há quase cinquenta anos, sendo indicados para o tratamento da doença de Chagas aguda e congênita, infecções reativadas e doenças crônicas em menores de 18 anos (Antinori et al. 2017; Pérez-Molina e Molina 2018). No entanto, a eficácia desses medicamentos varia conforme o estágio da infecção, sendo mais eficazes na fase aguda. Atualmente, o uso do nifurtimox para tratar a doença de Chagas é cada vez menor, sendo o benznidazol preferido devido ao seu melhor perfil de tolerabilidade e eficácia (Antinori et al. 2017; Pérez-Molina e Molina 2018; Yeung et al. 2021). O tratamento também apresenta diversos efeitos colaterais, o que reforça a importância do diagnóstico precoce e da intervenção terapêutica, independentemente da fase da infecção (Pérez-Molina e Molina 2018).

O acesso a novas tecnologias de saúde para populações em risco de contaminação está diretamente relacionado à disponibilidade, acessibilidade, adoção e implementação dessas tecnologias, fatores intrinsecamente ligados a aspectos sociopolíticos (Forsyth et al. 2019; Frost e Reich 2009). A situação atual, em que apenas uma pequena parcela dos casos de DC é detectada e tratada, contribui significativamente para a alta morbimortalidade, embora seja uma condição potencialmente evitável. Como descrito por Forsyth et al. (2019), essa realidade só mudará com uma ampla expansão de estratégias mais integradas, o que, por sua vez, exige o compromisso do governo e dos sistemas

de saúde pública, o aumento da investigação científica para o desenvolvimento de melhores ferramentas de tratamento e diagnóstico, maior acessibilidade a medicamentos, campanhas de conscientização direcionadas tanto aos pacientes quanto aos profissionais de saúde, e uma estratégia de tratamento abrangente que aborde os impactos biológicos, psicológicos e sociais da doença.

Desde a descoberta da infecção, a comunidade científica tem se empenhado com esforços significativos para controlar a doença de Chagas e mitigar os danos causados por ela. Diversas estratégias têm sido adotadas na busca por um tratamento seguro e eficaz contra *Trypanosoma cruzi*. Embora muitos novos medicamentos estejam em desenvolvimento para o combate à doença de Chagas (Vermelho et al. 2018, 2022; Yepes, Quintero- Saumeth, e Cardona- G 2020), sabe-se que a vacinação desempenha um papel crucial na erradicação ou restrição global de inúmeras doenças infecciosas. Não é surpreendente, portanto, que muitos protótipos de vacinas estejam atualmente em desenvolvimento (Bivona et al. 2020; Vekemans et al. 2021). No entanto, garantir uma proteção eficiente através da vacinação não é tarefa simples. Qualquer vacina em desenvolvimento deve considerar que o antígeno ideal precisa ser expresso nas diferentes fases do ciclo de vida do parasita e ser uma região bem conservada e presente em diversas cepas de *T. cruzi* (Bivona et al. 2020). Além disso, o mecanismo de entrega do antígeno deve ser capaz de apresentá-lo ao sistema imunológico do hospedeiro de forma rápida e sustentada, provocando uma resposta imunológica robusta e direcionada contra o parasita (Bivona et al. 2020).

Nesse contexto, uma das abordagens que têm mostrado resultados promissores é o uso de vacinas vetorizadas por bactérias (Bontempi et al. 2020; Matos et al. 2014; Quintana et al. 2018). Estas vacinas são definidas por sua base em uma cepa bacteriana atenuada, geneticamente modificada para ser segura, mas ainda imunogênica. A bactéria pode ser adicionalmente alterada para introduzir genes que codificam antígenos de patógenos de diferentes espécies. O produto resultante deve ser capaz de induzir respostas imunes protetoras tanto contra a bactéria vetor quanto contra os patógenos-alvo.

O uso de bactérias como vetores assegura uma entrega eficiente e confiável dos antígenos, potencialmente resultando em uma imunidade mais duradoura e eficaz do que as vacinas convencionais (Bivona et al. 2018). Essa

estratégia pode melhorar significativamente o combate à doença de Chagas, ao induzir uma resposta imune forte e específica, capaz de estimular tanto a imunidade humoral quanto a celular, essencial na defesa contra patógenos intracelulares como *T. cruzi*. Além disso, os vetores bacterianos podem ser projetados para expressar múltiplos antígenos simultaneamente, proporcionando uma proteção mais ampla contra diferentes cepas do parasita (Cazorla et al. 2015).

Interessantemente, a plataforma BCG (*Mycobacterium bovis* Bacillus Calmette-Guerin) como vetor para expressar antígenos de *T. cruzi* foi explorada apenas recentemente (Bontempi et al. 2020). O BCG, conhecido por sua capacidade adjuvante, promove imunidade contra microrganismos intracelulares (Bastos et al. 2009). Estudos epidemiológicos confirmaram que o BCG induz uma resposta imune tipo Th1 contra antígenos relacionados ou não ao microrganismo (Stanford 1989), um perfil essencial para o controle da infecção por *T. cruzi* (Tarleton 2015). Além disso, a eficácia do BCG em aumentar a resposta imune contra parasitas foi também comprovada no desenvolvimento de vacinas contra *Leishmania braziliensis* (Convit et al. 2004). Bontempi et al. (2020) observaram não apenas uma resposta Th1, mas também uma resposta Th17 em camundongos, um perfil desejado na imunização com BCG e considerado crítico para a proteção contra *T. cruzi* (Matos et al. 2017).

Inúmeras proteínas de *T. cruzi* desempenham papéis cruciais em seus processos biológicos, principalmente na facilitação da interação do parasita com seus hospedeiros vertebrados, sendo essenciais para a infecção, sobrevivência e proliferação. Essas proteínas, caracterizadas como fatores imunogênicos e de virulência, foram identificadas através de triagem imunológica de bibliotecas de expressão de cDNA utilizando soros de indivíduos afetados pela doença de Chagas (Engman, Dragon, e Donelson 1990; Maldonado et al. 2022). Diversas dessas proteínas demonstram potencial como antígenos para o desenvolvimento de vacinas recombinantes no combate à DC (Jiménez et al. 2019). Antígenos como cruzipain, Tc24, Tc52, TSA-1, PFR-3 e ASP-2, provenientes de diferentes estágios de desenvolvimento e regiões, como membrana e flagelo, do *T. cruzi*, já foram utilizados em várias formulações vacinais com o objetivo de desenvolver uma vacina profilática (Arce-Fonseca et al. 2018; Bivona et al. 2020; Dumonteil, Herrera, e Buekens 2019).

Dentre esses antígenos, alguns se destacam por apresentarem resultados significativos quando utilizados em protocolos de imunização profilática, seja em sua totalidade ou em regiões específicas com epítopos de interesse (Dumonteil et al. 2004; Machado et al. 2006). Exemplos notáveis são a proteína flagelar de ligação ao cálcio de 24 kDa (Tc24) e o antígeno de superfície de tripomastigota 1 (TSA-1), ambos expressos na fase tripomastigota do parasita e capazes de induzirem respostas imunes mediadas por células CD8+ (Limon-Flores et al. 2010; Quijano- Hernandez et al. 2008; Sanchez-Burgos et al. 2007). Além disso, proteínas da fase amastigota, como a proteína de superfície de amastigota 2 (ASP-2), também conferem proteção em linhagens de camundongos altamente suscetíveis à infecção, como demonstrado por Araújo et al. (2005).

A proteína flagelar de 24 kDa está localizada próxima à região flagelar do parasito, apresentando baixo polimorfismo e propriedades imunomoduladoras (Gunter et al. 2018). Possui propriedades imunoprotetoras, como o aumento da produção de IFN-γ e o recrutamento de células T CD8+ (Dumonteil et al. 2019). Anticorpos contra essa proteína foram detectados em pacientes com doença de Chagas, confirmando seu potencial imunogênico (Villanueva- Lizama et al. 2020). Pesquisas com camundongos demonstraram que a TC24, seja administrada por via oral a partir de microalgas marinhas expressando a proteína ou por vacinação direta, induzindo forte resposta imune humoral, produção de citocinas e redução na parasitemia (Ramos-Vega et al. 2024). Resultados consistentes com os de Poveda et al. (2023), que também observaram um aumento na resposta imune e redução da parasitemia, além da produção de células T CD8+ específicas e um perfil de citocinas equilibrado. Martinez-Campos et al. (2015) reportou uma resposta predominantemente Th1, caracterizada por altos níveis de IgG2a e INF-γ, sugerindo o potencial da TC24 como vacina preventiva.

O antígeno de superfície de tripomastigota é uma glicoproteína da família das transialidases, que desempenha um papel no reconhecimento entre as células do parasito e do hospedeiro, além de possuir propriedades imunoprotetoras em diversos modelos experimentais (De La Cruz et al. 2019; Dumonteil et al. 2019). Quijano-Hernández et al. (2013) sugere o uso de vacinas preventivas e terapêuticas com as proteínas TSA-1 e TC24 em cães, resultando

em menor parasitemia, redução da inflamação e danos cardíacos. Limon-Flores et al. (2010) obteve resultados semelhantes em camundongos, com a ativação de células T CD4+ e T CD8+ produtoras de INF-γ, auxiliando no controle da infecção. A TSA-1 também foi utilizada em vacinas de DNA, demonstrando aumento de células T CD4+ e T CD8+ específicas para INF-γ (Zapataestrella et al. 2006) e redução da parasitemia (Sanchez-Burgos et al. 2007). Em ensaios profiláticos em camundongos, De La Cruz et al. (2019) utilizou TSA-1 e TC24, observando diminuição da parasitemia e aumento na expressão de INF-γ e IL-4.

A proteína de superfície de amastigota nº 2 é encontrada na fase intracelular do parasito e pertence à família das transialidases. Sua estrutura proteica é bem conservada e possui ação imunogênica comprovada, promovendo o recrutamento de células T CD8+ citotóxicas no hospedeiro (Ribeiro et al. 2019). Araújo e colaboradores avaliaram a proteína recombinante ASP-2 como antígeno vacinal profilático em camundongos, resultando em uma sobrevivência superior a 65% após o desafio, com envolvimento de células T citotóxicas (Araújo et al. 2005). O gene que codifica para a proteína ASP-2 tem sido amplamente utilizado em vacinas baseadas em plasmídeos recombinantes, demonstrando aumento de linfócitos T citotóxicos, anticorpos, e redução da parasitemia e inflamação, além de maior sobrevivência (Garg e Tarleton 2002). Araújo et al. (2014) também exploraram vacinas profiláticas de DNA com plasmídeos de adenovírus recombinante contendo o gene ASP-2, resultando em aumento de IL-12 e proteção contra patologias agudas e crônicas. Vacinas de RNA mensageiro demonstraram potencial terapêutico, reduzindo a carga parasitária e inflamação (Mancino et al. 2024). Estratégias com vacinas vetorizadas expressando ASP-2, utilizando adenovírus recombinante, foram avaliadas tanto para prevenção quanto para tratamento, demostrando ativação de células T específicas e produção de INF-γ e TNF-α (Barbosa et al. 2013; Ribeiro et al. 2019). Contudo, mesmo apresentando perspectivas promissoras, muito resta a ser desenvolvido e avaliado até que de fato uma solução possa ser empregada em larga escala.

A presente tese ainda irá discutir de forma mais aprofundada o que fora abordado até este ponto. Para tal, a mesma foi dividida em capítulos para melhor entendimento e organização. Após deixar claro a hipótese e objetivos propostos para este trabalho, o primeiro capítulo discorre sobre protótipos vacinais,

desenvolvidos para o combate à doença de Chagas, através de dois manuscritos a serem submetidos a revistas científicas. Já os capítulos II e III descrevem os resultados outrora obtidos, respectivamente, através de patente submetida ao INPI e manuscrito a ser submetido a revista *Vaccine*; e os resultados obtidos não compilados em manuscritos, mas aqui descritos conforme as normas de redação científica. Por fim, uma breve conclusão geral encerra esta tese resumindo os resultados obtidos e abordando futuros passos a serem tomados em continuidade deste projeto.

2. Hipótese e Objetivos

2.1 Hipóteses

A formulação baseada em BCG recombinante expressando antígenos de *Trypanosoma cruzi* é capaz de induzir produção de anticorpos e citocinas em modelo murinho;

2.2 Objetivo geral

Avaliar o potencial de *M. bovis* BCG recombinante expressando os genes asp-2, tsa-1 e tc24 na indução da resposta imunológica celular e humoral específica em modelo murinho.

2.3 Objetivos específicos

- Compilar os resultados mais promissores de protótipos vacinais contra doença de Chagas presentes na literatura;
- Expressar as proteínas rASP-2, rTSA-1 e rTC24 em rBCG Pasteur;
- Expressar as proteínas rASP-2, rTSA-1 e rTC24 em *Escherichia coli*;
- Imunizar camundongos Balb/c com as diferentes formulações vacinais contendo BCG recombinante;
- Avaliar a resposta imune induzida pelas diferentes formulações vacinais.

3. Resultados

3.1) Manuscrito 1

Manuscrito a ser submetido a revista *Biotechnology Research and Innovation*

Vaccines for Chagas' disease: a synthetized up-to-date review

Guilherme Senna dos Santos[1], Bárbara da Rocha Fonseca[1], Laura Dall'Agno[1], Odir Antonio Dellagostin[2], Sibele Borsuk[1*]

1 Laboratório de Biotecnologia Infecto-Parasitária, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário s/n, Prédio 19, Pelotas, RS 96010-900, Brazil

2 Laboratório de Vacinologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário s/n, Prédio 19, Pelotas, RS 96010-900, Brazil

*Corresponding author: sibeleborsuk@gmail.com

Abstract

Chagas' disease (CD) is an infectious disease attacking an estimated 8 million people, mainly in rural areas of Latin America countries. CD has no effective treatment, evidencing the vaccination schedule as the best control strategy. Although some medicaments are available, none of them provides a solution for the infection nor are capable of inducing protection. They also have questionable safety levels and, in various cases, present side effects. In light of this, several experimental vaccines are in development in order to improve safety, reproducibility, and protective immune response against the etiologic agent of CD, *Trypanosoma cruzi*. In this review, we discuss aspects as antigen, adjuvant, routes of administration, protection level, animal models, and economic impact in CD vaccine development, as well the challenges and future perspectives for manufacturing and applying an effective product.

Keywords: *Trypanosoma cruzi* · Vaccine development · Adjuvant · Murine model · Immunoprophylaxis

Statements and Declarations

Autor's contributions:

All authors contributed to this review conception and design. Data collection and analysis were performed by Guilherme Senna dos Santos, Bárbara da Rocha Fonseca and Laura Dall'Agno. The first draft of the manuscript was written by Guilherme Senna dos Santos, Sibele Borsuk and Odir Antonio Dellagostin. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding:

This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoa de Nível Superior – Brasil (CAPES) – Finance Code 001

Interests:

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Introduction

First described by Carlos Chagas in 1909, Chagas' disease (CD) is a parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*. The infection occurs mainly from Triatomid insects, most commonly from *Triatoma infestans*, *Rhodnius prolixus* and *Triatoma dimediata*, which act as vectors (Galvão, 2014). Infected feces enter the host's bloodstream, where the parasite starts multiplying and establishing the infection. Although less frequent, other forms of infection can occur, such as congenitally, by blood transfusion, organ transplantation, or ingestion of contaminated food (Coura, 2015; Rassi et al., 2010).

CD has two different clinical phases: the acute phase, signaling the beginning of the infection and the first symptoms. The chronic phase, where abnormalities are developed in organs of the gastrointestinal tract or damage to the cardiovascular system, such as arrhythmias and thromboembolism, are detected. Cardiomegaly is also a symptom in the chronic phase. An indeterminate phase is characterized by the presence of the protozoan in the host organism, even though it does not present evident clinical signs (Guarner, 2019).

It is estimated that 6 to 8 million people are affected by this parasitosis, concentrated mainly in Latin American countries (WHO, 2022). CD is recognized by the World Health Organization as a neglected tropical disease, given the low investment in research and adequate prevention measures, affecting mainly rural areas, with difficult access to diagnosis and treatment (Fonseca et al., 2020; Martins-Melo et al., 2018). Drugs benzimidazole and nifurtimox are employed in the treatment against *T. cruzi*, however, the effective rates of both drugs vary according to the stage of infection, being more effective in the acute stage of the disease. The treatment also boasts several side effects, demonstrating the importance of early diagnosis and treatment intervention, independent of infection phase (Pérez-Molina & Molina, 2018).

To control CD and minimize the damage caused, different strategies have been adopted in order to find a safe and effective vaccine formulation against *T. cruzi*. Alternatives that use the attenuated or inactivated form of the parasite or from other non-pathogenic Trypanosoma species were evaluated. Advancements in modern biotechnology techniques have allowed new strategies

to be addressed, vaccines formulations that make use of recombinant subunits from the parasite or the genetic material of the organism in order to express a specific protein as antigen with the use of different viral or bacterial vectors (Cerny et al., 2020; Dumonteil et al., 2020; I. R. Pereira et al., 2015; Rodríguez-Morales et al., 2015). To ensure an efficient protection is achieved, any vaccine in development takes into account that an ideal antigen must present high immunogenicity; be expressed in different life stages; be a well-conserved region and present in different strains of *T. cruzi* (Bivona et al., 2020).

This present review presents a panorama of the main experimental vaccines already in development for CD and the different strategies adopted for each formulation. These points provide some discussion on the main obstacles found in the development of a 100% safe and effective vaccine against *T. cruzi* infection, as well key points for future research and development.

Experimental CD vaccines

Table 1 compiles data from publications addressing the experimental CD vaccines published so far. Data are summarized according to antigen, adjuvant, route of administration, protection efficacy and animal model used in each study.

Chagas' disease vaccine research and development are remarkable variable as time progresses. Primarily, the use of the live attenuated *T. cruzi* or a processed parasite were tested with adjuvant addition. These strategies were encouraged after studies using mice and guinea pigs proving that after primary *T. cruzi* infection, the animals acquire long-term immunity against secondary exposures (Lima et al., 1990). With technological development, a second phase was started; the use of recombinant DNA updated the immunization strategies: subunit, DNA, and vectorised vaccines gives reproducibility, safety, and target immune responses using specific epitopes or proteins (de la Cruz et al., 2018; Hegazy-Hassan et al., 2019; Moraschi et al., 2021).

Worth mentioning that the cost per protected individual will be determined by several factors like the cost of a dose, the time spawn in which it confers protection, the number of doses needed to achieve protection and the possibility of side effects (Lee et al., 2010). Recombinant subunit and DNA vaccines are currently not cost-effective methods of producing antigens that are free from the

exogenous materials that are associated with conventional vaccines (Bivona et al., 2020). Even so, when compared to annual economic losses, estimated to be $1.2 billion in productivity and $627.46 million in healthcare costs, both prophylactic and therapeutic vaccines against *T. cruzi* would be cost-effective in different predicted scenarios considering the risk of infection, the price of manufacture and efficacy of the vaccine (Conteh et al., 2010; Lee et al., 2010, 2012).

Whole parasite vaccines

The first generation vaccines against CD, attempted since the 1960 decade and still in experimentation to this date, are also described (Basombrío et al., 2002; Basso et al., 2014; Breganó et al., 2003; Collins et al., 2011; Lima et al., 1990; Pinge-Filho et al., 2005; Rosas-Jorquera et al., 2013; Sánchez-Valdéz et al., 2014; Zago et al., 2008). Comprised of whole organisms, either live, weakened or killed, these vaccines are able to induce killer T-cells or helper T-cells responses and antibody immunity (Basso et al., 2014). While more recent studies describe the protection levels and stimuli in humoral and cell-mediated immune responses in a detailed approach, it should be taken notice that analytical techniques for such cellular responses were not available for the first vaccine studies developed, which presented data in survivability and/or parasitemia only (McHardy, 1978). This, however, is not synonym of ineffectiveness. Rockland strain mice vaccinated with the formulation containing pressurized parasites reported a 100% protection against the virulent *T. cruzi* infection when compared with the unprotected control group (McHardy, 1978). A little more than two decades later, a formulation confectioned with non-infectious Clone 14 *T. cruzi* epimastigotes also conferred total protection to i.p. immunized BALB/c mice, although, it was discovered the immunization is time dependant, where the protection is only conferred if the vaccine is applied 5 weeks before the infection (Lima et al., 1990). Studies with first generation vaccines continued to be developed over the new millennium. *T. rangeli*-vaccinated guinea pigs infected with *T. cruzi* showed low histopathological alterations, only focal and scarce infiltration of mononuclear cells in epicardic area and absence of amastigote nests in tissue, consequences of a significantly lower parasitemia (Basso et al., 2014). Following the line of efficacy evaluation by tissue damage,

an attenuated *T. cruzi* TCC strain with a deletion of one copy of the TcCRT gene resulted in the generation of a safe live attenuated parasite with high experimental immunoprotective properties, indicated by inferior levels of parasitemia and splenomegaly in BALB/c mice, in comparison with unvaccinated control groups (Sánchez-Valdéz et al., 2014).

Subunit Vaccines

Different techniques were used to isolate fractions or even the whole of *T. cruzi* proteins, in order to identify antigens with immunodominant and protective characteristics (Araujo & Morein, 1991; Garcia et al., 2000b; Gomes et al., 1999; Miller et al., 1996) for the prophylaxis of CD. Formulated with one or more compounds extracted from the parasite, this type of vaccine is able to activate an immune response from the organism without the risk presented by using the whole pathogen. It has been almost three decades since the first isolated and characterized paraflagellar rod protein (PFR) from *T. cruzi* was confirmed to induce some degree of protection against the infection (Wrightsman et al., 1995). In the following years, BALB/c mice immunized with 40 µg of PFR emulsified with Freund's adjuvant, followed by two more doses of 20 µg, presented 100% survivability when vaccinated by i.p route (Miller et al., 1996) Other researchers made use of monoclonal antibodies for reactive assays against *T. cruzi* antigens in order to identify more specific proteins. The results indicate that concentrations as low as 5 µg of affinity purified antigens of the epimastigote stage of *T. cruzi* are sufficient to induce remarkable protection against challenge with blood form trypanosomes in mice, provided that the antigens are incorporated into the proper antigen delivery system (Araujo & Morein, 1991). In the 2000s, CBA/J mice were treated with *T. cruzi* soluble extract antigen (TCSE), increasing cellular proliferative response to Con A and the levels of IFN-ɣ, consequently reducing the number of circulating parasites. TCSE produced dose-dependent protective immunity, with 85% of the mice immunized with 400 µg of TCSE surviving and staying alive 150 days after infection while the mice receiving 600 µg of TCSE or BSA died on the 11th day after infection (Garcia et al., 2000a)

Subunit Recombinant vaccines

Studies have shown that the use of subunit recombinant vaccines against CD has been safe and effective, mainly due to their use of semi-pure antigens and the identification of potential protective effects identified by more advanced bioinformatics approaches (Trevisan et al., 2020). Currently, several papers described the use of recombinant vaccine strategy against Chagas' disease (Barry et al., 2019; de la Cruz et al., 2018; Dumonteil et al., 2020; Seid et al., 2016; Taibi et al., 1993). This vaccine technique is mainly focused on the recognition and use of parts of the pathogen's antigens, however, since the high purity level lowers the immunogenicity of the vaccine, the use of additional adjuvants is required to stimulate the immune response (de la Cruz et al., 2018). Various types of adjuvants have been tested in combination with recombinant proteins, which include CpG ODN (Cazorla et al., 2010), Freund's adjuvant (Flores-García et al., 2011), and aluminum hydroxide (Araújo et al., 2005; Luhrs et al., 2003).

A fragment of the ASP-2 protein as part of a recombinant vaccine was tested in A/Sn mice, increasing the number of $CD8^+$ T-cells and IFN-ɣ levels; which raised survivability to 100% after intraperitoneal challenge with *T. cruzi* (Araújo et al., 2005). To achieve a higher degree of protection, the formulation utilized aluminum hydroxide and CpG ODN 1826 as adjuvants, both of which could improve Th1 immune response and protective immunity to a *T. cruzi* infection (Frank et al., 2003). In another study, recombinant protein TSA-1 (rTSA-1) was capable to promote protection rates of 85 to 100% in BALB/c mice, when formulated with MPLA, E6020 or the stable emulsion of glucopyranosyl lipid (GLA-SE) as adjuvant, controlling the infection as indicated by lower parasitemia, cardiac inflammation and parasite burden (de la Cruz et al., 2018). In association with the synthetically produced TLR4 agonist, E6020, as an adjuvant, recombinant Tc24 (rTc24) was able to reduce the number of cardiac inflammatory cells and cardiac fibrosis in ICR mice. Sixty percent of vaccinated mice remained with undetectable systemic parasitemia at all time points post-vaccination, which could be linked to the induced TH1-skewed immune response through the TLR4 pathway, generating higher levels of IFN-ɣ in vaccinated animals (Barry et al., 2019).

Testing different adjuvants with the same recombinant protein might generate completely different results. The results presented by Bontempi et al.

(2015), shown that the vaccination of BALB/c mice with recombinant Trans-sialidase (rTS) achieved no significant protection. However, the combination of rTS with Freund's adjuvant raised survivability to 60% and to 100% when combined with ISCOMATRIX adjuvant, with increased levels of IgG, IFN-ɣ, and IL-10 when compared to the recombinant protein alone.

Different recombinant proteins in association have been pointed as alternative to improve protection and immunity. Dumonteil et al. (2020) were able to elevate the levels of CD4+ T-Cells' IFN-ɣ and CD8+ T-Cells' IL-2 in Rhesus macaques when immunized with vaccine formulations containing rTc24 and rTSA-1 proteins, expressed in *Escherichia coli*, and E6040 as adjuvant. Protection rate of 100% was obtained in C56BL/6J mice after challenge with the virulent strain Y of *T. cruzi* with the usage of equimolar mix of recombinant paraflagellar rod proteins (rPFR) 1, 2 and 3 in vaccine formulation. Even though formulations with isolated rPFR-2 also achieved 100% protection, animals vaccinated with mixed proteins had lower parasitemia 21 days post infection. Inhibition of parasite replication tested in IC-21 cells was also higher for mixed rPFRs (Luhrs et al., 2003).

DNA Vaccines

Based on the administration of a plasmid containing an antigen-encoding sequence within an expression cassette, DNA vaccines appears as alternative to recombinant proteins. This kind of vaccines favors the activation of CD8+ T-cells, the necessary effector mechanism against *T. cruzi*, with proteins being synthesized in the cytosol, the possibility of processing and presenting antigens through MHC class I molecules is higher (Padilla et al., 2009). With interest in developing a stronger response of the above mentioned cell machinery, several studies reported the use of DNA vaccine strategy against Chagas' disease (Aparicio-Burgos et al., 2015; Arce-Fonseca et al., 2018; Bhatia & Garg, 2008; Boscardin et al., 2003; Brandán et al., 2017; Caeiro et al., 2018; Cerny et al., 2016; Costa et al., 1998; Eickhoff et al., 2011; Gupta et al., 2019; Gupta & Garg, 2015; Hegazy-Hassan et al., 2019; Limon-Flores et al., 2010; Lokugamage et al., 2020; Rodrigues et al., 1999; Rodríguez-Morales et al., 2015; Wizel et al., 1998; Zapata-Estrella et al., 2006).

In murine model, BALB/c mice more specifically, Dumonteil et al. (2004) developed two DNA vaccines, one coding for the Tc24 protein and the other for the TSA-1 protein, using the pcDNA3 plasmid. Both vaccines were able to confer protection against a virulent *T. cruzi* H4 strain, with 100% and 70% of group survival being reported respectively, while pcDNA3/Tc24 was able to significantly reduce parasitemia 10 days before pcDNA3/TSA-1. However, the same vaccine formulations were not effective in inducing an immune response of the same magnitude when applied to ICR mice against the less virulent H1 strain of *T. cruzi*, with 85% survivability for pcDNA3/Tc24 and 65% for pcDNA3/TSA-1 (Sanchez-Burgos et al., 2007).

Other studies have tried to induce a higher protection with lower DNA fragments, usually by identifying domains responsible for specific cellular responses within antigen genes (Eickhoff et al., 2016; Fujimura et al., 2001; Garg & Tarleton, 2002). To evaluate the importance of the Kd1 epitope in Trans-sialidase (TS) for the immune response against Chagas' disease, a DNA vaccine encoding TS lacking this functional epitope (TSKd1 null) was tested in BALB/c mice. As expected, the protection of this vaccine was far lower (30% survivability) when compared to the DNA vaccine formulated with the consensus sequence of wild type TS (80%), even so, mice vaccinated with the wild type and TSKd1 null vaccines, surprisingly, developed similar total and $CD8^+$ T cell responses reactive with the entire wild type TS consensus catalytic domain (Eickhoff et al., 2016).

Vector-based vaccines

Vaccines containing vectors expressing heterologous genes proved to be a possibility for CD prophylaxis. Prokaryotic vectors transformed with eukaryotic expression plasmids or recombinant proteins encoded by viral vectors eliciting impressive cellular immune responses are some promising alternatives to naked DNA vaccines (Barbosa et al., 2013; Bivona et al., 2018; I. Bontempi et al., 2020; Cazorla et al., 2007, 2015; Cerny et al., 2020; De Alencar et al., 2009; Duan et al., 2009; Machado et al., 2006; Matos et al., 2014, 2016; Miyahira et al., 2005; Moraschi et al., 2021; Nogueira et al., 2013; I. R. Pereira et al., 2015; Quintana et al., 2018; Rigato et al., 2011; Schnapp et al., 2002). A good example is the application of attenuated *Salmonella enterica* carrying cruzipain gene (sCz). Via oral administration in C3H/HeN mice, followed or not by an I.D/I.N boost with rCz,

this vaccine was able to induce a strong humoral immune response characterized by the specific IgG, sIgA and T cells higher levels when compared to unvaccinated control group. It should be noted that vaccination with sCz alone generated weaker responses than booster vaccination, however, this group was also able to control the infection when challenged with RA strain of *T. cruzi*, presenting an even greater parasitemia reduction (Cazorla et al., 2007).

For viral vectors, the replication-deficient human type 5 recombinant adenoviruses (rAd) have an unsurpassed ability to induce type 1 immune responses, a necessity to achieve an effective protection against *T. cruzi* (Machado et al., 2006). Immunizations of mice with rAd encoding the amastigote surface protein 2 (ASP2) and TS induced high levels of serum anti-bodies specific for their recombinant products. In addition, both recombinant viruses were able to elicit a biased Th1 cellular immune response and a substantial $CD8^+$ T cell-mediated immune response. Moreover, individual immunization with rAdASP2 or rAdTS induced high levels of protection against a challenge with live parasites. $CD8^+$ T cells mediated, at least in part, such protection. Furthermore, when combined in the same inoculum, rAdTS plus rAdASP2 induced complete protection in all animals tested, even when challenges were performed 14 weeks after the last immunization (Machado et al., 2006). However, there are some counterpoints on the usage of Adenoviruses beyond the already known pronounced side effects, especially in first doses. Ad5 is a prevalent virus in human populations, which may decrease its efficiency as a vector for vaccines in seropositive patients. COVID-19 pandemic made this possibility extend to Ad26 and ChadOx (chimpanzee), as both were widely utilized in vaccines. Lastly, even though industrial scale production for adenovirus vaccines is feasible, recombinant protein in bacteria is far cheaper and accessible for middle-income countries, making it not the best option for a vaccine vector where a vaccine against *T. cruzi* is mostly needed (Castro et al., 2023).

A promising alternative among experimental vaccines is the one presented by Bontempi et al. (2020), where the use of bacillus Calmette-Guérin (BCG) as a recombinant vector vaccine expressing the N-terminal domain of TS gene (nTS) was evaluated. BCG is excellent for the formulation of a vector-based vaccine due to its adjuvant proprieties and low maintenance cost. When used to immunize BALB/c mice, the vaccine of recombinant *Mycobacteruim bovis* BCG expressing

the nTs protein achieved a 70% survival rate against challenge with the Tulahuen strain of *T. cruzi*. CD4+ cells presented significant proliferation, with increased IFN-ɣ and IL17 levels; the frequency of IFN-ɣ produced by CD8+ CD107+ T cells was also increased after vaccination (Bontempi et al., 2020).

Challenges and future perspectives in CD vaccine development

While vaccines are essential for CD control, the success of the vaccination program encompasses the correct use of vaccines alongside better health and hygiene conditions for population at risk [5]. In this context, to find a high-efficacy vaccine against CD is extremely desirable, but it is noteworthy that changing people's mentality to put down clinical, structural, systemic and psychosocial barriers that stand on the path for a better diagnosis and treatment of CD is essential (Forsyth et al., 2019).

Access to novel healthcare technologies for populations at risk of contamination is directly tied to availability, affordability, adoption, and architecture, ideas inherently connected to sociopolitical factors (Forsyth et al., 2019; Frost & Reich, 2009). The current situation, in which only a small portion of CD cases is detected and treated, leads to a great burden in morbidity and mortality, even though it is an avoidable scenario. As Forsyth et al., 2019 describes it, this is an unchangeable situation without wide scale-up of more integrated strategies, which in turn requires commitment from government and public health systems, increased scientific research for improved treatment and diagnostic tools, greater accessibility of medications, broad awareness campaigns targeting both patients and providers, and a comprehensive treatment strategy that addresses the biological, psychological, and social impacts of the disease.

To change this situation with an efficient protective/therapeutic vaccine is a challenge by itself. *T. cruzi* has several escape mechanisms, among them, a decoy antigenic domain to avoid the immune response against the portion of the antigens with important function for parasite survival (Bivona et al., 2020). It is well known that adjuvants portraits a crucial role to increase the immunogenicity of the antigen and orchestrate an adequate adaptive immune response against *T. cruzi* infection. Although Th1 responses have been strongly associated with protection against *T. cruzi* infection, last generation adjuvants that induce other immunological mechanisms/profiles have been also able to confer protection. For

example, Zygmunt et al. (2012) elicited a greater Th17 cell response with the usage of CDA adjuvant than when CpG-ODN was utilized, a response recently found to be even more protective than that of Th1 cells (Cai et al., 2016). Therefore, in order to achieve a formulation capable of effective counteract this mechanism, the research of new combinations between untested adjuvants and antigens is a must.

Achieving a low cost but efficient vaccine is imperative to change this reality. However, to reach a formulation that attends to these requisites, evaluation in the appropriate animal model is essential, as means to, more precisely, estimate the immune response generated in the human organism. Different animals have already been tested as models for experimentation, from mice to dogs to primate species (Dumonteil et al., 2020; Machado et al., 2006; Rodríguez-Morales et al., 2020). Mice strains are most commonly used as they portraits advantages such as: small size, short life spawn, fast reproduction and easy maintenance. Nonetheless, such aspects do not determine it as ideal model for this disease's study, due to susceptibility to the acute form of the disease, not progressing to a chronic phase. Other small rodents, such as rats, rabbits and hamsters have also been evaluated, however, they have the same limitations presented by murine model. An ideal experimental model should be focused on the use of primates that, due to their phylogenetic proximity, develop the infection in a similar way to humans. Among the most used, the highlighted capuchin monkey (*Cebus apella*) and the rhesus monkey (*Macaca mulatta*), presented cardiac and digestive alterations, parasitemia levels and immune responses very similar to human organism, making it ideal for immunological assays and immunodiagnostic techniques (Araújo-Jorge & Castro, 2000; Kennedy et al., 1997).

The research and development of a safe and effective vaccine against *T. cruzi* offers a positive economic impact to countries where the disease is endemic. After computational simulation, Lee et al. (2010) expects a high cost-benefit in the development of even a 25% effective vaccine, mainly when establishing a relationship between the cost related to the manufacture and application of doses, the levels of efficacy and the infection rate. Efficacy levels greater than 50% indicate a profitable situation, even if the cost per dose is higher. In some of the simulated cases, mass vaccination can also signal a

monetary gain for the country in question, even if the risk of infection is low. For endemic countries, even vaccines with higher production cost can be profitable with the right development policies and incentives for manufacturers (Lee et al., 2012).

Chagas disease is responsible for about 20% of problems related to heart failure in Latin America, while in some countries, like Brazil, this number can reach 30% (Santos & Menezes Falcão, 2020; Vieira et al., 2019). These aggravating factors have a high treatment cost and intensive medical care, causing an increase in budget demand for health systems. At this point, highlighted the importance of obtaining a safe and effective formulation against Chagas Disease, it is clear that vaccination provides benefits to population health, reduces economic losses and relieves health systems from onerous treatments (Bartsch et al., 2019; Dumonteil et al., 2012).

References

Aparicio-Burgos, J. E., Zepeda-Escobar, J. A., de Oca-Jimenez, R. M., Estrada-Franco, J. G., Barbabosa-Pliego, A., Ochoa-García, L., Alejandre-Aguilar, R., Rivas, N., Peñuelas-Rivas, G., Val-Arreola, M., Gupta, S., Salazar-García, F., Garg, N. J., & Vázquez-Chagoyán, J. C. (2015). Immune Protection against Trypanosoma cruzi Induced by TcVac4 in a Canine Model. *PLoS Neglected Tropical Diseases*, *9*(4). https://doi.org/10.1371/journal.pntd.0003625

Araújo, A. F. S., De Alencar, B. C. G., Vasconcelos, J. R. C., Hiyane, M. I., Marinho, C. R. F., Penido, M. L. O., Boscardin, S. B., Hoft, D. F., Gazzinelli, R. T., & Rodrigues, M. M. (2005). CD8+-T-cell-dependent control of Trypanosoma cruzi infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. *Infection and Immunity*, *73*(9), 6017–6025. https://doi.org/10.1128/IAI.73.9.6017-6025.2005

Araujo, F. G., & Morein, B. (1991). Immunization with Trypanosoma cruzi Epimastigote Antigens Incorporated into Iscoms Protects against Lethal Challenge in Mice. *INFECTION AND IMMUNITY*, *59*(9), 2909–2914. https://journals.asm.org/journal/iai

Araújo-Jorge, T., & Castro, S. (2000). *Doença de chagas: manual para experimentação animal*.

Arce-Fonseca, M., González-Vázquez, M. C., Rodríguez-Morales, O., Graullera-Rivera, V., Aranda-Fraustro, A., Reyes, P. A., Carabarin-Lima, A., & Rosales-Encina, J. L. (2018). Recombinant enolase of Trypanosoma cruzi as a novel vaccine candidate against Chagas disease in a mouse model of

acute infection. *Journal of Immunology Research*, *2018*. https://doi.org/10.1155/2018/8964085

Barbosa, R. P. A., Filho, B. G., Santos, L. I. dos, Junior, P. A. S., Marques, P. E., Pereira, R. V. S., Cara, D. C., Bruña-Romero, O., Rodrigues, M. M., Gazzinelli, R. T., & Machado, A. V. (2013). Vaccination Using Recombinants Influenza and Adenoviruses Encoding Amastigote Surface Protein-2 Are Highly Effective on Protection against Trypanosoma cruzi Infection. *PLoS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0061795

Barry, M. A., Versteeg, L., Wang, Q., Pollet, J., Zhan, B., Gusovsky, F., Bottazzi, M. E., Hotez, P. J., & Jones, K. M. (2019). A therapeutic vaccine prototype induces protective immunity and reduces cardiac fibrosis in a mouse model of chronic trypanosoma cruzi infection. *PLoS Neglected Tropical Diseases*, *13*(5). https://doi.org/10.1371/journal.pntd.0007413

Bartsch, S. M., Bottazzi, M. E., Asti, L., Strych, U., Meymandi, S., Falcón-Lezama, J. A., Randall, S., Hotez, P. J., & Lee, B. Y. (2019). Economic value of a therapeutic Chagas vaccine for indeterminate and Chagasic cardiomyopathy patients. *Vaccine*, *37*(28), 3704–3714. https://doi.org/10.1016/j.vaccine.2019.05.028

Basombrío, M. A., Gómez, L., Padilla, A. M., Ciaccio, M., Nozaki, T., & Cross, G. A. M. (2002). Targeted deletion of the GP72 gene decreases the infectivity of Trypanosoma cruzi for mice and insect vectors. *Journal of Parasitology*, *88*(3), 489–493. https://doi.org/10.1645/0022-3395(2002)088

Basso, B., Moretti, E., & Fretes, R. (2014). Vaccination with Trypanosoma rangeli induces resistance of guinea pigs to virulent Trypanosoma cruzi. *Veterinary Immunology and Immunopathology*, *157*(1–2), 119–123. https://doi.org/10.1016/j.vetimm.2013.10.011

Bhatia, V., & Garg, N. J. (2008). Previously unrecognized vaccine candidates control Trypanosoma cruzi infection and immunopathology in mice. *Clinical and Vaccine Immunology*, *15*(8), 1158–1164. https://doi.org/10.1128/CVI.00144-08

Bivona, A. E., Alberti, A. S., Cerny, N., Trinitario, S. N., & Malchiodi, E. L. (2020). Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control. In *Biochimica et Biophysica Acta - Molecular Basis of Disease* (Vol. 1866, Issue 5). Elsevier B.V. https://doi.org/10.1016/j.bbadis.2019.165658

Bivona, A. E., Sánchez Alberti, A., Matos, M. N., Cerny, N., Cardoso, A. C., Morales, C., González, G., Cazorla, S. I., & Malchiodi, E. L. (2018). Trypanosoma cruzi 80 kDa prolyl oligopeptidase (Tc80) as a novel immunogen for Chagas disease vaccine. *PLoS Neglected Tropical Diseases*, *12*(3). https://doi.org/10.1371/journal.pntd.0006384

Bontempi, I. A., Vicco, M. H., Cabrera, G., Villar, S. R., González, F. B., Roggero, E. A., Ameloot, P., Callewaert, N., Pérez, A. R., & Marcipar, I. S. (2015).

Efficacy of a trans-sialidase-ISCOMATRIX subunit vaccine candidate to protect against experimental Chagas disease. *Vaccine*, *33*(10), 1274–1283. https://doi.org/10.1016/j.vaccine.2015.01.044

Bontempi, I., Leal, K., Prochetto, E., Díaz, G., Cabrera, G., Bortolotti, A., Morbidoni, H. R., Borsuk, S., Dellagostin, O., & Marcipar, I. (2020). Recombinant Mycobacterium bovis BCG is a promising platform to develop vaccines against Trypansoma cruzi infection. *Clinical and Experimental Immunology*, *201*(3), 306–316. https://doi.org/10.1111/cei.13469

Boscardin, S. B., Kinoshita, S. S., Fujimura, A. E., & Rodrigues, M. M. (2003). Immunization with cDNA expressed by amastigotes of Trypanosoma cruzi elicits protective immune response against experimental infection. *Infection and Immunity*, *71*(5), 2744–2757. https://doi.org/10.1128/IAI.71.5.2744-2757.2003

Brandán, C. P., Mesías, A. C., Parodi, C., Cimino, R. O., Pérez Brandán, C., Diosque, P., & Basombrío, M. Á. (2017). Effects of IFN-γ coding plasmid supplementation in the immune response and protection elicited by Trypanosoma cruzi attenuated parasites. *BMC Infectious Diseases*, *17*(1). https://doi.org/10.1186/s12879-017-2834-6

Breganó, J. W., Picão, R. C., Graça, V. K., Menolli, R. A., Jankevicius, S. I., Filho, P. P., & Jankevicius, J. V. (2003). Phytomonas serpens, a tomato parasite, shares antigens with Trypanosoma cruzi that are recognized by human sera and induce protective immunity in mice. *FEMS Immunology and Medical Microbiology*, *39*(3), 257–264. https://doi.org/10.1016/S0928-8244(03)00256-6

Caeiro, L. D., Alba-Soto, C. D., Rizzi, M., Solana, M. E., Rodriguez, G., Chidichimo, A. M., Rodriguez, M. E., Sánchez, D. O., Levy, G. V., & Tekiel, V. (2018). The protein family TcTASV-C is a novel Trypanosoma cruzi virulence factor secreted in extracellular vesicles by trypomastigotes and highly expressed in bloodstream forms. *PLoS Neglected Tropical Diseases*, *12*(5). https://doi.org/10.1371/journal.pntd.0006475

Cai, C. W., Blase, J. R., Zhang, X., Eickhoff, C. S., & Hoft, D. F. (2016). Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. *PLoS Pathogens*, *12*(10). https://doi.org/10.1371/journal.ppat.1005902

Carabarin-Lima, A., González-Vázquez, M. C., Baylon-Pacheco, L., Tsutsumi, V., Talamás-Rohana, P., & Rosales-Encina, J. L. (2011). Immunization with the recombinant surface protein rTcSP2 alone or fused to the CHP or ATPase domain of TcHSP70 induces protection against acute Trypanosoma cruzi infection. *Journal of Vaccines and Vaccination*, *1*(3). https://doi.org/10.4172/2157-7560.1000110

Castro, J. T., Brito, R., Hojo-Souza, N. S., Azevedo, B., Salazar, N., Ferreira, C. P., Junqueira, C., Fernandes, A. P., Vasconcellos, R., Cardoso, J. M.,

Aguiar-Soares, R. D. O., Vieira, P. M. A., Carneiro, C. M., Valiate, B., Toledo, C., Salazar, A. M., Caballero, O., Lannes-Vieira, J., Teixeira, S. R., … Gazzinelli, R. T. (2023). ASP-2/Trans-sialidase chimeric protein induces robust protective immunity in experimental models of Chagas' disease. *Npj Vaccines*, *8*(1). https://doi.org/10.1038/s41541-023-00676-0

Cazorla, S. I., Becker, P. D., Frank, F. M., Ebensen, T., Sartori, M. J., Corral, R. S., Malchiodi, E. L., & Guzmán, C. A. (2007). Oral vaccination with Salmonella enterica as a cruzipain-DNA delivery system confers protective immunity against Trypanosoma cruzi. *Infection and Immunity*, *76*(1), 324–333. https://doi.org/10.1128/IAI.01163-07

Cazorla, S. I., Frank, F. M., Becker, P. D., Arnaiz, M., Mirkin, G. A., Corral, R. S., Guzmán, C. A., & Malchiodi, E. L. (2010). Redirection of the immune response to the functional catalytic domain of the cystein proteinase cruzipain improves protective immunity against trypanosoma cruzi infection. *Journal of Infectious Diseases*, *202*(1), 136–144. https://doi.org/10.1086/652872

Cazorla, S. I., Matos, M. N., Cerny, N., Ramirez, C., Alberti, A. S., Bivona, A. E., Morales, C., Guzmán, C. A., & Malchiodi, E. L. (2015). Oral multicomponent DNA vaccine delivered by attenuated Salmonella elicited immunoprotection against American trypanosomiasis. *Journal of Infectious Diseases*, *211*(5), 698–707. https://doi.org/10.1093/infdis/jiu480

Cerny, N., Bivona, A. E., Sanchez Alberti, A., Trinitario, S. N., Morales, C., Cardoso Landaburu, A., Cazorla, S. I., & Malchiodi, E. L. (2020). Cruzipain and Its Physiological Inhibitor, Chagasin, as a DNA-Based Therapeutic Vaccine Against Trypanosoma cruzi. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.565142

Cerny, N., Sánchez Alberti, A., Bivona, A. E., De Marzi, M. C., Frank, F. M., Cazorla, S. I., & Malchiodi, E. L. (2016). Coadministration of cruzipain and GM-CSF DNAs, a new immunotherapeutic vaccine against Trypanosoma cruzi infection. *Human Vaccines and Immunotherapeutics*, *12*(2), 438–450. https://doi.org/10.1080/21645515.2015.1078044

Collins, M. H., Craft, J. M., Bustamante, J. M., & Tarleton, R. L. (2011). Oral exposure to Trypanosoma cruzi elicits a systemic CD8+ T cell response and protection against heterotopic challenge. *Infection and Immunity*, *79*(8), 3397–3406. https://doi.org/10.1128/IAI.01080-10

Conteh, L., Engels, T., & Molyneux, D. H. (2010). Socioeconomic aspects of neglected tropical diseases. In *The Lancet* (Vol. 375, Issue 9710, pp. 239–247). Elsevier B.V. https://doi.org/10.1016/S0140-6736(09)61422-7

Costa, F., Franchin, G., Lucia Pereira-Chioccola, V., Schenkman, S., & Rodrigues, M. M. (1998). Immunization with a plasmid DNA containing the gene of &ns-sialidase reduces TIypanosoma cruzi infection in mice. In *Vaccine* (Vol. 16, Issue 8).

Coura, J. R. (2015). The main sceneries of chagas disease transmission. The vectors, blood and oral transmissions - A comprehensive review. *Memorias Do Instituto Oswaldo Cruz*, *110*(3), 277–282. https://doi.org/10.1590/0074-0276140362

De Alencar, B. C. G., Persechini, P. M., Haolla, F. A., De Oliveira, G., Silverio, J. C., Lannes-Vieira, J., Machado, A. V., Gazzinelli, R. T., Bruna-Romero, O., & Rodrigues, M. M. (2009). Perforin and gamma interferon expression are required for CD4+ and CD8+ T-cell-dependent protective immunity against a human parasite, Trypanosoma cruzi, elicited by heterologous plasmid DNA prime-recombinant adenovirus 5 boost vaccination. *Infection and Immunity*, *77*(10), 4383–4395. https://doi.org/10.1128/IAI.01459-08

de la Cruz, J. J., Villanueva-Lizama, L., Dzul-Huchim, V., Ramírez-Sierra, M. J., Martinez-Vega, P., Rosado-Vallado, M., Ortega-Lopez, J., Flores-Pucheta, C. I., Gillespie, P., Zhan, B., Bottazzi, M. E., Hotez, P. J., & Dumonteil, E. (2018). Production of recombinant TSA-1 and evaluation of its potential for the immuno-therapeutic control of Trypanosoma cruzi infection in mice. *Human Vaccines and Immunotherapeutics*, *15*(1), 210–219. https://doi.org/10.1080/21645515.2018.1520581

Duan, X., Yonemitsu, Y., Chou, B., Yoshida, K., Tanaka, S., Hasegawa, M., Tetsutani, K., Ishida, H., Himeno, K., & Hisaeda, H. (2009). Efficient protective immunity against Trypanosoma cruzi infection after nasal vaccination with recombinant Sendai virus vector expressing amastigote surface protein-2. *Vaccine*, *27*(44), 6154–6159. https://doi.org/10.1016/j.vaccine.2009.08.026

Dumonteil, E., Bottazzi, M. E., Zhan, B., Heffernan, M. J., Jones, K., Valenzuela, J. G., Kamhawi, S., Ortega, J., De Leon Rosales, S. P., Lee, B. Y., Bacon, K. M., Fleischer, B., Slingsby, B. T., Cravioto, M. B., Tapia-Conyer, R., & Hotez, P. J. (2012). Accelerating the development of a therapeutic vaccine for human Chagas disease: Rationale and prospects. In *Expert Review of Vaccines* (Vol. 11, Issue 9, pp. 1043–1055). https://doi.org/10.1586/erv.12.85

Dumonteil, E., Escobedo-Ortegon, J., Reyes-Rodriguez, N., Arjona-Torres, A., & Ramirez-Sierra, M. J. (2004). Immunotherapy of Trypanosoma cruzi Infection with DNA Vaccines in Mice. *Infection and Immunity*, *72*(1), 46–53. https://doi.org/10.1128/IAI.72.1.46-53.2004

Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P. A., Ortega-Lopez, J., Hotez, P. J., & Bottazzi, M. E. (2020). Safety and immunogenicity of a recombinant vaccine against Trypanosoma cruzi in Rhesus macaques. *Vaccine*, *38*(29), 4584–4591. https://doi.org/10.1016/j.vaccine.2020.05.010

Eickhoff, C. S., Vasconcelos, J. R., Sullivan, N. L., Blazevic, A., Bruna-Romero, O., Rodrigues, M. M., & Hoft, D. F. (2011). Co-administration of a plasmid DNA encoding IL-15 improves long-term protection of a genetic vaccine

635 against Trypanosoma cruzi. *PLoS Neglected Tropical Diseases*, *5*(3).
636 https://doi.org/10.1371/journal.pntd.0000983

637 Eickhoff, C. S., Zhang, X., Vasconcelos, J. R., Motz, R. G., Sullivan, N. L.,
638 O'Shea, K., Pozzi, N., Gohara, D. W., Blase, J. R., Di Cera, E., & Hoft, D.
639 F. (2016). Costimulatory Effects of an Immunodominant Parasite Antigen
640 Paradoxically Prevent Induction of Optimal CD8 T Cell Protective Immunity.
641 *PLoS Pathogens*, *12*(9). https://doi.org/10.1371/journal.ppat.1005896

642 Flores-García, Y., Rosales-Encina, J. L., Satoskar, A. R., Talamás-Rohana, P.,
643 & Rohana, P. T. (2011). IL-10-IFN-γ Double Producers CD4+ T Cells Are
644 Induced by Immunization with an Amastigote Stage Specific Derived
645 Recombinant Protein of Trypanosoma Cruzi. In *Int. J. Biol. Sci* (Vol. 7, Issue
646 8). http://www.biolsci.org1093

647 Fonseca, B. de P., Albuquerque, P. C., & Zicker, F. (2020). Neglected tropical
648 diseases in Brazil: lack of correlation between disease burden, research
649 funding and output. *Tropical Medicine and International Health*, *25*(11),
650 1373–1384. https://doi.org/10.1111/tmi.13478

651 Fontanella, G. H., De Vusser, K., Laroy, W., Daurelio, L., Nocito, A. L., Revelli,
652 S., & Contreras, R. (2008). Immunization with an engineered mutant trans-
653 sialidase highly protects mice from experimental Trypanosoma cruzi
654 infection: A vaccine candidate. *Vaccine*, *26*(19), 2322–2334.
655 https://doi.org/10.1016/j.vaccine.2008.02.060

656 Forsyth, C., Meymandi, S., Moss, I., Cone, J., Cohen, R., & Batista, C. (2019).
657 Proposed multidimensional framework for understanding Chagas disease
658 healthcare barriers in the United States. In *PLoS Neglected Tropical*
659 *Diseases* (Vol. 13, Issue 9). Public Library of Science.
660 https://doi.org/10.1371/journal.pntd.0007447

661 Frank, F. M., Petray, P. B., Cazorla, S. I., Muñoz, M. C., Corral, R. S., & Malchiodi,
662 E. L. (2003). Use of a purified Trypanosoma cruzi antigen and CpG
663 oligodeoxynucleotides for immunoprotection against a lethal challenge with
664 trypomastigotes. *Vaccine*, *22*(1), 77–86. https://doi.org/10.1016/S0264-
665 410X(03)00541-3

666 Frost, L. J., & Reich, M. R. (2009). Creating access to health technologies in poor
667 countries. In *Health Affairs* (Vol. 28, Issue 4, pp. 962–973).
668 https://doi.org/10.1377/hlthaff.28.4.962

669 Fujimura, A. E., Kinoshita, S. S., Pereira-Chioccola, V. L., & Rodrigues, M. M.
670 (2001). DNA sequences encoding CD4+ and CD8+ T-cell epitopes are
671 important for efficient protective immunity induced by DNA vaccination with
672 a Trypanosoma cruzi gene. *Infection and Immunity*, *69*(9), 5477–5486.
673 https://doi.org/10.1128/IAI.69.9.5477-5486.2001

674 Galvão, C. (2014). *Vetores da doença de Chagas no Brasil*. Sociedade Brasileira
675 de Zoologia.

Garcia, C. A. A. C., Oliveira, E. C., Sakurada, J. K., & Santos, L. M. B. (2000a). Protective immunity induced by a Trypanosoma cruzi soluble extract antigen in Experimental Chagas' Disease. Role of Interferon y. *Immunological Investigations*, *29*(1).

Garcia, C. A. A. C., Oliveira, E. C., Sakurada, J. K., & Santos, L. M. B. (2000b). Protective immunity induced by a Trypanosoma cruzi soluble extract antigen in Experimental Chagas' Disease. Role of Interferon y. *Immunological Investigations*, *29*(1).

Garg, N., & Tarleton, R. L. (2002). Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in Trypanosoma cruzi infection. *Infection and Immunity*, *70*(10), 5547–5555. https://doi.org/10.1128/IAI.70.10.5547-5555.2002

Gomes, Y. M., Abath, F. G., Nakazawa, M., Minoprio, P., Vouldoukis, I., & Monjour, L. (1999). Partial Protection of Mice against Trypanosoma cruzi after Immunizing with the TcY 72 Antigenic Preparation. *Mem Inst Oswaldo Cruz*, *94*(2), 167–172.

Gonzáles Cappa SM, Schmuñis GA, Traversa OC, et al (1968) Complement-fixation tests, skin tests, and experimental immunization with antigen of Trypanosoma Cruzi prepared under pressure. Am J Trop Med Hyg 17:709-715. https://doi.org/10.4269/ajtmh.1968.17.709

Guarner, J. (2019). Chagas disease as example of a reemerging parasite. In *Seminars in Diagnostic Pathology* (Vol. 36, Issue 3, pp. 164–169). W.B. Saunders. https://doi.org/10.1053/j.semdp.2019.04.008

Gupta, S., & Garg, N. J. (2015). A Two-Component DNA-Prime/Protein-Boost Vaccination Strategy for Eliciting Long-Term, Protective T Cell Immunity against Trypanosoma cruzi. *PLoS Pathogens*, *11*(5). https://doi.org/10.1371/journal.ppat.1004828

Gupta, S., Salgado-Jiménez, B., Lokugamage, N., Vázquez-Chagoyán, J. C., & Garg, N. J. (2019). TcG2/TcG4 DNA Vaccine Induces Th1 Immunity against Acute Trypanosoma cruzi Infection: Adjuvant and antigenic effects of heterologous T. Rangeli Booster Immunization. *Frontiers in Immunology*, *10*(JUN). https://doi.org/10.3389/fimmu.2019.01456

Gupta, S., Smith, C., Auclair, S., Delgadillo, A. D. J., & Garg, N. J. (2015). Therapeutic efficacy of a subunit vaccine in controlling chronic Trypanosoma cruzi infection and chagas disease is enhanced by glutathione peroxidase over-expression. *PLoS ONE*, *10*(6). https://doi.org/10.1371/journal.pone.0130562

Hegazy-Hassan, W., Zepeda-Escobar, J. A., Ochoa-García, L., Contreras-Ortíz, J. M. E., Tenorio-Borroto, E., Barbabosa-Pliego, A., Aparicio-Burgos, J. E., Oros-Pantoja, R., Rivas-Santiago, B., Díaz-Albiter, H., Garg, N. J., & Vázquez-Chagoyán, J. C. (2019). TcVac1 vaccine delivery by intradermal electroporation enhances vaccine induced immune protection against

718 Trypanosoma cruzi infection in mice. *Vaccine*, *37*(2), 248–257.
719 https://doi.org/10.1016/j.vaccine.2018.11.041

720 Introini MV, Basso B, Moretti E (1998) Experimental Chagas' disease: I. Study of
721 different immunization conditions in the infection course. Bol Chil Parasitol
722 53(3-4):45-51.

