UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM
IMUNOLOGIA E PARASITOLOGIA APLICADAS

A ausência da proteína P21 em *Trypanosoma cruzi* impacta a invasão celular por tripomastigotas e modula a multiplicação e a expressão gênica de amastigotas intracelular de cepas filogeneticamente distintas

ANNA CLARA AZEVEDO SILVEIRA

NOVEMBRO/2025

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Eu sou capaz.

LISTA DE ABREVIAÇÕES

% - percentagem

AM - Amastigota

BZN - benzonidazol

CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9

czp 2 - cruzipaína 2

Ca^{2+} - Via do cálcio

DEGs - diferencialmente expressos

DGF-1 -Dispersed Gene Family-1

DTU - Discrete Typing Unit

DNA - ácido desoxirribonucleico

EPI - Epimastigota

GAT3 - Transportado ABC glicossomal 3

GPI - Glicosilfosfatidilinositol

IL-4 - Interleucina-4

kDa - quilodalton

LAMP - Lysosome-Associated Membrane Protein

mRNA - messenger RNA

MT - Tripomastigota Metacíclico

MVK - Mevalonato quinase

NADH - Nicotinamida Adenina Dinucleotídeo na forma reduzida

NFX - nifurtimox

NTRs - nitroredutase tipo I

PFGE - Eletroforese em gel de campo pulsado

PI-PLC - Fosfolipase C endógena

P21 - Proteína de 21 de *T. cruzi*

RNA - ácido ribonucleico

RNA-seq - RNA sequencing

rP21 – proteína recombinante P21

sgRNA - single-guide RNA

TcMUC - mucinas de *T. cruzi*

TcMVK - proteína recombinate de mevalonato quinase

TcP21$^{-/-}$ - parasitas knockout para a P21

T. cruzi - *Trypanosoma cruzi*

TS- Trans-sialidase

TCT - Tripomastigota de corrente sanguinea

UTRs - Regiões Não Traduzidas

WT - Wild-type

$Zn^{2}+$ - Zinco

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RESUMO

Trypanosoma cruzi é um protozoário parasita causador da Doença de Chagas, uma enfermidade negligenciada com ampla distribuição nas Américas. Esse parasita apresenta elevada variabilidade genética, organizada em Unidades de Tipagem Discretas (DTUs), que refletem diferenças importantes em aspectos biológicos, clínicos e moleculares. Dentre os diversos fatores envolvidos na patogenicidade de *T. cruzi*, destaca-se a proteína P21, uma glicoproteína secretada associada à modulação da resposta inflamatória, adesão e invasão de células hospedeiras, além de possível regulação do ciclo celular do parasita. Este trabalho teve como objetivo geral investigar o papel funcional da proteína P21 por meio da sua deleção gênica em diferentes cepas de *T. cruzi* utilizando a ferramenta CRISPR/Cas9, com ênfase na análise do impacto transcriptômico da ausência dessa proteína. Foram geradas linhagens *knockout* para o gene P21 nas cepas Y (TcII) e G (TcI), representativas de DTUs distintas. Avaliou-se o impacto fenotípico da deleção em diferentes formas evolutivas do parasita e, em seguida, foi realizada análise global de expressão gênica por RNA-seq. A caracterização transcriptômica revelou perfis distintos entre as cepas *knockout*s, com genes diferencialmente expressos relacionados a processos-chave como adesão celular, sinalização, resposta ao estresse, modificação pós-traducional e expressão de proteínas de superfície envolvidas na virulência. Esses dados sugerem que a P21 exerce papel modulador na biologia de *T. cruzi*, com efeitos específicos em diferentes contextos genéticos. Os resultados obtidos contribuem para o entendimento da função da P21 no ciclo de vida do parasita e na interação parasita-hospedeiro, além de fortalecerem o uso da edição gênica como ferramenta estratégica para estudos funcionais em tripanossomatídeos.

Palavras-chave: *Trypanosoma cruzi*. P21. CRISPR/Cas9. RNA-seq. Transcriptoma.

ABSTRACT

Trypanosoma cruzi is a protozoan parasite and the causative agent of Chagas disease, a neglected tropical disease with wide distribution in the Americas. This parasite displays high genetic variability, classified into Discrete Typing Units (DTUs), which are associated with biological, clinical, and molecular differences. Among the various factors involved in *T. cruzi* pathogenicity, the P21 protein stands out as a secreted glycoprotein implicated in modulating the inflammatory response, promoting host cell adhesion and invasion, and potentially regulating the parasite cell cycle. This study aimed to investigate the functional role of the P21 protein through targeted gene knockout in different *T. cruzi* strains using the CRISPR/Cas9 genome editing system, with a special focus on transcriptomic changes resulting from its absence. Knockout lines were generated in Y (TcII) and G (TcI) strains, representing distinct DTUs. The phenotypic impact of the knockout was evaluated in different developmental forms of the parasite, followed by global gene expression analysis using RNA-seq. Transcriptomic profiling revealed distinct expression patterns between knockout strains, with differentially expressed genes related to key processes such as cell adhesion, signal transduction, stress response, post-translational modifications, and the expression of surface proteins involved in virulence. These findings suggest that P21 plays a modulatory role in *T. cruzi* biology, with strain-specific effects on host-parasite interactions. The results contribute to a better understanding of P21 function during the parasite life cycle and highlight the utility of genome editing as a powerful tool for functional studies in trypanosomatids.

Keywords: *Trypanosoma cruzi*; P21; CRISPR/Cas9; RNA-seq; Transcriptome;

CAPÍTULO I

Fundamentação Teórica

1. INTRODUÇÃO

1.1 Aspectos gerais da doença

A Doença de Chagas é uma doença tropical negligenciada com implicações significativas para a saúde global, causada pelo protozoário *Trypanosoma cruzi* (*T. cruzi*; ordem: Kinetoplastida; família Trypanosomatidae). O parasita é transmitido por insetos vetores triatomíneos (ordem Hemiptera; família Reduviidae) (Michel-Todó *et al.*, 2019; WHO, 2025). Endêmica em 21 países da América Latina, a distribuição geográfica da doença foi inicialmente determinada pela presença do inseto vetor. No entanto, devido à migração humana, o parasita expandiu-se para regiões não endêmicas, tornando-se uma ameaça crescente à saúde pública global. Estima-se que aproximadamente 7 milhões de pessoas no mundo esteja infectada (WHO, 2025). O parasita apresenta um ciclo de vida complexo que envolve tanto insetos vetores como hospedeiros mamíferos.

A transmissão da Doença de Chagas pode ocorrer por meio do inseto vetor; transfusão de sangue de doadores infectados; por transmissão congênita (de mãe para filho) durante a gravidez ou parto; transplante de órgãos de doadores infectados; ingestão de alimentos e bebidas contaminados com fezes de triatomíneo infectado; e por acidentes laboratoriais (Hughes *et al.*, 2012; Jackson, 2010).

Ao longo do seu ciclo de vida o *T. cruzi* transita entre hospedeiros vertebrados como mamíferos e humanos, e o inseto vetor, o triatomíneo. No vetor o ciclo se inicia quando este se alimentar de sangue de um hospedeiro infectado. No intestino médio do inseto vetor, o parasita assume a forma epimastigota (EPI) de morfologia alongada e de tamanho variado e onde se multiplica. Posteriormente, no intestino posterior, essas formas se diferenciam em tripomastigotas metacíclicos, morfologicamente alongadas e

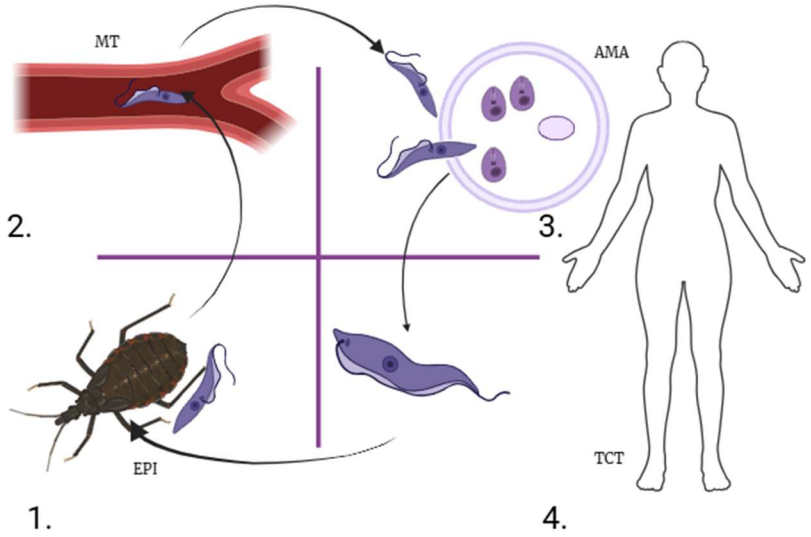

Figura 1.: Ciclo de vida *Trypanosoma cruzi*. 1.Inseto triatomíneo, conhecido como barbeiro, transmissor da doença de Chagas, abriga no seu intestino a forma epimastigota (EPI), que se diferencia em tripomastigotas metacíclicos (MT), a forma infectiva do parasita. 2. Durante o repasto sanguíneo, a forma MT é liberada e penetra nas células do hospedeiro vertebrado, onde se diferencia na forma amastigota (AMA). 3. No interior da célula, os amastigotas se multiplicam por divisão binária. 4. Após o ciclo replicativo, a célula se rompe, liberando tripomastigotas sanguíneos (TCT) na corrente sanguínea do hospedeiro, os quais podem invadir novas células ou serem ingeridos por um novo barbeiro durante a alimentação, reiniciando o ciclo. Fonte: Elaborado pelo autor com base em ferramentas do BioRender (2025).

fusiformas. Durante uma nova refeição sanguínea, o triatomíneo defeca próximo ao local da picada, liberando os tripomastigotas metacíclicos (MT), que podem penetrar nos organismos do hospedeiro através da pele lesionada, de mucosas ou de pequenos ferimentos. Uma vez no hospedeiro vertebrado, os MT invadem células próximas ao ponto de entrada e são inicialmente internalizados em uma vesícula, o vacúolo parasitóforo. Após escaparem dessa estrutura, diferenciam-se em formas amastigotas (AM), intracelular, arredondadas, aflageladas e replicativas. Após múltiplos ciclos de replicação intracelular, os amastigotas se diferenciam dentro da célula hospedeira em tripomastigotas de corrente sanguínea (TCT) formas móveis e flageladas que são liberadas no espaço extracelular, podendo atingir novos tecidos ou serem ingeridos por outros triatomíneos durante a alimentação (Clayton, 2010; Souza, De, 1984; WHO, 2022).

Trypanosoma cruzi é capaz de infectar quaisquer células nucleadas. No entanto, diferentes cepas demostram tropismos distintos durante a fase aguda da infecção, com preferência por órgãos como esôfago, fígado, baço, intestino, coração e músculo esquelético. Já na fase crônica, o tropismo tende a ser mais restrito e homogêneo, predominando em tecidos como o intestino, o músculo esquelético e o coração (Santi-Rocca *et al.*, 2017).

A doença de Chagas apresenta duas fases distintas: aguda e crônica. A fase aguda da infecção normalmente o paciente é assintomática ou pode manifestar sintomas leves e inespecíficos, semelhantes aos de outras síndromes infecciosas. Os danos observados nesta fase estão relacionados à infecção e multiplicação do parasita no miocárdio, assim como em outros tecidos, como o sistema nervoso e o trato digestivo. Com a remissão da parasitemia e das reações inflamatórias sistêmicas, o paciente normalmente progride para a fase crônica da doença (WHO, 2025).

Na fase crônica, podem surgir complicações cardíacas e digestivas que se manifestam após vários anos, comprometendo o coração, com arritmias e outras alterações; e o sistema digestório, com dilatações do esôfago e do cólon (Simões *et al.*, 2018; WHO, 2022).

O tratamento disponível atualmente se limita a duas drogas utilizadas há mais de 40 anos: o benzonidazol (BZN) (Rochagan® e Rodanil®, Roche) e nifurtimox (NFX) (Lampit®, Bayer) (Guedes *et al.*, 2011). Ambos os medicamentos apresentam cerca de 80% de eficácia na cura da doença quando administradas precocemente, logo após a infecção, incluindo casos de transmissão congênita (Clayton, 2010; OPAS, 2023). No entanto, sua eficácia diminui significativamente à medida que o tempo de infecção se prolonga, e seu uso pode estar associado a efeitos colaterais graves (Patterson e Wyllie, 2014).

O benzonidazol é o fármaco mais utilizado para o tratamento da doença de Chagas. A posologia recomendada é de 5 a 7 mg/kg/dia, por via oral, dividida em duas ou três doses diárias, durante 60 dias, em adultos. Esse composto é derivado do 2-nitroimidazol e atua como pró-fármaco, sendo ativado após

bioativação enzimática, resultando em metabólicos reativos com atividade tripanocida (Müller Kratz *et al.*, 2018).

A ativação do BZN é mediada por nitroredutase tipo I (NTRs) do parasita, uma classe de enzimas insensíveis ao oxigênio, presentes em diversos protozoários (Hall e Wilkinson, 2012). As NTRs do tipo I, dependentes de NADH (Nicotinamida Adenina Dinucleotídeo na forma reduzida), catalisam a redução do 2-nitroimidazol a uma hidroxilamina, a qual é posteriormente convertida, por transformações não enzimáticas, em dialdeído glioxal. Esse metabólito altamente reativo pode formar dutos com proteínas, ácidos nucleicos (DNA/RNA) e moléculas pequenas como a glutationa. Essas interações resultam em toxicidades para o parasita, promovendo um efeito tripanocida rápido e localizado, tanto contra formas intracelulares quanto extracelulares de *Trypanosoma cruzi* (Patterson e Wyllie, 2014). Além disso, estudos indicam que o BZN pode aumentar a fagocitose, a produção de citocinas e a síntese de intermediários reativos de nitrogênio, favorecendo a destruição dos parasitas intracelulares por células do sistema imune (Murta *et al.*, 1999). Apesar de serem as drogas de primeira escolha, BZN e NFX apresentam baixa eficácia na fase crônica da doença. Essa limitação terapêutica tem motivado o direcionamento de diversos estudos para o entendimento das interações entre o parasita e o hospedeiro, com o objetivo de identificar novas estratégias terapêuticas ou alternativas de controle da doença.

1.2 Biologia do *T. cruzi* e sua interação com o hospedeiro

Atualmente, existem diversas cepas geneticamente distintas de *Trypanosoma cruzi,* o que tem motivado pesquisadores a buscarem métodos de classificação baseados, principalmente, em diferenças biológicas e genômicas (Brenière, Waleckx e Barnabé, 2016). A classificação do parasita, entretanto, é dificultada por particularidades de seu ciclo celular: durante a mitose, por exemplo, o genoma de *T. cruzi* não se condensa em cromossomos visíveis, o que inviabiliza sua análise por técnicas citogenéticas convencionais. O cariótipo da espécie só pode ser determinado por meio de técnica de biologia molecular, como a eletroforese em gel de campo pulsado (PFGE), combinada ao método de *Southern blot*. Esses estudos revelaram grande variabilidade molecular quanto ao tamanho e número de cromossomos entre cepas, e até mesmo entre clones de uma mesma cepa (Henriksson *et al.*, 2002; Lima *et al.*, 2013; Souza *et al.*, 2011).

A primeira proposta formal de classificação das cepas de *T. cruzi* foi elaborada durante uma reunião satélite realizada na Fiocruz, em 1999, na qual um comitê de especialistas revisou os dados disponíveis e propôs dois grupos principais: *T. cruzi* I e *T. cruzi* II. A divisão foi baseada em características biológicas, bioquímicas e em dados moleculares (“Recommendations from a satellite meeting”, 1999).

A partir de análises filogenéticas baseadas em eletroforese enzimática multilocus (MLEE) e em marcadores RAPD (DNA polimórfico amplificado aleatoriamente), o grupo *T. cruzi* II foi subdividido em cinco DTUs: IIa a IIe (Brisse, Barnabé e Tibayrenc, 2000; Brisse, Verhoef e Tibayrenc, 2001). Com base nessa nova classificação, considerou-se que os DTUs I e IIb correspondiam aos grupos *T. cruzi* I e *T. cruzi* II definidos originalmente. Além disso, os DTUs I e IIb passaram a ser considerados linhagens ancestrais, enquanto os DTUs IId e IIe seriam produtos de eventos de hibridização recentes, e os DTUs IIa e IIc, híbridos ancestrais (Freitas, De *et al.*, 2006; Tomazi *et al.*, 2009; Westenberger *et al.*, 2005).

Dez anos depois em 2009, avanços no conhecimento da diversidade genética do parasita e o uso de análises de genotipagem multilocus levaram à proposição de seis unidades discretas de tipagem genéticas (DTUs) TcI a TcVI. As DTUs correspondem a conjuntos genéticos coesos, com maior similaridade entre si do que com outros agrupamentos, e são identificáveis por marcadores genéticos, moleculares ou imunológicos específico ("Recommendations from a satellite meeting", 1999; Zingales *et al*., 2009). Nessa proposta, TcI e TcII representam cepas ancestrais; TcIII e TcIV seriam híbridos homozigotos resultantes de recombinações entre TcI e TcII; e TcV e TcVI corresponderiam a híbridos heterozigotos entre TcII e TcIII. Posteriormente, uma nova cepa isolada de morcegos foi descrita e classificada como TcBat, considerada a sétima DTU (Lima *et al*., 2015; Marcili *et al*., 2009; Zingales *et al*., 2009).

A compreensão da diversidade genética de *Trypanosoma cruzi* e sua classificação em diferentes DTUs é fundamental para o delineamento de estudos funcionais. As cepas Y e G, pertencem a DTUs distintas, TcII e TcI, respectivamente, e representam populações com características genéticas e biológicas marcadamente diferentes. A cepa Y pertence ao grupo *T. cruzi* II (TcII), frequentemente associada às formas clínicas mais graves da doença de Chagas, incluindo manifestações cardíacas, e megaesôfago e megacólon concomitantes. Os hospedeiros e vetores naturais do TcII ainda não são totalmente compreendidos, e a maioria dos isolados foi obtida em fragmentos remanescentes da Mata Atlântica brasileira, a partir de primatas e, esporadicamente, de outras espécies de mamíferos (Fernandes *et al.*, 1999; Lisboa *et al.*, 2007; Roellig *et al.*, 2008; Zingales *et al.*, 1999).

Por outro lado, a cepa G pertence ao grupo *T. cruzi* I (TcI), a DTU mais abundante e amplamente distribuída nas Américas. É encontrada em praticamente toda a área de ocorrência dos vetores triatomíneos e está associada tanto a ciclos silvestres quanto domésticos. A infecção humana por TcI concentra-se principalmente no norte da América do Sul e na América Central, onde está associada à cardiomiopatia chagásica. Há relatos esporádicos de infecção humana por TcI em regiões ao sul da bacia amazônica e isolados selvagens também foram identificados no Alabama, nos Estados Unidos (Roellig *et al.*, 2008; Zingales *et al.*, 2012).

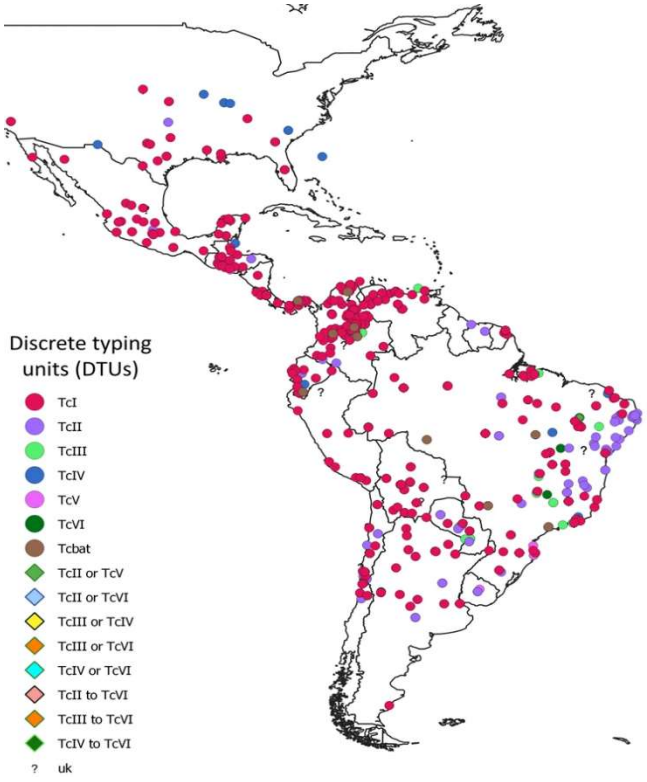

Figura 2.: Distribuição geográfica dos DTUs de *Trypanosoma cruzi* nas Américas. Fonte: Velásquez-Ortiz *et al.* (2022). Adaptado de: VELÁSQUEZ-ORTIZ, N. *et al*. Discrete typing units of *Trypanosoma cruzi*: Geographical and biological distribution in the Americas. Scientific Data, v. 9, p. 360, 2022. Disponível em: https://doi.org/10.1038/s41597-022-01452-w.

1.3 Interação parasita hospedeiro

Trypanosoma cruzi é um táxon geneticamente heterogêneo, cuja variabilidade é representada pelas distintas Unidades Discretas de Tipagem (DTUs) (Zingales *et al*., 2009). Essa diversidade genética se traduz em um genoma composto por um núcleo conservado de genes e múltiplas famílias de proteínas de superfície altamente variáveis entre as cepas (Berná *et al.*, 2018; Pablos, Luis M. De e Osuna, 2012). Famílias multigênicas, como trans-sialidases (TS), mucinas, MASP (proteínas de superfície associadas à mucina) e DGF-1 (Dispersed Gene Family-1), estão amplamente expandidas, conferindo uma diversidade funcional que favorece a adaptação e a virulência do parasita (El-Sayed *et al.*, 2005). Essa plasticidade genética permite ao parasita interagir com diferentes receptores presentes nas células

hospedeiras, sendo um fator essencial para o sucesso do processo infeccioso (Campetella *et al.*, 2020; Melo *et al.*, 2021).

A interação entre *T. cruzi* e o hospedeiro vertebrado pode ser dividida em quatro etapas principais: (1) adesão e reconhecimento celular, (2) internalização do parasita, (3) formação e maturação do vacúolo parasitóforo, e (4) sinalização intracelular que culmina na liberação do parasita no citosol. Esses processos envolvem mecanismos moleculares complexos mediados por glicoproteínas, glicolipídios e proteínas do tipo lectina, tanto do parasita quanto da célula hospedeira (Barrias, Carvalho, de e Souza, De, 2013).

A capacidade de *T. cruzi* de infectar, sobreviver e proliferar no hospedeiro vertebrado depende da atuação coordenada de diversas proteínas envolvidas em processos chave como adesão celular, evasão da resposta imune, replicação e diferenciação. Essas proteínas podem estar localizadas tanto na superfície do parasita quando em compartimentos intracelulares, sendo fundamentais para o estabelecimento e manutenção da infecção (Barrias, Carvalho, de e Souza, De, 2013).

1.4 Glicoproteínas e enzimas de modificação pós-traducional

Trypanosoma cruzi é revestido por diversas proteínas de superfície. A primeira análise do glicoproteoma da espécie, baseada em espectrometria de massas, identificou 690 glicoproteínas, revelando que cada forma evolutiva do parasita expressa um conjunto distinto de glicoproteínas específicas do estágio (Alba Soto e González Cappa, 2019; Alves *et al.*, 2017).

Entre as glicoproteínas envolvidas na adesão do *T. cruzi* à célula hospedeira temos a gp82, ancorada à membrana externa do parasita via glicosilfosfatilinositol (GPI). Essa ancoragem é suscetível à clivagem por fosfolipase C endógena (PI-PLC), resultando em sua liberação no meio extracelular. Durante a invasão pelas formas metacíclicas, moléculas de gp82, secretadas ou ainda ancoradas, interagem com receptores da célula hospedeira, desencadeando vias de sinalização que promovem aumento do cálcio intracelular e mobilização dos lisossomos (Bayer-Santos *et al.*, 2013; Manque *et al.*, 2003). A proteína LAMP-2 (Proteína de Membrana Associada ao Lisossomo) foi identificada como receptor hospedeiro de gp82, e anticorpos anti-Lamp2 inibem significativamente a invasão (Rodrigues *et al.*, 2019). Comparando-se os mecanismos de invasão das formas metacíclicas (MT) e tripomastigota sanguíneos (TCT), observa-se que os TCTs recrutam menos lisossomos e, nos estágios iniciais da infecção, adquirem principalmente marcadores de membrana plasmática. Ainda assim, proteína LAMP também estão envolvidas, e fibroblastos de camundongos deficientes em LAMP-1/2 apresentam menor suscetibilidade à invasão por TCTs (Albertti *et al.*, 2010).

A GP63 é uma importante glicoproteína de 63kDa ancorada por GPI, amplamente estudada em *Leishmania spp*., onde atua como metaloprotease Zn^{2+} dependente com papel na clivagem de receptores da célula hospedeira e na evasão imune (Bouvier, Etges e Bordiers, 1985; Button *et al.*, 1989; Etges,

Bouvier e Bordier, 1986). Em *T. cruzi,* segundo Cuevas *et al*. 2003, a GP63 também apresenta atividade proteolítica e possível papel na infecção, sendo codificadas por uma família multigênica. Dois grupos principais de genes, Tcgp63-I e Tcgp63– II, foram caracterizados. Embora suas funções em *T. cruzi* ainda não estejam totalmente elucidadas, há indícios de que a GP63 participa da invasão celular e interfere em vias de sinalização do hospedeiro (d'Avila-Levy *et al.*, 2014).

As trans-sialidases (TS), uma das maiores famílias gênicas de *T. cruzi* é composta por proteína que catalisam a transferência de resíduos de ácidos siálico de glicoconjugados do hospedeiro para glicoproteínas da superfície do parasita. Essa modificação do glicocálix promove adesão celular, camuflagem antigênica e evasão da resposta imune mediada por complemento (Burle-Caldas *et al.*, 2022). Algumas isoformas, como SAPA (do inglês Shed Acute Phase Antigen ou antígeno da fase aguda), são altamente expressas no início da infecção e liberadas no meio extracelular, circulando sistemicamente até que sejam neutralizadas por anticorpos (Buschiazzo *et al.*, 2012).

As TS atuam especialmente no vacúolo parasitóforo, rico em LAMPs sialiladas. Em ambiente ácido, as TS transferem resíduos de ácido siálico das LAMPs para a superfície do parasita, promovendo a dessialilação da membrana do vacúolo e facilitando a ação da toxina poraformadora Tc-Tox, o que permite a liberação do parasita no citoplasma (Albertti *et al.*, 2010; Hall *et al.*, 1992; Rubin-de-Celis *et al.*, 2006).

Outra proteína relevante é a gp85/TS, expressa nas formas infectantes e pertencente à família multigênica gp85/trans-sialidade (gp85/Td) (Abuin *et al.*, 1989; Colli, 1993; Manso Alves *et al.*, 1986; Mattos *et al.*, 2014). As proteínas do grupo I dessa família apresentam atividade trans-sialidásica; já a Tc85 pertence ao grupo II, sem atividade enzimática, está implicada na adesão e invasão celular (Mattos *et al.*, 2014; Pereira *et al.*, 1996). Tc85 interage com diversos receptores, como laminina, citoqueratina, vimentina, fibronectina, mucinas e o receptor de procineticina-2 (Giordano *et al.*, 1994; Marroquin-Quelopana *et al.*, 2004). O peptídeo derivado de gp85/TS, originado da sequência conservada VTVxNVxLYNRPLN, atua na ligação com citoqueratinas e na ativação da via de sinalização ERK1/2, aumentando a taxa de invasão (Magdesian *et al.*, 2001, 2007).

A δ-Amastina é uma glicoproteína transmembrana expressa predominantemente nas formas amastigotas intracelulares e associada à invasão e diferenciação das formas extracelulares (Cruz *et al.*, 2012). Foram identificadas 14 cópias do gene em duas cepas de *T. cruzi,* com localização de superfície confirmada por microscopia confocal e *Western Blot*. A cepa G, de baixa infectividade, apresenta níveis reduzidos de transcritos dessa proteína (Kangussu-Marcolino *et al.*, 2013). Ensaios com proteína recombinante demostraram sua ligação celular e capacidade de inibir a internalização, sugerindo papel direto na invasão (Cruz *et al.*, 2012). Os genes de amastina se alternam com genes de tuzin (família de gene que codificam pequenas proteínas de membrana), organizando-se em grandes blocos gênomicos

(Jackson, 2010). Filogeneticamente, dividem-se em quatro subfamílias (α, β, γ e δ), todas com quatro domínios transmembrana e caudas citoplasmáticas (Rochette *et al.*, 2005).

1.5 Mucinas e MASPs (Mucin-Associated Surface Proteins)

As mucinas e as proteínas associadas à mucina (MASPs) constituem importantes componentes estruturais da superfície de *Trypanosoma cruzi*, estando fortemente glicosiladas e desempenhando funções essenciais na proteção do parasita contra agressões do sistema imunológico.

As mucinas de *T. cruzi* (TcMUC) são glicoproteínas expressas na superfície do parasito ao longo de diferentes estágios do ciclo de vida, o que sugere um papel estratégico na evasão da resposta imune do hospedeiro (Buscaglia *et al.*, 2006). Essas moléculas formam uma barreira física e química que dificulta o reconhecimento imunológico e, além disso, participam da adesão às células do hospedeiro. As TcMUC também são os principais substratos da trans-sialidase, sendo sialiladas com resíduos de ácido siálico retirados de glicoconjugados do hospedeiro, promovendo camuflagem antigênica (Acosta-Serrano *et al.*, 2001). Além disso, as mucinas contribuem para a modulação da resposta inflamatória, sendo capazes de induzir a secreção de citocinas pró-inflamatórias e óxido nítrico por macrófagos ativados. A identificação dessas glicoproteínas como semelhantes às mucinas de mamíferos ocorreu em 1993, devido à semelhança na composição de açúcares e aminoácidos (Acosta-Serrano *et al.*, 2001; Almeida *et al.*, 1994; Previato *et al.*, 1994; Schenkman *et al.*, 1991).

As MASPs são uma grande família de proteínas de superfície codificada por aproximadamente 1.300 genes distribuídos por todo o genoma de *T. cruzi*, frequentemente agrupados com genes de mucinas e outras proteínas de superfície (Bartholomeu *et al.*, 2009; El-Sayed *et al.*, 2005). Essas proteínas são expressas predominantemente nas formas infecciosas do parasito (tripomastigotas metacíclicos e de corrente sanguínea) e podem ser secretadas para o meio extracelular durante o processo infeccioso (Pablos, De *et al.*, 2011; Pablos, Luis Miguel De e Osuna, 2012). A família MASP é caracterizada por domínios conservados nas regiões N-terminal (com um peptídeo sinal) e C-terminal (contendo o sítio de adição da âncora GPI), que direcionam sua localização para a superfície celular. A porção central das MASPs, por outro lado, é altamente variável em sequência e comprimento, composta por múltiplos motivos repetitivos peptídicos que são compartilhados entre diferentes membros da família. Essa diversidade sugere um repertório estendido de antígenos capazes de interagir com células hospedeiras e com o sistema imunológico (Bartholomeu *et al.*, 2009). Apesar da elevada variabilidade na região codificadora, os mRNAs dos genes *masp* apresentam regiões 5' e 3' não traduzidas (UTRs) altamente conservadas entre cepas, o que indica possível regulação coordenada da expressão gênica (Bartholomeu *et al.*, 2009; Pablos, Luis Miguel De e Osuna, 2012). Embora as funções específicas das MASPs ainda estejam sendo elucidadas, sua diversidade, padrão de expressão e localização sugerem

participação em processos de adesão, diferenciação e adaptação ao microambiente celular (Bradwell *et al.*, 2018).

1.6 Proteínas envolvidas em sinalização e resposta ao estresse

Diversas proteínas de sinalização e de resposta ao estresse estão envolvidas na interação entre *Trypanosoma cruzi* e seu hospedeiro. Entre essas, destacam-se as proteínas quinases, glicoproteínas de superfície como gp83 e gp90, além da protease cruzipaína.

A gp63 é uma importante glicoproteína de 63kDa ancorada por GPI, amplamente estudada em *Leishmania* spp., onde atua como metaloprotease Zn^{2+} dependente com papel na clivagem de receptores da célula hospedeira e na evasão imune (Bouvier, Etges e Bordiers, 1985; Button *et al.*, 1989; Etges, Bouvier e Bordier, 1986). Em *T. cruzi,* segundo Cuevas *et al*. (2003), a GP63 também apresenta atividade proteolítica e possível papel na infecção, sendo codificado por uma família multigênica. Dois grupos principais, Tcgp63-I e Tcgp63– II, foram caracterizados. Embora suas funções em *T. cruzi* ainda não estejam totalmente elucidadas, há indícios de que a GP63 participa da invasão celular e interfere em vias de sinalização do hospedeiro (d'Avila-Levy *et al.*, 2014).

A gp90 é uma glicoproteína de superfície expressa na forma metacíclica do parasita, com papel inibitório na invasão celular. Sua presença está inversamente correlacionada à capacidade invasiva de *T. cruzi.* A ligação da gp90 às células de mamíferos ocorre por meio de receptores, sem ativação da sinalização por cálcio intracelular (Málaga e Yoshida, 2001). Propõe-se que seu mecanismo de ação envolva a inibição da fusão lisossomal na célula hospedeira, regulando negativamente a invasão (Rodrigues *et al.*, 2017). Ainda, estudos com anticorpos monoclonais demostraram baixos níveis de expressão de gp90 em cepas altamente invasivas, como a cepa CL, enquanto cepas pouco invasivas, como a G, apresentam expressão elevada (Ruiz *et al.*, 1998).

A cruzipaína (ou cruzipain) é uma cisteíno protease expressa em todas as formas evolutivas de *T. cruzi,* sendo predominantemente localizada em organelas relacionadas ao lisossomo. O termo "cruzipaína" refere-se à enzima nativa, enquanto sua forma recombinante, desprovida da porção C-terminal, é conhecida como cruzaína (Cazzulo *et al.*, 1990; Murta *et al.*, 1990). Diferentes isoformas da enzima foram identificadas: a cruzipaína 1 (czp 1), considerada a forma clássica, e cruzipaína 2 (czp 2), descrita posteriormente (Ana Paula *et al.*, 1994). Essas isoformas exibem propriedades distintas quando à afinidade por substratos e inibidores. Análises por *Wester blot* mostraram que os epimastigotas da cepa DM28 (TcI) expressam predominante czp 1, enquanto os tripomastigotas expressam czp 2 (Lima *et al.*, 2001). Embora não seja essencial, a cruzipaína é necessária para a eficiência da invasão celular. Essa enzima está envolvida em diversos processos biológicos, como metaciclogênese, invasão do hospedeiro e modulação da resposta imune (Andrade *et al.*, 2012; Doyle *et al.*, 2011; Franke de Cazzulo *et al.*, 1994; Tomas, Miles e Kelly, 1997).

1.7 DGF-1: proteína da família disperso-1

A primeira descrição da família gênica *Dispersed Gene Family-1* (DGF-1) remonta a 1990, quando Wincker e colaboradores identificaram, na cepa DM28c de *T. cruzi,* uma sequência nuclear repetida presente em diversos cromossomos e associada a proteína de superfície (Wincker, Roizes e Goldenberg, 1990). Atualmente, sabe-se que DGF-1 constitui a terceira maior família gênica do genoma do parasita, com seus membros conservando motivos de adesinas, incluindo quatro segmentos com alta similaridade à intregrina humana β7. Por meio de ensaios de citometria de fluxo e biotinilação com anticorpos anti-DGF-1, demonstrou-se que membros dessa família são expressos na superfície dos tripomastigotas (Kawashita *et al.*, 2009). Essas proteínas apresentam entre oito e nove hélices hidrofóbicas transmembranares na extremidade C-terminal, além de motivos de ligação sugerem que a localização na superfície celular pode estar envolvida na interação célula-célula ou atuar como receptor de sinalização (Kawashita *et al.*, 2009; Kim *et al.*, 2005; Wincker, Roizes e Goldenberg, 1990). Estudos de expressão diferencial revelam que o gene DGF-1.2 é mais fortemente expresso durante a fase amastigota, onde a proteína se acumula no lado interno da membrana plasmática. Após a diferenciação para tripomastigota extracelular, a proteína é detectada no meio da cultura, sugerindo que é secretada durante a infecção da célula hospedeira (Lander *et al.*, 2010).

1.8 Mevalonato quinase (MVK)

A mevalonato quinase (MVK) é uma enzima essencial na via de biossíntese de isoprenoides esteróides, catalisando a conversão de ácido mevalônico em fosfomevalonato (Fu *et al*., 2002). Essa via fornece precursores fundamentais para a produção de moléculas bioativas, como colesterol em humanos ou ergosterol nos tripanossomatídeos. Os derivados isoprenoides são cruciais para modificações pós-traducionais de diversas proteínas envolvidas em processos celulares essenciais, como sinalização intracelular, expressão gênica, glicosilação proteica, montagem do citoesqueleto e diferenciação celular (Fu *et al.*, 2008; Goldstein e Brown, 1990).

A proteína recombinante TcMVK foi identificada em culturas de tripomastigotas metacíclicos e amastigotas extracelulares, sendo secretada no meio extracelular. Estudos demostram que a TcMVK é capaz de modular a sinalização celular do hospedeiro durante a invasão pelo parasita (Ferreira *et al.*, 2016).

1.9 Proteína P21

A proteína P21, com massa molecular de aproximadamente 21 kDa, é uma glicoproteína secretada expressa em todos os estágios de desenvolvimento de *Trypanosoma cruzi*, incluindo as formas amastigotas e tripomastigotas. Diversos estudos apontam para seu papel multifuncional na interação

parasita-hospedeiro, especialmente na adesão e modulação da resposta celular. Sua análise genômica revelou que o gene que codifica a P21 é de cópia única e não possui ortólogos em outros tripanossomatídeos, indicando um papel específico para *T. cruzi.* Sua forma recombinante (rP21) adere à células de mamíferos de maneira dose-dependente e atua na regulação positiva da fagocitose por células não fagocíticas (Silva, da *et al.*, 2009). A rP21 também promove a polimerização do citoesqueleto de actina em macrófagos murinos, dependente de sua interação com o receptor de quimiocina CXCR4 e da ativação da via de transdução de sinal mediada pela PI3-quinase (Rodrigues *et al.*, 2012). Em modelos experimentais *in vivo,* a administração de rP21 foi associada à redução da carga parasitária e da angiogêneses dependente da interação direta da proteína recombinante com o receptor CXCR4, além da indução de fibrose no tecido cardíaco de camundongos infectados (Teixeira *et al.*, 2017, 2015). Estudos adicionais mostraram que a proteína recombinante interfere na multiplicação de epimastigotas, inibe a replicação intracelular de amastigotas e modula o ciclo celular do parasito, promovendo o bloqueio na fase G1 (Teixeira *et al.*, 2015, 2019). Esses resultados sugerem que a P21 atua como reguladora da replicação do parasita no hospedeiro, favorece o equilíbrio entre a sobrevivência do parasita e a manutenção da integridade do hospedeiro. Em modelos de inflamação crônica induzida por implantes de esponja de poliéster, a rP21 promoveu o recrutamento de leucócitos, aumento da produção de IL-4, deposição de colágeno e inibição da formação de novos vasos sanguíneos, destacando sua atividade antiangiogênica (Teixeira *et al.*, 2017, 2015). Os estudos prévios com a proteína recombinante P21 (rP21) permitiram uma compreensão inicial de seu papel no *T. cruzi*. No entanto, a elucidação da função nativa da proteína é crucial para determinar sua relevância biológica. Para tal, empregou-se a técnica *CRISPR/Cas9* visando à deleção do gene p21 nas linhagens Y e G de *T. cruzi* (Teixeira *et al.*, 2022). Esta técnica já foi usada em outros trabalhos para realizar deleções simples do gene GAT3 (transportadores ABC glicossomal 3) resultando em um aumento nos níveis de GAT2, sem alterações nos níveis de GAT1 sugerindo que o GAT3 não é essencial para a sobrevivência de *T. cruzi* (Lima *et al.*, 2025). Também já foi realizado a deleção de três genes envolvidos nas vias de sinalização de cAMP e Ca^{2+} uma possível proteína quinase dependente de $Ca^{2+/calmodulina}$, proteína flagelar 6 e a proteína contendo o domínio de ligação a nucleotídeos cíclicos/domínio C2, o resultado deste trabalho permitiu observa que o TcCC2P é um gene essencial em epimastigota de *T. cruzi* mostrando que está ferramenta é importante para observa o impacto da deleção de genes no protozoário (Chiurillo *et al.*, 2023).

Os resultados obtidos com estes parasitas *knockout* são o foco desta tese de doutorado. Essa abordagem experimental contribui para um entendimento mais abrangente do papel funcional do gene P21, refletindo a diversidade biológica do parasita em diferentes regiões endêmicas da doença de Chagas.

2. JUSTIFICATIVA

A Doença de Chagas continua sendo um grave problema de saúde pública global, afetando milhões de pessoas e apresentando limitações terapêuticas significativas, especialmente em sua fase crônica. Apesar dos avanços no entendimento dos mecanismos de infecção e evasão imune de *Trypanosoma cruzi*, compreender os mecanismos envolvendo a proteína P21 é muito importante uma vez que ela e expressa em diferentes formas evolutivas de *T. cruzi*, tem sido apontada como um importante fator de virulência, com potencial envolvimento na regulação da invasão e na resposta imune do hospedeiro. No entanto, ainda são escassos os estudos que investigam como a ausência da P21 afetam o perfil transcriptômico do parasita, principalmente considerando a alta diversidade genética existente entre as cepas de *T. cruzi*.

As cepas Y (TcII) e G (TcI) representam modelos com diferenças marcantes de virulência, tropismo tecidual e resposta imune induzida. Compreender como a deleção da proteína P21 impacta o transcriptoma dessas duas cepas pode revelar mecanismos moleculares distintos associados à virulência, adaptação e resposta ao hospedeiro, contribuindo para elucidar aspectos fundamentais da biologia do parasita.

Dessa forma, este estudo se justifica pela necessidade de ampliar o entendimento sobre os mecanismos moleculares que sustentam a variabilidade fenotípica de *T. cruzi* e o papel regulatório da P21 nesses processos. Os resultados poderão fornecer subsídios para o desenvolvimento de novas estratégias terapêuticas e alvos moleculares, colaborando para o avanço do conhecimento sobre a patogênese da Doença de Chagas e o controle dessa enfermidade negligenciada.

3. OBJETIVOS

O presente trabalho teve como objetivo investigar o papel funcional da proteína P21 em *Trypanosoma cruzi*, por meio da deleção gênica utilizando a ferramenta CRISPR/Cas9, avaliando os efeitos fenotípicos e transcriptômicos nas cepas Y e G do parasita.

3.1 Objetivos específicos

- Avaliar a capacidade de invasão em células de mamíferos *in vivo* e *in vitro* de cepas pertencentes as linhagens filogeneticamente distintas G e Y, bem como de suas respectivas cepas com o gene P21 knockoutado TcP21$^{-/-}$;
- Avaliar a capacidade de invasão, multiplicação, e eclosão em células de mamíferos de ambas as linhagens celulares G e Y e suas respectivas cepas com o gene P21 knockoutado TcP21$^{-/-}$;
- Quantificar a carga parasitária por qPCR de sangue e coração de camundongos infectados com as linhagens celulares G e Y e suas respectivas cepas com o gene P21 knockoutado TcP21$^{-/-}$;

- Analisar as alterações histopatológicas no tecido cardíacos de camundongos infectados com as linhagens celulares G e Y e suas respectivas cepas com o gene P21 knockoutado TcP21$^{-/-}$;
- Analisar o perfil de expressão gênica global dos parasitas G e Y e suas respectivas cepas com o gene P21 knockoutado TcP21$^{-/-}$por meio de RNA-seq, visando identificar alterações no transcriptoma associadas à ausência da proteína P21;
- Investigar possíveis vias moleculares e mecanismos de regulação modulados por P21, especialmente aqueles relacionados à adesão, invasão e resposta ao estresse celular no hospedeiro;
- Analisar alterações em vias biológicas e processos celulares como ciclo celular e diferenciação celular afetados pela deleção do gene P21.

CAPÍTULO II

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The *Trypanosoma cruzi* pleiotropic protein P21 orchestrates the intracellular retention and *in-vivo* parasitism control of virulent Y strain parasites

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P21 is a protein secreted by all forms of *Trypanosoma cruzi* (*T. cruzi*) with recognized biological activities determined in studies using the recombinant form of the protein. In our recent study, we found that the ablation of P21 gene decreased Y strain axenic epimastigotes multiplication and increased intracellular replication of amastigotes in HeLa cells infected with metacyclic trypomastigotes. In the present study, we investigated the effect of P21 *in vitro* using C2C12 cell lines infected with tissue culture-derived trypomastigotes (TCT) of wild-type and P21 knockout (TcP21$^{-/-}$) Y strain, and *in vivo* using an experimental model of *T. cruzi* infection in BALB/c mice. Our *in-vitro* results showed a significant decrease in the host cell invasion rate by TcP21$^{-/-}$ parasites as measured by Giemsa staining and cell count in bright light microscope. Quantitative polymerase chain reaction (qPCR) analysis showed that TcP21$^{-/-}$ parasites multiplied intracellularly to a higher extent than the scrambled parasites at 72h post-infection. In addition, we observed a higher egress of TcP21$^{-/-}$ trypomastigotes from C2C12 cells at 144h and 168h post-infection. Mice infected with Y strain TcP21$^{-/-}$ trypomastigotes displayed higher systemic parasitemia, heart tissue parasite burden, and several histopathological alterations in heart tissues compared to control animals infected with

scrambled parasites. Therewith, we propose that P21 is important in the host–pathogen interaction during invasion, cell multiplication, and egress, and may be part of the mechanism that controls parasitism and promotes chronic infection without patent systemic parasitemia.

KEYWORDS

Trypanosoma cruzi, CRISPR/Cas9, parasite-host interaction, cell invasion, intracellular multiplication, virulence

1 Introduction

Trypanosoma cruzi (*T. cruzi*) is a flagellated protozoan endemic in Latin America and the etiological agent of Chagas disease. *T. cruzi* is morphologically characterized by having three distinct evolutionary stages: epimastigote, trypomastigote, and amastigote. Development from one stage to another is a complex process, involving ultrastructural, antigenic, and physiological changes (Brener, 2003; de Souza, 2007). During the process of cell invasion, infective forms of *T. cruzi* (metacyclic trypomastigote, bloodstream trypomastigote, and extracellular amastigote) use different molecules to interact with host cell components to overcome the obstacles imposed by the mammalian host.

The protein P21 binds to the host cell in a dose-dependent manner, is ubiquitously expressed and secreted, and is involved in host cell invasion by trypomastigotes and extracellular amastigotes (da Silva et al., 2009). The use of the recombinant form of P21 (rP21) revealed that the native protein may promote phagocytosis by binding to the CXCR4 receptor and has chemotactic activity for macrophages and neutrophils (Rodrigues et al., 2012). It has also been demonstrated that rP21-induced myeloperoxidase and IL-4 production and decreased blood vessel formation *in vitro* and *in vivo* (Teixeira et al., 2015). In addition, rP21 reduced the growth of epimastigotes, inhibited intracellular replication of amastigotes, and modulated the parasite cell cycle (Teixeira et al., 2019). Corroborating with these results, we observed that rP21 decreased the multiplication of *T. cruzi* (Y strain) in C2C12 myoblasts, a phenomenon associated with greater actin polymerization and higher expression of IL-4 (Martins et al., 2020).

We have generated parasites of Y strain that are knockout for P21 by CRISPR/Cas9. The ablation of P21 in these parasites inhibited epimastigotes multiplication and upregulated intracellular amastigotes replication in HeLa cells infected with metacyclic trypomastigotes. To assess potential additional roles of P21 in host cell invasion, multiplication, egress, and cardiac tissue parasite load, we used tissue culture-derived trypomastigotes (TCT) of Y strain that are knockout for P21 (TcP21$^{-/-}$) to infect C2C12 cell line *in vitro* and BALB/c mice *in vivo*. The results showed that Y strain TcP21$^{-/-}$ parasites invaded C2C12 cells to a lower extent, multiplied at higher levels at 72h post-infection, and egressed significantly more at 144h post-infection than scrambled parasites. *In vivo*, mice infected with Y strain TcP21$^{-/-}$ parasites showed higher parasitemia, cardiac tissue parasite load, and cardiac tissue histopathological alterations than those infected with scrambled parasites.

2 Materials and methods

2.1 Parasite and cell cultures

Epimastigotes of Y (DTU II) strain were grown at 28°C in liver infusion tryptose (LIT) medium supplemented with 20% fetal bovine serum (FBS; Invitrogen). To differentiate epimastigotes into metacyclic forms, the epimastigotes were maintained in LIT for 14 days, and metacyclic trypomastigotes were purified as previously described (Teixeira and Yoshida, 1986).

Vero and C2C12 cells (obtained from Instituto Adolfo Lutz, São Paulo, SP, Brazil) were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FBS (Cultilab, Campinas, SP, Brazil), 10 µg/ml streptomycin, 100 U/ml penicillin, and 40 µg/ml gentamycin at 37°C in a 5% CO_2 humid atmosphere.

Cultures of scrambled and TcP21$^{-/-}$ epimastigotes of Y strain in the stationary phase containing metacyclic trypomastigotes (Teixeira et al., 2022) were used to infect Vero cells to obtain TCT forms for *in-vitro* and *in-vivo* experiments.

2.2 Animals and ethics

Six- to eight-week-old male BALB/c mice (15 animals) were maintained under standard conditions on a 12h light-dark cycle in a temperature-controlled setting (25°C), with food and water *ad-libitum*. Maintenance and animal care complied with the guidelines of the Ethics Committee for the Use of Animals (CEUA). Animal euthanasia was performed based on international welfare grounds according to the American Veterinary Medical Association Guidelines on Euthanasia. For euthanasia, mice were anesthetized intraperitoneally with a solution containing ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/

kg) followed by cervical dislocation. This study was approved by CEUA-UFU, with protocol number: 23117.077543/2022–27.

2.3 Biosafety approval

This study was approved by the CTNBio for the use of genetically modified organisms with the process number: 01245.004217/2023–83 and extract number: 8739/2023.

2.4 Host cell invasion and egress assays

C2C12 cell invasion assay was performed by adding 500 µl of cell suspension (5×10^4 cells and 3×10^4, respectively) into 24 well plates containing sterile glass coverslips (13 mm) and left seeding overnight. TCT suspensions of scrambled and Y strain TcP21$^{-/-}$ parasites were added at a multiplicity of infection of 5 (five parasites per cell), and plates were incubated for 2h at 37°C in a CO_2 (5%) humidified incubator. After incubation, cells were gently washed 3 times with phosphate-buffered saline, fixed with Bouin, and stained with Giemsa. The number of internalized parasites were counted in a total of 300 cells.

For the egress assay, which follows the host cell invasion described above and determines the number of parasites that exit the cells post-infection, plates were washed after infection and complete medium replaced, and then they were incubated at 37°C in a CO_2 (5%) humidified incubator. After 72h and up to 10 days (240h) post-infection, the number of parasites in the supernatant was determined by counting trypomastigote and amastigote forms using a Neubauer chamber.

