INRS - Centre Armand-Frappier Santé Biotechnologie

REPROGRAMMING eIF4A-DEPENDENT mRNA TRANSLATION TO CONTROL *LEISHMANIA* INFECTION

Par

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Mémoire présenté pour l'obtention du grade de

Maître ès Sciences (M.Sc.)

en sciences de Virologie et Immunologie

Jury d'évaluation

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ACKNOWLEDGEMENTS

This document was written between the (not so) harsh Montreal 2024 winter and the (very) hot and stormy São Paulo 2024 summer. I experienced these two very distinct seasons, happening simultaneously, in opposite sides of the globe. They were equally as intense, both outside and within me. This 150-page document couldn't possibly comprise the past 2.5 years, the most challenging, life changing and unbelievably fun of my life. I am so proud of what I have accomplished, but I couldn't possibly have gotten here by myself: research is an inherently collective effort, and my community has been the most important part of my experience in Canada. This is my moment to thank everyone that has been there for me, a love letter to some of my favorite people in the world right now:

Before anything else, I need to thank my parents, Ana e Laercio, and it does not feel right to do this in any other language apart from our own. Muito obrigado, pai e mãe, por todo o apoio e cuidado. Vocês continuam me surpreendendo com o amor de vocês e esse porto-seguro é o melhor presente que vocês poderiam me dar. Espero um dia retribuir tudo o que vocês fizeram, fazem e ainda vão fazer por mim. Essa conquista é de vocês porque sem vocês eu não estaria aqui, literalmente. Obrigado por serem os melhores pais do mundo. While we're at it, I also need to thank my sister. Tata, I'm so grateful I chose to go through this experience while conveniently being a few hours away from you. I don't know if I would have made it if I didn't have you around, both physically and emotionally, te amo. Finally, I'd like to extend my gratitude to other members of my family that have showed me support over the years: my aunts Leila, Nina, e Ró; my cousins Victor, Rapha, Enzo e Marcus; my godparents Valéria e Alfredo and my great aunt Tia Jô. Obrigado por todo o carinho e apoio que vocês tiveram por mim ao longo desse mestrado. Essa conquista, a primeira na família, também é de vocês.

I have always been obsessed with my friends, and leaving Brasil made me value them even more. Evelyn, Gabriel, Leo, Lucas, Mariana, Marina, Rodes, Santiago, Thalita and Triz, I am so lucky to have you in my life, and without you I probably wouldn't have made it this far after moving away. Thank you so much for all the support, for trying to keep up with my (sometimes chaotic) life abroad, and for making yourselves present, sometimes daily, even though we are 9 000 km away. It means the world to me to know that I'll always have you back home, and that you are with me no matter where I go.

Montreal was the best surprise I could have discovered in North America, with all the amazing people I found here that have shaped me into who I am today: Andre, Berson, Christina,

Darinka, Doris, Lulu, Maria Gracia, Milla, Rummenigge and Thays, thank you for being my family for the past 2.5 years, for all the parties, the deep conversations and - more importantly - the very silly and unserious ones. You taught me so much and you don't even know it. A special thanks to Cami, the happiest coincidence that completely changed my experience in the city. You have been with me since day 0 in this craziness and watched me become who I am today. Another honorable mention goes to Camila, my favorite lab mate and probably the one single person in this planet that knows exactly what I have been through since I arrived in Montreal for my Master's, because she was going through the exact same. Only we know what these years were like, and I couldn't be happier to have lived them with you.

Thank you to my supervisor, Dr. Maritza Jaramillo, for all the support. Your love for research and your ambition are truly inspiring. I hope many more students under your supervision can witness the kind, understanding and competent professional you are. I am very proud of the work we did together, and extremely happy to have contributed to the amazing work you do in your lab. Thank you for believing in me, and for giving me the opportunity to live those past 2.5 years. I never thought such small parasites would take me so far. Furthermore, I need to thank the other current and past members of the Jaramillo lab: Andres, Aurore, Carolina, Laura, Louis-Philippe, and Visnu, for their help and support.

I obviously cannot finish this without thanking my late friend, Leonardo Takase, whose loss I still mourn and feel every single day. It hurts me to know you saw the beginning of this journey but you're not here to see the end. You supported my decision to do this and cheered for me as the first months of my new life unravelled. I may never forgive myself for not being physically there for you during your last days. As I once said, all my achievements are dedicated to you, your memory, and to all the wonderful things you would have accomplished if you were still with us. This is for you, Leo.

Writing this section alone took more effort than the rest of this document, and even though the details of my master's thesis will most likely escape me in the future, my gratitude will remain intact. Above my soon-to-be-awarded title, experiments, long hours in the lab, scientific conferences and classes, my biggest accomplishment during these years was realizing how much I am supported and shaped by my community. People are my biggest prize: those I brought with me from home, those I met along the way, those I'll still get to know, and the ones that, despite no longer being here, will be forever in my heart.

RÉSUMÉ

Les parasites protozoaires du genre *Leishmania* sont des agents responsables de la leishmaniose, un spectre de maladies tropicales négligées. L'absence de vaccins efficaces et les échecs thérapeutiques récurrents reflètent la nécessité d'identifier de nouvelles cibles thérapeutiques. Notre laboratoire a démontré que, lors d'une infection par *Leishmania*, la traduction des ARNm dépendants de l'hélicase eIF4A est activée dans les macrophages murins dérivés de la moelle osseuse (BMDMs). Les rocaglates, inhibiteurs sélectifs d'eIF4A, possèdent des propriétés immunomodulatrices associées à leur capacité à reguler les fonctions des macrophages. Par conséquent, nous postulons que l'inhibition des programmes traductionnels dépendants d'eIF4A contribue au contrôle de l'infection par *Leishmania*. Pour tester cette hypothèse, nous avons évalué l'activité leishmanicide d'un panel de rocaglates synthétiques ayant une affinité forte ou faible/inexistante pour eIF4A1 des mammifères (« weak » et « strong clampers » de eIF4A1, respectivement). Nous avons identifié des rocaglates synthétiques capables de réduire la persistance de *L. amazonensis* dans les BMDMs et les macrophages humains, ainsi la capacité de ces composés à affecter les parasites dans leur stade amastigote et à inhiber la traduction. Nous avons aussi caractérisé la dépendance de cet effet antiparasitaire sur l'eIF4A de l'hôte. Notre objectif à long terme est de fournir un aperçu de la base mécanistique et du potentiel thérapeutique de la modulation des programmes traductionnels dépendants d'eIF4A pour réduire la morbidité et la mortalité associées à la leishmaniose.

Mots-clés : *Leishmania*; macrophage; interaction hôte-parasite; traduction de ARNm; eIF4A; rocaglates.