723 Jha, B. K., Varikuti, S., Verma, C., Shivahare, R., Bishop, N., Dos Santos, G. P.,
724 McDonald, J., Sur, A., Myler, P. J., Schenkman, S., Satoskar, A. R., &
725 McGwire, B. S. (2023). Immunization with a Trypanosoma cruzi cyclophilin-
726 19 deletion mutant protects against acute Chagas disease in mice. *Npj*
727 *Vaccines*, *8*(1). https://doi.org/10.1038/s41541-023-00647-5

728 Kennedy, R. C., Shearer, M. H., & Hildebrand, W. (1997). Ekevier Nonhuman
729 primate models to evaluate vaccine safety and immunogenicity. In *Vaccine*
730 (Vol. 15, Issue 6).

731 Lee, B. Y., Bacon, K. M., Connor, D. L., Willig, A. M., & Bailey, R. R. (2010). The
732 potential economic value of a Trypanosoma cruzi (chagas disease) vaccine
733 in Latin America. *PLoS Neglected Tropical Diseases*, *4*(12), 1–8.
734 https://doi.org/10.1371/journal.pntd.0000916

735 Lee, B. Y., Bacon, K. M., Wateska, A. R., Bottazzi, M. E., Dumonteil, E., & Hotez,
736 P. J. (2012). Modeling the economic value of a Chagas' disease therapeutic
737 vaccine. *Human Vaccines and Immunotherapeutics*, *8*(9), 1293–1301.
738 https://doi.org/10.4161/hv.20966

739 Lima, M. T., Jansen, A. M., Rondinelli, E., & Gattass, C. R. (1990). Trypanosoma
740 cruzi: properties of a clone isolated from CL strain. *Parasitol Res*, *77*, 77–
741 81.

742 Limon-Flores, A. Y., Cervera-Cetina, R., Tzec-Arjona, J. L., Ek-Macias, L.,
743 Sánchez-Burgos, G., Ramirez-Sierra, M. J., Cruz-Chan, J. V.,
744 VanWynsberghe, N. R., & Dumonteil, E. (2010). Effect of a combination
745 DNA vaccine for the prevention and therapy of Trypanosoma cruzi infection
746 in mice: Role of CD4+ and CD8+ T cells. *Vaccine*, *28*(46), 7414–7419.
747 https://doi.org/10.1016/j.vaccine.2010.08.104

748 Lokugamage, N., Choudhuri, S., Davies, C., Chowdhury, I. H., & Garg, N. J.
749 (2020). Antigen-based nano-immunotherapy controls parasite persistence,
750 inflammatory and oxidative stress, and cardiac fibrosis, the hallmarks of
751 chronic chagas cardiomyopathy, in a mouse model of trypanosoma cruzi
752 infection. *Vaccines*, *8*(1). https://doi.org/10.3390/vaccines8010096

753 Luhrs, K. A., Fouts, D. L., & Manning, J. E. (2003). Immunization with
754 recombinant paraflagellar rod protein induces protective immunity against
755 Trypanosoma cruzi infection. *Vaccine*, *21*(21–22), 3058–3069.
756 https://doi.org/10.1016/S0264-410X(03)00108-7

757 Machado, A. V, Cardoso, J. E., Claser, C., Rodrigues, M. M., Gazzinelli, R. T., &
758 Bruna-Romero, O. (2006). Long-Term Protective Immunity Induced Against

759 Trypanosoma cruzi Infection After Vaccination with Recombinant
760 Adenoviruses Encoding Amastigote Surface Protein-2 and Trans-Sialidase
761 OVERVIEW SUMMARY. In *HUMAN GENE THERAPY* (Vol. 17).

762 Martinez-Campos, V., Martinez-Vega, P., Ramirez-Sierra, M. J., Rosado-Vallado,
763 M., Seid, C. A., Hudspeth, E. M., Wei, J., Liu, Z., Kwityn, C., Hammond, M.,
764 Ortega-López, J., Zhan, B., Hotez, P. J., Bottazzi, M. E., & Dumonteil, E.
765 (2015). Expression, purification, immunogenicity, and protective efficacy of
766 a recombinant Tc24 antigen as a vaccine against Trypanosoma cruzi
767 infection in mice. *Vaccine*, *33*(36), 4505–4512.
768 https://doi.org/10.1016/j.vaccine.2015.07.017

769 Martins-Melo, F. R., Carneiro, M., Ramos, A. N., Heukelbach, J., Ribeiro, A. L.
770 P., & Werneck, G. L. (2018). The burden of Neglected Tropical Diseases in
771 Brazil, 1990-2016: A subnational analysis from the Global Burden of
772 Disease Study 2016. *PLoS Neglected Tropical Diseases*, *12*(6).
773 https://doi.org/10.1371/journal.pntd.0006559

774 Matos, M. N., Cazorla, S. I., Bivona, A. E., Morales, C., Guzma´n, C. A., &
775 Malchiodi, E. L. (2014). Tc52 amino-terminal-domain DNA carried by
776 attenuated Salmonella enterica serovar typhimurium induces protection
777 against a trypanosoma cruzi lethal challenge. *Infection and Immunity*,
778 *82*(10), 4265–4275. https://doi.org/10.1128/IAI.02190-14

779 Matos, M. N., Sánchez Alberti, A., Morales, C., Cazorla, S. I., & Malchiodi, E. L.
780 (2016). A prime-boost immunization with Tc52 N-terminal domain DNA and
781 the recombinant protein expressed in Pichia pastoris protects against
782 Trypanosoma cruzi infection. *Vaccine*, *34*(28), 3243–3251.
783 https://doi.org/10.1016/j.vaccine.2016.05.011

784 McHardy N (1978) Immunization of mice against Trypanosoma cruzi: the effect
785 of chemical treatment or immune serum on an epimastigote vaccine.
786 Tropenmedzin und Parasitologie 29(2):215-222.

787 Miller, M. J., Wrightsman, R. A., & Manning, J. E. (1996). Trypanosoma cruzi:
788 Protective Immunity in Mice Immunized with Paraflagellar Rod Proteins Is
789 Associated with a T-Helper Type 1 Response. In *Trypanosoma cruzi*.

790 Miyahira, Y., Takashima, Y., Kobayashi, S., Matsumoto, Y., Takeuchi, T.,
791 Ohyanagi-Hara, M., Yoshida, A., Ohwada, A., Akiba, H., Yagita, H.,
792 Okumura, K., & Ogawa, H. (2005). Immune responses against a single
793 CD8+-T-cell epitope induced by virus vector vaccination can successfully
794 control trypanosoma cruzi infection. *Infection and Immunity*, *73*(11), 7356–
795 7365. https://doi.org/10.1128/IAI.73.11.7356-7365.2005

796 Moraschi, B. F., Noronha, I. H., Ferreira, C. P., Cariste, L. M., Monteiro, C. B.,
797 Denapoli, P., Vrechi, T., Pereira, G. J. S., Gazzinelli, R. T., Lannes-Vieira,
798 J., Rodrigues, M. M., Bortoluci, K. R., & Vasconcelos, J. R. C. (2021).
799 Rapamycin Improves the Response of Effector and Memory CD8+ T Cells
800 Induced by Immunization With ASP2 of Trypanosoma cruzi. *Frontiers in*

Cellular and Infection Microbiology, *11*. https://doi.org/10.3389/fcimb.2021.676183

Nogueira, R. T., Nogueira, A. R., Pereira, M. C. S., Rodrigues, M. M., Neves, P. C. da C., Galler, R., & Bonaldo, M. C. (2013). Recombinant Yellow Fever Viruses Elicit CD8+ T Cell Responses and Protective Immunity against Trypanosoma cruzi. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0059347

Padilla, A. M., Simpson, L. J., & Tarleton, R. L. (2009). Insufficient TLR Activation Contributes to the Slow Development of CD8 + T Cell Responses in Trypanosoma cruzi Infection . *The Journal of Immunology*, *183*(2), 1245–1252. https://doi.org/10.4049/jimmunol.0901178

Pereira, I. R., Vilar-Pereira, G., Marques, V., da Silva, A. A., Caetano, B., Moreira, O. C., Machado, A. V., Bruna-Romero, O., Rodrigues, M. M., Gazzinelli, R. T., & Lannes-Vieira, J. (2015). A Human Type 5 Adenovirus-Based Trypanosoma cruzi Therapeutic Vaccine Re-programs Immune Response and Reverses Chronic Cardiomyopathy. *PLoS Pathogens*, *11*(1), 1–26. https://doi.org/10.1371/journal.ppat.1004594

Pereira, V. R., Maria Lorena, V. B., Fernanda Verçosa, A. A., Silva, E. D., Ferreira, A. G., Montarroyos, U. R., Galvão Silva, A. P., & Gomes, Y. M. (2003). Antibody isotype responses in Balb/C Mic immunized with the cytoplasmatic repetitive antigen and flagellar repetitive antigen of Trypanosoma cruzi. *Mem Inst Oswaldo Cruz*, *98*(6), 823–825.

Pérez-Molina, J. A., & Molina, I. (2018). Chagas disease. *The Lancet*, *391*(10115), 82–94. https://doi.org/10.1016/S0140-6736(17)31612-4

Pinge-Filho, P., Peron, J. P. S., De Moura, T. R., Menolli, R. A., Graça, V. K., Estevão, D., Tadokoro, C. E., Jankevicius, J. V., & Rizzo, L. V. (2005). Protective immunity against Trypanosoma cruzi provided by oral immunization with Phytomonas serpens: Role of nitric oxide. *Immunology Letters*, *96*(2), 283–290. https://doi.org/10.1016/j.imlet.2004.09.010

Quintana, I., Espariz, M., Villar, S. R., González, F. B., Pacini, M. F., Cabrera, G., Bontempi, I., Prochetto, E., Stülke, J., Perez, A. R., Marcipar, I., Blancato, V., & Magni, C. (2018). Genetic engineering of Lactococcus lactis co-producing antigen and the mucosal adjuvant 3’ 5’- cyclic di Adenosine Monophosphate (c-di-AMP) as a design strategy to develop a mucosal vaccine prototype. *Frontiers in Microbiology*, *9*(SEP). https://doi.org/10.3389/fmicb.2018.02100

Ramos-Vega, A., Dzul-Huchim, V. M., Villanueva-Lizama, L. E., Dumonteil, E., Rosales-Mendoza, S., Bañuelos-Hernández, B., Angulo, M., Cruz-Chan, J. V., Monreal-Escalante, E., & Angulo, C. (2024). Protective efficacy of the oral vaccine Tc24:Co1 produced in Schizochytrium sp. against Trypanosoma cruzi infection in a mouse model. *Microbial Pathogenesis*, *186*. https://doi.org/10.1016/j.micpath.2023.106488

Rassi, A., Rassi, A., & Marin-Neto, J. A. (2010). Chagas disease. *The Lancet*, *375*(9723), 1388–1402. https://doi.org/10.1016/S0140-6736(10)60061-X

Rigato, P. O., de Alencar, B. C., de Vasconcelos, J. R. C., Dominguez, M. R., Araújo, A. F., Machado, A. V., Gazzinelli, R. T., Bruna-Romero, O., & Rodrigues, M. M. (2011). Heterologous plasmid DNA prime-recombinant human adenovirus 5 boost vaccination generates a stable pool of protective long-lived CD8+ T effector memory cells specific for a human parasite, Trypanosoma cruzi. *Infection and Immunity*, *79*(5), 2120–2130. https://doi.org/10.1128/IAI.01190-10

Rodrigues, M. M., Ribeira˜o, M., Ribeira˜o, R., Pereira-Chioccola, V., Renia, L., & Costa, F. (1999). Predominance of CD4 Th1 and CD8 Tc1 Cells Revealed by Characterization of the Cellular Immune Response Generated by Immunization with a DNA Vaccine Containing a Trypanosoma cruzi Gene. In *INFECTION AND IMMUNITY* (Vol. 67, Issue 8). https://journals.asm.org/journal/iai

Rodríguez-Morales, O., Monteón-Padilla, V., Carrillo-Sánchez, S. C., Rios-Castro, M., Martínez-Cruz, M., Carabarin-Lima, A., & Arce-Fonseca, M. (2015). Experimental Vaccines against Chagas Disease: A Journey through History. In *Journal of Immunology Research* (Vol. 2015). Hindawi Limited. https://doi.org/10.1155/2015/489758

Rodríguez-Morales, O., Roldán, F. J., Vargas-Barrón, J., Parra-Benítez, E., De Lourdes Medina-García, M., Vergara-Bello, E., & Arce-Fonseca, M. (2020). Echocardiographic findings in canine model of chagas disease immunized with DNA Trypanosoma cruzi genes. *Animals*, *10*(4). https://doi.org/10.3390/ani10040648

Rosas-Jorquera, C. E., Sardinha, L. R., Pretel, F. D., Bombeiro, A. L., Lima, M. R. D. I., & Alvarez, J. M. (2013). Challenge of chronically infected mice with homologous Trypanosoma cruzi parasites enhances the immune response but does not modify cardiopathy: Implications for the design of a therapeutic vaccine. *Clinical and Vaccine Immunology*, *20*(2), 248–254. https://doi.org/10.1128/CVI.00032-12

Sanchez-Burgos, G., Mezquita-Vega, R. G., Escobedo-Ortegon, J., Ramirez-Sierra, M. J., Arjona-Torres, A., Ouaissi, A., Rodrigues, M. M., & Dumonteil, E. (2007). Comparative evaluation of therapeutic DNA vaccines against Trypanosoma cruzi in mice. *FEMS Immunology and Medical Microbiology*, *50*(3), 333–341. https://doi.org/10.1111/j.1574-695X.2007.00251.x

Sánchez-Valdéz, F. J., Pérez Brandán, C., Ramírez, G., Uncos, A. D., Zago, M. P., Cimino, R. O., Cardozo, R. M., Marco, J. D., Ferreira, A., & Basombrío, M. Á. (2014). A Monoallelic Deletion of the TcCRT Gene Increases the Attenuation of a Cultured Trypanosoma cruzi Strain, Protecting against an In Vivo Virulent Challenge. *PLoS Neglected Tropical Diseases*, *8*(2). https://doi.org/10.1371/journal.pntd.0002696

Santos, É., & Menezes Falcão, L. (2020). Chagas cardiomyopathy and heart failure: From epidemiology to treatment. In *Revista Portuguesa de Cardiologia* (Vol. 39, Issue 5, pp. 279–289). Sociedade Portuguesa de Cardiologia. https://doi.org/10.1016/j.repc.2019.12.006

Schnapp, A. R., Eickhoff, C. S., Sizemore, D., Curtiss, R., & Hoft, D. F. (2002). Cruzipain induces both mucosal and systemic protection against Trypanosoma cruzi in mice. *Infection and Immunity*, *70*(9), 5065–5074. https://doi.org/10.1128/IAI.70.9.5065-5074.2002

Seid, C. A., Jones, K. M., Pollet, J., Keegan, B., Hudspeth, E., Hammond, M., Wei, J., McAtee, C. P., Versteeg, L., Gutierrez, A., Liu, Z., Zhan, B., Respress, J. L., Strych, U., Bottazzi, M. E., & Hotez, P. J. (2016). Cysteine mutagenesis improves the production without abrogating antigenicity of a recombinant protein vaccine candidate for human chagas disease. *Human Vaccines and Immunotherapeutics*, *13*(3), 621–633. https://doi.org/10.1080/21645515.2016.1242540

Taibi, A., Plumas-Marty, B., Guevara-Espinoza, A., Schoneck, R., Pessoa, H., Loyens, M., Piras, R., Aguirre, T., Gras-Masse, H., & Bossus, M. (1993). Trypanosoma cruzi: Immunity-induced in mice and rats by trypomastigote excretory-secretory antigens and identification of a peptide sequence containing a T cell epitope with protective activity. *J Immunol*, *151*(5), 2676–2689. http://www.jimmunol.org#otherarticles

Trevisan, R. O., Santos, M. M., Desidério, C. S., Alves, L. G., De Jesus Sousa, T., De Castro Oliveira, L., Jaiswal, A. K., Tiwari, S., Bovi, W. G., De Oliveira-Silva, M., Costa-Madeira, J. C., Castellano, L. R. C., Silva, M. V., Azevedo, V., Rodrigues, V., Oliveira, C. J. F., & De Castro Soares, S. (2020). In Silico Identification of New Targets for Diagnosis, Vaccine, and Drug Candidates against Trypanosoma cruzi. *Disease Markers*, *2020*. https://doi.org/10.1155/2020/9130719

Vasconcelos, J. R. C., Boscardin, S. B., Hiyane, M. I., Kinoshita, S. S., Fujimura, A. E., & Rodrigues, M. M. (2003). A DNA-priming protein-boosting regimen significantly improves type 1 immune response but not protective immunity to Trypanosoma cruzi infection in a highly susceptible mouse strain. *Immunology and Cell Biology*, *81*(2), 121–129. https://doi.org/10.1046/j.0818-9641.2002.01136.x

Vieira, J. L., Távora, F. R. F., Sobral, M. G. V., Vasconcelos, G. G., Almeida, G. P. L., Fernandes, J. R., da Escóssia Marinho, L. L., de Mendonça Trompieri, D. F., De Souza Neto, J. D., & Mejia, J. A. C. (2019). Chagas Cardiomyopathy in Latin America Review. In *Current Cardiology Reports* (Vol. 21, Issue 2). Current Medicine Group LLC 1. https://doi.org/10.1007/s11886-019-1095-y

Wizel, B., Garg, N., & Tarleton, R. L. (1998). Vaccination with Trypomastigote Surface Antigen 1-Encoding Plasmid DNA Confers Protection against

Lethal Trypanosoma cruzi Infection. In *INFECTION AND IMMUNITY* (Vol. 66, Issue 11). https://journals.asm.org/journal/iai

World Health Organization (2022) Chagas disease (also known as American trypanosomiasis). World Health Organization Newsletter. https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis). Accessed 21 May 2022

Wrightsman, R. A., Miller, M. J., Saborio, J. L., & Manning, J. E. (1995). Pure Paraflagellar Rod Protein Protects Mice against Trypanosoma cruzi Infection. In *INFECTION AND IMMUNITY* (Vol. 63, Issue 1).

Zago, M. P., Barrio, A. B., Cardozo, R. M., Duffy, T., Schijman, A. G., & Basombrío, M. A. (2008). Impairment of infectivity and immunoprotective effect of a LYT1 null mutant of Trypanosoma cruzi. *Infection and Immunity*, *76*(1), 443–451. https://doi.org/10.1128/IAI.00400-07

Zapata-Estrella, H., Hummel-Newell, C., Sanchez-Burgos, G., Escobedo-Ortegon, J., Ramirez-Sierra, M. J., Arjona-Torres, A., & Dumonteil, E. (2006). Control of Trypanosoma cruzi infection and changes in T-cell populations induced by a therapeutic DNA vaccine in mice. *Immunology Letters*, *103*(2), 186–191. https://doi.org/10.1016/j.imlet.2005.11.015

Zygmunt, B. M., Weissmann, S. F., & Guzman, C. A. (2012). NKT cell stimulation with α-galactosylceramide results in a block of Th17 differentiation after intranasal immunization in mice. *PLoS ONE*, *7*(1). https://doi.org/10.1371/journal.pone.0030382

971 **Table 1: Experimental vaccines developed against Chagas' disease.** The
972 table presents the vaccinal formulation, adjuvant of choice, route of
973 administration, protection rate and animal model used in each experiment.
974

Immunogen	Adjuvant	Route	% protection	Animal model	Author
Whole parasite vacines					
T. cruzi pressurized antigens	-	-	88-100	Rockland strain mice	(Gonzáles Cappa et al., 1968)
CL-14 *T. cruzi* trypomastigotes	-	I.P[1]	100	BALB/c mice	(Lima et al., 1990)
CL-14 *T. cruzi* trypomastigotes			100	C3H mice	
T. rangeli epimastigotes	SAP[10]	-	95	BALB/c mice	(Introini et al., 1998)
Y *T. cruzi gp-72* null epimastigotes	-	S.C[2]	-	Swiss mice	(Basombrío et al., 2002)
Phytomonas serpen	-	I.P/O.A[3]	40-100	BALB/c mice	(Breganó et al., 2003)
Phytomonas serpen	-	O.A	50	C57BL/6 mice	(Pinge-Filho et al., 2005)
L16 *T. cruzi LYT1* null epimastigotes	-	I.P	100	Swiss mice	(Zago et al., 2008)
CL *T. cruzi* CoA hydratase 1 null trypomastigotes	-	O.A I.P S.C	-	C57BL/6 mice	(Collins et al., 2011)
CL *T. cruzi* CoA hydratase 2 null trypomastigotes					
Sylvio X10/4 *T. cruzi*	-	I.P	-	C3H/HePAS mice	(Rosas-Jorquera et al., 2013)
T. rangeli epimastigotes	-	I.D[4]	-	Guinea pigs	(Basso et al., 2014)
TCC *T. cruzi* TcCRT null	-	I.P	100	BALB/c mice	(Sánchez-Valdéz et al., 2014)
T. cruzi overexpressing TcCRT TCC strain			100		
Inactivated *T. rangeli* epimastigotes	PBS[11]-SAP	S.C or I.D	-	Mongrel dogs	(Aparicio-Burgos et al., 2015)
CL Brener *T. cruzi* Cyp19 depleted	PBS	I.P	100	A/J mice; BALB/c mice	(Jha et al., 2023)
Subunit vaccines					
Immunoglobulin G3 monoclonal antibody	MEGA-10 detergent + Quil-A	-	100	BALB/c mice	(Araujo & Morein, 1991)
Immunoglobulin G3 monoclonal antibody	SAP		0		
PFR	F.C[12]	I.P	100		(Miller et al., 1996)

	Alum	S.C	83.3	BALB/cByJ mice	
Y *T. cruzi* 72 kDa protein	-	S.C	0	C57BL/10 mice	(Gomes et al., 1999)
T. cruzi soluble extract antigen	-	I.V[5]	85	CBA/J mice	(Garcia et al., 2000a)
Recombinant subunit vaccines					
ESA	-	-	60	BALB/c mice	(Taibi et al., 1993)
			100	Fischer rats	
rPFR-1	Alum + IL-12	-	100	C56BL/6J mice	(Luhrs et al., 2003)
rPFR-2	Alum + IL-12		100		
rPFR-3	Alum + IL-12		50		
rPFR-1 + rPFR-2 + rPFR-3	Alum + IL-12		100		
rCRA	F.C	S.C	-	BALB/c mice	(V. R. Pereira et al., 2003)
rFRA	F.I[13]		-		
rTS + p154/13	Alum	I.M[6]	20	A/Sn mice	(Vasconcelos et al., 2003)
rASP-2 (His-65kDa)	Alum + CpG ODN	I.M	73	A/Sn mice	(Araújo et al., 2005)
rASP-2 (His-25kDa)	Alum + CpG ODN		35		
rASP-2 (GST-P1-P3)	Alum + CpG ODN		10		
rASP-2 (GST-P4-P7)	Alum + CpG ODN		100		
rASP-2 (GST-P4-P5)	Alum + CpG ODN		100		
rASP-2 (GST-P6-P7)	Alum + CpG ODN		0		
rTS	F.C	S.C	100	BALB/c mice	(Fontanella et al., 2008)
rCz (N-terminal domain)	CpG ODN	-	-	C3H/HeN mice	(Cazorla et al., 2010)
rCz (C-terminal domain)					
rCz					
rTcSP2	Titer-Max®	I.P	100	BALB/c mice	(Carabarin-Lima et al., 2011)
rTcSP2-CHP	-		75		
rTCSP2-ATP			75		
rMBP::SSP4	F.C	I.P	-	C57BL/129 mice	(Flores-García et al., 2011)
mTS	-	S.C	20	BALB/c mice	(Bontempi et al., 2015)
mTS	F.C		60		
mTS	IMX[14]		100		
rTc24	MPLA[15]	I.M	50	BALB/c mice	(Martinez-Campos et al., 2015)

rTc24 E WT His + E6020	AddaVax™	S.C	90	BALB/c mice	(Seid et al., 2016)
rTc24 E C4 + E6020			80		
rTc80	CpG-ODN	I.M	30	C3H/HeN mice	(Bivona et al., 2018)
rTSA-1	MPLA	I.P	85-100	BALB/c mice	(de la Cruz et al., 2018)
rTSA-1	GLA-SE				
rTSA-1	E6020				
rTc24	E6020	-	-	ICR mice	(Barry et al., 2019)
rTc24	E6020	-	-	Rhesus macaques	(Dumonteil et al., 2020)
rTSA-1	E6020		-		
rTc24-C4	GLA-SE	S.C	-	C57BL/6 mice	(Poveda et al., 2023)
TRASP (TS+ASP-2 chimera)	CpG B344 + Alum[1] or Hiltonol[1,2]	S.C	100	[1,2]C57BL/6 mice; [2]Mongrel dogs	(Castro et al., 2023)
DNA vaccines					
pcDNA3.TS-cat7	-	I.M	100	BALB/c mice	(Costa et al., 1998)
VR1012 TSA1.7	-	I.M	100[1] 50[2]	[1]BALB/c mice [2]B6 mice	(Wizel et al., 1998)
VR1012 TSA2.1			100[1] 50[2]		
pcDNA3.TS	-	I.M	-	BALB/c mice	(Rodrigues et al., 1999)
pcDNA3.TS	-	I.M	100	BALB/c mice	(Fujimura et al., 2001)
pcDNA3.TSsignal pep+catalytic domain			90		
pcDNA3.TSsignal pep+part of catalytic domain			35		
pCMVI.UBF3/2.ASP-1 + pcDNA3.msp35, pcDNA3.msp40 [IL-12], and pCMVI.GM-CSF	-	I.M	55	C57BL/6 mice	(Garg & Tarleton, 2002)
pCMVI.UBF3/2.ASP-2 + pcDNA3.msp35, pcDNA3.msp40 [IL-12], and pCMVI.GM-CSF			75		
pCMVI.UBF3/2.TSA-1 + pcDNA3.msp35, pcDNA3.msp40 [IL-12], and pCMVI.GM-CSF			55		
pCMVI.UBF3/2.ASP-1			50		
pCMVI.UBF3/2.ASP-2			70		
pCMVI.UBF3/2.TSA-1			25		

pCMVI.UBF3/2.ASP-1 + pCMVI.UBF3/2.ASP-2 + pCMVI.UBF3/2.TSA-1			80		
pCMVI.UBF3/2.ASP-1 + pCMVI.UBF3/2.ASP-2 + pCMVI.UBF3/2.TSA-1 + pcDNA3.msp35, pcDNA3.msp40 [IL-12], and pCMVI.GM-CSF			80		
pcDNA3.ASP2-clone9	-	I.M	100	BALB/c mice	(Boscardin et al., 2003)
pSP.ASP2-clone9			100		
pcDNA3.TSA1	-	I.M	70	BALB/c mice	(Dumonteil et al., 2004)
pcDNA3.Tc24			100		
pcDNA3.1.TSA1	-	I.M	-	ICR mice	(Zapata-Estrella et al., 2006)
p154.TS	Alum	I.M	50	ICR mice	(Sanchez-Burgos et al., 2007)
plgSP.ASP2			50		
plgSP.ASP2 + p154.TS			55		
pcDNA3.Tc24			85		
pcDNA3.Tc52			75		
pcDNA3.TSA1			65		
pcDNA3.TcG1	-	I.M	-	C57BL/6 mice	(Bhatia & Garg, 2008)
pcDNA3.TcG2					
pcDNA3.TcG3					
pcDNA3.TcG1 + pcDNA3.TcG2 + pcDNA3.TcG3					
pcDNA3.1.TSA-1 + pcDNA3.1.Tc24	Alum		60-80	BALB/c mice	(Limon-Flores et al., 2010)
pcDNA.TS	PBS	I.M	100	BALB/c mice	(Eickhoff et al., 2011)
pVAX-1.IL-15opt			0		
pcDNA.TS + pVAX-1.IL-15opt			100		
pcDNA3.1.TcG1.TcG2.TcG4 + Inactivated *T. rangeli* epimastigotes*	*PBS-SAP	I.M or I.D + S.C or I.D	-	Mongrel dogs	(Aparicio-Burgos et al., 2015)
pcDNA3.TcG2 + (pcDNA3.msp35 + pcDNA3.msp40 + pCMVI.GM-CSF)$^{+}$ + rTcG2^{-}	-	$^{+}$I.M $^{-}$I.D	-	C57BL/6 mice	(Gupta et al., 2015)
pcDNA3.TcG4 + (pcDNA3.msp35 + pcDNA3.msp40 + pCMVI.GM-CSF)$^{+}$ + rTcG4^{-}					

pcDNA3.1.TcG2.TcG4^{+} + rTcG2 + rTcG4^{-*}	*PBS-SAP	$^{+}$I.M $^{-}$I.D	-	C57BL/6 mice	(Gupta & Garg, 2015)
pcDNA3.1.Cz	-	I.M	80	-	(Cerny et al., 2016)
pcDNA3.1.Cz + pcDNA3.1.GM-CSF			100		
pcDNA3.1.Cz	*Salmonella aro* A7207		0		
pcDNA3.1.Cz + pcDNA3.1.GM-CSF	-		70		
Wild type TS DNA	-	I.M	80	BALB/c mice	(Eickhoff et al., 2016)
TS Kd1 null DNA			30		
pVXVR-mIFN-ɣ + live attenuated *T. rangeli* TCC strain		I.P	100	C57BL/6J mice	(Brandán et al., 2017)
rTcEnolase* + rTcEnolase**	*F.C **F.I	I.P	75	BALB/c mice	(Arce-Fonseca et al., 2018)
pBK.TcEnolase	PBS	I.M	0		
(pCI_Not-TcTASV-C + VR10_GM-CSF) + (TcTASV-C_{GST} + TcTASV-C_{HIS})*	*Alum	I.M	25	C3H/HeN mice	(Caeiro et al., 2018)
pcDNA3.1.TcG2 + pcDNA3.1.TcG4 + fixated *T. rangeli* lysate	PBS	S.C	-	C57BL/6 mice	(Gupta et al., 2019)
pcDNA3.1.TcG2 + pcDNA3.1.TcG4 + quil A		I.M			
pcDNA3.1.TcG2 + pcDNA3.1.TcG4 + *T. rangeli* + quil A		S.C			
pcDNA3.1.TcG2 + pcDNA3.1.TcG4 + pcDNA3.1-msp35 + pcDNA3.1-msp40 + pcMVI.GM-CSF	-	I.M or I.D.E^{7}	100	BALB/c mice	(Hegazy-Hassan et al., 2019)
pCDNA3 carrying TcG2 + TcG4 genes	PBS	I.M	-	C57BL/6 mice	(Lokugamage et al., 2020)
NTC9385R carrying TcG2 + TcG4 genes					
pBK-CMV carrying TcSSP4 gene	-	I.M	-	Beagle dogs	(Rodríguez-Morales et al., 2020)
pBK-CMV carrying TcSP gene					

pcDNA3.TS + pcDNA3.ASP-2 (boosted with rAdTS + rAdASP-2)	-	I.M	100	C57BL/6 mice; Mongrel dogs	(Castro et al., 2023)
Vector-based vaccines					
Salmonella enterica expressing Cz	PBS	I.P	80	BALB/c mice	(Schnapp et al., 2002)
Ad-MANY (MVA-MANY + MVA-p3)	-	I.M	100	C57BL/6 mice	(Miyahira et al., 2005)
Ad-MANY (MVA-MANY + MVA-RANKL)			100		
rAdenovirus expressing ASP-2	RPMI[16]	S.C	80	BALB/c mice; C57BL/6 mice; C57BL/6 CD8+ KO mice	(Machado et al., 2006)
rAdenovirus expressing TS			50		
rAddenovirus ASP-2 + rAdenovirus TS			100		
Salmonella carrying Cz DNA	-	O.A	-	C3H/HeN mice	(Cazorla et al., 2007)
Salmonella carrying Cz DNA + rCz	CpG-ODN 1826	O.A + I.D	100		
Salmonella carrying Cz DNA + rCz	MALP[17]	O.A + I.N[9]	100		
plgSPCI.9 + Adenovirus expressing ASP-2	-	I.M	75-90	A/Sn mice; C57BL/6 mice	(De Alencar et al., 2009)
rSeV expressing ASP-2	PBS	I.N	95	C57BL/6 mice	(Duan et al., 2009)
rSeV expressing ASP-2 fusioned with ubiquitin			90		
plgSPCI.9 + Adenovirus expressing ASP-2	-	I.M	100	C57BL/6 mice; A/Sn mice	(Rigato et al., 2011)
plgSPCI.9 + Adenovirus expressing ASP-2 + MVA-GFP			90		
rFlu-C-ASP2 (rAd-ASP2)	PBS	I.N	100	C57BL/6 mice	(Barbosa et al., 2013)
rYF 17D virus expressing ASP-2	-	S.C	60	A/J mice	(Nogueira et al., 2013)
Salmonella carrying Tc52 DNA	PBS	O.A	60	C3H/HeN mice	(Matos et al., 2014)
Salmonella carrying Tc52-C-term DNA			25		
Salmonella carrying Tc52-N-term DNA			100		
Salmonella expressing Cz + Tc52 + Tc24	-	-	80	C3H/HeN mice	(Cazorla et al., 2015)
Salmonella expressing Cz + Tc52			50		

rAdenovirus ASP2 + rAdTS	-	S.C	70-85	C57BL/6 mice	(I. R. Pereira et al., 2015)
Salmonella carrying NTc52 + rNTc52	CpG-ODN 1826	O.A + I.D	80	C3H/HeN mice	(Matos et al., 2016)
rNTc52		I.N/I.D	60		
Salmonella expressing Tc80	-	O.A	85	C3H/HeN mice	(Bivona et al., 2018)
Salmonella expressing Tc80 + rTc80		O.A + I.M	70		
L. lactis expressing TS	CDA[18]	O.A	-	BALB/c mice	(Quintana et al., 2018)
M. bovis BCG expressing TS	-	-	70	BALB/cCmedc mice	(Bontempi et al., 2020)
M. bovis BCG expressing Cz			<70		
S. enterica expressing Cz	-	O.A	60	C3H/HeN mice	(Cerny et al., 2020)
S. enterica expressing Cz + *S. enterica* expressing Chg			80		
plgSPCI.9 + Adenovirus expressing ASP-2 + rapamycin	PBS	I.M	100	C57BL/6 mice; A/Sn mice	(Moraschi et al., 2021)
Schizochytrium sp expressing Tc24:Co1	PBS	O.A	-	BALB/c mice	(Ramos-Vega et al., 2024)

I.P: Intraperitoneal; S.C: Subcutaneous; O.A: Oral administration; I.D: Intradermal; I.V: Intravenous; I.M: Intramuscular; I.D.E: Intradermal electroporation; I.N: Intranasal; SAP: Saponin; PBS: Phosphate-buffered saline; F.C: Freund's complete; F.I: Freund's incomplete; IMX: ISCOMATRIX; MPLA: Monophosphoryl Lipid A; RPMI: Roswell Park Memorial Institute 1640 medium; MALP: Macrophage-activating lipopeptide from *M. fermentans;* CDA: cyclic di AMP.

3.2) Manuscrito 2

Manuscrito a ser submetido a revista *Microbiological Research*

Bacterial Vector-based Vaccines for Chagas' disease: a double immune response activation alternative.

Guilherme Senna dos Santos[1], Bárbara da Rocha Fonseca[1], Mirna Samara Dié Alves[1], Odir Antonio Dellagostin[2], Iván Marcipar[3], Sibele Borsuk[1*]

1 Laboratório de Biotecnologia Infecto-Parasitária, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário s/n, Prédio 19, Pelotas, RS 96010-900, Brazil

2 Laboratório de Vacinologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Universitário s/n, Prédio 19, Pelotas, RS 96010-900, Brazil

3

*Corresponding author: sibeleborsuk@gmail.com

Abstract

Chagas' disease (CD) is an infectious disease attacking an estimated 8 million people, mainly in rural areas of Latin America countries. CD has no effective treatment, evidencing the vaccination schedule as the best control strategy. Although some medicaments are available, none of them provides a solution for the infection nor are capable of inducing protection. They also have questionable safety levels and side effects. In light of this, several experimental vaccines are in development in order to improve safety, reproducibility, and protective immune response against the etiologic agent of CD, *Trypanosoma cruzi*. In this review, we discuss aspects as antigen, adjuvant, routes of administration, protection level, animal models, and economic impact in CD vaccine development, as well the challenges and future perspectives.

Key points

- *Chagas' disease (CD) does not have an appropriate treatment.*
- *Different experimental vaccines are in development aiming to induce total protection against Trypanosoma cruzi.*
- *An ideal vaccine for CD is necessary for disease control, with bacterial vector-based vaccines as a promising alternative.*

Keywords: *Trypanosoma cruzi* · Vaccine development · Adjuvant · Immunoprophylaxis

1. Introduction

First described more than a hundred years ago by Carlos Chagas, and titled with his name, the Chagas disease is caused by the parasitic protozoan *Trypanosoma cruzi* and is transmitted by hematophagous triatominae insects, usually *Triatoma infestans* (Yeung et al., 2021). It is endemic to 15 countries in Latin America, and as result of human migration, the disease appeared in other American countries, Western Pacific areas, and Europe, with an estimated 8–10 million people infected by *T. cruzi* worldwide (Antinori et al., 2017). Around 120 million people living in endemic areas are estimated to be at risk of infection. The disease has, usually, two different clinical phases, however, a third asymptomatic phase can be observed. The acute phase signals the beginning of the infection and where the first symptoms can be detected. About 30-40% of patients can evolve to a chronic phase, where abnormalities can be detected in organs of the gastrointestinal tract or damage to the cardiovascular system, such as arrhythmias, thromboembolism and cardiomegaly. The third, indeterminate phase is characterized by the presence of the protozoan in the host organism, even though it does not present evident clinical signs in the form of symptoms (Guarner, 2019; Pérez-Molina and Molina, 2018).

Even though research aiming for the eradication of this disease is by no means a recent topic, many hindrances still remain. Chagas disease is recognized by the World Health Organization (WHO) as a neglected tropical disease, given the low investment in combat and adequate prevention measures, affecting mainly rural areas, with difficult access to diagnosis and treatment (Fonseca et al., 2020; Martins-Melo et al., 2018). Not only the lack of the bare minimum for living is a severe issue in some endemic areas, but also the growing age of the population alongside many comorbidities or other pathologies, can influence the disease outcome.

Drugs benzimidazole and nifurtimox are employed in the treatment against *T. cruzi* for almost fifty years, they are used to treat acute and congenital Chagas disease, reactivated infections, and chronic disease in children under 18 years old (Antinori et al., 2017; Pérez-Molina and Molina, 2018; Yeung et al., 2021). However, the effective rates of both drugs vary according to the stage of infection,

being more effective in the acute stage of the disease. Nifurtimox is no longer recommended to treat Chagas disease and benzimidazole is preferred for its better tolerability profile and efficacy (Antinori et al., 2017; Pérez-Molina and Molina, 2018; Yeung et al., 2021). The treatment also boasts several side effects, demonstrating the importance of early diagnosis and treatment intervention, independent of infection phase (Pérez-Molina and Molina, 2018).

To control Chagas disease and minimize the damage caused, different strategies have been adopted in order to find a safe and effective treatment against *T. cruzi*. A plethora of new drugs are being developed against Chagas disease, even so, it is well known that vaccination is largely responsible for the worldwide eradication or restriction of numerous infectious diseases, being so, it is of no surprise that many vaccine prototypes are in development (Bivona et al., 2020; Vekemans et al., 2021). However, to ensure an efficient protection is achieved is no easy task, especially against a parasite with innumerous mechanisms to escape the host immune system. For this reason, any vaccine in development takes into account that an ideal antigen must present high immunogenicity; be expressed in different life stages; be a well-conserved region and present in different strains of *T. cruzi* (Bivona et al., 2020). Of equal relevance is the mechanism of delivery of said antigen, which should not only be able to present the antigen to the host immune system as quickly and for as long as possible, but also, ideally, be able to elicit an immune response that fortify those necessary to fight the parasite of interest.

In this review, we will present recent strategies to develop bacterial vector-based vaccines expressing antigen candidates, which in our understanding is a promising strategy, to obtain a vaccine against Chagas disease in an effort to restrict the parasite spreading and to prevent the clinical outcome of the disease. For a better understanding on the usage of said strategy, we will discuss about the mechanisms involved on *T. cruzi* infection, while also reviewing the choice of antigen, here we will present some of the more explored choices, how they are conserved among different strains and how can this be of impact while developing a vaccine.

2. Considerations to develop a vaccine against Chagas disease

Chagas disease is an inflammatory, infectious, systemic, life-long, and life-threatening illness for which treatment is not as effective as it needs to be and causes several side effects (Jiménez et al., 2019; Ramírez-Toloza et al., 2020). Prophylaxis against *T. cruzi* is a way to achieve protection and save lives. It has advantages such as low to no adverse reactions, higher efficacy to prevent cardiac and gastrointestinal complications, and potential use during pregnancy to prevent congenital Chagas disease (Lee et al., 2010). Another strategy is immunotherapy, such as a therapeutic vaccine, that could be administered to patients in the indeterminate phase or those in the chronic stage (Dumonteil et al., 2019, 2012).

Furthermore, a vaccine would help reduce the economic burden of the disease, which is estimated to cost billions of dollars each year in healthcare costs and lost productivity worldwide. Economically speaking, developing a therapeutic or prophylactic vaccine is highly cost-effective, could save numerous lives, and depending on vaccine cost and risk of infection (%), it could even save money that would have to be spent on treatments (Dumonteil et al., 2012; Lee et al., 2010). An ideal vaccine against Chagas should target all parasitic stages and work as a prophylactic or immunotherapeutic (Rios et al., 2019).

When developing a vaccine, researchers aim to successfully activate the host's immune system and promote long-term protection. That is only possible when the vaccine triggers effective humoral and cellular immune responses and generates effector memory cells to add to the host's defense repertoire against diseases. Before any vaccine development, researchers need to understand how the immune system behaves when in contact with the pathogen to design a vaccine strategy considering host-pathogen interactions (Bivona et al., 2020). Therefore, understanding what is known about the immune response against *Trypanosoma cruzi* is a step toward developing a vaccine against Chagas' disease.

Another essential step toward developing a vaccine is choosing which vaccine technology and target(s) will be used. Currently, vaccine prototypes

against Chagas disease can be divided into prophylactic and therapeutic. Several platforms have been applied to develop prophylactic formulations, such as live attenuated parasites (Williams et al., 2020), DNA vaccines (Gupta et al., 2019), subunit vaccines (Sanchez Alberti et al., 2020), viral vectors (Castro et al., 2023), and bacterial vectors (Bontempi et al., 2020). When using vectorized and subunit vaccines, choosing the most appropriate antigens as vaccine targets is essential to trigger the desired immune response.

Here, we review aspects relating to the immune responses activated against *T. cruzi* infection, the *T. cruzi* antigens used to design vaccines, and bacterial-vector vaccine technology.

2.1 Immune response against *Trypanosoma cruzi*

Chagas disease is multisystemic and multifactorial, with virulence factors, route of infection, invasion mechanisms, *T. cruzi* strains and discrete typing units (DTUs), parasitic load, and host immune factors playing a role in how the immune system is activated (Acevedo et al., 2018; Da Costa et al., 2014; Jiménez et al., 2019). Several immune activation profiles have been described in Chagas patients, and they are directly influenced by the factors described above (Acevedo et al., 2018).

Once infection is established, it triggers an innate immune response, giving start to what is known as the acute phase, which can either present symptoms or not. If untreated or uncleared by the host's immune system, the disease can evolve to the indeterminate phase, an asymptomatic chronic phase that can become permanent or evolve to a chronic phase in which cardiac, digestive, or cardio-digestive symptoms are present (Cristovão-Silva et al., 2021; Macaluso et al., 2023).

The pathophysiology of Chagas disease is quite complex and still not fully understood. Since immunological responses are, among other things, influenced by pathogenesis, it makes sense that the immune response to this multifactorial disease would be as complex. There is much that is still not known about the immune response against Chagas disease. Nonetheless, pathogen dissemination or elimination is intrinsically related to how the parasite's proliferative rate and the host's inflammatory and immune response are balanced (Acevedo et al., 2018; Cardillo et al., 2007; Macaluso et al., 2023).

Over the next two subtopics, we will swiftly discuss the basics of the immune response against Chagas disease to allow for a quick grasp on how host-parasite interactions trigger the immune response, which type of responses are triggered, and how *T. cruzi* evades the immune system to cause a persistent infection. Such understanding is essential when developing a vaccine and, therefore, needs to be discussed in this review. More detailed reviews have been published and should be explored for a deeper view of this topic (Acevedo et al., 2018; Cardillo et al., 2015; Cerbán et al., 2020; Cristovão-Silva et al., 2021).

2.1.1 Innate immune response

Innate immunity is the first line of immunological defense and is activated after the host's physical barriers are breached by the pathogen. Innate cells, especially macrophages (Mo), neutrophils (Nt), dendritic cells (DCs), and natural killer (NK) cells, are responsible for setting up such defense (Murphy; Weaver, 2017). The three complement system (CS) pathways [classical (CP), alternative (AP), and lectin (LP)] actively participate in immediate immunity, with its many plasma proteins guiding processes such as pathogen opsonization, phagocyte recruitment, and direct lysis (Murphy and Weaver, 2017).

For *T. cruzi* infection to properly trigger an innate immune response, the host pattern recognition receptors (PRRs) have to recognize *T. cruzi* pathogen-associated molecular patterns (PAMPs) and endogenous molecules known as damage-associated molecular patterns (DAMPs), which are released in response to tissue injury and cell death. The two main families of PRRs are Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). TLRs are a family of thirteen type 1 transmembrane receptors found in immune and non-immune cells and have two main cellular locations, the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) and endosomal membranes (TLR3, TLR7, TLR8, and TLR9) (Fitzgerald and Kagan, 2020). In the context of *T. cruzi* infection, TLR2 (dimerized with TLR6) and TLR4 are responsible for recognizing several TLR ligands, such as glycosylphosphatidylinositol (GPI) anchors and glycoinositolphospholipids (GIPLs), respectively, in the pathogen cell surface (Bafica et al., 2006; Oliveira et al., 2004). Meanwhile, TLR9 binds to unmethylated CpG motifs of *T. cruzi* DNA (Bafica et al., 2006), and *T. cruzi* RNA acts as a TLR7 agonist (Caetano et al., 2011). NLRs are cytoplasmic sensors,

and their function in modulating immune response against *T. cruzi* is not as well-known as that of TLRs. However, NOD1 (NLRC1), NLRP3, and NAIP/NLRC4 are involved in the defense against *T. cruzi* by activating pro-inflammatory signaling pathways and forming inflammasomes (Amaral et al., 2023; Silva et al., 2013, 2010). As will be briefly discussed in this section, *T. cruzi* infection activates innate immunity and leads to an inflammatory response, NK cell cytotoxicity, and initiates adaptive immune response.

An important aspect of innate immunity during the Chagas disease acute phase is the interplay between the host complement system and *T. cruzi* evasion mechanisms. Interestingly, recent findings show that most immune cells express CS receptors and proteins, suggesting that CS takes part in the immune response against Chagas in more ways than previously taught and might participate in T-cell modulation during the chronic phase (Caputo et al., 2022). The three CS pathways can be activated by all *T. cruzi* life forms through the recognition of PAMPs such as *N*-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) (Acevedo et al., 2018; Lidani et al., 2017). However, complement effector molecules only successfully lysate non-infectious epimastigotes and metacyclic trypomastigotes of some *T. cruzi* strains (Lidani et al., 2017). That is a result of effective immunomodulation that this pathogen developed to increase its survival. Several molecules have been described as mediators of resistance to CS activity (Cestari et al., 2009; Norris, 1998; Ramírez-Toloza et al., 2020) and they can act by inhibiting the first steps of the proteolytic cascade (Cestari et al., 2008) or by inhibiting the convertases of the three CS pathways, namely C3 and C5 convertases (Cestari et al., 2012).

One well-known molecule that inhibits CS initial activation steps is *T. cruzi* calreticulin (TcCalr, formerly TcCRT), a highly immunogenic molecule that binds to C1q, mannan-binding lectin (MBL) and L-ficolin tails and inhibits CP and LP (Ferreira et al., 2004; Ramírez-Toloza et al., 2020; Sosoniuk et al., 2014). Another evasion molecule is *T. cruzi* complement C2 receptor inhibitor trispanning protein (CRIT). This inhibitor binds to C2, inactivating it and inhibiting its cleavage and, by impairing C3 convertase formation, blocks the progression of the CP and LP (Cestari et al., 2009, 2008). Moreover, *T. cruzi* C regulatory protein (TcCRP or gp160) is an immunogenic molecule that inhibits the CP and AP by binding to C3b and C4b and preventing C3 convertase formation (Norris, 1998).

Immune cells, such as Mo, Nt, DCs, and NK cells, and non-immune cells, such as epithelial cells, are equipped with TLRs and NLRs and recognize *T. cruzi* PAMPs and DAMPs through them. Once receptor-PAMP/DAMP interactions take place, they trigger cytokine production and secretion, resulting in inflammation and activation of immune defense signaling pathways. Mo and Nt are the first immune cells to interact with *T. cruzi*, which they recognize and internalize to be processed in the phagolysosome. DCs also recognize and internalize *T. cruzi* (Acevedo et al., 2018; Cerbán et al., 2020; Cristovão-Silva et al., 2021). After these parasite-host interactions, a storm of pro-inflammatory cytokines, primarily IL-12, IL-1β, IL-6, and TNF-α, and chemokines, such as CCL2, CCL3, CCL4, CCL5, and CXCL10, are secreted by these immune cells. Transcription factors such as nuclear factor-kB (NF-kB), signal transducer and activator of transcription 6 (STAT6), and interferon regulatory factor (IRF), which induce inflammatory cytokine and chemokines expression, are also released (de Oliveira et al., 2016; Kayama and Takeda, 2010; Zanluqui, 2015). NK cells are activated by the IL-12 produced by macrophages, and besides directly killing parasites, NK cells recognize and kill infected host cells. Once activated, NK cells secrete high levels of IFN-γ. This cytokine activates macrophages and reinforces NK cells' immune activities. Activated macrophages produce reactive oxygen species (ROS) and antimicrobial molecules. Also, when these cells recognize *T. cruzi* GPIs via TLR2, they produce nitric oxide (NO), nitric oxide synthase (iNOS), TNF-α, and IL-12, strengthening inflammation while inducing a Th1 response (Acevedo et al., 2018). Interestingly, studies have shown that in *T. cruzi* infection, NK cells impact more in infection control by reducing parasite burden through direct parasite killing than by eliminating the infected cells, as might be expected considering *T. cruzi* is mostly an intracellular pathogen (Batalla et al., 2013; Lieke et al., 2004).

During *T. cruzi* infection, macrophages go through polarization in response to stimuli and form two profiles: M1 macrophages (M1 Mo) induced, among other things, by IL-12 and IFN-γ, and M2 macrophages (M2 Mo), which result from IL-4, IL-10, IL-13, and other molecules. These profiles matter because they help induce different outcomes for the infection. As such, Mo can play two roles during *T. cruzi* infection: destroy amastigotes (M1 Mo and Th1 immune profile) or, conversely, allow for parasite encapsulation and replication in its phagolysosomes (M2 Mo and Th2 immune profile) depending on how they are

balanced (Zanluqui, 2015). Although, the latter is more a result of *T. cruzi* evasion mechanisms and molecules, such as ascorbate-dependent haemoperoxidase (TcAPX), cytosolic and mitochondrial tryparedoxin peroxidases (TcCPX and TcMPX, respectively), which are part of an antioxidant system that protects the pathogen against the oxidant agents produced by activated macrophages (Acevedo et al., 2018).

Once DCs recognize *T. cruzi* antigens via TLRs and NLRs, they have two main functions: activating leukocytes by releasing IL-12 and TNF-α and activating T cells by presenting them with processed *T. cruzi* antigens via MHC class I or II (Acevedo et al., 2018). Interestingly, molecules secreted by *T. cruzi* (e.g., GIPLs) immunomodulate DCs and can induce a tolerogenic state that favors pathogen survival and infection persistence. Such molecules are known to (i) downregulate IL-6, IL-12, and TNF-α production while upregulating IL-10; (II) decrease MHC I and II and CD40 co-receptor molecules, impairing antigen presentation; and (iii) suppress CD8+ priming through the stimulation of regulatory TCD4+Foxp3+ cells, directly interfering with adaptive immune response (Ersching et al., 2016; Van Overtvelt et al., 2002). Moreover, DCs and macrophage cytokine profiles might also be different depending on the *T. cruzi* strain promoting the infection (Da Costa et al., 2014). DCs and macrophages have a role in *T. cruzi* infection that goes beyond innate immunity because these cells are professional antigen-presenting cells (APCs). As APCs, they activate T cells and, consequently, adaptive immunity by presenting *T. cruzi* antigens via MHC and inducing the expression of cytokines and co-stimulatory molecules. Therefore, these cells are the link between innate and adaptive responses.

More than understanding innate response itself, it is essential to be aware of *T. cruzi* molecules and their significance to either immune activation or evasion, particularly when aiming at vaccine development. For instance, Tc52 is a *T. cruzi* protein with glutathione transferase activity, and it is vital for *T. cruzi* virulence and survival (Allaoui et al., 2002). This antigen is an effective TLR2 agonist, has a role in DC maturation, and activates NF-κB, the expression of co-stimulatory molecules, and cytokine production (Allaoui et al., 2002; Cerbán et al., 2020; Kayama and Takeda, 2010). Moreover, trypomastigote surface antigen (TSA-1) belongs to the trans-sialidase (TS) family and binds to TLR4, leading to NF-kB activation and inflammation (de la Cruz et al., 2019). Information provided by their

immunological functions made Tc52 and TSA-1 potential vaccine targets, and they have been applied as such in many studies (Matos et al., 2017, 2016, 2014), as will be further discussed in topic 2.2. The same is true for TcCRP, which has been applied as a vaccine antigen because of its immunogenicity and immunomodulation abilities (Sepulveda et al., 2000), and other molecules as will be discussed.

All in all, to understand innate immunity and its intricate processes in response to *T. cruzi* infection, as well as *T. cruzi* evasion mechanisms, it is necessary to recognize how it triggers the adaptive system and which *T. cruzi* molecules are potential vaccine targets. Therefore, the abovementioned is essential for a clearer discussion of the following topics in this review.

2.1.2 Adaptative immune response

The previous topic has established how important innate immunity is in the fight Chagas' disease (CD). Nonetheless, unless adaptive immune is activated, there is no protection against *T. cruzi* infection. The innate response slows down pathogen multiplication through its quick initial response and it triggers critical signals that initiate adaptive response. Adaptive immune responses can be divided into the antigen recognition phase, the induction phase, when lymphocytes are activated, and the effector phase, when parasites are eliminated (Acosta Rodríguez et al., 2019). Studies have established that both B and T cell are needed to control Chagas' disease, with effector T cells being crucial, and cytokines are vital for coordinating humoral and cellular immune responses. To recapitulate, once macrophages recognize *T. cruzi* PAMPs, they produce TNF-α, IL-12, and other cytokines. Then, IL-12 stimulates and activates DC and NK cells, which release IL-12 and IFN-γ. Together, IFN-γ and TNF-α activate macrophages, which produce nitric oxide (NO) that acts in *T. cruzi* clearance and induce inflammation. Antigen presenting cells (APCs), such as DC and macrophages, are responsible for activating B and T cells by presenting them processed *T. cruzi* antigens via a major histocompatibility complex (MHC) (Acevedo et al., 2018; Cristovão-Silva et al., 2021; Macaluso et al., 2023).

Activated B cells produce antibodies, secrete cytokines, such as IL-17 and IL-10, and present antigens to T cells, helping to activate Th1 cells. Although the mechanism of action for antibodies has not been fully elucidated yet, they play a

significant role in controlling *T. cruzi* through its opsonization followed by phagocytosis and trypomastigote lysis, through antibody-dependent cellular cytotoxicity (ADCC), and trypomastigote agglutination (Krettli and Brener, 1976; Lages-Silva et al., 1987; Lima-Martins et al., 1985). The significance of anti-*T. cruzi* antibodies was shown in mutant mice that without antibodies were not able to stop pathogen growth and led to death during the acute phase (Kumar and Tarleton, 1998). However, when parasite load is too high, antibodies are not enough to control nor eliminate the infection. Such inefficient response might be due to *T. cruzi* antigenic variability, reduction of immature B cells in the bone marrow, and activation of non-specific polyclonal B-cells (Acosta Rodriguez et al., 2007; Bermejo et al., 2011; Pitcovsky et al., 2002). Nonetheless, anti-*T. cruzi* antibodies are essential to the immune response against Chagas' disease and knowing the possible reasons why humoral response is not effective is essential when developing a vaccine. For instance, to overcome the problem caused by antigenic variability vaccines might need to use a non-excessive number of immunodominant *T. cruzi* antigens.

Activated B cells help activate and regulate effector and memory $CD4^+$ and $CD8^+$ T cells to form the response against Chagas' disease. Although some studies indicate that B cells might not be essential for T cell priming, they are vital for maintaining $CD8^+$ T cell populations (Cardillo et al., 2007; Sullivan et al., 2015). Interestingly, B cells also immunomodulate the response against *T. cruzi* by producing IL-17, even more than Th17 cells, and with the discovery that high IL-17 has been correlated with improved cardiac function in Chagas disease patients B cells significance becomes clearer (Bermejo et al., 2013; Magalhães et al., 2013).