These experiments were performed in three technical replicates and three independent biological procedures.

2.5 *In-vivo* infection

BALB/c mice were randomized into three groups, each containing five mice. Group 1: animals not infected; Group 2: animals infected with scrambled parasites (control); and Group 3: animals infected with TcP21$^{-/-}$ parasites. Both scrambled and TcP21$^{-/-}$ were parasites of Y strain.

Animals were infected intraperitoneally with 10^5 parasites. A systemic parasitemia was determined from day 3 post-infection, and then every other day up to day 15 post-infection, by collecting 5 µl of blood from the animal's tail, and the parasites were counted under light microscopy. On day 15 post-infection, after performing parasitemia, animals were euthanized and their hearts were collected for histopathological analysis and quantification of parasite DNA by qPCR.

2.6 Parasite load determined by qPCR

The hearts collected after euthanasia were weighed (100 mg) and stored in liquid nitrogen. After maceration with the aid of a porcelain crucible, a lysis buffer containing 500 µl of nuclei lysis buffer, 16 µl of Sodium dodecyl-sulfate (SDS) at 10%, and 8 µl of Proteinase K solution was added following an incubation at 50°C overnight. Next, 150 µl of NaCl buffer was added to the lysed hearts, which were then vortexed for 15 s and placed on ice for 10 min. The supernatant was collected and transferred to an Eppendorf tube. After addition of 800 µl of absolute ethanol solution, the tube was mixed well by inversion and centrifuged at 12000 rpm for 15 min. After discarding the supernatant, 1 mL of ethanol (75%) was added to the sample pellet, mixed and centrifuged again at 12000 rpm for 5 min. The supernatant was discarded and the sample pellet was allowed to dry for 10 min. The pellet was resuspended with 15–200 µl of RNAse and DNAse free water.

The DNA was quantified by nanodrop and a quantitative PCR was performed on the ABI Prism 7500 Fast System (Applied Biosystems, Foster City, CA) using a final sample volume of 10 µl [4 µl of DNA, 5 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, EUA) and 1 µl of primers Diaz7 e Diaz8 (Diaz et al., 1992)].

The standard curve was obtained using serial dilutions of 100ng of DNA extracted from epimastigotes with a limit of 0.0001 fg as proposed by Diaz et al. (1992) (Diaz et al., 1992) and modified by De Oliveira et al. (2020) (de Oliveira et al., 2020). Positive, negative, and reagent internal controls were used in all qPCR reactions.

A similar procedure was applied to C2C12 cells infected with TCTs of Y strain in order to obtain the DNA from these cells during the kinetics of multiplication. In this case, a total of 1×10^5 infected cells were harvested and lysed. The experiment was performed three times in triplicate.

2.7 Inflammatory score

Heart samples were fixed in 10% buffered formalin solution, dehydrated in ethanol solution, diaphanized in xylene, and embedded in paraffin. Blocks containing hearts were sectioned at 5-µm thick sections, and then placed onto glass slides and stained. To evaluate the number of amastigote nests, inflammatory infiltrate, and damage tissue score, slides of cardiac tissue were stained with hematoxylin and eosin (HE). The amastigote nests in each slide were qualitatively measured under light microscopy. The inflammatory infiltrate and damage tissue were scored by intensity: (–) absent, (+) mild, (++) moderate, and (+ + +) intense as described by Da Silva et al. (2018) (Da Silva et al., 2018).

2.8 Statistical analysis

All data were presented as the mean ± standard error (mean ± SEM) of at least three independent experiments performed in triplicate. The normal distribution of the data was checked using a Shapiro–Wilk test. Then, the significant differences were determined by *t*-test, and a multiple comparison by Mann–Whitney test. For some data, the significant differences were determined using a two-way analysis of variance (ANOVA), and the multiple comparison by Bonferroni's test for parametric data and Sidak's test for non-parametric data, value of $p \leq 0.05$ were considered significant. All

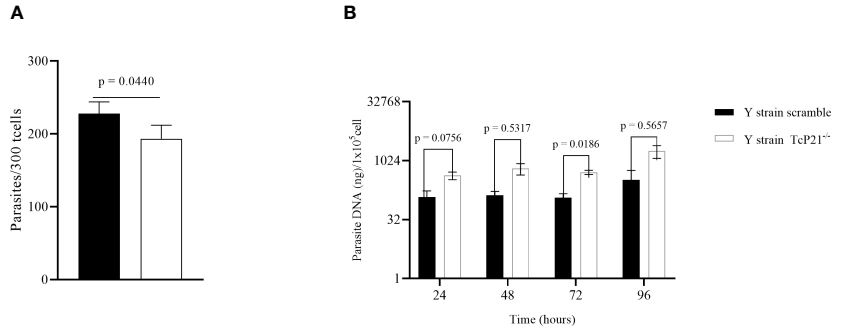

FIGURE 1
Cell invasion and intracellular multiplication of TcP21$^{-/-}$ and scrambled *T. cruzi* of Y strain in C2C12 cell lines. **(A)** C2C12 cell invasion—number of intracellular parasites in 300 cells. TcP21$^{-/-}$ parasites showed lower invasion rate compared to the scrambled group; **(B)** qPCR analysis showed that, at 72h post-infection, TcP21$^{-/-}$ parasites showed a higher multiplication rate compared to the scrambled group. All data were analyzed using GraphPad Prism software version 8.0.1. The graph shows the mean ± SEM of three experiments performed in triplicate. The comparison of invasion data between the knockout (TcP21$^{-/-}$) and control (scrambled) parasites was performed using *t*-test and Mann–Whitney test for multiple comparison. Statistical differences of intracellular multiplication were determined by two-way ANOVA and Bonferroni's test for multiple comparisons. In all analyses, the value of $p < 0.05$ was considered to be statistically significant.

the statistical analyses were performed using GraphPad Prism software version 8.0.1.

3 Results

3.1 Knockout of P21 in TCT from *T. cruzi* of Y strain affects the cell invasion and multiplication

TcP21$^{-/-}$ parasites showed a decrease in host cell invasion compared to control (scrambled) parasites in C2C12 cells ($p = 0.0440$) (Figure 1A). However, qPCR analysis showed that TcP21$^{-/-}$ multiplied at a higher level than the scrambled parasites at 72h post-infection ($p = 0.0186$) (Figure 1B).

3.2 The knockout of P21 affected the egress of Y-strain parasites

Regarding egress of trypomastigotes and amastigotes of Y-strain, we observed a higher number of TcP21$^{-/-}$ trypomastigotes in the supernatant of infected cells at 144 ($p = 0.0400$) and 168 ($p = 0.0500$) hours post-infection compared to scrambled trypomastigotes (Figure 2A). The release of amastigotes to the supernatant of C2C12 cells was similar between both groups (Figure 2B).

3.3 The knockout of P21 affected the parasitism in mice infected with Y strain

Animals infected with Y strain TcP21$^{-/-}$ showed a significantly higher systemic parasitemia compared to animals infected with scrambled parasites at days 3 and 6 post-infection ($p = 0.0001$) (Figure 3A). When heart samples were analyzed for parasite load by qPCR, we observed a higher parasite burden in animals infected with Y strain TcP21$^{-/-}$ parasites than mice infected with scrambled ones ($p = 0.0011$) (Figure 3B).

3.4 The knockout of P21 affected the inflammatory score in heart tissue from mice infected with TCT of Y strain

A qualitative analysis of heart tissue from animals infected with Y strain TcP21$^{-/-}$ parasites showed increased presence of neutrophilic leucocytes, eosinophils, macrophages, tissue damage, apoptotic bodies, and amastigote nests compared to tissues from mice infected with scrambled parasites (Table 1). Representative images are shown in Figure 3C.

4 Discussion

Our previous results using the recombinant form of P21 (rP21) have suggested that this *T. cruzi* ubiquitous and specific protein plays a role in the invasion and multiplication processes of the infective forms of the parasite. Although P21 is not conserved among eukaryotic species, we observed that its recombinant form induces phagocytosis of *Leishmania amazonensis* and *Toxoplasma gondii* (Rodrigues et al., 2012). In addition, we observed that rP21 induces *T. gondii* invasion and decreases its multiplication in BeWo cell line (de Souza et al., 2023).

In this scenario, P21 may be involved in the modulation of host cell invasion by the parasite, in its multiplication and egress from host cells. Therefore, we proposed that P21 maintains parasites intracellularly at low multiplication rate and away from host immune attack, leading the disease to the chronic phase without systemic parasitemia. In order to confirm this hypothesis, we first verified the impact of knocking out P21 from metacyclic trypomastigotes on the host cell invasion and multiplication using HeLa cell line *in vitro*. Corroborating with our

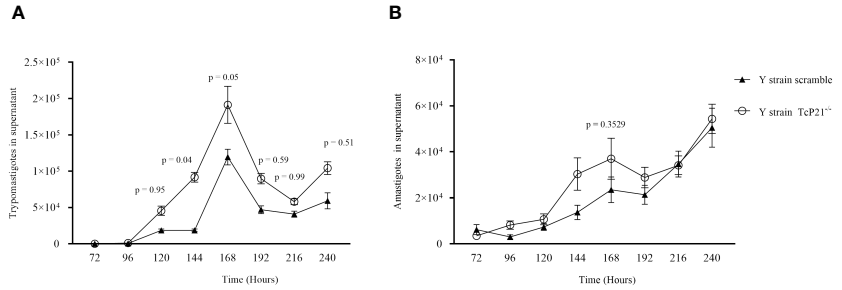

FIGURE 2
Egress of trypomastigotes and amastigotes from infected C2C12 cells along with the kinetics of parasite multiplication. **(A, B)** Egress of TcP21$^{-/-}$ and scrambled trypomastigotes and amastigotes of Y strain from C2C12 cells. All data were analyzed using GraphPad Prism software version 8.0.1. The graph shows the mean ± SEM of three experiments performed in triplicate. Statistical differences between the egress of knockout (TcP21$^{-/-}$) and control (scrambled) trypomastigotes and amastigotes were analyzed using two-way ANOVA and Sidak's test for multiple comparisons. In all analyzes the value of $p < 0.05$ was considered to be statistically significant.

hypothesis, results showed that the P21 knockout impaired parasite host cell invasion and induced parasite multiplication at 72h post-infection (Teixeira et al., 2022).

Here, we addressed the extended impact of knocking out P21 from the virulent strain (Y strain–DTU II) on cell invasion, multiplication, egress, systemic parasitemia, and cardiac tissue parasite load. The infective form used was TCT and the host cell was C2C12 cell line. Our results showed that Y strain TcP21$^{-/-}$ parasites invaded C2C12 cells at a lower rate than control (scrambled) parasites. These results confirmed the findings of our recently published study performed with knockout metacyclic trypomastigotes from the same strain (Teixeira et al., 2022).

qPCR procedure showed higher multiplication rate at 72h post-infection for TcP21$^{-/-}$parasites in comparison to scrambled ones.

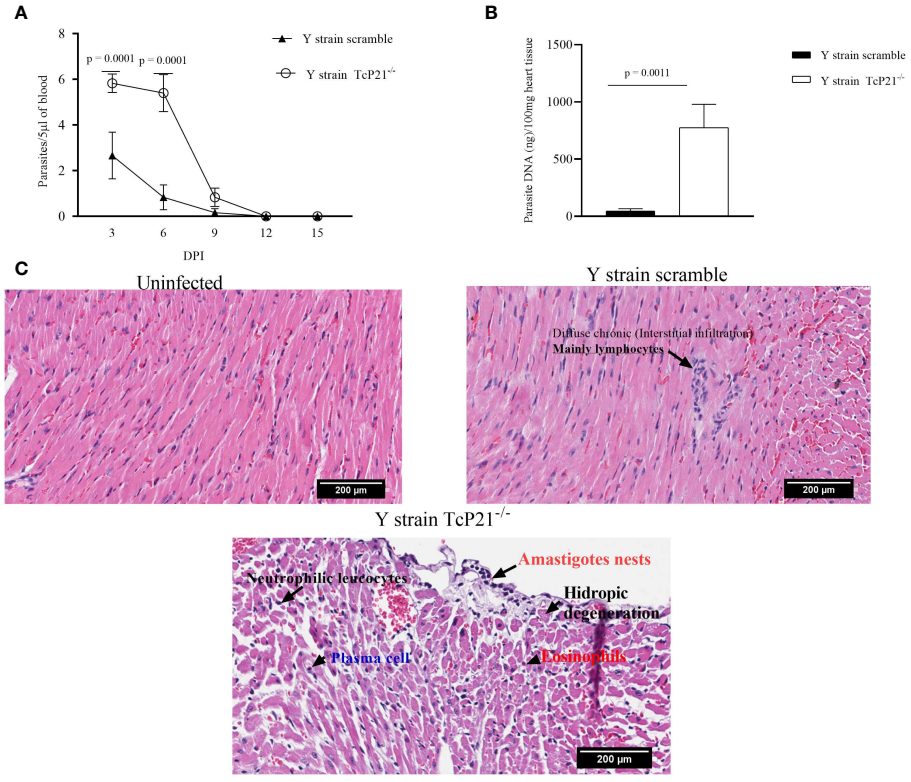

FIGURE 3
Systemic parasitism and Cardiac parasite load. **(A)** Systemic parasitism detected every other day along 15 days of BALB/c mice infection; **(B)** cardiac parasite load of BALB/c mice infected with parasites of Y strain; **(C)** representative images of the cardiac tissue inflammatory score of BALB/c mice infected with *T. cruzi* of Y strain. The histopathological alterations are indicated by black arrows. $n = 5$ animals per group. All data were analyzed using GraphPad Prism software version 8.0.1. The graph shows the mean ± SEM of three experiments performed in triplicate. The comparison of heart tissue parasite load data between the knockout (TcP21$^{-/-}$) and control (scrambled) parasites was performed using unpaired *t*-test for multiple comparisons. In all analyses, the value of $p < 0.05$ was considered to be statistically significant. Bars: 200µm.

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TABLE 1 Qualitative histological analyses of heart tissues from BALB/c mice at 15 days post-infection with TCT of *T. cruzi* (Y strain).

Histological criteria	Non infected (*n* = 5)	Scrambled (*n* = 5)	TcP21$^{-/-}$ (*n* = 5)
Inflammatory response	None	Low/mild	Moderate
Predominantly	–	Diffuse chronic (interstitial infiltration)	Diffuse chronic (interstitial infiltration)
Neutrophilic leucocytes	–	+	++
Eosinophils	–	–	+
Macrophages	–	+	++
Lymphocytes	–	++	+++
Plasma cells	–	+	+
Giant foreign body cells	–	–	–
Tissue damage	–	+	++
Hydropic degeneration	–	++	+++
Necrotic tissue	–	–	–
Apoptotic bodies	–	–	+
Edema	–	+	+++
Fibroblast	–	+	+
Fibrosis	–	+	+
Adipocyte	–	–	–
Epicardium calcification	–	–	–
Amastigotes nests	–	+	++

(–) absent, (+) mild, (++) moderate, and (+ + +) intense.

This is consistent with our previously raised hypothesis and with our recent study using metacyclic trypomastigotes of Y strain (Teixeira et al., 2022). The number of trypomastigotes in the supernatant was significantly higher in C2C12 cells infected by the knockout parasites compared to scrambled parasites at 144h and 168h post-infection. The higher egress of knockout parasites compared to scrambled ones may reflect the higher ability of these parasites to differentiate back into trypomastigote forms.

In order to verify the impact of P21 knockout in a complex system, we infected BALB/c mice with these parasites. Animals infected with Y strain TcP21$^{-/-}$ parasites showed higher systemic parasitemia and a higher parasite load in heart tissues compared to animals infected with scrambled parasites. This is the first time that we confirmed the ability of P21 in controlling the infectivity of the parasite *in vivo*, reinforcing our *in-vitro* data. The observed greater infection rate *in vivo* led by the absence of P21 highlights the role of P21 on host parasitism, which is likely a consequence of the combined effects of P21 observed *in vitro* such as the ability of the parasite to invade, multiply, differentiate, and egress from the host cells. Therefore, it is plausible to suggest that P21 may play an important role in the control of parasitism of Y-strain parasites.

The histopathological analysis of hearts obtained from mice infected with Y strain TcP21$^{-/-}$ parasites showed several pathological alterations, including presence of neutrophil and macrophage infiltrates, apoptotic bodies, and a high number of amastigotes nests. These results further support P21 as an important player in controlling infection by virulent strains in order to establish a chronic infection without much damage to the host.

Recently, authors have demonstrated that *T. cruzi* can enter a state of spontaneous dormancy. The dormant amastigotes are highly resistant to therapy both *in vivo* and *in vitro* (Sánchez-Valdéz et al., 2018). In addition, authors have shown the existence of an adaptive difference between *T. cruzi* strains to generate dormant cells, and that homologous recombination may be important for dormancy (Resende et al., 2020). Conversely, another research group suggested that *T. cruzi* persistence continues to involve regular cycles of replication, host cell lysis, and re-infection. They could find no evidence for wide-spread dormancy in parasites that persist in tissue reservoir (Ward et al., 2020). Although the dormancy in *T. cruzi* is still a matter of debates, we believe that P21 takes place in a machinery involved in the control of parasite multiplication, leading the disease to the chronic phase.

5 Conclusion

We conclude that some finely regulated mechanisms control parasite multiplication, differentiation, and egress during infection. As our results showed that P21 plays a role in cell invasion, intracellular multiplication, and egress *in vitro* and in the parasitism in *in-vivo* experiments, we propose that P21 may be a protagonist in the machinery that would be involved in the perpetuation of the disease in the infected host, since it seems to orchestrate the intracellular retention of the parasite from the virulent Y strain.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Comissão de Ética na Utilização de Animais/Universidade Federal de Uberlândia. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. AS: Investigation, Writing – original

draft. NU: Investigation, Writing – original draft. TV: Investigation, Writing – original draft. BB: Investigation, Writing – original draft. TT: Investigation, Writing – original draft. VA: Investigation, Writing – original draft. JT: Investigation, Writing – original draft. CP: Investigation, Writing – original draft. GS: Investigation, Writing – original draft. ST: Investigation, Writing – original draft. JS: Investigation, Writing – original draft. MS: Methodology, Resources, Writing – original draft. TM: Methodology, Resources, Writing – original draft. RR: Methodology, Resources, Writing – original draft. RM: Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. JDS: Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Trypanosoma cruzi P21 Is a Pleiotropic Protein That Is Involved in Parasite Host Cell Invasion and Intracellular Parasitism

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ABSTRACT

We characterized the secreted *Trypanosoma cruzi* P21 protein and hypothesized its role in parasite invasion and multiplication. To investigate the role of *T. cruzi* P21 protein in host-parasite interactions, specifically focusing on the low-virulence G strain. P21 knockout parasites were generated using CRISPR/Cas9. Cell invasion, multiplication, egress, and tissue parasitism were assessed in vitro and in vivo, comparing knockout and control parasites. P21 knockout significantly reduced parasite invasion and multiplication in Vero cells. *In vivo*, knockout parasites also showed reduced heart tissue parasitism in infected mice, despite no observable systemic parasitemia. Accordingly, P21 knockout trypomastigote egress was reduced in Vero cells. P21 plays a pleiotropic role in *T. cruzi* infection, differentially impacting parasite biology in the low-virulent G strain. In the G strain, P21 promotes invasion and persistence, potentially through mechanisms distinct from its role in the Y strain previously described. This highlights its potential as a therapeutic target for Chagas disease, warranting further investigation into strain-specific functions.

1 | Introduction

Trypanosoma cruzi, the etiological agent of Chagas disease, is a flagellated protozoan of significant public health concern in Latin America. Its complex life cycle involves distinct developmental stages, epimastigote, trypomastigote, and amastigote essential for successful host cell invasion, immune evasion, and the establishment of chronic infections (Brener 1973; de Souza 2007). During cell invasion, infective forms of *T. cruzi* (metacyclic trypomastigotes, bloodstream trypomastigotes, and extracellular amastigotes) employ diverse molecules to interact with host cell components, overcoming the mammalian host's barriers. While the role of P21 in the virulent Y strain has been previously investigated, its function in the low-virulence G strain and the potential strain-specific nuances of its pleiotropic activities remain largely unexplored, representing a significant gap in our understanding. This study addresses this gap by characterizing the role of P21 in the G strain and contrasting it with existing knowledge from the Y strain.

Nelsa Paula Inácio Uombe, Teresiama Velikkakam, and Anna Clara Azevedo Silveira contributed equally to this study.

T. cruzi P21 protein was identified in a comparative gene expression study between the low-virulence G and virulent CL strains. *In silico* analyses predicted a high probability of protein secretion. Previous studies using recombinant P21 (rP21) demonstrated dose-dependent adhesion to host cell surfaces, expression across all parasite life stages, secretion, and enhanced cellular invasion by extracellular amastigotes and metacyclic trypomastigotes (da Silva et al. 2009). Subsequently, rP21 was shown to induce macrophage phagocytosis, exhibit chemotactic activity for macrophages and neutrophils, and potentially bind the chemokine receptor CXCR4 (Rodrigues et al. 2012). In a polyester sponge-induced inflammation model, rP21 recruited immune cells, induced myeloperoxidase and IL-4 production, and reduced angiogenesis in vitro and in vivo (Martins et al. 2020). This capacity is likely linked to the modulation of actin expression and angiogenesis-associated genes (Teixeira et al. 2017).

Prior investigations revealed that rP21 treatment reduced parasite load (Y strain) and angiogenesis, while inducing fibrosis in the cardiac tissue of infected mice. Furthermore, rP21 diminished epimastigote growth, inhibited intracellular amastigote replication, and modulated the parasite cell cycle (Teixeira et al. 2019). Corroborating these findings, rP21 decreased *T. cruzi* (Y strain) multiplication in C2C12 myoblasts, associated with increased actin polymerization and IFN-γ, and elevated IL-4 expression. During experimental infection (Y strain), mice treated with rP21 exhibited fewer cardiac nests, reduced inflammatory infiltrate, and less fibrosis, correlating with high IFN-γ expression counterbalanced by elevated IL-10 levels, consistent with reduced cardiac tissue injury. It was also observed that under stress, such as IFN-γ exposure, *T. cruzi* upregulated P21 mRNA expression (Teixeira et al. 2015; Teixeira et al. 2022).

Collectively, these data suggest that native *T. cruzi* P21 protein plays a pivotal role in natural infection progression. The observation that recombinant protein induces cell invasion yet reduces intracellular multiplication, coupled with increased native protein expression under stress, implies P21's involvement in a complex mechanism for disease perpetuation. We hypothesize that P21 promotes intracellular parasite persistence and may be upregulated in response to parasite stress. To validate recombinant protein data, we generated P21 knockout parasites using CRISPR/Cas9 gene editing. Initially, we knocked out the P21 gene in the virulent Y strain, demonstrating that P21 knockout metacyclic trypomastigotes exhibited reduced HeLa cell invasion and increased multiplication compared to control parasites (Silveira et al. 2024; also for detailed information on knockout generation, see Silveira et al. 2025).

In this study, we targeted P21 knockout in the G strain, known for its low in vitro virulence and lack of parasitemia induction in experimental in vivo models (Rodrigues et al. 2012). Our strategy involved analyzing various aspects of host-pathogen interaction in vitro and in vivo using tissue culture-derived trypomastigotes (TCT) from P21 knockout G strain parasites compared to control (Cas9) parasites. We assessed the protein's effect on invasion, multiplication, and egress in vitro in Vero cell line. *In vivo*, we evaluated systemic parasitemia and inflammatory scores in heart tissue of C57BL/6 mice infected with P21 knockout G strain TCT compared to control parasites. This study aims to verify the strain-specific and pleiotropic roles of P21 in *T. cruzi* infection, contributing novel insights into the parasite's biology and potential therapeutic interventions.

2 | Materials and Methods

2.1 | Parasites and Cell Cultures

Epimastigotes from the G strain were cultured at 28°C in liver infusion tryptose (LIT) medium supplemented with 20% fetal bovine serum (FBS; Invitrogen). Metacyclic forms were obtained by maintaining epimastigotes in LIT for 14 days, and metacyclic trypomastigotes were purified using established protocols (Teixeira and Yoshida 1986). Vero and C2C12 cells (obtained from Instituto Adolfo Lutz, São Paulo, SP, Brazil) were cultured in Dulbecco's minimal essential medium (DMEM) (Sigma Chemical Co.) supplemented with 10% FBS (Cultilab), 10 μg/mL streptomycin, 100 U/mL penicillin, and 40 μg/mL gentamicin at 37°C in a 5% CO2 humidified atmosphere. Stationary phase epimastigote cultures containing metacyclic trypomastigotes from Cas9 and TcP21-/- (G strain) were used to infect Vero and C2C12 cells, generating tissue culture-derived trypomastigotes (TCT) for in vitro and in vivo experiments. For growth curves, epimastigote forms of the G Cas9 and TcP21-/- strains were cultured at a density of 1 × 10^7 parasites per mL. Parasites were harvested and counted using a Neubauer chamber every 7 days until day 12.

2.2 | Animals and Ethics

Six-to-8-week-old male C57BL/6 INFγ knockout mice (15 animals) were housed under standard conditions with a 12-h light-dark cycle at 25°C, with food and water ad libitum. Animal care and procedures adhered to the guidelines of the Ethics Committee for the Use of Animals (CEUA). Euthanasia was performed following international welfare standards as per the American Veterinary Medical Association Guidelines. The study was approved by CEUA-UFU, protocol number: 23117.077543/2022-27.

2.3 | Generation of P21 Knockout Parasites

Early-log phase epimastigotes were transfected with Cas9/pTREX-n (Addgene Plasmid #68708) (Lander et al. 2015). Selection was performed with G418 (250 μg/mL) 24 h post-transfection, and GFP-positive parasites were sorted 15 days posttransfection using BD FACSARIA II. SgRNA sequences were designed with EuPatGDT (Peng and Tarleton 2015). DNA templates for sgRNA in vitro transcription were generated by PCR. sgRNAs were transcribed in vitro using the MEGA-Shortscript T7 kit (Thermo Fisher Scientific). Donor DNA for homologous recombination was produced by PCR using 100 bp ultramers primers. For transfection, 1×10^7 early-log phase Cas9-GFP expressing epimastigotes were electroporated with sgRNAs and donor DNA. CRISPR mutant cell lines were maintained under selection with G418, blasticidin, and

hygromycin. Genomic DNA was extracted from Wild Type (WT), Cas9-GFP (Cas9), and knockout lineages (TcP21-/-) and analyzed by PCR. The detailed protocol for the generation of these P21 knockout parasites has been previously described in Silveira et al. 2025.

2.4 | Host Cell Invasion, Intracellular Multiplication, and Egress Assays

Vero cell invasion assays were performed in 24-well plates containing coverslips. TCT suspensions (MOI: 10:1) were added, and the plates were incubated for 2 h. After incubation, cells were washed, Bouin's fixed, and Giemsa stained. The number of internalized parasites was counted in 300 total cells. For multiplication assays, after invasion, plates were washed, and the medium was replaced. Cells were collected at 24-, 48-, and 72-h postinfection, and DNA was extracted. Experiments were performed in triplicate with three independent biological replicates. For egress assays, following host cell invasion, plates were washed, medium replaced, and incubated. After 72 h, the number of parasites in the supernatant was determined by counting trypomastigote and amastigote forms in a Neubauer chamber for 10 days (240 h) postinfection.

2.5 | In Vivo Infection

INFγ−/− C57BL/6 mice were randomized into three groups: uninfected, infected with Cas9 parasites (control), and infected with TcP21−/− parasites. Animals were infected intraperitoneally with 10^5 TCT/mL. Systemic parasitemia was determined from day 3 postinfection. On day 15, animals were euthanized, and hearts were collected for analysis.

2.6 | Parasite Load Determined by qPCR

Hearts were weighed, stored, macerated, and incubated with NLB buffer, SDS, and PK solution. NaCl buffer was added, and samples were vortexed and placed on ice. Supernatant was collected, ethanol was added, and samples were centrifuged. The pellet was resuspended, and DNA was quantified and analyzed by qPCR. The standard curve was obtained using serial dilutions of 100 ng of DNA extracted from epimastigotes of G strain, with a limit of 0.0001 fg, as proposed by Diaz et al. (1992) and modified by Tavares de Oliveira et al. (2020). Positive, negative, and reagent internal controls were used in all qPCR reactions. The pair of primers used is shown in Table S1. These procedures were also used to quantify parasite DNA in the in vitro multiplication assays.

2.7 | Inflammatory Score

Heart samples were fixed, processed, and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Amastigote nests, inflammatory infiltrate, and tissue damage were evaluated under light microscopy and scored by intensity: (−) absent, (+) mild, (++) moderate, (+ + +) intense as described by da Silva et al. (2018).

2.8 | Statistical Analysis

Data were presented as mean ± SEM. Normal distribution was checked using the Shapiro-Wilk test. Significant differences were determined by t-test and Mann-Whitney test. Two-way ANOVA with Bonferroni's or Sidak's test was used for some data. Analyses were performed using GraphPad Prism software. Outlier analysis was performed using Grubbs' test where applicable, but no data points were excluded.

3 | Results

3.1 | Knockout of P21 Affected Cell Invasion, Multiplication, and Tissue Parasitism by TCT From *T. cruzi* G Strain

The successful generation and comprehensive characterization of P21 knockout parasites from G strain validation, have been previously detailed (Silveira et al. 2025). Here, we addressed P21 knockout impact on cell invasion, multiplication, egress, and tissue parasitism in the low-virulence G strain. We used TCT as the infective form and Vero cell as host cell line. Our results corroborated previous data using rP21 indicating that P21 upregulates parasite cell invasion by *T. cruzi* infective forms. Vero cells infected with TcP21−/− parasites showed significantly lower infection rates compared to cells infected with Cas9 parasites ($p = 0.001$) (Figure 1A).

To assess P21 knockout impact on intracellular multiplication, we allowed Cas9 and TcP21−/− parasites to invade Vero cells for 2 h. qPCR quantification of parasite DNA revealed that Vero cells infected with TcP21−/− parasites had lower parasite DNA content compared to cells infected with Cas9 parasites at 24-,48-, and 72-h postinfection ($p = 0.0047$) (Figure 1B). Trypomastigote egress from Vero cells was significantly lower for G strain TcP21−/− parasites than Cas9 from 168 to 240 h postinfection (Figure 1D).

Previously, we observed that extracellular amastigotes from G strain only established patent infection in IFN-γ knockout mice (Rodrigues et al. 2012). We used these animals to investigate P21 knockout impact in vivo using TCT from G strain. Surprisingly, neither G strain TcP21−/− nor Cas9 parasite-infected mice exhibited systemic parasitemia, even in IFN-γ knockout mice (data not shown), suggesting susceptibility might be restricted to the extracellular amastigote infective form. However, qPCR analysis of heart tissue revealed that animals infected with G strain TcP21−/− showed a significantly reduced parasite load compared to those infected with Cas9 parasites ($p = 0.0271$) (Figure 1C). Histopathological analysis of heart tissue from mice infected with G strain TcP21−/− and Cas9 parasites revealed only mild to moderate alterations compared to control (Figure 1F; Table 1). Consistent with these findings, P21-deficient epimastigotes of the G strain also exhibited reduced growth compared to control parasites, statistically significant at 4, 6, 10, and 12 days of culture (Figure 1E).

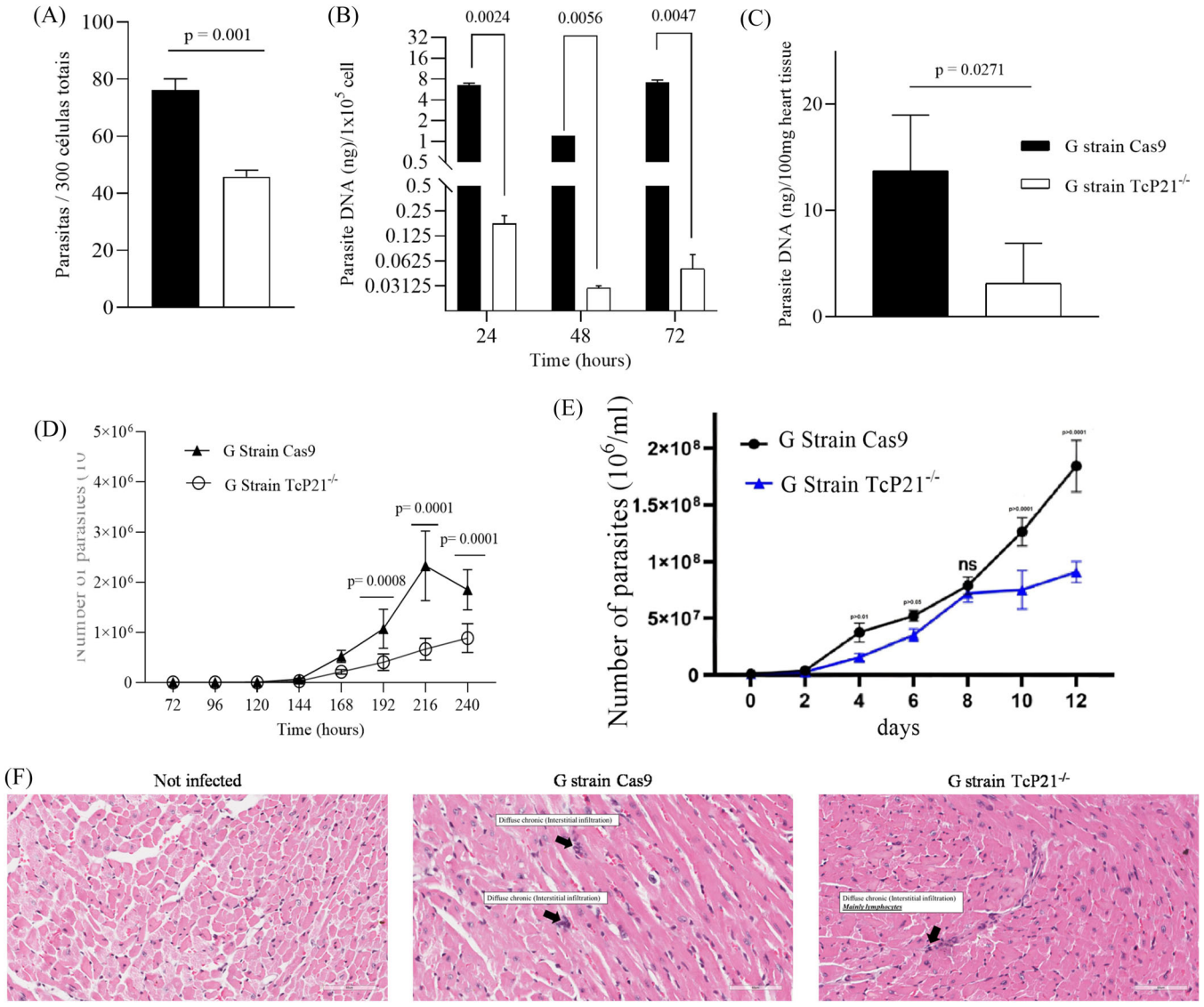

FIGURE 1 | Cell invasion, intracellular multiplication, and tissue parasitism of *T. cruzi* G strain TcP21-/- and Cas9 in the Vero cell line. (A) Vero cell invasion—number of parasites per 300 total cells. (B) Vero cell parasitism determined by the amount of parasite DNA amplified by qPCR. (C) Cardiac parasitism determined by the amount of parasite DNA amplified by qPCR. (D) Egress of trypomastigotes from Vero cells, counted in the supernatant over 10 days. (E) Growth curves of Cas9 and TcP21−/− epimastigotes during 12 days of culture. (F) Representative images of cardiac tissue stained with hematoxylin and eosin. Histopathological alterations are indicated by black arrows.

4 | Discussion

Our prior results with recombinant P21 suggested its involvement in invasion and multiplication processes of parasite infective forms. To confirm this, we initially verified P21 knockout impact on host cell invasion and multiplication by Y strain metacyclic trypomastigotes in HeLa cells. The results corroborated our hypothesis, with knockout impairing parasite host cell invasion and inducing parasite multiplication at 72 h postinfection (Silveira et al. 2024).

Here, we addressed P21 knockout impact on cell invasion, multiplication, egress, and tissue parasitism of the low virulent G strain. Using TCT as the infective form and Vero cell line, our results corroborated previous rP21 data that P21 upregulates parasite cell invasion by *T. cruzi* infective forms.

Considering multiplication rate, G strain knockout parasite multiplication ability was compromised in Vero cells. qPCR data revealed lower multiplication rates in Vero cells for TcP21−/− parasites. These results contrast with those from virulent Y strain infections (Silveira et al. 2024; Teixeira et al. 2022). We hypothesize that in the context of the low-virulent G strain, P21 might be involved in biological processes supporting parasite persistence within the mammalian host. P21 may exhibit pleiotropic activity, differing across parasite strains. In G strain infection, P21 could maintain a basal cell cycle ensuring perpetuation. Conversely, in Y strain infection, P21 might control multiplication to mitigate parasitism and tissue damage, also contributing to strain survival during infection.

We suggest P21 has a pleiotropic nature, with different activities depending on the strain. In G strain infection, P21 may ensure basal cell cycle for perpetuation, while in Y strain infection, P21

TABLE 1 | Qualitative analyses of heart tissues from INFγ knockout mice (C57/BL6) at 15 days postinfection with TCT of *T. cruzi* G strain.

Histological criteria	Noninfected ($n = 3$)	G strain Cas9 ($n = 6$)	G strain TcP21-/- ($n = 6$)
Inflammatory response	None	Low/Mild	Low/Mild
Neutrophilic leukocytes	−	+	+
Eosinophils	−	−	−
Macrophages	−	+	+
Lymphocytes	−	++	++
Plasma cells	−	+	+
Giant foreign body cells	−	−	−
Tissue damage	−	+	+
Hydropic degeneration	−	++	++
Necrotic tissue	−	−	−
Apoptotic bodies	−	−	−
Edema	−	+	+
Fibroblast	−	+	+
Fibrosis	−	+	+
Adipocyte	−	−	−
Epicardium calcification	−	−	−
Amastigotes nests	−	+	+

may control multiplication to reduce parasitism and host damage, also ensuring parasite survival. Thus, two biological activities with the same overarching purpose: parasite survival and perpetuation. We also analyzed trypomastigote egress from host cell line. G strain TcP21−/− trypomastigotes egressed from Vero cells in lower numbers than controls throughout the kinetics, consistent with impaired host cell invasion and reduced multiplication.

To verify P21 knockout impact in a complex system, we performed mouse infections. We previously observed that G strain extracellular amastigotes only produced patent infection in IFN-γ knockout mice (Rodrigues et al. 2012). We used these animals to verify P21 knockout impact in vivo using G strain TCT form. Surprisingly, we observed no parasitemia in either knockout or control parasite infections, suggesting that the susceptibility previously observed might be restricted to extracellular amastigotes infective forms. Consistently, qPCR analysis of heart tissue from TcP21−/− infected animals showed significantly lower parasite load compared to Cas9 infected animals. This in vivo finding, along with in vitro qPCR multiplication data, contrasts with our hypothesis that P21 controls parasite multiplication to maintain intracellular protection and appears to contradict previous findings with Y strain metacyclic trypomastigotes (Teixeira et al. 2022). This discrepancy highlights the strain-dependent pleiotropic nature of P21. In G strain infection, P21's role and molecular interactions might be for maintaining a regular intracellular amastigote multiplication cycle to perpetuate sub-patent infection. Histopathological analysis revealed no significant qualitative differences between infection groups compared to uninfected animals. The lack of significant difference in inflammatory scores between groups, despite reduced parasite load in TcP21−/− infected mice, is noteworthy. This contrasts with expectations based on rP21 studies and findings in the Y strain, where P21 appeared to modulate inflammation. In the context of the low-virulent G strain, P21's role in promoting persistence may be less linked to acute inflammation and more focused on long-term survival mechanisms. This further supports the hypothesis of strain-specific pleiotropic functions for P21.

Based on TcP21−/− parasite results from the G strain, we conclude that P21 from the G strain promotes host cell invasion in vitro and sustains in vitro and in vivo parasitism. P21's potential effect on host cardiac tissue parasitism may be related to a mechanism in this non-virulent strain promoting silent parasite perpetuation in the vertebrate mammalian host. However, it is important to acknowledge the limitations of this study. In vivo experiments were performed in IFNγ knockout mice. This model was chosen because our previous work (Rodrigues et al. 2012) showed that the G strain only establishes patent infection in these immunodeficient animals, allowing us to observe a clear infection phenotype. However, this model may not fully reflect the P21 role in immunocompetent hosts, where the immune response would be different. Furthermore, in vitro studies were limited to the Vero cell line, and further investigation in diverse cell types such as macrophages and cardiomyocytes, and in vivo models is warranted to fully elucidate the pleiotropic roles of P21 across different *T. cruzi* strains and infection contexts. However, it is necessary to acknowledge that the relatively small sample size ($n = 3$–6) for in vivo experiments may limit the statistical power and generalizability of our conclusions. Future studies with larger sample sizes would be beneficial to validate these findings. Nevertheless, this study significantly advances our understanding of P21 function, particularly in the context of the low-

virulence G strain and further establishes P21 as a potential therapeutic target for Chagas disease, emphasizing the need to consider strain-specific mechanisms in drug development.

Author Contributions

Claudio V. da Silva: conceptualization, supervision, project administration, writing – original draft, writing – review and editing. **José Franco da Silveira:** conceptualization, methodology, funding acquisition, writing – original draft, writing – review and editing. **Renato Arruda Mortara:** conceptualization, methodology, funding acquisition, writing – original draft, writing – review and editing. **Nelsa Paula Inácio Uombe:** investigation, data curation, writing – original draft, writing – review and editing. **Teresiama Velikkakam:** investigation, data curation, writing – original draft, writing – review and editing. **Anna Clara Azevedo Silveira:** investigation, data curation, writing – original draft, writing – review and editing. **Cassiano Costa Rodrigues:** investigation, data curation, writing – original draft, writing – review and editing. **Bruna Cristina Borges:** investigation, data curation, writing – original draft, writing – review and editing. **Thaise Lara Teixeira:** investigation, data curation, writing – original draft, writing – review and editing. **Cecília Luiza Pereira:** investigation, data curation, writing – original draft, writing – review and editing. **João Paulo Silva Servato:** investigation, data curation, writing – original draft, writing – review and editing. **Normanda Souza Melo:** investigation, data curation, writing – original draft, writing – review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

None declared.

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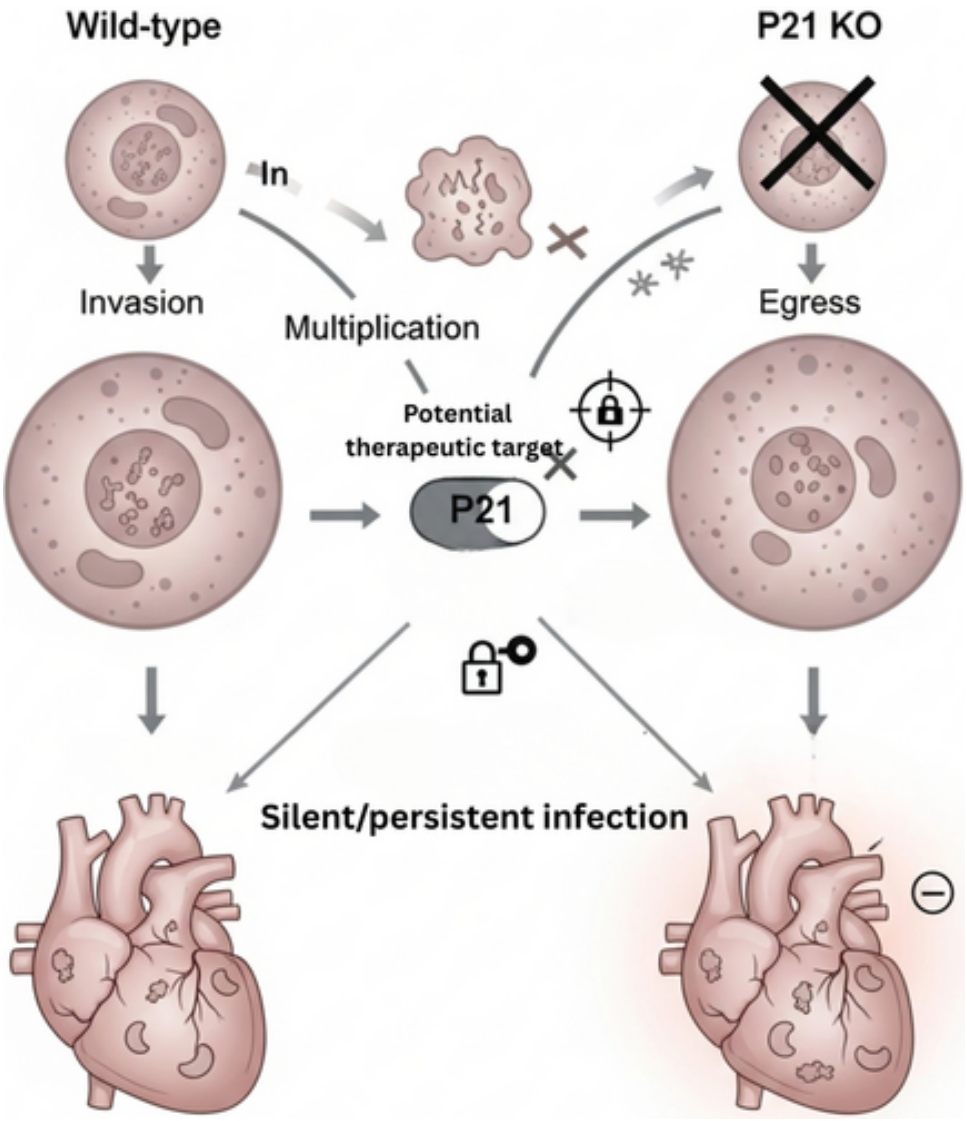
Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary Table 1. Primers used to generate CRISPR/Cas9 *T. cruzi* P21$^{-/-}$, confirmation of KO clones, RT-PCR and qPCR.

Graphical Abstract

This study reveals the pleiotropic role of *Trypanosoma cruzi* P21 protein in the low-virulence G strain. P21 knockout significantly impairs parasite invasion, intracellular multiplication, and egress in vitro. *In vivo*, P21 deficiency leads to reduced cardiac tissue parasitism, suggesting its role in establishing and maintaining silent infections. P21 influences parasite persistence and host-pathogen interactions, highlighting its potential as a therapeutic target with strain-specific implications.



Supporting Information

Filename	Description
mbo370154-sup-0001-Supplementary_Table_1.docx 15.3 KB	**Supplementary Table 1**. Primers used to generate CRISPR/Cas9 *T. cruzi* P21$^{-/-}$, confirmation of KO clones, RT-PCR and qPCR.

Supplementary Table 1. Primers used to generate CRISPR/Cas9 *T. cruzi* P21$^{-/-}$, confirmation of KO clones, RT-PCR and qPCR.

Primers		
1	sgRNA_36 Fw	GGAGGCCGGAGAATTGTAATACGACTCACTATAGGGAGAG**GCGCCTGCAGCGTGTCGGCCG**GTTTTAGAGCTAGAAATAGCAAG
2	sgRNA_424 Fw	GGAGGCCGGAGAATTGTAATACGACTCACTATAGGGAGAG**GTTCTACAAAGATACCGTGGT**GTTTTAGAGCTAGAAATAGCAAG
3	sgRNA_all genes Rv	CAGTGGATCCAAAAAAGCACCGACTCGGT
4	Bsd_ ultramer Fw	GTGTGAGAATAGGCTTTGTAAAAGGAATTTAATTTTACGGACACATCTCGCTAAACAGCAGCAACAACAGCAGGAGGAGC**ATGGCCAAGCCTTTGTCTCA**
5	Bsd_ultramer Rv	TCATTTTTCCATACAGTTGTCAGGCTGCCCCTTCTCTCCTCCTCTCCTGCAGCCGTGAAGAATCCCCCCATTCCGAGGTG**TTAGCCCTCCCACACATAAC**
6	Hygro_ ultramer Fw	GTGTGAGAATAGGCTTTGTAAAAGGAATTTAATTTTACGGACACATCTCGCTAAACAGCAGCAACAACAGCAGGAGGAGC**ATGAAAAAGCCTGAACTCAC**
7	Hygro_ ultramer Rv	TCATTTTTCCATACAGTTGTCAGGCTGCCCCTTCTCTCCTCCTCTCCTGCAGCCGTGAAGAATCCCCCCATTCCGAGGTG**CTATTCCTTTGCCCTCGGAC**
8	P21 Fw	GATACAACCACAAGGAGCC
9	P21 Rv	TTACTGGCGTCTGTGGAATC
10	UTR P21 Fw	GCCTCCATCCACATTTCATG
11	UTR P21 Rv	AACGTCCAATTAGGTCTTGTA
12	TcHGPRT Fw	CTACAAGGGAAAGGGTCTGC
13	TcHGPRT Rv	ACCGTAGCCAATCACAAAGG
14	TcMVK Fw	CGGCCGCGACATTTGGT
15	TcMVK Rv	GGCACTTCTAGGGCACGCAG
16	Diaz7	CGCAAACAGATATTGACAGAG
17	Diaz8	TGTTCACACACTGGACACCAA

Uberlândia, 19 de outubro de 2022

Ao Senhor Coordenador da Comissão de Ética na Utilização de Animais
Campus Umuarama Uberlândia - MG

ASSUNTO: Anuência para utilização da REBIR-UFU

Por meio deste, o Biotério Central da Rede de Biotérios de Roedores da Universidade Federal de Uberlândia confere anuência, mediante análise da demanda geral de usuários do Biotério Central, ao projeto de pesquisa intitulado: "O papel da P21 de *Trypanosoma cruzi* na infecção experimental *in vivo*", sob coordenação do (a) **Prof (a). Dr (a). Claudio Vieira da Silva,** a ser executado no período de 15/01/2023 à 14/01/2025. Tal anuência se dá em relação à:

Espaço Físico para desenvolvimento dos experimentos: Sim (X) Não ( ):

OBS 1: Ressalta-se que tais experimentos somente serão realizados nas dependências físicas da REBIR após aprovação pela CEUA;

OBS 2: Para realização de solicitação de animais ao Biotério Central da REBIR é necessário anexar o Certificado de Aprovação ao formulário de solicitação na página da REBIR.