ABSTRACT

Protozoan parasites of the genus *Leishmania* are causative agents of leishmaniasis, a spectrum of neglected tropical diseases. The lack of effective vaccines and recurrent treatment failure reflect the need to identify new therapeutic targets. Our laboratory has demonstrated that, during *Leishmania* infection, mRNA translation dependent of the eIF4A helicase is activated in mouse bone marrow-derived macrophages (BMDMs). Rocaglates, selective eIF4A inhibitors, have immunomodulatory properties associated with their ability to fine-tune macrophage functions. Therefore, we postulate that the inhibition of eIF4A-dependent translational programs contributes to the control of *Leishmania* infection. To test this hypothesis, we evaluated the leishmanicidal activity of a panel of synthetic rocaglates with strong or weak/no affinity for mammalian eIF4A1 ("strong clampers" and "weak clampers" of eIF4A1, respectively). We were able to identify synthetic rocaglates capable of reducing the persistence of *L. amazonensis* in BMDMs and human macrophages, as well as identify the ability of these compounds to affect amastigote stage of the parasite, inhibit translation and we characterized the dependency of this antiparasitic effect on host eIF4A. Our long-term goal is to provide insight into the mechanistic basis and therapeutic potential of modulating eIF4A-dependent translational programs to reduce morbidity and mortality associated with leishmaniasis.

Keywords : *Leishmania;* macrophage; host-parasite interactions; mRNA translation; eIF4A; rocaglates.

SOMMAIRE RÉCAPITULATIF

Introduction

La leishmaniose est un groupe de maladies tropicales négligées à transmission vectorielle, causées par au moins 20 espèces de parasites protozoaires *Leishmania* (Burza *et al.*, 2018). Différentes manifestations de la maladie peuvent apparaître chez les patients, notamment la leishmaniose cutanée (CL), la leishmaniose muco-cutanée (MCL) et la leishmaniose viscérale (VL) (Bari, 2012). La forme cutanée de la leishmaniose est endémique dans 90 pays (Figure 1.1) et provoque environ 1 million de cas dans le monde par année (Burza *et al.*, 2018). La leishmaniose cutanée est caractérisée par l'apparition d'une lésion unique à l'endroit de la piqûre du phlébotome (Burza *et al.*, 2018; Thomaidou *et al.*, 2015). Un faible pourcentage de patients infectés par des espèces du sous-genre *Viannia* responsables de la CL peuvent également développer une MCL en raison de la propagation des parasites par la circulation sanguine ou du tissu lymphatique (Marra et al., 2014). Le MCL est une manifestation très défigurante de la maladie, provoquant la destruction des tissus muqueux et cartilagineux. La leishmaniose viscérale est endémique dans 80 pays et touche 50 000 à 90 000 patients par année (Burza *et al.*, 2018), et est éventuellement mortelle si non traitée (van Griensven *et al.*, 2012; van Griensven *et al.*, 2019). Les symptômes courants associés à cette forme de la maladie sont la fièvre, l'organomégalie et la perte de poids (Guerin *et al.*, 2002).

La charge de morbidité due à la leishmaniose touche les populations les plus pauvres et mal nourries, avec de faibles revenus par habitant signalés chez les patients atteints de leishmaniose dans différents pays en développement (inférieurs à 1 $/jour) (Alvar *et al.*, 2006). De plus, les mauvaises conditions de logement sont des facteurs de risque connus de la leishmaniose, car elles constituent un environnement idéal pour les phlébotomes qui transmettent la maladie (Calderon-Anyosa *et al.*, 2018). La biologie du vecteur, les espèces de parasites et le statut/réponse immunitaire de l'hôte infecté détermineront la pathogenèse de la leishmaniose (Colmenares *et al.*, 2002).

Les leishmanioses sont causées par *Leishmania spp*., parasites protozoaires de la famille des *Trypanosomatidae*. Ce sont des parasites intracellulaires obligatoires et peuvent infecter différentes espèces, notamment les mammifères, les reptiles et les amphibiens. Les formes promastigotes du parasite sont mobiles, allongées et contiennent un flagelle visible, elles vivent et se reproduisent dans le tube digestif des phlébotomes (Phlebotominae) (Teixeira *et al.*, 2013).

Au cours du cycle de vie du parasite (Figure 1.2), des formes promastigotes métacycliques de *Leishmania* sont régurgitées dans les tissus cutanés de l'hôte, lors du repas de sang d'un phlébotome infecté (Bates, 2007; Turco *et al.*, 1992). Une fois ces parasites sont internalisés dans les vacuoles parasitophores des macrophages hôtes, le changement de pH et de température déclenchera la différenciation de *Leishmania* en forme amastigote (Bates, 1994; Saar *et al.*, 1998; Zilberstein, 2020). Les espèces de *Leishmania* du complexe *mexicana* (*L. amazonensis et L. mexicana*) sont connues pour former de grands espaces vacuolaires (vacuoles communales) à l'intérieur de la cellule hôte, alors que la majorité des autres espèces de parasites *Leishmania* (y compris *L. major* et *L. donovani*) se trouvent dans des espaces vacuolaires réduits (vacuoles individuelles), contenant un ou deux parasites (Batista *et al.*, 2020; Real *et al.*, 2012).

Différentes options de traitement sont aujourd'hui disponibles contre la leishmaniose, mais toutes présentent des désavantages, telles que la résistance aux médicaments, le coût et divers effets secondaires (Tableau 1.1). Pour cette raison, il est crucial de trouver d'autres options thérapeutiques pour traiter efficacement la maladie. Parmi les options thérapeutiques alternatives actuellement explorées, les thérapies dirigées vers l'hôte constituent une approche intéressante, car elles peuvent éviter le développement de résistance chez le parasite en ciblant l'hôte, soit en renforçant la réponse pour combattre le parasite, soit en évitant la réponse immunitaire exacerbée qui est, dans la plupart des cas, responsable pour la pathogenèse (Novais *et al.*, 2021).

La réponse immunitaire efficace contre la leishmaniose repose fortement sur un profil CD4+ *T helper 1* (Th1), concomitant à une inhibition de la réponse Th2 (Seyed *et al.*, 2021; Srivastava *et al.*, 2012). Les macrophages, les monocytes et les cellules dendritiques infectées éliminent le parasite en produisant de l'oxyde nitrique (NO) et des espèces réactives de l'oxygène (ROS) (Loria-Cervera *et al.*, 2020). L'excès de réponse Th1 peut également provoquer une pathogenèse, et pour cette raison un équilibre est crucial au cours de la progression de la maladie (Figures 1.3 et 1.4).