Both $CD4^+$ and $CD8^+$ T cells are part of the immune response against *T. cruzi* infection. Activated NK cells release IL-12 and IFN-γ stimulating $CD4^+$ and $CD8^+$ T cells expansion. Such effector T cells release IFN-γ, and together with TNF, further stimulate macrophages to produce NO. Also, IFN-γ induces chemokines and recruits T cells to the tissues during the acute phase of infection. Cytotoxic T cells (CTLs) and their activities are also triggered by IFN-γ and when increased this cytokine leads to a polarization towards Th1 profile and reinforces macrophages effector activities. Moreover, effector $CD4^+$ T cells act back by

priming and stimulating B cells to proliferate and produce antibodies (Acevedo et al., 2018; Acosta Rodríguez et al., 2019; Cristovão-Silva et al., 2021).

$CD4^+$ T cells are needed during the primary response and $CD8^+$ T cell, and more specifically CTL, response in infectious diseases. However, its functions and mechanisms of action during a *T. cruzi* infection are not fully understood and are still a matter of discussion. Nonetheless, $CD4^+$ T cells unequivocally needed to promote survival in this infection. Once activated, these cells can activate, modulate or enhance the immune response against *T. cruzi*. Activated $CD4^+$ T cells can release pro-inflammatory cytokines, such as IFN-γ and TNF-α, triggering NO synthesis and cytotoxic mechanisms and activating other immune cells. Depending on the Th profile in which they differentiated after infection these cells will secrete different cytokines. IL-17 and IL-21 can be released and induce ROS production, neutrophils recruitment and activate the effector mechanisms of $CD8^+$ T cells. IL-17 and IL-22 might be released and help modulate inflammation while IL-2 release can lead to proliferation, viability maintenance, and activation of other T cells. $CD4^+$ T cells can also modulate cytotoxic responses and control inflammation by releasing TGF-β and IL-10 (Acevedo et al., 2018; Cerbán et al., 2020; Padilla et al., 2007). Furthermore, $CD4^+$ T cells seem to modulate strain-specific immunodominance patterns during primary $CD8^+$ T cell response during *T. cruzi* infection (Acosta Rodríguez et al., 2019; Padilla et al., 2007).

$CD8^+$ T cells are the most well-understood cell subset when it comes to Chagas' disease (CD) immunity. The timing of the immune response against CD differs from other infectious diseases because the first *T. cruzi* inoculum that infects the host is not enough to promote parasite-specific cell activation. Consequently, the classic rapid expansion of parasite-specific $CD8^+$ T cells is delayed in *T. cruzi* infection. It is only after the first round of *T. cruzi* replication that pathogen antigens, and possibly adjuvant molecules, accumulate in levels that can trigger APCs maturation and allow antigen presentation to naïve $CD8^+$ T cells, resulting in a slow emergence of effector $CD8^+$ T cells. Such activation kinetics have been corroborated by studies that show that nonproliferating pathogens cannot trigger protective $CD8^+$ T cell responses while increasing parasite load in the first inoculum, using a fast-replicating *T. cruzi* strains or injecting adjuvant molecules reverts this scenario (Padilla et al., 2009; Tzelepis et al., 2007). During the acute phase of CD, parasite replication and host

susceptibility are increased when $CD8^+$ T cells are depleted. Meanwhile, the expansion of effector $CD8^+$ T cells is vital to control parasite load and prevent exacerbated heart tissue inflammation during the chronic phase of CD in mice (Tarleton, 1990; Tarleton et al., 1994). These findings show that $CD8^+$ T cells are critical for survival in the acute phase and for parasite control in the chronic phase. Therefore, an effective $CD8^+$ T cell response is indispensable in a vaccine candidate, and since $CD4^+$ T cells and B cells are directly or indirectly needed to elicit such response an appropriate vaccine candidate would have to elicit these cells as well.

An important aspect to consider when developing vaccines against Chagas' disease are the similarities and differences reported for $CD8^+$ T cell immunity in mice and humans. The relevance and immunodominance profile of this response is similar between these two organisms. However, there are differences between the functionality of $CD8^+$ T cells and their differentiation status between the two species (Acosta Rodríguez et al., 2019), therefore not every result found in mice can be transposed to humans. To exemplify, CD8+ T cell responses remain functional during the chronic phase of CD in mice while in chronic Chagas' patients these cells present dysfunctional characteristics, such as diminished effector function and increased susceptibility to apoptosis, that are associated with the clinical severity of the disease (Albareda et al., 2006; Mateus et al., 2015). Interestingly, using immunodominant epitopes as vaccine candidates has been shown to induce higher protection against *T. cruzi* infection than using both immunodominant and subdominant epitopes (Eickhoff et al., 2016). Showing that understanding the similarities between the experimental model and humans can help with developing improved vaccine formulations.

As IL-17 producers, Th17 cells, a subset of $CD4^+$ T cells, play a role in *T. cruzi* infection and have been shown to increase $CD8^+$ T cell proliferation and cytokine production. However, these cells have are found in limited numbers during *T. cruzi* infection, indicating that other IL-17 producing cells are vital for maintaining $CD8^+$ T cell responses. Nonetheless, Th17 cells reduced parasite load and prevented mortality after a lethal *T. cruzi* challenge in mice, participating in both systemic and mucosal immunity against *T. cruzi*, with the latter requiring NAPDH oxidase activity to induce protection. This enzyme helps by producing reactive oxygen species (ROS) which lead to the death of intracellular parasites

(Cai et al., 2021, 2016). The IL-17A cytokine produced by these cells activates colony stimulating factors and recruits and activates neutrophils and other granulocytes (Bettelli et al., 2008).

There is a strong interplay between B and T cells to generate an efficient response against Chagas' disease. B-cell deficient mice have an incomplete $CD4^+$ and $CD8^+$ T cell expansion, with memory T cell subsets being particularly affected, and reduced Th1, Th17, and Treg populations during *T. cruzi* infection. In addition, $CD4^+$ T cell expressing inhibitory receptors are found in higher numbers in B-cell deficient mice that wild type mice infected with *T. cruzi* (Cardillo et al., 2007; Fiocca Vernengo et al., 2019; Serrán et al., 2017; Sullivan et al., 2015). *T. cruzi* trypomastigotes modulate B cells, which in turn modulate $CD4^+$ T cells altering cytokine production, arresting cell division, and inducing apoptosis (Somoza et al., 2022). Studies have shown that mice lacking functional B cells have lower levels of proinflammatory cytokines (IFN-γ and IL-12) in the spleen and produce less parasite-specific $CD8^+$ T cells, with the ones produced having a lower effector activity. B cell reduction in mice generates a $CD8^+$ T cell response with lower quality and magnitude and reduces the frequency of IL-17A producing B cells and other cells. Parasite-specific $CD8^+$ T cell from such mice have lower effector function, higher apoptosis rates, and a partially arrested cell expansion that leads to premature contraction (Cardillo et al., 2007; Sullivan et al., 2015). Since B cells are known APCs to T cells their absence directly impacts cellular response. Together the lack of humoral response and the impaired cellular response are possibly why B-cell lacking mice die during the acute phase of the disease.

Cytokines are essential for modulating the immune response against Chagas and the response profile depends on the cytokines released during infection. As mentioned, different strains and DTUs of *T. cruzi* directly affect the response generated, including the cytokines released, and influence disease progression during the acute and chronic phases. For instance, the presence of the proinflammatory cytokines IFN-γ, TNF, and IL-6 (involved in Th1 profile) are associated with resistance to *T. cruzi* while IL-4 and a Th2 profile are associated with susceptibility to the disease. Interestingly, patients with chronic Chagas' disease with cardiac and digestive symptoms have a Th1 profile, characterized by IFN-γ, TNF, IL-2, IL-6, IL-9, IL-12, and low level Th2 profile, characterized by

IL-4, IL-5, IL-10, and IL-13 (Cardillo et al., 2007; Cerbán et al., 2020; Cristovão-Silva et al., 2021).

Understanding how certain cytokines modulate disease progression and adaptive immunity, and what type of immune profile favors parasite elimination allows for better planning during vaccine development. For instance, IL-6 regulates the production of IL-1β-induced NO by the inflammasome, helping control oxidative stress and protecting mice infected with *T. cruzi*. IL-6 has also been shown to uphold the survival and effector functions of human $CD8^+$ T cell from chagasic patients (Sanmarco et al., 2016). Moreover, Chagas' patients with cardiomyopathy had higher IL-6 levels than patients in the indeterminate phase of the disease (Gómez-Olarte et al., 2019; Sousa et al., 2014). Another vital cytokine in Chagas' disease is IFN-γ, which acts as an immunomodulator in innate and adaptive response and has contrasting functions in the acute and chronic phase of the disease. More specifically, an effective response in the acute phase is only possible when IFN-γ is present in significant levels; however, in the chronic phase excessive IFN-γ can cause damage to tissues and help trigger symptoms. The same is true for TNF. For instance, Chagas' patients with cardiac symptoms present higher IFN-γ levels than patients with the indeterminate form (Chevillard et al., 2018; Gómez-Olarte et al., 2019). In corroboration, a study showed that infected patients develop dysfunctional $CD8^+$ T cells when chronically exposed to inflammatory mediators, possibly because the nitration of *T. cruzi* surface proteins leads to unresponsiveness and apoptosis of these cells (Sanmarco et al., 2016).

Another important cytokine in Chagas' disease is IL-10. Although IL-10 is an anti-inflammatory cytokine, its expression is essential to prevent a pathologic immune response during *T. cruzi* infection. A study with IL-10 deficient mice showed that these animals produce less IFN-γ and IL-2, have an impaired $CD8^+$ T cell expansion and proliferation, a higher expression of inhibitory surface receptors, such as CTLA-4, LAG-3, and PD-1, in $CD8^+$ T cells, and decreased survival (Pino-Martínez et al., 2019). This cytokine was also shown to protect mice from fatal myocarditis and reduce parasite load in the acute phase of *T. cruzi* infection, with IL-10-producing $CD8^+$ T cells shown as the main source of the cytokine (Roffê et al., 2012). In addition, trypomastigote-stimulated B cells secreted IL-6, IL-10 and IL-21, with different B cell subsets producing IL-10. The

study suggested that IL-6 and IL-10 are involved in unspecific secretion of immunoglobulins, B cell activation and proliferation while IL-21 induces IL-10 secretion by B cells via MyD88 signaling (Somoza et al., 2022). The cytokine IL-21 may also play a role in *T. cruzi* infection because parasite-specific Th17 cells act through mechanisms that are dependent on this cytokine (Cai et al., 2016). In the context of chronic Chagas disease, IL-7 and IL-15 also seem to be significant because in heart biopsies increased local production of these cytokines is associated with $CD8^+$ T cell predominance, and $CD8^+$ T cells from these heart infiltrates have improved expansion and survival when stimulated these two cytokines (Fonseca et al., 2007).

The significance of IL-17 has been demonstrated in the experimental model and in Chagas patients. In one study, IL-17RA knockout mice were shown to have inefficient pathogen control in target tissues (sleep, liver, and heart), their effector $CD8^+$ T cells had a cell exhaustion profile, and because of premature $CD8^+$ T cell contraction the animals had an unsuccessful immune response (Boari et al., 2018). These results indicated that IL-17 promotes the survival of parasite-specific effector $CD8^+$ T cells. A recent study in mice found that Th17 cells might be more protective than Th1 cells against *T. cruzi*, in both extracellular and intracellular immunity (Cai et al., 2016). Interestingly, Chagas' patients with mild cardiomyopathy have higher IL-10 and IL-17 levels while patients with severe cardiomyopathy symptoms have lower IL-17 levels (Guedes et al., 2012). Moreover, cardiac Chagas' patients have less circulating Th17 cells than patients with the indeterminate form and uninfected patients (Magalhães et al., 2013). Overall, these data show that IL-17 producing cells, such as NK cells, B cells, Th17 cells, and IL-17-secreting $CD8^+$ T cells (Bermejo et al., 2013; Bontempi et al., 2020), are involved in maintaining and enhancing parasite-specific $CD8^+$ T cell immune responses.

Altogether, these data represent how complex is the immune response against Chagas' disease. Here, it becomes clear how a balanced Th1/Th2/Th17 profile, in which pro- and anti-inflammatory cytokines and chemokines are tightly controlled, leading to parasite elimination while avoiding inflammatory and fibrotic responses from the host might be the most effective response to fight CD. The balance between an anti- and pro-inflammatory response is key to eliminating the parasite without causing extensive damage to the host. Therefore, future vaccine

candidates should focus on inducing such immune responses to lead to protection against *T. cruzi*.

2.2. *Trypanosoma cruzi* Antigens

Numerous proteins of *T. cruzi* are known to be crucial for its biological processes, particularly in facilitating the parasite's interaction with vertebrate hosts, essential for infection, survival, and proliferation. These proteins have been characterized as both immunogenic and virulence factors, identified through immunological screening of cDNA expression libraries using sera from individuals affected by Chagas disease (Engman et al., 1990). Most of these antigens have been, recently, thoroughly review by (Maldonado et al., 2022) in regards of their molecular structure and classification. For this reason, this topic will cover said antigens in regard of their similarity when compared on different parasite strains and their potential to be applied as a vaccine component.

It is important to denote, that *T. cruzi* subpopulations are considerably large on biological, genetic and biochemical heterogeneity. As an attempt to classify such diversity, the species is subdivided in six Discrete Typing Units (DTUs), TcI to TcVI (Zingales et al., 2009) and a seventh one associated with the chiroptera order, TcBat (Brenière et al., 2016). Clones and subclones belonging to the same strain, usually, present similar biological behaviour and isoenzymic patterns, confirming the stability and clonal homogeneity of the strain (Campos and Andrade, 1996). That, however, seems to hold true only on small geographic areas, with great indication of different biological and clinical properties between isolates of distant regions (Higo et al., 2000).

T. cruzi presents several mechanisms of evolution in addition to an extraordinary plasticity of its genome, making a classification for the whole genome of a strain already a herculean task. Considered an isolated sequence coding for a single protein, to keep a relationship between strain similarity and DTUs is not always possible, on the contrary, it is mostly uncommon. To verify this pattern, we went through different antigens' protein sequences available at genetic databanks for various *T. cruzi* strains, to compare sequences of same antigens. This approach allows for a quickly visualization of the shared amino acid sequence of determinate antigen among different strains.

2.2.1 Mucins

The mucin family of proteins are widely distributed antigens on the parasite surface and are very often useful for serodiagnosis. Covering the parasite in all of its developmental stages, these molecules are glycoproteins and their sugar moieties are able to interact with mammalian cells. There are many types of mucins, but of most importance are those which play a key role not only in parasite protection, but infectivity and modulation of the host immune response as well (Buscaglia et al., 2006; De Marchi et al., 2011).

T. cruzi mucins can be divided into two types: TcMUC and TcSMUG. Some TcMUC proteins are targets for the O-glycosylation pathway in *T. cruzi*, possessing a C-terminal glycophosphatidylinositol (GPI)-anchor signal (Pech-Canul et al., 2017). However, as widely available on the parasite as these proteins are, at a molecular level, they do not keep a strong similarity among the various strains of *T. cruzi*. Opposing what happens for the first mucin family member, the TcSMUG, main acceptors of sialic acid from *T. cruzi* trans-sialidases on the parasite surface, retain greater levels of similarity when strains are compared (Nakayasu et al., 2009).

A simple BLAST between available protein sequences generates a profile of similarity between various strains (Figure 1). For TcMUC I the highest, of 86%, is present among the less virulent Dm28C and Berenice strains (GenBank access: ESS62912.1; KAF5214551.1). Dm28C maintains high similarity with Sylvio (85%; GB access: EKG01015.1) and G strains (83%, GB access: RNF02673.1). Even though the sequences for each strain vary in size, it can be inferred that most of the 210 aminoacids that compound the TcMUC I protein on the Dm28C strain are present for the afore mentioned strains. The same cannot be said for the CL (GB access: RNC32051.1) and CL Brenner (GB access: XP_802175.1) strains, like many other strain interactions for this protein, who do not reach the imposed 70% similarity threshold for relevant similarity.

Data for TcMUC II is far more available, with a higher number of strains having their proteins sequenced (Figure 2). However, sequence lengths and similarities are even more variable. A few exceptions being, first, the 100% similarity between the 183 amino acids of the Y clone 6 (GB access: KAF8281566.1) strain among the 353 ones of CL Brenner (GB access: XP_803227.1) strain. Dm28C (GB access: ESS62491.1) and Brazil clone 4 (GB

access: KAF8306510.1) strains share 89% of similarity among the 192 and 191 amino acids of their respective sequences. A little lower, the shared similarity between the 192 amino acids of the TCC (GB access: PWV02240.1) strain and the 191 of Brazil clone 4 strain is of 81%. CL strain (GB access: RNC51706.1) shares over 80% similarity with Y clone 6 and CL Brener, however, only Y clone 6 strain shares a close number of amino acids within the available sequence (169 and 183, for CL and Y respectively). With such a variable length the inferred profile among strains may be far from correct.

With the exception of TCC (GB access: PWV15054.1) and G (GB access: RNF21741.1) strains, who boasts more than 180 amino acids on their sequences, most of the available strains share a close number of components regarding the TcSMUG L protein. No strain presented less than 80% similarity when compared against any other of the available ones (Figure 3), making the TcSMUG L protein the best candidate, among mucins, to consider as an antigen for vaccine formulation, while comparing their similarity alone.

2.2.2 Cruzipain

Cruzipain (Cz) based vaccines can reduce parasitemia and provide high survival rates, while preventing chronic phase-related damage (Bivona et al., 2020; Cazorla et al., 2010), making this family of proteins one of the most recurrent studied as antigens for vaccine formulations. Czs are cysteine proteases of the parasite, with genes that encode several isoforms (Campetella et al., 1992), expressed on the surface of epimastigotes and trypomastigotes (Pech-Canul et al., 2017).

These proteins are associated to host cell internalization processes, but also, once inside the macrophage, they have participative function in the parasite's escape to the cytoplasm, where the infective trypomastigotes differentiate to amastigotes and can proliferate (Santos et al., 2021). The inhibition of Cz was proved to interfere with cell invasion and intracellular *T. cruzi* proliferation (Beaulieu et al., 2010).

Even though Czs are considered a large group of proteins, the aminoacid sequences for available strains retain over 95% similarity among them. CL Brener (GB access: MZ087255.1) and CL (GB access: RNC47525.1) strains have almost identical Czs, by sharing 99.79% of their sequence. Phylogenetically, the

Y (GB access: AF314929.1) and TCC (GB access: AF265227.1) strains are the closest regarding this protein, however their similarity is one of the lowest in terms of composition (Figure 4).

2.2.3 Trans-sialidase

The other superfamily of antigens, distributed along the cell body flagellum and flagellar pocket of the parasite, are the trans-sialidase (TS) gene products (Frasch, 2000). TSs belong to one of the most extensive super gene families found in *T. cruzi*, comprising hundreds of genes that are simultaneously expressed on the parasite surface. This collective expression may create a camouflage effect against the immune response. The transfer of sialic acid from the host glycoproteins to the parasite mucins of the trypomastigote cell surface is credited to TS activity; meanwhile, neuraminidase (TCNA) activity is detected when non-suitable acceptors for sialic acid are present, and sialic acid is transferred to water (Dc-Rubin and Schenkman, 2012). This process is crucial for *T. cruzi* viability and proliferation in the host, as it is thought that TS allows assimilation of the parasite and masks them as a mammalian protein to avoid recognition and parasite lysis (Freire-De-Lima et al., 2015). This group, in thesis, is responsible for what is called the “smoke screen effect”. However, information found by us regarding the variability of these protein compositions through different *T. cruzi* strains is negligible, with GenBank presenting only two entries: one for TCNA (GB access AAA30255.1) and one for SAPA (GB access CAA40511.1).

The TS group II is composed of members of surface glycoproteins, which have been implicated in host-cell attachment and invasion, including ASP1, ASP2, TSA1, Tc85, SA85, GP82 and GP90. ASP1, ASP2 (amastigote surface proteins) and TSA1 (trypomastigote surface protein) induce strong antibody responses and are targets of *T. cruzi*-specific $CD8^+$ cytotoxic T lymphocytes. The Tc85 glycoprotein molecule (85 kDa), present in blood trypomastigotes, is a ligand with the ability to bind to different host receptor molecules located on the cell surface of monocytes, neutrophils, and fibroblasts (Maldonado et al., 2022; Pech-Canul et al., 2017). SA85 is expressed in blood trypomastigotes and amastigotes, the latter being the only form that can express the mannose-binding protein ligand, which is probably involved in the opsonization of the parasite to

enhance infection capacity (Kahn et al., 1995). Finally, GP82 and GP90 are glycoproteins, expressed mainly at the plasma membrane of metacyclic trypomastigotes (Staquicini et al., 2010). However, GP90 is also present in mammalian blood trypomastigote and amastigote forms and has an antiphagocytic effect mediated by the removal of sugar residues necessary for *T. cruzi* internalization (Pech-Canul et al., 2017).

Group II comprises most of the available molecular information for the TS superfamily, with seven antigens presenting sequences for more than one strain and four among them with information capable of being compared between strains. ASP2 and TSA1 are especially worth mention, as all available strains data present over 70% and 80% similarity, respectively (Figure 5). Y (GB access AAO84044.1), Tulahen (GB access ABQ53591.1) and Colombian CL 22 (GB access ABQ53589.1) strains share 95% of their amino acid composition for the ASP2 protein, while keeping great proximity phylogenetically. Dm28C (GB access EF583446.1) strain shares the lowest percentage among available strains for comparison, with 80% against the CL Brener strain (GB access ABQ53588.1) and less than75% against all others.

Even though sample sizes vary over 100-200 amino acids, strains are far more similar for the TSA-1 antigen. With less than 10 amino acids differing their size, CL (GB access RNC44606.1) and CL Brener (GB access AID66712.1) strains share 93% similarity among their sequences. However, phylogenetically, this gene does not stand close for these strains; with CL being closer to Y Clone 6 (GB access KAF8296649.1) strain, and CL Brener closer to Peru (GB access AAA30259.1) strain (Figure 6). Dm28C (GB access PWU85144) and Sylvio X10 (GB access AAA18827.1) strains on the other hand share the second highest sequence similarity (98%) and the closest phylogenetically among samples, with only 30 amino acids differing their size.

The TS group III comprises several surface glycoproteins present in mammalian trypomastigotes, like CRP, CEA, TESA, and FL160, recognized by sera from chagasic patients. They are all able to inhibit the classical and alternative pathway of complement activation, which could work as a protection mechanism from host complement lysis of the parasite trypomastigote form (Beucher and Norris, 2008). The TS IV group is composed of genes that encode trypomastigote surface antigens, whose function is unknown yet.

2.2.4 Paraflagellar Rod Proteins

The PFRs (paraflagellar rod proteins) are a family specific to kinetoplastids and present at the *T. cruzi* flagellum. These proteins are structurally and immunologically distinct from any of the major filamentous systems of the host cell, including microfilaments, microtubules, or intermediate filaments (Saborio et al., 1989). The PFRs can be divided into four already described members, PARs 1 to 4, expressed through all different stages of the parasite (Fouts et al., 1998).

PFRs have a well-established role in flagellar motility (Portman and Gull, 2010), and is very likely to integrate and transmit to the cell body or to the axoneme, the external signals captured by the flagellum. The modifications of the phosphorylation status of flagellar protein may be involved in a hypothetical signal mediation by the flagellum when interacting with host cell extracellular matrix. However, the role of the phosphorylation/dephosphorylation cycle of paraflagellar rod proteins is yet unknown (Mattos et al., 2012), leaving the choice of composing a vaccine with it as an antigen much to the fact of its quick presentation to the host immune system.

A choice well rewarded, either by the use of the protein purified from epimastigotes (Wrightsman et al., 1995) or recombinant PFRs (Luhrs et al., 2003; Wrightsman and Manning, 2000), allowing vaccine projects to be capable of inducing 100% survival and significant reduction in parasitemia levels in immunized animals with an otherwise lethal challenge. A protection associated with a highly polarized type 1 cytokine production profile (Luhrs et al., 2003).

Based on the cds evaluated on this paper, PFR-2 does not keep a homogenous profile throughout the data available for strains of *T. cruzi*. While CL (GB access: RNC34307.1), Berenice (GB access: KAF5214366.1) and TCC (GB access: PWU97142.1) strains share over 99% of their amino acid composition for PFR-2, Brazil Clone 4 (GB access: KAF8276174.1) strain does not reach 60% similarity with any of the found sequences (Figure 7). The same cannot be said for PFR-3, which share >99% similarity between different strains (Figure 8), with all available sequences, with the exception of G strain (GB access: RNF16495.1), presenting the same size.

2.2.5 Tc52

Another set of intriguing antigens is produced by genes present in low numbers, Tc52, a protein known for its high conservation (Figure 9) and glutathione S-transferase activity, which possesses immunomodulatory properties crucial for parasite viability. Deletion of both alleles of Tc52 proves to be lethal for the kinetoplastid (Allaoui et al., 1999). This antigen is expressed across all stages of the parasite's development, with peak expression observed in epimastigotes and amastigotes (Bivona et al., 2020).

Tc52 presents very few differences between strains, with all available data presenting over 93% similarity at amino acid composition. However, phylogenetically, a result was unexpected: The Y strain (GB access: AAG08957.1) and its clone 7 (GB access: AAO63161.1) are the most distant sequences among the evaluated ones. Which can be explained either by the applied analytical procedure of utilizing only the Tc52 cds for comparison and not the whole genome of each strain, or perhaps, by a specific mutation of this gene derivate from the numerous cloning processes. Further studies are necessary to clarify this phylogenetic distance.

Immunization using Tc52 provided substantial protection against challenges from *T. cruzi*. Specifically, the N-terminal domain of Tc52 conferred superior protection compared to its C-terminal domain or the entire protein during both acute and chronic infection phases (Matos et al., 2016, 2014). Anti-Tc52 antibodies can induce trypomastigote lysis by complement activation, making them effective neutralizing antibodies and a promising candidate for combating *T. cruzi* infections (Bivona et al., 2020).

2.2.6 Tc80

Tc80, a prolyl oligopeptidase, has the capability to degrade host cell extracellular matrix components such as collagen and fibronectin, which is believed to facilitate invasion of host cells by the parasite (Santana et al., 1997). Vaccines based on Tc80 have demonstrated effectiveness in reducing parasite burden during both acute and chronic phases of Chagas disease, thereby improving survival rates in mice and preventing complications associated with the chronic phase. In this study, immunization with Tc80, combined with an adjuvant, induced a robust immune response involving both humoral and cell-mediated components. The humoral response resulted in the production of antibodies that

presented enzyme inhibition properties, neutralized *T. cruzi* infectivity, and facilitated complement-mediated lysis of trypomastigotes (Bivona et al., 2018).

These vaccines are further encouraged by Tc80 high amino acid sequence similarity kept by many *T. cruzi* strains. Most of the available strains data presents over 95% similarity (Figure 10), the only exception being the TCC strain sequence (GB access: PWV08312.1), which stays between 75% and 90% similarity when compared with other strains. One possible explanation for this high similarity, kept among different strains, is that Tc80 is expressed by all stages of the parasite (Bivona et al., 2020), being a conserved gene of high importance, even among different DTUs.

2.2.7 Tc24

The Tc24 antigen, a 24 kDa protein, is encoded by multiple gene copies arranged in tandem arrays and expressed in all developmental stages of *T. cruzi* (Gunter et al., 2016). This antigen is located on the cell membrane, at the flagellar pocket of the parasite, withholding many calcium-binding domains (Arnal et al., 2020). It is a highly conserved protein among *T. cruzi* strains, with no available sequence in data banks presenting similarity lower than 96%. The proximity is kept even phylogenetically, with CL (GB access: RNC34385.1) and CL Brener (GB access: XP_805575.1), much like in the Cruzipain case, being the only isolated strains (Figure 11).

Vaccines encoding and/or expressing Tc24 antigen can prevent Chagas disease progression both in murine, canine and macaque models (Arnal et al., 2020; Dumonteil et al., 2020). The administration of recombinant Tc24 protein can decrease parasitaemia and cardiac parasite burden in immunized animals compared to controls, making it another promising candidate for a vaccine against *T. cruzi.*

2.3. Bacteria Vector-Based Vaccines

Bacterial-based carrier vaccines can be defined by the concept of an attenuated strain of bacteria, genetically engineered to be safe yet still immunogenic, which can be further modified by the introduction of additional genes encoding variable antigens from pathogens of different species to kingdoms; the resulting product should then be capable of eliciting biologically

relevant protective immune responses against both the carrier vaccine itself as well as the additional target pathogens. Although simple in principle and elegant in terms of vaccine development, this novel approach has required refinement through various iterations over the last decades.

Utilizing bacteria as vectors ensures a reliable and efficient delivery of antigens, potentially leading to more enduring and effective immunity than conventional vaccines. A strategy that can improve the fight against Chagas disease by provoking a strong and specific immune response, which also have the capacity to induce both humoral and cellular immune responses, a necessary response when fighting intracellular pathogens like *T. cruzi*. Additionally, bacterial vectors can be modified to present multiple antigens at once, providing wider protection against different parasite strains.

Over the next subtopics, we will swiftly review the bacteria of choice for vectorizing vaccines against Chagas disease on past and recent years. We look to share why these bacteria are selected, how it can improve immune response against this parasite, what are the strong points of each strategy and the efficacy of each vaccine design attempt.

2.3.1 Salmonella

Live recombinant attenuated Salmonella-vectored vaccines show great promise for enhancing human health by providing long-lasting mucosal, humoral, and cellular immunity against a variety of non-Salmonella pathogens at a low cost. The approach is relatively straightforward. Protective antigen genes from a pathogen are cloned and expressed in attenuated *Salmonella enterica*. The recombinant Salmonella strain expressing the heterologous gene can then be administered orally to induce an immune response against the pathogen from which the heterologous gene was derived. While strains of other bacteria such as Escherichia coli, Listeria, Shigella, and Vibrio have been and continue to be evaluated as vaccines, the invasive nature of Salmonella makes it particularly effective at eliciting B and T cell memory responses, giving Salmonella the greatest potential to induce long-lasting immunity.

The human-restricted *Salmonella enterica* serovar Typhi is the Salmonella serovar of choice for human vaccines. As the causative agent of typhoid fever, S. Typhi is fully capable of invading human mucosal tissues and entering systemic

sites, ultimately targeting the host immune system to stimulate strong mucosal, humoral and cellular responses. Although many other pathogenic serovars of Salmonella, such as *Salmonella enterica* serovar Typhimurium, cause disease in humans, they have not been pursued as actively as S. Typhi (Galen et al., 2021).

The first report, found by us, for the usage of *S. enterica* as a carrier system for a *T. cruzi* antigen was published in 2002, where BALB/c mice were vaccinated four times intranasally with recombinant attenuated *S. enterica* serovar Typhimurium expressing cruzipain. Highly significant decreases in recoverable *T. cruzi* DNA and viable parasites were detected in vaccinated mice compared with unvaccinated and vaccinated control mice (Schnapp et al., 2002). However, the authors presented only immunologic data for mice vaccinated with isolated recombinant cruzipain, without clarifying if the vector contributed on cellular and humoral responses. Answers for the doubts left by the previous author soon arrived. Cazorla et al. (2008) orally immunized C3H/HeN mice with four doses of *Salmonella* carrying cruzipain gene. Results presented a strong mucosal response in the form of cruzipain specific sIgA and T cells in gut-associated lymphoid tissue. When compared with a heterologous strategy of *Salmonella* carrying cruzipain as prime and recombinant cruzipain + CpG as boost however, serum IgG levels, splenocyte proliferation and IFN-γ secretion were far weaker. Contrary to what was expected, even with weaker systemic responses, the vectored vaccine was able to better control the infection in challenge with *T. cruzi*, with greater reduction in parasitemia levels and chronic tissue damage.

Another antigen whose efficiency was evaluated under the *Salmonella* vector strategy is the Tc52. Constructions with plasmids encoding the full-length gene, its N-terminal and C-terminal domains were reported, with Salmonella carrying the N-terminal presenting better protection among the three groups (Matos et al., 2014). For this vaccine attempt, C3H/HeN mice orally immunized four times presented an immune response with activation of sIgA. $CD4^+$ Th1 cells and IFN-γ plus cytotoxic effect of $CD8^+$ T cells, with splenocytes secreting IL-10, which could down-regulate $CD8^+$ activity and/or prevent tissue damage. Against a lethal infection, the entire group vaccinated with the N-terminal construct survived through the whole period of evaluation. Protection was also elicited during the chronic phase of infection, with lower tissue damage reported on vaccinated animals.

Shortly after, Salmonella carrying the N-terminal portion of Tc52 was tested in a prime-boost strategy, with two oral doses followed by two intradermal doses of the recombinant N-terminal Tc52 protein adjuvanted with CpG-ODN. The immunized animals developed a predominant Th1 cellular immune response and strong humoral responses, as well as specific mucosal IgA, thus conferring a good protection in the acute and chronic stages of infection. This strategy was proven to be more efficient in answering the infection when compared with a single type vaccine, be it the plasmid encoding the gene or the recombinant protein (Matos et al., 2016).

Among vaccine formulations in research, the Tc80 is not a commonly applied antigen; however, an immunization protocol with *Salmonella* carrying Tc80 gene has proved itself quite efficient. Be it applied in solo, or in a prime/protein boost regime, the survival of infected mice was superior when immunized with the prokaryotic DNA delivery system protocol to with the subunit vaccine alone. Greater effective in this case could be linked to the ability of the Salmonella carrying Tc80 gene to induce polyfunctional $CD4^+$ IFN-γ^+ TFN-σ^+ T cells, which present high levels of cytokine production (Bivona et al., 2018).

The immunization with *Salmonella* carrying different antigens, co-administrated, was also evaluated. (Cazorla et al., (2015) tested the efficiency of 3 *Salmonella* carrying the plasmids that encode the antigens Cz, Tc52, and Tc24. SCz+STc52+STc24–immunized mice presented an increased antibody response against each antigen compared with those in the single antigen–immunized groups, as well as higher trypomastigotes antibody-mediated lyses and cell invasion inhibition compared with controls. The multi-antigen group also presented lower parasitaemia, no weight loss after infection and almost no abnormalities in muscle tissues. Is worth noting that even though the titer of antibodies was modest, which is expected for a DNA vaccine, they were able to induce significant complement-mediated killing of trypomastigotes in vitro and were also able to inhibit the invasion of trypomastigotes in mammalian Vero cells.

More recently, another coadministration protocol was tested. A DNA vaccine combining cruzipain and chagasin, which is the natural cruzipain inhibitor. The bicomponent vaccine based on *Salmonella* carrying cruzipain and chagasin was able to improve the protection obtained by each antigen as monocomponent therapeutic vaccine and significantly increased the titers of

antigen- and parasite-specific antibodies. More importantly, the bicomponent vaccine triggered a robust cellular response with IFN-γ secretion that rapidly reduced the parasitaemia during the acute phase and decreased the tissue damage in the chronic stage of the infection, suggesting it could be an effective tool to ameliorate the pathology associated to Chagas disease (Cerny et al., 2020).

2.3.2 BCG

Being a live attenuated vaccine that is widely used to prevent tuberculosis, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is uniquely suited to exert this function, as it is stable, safe, easy to apply, cost effective and presents strong adjuvant properties. Additionally, it replicates in macrophages and dendritic cells and, therefore, can effectively present antigens to the host immune system, conferring long-term immunity (Oliveira et al., 2019).

Several heterologous antigens have already been successfully expressed in recombinant BCG (rBCG) under the control of different promoters. The strength of promoters may affect the levels of antigen expression and strain stability in vivo (Oliveira et al., 2019). Antigens from parasites, bacteria and viruses that are expressed in BCG have been shown to induce humoral and cellular immune responses in several animals, including mice and hamsters (Bunde et al., 2023; Streit et al., 2000). When expressed in rBCG, the *Leishmania chagasi* LCR1 antigen induces the production of IFN-γ and protects against virulent *L. chagasi* challenge in mice (Streit et al., 2000). rBCG expressing the *T. gondii* GRA1 antigen induces specific cell-mediated responses and results in significant protection in sheep (Supply et al., 1999).

Surprisingly, the BCG platform was just recently applied to express *T. cruzi* antigens (Bontempi et al., 2020). The effects of BCG against CD however, are known for almost forty years. Although the initial immunization attempts resulted in failure, (Abrahamsohn et al., 1981) successfully reduced mortality of C57BL mice, previously vaccinated with BCG, against a lethal *T. cruzi* infection. Furthermore, decreased parasitemia during the acute phase and lower heart lesions for BCG vaccinated animals were described by (Bertelli et al., 1981). Decades later, it was described that individuals who have been vaccinated with BCG presented lower levels of antibodies induced by *T. cruzi*, including those

with pathological role such as anti-p2β and anti-B13 which cross react to host antigens (Peverengo et al., 2016; Vicco et al., 2014). This may be consequence of “trained immunity”, which confer a long-term non-specific immune memory of innate immunity improving its capacity to respond upon reinfections.

To our knowledge, Bontempi et al. (2020) presents the only vaccine formulation attempt utilising recombinant BCG against CD. In this work, different rBCG vaccines expressing fragments of the trans-sialidase and cruzipain antigens were constructed and evaluated. The C-terminal fraction of the trans-sialidase protein, not including the shed acute-phase antigen (SAPA) region, was expressed for one formulation, while the N-terminal fragment was expressed for another. Regarding cruzipain, only its N-terminal section was evaluated. Each gene fragment was inserted in two different plasmids: pUS977 and pUS2000, resulting in six vaccine prototypes tested by the authors. The antibody response was low but significant for all clones with respect to the controls. The humoral response obtained was not strong, but in accordance with what was expected for rBCG expressing foreign antigens, with low IgG response. However, the strong DTH response obtained, indicates a cellular activation compatible with BCG response. When mice were challenged with a 50% lethal dose of the parasite, pUS2000 tended to be more protective than pUS977, especially when combined with the N-terminal portion of trans-sialidase as antigen. In this same group of vaccinated mice, the frequency of IFN-γ produced by CD8+CD107+ T cells was increased after trans-sialidase stimulation, suggesting that this vaccine candidate favors the priming and activation of CD8 lymphocytes in a polyfunctional way. A mixed Th17 and Th1 profile was obtained by this protocol, a noted and desired characteristic by the BCG vaccine already reported in mice (Da Costa et al., 2014) and humans (Loxton et al., 2017), as Th17 response profile is critical for protection against *T. cruzi* (Matos et al., 2017).

2.3.3 Lactococcus lactis

Lactococcus lactis is one of the most frequently used microorganisms in the food industry across the world (De Vos, 2011; Smid and Kleerebezem, 2014). Moreover, recent reports that use *L. lactis* as a therapeutic agent for the treatment of different human and animal diseases have stimulated the interest of this microorganism by the pharmaceutical industry. The potential biotechnological

applications of this microorganism in the pharmaceutical drug production and the spectrum of possibilities it offers constitutes nowadays one of the most striking reasons for the investigation on *L. lactis* genetic manipulation (Cano-Garrido et al., 2015). In particular, the use of *L. lactis* as a live non-invasive mucosal vaccine seems a promising alternative due to their GRAS (Generally Recognized As Safe) status (Cano-Garrido et al., 2015; Kim et al., 2015; Mancha-Agresti et al., 2017).

L. lactis has been successfully employed to produce specific viral and bacterial antigens to cope infections or non-antigenic immunomodulatory proteins like cytokines or proteases to control infections or more complex inflammatory diseases such as the inflammatory bowel disease (Cano-Garrido et al., 2015; Kim et al., 2015; Mancha-Agresti et al., 2017; Marelli et al., 2011; Wells and Mercenier, 2008). Most importantly, it has been used for the expression and delivery of heterologous antigens to develop oral and mucosal vaccines (Cano-Garrido et al., 2015; Wells and Mercenier, 2008).

Recently, a vaccine prototype was developed focused on a *Lactococcus lactis* that can simultaneously produce a fragment of trans-sialidase and overproduce c-di-AMP, a previously applied adjuvant for CD vaccines on research (Quintana et al., 2018). This prototype was put to test in an immunization assay to evaluate its efficiency against a mucosal infection with *T. cruzi*. Alongside it, another vaccine formulation, very similar, but producing an ISCOMATRIX-like adjuvant (ISPA) instead of c-di-AMP, was evaluated. Even though both ISPA and c-di-AMP groups induced humoral and cellular responses, the formulation producing c-di-AMP was significantly more efficient at fighting the infection. Specific secretory IgA was more enhanced in c-di-AMP group and parasitemia control was only achieved by animals vaccinated with it, despite all vaccinated groups showing enhanced $CD8^{+}IFN\text{-}\gamma^{+}$ T cell numbers. Furthermore, during the acute phase, a significant reduction of tissue parasite load, clinical manifestations and tissue damage was observed. The better prophylactic capacity elicited by the c-di-AMP group was most likely related to the induction of neutralizing plasma antibodies and augmented levels of mucosal IgA, since both groups displayed similar immunogenicity and $CD8^{+}IFN\text{-}\gamma^{+}$ T-cell response (Pacini et al., 2022).

3. Conclusions

Recent advances in the research of vaccines vectorized by bacteria have shown promise in the fight against Chagas disease, one of the infectious diseases in which despite all efforts, does not possess a successful treatment. These bacterial vector vaccines leverage genetically modified bacteria to deliver antigens that stimulate an immune response against *T. cruzi*. This method can enhance the combat against Chagas disease by eliciting a robust and targeted immune response, potentially reducing the incidence and severity of infections. The use of bacteria as vectors allows for the stable and efficient delivery of antigens, which can result in a more sustained and effective immunity compared to traditional vaccines. Furthermore, bacterial vectors can be engineered to express multiple antigens simultaneously, offering broader protection against various strains of the parasite.

The strategy of using bacterial vector vaccines is favoured over other vaccine types due to several key advantages. Unlike live-attenuated or inactivated vaccines, which may pose safety concerns or require complex production processes, bacterial vector vaccines can be produced relatively easily and safely. They also have the capacity to induce both humoral and cellular immune responses, crucial for combating intracellular pathogens like *T. cruzi*. Additionally, bacterial vectors can be designed to mimic natural infections, thereby providing a more comprehensive immune response. Future research should continue to pursue this promising avenue, focusing on optimizing vector design, enhancing antigen expression, and conducting extensive trials to ensure safety and efficacy. Advancing this innovative vaccine strategy, aligned with a thoroughly thought antigen strategy, could significantly contribute to control Chagas disease.

Author Contributions

Conceptualization: Sibele Borsuk, Ivan Marcipar and Odir Dellagostin.; Investigation: Guilherme Senna dos Santos, Bárbara da Rocha Fonseca and Sibele Borsuk; Writing—Original Draft Preparation: Guilherme Senna dos Santos; Writing—Review and Editing: Guilherme Senna dos Santos, Bárbara

da Rocha Fonseca, Mirna Samara Dié Alves, Sibele Borsuk, Ivan Marcipar and Odir Dellagostin; Visualization: Sibele Borsuk and Ivan Marcipar. All authors have read and agreed to the published version of the manuscript.

References

Abrahamsohn, I.A., Blotta, M.H.S.L., Curotto, M.A., 1981. Enhancement of delayed-type hypersensitivity to *Trypanosoma cruzi* in mice treated with *Mycobacterium bovis* BCG and cyclophosphamide. Infect Immun 31, 1145–1151. https://doi.org/10.1128/IAI.31.3.1145-1151.1981

Acevedo, G.R., Girard, M.C., Gómez, K.A., 2018. The unsolved jigsaw puzzle of the immune response in Chagas disease. Front Immunol 9, 1929. https://doi.org/10.3389/fimmu.2018.01929

Acosta Rodríguez, E. V., Araujo Furlan, C.L., Fiocca Vernengo, F., Montes, C.L., Gruppi, A., 2019. Understanding CD8+ T cell immunity to *Trypanosoma cruzi* and how to improve it. Trends Parasitol 35, 899–917. https://doi.org/10.1016/j.pt.2019.08.006

Acosta Rodriguez, E. V., Zuniga, E.I., Montes, C.L., Merino, M.C., Bermejo, D.A., Amezcua Vesely, M.C., Motran, C.C., Gruppi, A., 2007. *Trypanosoma cruzi* infection beats the B-cell compartment favouring parasite establishment: Can we strike first? Scand J Immunol 66, 137–142. https://doi.org/10.1111/J.1365-3083.2007.01968.X

Albareda, M.C., Laucella, S.A., Alvarez, M.G., Armenti, A.H., Bertochi, G., Tarleton, R.L., Postan, M., 2006. *Trypanosoma cruzi* modulates the profile of memory CD8+ T cells in chronic Chagas' disease patients. Int Immunol 18, 465–471. https://doi.org/10.1093/INTIMM/DXH387

Allaoui, A., François, C., Zemzoumi, K., Guilvard, E., Ouaissi, A., 2002. Intracellular growth and metacyclogenesis defects in *Trypanosoma cruzi* carrying a targeted deletion of a Tc52 protein-encoding allele. Mol Microbiol 32, 1273–1286. https://doi.org/10.1046/j.1365-2958.1999.01440.x

Allaoui, A., François, C., Zemzoumi, K., Guilvard, E., Ouaissi, A., 1999. Intracellular growth and metacyclogenesis defects in *Trypanosoma cruzi* carrying a targeted deletion of a Tc52 protein-encoding allele. Mol Microbiol 32, 1273–1286. https://doi.org/10.1046/J.1365-2958.1999.01440.X

Amaral, M.P., Cardoso, F.D., de Farias, I.S., de Souza, R.Q., Matteucci, K.C., Torrecilhas, A.C., Bortoluci, K.R., 2023. NAIP/NLRC4 inflammasome participates in macrophage responses to *Trypanosoma cruzi* by a mechanism that relies on cathepsin-dependent caspase-1 cleavage. Front Immunol 14. https://doi.org/10.3389/fimmu.2023.1282856

Antinori, S., Galimberti, L., Bianco, R., Grande, R., Galli, M., Corbellino, M., 2017. Chagas disease in Europe: A review for the internist in the globalized world. Eur J Intern Med 43, 6–15. https://doi.org/10.1016/J.EJIM.2017.05.001

Arnal, A., Villanueva-Lizama, L., Teh-Poot, C., Herrera, C., Dumonteil, E., 2020. Extent of polymorphism and selection pressure on the *Trypanosoma cruzi* vaccine candidate antigen Tc24. Evol Appl 13, 2663–2672. https://doi.org/10.1111/EVA.13068

Bafica, A., Santiago, H.C., Goldszmid, R., Ropert, C., Gazzinelli, R.T., Sher, A., 2006. Cutting Edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. The Journal of Immunology 177, 3515–3519. https://doi.org/10.4049/jimmunol.177.6.3515

Batalla, E.I., Pino Martínez, A.M., Poncini, C. V., Duffy, T., Schijman, A.G., González Cappa, S.M., Soto, C.D.A., 2013. Impairment in natural killer cells editing of immature dendritic cells by infection with a virulent *Trypanosoma cruzi* population. J Innate Immun 5, 494–504. https://doi.org/10.1159/000350242

Beaulieu, C., Isabel, E., Fortier, A., Massé, F., Mellon, C., Méthot, N., Ndao, M., Nicoll-Griffith, D., Lee, D., Park, H., Black, W.C., 2010. Identification of potent and reversible cruzipain inhibitors for the treatment of Chagas disease. Bioorg Med Chem Lett 20, 7444–7449. https://doi.org/10.1016/J.BMCL.2010.10.015

Bermejo, D.A., Amezcua Vesely, M.C., Khan, M., Acosta Rodríguez, E. V., Montes, C.L., Amezcua Vesely, M.C., Toellner, K.M., Mohr, E., Taylor, D., Cunningham, A.F., Gruppi, A., 2011. *Trypanosoma cruzi* infection induces a massive extrafollicular and follicular splenic B-cell response which is a high source of non-parasite-specific antibodies. Immunology 132, 123–133. https://doi.org/10.1111/J.1365-2567.2010.03347.X

Bermejo, D.A., Jackson, S.W., Gorosito-Serran, M., Acosta-Rodriguez, E. V., Amezcua Vesely, M.C., Sather, B.D., Singh, A.K., Khim, S., Mucci, J., Liggitt, D., Campetella, O., Oukka, M., Gruppi, A., Rawlings, D.J., 2013. *Trypanosoma cruzi* trans-sialidase initiates a program independent of the transcription factors RORγt and Ahr that leads to IL-17 production by activated B cells. Nature Immunology 2013 14:5 14, 514–522. https://doi.org/10.1038/ni.2569

Bertelli, M.S., Alcantara, A., Brener, Z., 1981. BCG-induced resistance in *Trypanosoma cruzi* experimental infections. Tropenmed Parasitol 32, 93–96.

Bettelli, E., Korn, T., Oukka, M., Kuchroo, V.K., 2008. Induction and effector functions of TH17 cells. Nature 2008 453:7198 453, 1051–1057. https://doi.org/10.1038/nature07036

Beucher, M., Norris, K.A., 2008. Sequence diversity of the *Trypanosoma cruzi* complement regulatory protein family. Infect Immun 76, 750–758. https://doi.org/10.1128/IAI.01104-07

Bivona, A.E., Alberti, A.S., Cerny, N., Trinitario, S.N., Malchiodi, E.L., 2020. Chagas disease vaccine design: the search for an efficient *Trypanosoma cruzi* immune-mediated control. Biochim Biophys Acta Mol Basis Dis 1866. https://doi.org/10.1016/j.bbadis.2019.165658

Bivona, A.E., Sánchez Alberti, A., Matos, M.N., Cerny, N., Cardoso, A.C., Morales, C., González, G., Cazorla, S.I., Malchiodi, E.L., 2018. *Trypanosoma cruzi* 80 kDa prolyl oligopeptidase (Tc80) as a novel immunogen for Chagas disease vaccine. PLoS Negl Trop Dis 12, e0006384. https://doi.org/10.1371/JOURNAL.PNTD.0006384

Boari, J.T., Araujo Furlan, C.L., Vernengo, F.F., Rodriguez, C., Ramello, M.C., Amezcua Vesely, M.C., Serrán, M.G., Nuñez, N.G., Richer, W., Piaggio, E., Montes, C.L., Gruppi, A., Acosta Rodríguez, E. V., 2018. IL-17RA-signaling modulates CD8+ T Cell survival and exhaustion during *Trypanosoma cruzi* infection. Front Immunol 9, 403363. https://doi.org/10.3389/FIMMU.2018.02347

Bontempi, I., Leal, K., Prochetto, E., Díaz, G., Cabrera, G., Bortolotti, A., Morbidoni, H.R., Borsuk, S., Dellagostin, O., Marcipar, I., 2020. Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypansoma cruzi* infection. Clin Exp Immunol 201, 306–316. https://doi.org/10.1111/cei.13469

Brenière, S.F., Waleckx, E., Barnabé, C., 2016. Over six thousand *Trypanosoma cruzi* strains classified into Discrete Typing Units (DTUs): Attempt at an inventory. PLoS Negl Trop Dis 10. https://doi.org/10.1371/JOURNAL.PNTD.0004792

Bunde, T.T., de Oliveira, N.R., Santos, F.D.S., Pedra, A.C.K., Maia, M.A.C., Dellagostin, O.A., Oliveira Bohn, T.L., 2023. Characterization of cellular immune response in hamsters immunized with recombinant vaccines against leptospirosis based on LipL32:LemA:LigAni chimeric protein. Microb Pathog 184, 106378. https://doi.org/10.1016/J.MICPATH.2023.106378

Buscaglia, C.A., Campo, V.A., Frasch, A.C.C., Di Noia, J.M., 2006. *Trypanosoma cruzi* surface mucins: Host-dependent coat diversity. Nature Reviews Microbiology 2006 4:3 4, 229–236. https://doi.org/10.1038/nrmicro1351

Caetano, B.C., Carmo, B.B., Melo, M.B., Cerny, A., dos Santos, S.L., Bartholomeu, D.C., Golenbock, D.T., Gazzinelli, R.T., 2011. Requirement of UNC93B1 reveals a critical role for TLR7 in host resistance to primary infection with *Trypanosoma cruzi*. The Journal of Immunology 187, 1903–1911. https://doi.org/10.4049/jimmunol.1003911

Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F., 2016. Th17 Cells are more protective than Th1 cells against the intracellular parasite *Trypanosoma cruzi*. PLoS Pathog 12, e1005902. https://doi.org/10.1371/JOURNAL.PPAT.1005902

Cai, C.W., Eickhoff, C.S., Meza, K.A., Blase, J.R., Audette, R.E., Chan, D.H., Bockerstett, K.A., DiPaolo, R.J., Hoft, D.F., 2021. Th17 cells provide mucosal protection against gastric *Trypanosoma cruzi* infection. Infect Immun 89. https://doi.org/10.1128/IAI.00738-20

Campetella, O., Henriksson, J., Åslund, U., Frasch, A.C.C., Pettersson, U., Cazzulo, J.J., 1992. The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic tandemly organized genes located on different chromosomes. Mol Biochem Parasitol 50, 225–234. https://doi.org/10.1016/0166-6851(92)90219-A

Campos, R.M.F., Andrade, S.G., 1996. Characterization of subpopulations (clones and subclones) of the 21 SF strain of *Trypanosoma cruzi* after long lasting maintenance in the laboratory. Mem Inst Oswaldo Cruz 91, 794–800. https://doi.org/10.1590/S0074-02761996000600029

Cano-Garrido, O., Seras-Franzoso, J., Garcia-Fruitós, E., 2015. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microbial Cell Factories 2015 14:1 14, 1–12. https://doi.org/10.1186/S12934-015-0313-6

Caputo, M.B., Elias, J., Cesar, G., Alvarez, M.G., Laucella, S.A., Albareda, M.C., 2022. Role of the complement system in the modulation of T-cell responses in chronic Chagas disease. Front Cell Infect Microbiol 12. https://doi.org/10.3389/fcimb.2022.910854

Cardillo, F., De Pinho, R.T., Antas, P.R.Z., Mengel, J., 2015. Immunity and immune modulation in *Trypanosoma cruzi* infection. Pathog Dis 73. https://doi.org/10.1093/femspd/ftv082

Cardillo, F., Postol, E., Nihei, J., Aroeira, L.S., Nomizo, A., Mengel, J., 2007. B cells modulate T cells so as to favour T helper type 1 and CD8 + T-cell responses in the acute phase of *Trypanosoma cruzi* infection. Immunology 122, 584–595. https://doi.org/10.1111/j.1365-2567.2007.02677.x

Castro, J.T., Brito, R., Hojo-Souza, N.S., Azevedo, B., Salazar, N., Ferreira, C.P., Junqueira, C., Fernandes, A.P., Vasconcellos, R., Cardoso, J.M., Aguiar-Soares, R.D.O., Vieira, P.M.A., Carneiro, C.M., Valiate, B., Toledo, C., Salazar, A.M., Caballero, O., Lannes-Vieira, J., Teixeira, S.R., Reis, A.B., Gazzinelli, R.T., 2023. ASP-2/Trans-sialidase chimeric protein induces robust protective immunity in experimental models of Chagas' disease. npj Vaccines 2023 8:1 8, 1–14. https://doi.org/10.1038/s41541-023-00676-0

Cazorla, S.I., Becker, P.D., Frank, F.M., Ebensen, T., Sartori, M.J., Corral, R.S., Malchiodi, E.L., Guzmán, C.A., 2008. Oral vaccination with *Salmonella enterica* as a cruzipain-DNA delivery system confers protective immunity

against *Trypanosoma cruzi*. Infect Immun 76, 324–33. https://doi.org/10.1128/IAI.01163-07

Cazorla, S.I., Frank, F.M., Becker, P.D., Arnaiz, M., Mirkin, G.A., Corral, R.S., Guzmán, C.A., Malchiodi, E.L., 2010. Redirection of the immune response to the functional catalytic domain of the cystein proteinase cruzipain improves protective immunity against *Trypanosoma cruzi* infection. J Infect Dis 202, 136–144. https://doi.org/10.1086/652872

Cazorla, S.I., Matos, M.N., Cerny, N., Ramirez, C., Alberti, A.S., Bivona, A.E., Morales, C., Guzman, C.A., Malchiodi, E.L., 2015. Oral Multicomponent DNA Vaccine Delivered by Attenuated Salmonella Elicited Immunoprotection Against American Trypanosomiasis. Journal of Infectious Diseases 211, 698–707. https://doi.org/10.1093/infdis/jiu480

Cerbán, F.M., Stempin, C.C., Volpini, X., Carrera Silva, E.A., Gea, S., Motran, C.C., 2020. Signaling pathways that regulate *Trypanosoma cruzi* infection and immune response. Biochim Biophys Acta Mol Basis Dis 1866. https://doi.org/10.1016/j.bbadis.2020.165707

Cerny, N., Bivona, A.E., Sanchez Alberti, A., Trinitario, S.N., Morales, C., Cardoso Landaburu, A., Cazorla, S.I., Malchiodi, E.L., 2020. Cruzipain and Its Physiological Inhibitor, Chagasin, as a DNA-Based Therapeutic Vaccine Against *Trypanosoma cruzi*. Front Immunol 11, 565142. https://doi.org/10.3389/FIMMU.2020.565142

Cestari, I., Ansa-Addo, E., Deolindo, P., Inal, J.M., Ramirez, M.I., 2012. *Trypanosoma cruzi* immune evasion mediated by host cell-derived microvesicles. The Journal of Immunology 188, 1942–1952. https://doi.org/10.4049/jimmunol.1102053

Cestari, I. dos S., Krarup, A., Sim, R.B., Inal, J.M., Ramirez, M.I., 2009. Role of early lectin pathway activation in the complement-mediated killing of *Trypanosoma cruzi*. Mol Immunol 47, 426–437. https://doi.org/10.1016/j.molimm.2009.08.030

Cestari, I.D.S., Evans-Osses, I., Freitas, J.C., Inal, J.M., Ramirez, M.I., 2008. Complement C2 receptor inhibitor trispanning confers an increased ability to resist complement-mediated lysis in *Trypanosoma cruzi*. Journal of Infectious Diseases 198, 1276–1283. https://doi.org/10.1086/592167

Chevillard, C., Nunes, J.P.S., Frade, A.F., Almeida, R.R., Pandey, R.P., Nascimento, M.S., Kalil, J., Cunha-Neto, E., 2018. Disease tolerance and pathogen resistance genes may underlie *Trypanosoma cruzi* persistence and differential progression to Chagas disease cardiomyopathy. Front Immunol 9, 411639. https://doi.org/10.3389/FIMMU.2018.02791

Cristovão-Silva, A.C., Brelaz-de-Castro, M.C.A., Hernandes, M.Z., Pereira, V.R.A., 2021. Chagas disease: Immunology of the disease at a glance. Cytokine Growth Factor Rev 62, 15–22. https://doi.org/10.1016/j.cytogfr.2021.10.001

Da Costa, A.C., Costa, A.D.O., De Oliveira, F.M., Nogueira, S.V., Rosa, J.D., Resende, D.P., Kipnis, A., Junqueira-Kipnis, A.P., 2014. A new recombinant BCG vaccine induces specific Th17 and Th1 effector cells with higher protective efficacy against tuberculosis. PLoS One 9, e112848. https://doi.org/10.1371/JOURNAL.PONE.0112848

Dc-Rubin, S.S.C., Schenkman, S., 2012. *Trypanosoma cruzi* trans-sialidase as a multifunctional enzyme in Chagas' disease. Cell Microbiol 14, 1522–1530. https://doi.org/10.1111/J.1462-5822.2012.01831.X

de la Cruz, J.J., Villanueva-Lizama, L., Dzul-Huchim, V., Ramírez-Sierra, M.J., Martinez-Vega, P., Rosado-Vallado, M., Ortega-Lopez, J., Flores-Pucheta, C.I., Gillespie, P., Zhan, B., Bottazzi, M.E., Hotez, P.J., Dumonteil, E., 2019. Production of recombinant TSA-1 and evaluation of its potential for the immuno-therapeutic control of *Trypanosoma cruzi* infection in mice. Hum Vaccin Immunother 15, 210–219. https://doi.org/10.1080/21645515.2018.1520581

De Marchi, C.R., Di Noia, J.M., Frasch, A.C.C., Neto, V.A., Almeid, I.C., Buscaglia, C.A., 2011. Evaluation of a recombinant *Trypanosoma cruzi* mucin-like antigen for serodiagnosis of Chagas' disease. Clinical and Vaccine Immunology 18, 1850–1855. https://doi.org/10.1128/CVI.05289-11

de Oliveira, A.P., Ayo, C.M., Bestetti, R.B., Brandão de Mattos, C.C., Cavasini, C.E., de Mattos, L.C., 2016. The role of CCR5 in Chagas disease - a systematic review. Infection, Genetics and Evolution 45, 132–137. https://doi.org/10.1016/j.meegid.2016.08.012

De Vos, W.M., 2011. Systems solutions by lactic acid bacteria: From paradigms to practice. Microb Cell Fact 10, 1–13. https://doi.org/10.1186/1475-2859-10-S1-S2

Dumonteil, E., Bottazzi, M.E., Zhan, B., Heffernan, M.J., Jones, K., Valenzuela, J.G., Kamhawi, S., Ortega, J., De Leon Rosales, S.P., Lee, B.Y., Bacon, K.M., Fleischer, B., Slingsby, B.T., Cravioto, M.B., Tapia-Conyer, R., Hotez, P.J., 2012. Accelerating the development of a therapeutic vaccine for human Chagas disease: Rationale and prospects. Expert Rev Vaccines 11, 1043–1055. https://doi.org/10.1586/erv.12.85

Dumonteil, E., Herrera, C., Buekens, P., 2019. A therapeutic preconceptional vaccine against Chagas disease: A novel indication that could reduce congenital transmission and accelerate vaccine development. PLoS Negl Trop Dis 13. https://doi.org/10.1371/journal.pntd.0006985

Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P.A., Ortega-Lopez, J., Hotez, P.J., Bottazzi, M.E., 2020. Safety and immunogenicity of a recombinant vaccine against *Trypanosoma cruzi* in Rhesus macaques. Vaccine 38, 4584–4591. https://doi.org/10.1016/J.VACCINE.2020.05.010

Eickhoff, C.S., Zhang, X., Vasconcelos, J.R., Motz, R.G., Sullivan, N.L., O'Shea, K., Pozzi, N., Gohara, D.W., Blase, J.R., Di Cera, E., Hoft, D.F., 2016. Costimulatory effects of an immunodominant parasite antigen paradoxically prevent induction of optimal CD8 T cell protective immunity. PLoS Pathog 12, e1005896. https://doi.org/10.1371/JOURNAL.PPAT.1005896

Engman, D.M., Dragon, E.A., Donelson, J.E., 1990. Human humoral immunity to hsp70 during *Trypanosoma cruzi* infection. J Immunol 144, 3987–91.