Animais: Sim (X) Não ( ):

Linhagem/Colônia	**BALB/c**	**IFN-g-/-**		
Idade	5-6 semanas	5-6 semanas		
Peso aproximado	15-20g	15-20g		
Sexo	Macho (15); Fêmea (--); **Subtotal: 15**	Macho (15); Fêmea (--); **Subtotal: 15**		
Total	**30**			

Atenciosamente,

Loyane Bertagnolli Coutinho
Coordenadora Área Experimentação REBIR-UFU
Portaria PROPP Nº 19 de 06.06.2018

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Research Article

P21 Ablation Unveils Strain-Specific Transcriptional Reprogramming in *Trypanosoma cruzi* Amastigotes

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Trypanosoma cruzi is the causative agent of Chagas disease and is capable of invading any nucleated cell in the vertebrate host. The parasite utilizes various virulence factors during cell invasion, including the P21 protein. P21 is encoded by a single-copy, nonconserved gene expressed across all *T. cruzi* life cycle stages. Its sequence codes for a protein implicated in cell invasion and parasite multiplication. Given the significant differences in biological behavior between distinct strains of *T. cruzi*, we ablated the P21-coding gene in two phylogenetically distant strains (G and Y strains) and assessed its impact on the transcriptome profile of intracellular amastigotes. Our findings revealed that P21 depletion affected the transcription of different genes in the G and Y strains, with each strain exhibiting enrichment for distinct biological processes. Notably, protein translation was the major biological process impacted by P21 depletion, showing upregulation in the G strain and downregulation in the Y strain. In conclusion, our findings demonstrate that P21 gene ablation induces strain-specific transcriptional reprogramming in *T. cruzi* amastigotes, revealing divergent roles for P21 in modulating fundamental cellular processes like protein translation and potentially influencing host–parasite interactions, contingent upon the parasite's genetic background.

Keywords: DTUs; P21 protein; RNA-seq; strain-specific responses; transcription; *Trypanosoma cruzi*

1. Introduction

Trypanosoma cruzi (*T. cruzi*), the etiological agent of Chagas disease, is an intracellular protozoan parasite capable of invading a wide range of nucleated cells within the vertebrate host. The parasite employs a variety of membrane-anchored and secreted proteins to facilitate cellular invasion [1, 2]. Among these, the P21 protein has been identified as a key player in the invasion process [3]. P21 is encoded by a single-copy gene in the *T. cruzi* genome and is expressed throughout the parasite's life cycle [3]. Previous studies have demonstrated that the ablation of the P21-coding gene leads to reduced cellular invasion and increased multiplication in the Y strain of *T. cruzi* [4, 5]. These observations suggest that P21 may function to maintain intracellular persistence of the parasite, potentially by shielding it from host immune responses and modulating the cell cycle of intracellular amastigotes, thus contributing to the establishment of chronic infection.

T. cruzi exhibits remarkable genetic diversity and is currently classified into six discrete typing units (DTUs), TcI–TcVI. These DTUs exhibit significant variations in biological characteristics, including virulence and the expression of proteins involved in host cell invasion [2, 6]. This inherent diversity underscores the importance of strain-specific studies to fully understand *T. cruzi* biology and pathogenesis. For

instance, highly virulent strains (DTU II and VI) express and shed larger quantities of active trans-sialidase, leading to severe damage to the thymus and spleen, whereas low-virulent DTU I strains cause milder effects [6]. Furthermore, proteomic analysis has revealed that trypanolytic factors in the hemolymph and salivary glands of *Rhodnius prolixus* can lyse epimastigotes and trypomastigotes of the Y strain (TcII) but not of the Dm28c strain (TcI) [7]. Transcriptomic studies have also highlighted differences between TcI and TcII strains in their response to temperature stress, with TcII being less affected and showing increased expression of the surface metalloprotease GP63 [8]. Given this established strain diversity and the known involvement of P21 in *T. cruzi* virulence, we hypothesized that P21's functional role and regulatory impact might also diverge across different strains. For instance, strain-specific differences in tissue tropism, disease severity, and drug susceptibility have been well-documented in Chagas disease. Understanding the molecular basis of such strain variation is critical for developing broadly effective diagnostic and therapeutic strategies. Therefore, in the present work, we sought to investigate the impact of P21 gene deletion on the transcriptome of intracellular amastigotes from two phylogenetically distant *T. cruzi* strains, G and Y. The amastigote stage was specifically chosen for this study as it is the intracellular replicative form of the parasite, and our previous data indicated that P21 impact on multiplication varies between strains. While we acknowledge the importance of examining gene expression in the trypomastigote stage, which is responsible for host cell invasion, our primary focus in this study was to explore the strain-specific effects of P21 on the critical intracellular multiplication phase. By comparing the transcriptomic profiles of P21 knockout and Cas9 amastigotes from both strains, we aimed to identify genes and pathways differentially regulated in response to P21 ablation. This approach allowed us to gain insights into the diverse roles of P21 in different *T. cruzi* strains and its potential contribution to the distinct virulence phenotypes observed.

2. Material and Methods

2.1. Parasite and Cell Cultures. T. cruzi epimastigotes of both the Cas9 (parental) and TcP21-/- (P21 knockout) lines, belonging to the G (DTU I) and Y (DTU II) strains, were cultured at 28°C in liver infusion tryptose (LIT) medium (composition: 0.05% liver infusion, 0.5% tryptose, 0.004% hemin, 0.4% NaCl, 0.042% KCl, 0.8% Na_2HPO_4, 0.2% glucose, pH 7.3) supplemented with 20% fetal bovine serum (FBS; Invitrogen). Metacyclogenesis was induced by maintaining epimastigotes in LIT for 14 days, utilizing nutritional stress to promote differentiation, followed by purification of metacyclic trypomastigotes as previously described [9]. P21 knockout parasites from the G strain were generated upon early-log phase epimastigotes transfection with Cas9/pTREX-n (Addgene Plasmid #68708) [10]. Selection was performed with G418 (250 μg/mL) 24 h post-transfection, and GFP-positive parasites were sorted 15 days post-transfection using BD FACSARIA II. sgRNA sequences were designed with EuPatGDT [11]. DNA templates for sgRNA in vitro transcription were generated by PCR. sgRNAs were transcribed in vitro using the MEGAShortscript T7 kit (Thermo Fisher Scientific). Donor DNA for homologous recombination was produced by PCR using 100 bp ultramer primers. For transfection, 1×10^7 early-log phase Cas9-GFP expressing epimastigotes were electroporated with sgRNAs and donor DNA. CRISPR mutant cell lines were maintained under selection with G418, blasticidin, and hygromycin. Genomic DNA was extracted from wild-type (WT), Cas9-GFP (Cas9), and knockout lineages (TcP21-/-) and analyzed by PCR. Total RNA from WT, Cas9, and TcP21-/- parasites was extracted and treated with DNAse I. First-strand cDNA was synthesized from total RNA using the Superscript III First-Strand Synthesis System. PCR was performed to amplify P21 and the endogenous controls TcHGPRT and TcMVK [12]. WT and TcP21-/- parasites were fixed, washed, and incubated with anti-P21 antibodies. Subsequently, they were incubated with anti-mouse conjugated to Alexa Fluor 568 and DAPI. Images were acquired using confocal microscopy and analyzed with Imaris software (Supporting Information 1: Figure S1).

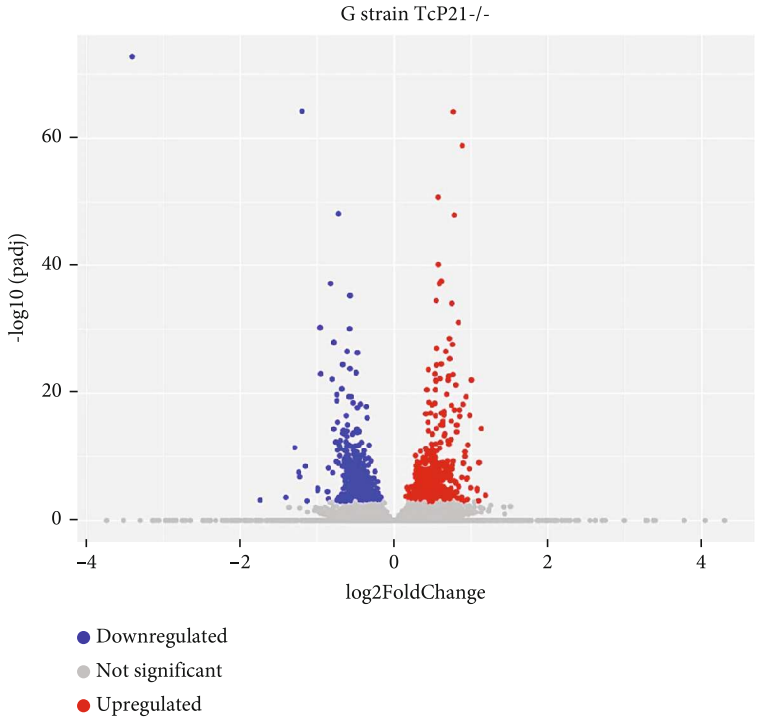
Vero cells (obtained from Instituto Adolfo Lutz) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Chemical Co.) supplemented with 10% FBS (Cultilab), 10 μg/mL streptomycin, 100 U/mL penicillin, and 40 μg/mL gentamicin. The Vero cell line was obtained from Instituto Adolfo Lutz, and cell line identity was confirmed by method of authentication, for example, STR profiling. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. To generate tissue culture–derived trypomastigotes (TCTs), Vero cells were infected with purified metacyclic trypomastigotes from both the parental Cas9 and TcP21-/- lines of the G and Y strains. Vero cells (0.7×10^6) were seeded in 75 cm^2 culture flasks and incubated at 37°C with 5% CO_2. Infection was carried out using TCTs present in the supernatant of pre-established cultures of knockout and control parasites. To ensure that parasites were collected at a similar stage, the time of infection was adjusted based on the known growth rates of the different strains and their respective P21 deletion mutants. After overnight infection, cells were washed with PBS to remove extracellular parasites and cultured for an additional 6 days to allow intracellular amastigote development. Infected cells were then harvested by washing with ice-cold Krebs–Henseleit buffer (KHB) containing 0.5 mM glucose, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$, followed by detachment using a cell scraper and 1 mL of KHB. The detached cells were collected in a final volume of 10 mL of KHB +0.5 mM glucose, centrifuged, and then the supernatant was discarded. The cell pellet was resuspended in 1 mL of KHB +0.5 mM glucose, transferred to an Eppendorf tube, vortexed for 45 s, and passed 20 times through a 1 mL syringe with a 27G needle to release the amastigotes. Infected cells and debris were pelleted by centrifugation at 100 g for 5 min, and the supernatant containing amastigotes was further centrifuged at 1000 g for 10 min at room temperature to pellet the amastigotes. Amastigotes were counted and stored in RNAlater solution until RNA extraction.

2.2. RNA Extraction and RNA-Seq. Total RNA was extracted from the amastigotes using the Qiagen RNeasy Mini Kit. Amastigotes were resuspended in 350 μL of RLT buffer with 0.1% β-mercaptoethanol, homogenized, and vortexed. The samples were centrifuged for 2 min at maximum speed at 4°C. The supernatant was collected and mixed with 350 μL of 70% ethanol, and 700 μL of this mixture was added to the column. RNA extraction was then performed according to the manufacturer's instructions. RNA quality was assessed using a NanoDrop 2000 spectrophotometer and an Agilent Bioanalyzer, confirming RNA Integrity Numbers (RIN) > 8.0 for all samples, indicating high RNA quality suitable for RNA-seq. The extracted RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and stored at −80°C. RNA samples were obtained from both Cas9 and TcP21-/- groups for each strain (Y and G), resulting in a total of four groups. Each group had three replicates, yielding a total of 12 samples for the experimental design. mRNA sequencing was performed by the Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida (LaCTAD) using an Illumina HiSeq2500 sequencer. Three independent replicates of each condition were sequenced, generating paired-end 2 × 100 bp reads (30 million reads per sample).

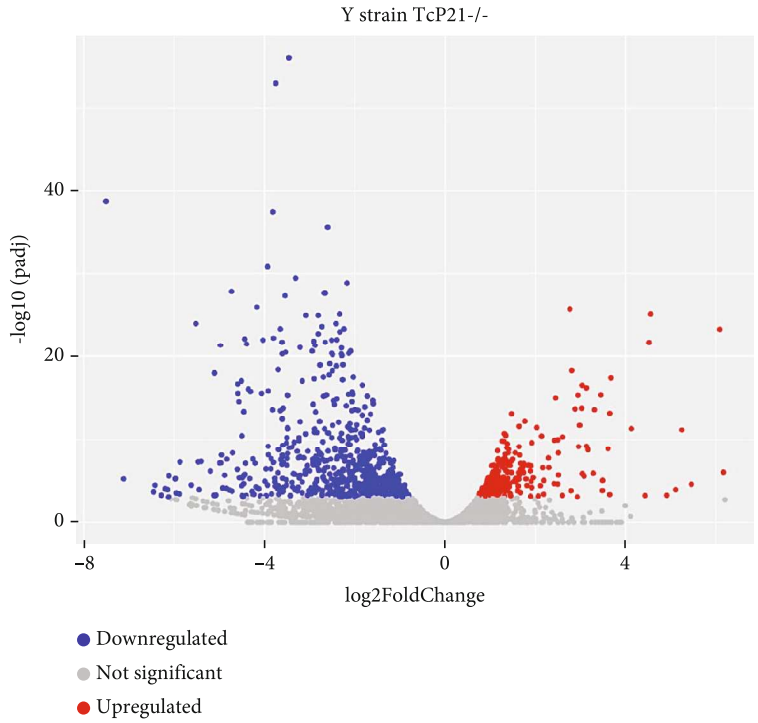
2.3. Data Processing. Fastq files were processed using the fastp program to remove low-quality reads and Illumina adapters [13]. Kraken2 was used for taxonomic classification and to filter out reads derived from Vero cells [14]. The Kraken2 database was built using the EuPathDB database [15]. The mean percentage of classified reads was 35.8% and 42.3% for G and Y strains, respectively. Of these, the mean percentage annotated to the *T. cruzi* genome was 97.6% and 96.4% for G and Y strains, respectively. The classified reads were aligned to the *T. cruzi* G strain genome using STAR, using the genome-guided alignment approach with default parameters and gene annotation from TriTrypDB (Version 64). The G strain genomic sequence (Version 64) and annotation files were obtained from TriTrypDB [16, 17]. Gene expression quantification using featureCounts was performed at the gene level, counting reads that uniquely mapped to exons according to the gene annotation file. The featureCounts program [18] was used to quantify reads at the gene level and generate gene expression count tables. The preprocessing was implemented as a Nextflow pipeline (available at https://github.com/iaradsouza1/gene_exp_tcruzi/tree/add-fastp). PCA of gene expression was performed using the "prcomp" function in R. Differential expression analysis was performed using DESeq2 in R/Bioconductor, employing a design formula of "~ strain + condition" to model gene expression, where "strain" accounts for strain-specific baseline differences and "condition" represents the comparison between TcP21-/- and Cas9 within each strain [19]. Raw counts of 13,504 protein-coding genes were tested. Genes with FDR < 0.001 were considered differentially expressed. Functional enrichment analysis was performed using the GOstats package in R, using the Gene Ontology database as a reference. GO term enrichment was assessed using Fisher's exact test, with p values adjusted for multiple testing using the Benjamini–Hochberg FDR method. Terms with at least three genes in each ontology were considered. p values were adjusted, and enriched terms with FDR < 0.05 were identified. Raw sequencing data is available at the National Library of Medicine under accession number PRJNA1156032. Moreover, Supporting Information 8: Table S7 and Supporting Information 9: Table S8 listing all differentially expressed genes between G (Cas9 vs. P21−/−) and Y (Cas9 vs. P21−/−), respectively, including fold changes, are provided.

3. Results

RNA sequencing was performed on samples from Cas9 (parental) and P21 knockout (TcP21-/-) intracellular amastigotes of both G and Y strains. A total of 1019 and 1060 transcripts were differentially expressed in TcP21-/- amastigotes of G and Y strains, respectively, compared to their Cas9 counterparts. Of these, 866 transcripts were specifically differentially expressed in TcP21-/- parasites of the G strain, while 907 transcripts were uniquely differentially expressed in TcP21-/- parasites of the Y strain. One hundred fifty-three transcripts were differentially expressed in both strains (Figures 1a, 1b, and 1c). A group of 442 genes from TcP21-/- amastigotes of the G strain and 350 genes from the TcP21-/- amastigotes of the Y strain were associated with enriched biological processes. Functional enrichment analysis of the differentially expressed genes in amastigotes of both strains revealed the biological terms potentially altered by P21 knockout. Two biological processes were enriched in TcP21-/- samples of the G strain, while eight were enriched in TcP21-/- samples of the Y strain. Translation was upregulated, and protein phosphorylation was downregulated in TcP21-/- parasites of the G strain. In contrast, translation, cell adhesion, protein glycosylation, translational elongation, intracellular signal transduction, and cyclic nucleotide biosynthetic processes were downregulated in TcP21-/- parasites of the Y strain, while tRNA aminoacylation for protein translation and protein import into the nucleus were upregulated. Notably, translation, a fundamental process for cellular growth and metabolism, exhibited strikingly divergent regulation: it was upregulated in the G strain TcP21-/- parasites while being downregulated in the Y strain TcP21-/- parasites, suggesting distinct impacts of P21 ablation on fundamental cellular processes in these strains. Forty-four transcripts related to translation were upregulated in TcP21-/- parasites of the G strain. The modulation of 40 of these 44 transcripts was exclusive to the G strain. Fifteen downregulated transcripts were related to protein phosphorylation. In contrast, 39 transcripts related to translation were downregulated in TcP21-/- parasites of the Y strain. Ten transcripts were related to protein glycosylation, 14 to cell adhesion, six to translational elongation, five to cyclic nucleotide biosynthetic process and intracellular signal transduction, and five to tRNA aminoacylation for protein translation. Three transcripts were related to protein import into the nucleus (Figure 2a,b). The modulation of 36 of these 39 transcripts was exclusive to TcP21-/- amastigotes of the Y strain. The genes associated with translation

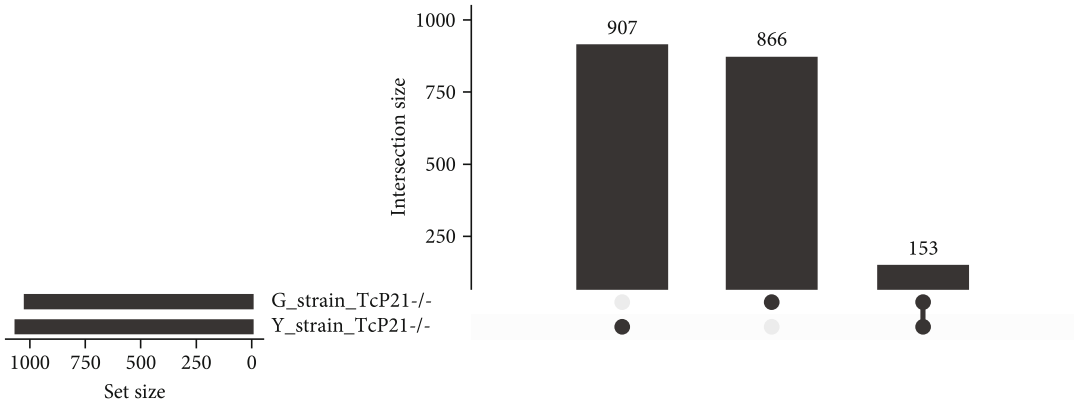


(a)



(b)

Figure 1: Continued.



(c)

Figure 1: (a, b) Volcano plots depicting differentially expressed genes (DEGs) in TcP21-/- intracellular amastigotes compared to Cas9 controls for G and Y strains, respectively. Upregulated genes are shown in red, and downregulated genes are shown in blue. The *x*-axis represents the $\log_2$ fold change in expression, and the *y*-axis represents the $-\log_{10}$ adjusted *p* value. (c) Venn diagram illustrating the overlap of DEGs between TcP21-/- amastigotes of G and Y strains compared to their respective Cas9 controls. Numbers within each section represent the number of genes uniquely differentially expressed in each strain or shared between them.

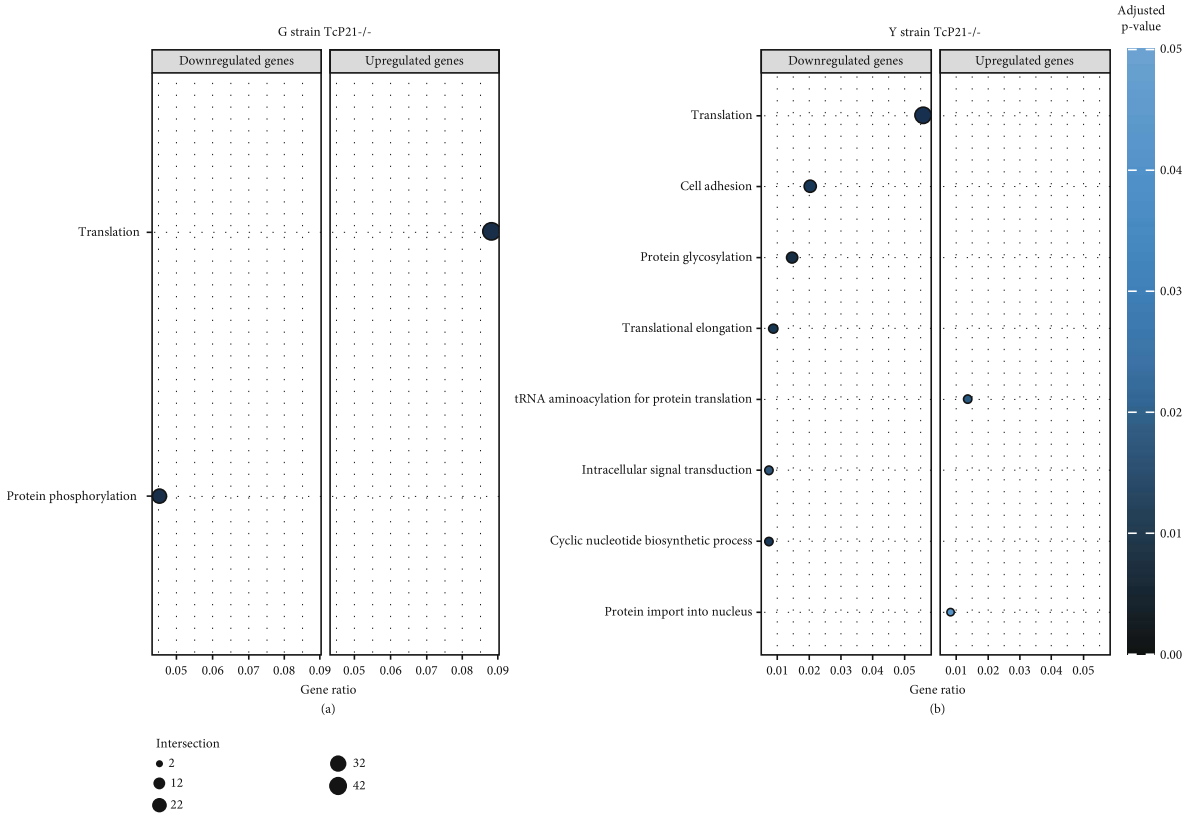


Figure 2: Biological processes enriched in TcP21-/- intracellular amastigotes of G and Y strains. (a, b) Bar plots showing the enrichment of differentially expressed genes associated with various biological processes in G and Y strain TcP21-/- amastigotes, respectively, compared to Cas9 controls. The *x*-axis represents the biological processes, and the *y*-axis represents the $-\log_{10}$ of the adjusted *p* value for enrichment. The color intensity of the bars corresponds to the level of enrichment, with darker colors indicating higher significance.

encode ribosomal proteins such as 60S ribosomal protein L11 (TcG_07781), 60S ribosomal subunit protein L31 (TcG_00575), 60S ribosomal protein L17 (TcG_00791), 40S ribosomal protein S14 (TcG_00946), and 40S ribosomal protein S15a (TcG_01906). The consistent upregulation of multiple ribosomal protein transcripts in the G strain TcP21-/- parasites indicates a potential increase in ribosome biogenesis or translational capacity in response to P21 ablation in this strain. Genes encoding for protein phosphorylation are represented by putative mitogen-activated protein

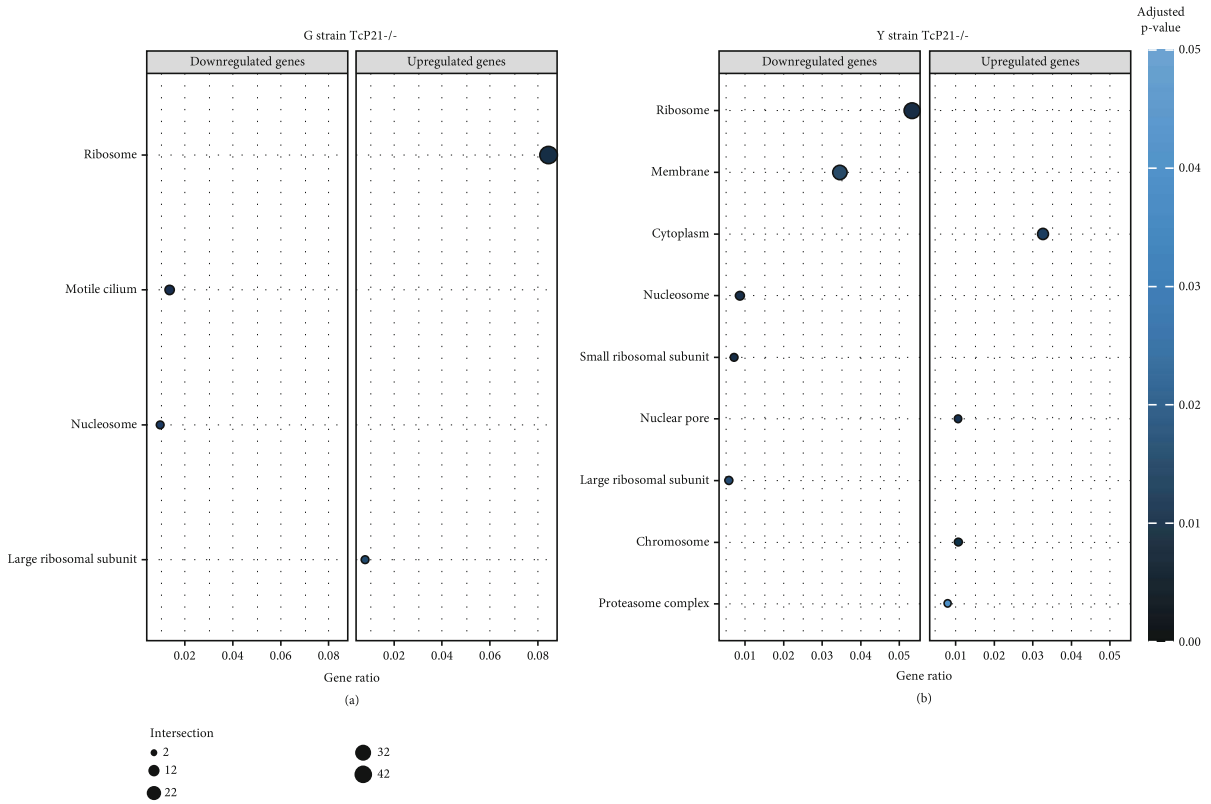


FIGURE 3: Cellular components enriched in TcP21-/- intracellular amastigotes of G and Y strains. (a, b) Bar plots showing the enrichment of differentially expressed genes associated with various cellular components in G and Y strain TcP21-/- amastigotes, respectively, compared to Cas9 controls. The *x*-axis represents the cellular components, and the *y*-axis represents the $-\log_{10}$ of the adjusted *p* value for enrichment. The color intensity of the bars corresponds to the level of enrichment, with darker colors indicating higher significance.

kinase (TcG_00063), putative serine/threonine protein kinase (TcG_00884), and casein kinase II, alpha chain (TcG_03076). Regarding cell adhesion, several copies of GP63 and putative GP63 genes were downregulated in TcP21-/- parasites of the Y strain, such as surface protease GP63 (TcG_07731) and GP63 Group II protein (TcG_08787). The downregulation of GP63, a well-established *T. cruzi* virulence factor involved in host cell invasion and immune modulation, in the Y strain TcP21-/- parasites, suggests that P21 may indirectly influence the expression of key surface proteins relevant to virulence in a strain-specific manner. Some representative transcripts of protein glycosylation are UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase (TcG_12364) and alpha-(1,3)-fucosyltransferase, family GT10 (TcG_11677). Representative transcripts of translational elongation are 60S acidic ribosomal protein P2 (TcG_00916) and elongation factor-1 alpha (TcG_02589). For the cyclic nucleotide biosynthetic process and intracellular signal transduction, differentially expressed genes are represented by receptor-type adenylate cyclase (TcG_01431) and adenylyl cyclase (TcG_01432). Valyl-tRNA synthetase (TcG_00996) and isoleucine-tRNA ligase (TcG_04183) are the representative transcripts of tRNA aminoacylation for protein translation. Karyopherin beta (TcG_06807) is an example of protein import into the nucleus. For the complete list of differentially expressed transcripts in each strain, see Supporting Information 2: Table S1 and Supporting Information 3: Table S2. Four cellular components in the G strain and nine in the Y strain were significantly affected by the absence of P21 gene expression (Figure 3a,b). TcP21-/- parasites of both G and Y strains displayed increased differential gene expression of ribosome, motile cilium, nucleosome components, and large ribosomal subunit cellular components compared to Cas9 parasites. Additionally, TcP21-/- parasites of the Y strain also showed significant differential expression of membrane, cytoplasm, small ribosomal subunits, nuclear pore, chromosome, and proteasome complex cellular components. Ribosomal transcripts were upregulated in TcP21-/- parasites of the G strain but downregulated in those of the Y strain, consistent with the biological process analysis. The same strain-specific transcripts described previously were also observed in this analysis. Regarding nucleosomes, transcripts such as histone H2A (TcG_03830) were downregulated in TcP21-/- parasites of both G and Y strains. In relation to motile cilium enriched in the G strain, the following transcripts were downregulated: intraflagellar transport 172-like protein (TcG_07868), paraflagellar rod component (TcG_05176), and paraflagellar rod protein 2C (TcG_02132). Transcripts related to the small ribosomal subunit, for example, 40S ribosomal protein S15 (TcG_06395) and 40S ribosomal protein AS (TcG_09354), were downregulated in Y strain TcP21-/- parasites. Transcripts related to the nuclear pore, for example, putative nuclear pore complex protein (NUP155) (TcG_04832) and putative ATP-dependent RNA

helicase (TcG_05048), were upregulated in Y strain TcP21-/- parasites. Large ribosomal subunit transcripts, such as 60S ribosomal protein L17 (TcG_01077) and 60S ribosomal protein L26 (TcG_02092), were upregulated in the G strain and downregulated in the Y strain TcP21-/- parasites. Chromosome-related transcripts, for example, putative structural maintenance of chromosomes (SMC) family protein (TcG_00603), were upregulated in Y strain TcP21-/- parasites. The downregulated transcripts related to the membrane are those encoding for GP63 and UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase (cell adhesion and glycosylation biological processes). Some upregulated transcripts related to the cytoplasm are valyl-tRNA synthetase (TcG_00996) and karyopherin beta (TcG_06807). Proteasome complex transcripts were upregulated in Y strain TcP21-/- parasites. Putative proteasome regulatory non-ATPase subunit (TcG_01492) is an example of a gene transcript of this cellular component. For the complete list of transcripts, see Supporting Information 4: Table S3 and Supporting Information 5: Table S4. Next, we analyzed the differential gene expression associated with molecular function. The results revealed 11 molecular functions differently expressed in the G strain and nine in the Y strain TcP21-/- parasites. ATP-binding transcripts were both upregulated and downregulated in the G strain and upregulated in the Y strain TcP21-/-, exemplified by ATP-dependent DEAD/H RNA helicase (TcG_00892) and ATP-binding cassette protein subfamily B, Member 1 (TcG_02742). Protein binding was downregulated in the G strain TcP21-/-. Representative transcripts include leucine-rich repeat protein (TcG_00090), putative protein transport protein Sec31 (TcG_01901), and putative eukaryotic translation initiation factor 4 gamma (TcG_02191). Transcripts related to structural constituents of ribosomes were upregulated in the G strain and downregulated in the Y strain TcP21-/-, as previously noted in the translation biological process and ribosome cellular component sections. Protein kinase activity was downregulated in the G strain TcP21-/-, as mentioned in the protein phosphorylation section. Nucleotide binding transcripts were downregulated in the G strain and upregulated in the Y strain TcP21-/-, including succinyl-CoA synthetase alpha subunit (TcG_06609) and isoleucine–tRNA ligase (TcG_04183). Metal ion binding was downregulated in the G strain TcP21-/-, such as the putative zinc finger protein (TcG00146). ATP hydrolysis activity transcripts were upregulated in the Y strain TcP21-/-, such as ATP-binding cassette protein subfamily A, Member 10 (TcG_01541). Catalytic activity was upregulated in G strain TcP21-/- parasites with the transcription of triosephosphate isomerase (TcG_00710) and 2-amino-3-ketobutyrate coenzyme A ligase (TcG_07370). Oxidoreductase activity transcripts were upregulated in both strains, for example, tryparedoxin peroxidase (TcG_08583 in G strain; TcG_08077 in Y strain). Methyltransferase activity was upregulated in the G strain TcP21-/- with the transcription of putative FtsJ cell division protein (TcG_01406) and tRNA guanosine-2-O-methyltransferase TRM13 (TcG_04372). G strain TcP21-/- data were also enriched in protein heterodimerization activity and calmodulin binding. The transcripts for these molecular functions were histones and paraflagellar rod proteins, respectively. The Y strain TcP21-/- showed the downregulation of metalloendopeptidase activity with transcripts that encode for GP63. In addition, the Y strain TcP21-/- showed the upregulation of aminoacyl-tRNA ligase activity, aminoacyl-tRNA editing activity, and structural constituent of the nuclear pore. The transcripts for aminoacyl-tRNA ligase and editing activities were those related to valyl-tRNA synthetase, isoleucine–tRNA ligase, and others. Regarding the constituent of the nuclear pore, putative nuclear pore complex protein (NUP155) (TcG_04832) is an example of a transcript (Figure 4a,b). The complete list of transcripts can be found in Supporting Information 6: Table S5 and Supporting Information 7: Table S6. Transcripts associated with plasma membrane and secreted proteins, likely involved in parasite virulence, were also analyzed. Transcripts of trans-sialidase were both upregulated and downregulated in the G and Y strains of TcP21-/- parasites. Dispersed gene protein family-1 (DGF-1) transcripts were upregulated in parasites from the G strain and downregulated in parasites from the Y strain. Mucin-associated surface protein (MASP) and mucin transcripts were downregulated in both strains. Mucin-like glycoprotein and GP63 transcripts were both upregulated and downregulated in the G strain but only downregulated in the Y strain. Mevalonate kinase transcripts were upregulated in the G strain. Surface protein-2 transcripts were downregulated in the G strain, and amastigote surface protein-4 transcripts were downregulated in the Y strain (Figure 5a,b). Supporting Information 8: Table S7 and Supporting Information 9: Table S8 provide all differentially expressed genes between G (Cas9 vs. P21−/−) and Y (Cas9 vs. P21−/−), respectively, including fold changes.

4. Discussion

The dynamic interplay between *T. cruzi* and its host cell involves intricate molecular mechanisms that govern parasite invasion, intracellular survival, and ultimately, the establishment of chronic infection. To address the relevance of strain-specific characteristics, it is important to note that while the G and Y strains exhibit well-documented differences in virulence and infectivity, there is no conclusive published evidence for distinct tissue tropisms. Therefore, the divergent outcomes observed in this study are more likely attributable to intrinsic functional differences in virulence factors between the strains rather than a predetermined preference for different host cell environments. Previous research by Li et al. [20] has shed light on the extensive transcriptome remodeling that occurs in both the parasite and the host cell during infection, highlighting the complex network of host–parasite interactions. By using a deconvolution technique, authors identified six distinct subpopulations of intracellular amastigotes, confirming their heterogeneity [21]. Our study builds upon this foundation by focusing on the role of the P21 protein, a secreted factor implicated in parasite virulence. We specifically investigated the transcriptomic consequences of P21 deletion in intracellular amastigotes collected between 5 and 8 days postinfection.

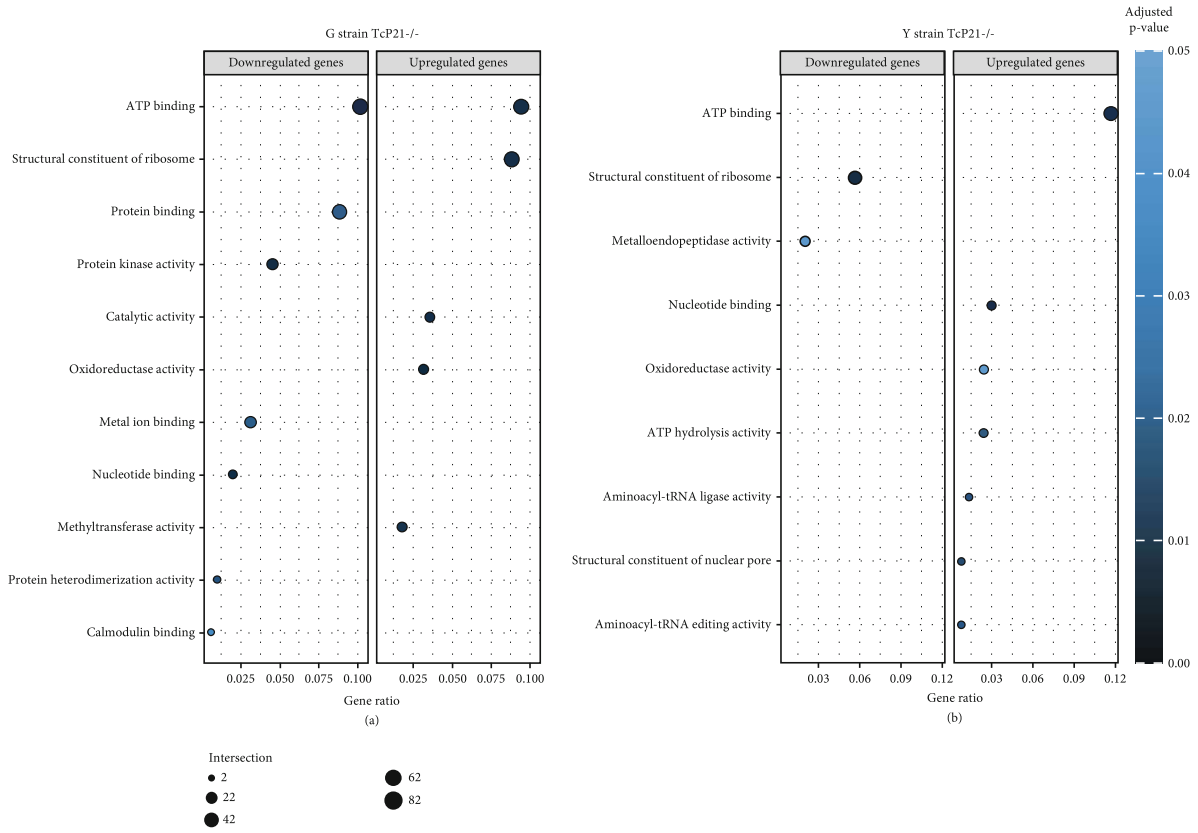


FIGURE 4: Molecular functions enriched in TcP21-/- intracellular amastigotes of G and Y strains. (a, b) Bar plots showing the enrichment of differentially expressed genes associated with various molecular functions in G and Y strain TcP21-/- amastigotes, respectively, compared to Cas9 controls. The *x*-axis represents the molecular functions, and the *y*-axis represents the $-\log_{10}$ of the adjusted *p* value for enrichment. The color intensity of the bars corresponds to the level of enrichment, with darker colors indicating higher significance.

This timeframe corresponds to the period of active amastigote replication allowing us to capture the transcriptional changes associated with this crucial process. The timing of collection was carefully adjusted based on the growth rates of each strain and its P21 knockout counterpart to ensure that parasites were at a comparable stage of intracellular development, minimizing potential confounding effects due to differences in the parasite's life cycle progression. Highlighting amastigote heterogeneity is relevant as it provides crucial context for interpreting our bulk RNA-seq data. Our approach captures the net effect of P21 deletion across the entire intracellular population, thereby establishing a foundational overview that complements future single-cell resolution studies.

In the G strain, P21 deletion led to the upregulation of translation-related processes, suggesting a role for P21 in controlling protein synthesis in this low-virulence strain. This might contribute to the slower replication rate observed in G strain TcP21-/- parasites. In contrast, the Y strain showed the downregulation of translation-related genes, indicating a different regulatory mechanism for P21 in this more virulent strain. The opposing regulation of translation in the G and Y strains TcP21-/- parasites may offer insights into the strain-specific effects of P21 on parasite multiplication. In the low-virulence G strain, the observed upregulation of translation, while seemingly paradoxical given the reported slower replication rate in the knockout, could represent an inefficient or unbalanced compensatory response. This increased translational activity might be metabolically costly or lead to the production of proteins that are not optimally required for replication, ultimately hindering parasite growth. Conversely, the downregulation of translation in the more virulent Y strain TcP21-/- parasites, which show increased multiplication in previous studies, might indicate a more efficient or targeted shift in protein synthesis, prioritizing the production of specific proteins required for rapid intracellular replication at the expense of overall translational output.

Protein kinase activity was downregulated in G strain TcP21-/- parasites, aligning with the results obtained for the protein phosphorylation biological process. The downregulation of signaling events dependent on protein phosphorylation by protein kinases may disrupt the parasites' ability to invade host cells and multiply intracellularly. Serine–threonine kinases are crucial enzymes in cellular proliferation and differentiation. Protein phosphorylation plays a significant role in cell signaling, gene expression, differentiation, and global control of DNA/RNA-mediated processes [22].

Oxidoreductase activity was upregulated in TcP21-/- parasites of both strains. Among the transcripts, a common one is tryparedoxin peroxidase. *T. cruzi* cytosolic tryparedoxin peroxidase is a 2-Cys peroxiredoxin with a vital role in detoxifying host cell; its overexpression enhances parasite infectivity and resistance to exogenous oxidation.

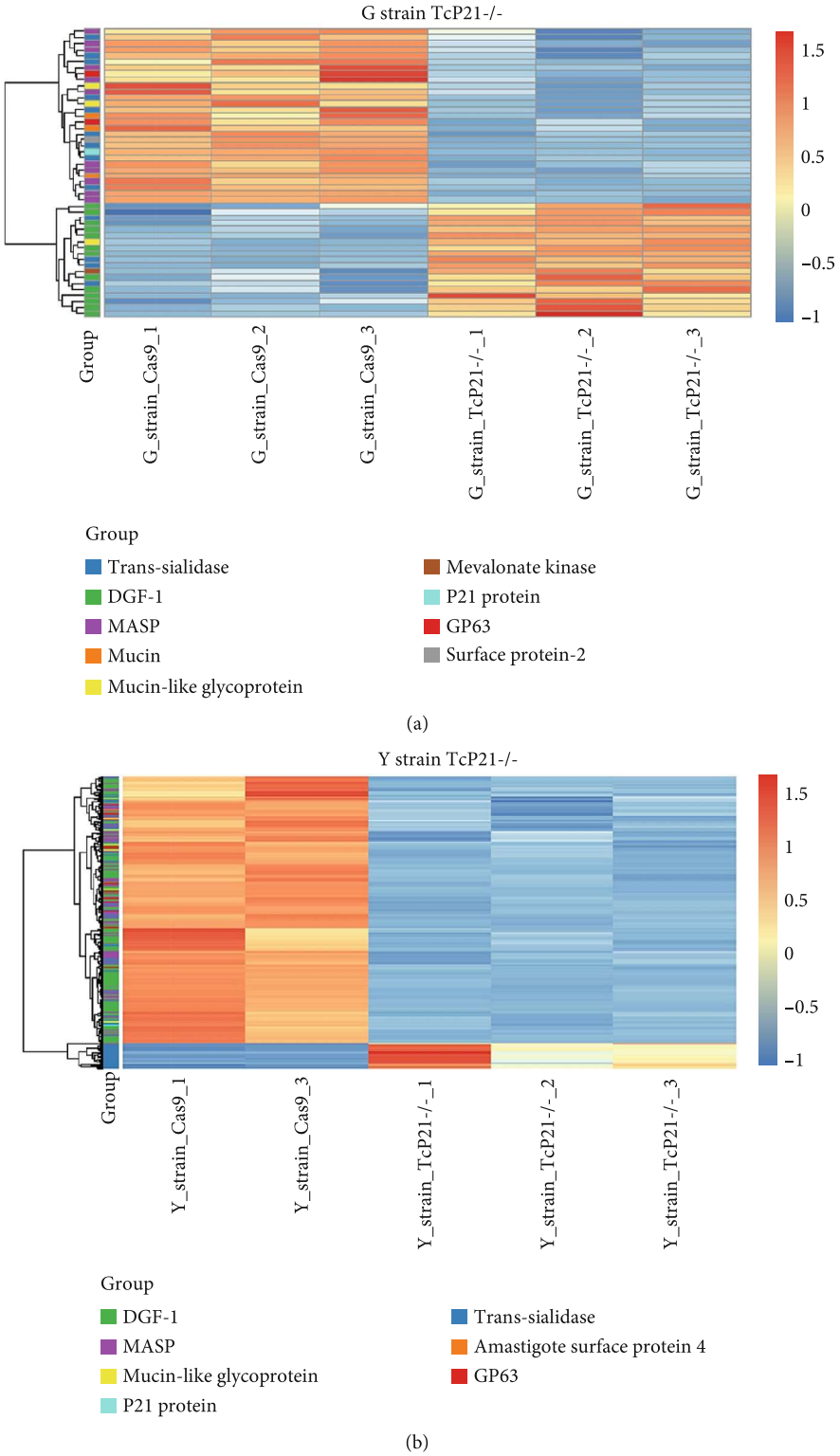


Figure 5: Heatmap of differentially expressed genes encoding membrane and secreted proteins in TcP21-/- amastigotes. (a, b) Heatmap visualization of the expression levels of genes coding for membrane and secreted proteins in TcP21-/- intracellular amastigotes compared to Cas9 controls for G and Y strains, respectively. Red indicates upregulation, and blue indicates downregulation. The color intensity corresponds to the magnitude of the $\log_2$ fold change.

Tryparedoxin peroxidase induces the recruitment of IL-12/23p40-producing innate antigen-presenting cells and promotes a strong specific Th1 immune response, also inducing proliferation and high levels of IFN-γ secretion in PBMCs from chronic patients without clear cardiac manifestations [23]. Studies have shown that the cytosolic tryparedoxin

peroxidase of *T. cruzi* is secreted by epimastigotes and trypomastigotes associated with extracellular vesicles and as a vesicle-free protein. Transcriptomic analysis revealed that cytosolic tryparedoxin peroxidase induces endoplasmic reticulum stress and interleukin-8 expression in epithelial cells. Moreover, the enzyme exhibited a mitogenic, proliferative, and proinflammatory effect on these cells in a dose-dependent manner and acts as a paracrine virulence factor, increasing the susceptibility to infection in pretreated epithelial cells [24].

Methyltransferase activity was an upregulated molecular function in G strain TcP21-/- parasites. Methyltransferases catalyze the transfer of a methyl group from S-adenosyl-l-methionine to their substrates. It has been shown that trimethylation of histone H3K76 by Dot1B enhances cell cycle progression after mitosis in *T. cruzi* [25]. These strain-specific differences suggest that P21 function extends beyond simply controlling replication and may involve modulating diverse cellular processes critical for parasite survival and virulence (Figure 4a,b). The divergent effects of P21 deletion in G and Y strains can be interpreted through several nonexclusive mechanisms. Firstly, the P21 protein sequences themselves are known to differ between these strains. This genetic divergence likely translates into structural variations that could alter the protein's stability, enzymatic activity, or its affinity for binding partners within the parasite, thus leading to distinct downstream regulatory consequences. Secondly, as P21 is a secreted factor, its influence may not be entirely cell-autonomous. P21 could modulate the host cell environment, for instance, by altering host signaling pathways or metabolic states. This altered host milieu would then exert a secondary, indirect effect on the intracellular amastigotes, contributing to the observed strain-specific transcriptomic shifts. This possibility underscores the complexity of the three-way interaction between the parasite genotype, the virulence factor's function, and the host cell response. The observed upregulation of translation-related genes in the G strain TcP21-/- parasites is intriguing. One possible mechanism could involve P21 acting as a repressor of translation in the G strain under normal conditions. P21 deletion might thus relieve this repression, leading to a compensatory upregulation of translation. Alternatively, P21 could be involved in signaling pathways that indirectly influence translation initiation or ribosome biogenesis. Future studies could investigate whether P21 interacts with known translational regulators or affects the activity of key signaling kinases or phosphatases involved in translational control.

The downregulation of GP63 transcripts in the Y strain TcP21-/- amastigotes raises questions about the role of P21 in regulating surface protein expression. GP63 is crucial for host cell invasion, parasite survival within macrophages, and modulation of the host immune response. Reduced GP63 levels in the knockout parasites could potentially impact their virulence, although the precise consequences require further investigation. It is possible that P21, in the Y strain, is involved in pathways that positively regulate GP63 expression, or that its absence indirectly affects GP63 transcription through broader changes in cellular signaling or regulatory networks.

Finally, we addressed the differential expression of transcripts coding for parasite membrane and secreted proteins. Surface proteins play diverse roles in parasite life cycle progression, host-cell interplay, immune system evasion, and parasite persistence [26]. An interesting observation was the downregulation of mucin and MASP transcripts in TcP21-/- parasites of both G and Y strains. Generally, P21 ablation resulted in the differential regulation of genes and biological processes in both strains. When the same biological process was regulated in both strains, this regulation was antagonistic. The consistency of similar transcriptional regulation of mucin-type proteins in both strains suggests that P21 directly controls the transcription of these genes. Mevalonate kinase is a glycosomal and secreted enzyme involved in parasite host cell invasion [12]. Transcripts coding for this enzyme were upregulated in G strain TcP21-/- intracellular amastigotes. The mevalonate pathway is highly conserved and mediates the production of metabolites essential for cellular metabolism, growth, and differentiation, such as isoprenoids, which feed into biosynthetic pathways for sterols, dolichol, ubiquinone, heme, isopentenyl adenine, and prenylated proteins [27] (Figure 5a,b).

5. Conclusion

Our findings provide novel insights into the complex and strain-specific functions of the *T. cruzi* P21 protein. This knowledge enhances our understanding of parasite adaptability and its strategies for establishing chronic infections. Future research should prioritize investigating the proteomic consequences of P21 ablation in both G and Y strains to validate the observed transcriptional changes and assess post-transcriptional regulation. Functional studies are necessary to dissect the molecular mechanisms by which P21 differentially regulates translation in these strains, potentially through interactions with translational machinery or signaling pathways. Furthermore, in vivo studies are warranted to determine how these strain-specific transcriptional responses and P21 function ultimately impact parasite virulence, chronic infection establishment, and interaction with the host immune system in the context of different *T. cruzi* strains. In conclusion, this study emphasizes that dissecting the strain-specific nuances of *T. cruzi* virulence factors like P21 is not merely an academic exercise but a fundamental imperative for advancing our understanding of Chagas disease pathogenesis and for the development of truly effective and broadly applicable therapeutic strategies in the face of *T. cruzi*'s remarkable genetic and phenotypic diversity.