Les macrophages sont des cellules immunitaires caractérisées par leur capacité phagocytaire. Ce sont des cellules très diverses et plastiques, ayant des fonctions spécialisées dans différents tissus, comme les poumons, le foie, le système nerveux central, les os, etc. (Aegerter *et al.*, 2022; Lalancette-Hebert *et al.*, 2007; Michaelson *et al.*, 1996; Paolicelli *et al.*, 2011; Scott *et al.*, 2018; Vilhardt, 2005; Yao *et al.*, 2021). En tant que phagocytes professionnels, les macrophages internalisent les particules externes, les cellules ou les agents pathogènes dans des vacuoles dérivées de la membrane appelées phagosomes. Ces compartiments naissants

acquièrent les conditions nécessaires pour éliminer le pathogène grâce à un processus appelé maturation du phagosome (Vieira *et al.*, 2002).

Les différents profils de polarisation des macrophages ont été initialement décrits en ce qui concerne leur métabolisme de l'arginine: les macrophages classiquement activés (M1) produisent du NO à partir de la L-arginine, alors que les macrophages activés alternativement (M2) ont un métabolisme d'arginine modifié pour produire de l'ornithine et des polyamines (Mills *et al.*, 2000) (Figure 1.7). Les macrophages M1 sont connus pour avoir un fort profil cytotoxique, produisant du NO et des ROS, en plus d'une réponse pro-inflammatoire Th1 (IL-1, IL-6, IL-12, IL-23, TNF-α) (Funes *et al.*, 2018; Zhang *et al.*, 2008). Les macrophages M2 présentent un profil anti-inflammatoire cicatrisant, produisant des médiateurs de l'angiogenèse, tels que le TGF-β, le VEGF et l'EGF (Laskin *et al.*, 2011). Différents sous-types de polarisation ont différentes fonctions proposées, décrites ici (Tableau 1.2).

Les parasites *Leishmania* peuvent perturber plusieurs fonctions des macrophages pour réussir à établir l'infection. Les interactions entre les parasites *Leishmania* et les macrophages sont nombreuses et comprennent toutes les étapes de la vie du parasite au sein de l'hôte : dès la reconnaissance et l'intériorisation (Vinet *et al.*, 2009), à la différenciation (Mittra *et al.*, 2013a; Mittra *et al.*, 2013b), à l'acquisition de nutriments (McConville *et al.*, 2007; Naderer *et al.*, 2008; Podinovskaia *et al.*, 2015), etc. Certains facteurs de virulence parasitaire jouent un rôle très important dans ces effets induits par *Leishmania*, comme la métalloprotéase GP63 (Figure 1.6). Lorsque GP63 accède à l'intérieur des macrophages, il peut cliver plusieurs substrats (Figure 1.6), modifiant ainsi la signalisation de l'hôte et l'expression des gènes pour favoriser le parasite (Olivier *et al.*, 2012).

La perturbation des fonctions des cellules hôtes causée par *Leishmania* peut être due à des altérations induites par les parasites dans différents niveaux d'expression génétique de l'hôte, tels que les niveaux épigénétique (Calegari-Silva *et al.*, 2018; Parmar *et al.*, 2020), transcriptionnel et post-transcriptionnel (Chaparro *et al.*, 2022; Dillon *et al.*, 2015; Fernandes *et al.*, 2024; Gregory *et al.*, 2008; Muxel *et al.*, 2018; Shadab *et al.*, 2019). En effectuant une analyse du translatome à l'échelle du transcriptome dans les macrophages murins infectés par *L. donovani*, notre groupe a identifié que le parasite pourrait également favoriser des altérations de la traduction de plusieurs ARN messagers (ARNm) lors de l'infection (Chaparro *et al.*, 2020) (Figure 1.8).

La traduction est le processus par lequel une molécule d'ARNm mature est décodée en polypeptide par les ribosomes. Elle peut être divisée en différentes étapes: initiation, élongation,

terminaison et recyclage des ribosomes, bien que l'essentiel de la régulation se produise au stade d'initiation (Sonenberg *et al.*, 2009).

L'initiation de la traduction peut être dépendant des sites d'entrée internes du ribosome (IRES) ou de la coiffe 5' présente dans les ARNm matures (Fitzgerald *et al.*, 2009). Au cours de l'initiation de la traduction dépendante de la coiffe, un complexe de pré-initiation (43S PIC) composé de la partie 40S du ribosome, de l'ARN de transfert de méthionyle initiateur (complexe 40S-Met-tRNAi) et de différents facteurs d'initiation de la traduction des eucaryotes (eIF, *eukaryotic initiation factor*) se lie à l'extrémité 5' d'un ARNm coiffé par m^7G (Figure 1.9) (Pestova *et al.*, 2002). Ce complexe scanne la molécule d'ARNm jusqu'à ce qu'elle atteigne le codon d'initiation (Hershey *et al.*, 2012; Merrick *et al.*, 2018). Une fois le codon d'initiation identifié, la sous-unité ribosomale 60S rejoint le 48S résultant, formant le ribosome mature capable de démarrer l'étape d'élongation de la traduction (Gingras *et al.*, 1999; Kozak, 1991).

Dans ce contexte, le complexe eIF4F joue un rôle en facilitant le recrutement du 43S PIC sur la coiffe 5' d'un ARNm qui sera traduit (Banerjee, 1980; Gingras *et al.*, 1999; Sonenberg *et al.*, 2009). Le complexe eIF4F est composé d'eIF4E, une protéine liant la coiffe, d'eIF4A, une hélicase à ARN, et d'eIF4G, une protéine d'échafaudage qui lie tous les autres composants du complexe et l'ARNm (Fitzgerald *et al.*, 2009; Pestova *et al.*, 2002). Il a été démontré que les formes mutantes d'eIF4A arrêtaient le complexe eIF4F dans un état inactif, inhibant la traduction indépendante et dépendante de la coiffe (Pause *et al.*, 1994b; Svitkin *et al.*, 2001) et démontrant l'importance du complexe eIF4F et spécifiquement d'eIF4A pour initiation de la traduction.

La région 5' non-traduite (« UTR, untranslated region ») de certains ARNm matures forme des structures secondaires qui bloquent l'initiation de la traduction de cette molécule (Hinnebusch *et al.*, 2016). Ces régions 5' UTR peuvent réguler négativement la traduction en fonction de leur complexité et de leur organisation structurelle (Grayeski *et al.*, 2022; Wang *et al.*, 2022). En tant qu'hélicase à ARN, eIF4A est connue pour favoriser une restructuration dépendante de l'ATP de la région 5' UTR des ARNm, générant une molécule d'ARNm simple brin, afin de faciliter la liaison du PIC 43S et la recherche du codon d'initiation (Hinnebusch *et al.*, 2012; Pestova *et al.*, 2002; Ray *et al.*, 1985; Sonenberg *et al.*, 2009). Plus précisément chez les mammifères, les molécules d'ARNm dépendantes d'eIF4A sont caractérisées par de longues régions 5'UTR qui forment des structures secondaires stables et par la présence de structures appelées G-quadruplexes (Rubio *et al.*, 2014; Svitkin *et al.*, 2001; Wolfe *et al.*, 2014).