Ersching, J., Basso, A.S., Kalich, V.L.G., Bortoluci, K.R., Rodrigues, M.M., 2016. A human trypanosome suppresses CD8+ T cell priming by dendritic cells through the induction of immune regulatory CD4+ Foxp3+ T cells. PLoS Pathog 12. https://doi.org/10.1371/journal.ppat.1005698

Ferreira, V., Valck, C., Sánchez, G., Gingras, A., Tzima, S., Carmen Molina, M., Sim, R., Schwaeble, W., Ferreira, A., 2004. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi* 1. The Journal of Immunology 172, 3042–3050.

Fiocca Vernengo, F., Beccaria, C.G., Araujo Furlan, C.L., Tosello, J., Almada, L., Gorosito Serrán, M., Gazzoni, Y., Montes, C.L., Acosta Rodríguez, V., Gruppi, A., Tosello Boari, J., TransImm, S., 2019. CD8+ T cell immunity is compromised by anti-CD20 treatment and rescued by IL-17A. bioRxiv 642801. https://doi.org/10.1101/642801

Fitzgerald, K.A., Kagan, J.C., 2020. Toll-like receptors and the control of immunity. Cell 180, 1044–1066. https://doi.org/10.1016/j.cell.2020.02.041

Fonseca, B. de P., Albuquerque, P.C., Zicker, F., 2020. Neglected tropical diseases in Brazil: lack of correlation between disease burden, research funding and output. Tropical Medicine & International Health 25, 1373–1384. https://doi.org/10.1111/TMI.13478

Fonseca, S.G., Reis, M.M., Coelho, V., Nogueira, L.G., Monteiro, S.M., Mairena, E.C., Bacal, F., Bocchi, E., Guilherme, L., Zheng, X.X., Liew, F.Y., Higuchi, M.L., Kalil, J., Cunha-Neto, E., 2007. Locally Produced Survival Cytokines IL-15 and IL-7 may be Associated to the Predominance of CD8+ T cells at Heart Lesions of Human Chronic Chagas Disease Cardiomyopathy. Scand J Immunol 66, 362–371. https://doi.org/10.1111/J.1365-3083.2007.01987.X

Fouts, D.L., Stryker, G.A., Gorski, K.S., Miller, M.J., Nguyen, T. V., Wrightsman, R.A., Manning, J.E., 1998. Evidence for four distinct major protein components in the paraflagellar rod of *Trypanosoma cruzi*. Journal of Biological Chemistry 273, 21846–21855. https://doi.org/10.1074/JBC.273.34.21846

Frasch, A.C.C., 2000. Functional diversity in the trans-sialidase and mucin families in *Trypanosoma cruzi*. Parasitology Today 16, 282–286. https://doi.org/10.1016/S0169-4758(00)01698-7

Freire-De-Lima, L., Fonseca, L.M., Oeltmann, T., Mendonca̧-Previato, L., Previato, J.O., 2015. The trans-sialidase, the major *Trypanosoma cruzi* virulence factor: Three decades of studies. Glycobiology 25, 1142–1149. https://doi.org/10.1093/GLYCOB/CWV057

Galen, J.E., Wahid, R., Buskirk, A.D., 2021. Strategies for enhancement of live-attenuated Salmonella-based carrier vaccine immunogenicity. Vaccines 2021, Vol. 9, Page 162 9, 162. https://doi.org/10.3390/VACCINES9020162

Gómez-Olarte, S., Bolaños, N.I., Echeverry, M., Rodríguez, A.N., Cuéllar, A., Puerta, C.J., Mariño, A., González, J.M., 2019. Intermediate monocytes and cytokine production associated with severe forms of Chagas disease. Front Immunol 10, 1671. https://doi.org/10.3389/FIMMU.2019.01671

Guarner, J., 2019. Chagas disease as example of a reemerging parasite. Semin Diagn Pathol 36, 164–169. https://doi.org/10.1053/J.SEMDP.2019.04.008

Guedes, P.M.M., Gutierrez, F.R.S., Silva, G.K., Dellalibera-Joviliano, R., Rodrigues, G.J., Bendhack, L.M., Rassi, A., Rassi, A., Schmidt, A., Maciel, B.C., Marin Neto, J.A., Silva, J.S., 2012. Deficient Regulatory T Cell Activity and Low Frequency of IL-17-Producing T Cells Correlate with the Extent of Cardiomyopathy in Human Chagas' Disease. PLoS Negl Trop Dis 6, e1630. https://doi.org/10.1371/JOURNAL.PNTD.0001630

Gunter, S.M., Jones, K.M., Zhan, B., Essigmann, H.T., Murray, K.O., Garcia, M.N., Gorchakov, R., Bottazzi, M.E., Hotez, P.J., Brown, E.L., 2016. Identification and characterization of the *Trypanosoma cruzi* B-cell superantigen Tc24. Am J Trop Med Hyg 94, 114–121. https://doi.org/10.4269/AJTMH.15-0438

Gupta, S., Salgado-Jiménez, B., Lokugamage, N., Vázquez-Chagoyán, J.C., Garg, N.J., 2019. TcG2/TcG4 DNA vaccine induces Th1 immunity against acute *Trypanosoma cruzi* infection: Adjuvant and antigenic effects of heterologous T. rangeli booster immunization. Front Immunol 10, 458314. https://doi.org/10.3389/FIMMU.2019.01456

Higo, H., Yanagi, T., Matta, V., Agatsuma, T., Cruz-Reyes, A., Uyema, N., Monroy, C., Kanbara, H., Tada, I., 2000. Genetic structure of *Trypanosoma cruzi* in American continents: special emphasis on sexual reproduction in Central America. Parasitology 121, 403–408. https://doi.org/10.1017/S0031182099006514

Jiménez, P., Jaimes, J., Poveda, C., Ramírez, J.D., 2019. A systematic review of the *Trypanosoma cruzi* genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. Parasitology 146, 269–283. https://doi.org/10.1017/S0031182018001506

Kahn, S., Wleklinski, M., Aruffo, A., Farr, A., Coder, D., Kahn, M., 1995. *Trypanosoma cruzi* amastigote adhesion to macrophages is facilitated by

the mannose receptor. Journal of Experimental Medicine 182, 1243–1258. https://doi.org/10.1084/JEM.182.5.1243

Kayama, H., Takeda, K., 2010. The innate immune response to *Trypanosoma cruzi* infection. Microbes Infect 12, 511–517. https://doi.org/10.1016/j.micinf.2010.03.005

Kim, J.I., Park, T.E., Maharjan, S., Li, H.S., Lee, H. Bin, Kim, I.S., Piao, D., Lee, J.Y., Cho, C.S., Bok, J.D., Hong, Z.S., Kang, S.K., Choi, Y.J., 2015. Soluble RANKL expression in *Lactococcus lactis* and investigation of its potential as an oral vaccine adjuvant. BMC Immunol 16, 1–11. https://doi.org/10.1186/S12865-015-0132-X

Krettli, A.U., Brener, Z., 1976. Protective effects of specific antibodies in *Trypanosoma cruzi* infections. J Immunol 116, 755–60.

Kumar, S., Tarleton, R.L., 1998. The relative contribution of antibody production and CD8+ T cell function to immune control of *Trypanosoma cruzi*. Parasite Immunol 20, 207–216. https://doi.org/10.1046/J.1365-3024.1998.00154.X

Lages-Silva, E., Ramirez, L.E., Krettli, A.U., Brener, Z., 1987. Effect of protective and non-protective antibodies in the phagocytosis rate of *Trypanosoma cruzi* blood forms by mouse peritoneal macrophages. Parasite Immunol 9, 21–30. https://doi.org/10.1111/J.1365-3024.1987.TB00485.X

Lee, B.Y., Bacon, K.M., Connor, D.L., Willig, A.M., Bailey, R.R., 2010. The potential economic value of a *Trypanosoma cruzi* (Chagas disease) vaccine in Latin America. PLoS Negl Trop Dis 4, 1–8. https://doi.org/10.1371/journal.pntd.0000916

Lidani, K.C.F., Bavia, L., Ambrosio, A.R., de Messias-Reason, I.J., 2017. The complement system: A prey of *Trypanosoma cruzi*. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.00607

Lieke, T., Graefe, S.E.B., Klauenberg, U., Fleischer, B., Jacobs, T., 2004. NK cells contribute to the control of *Trypanosoma cruzi* infection by killing free parasites by perforin-independent mechanisms. Infect Immun 72, 6817–6825. https://doi.org/10.1128/IAI.72.12.6817-6825.2004

Lima-Martins, M.V.C., Sanchez, G.A., Krettli, A.U., Brener, Z., 1985. Antibody-dependent cell cytotoxicity against *Trypanosoma cruzi* is only mediated by protective antibodies. Parasite Immunol 7, 367–376. https://doi.org/10.1111/J.1365-3024.1985.TB00083.X

Loxton, A.G., Knaul, J.K., Grode, L., Gutschmidt, A., Meller, C., Eisele, B., Johnstone, H., Van Der Spuy, G., Maertzdorf, J., Kaufmann, S.H.E., Hesseling, A.C., Walzl, G., Cotton, M.F., 2017. Safety and immunogenicity of the recombinant *Mycobacterium bovis* BCG vaccine VPM1002 in HIV-unexposed newborn infants in South Africa. Clin Vaccine Immunol 24. https://doi.org/10.1128/CVI.00439-16

Luhrs, K.A., Fouts, D.L., Manning, J.E., 2003. Immunization with recombinant paraflagellar rod protein induces protective immunity against *Trypanosoma cruzi* infection. Vaccine 21, 3058–3069. https://doi.org/10.1016/S0264-410X(03)00108-7

Macaluso, G., Grippi, F., Di Bella, S., Blanda, V., Gucciardi, F., Torina, A., Guercio, A., Cannella, V., 2023. A Review on the immunological response against *Trypanosoma cruzi*. Pathogens 12. https://doi.org/10.3390/pathogens12020282

Magalhães, L.M.D., Villani, F.N.A., Nunes, M. do C.P., Gollob, K.J., Rocha, M.O.C., Dutra, W.O., 2013. High interleukin 17 expression is correlated with better cardiac function in human Chagas disease. J Infect Dis 207, 661–665. https://doi.org/10.1093/INFDIS/JIS724

Maldonado, E., Morales-Pison, S., Urbina, F., Solari, A., 2022. Vaccine design against Chagas disease focused on the use of nucleic acids. Vaccines 2022, Vol. 10, Page 587 10, 587. https://doi.org/10.3390/VACCINES10040587

Mancha-Agresti, P., de Castro, C.P., dos Santos, J.S.C., Araujo, M.A., Pereira, V.B., LeBlanc, J.G., Leclercq, S.Y., Azevedo, V., 2017. Recombinant invasive *Lactococcus lactis* carrying a DNA vaccine coding the Ag85A antigen increases INF-γ, IL-6, and TNF-α cytokines after intranasal immunization. Front Microbiol 8, 268017. https://doi.org/10.3389/FMICB.2017.01263

Marelli, B., Perez, A.R., Banchio, C., de Mendoza, D., Magni, C., 2011. Oral immunization with live *Lactococcus lactis* expressing rotavirus VP8* subunit induces specific immune response in mice. J Virol Methods 175, 28–37. https://doi.org/10.1016/J.JVIROMET.2011.04.011

Martins-Melo, F.R., Carneiro, M., Ramos, A.N., Heukelbach, J., Ribeiro, A.L.P., Werneck, G.L., 2018. The burden of Neglected Tropical Diseases in Brazil, 1990-2016: A subnational analysis from the Global Burden of Disease Study 2016. PLoS Negl Trop Dis 12, e0006559. https://doi.org/10.1371/JOURNAL.PNTD.0006559

Mateus, J., Lasso, P., Pavia, P., Rosas, F., Roa, N., Valencia-Hernández, C.A., González, J.M., Puerta, C.J., Cuéllar, A., 2015. Low frequency of circulating CD8+ T stem cell memory cells in chronic chagasic patients with severe forms of the disease. PLoS Negl Trop Dis 9, e3432. https://doi.org/10.1371/JOURNAL.PNTD.0003432

Matos, M.N., Cazorla, S.I., Bivona, A.E., Morales, C., Guzma´n, C.A., Malchiodi, E.L., 2014. Tc52 amino-terminal-domain DNA carried by attenuated *Salmonella enterica* serovar typhimurium induces protection against a *Trypanosoma cruzi* lethal challenge. Infect Immun 82, 4265–4275. https://doi.org/10.1128/IAI.02190-14

Matos, M.N., Cazorla, S.I., Schulze, K., Ebensen, T., Guzmán, C.A., Malchiodi, E.L., 2017. Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-AMP induces Th17+Th1 specific immune responses and confers protection against Trypanosoma cruzi. PLoS Negl Trop Dis 11. https://doi.org/10.1371/journal.pntd.0005300

Matos, M.N., Sánchez Alberti, A., Morales, C., Cazorla, S.I., Malchiodi, E.L., 2016. A prime-boost immunization with Tc52 N-terminal domain DNA and the recombinant protein expressed in Pichia pastoris protects against *Trypanosoma cruzi* infection. Vaccine 34, 3243–3251. https://doi.org/10.1016/j.vaccine.2016.05.011

Mattos, E.C., Schumacher, R.I., Colli, W., Alves, M.J.M., 2012. Adhesion of *Trypanosoma cruzi* trypomastigotes to fibronectin or laminin modifies tubulin and paraflagellar Rod protein phosphorylation. PLoS One 7. https://doi.org/10.1371/JOURNAL.PONE.0046767

Murphy, K., Weaver, C., 2017. Janeway's Immunobiology, 9th ed. Garland Science, New York.

Nakayasu, E.S., Yashunsky, D. V., Nohara, L.L., Torrecilhas, A.C.T., Nikolaev, A. V., Almeida, I.C., 2009. GPIomics: Global analysis of glycosylphosphatidylinositol-anchored molecules of *Trypanosoma cruzi*. Mol Syst Biol 5. https://doi.org/10.1038/MSB.2009.13

Norris, K.A., 1998. Stable transfection of *Trypanosoma cruzi* epimastigotes with the trypomastigote-specific complement regulatory protein cDNA confers complement resistance. Infect Immun 66, 2460–2465.

Oliveira, A.-C., Peixoto, J.R., de Arruda, L.B., Campos, M.A., Gazzinelli, R.T., Golenbock, D.T., Akira, S., Previato, J.O., Mendonça-Previato, L., Nobrega, A., Bellio, M., 2004. Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. The Journal of Immunology 173, 5688–5696. https://doi.org/10.4049/jimmunol.173.9.5688

Oliveira, T.L., Rizzi, C., da Cunha, C.E.P., Dorneles, J., Seixas Neto, A.C.P., Amaral, M.G., Hartwig, D.D., Dellagostin, O.A., 2019. Recombinant BCG strains expressing chimeric proteins derived from Leptospira protect hamsters against leptospirosis. Vaccine 37, 776–782. https://doi.org/10.1016/J.VACCINE.2018.12.050

Pacini, M.F., González, F.B., Dinatale, B., Bulfoni Balbi, C., Villar, S.R., Farré, C., Lupi, G., Espariz, M., Blancato, V.S., Magni, C., Marcipar, I., Pérez, A.R., 2022. Nasal immunization with a *L. lactis*-derived trans-sialidase antigen plus c-di-AMP protects against acute oral *T. cruzi* infection. Vaccine 40, 2311–2323. https://doi.org/10.1016/J.VACCINE.2022.02.071

Padilla, A., Xu, D., Martin, D., Tarleton, R., 2007. Limited role for CD4+ T-cell help in the initial priming of *Trypanosoma cruzi*-specific CD8+ T cells. Infect Immun 75, 231–235. https://doi.org/10.1128/IAI.01245-06

Padilla, A.M., Simpson, L.J., Tarleton, R.L., 2009. Insufficient TLR activation contributes to the slow development of CD8+ T cell responses in *Trypanosoma cruzi* infection. The Journal of Immunology 183, 1245–1252. https://doi.org/10.4049/JIMMUNOL.0901178

Pech-Canul, Á.D.L.C., Monteón, V., Solís-Oviedo, R.L., 2017. A Brief view of the surface membrane proteins from *Trypanosoma cruzi*. J Parasitol Res 2017, 3751403. https://doi.org/10.1155/2017/3751403

Pérez-Molina, J.A., Molina, I., 2018. Chagas disease. The Lancet 391, 82–94. https://doi.org/10.1016/S0140-6736(17)31612-4

Peverengo, L., Prochetto, E., Rodeles, L., Valenzuela, I., Marcipar, I.S., Bottasso, O., Vicco, M.H., 2016. Antibody profiles induced by *Trypanosoma cruzi* in chagasic patients with previous or current exposure to mycobacteria. Pathog Dis 74, 109. https://doi.org/10.1093/FEMSPD/FTW109

Pino-Martínez, A.M., Miranda, C.G., Batalla, E.I., González-Cappa, S.M., Alba Soto, C.D., 2019. IL-10 participates in the expansion and functional activation of CD8+ T cells during acute infection with *Trypanosoma cruzi*. J Leukoc Biol 105, 163–175. https://doi.org/10.1002/JLB.3A0318-111RR

Pitcovsky, T.A., Buscaglia, C.A., Mucci, J., Campetella, O., 2002. A functional network of intramolecular cross-reacting epitopes delays the elicitation of neutralizing antibodies to *Trypanosoma cruzi* trans-sialidase. J Infect Dis 186, 397–404. https://doi.org/10.1086/341463

Portman, N., Gull, K., 2010. The paraflagellar rod of kinetoplastid parasites: From structure to components and function. Int J Parasitol 40, 135. https://doi.org/10.1016/J.IJPARA.2009.10.005

Quintana, I., Espariz, M., Villar, S.R., González, F.B., Pacini, M.F., Cabrera, G., Bontempi, I., Prochetto, E., Stülke, J., Perez, A.R., Marcipar, I., Blancato, V., Magni, C., 2018. Genetic engineering of *Lactococcus lactis* co-producing antigen and the mucosal adjuvant 3’ 5’- cyclic di Adenosine Monophosphate (c-di-AMP) as a design strategy to develop a mucosal vaccine prototype. Front Microbiol 9, 410602. https://doi.org/10.3389/FMICB.2018.02100

Ramírez-Toloza, G., Aguilar-Guzmán, L., Valck, C., Ferreira, V.P., Ferreira, A., 2020. The interactions of parasite calreticulin with initial complement components: Consequences in immunity and virulence. Front Immunol 11, 1561. https://doi.org/10.3389/fimmu.2020.01561

Rios, L.E., Vázquez-Chagoyán, J.C., Pacheco, A.O., Zago, M.P., Garg, N.J., 2019. Immunity and vaccine development efforts against *Trypanosoma cruzi*. Acta Trop 200. https://doi.org/10.1016/j.actatropica.2019.105168

Roffê, E., Rothfuchs, A.G., Santiago, H.C., Paula, A., Marino, M.P., Ribeiro-Gomes, F.L., Eckhaus, M., Lis, Ⅱ, Antonelli, R. V, Murphy, P.M., 2012. IL-10 limits parasite burden and protects against fatal myocarditis in a mouse

model of *Trypanosoma cruzi* infection. The Journal of Immunology 188, 649–660. https://doi.org/10.4049/JIMMUNOL.1003845

Saborio, J.L., Hernandez, J.M., Narayanswami, S., Wrightsman, R., Palmer, E., Manning, J., 1989. Isolation and characterization of paraflagellar proteins from *Trypanosoma cruzi*. Journal of Biological Chemistry 264, 4071–4075. https://doi.org/10.1016/S0021-9258(19)84963-3

Sanchez Alberti, A., Bivona, A.E., Matos, M.N., Cerny, N., Schulze, K., Weißmann, S., Ebensen, T., González, G., Morales, C., Cardoso, A.C., Cazorla, S.I., Guzmán, C.A., Malchiodi, E.L., 2020. Mucosal Heterologous Prime/Boost Vaccination Induces Polyfunctional Systemic Immunity, Improving Protection Against *Trypanosoma cruzi*. Front Immunol 11, 487548. https://doi.org/10.3389/FIMMU.2020.00128

Sanmarco, L.M., Visconti, L.M., Eberhardt, N., Ramello, M.C., Ponce, N.E., Spitale, N.B., Vozza, M.L., Bernardi, G.A., Gea, S., Minguez, A.R., Aoki, M.P., 2016. IL-6 improves the nitric oxide-induced cytotoxic CD8+ T cell dysfunction in human Chagas disease. Front Immunol 7, 219809. https://doi.org/10.3389/FIMMU.2016.00626

Santana, J.M., Grellier, P., Schrével, J., Teixeira, A.R.L., 1997. A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. Biochemical Journal 325, 129–137. https://doi.org/10.1042/BJ3250129

Santos, V.C., Oliveira, A.E.R., Campos, A.C.B., Reis-Cunha, J.L., Bartholomeu, D.C., Teixeira, S.M.R., Lima, A.P.C.A., Ferreira, R.S., 2021. The gene repertoire of the main cysteine protease of *Trypanosoma cruzi*, cruzipain, reveals four sub-types with distinct active sites. Scientific Reports 2021 11:1 11, 1–14. https://doi.org/10.1038/s41598-021-97490-2

Schnapp, A.R., Eickhoff, C.S., Sizemore, D., Curtiss, R., Hoft, D.F., 2002. Cruzipain induces both mucosal and systemic protection against *Trypanosoma cruzi* in mice. Infect Immun 70, 5065–5074. https://doi.org/10.1128/IAI.70.9.5065-5074.2002

Sepulveda, P., Hontebeyrie, M., Liegeard, P., Mascilli, A., Norris, K.A., 2000. DNA-based immunization with *Trypanosoma cruzi* complement regulatory protein elicits complement lytic antibodies and confers protection against Trypanosoma cruzi infection. Infect Immun 68, 4986–4991.

Serrán, M.G., Boari, J.T., Vernengo, F.F., Beccaría, C.G., Ramello, M.C., Bermejo, D.A., Cook, A.G., Vinuesa, C.G., Montes, C.L., Rodriguez, E.V.A., Gruppi, A., 2017. Unconventional pro-inflammatory CD4+ T cell response in B cell-deficient mice infected with *Trypanosoma cruzi*. Front Immunol 8, 289338. https://doi.org/10.3389/FIMMU.2017.01548

Silva, G.K., Costa, R.S., Silveira, T.N., Caetano, B.C., Horta, C.V., Gutierrez, F.R.S., Guedes, P.M. da M., Andrade, W.A., De Niz, M., Gazzinelli, R.T., Zamboni, D.S., Silva, J.S., 2013. Apoptosis-associated Speck–like protein

containing a caspase recruitment domain inflammasomes mediate IL-1β response and host resistance to *Trypanosoma cruzi* infection. The Journal of Immunology 191, 3373–3383. https://doi.org/10.4049/jimmunol.1203293

Silva, G.K., Gutierrez, F.R.S., Guedes, P.M.M., Horta, C. V., Cunha, L.D., Mineo, T.W.P., Santiago-Silva, J., Kobayashi, K.S., Flavell, R.A., Silva, J.S., Zamboni, D.S., 2010. Cutting edge: Nucleotide-binding oligomerization domain 1-dependent responses account for murine resistance against *Trypanosoma cruzi* infection. The Journal of Immunology 184, 1148–1152. https://doi.org/10.4049/jimmunol.0902254

Smid, E.J., Kleerebezem, M., 2014. Production of aroma compounds in lactic fermentations. Annu Rev Food Sci Technol 5, 313–326. https://doi.org/10.1146/ANNUREV-FOOD-030713-092339/CITE/REFWORKS

Somoza, M., Bertelli, A., Pratto, C.A., Verdun, R.E., Campetella, O., Mucci, J., 2022. *Trypanosoma cruzi* induces B cells that regulate the CD4+ T cell response. Front Cell Infect Microbiol 11. https://doi.org/10.3389/fcimb.2021.789373

Sosoniuk, E., Vallejos, G., Kenawy, H., Gaboriaud, C., Thielens, N., Fujita, T., Schwaeble, W., Ferreira, A., Valck, C., 2014. *Trypanosoma cruzi* calreticulin inhibits the complement lectin pathway activation by direct interaction with L-Ficolin. Mol Immunol 60, 80–85. https://doi.org/10.1016/j.molimm.2014.03.014

Sousa, G.R., Gomes, J.A.S., Fares, R.C.G., Damásio, M.P.D.S., Chaves, A.T., Ferreira, K.S., Nunes, M.C.P., Medeiros, N.I., Valente, V.A.A., Corrêa-Oliveira, R., Rocha, M.O.D.C., 2014. Plasma cytokine expression is associated with cardiac morbidity in Chagas disease. PLoS One 9, e87082. https://doi.org/10.1371/JOURNAL.PONE.0087082

Staquicini, D.I., Martins, R.M., Macedo, S., Sasso, G.R.S., Atayde, V.D., Juliano, M.A., Yoshida, N., 2010. Role of GP82 in the selective binding to gastric mucin during oral infection with *Trypanosoma cruzi*. PLoS Negl Trop Dis 4. https://doi.org/10.1371/JOURNAL.PNTD.0000613

Streit, J.A., Recker, T.J., Donelson, J.E., Wilson, M.E., 2000. BCG expressing LCR1 of *Leishmania chagasi* induces protective immunity in susceptible mice. Exp Parasitol 94, 33–41. https://doi.org/10.1006/EXPR.1999.4459

Sullivan, N.L., Eickhoff, C.S., Sagartz, J., Hoft, D.F., 2015. Deficiency of antigen-specific B cells results in decreased *Trypanosoma cruzi* systemic but not mucosal immunity due to CD8 T cell exhaustion. The Journal of Immunology 194, 1806–1818. https://doi.org/10.4049/JIMMUNOL.1303163

Supply, P., Sutton, P., Coughlan, S.N., Bilo, K., Saman, E., Trees, A.J., Cesbron Delauw, M.F., Locht, C., 1999. Immunogenicity of recombinant BCG producing the GRA1 antigen from *Toxoplasma gondii*. Vaccine 17, 705–714. https://doi.org/10.1016/S0264-410X(98)00255-2

Tarleton, R.L., 1990. Depletion of CD8+ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. J Immunol 144, 717–24.

Tarleton, R.L., Sun, J., Zhang, L., Postan, M., 1994. Depletion of T-cell subpopulations results in exacerbation of myocarditis and parasitism in experimental Chagas' disease. Infect Immun 62, 1820. https://doi.org/10.1128/IAI.62.5.1820-1829.1994

Tzelepis, F., Persechini, P.M., Rodrigues, M.M., 2007. Modulation of CD4+ T cell-dependent specific cytotoxic CD8+ T cells differentiation and proliferation by the timing of increase in the pathogen load. PLoS One 2, e393. https://doi.org/10.1371/JOURNAL.PONE.0000393

Van Overtvelt, L., Andrieu, M., Rie Verhasselt, V.Â., Connan, F., Choppin, J., Vercruysse, V., Goldman, M., Hosmalin, A., Vray, B., 2002. *Trypanosoma cruzi* down-regulates lipopolysaccharide-induced MHC class I on human dendritic cells and impairs antigen presentation to speci®c CD8 + T lymphocytes. Int Immunol 14, 1135–1144.

Vekemans, J., Hasso-Agopsowicz, M., Kang, G., Hausdorff, W.P., Fiore, A., Tayler, E., Klemm, E.J., Laxminarayan, R., Srikantiah, P., Friede, M., Lipsitch, M., 2021. Leveraging vaccines to reduce antibiotic use and prevent antimicrobial resistance: A World Health Organization action framework. Clinical Infectious Diseases 73, e1011–e1017. https://doi.org/10.1093/CID/CIAB062

Vicco, M.H., Bontempi, I.A., Rodeles, L., Yodice, A., Marcipar, I.S., Bottasso, O., 2014. Decreased level of antibodies and cardiac involvement in patients with chronic Chagas heart disease vaccinated with BCG. Med Microbiol Immunol 203, 133–139. https://doi.org/10.1007/S00430-013-0326-X/TABLES/4

Wells, J.M., Mercenier, A., 2008. Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nature Reviews Microbiology 2008 6:5 6, 349–362. https://doi.org/10.1038/nrmicro1840

Williams, T., Guerrero-Ros, I., Ma, Y., Dos Santos, F.M., Scherer, P.E., Gordillo, R., Horta, A., Macian, F., Weiss, L.M., Huang, H., 2020. Induction of effective immunity against *Trypanosoma cruzi*. Infect Immun 88. https://doi.org/10.1128/IAI.00908-19

Wrightsman, R.A., Manning, J.E., 2000. Paraflagellar rod proteins administered with alum and IL-12 or recombinant adenovirus expressing IL-12 generates antigen-specific responses and protective immunity in mice against *Trypanosoma cruzi*. Vaccine 18, 1419–1427. https://doi.org/10.1016/S0264-410X(99)00380-1

Wrightsman, R.A., Miller, M.J., Saborio, J.L., Manning, J.E., 1995. Pure paraflagellar rod protein protects mice against *Trypanosoma cruzi* infection. Infect Immun 63, 122. https://doi.org/10.1128/IAI.63.1.122-125.1995

Yeung, C., Mendoza, I., Echeverria, L.E., Baranchuk, A., 2021. Chagas' cardiomyopathy and Lyme carditis: Lessons learned from two infectious diseases affecting the heart. Trends Cardiovasc Med 31, 233–239. https://doi.org/10.1016/J.TCM.2020.04.004

Zanluqui, N.G., 2015. Macrophage polarization in Chagas disease. J Clin Cell Immunol 06. https://doi.org/10.4172/2155-9899.1000317

Zingales, B., Andrade, S.G., Briones, M.R.S., Campbell, D.A., Chiari, E., Fernandes, O., Guhl, F., Lages-Silva, E., Macedo, A.M., Machado, C.R., Miles, M.A., Romanha, A.J., Sturm, N.R., Tibayrenc, M., Schijman, A.G., 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz 104, 1051–1054. https://doi.org/10.1590/S0074-02762009000700021

Figures

A

Percent similarity

	CL Brener	CL	Sylvio X10/1	G	Berenice	Dm28C
CL Brener		59.74	54.12	51.46	47.14	53.51
CL	40.26		64.07	54.17	62.59	63.75
Sylvio X10/1	45.88	35.93		73.77	61.08	85.14
G	48.54	45.83	26.23		74.17	82.79
Berenice	52.86	37.41	38.92	25.83		86.69
Dm28C	46.49	36.25	14.86	17.21	13.31	

Percent dissimilarity

B

Figure 1: A: comparison of amino acid similarity for TcMUC I protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the TcMUC I protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table I and Figure 1 of the supplementary material.

A

Percent similarity

	CL Brener	Y Clone 6	Brazil Clone A4	TCC	Berenice	CL	Dm28C	Sylvio X10/1
CL Brener		100.00	74.16	75.28	77.24	82.29	71.99	74.10
Y Clone 6	0.00		74.16	75.28	17.32	17.29	18.93	18.97
Brazil Clone A4	25.84	25.84		83.25	12.30	13.87	14.62	14.77
TCC	24.72	24.72	16.75		12.83	13.77	14.53	13.07
Berenice	22.76	82.68	87.70	87.17		55.76	56.60	66.52
CL	17.71	82.71	86.13	86.23	44.24		75.16	63.86
Dm28C	28.01	81.07	85.38	85.47	43.40	24.84		74.09
Sylvio X10/1	25.90	81.03	85.23	86.93	33.48	36.14	25.91	

Percent dissimilarity

B

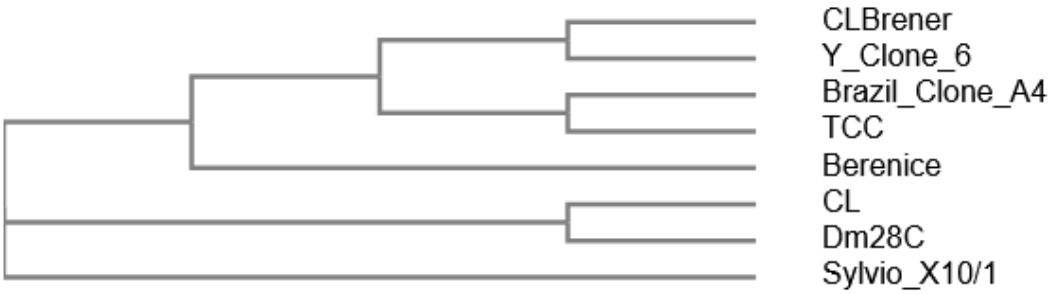


Figure 2: A: comparison of amino acid similarity for TcMUC II protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the TcMUC II protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table I and Figure 2 of the supplementary material.

A

Percent similarity

	TCC	CL	CL Brener	Tula Clone 2	Y Clone 6	Dm28C	G	Brazil Clone A4
TCC		93.20	88.72	92.16	87.90	88.00	92.02	96.12
CL	6.80		97.09	98.02	98.06	94.17	96.08	96.08
CL Brener	11.28	2.91		95.10	92.74	86.40	97.09	97.09
Tula Clone 2	7.84	1.98	4.90		98.04	94.12	96.04	96.04
Y Clone 6	12.10	1.94	7.26	1.96		96.64	98.06	98.06
Dm28C	12.00	5.83	13.60	5.88	3.36		98.06	98.06
G	7.98	3.92	2.91	3.96	1.94	1.94		100.00
Brazil Clone A4	3.88	3.92	2.91	3.96	1.94	1.94	0.00	

Percent dissimilarity

B

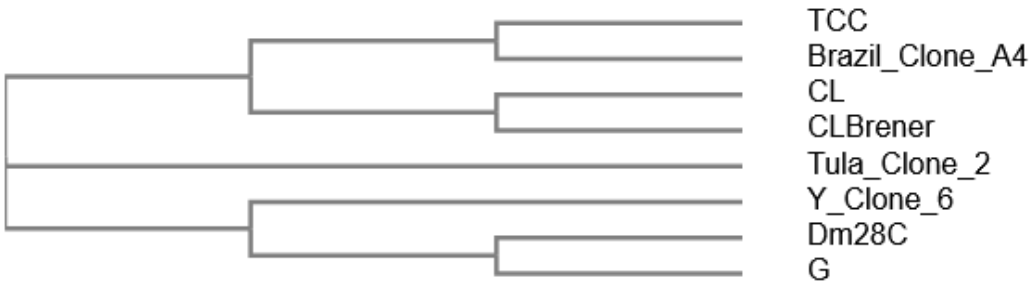


Figure 3: A: comparison of amino acid similarity for TcSMUG L protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the TcSMUG L protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table I and Figure 3 of the supplementary material.

A

Percent similarity

	TCC	Y	TUL	CL Brener	CL
TCC		95.50	95.72	95.72	95.93
Y	4.50		97.22	97.43	97.64
TUL	4.28	2.78		99.36	99.14
CL Brener	4.28	2.57	0.64		99.79
CL	4.07	2.36	0.86	0.21	

Percent dissimilarity

B

Figure 4: A: comparison of amino acid similarity for Cz protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the Cz protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table II and Figure 4 of the supplementary material.

A

Percent similarity

	Dm28C	Tulahuen	Y	Colombian Clone 22	Brazil	CL Brener
Dm28C		72.00	71.56	70.73	74.67	80.44
Tulahuen	28.00		96.83	95.81	84.54	83.82
Y	28.44	3.17		97.54	82.80	84.10
Colombian Clone 22	29.27	4.19	2.46		82.46	83.48
Brazil	25.33	15.46	17.20	17.54		85.69
CL Brener	19.56	16.18	16.52	16.52	14.31	

Percent dissimilarity

B

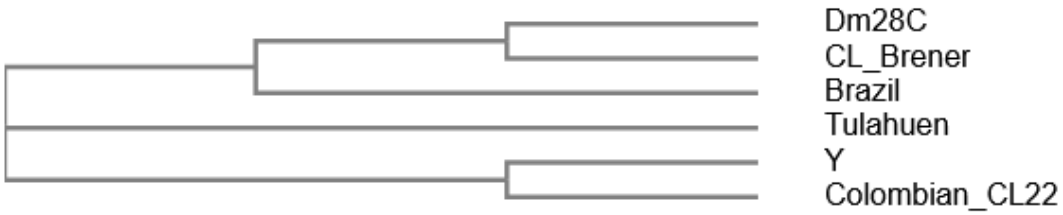


Figure 5: A: comparison of amino acid similarity for ASP-2 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the ASP-2 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table III and Figure 5 of the supplementary material.

A

Percent similarity

	Dm28C	Sylvio X10	Esmeraldo	CL	Y Clone 6	CL Brener	Peru
Dm28C		98.18	87.69	88.38	87.12	86.39	83.05
Sylvio X10	1.82		87.11	87.84	87.15	86.22	82.83
Esmeraldo	12.31	12.89		98.46	98.73	93.12	91.97
CL	11.62	12.16	1.54		99.83	93.43	92.15
Y Clone 6	12.88	12.85	1.27	0.17		93.46	91.52
CL Brener	13.61	13.78	6.88	6.57	6.54		94.46
Peru	16.95	17.17	8.03	7.85	8.48	5.54	

Percent dissimilarity

B

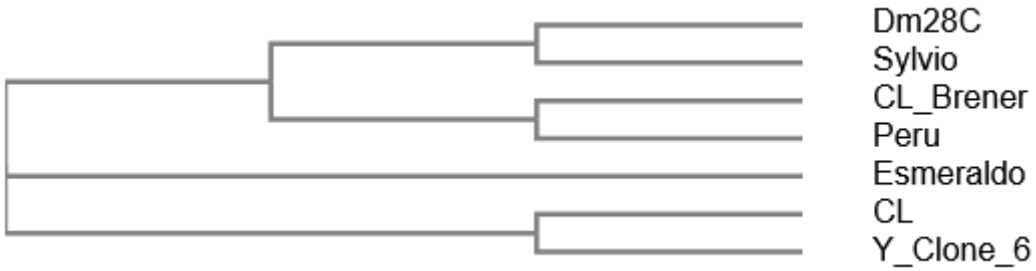


Figure 6: A: comparison of amino acid similarity for TSA-1 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the TSA-1 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table III and Figure 6 of the supplementary material.

A

Percent similarity

	Brazil Clone 4	Dm28C	TCC	Berenice	CL
Brazil Clone 4		55.48	55.48	55.48	55.86
Dm28C	44.52		91.64	91.64	92.94
TCC	44.52	8.36		99.17	99.76
Berenice	44.52	8.36	0.83		100.00
CL	44.14	7.06	0.24	0.00	

Percent dissimilarity

B

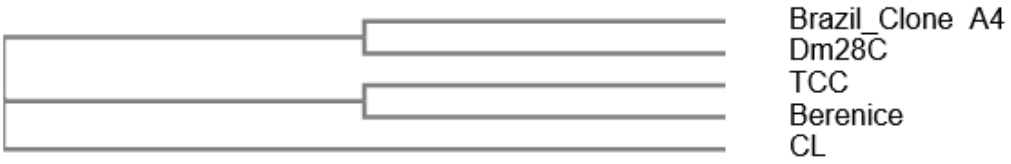


Figure 7: A: comparison of amino acid similarity for PFR-2 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the PFR-2 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table IV and Figure 7 of the supplementary material.

A

Percent similarity

	CL Brener	Dm28C	Sylvio	Esmeraldo	G
CL Brener		99.83	99.66	99.23	100.00
Dm28C	0.17		99.83	99.66	100.00
Sylvio	0.34	0.17		99.49	100.00
Esmeraldo	0.77	0.34	0.51		100.00
G	0.00	0.00	0.00	0.00	

Percent dissimilarity

B

Figure 8: A: comparison of amino acid similarity for PFR-3 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the PFR-3 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table IV and Figure 8 of the supplementary material.

A

Percent similarity

	Y	G	Y Clone 7	CL Brener	Tula Clone 2
Y		99.10	93.43	93.22	94.46
G	0.90		93.43	93.22	94.46
Y Clone 7	6.57	6.57		96.71	97.59
CL Brener	6.78	6.78	3.29		97.59
Tula Clone 2	5.54	5.54	2.41	2.41	

Percent dissimilarity

B

Figure 9: A: comparison of amino acid similarity for Tc52 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the Tc52 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table V and Figure 9 of the supplementary material.

A

Percent similarity

	Sylvio X10/1	Brazil Clone A4	Dm28C	Berenice	Tulahuen	Y Clone 6	CL Brener	TCC	CL
Sylvio X10/1		99.71	96.67	97.99	97.56	97.85	98.57	80.00	98.07
Brazil Clone A4	0.29		100.00	98.13	97.70	97.99	98.71	80.16	98.34
Dm28C	3.33	0.00		97.99	97.49	97.83	98.66	76.62	98.18
Berenice	2.01	1.87	2.01		99.00	99.28	99.14	81.44	99.17
Tulahuen	2.44	2.30	2.51	1.00		99.71	98.71	81.12	98.62
Y Clone 6	2.15	2.01	2.17	0.72	0.29		99.00	81.12	98.90
CL Brener	1.43	1.29	1.34	0.86	1.29	1.00		81.44	99.17
TCC	20.00	19.84	23.38	18.56	18.88	18.88	18.56		99.45
CL	1.93	1.66	1.82	1.38	1.38	1.10	0.83	0.55	

Percent dissimilarity

B

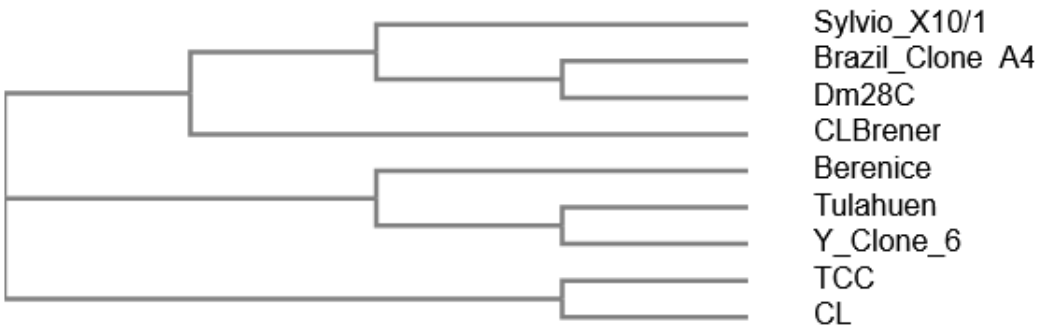


Figure 10: A: comparison of amino acid similarity for Tc80 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the Tc80 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table VI and Figure 10 of the supplementary material.

A

Percent similarity

	G	Berenice	Y Clone 6	Brazil Clone A4	Dm28C	Sylvio X10/1	TCC	Y	CL Brener	CL
G		97.71	96.95	97.71	97.14	96.95	97.71	97.71	97.71	97.71
Berenice	2.29		99.53	98.10	97.18	97.63	98.10	98.10	98.10	98.58
Y Clone 6	3.05	0.47		97.63	96.48	97.16	97.63	97.63	97.63	98.10
Brazil Clone A4	2.29	1.90	2.37		99.30	99.53	99.05	99.05	99.05	99.53
Dm28C	2.86	2.82	3.52	0.70		100.00	98.59	98.59	98.59	98.59
Sylvio X10/1	3.05	2.37	2.84	0.47	0.00		98.58	98.58	98.58	99.05
TCC	2.29	1.90	2.37	0.95	1.41	1.42		99.53	99.05	99.53
Y	2.29	1.90	2.37	0.95	1.41	1.42	0.47		99.05	99.53
CL Brener	2.29	1.90	2.37	0.95	1.41	1.42	0.95	0.95		99.53
CL	2.29	1.42	1.90	0.47	1.41	0.95	0.47	0.47	0.47	

Percent dissimilarity

B

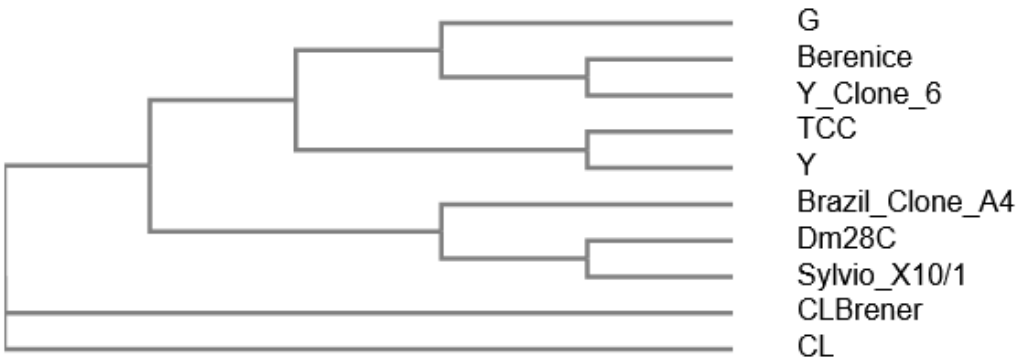


Figure 11: A: comparison of amino acid similarity for Tc24 protein of various *T. cruzi* strains. B: dendrogram showing the relative sequence distances of the Tc24 protein of various *T. cruzi* strains. The individual protein characteristics and sequences are defined, respectively, in Table VII and Figure 11 of the supplementary material.

Supplementary material

Table I – Mucin proteins characteristics for different strains of *T. cruzi.*

Protein	Strain	GB Access	Molecular weight	#AA
TcMUC I	CL	RNC32051.1	20.88*	190
	CL	RNC32949.1	18.64	169
	Sylvio X10/1	EKG01015.1	26.35	239
	Dm28C	ESS62912.1	23.10	210
	Berenice	KAF5214551.1	39.94	370
	G	RNF02673.1	13.67	122
	CL Brener	XP_802175.1	21.59	198
TcMUC II	CL Brener	XP_803227.1	37.60	353
	Y Clone 6	KAF8281566.1	18.49	183
	TCC	PWV02240.1	19.43	192
	Dm28C	ESS62816.1	26.89	240
	Dm28C	ESS62491.1	19.57	192
	CL	RNC35393.1	19.44	173
	CL	RNC51706.1	17.09	169
	Sylvio X10/1	EKF98774.1	26.15	233
	Sylvio X10/1	EKG02248.1	29.43	266
	Berenice	KAF5219833.1	31.51	297
	Berenice	KAF5214609.1	22.26	200
	Brazil Clone 4	KAF8306510.1	19.36	191
TcSMUG L	CL Brener	XP_808662.1	13.56	133
	CL	RNC53459.1	10.52	103
	Y Clone 6	KAF8280200.1	12.70	124
	TCC	PWV15054.1	24.72	239
	Dm28C	PBJ76201.1	12.89	125
	Brazil Clone A4	KAF8290903.1	10.57	103
	Tula Clone 2	AEI25746.1	10.53	102
	G	RNF21741.1	19.31	188

*calculated molecular weight based on the available amino acid sequence

Table II – Cruzipain proteins characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
Cz	CL Brener	UBT23817.1	49.78*	467
	CL	RNC47525.1	53.33	500
	Y	AAG35357.1	49.82	467
	TCC	AAF75547.1	49.72	467
	TUL	AAF75546.1	49.67	467

*calculated molecular weight based on the available amino acid sequence

Table III – Trans-sialidase proteins characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
ASP-2	CL Brener	ABQ53588.1	75.92*	692
	Y	AAO84044.1	76.02	694
	Dm28C	EF583446.1	75.90	693
	Brazil	AAC47720.1	77.03	706
	Tulahuen	ABQ53591.1	76.37	694
	Colombian Clone 22	ABQ53589.1	75.56	692
TSA-1	CL Brener	AID66712.1	65.63	596
	CL	RNC44606.1	64.37	587
	Y Clone 6	KAF8296649.1	83.31	759
	Dm28C	PWU85144	82.23	753
	Peru	AAA30259.1	90.45	853
	Esmeraldo	AAD10620.1	77.89	711
	Sylvio X10	AAA18827.1	78.84	723

*calculated molecular weight based on the available amino acid sequence

Table IV – Paraflagellar rod proteins characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
PFR-2	Brazil Clone A4	KAF8276174.1	16.79*	146
	TCC	PWU97142.1	19.75	167
	TCC	PWV20566.1	65.57	600
	Dm28C	PWU89883.1	40.09	359
	Berenice	KAF5214366.1	65.50	600
	CL	RNC34307.1	48.93	425
PFR-3	CL Brener	XP_809830.1	68.68	589
	Dm28C	ESS64222.1	68.74	589
	G	RNF16495.1	23.62	207
	Sylvio X10	EKG04606.1	68.75	589
	Esmeraldo	AAC32018.1	68.76	589

*calculated molecular weight based on the available amino acid sequence

Table V – Tc52 protein characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
Tc52	Tula Clone 2	AAO63167.1	46.66*	415
	Y Clone 7	AAO63161.1	48.34	426
	CL Brener	AAO63166.1	47.41	428
	Y	AAG08957.1	50.68	445
	G	RNF14946.1	50.67	445

*calculated molecular weight based on the available amino acid sequence

Table VI – Tc80 protein characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
Tc80	Tulahuen	AAQ04681.1	78.83*	697
	Y Clone 6	KAF8291677.1	78.30	697
	Berenice	KAF5226303.1	78.25	697
	CL Brener	XP_820337.1	78.32	697
	Brazil Clone 4	KAF8288273.1	78.29	697
	Dm28C	ESS65583.1	66.57	598
	TCC	PWV08312.1	71.00	625
	CL	RNC31333.1	41.07	362
	Sylvio X10/1	EKG04331.1	78.22	697

*calculated molecular weight based on the available amino acid sequence

Table VII – Tc24 protein characteristics for different strains of *T. cruzi*.

Protein	Strain	GB Access	Molecular weight	#AA
Tc24	Dm28C	UYL40307.1	16.39*	142
	Sylvio X10/1	EKG06658.1	23.77	211
	Brazil Clone A4	KAF8288663.1	23.78	211
	CL Brener	XP_805575.1	23.82	211
	TCC	PWV20463.1	23.79	211
	Y	BAA13411.1	23.74	211
	CL	RNC34385.1	28.59	257
	Berenice	KAF5223631.1	23.79	211
	Y Clone 6	KAF8301209.1	23.81	211
	G	RNF13547.1	14.84	131

*calculated molecular weight based on the available amino acid sequence

Figure 1 – Alignment of TcMUC I protein sequences.

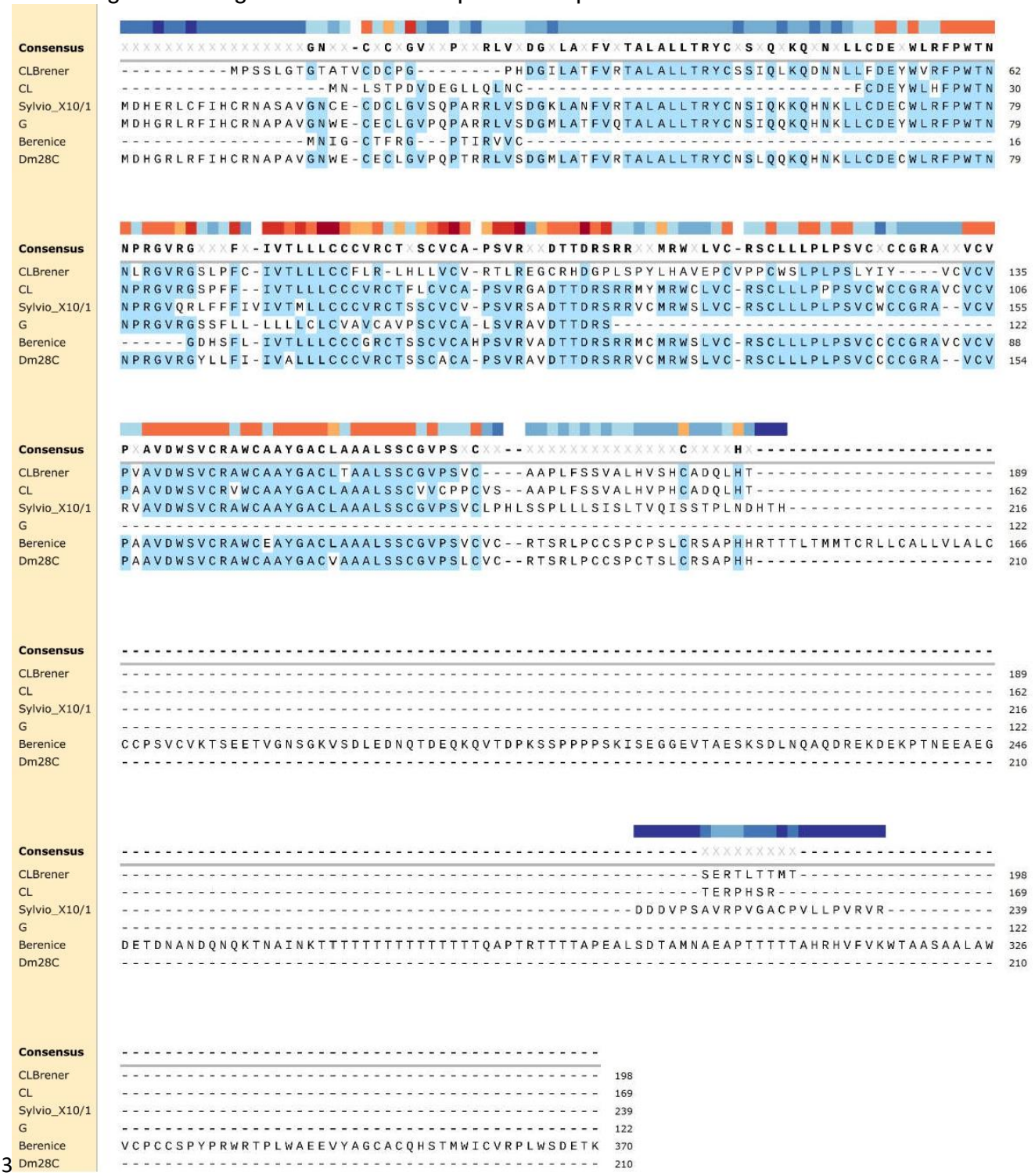


Figure 2 – Alignment of TcMUC II protein sequences.