Nomenclature

DTU	discrete typing unit
LIT	liver infusion tryptose
TCT	tissue culture–derived trypomastigote
DGF-1	dispersed gene protein family-1
MASP	mucin-associated surface protein

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information 1. Figure S1: Strategy and confirmation of P21 knockout clones. (A) Schematic representation of the cloning strategy. (B) sgRNA transcripts obtained by in vitro transcription of DNA_sgRNA using the MEGAShortscript T7 kit. pTRI-RNA (control); sgRNA_36 and sgRNA_424 target Cas9 for cleavage at nucleotides 36 and 424 of the P21 gene, respectively. (C) Donor DNAs obtained by PCR using ultramer primers (Table S1) and pGEM-Bsd or TOPO_hygro vectors as templates. (D) PCR of gDNA isolated from knockout clones and controls (WT and Cas9) using specific primers for P21 and the UTR region of the P21 gene. (E) PCR of cDNA from knockout clones and controls (WT and Cas9) analyzing the expression of P21 and endogenous TcMVK and TcHGPRT. (F) Epimastigotes were incubated with polyclonal anti-P21 antibodies (mouse) and anti-mouse antibodies conjugated to Alexa Fluor 568 (red). Nuclei were labeled with DAPI (blue). DIC, differential interference contrast. Single-plane images were acquired by confocal microscopy. Scale bars represent 3 μm.

Supporting Information 2. Table S1: Biological process transcripts enriched in G strain TcP21-/intracellular amastigotes.

Supporting Information 3. Table S2: Biological process transcripts enriched in Y strain TcP21-/intracellular amastigotes.

Supporting Information 4. Table S3: Cellular component transcripts enriched in G strain TcP21-/- intracellular amastigotes.

Supporting Information 5. Table S4: Cellular component transcripts enriched in Y strain TcP21-/- intracellular amastigotes.

Supporting Information 6. Table S5: Molecular function transcripts enriched in G strain TcP21-/- intracellular amastigotes.

Supporting Information 7. Table S6: Molecular function transcripts enriched in Y strain TcP21-/- intracellular amastigotes.

Supporting Information 8. Table S7: Comprehensive table listing all differentially expressed genes by G strain (Cas9 vs. P21−/−) amastigotes along with the fold change values.

Supporting Information 9. Table S8: Comprehensive table listing all differentially expressed genes by Y strain (Cas9 vs. P21−/−) amastigotes along with the fold change values.

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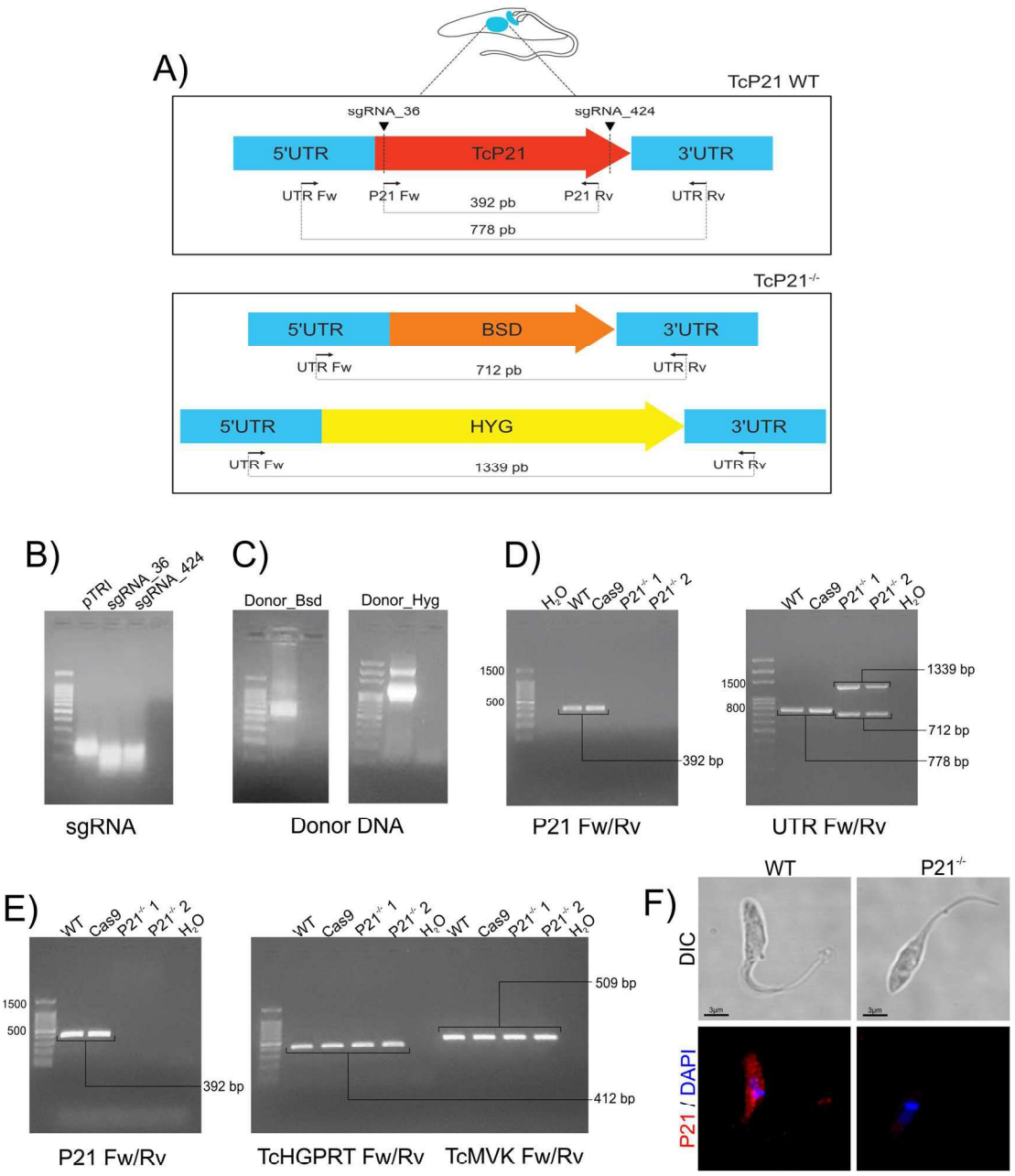
Supplementary Figure 1: G strain P21 *gene* knockout strategy

T. cruzi G parasites were transfected with Cas9/pTREX-n (Lander et al., 2015) to generate a lineage expressing Cas9 protein fused to eGFP. Following selection and enrichment for eGFP-positive cells, these Cas9-eGFP expressing parasites were transfected with two single guide RNAs (sgRNAs) targeting P21 (at nucleotides 36 and 424) (Fig. 1A and B) along with donor DNA harboring the Bsd resistance gene (Fig 1C) and selected with 50 µg/mL blasticidin and 250 µg/mL G418.

The selected parasites were cloned by limiting dilution, and genomic DNA from grown clones was extracted and analyzed by PCR. We identified P21+/- clones, containing one allele with the intact P21 gene and the other allele harboring the Bsd gene. To generate homozygous knockout (P21-/-) clones, the P21+/- parasites were electroporated with sgRNAs and donor DNA containing the hygromycin resistance gene, selected using 50 µg/mL blasticidin and 300 µg/mL hygromycin B, and cloned again by limiting dilution. Genomic DNA from these clones was extracted and analyzed by PCR with primers specific for P21 and the UTR regions (Fig. 1D). Our results indicated the absence of P21 gene amplification in these clones (Fig. 1D - gel 1). Conversely, the Bsd and Hygro resistance genes were amplified at the P21 genomic locus (Fig. 1D – gel 2), confirming the successful generation of two P21-/- clones. To verify the absence of P21 transcripts in knockout clones, cDNA synthesized by RT-PCR from total RNA was analyzed using primers for the P21 coding sequence. Two housekeeping genes, *T. cruzi* mevalonate kinase (TcMVK) (Ferreira et al., 2016) and hypoxanthine-guanine phosphoribosyltransferase (TcHGPRT) (Murta et al., 2006), served as internal controls. The results demonstrated the absence of P21 transcripts in knockout clones, while both clones expressed TcMVK and HGPRT transcripts (Fig. 1E).

The presence of P21 protein in epimastigotes was further assessed by immunofluorescence assay using confocal microscopy. In WT epimastigotes, P21 protein exhibited a clustered distribution at multiple cytoplasmic locations. However, no P21 protein labeling was observed in knockout parasites (Fig. 1F). Collectively, these data confirm the successful knockout of P21 protein using the employed strategy. We opted not to generate add-back parasites to maintain a clear experimental system and avoid potential confounding factors from gene re-introduction, focusing on a thorough analysis of the knockout phenotype within our resource constraints.


A)
TcP21 WT
sgRNA_36
sgRNA_424
5'UTR
TcP21
3'UTR
UTR Fw
P21 Fw
392 pb
P21 Rv
UTR Rv
778 pb
TcP21-/-
5'UTR
BSD
3'UTR
UTR Fw
712 pb
UTR Rv
5'UTR
HYG
3'UTR
UTR Fw
1339 pb
UTR Rv
B)
pTRI
sgRNA_36
sgRNA_424
sgRNA
C)
Donor_Bsd
Donor_Hyg
Donor DNA
D)
H2O
WT
Cas9
P21-/- 1
P21-/- 2
1500
500
392 bp
P21 Fw/Rv
WT
Cas9
P21-/- 1
P21-/- 2
H2O
1500
800
1339 bp
712 bp
778 bp
UTR Fw/Rv
E)
WT
Cas9
P21-/- 1
P21-/- 2
H2O
1500
500
392 bp
P21 Fw/Rv
WT
Cas9
P21-/- 1
P21-/- 2
H2O
WT
Cas9
P21-/- 1
P21-/- 2
H2O
509 bp
412 bp
TcHGPRT Fw/Rv
TcMVK Fw/Rv
F)
WT
P21-/-
DIC
3µm
3µm
P21 / DAPI

Supplementary Table 1: Biological processes transcripts enriched in G strain TcP21$^{-/-}$ intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED TRANSLATION	
TCG_00575	60S ribosomal subunit protein L31
TCG_00764	methyltransferase
TCG_00791	60S ribosomal protein L17
TCG_00940	ribosomal protein L15
TCG_00946	40S ribosomal protein S14
TCG_00970	putative 60S ribosomal protein L9
TCG_01380	ribosomal protein L21E (60S)
TCG_01628	putative 60S ribosomal protein L23a
TCG_01770	ribosomal protein S29
TCG_01906	40S ribosomal protein S15a
TCG_02057	60S ribosomal protein L12
TCG_02092	60S ribosomal protein L26
TCG_02510	60S ribosomal protein L13a
TCG_02639	ribosomal protein S19
TCG_02649	putative ribosomal protein L3
TCG_02796	ribosomal protein L35A
TCG_02966	40S ribosomal protein S24E
TCG_03508	putative ribosomal protein S7
TCG_03549	hypothetical protein
TCG_03847	40S ribosomal protein S3A
TCG_04538	60S acidic ribosomal protein P2
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05529	60S ribosomal protein L26
TCG_05966	60S ribosomal protein L14
TCG_06224	ribosomal proteins L36
TCG_06314	40S ribosomal protein S15a
TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07213	60S ribosomal protein L35
TCG_07214	60S ribosomal protein L35
TCG_07369	60S ribosomal protein L12
TCG_07781	60S ribosomal protein L11
TCG_08072	60S ribosomal protein L6
TCG_08129	40S ribosomal protein S14
TCG_08135	ribosomal protein S20
TCG_08281	40S ribosomal protein S12
TCG_08913	60S ribosomal protein L44
TCG_08967	60S ribosomal protein L2
TCG_09183	60S ribosomal protein L6

TCG_09273	40S ribosomal protein S33
TCG_10488	40S ribosomal protein S13
TCG_11208	60S ribosomal protein L34
TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
PROTEIN PHOSPHORYLATION	
DOWNREGULATED	
TCG_00063	putative mitogen-activated protein kinase, putative,kinase
TCG_00242	transferase
TCG_00884	putative serine/threonine protein kinase, putative,protein kinase
TCG_01098	putative protein kinase
TCG_01099	putative protein kinase
TCG_01102	putative protein kinase
TCG_01148	putative protein kinase, putative,serine/threonine protein kinase
TCG_01179	putative protein kinase
TCG_02217	putative protein kinase
TCG_02514	putative serine/threonine protein kinase, putative,protein kinase
TCG_03076	casein kinase II, alpha chain
TCG_03170	putative protein kinase
TCG_04057	putative protein kinase
TCG_04220	putative protein kinase, putative,serine/threonine-protein kinase Nek1
TCG_04236	putative protein kinase, putative,serine/threonine protein kinase
TCG_05071	putative serine/threonine protein kinase
TCG_05295	putative protein kinase
TCG_06077	putative serine/threonine protein kinase, putative,protein kinase
TCG_06667	putative mitogen-activated protein kinase
TCG_07418	putative protein kinase
TCG_08019	putative mitogen-activated protein kinase 3
TCG_08097	putative protein kinase
TCG_09911	putative protein kinase

Supplementary Table 2: Biological processes transcripts enriched in Y strain TcP21-/- intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED	
tRNA aminoacylation for protein translation	
TCG_00996	valyl-tRNA synthetase
TCG_01485	putative tryptophanyl-tRNA synthetase
TCG_04183	isoleucine--tRNA ligase
TCG_06042	uncharacterized protein
TCG_06182	putative arginyl-tRNA synthetase
UPREGULATED	
Protein import into nucleus	
TCG_00380	putative importin alpha
TCG_04944	putative importin beta-1 subunit
TCG_06807	karyopherin beta
DOWNREGULATED	
Translation	
TCG_00575	60S ribosomal subunit protein L31
TCG_00916	60S acidic ribosomal protein P2
TCG_00931	60S acidic ribosomal protein P2 beta (H6.4)
TCG_01077	60S ribosomal protein L17
TCG_01080	putative 40S ribosomal protein S2
TCG_01091	putative 40S ribosomal protein S2
TCG_01258	small subunit ribosomal protein S9e
TCG_01290	40S ribosomal protein S18
TCG_01628	putative 60S ribosomal protein L23a
TCG_01758	40S ribosomal protein S17
TCG_01858	40S ribosomal protein S21
TCG_02092	60S ribosomal protein L26
TCG_02464	ubiquitin/ribosomal protein S27a
TCG_02870	putative 60S ribosomal protein L4
TCG_03508	putative ribosomal protein S7
TCG_03960	60S ribosomal protein
TCG_04156	60S ribosomal protein L2
TCG_04512	ubiquitin/ribosomal protein S27a
TCG_04538	60S acidic ribosomal protein P2
TCG_04928	60S ribosomal protein L32
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05510	60S ribosomal protein L13a
TCG_05529	60S ribosomal protein L26
TCG_06155	polyubiquitin
TCG_06224	ribosomal proteins L36

TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07214	60S ribosomal protein L35
TCG_07781	60S ribosomal protein L11
TCG_08004	putative 60S ribosomal protein L2
TCG_08072	60S ribosomal protein L6
TCG_08443	60S ribosomal protein L34
TCG_08967	60S ribosomal protein L2
TCG_09354	40S ribosomal protein SA
TCG_11208	60S ribosomal protein L34
TCG_12209	putative ribosomal protein L11
TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
DOWNREGULATED	
Cell adhesion	
TCG_07731	surface protease GP63
TCG_07894	putative surface protease GP63
TCG_08211	surface protease GP63
TCG_08787	GP63 group II protein
TCG_08789	surface protease GP63
TCG_08836	surface protease GP63
TCG_08837	surface protease GP63
TCG_09033	putative surface protease GP63
TCG_09600	surface protease GP63
TCG_10132	putative surface protease GP63
TCG_11623	putative surface protease GP63
TCG_11823	putative surface protease GP63
TCG_12560	surface protease GP63
TCG_12563	surface protease GP63
DOWNREGULATED	
Protein glycosylation	
TCG_07267	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_07540	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_10088	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_10095	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_10794	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_11677	Alpha-(1,3)-fucosyltransferase, family GT10
TCG_11727	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_12364	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase

TCG_12471	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_13295	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
DOWNREGULATED	
Translational elongation	
TCG_00916	60S acidic ribosomal protein P2
TCG_00931	60S acidic ribosomal protein P2 beta (H6.4)
TCG_02589	elongation factor-1 alpha
TCG_04538	60S acidic ribosomal protein P2
TCG_09233	putative elongation factor 1-gamma (EF-1-gamma)
TCG_11789	elongation factor 1-gamma (EF-1-gamma)
DOWNREGULATED	
Intracellular signal transduction	
TCG_01431	receptor-type adenylate cyclase
TCG_01432	adenylyl cyclase
TCG_06813	adenylate cyclase
TCG_10649	receptor-type adenylate cyclase
TCG_13155	receptor-type adenylate cyclase
DOWNREGULATED	
Cyclic nucleotide biosynthetic process	
TCG_01431	receptor-type adenylate cyclase
TCG_01432	adenylyl cyclase
TCG_06813	adenylate cyclase
TCG_10649	receptor-type adenylate cyclase
TCG_13155	receptor-type adenylate cyclase

Supplementary Table 3: Cellular components transcripts enriched in G strain TcP21-/- intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED	
Ribosome	
TCG_00575	60S ribosomal subunit protein L31
TCG_00791	60S ribosomal protein L17
TCG_00940	ribosomal protein L15
TCG_00946	40S ribosomal protein S14
TCG_00970	putative 60S ribosomal protein L9
TCG_01380	ribosomal protein L21E (60S)
TCG_01468	hypothetical protein
TCG_01628	putative 60S ribosomal protein L23a
TCG_01770	ribosomal protein S29
TCG_01906	40S ribosomal protein S15a
TCG_02057	60S ribosomal protein L12
TCG_02510	60S ribosomal protein L13a
TCG_02639	ribosomal protein S19
TCG_02649	putative ribosomal protein L3
TCG_02796	ribosomal protein L35A
TCG_02966	40S ribosomal protein S24E
TCG_03508	putative ribosomal protein S7
TCG_03549	hypothetical protein
TCG_03847	40S ribosomal protein S3A
TCG_04538	60S acidic ribosomal protein P2
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05966	60S ribosomal protein L14
TCG_06224	ribosomal proteins L36
TCG_06314	40S ribosomal protein S15a
TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07213	60S ribosomal protein L35
TCG_07214	60S ribosomal protein L35
TCG_07369	60S ribosomal protein L12
TCG_07781	60S ribosomal protein L11
TCG_08072	60S ribosomal protein L6
TCG_08129	40S ribosomal protein S14
TCG_08135	ribosomal protein S20
TCG_08281	40S ribosomal protein S12
TCG_08913	60S ribosomal protein L44
TCG_08967	60S ribosomal protein L2
TCG_09183	60S ribosomal protein L6
TCG_09273	40S ribosomal protein S33
TCG_10488	40S ribosomal protein S13
TCG_11208	60S ribosomal protein L34

TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
UPREGULATED	
Large ribosomal subunit	
TCG_00791	60S ribosomal protein L17
TCG_02092	60S ribosomal protein L26
TCG_02510	60S ribosomal protein L13a
TCG_05529	60S ribosomal protein L26
DOWNREGULATED	
Motile cilium	
TCG_01546	putative flagellar radial spoke protein-like
TCG_02132	paraflagellar rod protein 2C
TCG_05176	paraflagellar rod component
TCG_06311	putative paraflagellar rod protein 1D
TCG_06474	putative intraflagellar transport protein 57
TCG_07868	intraflagellar transport 172-like protein
TCG_08330	putative flagellar calcium-binding protein
DOWNREGULATED	
Nucleosome	
TCG_01152	histone H3 variant
TCG_03830	histone H2A
TCG_03832	histone H2A
TCG_05567	histone H2A
TCG_08085	histone H2B

Supplementary Table 4: Cellular components transcripts enriched in Y strain TcP21-/- intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED	
Cyyoplasm	
TCG_00380	putative importin alpha
TCG_00907	putative 26S protease regulatory subunit
TCG_00996	valyl-tRNA synthetase
TCG_03161	putative asparagine synthetase a
TCG_03554	putative cytosolic leucyl aminopeptidase, putative,metallo-peptidase, Clan MF, Family M17
TCG_03894	eukaryotic peptide chain release factor subunit 1
TCG_03916	putative nucleotide-binding protein
TCG_06042	uncharacterized protein
TCG_06182	putative arginyl-tRNA synthetase
TCG_06807	karyopherin beta
TCG_07033	putative T-complex protein 1, delta subunit
TCG_07402	putative phenylalanyl-tRNA synthetase
UPREGULATED	
Nuclear pore	
TCG_01972	hypothetical protein
TCG_04832	putative nuclear pore complex protein (NUP155)
TCG_05048	putative ATP-dependent RNA helicase
TCG_06764	hypothetical protein
UPREGULATED	
Chromosome	
TCG_00603	putative structural maintenance of chromosome (SMC) family protein
TCG_02709	DNA topoisomerase IB, large subunit
TCG_07879	putative structural maintenance of chromosome protein 4
TCG_08098	putative structural maintenance of chromosome (SMC)
UPREGULATED	
Proteasome complex	

TCG_00907	putative 26S protease regulatory subunit
TCG_01492	putative proteasome regulatory non-ATPase subunit
TCG_04767	putative proteasome regulatory non-ATP-ase subunit 2
DOWNREGULATED	
Ribosome	
TCG_00575	60S ribosomal subunit protein L31
TCG_00916	60S acidic ribosomal protein P2
TCG_00931	60S acidic ribosomal protein P2 beta (H6.4)
TCG_01077	60S ribosomal protein L17
TCG_01080	putative 40S ribosomal protein S2
TCG_01091	putative 40S ribosomal protein S2
TCG_01284	40S ribosomal protein S10
TCG_01290	40S ribosomal protein S18
TCG_01628	putative 60S ribosomal protein L23a
TCG_01758	40S ribosomal protein S17
TCG_01858	40S ribosomal protein S21
TCG_02464	ubiquitin/ribosomal protein S27a
TCG_02870	putative 60S ribosomal protein L4
TCG_03508	putative ribosomal protein S7
TCG_03960	60S ribosomal protein
TCG_04156	60S ribosomal protein L2
TCG_04512	ubiquitin/ribosomal protein S27a
TCG_04538	60S acidic ribosomal protein P2
TCG_04928	60S ribosomal protein L32
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05510	60S ribosomal protein L13a
TCG_06155	polyubiquitin
TCG_06224	ribosomal proteins L36
TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07214	60S ribosomal protein L35
TCG_07781	60S ribosomal protein L11
TCG_08004	putative 60S ribosomal protein L2
TCG_08072	60S ribosomal protein L6
TCG_08443	60S ribosomal protein L34
TCG_08967	60S ribosomal protein L2
TCG_09354	40S ribosomal protein SA

TCG_11208	60S ribosomal protein L34
TCG_12209	putative ribosomal protein L11
TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
DOWNREGULATED	
Membrane	
TCG_07267	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_07540	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_07731	surface protease GP63
TCG_07894	putative surface protease GP63
TCG_08211	surface protease GP63
TCG_08787	GP63 group II protein
TCG_08789	surface protease GP63
TCG_08836	surface protease GP63
TCG_08837	surface protease GP63
TCG_09033	putative surface protease GP63
TCG_09600	surface protease GP63
TCG_10088	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_10095	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_10132	putative surface protease GP63
TCG_10794	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_11623	putative surface protease GP63
TCG_11677	Alpha-(1,3)-fucosyltransferase, family GT10
TCG_11727	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_11823	putative surface protease GP63
TCG_12364	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_12471	putative UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
TCG_12560	surface protease GP63
TCG_12563	surface protease GP63
TCG_13295	UDP-Gal or UDP-GlcNAc-dependent glycosyltransferase
DOWNREGULATED	
Nucleosome	

TCG_01628	putative 60S ribosomal protein L23a
TCG_03830	histone H2A
TCG_03831	histone H2A
TCG_03832	histone H2A
TCG_07837	histone H4
TCG_08085	histone H2B
DOWNREGULATED	
Small ribosomal subunit	
TCG_01080	putative 40S ribosomal protein S2
TCG_01258	small subunit ribosomal protein S9e
TCG_06395	40S ribosomal protein S15
TCG_09354	40S ribosomal protein SA
TCG_13471	putative 40S ribosomal protein S23
DOWNREGULATED	
Large ribosomal subunit	
TCG_01077	60S ribosomal protein L17
TCG_02092	60S ribosomal protein L26
TCG_05510	60S ribosomal protein L13a
TCG_05529	60S ribosomal protein L26

Suplementary Table 5: Molecular functions transcripts enriched in G strain TcP21-/- intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED	
ATP binding	
TCG_00218	putative DEAD/DEAH box helicase-like protein
TCG_00682	putative pre-mRNA splicing factor
TCG_00686	putative helicase-like protein
TCG_00696	putative replication factor C, subunit 1
TCG_00704	putative minchromosome maintenance (MCM) complex subunit
TCG_00719	putative DNA repair and recombination protein RAD54
TCG_00730	putative protein kinase
TCG_00739	putative DNA helicase
TCG_00772	ATP-dependent RNA helicase
TCG_01002	putative homoserine kinase
TCG_01407	transferase
TCG_01450	ATP-binding cassette protein subfamily C, member 1
TCG_01454	rac serine-threonine kinase
TCG_01467	putative protein kinase
TCG_01886	pyruvate phosphate dikinase 1
TCG_02051	protein kinase
TCG_02955	DNA dependent protein kinase catalytic subunit
TCG_03414	putative DNA repair protein
TCG_03493	REL1 protein
TCG_03525	putative RNA helicase
TCG_03546	putative T-complex protein 1, gamma subunit
TCG_04349	putative ATP-dependent RNA helicase
TCG_04374	glycosomal ABC transporter member 1
TCG_04376	putative mevalonate kinase
TCG_04462	acetyl-CoA carboxylase
TCG_04472	putative protein kinase

TCG_04606	putative glycosomal phosphoenolpyruvate carboxykinase
TCG_05140	putative ATP-dependent RNA helicase
TCG_05447	transferase
TCG_05452	mismatch repair protein MLH1
TCG_05453	putative kinesin
TCG_05466	putative ATP-dependent DEAD/H DNA helicase recQ
TCG_05659	putative kinesin
TCG_06906	putative mismatch repair protein MSH4
TCG_07101	putative protein kinase
TCG_07108	putative cell division cycle protein
TCG_07307	putative serine/threonine protein kinase
TCG_07368	hypothetical protein
TCG_07416	Hsp90
TCG_07567	101 kDa heat shock protein
TCG_08163	chaperonin HSP60, mitochondrial precursor
TCG_08332	myosin heavy chain kinase A
TCG_08951	putative protein kinase
TCG_09372	putative phosphatidylinositol-4-phosphate 5-kinase-like protein
TCG_09682	hypothetical protein
TCG_09684	putative tryptophanyl-tRNA synthetase
TCG_09692	ATP-binding cassette protein subfamily C, member 2
TCG_12127	ATP-binding cassette protein subfamily C, member 1
UPREGULATED	
Structural constituent of ribosome	
TCG_00575	60S ribosomal subunit protein L31
TCG_00791	60S ribosomal protein L17
TCG_00940	ribosomal protein L15
TCG_00946	40S ribosomal protein S14

TCG_00970	putative 60S ribosomal protein L9
TCG_01380	ribosomal protein L21E (60S)
TCG_01468	hypothetical protein
TCG_01628	putative 60S ribosomal protein L23a
TCG_01770	ribosomal protein S29
TCG_01906	40S ribosomal protein S15a
TCG_02057	60S ribosomal protein L12
TCG_02092	60S ribosomal protein L26
TCG_02510	60S ribosomal protein L13a
TCG_02639	ribosomal protein S19
TCG_02649	putative ribosomal protein L3
TCG_02796	ribosomal protein L35A
TCG_02966	40S ribosomal protein S24E
TCG_03508	putative ribosomal protein S7
TCG_03549	hypothetical protein
TCG_03847	40S ribosomal protein S3A
TCG_04538	60S acidic ribosomal protein P2
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05529	60S ribosomal protein L26
TCG_05966	60S ribosomal protein L14
TCG_06224	ribosomal proteins L36
TCG_06314	40S ribosomal protein S15a
TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07213	60S ribosomal protein L35
TCG_07214	60S ribosomal protein L35
TCG_07369	60S ribosomal protein L12
TCG_07781	60S ribosomal protein L11
TCG_08072	60S ribosomal protein L6
TCG_08129	40S ribosomal protein S14

TCG_08135	ribosomal protein S20
TCG_08281	40S ribosomal protein S12
TCG_08913	60S ribosomal protein L44
TCG_08967	60S ribosomal protein L2
TCG_09183	60S ribosomal protein L6
TCG_09273	40S ribosomal protein S33
TCG_10488	40S ribosomal protein S13
TCG_11208	60S ribosomal protein L34
TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
UPREGULATED	
Catalytic activity	
TCG_00400	putative dTDP-glucose 4,6-dehydratase
TCG_00710	triosephosphate isomerase
TCG_00760	putative aspartate aminotransferase, mitochondrial
TCG_00765	putative fumarate hydratase
TCG_01886	pyruvate phosphate dikinase 1
TCG_02805	protein G6
TCG_03529	acetyltransferase-like protein
TCG_04681	prostaglandin F2alpha synthase
TCG_04847	putative histidine ammonia-lyase
TCG_05528	transketolase 1
TCG_05968	putative cytosolic malate dehydrogenase
TCG_06686	putative aldose 1-epimerase-like protein
TCG_07370	2-amino-3-ketobutyrate coenzyme A ligase
TCG_07373	putative 2-amino-3-ketobutyrate coenzyme A ligase
TCG_07854	actin interacting protein-like protein
TCG_08739	putative 3,2-trans-enoyl-CoA isomerase, mitochondrial precursor
TCG_09067	putative dihydrouridine synthase (Dus)

TCG_12830	tyrosine aminotransferase
UPREGULATED	
Oxidoreductase activity	
TCG_01462	hypothetical protein
TCG_03535	putative lathosterol oxidase
TCG_04378	putative C-5 sterol desaturase
TCG_04681	prostaglandin F2alpha synthase
TCG_05330	putative glycerol-3-phosphate dehydrogenase (FAD-dependent)
TCG_05968	putative cytosolic malate dehydrogenase
TCG_06704	putative oxidoreductase
TCG_06818	putative delta-4 fatty acid desaturase
TCG_07244	hypothetical protein
TCG_07854	actin interacting protein-like protein
TCG_08449	NADH-cytochrome b5 reductase
TCG_08583	tryparedoxin peroxidase
TCG_09163	putative dihydrolipoamide dehydrogenase
TCG_09691	putative NAD(P)-dependent oxidoreductase
TCG_09726	putative NADP-dependent alcohol hydrogenase
TCG_09949	hypothetical protein
UPREGULATED	
Methyltransferase activity	
TCG_00371	putative DREV methyltransferase
TCG_00764	methyltransferase
TCG_01406	putative FtsJ cell division protein
TCG_01473	putative diphthine synthase
TCG_03547	putative S-adenosyl-methyltransferase mraW-like protein
TCG_04372	tRNA guanosine-2-O-methyltransferase TRM13
TCG_06697	putative ribosomal RNA methyltransferase

TCG_06839	putative sterol 24-c-methyltransferase
TCG_08984	putative nucleolar protein
DOWNREGULATED	
ATP binding	
TCG_00059	putative chaperonin alpha subunit
TCG_00063	putative mitogen-activated protein kinase, putative,kinase
TCG_00156	ATP-binding cassette protein subfamily F, member 2
TCG_00242	transferase
TCG_00316	putative OSM3-like kinesin
TCG_00882	putative 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
TCG_00884	putative serine/threonine protein kinase, putative,protein kinase
TCG_00907	putative 26S protease regulatory subunit
TCG_00953	S-adenosylmethionine synthetase
TCG_01073	putative ATP-dependent RNA helicase
TCG_01098	putative protein kinase
TCG_01099	putative protein kinase
TCG_01102	putative protein kinase
TCG_01110	C-terminal kinesin KIFC1
TCG_01148	putative protein kinase, putative,serine/threonine protein kinase
TCG_01158	putative kinesin
TCG_01179	putative protein kinase
TCG_01541	ATP-binding cassette protein subfamily A, member 10
TCG_01559	putative glucose regulated protein 94
TCG_01717	putative vesicular-fusion ATPase-like protein
TCG_01741	T-complex protein 1 subunit beta
TCG_02127	putative kinesin
TCG_02217	putative protein kinase
TCG_02514	putative serine/threonine protein kinase, putative,protein kinase

TCG_02924	mitochondrial ATP-dependent zinc metallopeptidase
TCG_03076	casein kinase II, alpha chain
TCG_03140	dynein, axonemal, heavy polypeptide 1
TCG_03170	putative protein kinase
TCG_03307	putative MCAK-like kinesin
TCG_03399	putative seryl-tRNA synthetase
TCG_03580	ATPase beta subunit
TCG_03750	putative cation transporting ATPase
TCG_04057	putative protein kinase
TCG_04183	isoleucine--tRNA ligase
TCG_04220	putative protein kinase, putative,serine/threonine-protein kinase Nek1
TCG_04236	putative protein kinase, putative,serine/threonine protein kinase
TCG_04588	hypothetical protein
TCG_05071	putative serine/threonine protein kinase
TCG_05295	putative protein kinase
TCG_05802	putative phospholipid-translocating P-type ATPase (flippase)
TCG_06042	uncharacterized protein
TCG_06077	putative serine/threonine protein kinase, putative,protein kinase
TCG_06214	putative methionyl-tRNA synthetase
TCG_06667	putative mitogen-activated protein kinase
TCG_07418	putative protein kinase
TCG_08019	putative mitogen-activated protein kinase 3
TCG_08097	putative protein kinase
TCG_08705	pyruvate kinase
TCG_09911	putative protein kinase
TCG_10163	metallo-peptidase, Clan MA(E), Family M41
TCG_10652	putative proteasome regulatory ATPase subunit 2
TCG_11092	ubiquitin-conjugating enzyme E2

DOWNREGULATED	
Protein binding	
TCG_00073	hypothetical protein
TCG_00090	leucine-rich repeat protein
TCG_00267	putative dynein
TCG_00476	putative paraflagellar rod component
TCG_00882	putative 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
TCG_01084	hypothetical protein
TCG_01092	putative peroxin 13
TCG_01901	putative protein transport protein Sec31
TCG_02191	putative eukaryotic translation initiation factor 4 gamma
TCG_02210	hypothetical protein
TCG_02218	hypothetical protein
TCG_02223	WD domain-containing protein
TCG_02343	hypothetical protein
TCG_02520	ribonucleoprotein p18
TCG_02905	hypothetical protein
TCG_03044	hypothetical protein
TCG_03180	flagellar associated protein
TCG_03277	hypothetical protein
TCG_03307	putative MCAK-like kinesin
TCG_03649	putative intraflagellar transport protein IFT88
TCG_03736	hypothetical protein
TCG_04215	Intraflagellar Transport Protein 140
TCG_04216	putative leucine-rich repeat protein (LRRP)
TCG_04234	vacuolar protein 8
TCG_04411	hypothetical protein
TCG_04957	putative eukaryotic translation initiation factor 5
TCG_05035	putative immunodominant antigen, putative,tc40 antigen-like
TCG_05904	pf20-like protein
TCG_05911	Protein XRP2
TCG_06028	putative vacuolar protein sorting complex subunit
TCG_06068	hypothetical protein
TCG_06486	nuclear protein Tc22

TCG_06664	putative leucine-rich repeat protein
TCG_07254	flagellar associated protein
TCG_07389	flagellar inner dynein arm I1 intermediate chain IC140
TCG_07390	programmed cell death 6-interacting protein
TCG_07555	hypothetical protein
TCG_07773	tetratricopeptide repeat protein 21B isoform a
TCG_07868	intraflagellar transport 172-like protein
TCG_07891	hypothetical protein
TCG_08168	putative vacuolar protein sorting-associated protein 41
TCG_08342	putative calreticulin
TCG_08642	WDdomain 60
TCG_08698	hypothetical protein
TCG_09148	hypothetical protein
DOWNREGULATED	
Protein kinase activity	
TCG_00063	putative mitogen-activated protein kinase, putative,kinase
TCG_00242	transferase
TCG_00884	putative serine/threonine protein kinase, putative,protein kinase
TCG_01098	putative protein kinase
TCG_01099	putative protein kinase
TCG_01102	putative protein kinase
TCG_01148	putative protein kinase, putative,serine/threonine protein kinase
TCG_01179	putative protein kinase
TCG_02217	putative protein kinase
TCG_02514	putative serine/threonine protein kinase, putative,protein kinase
TCG_03076	casein kinase II, alpha chain
TCG_03170	putative protein kinase
TCG_04057	putative protein kinase
TCG_04220	putative protein kinase, putative,serine/threonine-protein kinase Nek1

TCG_04236	putative protein kinase, putative,serine/threonine protein kinase
TCG_05071	putative serine/threonine protein kinase
TCG_05295	putative protein kinase
TCG_06077	putative serine/threonine protein kinase, putative,protein kinase
TCG_06667	putative mitogen-activated protein kinase
TCG_07418	putative protein kinase
TCG_08019	putative mitogen-activated protein kinase 3
TCG_08097	putative protein kinase
TCG_09911	putative protein kinase
DOWNREGULATED	
Metal ion binding	
TCG_00146	putative zinc finger protein
TCG_00953	S-adenosylmethionine synthetase
TCG_01116	putative RNA-binding protein
TCG_02076	putative reiske iron-sulfur protein precursor
TCG_02236	hypothetical protein
TCG_02241	endonuclease G
TCG_02474	hypothetical protein
TCG_02486	putative zinc finger protein family member
TCG_02504	hypothetical protein
TCG_03111	zinc finger protein
TCG_03694	putative fructose-1,6-bisphosphatase, cytosolic
TCG_03750	putative cation transporting ATPase
TCG_05909	cytosolic aconitase
TCG_06183	hypothetical protein
TCG_08438	zinc finger protein family memeber
TCG_10161	putative FYVE, RhoGEF and PH domain-containing protein 2
DOWNREGULATED	
Nucleotide binding	
TCG_03399	putative seryl-tRNA synthetase
TCG_03700	P-ATPase family transporter: proton

TCG_03750	putative cation transporting ATPase
TCG_04024	sarcoplasmic/endoplasmic reticulum calcium ATPase 3
TCG_04183	isoleucine--tRNA ligase
TCG_05802	putative phospholipid-translocating P-type ATPase (flippase)
TCG_06042	uncharacterized protein
TCG_06214	putative methionyl-tRNA synthetase
TCG_06609	succinyl-CoA synthetase alpha subunit
TCG_13098	putative P-type H+-ATPase
DOWNREGULATED	
Protein heterodimerization activity	
TCG_01152	histone H3 variant
TCG_03830	histone H2A
TCG_03832	histone H2A
TCG_05567	histone H2A
TCG_08085	histone H2B
DOWNREGULATED	
Calmodulin binding	
TCG_02132	paraflagellar rod protein 2C
TCG_05176	paraflagellar rod component
TCG_06311	putative paraflagellar rod protein 1D

Supplementary Table 6: Molecular functions transcripts enriched in Y strain TcP21-/- intracellular amastigotes

ID	*DESCRIPTION*
UPREGULATED	
ATP binding	
TCG_00254	putative ABC transporter
TCG_00273	T-complex protein 1 subunit epsilon
TCG_00603	putative structural maintenance of chromosome (SMC) family protein
TCG_00825	putative protein kinase
TCG_00892	ATP-dependent DEAD/H RNA helicase
TCG_00907	putative 26S protease regulatory subunit
TCG_00947	putative protein kinase
TCG_00996	valyl-tRNA synthetase
TCG_01005	putative kinesin
TCG_01320	cytoplasmic dynein 2 heavy chain 1 isoform X1
TCG_01485	putative tryptophanyl-tRNA synthetase
TCG_01541	ATP-binding cassette protein subfamily A, member 10
TCG_01717	putative vesicular-fusion ATPase-like protein
TCG_02689	putative RNA editing associated helicase 2,putative
TCG_02715	ruvB-like 1
TCG_02742	ATP-binding cassette protein subfamily B, member 1
TCG_02833	putative protein kinase
TCG_03307	putative MCAK-like kinesin
TCG_03474	putative eukaryotic initiation factor 4a
TCG_03750	putative cation transporting ATPase
TCG_03916	putative nucleotide-binding protein
TCG_04057	putative protein kinase
TCG_04091	putative heat shock protein
TCG_04183	isoleucine--tRNA ligase
TCG_04349	putative ATP-dependent RNA helicase
TCG_04462	acetyl-CoA carboxylase
TCG_04548	topoisomerase
TCG_04791	cell division control protein 48-like protein E
TCG_04899	putative RNA helicase

TCG_05481	mitochondrial ATP-dependent zinc metallopeptidase
TCG_05829	putative DNA ligase
TCG_05830	DNA ligase
TCG_06042	uncharacterized protein
TCG_06182	putative arginyl-tRNA synthetase
TCG_07033	putative T-complex protein 1, delta subunit
TCG_07402	putative phenylalanyl-tRNA synthetase
TCG_07411	putative mismatch repair protein MSH2
TCG_07417	ATP-binding cassette protein subfamily F, member 3
TCG_07445	ATP-binding cassette protein subfamily F, member 1
TCG_07604	uncharacterized protein
TCG_07874	putative kinesin
TCG_07879	putative structural maintenance of chromosome protein 4
TCG_08098	putative structural maintenance of chromosome (SMC)
UPREGULATED	
Nucleotide binding	
TCG_00996	valyl-tRNA synthetase
TCG_01485	putative tryptophanyl-tRNA synthetase
TCG_03750	putative cation transporting ATPase
TCG_04183	isoleucine--tRNA ligase
TCG_04663	DNA polymerase epsilon catalytic subunit A
TCG_05256	putative calcium-transporting ATPase
TCG_05402	putative calcium motive p-type ATPase
TCG_06042	uncharacterized protein
TCG_06182	putative arginyl-tRNA synthetase
TCG_07200	glutamate dehydrogenase
TCG_07402	putative phenylalanyl-tRNA synthetase
UPREGULATED	
Oxidoreductase activity	
TCG_01027	putative aldehyde dehydrogenase
TCG_03914	oxidoreductase
TCG_03941	hypothetical protein

TCG_04256	alkyldihydroxyacetonephosphate synthase
TCG_06131	putative ribonucleoside-diphosphate reductase small chain
TCG_07200	glutamate dehydrogenase
TCG_08073	glutamate dehydrogenase
TCG_08077	tryparedoxin peroxidase
TCG_11294	trifunctional enzyme alpha subunit, mitochondrial precursor-like protein
UPREGULATED	
ATP hydrolysis activity	
TCG_00907	putative 26S protease regulatory subunit
TCG_01541	ATP-binding cassette protein subfamily A, member 10
TCG_01717	putative vesicular-fusion ATPase-like protein
TCG_02742	ATP-binding cassette protein subfamily B, member 1
TCG_03750	putative cation transporting ATPase
TCG_04791	cell division control protein 48-like protein E
TCG_05481	mitochondrial ATP-dependent zinc metallopeptidase
TCG_07417	ATP-binding cassette protein subfamily F, member 3
TCG_07445	ATP-binding cassette protein subfamily F, member 1
UPREGULATED	
Aminoacyl-trna ligase activity	
TCG_00996	valyl-tRNA synthetase
TCG_01485	putative tryptophanyl-tRNA synthetase
TCG_04183	isoleucine--tRNA ligase
TCG_06042	uncharacterized protein
TCG_06182	putative arginyl-tRNA synthetase
UPREGULATED	
Aminoacyl-trna editing activity	
TCG_00996	valyl-tRNA synthetase
TCG_04183	isoleucine--tRNA ligase
TCG_06042	uncharacterized protein
UPREGULATED	
Structural constituent of nuclear pore	

TCG_04832	putative nuclear pore complex protein (NUP155)
TCG_05048	putative ATP-dependent RNA helicase
TCG_06764	hypothetical protein
DOWNREGULATED	
Structural constituent of ribosome	
TCG_00575	60S ribosomal subunit protein L31
TCG_00916	60S acidic ribosomal protein P2
TCG_00931	60S acidic ribosomal protein P2 beta (H6.4)
TCG_01077	60S ribosomal protein L17
TCG_01080	putative 40S ribosomal protein S2
TCG_01091	putative 40S ribosomal protein S2
TCG_01258	small subunit ribosomal protein S9e
TCG_01290	40S ribosomal protein S18
TCG_01628	putative 60S ribosomal protein L23a
TCG_01758	40S ribosomal protein S17
TCG_01858	40S ribosomal protein S21
TCG_02092	60S ribosomal protein L26
TCG_02464	ubiquitin/ribosomal protein S27a
TCG_02870	putative 60S ribosomal protein L4
TCG_03508	putative ribosomal protein S7
TCG_03960	60S ribosomal protein
TCG_04156	60S ribosomal protein L2
TCG_04512	ubiquitin/ribosomal protein S27a
TCG_04538	60S acidic ribosomal protein P2
TCG_04928	60S ribosomal protein L32
TCG_04979	ribosomal protein S26
TCG_05410	40S ribosomal protein S6
TCG_05510	60S ribosomal protein L13a
TCG_05529	60S ribosomal protein L26
TCG_06155	polyubiquitin
TCG_06224	ribosomal proteins L36
TCG_06395	40S ribosomal protein S15
TCG_06732	40S ribosomal protein L14
TCG_07214	60S ribosomal protein L35
TCG_07781	60S ribosomal protein L11
TCG_08004	putative 60S ribosomal protein L2
TCG_08072	60S ribosomal protein L6
TCG_08443	60S ribosomal protein L34

TCG_08967	60S ribosomal protein L2
TCG_09354	40S ribosomal protein SA
TCG_11208	60S ribosomal protein L34
TCG_12209	putative ribosomal protein L11
TCG_13465	40S ribosomal protein S8
TCG_13471	putative 40S ribosomal protein S23
DOWNREGULATED	
Metalloendopeptidase activity	
TCG_07731	surface protease GP63
TCG_07894	putative surface protease GP63
TCG_08211	surface protease GP63
TCG_08787	GP63 group II protein
TCG_08789	surface protease GP63
TCG_08836	surface protease GP63
TCG_08837	surface protease GP63
TCG_09033	putative surface protease GP63
TCG_09600	surface protease GP63
TCG_10132	putative surface protease GP63
TCG_11623	putative surface protease GP63
TCG_11823	putative surface protease GP63
TCG_12560	surface protease GP63
TCG_12563	surface protease GP63

4. DISCUSSÃO

A diversidade genética de *Trypanosoma cruzi* é o principal motor das variações fenotípicas observadas entre as Unidades Discretas de Tipagem (DTUs), refletindo-se diretamente na patogenicidade e no tropismo tecidual (Velásquez-Ortiz *et al.*, 2022). Em nosso estudo com as linhagens *knockou*t geradas por CRISPR/Cas9 nas cepas Y (TcII) e G (TcI) (Teixeira *et al.*, 2022), sugerimos que a P21 possui natureza pleiotrópica, exercendo diferentes funções moduladoras conforme o contexto genético da cepa.

Nos experimentos realizados com a cepa Y e G, observou-se que a ausência de P21 reduziu significativamente a taxa de invasão celular, configurando a proteína como um promotor de invasão celular *in vitro*. A trans-sialidase (TS), expressa e liberada em níveis significativos maiores na cepa Y do que na cepa G, é um fator conhecido por induzir alterações teciduais e processos inflamatórios correlacionando-se diretamente com a virulência *in vivo* (Risso *et al*., 2004).Além disso, a P21 em sua forma recombinante (rP21) apresenta um potencial indutor de polimerização da actina e fagocitose, mecanismos essenciais para a internalização em células não-fagocíticas (Martins *et al.*, 2020).

Na cepa Y, na ausência da P21, os parasitas apresentaram maior multiplicação intracelular e saída antecipada das células hospedeiras. Essa função de restrição da replicação mediada pela P21 é uma estratégia evolutiva que visa ao controle do parasita para evitar a eliminação precoce do hospedeiro por uma resposta imune exacerbada. Esse conceito de "controle" da replicação é corroborado por estudos que mostram a modulação da resposta imune por rP21, que induz maior expressão de IFN-γ e IL-4, bem como altos níveis de IL-10, sugerindo um mecanismo de imunomodulação que favorece a latência e a sobrevivência do hospedeiro ao longo prazo (Martins *et al.*, 2020). Já na cepa G, a P21 é necessária para sustentar a multiplicação basal e a persistência silenciosa *in vivo*. O fenótipo do parasita TcP21$^{-/-}$ nessa cepa se assemelha ao de um parasita deficiente em fatores essenciais de ciclo celular, sugerindo que a P21 pode estar envolvida em processos que asseguram a homeostase do amastigota intracelular, conceito que dialoga com a complexidade da heterogeneidade de amastigotas observada por estudos transcriptômicos (Li *et al*., 2016).

Do ponto de vista da persistência e da resposta terapêutica, os resultados reforçam uma hipótese funcional relevante: P21 poderia contribuir para manter populações intracelulares em um estado de multiplicação basal ou controlada, favorecendo a cronificação com baixa parasitemia e reduzir dano agudo ao hospedeiro, uma estratégia adaptativa que, ao mesmo tempo, tornaria os parasitas menos visíveis ao sistema imune e potencialmente mais resistente a tratamentos. Essa interpretação encontra suporte em estudos que descrevem formas dormentes de *T. cruzi* resistentes a tratamento e em trabalhos que apontam diferenças entre linhagens na capacidade de gerar células dormentes. A existência desse estado de dormência ainda se encontra em debate, mas é provável que a P21 atue em conjunto com

mecanismos de recombinação e resposta ao estresse para modular a entrada ou saída desse estado (Resende *et al*., 2020; Sánchez-Valdéz *et al*., 2018).

A regulação contraria de vias molecular (tradução/síntese proteica) demostrada pela análise transcriptômica entre as cepas TcP21$^{-/-}$ Y e G enfatiza que a P21 é um ponto de convergência regulatório que sustente a sobrevivência do parasita por rotas distintas, dependendo do genótipo. Outros fatores de virulência, como as Mucinas e Maps, também são expressos diferencialmente entre as DTUs e influenciam o tropismo tecidual e a evasão imune (Ferri e Edreira, 2021). A P21 se insere neste grupo de moléculas críticas, mas com um papel regulatório que parece influenciar o sucesso da infecção em um nível mais fundamental.

Embora as atividades biológicas sejam diferentes, os estudos sugerem que ambas as funções servem ao mesmo propósito final: a sobrevivência e perpetuação do parasita no hospedeiro. Em conjunto, os dados indicam que a P21 é uma proteína multifuncional com papel modulador da virulência, cuja função varia conforme o contexto genético da cepa. Enquanto na cepa G ela favorece a persistência tecidual, na cepa Y exerce papel homeostático, limitando a replicação. Esses achados reforçam a importância da P21 na adaptação de *T. cruzi* ao hospedeiro, além de apontar essa proteína com um potencial alvo de intervenção, desde que considerada a variabilidade entre cepas.

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6. ANEXOS

Ao longo do doutorado, tive a oportunidade de participar de diferentes projetos de pesquisa que resultaram em publicações científicas. Esses trabalhos não apenas ampliaram meu entendimento sobre a biologia do *Trypanosoma cruzi*, como também me proporcionaram experiências valiosas no desenvolvimento experimental, na análise crítica de dados e na escrita científica.