La cible mécanistique de la rapamycine (« mTOR, mammalian target of rapamycin ») est une kinase sérine/thréonine dont le rôle est bien établi dans la régulation de la traduction des

cellules eucaryotes supérieures. Le complexe mTOR 1 (mTORC1) est formé par mTOR, la protéine régulatrice associée à mTOR (Raptor), et la « mammalian lethal » protéine Sec13 8 (mLST8) (Kim *et al.*, 2002; Melick *et al.*, 2020). Il détecte les stimuli externes, les changements dans la disponibilité des nutriments et reprogramme la cellule de différentes manières. La régulation translationnelle dépendante de mTORC1 repose principalement sur la phosphorylation des protéines liant eIF4E (« 4E-BP, eIF4E-binding proteins ») (Dowling *et al.*, 2010; Gingras *et al.*, 1998), de la protéine ribosomale S6 kinase (S6K) (Magnuson *et al.*, 2012), et les protéines 1 liées à La (LARP1) (Figure 1.11). Compte tenu de son importance dans la régulation de la traduction et d'autres processus cellulaires essentiels, tels que l'autophagie, mTOR est un facteur couramment dérégulé dans différentes maladies infectieuses, ayant des effets indépendants et dépendants de la traduction: différentes infections virales et bactériennes sont décrites pour moduler l'activité de mTOR (Akbay *et al.*, 2020; Clippinger *et al.*, 2011; Garg *et al.*, 2020; Shariq *et al.*, 2023; Sokolova *et al.*, 2014; Spangle *et al.*, 2010). Notre groupe a identifié une activation de la traduction de l'ARNm sensible à mTOR lors d'une infection par *Toxoplasma gondii* et *Leishmania donovani* (Chaparro *et al.*, 2020; Holmes *et al.*, 2019; Leroux *et al.*, 2018).

Comme indiqué précédemment, l'assemblage du complexe eIF4F est sous régulation dépendante de mTOR, (Pelletier *et al.*, 2019). Dans les macrophages murins primaires (« BMDMs, bone marrow-derived macrophages ») infectés par *L. donovani,* notre groupe a identifié une régulation positive dans la traduction des transcrits sensibles à eIF4A (Figure 1.12A), après une analyse *in silico* (basée sur une compilation de transcrits sensibles à eIF4A précédemment signalés, identifiés à l'aide d'inhibiteurs d'eIF4A1 ou de l'inactivation d'eIF4A1 dans les cellules) (Chaparro *et al.*, 2020). Des rapports antérieurs ont montré que la région 5' UTR contenant des motifs CGG (également connus sous le nom de structures « G-quadruplex ») étaient une signature de la sensibilité à eIF4A dans les ARNm (Wolfe *et al.*, 2014), et notre groupe a identifié que les ARNm contenant ces motifs présentaient également une augmentation d'efficacité de la traduction lors d'une infection par *L. donovani* (Figure 1.12B).

Dans les macrophages, l'inhibition d'eIF4A à l'aide de composés appelés rocaglates peut avoir des effets immunomodulateurs, favorisant une reprogrammation de la polarisation des macrophages qui a été associée à des effets antimicrobiens contre différents agents pathogènes (Bhattacharya *et al.*, 2016; Blum *et al.*, 2020; Chatterjee *et al.*, 2021; Schiffmann *et al.*, 2023). Les rocaglates sont une classe diversifiée de composés naturels et synthétiques qui partagent un cycle cyclopenta[b] benzofurane, isolé à l'origine du genre d'angiospermes *Aglaia* (King *et al.*, 1982). Les rocaglates favorisent un gain de fonction de la protéine eIF4A, améliorant la liaison

(serrage) des deux paralogues (eIF4A1 et eIF4A2) à la molécule d'ARNm (Chu *et al.*, 2019; Iwasaki *et al.*, 2016). La stabilisation de l'interaction ARNm/eIF4A par les rocaglates entrave la liaison du PIC 43S à l'ARNm et la recherche du codon d'initiation, inhibant ainsi la traduction (Iwasaki *et al.*, 2016; Iwasaki *et al.*, 2019). De plus, la rétention plus longue du complexe eIF4F au niveau de la coiffe favorisée par le traitement rocaglate peut avoir un effet inhibiteur supplémentaire sur la traduction globale (Chu *et al.*, 2020).

Nous avons observé que le Silvestrol (un inhibiteur connu d'eIF4A appartenant à la classe des rocaglates) réduisait la persistance du parasite dans les BMDMs (ajouter référence : Chaparro *et al.*, 2020). Cela suggère que l'augmentation de la traduction dépendante d'eIF4A est exploitée par le parasite pour favoriser l'infection (Figure 1.13). Sur la base de ces résultats, nous avons émis l'hypothèse que l'inhibition des programmes de traduction des ARNm de la cellule hôte dépendants d'eIF4A à l'aide de rocaglates pourrait contrôler l'infection à *Leishmania in vitro*. Pour tester cette hypothèse, nous avons évalué la capacité de 40 rocaglates synthétiques ayant une affinité forte ou faible/inexistante pour eIF4A1 des mammifères (« weak » et « strong clampers » d'eIF4A1, respectivement), fournis par le Centre de découverte moléculaire de l'Université de Boston (« BU-CMD, Boston University Center for Molecular Discovery »), à contrôler la réplication du parasite dans les BMDMs.

Notre objectif principal avec ce projet était d'étudier le potentiel thérapeutique du ciblage de la traduction de l'ARNm dépendante d'eIF4A lors d'une infection à *Leishmania in vitro*. Nos objectifs spécifiques étaient divisés comme suit:

1. Identifier les inhibiteurs d'eIF4A parmi une librairie de rocaglates ayant une activité anti-*Leishmania in vitro*, et

2. Caractériser les mécanismes par lesquels ces inhibiteurs d'eIF4A peuvent contrôler l'infection causée par le parasite *Leishmania in vitro*.