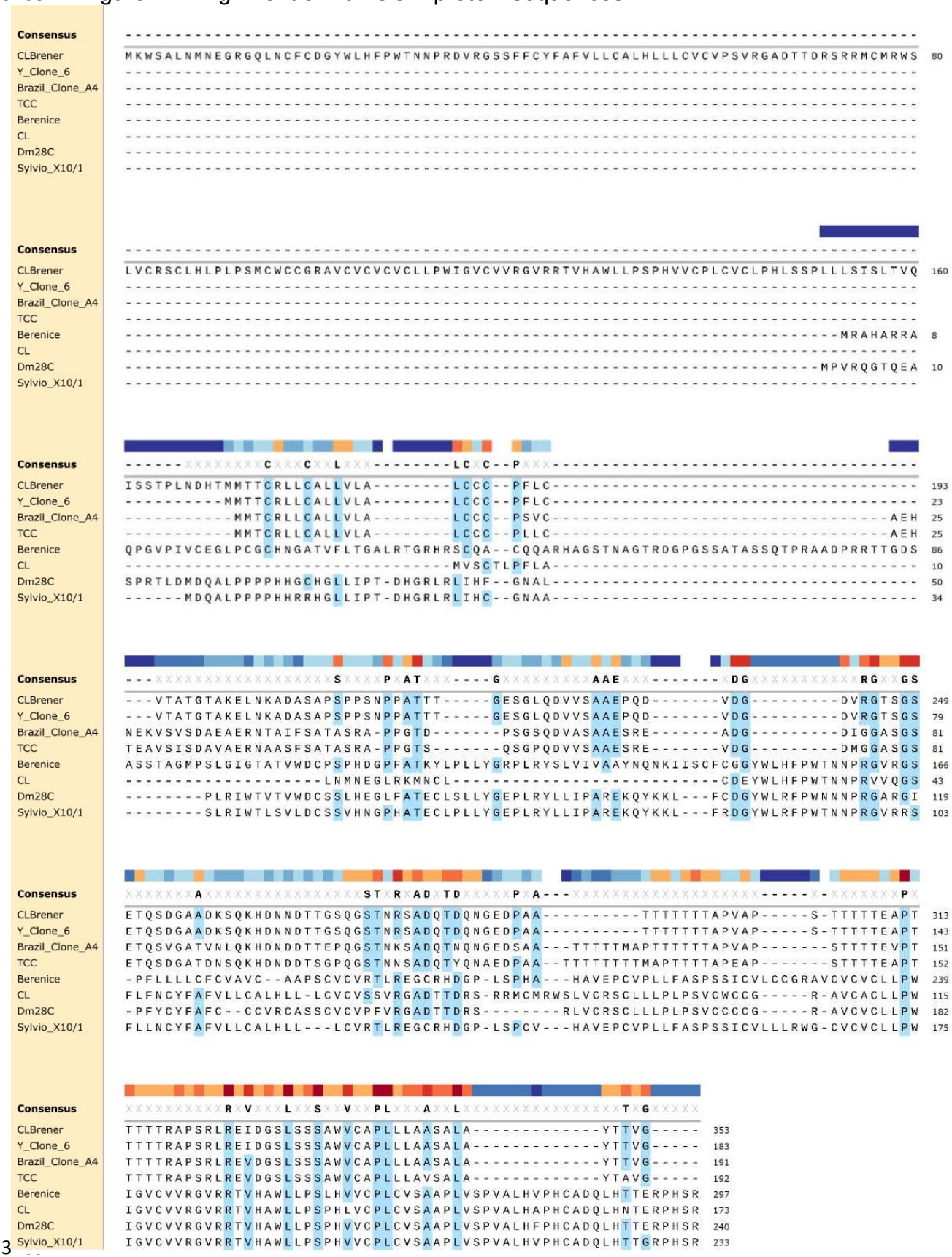


Figure 3 – Alignment of TcSMUG L protein sequences.

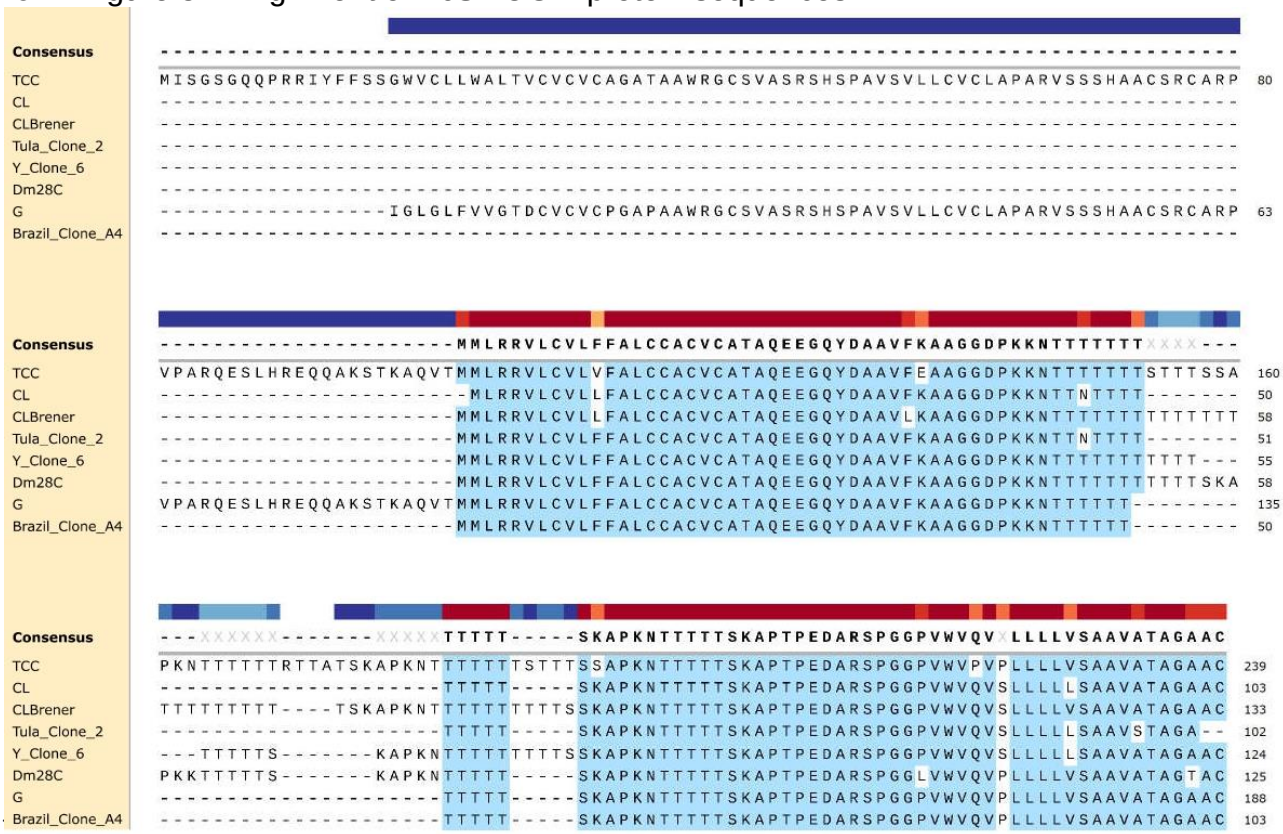


Figure 4 – Alignment of Cruzipain protein sequences.

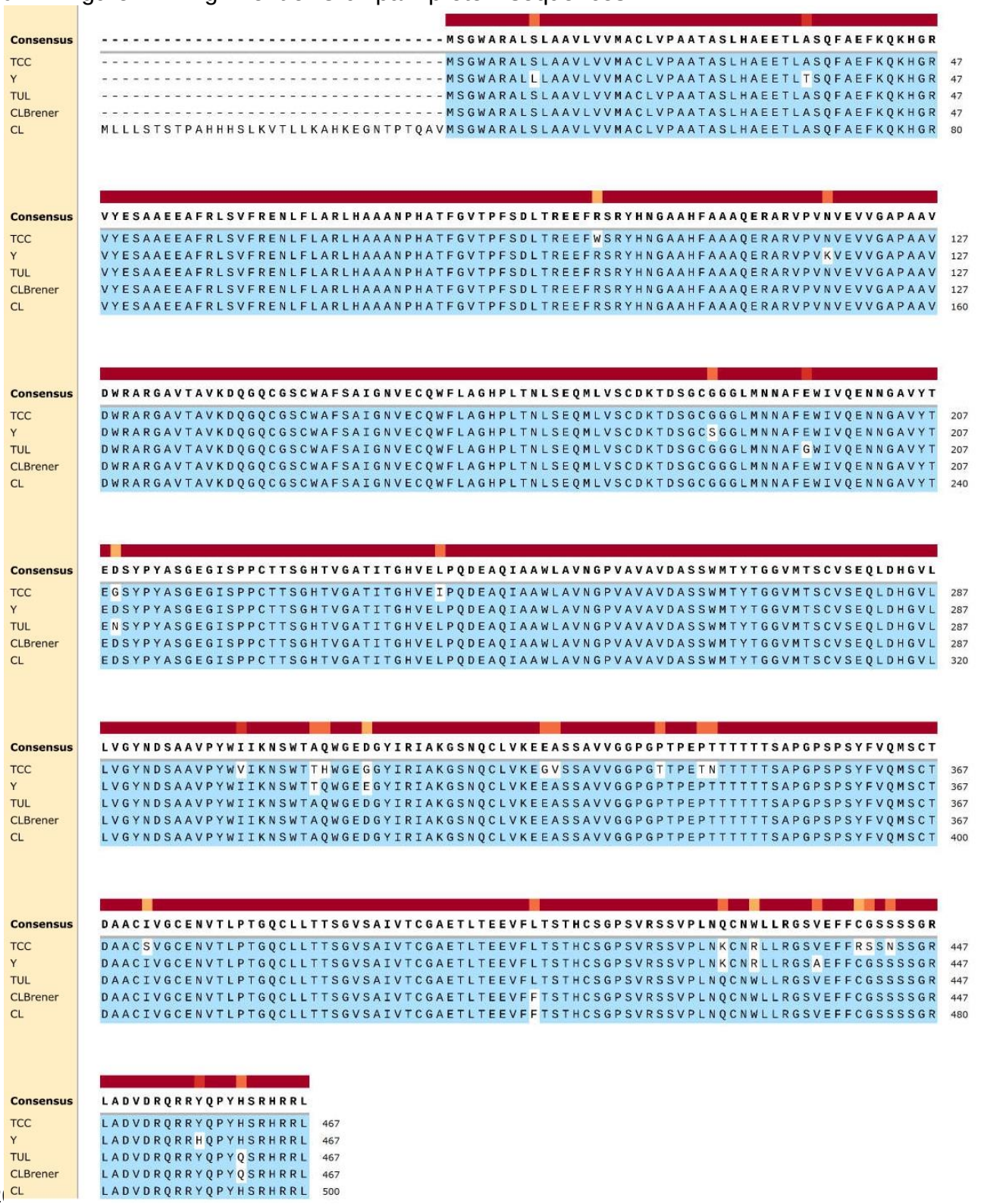


Figure 5 – Alignment of ASP-2 protein sequences

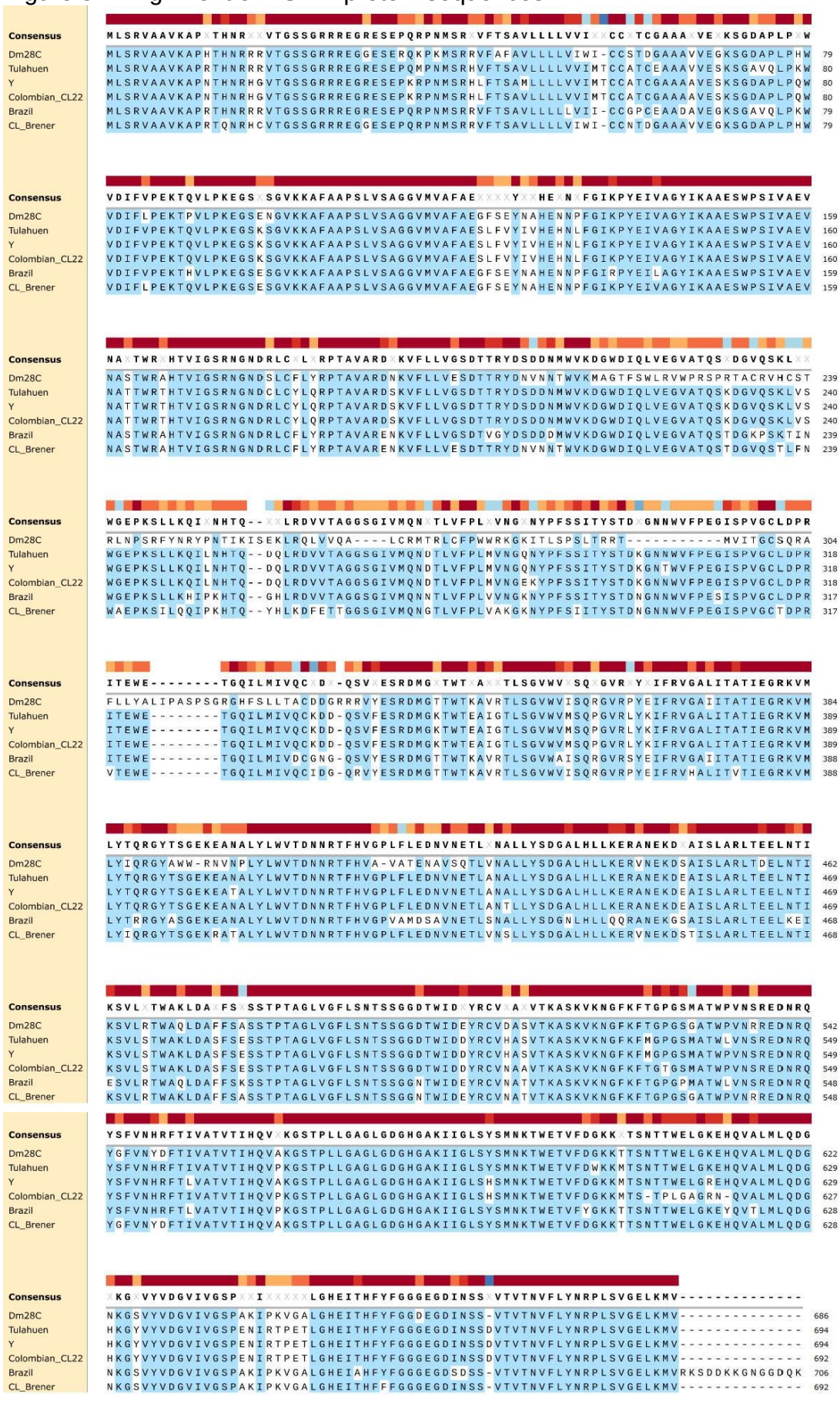
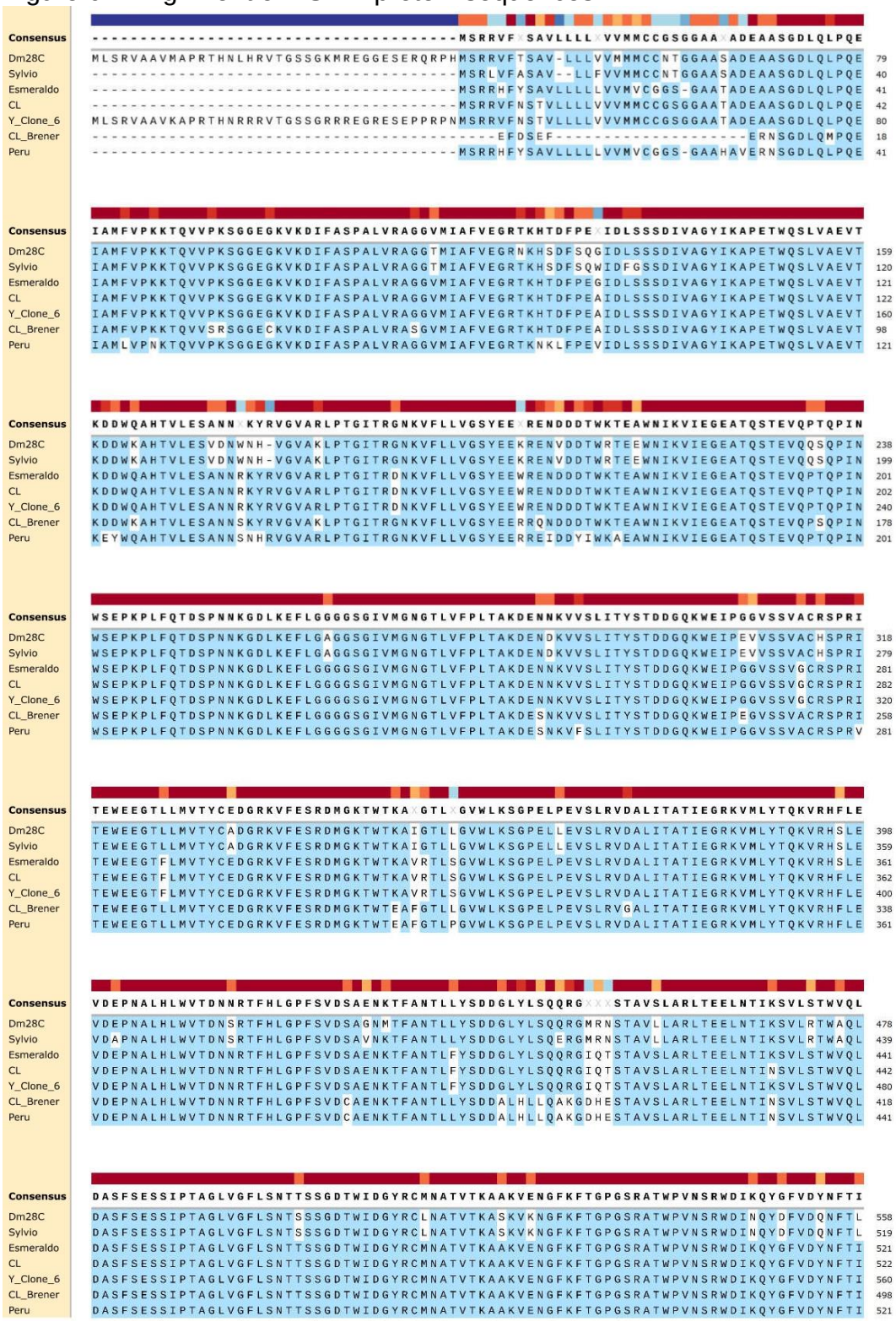


Figure 6 – Alignment of TSA-1 protein sequences.




Consensus
VAMATIHQVPSESTPLLGASLRGNKRTKLIGLSYGAGGKWETVYDGTKTVQGGTWEPGREYQVALMLQDGNKGFVYVDGV
Dm28C
Sylvio
Esmeraldo
CL
Y_Clone_6
CL_Brener
Peru
Consensus
LVGNPAMLPTPEERWTE S FYFGGDEGDSGS AT DVFLY RPLS ELKMIK V EDK
Consensus
KE SGD EDKK SGD EDKK SGD EDKKGSGDGSMREGMSRVL
Consensus
LLLLGMWGIAAI

Figure 7 – Alignment of PFR-2 protein sequences.

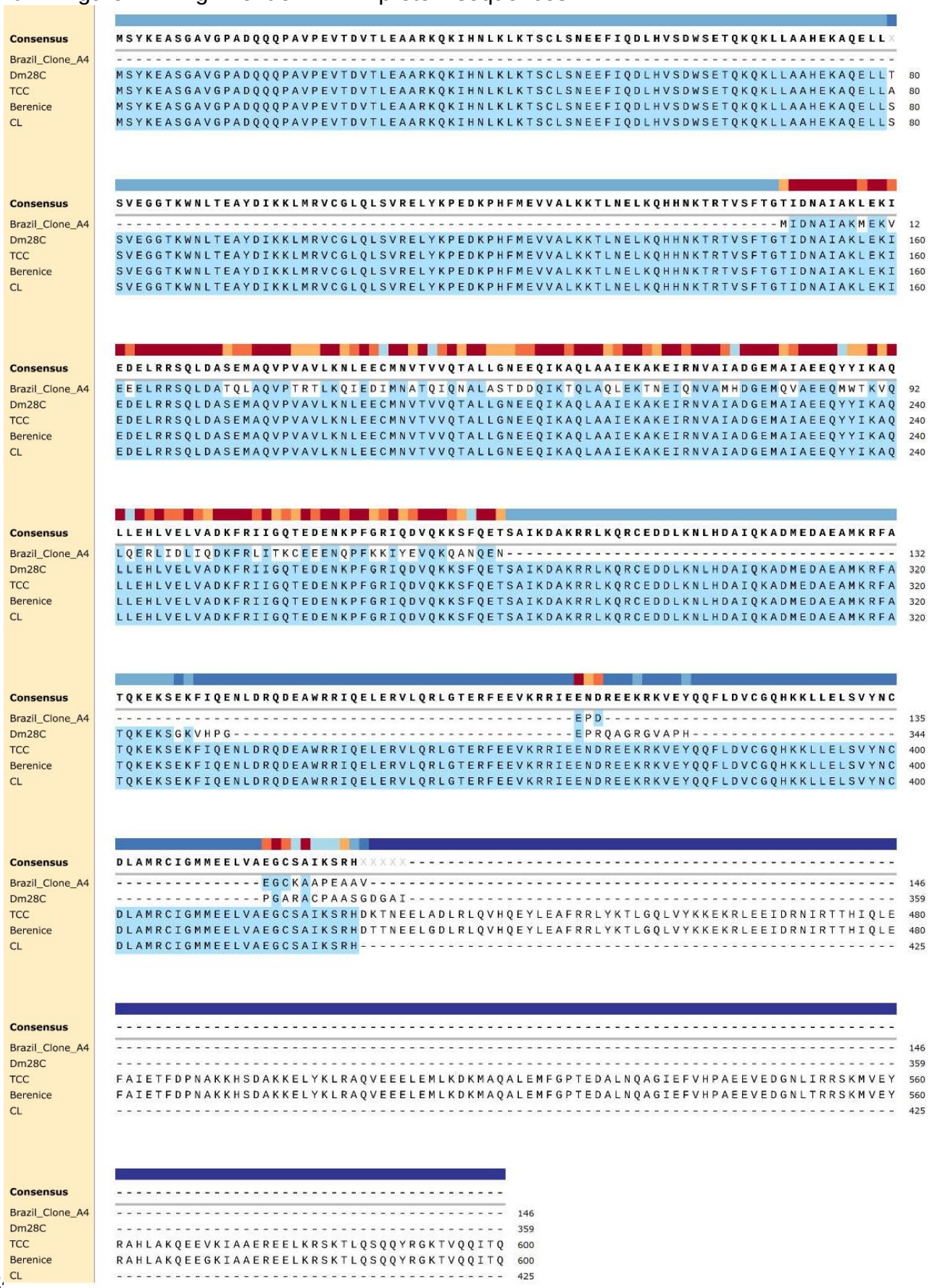


Figure 8 – Alignment of PFR-3 protein sequences.

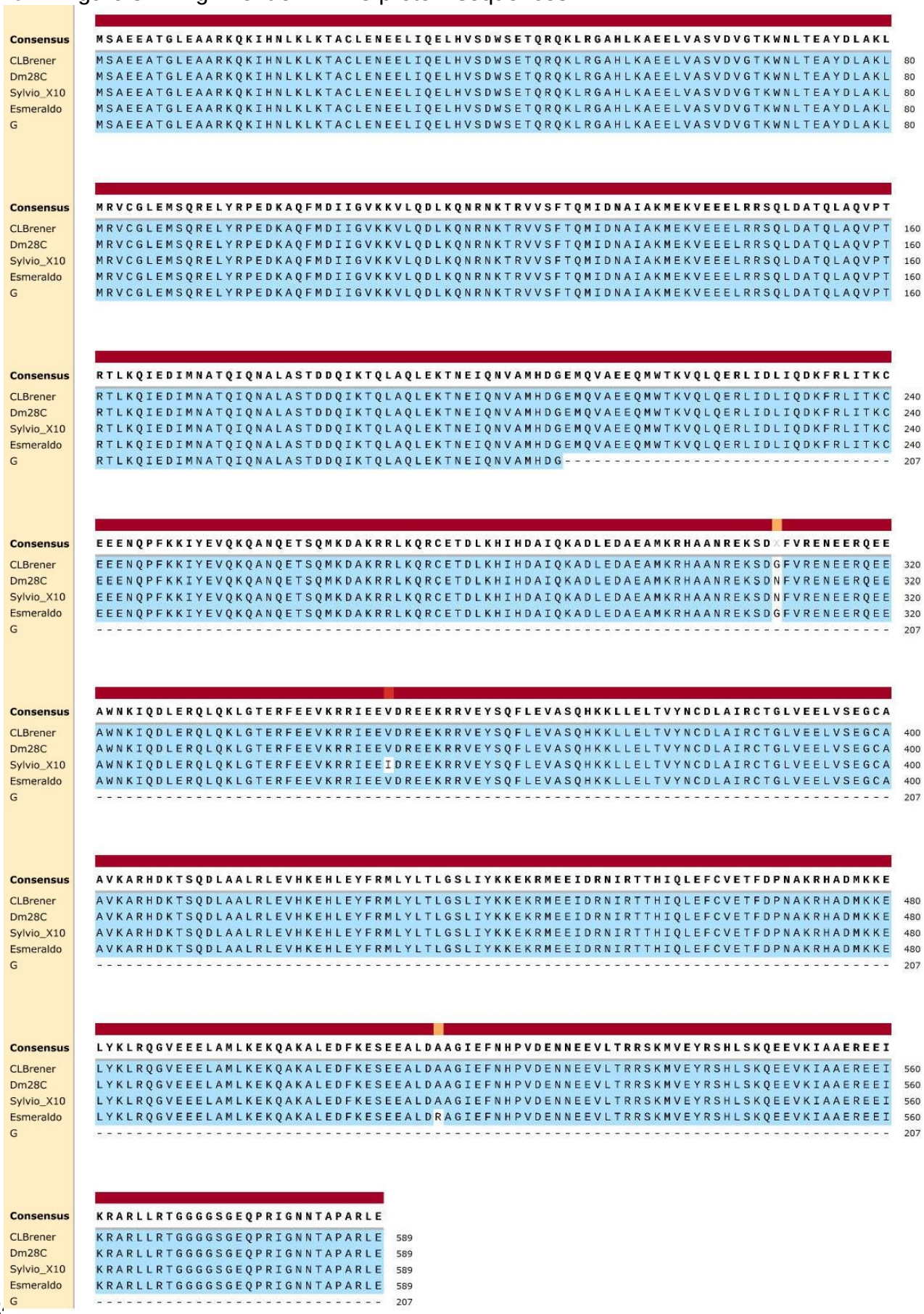


Figure 9 – Alignment of Tc52 protein sequences.

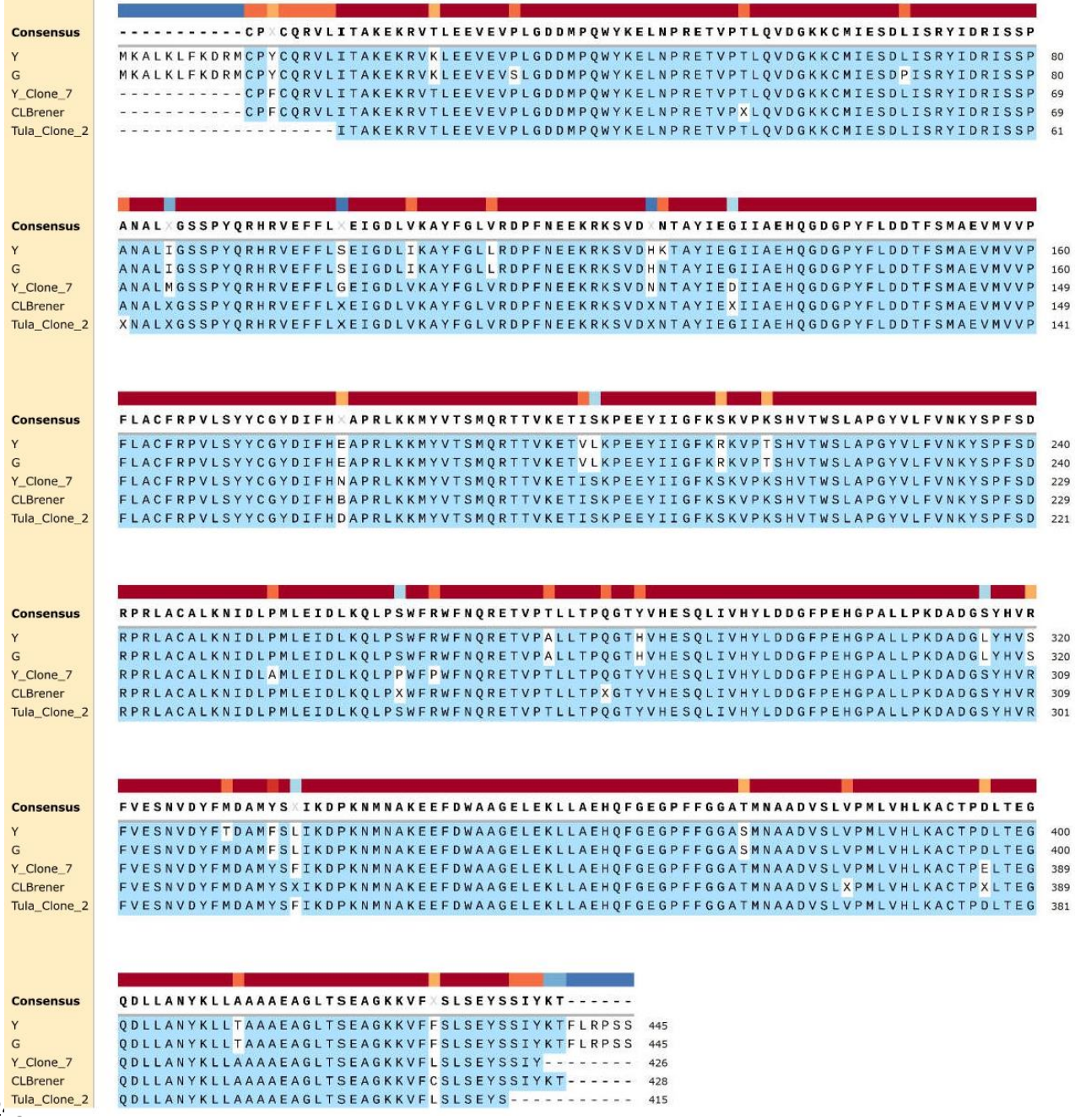
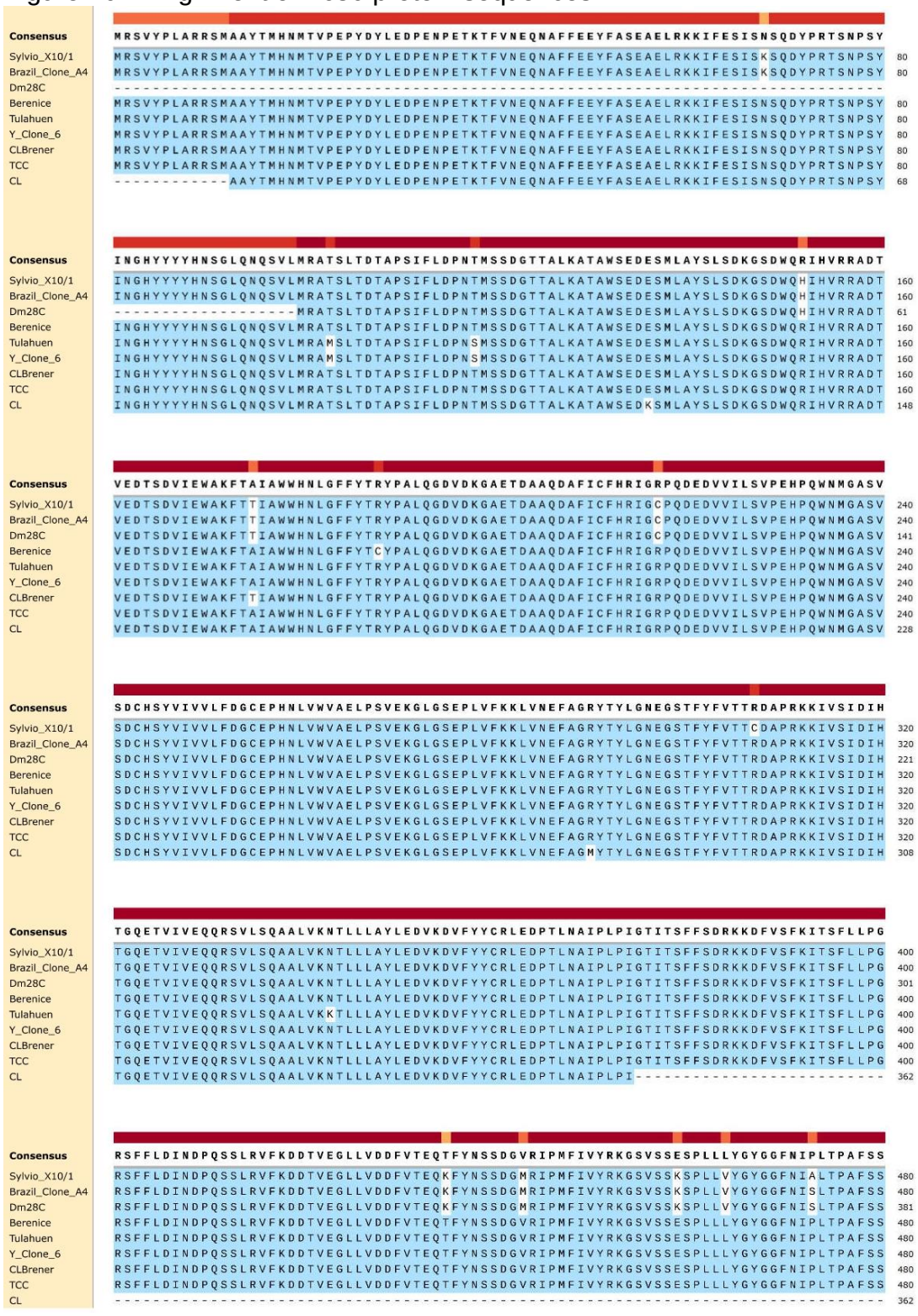


Figure 10 – Alignment of Tc80 protein sequences.



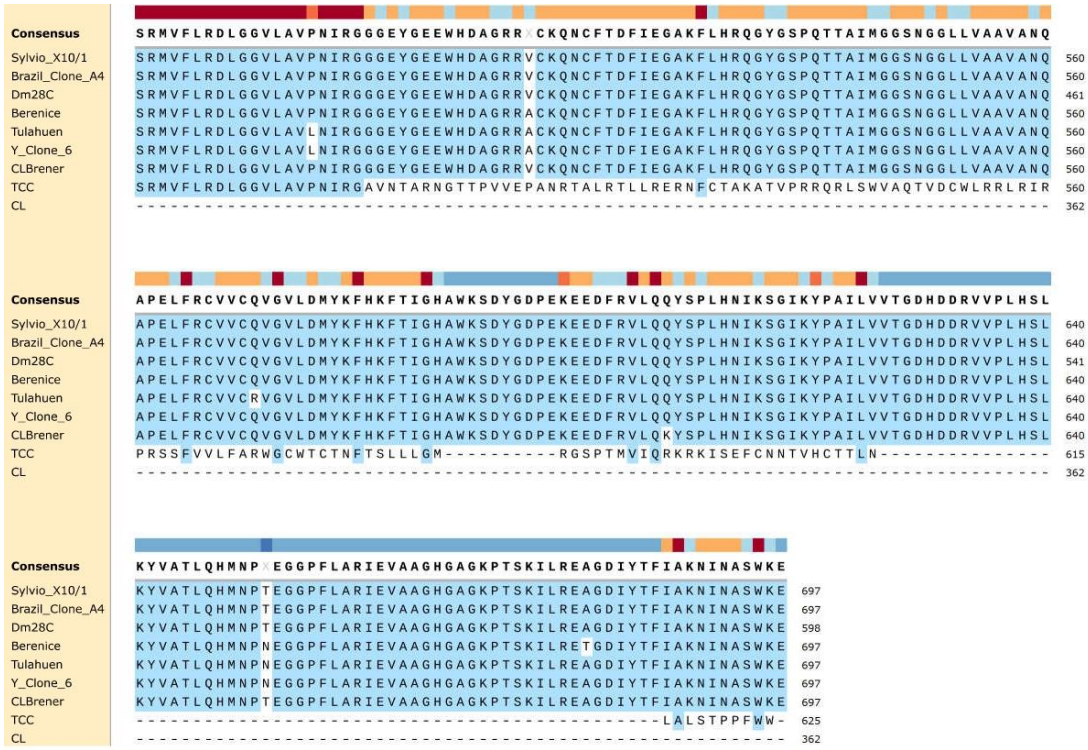
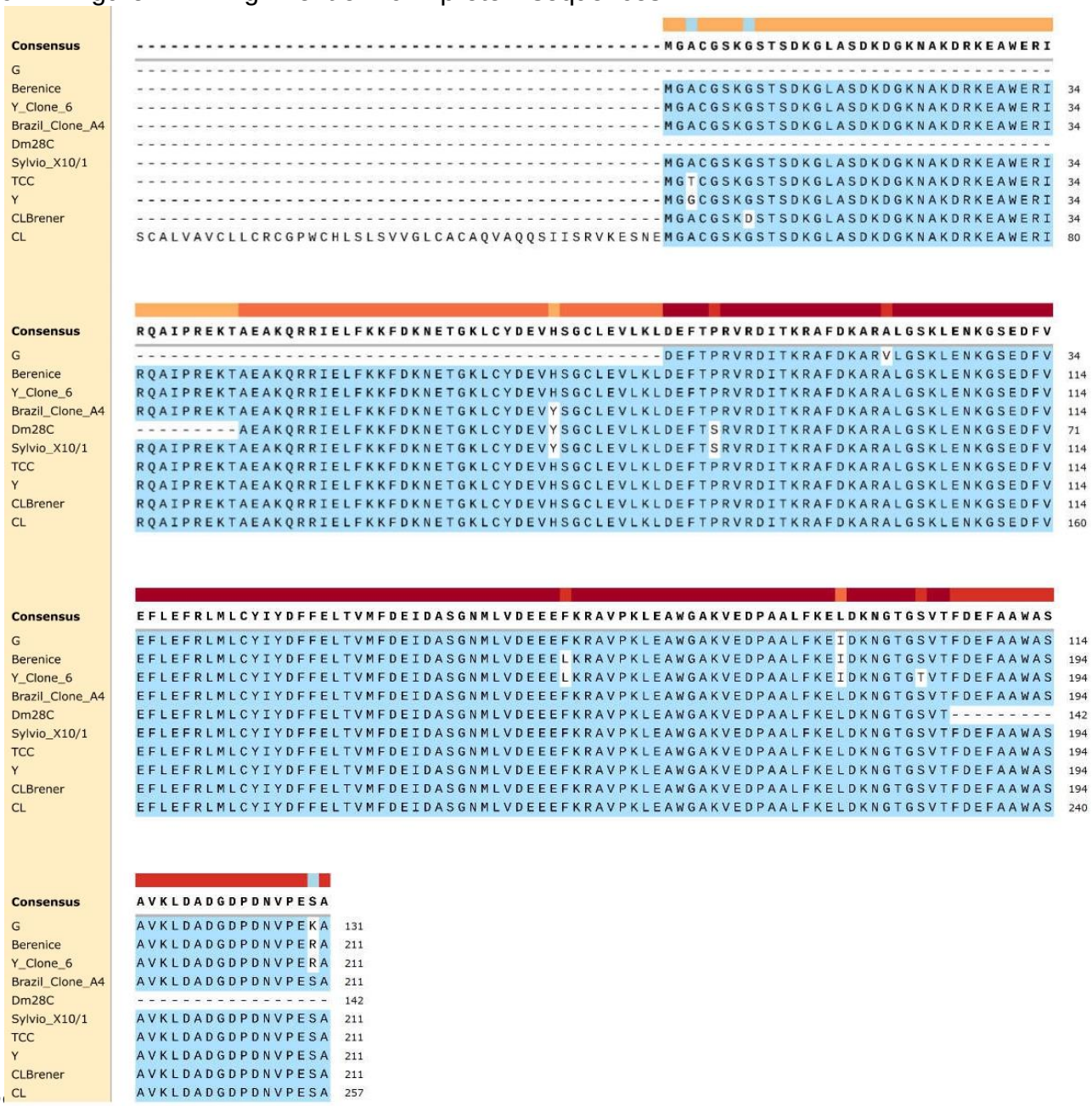

Consensus SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRXCKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ
Sylvio_X10/1 SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRVCKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
Brazil_Clone_A4 SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRVCKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
Dm28C SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRVCKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 461
Berenice SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRACKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
Tulahuen SRMVFLRDLGGVLAVLNIRGGGEYGEEWHDAGRRACKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
Y_Clone_6 SRMVFLRDLGGVLAVLNIRGGGEYGEEWHDAGRRACKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
CLBrener SRMVFLRDLGGVLAVPNIRGGGEYGEEWHDAGRRVCKQNCFTDFIEGAKFLHRQGYGSPQTTAIMGGSNGGLLVAAVANQ 560
TCC SRMVFLRDLGGVLAVPNIRGAVNTARNGTTPVVEPANRTALRTLLRERNFCTAKATVPRRQRLSWVAQTVDCWLRRLRIR 560
CL ---------- 362
Consensus APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL
Sylvio_X10/1 APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
Brazil_Clone_A4 APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
Dm28C APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 541
Berenice APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
Tulahuen APELFRCVVCRVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
Y_Clone_6 APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQQYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
CLBrener APELFRCVVCQVGVLDMYKFHKFTIGHAWKSDYGDPEKEEDFRVLQKYSPLHNIKSGIKYPAILVVTGDHDDRVVPLHSL 640
TCC PRSSFVVLFARWGCWTCTNFTSLLLGM---------RGSPTMVIQRKRKISEFCNNTVHCTTLN--------- 615
CL ---------- 362
Consensus KYVATLQHMNPXEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE
Sylvio_X10/1 KYVATLQHMNPTEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 697
Brazil_Clone_A4 KYVATLQHMNPTEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 697
Dm28C KYVATLQHMNPTEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 598
Berenice KYVATLQHMNPNEGGPFLARIEVAAGHGAGKPTSKILRETGDIYTFIAKNINASWKE 697
Tulahuen KYVATLQHMNPNEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 697
Y_Clone_6 KYVATLQHMNPNEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 697
CLBrener KYVATLQHMNPTEGGPFLARIEVAAGHGAGKPTSKILREAGDIYTFIAKNINASWKE 697
TCC ----------LALSTPPFWW- 625
CL ---------- 362

Figure 11 – Alignment of Tc24 protein sequences.



3.3) Patente 1

CONSTRUÇÃO DE BCG RECOMBINANTE EXPRESSANDO ANTÍGENOS DE *Trypanosoma cruzi*

Patente depositada no INPI: BR 10 2023 003276 1

RELATÓRIO DESCRITIVO

FUNDAMENTOS DA INVENÇÃO

CAMPO DA INVENÇÃO

[001] A presente invenção descreve a construção de nove protótipos vacinais contra Doença de Chagas compostos pelas cepas de *Mycobacterium bovis* BCG Pasteur (vetores pUS977 e pUS2000) recombinantes expressando a porção final (a partir do aminoácido 261) da proteína de superfície de amastigota n° 2 (ASP-2) e o segmento do aminoácido 1 a 617 do antígeno de superfície de trypomastigota n° 1 (TSA-1), bem como a sequência completa da proteína flagelar de ligação ao cálcio de 24 kDa (Tc24)sintéticas de *Trypanosoma cruzi*. A presente invenção refere-se a seleção das sequências nucleotídicas que codificam para estas proteínas a partir do NCBI www.ncbi.nlm.nih.gov (AAC47720.1; KJ668043.1; AAB08762.1), clonagem dos genes no vetor PUC57 e expressão das proteínas em *M. bovis* BCG Pasteur.

DESCRIÇÃO DO ESTADO DA TÉCNICA

[002] A doença de Chagas (DC), ou tripanossomíase americana, tem como causa o protozoário flagelado *Trypanosoma cruzi*, persistindo em 21 países da America Latina, sendo endêmica em pelo menos 15. Transmitida principalmente por meio do contato das mucosas ou de lesões cutâneas com as fezes de insetos triatomíneos da família

Reduvidae, infectados pelo *T. cruzi*. Além da transmissão vetorial, ainda pode haver a infecção por meio de transfusões sanguíneas, de transplantes de órgãos ou por transmissão materno-fetal. Estima-se que pelo menos 28 milhões de pessoas estejam sob risco de contaminação na América Latina, e mesmo em países desenvolvidos a DC vem sendo considera um problema em potencial, devido a imigração de populações oriundas de áreas endêmicas **(Moncayo, A., Silveira, A.C. 2009. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. Mem Inst Oswaldo Cruz. 104 Suppl 1:17-30. doi: 10.1590/S0074-02762009000900005. PMID: 19753454.).**

[003] O *T. cruzi* é um organismo digenético, apresentando um hospedeiro vertebrado e um invertebrado. Seu ciclo de vida é complexo, onde o vetor invertebrado quando infectado libera tripomastigotas em suas fezes próximo ao local da picada, ao se alimentar de sangue do hospedeiro. No interior do novo hospedeiro, os tripomastigotas invadem as células próximas ao local da inoculação, onde se diferenciam em amastigotas intracelulares. Os amastigotas se multiplicam por fissão binária e se diferenciam em tripomastigotas, sendo então liberados na corrente sanguínea. Os tripomastigotas infectam células de uma variedade de tecidos e se transformam em amastigotas intracelulares em novos locais de infecção. Manifestações clínicas podem resultar desse ciclo infeccioso. Os tripomastigotas da corrente sanguínea não se replicam, a replicação recomeça apenas quando os parasitas entram em outra célula ou são ingeridos por outro vetor. O inseto é infectado ao se alimentar de sangue humano ou animal que contém parasitas circulantes. Os tripomastigotas ingeridos se transformam em epimastigotas no intestino médio do vetor e se diferenciam em tripomastigotas metacíclicos infectantes no intestino posterior **(Macedo, A.M., Oliveira, R.P., Pena, S.D. 2002. Chagas disease: role of parasite**

genetic variation in pathogenesis. Expert Rev Mol Med. 4:1-16. doi: 10.1017/S1462399402004118. PMID: 14987389.). O *Trypanosoma* infecta outros vertebrados além do homem, como mamíferos placentados e marsupiais, os quais servem como reservatório para o protozoário, impossibilitando a erradicação da doença **(Dias, J.C. 2009. Elimination of Chagas' disease transmission: perspectives. Mem Inst Oswaldo Cruz. 104 Suppl 1:41-45. doi: 10.1590/s0074-02762009000900007. PMID: 19753456.).**

[004] Após o contato com o parasita, o hospedeiro vertebrado desenvolve a fase aguda da doença, que pode durar semanas ou meses, podendo ser sintomática ou assintomática. Após o controle da parasitemia pelo sistema imune, o indivíduo passa então à fase crônica da doença, a qual é caracterizada por uma parasitemia subpatente. Como o parasita nunca é eliminado do organismo, a fase crônica da doença de Chagas perdura por toda a vida do indivíduo. Em cerca de 66% dos casos, as pessoas infectadas permanecem assintomáticas, já em 34% dos casos, após anos, pode haver o desenvolvimento de sintomas do trato digestivo, do sistema nervoso periférico ou cardíacos, podendo em muitos casos levar à morte **(Coura, J.R., de Abreu, L.L., Pereira, J.B., Willcox, H.P. 1985. Morbidity in Chagas' disease. IV. Longitudinal study of 10 years in Pains and Iguatama, Minas Gerais, Brazil. Mem Inst Oswaldo Cruz. 80(1):73-80. doi: 10.1590/s0074-02761985000100011. PMID: 3937015.).**

[005] Atualmente existem apenas duas drogas disponíveis para o tratamento da doença de Chagas: o benzonidazol e o nifurtimox. Ambas as drogas, no entanto, frequentemente causam efeitos colaterais que levam parte dos pacientes a abandonar o tratamento. O nifurtimox induz reações adversas em cerca de 40% dos indivíduos tratados, incluindo náuseas, vômitos, dores abdominais, perda de peso, anorexia severa e complicações neurológicas **(Marin-Neto,**

J.A., Rassi, A.Jr., Avezum, A.Jr., Mattos, A.C., Rassi, A., Morillo, C.A., Sosa-Estani, S., Yusuf, S., BENEFIT Investigators. 2009. The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. Mem Inst Oswaldo Cruz. 104 Suppl 1:319-24. doi: 10.1590/s0074-02762009000900042. PMID: 19753491.). O benzonidazol induz efeitos colaterais em uma percentagem menor de indivíduos, incluindo edema, febre, rash cutâneo, dor muscular, neuropatia periférica e neutropenia **(McKerrow, J.H., Doyle, P.S., Engel, J.C., Podust, L.M., Robertson, S.A., Ferreira, R., Saxton, T., Arkin, M., Kerr, I.D., Brinen, L.S., Craik, C.S. 2009. Two approaches to discovering and developing new drugs for Chagas disease. Mem Inst Oswaldo Cruz. 104 Suppl 1(0 1):263-9. doi: 10.1590/s0074-02762009000900034. PMID: 19753483. PMCID: PMC4156466.).**

[006] Apesar de vacinas desenvolvidas e avaliadas em estágio pré-clínico, como vacinas recombinantes, de subunidade ou de DNA (**Bivona, A.E., Alberti, A.S., Cerny, N., Trinitario, S.N., Malchiodi, E.L. 2020. Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control. Biochim Biophys Acta Mol Basis Dis. 1;1866(5):165658. doi: 10.1016/j.bbadis.2019.165658. PMID: 31904415.**), dada a complexidade da infecção, ainda não há uma vacina eficaz no tratamento ou imunização à doença. Entretanto, diversas proteínas podem apresentar potencial na sua utilização como antígenos para o desenvolvimento de vacinas recombinantes no combate a DC (**Jiménez, P., Jaimes, J., Poveda, C., Ramírez, J.D. 2019. A systematic review of the Trypanosoma cruzi genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. Parasitology. 146(3):269-283. doi: 10.1017/S0031182018001506. PMID: 30210012.**). Proteínas consideradas fatores de virulência do *T. cruzi*, como a

cruzipain, trans-sialidase, Tc24, TcG2 e TSA-1, foram utilizadas como antígenos em diferentes formulações vacinais, na tentativa de obter uma vacina profilática (**Arce-Fonseca, M., Carbajal-Hernández, A.C., Lozano-Camacho, M., Carrillo-Sánchez, S.D.C., Roldán, F.J., Aranda-Fraustro, A., Rosales-Encina, J.L., Rodríguez-Morales, O. 2020. DNA Vaccine Treatment in Dogs Experimentally Infected with Trypanosoma cruzi. J Immunol Res. 2020:9794575. doi: 10.1155/2020/9794575. PMID: 32455143. PMCID: PMC7222601.; Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P.A., Ortega-Lopez, J., Hotez, P.J., Bottazzi, M.E. 2020. Safety and immunogenicity of a recombinant vaccine against Trypanosoma cruzi in Rhesus macaques. Vaccine. 38(29):4584-4591. doi: 10.1016/j.vaccine.2020.05.010. PMID: 32417142. PMCID: PMC7310587.**). Dentre os antígenos já descritos, Tc24 e TSA-1, proteínas expressas na fase tripomastigota do parasita, apresentaram evidências como vacina terapêutica, baseando-se na imunização de camundongos e cães (**Sanchez-Burgos, G., Mezquita-Vega, R.G., Escobedo-Ortegon, J., Ramirez-Sierra, M.J., Arjona-Torres, A., Ouaissi, A., Rodrigues, M.M., Dumonteil, E. 2007. Comparative evaluation of therapeutic DNA vaccines against Trypanosoma cruzi in mice. FEMS Immunol Med Microbiol. 50(3):333-41. doi: 10.1111/j.1574-695X.2007.00251.x. PMID: 17521394.; Quijano-Hernandez, I.A., Bolio-González, M.E., Rodríguez-Buenfil, J.C., Ramirez-Sierra, M.J., Dumonteil, E. 2008. Therapeutic DNA vaccine against Trypanosoma cruzi infection in dogs. Ann N Y Acad Sci. 1149:343-6. doi: 10.1196/annals.1428.098. PMID: 19120245.**), ao gerar respostas imunes de células CD8+ (**Limon-Flores, A.Y., Cervera-Cetina, R., Tzec-Arjona, J.L., Ek-Macias, L., Sánchez-Burgos, G., Ramirez-Sierra, M.J., Cruz-Chan, J.V., VanWynsberghe, N.R., Dumonteil, E. 2010. Effect of a combination DNA vaccine for the prevention and therapy**

of Trypanosoma cruzi infection in mice: role of CD4+ and CD8+ T cells. Vaccine. 28(46):7414-9. doi: 10.1016/j.vaccine.2010.08.104. PMID: 20850536.). Da mesma forma, foi reportada a alta eficiência da imunização com PRF (proteína paraflagelar da haste), ao reduzir a parasitemia aguda e proteger os camundongos C57BL/6J em desafio, outrora letal, contra cepa altamente virulenta de *T. cruzi* (**Wrightsman, R.A., Manning, J.E. 2000. Paraflagellar rod proteins administered with alum and IL-12 or recombinant adenovirus expressing IL-12 generates antigen-specific responses and protective immunity in mice against Trypanosoma cruzi. Vaccine. 18(14):1419-27. doi: 10.1016/s0264-410x(99)00380-1. PMID: 10618540.**). Mesmo proteínas oriundas da fase amastigota do patógeno, como a ASP-2, são capazes de conferir proteção a linhagem de camundongos altamente suscetível a infecção, quando confeccionados em vacina (**Araújo, A.F., de Alencar, B.C., Vasconcelos, J.R., Hiyane, M.I., Marinho, C.R., Penido, M.L., Boscardin, S.B., Hoft, D.F., Gazzinelli, R.T., Rodrigues, M.M. 2005. CD8+-T-cell-dependent control of Trypanosoma cruzi infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect Immun. 73(9):6017-25. doi: 10.1128/IAI.73.9.6017-6025.2005. PMID: 16113322. PMCID: PMC1231112.**).

[007] Sabe-se que, em geral, o combate às infecções virais é coordenado por uma resposta imune do tipo Th1 somado à indução de anticorpos neutralizantes, e para protozoários como o *T. cruzi*, o processo não é diferente (**Frank, F.M., Petray, P.B., Cazorla, S.I., Muñoz, M.C., Corral, R.S., Malchiodi, E.L. 2003. Use of a purified Trypanosoma cruzi antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. Vaccine. 22(1):77-86. doi: 10.1016/s0264-410x(03)00541-3. PMID:**

14604574.; Machado, A.V., Cardoso, J.E., Claser, C., Rodrigues, M.M., Gazzinelli, R.T., Bruna-Romero, O. 2006. Long-term protective immunity induced against Trypanosoma cruzi infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. Hum Gene Ther. 17(9):898-908. doi: 10.1089/hum.2006.17.898. PMID: 16972758.; Barry, M.A., Versteeg, L., Wang, Q., Pollet, J., Zhan, B., Gusovsky, F., Bottazzi, M.E., Hotez, P.J., Jones, K.M. 2019. A therapeutic vaccine prototype induces protective immunity and reduces cardiac fibrosis in a mouse model of chronic Trypanosoma cruzi infection. PLoS Negl Trop Dis. 13(5):e0007413. doi: 10.1371/journal.pntd.0007413. PMID: 31145733. PMCID: PMC6542517.).

Assim, o uso de estratégias vacinais capazes de estimular esse tipo de resposta são abordagens promissoras. A vacina BCG, uma cepa atenuada de *Mycobacterium bovis*, é mundialmente utilizada contra tuberculose (TB) (**Bannon;, M.J., Finn, A., 1999. BCG and tuberculosis Commentary. Arch. Dis. Child. 80, 80–83.; Li, J., Zhao, A., Tang, J., Wang, G., Shi, Y., Zhan, L., Qin, C., 2020. Tuberculosis vaccine development: from classic to clinical candidates. Eur. J. Clin. Microbiol. Infect. Dis.**) e também empregada como um dos tratamentos de maior eficácia contra câncer superficial de bexiga (**Alhunaidi, O., Zlotta, A.R., 2019. The use of intravesical BCG in urothelial carcinoma of the bladder. E cancer medical science 13.**). A vacinação com BCG induz, majoritariamente, uma resposta celular mediada por linfócitos Th1, além de aumentar significativamente a indução da resposta de células Th17, a qual encontrada, recentemente, como sendo ainda mais protetora do que a resposta de células Th1 contra a infecção por *T. cruzi* (**Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J.,**

Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.; Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F. 2016. Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. PLoS Pathog. 12(10):e1005902. doi: 10.1371/journal.ppat.1005902. PMID: 27695083. PMCID: PMC5047564.; O'Neill LAJ, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nat Rev Immunol. 2020 Jun;20(6):335-337. doi: 10.1038/s41577-020-0337-y. PMID: 32393823; PMCID: PMC7212510.).