Iniciei minha contribuição com o artigo "*Trypanosoma cruzi* infection induces proliferation and impairs migration of a human breast cancer cell line" (Experimental Parasitology, 2023). Este trabalho foi de grande importância, pois permitiu investigar a complexa interação entre o *Trypanosoma cruzi* e células de câncer de mama, demonstrando que a infecção pelo parasita foi capaz de induzir a proliferação e, ao mesmo tempo, prejudicar a migração dessas células tumorais. Posteriormente, contribuí com o artigo de revisão "Subversion strategies of lysosomal killing by intracellular pathogens" (Microbiological Research, 2023). Esta colaboração permitiu um aprofundamento nos mecanismos de evasão utilizados por diferentes patógenos intracelulares, como bactérias, fungos e protozoários, para escapar da destruição pelos lisossomos da célula hospedeira, um aspecto central da sobrevivência parasitária.

Por fim, participei também da revisão "Cellular dormancy: A widespread phenomenon that perpetuates infectious diseases" (Journal of Basic Microbiology, 2024). Este estudo abordou como a dormência celular em microrganismos, incluindo protozoários, é um fator determinante para a persistência de doenças infecciosas e para o fracasso de terapias.

Esses três trabalhos representam marcos importantes na minha trajetória acadêmica, cada um deles trazendo aprendizados que ultrapassam os resultados científicos. Por meio deles, aprendi a valorizar o rigor experimental, o trabalho colaborativo e a importância da persistência diante dos desafios da pesquisa. Cada projeto foi uma etapa de crescimento científico, técnico e pessoa que consolidou minha identidade como pesquisadora.

Em conjunto, a participação nestes projetos foi essencial para o desenvolvimento de novas competências técnicas, para o aprimoramento da análise crítica e para a consolidação das bases metodológicas e conceituais que fundamentam a presente tese.

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Trypanosoma cruzi infection induces proliferation and impairs migration of a human breast cancer cell line

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ABSTRACT

Breast cancer is considered the type of cancer that most affects women in the world. The triple negative breast cancer is considered aggressive with poor prognosis. In the 1930s Russian researchers observed that *T. cruzi* has tropism for tumor cells. Since then, this research field has been subject of a numerous of researches. Here, we proposed to investigate the impact of *T. cruzi* infection on proliferation and migration of triple negative breast cancer cell line (MDA-MB-231). *T. cruzi* showed high invasion and multiplication rate in MDA-MB-231 cell line. The infection promoted the multiplication of MDA-MB-231 cell, continuous cell lysis throughout of days of *in vitro* infection and impaired MDA-MB-231 cell migration. Taken together, these results demonstrated the high susceptibility of MDA-MB-231 cell to *T. cruzi* and suggested that molecules from *T. cruzi* may impair host cell migration with potential use to avoid metastasis.

1. Introduction

Trypanosoma cruzi, agent of Chagas disease, possesses anticancer activities. This proposal was first demonstrated by Soviet researchers during the years of 1930 when they observed that the parasite showed tropism to tumor cells(Roskin and Exempliarskaia, 1931). In 1946, Nina Kliueva and Grigorii Roskin discovered that the use of a "toxic substance" secreted by the parasite had a biotherapeutic effect in the treatment of carcinomas in mice (Krementsov, 2009). Moreover, different research groups have proposed that the immune response against *T. cruzi* cross-reacts against tumor cells. These results suggested the potential use of parasite proteins for anti-cancer protection (Cabral, 2000; Kallinikova et al.,n.d.; López et al., 2010; Oliveira et al., 2001; Ramírez et al., 2012; Sheklakova et al., 2003; Zhigunova et al., 2013).

Multiple parasite molecules and mechanisms are involved in the tumor resistance mediated by T. cruzi infection. The recombinant form of GP82 protein, specific for the metacyclic trypomastigote form of *T. cruzi*, is capable of inducing apoptosis of melanoma cells *in vitro* and reducing tumors *in vivo* (Atayde et al., 2008). It was also demonstrated that the molecular chaperone calreticulin from *T. cruzi* (TcCRT) is able to translocate to the parasite plasma membrane and inhibit the complement system activation cascades, favoring the infection. In addition, calreticulin has a fragment in the n-terminal portion, called vasostatin, which is capable of preventing the binding of endothelial cells to the extracellular matrix. This characteristic guarantees calreticulin an antiangiogenic activity (López et al., 2010; Peña Álvarez et al., 2020; Ramírez-Toloza et al., 2016, 2020; Ramírez et al., 2012). *T. cruzi* P21 protein is secreted by the parasite and plays an important role in cell invasion by the pathogen (da Silva et al., 2009). Using the recombinant form of protein P21 (rP21) it was possible to determine several biological properties potentially performed by its native form. In this context, rP21 has chemotactic activity for leukocytes, interacts with the chemokine receptor CXCR4 (Rodrigues et al., 2012), has anti-angiogenic activity (Teixeira et al., 2017) and prevents the invasion of triple negative (TN) breast tumor cells(Borges et al., 2020).

Cellular proliferation in a hallmark in cancer spreading. The impact of *T. cruzi* infection on host cell multiplication has already been investigated highlighting conflicting results. In this sense, authors found that

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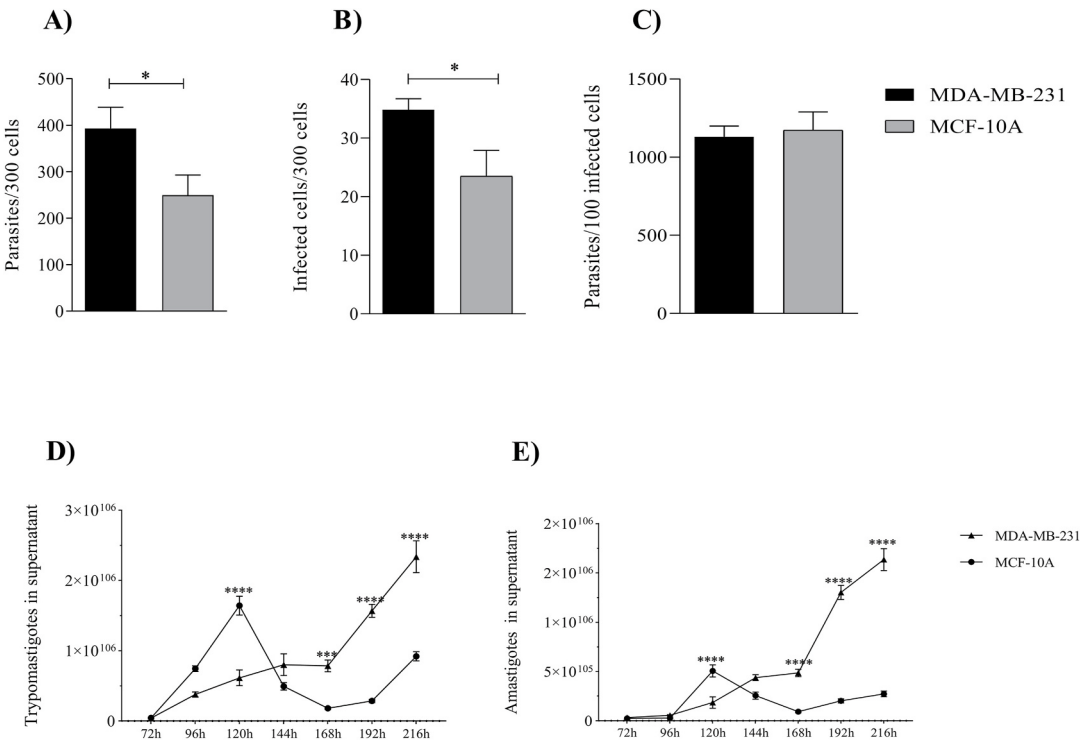

Fig. 1. MDA-MB-231 cell is more susceptible to *T. cruzi* infection than MCF-10A. A: *T. cruzi* was able to invade more MDA-MB-231 cells than MCF-10 A. B: *T. cruzi* invaded higher number of MDA-MB-231 cell. C: *T. cruzi* multiplied in MDA-MB-231 and MCF-10 A in a similar extent. D, E: The number of trypomastigote and amastigote in the supernatant of MCF10-A was higher in the initial time points of collection. However, this number was superior in MDA-MB-231 at later time points. *p < 0.05; ****p < 0.0001.

T. cruzi infection causes a block in the host cell cycle, at the level of cytokinesis (Costales et al., 2009). However, other authors have shown that *T. cruzi* infection induces the expression of Cyclin D1 by the host cell (Bouzahzah et al., 2008), promotes the proliferation of a human trophoblast cell line (Droguett et al., 2017) and induces the proliferation of vascular smooth cells (Hassan et al., 2006). Considering cancer cells, authors demonstrated that the recombinant form of TcCRT had the ability to inhibit endothelial cell proliferation and that translocated/externalized natural TcCRT was responsible for at least an important part of the anti mammary tumor effect during experimental infection with *T. cruzi* (Abello-Cáceres et al., 2016).

Breast cancer is annually diagnosed in more than 2.1 million women, with a rate of 650,000 deaths, being considered as the type of cancer that most affects women in the world (Vafaizadeh et al., 2020). It is classified into four divisions according to its molecular, diagnostic and treatment particularities, being categorized as Luminal A, Luminal B, HER2 and TN (Ma et al., 2011). TN is considered aggressive and has a poor prognosis. It does not have estrogen or progesterone receptors and does not have an increase in the HER2 protein. This becomes an obstacle in diagnosis, as these receptors are fundamental structures in tumor detection, in addition to being targets for drug therapies (Medina et al., 2020).

CXCR4 and its ligand CXCL12 can promote the proliferation, survival, and invasion of cancer cells (Uygur and Wu, 2011; Wald et al., 2013; Wang et al. n.d.) They have been shown to play an important role in regulating metastasis of breast cancer to specific organs. High CXCR4 expression was also correlated to poor clinical outcome (Mirisola et al., 2009; Müller et al., 2001; Prat and Perou, 2011; Richmond et al., 2004) Authors findings underlined that the CXCL12-CXCR4 axis can increase the invasion and apoptosis of MDA-MB-231 simultaneously. These data strongly support the hypothesis that CXCL12-CXCR4 axis promotes the natural selection of breast cancer cell metastasis (Sun et al., 2014).

In this context, this study aimed to evaluate the susceptibility of TN breast tumor cell line (MDA-MB-231) to *T. cruzi* infection and the impact on host cell proliferation and migration.

2. Material and methods

2.1. Cell culture

Non-tumorigenic human breast cells (MCF-10 A) and human triple-negative breast tumoral cells (MDA-MB-231) were purchased from Banco de Células do Rio de Janeiro. MCF-10 A cells were cultivated in Dulbecco's modified Eagle's/Ham's Nutrient Mixture F12 (DMEM/F12; Life Technologies, Carlsbad, CA, United States) supplemented with epidermal factor growth (20 ng/ml), insulin from bovine pancreas (10 μg/ml), hydrocortisone (0.5 μg/ml), and 5% fetal bovine serum (FBS). MDA-MB-231 cells were cultivated in DMEM medium (Sigma-Aldrich, MO, United States) supplemented with 10% FBS and 2 mM sodium bicarbonate. Vero cell line was purchased from Banco de Células do Rio de Janeiro and was used to maintain *in vitro T. cruzi* infection. Here we used *T. cruzi* Y strain that was isolated from a young patient in 1953 at Hospital de Clínicas de São Paulo, Brazil (Neto, 2010). The supernatants of Vero cell containing tissue culture trypomastigotes from Y strain of *T. cruzi* was used during the experimental procedures involving cell infection. Cells were maintained with 100 U/ml penicillin and 100 μg/ml streptomycin. Incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Parasite cell invasion, intracellular multiplication and supernatant release

In order to determine the ability of *T. cruzi* to invade host cell, 1×10^5 MCF-10 A and MDA-MB-231 cell lines were seeded in a 24 wells culture plaque. After 24 h of incubation, tissue culture trypomastigotes from Y strain of *T. cruzi* were put to invade cell in a proportion of 20 parasites/cell. After 2 h of infection, cells were washed with PBS, fixed with bouin and Giemsa stained. The number of invading parasites and the number of infected cells were determined in 300 total cells. To determine intracellular multiplication, coverslips were washed with PBS

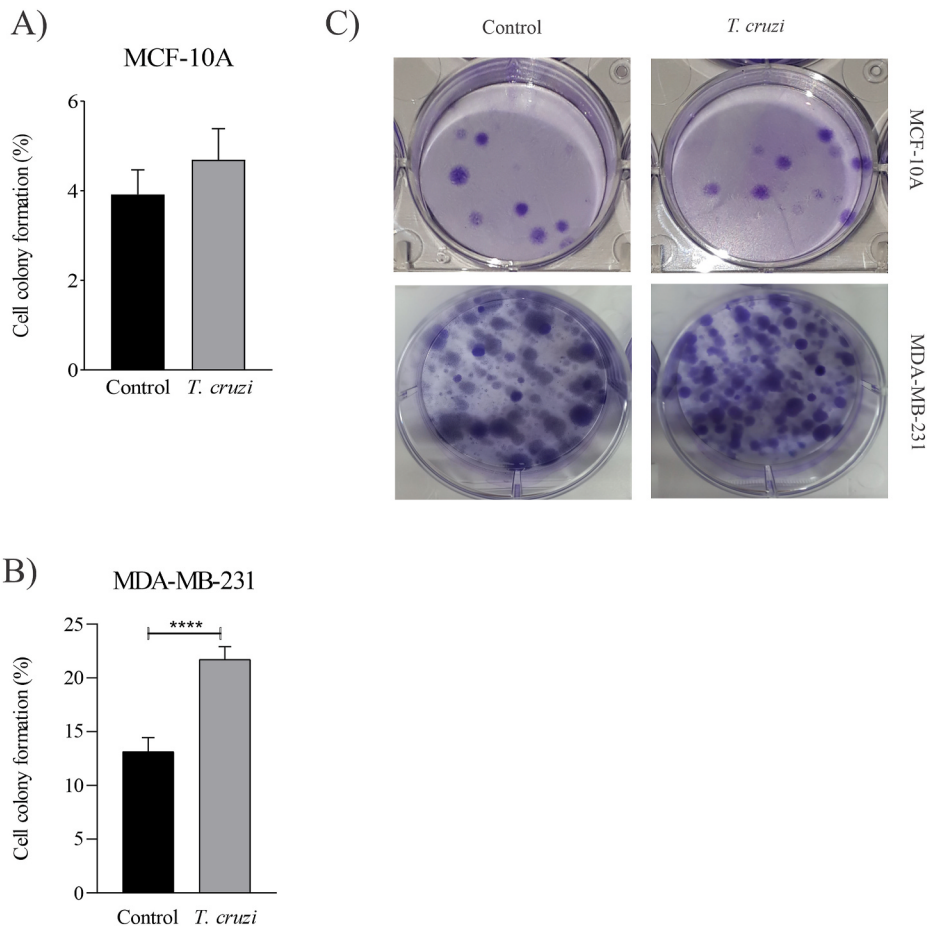

Fig. 2. *T. cruzi* infection increased the clonogenic ability of MDA-MB-231 cell line. A: *T. cruzi* infection did not alter the percentage of colony formation of MCF-10 A cell line: B: *T. cruzi* infection promoted the colony formation of MDA-MB-231 cell line. C: representative images of colony formation for both cell lines and controls. ****p < 0.0001.

after 2 h of invasion process and complete medium was replaced. Coverslips were bouin fixed and Giemsa stained after 72 h post-infection. The number of intracellular parasites per 100 infected cells was determined. In order to determine the kinetic of parasites free in the cytosol of infected cells, both cell lines were infected for 2 h. After, cells were washed with PBS and complete medium was replaced. At the time point of 72 h to the 216 h post-infection the number of parasites in the supernatant was determined in neubauer chamber.

2.3. Clonogenic assay

In order to evaluate if invasion interferes in cells proliferation, 1 × 10^5 cells MCF-10 A and MDA-MB-231 were plated in 24-well culture plates. Next day, the cells were incubated with *T. cruzi* trypomastigotes forms in the proportion of 10 parasites/cell during 2 h. Besides that, the cells were collected by enzymatic digestion (Trypsin/EDTA), centrifuged at 1500 rpm for 5 min and seeded (200 cells/well) in 6-well plates containing complete medium for 15 days. Every 5 days, medium was changed. Finally, the colonies were fixed with acetone and metanol (1:1) for 30 min, stained with crystal violet 0.25% *overnight*. Only the colonies with >50 cells were counted by direct visual inspection. To calculate colony formation, the number of colonies formed/number of cells seeded × 100%.

2.4. Mitotic index

Control and infected MDA-MB-231 and MCF-10 A cell lines were fixed with 4% formaldehyde for 1 h, washed three times with PBS. After, cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 15 min and after washes, slides were mounted using 1,2-phenylenediamine (PPD) and analyzed by confocal microscopy. The mitotic figures were identified, and at least 500 cells per condition were counted. The mitotic index was determined using the following formula: mitotic index ¼ (M/N) * 100, where "M" corresponds to the sum of the cells in the M phase of the cell cycle, and "N" corresponds to the total number of cells (Baak et al., 2009).

2.5. Migration assay

Migration assay was performed using transwells with 8 μm pores (Costar, Corning, USA). The upper chamber contained cells in the culture medium (1 × 10^5/mL) and the lower chamber contained the culture medium (negative control) or 20 ng/mL CXCL12. The CXCL12 concentration of use was previously determined (Borges et al., 2020). Cells were infected during 2 h with *T. cruzi* and added to the upper chamber. Cells were incubated for 6 h at 37 °C in 5% CO2. Non-migrated cells were removed from the upper surface of the membrane with a cotton swab and the migrated cells remaining on the lower surface were counted after staining with 0.5% crystal violet. Cell counts were performed using a Leica DM 500 microscope at 10× magnification. The images were used to count the number of cells using ImageJ software.

2.6. CXCR4 subcellular distribution and T. cruzi infection kinetic

In order to determine the subcellular distribution of CXCR4 receptor during kinetic of *T. cruzi* infection, 1 × 10^5 MDA-MB-231 cells were

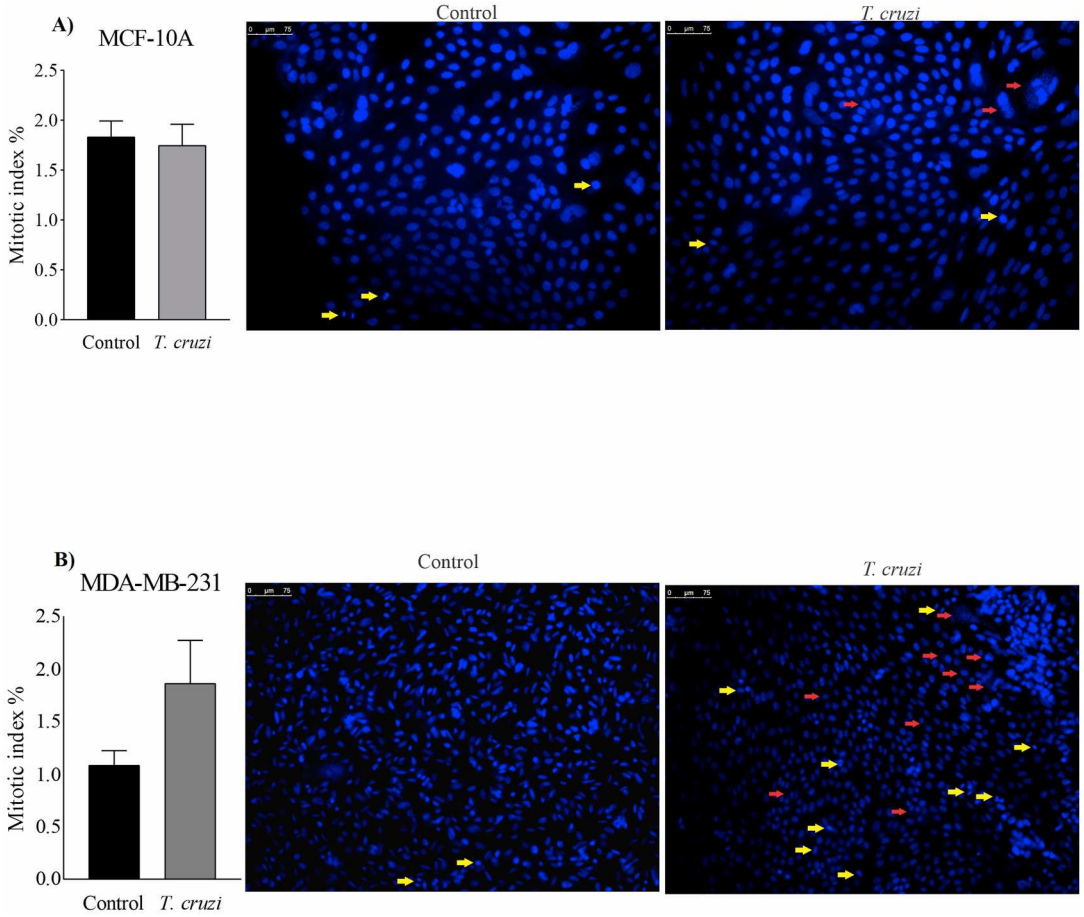

Fig. 3. The number of mitotic cells showed a tendency to be higher in MDA-MB-231 cell line. The mitotic index of MCF-10 A (A) and MDA-MB-231 (B) cell lines was determined in infected and non-infected cells by staining nuclei with DAPI and analysis in confocal microscope. Representative DAPI stained cells are shown. Yellow arrows indicate mitotic cell figures and red arrows indicate the presence of the parasite.

seeded in 24-well coverslips. Next, the cells were incubated with *T. cruzi* trypomastigotes forms in the proportion of 10 parasites/cell during 1 h. At the time points of 3 and 6 h post-infection, coverslips were washed with PBS, and fixed with 4% formaldehyde for 1 h and washed three times with PBS. Then, cells were permeabilized and blocked with PGN plus saponin and labeled with rabbit anti-CXCR4 antibody (SIGMA ALDRICH diluted 1:100) overnight at 4 °C. Next, cells were washed with PBS, incubated with goat anti-rabbit IgG conjugated with PE (One Lambda diluted 1:200) and 4',6-diamidino-2-phenylindole (DAPI). After washes, slides were mounted using 1,2-phenylenediamine (PPD) and analyzed by confocal microscopy.

2.7. Statistical analysis

All data are presented as the mean ± standard error of the mean of experiments performed at least three times in triplicate. All data were first checked for normal distribution. Significant differences were determined by one-way ANOVA, Tukey's multiple comparisons test, and Student's *t*-test (two-sided) for parametric data or the Mann–Whitney test for non-parametric data according to the experimental design. $P < 0.05$ was considered significant. All the statistical analyses were performed using GraphPad Prism software version 8.0.

3. Results and discussion

To assess the susceptibility of MDA-MB-231 tumor cell line to *T. cruzi* infection, we performed cell invasion and intracellular multiplication assays of the parasite in comparison to the infection in the non-tumor cell line, MCF-10 A. The results showed that *T. cruzi* had a higher rate of cell invasion in MDA-MB-231 cell line (393.2 ± 45.27) than in MCF-10 A (249.7 ± 43.07), $P < 0.05$. Moreover, parasites infected a greater number of cells in MDA-MB-231 cell line (34.83 ± 1.87) when compared to MCF-10 A cell line (23.5 ± 4.41), $P < 0.05$ (Fig. 1A and B). In this context, we point out that the literature provides data that demonstrate the high susceptibility of tumor cell lines to *T. cruzi* infection (Vargas-Zambrano et al., 2013). Conversely, the rate of *T. cruzi* infection in non-tumor cell lines such as Vero cells (Pires et al., 2008) and BESM (bovine muscle) cells is lower (DVORAK and HOWE, 1976). Although *T. cruzi* showed a higher rate of invasion in MDA-MB-231 cells, intracellular multiplication assays showed that the parasite multiplied up to 72 h post-infection, similarly in MDA-MB-231 (1131 ± 68.58) and MCF-10 A (1174 ± 115.5) cell lines (Fig. 1C).

Subsequently, we investigated the release of parasites to the supernatant of infected cells. For this, we collected the supernatant during nine days post-infection and counted the number of trypomastigotes and amastigotes in the supernatant. The presence of amastigote in the supernatant is indicative of premature lysis of the infected cell, since the amastigote form is replicative and differentiates into trypomastigote form, which in turn ruptures the plasma membrane of the infected cell. The results showed that at 120 h post-infection, MCF-10 A cell showed a peak of trypomastigote (mean: 1.6×10^6) and amastigote (mean: 5×10^5) forms in the supernatant. However, this number decreased significantly in subsequent time points. This result suggests that as MCF-10 A cell had a lower infection rate, the parasites that were released within 120 h infected new cells and resumed the cycle, which could explain the decrease in free parasites in the supernatant. MDA-MB-231 cell showed

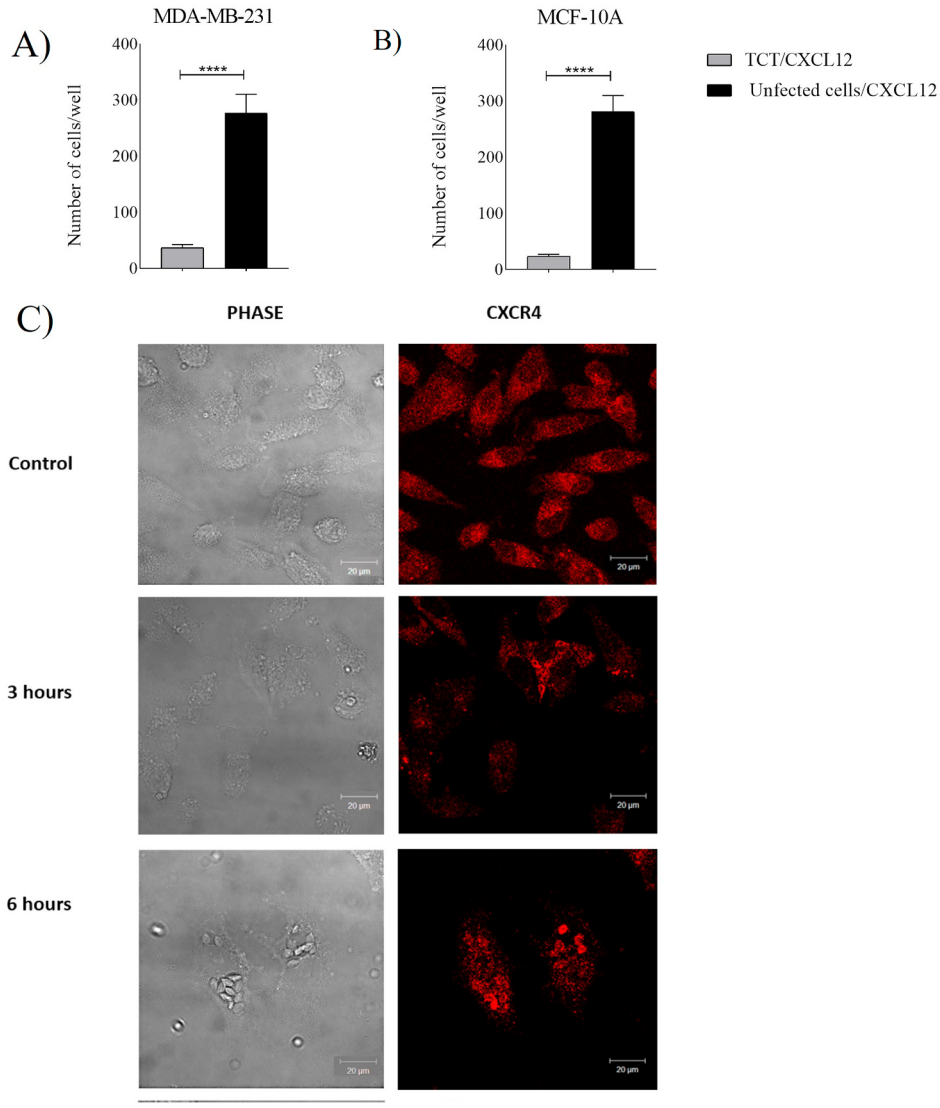

Fig. 4. Infection by *T. cruzi* impaired migration of both MDA-MB-231 and MCF-10A cell lines by subcellular redistribution of CXCR4 receptor. A, B: MDA-MB-231 and MCF-10 A cell lines infected by *T. cruzi* showed lower migration rate than non-infected controls in transwell stimulated with CXCL-12 chemokine. C: representative images of CXCR4 redistribution (red staining) upon infection by *T. cruzi* at the time points of 3 and 6 h post-infection. ****p < 0.0001. TCT: Tissue Culture Trypomastigote.

a significant increase in the number of trypomastigotes and amastigotes in the supernatant starting at 168 h post-infection and with a peak at the time of 216 h post-infection. Surprisingly, at the time of 216 h, the number of amastigote in the supernatant (mean: 1.6×10^6) was high as the number of trypomastigote (mean: 2.3×10^6), suggesting that MDA-MB-231 cells do not withstand infection and undergo premature lysis (Fig. 1D and E). Supplementary Video 1 (MCF-10 A) and 2 (MDA-MB-231) show the infection at the time point of 216 h. We can observe that MCF-10 A cells present in a confluent form, with most of the infected cells harboring mainly trypomastigote (motile forms). In the supernatant, there is a reduced number of trypomastigote and amastigote forms (round shaped forms). Conversely, MDA-MB-231 cells are semi-confluent, infected mostly by amastigote forms and in the supernatant there is a large amount of trypomastigote and amastigote. Many dead cells are also seen in the cell culture supernatant. As MDA-MB-231 cells showed greater susceptibility to infection with the premature release of amastigote forms, it was expected that at 216 h post-infection there would be few cells on the coverslips and not semi-confluent cells infected with amastigote forms in replication. In this context, *T. cruzi* infection can alter different genes expression in the host cell, as cell cycle regulators (Li et al., 2016). This fact may be one of the possible explanations for the different behavior of infection between these cell types and for the persistence of MDA-MB-231 monolayer during nine days post-infection. Therefore, we performed clonogenic assays to assess the impact of *T. cruzi* infection on the multiplication capacity of both cell lines. We observed that *T. cruzi* infection increased the clonogenic capacity (multiplication ability) of MDA-MB-231 cells. This assay showed that non-infected MCF-10 A cell had a colony formation percentage of 3.9 ± 0.5 and infected group displayed a range of 4.7 ± 0.7. Conversely, non-infected MDA-MB-231 cells had 13.1 ± 1.3 percentage of cell colony formation, while this value was 21.7 ± 1.2 for infected cells, $P < 0.0001$ (Fig. 2A, B and C). Thus, we believe that the infection promotes mitosis of MDA-MB-231 cells. In this sense, growing cells are infected by the parasites from the supernatant establishing a cycle of cell multiplication, infection and premature lysis that culminates in the release of a large amount of parasites to the supernatant. For MCF-10 A cells, the infection did not change the clonogenic ability, which even in uninfected cells was low when compared to MDA-MB-231. To confirm the results of the clonogenic assays, we performed experiments to determine the mitotic index of both cell lines infected or not infected. Although a tendency of larger amounts of mitotic figures was observed for infected MDA-MB-231 cell line (1.9 ± 0.4), no statistically significant difference was observed compared to non-infected control cell (1.1 ± 0.1). Considering MCF-10 A cell line similar amounts of mitotic figures were observed when cell were infected or not with *T. cruzi* (Fig. 3A, B and C).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.exppara.2022.108443

The impact of *T. cruzi* infection on host cell multiplication has

already been investigated in the literature gathering conflicting results (Abello-Cáceres et al., 2016; Bouzahzah et al., 2008; Costales et al., 2009; Droguett et al., 2017; Hassan et al., 2006). The comprehension of the mechanism associated to induction of tumor cell line proliferation by *T. cruzi* is intriguing and deserves further investigations. We cannot rule out that the malignant phenotype of MDA-MB-231 may have some impact on promoting the multiplication of infected cells.

The induction of cell proliferation by *T. cruzi* led us to question the impact of infection on the migration of host cells. The CXCR4-CXCL12 axis is of great importance in tumor cell migration (Teicher and Fricker, 2010). They have been shown to play an important role in regulating metastasis of breast cancer to specific organs, they are correlated to poor clinical outcome and promote the natural selection of breast cancer cell metastasis (Mirisola et al., 2009; Müller et al., 2001; Prat and Perou, 2011; Richmond et al., 2004; Sun et al., 2014). Thus, we evaluated the vertical migration of cells during 6 h post-infection towards a gradient containing the chemokine CXCL12. The results showed that both MCF-10 A and MDA-MB-231 cells infected by *T. cruzi* migrated less when compared to uninfected controls. We observed that the median and standard error of the number of migrated cells from MCF-10 A unifected group was 280.7 ± 29.0 and from infected group was 23.4 ± 3.8, $P < 0.0001$. For MDA-MB-231 cell line, the median in control group was 276.8 ± 33.5, while in infected group, it was 36.7 ± 5.7, $P < 0.0001$ (Fig. 4A and B). This result suggests that the infection may cause some change in the subcellular location of the CXCR4 receptor. To check this hypothesis, we performed cell invasion by *T. cruzi* kinetics and immunofluorescence staining to determine the subcellular distribution of CXCR4 receptor on infected MDA-MB-231 cell. The results showed accumulation of the receptor around the parasitophorous vacuole of the parasite (Fig. 4C). We believe that the process of cell invasion by *T. cruzi* recruited CXCR4 receptor to the nascent parasitophorous vacuole. Thus, CXCR4 would be unavailable to respond to the stimulus promoted by the chemokine CXCL12 during the 6 h of cell migration.

4. Conclusion

We concluded that *T. cruzi* highly infected and multiplied in MDA-MB-231 cell line. The infection contributed to the perpetuation of a cycle of MDA-MB-231 cell proliferation, parasite cell invasion, continuous infected cell lysis and impaired host cell migration.

Declaration of competing interest

Authors declare that no conflict of interest exists. The manuscript has not been published and is not under consideration elsewhere.

Data availability

Data will be made available on request.

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Subversion strategies of lysosomal killing by intracellular pathogens

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ABSTRACT

Many pathogenic organisms need to reach either an intracellular compartment or the cytoplasm of a target cell for their survival, replication or immune system evasion. Intracellular pathogens frequently penetrate into the cell through the endocytic and phagocytic pathways (clathrin-mediated endocytosis, phagocytosis and macropinocytosis) that culminates in fusion with lysosomes. However, several mechanisms are triggered by pathogenic microorganisms – protozoan, bacteria, virus and fungus - to avoid destruction by lysosome fusion, such as rupture of the phagosome and thereby release into the cytoplasm, avoidance of autophagy, delaying in both phagolysosome biogenesis and phagosomal maturation and survival/replication inside the phagolysosome. Here we reviewed the main data dealing with phagosome maturation and evasion from lysosomal killing by different bacteria, protozoa, fungi and virus.

Phagocytosis is an essential process by which specialized cells engulf invading pathogens and is probably the most important mechanism of foreign particles uptake. This process triggers the activation of multiple transmembrane signaling pathways that lead to the formation of a phagosome. This vesicle undergoes a maturation process, associated with alterations of the surrounding membrane and vacuolar content by multiple transient interactions with endosomal compartments, including lysosomes, ultimately leading to the formation of a specialized organelle: the phagolysosome (Desjardins, 1995; Roche and Furuta, 2015).

Phagosomes are not mere membrane bags around a particle. They are organelles with a mission, and their contents are expected to reflect this and to influence their destiny. A phagosome is formed when the phagocyte wraps a portion of its plasma membrane around the particle, followed by plasma membrane fusion at the tip of the particle and ingestion of the newly produced membrane bag containing the particle. The process of ingestion in most cases follows the 'zipper mode', i.e. particle-ligand macrophage–receptor interactions all around the particle

Abbreviations: HME, human monocytic ehrlichiosis; HE, human granulocytic ehrlichiosis; CD, cluster of differentiation; LAMP-1, lysosome-associated membrane protein 1; LAMP-2, lysosome-associated membrane protein 2; LAMP-3, lysosome-associated membrane protein 3; EEA1, early endosome antigen 1; M6PR, mannone-6-phosphate receptor; ECV, *Ehrlichia*-containing vacuole; TRP, tandem repeat protein; Etf, *Ehrlichia* translocated factor; LC3, microtubule-associated protein 1 light chain 3; TFEB, transcription factor EB; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; Inl, internalin; LLO, listeriolysin O; PLC, phospholipase C; PTF, pore-forming toxin; CDC, cholesterol-dependent cytolysin; LisCV, *Listeria*-containing vacuole; VBNC, viable but non-culturable; Steap 3, six-transmembrane ephitelial antigen of the prostate 3; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system; SCV, *Salmonella*-containing vacuole; TfR, transferrin receptor; LBPA, lysobisphosphatidic acid; ARF, ADP-ribosylation factor; IFN-I, type I IFN; CCV, *Campylobacter*-containing vacuole; PV, parasitophorous vacuole; hiPSCCM, iPSC-derived cardiomyocyte; MT, metacyclic trypomastigote; TCT, tissue culture trypomastigote; WT, wild type; KO, knockout; ERK, extracellular signal regulated kinase; DC, dendritic cell; MIC, micronemal protein; ROP, protein derived from rhoptries; LAMTOR1, mammalian target of rapamycin complex 1; PCM, paracoccidioidomycosis; PAMP, pathogen associated molecular pattern; TLR, Toll-like receptor; CLR, C-type lectin receptor; HBV, human hepatitis B virus; HCC, hepatocellular carcinoma; HBx, x protein of hepatitis B virus; SHB, small surface protein; EDEM, mannosidase-like protein; HSV, herpes simplex virus; SPP, signal peptide peptidase.

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lead to a close apposition of the phagosome membrane. The development of the phagosome into a final, degradative phagolysosome is a neatly ordered process and parallels endosome maturation. The newly formed early phagosome develops into a late phagosome after fusion with late endosomes and finally into a phagolysosome by fusion with lysosomes (For review: Haas, 2007).

Efficient sorting of the material internalized by endocytosis is essential for key cellular functions and represents a, if not the, major trafficking pathway in mammalian cells. Incoming material - solutes, receptors and cargos, lipids and even pathogenic agents - are routed to various destinations within mammalian cells at two major sorting stations: the early and late endosome. The early endosome receives all manner of incoming material from the plasma membrane, as well as from the Golgi, and serves as an initial sorting nexus routing molecules back to the cell surface through recycling endosomes, to the trans-Golgi network by retrograde transport, or on to the late endosome/lysosome. The early endosome also regulates cell signaling, through the down-regulation of internalized receptors, which are packaged into intralumenal vesicles that arise from inward invaginations of the limiting membrane. These multivesicular regions detach or mature from early endosomes and become free endocytic carrier vesicle/multivesicular body, which transports cargoes to late endosomes. The late endosome provides a central hub for incoming traffic from the endocytic, biosynthetic and autophagic pathways and outgoing traffic to the lysosomes, the Golgi complex or the plasma membrane. They also function as a key sensing/signaling platform that inform the cell about the nutrient situation (For review: Scott et al., 2014).

Vacuole is a more generic and unspecific definition of the membrane-bound organelle that surrounds the internalized pathogen. A vacuole can be anything from endosome to lysosome independent of the fact, if there is a pathogen residing in it or not. In this review we used the term phagosome to properly denominate the membrane surrounding the pathogens, besides keeping the vacuolar denomination in cases of the original article refers the organelle as vacuole.

Lysosomes are ubiquitous membrane-bound intracellular organelles with an acidic interior. A well-known function of lysosomes is degradation. The 60 lysosome-resident hydrolases (including proteases, peptidases, phosphatases, nucleases, glycosidases, sulfatases, and lipases) have different target substrates, and their collective action permits the degradation of all types of macromolecules. They also play important role in plasma membrane repair, cholesterol homeostasis, bone remodeling, antigen presentation, cell death and contribute in the pathogenesis of storage disorders, cancer, neurodegenerative and cardiovascular diseases (For review: Appelqvist et al., 2013; Aits and Jäättelä, 2013).

A remarkable characteristic during infection is a respiratory burst process inside the macrophage, a process characterized by increased oxygen uptake, reactive oxygen species (ROS) and reactive nitrogen species (RNS) production. The most common ROS and RNS generated inside the phagolysosome are nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^{-}$), superoxide anion radical (O^{2-}) and hydroxyl radical (OH^{-}) (Parente et al., 2015).

During pathogen-host cell invasion, cellular entry usually requires the use of endocytic and phagocytic pathways that terminate in fusion with lysosomes for degradation. However, intracellular pathogens have evolved several strategies to evade lysosome-mediated lysis, aiding their intracellular survival (Luzio et al., 2007). This review focuses on the strategies developed by bacteria, protozoa, fungi and virus to avoid lysosomal fusion, escape from or modulate phagolysosome properties in order to survival and perpetuate infection. We have selected different pathogens that evolved different strategies of survival inside host cell.

1. Bacteria

1.1. Anaplastamaceae

Obligate intracellular bacteria with unique host cell specificities, such as members of the family Anaplasmataceae, have developed several mechanisms to ensure immune evasion of host cellular defenses. These mechanisms involve adaptations for survival and replication within nonlysosomal intracellular phagosomes, which are host cell membrane-bound inclusions called morulae (Hackstadt, 1998). This is particularly important for these bacteria because they exclusively reside in professional phagocytes that have as their main function the destruction of engulfed pathogens through lysosomal degradation (Rikihisa, 2006; Cheng et al., 2014).

The best known bacteria whose cytoplasmic inclusions do not fuse with lysosomes and which are currently included in this family are *Ehrlichia* spp, *Anaplasma* spp. and *Neorickettsia* spp. (Wells and Rikihisa, 1988; Barnewall et al., 1997; Mott et al., 1999).

Members of the genus *Ehrlichia* are increasingly being recognized as pathogens of human disease in the United States and other parts of the world. Two emerging infectious diseases, human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis* and human granulocytic ehrlichiosis (HGE) caused by *Anaplasma phagocytophilum* (formerly *E. equi* and HGE agent), have only been recognized in the last years (Hackstadt, 1998; Rikihisa, 2003). Beyond that, the global canine pathogen *Ehrlichia canis* has been isolated from a human in Venezuela, and several patients with clinical signs similar to HME were found to be infected with *E. canis* at the same country (Perez et al., 1996; Perez et al., 2006).

Caveolae-or lipid raft-mediated endocytosis is a vesicle trafficking system that bypasses phagolysosomal pathways, and is thus utilized by a wide variety of pathogenic microorganisms to invade host cells (Lafont and Van Der Goot, 2005). The entry and intracellular infection of *E. chaffeensis* and *A. phagocytophilum* involve cholesterol-rich lipid rafts or caveolae and glycosylphosphatidylinositol (GPI)-anchored proteins. By fluorescence microscopy, caveolar marker protein caveolin-1 was co-localized with both early and replicative bacterial inclusions. Additionally, tyrosine-phosphorylated proteins and PLC-gamma2 were found in bacterial early inclusions. In contrast, clathrin was not found in any inclusions from either bacterium. An early endosomal marker, transferrin receptor, was not present in the early inclusions of *E. chaffeensis*, but was found in replicative inclusions of *E. chaffeensis*. Furthermore, several bacterial proteins from *E. chaffeensis* and *A. phagocytophilum* were co-fractionated with Triton X-100-insoluble raft fractions. The formation of bacteria-encapsulating caveolae, which assemble and retain signalling molecules essential for bacterial entry and interact with the recycling endosome pathway, may ensure the survival of these obligatory intracellular bacteria in primary host defensive cells. In contrast to other bacteria that use caveolae only for their entry, their results showed the involvement of caveolae throughout the intraleucocyte stage of the life cycles of *E. chaffeensis* and *A. phagocytophilum* by retaining signalling molecules and interacting with endosome recycling pathways. This study suggested that caveolae are the sites at which the early signalling molecules (PLC-γ2 and tyrosine-phosphorylated proteins) that are essential for bacterial entry were initially assembled. It is very likely that the binding of *E. chaffeensis* or *A. phagocytophilum* to unidentified receptors activates some (receptor) tyrosine kinases, which in turn phosphorylate proteins located in caveolae. PLC-γ 2 is one of the rapidly tyrosine-phosphorylated proteins in *E. chaffeensis*-infected host cells. As the substrate of PLC-γ 2, phosphatidylinositol 4,5-bisphosphate (PIP2), is also enriched in caveolae. Caveolae may facilitate PLC-γ 2 enzymatic action, leading to an increase in the intracellular Ca2 + level that is essential for bacterial infection. Moreover, *E. chaffeensis* and *A. phagocytophilum* inclusions were not colocalized with CD63 or LAMP-1 (lysosome-associated membrane protein-1), lysosomes membrane glycoproteins, which can be used as markers of lysosomal fusion (Lin and Rikihisa, 2003).

The absence of these lysosomal markers on ehrlichial inclusions indicates these inclusions do not fuse with lysosomes (Chen et al., 1986; Barnewall et al., 1997; Webster et al., 1998; Mott et al., 1999). Nevertheless, these bacteria use different strategies to avoid lysosomal fusion and create their safe havens. For example, when a human promyelocytic leukemia cell line HL-60 is coinfected with *E. chaffeensis* and *A. phagocytophilum*, they resided in separate inclusion compartments with different characteristics within the same cell (Mott et al., 1999).

Cytoplasmic inclusions containing *E. chaffeensis* in human promyelocytic leukemia cell line THP-1 and HL-60 have characteristics of early endosomes (early phagosome), presenting the markers Rab5, early endosome antigen 1 (EEA1) and transferrin receptor (TfR) (Barnewall et al., 1997; Mott et al., 1999). Furthermore, minimal accumulation of the acidotropic base 3-(2,4-dinitroanilino)− 3′-amino--*N*-methyldipropylamine and the vacuolar H^+ ATPase within *E. chaffeensis* morulae suggested that the vesicle is only weakly acidic (Barnewall et al., 1997; Cheng et al., 2014). *E. chaffeensis* thus appear to block maturation of endosomes and remain in an early endosomal compartment, thereby avoiding lysosomal fusion. In contrast, the inclusion compartment of *A. phagocytophilum* do not possess these early endosome characteristics (Webster et al., 1998; Mott et al., 1999). Moreover, phagosomes from HL-60 cell line containing these bacteria incorporated endocytosed colloidal gold particles and were labeled to the cation-dependent mannose-6-phosphate receptor (M6PR). The M6PR is involved with delivery of lysosomal enzymes to late endosomes and lysosomes recycle from the Golgi apparatus to endosomal compartments and back again. Therefore, *A. phagocytophilum* resides in compartments belonging to endocytic pathway (Webster et al., 1998).

The cytochemical staining for acid phosphatase marks late endosomes and lysosomes, as well as phagosomes that merge with these organelles (Hackstadt, 1998). Previous studies indicated that the vacuole that contains *N. risticii* or *A. phagocytophilum* showed no labelling for acid phosphatase activity in cells not treated with the antibiotic oxytetracycline. Once treated, the cells showed a significant increase in the co-localization of lysosomal markers and phagosomes that contains the bacteria, suggesting that this drug affect the ability of these bacteria to inhibit lysosomal fusion (Wells and Rikihisa, 1988; Gokce et al., 1999). Therefore, inhibition of ehrlichial protein synthesis by oxytetracycline causes a failure to inhibit the maturation of endosomes to lysosomes, with resultant destruction of the parasites. Similarly, Alves et al. (2014) demonstrated that intact cytoplasmic inclusions of *E. canis* are rarely labelled with acid phosphatase compared to deteriorated inclusions suggesting that the spreading process of *E. canis* in vitro is dependent on lysosomal evasion. These data indicate that inactive or dead intracellular microorganisms lose their ability to inhibit phagosome-lysosome fusion. Another study showed that lysosomal proteins such as cathepsin D, cathepsin S, and lysosomal acid phosphatase were not detected in *E. chaffeensis* phagosome preparations by proteomics methods (Cheng et al., 2014). Moreover, the inhibition of lysosomal fusion is specific to phagosomes-containing the bacterias, as intracellular *N. risticii* or *A. phagocytophilum* or *E. chaffeensis* do not inhibit lysosomal fusion with phagosomes containing latex particles ingested by the same cell (Wells and Rikihisa, 1988; Gokce et al., 1999; Cheng et al., 2014).

Despite the mentioned studies demonstrated that *Ehrlichia*-containing vacuole (ECV) (phagosome) does not fuse with lysosomes, an essential condition for *Ehrlichia* to survive inside phagocytes, the mechanism of inhibiting the fusion of the phagosome with lysosomes is not clear. Cheng et al. (2014) detected in DH 82 cell line, Rab7, a late endosomal marker, in *E. chaffeensis* phagosomes by proteomic and immunofluorescence analysis. Beyond that, these phagosomes were acidified at approximately pH 5.2, suggesting that the *E. chaffeensis* phagosome was a late phagosome. Thereby, *E. chaffeensis* phagosomes were capable of fusing with early endosomes and maturing into late endosomes, without lysosome fusion. This phenomenon by which *E. chaffeensis* inhibits phagosome-lysosome fusion is to modify its phagosome membrane composition, rather than by regulating the expression of host genes involved in trafficking.

Despite being based on different strategies according to the species of *Ehrlichia* spp., the evasion of lysosomal fusion by ehrlichial inclusions is fundamental to the survival and replication of this pathogen (Rikihisa, 2003). Additional analyses of the ECV molecular composition could decipher the mechanism by which *Ehrlichia* inhibits phagosome-lysosome fusion in the host cell and may facilitate the development of new therapeutic strategies.

E. chaffeensis, hijacks host cell processes of the mononuclear phagocyte to evade host defenses through mechanisms executed in part by tandem repeat protein (TRP) effectors secreted by the type 1 secretion system. TRP120 has emerged as a model moonlighting effector, acting as a ligand mimetic, nucleomodulin and ubiquitin ligase. These defined functions illuminate the diverse roles TRP120 plays in exploiting and manipulating host cell processes, including cytoskeletal organization, vesicle trafficking, cell signaling, transcriptional regulation, post-translational modifications, autophagy and apoptosis (For review Byerly et al., 2021).

E. chaffeensis enters human cells via the binding of its unique outer-membrane invasin EtpE to the cognate receptor DNase X on the host-cell plasma membrane; this triggers actin polymerization and filopodia formation at the site of *E. chaffeensis* binding, and blocks activation of phagocyte NADPH oxidase that catalyzes the generation of microbicidal reactive oxygen species. Subsequently, the bacterium replicates by hijacking/dysregulating host-cell functions using Type IV secretion effectors. *Ehrlichia* translocated factor (Etf)− 1 enters mitochondria and inhibits mitochondria-mediated apoptosis of host cells. Etf-1 also induces autophagy mediated by the small GTPase RAB5, the result being the liberation of catabolites for proliferation inside host cells. Moreover, Etf-2 competes with the RAB5 GTPase-activating protein, for binding to RAB5-GTP on the surface of *E. chaffeensis* inclusions, which blocks GTP hydrolysis and consequently prevents the fusion of inclusions with host-cell lysosomes. Etf-3 binds ferritin light chain to induce ferritinophagy to obtain intracellular iron. To enable *E. chaffeensis* to rapidly adapt to the host environment and proliferate, the bacterium must acquire host membrane cholesterol and glycerophospholipids for the purpose of producing large amounts of its own membrane (For review: Rikihisa, 2022). Within inclusions, *Ehrlichia* obtains host-derived nutrients by inducing RAB5-regulated autophagy using *Ehrlichia* translocated factor-1 deployed by its type IV secretion system. This manipulation of RAB5 by a bacterial molecule offers a simple strategy for *Ehrlichia* to avoid destruction in lysosomes and obtain nutrients, membrane components, and a homeostatic intra-host-cell environment in which to grow (For review: Rikihisa, 2019).