Résultats

Nous avons effectué un premier criblage pour évaluer rapidement l'effet potentiel anti-*Leishmania* de l'ensemble des rocaglates obtenus à partir de BU-CMD, tout en obtenant également un aperçu préliminaire de la toxicité de ces composés pour la cellule hôte. Pour ce faire, les macrophages ont été infectés par des promastigotes métacycliques de *L. amazonensis* (MOI 10: 1) et traités 24 h plus tard avec 2 concentrations différentes de chacun des 40 rocaglates (100 nM et 20 nM). Après 72 h de traitement, les cellules ont été collectées et l'indice d'infection a été calculé (Figure 4.1). Les données sont présentées dans deux graphiques, organisés entre les « clampers » d'eIF4A1 faibles (C1-C20) (Figure 4.1A) et les « clampers » forts d'eIF4A1 (C21-C40) (Figure 4.1B). À l'origine, des « clampers » faibles ont été ajoutés comme contrôles dans nos expériences, mais certains de ces composés ont étonnamment présenté un effet antiparasitaire.

Comme critères de sélection pour les « clampers » forts (Figure 4.1A), nous avons choisi des composés qui n'étaient pas toxiques pour les macrophages à 100 nM (sur la base de l'observation au microscope de la morphologie cellulaire, Figure 4.2) et/ou qui étaient capables de réduire l'indice d'infection 72 h après le traitement d'environ 50 % ou plus. Pour les « clampers » faibles (Figure 4.1B), étant donné qu'aucun de ces composés n'était toxique pour les cellules aux concentrations testées, nous avons choisi composés capables de réduire l'indice d'infection 72 h après le traitement d'environ 50 % ou plus. Notre premier criblage nous a permis de réduire de 40 à 9 rocaglates, un nombre beaucoup plus restreint de composés afin de faciliter leur caractérisation ultérieure.

Ensuite, nous avons cherché à évaluer l'effet des composés sélectionnés lors de notre expérience pilote sur des cultures extracellulaires de promastigotes *Leishmania*. Nous avons observé qu'aucun des « clampers » forts et faibles aux concentrations testées n'avait d'effet sur les cultures des promastigotes extracellulaires de *L. amazonensis* après 72 heures de traitement (Figures 4.3 et 4.4, respectivement). Ces résultats suggèrent que le sous-ensemble de rocaglates testés ne cible pas les promastigotes de *L. amazonensis* LV79.

Nous avons ensuite procédé à l'évaluation du profil de toxicité des rocaglates sélectionnés en 4.1 dans les BMDMs (Figures 4.5 et 4.6). Pour les « clampers » faibles, nous avons observé que les composés 3, 7, 11 et 15 peuvent être toxiques pour les BMDMs à des concentrations élevées sur la plage micromolaire, et que les composés 18 et 20 n'ont jamais affecté la viabilité de plus de 40% des cellules aux concentrations testées. Les « clampers » forts ont eu un effet toxique beaucoup plus net sur les BMDMs aux concentrations testées. Une courbe dose-réponse

claire a été obtenue pour ces composés, avec une viabilité cellulaire diminuée à mesure que la concentration de rocaglate augmentait (Figure 4.6). Grâce à ces données, nous avons pu générer les CC_{50} de ces composés (tableau 4.4.1).

Puisque l'objectif du projet était d'évaluer l'effet de la traduction dépendante de eIF4A sur l'infection par *Leishmania*, nous avons choisi d'avancer uniquement avec les 2 composés sélectionnés du groupe des « clampers » forts (C26, C37). Nous avons conservé deux « clampers » faibles (C3 et C18) dans nos expériences comme contrôles, et l'effet des « clampers » faibles dans l'infection par *Leishmania* est ensuite devenu le sujet d'un autre projet de maîtrise au laboratoire.

Nous avons ensuite voulu comprendre si les rocaglates pouvaient cibler le stade amastigote du parasite. Pour cela, nous avons réalisé ce que nous appelons une expérience de réinfection: les BMDMs ont été infectés et traités avec des rocaglates sélectionnés (C3, C18, C26 et C37) comme décrit dans la section méthodologie. Après 72 h de traitement, nous avons extrait les amastigotes des macrophages infectés et utilisé ces parasites pour infecter des nouveaux BMDMs non traités. Nous avons observé qu'entre 24 h et 72 h après la réinfection, les parasites extraits des BMDMs préalablement traités avec du DMSO, C18, C26 ou C37 étaient capables de se répliquer normalement dans des cellules non traitées (Figure 4.7). Cependant, avec C3, nous avons observé que ces parasites étaient incapables de se répliquer et d'augmenter l'indice d'infection. Ceci suggère que le composé 3 pourrait spécifiquement cibler le stade amastigote du parasite et affecter sa capacité réplicative (« fitness ») même lors d'une infection ultérieure, dans des cellules non traitées.

Nous avons procédé à l'évaluation de l'effet des rocaglates sélectionnés dans les BMDMs infectés et des macrophages dérivés de la lignée cellulaire humaine THP-1 (« monocyte-like ») sur trois expériences indépendantes (Figure 4.9). Tous les rocaglates sélectionnés (C3, C18, C26 et C37) ont réduit l'indice d'infection d'environ 50 % dans les BMDMs (Figure 4.9A) et les THP-1 (Figure 4.9B), comme observé précédemment lors de notre expérience pilote dans les BMDMs (Figure 4.1). Cela a validé nos premiers résultats et nous a permis d'établir que la diminution de l'indice d'infection lors du traitement par les rocaglates testés est statistiquement significative.

Parmi les rocaglates utilisés dans les expériences précédentes, C37 a été sélectionné pour caractériser la cinétique du traitement lors d'une infection par *L. amazonensis* parce qu'il a eu le meilleur effet pour réduire l'indice d'infection dans les BMDMs (Figure 4.9A). Nous avons observé que même si les parasites se répliquaient dans les cellules traitées au DMSO, l'indice d'infection restait le même tout au long de l'infection dans les cellules traitées au C37 (Figure 4.11). Ces

données suggèrent que le traitement au C37 favorise le blocage de la réplication du parasite plutôt que son élimination à l'intérieur de sa cellule hôte.

Nous avons ensuite cherché à évaluer si l'effet des rocaglates dépendait d'eIF4A de l'hôte. Pour ce faire, nous avons infecté et traité des BMDMs obtenus à partir de souris *EIF4A1*$^{+/-}$ et *EIF4A2*$^{+/-}$. Nous avons observé que l'effet antiparasitaire des « clampers » forts (C26, C37, C38) était perdu lorsque les cellules hôtes étaient déficientes en eIF4A1 (*EIF4A1*$^{+/-}$). (Figure 4.11). En revanche, l'effet anti-*Leishmania* a été observé dans les tous les groupes de BMDMs pour les « clampers » faibles testés (C3 et C18), indiquant que cet effet est indépendant d'eIF4A1 et d'eIF4A2. En conséquence, nous émettons l'hypothèse que pour les « clampers » faibles, l'effet est dû à un ou plusieurs autres facteurs de l'hôte et/ou du parasite, encore inconnus. Enfin, le traitement avec des rocaglates sectionnés dans les BMDMs *EIF4A2*$^{+/-}$ a montré un effet similaire à celui observé dans les BMDMs de type sauvage (Figure 4.11).