[008] Estudos têm sugerido que algumas das vacinas administradas rotineiramente em bebês e crianças, como a BCG, também tem efeitos não-específicos sobre o risco de doença e morte por outras condições, além daquelas para as quais as vacinas foram projetadas para prevenir. No caso de BCG, sua administração foi associada ao menor risco subsequente de doença e morte por outras causas, fato decorrente de mecanismos conhecidos por "trained immunity" e imunidade heteróloga (**Higgins, J.P.T., Soares-Weiser, K., López-López, J.A., Kakourou, A., Chaplin, K., Christensen, H., Martin, N.K., Sterne, J.A.C., Reingold, A.L., 2016. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ 355, i5170.; Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J., Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.**). Um estudo demonstrou que a vacinação com BCG induziu reprogramação epigenética *in vivo* de monócitos contra infecção experimental com uma vacina atenuada contra o vírus da febre amarela com papel

fundamental da IL-1b como mediador dessa resposta (**Arts, R.J.W., Moorlag, S.J.C.F.M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., Kumar, V., Xavier, R.J., Wijmenga, C., Joosten, L.A.B., Reusken, C.B.E.M., Benn, C.S., Aaby, P., Koopmans, M.P., Stunnenberg, H.G., van Crevel, R., Netea, M.G., 2018. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. Cell Host Microbe 23, 89-100.e5.**).

[009] A construção de cepas recombinantes de BCG que proporcionem maior estímulo do sistema imune tem sido explorada para melhorar sua eficácia contra TB (**Nieuwenhuizen, N.E., Kaufmann, S.H.E., 2018. Next-Generation Vaccines Based on Bacille Calmette–Guérin. Front. Immunol. 9, 121.**), aumentar seu efeito antitumoral na terapêutica de tumores de bexiga (**Begnini, K.R., Buss, J.H., Collares, T., Seixas, F.K., 2015. Recombinant *Mycobacterium bovis* BCG for immunotherapy in nonmuscle invasive bladder cancer. Appl. Microbiol. Biotechnol. 99, 3741–3754.)**, e expressar antígenos de diferentes patógenos para emprego como vetor vacinal (**Bastos, R.G., Borsuk, S., Seixas, F.K., Dellagostin, O.A., 2009. Recombinant *Mycobacterium bovis* BCG. Vaccine.; Zheng, Y., Naguib, Y.W., Dong, Y., Shi, Y., Bou, S., Cui, Z., 2015. Applications of bacillus Calmette-Guerin and recombinant bacillus Calmette-Guerin in vaccine development and tumor immunotherapy. Expert Rev. Vaccines 14, 1255–75. Marques-Neto LM, Piwowarska Z, Kanno AI, Moraes L, Trentini MM, Rodriguez D, Silva JLSC, Leite LCC. Thirty years of recombinant BCG: new trends for a centenary vaccine. Expert Rev Vaccines. 2021 Aug;20(8):1001-1011. doi: 10.1080/14760584.2021.1951243. Epub 2021 Jul 13. PMID: 34224293.**). Nesse contexto, salienta-se o potencial da construção e do uso de BCG como vetor vacinal expressando

antígenos de *T. cruzi* como ferramenta profilática contra doença de Chagas.

[010] Em uma pesquisa prévia realizada nos bancos de dados mundiais de depósito de patentes, é notória a quantidade de documentos que utilizam a plataforma BCG como fórmula vacinal contra uma gama de enfermidades. Encontramos como resultado o documento **US 6471967 B1**, referente a construção de BCG recombinante tendo uma sequência de ácido nucleico extracromossômico compreendendo gene que codifica uma proteína extracelular de 30 kDa de *Mycobacteria tuberculosis*. Como já foi descrito, nosso produto, apesar da semelhança metodológica, não será empregado contra tuberculose, mas sim contra doença de Chagas, fazendo uso de proteínas diferentes da reivindicada pela patente citada.

[011] Nossa busca contemplou ainda a reivindicação por uma composição imunogénica compreendendo BCG recombinante, em que o referido expressa pelo menos uma proteína extracelular de *Mycobacteria major* seleccionada entre proteína de 23,5 kDa, proteína de 30 kDa, proteína de 32A kDa e proteína de 32B kDa; em que uma sequência de ácido nucleico que codifica para pelo menos uma proteína extracelular de micobactéria é incorporada no(s) cromossomo(s) do BCG recombinante sob um promotor forte, de modo que a proteína é superexpressa e o BCG recombinante não abriga um marcador de resistência a antibiótico (**US 8932846 B2**). Novamente, a composição deste produto pouco se assemelha com nossa invenção, uma vez que nossa estratégia objetiva a expressão heteróloga de genes de *T. cruzi* em *M. bovis* BCG como vacina contra DC.

[012] No entanto, alguns documentos se assemelham com nosso produto. Como por exemplo, a patente que reivindica a construção de vacinas de BCG recombinantes que expressam DNA de interesse, incorporado na micobactéria, sob o controle de um promotor. Referindo-se particularmente a *M. bovis*-BCG

recombinante em que o DNA de interesse é expresso extracromossomicamente sob o controle de um promotor hsp micobacteriano, tal como hsp70 e hsp60 (**WO 1995003418 A3**). Nossa estratégia se baseia sim nesta mesma forma de construção, no entanto, nossa invenção objetiva a adição de genes de *T. cruzi* na cepa Pasteur de *M. bovis* BCG para uso como vacina vetorizada utilizando promotores diferentes dos mencionados na referida patente.

[013] Identificamos também a existência de um estudo utilizando BCG como imunizante contra doença de Chagas, intitulado **"The effect of BCG on the course of experimental Chagas' disease in mice"**. Esse trabalho visa a observação dos efeitos do tratamento com BCG não recombinante sobre a infecção de *T. cruzi* em camundongos C3H(He). Apesar de também fazer uso de *M. bovis* BCG no combate a DC, nosso invento baseia-se na utilização de uma cepa de BCG que irá expressar proteínas de *T. cruzi* (sem combinação com outros antígenos) cujas sequências gênicas foram especificamente selecionadas para clonagem em vetores de expressão em micobactérias, não contando apenas com a atividade imunológica gerada pelo BCG.

[014] O artigo intitulado **"Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypanosoma cruzi* infection"**, publicado por nosso grupo, também foi identificado em nossa busca. Ele demonstra a expressão dos fragmentos N-terminal e C-terminal da proteína transialidase e da proteína cruzipain de *T. cruzi, em M. bovis*-BCG, para utilização como imunizante. O mesmo além de reforçar o pioneirismo na utilização de BCG como vetor vacinal no combate a doença de Chagas por parte de nosso grupo, demonstra ainda a expertise do mesmo na utilização de BCG recombinante na superexpressão de antígenos. A particularidade de nossas sequências e consequentemente de

nossa construção, garante o caráter ÚNICO e INOVADOR de nosso produto.

SUMÁRIO DA INVENÇÃO

[015] A presente invenção refere-se a processos de construção, clonagem e expressão de proteínas de *Trypanosoma cruzi* na cepa de *M. bovis* BCG Pasteur. A invenção refere-se mais precisamente a busca, seleção, desenho e montagem de sequências gênicas sinteticas de *Trypanosoma cruzi* para ligação em vetores de expressão em BCG. O invento ainda se refere a síntese dos genes eleitos em vetores pUC57.

DESCRIÇÃO DETALHADA DO INVENTO

[016] A presente invenção destina-se a construção de cepas de *Mycobacterium bovis* BCG Pasteur recombinante expressando as proteínas ASP-2, TSA-1 e Tc24 de *Trypanosoma cruzi* para utilização em protótipos vacinais para Doença de Chagas. O invento é descrito em mais detalhes a seguir:

Seleção dos alvos vacinais

[017] As sequências nucleotídicas que codificam para a proteína ASP-2, a proteína TSA-1 e a proteína Tc24 de *Trypanosoma cruzi* são obtidas do NCBI www.ncbi.nlm.nih.gov (AAC47720.1; KJ668043.1; AAB08762.1). Genes sintéticos são desenhados visando a clonagem das sequência completa da proteína Tc24, além das regiões previamente descritas das proteínas ASP-2 e TSA-1. Todos os genes sintéticos são projetados com o auxílio do software Vector NTI 11 (Invitrogen™). Sítios de restrição enzimáticos (XbaI, BamHI e HindIII) são adicionados nas extremidades 5’ e 3’ das sequências, visando a clonagem no vetor pAE de expressão em

E. coli (**Ramos, C.R.R., Abreu, P.A.E., Nascimento, A.L.T.O., Ho, P.L., 2004. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal his-tagged fusion peptide. Brazilian J. Med. Biol. Res. 37, 1103–1109.**), e nos vetores de expressão em M. bovis BCG (pUS2000 e pUS977) (**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**).

Construção de BCG expressando as proteínas ASP-2, TSA-1 e Tc24 de *Trypanosoma cruzi*

[018] O gene que codofica para as regiões das proteínas ASP-2 e TSA-1, bem como a proteína Tc24 são sintetizados (Genome) e após subclonados nos vetores de expressão em *M. bovis* BCG Pasteur (pUS2000 e pUS977)(**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**). Em seguida, o produto da ligação é transformado por eletroporação em *E. coli* TOP10 para obtenção dos clones recombinantes. Após caracterização enzimática e por sequenciamento de DNA, os clones recombinantes são utilizados para transformação de *M. bovis* BCG Pasteur por eletroporação.

Cepas e condições de cultivo

[019] *M. bovis* BCG Pasteur é cultivado em meio 7H9 (Middlebrook 7H9 Broth Base) (líquido) ou 7H10 (Middlebrook 7H10 Broth Base)(sólido) com suplementação de 10% de OADC (Oleic Albumin Dextrose Catalase) e adição do antibiótico canamicina (50 mg/mL) quando necessário. O cultivo é mantido

a 37°C por 7 dias em meio 7H9 ou por 21 dias em meio 7H10. *E. coli* é cultivada em meio LB líquido ou LB-Ágar à 37°C por 16 h, com suplementação do antibiótico canamicina (50 mg/mL) quando necessário.

BREVE DESCRIÇÃO DAS FIGURAS

[0201] **Figura 1:** A figura 1 demonstra o mapa do vetor pUS2000/ASP-2-261 construído contendo a sequência sintética da porção do gene ASP-2.

[021] **Figura 2:** A figura 1 demonstra o mapa do vetor pUS2000/TSA-1-617 construído contendo a sequência sintética do fragmento do gene TSA-1.

[022] **Figura 3:** A figura 1 demonstra o mapa do vetor pUS2000/TC24 construído contendo a sequência sintética do gene Tc24.

[023] **Figura 4**: A figura 4 demonstra a caracterização dos clones recombinantes (pUS2000/ASP-2-261; pUS977/ASP-2-261; pUS2000/TSA-1-617; pUS977/TSA-1-617) através de digestão enzimática utilizando as enzimas de restrição XbaI e HindIII, e visualização em gel de agarose. Em A: pUS977/ASP-2-261, B: pUS2000/ASP-2-261, C: pUS977/TSA-1-617, D: pUS2000/TSA-1-617.

EXEMPLO

EXEMPLO 1 – Avaliação da construção dos vetores

[024] Este exemplo ilustra a construção dos plasmídeos pUS977 e pUS2000 contendo os genes sintéticos que codificam para as proteínas ASP-2 e TSA-1 de *T. cruzi*. A caracterização da construção é realizada por digestão enzimática com as enzimas de restrição XbaI e HindIII (**figura 4**), onde pode

ser observado um fragmento de 1.354 pares de bases (pb) que corresponde ao tamanho do gene sintético ASP-2 para os plasmídeos pUS2000/ASP-2-261 e pUS977/ASP-2-261. Para o gene TSA-1 o fragmento gerado após a digestão do DNA dos plasmídeos pUS2000/TSA-1-617 e pUS977/TSA-1-617, que corresponde ao gene é de 1.864 pb. Os resutados da caracterização enzimática mostram que os plasmídeos pUS977 e pUS2000 contem as sequências gênicas do gene ASP-2 e TSA-1.

FIGURAS

Figura 1.

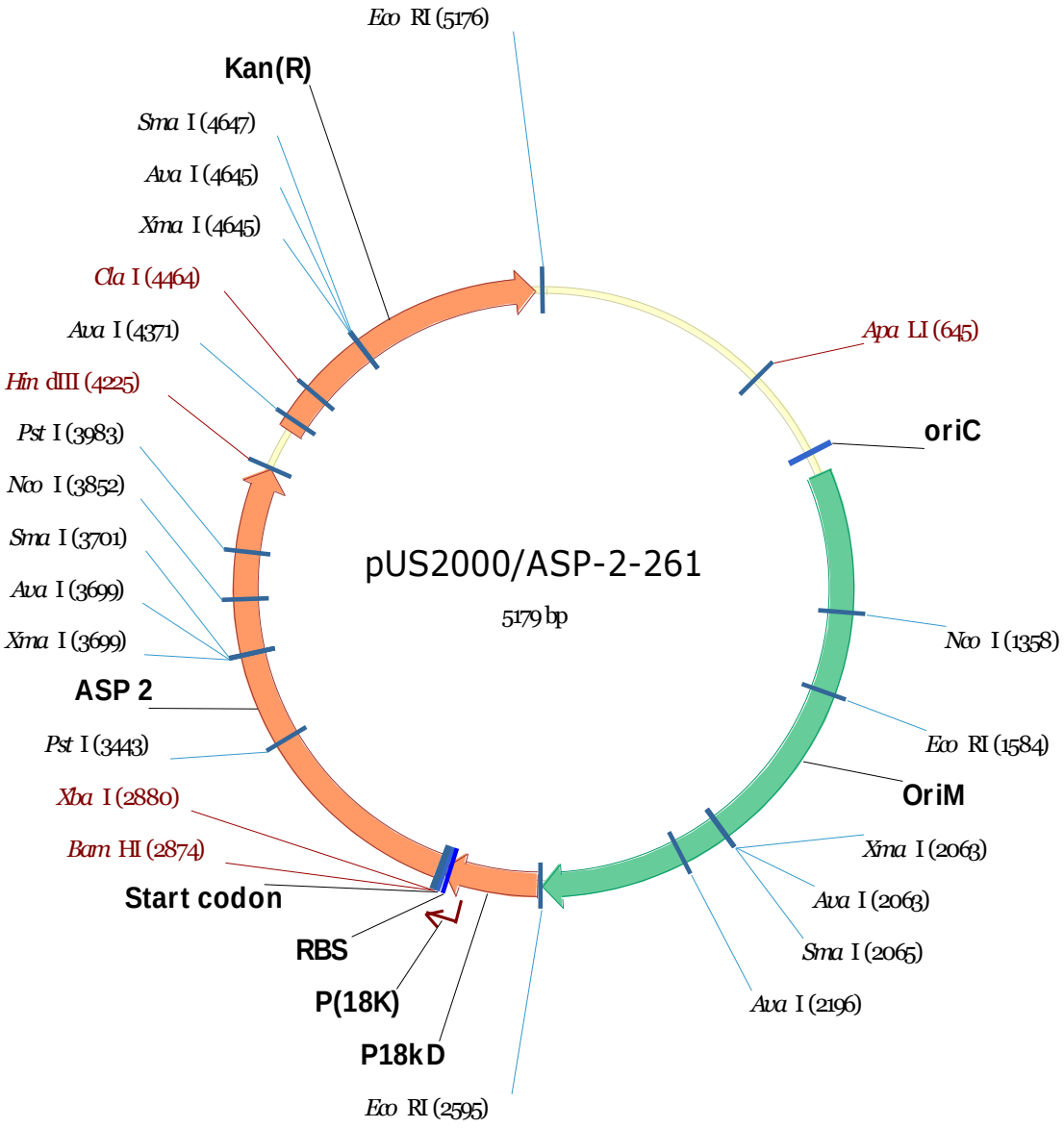


Figura 2.

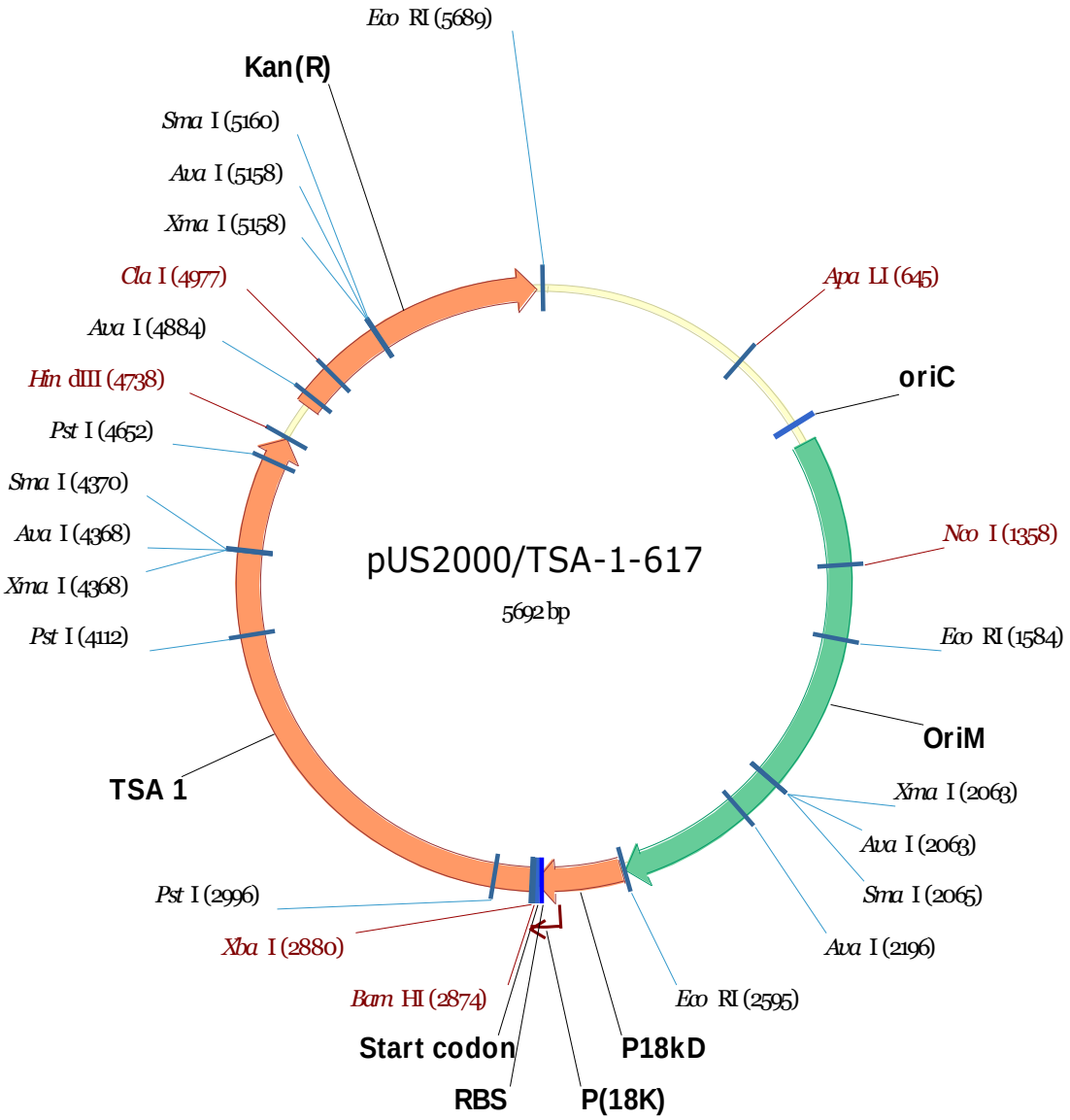

Eco RI (5689)
Kan(R)
Sma I (5160)
Ava I (5158)
Xma I (5158)
Cla I (4977)
Ava I (4884)
Hin dIII (4738)
Pst I (4652)
Sma I (4370)
Ava I (4368)
Xma I (4368)
Pst I (4112)
TSA 1
Pst I (2996)
Xba I (2880)
Bam HI (2874)
Start codon
RBS
P(18K)
P18kD
Eco RI (2595)
Ava I (2196)
Sma I (2065)
Ava I (2063)
Xma I (2063)
OriM
Eco RI (1584)
Nco I (1358)
oriC
Apa LI (645)
pUS2000/TSA-1-617
5692 bp

Figura 3.

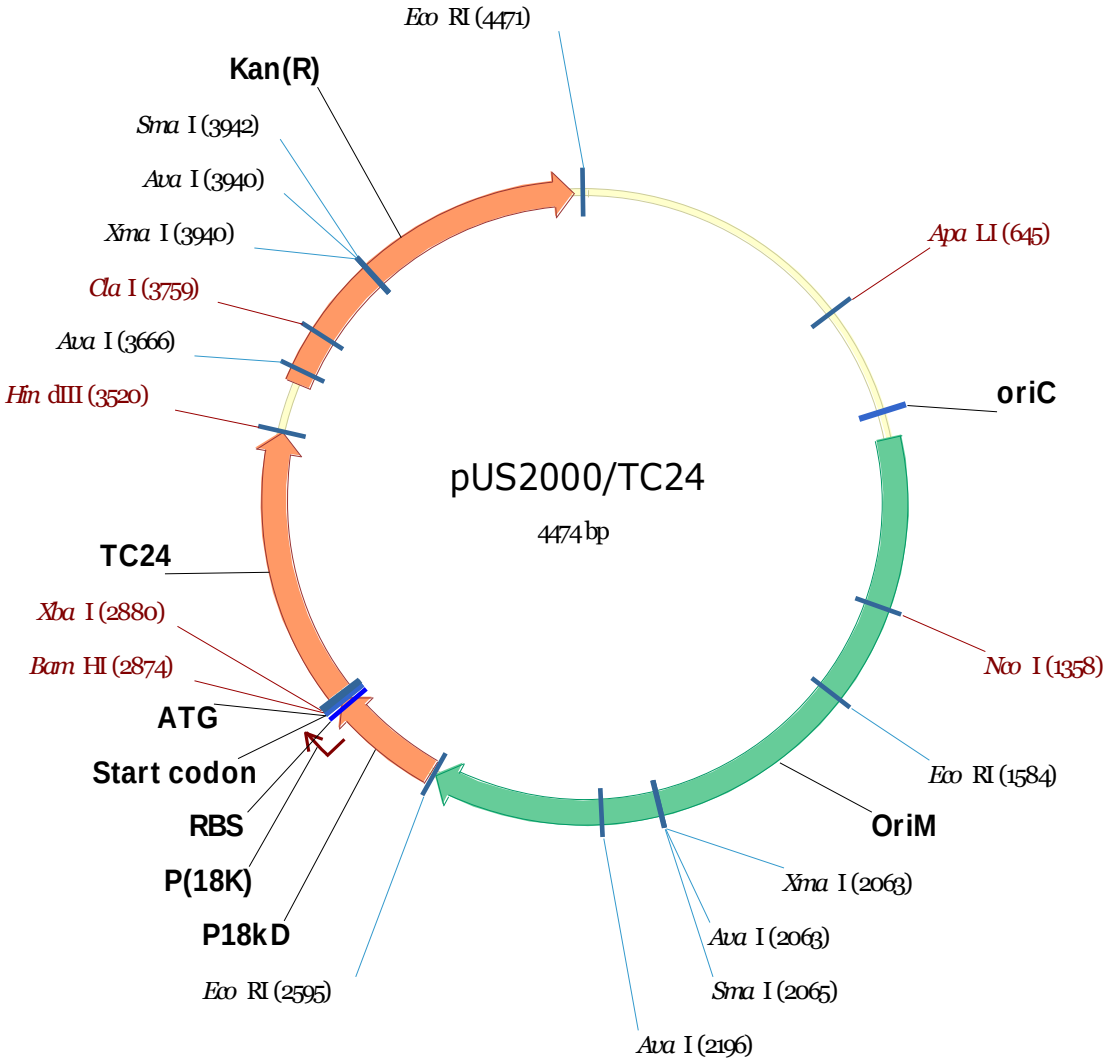
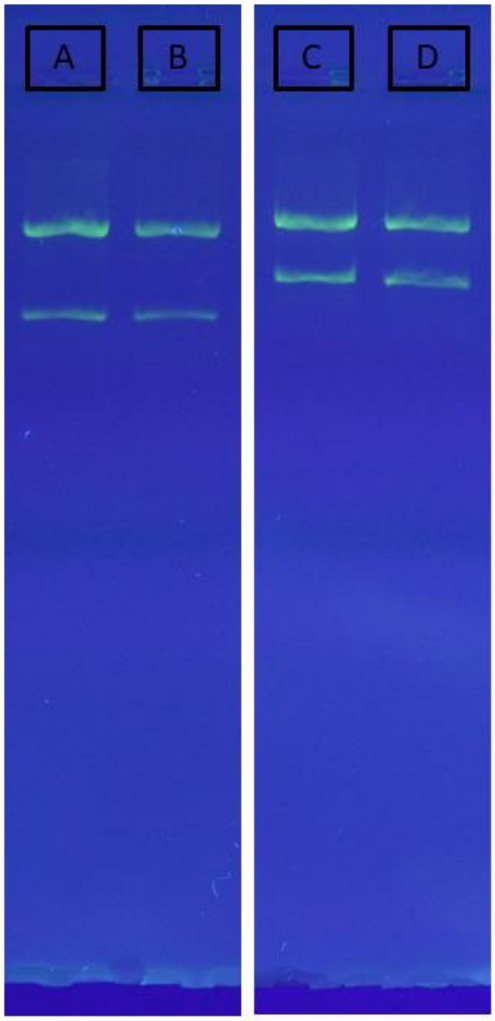

Eco RI (4471)
Kan(R)
Sma I (3942)
Ava I (3940)
Xma I (3940)
Cla I (3759)
Ava I (3666)
Hin dIII (3520)
TC24
Xba I (2880)
Bam HI (2874)
ATG
Start codon
RBS
P(18K)
P18kD
Eco RI (2595)
pUS2000/TC24
4474 bp
Apa LI (645)
oriC
Nco I (1358)
Eco RI (1584)
OriM
Xma I (2063)
Ava I (2063)
Sma I (2065)
Ava I (2196)

Figura 4.



A - pUS977/ASP-2-261
B - pUS2000/ASP-2-261
C - pUS977/TSA-1-617
D - pUS2000/TSA-1-617

3.4) Manuscrito 3

Manuscrito a ser submetido a revista *Vaccine*

Recombinant *Mycobacterium bovis* BCG expressing the ASP-2 and TC24 proteins from *Trypanosoma cruzi* as a vaccine strategy against Chagas disease

Guilherme Senna dos Santos[1], Bárbara da Rocha Fonseca[1], Fernanda Severo Sabedra Sousa[2], Fabiana Kommling Seixas[2], Sibele Borsuk[1]*

[1] Laboratório de Biotecnologia Infecto-parasitária, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brasil, 96010–900

[2] Laboratório de Biotecnologia do Câncer, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, RS, Brasil, 96010–900

*Corresponding author: Laboratório de Biotecnologia Infecto-Parasitária, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas (UFPel) – Campus Universitário s/n, Prédio 19 –, Pelotas, RS, Brazil, 96010-900. Phone: +55 53 3275 7350. E-mail: sibeleborsuk@gmail.com

Abstract

Although drugs have been employed over the past years to treat Chagas disease, they work mostly on the acute phase of infection, where diagnosis is hardly ever made, and present a plethora of negative side effects that ends in discontinuation of treatment. Consequently, to deal with this disease, prophylaxis seems to be a better strategy, with recombinant subunit vaccines showing promising results. Among those, *Mycobaterium bovis* Bacillus Calmette-Guérin (BCG) has recently been employed as vector for delivering *T. cruzi* antigens with positive results on immune response stimulus and protection against the infection. Following this perspective, this study aimed to characterize the immune response elicited by recombinant BCG expressing a fraction of the amastigote surface protein 2 (ASP-2) and the 24 kDa flagellar calcium-binding protein (TC24) of *T. cruzi*. To accomplish this, four groups of BALB/c female mice (n = 10) were vaccinated with 0.9% saline solution (Group A), non-transformed BCG Pasteur (Group B), rBCG/pUS977/asp-2 (Group C) or rBCG/pUS977/tc24 (Group D). Cellular responses, assessed by cytokine expression from cultured and protein stimulated splenocytes, were statistically higher for both vaccinal formulations when compared with basal levels (Group A) and non-transformed BCG (Group B). Group D achieved better results for interleukins 10 and 17, while interferon γ was greatly stimulated by vaccination with Group C. Even though further analyses are needed to evaluate the full efficacy of the constructions, the here presented results exhibit the potential of BCG vectored vaccines in eliciting Th1/Th2/Th17 mixed immune responses.

Keywords: Recombinant vaccine; American trypanosomiasis; subunit vaccine; bacterial vector; immune response.

1. INTRODUCTION

Chagas disease is a condition triggered by the protozoan *Trypanosoma cruzi*, which transmission occurs through the faeces of triatomine insects, commonly called kissing bugs (Centers for Disease Control and Prevention of the U.S., 2021). According to the World Health Organization, Chagas disease ranks among the most significant neglected tropical diseases globally, largely due to the disease's complexity, the difficulties in diagnosis and treatment, the high levels of underreporting, and the limited investment in research (De Fuentes-Vicente et al., 2023). Drugs benznidazole and nifurtimox have been employed to treat *T. cruzi* infections, however, their effectiveness varies depending on the stage of infection. The treatment is also associated with various side effects, highlighting the critical need for early diagnosis and timely treatment, regardless of the infection stage (Pérez-Molina and Molina, 2018). To mitigate the impact of Chagas disease, a range of strategies have been pursued to develop safe and effective treatments against it. While new drugs are being developed, it is widely recognized that vaccines play a crucial role in the global elimination or control of many infectious diseases (Rodrigues and Plotkin, 2020). Consequently, several vaccine prototypes for Chagas disease are under development. However, creating a vaccine that provides robust protection is challenging, especially against a parasite that has numerous strategies to evade the host's immune system (Bivona et al., 2020; Maldonado et al., 2022).

Various approaches are being investigated to develop effective control measures and reduce the damage caused by the disease, such as vectorized bacterial vaccines (Bivona et al., 2020). Using bacteria as vectors ensures a reliable and effective delivery of antigens, potentially leading to more durable and potent immunity compared to traditional vaccines. This approach could enhance the fight against Chagas disease by inducing a strong and specific immune response, capable of triggering both humoral and cellular immunity, which is essential for combating intracellular pathogens like *T. cruzi*. Additionally, bacterial vectors can be engineered to present multiple antigens simultaneously, offering broader protection against different strains of the parasite (Bivona et al., 2018; Bontempi et al., 2020; Quintana et al., 2018).

A promising strategy recently explored involves using *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) as a vector to express antigens against *T. cruzi*. BCG, a live attenuated vaccine widely used to prevent tuberculosis, is particularly well-suited for this role due to its stability, safety, ease of administration, cost-effectiveness, and strong adjuvant properties (Tanner and McShane, 2020). Furthermore, BCG replicates in macrophages and dendritic cells, enabling it to effectively present antigens to the host immune system and confer long-term immunity (Oliveira et al., 2019). Another interesting characteristic of BCG is its capability of inducing a “trained immunity.” While the exact mechanisms remain unknown, data suggests that cross protection may trigger “heightened” alertness in the innate immune system against non-tuberculous diseases (Cho et al., 2021). Although the humoral response was relatively weak, Bontempi et al. (2020) observed a strong delayed-type hypersensitivity (DTH) response after vaccinating with recombinant BCG expressing the *T. cruzi* trans-sialidase protein, indicating a cellular response compatible with BCG. When mice were challenged, there was an increase in IFN-γ production by CD8+CD107+ T cells, suggesting the priming and activation of CD8 lymphocytes in a polyfunctional manner. This protocol produced a mixed Th17 and Th1 response, a desirable characteristic of the BCG vaccine previously reported in both mice (Costa et al., 2014) and humans (Loxton et al., 2017), as the Th17 response is crucial for protection against *T. cruzi* (Matos et al., 2017).

To achieve a more robust and effective immune response, the study aimed to use a similar strategy to Bontempi et al. (2020), but with a recombinant *M. bovis* BCG Pasteur vaccine expressing a fragment of the ASP-2 or the Tc24 protein of *Trypanosoma cruzi* as the chosen antigen. ASP-2 is known to induce strong antibody responses and is a target of *T. cruzi*-specific CD8+ cytotoxic T lymphocytes when used alone (Ribeiro et al., 2019), while TC24 as an antigen can decrease parasitaemia and cardiac burden in immunized animals (Dumonteil et al., 2020). This approach seeks to maximize the immune response generated by the recombinant BCG vaccine against Chagas disease by utilizing a more defined, well-characterized, and conserved protein across different *T. cruzi* strains as the antigen, in conjunction with the immune response already elicited by BCG.

2. MATERIALS AND METHODS

2.1 Strains and culture conditions

Escherichia coli BL21 (DE3) Star and TOP10 were grown in liquid Luria-Bertani (LB) medium or LB-Agar, 100 µg/mL kanamycin was added when necessary. The bacteria were grown in a bacteriological oven or under agitation in a shaker for 16 h at 37 ºC. *M. bovis* BCG Pasteur was grown in 7H9 medium plus OADC (10%) and Tween 80 (20%) along with 25 µg/mL kanamycin, when necessary. The BCG was grown in cylindric glass flasks under constant agitation in a shaker for seven days and in cultivation bottles placed in bacteriological oven, both at 37 ºC.

2.2 Cloning of the *asp-2* gene in the *pUS* vector and transformation into M*. bovis* BCG Pasteur

The genes of interest, *asp-2* and *tc24*, were previously synthesized by GenOne, conform designed (from amino acid 261 to 706 from the cds of the ASP-2 protein access AAC47720.1 at GenBank NCBI https://www.ncbi.nlm.nih.gov/protein/1684907 and the whole cds of the TC24 protein access AAB08762.1 at GenBank NCBI https://www.ncbi.nlm.nih.gov/protein/1556494), and sent inserted in *pUC57* carrier vector. To remove the gene from the carrier plasmid a digestion with the restriction enzymes *XbaI* and *HindIII* was performed. Shortly after, purification of the *asp-2* gene was performed using GFX PCR DNA (Invitrogen). The purified *asp-2* gene was ligated to the *XbaI* and *HindIII* sites of the *pUS977* vector (Dellagostin et al., 1993). The ligation was then transformed into electrocompetent *E. coli* TOP10 strain cells and resultant transformants were plated on LB agar medium containing 50 mg/mL kanamycin. The obtained recombinant plasmid was characterized by digestion with the same restriction enzymes, followed by DNA sequencing.

The recombinant plasmids *pUS977/asp-2* and *pUS977/tc24* were transformed into competent *M. bovis* BCG Pasteur cells by electroporation, as described by Leal et al. (2018). The resulting cells were plated on 7H10 medium containing 25 mg/mL kanamycin and incubated at 37 ºC for 28 days.

2.3 Recombinant rASP-2 and rTC24 expression

The previously constructed and characterized pAE/asp-2 and pAE/tc24 plasmids were transformed into *E. coli* strain BL21 (DE3) Star by heat shock. The transformed bacteria were cultured in liquid LB medium with 100 mg/mL ampicillin for the selection of recombinant clones. Incubation was carried out in an orbital shaker at 37 ºC until optical density reached 0.6 – 0.8 DO_{600}. Protein expression was induced with 1 mM isopropyl α-D-thiogalactoside (IPTG), followed by 3 hours of incubation at 37 ºC. Western blotting assays were performed using horse-radish peroxidase (HRP)-conjugated anti-6 x His tag monoclonal anti-body (Sigma-Aldrich) to confirm the expression of the recombinant proteins rASP-2 and rTC24. The proteins were purified via nickel-affinity chromatography on Sepharose column (GE Healthcare). The purity of the proteins was determined by 12% SDS-PAGE, and their concentration was estimated using a commercial kit (BCA; Pierce).

2.4 Production of polyclonal serum

Female Balb/c mice (n = 8; 6-8 weeks old) were inoculated, subcutaneously, with 300 µg of the recombinant rASP-2 or rTC24 purified protein associated with aluminum hydroxide adjuvant (15%). A booster dose was administered after 21 days for two immunization events in total. Blood was collected from the submandibular plexus vein, and the serum was collected and stored at -20 ºC.

2.5 Evaluation of protein expression in rBCG

To evaluate the expression of the protein in rBCG, 10 mL of the culture of rBCG transformed with *pUS977/asp-2* and *pUS977/tc24* were centrifuged at 4000 rpm for 10 min. The resulting pellet was suspended in 1 mL of Tris-HCl (50 mM) and treated with a ribolyser six times for 30 s with glass pearls of 0.1 µm. Samples were centrifuged at 6000 g for 2 min and the pellets were resuspended in 100 µL of 5X SDS-PAGE buffer. The samples were, then, boiled and analyzed via 12% SDS-PAGE. Proteins in the gel samples were electro-transferred to a nitrocellulose membrane (Nitrocellulose Blotting Membrane – GE Healthcare).

Next, polyclonal antibodies specific for rASP-2 proteins (1:50) were added to the membrane for 1 h at room temperature and washed thrice with PBS-T (0.05% Tween 20). HRP-conjugated anti-mouse total IgG antibody (Sigma-Aldrich) diluted 1:4000 in PBS-T was added to the membrane, followed by incubation for 1 h. The reaction was detected by the addition of a chemiluminescent substrate for peroxidase ECL kit (GE Healthcare), and subsequent exposure of the membrane to an X-ray film.

2.6 Vaccination protocol

To formulate the vaccines, the optical density of the rBCG cultures was measured and the volume was adjusted to obtain 10^7 CFU/mL in 1X PBS. For the immunization experiment, female Balb/c mice (n = 40; 6-8 weeks old) were placed in four groups (G1, G2, G3 and G4; n = 10 animals per group) as shown in the following table. The vaccinated mice received two doses (100 µL per dose) subcutaneously, with 21 days of interval between them. No animal was challenged in this experiment.

Group	First dose	Second dose
G1: 0.9% Saline solution	100 µL 0.9% Saline solution (S.C.)	100 µL 0.9% Saline solution (S.C.)
G2: BCG Pasteur	100 µL 10^7 UFC BCG Pasteur (S.C.)	100 µL 10^7 UFC BCG Pasteur (S.C.)
G3: rBCG/pUS977/ASP-2	100 µL 10^7 UFC rBCG/pUS977/ASP-2 (S.C.)	100 µL 10^7 UFC rBCG/pUS977/ASP-2 (S.C.)
G4: rBCG/pUS977/TC24	100 µL 10^7 UFC rBCG/pUS977/TC24 (S.C.)	100 µL 10^7 UFC rBCG/pUS977/TC24 (S.C.)

*(S.C.) = Subcutaneous.

The protocol represented here described was approved by the ethics committee on animal experimentation of the Federal University of Pelotas (CEEA-UFPel), under permit number 23110.031479/2021-17.

2.7 Blood collection and humoral immune response

Blood was collected one day prior to vaccination (day 0), 21 days after administering the first dose (day 21) and 21 days after administering the second dose (day 42). Blood samples were used to obtain serum, utilized to perform indirect ELISA in order to determine the humoral immune response through IgG antibody levels produced in each group.

In this assay, polystyrene 96-well flat-bottom plates (TPP – Techno Plastic Products) were coated with 0.5 mg/mL rASP-2 or rTC24 in bicarbonate buffer (pH = 9.8) and incubated at 4 °C for 16 h. Then, each well was washed with PBS-T (0.05% Tween 20) and blocked with 100 µL of 5% skim milk powder diluted in PBS, followed by another incubation at 37 °C for 1 h. After, the plates were washed with PBS-T thrice, and the mouse serum samples were added (100 µL/well) at a dilution of 1:50, followed by new incubation at 37 °C for 1 h and three washes with PBS-T. HRP-conjugated anti-mouse total IgG antibodies (Sigma-Aldrich) were added (100 µL/well) at a dilution of 1:5000 in PBS-T. Following incubation at 37 °C for 1 h and five washes with PBS-T, the revelation solution (composed of 200 mol of ortho-phenylenediamine [OPD, Sigma Aldrich] dissolved in 10 mL of citrate–phosphate buffer at pH 7.6, and 50 µL of H2O2) was added 100 µL/well. A final incubation at room temperature and absence of light was performed for 15 minutes. Lastly, the absorbance was measured with help of an ELISA plate reader (Mindray) at 450 nm. All samples were analyzed in duplicate.

2.8 Collection of splenocytes and cellular immune response

The same animals used for the humoral immune response evaluation were used for the analysis of the cellular immune response. At the end of experiment (day 43). The mice were euthanized, and their spleens were collected in a sterile environment. Spleen cell suspensions extracted from each group were cultivated

using the Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (LGC Biotec). The cells were counted in a Neubauer chamber and cell density was standardized to 5 × 106 cells/mL. The viability of these cells was assessed using the Trypan Blue dye (Vetec) exclusion method. Next, 12-well plates (MaxiSorp, Nunc) were filled with 3 mL of the standardized cell density solution in triplicate. The cells were stimulated with the culture medium (G1), 5 µg/mL of each rASP-2 and rTC24 (G2), 10 µg/mL of rASP-2 (G3) and 10 µg/mL of rTC24 (G4), or 8 µg/mL concanavalin A mitogen (positive control). Cells were then incubated with 5% CO2 at 37 °C for 48 h.

After harvest, RNA was extracted from the cells using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. The cDNA was synthesized with 1 µg of total RNA using the cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's protocol. The cDNA samples were used to quantify the cytokines IFN-γ, IL-4, IL-6, IL-10, and IL-17 through Statagene Mx3005P Real-Time PCR System (Agilent Technologies) and the SYBR Green reagent (Applied Biosystems). The primer sequences used were F 5′ GCG TCA TTG AAT CAC ACC TG 3′ and R 5′ TGA GCT CAT TGA ATG CTT GG 3′ for IFN-γ, F 5' CCA AGG TGC TTC GCA TAT TTF 3' and R 5' ATC GAA AAG CCC GAA AGA GT 3' for IL-4, F 5' CCA GAA ACC GCT ATG AAG 3' and R 5' CAC CAG CAT CAG TCC CAA GA 3' for IL-6, F 5′ TTT GAA TTC CCT GGG TGA GAA 3′ and R 5′ ACA GGG GAG AAA TCG ATG ACA 3′ for IL-10, and F 5′ GCT CCA GAA GGC CCT CAG A 3′ and R 5′ AGC TTT CCC TCC GCA TTG A 3′ for IL-17. The results were normalized using the glycerol-3-phosphate dehydrogenase (GPDH) gene and all samples were analyzed in duplicate.

2.9 Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.) and analyzed using GraphPad Prism (version 7.0) for Windows (Graph-Pad Software, San Diego, CA). The differences between the IgG and cytokines levels in different groups were analyzed by one-way ANOVA, followed by Tukey's post hoc tests. All differences among and between groups were considered to be statistically significant at $p < 0.05$.

3. RESULTS

3.1 Expression of recombinant ASP-2 and TC24 in *E. coli*

The recombinant proteins rASP-2 and rTC24 were expressed in *E. coli* and purified, yielding 200 mg/L. To confirm the identity of the recombinant protein, a Western blotting assay was performed using anti-histidine monoclonal antibody. One of the proteins showed a band of approximately 50 kDa, which was expected for rASP-2 (Figure 1A), while the other presented a band of approximately 25 kDa, which was expected for rTC24 (Figure 1B).

Figure 1. A. Western blotting utilizing monoclonal antibody anti-6x His tag (Sigma Aldrich): (1) purified rASP-2, approximately 50 kDa; (2) Molecular weight standard. B. Western blotting utilizing monoclonal antibody anti-6x His tag (Sigma Aldrich): (1) Molecular weight standard; (2) purified rTC24, approximately 25 kDa.

3.2 Expression of recombinant ASP-2 and TC24 in rBCG

The expression of the recombinant rBCG was confirmed by the Western blotting assays using polyclonal antiserum anti-rASP-2 or anti-rTC24. The proteins expressed in *M. bovis* BCG exhibited the expected size of 50 kDa and 24 kDa, while no protein expression was detected in *M. bovis* BCG Pasteur (non-transformed cells), which confirmed the expression in recombinant BCG (transformed with pUS977/asp-2 or pUS977/tc24) (Figure 2 A and B).

Figure 2. Western blotting assay, performed to assess the expression in rBCG, using: (A) the anti-rASP-2 antibody: (1) purified rASP-2, showing approximately 50 kDa; (2) untransformed BCG Pasteur (negative control); (3) rBCG/pUS977/asp-2; (B) the anti-rTC24 antibody: (1) purified rTC24, showing approximately 25 kDa; (2) untransformed BCG Pasteur (negative control); (3) molecular weight standard; (4) rBCG/pUS977/tc24.

3.3 Humoral immune response

The production of total IgG antibodies against the rASP-2 and rTC24 proteins were evaluated using indirect ELISA, where results showed that the specific total IgG production rates among the vaccinated groups (G2, G3 and G4) were not statistically significant on days 0, 21 and 42 (Figures 3 and 4).

Figure 3. Total IgG antibody level induced by rBCG/pUS977/asp-2 vaccine formulation in BALB/c mice (n = 10/group) at days 0, 21 and 42 of experimentation. Results are presented as mean value (bars) ± standard deviation of absorbance (nm), obtained by indirect ELISA assay. “*” indicate statistical difference between groups (p > 0,05).

Figure 4. Total IgG antibody level induced by rBCG/pUS977/tc24 vaccine formulation in BALB/c mice (n = 10/group) at days 0, 21 and 42 of experimentation. Results are presented as mean value (bars) ± standard deviation of absorbance (nm), obtained by indirect ELISA assay. “*” indicate statistical difference between groups (p > 0,05).

3.4 Cellular immune response

The production of specific anti-ASP-2 and anti-TC24 cytokines were assessed using real-time PCR, with the results displayed in Figure 5. It can be seen that the experimental group C exhibited a marked increase in the levels of Interferon γ (INF-γ) and Interleukin 4 (IL-4) (Fig. 5A and D). In contrast, the other formulation containing the rTC24 protein (group C) did not show a significant production of IFN-γ, with levels comparable to the basal amounts produced by the animals in the control group (group A). However, group C did promote the most substantial increase in IL-10 and IL-17 levels (Fig.5B e F).

Apart from IFN-γ, group B influenced the levels of other cytokines, leading to an increase in IL-4, IL-10, and IL-17 expression. Despite this, the immune response induced by group B was less pronounced than that of groups C or D, which achieved a higher expression of these cytokines. The only cytokine that showed similar patterns across groups B, C, and D was Interleukin 6. All experimental groups exhibited a significant reduction in IL-6 levels compared to the basal levels observed in the control group (group A).

Figure 5. Cytokine level induced by the different vaccine formulations in Balb/c mice (n = 10/group) after 42 days of experimentation. Results are presented as mean value (bars) ± standard deviation, obtained through qPCR. "*" indicate statistical difference between groups (p > 0,05).

4. DISCUSSION

Recently, several studies (Bastos et al., 2009; Bontempi et al., 2020; Kilpeläinen et al., 2018; Oliveira et al., 2019) have reported the use of recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) as a delivery platform for subunit protein vaccines, demonstrating its ability to confer immune protection against various pathogens. This system induces a T-cell-mediated immune response, which is essential for combating intracellular pathogens (Bastos et al., 2009). In the context of *Trypanosoma cruzi* infection, this immune response profile is particularly desirable for effective infection control (Tarleton, 2015). In this study, we constructed and evaluated two different recombinant BCG vaccines, one expressing a fragment of the ASP-2 antigen and another expressing the whole sequence of the TC24 protein, targeting this specific immune response profile.

Chagas disease is a chronic and life-threatening condition for which current treatments are suboptimal in terms of efficacy (Jiménez et al., 2019; Ramírez-Toloza et al., 2020). Prophylactic measures against *T. cruzi* offer a promising avenue for achieving the desired protective outcomes. The advantages include the ability to induce minimal to no adverse reactions, prevent cardiac and gastrointestinal complications, and enable usage during pregnancy to avert congenital Chagas disease—benefits that are particularly noteworthy (Lee et al., 2010). Additionally, the dual functionality of such interventions, potentially serving as both prophylactic and therapeutic vaccines for patients in the indeterminate or chronic stages of the disease, further highlight their importance (Dumonteil et al., 2019, 2012).

To characterize the cellular immune response elicited by the vaccine formulations and to assess the potential efficacy of each, the mRNA expression levels of five cytokines (IFN-γ, IL-4, IL-6, IL-10, and IL-17) were quantified. Group

C, consisting of mice vaccinated with two doses of rBCG/pUS977/asp2, exhibited the highest induced levels of interferon γ (IFN-γ), a critical proinflammatory cytokine in infection management (Acevedo et al., 2018). IFN-γ plays a pivotal role in orchestrating the Th1 immune response, directly facilitating the activation of macrophages, which produce nitric oxide crucial for *Trypanosoma cruzi* clearance (Cristovão-Silva et al., 2021). Additionally, IFN-γ promotes the differentiation of CD4+ and CD8+ T cells and induces chemokine production, thereby recruiting T cells to tissues during the acute phase of infection (Macaluso et al., 2023). Surprisingly, all vaccinated groups, including Group C, demonstrated statistically relevant induced levels of interleukin-4 (IL-4) compared to the saline control group. Although the role of IL-4 in Chagas disease is not fully elucidated, it is predominantly associated with increased susceptibility to infection and a Th2-type response, acting as a regulator and suppressor of IFN-γ production (Soares et al., 2001).

Interleukin-10 (IL-10) is another cytokine of significance in the context of Chagas disease. Despite its anti-inflammatory properties, IL-10 expression is crucial in preventing pathological immune responses during *T. cruzi* infection. Research involving IL-10-deficient mice has shown that these animals produce lower levels of IFN-γ and IL-2, exhibit impaired CD8+ T cell expansion and proliferation, display higher expression of inhibitory surface receptors on CD8+ T cells, and have reduced survival rates (Pino-Martínez et al., 2018). Furthermore, IL-10 has been demonstrated to protect mice from fatal myocarditis and reduce parasitic burden during the acute phase of *T. cruzi* infection, with IL-10-producing CD8+ T cells identified as the primary source of this cytokine (Roffê et al., 2012). Notably, Group D produced the highest levels of interleukin-10, supporting the coordinated role of these cytokines in the immune response observed.

Group D, consisting of mice that received two vaccinations with rBCG/pUS977/tc24, exhibited an immune response profile that was markedly different from Group C. The levels of IFN-γ in this group were not statistically different from those observed in Group A, indicating that the immune response in Group D remained at basal levels following vaccination with this formulation. However, this should not be interpreted as a negative outcome. The immune response to Chagas disease is highly complex, with evidence suggesting that an optimal response involves a balanced Th1/Th2/Th17 profile. In such a balanced

immune response, the tightly regulated production of pro-inflammatory and anti-inflammatory cytokines and chemokines facilitates parasite elimination while minimizing inflammatory and fibrotic damage to the host. The equilibrium between pro-inflammatory and anti-inflammatory responses is critical for effective parasite clearance without causing excessive host tissue damage. For example, while high levels of IFN-γ are beneficial during the early stages of infection, excessive IFN-γ during the chronic phase can lead to tissue damage and exacerbate symptoms (De Alba-Alvarado et al., 2023). Notably, Chagas patients with cardiac manifestations exhibit higher levels of IFN-γ compared to those with the indeterminate form of the disease (Chevillard et al., 2018; Gómez-Olarte et al., 2019). Furthermore, studies have demonstrated that chronic exposure to inflammatory mediators in infected patients can result in dysfunctional CD8+ T cells, potentially due to the nitration of *Trypanosoma cruzi* surface proteins, which leads to the unresponsiveness and apoptosis of these cells (Sanmarco et al., 2016).

Both recombinant BCG groups (C and D) exhibited reduced levels of IL-6 compared to Group A. Interleukin-6 plays a key role in regulating the production of IL-1β-induced nitric oxide via the inflammasome (Gomes Dos Santos et al., 2020), which helps control oxidative stress and protect mice infected with *T. cruzi*. IL-6 has also been shown to support the survival and effector functions of human CD8+ T cells in Chagas patients, characterizing a Th1-type immune response (Sanmarco et al., 2016). While the repression of IL-6 observed in the rBCG/pUS977/tc24 group aligns with its overall Th2 profile, it was unexpected in the other vaccine formulation, which exhibited a tendency towards a Th1-oriented response. Conversely, Group B, composed of mice vaccinated with non-transformed *M. bovis* BCG Pasteur, also displayed lower levels of IL-6 than the saline control group (A), suggesting that the observed IL-6 repression may be an effect of the bacterial vector rather than the antigen itself.

The induction of IL-17 expression, which plays a crucial role in the survival of parasite-specific effector CD8+ T cells (Sousa et al., 2017), was also evaluated. Th17 cells have been hypothesized to provide greater protection against *T. cruzi* compared to Th1 cells, contributing to both extracellular and intracellular immunity (Cai et al., 2016). Notably, patients with Chagas disease who exhibit mild cardiomyopathy have been found to have higher levels of IL-10

and IL-17, whereas those with severe cardiomyopathy symptoms show reduced IL-17 levels (Guedes et al., 2012). Additionally, patients with cardiac manifestations of Chagas disease have fewer circulating Th17 cells compared to those with the indeterminate form and uninfected individuals (Magalhães et al., 2013). Collectively, these findings suggest that IL-17-producing cells—including NK cells, B cells, Th17 cells, and IL-17-secreting CD8+ T cells (Bermejo et al., 2013; Bontempi et al., 2020)—are integral to maintaining and enhancing parasite-specific CD8+ T cell immune responses. This immune profile is particularly stimulated by vaccination with rBCG/pUS977/tc24 (Group D), which exhibited significantly higher IL-17 levels compared to any other group. In contrast, Group C elicit a far lower induction of this cytokine, while Group B, consisting of non-transformed BCG, was able to induce IL-17 to the same extent as Group C.

The amastigote surface protein 2 (ASP-2) is expected to be surface-anchored via glycosylphosphatidylinositol (GPI) structures and may be released into the host cell cytoplasm during the intracellular development cycle of the parasite (Rodríguez-Bejarano et al., 2021). ASP-2 contains numerous major histocompatibility complex (MHC) class I-restricted epitopes that are recognized by specific cytotoxic T cells derived from both mice and humans. These CD8+ T cells, targeting the amastigote forms of the parasite, can lyse *T. cruzi*-infected cells and produce critical pro-inflammatory cytokines such as IFN-γ, which contribute to the elimination of intracellular parasites. Moreover, immunization with ASP-2 fragments or peptides has been shown to generate specific type 1 IFN-γ-producing and cytotoxic T cells in vaccinated mice (Nogueira et al., 2013). Consequently, ASP-2 is considered one of the most effective targets for eliciting a robust host immune response, as evidenced by the responses generated in Group C.

On the other hand, regarding the 24 kDa flagellar calcium-binding protein (TC24), despite the promising results of this vaccine formulation, which induces a Th2/Th17 profile with crucial cytokines for combating Chagas disease, such as IL-10 and IL-17, it is important to note the divergence in relation to the observed levels of expressed interferon γ and data present in other papers. Recombinant subunit vaccines (Barry et al., 2019; Martinez-Campos et al., 2015; Seid et al., 2017) and DNA vaccines (Dumonteil et al., 2004; Sanchez-Burgos et al., 2007) using TC24 as antigen were unanimous in inducing a significant increase in the

expression of IFN-γ. However, this induction, together with the achievement of a Th1 profile, is associated with several factors in addition to the antigen itself. Both (Martinez-Campos et al., 2015) and (Seid et al., 2017) highlight the influence of the adjuvant on the immune response profile when evaluating the induction of cytokines. For example, the use of the recombinant protein in emulsion with the E6020 adjuvant resulted in a protective immune response with IFN-γ+ and a Th1 profile, while the use of aluminum hydroxide as an adjuvant produced a predominantly Th2 response (Barry et al., 2019). Regarding DNA vaccines, (Sanchez-Burgos et al., 2007) reports a tendency towards a significant Th1 bias. Thus, it is clear that the assessment of the immunological response must consider the vaccine formulation as a whole, observing the interactions between antigen and vector that can result in a more effective immunological profile in combating infection.

Another hypothesis for the observed deviation in induced cytokine profiles involves *in vivo* protein expression. A limitation of the rBCG platform is that the *in vivo* expression of the recombinant protein is estimated to be between 1 and 20 ng per dose of 10^6 CFU, but this amount cannot be precisely determined (Dennehy and Williamson, 2005). In addition to presenting a significantly lower antigen quantity to the immune system compared to other recombinant subunit vaccines, there is also no assurance that the antigen is being correctly expressed as intended. The possibility of erroneous synthesis, potentially lacking one or more epitopes, cannot be excluded. Further studies are needed to confirm this hypothesis and to assess the cytokine profiles that may be achieved by adjusting the concentration of recombinant BCG in the formulations. Additionally, challenging the vaccinated animals with a virulent strain of *T. cruzi* should help determine whether one or both of these immune profiles are effective, based on their observed impacts on parasitemia, disease progression or regression, and tissue damage.

5. CONCLUSION

The results presented in this study demonstrate the immunogenic properties of recombinant *Mycobacterium bovis* BCG expressing the ASP-2 and TC24 proteins when used in vaccine formulations against *Trypanosoma cruzi*,

employing the pUS977 expression vector. Despite the relatively low level of antigen expression in the rBCG platform, the vaccination elicited a significant immune response, characterized by higher production of specific cytokines and consequent enhancement of the immune response. Further studies, however, are necessary to fully characterize these vaccine formulations, particularly to assess their efficacy against challenges with virulent *T. cruzi* strains.

Funding information

This study was partially financed by the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) (Grant number: 21/2551-0001959-0).

Ethical Approval

Animal experimentations were conducted accordingly to the Brazilian Council for Animal Experimentation Control. It was also approved by the Ethics Commission on Animal Experimentation from Federal University of Pelotas (CEEA- UFPel), under protocol number 23110.031479/2021-17.

Conflicts of Interest

The authors declare that they have no knowledge of competing financial interests or personal relationships that could have any kind of influence to the work reported in this paper.

Authors' Contributions

GSS: investigation, methodology, data analyses, writing-original draft; BRF: investigation, methodology, data analyses; FSSS: data analyses; FKS: funding acquisition, review, editing; SB: supervision, funding acquisition, conceptualization, writing-review and editing.