A study using human monocytic leukemia cells (THP-1) revealed that Wnt signaling plays a crucial role in inhibition of lysosomal fusion and autolysosomal destruction of ehrlichiae. During early infection, autophagosomes fuse with ehrlichial phagosomes to form an amphisome indicated by the presence of autophagy markers such as LC3 (microtubule-associated protein 1 light chain 3), Beclin-1, and p62. LC3 colocalized with ehrlichial morulae on days 1, 2, and 3 postinfection, and increased LC3II levels were detected during infection, reaching a maximal level on day 3. Ehrlichial phagosomes did not colocalize with the lysosomal marker LAMP2, and lysosomes were redistributed and dramatically reduced in level in the infected cells. An inhibitor specific for the Wnt receptor signaling component dishevelled induced lysosomal fusion with ehrlichial inclusions corresponding to p62 degradation and promoted transcription factor EB (TFEB) nuclear localization. *E. chaffeensis* infection activated the phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR pathway, and activation was induced by TRP120, TRP32, and TRP47 ehrlichial effectors, with TRP120 inducing the strongest activation. Moreover, induction of glycogen synthase kinase-3 (GSK3) performed using a Wnt inhibitor and small interfering RNA (siRNA) knockdown of critical components of PI3K-GSK3-mTOR signaling decreased ehrlichial survival (Lina et al., 2017). This report

showed *Ehrlichia* exploitation of the evolutionarily conserved Wnt pathway to inhibit autolysosome generation, thus, leading to evasion of this innate immune defense mechanism. (Fig. 1).

1.2. Listeria monocytogenes

Listeria monocytogenes is a gram-positive facultative intracellular pathogen and is the etiologic agent of listeriosis, a gastroenteritis that is self-limiting in healthy individuals, but in pregnant women, neonates or immunocompromised individuals may be the cause of serious infections (Rocourt and Bille, 1997; Vázquez-Boland et al., 2001). *L. monocytogenes* can infect phagocytic and nonphagocytic cells and may mediate its internalization via the bacterial internalins InlA and InlB (Birmingham et al., 2007). The internalization occurs by a 'zippering' mechanism, which leads to the formation of a phagocytic vacuole that is rapidly lysed. After this, occurs the release of bacteria in the cytosol, where it replicates and moves via polymerization of host cell actin cytoskeleton. After internalization into mouse RAW 264.7 macrophages, phagosomal escape is mediated by toxins such listeriolysin O (LLO) and two phospholipase C enzymes (PLCs), with substrate preferences for phosphatidylinositol (PI-PLC, encoded by *plcA*), or phosphatidylcholine and other phosphoinositides (PC-PLC, encoded by *plcB*) (Birmingham et al., 2007; Silva et al., 2012). The activity of these factors, LLO and PC-PLC, is regulated by vacuolar pH (Marquis et al., 1997; Marquis and Hager, 2000; Schuerch et al., 2005), where in the level adjustment is defined by the virulence of *L. monocytogenes* (Glomski et al., 2002; Yeung et al., 2007). The PC-PLC, and its activating enzyme, the metalloprotease of *L. monocytogenes* (Mpl), are generated as inactive proenzymes (Forster et al., 2014). *L. monocytogenes* temporarily resides in phagosomes that acidify, leading to the autocatalysis of Mpl and proteolytic activation of PC-PLC that in the active form is not generated outside the phagosomal environment (Marquis et al., 1997).

Pore-forming toxins (PFTs) that disrupt the plasma membrane of mammalian cells are the most common bacterial virulence factors (Köster et al., 2014). Listeriolysin O is a member of the family of cholesterol-dependent cytolysins (CDC), which include the largest family among bacterial PFTs (Alouf, 2001). Escape from the phagosome of mouse RAW 264.7 macrophages is mediated principally by LLO, a pore-forming protein encoded by the *hly* gene (Birmingham et al., 2007). This toxin alone is sufficient to allow phagosome escape by nonpathogenic bacteria (Monack and Theriot, 2001; Bielecki et al., 1990) and can disrupt pH and calcium gradients across phagosomal membranes, which delays the fusion of phagosomes containing bacteria with LAMP-1–-positive lysosomes (Henry et al., 2006; Shaughnessy et al., 2006). Thus, the bacteria typically escape macrophage phagosomes prior to phagosome–lysosome fusion (Henry et al., 2006).

Upon entry into the cytosol of mouse RAW 264.7 macrophages, *L. monocytogenes* multiplies and use the bacterial protein ActA to recruit actin regulatory factors of the host cell. This recruitment moves intracellular and intercellular to invade neighboring cells via induction of polymerization of host cell actin cytoskeleton (Birmingham et al., 2007; Silva et al., 2012; Davis et al., 2012). All virulence factors of *L. monocytogenes*, including ActA, LLO, PI-PLC and PC-PLC, are controlled by the bacterial transcriptional regulator PrfA (Hamon et al., 2006; Portnoy et al., 2002). *L. monocytogenes* utilizes multiple PrfA-regulated mechanisms, including ActA-dependent actin polymerization and bacterial PLC expression, to avoid destruction by both the autophagy system and lysosomal fusion (Birmingham et al., 2007) (Fig. 1).

During several days of infection in human hepatocytes or trophoblast cells, *L. monocytogenes* switches from this active motile lifestyle to a stage of persistence in vacuoles. Upon intercellular spread, bacteria gradually stopped producing ActA and became trapped in lysosome-like vacuoles termed *Listeria*-Containing Vacuoles (LisCVs). Subpopulations of bacteria resisted degradation in LisCVs and entered a slow/non-replicative state. During the subculture of host cells harboring LisCVs, bacteria showed a capacity to cycle between the vacuolar and the actin-based motility stages. When ActA was absent, such as in ΔactA mutants, vacuolar bacteria parasitized host cells in the so-called "viable but non-culturable" state (VBNC). The exposure of infected cells to high doses of gentamicin did not trigger the formation of LisCVs, but selected for vacuolar and VBNC bacteria. Together, these results reveal the ability of *L. monocytogenes* to enter a persistent state in a subset of epithelial cells, which may favor the asymptomatic carriage of this pathogen, lengthen the incubation period of listeriosis, and promote bacterial survival during antibiotic therapy (Kortebi et al., 2017).

Authors have reported that the protein abundance of the Six-transmembrane epithelial antigen of the prostate 3 (Steap3) was decreased upon *L. monocytogenes* infection compared to uninfected Raw264.7 cells (murine macrophage cell line). However, the decreased Steap3 abundance was not regulated by the host but was caused by LLO. Ablation of Steap3 facilitated entry of *L. monocytogenes* from the phagosome into the cytosol. Then, the comprehensive proteomic analysis revealed that the deletion of Steap3 could affect the proteins abundance of the lysosomal signaling pathway in Raw264.7 cells. Among these proteins affected by Steap3, They discovered that only the Ganglioside GM2 activator (Gm2a) inhibited the phagosomal escape of *L. monocytogenes* as Steap3. In summary, They found that the Steap3-Gm2a axis could restrict the phagosomal escape of *L. monocytogenes* and serve as a potential molecular drug target for antibacterial treatment (Yuan et al., 2022).

1.3. Salmonellae

Salmonellae represent a group of gram-negative facultative anaerobic pathogenic bacteria that contaminate food and water and cause a variety of disease syndromes reaching 10 million cases annually worldwide (Crump et al., 2004; Majowicz et al., 2010; Garai et al., 2012). Although the disease elicited by *Salmonella* is dependent upon the serological variety (serovar) of the pathogen as well as the characteristics of the host, infections range from self-limiting gastrointestinal inflammation to disseminated systemic disease (Behnsen et al., 2015). The human restricted *S. enterica* serovar Typhi (*S. typhi*) and Paratyphi (*S. paratyphi*) are the causative agents of typhoid fever, a life-threatening systemic disease that primarily affects liver and spleen. On the other hand, non-typhoid serovars, like Typhimurium (*S. typhimurium*) causes a self-limiting gastroenteritis in humans as a result of the bacterium invading the intestinal mucosa (Broz et al., 2012; LaRock et al., 2015).

The typhoidal and non-typhoidal serovars of *Salmonella* are prime examples of how host immunity is a Double-edged sword: on one edge, aspects of the host immune response are of crucial importance because they limit *Salmonella* replication and systemic dissemination; on the other edge, *Salmonella* can evade, manipulate, and exploit aspects of host immunity to replicate and establish a persistent infection (Behnsen et al., 2015). Furthermore, survival at extra intestinal sites involve a complex interplay between this bacterium and immune cells, primarily macrophages, which are permissive for pathogen replication and constitute a niche that promotes *Salmonella* persistence within the host (Ruby et al., 2012; Behnsen et al., 2015).

The pathogenesis of diseases by *Salmonella* depends on the coordinated function of various sets of virulence proteins encoded by genes clusters on the virulence plasmid or by specific chromosomal loci, referred to as *Salmonella* pathogenicity islands (SPI) (Jantsch et al., 2011). The SPI1 and SPI2 encode two distinct type III secretion systems (T3SS) that are present on the cell wall and translocate a specific group of bacterial effector proteins into host cells (Garai et al., 2012; Ruby et al., 2012; LaRock et al., 2015). While SPI1 is required for the invasion of non-phagocytic host cells and elicitation of diarrheal disease, SPI2 is essential for the intracellular survival and replication of the bacteria (Lahiri et al., 2010; Jantsch et al., 2011).

Following entry into host cells that can occur either via bacteria-mediated invasion or by phagocytosis, Salmonellae express their

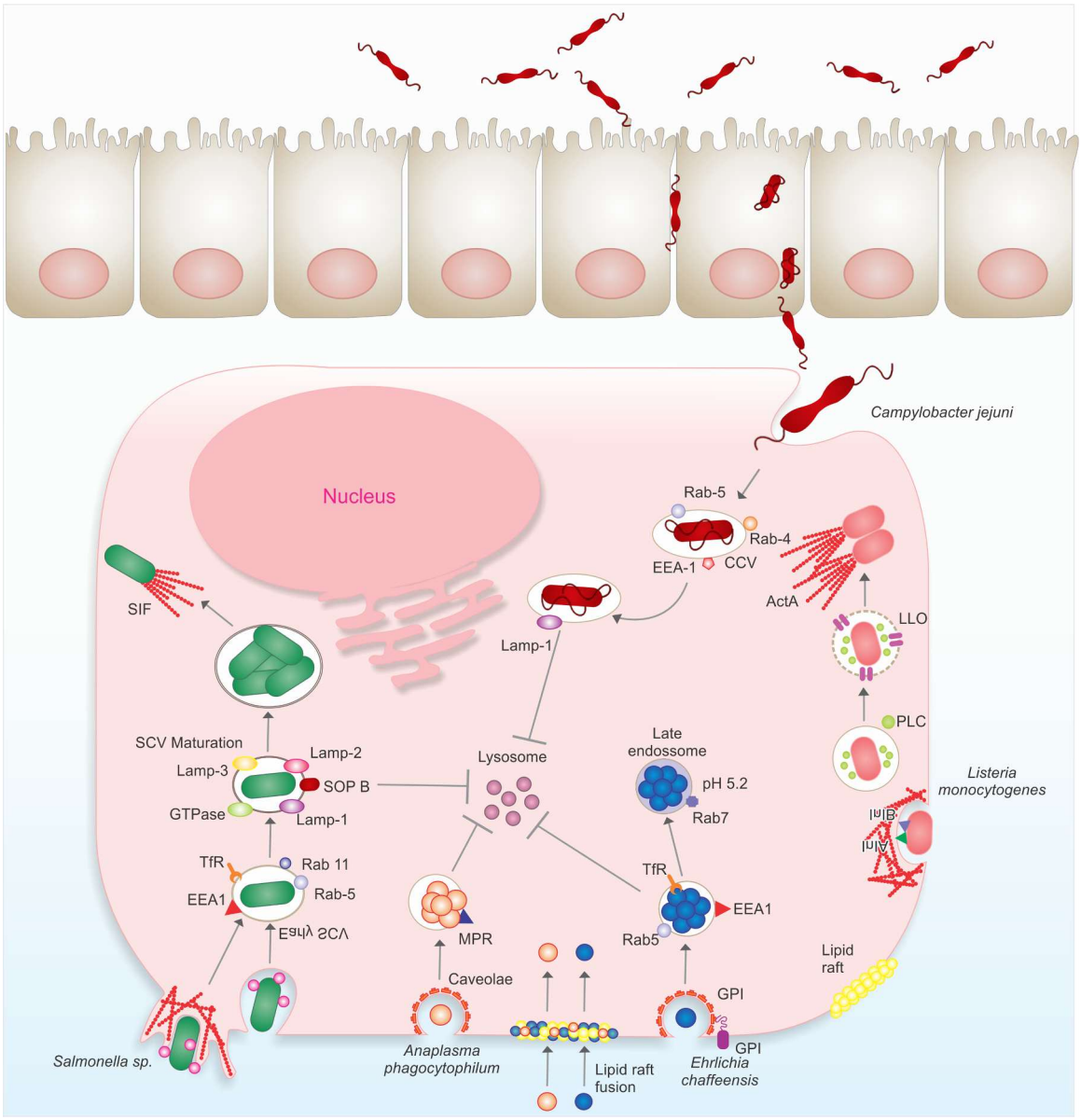

Fig. 1. Subversion mechanisms of lysosomal killing by bacteria - a. The entry of members Anaplasmataceae family in host cells such as *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* involves lipid rafts or caveolae and glycosylphosphatidylinositol (GPI)-anchored proteins mediated endocytosis. Cytoplasmic inclusions containing *E. chaffeensis* presents early endosomes features such as Rab 5, early endosome antigen 1 (EEA1) and trasnferrin receptor (TfR) and it seems that this pathogen blocks the maturation of endosomes, with weakly acidic characteristics. In a different way, *A. phagocytophilum*-containing compartment do not possess early endosome characteristics, but this vesicle were labeled to the cation-dependent mannose-6-phosphate receptor (MPR6) involved in the delivery of lysosomal enzymes in late endosomes and lysosomal recycling. This indicates that it belongs to via endocytic vesicle. b. *Listeria monocytogenes* infects phagocytic and non-phagocytic cells via bacterial internalins InlA and InlB, which are the surface proteins responsible for mediating the binding to the host cell. The internalization occurs by a 'zippering' mechanism, which leads to the formation phagocytic vacuole that is rapidly lysed by toxins such as listeriolysin O (LLO) and two phospholipase C enzymes (PI-PLC and PC-PLC). After escape to the cytosol, *L. monocytogenes* multiplies and use another virulence factor, the protein ActA, to recruit actin regulatory factors of the host cell. This allows movement within the bacterial cell and also in the neighboring cells invading by induction of actin cytoskeleton polymerization of host cell. c. The *Salmonella* enter the host cell can occur via bacteria-mediated invasion or by phagocytosis. This bacterium is established specifically within *Salmonella* containing vacuole (SCV), through the expression of virulence factors as SPI1–2 T3SS. The SCV follows the host endosomal pathway and interacts transiently with early endosomes recruits and loses early endocytic markers such as EEA1, TfR, Rab 5 and 11. After 15–60 min, other proteins common to late endosome such as Lamp 1, Lamp 2, Lamp 3, Rab 7 and vATPase are presents. After perinuclear positioning of SVC, the bacteria start to replicate and are found associated with extend tubular network known '*Salmonella* induced filaments' (SIF), which it is important to ensure SCV integrity during intracellular proliferation of *Salmonella*. Another virulence factor, SopB has presented manipulate the SCV surface charge resulting in the inhibition of SCV-lysosome fusion. All the effector proteins play also important role in protecting bacteria against reactive oxygen and nitrogen intermediates production, ensuring its survival. d. *Campylobacter jejuni* colonize the epithelial cells of ileum and colon. *C. jejuni* can enter into the cells by actin-dependent and microtubule-dependent uptake into eukaryotic cells. Inside the cells, *C. jejuni* resides within endolysosomal compartments called Campylobacter-containing vacuoles (CCV). These compartments interact with early endosomes presenting markers EEA1, Rab 4 and Rab5, but not follow the endocytic pathway canonical consequently not fuse with lysosomes. On the other hand, it was seen that CCV might contain Lamp-1, which should indicate a short contact with late endosomes.

virulence-associated SPI1–2 T3SS to establish themselves in a specialized intracellular phagosome termed as *Salmonella* containing vacuole (SCV). Within this compartment, *Salmonella* can replicate to high numbers before exiting the cell and infecting new host cells (Haraga et al., 2008; Jantsch et al., 2011). In spite of SCV arrests the host endosomal pathway at the late endosome stage and has some features in common with this endosome, such as the presence of lysosomal glycoproteins and the acidic luminal pH. Other properties are unique and may be the result of a modulation of normal host cell functions (Kumar and Valdivia, 2009; Jantsch et al., 2011). Within the SCV, the bacteria can persist intracellular for a long time, making it a unique compartment with respect to the normal progression of phagolysosomal recycling and maturation. Although there has been some controversy within the field, a variety of studies has shown that *Salmonella* can survive within macrophages in which their SCV has fused with the lysosomal compartments (Buchmeier and Heffron, 1991; Drecktrah et al., 2007; Ishibashi and Arai, 1995).

Consequently, the avoidance of phagolysosomal fusion is unlikely to be a major evasion pathogenic strategy of *Salmonella*. The SCV interacts transiently with the early endocytic pathway and quickly recruits and loses early endocytic markers, such as EEA1, TfR, and the early endocytic trafficking guanosine triphosphatases (GTPases) Rab5 and Rab11 (Smith et al., 2007; Haraga et al., 2008; Lahiri et al., 2010; Jantsch et al., 2011). Within 15–60 min, early endosomal proteins are replaced by proteins normally found on the late endosome or lysosomes, such as LAMP1, LAMP2, LAMP3, GTPase Rab7 and vATPase (vacuolar type ATPase) (Drecktrah et al., 2007; Haraga et al., 2008; Kumar and Valdivia, 2009; Lahiri et al., 2010; Jantsch et al., 2011). There is contradictory data on the presence of M6PR, LBPA (lysobisphosphatidic acid) and the hydrolase cathepsin D on the SCV (Drecktrah et al., 2007; Haraga et al., 2008; Jantsch et al., 2011; Lahiri et al., 2010). Thus, the presence or absence of different markers on the persistent SCV may indicate that they are variably detected rather than reflect whether or not the SCV has matured through a normal endocytic pathway (Haraga et al., 2008) (Fig. 1).

DNA microarray analysis identified LAMP-3 as one of the genes responding to LPS stimulation in THP-1 macrophage cells. Additional analyses revealed that LPS and *Salmonella* induced the expression of LAMP-3 at both the transcriptional and translational levels. LAMP-3, like LAMP-2, shifts its localization from the cell surface to alongside *Salmonella.* Knockdown of LAMP-3 by specific siRNAs decreased the number of *Salmonella* recovered from the infected cells. Thus, authors concluded that LAMP-3 is induced by *Salmonella* infection and recruited to the *Salmonella* pathogen for intracellular proliferation (Lee et al., 2016).

For establishing a successful lifecycle within the SCV, *Salmonella* SPI2 T3SS is induced within the SCV and translocates effector proteins across the phagosomal membrane several hours after phagocytosis (Haraga et al., 2008). After juxtanuclear positioning of the SCV by the balanced activities of the kinesin and dynein motor proteins and a lag phase, the bacteria start to replicate and at the same time, the SCV are found associated with an extended tubular network called '*Salmonella* induced filaments' (SIF) (Haraga et al., 2008; Lahiri et al., 2010; Jantsch et al., 2011). The SPI2-T3SS effector SifA is required for the maintenance of the SCV integrity during intracellular proliferation of *Salmonella*, as well as for the induction of SIF (Stein et al., 1996; Beuzón et al., 2000). SifA subverts Rab9-dependent retrograde trafficking of M6PRs, thereby attenuating lysosome function. This required binding of SifA to its host cell target SKIP/PLEKHM2. Translocated SifA formes a stable complex with SKIP and Rab9 in infected cells. Sequestration of Rab9 by SifA-SKIP accounts for the effect of SifA on MPR transport and lysosome function. Growth of *Salmonella* increases in cells with reduced lysosomal activity and decreases in cells with higher lysosomal activity. These results suggest that *Salmonella* vacuoles undergo fusion with lysosomes whose potency has been reduced by SifA (McGourty et al., 2012). The Pleckstrin homology domain-containing protein family member 1 (PLEKHM1), a lysosomal adaptor, is targeted by *Salmonella* through direct interaction with SifA. By binding the PLEKHM1 PH2 domain, *Salmonella* utilize a complex containing PLEKHM1, Rab7, and the HOPS tethering complex to mobilize phagolysosomal membranes to the SCV (McEwan et al., 2015).

Salmonella effector protein, SipC, specifically binds with host Syntaxin6 and recruits Syntaxin6 and other accessory molecules like VAMP2, Rab6, and Rab8 on *Salmonella*-containing phagosomes and acquires LAMP1 by fusing with LAMP1-containing Golgi-derived vesicles (Madan et al., 2012).

SopB, a SPI1-T3SS effector acting as bacterial phosphoinositide phosphatase, was found to manipulate the SCV surface charge resulting in the inhibition of SCV-lysosome fusion (Bakowski et al., 2010). Furthermore, the secreted effector proteins also play an important role in evading the effects of antimicrobial compounds such as reactive oxygen and nitrogen species (ROS and RNS, respectively); giving the ability of *Salmonella* to survive exposure to lysosomal contents (Jantsch et al., 2011).

SopF is an ADP-ribosyltransferase specifically modifying Gln124 of ATP6V0C in V-ATPase. Authors identify GTP-bound ADP-ribosylation factor (ARF) GTPases as a cofactor required for SopF functioning. Moreover, lysosome or Golgi damage-induced autophagic LC3 activation is inhibited by SopF (Xu et al., 2022). In this sense, ARF GTPases activates *Salmonella* SopF to ADP ribosylate host V-ATPase and inhibit endomembrane damage-induced xenophagy.

The SCV is considered the primary intracellular niche for *Salmonella* nevertheless the bacteria can also be found in the cytosol. In some cells, such as macrophages, the cytosol is a lethal environment for *Salmonella*, however, in epithelial cells the cytosol supports growth (Beuzón et al., 2002; Brumell et al., 2002; Knodler et al., 2010). Besides the bacterial replication into the cytosol far exceeds that occurring within SCVs and is important for the infectious cycle (Malik-Kale et al., 2011).

A genome-scale CRISPR/Cas9 screen in intestinal epithelial cells with the prototypical intracellular bacterial pathogen *Salmonella* led authors to discover that type I IFN (IFN-I) remodels lysosomes. IFN-I-dependent lysosome acidification was associated with elevated intracellular *Salmonella* virulence gene expression, rupture of the *Salmonella*-containing vacuole, and host cell death. In addition, IFN-I signaling promoted in vivo *Salmonella* pathogenesis in the intestinal epithelium where *Salmonella* initiates infection, indicating that IFN-I signaling can modify innate defense in the epithelial compartment. Authors proposed that IFN-I control of lysosome function broadly impacts host defense against diverse viral and microbial pathogens (Zhang et al., 2020).

S. Typhimurium impairs glycolysis and its modulators such as insulin-signaling to impair macrophage defense. Glycolysis facilitates glycolytic enzyme aldolase A mediated v-ATPase assembly and the acidification of phagosomes which is critical for lysosomal degradation. Thus, impairment in the glycolytic machinery leads to decreased bacterial clearance and antigen presentation in murine macrophages. These results highlight a crucial molecular link between metabolic adaptation and phagosome maturation in macrophages, which is targeted by *S. Typhimurium* to avoid cell-autonomous defense (Gutiérrez et al., 2021).

In summary, the aforementioned studies indicate that Salmonellae may not avoid fusion of the SCV with endocytic compartments but, can control recycling pathways in the host cell to remove undesirable factors (Fig. 1).

1.4. *Campylobacter jejuni*

Campylobacter jejuni is a wide spread gram-negative bacterium considered as a classical zoonotic pathogen and is found in the normal intestinal flora in several birds and mammals. *C. jejuni* colonizes various animals, it can contaminate food products during processing and the surface water (Friedman et al., 2000). Curiously, *C. jejuni* displays commensal behavior in chicken while in the human intestine this bacteria penetrates the mucus and colonizes the intestinal crypts in a very

efficient manner. The molecular basis of the difference in pathogenicity of *C. jejuni* in human and chicken still remains unclear (Backert et al., 2013).

C. jejuni, shows chemotactically controlled motility in viscous milieu that allows targeted navigation to intestinal mucus and colonization. By phase variation, quorum sensing, extensive O-and N-glycosylation and use of the flagellum as type-3-secretion system *C. jejuni* adapts effectively to environmental conditions. *C. jejuni* utilizes proteases to open cell-cell junctions and subsequently transmigrates paracellularly. Fibronectin at the basolateral side of polarized epithelial cells serves as binding site for adhesins CadF and FlpA, leading to intracellular signaling, which triggers membrane ruffling and reduced host cell migration by focal adhesion. Cell contacts of *C. jejuni* results in its secretion of invasion antigens, which induce membrane ruffling by paxillin-independent pathway. In addition to fibronectin-binding proteins, other adhesins with other target structures and lectins and their corresponding sugar structures are involved in host-pathogen interaction. Invasion into the intestinal epithelial cell depends on host cell structures. Fibronectin, clathrin, and dynein influence cytoskeletal restructuring, endocytosis, and vesicular transport, through different mechanisms. *C. jejuni* can persist over a 72-h period in the cell. *Campylobacter*-containing vacuoles (phagosomes), avoid fusion with lysosomes and enter the perinuclear space via dynein, inducing signaling pathways. Secretion of cytolethal distending toxin directs the cell into programmed cell death, including the pyroptotic release of proinflammatory substances from the destroyed cell compartments. The immune system reacts with an inflammatory cascade. The development of autoantibodies, directed not only against lipooligosaccharides, but also against endogenous gangliosides, triggers autoimmune diseases. Lesions of the epithelium result in loss of electrolytes, water, and blood, leading to diarrhea, which flushes out mucus containing *C. jejuni*. Together with the response of the immune system, this limits infection time (For review: Kemper, Hensel, 2023). The clinical course of *C. jejuni* infection varies from mild, non-inflammatory, self-limiting diarrhoea to severe, inflammatory, bloody diarrhoea that can continue for few weeks (Young et al., 2007; Van Putten et al., 2009; Dasti et al., 2010; Oyarzabal and Backert, 2011). In some cases, the infection by C. *jejuni* can be also associated with the development of reactive arthritis and peripheral neuropathies, known as Miller–Fisher and Guillain–Barrè syndromes (Nachamkin et al., 2008; Zilbauer et al., 2008; Backert et al., 2013).

Following ingestion by host, these bacteria use their flagella-driven motility to colonize the epithelial cells of the ileum and colon. The crypts seem to be an optimal growth environment for *C. jejuni* (Young et al., 2007; Nachamkin et al., 2008; Boehm et al., 2012). Experimental studies using cell culture models indicate that *C. jejuni* can enter into the cells by different routes. Both actin-dependent and microtubule-dependent uptake into eukaryotic cells have been reported (Olschlaeger et al., 1993; Russell and Blake, 1994; Hu and Kopecko, 1999; Kopecko et al., 2001; Monteville et al., 2003). The uptake process may require cellular factors such as caveolin-1 and the small Rho GTPases Rac1 and Cdc42, but not dynamin (Olschlaeger et al., 1993; Russell and Blake, 1994; Hu and Kopecko, 1999; Kopecko et al., 2001; Monteville et al., 2003; Hu et al., 2006; Krause-Gruszczynska et al., 2007; Watson and Galán, 2008; Bouwman et al., 2013).

Once inside T84, a human intestinal epithelial cell line, and Cos-1, a monkey kidney epithelial cell line, *C. jejuni* resides within a membrane-bound compartment called *Campylobacter*-containing vacuoles (CCV) (Watson and Galán, 2008; Garcia-Del Portillo et al., 2008). It was found that the Campylobacter-containing vacuole (CCV) deviates from the canonical endocytic pathway immediately after host cell entry, thus avoiding delivery into lysosomes. The CCV appears to interact with early endosomal compartments because it associates with early endosomal marker protein EEA1 and two trafficking GTPases, Rab4, and Rab5. However, this interaction seems only transient and does not progress inside the canonical endocytic pathway (Watson and Galán, 2008; Ocroinin and Backert, 2012).

The CCV can be also stained with LAMP-1 although this compartment appears to be unique and clearly distinct from lysosomes. CCVs were not stainable with the lysosomal marker protein cathepsin B and it is also not accessible to certain endocytic tracers (Watson and Galán, 2008). Taken together, the acquisition of LAMP-1 occurring very early during CCVs maturation, it appears to proceed by an unusual pathway not requiring the GTPases Rab5 or Rab7, although recruited to the CCV. More studies are required to elucidate the mechanism by which *C. jejuni* modulates intracellular trafficking and survival inside the host cell (Ocroinin and Backert, 2012).

The subset of *C. jejuni* genes, which are important for intracellular trafficking and survival are widely unknown, but a couple of potential factors is emerging. One of these factors is the CiaI, a reported secreted protein (Buelow et al., 2011). *C. jejuni* CiaI seemed be involved with the bacteria survival in CCV (Buelow et al., 2011; Ocroinin and Backert, 2012; Lugert et al., 2015). Some experiments using *C. jejuni*-specific antibodies revealed the presence of this pathogen with changed morphology in CCV. Interestingly, have been demonstrated in some cellular types a subset of intracellular *C. jejuni* population are able to survive and metabolically active after 48 h of infection, suggesting a successful intracellular lifestyle (Bouwman et al., 2013) (Fig. 1).

During infection of HeLa cells and a human intestinal epithelial cell line, Caco-2 cells, *C. jejuni* activates the Rho family small GTPase Rac1 signaling pathway, which modulates actin remodeling and promotes the internalization of this bacteria. LC3 contribute to *C. jejuni* invasion signaling via the Rac1. LC3 is recruited to bacterial entry site depending on Rac1 GTPase activation just at the early step of the infection. *C. jejuni* infection induced LC3-II conversion, and autophagy induction facilitate *C. jejuni* internalization. Conversely, autophagy inhibition attenuates *C. jejuni* invasion step. In addition, Rac1 recruites LC3 to the cellular membrane, activating the invasion of *C. jejuni* (Fukushima et al., 2022). Taken together, these findings provide insights into the new function of LC3 in bacterial host cell invasion. Authors found the crosstalk between the Rho family small GTPase, Rac1, and autophagy-associated protein, LC3.

A study investigated whether sialylation of *C. jejuni* lipooligosaccharide (LOS) structures, generating human nerve ganglioside mimics, is important for intestinal epithelial translocation. It was shown that *C. jejuni* isolates expressing ganglioside-like LOS bound in larger numbers to the Caco-2 intestinal epithelial cells than *C. jejuni* isolates lacking such structures. Ganglioside-like LOS facilitated endocytosis of bacteria into Caco-2 cells by the recruitment of EEA1, Rab5, and LAMP-1. This increased endocytosis was associated with larger numbers of surviving and translocating bacteria. Authors concluded that *C. jejuni* translocation across Caco-2 cells is facilitated by ganglioside-like LOS, which is of clinical relevance since *C. jejuni* ganglioside-like LOS-expressing isolates are linked with severe gastroenteritis and bloody stools in *C. jejuni*-infected patients (Louwen et al., 2012).

2. Protozoan

2.1. *Trypanosoma cruzi*

Trypanosoma cruzi, etiologic agent of Chagas disease, also known as American tripanosomiasis, is a flagellate protozoan of great importance in Latin America. Chagas disease is a chronic, systemic parasitic infection. It is considered one of the major neglected tropical diseases around the world (Malik et al., 2015). *T. cruzi* is able to actively invade several cell types, including phagocytic and non-phagocytic cells. Trypomastigotes are the main infective forms of the parasite. However, amastigotes can trigger experimental infection both in vitro and in vivo. (Mortara et al., 2005; Rodrigues et al., 2012; Walker et al., 2013).

The process of invasion by trypomastigotes is dynamic and complex. It involves a tight association between several parasite and host factors. Different strains of the parasite activate different proteins and invasion routes during infection (Maeda et al., 2012; Nagajyothi et al., 2012).

T. cruzi cell invasion is considered an evasion mechanism from host immune response and is necessary for the survival and replication of the parasite (Zhang and Tarleton, 1999; Schijman et al., 2004; Andrade and Andrews, 2005). During this process *T. cruzi* may trigger different entry pathways: a lysosomal-dependent pathway where lysosomes are gradually fused with the surface membrane around the parasite, offering membrane for formation of the lysosomal-based endosomes (parasitophorous vacuole) (Tardieux et al., 1992; Rodrigues et al., 1999). In this context, lysosomes are recruited along microtubules in a kinesin-dependent manner to the site of parasite entry in a calcium-dependent process (Rodriguez et al., 1996). Increased expression of the lysosomal membrane glycoprotein Lamp-1 at the cell surface renders CHO cells more susceptible to trypomastigote invasion. Mutation of critical residues in the lysosome-targeting motif of Lamp-1 abolished the enhancement of *T. cruzi* invasion. This suggests that interactions dependent on Lamp-1 cytoplasmic tail motifs, and not the surface-exposed luminal domain, modulate *T. cruzi* entry. Measurements of Ca2 + -triggered exocytosis of lysosomes in these cell lines revealed an enhancement of beta-hexosaminidase release in cells expressing wild-type Lamp-1 on the plasma membrane; this effect was not observed in cell lines transfected with Lamp-1 cytoplasmic tail mutants (Kima et al., 2000). These results also implicate Ca2 + -regulated lysosome exocytosis in cell invasion by *T. cruzi* and indicate a role for the Lamp-1 cytosolic domain in promoting more efficient fusion of lysosomes with the plasma membrane.

A lysosomal-independent pathway which involves the internalization of enveloped parasites by invagination of host cell surface membrane, in a host cell actin-independent mechanism and PI3-kinase pathway dependent process (Woolsey et al., 2003); finally a host cell actin-dependent route when *T. cruzi* trypomastigotes penetrate the cell by expanding the plasma membrane, with significant participation of the host cell actin cytoskeleton, culminating in the formation of parasitophorous vacuole (Woolsey and Burleigh, 2004; Burleigh, 2005; De Souza et al., 2010).

Regardless of the mechanism used for invasion by *T. cruzi*, as such mentioned above, the parasite will be located within a phagolysosome enriched in lysosomal markers (Andrade and Andrews, 2004; Woolsey and Burleigh, 2004). Trypomastigotes before differentiating in amastigotes lyses (escape) the parasitophorous vacuole (Dvorak and Hyde, 1973). Free in host cell cytosol trypomastigotes differentiate into replicative and non-flagellated amastigote forms (Tomlinson et al., 1995).

Different molecules mediate the escape from the phagolysosome, such as trans-sialidase/neuraminidase, which are secreted enzymes by

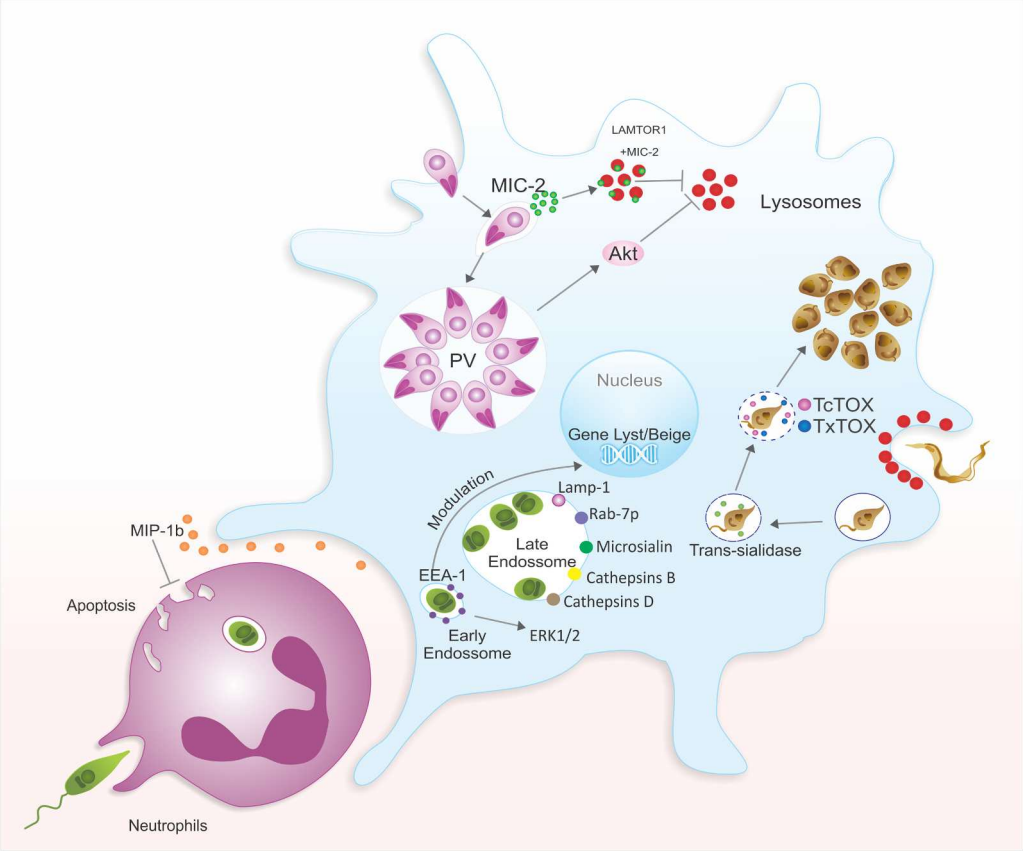

Fig. 2. Subversion mechanisms of lysosomal killing by protozoa - During the invasion by *T. cruzi* several molecules from parasite and host cell are essential for their internalization, which triggers different entry pathways. Following invasion, this parasite can be seen into the vacuole enriched in lysosomal markers. Trypomastigotes are able to secrete trans-sialidase/neuraminidase that will eliminate sialic residues from the parasitophorous vacuole (PV) membrane, which allow the action of two enzymes from *T. cruzi*, the TcTOX (enzyme with hemolysin activity and a C9-like protein) and TxTOX (enzyme encoded by a lytic factor LYT1). The together action of these enzymes induce the PV-membrane fragmentation, which culminates in the phagolysosome lyses. Free in the cytosol, trypomastigotes can differentiate into replicative and non-flagellated amastigote forms. *T. gondii* cannot survive in the lysosomal environment, thus non-fusogenic nature of the PV is essential. The immune system can kill the parasite by disrupting the PV membrane or by redirection the PV to the lysosomes through the autophagy machinery. However, *T. gondii* uses different mechanisms to escape of autophagic killing. This pathogen can induce activation of EGFR-Akt that avoid the autophagy protein-dependent vacuole-lysosomal fusion which ensure the non-fusogenic nature of PV. Besides that, this parasite also is capable to modulates the host-cell microtubules leading to the formation of a barrier for lysosomal fusion. Other escape mechanism used by *T.gondii* is related to the interaction between micronemal protein-2 (MIC-2) and LAMTOR1 (late endosomal/lysosomal adaptor MAPK and mTOR activator 1). The MIC-2 binding to LAMPTOR1 seem to modulate some pathways that prevent the terminal maturation of lysosomes and lysosome-mediated degradation of the parasite, allowing their survival.

parasite that will eliminate sialic residues from the phagosome membrane (Hall et al., 1992). It is becoming the phagosome membrane much sensitive to the action of TcTOX (enzyme with hemolysin activity and a C9-like protein) and TxTOX (enzyme encoded by a lytic factor LYT1) (Andrews et al., 1990; Manning-Cela et al., 2001). It has been shown that these enzymes induce the formation of the small pores in the phagosome membrane, promoting phagosome fragmentation (De Souza et al., 2010).

Andrade and Andrews (2004) have shown lysosomal fusion is essential for the retention of *T. cruzi* inside host cells. It was observed when lysosome-mediated parasite invasion is blocked trough phosophoinositide 3-kinase inhibition, an important fraction of the internalized parasites are not retained inside host cell for a success infection. Thus, a tight correlation can be seen between the lysosomal fusion rates after invasion and the intracellular retention of parasite. However, the role of this residence within lysosomes in the intracellular cycle of *T. cruzi* has continued unclear (Fig. 2).

mTOR pathway is activated during iPSC derived cardiomyocytes (hiPSCCM) *T. cruzi* infection, and the inhibition of mTOR with rapamycin reduced number of *T. cruzi* 48 h post infection. Rapamycin treatment also reduced lysosome migration from nuclei region to cell periphery resulting in less *T. cruzi* inside the phagolysosome in the first hour of infection. Additionally, the number of parasites leaving the phagolysosome to the cytosol to replicate in later times of infection was also lower after rapamycin treatment. Taken together, these data suggest that host mTOR activation concomitant with parasite infection modulates lysosome migration and that *T. cruzi* uses this mechanism to achieve infection and replication. Modulating this mechanism with rapamycin impaired the success of *T. cruzi* life cycle independent of mitophagy (Alvim et al., 2023).

Two main metacyclic stage-specific surface molecules, gp82 and gp90, play determinant roles in target HeLa cell invasion in vitro and in oral *T. cruzi* infection in mice. gp90, purified from poorly invasive G strain metacyclic trypomastigote (MT) and expressing gp90 at high levels, inhibited HeLa cell lysosome spreading and the gp82-mediated internalization of a highly invasive CL strain MT expressing low levels of a diverse gp90 molecule. A recombinant protein containing the conserved C-terminal domain of gp90 exhibited the same properties as the native G strain gp90. These data, plus the findings that lysosome spreading was induced upon HeLa cell interaction with CL strain MT, but not with G strain MT, and that in mixed infection CL strain MT internalization was inhibited by G strain MT, suggest that the inhibition of target cell lysosome spreading is the mechanism by which the gp90 molecule exerts its downregulatory role (Rodrigues et al., 2017).

Authors have shown that cholesterol depletion led to unregulated exocytic events, reducing lysosome availability at the cell cortex and consequently compromised TCT from *T. cruzi* entry into host cells. The results also suggest that cholesterol depletion may modulate the fusion of pre-docked lysosomes at the cell cortex with the invading parasite (Hissa et al., 2012).

Caradonna and Burleigh (2011) proposed that: one of the more accepted concepts in our understanding of the biology of early *T. cruzi*-host cell interactions is that the mammalian-infective trypomastigote forms of the parasite must transit the host cell lysosomal compartment in order to establish a productive intracellular infection. The acidic environment of the lysosome provides the appropriate conditions for parasite-mediated disruption of the phagolysosome and release of *T. cruzi* into the host cell cytosol, where replication of intracellular amastigotes occurs. Recent findings indicate a level of redundancy in the lysosome-targeting process where *T. cruzi* trypomastigotes exploit different cellular pathways to access host cell lysosomes in non-professional phagocytic cells. In addition, the reversible nature of the host cell penetration process was recently demonstrated when conditions for fusion of the nascent parasite vacuole with the host endosomal-lysosomal system were not met. Thus, the concept of parasite retention as a critical component of the *T. cruzi* invasion process was introduced. Although it is clear that host cell recognition, attachment and signalling are required to initiate invasion, integration of this knowledge with our understanding of the different routes of parasite entry is largely lacking.

Murine fibroblast cells lacking LAMP1 and LAMP2 are less permissive to parasite invasion but more prone to parasite intracellular multiplication. Parasites released from LAMP2 KO cells (TcY-L2$^{-/-}$) showed higher invasion, calcium signaling, and membrane injury rates when compared to those released from WT (TcY-WT) or LAMP1/2 KO cells (TcY-L1/2$^{-/-}$). TcY-WT presented an intermediary invasion and calcium signaling rates, compared to the others, in WT fibroblasts, parasites induced lower levels of injury, which reinforces that signals mediated by surface membrane protein interactions also have a significant contribution to trigger host cell calcium signals. These results show that parasites released from WT or LAMP KO cells are distinct from each other. In addition, these parasites ability to invade the cell may be distinct depending on which cell type they interact with. Since these alterations most likely would reflect differences among parasite surface molecules, authors evaluated their proteome. They identified few protein complexes, membrane, and secreted proteins regulated. Among those are some members of MASP, mucins, trans-sialidases, and gp63 proteins family, which are known to play an important role during parasite infection (Oliveira et al., 2022).

2.2. *Leishmania amazonensis*

Leishmania spp are obligate intracellular parasites from Trypanosomatidae family that cause a broad spectrum of human diseases throughout the world. Depending on the parasite species, on the genetic and immunological background of the mammalian host, the clinical form can range from self-healing cutaneous lesions to severe visceralizing disease (WHO, 2016; Hartley et al., 2014).

An important question to a better comprehension of *Leishmania* spp. pathogenesis is to understand the mechanisms underlying parasites persistence in mammalian host, even after the development of specific immune response (Osorio et al., 2007; Arango Duque and Descoteaux, 2015). In this context, the mechanisms of *L. amazonensis* persistence are of particular interest to our research group.

The flagellated, motile forms of *Leishmania* spp. are called promastigotes. They suffer consecutive changes to differentiate into non-dividing infectious 'metacyclic' promastigotes, found within the proboscides of infected sandflies. These parasites enter in mammalian host cells during insect blood meal and differentiate into aflagellate amastigotes. Amastigotes live as intracellular parasites in a variety of mammalian cells, mainly in professional phagocytes (Moradin and Descoteaux, 2012).

Like other pathogens, *Leishmania* spp are often first met by neutrophils (Kaye and Scott, 2011). Data in the literature indicate that neutrophils are not useful in the immune response against *Leishmania*. They have been proposed for establishing infection as a 'Trojan Horse'. Neutrophils ingest *Leishmania* parasites, delaying neutrophil apoptosis (Laskay et al., 2003). During this delay, of about 2 days, neutrophils secrete MIP-1β, a chemokine that attracts macrophages to the infection site. After the arrival of macrophages, occurs phagocytosis *Leishmania*-infected apoptotic neutrophils, so not active inflammatory responses. As a result, *Leishmania* was able to silently enter into their definitive host and establish infection (Van Zandbergen et al., 2004).

On the other hand, parasites can be phagocytosed directly through interaction with some receptors as the mannose receptor, first/three complement receptors, Fcγ-receptor, fibronectin receptors or by endocytosis (Wyler et al., 1985; Ueno and Wilson, 2012). Inside RAW 264.7 murine macrophage cell line, intracellular amastigotes replicate within a phagosome that share several properties with late endosomes/lysosomes, including low luminal pH and the presence of lysosome-specific membrane proteins and acidic hydrolases (Ndjamen et al., 2010).

The phagosome biogenesis involves markers present in late endosomes of the host, including LAMPs, MHCII molecules and Rabs (Antoine et al., 1998). To better understand the kinetics of formation of phagosome, Courret et al. (2002) showed that both metacyclic and amastigotes-containing phagosome acquire rapidly, about 30 min, markers of late endosomes as LAMP-1, microsialin, cathepsins B and D and Rab-7p after infection. Otherwise, these markers late endosomes/lysosomes were acquired through fusion with late endocytic compartments. The early endosome markers, EEA1 and the transferrin receptor, were hardly detected in parasite-containing compartments regardless of the parasitic stage and the time after infection. The mechanisms related to parasite residence into phagolysomes are not completely understood.

Intriguingly, *L. amazonensis* amastigotes replicate within very large phagosomes that continuously undergo fusion with lysosomes and phagolysosomes. Unlikely, phagosomes containing other *Leishmania* species (*L. major* and *L. donovani*) (Real and Mortara, 2012) are formed tightly adhered to individual amastigotes. It has been suggested that phagosome expansion might protect *L. amazonensis* from host killing mechanisms, by diluting microbicidal molecules (Antoine et al., 1998). However, comparing with other species questions can be raised upon this putative explanation for phagosome expansion. Would *L. amazonensis* be more susceptible to microbicidal host molecules? What would be the actual reason for this highly different way of life comparing to other species from this gender? (Fig. 2).

Localization of cathepsins B, H, L, and D was investigated in a study by using specific immunoglobulins. In uninfected rat bone marrow cells, these enzymes were located in perinuclear granules (most of them were probably secondary lysosomes) which, after infection, disappeared progressively. In infected macrophages, cathepsins were detected mainly in the phagosome, suggesting that the missing secondary lysosomes had fused with these organelles. Biochemical assays of various proteases (cathepsins B, H, and D and dipeptidyl peptidases I and II) showed that infection was accompanied by a progressive increase of all activities tested, except that of dipeptidyl peptidase II, which remained constant. No more than 1–10% of these activities could be attributed to amastigotes. These data indicate that (i) *Leishmania* infection is followed by an increased synthesis and/or a reduced catabolism of host lysosomal proteases, and (ii) amastigotes grow in a compartment rich in apparently fully active proteases. Unexpectedly, it was found that infected and uninfected macrophages degraded endocytosed proteins similarly. The lack of correlation in infected macrophages between increase of protease activities and catabolism of exogenous proteins could be linked to the huge increase in volume of the lysosomal compartment (Prina et al., 1990).

Some attempts to understand this complex life style, Wilson et al. (2008) showed that host cells (murine bone marrow-derived macrophages and mouse embryonic fribroblasts, MEF cell line) infected with *L. amazonensis* upregulate transcription of LYST/Beige, a gene known to regulated lysosome size. Mutations in LYST/Beige caused further phagosome expansion and enhanced *L. amazonensis* replication. In contrast, LYST/Beige overexpression led to small phagosomes that did not sustain parasite growth. Taking together, authors concluded that upregulation of LYST/Beige in infected cells functions as a host innate response to limit parasite growth, by reducing phagosome volume and inhibiting intracellular survival.

Furthermore, after *L. amazonensis* infection, there is an increase in MAPK extracellular signal regulated kinase (ERK1/2) phosphorylation within both macrophages and dendritic cells (Boggiatto et al., 2009; Mukbel et al., 2007). This ERK1/2 phosphorylation promotes an immature dendritic cell phenotype and macrophages unable to destroy intracellular *L. amazonensis* (Mukbel et al., 2007). In MAPKs pathway, upon binding of MP1 with MEK1/2, ERK1/2 is tethered to the *L. amazonensis* phagosome and promotes further pro-retention signals parasite. *L. amazonensis* use of the phagosome/endosomal-specific p14/MP1 complex scaffold promotes sustained ERK1/2 phosphorylation (Boggiatto et al., 2014), alteration of the phagocyte oxidative burst (Mukbel et al., 2007) and leads to chronic infection (Boggiatto et al., 2014; Boggiatto et al., 2009; Gibson-Corley et al., 2012). And also, *L. amazonensis*-mediated ERK1/2 activation ROS prevents production, possibly through blockage of Akt phosphorylation. This would prevent p47phox phosphorylation, NADPH complex formation, a lack of superoxide production, and pathogen clearance (Mukbel et al., 2007; Martinez and Petersen, 2014) (Fig. 2).