Enfin, pour évaluer si les rocaglates sélectionnés peuvent exercer un effet sur la traduction globale des cellules, nous avons traité une lignée de BMDMs immortalisés (iBMDMs) avec C3, C18, C26 et C37 pendant 2 h et évalué leurs profils de traçage de polysomes (« polysome tracings »). Pour la première expérience, nous avons également utilisé le Silvestrol comme contrôle de l'inhibition de la traduction dépendante d'eIF4A par un rocaglate commercial. Nous avons observé l'effet différentiel que les rocaglates « clampers » forts et faibles peuvent avoir sur la traduction globale des iBMDMs (Figure 4.12) : le traitement avec les « clampers » forts C37 et C26 a entraîné une forte inhibition de la traduction à 10 nM et 80 nM, respectivement. Le « clamper » faible C3 a montré une inhibition similaire de la traduction globale à une concentration plus élevée (100 nM) tandis que le « clamper » faible C18 n'a montré aucun effet sur la traduction, même à la concentration élevée utilisée (500 nM). Cela fait allusion au mécanisme antiparasitaire différentiel ces classes de rocaglates pourraient avoir dans les BMDM infectés (dépendant ou indépendant de la traduction de l'hôte), et est conforme à nos données montrant que l'effet antiparasitaire des « clampers » forts, dépendait de eIF4A1, mais pas celui des « clampers » faibles.

Discussion et conclusion

Dans ce projet, nous avons pu débuter l'évaluation des effets thérapeutiques potentiels des rocaglates synthétiques lors d'une infection à *Leishmania in vitro*. Les données obtenues ont soulevé de nouvelles questions qui seront abordées et développées plus en détail dans cette section.

L'analyse *in silico* de l'homologue eIF4A de *L. amazonensis* (LeIF4A) a révélé un profil d'acides aminés associé à la résistance aux rocaglates (Obermann *et al.*, 2023). Cela est conforme à nos résultats qui n'ont montré aucun effet des rocaglates « clampers » forts testés dans les cultures de promastigotes extracellulaires (Figure 4.3). Nous ne pouvons cependant pas exclure que les rocaglates, notamment les « clampers » faibles, puissent cibler d'autres protéines du parasite. C'est pour çela que nous avons évalué la capacité des amastigotes extraits des BMDMs préalablement traites avec des rocaglates sélectionnées à se répliquer dans des BMDMs non traités (Figure 4.7). Ces expériences indiquent que C3 semble influencer la réplication des amastigotes par un mécanisme qui reste encore à déterminer.

En plus de LeIF4A, d'autres hélicases à ARN ont été décrites chez les parasites *Leishmania* (Afonso-Lehmann *et al.*, 2015; Dhalia *et al.*, 2005; Marchat *et al.*, 2015; Mokdadi *et al.*, 2021) qui pourraient être ciblées par des rocaglates « clampers » faibles incluant C3, ce qui pourrait expliquer l'effet observé dans note étude. En explorant la possibilité que C3 cible une protéine spécifique de l'amastigote, son modèle d'expression devrait suivre ce qui a été décrit pour LeishDED1-1, une hélicase à ARN parasitaire connue pour être davantage exprimée au stade amastigote (Marchat *et al.*, 2015; Zinoviev *et al.*, 2012), puisque la viabilité des promastigotes n'est pas affectée par des concentrations allant jusqu'à 10 µM de C3 (Figure 4.4). Les rocaglates sont également décrits pour cibler des hélicases à ARN autres que eIF4A chez l'humain, notamment DDX3 (Chen et al., 2021 ; DiVita et al., 2021). Des expériences de polarisation de fluorescence (FP) ont été réalisées par notre collaboratrice, Dre Regina Cencic (laboratoires du Dr Jerry Pelletier et du Dr Sidong Hoang, Université McGill, Montréal, QC, Canada), montrant que le composé 3 se lie au eIF4A1 humain recombinant in vitro, mais n'interagit pas avec le DDX3X humain recombinant (Figure 5.1).

Au cours de mon projet de maîtrise, nous avons également évalué l'effet du traitement des BMDMs avec des rocaglates avant l'infection, afin d'obtenir des informations mécanistiques sur les rocaglates et de comprendre si les rocaglates pourraient être explorés comme option prophylactique. Nous avons observé que le prétraitement est également efficace pour réduire l'indice d'infection après 72 h d'infection chez les BMDMs (Figure 5.2).

Nos données de traçage de polysomes réalisées dans les iBMDMs ont révélé que les rocaglates sélectionnés pouvaient moduler de manière différentielle la traduction globale. En corrélant ces informations avec nos données antiparasitaires dans les BMDMs infectés, nous pouvons observer que la réduction de l'indice d'infection causée par le traitement est associée à une inhibition de la traduction pour C3, C26 et C37 mais pas pour C18 (Figure 4.12). Le modèle des iBMDMs a été utilisé pour ces expériences car la technique de traçage des polysomes nécessite une certaine quantité d'ARN beaucoup plus facile à obtenir à l'aide de ces cellules immortalisées, qui se répliquent en culture. Les iBMDMs peuvent également être génétiquement modifiés, ce qui nous aiderait à comprendre davantage le mécanisme par lequel les rocaglates inhibent la traduction. Pour cela, nous prévoyons de générer des lignées cellulaires iBMDMs déficientes (« knockdown » ou « knockout ») en eIF4A1 ou eIF4A2 et/ou des lignées cellulaires iBMDMs exprimant une forme d'eIF4A1 résistante aux rocaglates.

Étant donné que le NO est le principal mécanisme anti-*Leishmania* du macrophage infecté, nous avons évalué si la production du NO était augmentée lors du traitement aux rocaglates sélectionés (C3, C18, C26, C37) mais nous n'avons trouvé aucune différence dans les niveaux de NO lors du traitement des BMDMs infectés et non infectés et des macrophages dérivés de THP-1 (données non présentées). L'absence d'augmentation du principal mécanisme antiparasitaire du macrophage est conforme à nos données montrant que le rocaglate C37 favorise le blocage de la réplication, au lieu de l'élimination des parasites (Figure 4.10). La production de NO est une caractéristique de la polarisation des macrophages M1 (Funes *et al.*, 2018; Zhang *et al.*, 2008). Malgré ce résultat préliminaire négatif, des recherches plus approfondies sont nécessaires pour déterminer si les rocaglates peuvent polariser les macrophages (par exemple, surveiller d'autres marqueurs M1). Si les rocaglates testés ne polarisent effectivement pas les BMDM vers le phénotype M1, comme décrit précédemment (Bhattacharya *et al.*, 2016; Chatterjee *et al.*, 2021), un autre aspect de la réponse de la cellule hôte à *Leishmania* dépendant de l'eIF4A doit être affecté par le traitement. En évaluant la liste des transcrits régulées par la infection des BMDMs par *L. donovani* (Chaparro *et al.*, 2020), nous pouvons observer différentes catégories d'ARNm codant pour des protéines impliquées dans la signalisation cellulaire, le métabolisme de l'ARN ainsi que des protéines structurelles et impliquées dans le trafic cellulaire (Figure 1.8). Nous pourrions donc en déduire que l'altération de la traduction de certains de ces gènes dépendants de eIF4A décrits est préjudiciable à l'infection de *Leishmania* dans le macrophage.