Data Availability

The data used to support the findings of the current study are available from the corresponding author upon request.

REFERENCES

Acevedo, G.R., Girard, M.C., Gómez, K.A., 2018. The Unsolved Jigsaw Puzzle of the Immune Response in Chagas Disease. Front. Immunol. 9, 1929. https://doi.org/10.3389/fimmu.2018.01929

Barry, M.A., Versteeg, L., Wang, Q., Pollet, J., Zhan, B., Gusovsky, F., Bottazzi, M.E., Hotez, P.J., Jones, K.M., 2019. A therapeutic vaccine prototype induces protective immunity and reduces cardiac fibrosis in a mouse model of chronic Trypanosoma cruzi infection. PLoS Negl. Trop. Dis. 13, e0007413. https://doi.org/10.1371/journal.pntd.0007413

Bastos, R.G., Borsuk, S., Seixas, F.K., Dellagostin, O.A., 2009. Recombinant Mycobacterium bovis BCG. Vaccine 27, 6495–6503. https://doi.org/10.1016/j.vaccine.2009.08.044

Bermejo, D.A., Jackson, S.W., Gorosito-Serran, M., Acosta-Rodriguez, E.V., Amezcua-Vesely, M.C., Sather, B.D., Singh, A.K., Khim, S., Mucci, J., Liggitt, D., Campetella, O., Oukka, M., Gruppi, A., Rawlings, D.J., 2013. Trypanosoma cruzi trans-sialidase initiates a program independent of the transcription factors RORγt and Ahr that leads to IL-17 production by activated B cells. Nat. Immunol. 14, 514–522. https://doi.org/10.1038/ni.2569

Bivona, A.E., Alberti, A.S., Cerny, N., Trinitario, S.N., Malchiodi, E.L., 2020. Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control. Biochim. Biophys. Acta BBA - Mol. Basis Dis. 1866, 165658. https://doi.org/10.1016/j.bbadis.2019.165658

Bivona, A.E., Sánchez Alberti, A., Matos, M.N., Cerny, N., Cardoso, A.C., Morales, C., González, G., Cazorla, S.I., Malchiodi, E.L., 2018. Trypanosoma cruzi 80 kDa prolyl oligopeptidase (Tc80) as a novel immunogen for Chagas disease vaccine. PLoS Negl. Trop. Dis. 12, e0006384. https://doi.org/10.1371/journal.pntd.0006384

Bontempi, I., Leal, K., Prochetto, E., Díaz, G., Cabrera, G., Bortolotti, A., Morbidoni, H.R., Borsuk, S., Dellagostin, O., Marcipar, I., 2020. Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypansoma cruzi* infection. Clin. Exp. Immunol. 201, 306–316. https://doi.org/10.1111/cei.13469

Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F., 2016. Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. PLOS Pathog. 12, e1005902. https://doi.org/10.1371/journal.ppat.1005902

Centers for Disease Control and Prevention of the U.S., 2021. American Trypanosomiasis. URL https://www.cdc.gov/dpdx/trypanosomiasisamerican/index.html (accessed 8.15.24).

Chevillard, C., Nunes, J.P.S., Frade, A.F., Almeida, R.R., Pandey, R.P., Nascimento, M.S., Kalil, J., Cunha-Neto, E., 2018. Disease Tolerance and Pathogen Resistance Genes May Underlie Trypanosoma cruzi Persistence and Differential Progression to Chagas Disease Cardiomyopathy. Front. Immunol. 9, 2791. https://doi.org/10.3389/fimmu.2018.02791

Cho, T., Khatchadourian, C., Nguyen, H., Dara, Y., Jung, S., Venketaraman, V., 2021. A review of the BCG vaccine and other approaches toward tuberculosis eradication. Hum. Vaccines Immunother. 17, 2454–2470. https://doi.org/10.1080/21645515.2021.1885280

Costa, A.C.D., Costa-Júnior, A.D.O., Oliveira, F.M.D., Nogueira, S.V., Rosa, J.D., Resende, D.P., Kipnis, A., Junqueira-Kipnis, A.P., 2014. A New Recombinant BCG Vaccine Induces Specific Th17 and Th1 Effector Cells with Higher Protective Efficacy against Tuberculosis. PLoS ONE 9, e112848. https://doi.org/10.1371/journal.pone.0112848

Cristovão-Silva, A.C., Brelaz-de-Castro, M.C.A., Hernandes, M.Z., Pereira, V.R.A., 2021. Chagas disease: Immunology of the disease at a glance. Cytokine Growth Factor Rev. 62, 15–22. https://doi.org/10.1016/j.cytogfr.2021.10.001

De Alba-Alvarado, M.C., Torres-Gutiérrez, E., Reynoso-Ducoing, O.A., Zenteno-Galindo, E., Cabrera-Bravo, M., Guevara-Gómez, Y., Salazar-Schettino, P.M., Rivera-Fernández, N., Bucio-Torres, M.I., 2023. Immunopathological Mechanisms Underlying Cardiac Damage in Chagas Disease. Pathogens 12, 335. https://doi.org/10.3390/pathogens12020335

De Fuentes-Vicente, J.A., Santos-Hernández, N.G., Ruiz-Castillejos, C., Espinoza-Medinilla, E.E., Flores-Villegas, A.L., De Alba-Alvarado, M., Cabrera-Bravo, M., Moreno-Rodríguez, A., Vidal-López, D.G., 2023. What Do You Need to Know before Studying Chagas Disease? A Beginner's Guide. Trop. Med. Infect. Dis. 8, 360. https://doi.org/10.3390/tropicalmed8070360

Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–993. https://doi.org/10.1111/j.1365-2958.1993.tb00970.x

Dennehy, M., Williamson, A.-L., 2005. Factors influencing the immune response to foreign antigen expressed in recombinant BCG vaccines. Vaccine 23, 1209–1224. https://doi.org/10.1016/j.vaccine.2004.08.039

Dumonteil, E., Bottazzi, M.E., Zhan, B., Heffernan, M.J., Jones, K., Valenzuela, J.G., Kamhawi, S., Ortega, J., Rosales, S.P.D.L., Lee, B.Y., Bacon, K.M., Fleischer, B., Slingsby, B., Cravioto, M.B., Tapia-Conyer, R., Hotez, P.J., 2012. Accelerating the development of a therapeutic vaccine for human

Chagas disease: rationale and prospects. Expert Rev. Vaccines 11, 1043–1055. https://doi.org/10.1586/erv.12.85

Dumonteil, E., Escobedo-Ortegon, J., Reyes-Rodriguez, N., Arjona-Torres, A., Ramirez-Sierra, M.J., 2004. Immunotherapy of *Trypanosoma cruzi* Infection with DNA Vaccines in Mice. Infect. Immun. 72, 46–53. https://doi.org/10.1128/IAI.72.1.46-53.2004

Dumonteil, E., Herrera, C., Buekens, P., 2019. A therapeutic preconceptional vaccine against Chagas disease: A novel indication that could reduce congenital transmission and accelerate vaccine development. PLoS Negl. Trop. Dis. 13, e0006985. https://doi.org/10.1371/journal.pntd.0006985

Gomes Dos Santos, A., Watanabe, E.H., Ferreira, D.T., Oliveira, J., Nakanishi, É.S., Oliveira, C.S., Bocchi, E., Novaes, C.T.G., Cruz, F., Carvalho, N.B., Sato, P.K., Yamashiro-Kanashiro, E.H., Pontillo, A., De Freitas, V.L.T., Onuchic, L.F., Shikanai-Yasuda, M.A., 2020. A Specific IL6 Polymorphic Genotype Modulates the Risk of Trypanosoma cruzi Parasitemia While IL18, IL17A, and IL1B Variant Profiles and HIV Infection Protect Against Cardiomyopathy in Chagas Disease. Front. Immunol. 11, 521409. https://doi.org/10.3389/fimmu.2020.521409

Gómez-Olarte, S., Bolaños, N.I., Echeverry, M., Rodríguez, A.N., Cuéllar, A., Puerta, C.J., Mariño, A., González, J.M., 2019. Intermediate Monocytes and Cytokine Production Associated With Severe Forms of Chagas Disease. Front. Immunol. 10, 1671. https://doi.org/10.3389/fimmu.2019.01671

Guedes, P.M.M., Gutierrez, F.R.S., Silva, G.K., Dellalibera-Joviliano, R., Rodrigues, G.J., Bendhack, L.M., Rassi, A., Rassi, A., Schmidt, A., Maciel, B.C., Marin Neto, J.A., Silva, J.S., 2012. Deficient Regulatory T Cell Activity and Low Frequency of IL-17-Producing T Cells Correlate with the Extent of Cardiomyopathy in Human Chagas' Disease. PLoS Negl. Trop. Dis. 6, e1630. https://doi.org/10.1371/journal.pntd.0001630

Jiménez, P., Jaimes, J., Poveda, C., Ramírez, J.D., 2019. A systematic review of the *Trypanosoma cruzi* genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. Parasitology 146, 269–283. https://doi.org/10.1017/S0031182018001506

Kilpeläinen, A., Maya-Hoyos, M., Saubí, N., Soto, C.Y., Joseph, J., 2018. Advances and challenges in recombinant *Mycobacterium bovis* BCG-based HIV vaccine development: lessons learned. Expert Rev. Vaccines 17, 1005–1020. https://doi.org/10.1080/14760584.2018.1534588

Leal, K.S., De Oliveira Silva, M.T., De Fátima Silva Rezende, A., Bezerra, F.S.B., Begnini, K., Seixas, F., Collares, T., Dellagostin, O., Portela, R.W., De Carvalho Azevedo, V.A., Borsuk, S., 2018. Recombinant M. bovis BCG expressing the PLD protein promotes survival in mice

challenged with a C. pseudotuberculosis virulent strain. Vaccine 36, 3578–3583. https://doi.org/10.1016/j.vaccine.2018.05.049

Lee, B.Y., Bacon, K.M., Connor, D.L., Willig, A.M., Bailey, R.R., 2010. The Potential Economic Value of a Trypanosoma cruzi (Chagas Disease) Vaccine in Latin America. PLoS Negl. Trop. Dis. 4, e916. https://doi.org/10.1371/journal.pntd.0000916

Loxton, A.G., Knaul, J.K., Grode, L., Gutschmidt, A., Meller, C., Eisele, B., Johnstone, H., Van Der Spuy, G., Maertzdorf, J., Kaufmann, S.H.E., Hesseling, A.C., Walzl, G., Cotton, M.F., 2017. Safety and Immunogenicity of the Recombinant Mycobacterium bovis BCG Vaccine VPM1002 in HIV-Unexposed Newborn Infants in South Africa. Clin. Vaccine Immunol. 24. https://doi.org/10.1128/CVI.00439-16

Macaluso, G., Grippi, F., Di Bella, S., Blanda, V., Gucciardi, F., Torina, A., Guercio, A., Cannella, V., 2023. A Review on the Immunological Response against Trypanosoma cruzi. Pathogens 12, 282. https://doi.org/10.3390/pathogens12020282

Magalhães, L.M.D., Villani, F.N.A., Nunes, M.D.C.P., Gollob, K.J., Rocha, M.O.C., Dutra, W.O., 2013. High Interleukin 17 Expression Is Correlated With Better Cardiac Function in Human Chagas Disease. J. Infect. Dis. 207, 661–665. https://doi.org/10.1093/infdis/jis724

Maldonado, E., Morales-Pison, S., Urbina, F., Solari, A., 2022. Vaccine Design against Chagas Disease Focused on the Use of Nucleic Acids. Vaccines 10, 587. https://doi.org/10.3390/vaccines10040587

Martinez-Campos, V., Martinez-Vega, P., Ramirez-Sierra, M.J., Rosado-Vallado, M., Seid, C.A., Hudspeth, E.M., Wei, J., Liu, Z., Kwityn, C., Hammond, M., Ortega-López, J., Zhan, B., Hotez, P.J., Bottazzi, M.E., Dumonteil, E., 2015. Expression, purification, immunogenicity, and protective efficacy of a recombinant Tc24 antigen as a vaccine against Trypanosoma cruzi infection in mice. Vaccine 33, 4505–4512. https://doi.org/10.1016/j.vaccine.2015.07.017

Matos, M.N., Cazorla, S.I., Schulze, K., Ebensen, T., Guzmán, C.A., Malchiodi, E.L., 2017. Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-AMP induces Th17+Th1 specific immune responses and confers protection against Trypanosoma cruzi. PLoS Negl. Trop. Dis. 11, e0005300. https://doi.org/10.1371/journal.pntd.0005300

Nogueira, R.T., Nogueira, A.R., Pereira, M.C.S., Rodrigues, M.M., Neves, P.C.D.C., Galler, R., Bonaldo, M.C., 2013. Recombinant Yellow Fever Viruses Elicit CD8+ T Cell Responses and Protective Immunity against Trypanosoma cruzi. PLoS ONE 8, e59347. https://doi.org/10.1371/journal.pone.0059347

Oliveira, T.L., Rizzi, C., Da Cunha, C.E.P., Dorneles, J., Seixas Neto, A.C.P., Amaral, M.G., Hartwig, D.D., Dellagostin, O.A., 2019. Recombinant BCG

strains expressing chimeric proteins derived from Leptospira protect hamsters against leptospirosis. Vaccine 37, 776–782. https://doi.org/10.1016/j.vaccine.2018.12.050

Pérez-Molina, J.A., Molina, I., 2018. Chagas disease. The Lancet 391, 82–94. https://doi.org/10.1016/S0140-6736(17)31612-4

Pino-Martínez, A.M., Miranda, C.G., Batalla, E.I., González-Cappa, S.M., Alba Soto, C.D., 2018. IL-10 participates in the expansion and functional activation of CD8+ T cells during acute infection with *Trypanosoma cruzi*. J. Leukoc. Biol. 105, 163–175. https://doi.org/10.1002/JLB.3A0318-111RR

Quintana, I., Espariz, M., Villar, S.R., González, F.B., Pacini, M.F., Cabrera, G., Bontempi, I., Prochetto, E., Stülke, J., Perez, A.R., Marcipar, I., Blancato, V., Magni, C., 2018. Genetic Engineering of Lactococcus lactis Co-producing Antigen and the Mucosal Adjuvant 3′ 5′- cyclic di Adenosine Monophosphate (c-di-AMP) as a Design Strategy to Develop a Mucosal Vaccine Prototype. Front. Microbiol. 9, 2100. https://doi.org/10.3389/fmicb.2018.02100

Ramírez-Toloza, G., Aguilar-Guzmán, L., Valck, C., Ferreira, V.P., Ferreira, A., 2020. The Interactions of Parasite Calreticulin With Initial Complement Components: Consequences in Immunity and Virulence. Front. Immunol. 11, 1561. https://doi.org/10.3389/fimmu.2020.01561

Ribeiro, F.A.P., Pontes, C., Gazzinelli, R.T., Romero, O.-B., Lazzarin, M.C., Dos Santos, J.F., De Oliveira, F., Pisani, L.P., De Vasconcelos, J.R.C., Ribeiro, D.A., 2019. Therapeutic effects of vaccine derived from amastigote surface protein-2 (ASP-2) against Chagas disease in mouse liver. Cytokine 113, 285–290. https://doi.org/10.1016/j.cyto.2018.07.017

Rodrigues, C.M.C., Plotkin, S.A., 2020. Impact of Vaccines; Health, Economic and Social Perspectives. Front. Microbiol. 11, 1526. https://doi.org/10.3389/fmicb.2020.01526

Rodríguez-Bejarano, O.H., Avendaño, C., Patarroyo, M.A., 2021. Mechanisms Associated with Trypanosoma cruzi Host Target Cell Adhesion, Recognition and Internalization. Life 11, 534. https://doi.org/10.3390/life11060534

Roffê, E., Rothfuchs, A.G., Santiago, H.C., Marino, A.P.M.P., Ribeiro-Gomes, F.L., Eckhaus, M., Antonelli, L.R.V., Murphy, P.M., 2012. IL-10 Limits Parasite Burden and Protects against Fatal Myocarditis in a Mouse Model of *Trypanosoma cruzi* Infection. J. Immunol. 188, 649–660. https://doi.org/10.4049/jimmunol.1003845

Sanchez-Burgos, G., Mezquita-Vega, R.G., Escobedo-Ortegon, J., Ramirez-Sierra, M.J., Arjona-Torres, A., Ouaissi, A., Rodrigues, M.M., Dumonteil, E., 2007. Comparative evaluation of therapeutic DNA vaccines against

Trypanosoma cruzi in mice. FEMS Immunol. Med. Microbiol. 50, 333–341. https://doi.org/10.1111/j.1574-695X.2007.00251.x

Sanmarco, L.M., Visconti, L.M., Eberhardt, N., Ramello, M.C., Ponce, N.E., Spitale, N.B., Vozza, M.L., Bernardi, G.A., Gea, S., Minguez, A.R., Aoki, M.P., 2016. IL-6 Improves the Nitric Oxide-Induced Cytotoxic CD8+ T Cell Dysfunction in Human Chagas Disease. Front. Immunol. 7. https://doi.org/10.3389/fimmu.2016.00626

Seid, C.A., Jones, K.M., Pollet, J., Keegan, B., Hudspeth, E., Hammond, M., Wei, J., McAtee, C.P., Versteeg, L., Gutierrez, A., Liu, Z., Zhan, B., Respress, J.L., Strych, U., Bottazzi, M.E., Hotez, P.J., 2017. Cysteine mutagenesis improves the production without abrogating antigenicity of a recombinant protein vaccine candidate for human chagas disease. Hum. Vaccines Immunother. 13, 621–633. https://doi.org/10.1080/21645515.2016.1242540

Soares, M.B.P., Silva-Mota, K.N., Lima, R.S., Bellintani, M.C., Pontes-de-Carvalho, L., Ribeiro-dos-Santos, R., 2001. Modulation of Chagasic Cardiomyopathy by Interleukin-4. Am. J. Pathol. 159, 703–709. https://doi.org/10.1016/S0002-9440(10)61741-5

Sousa, G.R., Gomes, J.A.S., Damasio, M.P.S., Nunes, M.C.P., Costa, H.S., Medeiros, N.I., Fares, R.C.G., Chaves, A.T., Corrêa-Oliveira, R., Rocha, M.O.C., 2017. The role of interleukin 17-mediated immune response in Chagas disease: High level is correlated with better left ventricular function. PLOS ONE 12, e0172833. https://doi.org/10.1371/journal.pone.0172833

Tanner, R., McShane, H., 2020. BCG and Other Vaccines, in: Friedman, L.N., Dedicoat, M., Davies, P.D.O. (Eds.), Clinical Tuberculosis. CRC Press, Sixth edition. | Boca Raton, FL : CRC Press/Taylor & Francis Group, 2020., pp. 217–233. https://doi.org/10.1201/9781351249980-12

Tarleton, R.L., 2015. CD8+ T cells in Trypanosoma cruzi infection. Semin. Immunopathol. 37, 233–238. https://doi.org/10.1007/s00281-015-0481-9

Fig 1. Santos et al.

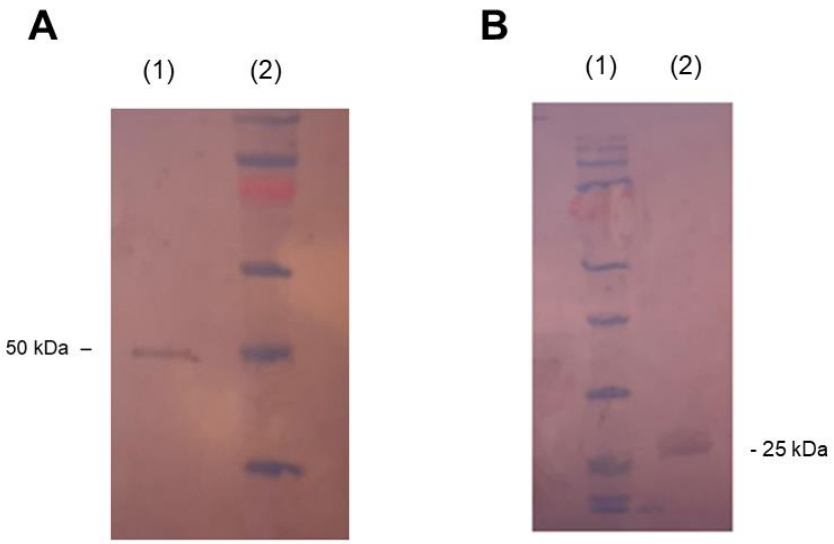


Fig 2. Santos et al.

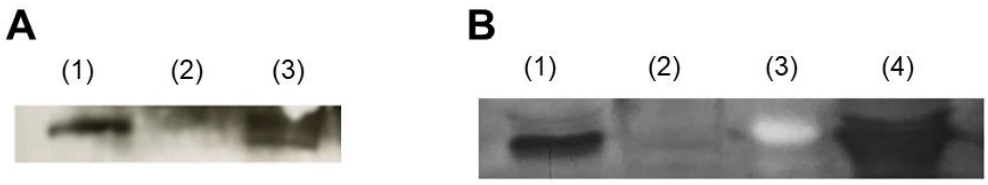


Fig 3. Santos et al.

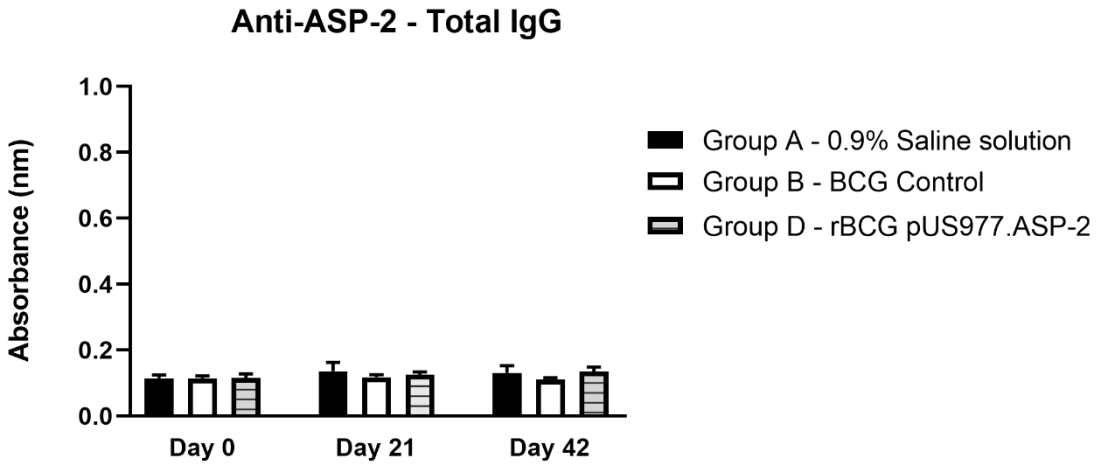


Fig 4. Santos et al.

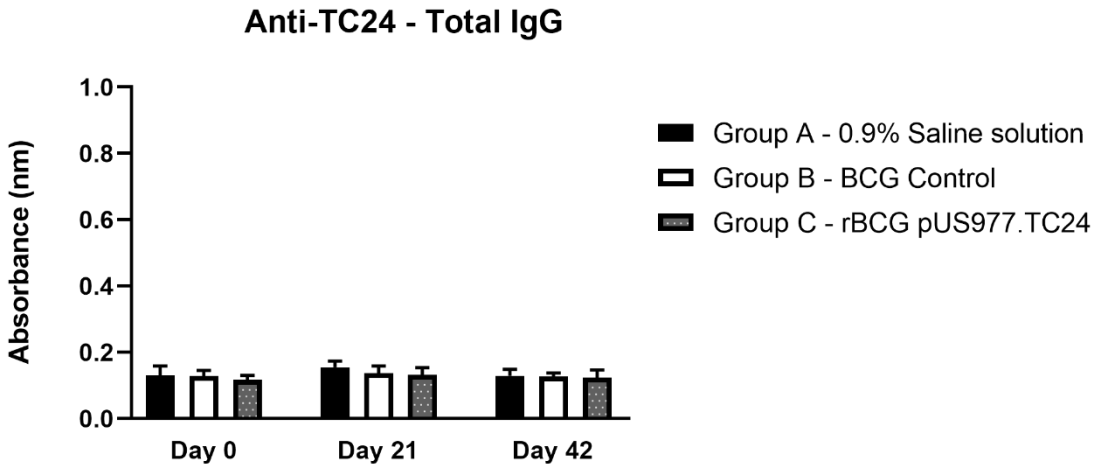
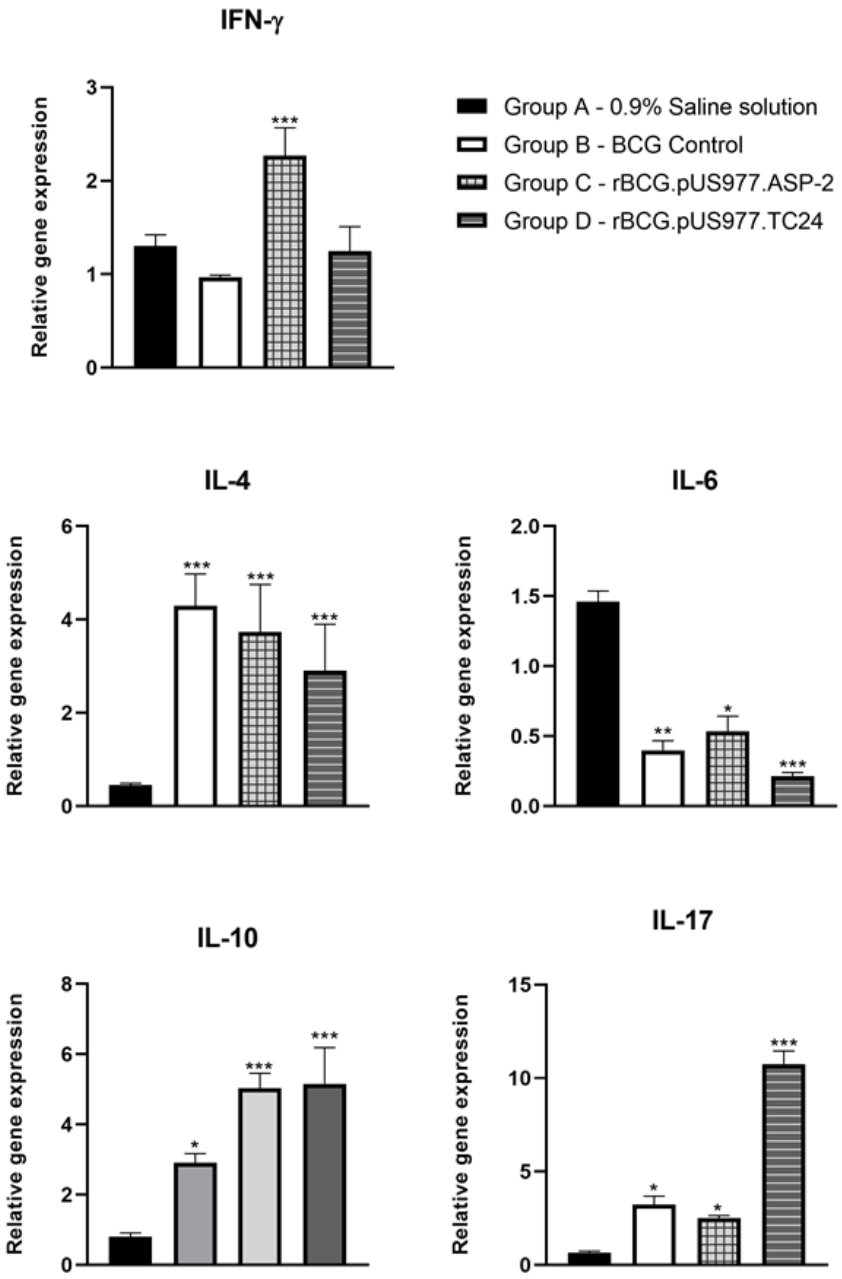


Fig 5. Santos et al.



3.5) Patente 2

Patente a ser submetida ao INPI

Construção vacinal baseada em BCG recombinante expressando antígeno ASP-2 de *Trypanosoma cruzi*

Campo da invenção

[001] A presente invenção descreve a construção de dois protótipos vacinais contra Doença de Chagas compostos pelas cepas de *Mycobacterium bovis* BCG Pasteur (vetores pUS977 e pUS2000) recombinantes expressando a porção final (a partir do aminoácido 261) da proteína de superfície de amastigota nº 2 (ASP-2) sintética de *Trypanosoma cruzi*. A presente invenção refere-se a expressão da proteína de superfície de amastigota nº 2 (ASP-2) sintética de *Trypanosoma cruzi* em *M. bovis* BCG Pasteur, imunização de modelo murino e indução de citocinas confirmando a efetividade do invento.

Fundamentos da invenção

[002] A doença de Chagas (DC), ou tripanossomíase americana, tem como causa o protozoário flagelado *Trypanosoma cruzi*, persistindo em 21 países da America Latina, sendo endêmica em pelo menos 15. Transmitida principalmente por meio do contato das mucosas ou de lesões cutâneas com as fezes de insetos triatomíneos da família *Reduvidae*, infectados pelo *T. cruzi*. Além da transmissão vetorial, ainda pode haver a infecção por meio de transfusões sanguíneas, de transplantes de órgãos ou por transmissão materno-fetal. Estima-se que pelo menos 28 milhões de pessoas estejam sob risco de contaminação na América Latina, e mesmo em países desenvolvidos a DC vem sendo considera um problema em potencial, devido a imigração

de populações oriundas de áreas endêmicas **(Moncayo, A., Silveira, A.C. 2009. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. Mem Inst Oswaldo Cruz. 104 Suppl 1:17-30. doi: 10.1590/S0074-02762009000900005. PMID: 19753454.)**.

[003] O *T. cruzi* é um organismo digenético, apresentando um hospedeiro vertebrado e um invertebrado. Seu ciclo de vida é complexo, onde o vetor invertebrado quando infectado libera tripomastigotas em suas fezes próximo ao local da picada, ao se alimentar de sangue do hospedeiro. No interior do novo hospedeiro, os tripomastigotas invadem as células próximas ao local da inoculação, onde se diferenciam em amastigotas intracelulares. Os amastigotas se multiplicam por fissão binária e se diferenciam em tripomastigotas, sendo então liberados na corrente sanguínea. Os tripomastigotas infectam células de uma variedade de tecidos e se transformam em amastigotas intracelulares em novos locais de infecção. Manifestações clínicas podem resultar desse ciclo infeccioso. Os tripomastigotas da corrente sanguínea não se replicam, a replicação recomeça apenas quando os parasitas entram em outra célula ou são ingeridos por outro vetor. O inseto é infectado ao se alimentar de sangue humano ou animal que contém parasitas circulantes. Os tripomastigotas ingeridos se transformam em epimastigotas no intestino médio do vetor e se diferenciam em tripomastigotas metacíclicos infectantes no intestino posterior **(Macedo, A.M., Oliveira, R.P., Pena, S.D. 2002. Chagas disease: role of parasite genetic variation in pathogenesis. Expert Rev Mol Med. 4:1-16. doi: 10.1017/S1462399402004118. PMID: 14987389.)**. O *Trypanosoma* infecta outros vertebrados além do homem, como mamíferos placentados e marsupiais, os quais servem como reservatório para o protozoário, impossibilitando a erradicação da doença **(Dias, J.C. 2009. Elimination of Chagas' disease transmission: perspectives. Mem Inst**

Oswaldo Cruz. 104 Suppl 1:41-45. doi: 10.1590/s0074-02762009000900007. PMID: 19753456.).

[004] Após o contato com o parasita, o hospedeiro vertebrado desenvolve a fase aguda da doença, que pode durar semanas ou meses, podendo ser sintomática ou assintomática. Após o controle da parasitemia pelo sistema imune, o indivíduo passa então à fase crônica da doença, a qual é caracterizada por uma parasitemia subpatente. Como o parasita nunca é eliminado do organismo, a fase crônica da doença de Chagas perdura por toda a vida do indivíduo. Em cerca de 66% dos casos, as pessoas infectadas permanecem assintomáticas, já em 34% dos casos, após anos, pode haver o desenvolvimento de sintomas do trato digestivo, do sistema nervoso periférico ou cardíacos, podendo em muitos casos levar à morte **(Coura, J.R., de Abreu, L.L., Pereira, J.B., Willcox, H.P. 1985. Morbidity in Chagas' disease. IV. Longitudinal study of 10 years in Pains and Iguatama, Minas Gerais, Brazil. Mem Inst Oswaldo Cruz. 80(1):73-80. doi: 10.1590/s0074-02761985000100011. PMID: 3937015.)**.

[005] Atualmente existem apenas duas drogas disponíveis para o tratamento da doença de Chagas: o benzonidazol e o nifurtimox. Ambas as drogas, no entanto, frequentemente causam efeitos colaterais que levam parte dos pacientes a abandonar o tratamento. O nifurtimox induz reações adversas em cerca de 40% dos indivíduos tratados, incluindo náuseas, vômitos, dores abdominais, perda de peso, anorexia severa e complicações neurológicas **(Marin-Neto, J.A., Rassi, A.Jr., Avezum, A.Jr., Mattos, A.C., Rassi, A., Morillo, C.A., Sosa-Estani, S., Yusuf, S., BENEFIT Investigators. 2009. The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. Mem Inst Oswaldo Cruz. 104 Suppl 1:319-24. doi: 10.1590/s0074-02762009000900042. PMID: 19753491.)**. O benzonidazol induz efeitos colaterais em uma percentagem menor de indivíduos, incluindo edema,

febre, rash cutâneo, dor muscular, neuropatia periférica e neutropenia **(McKerrow, J.H., Doyle, P.S., Engel, J.C., Podust, L.M., Robertson, S.A., Ferreira, R., Saxton, T., Arkin, M., Kerr, I.D., Brinen, L.S., Craik, C.S. 2009. Two approaches to discovering and developing new drugs for Chagas disease. Mem Inst Oswaldo Cruz. 104 Suppl 1(0 1):263-9. doi: 10.1590/s0074-02762009000900034. PMID: 19753483. PMCID: PMC4156466.)**.

[006] Apesar de vacinas desenvolvidas e avaliadas em estágio pré-clínico, como vacinas recombinantes, de subunidade ou de DNA (**Bivona, A.E., Alberti, A.S., Cerny, N., Trinitario, S.N., Malchiodi, E.L. 2020. Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control. Biochim Biophys Acta Mol Basis Dis. 1;1866(5):165658. doi: 10.1016/j.bbadis.2019.165658. PMID: 31904415.**), dada a complexidade da infecção, ainda não há uma vacina eficaz no tratamento ou imunização à doença. Entretanto, diversas proteínas podem apresentar potencial na sua utilização como antígenos para o desenvolvimento de vacinas recombinantes no combate a DC (**Jiménez, P., Jaimes, J., Poveda, C., Ramírez, J.D. 2019. A systematic review of the Trypanosoma cruzi genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. Parasitology. 146(3):269-283. doi: 10.1017/S0031182018001506. PMID: 30210012.**). Proteínas consideradas fatores de virulência do *T. cruzi*, como a cruzipain, trans-sialidase, Tc24, TcG2 e TSA-1, foram utilizadas como antígenos em diferentes formulações vacinais, na tentativa de obter uma vacina profilática (**Arce-Fonseca, M., Carbajal-Hernández, A.C., Lozano-Camacho, M., Carrillo-Sánchez, S.D.C., Roldán, F.J., Aranda-Fraustro, A., Rosales-Encina, J.L., Rodríguez-Morales, O. 2020. DNA Vaccine Treatment in Dogs Experimentally Infected with Trypanosoma cruzi. J Immunol Res. 2020:9794575. doi: 10.1155/2020/9794575. PMID:**

32455143. PMCID: PMC7222601.; **Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P.A., Ortega-Lopez, J., Hotez, P.J., Bottazzi, M.E. 2020. Safety and immunogenicity of a recombinant vaccine against Trypanosoma cruzi in Rhesus macaques. Vaccine. 38(29):4584-4591. doi: 10.1016/j.vaccine.2020.05.010. PMID: 32417142. PMCID: PMC7310587.**). Dentre os antígenos já descritos, proteínas oriundas da fase amastigota do patógeno, como a ASP-2, são capazes de conferir proteção a linhagem de camundongos altamente suscetível a infecção, quando confeccionados em vacina (**Araújo, A.F., de Alencar, B.C., Vasconcelos, J.R., Hiyane, M.I., Marinho, C.R., Penido, M.L., Boscardin, S.B., Hoft, D.F., Gazzinelli, R.T., Rodrigues, M.M. 2005. CD8+-T-cell-dependent control of Trypanosoma cruzi infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect Immun. 73(9):6017-25. doi: 10.1128/IAI.73.9.6017-6025.2005. PMID: 16113322. PMCID: PMC1231112.**).

[007] Sabe-se que, em geral, o combate às infecções virais é coordenado por uma resposta imune do tipo Th1 somado à indução de anticorpos neutralizantes, e para protozoários como o *T. cruzi*, o processo não é diferente (**Frank, F.M., Petray, P.B., Cazorla, S.I., Muñoz, M.C., Corral, R.S., Malchiodi, E.L. 2003. Use of a purified Trypanosoma cruzi antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. Vaccine. 22(1):77-86. doi: 10.1016/s0264-410x(03)00541-3. PMID: 14604574.**; **Machado, A.V., Cardoso, J.E., Claser, C., Rodrigues, M.M., Gazzinelli, R.T., Bruna-Romero, O. 2006. Long-term protective immunity induced against Trypanosoma cruzi infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. Hum Gene Ther. 17(9):898-908. doi: 10.1089/hum.2006.17.898. PMID:**

16972758.; Barry, M.A., Versteeg, L., Wang, Q., Pollet, J., Zhan, B., Gusovsky, F., Bottazzi, M.E., Hotez, P.J., Jones, K.M. 2019. A therapeutic vaccine prototype induces protective immunity and reduces cardiac fibrosis in a mouse model of chronic Trypanosoma cruzi infection. PLoS Negl Trop Dis. 13(5):e0007413. doi: 10.1371/journal.pntd.0007413. PMID: 31145733. PMCID: PMC6542517.).

[008] Assim, o uso de estratégias vacinais capazes de estimular esse tipo de resposta são abordagens promissoras. A vacina BCG, uma cepa atenuada de *Mycobacterium bovis*, é mundialmente utilizada contra tuberculose (TB) (**Bannon;, M.J., Finn, A., 1999. BCG and tuberculosis Commentary. Arch. Dis. Child. 80, 80–83.**; **Li, J., Zhao, A., Tang, J., Wang, G., Shi, Y., Zhan, L., Qin, C., 2020. Tuberculosis vaccine development: from classic to clinical candidates. Eur. J. Clin. Microbiol. Infect. Dis.**) e também empregada como um dos tratamentos de maior eficácia contra câncer superficial de bexiga (**Alhunaidi, O., Zlotta, A.R., 2019. The use of intravesical BCG in urothelial carcinoma of the bladder. E cancer medical science 13.**). A vacinação com BCG induz, majoritariamente, uma resposta celular mediada por linfócitos Th1, além de aumentar significativamente a indução da resposta de células Th17, a qual encontrada, recentemente, como sendo ainda mais protetora do que a resposta de células Th1 contra a infecção por *T. cruzi* (**Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J., Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.; Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F. 2016. Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. PLoS Pathog.**

12(10):e1005902. doi: 10.1371/journal.ppat.1005902. PMID: 27695083. PMCID: PMC5047564.; O'Neill LAJ, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nat Rev Immunol. 2020 Jun;20(6):335-337. doi: 10.1038/s41577-020-0337-y. PMID: 32393823; PMCID: PMC7212510.).

[009] Estudos têm sugerido que algumas das vacinas administradas rotineiramente em bebês e crianças, como a BCG, também tem efeitos não-específicos sobre o risco de doença e morte por outras condições, além daquelas para as quais as vacinas foram projetadas para prevenir. No caso de BCG, sua administração foi associada ao menor risco subsequente de doença e morte por outras causas, fato decorrente de mecanismos conhecidos por "trained immunity" e imunidade heteróloga (**Higgins, J.P.T., Soares-Weiser, K., López-López, J.A., Kakourou, A., Chaplin, K., Christensen, H., Martin, N.K., Sterne, J.A.C., Reingold, A.L., 2016. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ 355, i5170.; Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J., Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.**). Um estudo demonstrou que a vacinação com BCG induziu reprogramação epigenética *in vivo* de monócitos contra infecção experimental com uma vacina atenuada contra o vírus da febre amarela com papel fundamental da IL-1b como mediador dessa resposta (**Arts, R.J.W., Moorlag, S.J.C.F.M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., Kumar, V., Xavier, R.J., Wijmenga, C., Joosten, L.A.B., Reusken, C.B.E.M., Benn, C.S., Aaby, P., Koopmans, M.P., Stunnenberg, H.G., van Crevel, R., Netea, M.G., 2018. BCG Vaccination Protects against Experimental Viral Infection in Humans through the**

Induction of Cytokines Associated with Trained Immunity. Cell Host Microbe 23, 89-100.e5.).

[0010] A construção de cepas recombinantes de BCG que proporcionem maior estímulo do sistema imune tem sido explorada para melhorar sua eficácia contra TB (**Nieuwenhuizen, N.E., Kaufmann, S.H.E., 2018. Next-Generation Vaccines Based on Bacille Calmette–Guérin. Front. Immunol. 9, 121.**), aumentar seu efeito antitumoral na terapêutica de tumores de bexiga (**Begnini, K.R., Buss, J.H., Collares, T., Seixas, F.K., 2015. Recombinant *Mycobacterium bovis* BCG for immunotherapy in nonmuscle invasive bladder cancer. Appl. Microbiol. Biotechnol. 99, 3741–3754.),** e expressar antígenos de diferentes patógenos para emprego como vetor vacinal (**Bastos, R.G., Borsuk, S., Seixas, F.K., Dellagostin, O.A., 2009. Recombinant *Mycobacterium bovis* BCG. Vaccine.; Zheng, Y., Naguib, Y.W., Dong, Y., Shi, Y., Bou, S., Cui, Z., 2015. Applications of bacillus Calmette-Guerin and recombinant bacillus Calmette-Guerin in vaccine development and tumor immunotherapy. Expert Rev. Vaccines 14, 1255–75. Marques-Neto LM, Piwowarska Z, Kanno AI, Moraes L, Trentini MM, Rodriguez D, Silva JLSC, Leite LCC. Thirty years of recombinant BCG: new trends for a centenary vaccine. Expert Rev Vaccines. 2021 Aug;20(8):1001-1011. doi: 10.1080/14760584.2021.1951243. Epub 2021 Jul 13. PMID: 34224293.**). Nesse contexto, salienta-se o potencial da construção e do uso de BCG como vetor vacinal expressando antígenos de *T. cruzi* como ferramenta profilática contra doença de Chagas.

[0011] Em uma pesquisa prévia realizada nos bancos de dados mundiais de depósito de patentes, é notória a quantidade de documentos que utilizam a plataforma BCG como fórmula vacinal contra uma gama de enfermidades. Encontramos como resultado o documento **US 6471967 B1**, referente a construção de BCG recombinante tendo uma sequência de ácido nucleico

extracromossômico compreendendo gene que codifica uma proteína extracelular de 30 kDa de *Mycobacteria tuberculosis*. Como já foi descrito, nosso produto, apesar da semelhança metodológica, não será empregado contra tuberculose, mas sim contra doença de Chagas, fazendo uso de proteínas diferentes da reivindicada pela patente citada.

[0012] Nossa busca contemplou ainda a reivindicação por uma composição imunogénica compreendendo BCG recombinante, em que o referido expressa pelo menos uma proteína extracelular de *Mycobacteria major* seleccionada entre proteína de 23,5 kDa, proteína de 30 kDa, proteína de 32A kDa e proteína de 32B kDa; em que uma sequência de ácido nucleico que codifica para pelo menos uma proteína extracelular de micobactéria é incorporada no(s) cromossomo(s) do BCG recombinante sob um promotor forte, de modo que a proteína é superexpressa e o BCG recombinante não abriga um marcador de resistência a antibiótico (**US 8932846 B2**). Novamente, a composição deste produto pouco se assemelha com nossa invenção, uma vez que nossa estratégia objetiva a expressão heteróloga de genes de *T. cruzi* em *M. bovis* BCG como vacina contra DC.

[0013] No entanto, alguns documentos se assemelham com nosso produto. Como por exemplo, a patente que reivindica a construção de vacinas de BCG recombinantes que expressam DNA de interesse, incorporado na micobactéria, sob o controle de um promotor. Referindo-se particularmente a *M. bovis*-BCG recombinante em que o DNA de interesse é expresso extracromossomicamente sob o controle de um promotor hsp micobacteriano, tal como hsp70 e hsp60 (**WO 1995003418 A3**). Nossa estratégia se baseia sim nesta mesma forma de construção, no entanto, nossa invenção objetiva a adição de genes de *T. cruzi* na cepa Pasteur de *M. bovis* BCG para uso como vacina vetorizada utilizando promotores diferentes dos mencionados na referida patente.

[0014] Em relação ao antígeno selecionado para ser expresso pelo BCG recombinante, alguns documentos foram encontrados que também fazem uso da proteína de superfície de amastigota (ASP-2) de *Trypanosoma cruzi* em suas formulações vacinais. Vírus já foram utilizados para expressar sequências específicas de ASP-2 e outros antígenos como proposta vacinal (**WO 2009103133 A1**). Entretanto, as duas sequencias de ASP-2 propostas para uso pelo autor não correspondem a aqui presente selecionada. Além de tal, na patente encontrada, o veículo de expressão utilizado em nada se assemelha, uma vez que no evento aqui proposto utiliza-se um vetor bacteriano para expressão do antígeno e não um viral.

[0015] Identificamos também a existência de um estudo utilizando BCG como imunizante contra doença de Chagas, intitulado **"The effect of BCG on the course of experimental Chagas' disease in mice"**. Esse trabalho visa a observação dos efeitos do tratamento com BCG não recombinante sobre a infecção de *T. cruzi* em camundongos C3H(He). Apesar de também fazer uso de *M. bovis* BCG no combate a DC, nosso invento baseia-se na utilização de uma cepa de BCG que irá expressar proteínas de *T. cruzi* (sem combinação com outros antígenos) cujas sequências gênicas foram especificamente selecionadas para clonagem em vetores de expressão em micobactérias, não contando apenas com a atividade imunológica gerada pelo BCG.

[0016] O artigo intitulado **"Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypanosoma cruzi* infection",** publicado por nosso grupo, também foi identificado em nossa busca. Ele demonstra a expressão dos fragmentos N-terminal e C-terminal da proteína transialidase e da proteína cruzipain de *T. cruzi, em M. bovis*-BCG, para utilização como imunizante. O mesmo além de reforçar o pioneirismo na utilização de BCG como vetor vacinal no combate a doença de Chagas por parte de nosso grupo, demonstra ainda a expertise do mesmo

na utilização de BCG recombinante na superexpressão de antígenos. A particularidade de nossas sequências e consequentemente de nossa construção, garante o caráter ÚNICO e INOVADOR de nosso produto.

Breve descrição dos desenhos

A Figura 1 apresenta os níveis de citocinas INF-γ, Interleucina 4, 6, 10 e 17 produzidos pelos camundongos vacinados com as construções vacinais rBCG/pUS2000/ASP-2 e rBCG/pUS977/ASP-2.

Descrição da invenção

[0017] Tendo em vista os pontos abordados e os impactos gerados pela infecção por *T. cruzi*, em um contexto socioeconômico, ressalta-se a necessidade por novas formulações vacinais eficientes e seguras, como forma de minimizar os danos causados pela Doença de Chagas. Dessa forma, a presente invenção destina-se a construção de cepa de *Mycobacterium bovis* BCG Pasteur recombinante expressando a porção final (a partir do aminoácido 261) da proteína de superfície de amastigota nº 2 (ASP-2) de *Trypanosoma cruzi*, para uso em vacinas experimentais e comerciais contra a doença de Chagas. A seguir, descreve-se o invento em mais detalhes:

Cultivo de M. bovis BCG Pasteur e E. coli

[0018] *M. bovis* BCG Pasteur é cultivado em meio 7H9 (Middlebrook 7H9 Broth Base) (líquido) ou 7H10 (Middlebrook 7H10 Broth Base)(sólido) com suplementação de 10% de OADC (Oleic Albumin Dextrose Catalase) e adição do antibiótico canamicina (50 mg/mL) quando necessário. O cultivo é mantido a 37ºC por 7 dias em meio 7H9 ou por 21 dias em meio 7H10. *E. coli* é cultivada em meio Luria Bertani (LB) líquido ou LB-Ágar à 37°C por

16 h, com suplementação do antibiótico canamicina (50 mg/mL) quando necessário.

Seleção e clonagem do alvo vacinal

[0019] As sequências nucleotídicas que codificam para a proteínas ASP-2 de T*rypanosoma cruzi* foi obtida a partir do NCBI www.ncbi.nlm.nih.gov (AAC47720.1). A partir destas, foi possível realizar o desenho dos genes sintéticos *asp2* (SEQ ID NO:1) visando posterior clonagem nos vetores de expressão em *BCG* pUS2000 e pUS977, para obtenção dos construtos recombinantes rBCG/pUS2000/ASP-2 e rBCG/pUS977/ASP-2, respectivamente. Todos os genes sintéticos foram projetados com o auxílio do software Vector NTI 11 (Invitrogen™). Sítios de restrição enzimáticos (XbaI, BamHI e HindIII) foram adicionados nas extremidades 5' e 3' das sequências, visando a clonagem no vetor pAE de expressão em *E. coli* (**Ramos, C.R.R., Abreu, P.A.E., Nascimento, A.L.T.O., Ho, P.L., 2004. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal his-tagged fusion peptide. Brazilian J. Med. Biol. Res. 37, 1103–1109.**), e nos vetores de expressão em *M. bovis* BCG (pUS2000 e pUS977) (**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**).

[0020] O gene que codofica para a região da proteína ASP-2 foi sintetizado (Genome) e após subclonado nos vetores de expressão em *M. bovis* BCG Pasteur (pUS2000 e pUS977)(**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**). Em seguida, o produto da ligação foi transformado

por eletroporação em *E. coli* TOP10 para obtenção dos clones recombinantes. Após caracterização enzimática e por sequenciamento de DNA, os clones recombinantes foram utilizados para transformação de *M. bovis* BCG Pasteur por eletroporação.

Expressão da proteína recombinante rASP-2 e obtenção do soro policlonal

[0021] A expressão da proteína recombinante foi realizada em cepa de *E. coli* BL21 Star, cultivada em meio Luria Bertani (LB) com adição de 1,5% de ágar bacteriológico durante 16 horas à 37ºC, suplementado com 100 μg/mL de ampicilina, quando necessário. Essas células foram transformadas com os plasmídeos pAE/*asp2* por choque térmico, com posterior incubação. A indução da expressão dessas proteínas ocorreu pela adição de Isopropil-β-D-1-tiogalactopiranósido (IPTG) a 1mM e incubação por 3 horas. Ao final do processo, a purificação das proteínas se deu através de cromatografia de afinidade ao níquel, em coluna de Sepharose HisTRAP (GE). Por fim, a corrida eletroforética em gel de SDS-PAGE e posterior *Western Blot* com anticorpo monoclonal anti-histidina permitiu a confirmação da identidade das proteínas expressas.

[0022] Obtendo a confirmação da identidade das proteínas expressas, através de *Western Blot* com anticorpo monoclonal anti-histidina, foi possível dar início ao protocolo de inoculação para obtenção de soro policlonal, afim de avaliar a expressão da proteína pelo BCG recombinante. Para isso, 20 camundongos BALB/c fêmeas foram alocados em dois grupos, sendo: Grupo A: Solução salina 0,9% (Controle); e Grupo B: proteína recombinante rASP-2 + adjuvante hidróxido de alumínio. Todos os experimentos com animais aqui descritos foram aprovados pelo Comitê de Ética em Experimentação Animal da Universidade Federal de Pelotas (CEEA-UFPel), sob número de protocolo 23110.031479/2021-17.

[0023] A formulação para o grupo B caracterizou-se pela inoculação de 25 μg da proteína recombinante (rASP-2). O adjuvante hidróxido de alumínio foi acrescentado na concentração de 15%. Todas as aplicações foram realizadas por via subcutânea, em duas doses, com intervalo de 21 dias entre elas. Para obtenção do soro, após a segunda dose (dia 42), foi realizada coleta de sangue, que foi então centrifugado para separação do soro.

rBCG/pUS2000/ASP-2 e rBCG/pUS977/ASP-2

[0024] Posteriormente, foi realizada nova corrida eletroforética em gel de SDS-PAGE, seguida de *Western Blot*, desta vez, avaliando as construções vacinais rBCG/pUS2000/ASP-2 e rBCG/pUS977/ASP-2. Contrariamente ao processo realizado anteriormente, que utilizou o anticorpo monoclonal anti-histidina, aqui fez-se uso do soro policlonal obtido dos camundongos inoculados com a proteína recombinante isolada, o que permitiu a confirmação da expressão e identidade das proteínas em *M. bovis* BCG recombinante.

[0025] Obtidas as construções vacinais, deu-se início ao protocolo de imunização dos camundongos. Para tal, 40 camundongos BALB/c fêmeas foram alocados em quatro grupos, organizados da seguinte forma: Grupo A: Solução salina 0,9% (Controle); Grupo B: *M. bovis* BCG Pasteur não transformada; Grupo C: rBCG/pUS2000/ASP-2; e rBCG/pUS977/ASP-2.

[0026] As formulações para os grupos B, C e D caracterizaram-se pela inoculação de $1x10^7$ UFC/mL. Para tal, o cultivo teve sua densidade óptica aferida, onde o volume de cultivo em meio 7H9 equivalente a concentração necessária fora alocado em novo recipiente, centrifugado, separado do meio e ressuspendido em 10 mL de PBS. Todas as aplicações foram realizadas por via subcutânea, em duas doses, com intervalo de 21 dias entre elas.

Resposta imune celular

[0027] Para que fosse possível projetar a efetividade das vacinas, foi avaliada a resposta imune celular. Realizada a eutanásia dos 40 animais previamente imunizados, coletou-se o baço de cada camundongo, em ambiente estéril. A cultura dos esplenócitos foi realizada em Dulbecco's modified Eagle's médium (DMEM), contendo alta glicose e suplementado com 10% de soro fetal bovino (SFB), sendo adicionada a placas de cultivo de 24 cavidades, com fundo chato. O cultivo foi incubado por 24 horas a 37ºC em estufa com 5% de CO_2.

[0028] Após o período de incubação, foi realizado o estímulo contendo 10 μg da proteína recombinante nos grupos C e D. O grupo referente ao controle negativo foi adicionado apenas solução salina, enquanto o controle positivo foi estimulado com 10 μg de concavalina A, induzindo a produção de citocinas para quantificação. Nova incubação foi realizada, sob as mesmas condições citadas anteriormente.

[0029] Após as 24 horas subsequentes, a coleta das células foi efetuada, permitindo a extração de RNA total a partir do método do reagente Trizol (Invitrogen). Obtendo essa porção de material genético, a síntese de cDNA foi realizada a partir do kit comercial High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), utilizando 1 μL do RNA extraído para sua confecção. A partir das amostras de cDNA sintetizadas, foi possível avaliar a quantificação das citocinas INF-γ, TNF-α e Interleucina 17, através de PCR em tempo real, utilizando primers específicos.

[0030] Por fim, as análises estatísticas foram obtidas a partir do Software GraphPad Prism 7.0, a partir de análise unidirecional de variância (ANOVA), seguida pelo pós-teste de Tukey. O valor de significância observado como parâmetro foi de $p<0,05$, indicando diferença estatística.

Exemplos de concretizações da invenção

Exemplo 1:

BCG recombinante expressão antígeno de *T. cruzi* induz aumento na resposta imune celular em modelo murino

[001] A avaliação da resposta imune celular induzida a partir do cultivo de esplenócitos indicou uma produção balanceada entre citocinas pró e anti-inflamatórias. Nesse aspecto, observou-se um aumento significativo na expressão das citocinas INF-γ, Interleucina 4, 10 e 17, por parte de pelo menos uma das construções vacinais, enquanto observa-se uma repressão da citocina Interleucina 6 (Figura 1).

[002] Apesar de apresentarem divergências entre os perfis de citocinas de acordo com o vetor de expressão utilizado, ambas as construções apresentaram padrões balanceados entre respostas pró e anti-inflamatórias. Resposta desejada no combate à doença de Chagas. Enquanto o grupo D apresentou expressão estatisticamente significativa, ainda que pouco acentuada, para a citocina pró-inflamatória IFN-y, os demais grupos não apresentaram expressão quando comparados ao grupo controle A. Entretanto, apesar de parecer pouco propício para um modelo vacinal, altos níveis de expressão de IFN-y estão diretamente associados com maiores níveis de dano tecidual em pacientes positivados para Chagas em estágio crônico.