Concurrently, *L. amazonensis* has been shown to inhabit the perinuclear region within its phagosome (Wilson et al., 2008; Cortez et al., 2011). This can be beneficial to the parasite, because of the presence of nutrients near the Golgi complex. *L. amazonensis* secretes the signal indicates for ERK1/2 to begin to phosphorylate the intermediate chain (IC) of dynein at an increased level, thereby increasing its own chances to find the vacuole it has just entered toward the nucleus. This seems to support the idea that phosphorylated ERK1/2 that is close to the nucleus promotes activation of substrates that are beneficial for its survival. Modulating the spatial location may be used for the benefit of infection through which signaling molecule are able to target signal transduction (Chang and Karin, 2001). From these data, it is clear the importance of host cell modulation by the parasite in order to survive and perpetuate infection.

An interesting novel finding is that *L. amazonensis* can induce their own entry into fibroblasts independently of actin cytoskeleton activity, and, thus, through a mechanism that is distinct from phagocytosis. Invasion involves subversion of host cell functions, such as Ca^{2+} signaling and recruitment and exocytosis of host cell lysosomes involved in plasma membrane repair (Cavalcante-Costa et al., 2019). This means to be a similar behavior to *T. cruzi* host cell invasion.

2.3. *Toxoplasma gondii*

Toxoplasma gondii is an intracellular protozoan of the phylum Apicomplexa and etiologic agent of toxoplasmosis (Tenter et al., 2000). This parasite has a successful invasion mechanism and can infect a wide variety of warm-blooded animals, including humans (Dubey, 2010; Sibley, 2010). Adhesion to the host cell is initiated by surface antigens (SAGs) and micronemal proteins (MICs) of *T. gondii* (Zhou et al., 2005; Pollard et al., 2008; Blader and Saeij, 2009). Once secreted, MICs are present at the parasite surface membrane and can interact with host cell receptors (Carruthers and Tomley, 2008). After joining, *T. gondii* utilizes a specific and unique mode of locomotion named “gliding motility”. This movement is dependent on parasite actin and myosin and together with the binding to host cell receptor by MICs enables the parasite to get close to and entry into the cell (Sibley, 2010). Subsequently, occurs release proteins derived from rhoptries (ROPs) which form complexes capable of delivering the parasite into the host cell, and finally there is the formation of parasitophorous vacuole (PV) (Straub et al., 2009).

Tachyzoites of *T. gondii* resides and survive inside the phagosome of COS-1 cell line that is basically derived of invagination of the host plasma membrane (Suss-Toby et al., 1996). During the penetration into HFF cell line, the tachyzoites excludes some host cell surface proteins, like determinants that target the vacuole for endocytic process, and insert parasite proteins, ensuring the stay alive and replication (Håkansson et al., 2001; Joiner and Roos, 2002).

Using mouse peritoneal macrophages and the HFF1 fibroblast cell line and inhibitors of distinct endocytic pathways, a research group showed that treatment of host cells with compounds that interfere with clathrin-mediated endocytosis. Additionally, treatments that interfere with macropinocytosis, such as incubation with amiloride or IPA-3, increased parasite binding to the host cell surface but blocked parasite internalization. Immunofluorescence microscopy showed that markers of macropinocytosis, such as the Rab5 effector rabankyrin 5 and Pak1, are associated with parasite-containing cytoplasmic vacuoles. These results indicate that entrance of *T. gondii* into mammalian cells can take place both by the well-characterized interaction of parasite and host cell

endocytic machinery and other processes, such as the clathrin-mediated endocytosis, and macropinocytosis (Portes et al., 2020).

T. gondii cannot survive in the lysosomal environment, thus non-fusogenic nature of the phagosome is essential for replication and maintenance of the parasite inside primary cultures of murine astrocytes and mouse peritoneal exudate cells. The immune system can kill the parasite by disrupting the phagosome membrane through the effects of IFN-γ/Immunity related GTPases (Martens et al., 2005; Zhao et al., 2008) and by making the phagosome fusogenic through the effects of CD40 ligation that re-routes the phagosome to the lysosomes through the autophagy machinery (Andrade et al., 2006; Van Grol et al., 2013; Portillo et al., 2010). It was seen that 25–35% of $CD40^{+}$ cells subjected to CD40 ligation are unable to promote death of T. gondii, suggesting that the parasite may utilize mechanisms to prevent induction of autophagic killing (Muniz-Feliciano et al., 2013).

Muniz-Feliciano et al. (2013) showed an evasion mechanism that is based on the fact that *T. gondii* can induce activation of EGFR-Akt, a signaling cascade that prevents autophagy protein-dependent vacuole-lysosomal fusion, avert targeting of the parasite by $LC3^{+}$ structures and pathogen killing. The parasite proteins MICs containing EGF domains (EGF-MICs) appeared to promote EGFR activation and upstream of Akt phosphorylation and thus maintain the non-fusogenic nature of the phagosome. Furthermore, after internalization the phagosome is involved by endoplasmic reticulum and mitochondria and within 18 h there is the presence of microtubules around the phagosome, suggesting the formation of a barrier for lysosomal fusion (Andrade et al., 2001).

Previous studies have demonstrated that the interaction between the integrin-like A domain of MIC-2 and the host protein LAMTOR1 (late endosomal/lysosomal adaptor MAPK and mTOR activator 1) may be considered as one of the parasite evasion mechanisms. LAMTOR1 is a membrane adaptor protein localized exclusively to the surface of lysosomes and late endosomes/lysosomes that is involved in promoting lysosome-mediated degradation of cellular components (Nada et al., 2009; Wang et al., 2014). Formation of LAMTOR1-p14-MP1 complex is important for regulating the activity of the mammalian target of rapamycin complex 1 (mTORC1), that is crucial for the terminal maturation of lysosomes and late endosome–lysosome fusion (Takahashi et al., 2012; Wang et al., 2014). Then, MIC-2 may play roles in modulating biological processes by binding to LAMTOR1 and avoid lysosomal fusion.

T. gondii is an example of a pathogen that utilizes strategies to manipulate host cell signaling, vesicular trafficking and avoids eradication. One of the evasion mechanisms is the maintenance inside the phagosome and avoidance of the fusion with lysosome, allowing its survival (Fig. 2).

3. Fungi

3.1. Candida albicans

Candida albicans is a member of the human microbiota and colonizes the oral cavity, gastrointestinal and urogenitary tract of up to 70% or more of the population. (Mavor et al., 2005). *C. albicans* disseminates through the epithelial barriers reaching the bloodstream, it can cause life-threatening systemic infections. During both commensal and pathogenic relationships, attachment to the epithelial cells is a most important step to initiate the interaction. Host cell invasion and damage are likely critical virulence attributes of *C. albicans* (Filler et al., 1995; Park et al., 2005).

Oral epithelial cells are the first cells that conects with *C. albicans* during the establishment of oropharyngeal candidiasis. Following initial adhesion, *C. albicans* invades oral epithelial cells. Authors examined the trafficking of *C. albicans* through immortalized normal human oral keratinocyte cell line OKF6/TERT2 and primary human oral epithelial cells endocytic compartments. They showed evidence that *C. albicans* is internalized by oral epithelial cells through actin-dependent clathrin-mediated endocytosis and is taken into vacuolar compartments immediately following its internalization. *C. albicans*-containing endosomes transiently acquired early endosomal marker EEA1, but showed marked defects in acquisition of late endosomal marker LAMP1 and lysosomal marker cathepsin D (Zhao, Villar, 2011). Defects on endolysosomal maturation may partially explain the inability of oral epithelial cells to eliminate *C. albicans*.

Infection of RAW264.7 macrophages with *C. albicans* induce inhibition of the phagosome fusion with compartments enriched in the lysobisphosphatidic acid and the vATPase, and thereby the acquisition of a low pH from the outset of infection. Besides, the pathogen displays of additional specific survival strategies to prevent its targeting to compartments displaying late endosomal/lysosomal features, such as induction of active recycling out of phagosomes of LAMP-1 and the lysosomal protease cathepsin D (Fernández-Arenas et al., 2009).

Studies have found that this fungus is also able to invade endothelial cells in vitro by inducing its own endocytosis (Filler et al., 1995; Park et al., 2005). During invasion, fungus proteins binding to the surface molecules from host cell, which trigger a strong microfilament rearrangement and pseudopods formation that engulf the organism and pull it into the cell. Whereas active penetration relies on fungal viability, that represents the dominant invasion mechanism (Phan et al., 2005; Wachtler et al., 2012). Following internalization, phagosomes containing *C. albicans* acquired Rab14 shortly after uptake and this GTPase remains associated with phagosome for a little time. In this context, Okai et al. (2015) have showed that the silencing of Rab14 had no effect on markers of early phagosome maturation and did not significantly affect Rab5/Rab7 conversion but delayed the acquisition of key markers of late-stage maturation process in infected RAW 264.7 and J774.1 murine macrophage cell lines. Interestingly, this delay during the late-stage phagosome maturation had been seen related to the pathogen-escape mechanism from the microbicidal activity (Okai et al., 2015). Then, *C. albicans* is able to manipulate phagosome maturation, resist killing by macrophages and replicates intracellularly (Tavanti et al., 2006).

Other escape mechanism by *C. albicans* occurs during the yeast-to-hyphae transition that plays a pivotal role in facilitating escape from phagocytes, by production of carbon dioxide (CO_2) that induces hyphal growth allowing piercing and killing macrophages. In summary, phagocytosed fungal cells have the ability to precisely sense the surrounding environment and appropriately modify their transcriptional profile. Although intracellular survival strategies allow persistence within phagocytes, pathogens eventually escape to spread and infect distant tissue. (Seider et al., 2010; Marcil et al., 2002). In conclusion, the attachment in epithelial cell is very important to establish the infection, the morphology of *C. albicans* and Rab GTPases has a pivotal role to allow the escape from phagocytes and reach other tissues (Fig. 3).

Invading hyphae of *C. albicans* secrete candidalysin, a pore-forming peptide toxin. To prevent cell death, epithelial cells must protect themselves from direct damage induced by candidalysin and by the mechanical forces exerted by expanding hyphae. Authors have identified two key Ca^{2+}-dependent repair mechanisms employed by TR146 human oral epithelial cell line to withstand candidalysin-producing hyphae. They demonstrated candidalysin secretion directly into the invasion pockets induced by elongating *C. albicans* hyphae. The toxin induces oscillatory increases in cytosolic [Ca^{2+}], which cause hydrolysis of PtdIns(4,5)P_2 and loss of cortical actin. Epithelial cells dispose of damaged membrane regions containing candidalysin by an Alg-2/Alix/ESCRT-III-dependent blebbing process. At later stages, plasmalemmal tears induced mechanically by invading hyphae are repaired by exocytic insertion of lysosomal membranes. These two repair mechanisms maintain epithelial integrity and prevent mucosal damage during both commensal growth and infection by *C. albicans* (Westman et al., 2022).

Autophagy is known to be involved in the epithelial barrier maintenance, especially the intestinal barrier that is continuously challenged by exposure to the gut microbiota or to xenobiotics. Key proteins of the autophagy and vesicles presenting features of autophagosomes are

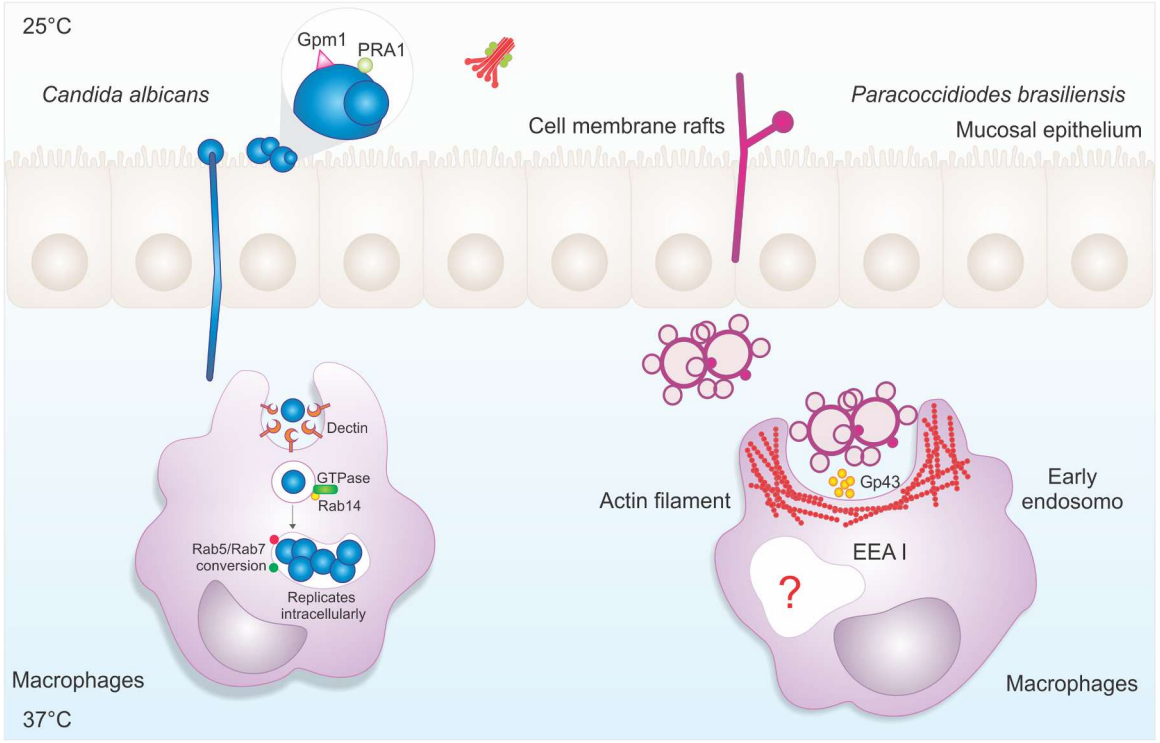

Fig. 3. Subversion mechanisms of lysosomal killing by fungi -The pH regulates antigen 1 (Pra1) and a phosphoglycerate mutase (Gpm1) are proteins used by *C. albicans* to evade from immune system by complement-mediated uptake modulation. After uptake, endosomes containing *C. albicans* acquired transiently Rab14 for a short time. During the late-stage phagosome maturation process, *C. albicans* promotes the modulation of acquisition of key lysosomal markers to evade from lysosomal killing. *P. brasiliensis* during the invasion, is able to secrete the gp43 (antigenic glycoprotein) that has the capacity to trigger host-cells signals, such as rearrangement of the cytoskeleton, clustering of epithelial cell membrane rafts, which are important events during the establishment of infection. Few hours after infection, ingested *P. brasiliensis* inside the endosome promotes strongly decrease of Early Endosome Antigen I (EEAI) – present in the surrounding of endosome membrane – that culminates in a block of endosome-lysosome fusion.

recruited at *C. albicans* invasion sites. These events are associated with host plasma membrane damage caused by the active penetration of *C. albicans*. ATG5 and ATG16L1 proteins contribute to plasma membrane repair mediated by lysosome exocytosis and participate in protection of epithelial cells integrity against *C. albicans*-induced cell death (Lapaquette et al., 2022).

Recently, authors have studied whether phagosomes from mouse bone marrow derived macrophages regulate their size to preserve integrity during infection with *C. albicans*. Phagosomes release calcium as *C. albicans* hyphae elongate, inducing lysosome recruitment and insertion, thereby increasing the phagosomal surface area. As hyphae grow, the expanding phagosome consumes the majority of free lysosomes. Simultaneously, lysosome biosynthesis is stimulated by activation of TFEB. Preventing lysosomal insertion causes phagosomal rupture, NLRP3 inflammasome activation, IL-1β secretion and host-cell death. Whole-genome transcriptomic analysis demonstrate that stress responses elicited in *C. albicans* upon internalization are reversed if phagosome expansion is prevented (Westman et al., 2020). Their data revealed a mechanism whereby phagosomes keep integrity while expanding, ensuring that growing *C. albican* remain entrapped within this microbicidal compartment.

3.2. *Paracoccidioides brasiliensis*

The fungus *Paracoccidioides brasiliensis* is the etiologic agent of paracoccidioidomycosis (PCM), a systemic granulomatous mycosis that is endemic to South America (San-Blas and Burger, 2011; Restrepo et al., 2012). PCM is probably acquired by inhalation of saprophytic mycelial structures into the lungs where the fungus can differentiate into the pathogenic yeast form (Tavares et al., 2013). Once in the lungs, *P. brasiliensis* is able to adhere, invade and disrupt the barriers imposed by the host tissues and can be internalized by professional phagocytes such as neutrophils, alveolar macrophages, dendritic cells and epithelial cells (Brito et al., 1973; Mendes- Giannini et al., 2000).

The first line of host defense is based on the recognition-pathogen associated molecular patterns (PAMPs), by Toll-like receptors (TLRs) such as TLR-2, TLR-4 and TLR-9 as well as the C-type lectin receptor (CLR) dectin-1 (Bonfim et al., 2009; Loures et al., 2010; Tavares et al., 2013; Menino et al., 2013). During the fungus-host cell interaction, *P. brasiliensis*, a facultative intracellular human pathogen, has the capacity to trigger host-cells signals, such as rearrangement of the cytoskeleton, clustering of epithelial cell membrane rafts, which can be a crucial step for the establishment of infection in mammalian host cells (Silva et al., 2006; Maza et al., 2008). Moreover, evidences suggesting that gp43, is a secreted antigenic glycoprotein by fungus and have a crucial role during the invasion (Popi et al., 2002; Silva et al., 2006).

Several microscopic studies showed that monocytes and monocyte-derived macrophages support the intracellular replication of ingested *P. brasiliensis* (Brummer et al., 1988; Brummer et al., 1989; Moscardi-Bacchi et al., 1994). However, little is known about the factors required for intracellular persistence of this fungus and still remains unclear the precise mechanisms governing maturation of phagosomes into phagolysosomes during PCM. (Derengowski et al., 2008; Voltan et al., 2013).

In the last years, some studies have showed that *P. brasiliensis* uses a complex transcriptional and translational program to survive inside the endosome (Grossklaus et al., 2013; Lima et al., 2014; Parente et al., 2015). Interestingly, when exposed to nitrosative and oxidative stresses, *P. brasiliensis* presented a global activation of antioxidant enzymes, such as catalases, superoxide dismutase, cytochrome c peroxidase and

thioredoxin, showing their resistance against the host immune response (Campos et al., 2005).

Other important escape mechanism used by *P. brasiliensis* to avoid the destruction by immune response is related with the modulation of EEAI, a protein responsible for vesicle budding, transporting, tethering, docking, fusion membrane, and functional organelle identity events in the early endosomes. During the infection of alveolar macrophages murine line AMJ2-C11 by *P. brasiliensis* is observed the expression and localization of EEAI in early endosomes on the first hours. Interestingly, few hours after infection this fungus promotes intense decrease of EEAI. Alterations in phagosome maturation featured by the EEAI losing provides strong evidence to a block in trafficking from the trans-Golgi network to phagosomes. Therefore, the decrease of EEAI can be considered a survival strategy used by *P. brasiliensis* consisting in inhibiting phagosome-endosome fusion, which also is observed in other parasites (Voltan et al., 2013) (Fig. 3).

4. Virus

4.1. Hepatitis B virus

The human hepatitis B virus (HBV) is a small enveloped DNA virus, considered the most significant human pathogen, with an estimated two billion people infected worldwide, and 350 million are chronic carriers. HBV is member of a family called hepadnaviruses, which infect a restricted number of mammals and birds. These viruses share a narrow host range and preferential tropism for hepatocytes (Neuveut et al., 2010). Chronic hepatitis B is a major risk factor for serious liver disease, including cirrhosis and hepatocellular carcinoma (HCC). HCC is the fifth most common cancer and the third leading cause of cancer death worldwide (Neuveut et al., 2010; El-Serag, 2012).

A major progress for the understanding of HBV life cycle was the identification of sodium taurocholate cotransporting polypeptide (NTCP) as entry relevant factor of HBV. The identification of NTCP as an entry relevant factor was associated with the observation that myristoylated peptides covering the N-terminus of the PreS1 domain efficiently block HBV infection (Yan et al., 2012). Years before NTCP was identified as entry factor, there was already evidence that HBV enters the cell by receptor-mediated endocytosis. This step depends on cellular factors, such as caveolin-1 and dynamin-2, as significant reduction of HBV transcripts and antigens was observed in HepaRG cells expressing mutants of caveolin-1 and dynamin-2. HBV entry is independent on lipid raft-/caveolin-mediated endocytosis, but that clathrin-mediated endocytosis mediates the NTCP-dependent entry of HBV. Knockdown of clathrin heavy chain (CHC), dynamin-2 and clathrin adaptor protein AP-2 reduced the susceptibility to HBV (Herrscher et al., 2020).

Furthermore, endosome-associated cellular Rab GTPases Rab5A and Rab7A have been demonstrated to be involved in transporting of HBV viral particles from early endosomes (rich of Rab5) to late endosomes (rich of Rab7). Silencing of either Rab5A or Rab7A leads to a significant inhibition of the early HBV infection, and a considerable number of virions were found in the late endosomes in a time-dependent manner. In contrast to this, silencing of Rab9 and Rab11, that are involved in the transport of the endocytotic vesicle to the recycling endosome or the trans Golgi complex, has no effect on the HBV entry. Although HBV viral particles are further transported to lysosome compartment, viral infectivity does not depend on the lysosomal activity. The machinery for endocytosis of epidermal growth factor receptor (EGFR) coordinates the transport of incoming hepatitis B virus to the endosomal network. This process involves EGF-R phosphorylation and the subsequent recruitment of adaptor molecules such as AP2A1 and Eps15. The EGFR-sorting machinery coordinates HBV transport in the endosomal network (early and late endosome and lysosome). In accordance to this, suppression of EGFR ubiquitination impairs HBV infection. These observations imply that translocation of the virus to late endosomes is critical for a successful infection. However, how the viral particles deliver their nucleocapsids from endosomal compartments into the cytoplasm was not clearly elucidated in these studies (For review: Jiang and Hildt, 2020).

Autophagy is a mechanism that mediates the removal of macromolecules and organelles damaged through a lysosomal degradation pathway. It is used also by cells to eliminate invading pathogens. However, in infected cells by HBV virus, the mechanisms responsible for induction autophagic and the viral replication steps affected by autophagy are still controversial (Tang et al., 2012; Yang et al., 2015). Therefore, studies have shown that HBV can enhance the autophagic process in hepatoma cells.

The X protein of hepatitis B virus (HBx), a small soluble cytoplasmic protein, has been seen as an oncoprotein in viral carcinogenesis. To promote virus replication, HBx subverts cellular activities such as signal transduction, transcription, autophagy, and proliferation (Sir et al., 2010; Czaja, 2011; Tang et al., 2012; Yang et al., 2015). The HBx apparently induces the accumulation of cellular dysfunctions and damage to the benefit of the virus, suggests that autophagy induction mediated by HBx uses the route of class III phosphatidylinositol 3-kinase (PI3K-C3) to increase its activity, induce autophagy and promote their replication (Yang et al., 2015).

Others studies have shown the HBx protein promotes beclin-1 activation, in contrast, when such gene is silenced by RNAi induction of autophagy is blocked by HBx (Tang et al., 2009; Ni et al., 2012; Tang et al., 2012). It was also observed in knockout mice for Atg5 gene a decrease HBV DNA replication thereby confirming that autophagy is required for efficient replication of HBV DNA (Tian et al., 2011; Silva and Jung, 2013).

On the contrary, another study showed that the deletion of small surface proteins (SHBs), synthesized as a transmembrane protein spanning the membrane of the ER, abrogated the HBV-induced autophagy while the deletion of HBx did not, suggests that the enhancement of the autophagic response by HBV was dependent on SHBs. In addition, overexpression of SHBs triggered a wrong unfolded protein response (UPR) by increasing the autophagosome production (Li et al., 2011; Ni et al., 2012; Tang et al., 2012; Yang et al., 2015). The stress induction of endoplasmic reticulum (ER) caused by SHBs is autophagy-inducing and activates signaling pathways; Perk, ATF6 and IRE1 and that blocking any of pathways inhibits autophagy (Tang et al., 2012; Yang et al., 2015).

HBx-induced autophagosome formation is accompanied by unchanged MTOR (mechanistic target of rapamycin) activity and decreased degradation of LC3 and SQSTM1/p62, the typical autophagic cargo proteins. HBx impairs lysosomal acidification leading to a drop in lysosomal degradative capacity and the accumulation of immature lysosomes possibly through interaction with V-ATPase affecting its lysosome targeting. In addition, clinical specimen test showed increased SQSTM1 and immature lysosomal hydrolase cathepsin D in human liver tissues with chronic HBV infection and HBV-associated liver cancer. These data suggest that a repressive effect of HBx on lysosomal function is responsible for the inhibition of autophagic degradation (Liu et al., 2014).

Mannosidase-like proteins (EDEMs) function as lectins that recognize terminally misfolded glycoproteins and have an important role in relieving the ER stress during HBV life cycle. It has been found that the synthesis of EDEMs (EDEM1 and homologs thereof, EDEM2 and EDEM3) is significantly overexpressed in cells with transient or persistent HBV replication (Lazar et al., 2012). The presence of viral surface proteins EDEM1 led to the degradation of the viral envelope by autophagy, suggesting that it may well be the mechanism for HBV controlling the amount of virus produced in infected cells and to establish a chronic infection (Lazar et al., 2012; Yang et al., 2015). Overall HBV appears to subvert for its favor the autophagic machinery, inducing their action to their intracellular replication and permanence (Fig. 4).

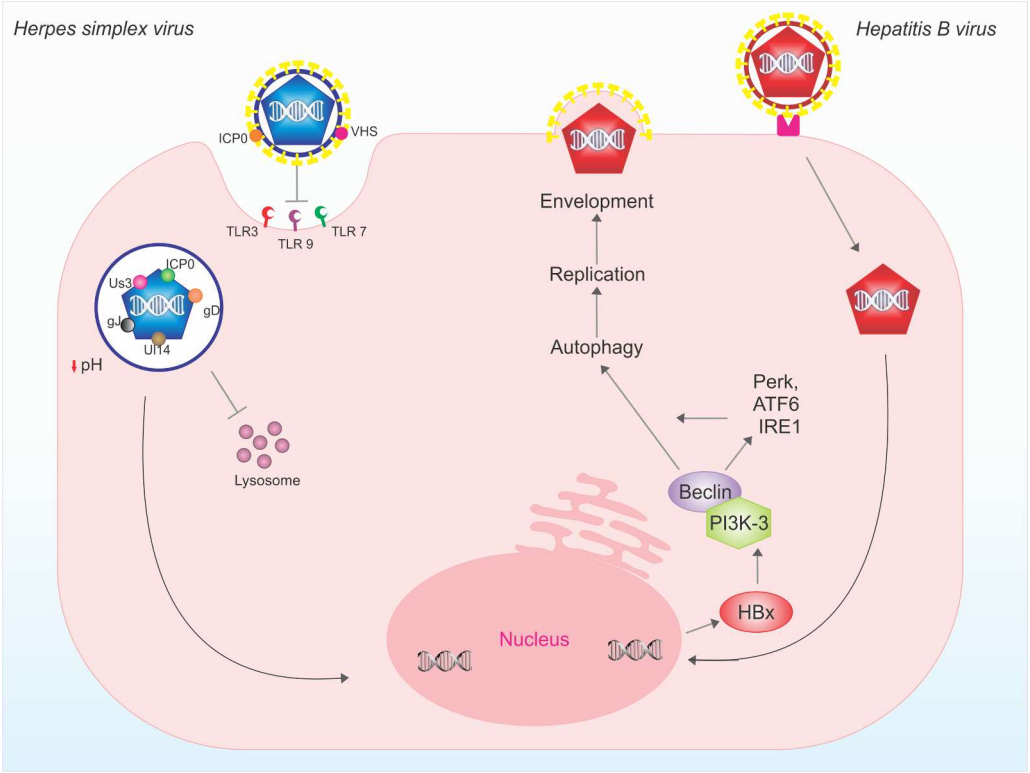

Fig. 4. Subversion mechanisms of lysosomal killing by virus - X protein of hepatitis B virus (HBx) is a viral oncoprotein involved in the host-cell autophagy activation through the phosphatidylinositol 3-kinase III (PI3K-C3) pathway. HBx protein promotes beclin-1 activation, which interacts with PI3K-C3 playing an up-regulation of autophagy. Small surface proteins (SHBs) HBV promote a stress induction of endoplasmic reticulum (ER) triggered a strong autophagosome production. Although the autophagy pathway is emerging as a component of host defense, HBV have developed strategies to counteract these antiviral mechanisms, which appear that cooperation with autophagy machinery as proviral host factors favoring viral replication. Regarding herpes simplex virus (HSV), internalized HSV are enveloped and located in large vesicles that mimic early stage endosomes, to facilitate its transit into the cytosol. Their traffic through the endocytic pathway is regulated at different stages by Rab 5 and Rab 11 (trafficking from plasmatic membrane to the early endosome); Rab 9 (early endosomes maturation to late endosomes) and Rab 7 (trafficking from Trans Golgi). The ICP34.5, a HSV viral protein, interacts with beclin-1, which allow evading the autophagy process. Other escape mechanism used by HSV is related to the gD expression in endosome. This glycoprotein seems interacts with mannose 6-phosphate (M6P) and is able to bind to M6P receptors in endosomes blocking the modified of the soluble lysosomal enzymes by the phosphotransferase M6P and altered of early endosomes maturation. Therefore, the gD can bind to signal peptide peptidase (SPP), affecting the lysosomes fusion and the endoplasmic reticulum (ER) responses.

4.2. Herperviridae

The Herpesviridae family includes more than 200 species closely related pathogens of man and animal and the largest subfamily of the herpesviruses is the Alpha-herpes-viruses that include the herpes simplex virus (HSV) (Kukhanova et al., 2014). These viruses, in general, present the ability to persist in a non-replicative, latent state in their host during its entire lifetime, infecting the nerve system of skin (Iannello et al., 2011).

To gain access to cells, the herpes simplex viruses (HSVs) must replicate and block immediately the host responses. The expression of ICP0 protein (Gianni et al., 2012) and VHS protein (Yao and Rosenthal, 2011) can block signaling mediated through TLR pathways (TLR3, TLR9 and TLR7) and non-TLR pathways (RIG-1/MDA5), respectively, interfering in host detection of viral determinants. Besides this, the protein expression of HSVs, such as gD, gJ, UL14, ICP0 and US3, can interfere in cell viability blocking apoptosis and the autophagosome is inhibited by the viral protein γ34.5 (Suazo et al., 2015).

Entry of herpes simplex virus type 1 (HSV-1) and HSV-2 is a complex process between the viral envelope, especially glycoprotein gD, and the cell surface molecules (Fuller and Lee, 1992). Kukhanova and colleagues (2014), proposed two HSV-1 entry pathways, the first involved the fusion of the viral envelope with the plasma membrane and transport of the viral capsid to the nucleus. The other pathway by which the virus enters the cell is endocytosis of the enveloped virion followed by fusion of the envelope with intracellular vesicles.

Clement et al. (2006) demonstrated that endocytosis of the HSV-1 mimic many features of phagocytosis involving rearrangement of actin cytoskeleton and trafficking of the virions. The internalized HSV is located in large vesicles but not in clathrin-coated pits as seen in typical endocytosis. HSV-1–infected primary human eye tissues cells presents relatively few protrusions, depending on the dynamin 2 and low pH.

The virus appears to stabilize vesicles in Vero, HFFF-2 and HeLa cells that mimic early stage endosomes, to facilitate its transit in the cytoplasm. Cargo movement through the endocytic pathway is regulated at different stages by a number of Rab proteins and depletion of Rab 5 and Rab 11, involved in trafficking from the plasmatic membrane to the early endosome, significantly reduced virus yield while depletion of Rab9 or Rab7, involved in maturation of early endosomes to late endosomes and trafficking from to the Trans Golgi, respectively, had limited effect on virus yield. Evidences that the late endocytic pathway is not involved in virus production (Hollinshead et al., 2012).

HSV has evolved a mechanism to evade the autophagy process via interaction of ICP34.5 protein with the essential autophagy protein Beclin-1, and besides infection induces an autophagy response, this is antagonized by the HSV-1 neurovirulence gene product, ICP34.5 (Orvedahl et al., 2007; Leib et al., 2009).

Herpes simplex virus (HSV) glycoprotein D (gD) is modified with mannose 6-phosphate (M6P) and binds to M6P receptors (MPRs). MPRs are involved in the well-characterized pathway by which lysosomal enzymes are directed to lysosomes via a network of endosomal membranes. Based on the impaired ability of HSV to form plaques under

conditions in which glycoproteins could not interact with MPRs, authors proposed that MPRs may function during HSV egress or cell-to-cell spread (Brunetti et al., 1995). To further analyze M6P modification and intracellular trafficking of gD in the absence of other HSV proteins, adenovirus (Ad) vectors were used to express soluble and membrane-anchored forms of gD. Both membrane-bound and soluble gD were modified with M6P residues and were localized to endosomes that contained the 275-kDa MPR or the transferrin receptor. Similar results were observed in HSV-infected cells. Cell fractionation experiments showed that gD was not present in lysosomes. However, a mutant form of gD and another HSV glycoprotein, gI, that were not modified with M6P were also found in endosomes in HSV-infected cells. Moreover, a substantial fraction of the HSV nucleocapsid protein VP6 was found in endosomes, consistent with accumulation of virions in an endosomal compartment. Therefore, it appears that HSV glycoproteins and virions are directed to endosomes, by M6P-dependent as well as by M6P-independent mechanisms, either as part of the virus egress pathway or by endocytosis from the cell surface (Brunetti et al., 1998).

Glycoprotein K (gK) is a virion envelope protein of herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), which plays important roles in virion entry, morphogenesis and egress. Two-hybrid and pull-down assays were utilized to demonstrate that gK and no other HSV-1 genes specifically binds to signal peptide peptidase (SPP), also known as minor histocompatibility antigen H13. SPP dominant negative mutants, shRNA against SPP significantly reduced HSV-1 replication in vitro. SPP also affected lysosomes and ER responses to HSV-1 infection. Thus, gK, despite its role in fusion and egress, is also involved in binding the cytoplasmic protein SPP. These results also suggest that SPP plays an important role in viral replication and possibly virus pathogenesis. This makes SPP unique in that its function appears to be required by the virus as no other protein can compensate its loss in terms of viral replication. (Allen et al., 2014).

HSV-encoded glycoprotein B (gB) is the most abundant protein in the viral envelope and promotes fusion of the virus with the cellular membrane. Authors have found that gB impacts on the major histocompatibility complex (MHC)-II pathway of antigen presentation by fostering homotypic fusion of early endosomes and trapping MHC-II molecules in these altered endosomes. By using an overexpression approach, they demonstrated that transient expression of gB induces giant vesicles of early endosomal origin, which contained Rab5, EEA1, and large amounts of MHC-II molecules, but no CD63. In HSV-1-infected and stably transfected cell lines that expressed lower amounts of gB, giant endosomes were not observed, but strongly increased amounts of HLA-DR and HLA-DM were found in EEA1 + early endosomes. They used these giant vesicles as a model system and revealed that gB interacts with Rab5 and EEA1, and that gB-induced homotypic fusion of early endosomes to giant endosomes requires phosphatidylinositol 3-phosphate, the activity of soluble N-ethylmaleimide-sensitive factor attachment protein receptors, and the cytosolic gB sequence 889YTQVPN894. They conclude that gB expression alters trafficking of molecules of the HLA-II processing pathway, which leads to increased retention of MHC-II molecules in early endosomal compartments, thereby intercepting antigen presentation. Authors have used the following cell lines: COS-7, HeLa, MelJuSo and IMR90S (Niazy et al., 2017).

The biogenesis of multivesicular bodies (MVBs) is topologically equivalent to virion budding. Hence, a number of viruses exploit the MVB pathway to build their envelope and exit from the cell. By expression of dominant negative forms of Vps4 and Vps24, two components of the MVB pathway, authors observed an impairment in infectious HSV assembly/egress in Vero and 293 T cell lines. Furthermore, HSV infection resulted in morphological changes to MVBs. Glycoprotein B (gB), one of the most highly conserved glycoproteins across the Herpesviridae family, was sorted to MVB membranes. In cells expressing the dominant negative form of Vps4, the site of intracellular gB accumulation was altered; part of gB accumulated as an endoglycosidase H-sensitive immature form at a calreticulin-positive compartment, indicating that gB traffic was dependent on a functional MVB pathway. gB was ubiquitinated in both infected and transfected cells. Ubiquitination was in part dependent on ubiquitin lysine 63, a signal for cargo sorting to MVBs. Partial deletion of the gB cytoplasmic tail resulted in a dramatic reduction of ubiquitination, as well as of progeny virus assembly and release to the extracellular compartment. Thus, HSV envelopment/egress and gB intracellular trafficking are dependent on functional MVB biogenesis. These data support the view that the sorting of gB to MVB membranes may represent a critical step in HSV envelopment and egress and that modified MVB membranes constitute a platform for HSV cytoplasmic envelopment or that MVB components are recruited to the site(s) of envelopment (Calistri et al., 2007). (Fig. 4).

5. Inhibition/induction of lysosome biogenesis by pathogens

The mechanisms regulating cellular lysosomal biogenesis are becoming clear in the recent years. Transcription factor EB (TFEB), a basic helix-loop-helix-leucine zipper transcription factor of the microphthalmia family, regulates transcription of the lysosomal genes and subsequently lysosomal biogenesis in cell. Multiple kinases including mTORC1, ERK2, AKT, GSKβ and PKCβ phosphorylate TFEB at different residues and regulate its subcellular localisation. In nutrient rich conditions, activated mTORC1 on the lysosomal membrane phosphorylates TFEB at Ser142 and Ser211, which promotes the binding of TFEB with the 14–3–3 cytosolic chaperon and favors its cytoplasm retention. Conversely, mTORC1 inactivation upon starvation leads to nuclear translocation of TFEB. Dephosphorylation of TFEB by calcium-activated calcineurin and protein phosphatase 2 A also induces nuclear translocation of TFEB. In the nucleus, TFEB binds to Coordinated Lysosomal Expression and Regulation (CLEAR) element in promoter region of the lysosomal genes and subsequently induces their transcription. Thus, signals from different cascades integrate at TFEB to regulate the lysosomal biogenesis and homeostasis in cells (For review: Sachdeva, Sundaramurthy, 2020).

Despite TFEB participating in critical mechanisms of pathogen recognition and in the transcriptional response to infection in mammalian macrophages, little is known about its roles in the infected epithelium or infected nonimmune cells in general. In this sense, authors demonstrated that TFEB is activated in nonimmune cells (HeLa and HEK-293 cells) upon infection with bacterial pathogens through a pathway dependent on mTORC1 inhibition and RAG-GTPase activity, reflecting the importance of membrane damage and amino acid starvation responses during infection. Additionally, they presented data demonstrating that although TFEB does not affect bacterial killing or load in nonimmune cells, it alters the host transcriptome upon infection, thus promoting an antibacterial transcriptomic landscape. Elucidating the roles of TFEB in infected nonimmune cells and the upstream signaling cascade provides critical insight into understanding how cells recognize and respond to bacterial pathogens (Cabral-Fernandes et al., 2022).

Authors have shown that the non-enveloped picornavirus echovirus 1 (EV1) that clusters its receptor α2β1 integrin and causes their internalization and accumulation in α2β1 integrin enriched multivesicular bodies (α2-MVBs) depends on biogenesis of novel multivesicular structures for successful infection (Karjalainen et al., 2011).

Salmonella survives in and utilises macrophages for effective dissemination, ultimately leading to systemic infection. Bacterial xenophagy or macro-autophagy is an important host defense mechanism in macrophages. *Salmonella* pathogenicity island-1 (SPI-1) effector SopB is involved in subverting host autophagy via dual mechanisms. SopB is a phosphoinositide phosphatase capable of altering the phosphoinositide dynamics of the host cell. SopB mediates escape from autophagy by inhibiting the terminal fusion of SCVs with lysosomes and/or autophagosomes. SopB downregulates overall lysosomal biogenesis by modulating the Akt-TFEB axis via restricting the latter's nuclear localisation. This reduces the overall lysosome content inside host macrophages,

further facilitating the survival of *Salmonella* in macrophages and systemic dissemination of *Salmonella* (Chatterjee et al., 2023).

In addition, Salmonella infection depletes acidic and catalytically active lysosomes in the host cells. Salmonella effector PipB2 instigate tubulations of late endosome late endosome/lysosomes to form Sifs. Ectopic expression of PipB2 induces the dispersal of late endosome/lysosomes toward the cell periphery by increasing their net anterograde movement. In addition, ectopic expression of SifA, SpiC, and SopD2 in mammalian cells also induces aggregation of late endosome/lysosomes. Few other studies have also reported remodeling of the endosomal system in Salmonella infection and propose that it facilitates the nutrient acquisition for the bacteria. Furthermore, Salmonella-effector SifA makes a stable complex with the host SKIP and Rab9 in infected cells and subverts the retrograde trafficking of mannose-6-phosphate receptors (MPRs). Subsequently, subverted MPR trafficking leads to misrouting of the lysosomal enzymes in the cell, which ultimately abolishes lysosomal catalytic activity. Ectopic expression of SifA is enough to alter MPR trafficking and lysosomal function in HeLa cells (For review: Sachdeva, Sundaramurthy, 2020).

Treponema pallidum (Tp) has a well-known ability to evade the immune system and can cause neurosyphilis by invading the central nervous system (CNS). Microglia are resident macrophages of the CNS that are essential for host defense against pathogens. Tp can exert significant toxic effects on microglia in vivo in Tg (mpeg1: EGFP) transgenic zebrafish embryos. Single-cell RNA sequencing results showed that Tp downregulated autophagy-related genes in human HMC3 microglial cells, which is negatively associated with apoptotic gene expression. Biochemical and cell biology assays further established that Tp inhibits microglial autophagy by interfering with the autophagosome-lysosome fusion process. Tp activates the mTORC1 signaling to inhibit the nuclear translocation of TFEB, leading to decreased lysosomal biogenesis and accumulated autophagosome. Importantly, the inhibition of autophagosome formation reversed Tp-induced apoptosis and promoted microglial clearance of Tp. Taken together, these findings show that Tp blocks autophagic flux by inhibiting TFEB-mediated lysosomal biosynthesis in human microglia. Autophagosome accumulation was demonstrated to be a key mechanism underlying the effects of Tp in promoting apoptosis and preventing itself from clearing by human microglia (Hu et al., 2023).

Authors analysed the cell invasion capacity of metacyclic trypomastigotes (MT) and tissue culture trypomastigotes (TCT) from *T. cruzi* under diverse conditions. Incubation of parasites for 1 h with HeLa cells in nutrient-deprived medium, a condition that triggered lysosome biogenesis and scattering, increased MT invasion and reduced TCT entry into cells. Sucrose-induced lysosome biogenesis increased HeLa cell susceptibility to MT and resistance to TCT. Treatment of cells with rapamycin, which inhibits mTOR, induced perinuclear lysosome accumulation and reduced MT invasion while augmenting TCT invasion. Metacylic trypomastigotes, but not TCT, induced mTOR dephosphorylation and TFEB. Lysosome biogenesis/scattering was stimulated upon HeLa cell interaction with MT but not with TCT. Internalized MT, but not TCT, were surrounded by colocalized lysosome marker LAMP2 and mTOR. The recombinant gp82 protein induced mTOR dephosphorylation, nuclear TFEB translocation and lysosome biogenesis/scattering. Taken together, these data clearly indicate that MT invasion is mainly lysosome-dependent, whereas TCT entry is predominantly lysosome-independent (Cortez et al., 2016). Table 1 highlights the mechanisms of pathogens evasion from lysosomal killing. Table 2 shows the intervention strategies that have been used to allow maturation of phagosomes and fusion with lysosomes and to determine the infectivity of the pathogens reviewed.

Table 1
Mechanisms of pathogens evasion from lysosomal killing.

Pathogens	Mechanism of evasion	References
Anaplastamaceae	Avoids lysosomal fusion to the phagosome	Webster et al. (1998);Mott et al. (1999)
Listeria monocytogenes	Escapes from phagosome	Marquis et al. (1997);Birmingham et al. (2007)
Salmonellae	Arrests the host endosomal pathway at the late endosome stage and modulate its content	Kumar and Valdivia (2009)Jantsch et al. (2011)
Campylobacter jejuni	Avoids delivery into lysosomes	Watson and Galan, 2008
Trypanosoma cruzi	Escapes from phagolysosome	Dvorak and Hyde (1973)
Leishmania amazonensis	Replicates within a phagosome that share several properties with late endosomes/lysosomes	Ndjamen et al. (2010)
Toxoplasma gondii	Replicates within a non-fusogenic vacuole that excludes some host cell surface proteins, like determinants that target the vacuole for endocytic process	Håkansson et al. (2001);Joiner and Roos (2002)
Candida albicans	Transiently acquires early endosomal marker EEA1, but shows marked defects in acquisition of late endosomal marker LAMP1 and lysosomal marker cathepsin D	Zhao, Villar (2011)
Paracoccidioides brasiliensis	Promotes intense decrease of EEAI that consists in inhibiting phagosome-endosome fusion	Voltan et al. (2013)
Human hepatitis B virus	Viral particles are transported to lysosomes with no impact on infection	For review:Jiang and Hildt (2020)
Herpesviridae Human herpes simplex virus	Evades the autophagy process via interaction of ICP34.5 protein with the essential autophagy protein Beclin-1	Orvedahl et al. (2007);Leib et al. (2009)

6. Final considerations and conclusion

By means of several methodological approaches with a broad range of cell lines, authors have shown that different pathogens have evolved diverse strategies to subvert intracellular trafficking in order to avoid lysosomal killing. In this context, pathogens promote the rupture of the phagosome and thereby reside into the cytosol, avoid autophagy, delay both phagolysosome biogenesis and phagosomal maturation and survive/replicate inside the phagolysosome. These strategies are used by pathogens regardless they are bacteria, protozoa, virus or fungi. Pathogens impose global alteration on the host lysosomal system by manipulating the lysosomal signaling cascades in cells. These pathogens induce alterations in the host lysosomal landscape including enrichment, depletion and redistribution of the lysosomes. For several intracellular pathogens, as *Listeria monocytogenes*, survival inside host cells depends on a rapid escape from endosomes before lysosomal fusion and can replicate free in the cytosol. Escape from the phagosome of is mediated principally by LLO, a pore-forming protein encoded by the *hly* gene. This mechanism has shown that preventing lysosomal fusion is necessary for the survival of some pathogens, which grow in the cytosol. In this context, the protozoa parasite *T. cruzi* also escape from the phagolysosome to reside free in the cytosol. However, in contrast to *L. monocytogenes*, *T. cruzi* can be seen inside lysosomes for a significant period before achieving into the cytosol, fostering questions looking for the comprehension of the role played by lysosomal fusion to *T. cruzi* phagosome rather than being only a step necessary for intracellular parasite retention.

On the other hand, *Salmonella* arrests the host endosomal pathway at the late endosome stage and has some features such as the presence of lysosomal glycoproteins and the acidic luminal pH. Other properties are unique and may be the result of a modulation of normal host cell functions creating an adequate niche for the bacteria survival. To induce this phenotype *Salmonella* depends on virulence proteins encoded by genes clusters on the virulence plasmid or by specific chromosomal loci,

Table 2
Intervention strategies that have been used to allow maturation of phagosomes and fusion with lysosomes.

Intervention strategies	Effects	References
Wnt inhibitor (Dvl-PDZ domain inhibitor)	lysosomal fusion with ehrlichial vacuoles	Lina et al. (2017)
Pyrvinium (Akt inhibitor)	ehrlichial survival	Lina et al. (2017)
siRNA of Rheb and p70 S6 kinase	decreases E. chaffeensis infection	Lina et al. (2017)
Ganglioside GM2 activator	inhibits the phagosomal escape of *L. monocytogenes*	Yuan et al. (2022)
siRNA of LAMP-3	decreases the number of *Salmonella* recovered from the infected cells	Lee et al. (2016)
Rapamycin	Reduces lysosome migration from nuclei region to cell periphery resulting in less *T. cruzi* inside the phagolysosome	Alvim et al. (2023)
Gp 90 from *T. cruzi*	Inhibites lysosome spreading	Rodrigues et al. (2017)
cholesterol depletion	reduces lysosome availability at the cell cortex	Hissa et al. (2012)
Mutations in LYST/ Beige	causes further phagosome expansion and enhanced *L. amazonensis* replication	Wilson et al. (2008)
siRNA of Rab5A and Rab7A	inhibits the early HBV infection, and a considerable number of virions were found in the late endosomes in a time-dependent manner	For review:Jiang and Hildt (2020)
suppression of EGFR ubiquitination	impairs HBV infection, implying that translocation of the virus to late endosomes is critical for a successful infection	For review:Jiang and Hildt (2020)
knockout mice for Atg5	decreases HBV DNA replication	Tian et al. (2011); Silva and Jung (2013)
depletion of Rab 5 and Rab 11	Reduce HSV load	Hollinshead et al. (2012)
shRNA against SPP	Reduces HSV-1 replication in vitro and affects lysosomes and ER responses to HSV-1 infection	Allen et al. (2014)
Transient overexpression of gB	Induces giant vesicles of early endosomal origin, which contained Rab5, EEA1, and large amounts of MHC-II molecules in HSV infections	Niazy et al. (2017)
dominant negative forms of Vps4 and Vps24	impairment in infectious HSV assembly/egress	Calistri et al. (2007)
Ectopic expression of PipB2 (*Salmonella*)	induces the dispersal of late endosome/lysosomes toward the cell periphery by increasing their net anterograde movement	For review:Sachdeva, Sundaramurthy (2020)
Ectopic expression of SifA, SpiC, and SopD2 (*Salmonella*)	induces aggregation of late endosome/lysosomes	For review:Sachdeva, Sundaramurthy (2020)
nutrient-deprived cell culture medium Sucrose	lysosome biogenesis and scattering	Cortez et al. (2016)
recombinant gp82 from *T. cruzi*	induces mTOR dephosphorylation, nuclear TFEB translocation and lysosome biogenesis/scattering	Cortez et al. (2016)

referred to as *Salmonella* pathogenicity islands (SPI). The SPI1 and SPI2 encode two distinct type III secretion systems (T3SS) that are present on the cell wall and translocate a specific group of bacterial effector proteins into host cells. In a similar milieu, *L. amazonensis* resides inside a very large phagosomes that continuously undergo fusion with lysosomes and phagolysosomes that might protect *L. amazonensis* from host killing mechanisms, by diluting microbicidal molecules. In this context, *Salmonella* and *L. amazonensis* seems to modulate the vacuolar contents in order to establish a favorable nich to survive.

A broad comprehension of the mechanisms triggered during lysosomal killing evasion may contribute to the generation of novel therapeutical approaches against microbe infections. Novel studies are needed for a broad comprehension of the subverting strategies adopted by the different pathogens reviewed in this manuscript. A special attention should be given to the fungi and virus reviewed which substantially lack recent researches in the field.

Declaration of Competing Interest

On behalf of all author, I declare that no conflict of interest exists.

Data Availability

No data was used for the research described in the article.

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REVIEW

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Cellular dormancy: A widespread phenomenon that perpetuates infectious diseases

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Abstract

Under adverse environmental conditions, microorganisms are able to enter a state of cellular dormancy which consists of cell cycle arrest and interruption of multiplication. This process ensures their perpetuation in the infected host organism and enables the spread of disease. Throughout biological evolution, dormancy allowed microorganisms to persist in a harsh niche until favorable conditions for their reactivation were re-established. Here, we propose to discuss the dormancy of bacteria and protozoa pathogens focusing on the potential mechanisms and components associated with dormancy.