Enfin, étant donné que le « clamper » fort C37 était notre meilleur candidat pour réduire l'indice d'infection dans les BMDMs infectés par *L. amazonensis* d'une manière dépendante d'eIF4A1, nous avons choisi ce composé pour poursuivre des expériences protéomiques. L'objectif est de réaliser un double protéome pour obtenir des informations sur les protéines du parasite et de l'hôte au cours de l'infection en présence ou en absence du C37. Avec cette expérience, nous esperons comprendre quelles protéines ont été ciblées par le traitement et comment elles sont importantes pour la persistance du parasite, ainsi que de comprendre si le traitement par ce rocaglate a un effet sur l'expression de protéines de l'amastigote.

Sur la base de nos découvertes et des informations fournies dans la discussion de ce mémoire, le « clamper » faible C3 semble être le meilleur candidat pour un rocaglate à double ciblage (c.-à-d., rocaglate qui pourrait cibler à la fois des ARNm parasitaires et de la cellule hôte), bien que les cibles exactes chez le parasite et l'hôte soient inconnues. Le « clamper » fort C37 semble cependant favoriser une réponse anti-*Leishmania* dépendante d'eIF4A dans les BMDMs. De façon intéressante, nos données indiquent que C3 et C37 peuvent affecter la traduction globale des iBMDMs.

Ce projet a servi de preuve de concept afin de poursuivre l'étude de l'utilisation de rocaglates pour contrôler l'infection par *Leishmania in vitro* et *in vivo.* En parallèle, les résultats générés dans le cadre de ce projet de recherche ont accru nos connaissances sur la manière dont différentes classes et sous-types de rocaglates peuvent avoir un effet anti-*Leishmania* dans les BMDMs (modèle de souris) et les macrophages dérivés de la lignée cellulaireTHP-1 (modèle humain).

La question qui reste est de determiner quels programmes traductionnels de l'hôte sont affectés par l'utilisation de C37 et pourquoi ils peuvent empêcher la réplication du parasite. Pour répondre à cette question, une étude multi-omique sera réalisée avec des cellules infectées et traitées seront réalisés dans notre laboratoire (transcriptome, translatome, et protéome). Nous avons également l'intention d'explorer davantage le potentiel anti-*Leishmania* du rocaglate C37, en évaluant son effet dans des isolats cliniques et *in vivo* à l'aide d'un modèle expérimental murin de leishmaniose cutanée. Puisque la leishmaniose viscérale peut être mortelle si elle n'est pas traitée, des expériences sur des modèles expérimentaux de leishmaniose viscérale sont également proposées pour évaluer l'effet des rocaglates sur cette manifestation de la maladie.

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1 INTRODUCTION

1.1 Leishmaniasis and *Leishmania* parasites

1.1.1 Leishmaniasis

Leishmaniasis is a group of vector-borne tropical neglected diseases, caused by at least 20 species of *Leishmania* protozoan parasites (Burza *et al.*, 2018). Different manifestations of the disease may emerge in patients: from subclinical (non-apparent) infections, to self-resolving localized manifestations, and potentially deadly disseminated infections (Bari, 2012). The burden of the disease affects the poorest and malnourished populations, with low per capita incomes (below 1$/day) reported in leishmaniasis patients in different developing countries (Alvar *et al.*, 2006). Additionally, poor housing conditions (walls with cracks, humidity, and darkness) are known risk factors to leishmaniasis, as they provide the ideal environment for sandflies that transmit the disease (Calderon-Anyosa *et al.*, 2018). Given that the disease disproportionally affects populations with little political power and low economic status to afford health care (Desjeux, 1996), leishmaniasis and poverty interact in a vicious cycle, mutually reinforcing each other (Alvar *et al.*, 2006).

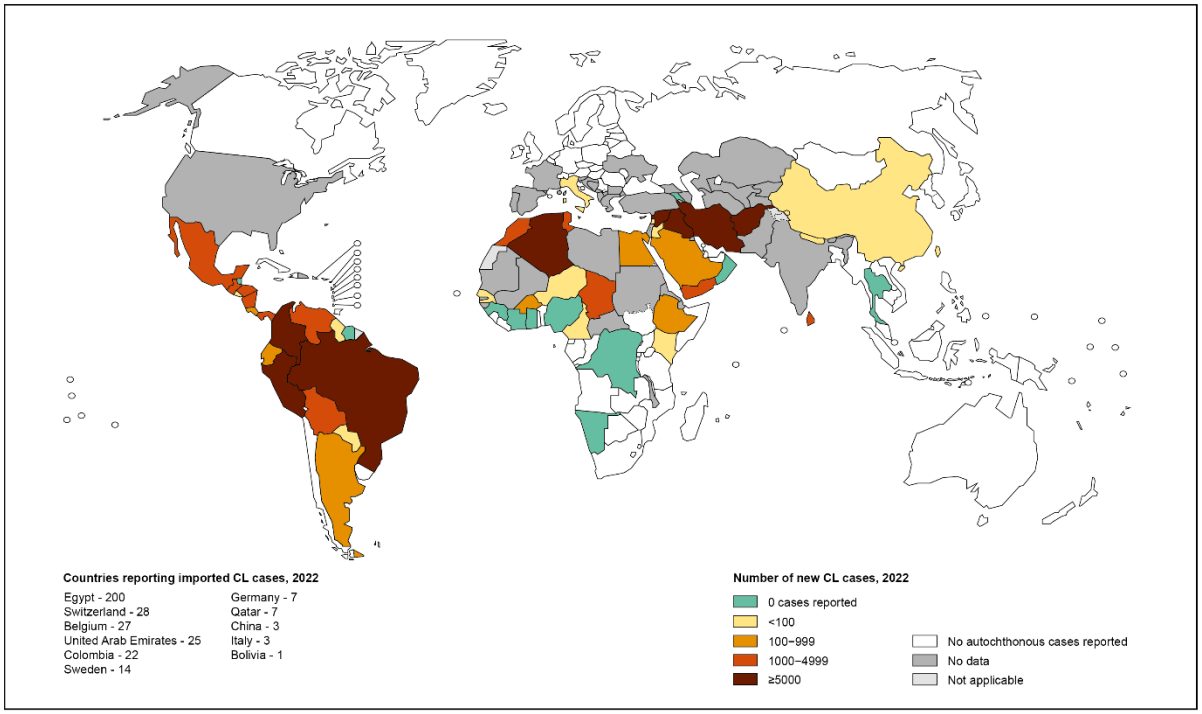
Vector biology, parasite species, and immune status/response of the infected host will determine the pathogenesis of leishmaniasis (Colmenares *et al.*, 2002). Four main clinical manifestations of the disease will be explored in the following paragraphs: cutaneous, muco-cutaneous, diffuse cutaneous and visceral leishmaniasis.