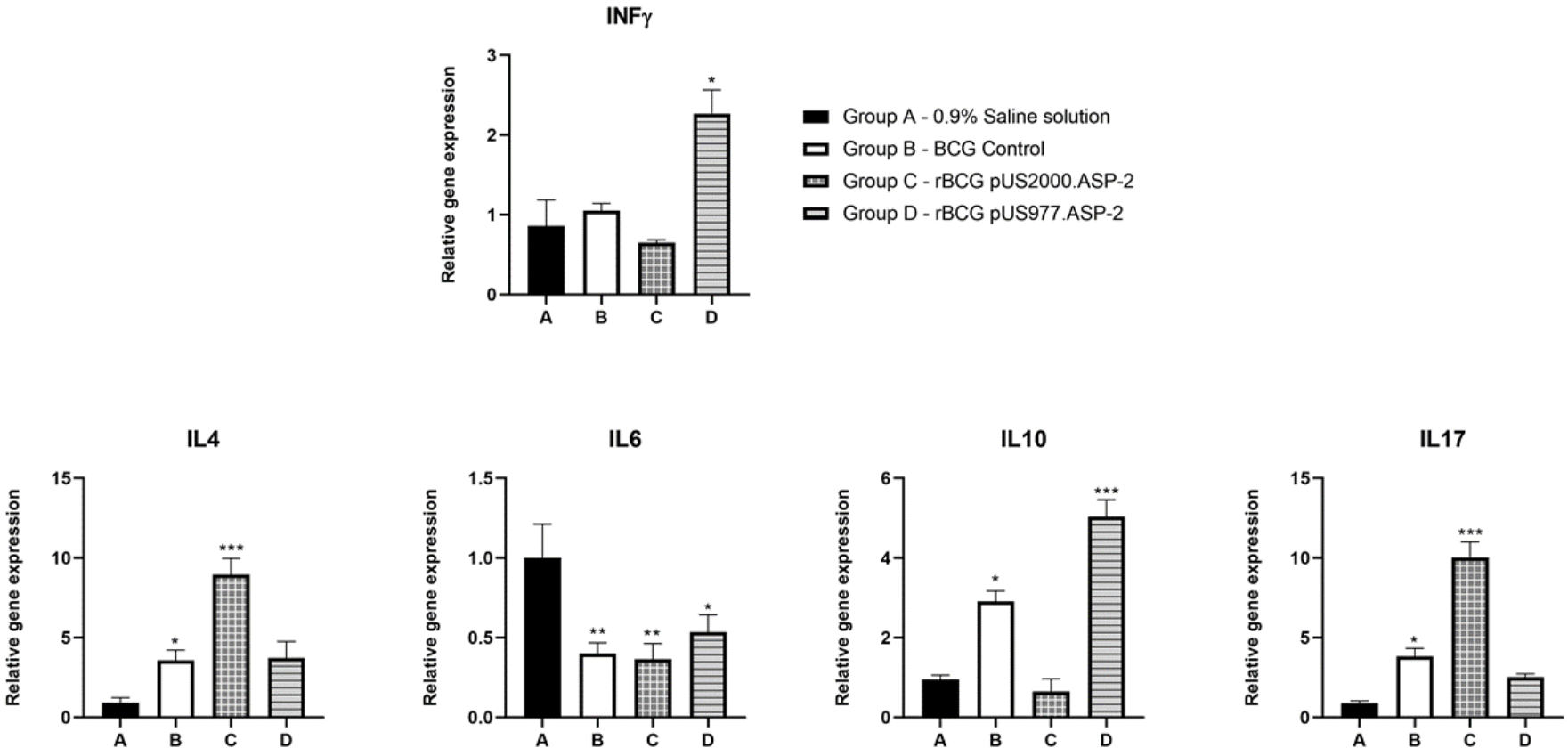
[003] Em contraponto, outras citocinas pró-inflamatórias são de grande interesse quando sua expressão é induzida em altos níveis, como é o caso da Interleucina 17. Esta é considerada uma das mais importantes na resposta imune contra a infeção por *T. cruzi*, pois está diretamente relacionada a

imunidade contra patógenos intracelulares, recrutamento de neutrófilos e respostas Th17, fundamentais na defesa contra parasitoses. Quando comparado ao grupo salina, apenas a imunização com *M. bovis* BCG não transformado (grupo B) já é capaz de aumentar significativamente a expressão dessa citocina, mas a indução é superior quando se considera a inoculação de rBCG/pUS2000/ASP-2 (grupo C).

[004] Como mencionado anteriormente, indução de respostas anti-inflamatórias são igualmente importantes no combate a Chagas. Dessa forma, os resultados obtidos para os grupos C e D na indução da expressão da Interleucina 4 e da repressão da Interleucina 6 corroboram com o cenário de uma amplificação de uma resposta imune balanceada quando comparado ao grupo não vacinado. Dessa forma, o uso de BCG recombinante expressando o antígeno ASP-2 induz uma resposta imune com um caráter mais robusto, propiciando ao organismo melhor preparo para impedir a infecção e/ou melhor combate-la uma vez instaurada.

5230

DESENHOS



5231
5232

Figura 1

3.6) Patente 3

Patente a ser submetida ao INPI

Construção vacinal baseada em BCG recombinante expressando antígeno Tc24 de *Trypanosoma cruzi*

Campo da invenção

[0031] A presente invenção descreve a construção de um protótipo vacinal contra Doença de Chagas composto pela cepa de *Mycobacterium bovis* BCG Pasteur recombinantes expressando a proteína flagelar de ligação ao cálcio de 24 kDa (Tc24) sintética de *Trypanosoma cruzi*. A presente invenção refere-se a expressão da proteína flagelar de ligação ao cálcio de 24 kDa (Tc24) sintética de *Trypanosoma cruzi* em *M. bovis* BCG Pasteur, imunização de modelo murino e indução de citocinas confirmando a efetividade do invento.

Fundamentos da invenção

[0032] A doença de Chagas (DC), ou tripanossomíase americana, tem como causa o protozoário flagelado *Trypanosoma cruzi*, persistindo em 21 países da America Latina, sendo endêmica em pelo menos 15. Transmitida principalmente por meio do contato das mucosas ou de lesões cutâneas com as fezes de insetos triatomíneos da família *Reduvidae*, infectados pelo *T. cruzi*. Além da transmissão vetorial, ainda pode haver a infecção por meio de transfusões sanguíneas, de transplantes de órgãos ou por transmissão materno-fetal. Estima-se que pelo menos 28 milhões de pessoas estejam sob risco de contaminação na América Latina, e mesmo em países desenvolvidos a DC vem sendo considera um problema em potencial, devido a imigração de populações oriundas de áreas endêmicas **(Moncayo, A., Silveira, A.C.**

2009. Current epidemiological trends for Chagas disease in Latin America and future challenges in epidemiology, surveillance and health policy. Mem Inst Oswaldo Cruz. 104 Suppl 1:17-30. doi: 10.1590/S0074-02762009000900005. PMID: 19753454.).

[0033] O *T. cruzi* é um organismo digenético, apresentando um hospedeiro vertebrado e um invertebrado. Seu ciclo de vida é complexo, onde o vetor invertebrado quando infectado libera tripomastigotas em suas fezes próximo ao local da picada, ao se alimentar de sangue do hospedeiro. No interior do novo hospedeiro, os tripomastigotas invadem as células próximas ao local da inoculação, onde se diferenciam em amastigotas intracelulares. Os amastigotas se multiplicam por fissão binária e se diferenciam em tripomastigotas, sendo então liberados na corrente sanguínea. Os tripomastigotas infectam células de uma variedade de tecidos e se transformam em amastigotas intracelulares em novos locais de infecção. Manifestações clínicas podem resultar desse ciclo infeccioso.

[0034] Os tripomastigotas da corrente sanguínea não se replicam, a replicação recomeça apenas quando os parasitas entram em outra célula ou são ingeridos por outro vetor. O inseto é infectado ao se alimentar de sangue humano ou animal que contém parasitas circulantes. Os tripomastigotas ingeridos se transformam em epimastigotas no intestino médio do vetor e se diferenciam em tripomastigotas metacíclicos infectantes no intestino posterior **(Macedo, A.M., Oliveira, R.P., Pena, S.D. 2002. Chagas disease: role of parasite genetic variation in pathogenesis. Expert Rev Mol Med. 4:1-16. doi: 10.1017/S1462399402004118. PMID: 14987389.)**.

[0035] O *Trypanosoma* infecta outros vertebrados além do homem, como mamíferos placentados e marsupiais, os quais servem como reservatório para o protozoário, impossibilitando a erradicação da doença **(Dias, J.C. 2009. Elimination of Chagas' disease transmission: perspectives. Mem Inst**

Oswaldo Cruz. 104 Suppl 1:41-45. doi: 10.1590/s0074-02762009000900007. PMID: 19753456.).

[0036] Após o contato com o parasita, o hospedeiro vertebrado desenvolve a fase aguda da doença, que pode durar semanas ou meses, podendo ser sintomática ou assintomática. Após o controle da parasitemia pelo sistema imune, o indivíduo passa então à fase crônica da doença, a qual é caracterizada por uma parasitemia subpatente. Como o parasita nunca é eliminado do organismo, a fase crônica da doença de Chagas perdura por toda a vida do indivíduo. Em cerca de 66% dos casos, as pessoas infectadas permanecem assintomáticas, já em 34% dos casos, após anos, pode haver o desenvolvimento de sintomas do trato digestivo, do sistema nervoso periférico ou cardíacos, podendo em muitos casos levar à morte **(Coura, J.R., de Abreu, L.L., Pereira, J.B., Willcox, H.P. 1985. Morbidity in Chagas' disease. IV. Longitudinal study of 10 years in Pains and Iguatama, Minas Gerais, Brazil. Mem Inst Oswaldo Cruz. 80(1):73-80. doi: 10.1590/s0074-02761985000100011. PMID: 3937015.)**.

[0037] Atualmente existem apenas duas drogas disponíveis para o tratamento da doença de Chagas: o benzonidazol e o nifurtimox. Ambas as drogas, no entanto, frequentemente causam efeitos colaterais que levam parte dos pacientes a abandonar o tratamento. O nifurtimox induz reações adversas em cerca de 40% dos indivíduos tratados, incluindo náuseas, vômitos, dores abdominais, perda de peso, anorexia severa e complicações neurológicas **(Marin-Neto, J.A., Rassi, A.Jr., Avezum, A.Jr., Mattos, A.C., Rassi, A., Morillo, C.A., Sosa-Estani, S., Yusuf, S., BENEFIT Investigators. 2009. The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. Mem Inst Oswaldo Cruz. 104 Suppl 1:319-24. doi: 10.1590/s0074-02762009000900042. PMID: 19753491.)**.

[0038] O benzonidazol induz efeitos colaterais em uma percentagem menor de indivíduos, incluindo edema, febre, rash cutâneo, dor muscular, neuropatia periférica e neutropenia **(McKerrow, J.H., Doyle, P.S., Engel, J.C., Podust, L.M., Robertson, S.A., Ferreira, R., Saxton, T., Arkin, M., Kerr, I.D., Brinen, L.S., Craik, C.S. 2009. Two approaches to discovering and developing new drugs for Chagas disease. Mem Inst Oswaldo Cruz. 104 Suppl 1(0 1):263-9. doi: 10.1590/s0074-02762009000900034. PMID: 19753483. PMCID: PMC4156466.)**.

[0039] Apesar de vacinas desenvolvidas e avaliadas em estágio pré-clínico, como vacinas recombinantes, de subunidade ou de DNA (**Bivona, A.E., Alberti, A.S., Cerny, N., Trinitario, S.N., Malchiodi, E.L. 2020. Chagas disease vaccine design: the search for an efficient Trypanosoma cruzi immune-mediated control. Biochim Biophys Acta Mol Basis Dis. 1;1866(5):165658. doi: 10.1016/j.bbadis.2019.165658. PMID: 31904415.**), dada a complexidade da infecção, ainda não há uma vacina eficaz na imunização à doença. Entretanto, diversas proteínas podem apresentar potencial na sua utilização como antígenos para o desenvolvimento de vacinas recombinantes no combate a DC (**Jiménez, P., Jaimes, J., Poveda, C., Ramírez, J.D. 2019. A systematic review of the Trypanosoma cruzi genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. Parasitology. 146(3):269-283. doi: 10.1017/S0031182018001506. PMID: 30210012.**). Proteínas consideradas fatores de virulência do *T. cruzi* foram utilizadas como antígenos em diferentes formulações vacinais, na tentativa de obter uma vacina profilática (**Arce-Fonseca, M., Carbajal-Hernández, A.C., Lozano-Camacho, M., Carrillo-Sánchez, S.D.C., Roldán, F.J., Aranda-Fraustro, A., Rosales-Encina, J.L., Rodríguez-Morales, O. 2020. DNA Vaccine Treatment in Dogs Experimentally Infected with Trypanosoma cruzi. J Immunol Res. 2020:9794575. doi:**

10.1155/2020/9794575. PMID: 32455143. PMCID: PMC7222601.; **Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P.A., Ortega-Lopez, J., Hotez, P.J., Bottazzi, M.E. 2020. Safety and immunogenicity of a recombinant vaccine against Trypanosoma cruzi in Rhesus macaques. Vaccine. 38(29):4584-4591. doi: 10.1016/j.vaccine.2020.05.010. PMID: 32417142. PMCID: PMC7310587.**).

[0040] Dentre os antígenos já descritos, proteínas flagelares do patógeno, como a Tc24, são capazes de conferir proteção a linhagem de camundongos, cães e macacos, quando confeccionados em vacina **(Arnal, A., Villanueva-Lizama, L., Teh-Poot, C., Herrera, C., Dumonteil, E., 2020. Extent of polymorphism and selection pressure on the Trypanosoma cruzi vaccine candidate antigen Tc24. Evol Appl 13, 2663–2672. Doi: 10.1111/EVA.13068. PMID: 33294015 PMCID: PMC7691455; Dumonteil, E., Herrera, C., Tu, W., Goff, K., Fahlberg, M., Haupt, E., Kaur, A., Marx, P.A., Ortega-Lopez, J., Hotez, P.J., Bottazzi, M.E., 2020. Safety and immunogenicity of a recombinant vaccine against Trypanosoma cruzi in Rhesus macaques. Vaccine 38, 4584–4591. Doi: 10.1016/J.VACCINE.2020.05.010. PMID: 32417142 PMCID: PMC7310587)**.

[0041] Sabe-se que, em geral, o combate às infecções virais é coordenado por uma resposta imune do tipo Th1 somado à indução de anticorpos neutralizantes, e para protozoários como o *T. cruzi*, o processo não é diferente (**Barry, M.A., Versteeg, L., Wang, Q., Pollet, J., Zhan, B., Gusovsky, F., Bottazzi, M.E., Hotez, P.J., Jones, K.M. 2019. A therapeutic vaccine prototype induces protective immunity and reduces cardiac fibrosis in a mouse model of chronic Trypanosoma cruzi infection. PLoS Negl Trop Dis. 13(5):e0007413. doi: 10.1371/journal.pntd.0007413. PMID: 31145733. PMCID:**

PMC6542517.). Assim, o uso de estratégias vacinais capazes de estimular esse tipo de resposta são abordagens promissoras.

[0042] A vacina BCG, uma cepa atenuada de *Mycobacterium bovis*, é mundialmente utilizada contra tuberculose (TB) (**Bannon;, M.J., Finn, A., 1999. BCG and tuberculosis Commentary. Arch. Dis. Child. 80, 80–83.**; **Li, J., Zhao, A., Tang, J., Wang, G., Shi, Y., Zhan, L., Qin, C., 2020. Tuberculosis vaccine development: from classic to clinical candidates. Eur. J. Clin. Microbiol. Infect. Dis.**) e também empregada como um dos tratamentos de maior eficácia contra câncer superficial de bexiga. A vacinação com BCG induz, majoritariamente, uma resposta celular mediada por linfócitos Th1, além de aumentar significativamente a indução da resposta de células Th17, considerada como sendo ainda mais protetora contra a infecção por *T. cruzi* (**Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J., Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.; Cai, C.W., Blase, J.R., Zhang, X., Eickhoff, C.S., Hoft, D.F. 2016. Th17 Cells Are More Protective Than Th1 Cells Against the Intracellular Parasite Trypanosoma cruzi. PLoS Pathog. 12(10):e1005902. doi: 10.1371/journal.ppat.1005902. PMID: 27695083. PMCID: PMC5047564.; O'Neill LAJ, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nat Rev Immunol. 2020 Jun;20(6):335-337. doi: 10.1038/s41577-020-0337-y. PMID: 32393823; PMCID: PMC7212510.**).

[0043] Estudos têm sugerido que algumas das vacinas administradas rotineiramente em bebês e crianças, como a BCG, também tem efeitos não-específicos sobre o risco de doença e morte por outras condições, além daquelas para as quais as vacinas foram projetadas para prevenir. No caso de

BCG, sua administração foi associada ao menor risco subsequente de doença e morte por outras causas, fato decorrente de mecanismos conhecidos por "trained immunity" e imunidade heteróloga (**Higgins, J.P.T., Soares-Weiser, K., López-López, J.A., Kakourou, A., Chaplin, K., Christensen, H., Martin, N.K., Sterne, J.A.C., Reingold, A.L., 2016. Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. BMJ 355, i5170.; Kleinnijenhuis, J., Quintin, J., Preijers, F., Benn, C.S., Joosten, L.A.B., Jacobs, C., van Loenhout, J., Xavier, R.J., Aaby, P., van der Meer, J.W.M., van Crevel, R., Netea, M.G., 2014. Long-Lasting Effects of BCG Vaccination on Both Heterologous Th1/Th17 Responses and Innate Trained Immunity. J. Innate Immun. 6, 152–158.**). Um estudo demonstrou que a vacinação com BCG induziu reprogramação epigenética *in vivo* de monócitos contra infecção experimental com uma vacina atenuada contra o vírus da febre amarela com papel fundamental da IL-1b como mediador dessa resposta (**Arts, R.J.W., Moorlag, S.J.C.F.M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., Kumar, V., Xavier, R.J., Wijmenga, C., Joosten, L.A.B., Reusken, C.B.E.M., Benn, C.S., Aaby, P., Koopmans, M.P., Stunnenberg, H.G., van Crevel, R., Netea, M.G., 2018. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. Cell Host Microbe 23, 89-100.e5.**).

[0044] A construção de cepas recombinantes de BCG que proporcionem maior estímulo do sistema imune tem sido explorada para melhorar sua eficácia contra TB (**Nieuwenhuizen, N.E., Kaufmann, S.H.E., 2018. Next-Generation Vaccines Based on Bacille Calmette–Guérin. Front. Immunol. 9, 121.**), aumentar seu efeito antitumoral na terapêutica de tumores de bexiga (**Begnini, K.R., Buss, J.H., Collares, T., Seixas, F.K., 2015. Recombinant *Mycobacterium bovis* BCG for immunotherapy in**

nonmuscle invasive bladder cancer. Appl. Microbiol. Biotechnol. 99, 3741–3754.), e expressar antígenos de diferentes patógenos para emprego como vetor vacinal (**Bastos, R.G., Borsuk, S., Seixas, F.K., Dellagostin, O.A., 2009. Recombinant *Mycobacterium bovis* BCG. Vaccine.; Zheng, Y., Naguib, Y.W., Dong, Y., Shi, Y., Bou, S., Cui, Z., 2015. Applications of bacillus Calmette-Guerin and recombinant bacillus Calmette-Guerin in vaccine development and tumor immunotherapy. Expert Rev. Vaccines 14, 1255–75. Marques-Neto LM, Piwowarska Z, Kanno AI, Moraes L, Trentini MM, Rodriguez D, Silva JLSC, Leite LCC. Thirty years of recombinant BCG: new trends for a centenary vaccine. Expert Rev Vaccines. 2021 Aug;20(8):1001-1011. doi: 10.1080/14760584.2021.1951243. Epub 2021 Jul 13. PMID: 34224293.**). Nesse contexto, salienta-se o potencial da construção e do uso de BCG como vetor vacinal expressando antígenos de *T. cruzi* como ferramenta profilática contra doença de Chagas.

[0045] Em uma pesquisa prévia realizada nos bancos de dados mundiais de depósito de patentes, é notória a quantidade de documentos que utilizam a plataforma BCG como fórmula vacinal contra uma gama de enfermidades. Encontramos como resultado o documento **US 6471967 B1**, referente a construção de BCG recombinante tendo uma sequência de ácido nucleico extracromossômico compreendendo gene que codifica uma proteína extracelular de 30 kDa de *Mycobacteria tuberculosis*. Como já foi descrito, nosso produto, apesar da semelhança metodológica, não será empregado contra tuberculose, mas sim contra doença de Chagas, fazendo uso de proteínas diferentes da reivindicada pela patente citada.

[0046] Nossa busca contemplou ainda a reivindicação por uma composição imunogénica compreendendo BCG recombinante, em que o referido expressa pelo menos uma proteína extracelular de *Mycobacteria major* seleccionada entre proteína de 23,5 kDa, proteína de 30 kDa, proteína de

32A kDa e proteína de 32B kDa; em que uma sequência de ácido nucleico que codifica para pelo menos uma proteína extracelular de micobactéria é incorporada no(s) cromossomo(s) do BCG recombinante sob um promotor forte, de modo que a proteína é superexpressa e o BCG recombinante não abriga um marcador de resistência a antibiótico (**US 8932846 B2**). Novamente, a composição deste produto pouco se assemelha com nossa invenção, uma vez que nossa estratégia objetiva a expressão heteróloga de genes de *T. cruzi* em *M. bovis* BCG como vacina contra DC.

[0047] No entanto, alguns documentos se assemelham com nosso produto. Como por exemplo, a patente que reivindica a construção de vacinas de BCG recombinantes que expressam DNA de interesse, incorporado na micobactéria, sob o controle de um promotor. Referindo-se particularmente a *M. bovis*-BCG recombinante em que o DNA de interesse é expresso extracromossomicamente sob o controle de um promotor hsp micobacteriano, tal como hsp70 e hsp60 (**WO 1995003418 A3**). Nossa estratégia se baseia sim nesta mesma forma de construção, no entanto, nossa invenção objetiva a adição de genes de *T. cruzi* na cepa Pasteur de *M. bovis* BCG para uso como vacina vetorizada utilizando promotores diferentes dos mencionados na referida patente.

[0048] Em relação ao antígeno selecionado para ser expresso pelo BCG recombinante, alguns documentos foram encontrados que também fazem uso da proteína flagelar de ligação ao cálcio de 24 kDa (Tc24) de *Trypanosoma cruzi* em suas formulações vacinais. Polipeptídeos de Tc24 sem resíduos de cisteínas em sua composição foram utilizados como protótipos vacinais (**WO 2017160849 A1**). Entretanto, as sequências de Tc24 propostas para uso pelo autor não correspondem a aqui presente selecionada, na qual utilizamos a proteína em sua integralidade. Além de tal, na patente encontrada, há a reivindicação da utilização de adjuvantes sintéticos na composição vacinal, enquanto o evento aqui proposto utiliza-se de um vetor bacteriano para

expressão do antígeno sem a necessidade de um adjuvante em sua composição.

[0049] Identificamos também a existência de um estudo utilizando BCG como imunizante contra doença de Chagas, intitulado **"The effect of BCG on the course of experimental Chagas' disease in mice"**. Esse trabalho visa a observação dos efeitos do tratamento com BCG não recombinante sobre a infecção de *T. cruzi* em camundongos C3H(He). Apesar de também fazer uso de *M. bovis* BCG no combate a DC, nosso invento baseia-se na utilização de uma cepa de BCG que irá expressar proteínas de *T. cruzi* (sem combinação com outros antígenos) cujas sequências gênicas foram especificamente selecionadas para clonagem em vetores de expressão em micobactérias, não contando apenas com a atividade imunológica gerada pelo BCG.

[0050] O artigo intitulado **"Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypanosoma cruzi* infection",** publicado por nosso grupo, também foi identificado em nossa busca. Ele demonstra a expressão dos fragmentos N-terminal e C-terminal da proteína transialidase e da proteína cruzipain de *T. cruzi, em M. bovis*-BCG, para utilização como imunizante. O mesmo além de reforçar o pioneirismo na utilização de BCG como vetor vacinal no combate a doença de Chagas por parte de nosso grupo, demonstra ainda a expertise do mesmo na utilização de BCG recombinante na superexpressão de antígenos. A particularidade de nossas sequências e consequentemente de nossa construção, garante o caráter ÚNICO e INOVADOR de nosso produto.

Breve descrição dos desenhos

[0051] A Figura 1 apresenta os níveis de citocinas INF-γ, Interleucina 4, 6, 10 e 17 produzidos pelos camundongos vacinados com a construção vacinal rBCG/pUS977/TC24.

Descrição da invenção

[0052] Tendo em vista os pontos abordados e os impactos gerados pela infecção por *T. cruzi*, em um contexto socioeconômico, ressalta-se a necessidade por novas formulações vacinais eficientes e seguras, como forma de minimizar os danos causados pela Doença de Chagas. Dessa forma, a presente invenção destina-se a construção de cepa de *Mycobacterium bovis* BCG Pasteur recombinante expressando a proteína flagelar de ligação ao cálcio de 24 kDa (Tc24) de *Trypanosoma cruzi*, para uso em vacinas experimentais e comerciais contra a doença de Chagas. A seguir, descreve-se o invento em mais detalhes:

Cultivo de M. bovis BCG Pasteur e E. coli

[0053] *M. bovis* BCG Pasteur é cultivado em meio 7H9 (Middlebrook 7H9 Broth Base) (líquido) ou 7H10 (Middlebrook 7H10 Broth Base)(sólido) com suplementação de 10% de OADC (Oleic Albumin Dextrose Catalase) e adição do antibiótico canamicina (50 mg/mL) quando necessário. O cultivo é mantido a 37ºC por 7 dias em meio 7H9 ou por 21 dias em meio 7H10. *E. coli* é cultivada em meio Luria Bertani (LB) líquido ou LB-Ágar à 37°C por 16 h, com suplementação do antibiótico canamicina (50 mg/mL) quando necessário.

Seleção e clonagem do alvo vacinal

[0054] As sequências nucleotídicas que codificam para a proteínas Tc24 de T*rypanosoma cruzi* foi obtida a partir do NCBI www.ncbi.nlm.nih.gov (U70035). A partir destas, foi possível realizar o desenho dos genes sintéticos *tc24* (SEQ ID NO:1) visando posterior clonagem nos vetores de expressão em *BCG*, para obtenção do construto recombinante rBCG/pUS977/TC24. Todos os genes sintéticos foram projetados com o auxílio do software Vector NTI 11 (Invitrogen™). Sítios de restrição enzimáticos (XbaI, BamHI e HindIII) foram adicionados nas extremidades 5' e 3' das sequências, visando a clonagem no vetor pAE de expressão em *E. coli* (**Ramos, C.R.R., Abreu, P.A.E., Nascimento, A.L.T.O., Ho, P.L., 2004. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal his-tagged fusion peptide. Brazilian J. Med. Biol. Res. 37, 1103–1109.**), e nos vetores de expressão em *M. bovis* BCG (**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**).

[0055] O gene que codofica para a proteína Tc24 foi sintetizado (Genome) e após subclonado no vetor de expressão em *M. bovis* BCG Pasteur (pUS977) (**Dellagostin, O.A., Wall, S., Norman, E., O'Shaughnessy, T., Dale, J.W., McFadden, J., 1993. Construction and use of integrative vectors to express foreign genes in mycobacteria. Mol. Microbiol. 10, 983–93.**). Em seguida, o produto da ligação foi transformado por eletroporação em *E. coli* TOP10 para obtenção dos clones recombinantes. Após caracterização enzimática e por sequenciamento de DNA, os clones recombinantes foram utilizados para transformação de *M. bovis* BCG Pasteur por eletroporação.

Expressão da proteína recombinante rTc24 e obtenção do soro policlonal

[0056] A expressão da proteína recombinante foi realizada em cepa de *E. coli* BL21 Star, cultivada em meio Luria Bertani (LB) com adição de 1,5% de ágar bacteriológico durante 16 horas à 37ºC, suplementado com 100 μg/mL de ampicilina, quando necessário. Essas células foram transformadas com os plasmídeos pAE/*tc24* por choque térmico, com posterior incubação. A indução da expressão dessas proteínas ocorreu pela adição de Isopropil-β-D-1-tiogalactopiranósido (IPTG) a 1mM e incubação por 3 horas. Ao final do processo, a purificação das proteínas se deu através de cromatografia de afinidade ao níquel, em coluna de Sepharose HisTRAP (GE). Por fim, a corrida eletroforética em gel de SDS-PAGE e posterior *Western Blot* com anticorpo monoclonal anti-histidina permitiu a confirmação da identidade das proteínas expressas.

[0057] Obtendo a confirmação da identidade das proteínas expressas, através de *Western Blot* com anticorpo monoclonal anti-histidina, foi possível dar início ao protocolo de inoculação para obtenção de soro policlonal, afim de avaliar a expressão da proteína pelo BCG recombinante. Para isso, 20 camundongos BALB/c fêmeas foram alocados em dois grupos, sendo: Grupo A: Solução salina 0,9% (Controle); e Grupo B: proteína recombinante rTc24 + adjuvante hidróxido de alumínio. Todos os experimentos com animais aqui descritos foram aprovados pelo Comitê de Ética em Experimentação Animal da Universidade Federal de Pelotas (CEEA-UFPel), sob número de protocolo 23110.031479/2021-17.

[0058] A formulação para o grupo B caracterizou-se pela inoculação de 25 μg da proteína recombinante (rTc24). O adjuvante hidróxido de alumínio foi acrescentado na concentração de 15%. Todas as aplicações foram realizadas por via subcutânea, em duas doses, com intervalo de 21 dias entre elas. Para obtenção do soro, após a segunda dose (dia 42), foi realizada coleta de sangue, que foi então centrifugado para separação do soro.

rBCG/pUS977/Tc24

[0059] Posteriormente, foi realizada nova corrida eletroforética em gel de SDS-PAGE, seguida de *Western Blot*, desta vez, avaliando a construção vacinal rBCG/pUS977/TC24. Contrariamente ao processo realizado anteriormente, que utilizou o anticorpo monoclonal anti-histidina, aqui fez-se uso do soro policlonal obtido dos camundongos inoculados com a proteína recombinante isolada, o que permitiu a confirmação da expressão e identidade da proteína em *M. bovis* BCG recombinante.

[0060] Obtida a construção vacinal, deu-se início ao protocolo de imunização dos camundongos. Para tal, 30 camundongos BALB/c fêmeas foram alocados em três grupos, organizados da seguinte forma: Grupo A: Solução salina 0,9% (Controle); Grupo B: *M. bovis* BCG Pasteur não transformada; Grupo C: rBCG/pUS977/TC24.

[0061] As formulações para os grupos B e C caracterizaram-se pela inoculação de $1x10^7$ UFC/mL. Para tal, o cultivo teve sua densidade óptica aferida, onde o volume de cultivo em meio 7H9 equivalente a concentração necessária fora alocado em novo recipiente, centrifugado, separado do meio e ressuspendido em 10 mL de PBS. Todas as aplicações foram realizadas por via subcutânea, em duas doses, com intervalo de 21 dias entre elas.

Resposta imune celular

[0062] Para que fosse possível projetar a efetividade da vacina, foi avaliada a resposta imune celular. Realizada a eutanásia dos 30 animais previamente imunizados, coletou-se o baço de cada camundongo, em ambiente estéril. A cultura dos esplenócitos foi realizada em Dulbecco’s modified Eagle’s médium (DMEM), contendo alta glicose e suplementado com 10% de soro fetal bovino (SFB), sendo adicionada a placas de cultivo de 24 cavidades,

com fundo chato. O cultivo foi incubado por 24 horas a 37ºC em estufa com 5% de CO_2.

[0063] Após o período de incubação, foi realizado o estímulo contendo 10 μg da proteína recombinante no grupo C. O grupo referente ao controle negativo foi adicionado apenas solução salina, enquanto o controle positivo foi estimulado com 10 μg de concavalina A, induzindo a produção de citocinas para quantificação. Nova incubação foi realizada, sob as mesmas condições citadas anteriormente.

[0064] Após as 24 horas subsequentes, a coleta das células foi efetuada, permitindo a extração de RNA total a partir do método do reagente Trizol (Invitrogen). Obtendo essa porção de material genético, a síntese de cDNA foi realizada a partir do kit comercial High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), utilizando 1 μL do RNA extraído para sua confecção. A partir das amostras de cDNA sintetizadas, foi possível avaliar a quantificação das citocinas INF-γ, Interleucina 4, 6, 10 e 17, através de PCR em tempo real, utilizando primers específicos.

[0065] Por fim, as análises estatísticas foram obtidas a partir do Software GraphPad Prism 7.0, a partir de análise unidirecional de variância (ANOVA), seguida pelo pós-teste de Tukey. O valor de significância observado como parâmetro foi de $p<0,05$, indicando diferença estatística.

Exemplos de concretizações da invenção

Exemplo 1:

BCG recombinante expressão antígeno de *T. cruzi* induz aumento na resposta imune celular em modelo murino

[0066] A avaliação da resposta imune celular induzida a partir do cultivo de esplenócitos indicou uma produção balanceada entre citocinas pró e anti-inflamatórias. Nesse aspecto, observou-se um aumento significativo na

expressão das citocinas Interleucina 4, 10 e 17, enquanto observa-se uma repressão da citocina Interleucina 6 (Figura 1).

[0067] A construção apresentou padrões balanceados entre respostas pró e anti-inflamatórias, resposta desejada no combate à doença de Chagas. Não houve expressão estatisticamente significativa para a citocina pró-inflamatória IFN-y quando comparados ao grupo controle A. Entretanto, apesar de parecer pouco propício para um modelo vacinal, altos níveis de expressão de IFN-y estão diretamente associados com maiores níveis de dano tecidual em pacientes positivados para Chagas em estágio crônico.

[0068] Em contraponto, outras citocinas pró-inflamatórias são de grande interesse quando sua expressão é induzida em altos níveis, como é o caso da Interleucina 17. Esta é considerada uma das mais importantes na resposta imune contra a infeção por *T. cruzi*, pois está diretamente relacionada a imunidade contra patógenos intracelulares, recrutamento de neutrófilos e respostas Th17, fundamentais na defesa contra parasitoses. Quando comparado ao grupo salina, apenas a imunização com *M. bovis* BCG não transformado (grupo B) já é capaz de aumentar significativamente a expressão dessa citocina, mas a indução é superior quando se considera a inoculação de rBCG/pUS977/TC24 (grupo C).

[0069] Como mencionado anteriormente, indução de respostas anti-inflamatórias são igualmente importantes no combate a Chagas. Dessa forma, os resultados obtidos para o grupo C na indução da expressão da Interleucina 4 e da repressão da Interleucina 6 corroboram com o cenário de uma amplificação de uma resposta imune balanceada quando comparado ao grupo não vacinado. Dessa forma, o uso de BCG recombinante expressando o antígeno Tc24 induz uma resposta imune com um caráter mais robusto, propiciando ao organismo melhor preparo para impedir a infecção e/ou melhor combate-la uma vez instaurada.

DESENHOS

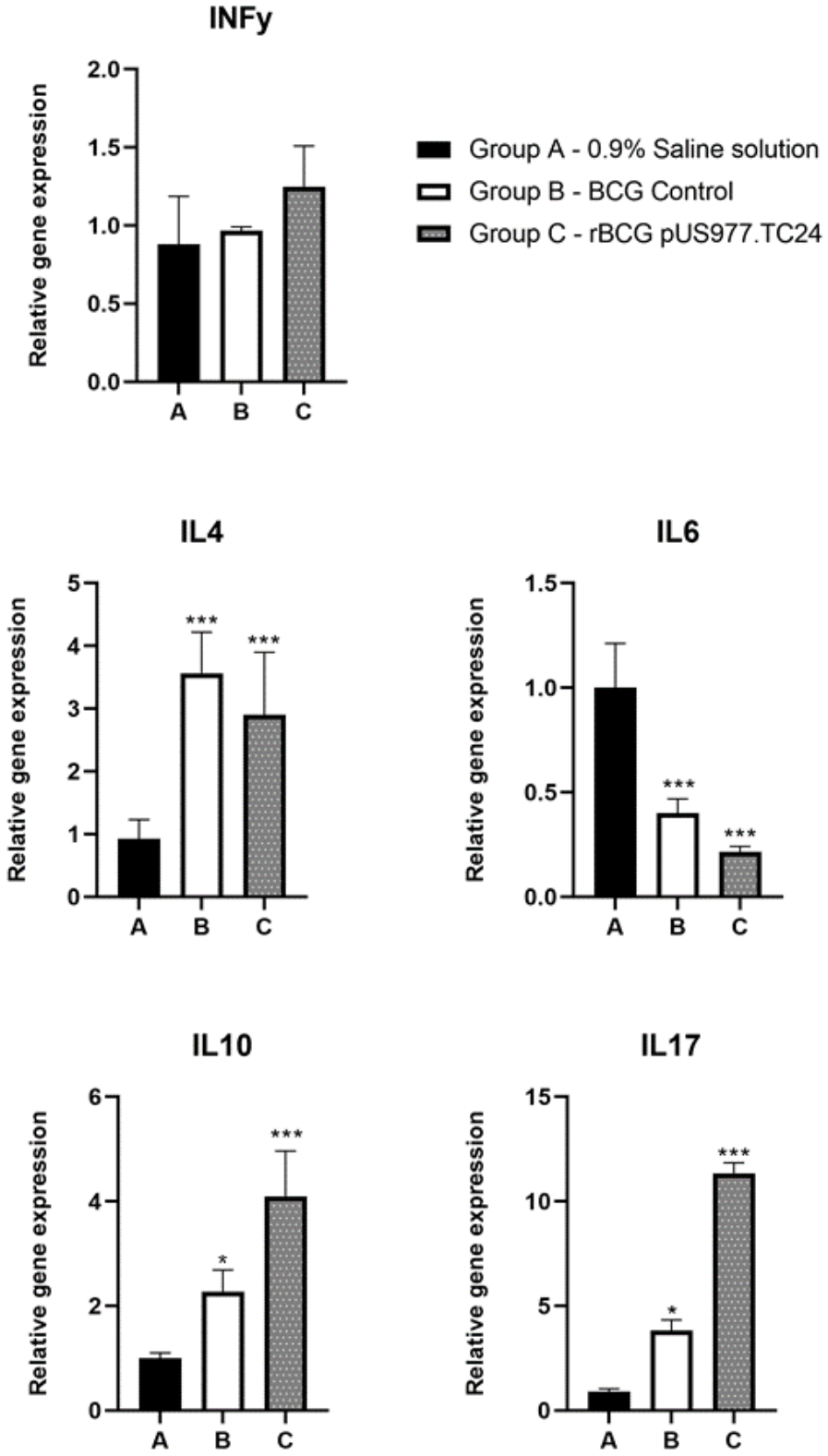


Figura 1

4. Conclusões

- A doença de Chagas (DC) não possui vacina, e diante disso, diferentes estratégias vacinais experimentais estão em desenvolvimento com o objetivo de proteger contra o patógeno *Trypanosoma cruzi*, tendo as vacinas vetorizadas por bactérias mostrado resultados bastante promissores;
- As estratégias vacinais utilizando rBCG elevaram os níveis das interleucinas IFN-y no Grupo C (rBCG/pUS977/asp-2) e IL-17 no Grupo D (rBCG/pUS977/tc24), enquanto os níveis das interleucinas IL-4 e IL-10 foram elevados por ambos os grupos, em relação aos controles basais (Grupo A) e BCG Pasteur não transformado (Grupo B).
- Para confirmar a efetividade das construções vacinais baseadas em rBCG, experimentos de desafio em modelo animal, utilizando cepa virulenta de *Trypanosoma cruzi* ainda serão necessários, com o intuito de avaliar taxas de sobrevivência.

Referências

Antinori, S., Galimberti, L., Bianco, R., Grande, R., Galli, M., & Corbellino, M. (2017). Chagas disease in Europe: A review for the internist in the globalized world. *European Journal of Internal Medicine*, *43*, 6–15. https://doi.org/10.1016/j.ejim.2017.05.001

Araújo, A. F., De Oliveira, G., Vasconcelos, J. F., Ersching, J., Dominguez, M. R., Vasconcelos, J. R., Machado, A. V., Gazzinelli, R. T., Bruna-Romero, O., Soares, M. B., & Rodrigues, M. M. (2014). Genetic Vaccination against Experimental Infection with Myotropic Parasite Strains of *Trypanosoma cruzi*. *Mediators of Inflammation*, *2014*, 1–13. https://doi.org/10.1155/2014/605023

Araújo, A. F. S., De Alencar, B. C. G., Vasconcelos, J. R. C., Hiyane, M. I., Marinho, C. R. F., Penido, M. L. O., Boscardin, S. B., Hoft, D. F., Gazzinelli, R. T., & Rodrigues, M. M. (2005). $CD8^+$-T-Cell-Dependent Control of *Trypanosoma cruzi* Infection in a Highly Susceptible Mouse Strain after Immunization with Recombinant Proteins Based on Amastigote Surface Protein 2. *Infection and Immunity*, *73*(9), 6017–6025. https://doi.org/10.1128/IAI.73.9.6017-6025.2005

Arce-Fonseca, M., González-Vázquez, M. C., Rodríguez-Morales, O., Graullera-Rivera, V., Aranda-Fraustro, A., Reyes, P. A., Carabarin-Lima, A., & Rosales-Encina, J. L. (2018). Recombinant Enolase of *Trypanosoma cruzi* as a Novel Vaccine Candidate against Chagas Disease in a Mouse Model of Acute Infection. *Journal of Immunology Research*, *2018*, 1–14. https://doi.org/10.1155/2018/8964085

Barbosa, R. P. A., Filho, B. G., Santos, L. I. D., Junior, P. A. S., Marques, P. E., Pereira, R. V. S., Cara, D. C., Bruña-Romero, O., Rodrigues, M. M., Gazzinelli, R. T., & Machado, A. V. (2013). Vaccination Using Recombinants Influenza and Adenoviruses Encoding Amastigote Surface Protein-2 Are Highly Effective on Protection against Trypanosoma cruzi Infection. *PLoS ONE*, *8*(4), e61795. https://doi.org/10.1371/journal.pone.0061795

Bastos, R. G., Borsuk, S., Seixas, F. K., & Dellagostin, O. A. (2009). Recombinant Mycobacterium bovis BCG. *Vaccine*, *27*(47), 6495–6503. https://doi.org/10.1016/j.vaccine.2009.08.044

Bivona, A. E., Alberti, A. S., Cerny, N., Trinitario, S. N., & Malchiodi, E. L. (2020). Chagas disease vaccine design: The search for an efficient Trypanosoma cruzi immune-mediated control. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1866*(5), 165658. https://doi.org/10.1016/j.bbadis.2019.165658

Bivona, A. E., Sánchez Alberti, A., Matos, M. N., Cerny, N., Cardoso, A. C., Morales, C., González, G., Cazorla, S. I., & Malchiodi, E. L. (2018). Trypanosoma cruzi 80 kDa prolyl oligopeptidase (Tc80) as a novel immunogen for Chagas disease vaccine. *PLOS Neglected Tropical Diseases*, *12*(3), e0006384. https://doi.org/10.1371/journal.pntd.0006384

Bontempi, I., Leal, K., Prochetto, E., Díaz, G., Cabrera, G., Bortolotti, A., Morbidoni, H. R., Borsuk, S., Dellagostin, O., & Marcipar, I. (2020). Recombinant *Mycobacterium bovis* BCG is a promising platform to develop vaccines against *Trypansoma cruzi* infection. *Clinical and Experimental Immunology*, *201*(3), 306–316. https://doi.org/10.1111/cei.13469

Cazorla, S. I., Matos, M. N., Cerny, N., Ramirez, C., Alberti, A. S., Bivona, A. E., Morales, C., Guzman, C. A., & Malchiodi, E. L. (2015). Oral Multicomponent DNA Vaccine Delivered by Attenuated Salmonella Elicited Immunoprotection Against American Trypanosomiasis. *Journal of Infectious Diseases*, *211*(5), 698–707. https://doi.org/10.1093/infdis/jiu480

Centers for Disease Control and Prevention of the U.S. (2021, junho 16). *American Trypanosomiasis*. https://www.cdc.gov/dpdx/trypanosomiasisamerican/index.html

Convit, J., Ulrich, M., Polegre, M. A., Avila, A., Rodríguez, N., Mazzedo, M. I., & Blanco, B. (2004). Therapy of Venezuelan patients with severe mucocutaneous or early lesions of diffuse cutaneous leishmaniasis with a vaccine containing pasteurized Leishmania promastigotes and bacillus Calmette-Guerin: Preliminary report. *Memórias do Instituto Oswaldo Cruz*, *99*(1), 57–62. https://doi.org/10.1590/S0074-02762004000100010

De La Cruz, J. J., Villanueva-Lizama, L., Dzul-Huchim, V., Ramírez-Sierra, M.-J., Martinez-Vega, P., Rosado-Vallado, M., Ortega-Lopez, J., Flores-Pucheta, C. I., Gillespie, P., Zhan, B., Bottazzi, M. E., Hotez, P. J., & Dumonteil, E. (2019). Production of recombinant TSA-1 and evaluation of its potential for the immuno-therapeutic control of *Trypanosoma cruzi* infection in mice. *Human Vaccines & Immunotherapeutics*, *15*(1), 210–219. https://doi.org/10.1080/21645515.2018.1520581

Dumonteil, E., Escobedo-Ortegon, J., Reyes-Rodriguez, N., Arjona-Torres, A., & Ramirez-Sierra, M. J. (2004). Immunotherapy of *Trypanosoma cruzi* Infection with DNA Vaccines in Mice. *Infection and Immunity*, *72*(1), 46–53. https://doi.org/10.1128/IAI.72.1.46-53.2004

Dumonteil, E., Herrera, C., & Buekens, P. (2019). A therapeutic preconceptional vaccine against Chagas disease: A novel indication that could reduce congenital transmission and accelerate vaccine development. *PLOS Neglected Tropical Diseases*, *13*(1), e0006985. https://doi.org/10.1371/journal.pntd.0006985

Engman, D. M., Dragon, E. A., & Donelson, J. E. (1990). Human humoral immunity to hsp70 during Trypanosoma cruzi infection. *Journal of Immunology (Baltimore, Md.: 1950)*, *144*(10), 3987–3991.

Forsyth, C., Meymandi, S., Moss, I., Cone, J., Cohen, R., & Batista, C. (2019). Proposed multidimensional framework for understanding Chagas disease healthcare barriers in the United States. *PLOS Neglected Tropical Diseases*, *13*(9), e0007447. https://doi.org/10.1371/journal.pntd.0007447

Freire-de-Lima, L., Fonseca, L., Oeltmann, T., Mendonça-Previato, L., & Previato, J. (2015). The *trans* -sialidase, the major *Trypanosoma cruzi* virulence factor: Three decades of studies. *Glycobiology*, *25*(11), 1142–1149. https://doi.org/10.1093/glycob/cwv057

Frost, L. J., & Reich, M. R. (2009). Creating Access To Health Technologies In Poor Countries. *Health Affairs*, *28*(4), 962–973. https://doi.org/10.1377/hlthaff.28.4.962

Garg, N., & Tarleton, R. L. (2002). Genetic Immunization Elicits Antigen-Specific Protective Immune Responses and Decreases Disease Severity in *Trypanosoma cruzi* Infection. *Infection and Immunity*, *70*(10), 5547–5555. https://doi.org/10.1128/IAI.70.10.5547-5555.2002

Guarner, J. (2019). Chagas disease as example of a reemerging parasite. *Seminars in Diagnostic Pathology*, *36*(3), 164–169. https://doi.org/10.1053/j.semdp.2019.04.008

Gunter, S. M., Versteeg, L., Jones, K. M., Keegan, B. P., Strych, U., Bottazzi, M. E., Hotez, P. J., & Brown, E. L. (2018). Covalent vaccination with *Trypanosoma cruzi* Tc24 induces catalytic antibody production. *Parasite Immunology*, *40*(11), e12585. https://doi.org/10.1111/pim.12585

Jiménez, P., Jaimes, J., Poveda, C., & Ramírez, J. D. (2019). A systematic review of the *Trypanosoma cruzi* genetic heterogeneity, host immune response and genetic factors as plausible drivers of chronic chagasic cardiomyopathy. *Parasitology*, *146*(3), 269–283. https://doi.org/10.1017/S0031182018001506

Limon-Flores, A. Y., Cervera-Cetina, R., Tzec-Arjona, J. L., Ek-Macias, L., Sánchez-Burgos, G., Ramirez-Sierra, M. J., Cruz-Chan, J. V., VanWynsberghe, N. R., & Dumonteil, E. (2010). Effect of a combination DNA vaccine for the prevention and therapy of Trypanosoma cruzi infection in mice: Role of CD4+ and CD8+ T cells. *Vaccine*, *28*(46), 7414–7419. https://doi.org/10.1016/j.vaccine.2010.08.104

Macaluso, G., Grippi, F., Di Bella, S., Blanda, V., Gucciardi, F., Torina, A., Guercio, A., & Cannella, V. (2023). A Review on the Immunological Response against Trypanosoma cruzi. *Pathogens*, *12*(2), 282. https://doi.org/10.3390/pathogens12020282

Machado, A. V., Cardoso, J. E., Claser, C., Rodrigues, M. M., Gazzinelli, R. T., & Bruna-Romero, O. (2006). Long-Term Protective Immunity Induced Against *Trypanosoma cruzi* Infection After Vaccination with Recombinant Adenoviruses Encoding Amastigote Surface Protein-2 and *Trans*- Sialidase. *Human Gene Therapy*, *17*(9), 898–908. https://doi.org/10.1089/hum.2006.17.898

Maldonado, E., Morales-Pison, S., Urbina, F., & Solari, A. (2022). Vaccine Design against Chagas Disease Focused on the Use of Nucleic Acids. *Vaccines*, *10*(4), 587. https://doi.org/10.3390/vaccines10040587

Mancino, C., Pollet, J., Zinger, A., Jones, K. M., Villar, M. J., Leao, A. C., Adhikari, R., Versteeg, L., Tyagi Kundu, R., Strych, U., Giordano, F., Hotez, P. J., Bottazzi, M. E., Taraballi, F., & Poveda, C. (2024). Harnessing RNA Technology to Advance Therapeutic Vaccine Antigens against Chagas Disease. *ACS Applied Materials & Interfaces*, *16*(13), 15832–15846. https://doi.org/10.1021/acsami.3c18830

Martinez-Campos, V., Martinez-Vega, P., Ramirez-Sierra, M. J., Rosado-Vallado, M., Seid, C. A., Hudspeth, E. M., Wei, J., Liu, Z., Kwityn, C., Hammond, M., Ortega-López, J., Zhan, B., Hotez, P. J., Bottazzi, M. E., & Dumonteil, E. (2015). Expression, purification, immunogenicity, and protective efficacy of a recombinant Tc24 antigen as a vaccine against Trypanosoma cruzi infection in mice. *Vaccine*, *33*(36), 4505–4512. https://doi.org/10.1016/j.vaccine.2015.07.017

Matos, M. N., Cazorla, S. I., Bivona, A. E., Morales, C., Guzmán, C. A., & Malchiodi, E. L. (2014). Tc52 Amino-Terminal-Domain DNA Carried by Attenuated Salmonella enterica Serovar Typhimurium Induces Protection against a Trypanosoma cruzi Lethal Challenge. *Infection and Immunity*, *82*(10), 4265–4275. https://doi.org/10.1128/IAI.02190-14

Matos, M. N., Cazorla, S. I., Schulze, K., Ebensen, T., Guzmán, C. A., & Malchiodi, E. L. (2017). Immunization with Tc52 or its amino terminal domain adjuvanted with c-di-AMP induces Th17+Th1 specific immune responses and confers protection against Trypanosoma cruzi. *PLOS Neglected Tropical Diseases*, *11*(2), e0005300. https://doi.org/10.1371/journal.pntd.0005300

Pérez-Molina, J. A., & Molina, I. (2018). Chagas disease. *The Lancet*, *391*(10115), 82–94. https://doi.org/10.1016/S0140-6736(17)31612-4

Poveda, C., Leão, A. C., Mancino, C., Taraballi, F., Chen, Y.-L., Adhikari, R., Villar, M. J., Kundu, R., Nguyen, D. M., Versteeg, L., Strych, U., Hotez, P. J., Bottazzi, M. E., Pollet, J., & Jones, K. M. (2023). Heterologous mRNA-protein vaccination with Tc24 induces a robust cellular immune response against Trypanosoma cruzi, characterized by an increased level of polyfunctional CD8+ T-cells. *Current Research in Immunology*, *4*, 100066. https://doi.org/10.1016/j.crimmu.2023.100066

Quijano-Hernandez, I. A., Bolio-González, M. E., Rodríguez-Buenfil, J. C., Ramirez-Sierra, M. J., & Dumonteil, E. (2008). Therapeutic DNA Vaccine against *Trypanosoma cruzi* Infection in Dogs: A Pilot Clinical Trial. *Annals of the New York Academy of Sciences*, *1149*(1), 343–346. https://doi.org/10.1196/annals.1428.098

Quijano-Hernández, I. A., Castro-Barcena, A., Vázquez-Chagoyán, J. C., Bolio-González, M. E., Ortega-López, J., & Dumonteil, E. (2013). Preventive and therapeutic DNA vaccination partially protect dogs against an infectious challenge with Trypanosoma cruzi. *Vaccine*, *31*(18), 2246–2252. https://doi.org/10.1016/j.vaccine.2013.03.005

Quintana, I., Espariz, M., Villar, S. R., González, F. B., Pacini, M. F., Cabrera, G., Bontempi, I., Prochetto, E., Stülke, J., Perez, A. R., Marcipar, I., Blancato, V., & Magni, C. (2018). Genetic Engineering of Lactococcus lactis Co-producing Antigen and the Mucosal Adjuvant 3’ 5’- cyclic di Adenosine Monophosphate (c-di-AMP) as a Design Strategy to Develop a Mucosal Vaccine Prototype. *Frontiers in Microbiology*, *9*, 2100. https://doi.org/10.3389/fmicb.2018.02100

Ramos-Vega, A., Dzul-Huchim, V. M., Villanueva-Lizama, L. E., Dumonteil, E., Rosales-Mendoza, S., Bañuelos-Hernández, B., Angulo, M., Cruz-Chan, J. V., Monreal-Escalante, E., & Angulo, C. (2024). Protective efficacy of the oral vaccine Tc24:Co1 produced in Schizochytrium sp. Against Trypanosoma cruzi infection in a mouse model. *Microbial Pathogenesis*, *186*, 106488. https://doi.org/10.1016/j.micpath.2023.106488

Rassi, A., Rassi, A., & Marin-Neto, J. A. (2010). Chagas disease. *The Lancet*, *375*(9723), 1388–1402. https://doi.org/10.1016/S0140-6736(10)60061-X

Ribeiro, F. A. P., Pontes, C., Gazzinelli, R. T., Romero, O.-B., Lazzarin, M. C., Dos Santos, J. F., De Oliveira, F., Pisani, L. P., De Vasconcelos, J. R. C., & Ribeiro, D. A. (2019). Therapeutic effects of vaccine derived from amastigote surface protein-2 (ASP-2) against Chagas disease in mouse liver. *Cytokine*, *113*, 285–290. https://doi.org/10.1016/j.cyto.2018.07.017

Sanchez-Burgos, G., Mezquita-Vega, R. G., Escobedo-Ortegon, J., Ramirez-Sierra, M. J., Arjona-Torres, A., Ouaissi, A., Rodrigues, M. M., & Dumonteil, E. (2007). Comparative evaluation of therapeutic DNA vaccines against *Trypanosoma cruzi* in mice. *FEMS Immunology & Medical Microbiology*, *50*(3), 333–341. https://doi.org/10.1111/j.1574-695X.2007.00251.x

Stanford, J. L. (1989). Immunotherapy for leprosy and tuberculosis. Em E. Jucker (Org.), *Progress in Drug Research* (p. 415–448). Birkhäuser Basel. https://doi.org/10.1007/978-3-0348-9146-2_13

Tarleton, R. L. (2015). CD8+ T cells in Trypanosoma cruzi infection. *Seminars in Immunopathology*, *37*(3), 233–238. https://doi.org/10.1007/s00281-015-0481-9

Vekemans, J., Hasso-Agopsowicz, M., Kang, G., Hausdorff, W. P., Fiore, A., Tayler, E., Klemm, E. J., Laxminarayan, R., Srikantiah, P., Friede, M., & Lipsitch, M. (2021). Leveraging Vaccines to Reduce Antibiotic Use and Prevent Antimicrobial Resistance: A World Health Organization Action Framework. *Clinical Infectious Diseases*, *73*(4), e1011–e1017. https://doi.org/10.1093/cid/ciab062

Vermelho, A. B., Cardoso, V., Mansoldo, F. R. P., Supuran, C. T., Cedrola, S. M. L., Rodrigues, I. A., & Rodrigues, G. C. (2022). Chagas Disease: Drug Development and Parasite Targets. Em A. B. Vermelho & C. T. Supuran (Orgs.), *Antiprotozoal Drug Development and Delivery* (Vol. 39, p. 49–81). Springer International Publishing. https://doi.org/10.1007/7355_2021_143

Vermelho, A. B., Da Silva Cardoso, V., Ricci Junior, E., Dos Santos, E. P., & Supuran, C. T. (2018). Nanoemulsions of sulfonamide carbonic anhydrase inhibitors strongly inhibit the growth of *Trypanosoma cruzi*. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *33*(1), 139–146. https://doi.org/10.1080/14756366.2017.1405264

Villanueva-Lizama, L. E., Cruz-Chan, J. V., Versteeg, L., Teh-Poot, C. F., Hoffman, K., Kendricks, A., Keegan, B., Pollet, J., Gusovsky, F., Hotez, P. J., Bottazzi, M. E., & Jones, K. M. (2020). TLR4 agonist protects against *Trypanosoma cruzi* acute lethal infection by decreasing cardiac parasite burdens. *Parasite Immunology*, *42*(10), e12769. https://doi.org/10.1111/pim.12769

World Health Organization. (2024). *Chagas disease (also known as American trypanosomiasis)*. https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis)

Yepes, A. F., Quintero-Saumeth, J., & Cardona-G, W. (2020). Chalcone-Quinoline Conjugates as Potential *T. cruzi* Cruzipain Inhibitors: Docking Studies, Molecular Dynamics and Evaluation of Drug-Likeness. *ChemistrySelect*, *5*(23), 7104–7112. https://doi.org/10.1002/slct.202000777

Yeung, C., Mendoza, I., Echeverria, L. E., & Baranchuk, A. (2021). Chagas' cardiomyopathy and Lyme carditis: Lessons learned from two infectious diseases affecting the heart. *Trends in Cardiovascular Medicine*, *31*(4), 233–239. https://doi.org/10.1016/j.tcm.2020.04.004

Zapataestrella, H., Hummelnewell, C., Sanchezburgos, G., Escobedoortegon, J., Ramirezsierra, M., Arjonatorres, A., & Dumonteil, E. (2006). Control of Trypanosoma cruzi infection and changes in T-cell populations induced by a therapeutic DNA vaccine in mice. *Immunology Letters*, *103*(2), 186–191. https://doi.org/10.1016/j.imlet.2005.11.015