KEYWORDS

bacteria, cell dormancy, *E. coli*, *Mycobacterium tuberculosis*, *Plasmodium*, *Toxoplasma gondii*, *Trypanosoma cruzi*

1 | INTRODUCTION

Unicellular organisms and metazoans have developed, throughout evolution, strategies to face adverse conditions and inhospitable environments. These strategies consist of arrests in their cell cycles and/or their development as a survival strategy to overcome their inability to resist various stressful conditions. Thus, they evolve into a state called cellular dormancy. Cellular dormancy requires the organism to acquire a physiological response characterized by a repression of cellular metabolism but preserving mitochondrial respiration to maintain ATP homeostasis and genomic integrity [1]. Cellular dormancy allows microorganisms to persist in unfavorable ecological niches until favorable conditions are established for them to reactivate.

"Scout model" is a strategy used by microorganisms to reactivate cell growth in a fraction of dormant cells in a stochastic manner, regardless of environmental conditions [2]. When leaving the state of dormancy, active microorganisms can cause an imbalance in their homeostasis with the host, which can lead to a state of disease and consequently, the activation of an immunological response and the search for treatment. Thus, it is plausible to consider that the state of dormancy generates

Abbreviations: AP2, apetala2; ATP, adenosine triphosphate; eIF2, eukaryotic initiation factor 2; gPKAc3, *Toxoplasma gondii* protein kinase A catalytic subunit 3; Hpf, hibernation promoting factor; LDH1, lactate dehydrogenase 1; mRNA, messenger ribonucleic acid; PP2A, phosphatase 2A; ppGpp, guanosine tetraphosphate nucleotide; RaiA, ribosome associated inhibitor; RMF, ribosome modulation factor; RNA, ribonucleic acid; TA, toxin antitoxin; TgCDPK1, *Toxoplasma gondii* calcium-dependent protein kinase 1; TgMAPKL1, *Toxoplasma gondii* mitogen-activated protein kinase like 1; tRNA, transfer RNA.

a certain balance between the microorganism and its host, promoting a favorable solution for both [3].

It is important to highlight that from an epidemiological point of view, the dormancy of the microorganism can have some impacts, for example, the carrier host, but not the sick one, can hinder the diagnosis. This affects the application of preventive and therapeutic methods, making the carrier individual a natural reservoir of the microorganism, increasing the transmission of the disease. The limited understanding of the mechanisms underlying cellular dormancy in microorganisms is a determining factor in the persistent therapeutic failure observed in some infectious diseases.

Here we propose to discuss the dormancy of bacteria (*Escherichia coli* and *Mycobacterium tuberculosis*) and protozoa pathogens (*Plasmodium*, *Toxoplasma gondii*, and *Trypanosoma cruzi)* focusing on the potential mechanisms and components associated to dormancy. We chose these bacteria and intracellular parasitic protozoa because they are some of the most important pathogens with an impact on global public health and because they present a greater number of scientific research related to cellular dormancy. We think that putting together different pathogens, prokaryotes and eukaryotes, it makes easier the comprehension that despite being phylogenetically distant from each other, they display mechanisms that leads to a final common state of cellular dormancy in stress conditions.

2 | BACTERIA

Due to the broad literature in the field of bacterial dormancy, this section will focus on some potential mechanisms of dormancy triggered only by *E. coli* and *M. tuberculosis*. Two distinct bacteria that differs in different aspects of living and host infection but share the dormancy as a common mechanism to support stressful conditions.

2.1 | Bacteria dormancy concepts

Bacterial dormancy refers to a bacterial population with a non-heritable antibiotic resistance phenotype that arises due to pressure caused by antibiotics through stochastic or deterministic epigenetic factors. After removal of the antibiotic stressor, these bacteria may even give rise to an antibiotic-sensitive bacterial population. Persistence mechanisms are based on survival strategies that involve efflux pumps, biofilm formation, and changes in metabolism [4].

The etiological agent of tuberculosis, *M. tuberculosis*, is an example of a bacterium that uses the cellular dormancy strategy to survive in inhospitable conditions. This bacterium negatively regulates or stops its cellular growth for long periods of time [5], which allows its survival in stressful environments [6].

Bacterial cell dormancy is a common mechanism shared by different pathogenic and nonpathogenic bacteria to evade environmental threats. Antibiotic tolerance has been associated with bacterial dormancy, as lethal doses of antibiotics are not effective against dormant bacteria. The extremely low metabolism of these microorganisms plays a critical role in the observed tolerance to antibiotics [7]. In this sense, the emergence of super-bacteria is not the only threat to human health. Many patients already suffer from untreatable diseases due to the innate mechanism of bacterial persistence. Asymptomatic carriers of dormant bacteria can hinder infection control and promote bacterial perpetuation in the environment.

2.2 | Screening of dormant *E. coli*: One phenotype/one mechanism model?

First, it is important to highlight that we are dealing with bacteria persistancy in a dormant stage different from the sporulation process of gram+ bacteria. Sporulation is the process by which a vegetative cell undergoes a developmental change to form a metabolically inactive and highly resistant endospore.

Screening bacterial libraries for transposon-inserted mutations was successfully used for different studies and objectives, revealing panels of candidate genes whose analyzes led to the description of different pathways and mechanisms. Thus, this strategy was the first used to identify genes associated with dormancy in *E. coli*. However, this strategy did not bring good results, since attempts to verify the bacteria ability to tolerate high doses of antibiotics did not lead to the appearance of any mutant cells without the presence of dormant bacteria. In this sense, the idea that there was no mechanism underling the formation of dormant bacteria seemed to be a good hypothesis. Ectopic expression of the *E. coli* chaperone, DnaJ or the expression of PmrC, an enzyme from *Salmonella enterica*, were both toxic when produced in excess by *E. coli* and inhibited cell growth resulting in tolerance to different compounds. Thus, this result corroborates the hypothesis that no mechanism is activated during the process of formation of dormant bacteria. However, this explanation is contrary to what is observed during the dynamics of formation of dormant bacteria. At the beginning of exponential growth, few dormant bacteria are seen in the culture and this number increases significantly near the mid-exponential state.

Maintaining the bacterial culture in the initial phase of exponential growth results in the complete elimination of dormant bacteria. In this way, no dormant bacteria are generated during the initial exponential phase of growth. It can therefore be concluded that the formation of dormant bacteria is the result of a dedicated mechanism rather than only by random errors in protein misfolding [8]. It was only after the generation of a complete library of knockout genes in *E. coli* that it became feasible to revisit screening studies of dormant bacteria. However, this advanced screening did not produce dormant bacteria with a single mutation. Thus, suggesting a high redundancy in the molecules that participate in the dormancy process in *E. coli*. The majority of the hits were based on global regulators, such as, DksA, DnaKJ, HupAB, and IhfAB. A global regulator interferes with the expression of several persistence-related genes simultaneously which results in dormancy of the bacteria.

The screening revealed two other candidate genes that may be more directly associated with entering dormancy. These genes are YgfA, which inhibits nucleotide synthesis and YigB, which blocks cellular metabolism through depletion of a pool of falvin mononucleotide. The major conclusion of this study is that the formation of dormant bacteria is not a simple regulatory pathway. Rather, it appears that the formation of dormant bacteria consists of a number of parallel independent mechanisms [8].

2.3 | SOS response, hyperphosphorylated guanosine derivatives, (p)ppGpp, and aggresome formation

Bacteria enter a state of cellular dormancy in a stochastic manner. However, this phenomenon can be induced by environmental factors that are related to imminent threats to the microorganism. In this context, stress-related signaling pathways, such as the general stress response or SOS (distress and alert signal) response together with second messenger (p)ppGpp control the size and composition of dormant bacteria [9, 10].

Cashel and Gallant [11] discovered guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp) and collectively referred to as (p)ppGpp or "alarmones." Since this first description, the synthesis and degradation of (p)ppGpp as well as the (p)ppGpp-mediated response to nutrient starvation, a phenomenon known as the "stringent response," have been extensively studied by different research groups. Members of the RelA/SpoT homology (RSH)-type protein family play a central role in the metabolism of (p)ppGpp [12]. In nutrient-poor environments, RSH proteins use ATP as a donor substrate and transfer its β- and β-phosphates onto the 3′-hydroxy group of the acceptor substrate guanosine 5′-diphosphate (GDP) or guanosine 5′-triphosphate (GTP) to generate ppGpp or pppGpp, respectively [13]. RSH proteins also degrade (p)ppGpp through removal of the 3′-pyrophosphate moiety of (p)ppGpp, thus, regenerating GDP/GTP [14].

E. coli was demonstrated, which controls the lag time for bacterial resuscitation after antibiotic removal. The authors identified a collection of endogenous protein aggregates, which were called aggresome. These aggregates constitute important indicators of the level of bacterial dormancy and their formation occurs after a decrease in cellular ATP levels. The elimination of aggresomes is essential for the bacteria to leave the dormant state. The authors also showed that recruitment of the DnaK-ClpB machinery facilitates ATP-dependent protein disaggregation and determined the lag time for the bacteria to grow again. DnaK binds directly to the aggregates and recruits ClpB that acts promoting disaggregation (Figure 1).

2.4 | *E. coli* toxin and antitoxin (TA) modules

TA modules are found on plasmids, where they constitute a maintenance mechanism. The toxin is a protein that inhibits an important cell function such as translation or replication and forms an inactive complex with the antitoxin. The toxin is stable, while the antitoxin is degradable. If a daughter cell does not receive a plasmid after segregation, antitoxin levels decrease due to proteolysis, leaving the toxin that either kills the cell or inhibits its propagation [15].

In *E. coli*, 10 TA systems were identified, the toxins of which all behave as mRNA endonucleases (mRNases) and can be splited into superfamilies based on the cleaving site target of the mRNases [16]. RelE, YoeB, HigB, YhavV, YafO, and YafQ cleave mRNA at the ribosomal A site. On the other hand, MazF, ChpB, MqsR, and HicA cut RNA site, in a specific and independent way of the ribosome. All of the act decreasing protein translation [16].

MazF and an unrelated toxin, RelE, induce stasis by the cleavage of mRNA, which inhibits translation. This phenotype can be reversed by the expression of the corresponding antitoxin [17, 18]. This property of toxins makes them excellent candidates for dormancy genes.

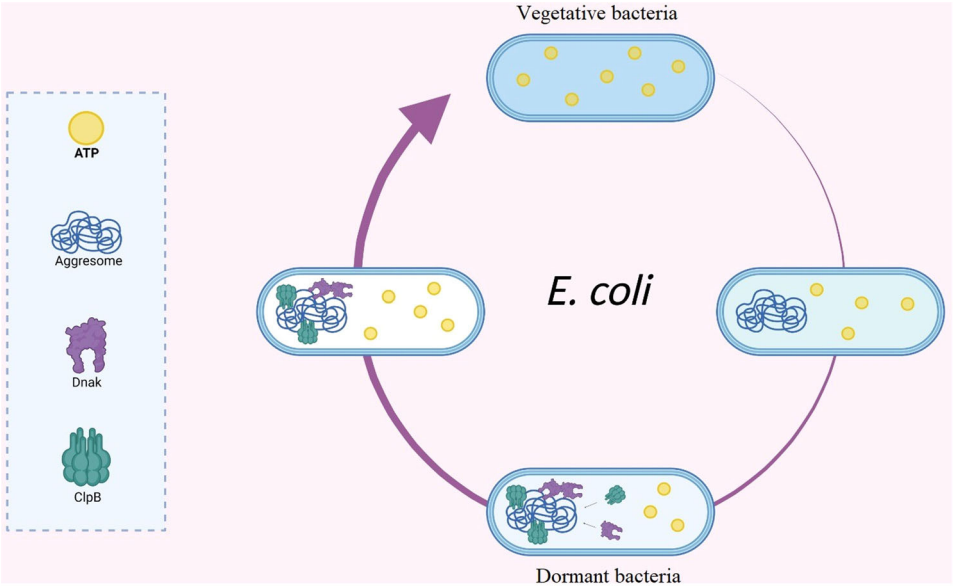

FIGURE 1 Protein aggresome is an important indicator of the level of *Escherichia coli* dormancy. The elimination of protein aggresome and the re-establishment of proteostasis are essential for bacteria to leave the dormant state and resuscitate. The functional recruitment of the DnaK-ClpB machinery facilitates adenosine triphosphate (ATP)-dependent disaggregation for bacterial regrowth. DnaK binds directly to the aggregates and recruits ClpB that acts promoting disaggregation.

Ectopic expression of RelE [19] or MazF [20] significantly increases antibiotic tolerance.

hipA is the first dormancy-related gene identified in *E. coli*. It is a toxin whose ectopic expression causes multidrug tolerance [20–23]. A gene variant that confers high-persistent phenotype is *hipA7*. Point mutations at two separate points in the *hipA* gene confer the observed phenotype [24]. How these mutations lead to high levels of dormancy is not completely understood. One mutation appears to render the hipA protein nontoxic since its overexpression moderately inhibits growth and translation compared to the wild-type protein, while the other is required for the high-persistence phenotype [25]. The non-toxicity is attributed to the reduced inability of the hipA7 protein to phosphorylate targets such as glutamate-tRNA (transfer RNA)–ligase (GltX) and several other proteins involved in transcriptional regulation.

It also has low binding affinity to the antitoxin. Paradoxically, overexpression of the hipA7 allele causes an increase in GltX phosphorylation [26]. Researchers have proposed a model to explain these seemingly contradictory findings. HipA7 expressed on the chromosome exists in greater abundance in its unbound form when compared to hipA. This allows the phosphorylation of higher levels of GltX. As a result, there is a reduction in the growth rate of strains expressing hipA7. HipA also phosphorylates other targets that contribute to its toxicity and ability to induce viable but non-culturable cells [25].

hipA and its upstream counterpart *hipB*, form the complex hipBA [24]. When stress results in *hipA* levels greater than those of *hipB*, *hipA* is able to phosphorylate GltX which avoids the transfer of glutamate to $tRNA^{Glu}$. The accumulation of uncharged $tRNA^{Glu}$ in the ribosomal A site activates the ribosome-associated guanosine tetra- and pentaphosphate ((p)ppGpp) synthase, RelA; the (p)ppGpp formed is believed to act as an alarmone and activate the release of toxins from other TA systems [26]. These toxins are then able to target other protein-encoding genes to decrease synthesis to reduce metabolic activity and finally enter in a state of cell dormancy.

TA modules pathway controlled by (p)ppGpp is involved in the formation of dormant *E. coli* K-12. This mechanism involves HokB toxin and several mRNA endonuclease toxins. In this context, the activation of HokB toxin depends on Obg guanosine triphosphatase and promotes the formation of dormant bacteria by abolishing the proton-motive force. On the other hand, mRNA endonuclease toxins are activated by the degradation of antitoxin by the Lon protease, which is involved in inducing global translation inhibition. In the same context, DNA damage activates the TisB toxin response by triggering the SOS response, which induces the formation of dormant bacteria in the same way as HokB [8, 16, 27].

In addition, (p)ppGpp is also involved in reducing protein synthesis by a mechanism dependent on the interaction with RNA polymerase. It is capable of directly

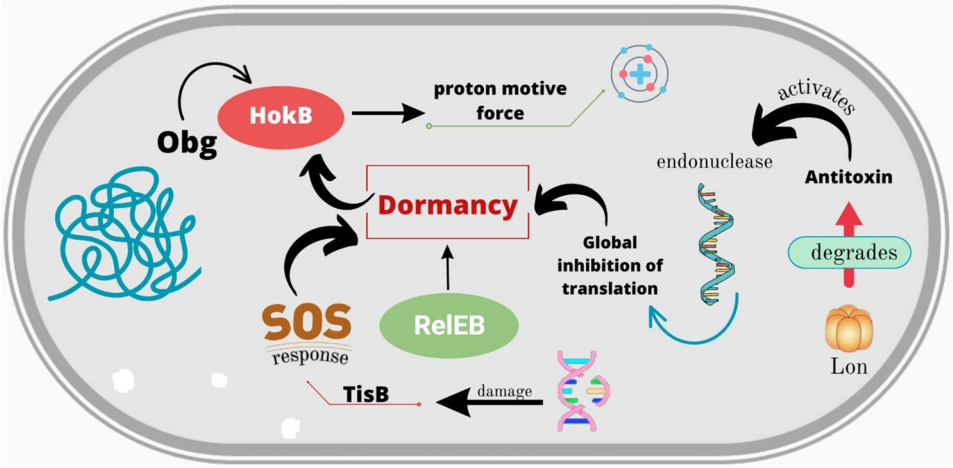

FIGURE 2 Activation of HokB toxin in *Escherichia coli* depends on Obg guanosine triphosphatase. The activation of Obg causes the formation of dormant bacteria by abolishing the proton-motive force. mRNA endonuclease toxins are activated by degradation of antitoxin by the Lon protease which induces global inhibition of translation. TisB toxin is activated in response to DNA damage by the SOS response and induces the formation of dormant bacteria similar to HokB. RelEB promotes the transition to dormant state of *E. coli* in a cell density-dependent manner.

modulating gene transcription through the activation of two crucial stress responses. These stress responses are the stress response for stationary phase (RpoS) and the stress response for misfolded proteins (RpoE), which act to promote a reduction in the generation of ribosomes [28]. Additionally, (p)ppGpp activates the production of ribosome modulation factor (RMF), hibernation promoting factor (Hpf), and ribosome associated inhibitor (RaiA). RMF converts active 70S ribosomes into inactive 100S ribosomes via an inactive 90S dimer complex, Hpf converts 90S ribosomes into 100S ribosomes and RaiA inactivates 70S ribosomes impairing the translation to occur [28].

RelEB is one of the major TA systems in *E. coli* and has been reported to mediate the process of transition to dormancy in a cell density-dependent manner. Thus, *E. coli* that grows at high density under the action of RelE toxin shows a higher rate of transition to the dormancy state [29] (Figure 2). Dormancy in *E. coli* MG1655 has also been triggered by mechanical stress of aerosolization with continued synthesis of vital proteins for bacterial survival [30]. This study showed that the speed by which airborne bacteria are spread, affects their ability to grow and their potential to develop antimicrobial resistance.

2.5 | The impact of epigenetic variation on *E. coli* growth

Different genes can influence bacterial growth. In this context, mutations or changes induced in gene expression are directly related to changes in growth rates. Stochastic variation in the expression of various genes appears to cause stochastic variation in bacterial growth [31]. The high persister phenotype of hipQ is directly associated with the new phenotype of reduced phenotypic inheritance which has the characteristics of reduced correlation of growth parameters such as division time, size at birth or cell elongation rate, either between mothers and daughter cells, or between sister cells. These results suggested that genes related to the epigenetic inheritance play a role in dormant cell induction. This study also demonstrated the *locus* of the hipQ phenotype as a mutation in a gene, *ydcI*, that encodes a putative transcription factor [32].

2.6 | *M. tuberculosis* toxin and antotixin modules

M. tuberculosis is the bacteria that causes tuberculosis, its pathology is complex and involves different forms of evasion of the host's immune response that promotes its persistence in the infected individual's body and can establish a reactivation process when environmental conditions are favorable. The bacterial TA system for resistance to antibiotic therapy is encoded by two genes, which are formed by two proteins. These proteins are long-lived protein, which corresponds to the toxin, and short-lived protein, the antitoxin. Under normal physiological conditions of the bacteria, the toxin is neutralized by the antitoxin. However, under unfavorable conditions *M. tuberculosis* represses the expression of the antitoxin. This repression promotes the accumulation of toxin,

keeping the bacteria in a dormant state. The toxin's mechanism of action acts similarly to a ribonuclease that cleaves free ribosomal bound single-stranded mRNA. This process promotes the inhibition of protein synthesis and consequently inhibits bacterial growth, allowing the bacteria to survive for long periods of time without presenting signs of the infection in the infected individual.

2.7 | Genes that regulate *M. tuberculosis* dormancy

Approximately 50 genes regulate the dormancy state in *M. tuberculosis*, and these genes belong to the DosR regulon that acts under the control of the dormancy survival regulator, dosR-dosS (dosT), which consists of a transcription factor. Evolutionarily, these genes emerged to help bacteria adapt to anaerobic conditions that are generated due to the formation of granulomas in the host's tissues. Thus, some factors such as hypoxia and environmental conditions not conducive to the growth of mycobacteria due to external growth factors constitute elements that activate the expression of these genes [33–35]. This system is a potential target for further investigation as it may make possible a better understanding of the mechanisms associated to the *M. tuberculosis* dormancy (Figure 3). RafH is an example of a DosR-regulated protein that contributes to the survival of the mycobacteria under hypoxic conditions by stabilizing ribosomes in their associated forms [36].

Authors verified by gene knockout that DosR is not an essential gene for the mycobacteria, since its deletion caused only an insignificant decrease in bacterial viability under hypoxic conditions [37, 38]. Thus, a hypothesis was proposed in which DosR would be a general adaptation strategy for bacteria to inappropriate environmental conditions, such as necrotic tissue rich in free active radicals generated by the host organism [39]. Additionally, dormant *M. tuberculosis* grown in aerobic conditions under potassium deficiency also showed increased Dos-regulon transcription [40, 41]. The same was seen in an experimental murine model of artificial granuloma [42]. These results support the notion that Dos-regulon activation may occur under various stress conditions rather than only under hypoxia.

2.8 | Stringent response and *M. tuberculosis* dormancy

In response to bacterial starvation, a stress signaling pathway is activated resulting in metabolic remodeling that leads to low growth rates and energy requirements. This established response is called stringent response. A classic example of stringent response is the low growth rate of the mycobacteria in medium containing low levels of phosphate. Among the characteristics of the stringent response, one can mention the major one, which consists of the negative regulation of rRNA and the synthesis of ribosomal protein concomitantly associated with the increase of amino acid biosynthetic operons to supply the needs of amino acids for the bacterial survival. Authors have demonstrated that the absence of Rel results in a disadvantage for the survival of the mycobacteria under stressful conditions. In this sense, Rel protein was considered the principal mediator of the stringent response in *M. tuberculosis*. Other important response

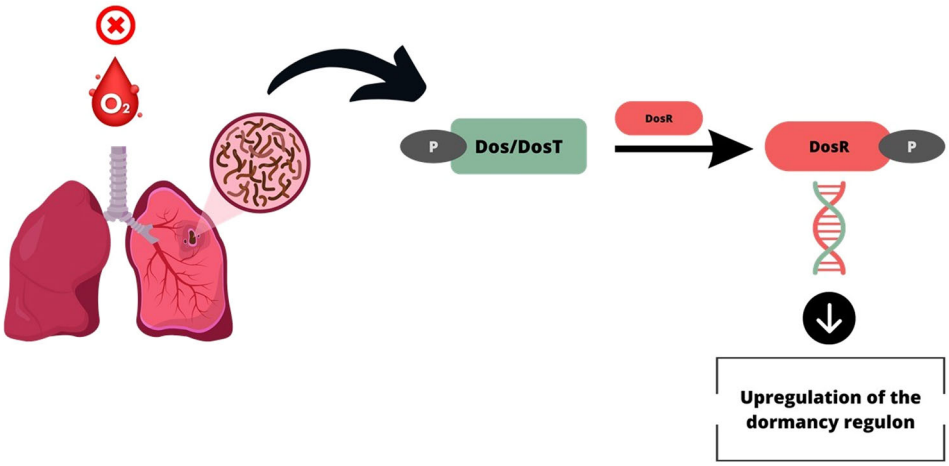

FIGURE 3 *Mycobacterium tuberculosis* dormancy is regulated by genes belonging to the DosR regulon under the fine control of the dormancy survival regulator, the transcription factor dosR-dosS (dosT). These genes arose to help *M. tuberculosis* to adapt to anaerobic conditions, allowing it to survive in the granuloma formed in host tissues.

regulators are CarD-based regulation and inorganic polyphosphate-based regulation [43].

Additionally, authors have found that the extracytoplasmic function sigma factor, SigE, plays a key role in stringent response. This component is not directly involved in the response activation process, but rather in bacterial protection against stress caused by the low exposure to phosphate and the activation of the stringent response [44]. In other words, the role of SigE is to help the bacteria to handle the metabolic stress related to the process of adaptation to low phosphate levels and activation of the stringent response. Taken together, these results make great contributions to the comprehension of the mechanisms associated with the activation of the stringent response and induction of dormancy in the mycobacteria.

2.9 | Proteomics of *M. tuberculosis* during hypoxia-induced cell dormancy

The search for more information about potential components involved in *M. tuberculosis* dormancy is constant and several experimental models are tested to identify new targets. In this context, proteomics studies were carried out using hypoxia-induced dormancy as an experimental model. Authors have found an increase in the expression of different proteins, such as DosR regulon proteins, proteins related to cell wall synthesis, lipid metabolism, the copper stress-related enzymes MymT (copper toxicity protection) and CsoR (copper-sensitive operon repressor). Various enzyme subnetworks of menaquinone metabolism, cholesterol degradation, fatty acid metabolism, mycolate biosynthesis, branched-chain fatty acid, sulfur compound metabolism were upregulated during hypoxia. Enzymes involved in the anaerobic electron transport chain (narX, narK2, ndh2 and cytochrome bd oxidase) were also upregulated [45]. These data suggest that in addition to proteins belonging to the DosR regulon, other molecules may be associated with the entry of the mycobacterium into a state of dormancy. This scenario opens new avenues of investigation for a deep understanding of the dormancy process.

3 | PROTOZOA

To show that phylogenetically distant eukaryotic protozoa from bacteria also display mechanisms to get into dormant state. We selected two Apicomplexa parasites of high importance worldwide that are known to possess resistant life cycle forms and a Trypanosomatidae, *T. cruzi*, which is endemic in Latin America. Its entrance in dormant state is a recent discovered issue but it is still a matter of debate.

3.1 | Plasmodium

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*, and is considered the most important parasitic disease in the world. The main species causing human infection are *Plasmodium falciparum* and *Plasmodium vivax*. These parasites feature great genetic variability, which provides great adaptability to establish themselves in hostile environments. Studies have shown that the *P. vivax* genome is more polymorphic than the *P. falciparum* genome even though it is in the same geographic region [46, 47].

P. vivax presents as a very important characteristic the ability to differentiate into dormant evolutionary stages in the liver. These evolutionary forms are called hypnozoites, which are responsible for relapsing episodes of infection that can occur at any time after the primary infection. This ability makes it even more difficult to establish disease control strategies [48, 49]. The mechanisms involved in dormancy and in the activation of hypnozoites are unknown.

3.1.1 | Genes associated with *P. vivax* dormancy

From the sequencing of the genomes of *P. vivax* and *Plasmodium cynomolgi* (also a relapsing parasite) it was possible to identify several genes that are probably involved in the entry into a state of dormancy. The strategy adopted to identify these genes was the comparison with orthologous genes presented in yeast and *Dictyostelium*, that were also related to dormancy in these organisms, as well as involved in different cellular processes, such as nucleocytoplasmic transport, GTP-binding proteins, GTPase activation, DNA-binding proteins and proteins related to the control of intracellular signaling [50, 51].

de Souza Ribeiro et al. [52] proposed a hypothetical mechanism for dormancy in *P. vivax* through a study based on in silico analysis of genes that showed great potential for involvement in the dormancy process. The authors also proposed the putative interactions among their encoded proteins. The proposed mechanism would involve protein products from 15 different genes. Among these genes, there would be three Ras-related proteins (Rab-5A, Rab-5B, and Rab-11B), one conserved hypothetical protein, three protein kinases, two GTP-binding

proteins, two translation initiation factors IF-2, one phosphatase, one ADP-ribosylation factor, one RNA helicase, and one beta unit of signal recognition particle. The criteria adopted for the selection of these genes were based on the low genetic variability among *P. vivax*, low genetic variability in relapsing parasites than in non-relapsing parasites and because they are under negative selective pressure. The hypothetical mechanism revealed that the putative crk2 protein kinase would be the hub molecule, being central in the network of interactions of the 15 proteins encoded by the genes selected in the study. Crk2 protein kinase is involved in cell cycle control and in this model, proteins related to three cellular activities are connected by crk2 protein kinase. Thus, cell cycle regulation, protein biosynthesis regulation, and vesicular transport would be the three key functional cellular activities involved. However, the proposed mechanism has not been empirically tested. It may be possible that the referred genes act in a coordinated way when receiving an external signal from the host and activate a signaling cascade of kinases and phosphatases to arrest cell cycle and protein translation [52].

3.1.2 | Transcriptome, proteomics, and in silico analyzes of *Plasmodium*

The formation of hypnozoites occurs in the early stages of liver infection and can be established in sporozoites even before cell invasion. Transcriptome and histone epigenetic characterization studies of sporozoites revealed that genes associated with functions necessary for the establishment of infection in early stages in mammals, despite being highly transcribed, are not translated and thus may be under a translational repression mechanism. The different translation control mechanisms, which are transcriptional, post-transcriptional and posttranslational, appear to be active in sporozoites but to a lesser extent in hypnozoites and absent in schizonts and mixed blood stages. The hypnozoite forms appear to figure as a transition point between sporozoites and replicating zchizonts. In this context, hypnozoites express several of the dominant transcripts in sporozoite forms but maintain high transcription rates of genes regulating pathways involved in transcription, translation, and chromatin settings [53].

Data from transcriptomics and FISH suggest that *P. cynomolgi* hypnozoites express a lower number of genes than schizonts due to the significant reduction in gene transcription observed in this evolutive form [54]. These results corroborate with findings from Muller et al. [53] which showed, by molecular tools, a refined regulation of gene transcription and translation in *P. vivax*. Moreover, authors demonstrated that different physiological characteristics related to quiescence are maintained by *P. cynomolgi*. Thus, the pathogen preserves membrane potential, ATP biosynthesis, and genomic integrity. Hypnozoites have a high expression rate of most mitochondrial electron flow genes and ATP production enzymes. Moreover, hypnozoites highly express genes related to the maintenance of the nucleus, chromatin, homologous recombination repair enzymes, and genes required for the maintenance of epigenetic markers [54]. In addition to the work by Wel et al. [54], Bertschi et al. [55], described that the process of maturation of hypnozoites along time leads to a down-regulation in gene transcription. Among the expressed genes are housekeeping genes and those involved in quiescence, energy metabolism, and maintenance of genomic integrity.

Transcriptome, proteomics, and in silico analyzes studies available in the literature are important pillars to explore the intricate mechanism that leads to the formation of hypnozoites. Understanding this mechanism is crucial for the glimpse of new therapeutics targeting both toxicity against the parasite and the inhibition of dormancy. Combined therapies may be a successful strategy to obtain clearance of infection. Focusing on specific inhibitor compounds of key molecules involved in the dormancy pathway may also be an alternative. Considering the potential role of crk2 protein kinase in *P. vivax* dormancy proposed by de Souza Ribeiro et al. [52] in their in silico work, we emphasize here the importance of additional proof of concept studies to confirm the critical role of this protein. This empiric evidence would strongly support crk2 as a potential target for the development of inhibitors.

P. vivax specific transcriptomic signature has been revealed by single-cell RNA profiling. Hypnozoites show higher expression of genes that encode for proteases, membrane proteins, transcription factors, and DNA-/RNA-regulating proteins. Hypnozoites present higher expression of genes encoding for proteases vivapain-1, vivapain-2, and plasmepsin IV than schizonts [56]. Few research have shown the activities of these gene products in *P. vivax* [57]. However, studies with other *Plasmodium* spp. have shown that they localize to the food vacuole and play an important role in hemoglobin catabolism during the blood stages of the parasite [58, 59]. These results add potential novel players in the dormancy of *P. vivax* hypnozoites.

A screen of the Repurposing, Focused Rescue, and Accelerated Medchem library against *P. vivax* liver stages has identified epigenetic inhibitors as potential drugs against hypnozoites. These *P. vivax* forms are highly sensitive to histone acetyltransferase and methyltransferase inhibitors, indicating that several epigenetic mechanisms are likely modulating hypnozoite persistence [60].

3.2 | *T. gondii*

T. gondii is the etiological agent of toxoplasmosis that causes great morbidity to the infected individual. Transmission of this protozoan occurs through tissue cysts containing the bradyzoite forms or through oocysts containing sporozoites. The disease spreads after ingestion of one of these evolutionary forms that differentiate into tachyzoites. These tachyzoites quickly spread throughout the host's body causing infection. *T. gondii* has the capacity to infect and replicate in virtually any warm-blooded host [61].

The evolutionary form, bradyzoites, is a form of resistance that persists in tissue cysts serving as reservoirs for exacerbation of the disease in immunocompromised individuals. Studies aimed at understanding the mechanisms associated with the formation of the resistance form of the parasite with the discovery of new therapeutic targets present in the bradyzoite forms can lead to the development of new drugs capable of curing the latent disease and thus preventing its reactivation. However, the molecular bases of the differentiation process from tachyzoites to bradyzoites are still unknown. There is an extensive literature addressing proteins specifically expressed in each parasite life stage. However, there is still no study relating these proteins to the differentiation process in bradyzoites. In this sense, the review of these differently expressed proteins is outside the scope of this review. Here we address advances in biological processes that in some way may be involved in parasite differentiation.

3.2.1 | *T. gondii* transcription factors related to bradyzoites formation

Studies carried out with *T. gondii* transcription factors, which contain the plant-related DNA binding domain Apetala2 (AP2), showed roles for different AP2s during the bradyzoite differentiation process. The binding motifs of some of these AP2 transcriptional factors have been shown to bind to the promoter regions of bradyzoite markers BAG1 and B-NTPase. This result suggested that these AP2 proteins are transregulators of bradyzoite genes. Some AP2 proteins act as bradyzoite activators while others as repressors [62–66]. Given the antagonistic activities of AP2 proteins, it can be suggested that the formation of bradyzoites is a standard phenomenon during the parasite differentiation process and that AP2 repressive proteins act to allow the continuity of tachyzoite gene expression. This facilitates acute infection and allows drugs effective in the acute phase to act against these evolutionary forms of the parasite. Understanding the upstream regulators of these AP2 transcription factors would further elucidate the genetic pathway that regulates bradyzoite differentiation. In addition, the discovery of additional genes regulated by these AP2 proteins will increase our understanding of the molecular basis involved in bradyzoite differentiation. Thus, one can suggest the possibility that the cure of toxoplasmosis may involve inhibition of the formation of tissue cysts, maintaining the presence only of tachyzoite forms that are sensitive to existing drugs. Thus, AP2 proteins may constitute important candidate targets for such attempts.

Using the same reasoning, one can mention the involvement of eukaryotic initiation factor 2 (eIF2) kinases in posttranslational modifications of proteins to control the differentiation of tachyzoites into bradyzoites. The phosphorylation of eIF2a is related to a decrease in global protein synthesis in favor of transcription factors associated with the stress response translation. In the case of *T. gondii*, TgIF2a phosphorylation is activated by environmental conditions that favor the translation of AP2 factors in response to endoplasmic reticulum stress. Phosphorylation of TgIF2a remains even after the differentiation process into bradyzoites has been completed. In vitro inhibition of TgIF2a dephosphorylation promotes, in turn, a downregulation in tachyzoite replication and leads to an upregulation in cyst formation with low reactivation rates [67–70].

The identification of a Myb-like transcription factor (BFD1) brought new perspectives to the definition of the characteristics of bradyzoite-specific promoters. This transcription factor is necessary for parasite differentiation in vitro and in vivo. BFD1 accumulates during stressful conditions for the parasite and the synthetic expression of its form is sufficient to induce differentiation. BFD1 has the ability to bind to promoters of many stage-specific genes and represents a counterpoint to the ApiAP2 factors [71].

3.2.2 | *T. gondii* proteins involved in bradyzoite differentiation

Bradyzoites express a Puf homolog with RNA-binding activity, however, the role of this protein in the differentiation remains to be elucidated [72]. The expression of TgAlba is crucial for cyst formation. This protein has the ability to bind to RNA and to interact with proteins that control translation [73]. Translation during bradyzoite differentiation may be modulated by additional RNA-binding proteins such as, Argonautes,

DEAD-box helicases, and KH-type splicing regulatory proteins [74, 75].

It is reasonable to speculate that upon differentiation, tachzoite transcripts are translationally repressed within bradyzoites and kept by ribonucleoproteins within granules awaiting translation upon reversion back to tachyzoites. Thus, the biological activity of tachyzoite-associated lactate dehydrogenase 1 (LDH1) mRNA corroborates this hypothesis since this mRNA contains a cis-acting regulatory element in the form of an RNA hairpin [76]. This hairpin has the ability to repress translation, most likely through binding of the 5′UTR to a non-identified trans-element for keeping away from the translation pool. These cis-acting elements are a mechanism that *T. gondii* may use to block tachyzoite gene translation after differentiation into bradyzoites [61, 77].

3.2.3 | *T. gondii* protein kinase and phosphatase effects on bradyzoite differentiation

Sugi et al. [78] have characterized the protein kinase A catalytic subunit 3 (TgPKAc3) and also described its biological activities related to bradyzoites. In general, PKAc isoforms are part of the modulation of different biological processes. Thus, PKAc regulates the response to starvation and respiratory functions through the phosphorylation of its substrates. As for the *T. gondii* protein, authors have demonstrated that it plays a role in regulating cell division [79] and in the differentiation of bradyzoites in response to increased cAMP levels [80]. In addition, while TgPKAc3 seemed to downregulate parasite differentiation, it may be involved in the formation of high number of cysts within the murine brain [78]. Understanding the substrates of TgPKAc3 will help to clarify its role in the mechanism of bradyzoite differentiation [61].

Calcium-dependent protein kinase 1 (TgCDPK1) and mitogen-activated protein kinase like 1 (TgMAPKL1) are some other kinases that authors have proposed to play a role in *T. gondii* differentiation. Bradyzoite markers are upregulated by inhibitors of bump kinases. These inhibitors also interfere in parasite multiplication through interactions with TgCDPK1 and TgMAPKL1 [78, 81]. Transcription of TgMAPKL1 is upregulated during early bradyzoite differentiation and its ablation leads to the downregulation of bradyzoite markers and interferes in parasite attachment and replication [82].

Authors have recently demonstrated that *T. gondii* phosphatase 2A (PP2A) holoenzyme is formed of a catalytic subunit PP2A-C, a scaffold subunit PP2A-A and a regulatory subunit PP2A-B. Ablation of any of these subunits upregulated starch accumulation and interrupted the tachyzoite-to-bradyzoite differentiation. PP2A favors the amylopectin regulation metabolism by dephosphorylation of calcium-dependent protein kinase 2. These results brought novel insight into the role of PP2A as a key regulator of starch metabolism and bradyzoite differentiation [83] (Figure 4).

3.3 | *T. cruzi*

Chagas disease, one of the most important tropical diseases in the Americas, is caused by *T. cruzi*. It affects millions of people in Latin America and worldwide. This disease is considered a neglected tropical disease because few effective treatments and preventive methods are routinely used and few efforts from the pharmaceutical industry are made to generate more effective drugs to cure the disease, specially during its chronic phase.

3.3.1 | Drug-induce *T. cruzi* dormancy

Currently, there are two drugs available for the treatment of Chagas disease. However, they are only effective for treating the acute phase of the disease and often fail to cure the infection. The reasons for therapeutic failure are unknown. One of the possible explanations for the therapeutic failure was recently suggested, in which the parasite would hide inside the host cell, entering a state of dormancy. Sánchez-Valdéz et al. [84] identified evolutionary forms of *T. cruzi* in a state of dormancy that led to parasite persistence after treatment. They observed the presence of nonreplicating amastigotes in in vivo and in vitro infections. These dormant amastigotes preserved their ability to differentiate into trypomastigotes and to reestablish the cell cycle and multiply later in the infection. In addition, dormant amastigotes survived specific treatment and were able to reestablish the infection after treatment.

It was proposed by research that therapeutic failure against Chagas disease could be the therapeutic protocol. In this context, the protocol does not consider the resistance of transiently dormant amastigotes to the drugs available for treatment. In support of this concept, these researchers demonstrated that the alternative use of a therapeutic regimen, employing high individual doses given less frequently over an extended period of time, eliminated the parasite in three experimental murine models of Chagas disease [85].

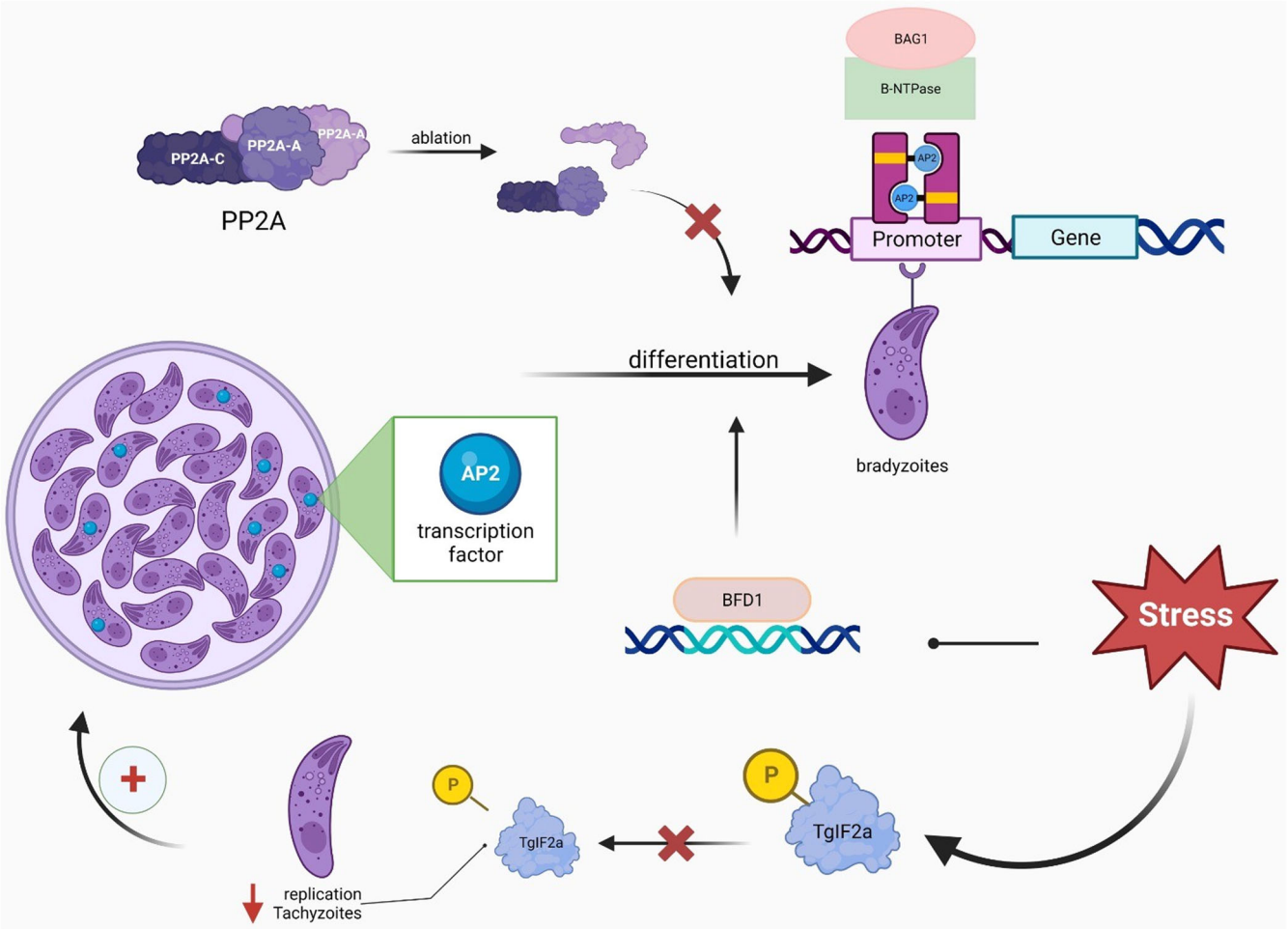

FIGURE 4 Aapetala2 (AP2) proteins from *Toxoplasma gondii* are transregutators of bradyzoite genes. AP2 transcriptional factors have been shown to bind to promoter regions of bradyzoite markers BAG1 and B-NTPase. Stressful conditions lead to phosphorylation of TgIF2a. TgIF2a phosphorylation remains even after differentiation into bradyzoites; Inhibition of TgIF2a dephosphorylation leads to decreased tachyzoite replication and increased cyst formation with low reactivation rate. Myb-like transcription factor (BFD1) is necessary for bradyzoite differentiation under stress condition. *T. gondii* phosphatase 2A (PP2A) holoenzyme is formed of a catalytic subunit PP2A-C, a scaffold subunit PP2A-A and a regulatory subunit PP2A-B. Ablation of any of these subunits upregulates starch accumulation and interruptes the tachyzoite-to-bradyzoite differentiation.

This story, in fact, began when research carried out in 2018 showed that intracellular amastigotes exposed to nutritional stress or exposed to therapeutic drugs demonstrated a rapid, but reversible proliferation suppression accompanied by the accumulation of parasites in the G1 phase of the cell cycle [86]. Thus, the debilitating and potentially fatal infection caused by *T. cruzi* can occur as the parasite has the ability to persist in the host for long periods of time. Taken together, tissue-resident *T. cruzi* amastigote forms are refractory to immune response and drug treatment, suggesting that in addition to evading immune response, amastigotes may facilitate their survival by flexibly adapting to diverse stressful niches.

3.3.2 | Hybrid *T. cruzi* are likely more susceptible to get into dormancy and homologous recombination may be involved in the process

From the initial studies, issues related to dormancy in *T. cruzi* became of great interest in academia, and other highly relevant studies were carried out. In this sense, authors observed that hybrid strains of *T. cruzi* had a greater number of dormant parasites when compared to non-hybrid strains. These same authors verified that the process of homologous recombination is involved in determining the entry into dormancy. To do so, they irradiated the parasites with gamma radiation and

observed that the percentage of dormant parasites was higher in irradiated cells [87].

3.3.3 | TcALBA30 aggregates into stress granules in *T. cruzi* submitted to nutritional stress. Would this protein be involved in dormancy of the parasite?

T. cruzi RNA-binding protein TcALBA30 was recently characterized in parasites subjected to nutritional stress conditions. The authors demonstrated that this protein resides in the cytoplasm under normal conditions but is recruited into the cytoplasmic foci after nutritional starvation. In addition, experiments performed with the recombinant form of the protein (rTcALBA30) during the stationary phase of parasite growth also showed the recruitment of this protein to cytoplasmic foci. These data indicate that TcALBA30 aggregates into stress granules in parasites subjected to nutritional stress [88]. Taking into account that RNA-binding proteins play an important role in *T. gondii* bradyzoite dormancy and that the formation of protein aggregates—aggresomes has an impact on the induction of dormancy in *E. coli* bacteria, we figured out if the cytoplasmic foci of protein and mRNA aggregates formed in *T. cruzi* cytoplasm under stress conditions would have any role during parasite dormancy. Would these cytoplasmic foci be upregulated during dormancy? These are interesting possible line of investigation to confirm the state of dormancy in *T. cruzi*.

3.3.4 | *T. cruzi* get into dormancy or maintain regular cycles of multiplication?

It was not long before controversies about *T. cruzi* ability to enter dormancy were sparked. A study demonstrated that *T. cruzi* spends more time in the S phase of the cell cycle in the acute phase than in the chronic phase of the disease. However, few host cells can survive infection for more than 14 days. This result suggests that parasite persistence involves regular cycles of replication, host cell lysis, and reinfection [89].

3.3.5 | The caution needed in the interpretation of the results gotten by the methodologies used to determine dormancy in *T. cruzi*

Following the same perspective, a DNDi publication raised important points about the interpretation of dormancy data in *T. cruzi*, especially regarding the methodologies and reagents used to determine the state of dormancy. In the text, the authors suggest caution when interpreting the results obtained with CellTrace Violet or EdU, which in themselves compromise the multiplication of the parasite. They comment on the complexity of parasite multiplication and differentiation and on the existence of parasites in vivo with a nonclassical morphology. Thus, they emphasize that infected host cells contain parasites within a variety of metabolic conditions, which may reflect heterogeneity in drug susceptibility [90]. Therefore, there is no evidence of a broad spectrum of dormancy in parasites that persist in these tissue reservoirs. While *T. cruzi* dormancy is still a matter of debate, additional studies are needed to better elucidate this process, and the molecular basis involved in the reduced parasite multiplication rate detected in the chronic phase of Chagas disease.

4 | FINAL CONSIDERATIONS AND CONCLUSION

Cell dormancy in bacteria and protozoa consists of a complex mechanism that involves different components of the cellular machinery. Its aim is to decrease metabolism and to interrupt cell cycle without killing the cell. Bacteria employ intricate mechanisms to induce cell dormancy triggered by a stressful environment such as antibiotic treatment. *E. coli* forms protein aggregates, called aggressomes that are important indicators of bacterial dormancy. The bacterial TA machinery allows bacteria to persist in the infected microenvironment even without acquiring antibiotic resistance plasmids. The formation of dormant *E. coli* has been shown to depend on the TA modules pathway controlled by (p)ppGpp, which involves HokB, TisB, RelEB toxins, and a panel of mRNA endonuclease toxins. Under favorable environmental conditions, these bacteria can resume their growth. In addition to the TA machinery, bacteria have an arsenal of molecules that together operate to establish dormancy. Among them, DosR regulon in *M. tuberculosis* is a potential target for the development of drugs that interfere with bacterial dormancy and favor its elimination.

In the case of *P. vivax* infection, hypnozoite forms are largely responsible for disease recurrences. Although the mechanisms associated with the dormant state of these evolutionary forms of the parasite remain unknown, studies have shown the potential role of several molecules in the dormancy process. Transcriptome and proteomics studies pointed to a set of molecules that may

have an important impact on the rhizome underlying the entry into dormancy. For *T. gondii*, studies have identified RNA-binding proteins, protein kinases and transcription factors that may elucidate the genetic and signaling pathways that regulates bradyzoite differentiation and dormancy onset. Little is known about the mechanisms by which *T. cruzi* becomes dormant. Data are still contradictory and no molecule potentially involved in the negative regulation of parasite multiplication during the chronic phase of the disease has been identified.

Finally, we understand that investment in research focused on detailed comprehension of the molecular basis for pathogens getting into dormancy is critical for the eradication of these infectious diseases. As parasite persistence means chronic carrier, chronic disease, chronic inflammation, and potential reservoir, the only weapon to fight against this hide-and-seek game is the full comprehension of dormancy. This knowledge is critical to aid in the identification of potential drugs able to turn pathogens visible to the immune system, and cytotoxic compounds and/or specific compounds that are toxic to the dormant form of the parasite, which will prevent disease reactivation.

AUTHOR CONTRIBUTIONS

Claudio V. da Silva: Conceptualization; project administration; supervision; writing—original draft; writing—review and editing. **Teresiama Velikkakam**: Writing—original draft. **Elida C. M. de Oliveira**: Writing—original draft. **Anna C. A. Silveira**: Writing—original draft. **Joed P. de Lima Júnior**: Writing—original draft. **Nelsa P. I. Uombe**: Writing—original draft. **Paulo H. R. da Silva**: Writing—original draft. **Bruna C. Borges**: Writing—original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

No data are available.

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