1.1.1.1 Cutaneous leishmaniasis (CL)

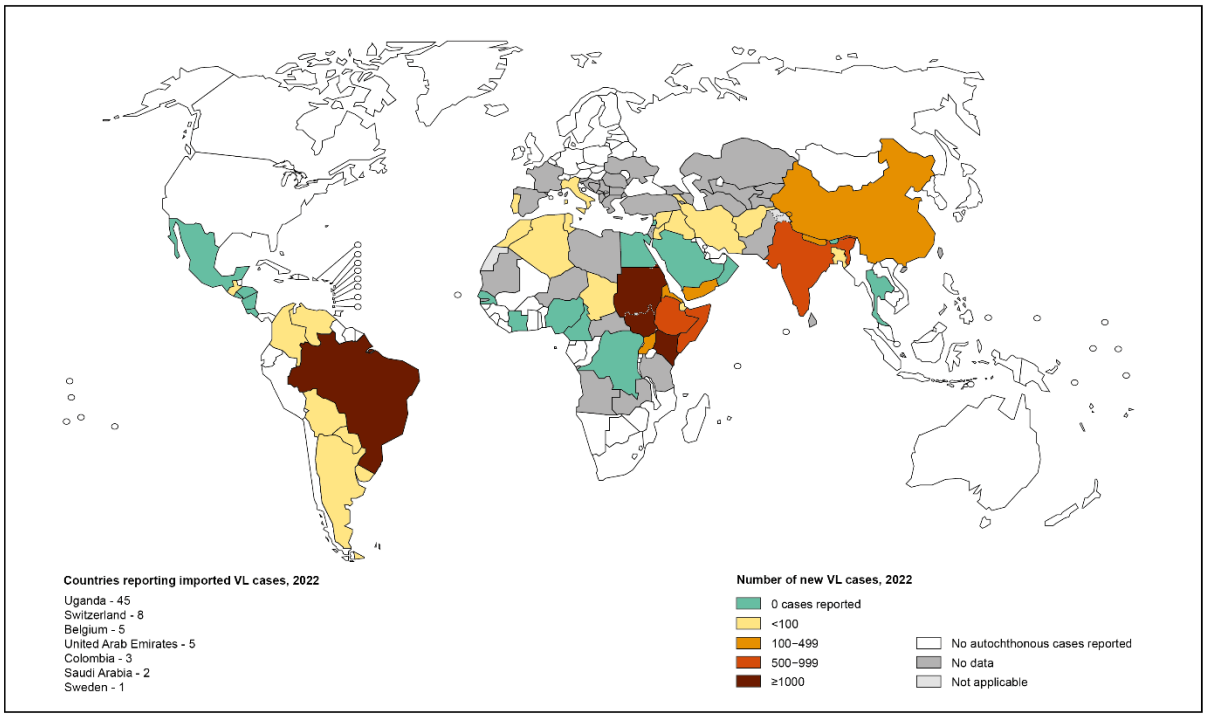
The cutaneous form of leishmaniasis is endemic in 90 countries (Figure 1.1) and causes approximately 1 million cases worldwide per year (Burza *et al.*, 2018), with 90% of these cases coming from 9 endemic countries (Desjeux, 2004). It is the most recurring form of the disease, and it is characterized by the appearance of a single lesion on the site of the sandfly bite, although multiple lesions have been observed in patients due to several sandfly bites or lymphatic parasite dissemination (Burza *et al.*, 2018; Thomaidou *et al.*, 2015). Different species of *Leishmania* can cause CL, importantly *L. major*, *L. tropica*, and *L. aethiopica*, in southwestern Asia and northern Africa, and *L. amazonensis*, *L. mexicana* and *L. braziliensis*, endemic in Central and South America (Burza *et al.*, 2018; Scorza *et al.*, 2017).

Imported cases of CL are increasingly becoming a burden in non-endemic countries, originating from travelers and populational displacement/immigration from endemic countries (Pavli *et al.*, 2010). Even though CL is not life-threatening and usually self-healing (Burza *et al.*, 2018; Scorza *et al.*, 2017), the lesions and scars caused by the disease are associated with a social and cosmetic stigma, and psychological burden on patients (Yanik *et al.*, 2004). Women are specially affected by the stigma of CL scars in endemic areas of the disease, with reported impact on their social lives and daily activities such as breastfeeding and working (Bilgic-Temel *et al.*, 2019; Hamdam, 2020).

Status of endemicity of cutaneous leishmaniasis (CL) worldwide, 2022 (as reported by November 2023)



Status of endemicity of visceral leishmaniasis (VL) worldwide, 2022 (as reported by November 2023)



Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

Figure 1.1 Status of endemicity of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) worldwide.
With over a million new cases every year and 1 billion people living in regions at risk, leishmaniasis is amongst the most important emerging vector-borne protozoan diseases. Taken from (World Health Organization, 2022)

1.1.1.2 Mucocutaneous leishmaniasis (MCL)

A small percentage of patients infected with species of the *Viannia* subgenus causative of CL (*L. braziliensis*, *L. amazonensis*, *L. panamensis,* and *L. guyanensis*) can also develop mucocutaneous leishmaniasis (MCL) due to the spread of the parasites via bloodstream or lymphatic tissue (Marra *et al.*, 2014). MCL is a highly disfiguring manifestation of the disease, causing the destruction of mucous and cartilaginous tissues. The nasal and oral mucosa are the most commonly affected areas, potentially affecting the vocal chords and this manifestation can be lethal if untreated (Abadias-Granado *et al.*, 2021).

1.1.1.3 Diffuse cutaneous leishmaniasis (DCL)

DCL, a rare variant of CL, presents itself as disseminated non-ulcerating papules and nodule lesions through the body (Goto *et al.*, 2010; Scorza *et al.*, 2017), as opposed to the single lesion on the site of the sandfly bite usually observed for CL. Regular CL can become diffuse in immunocompromised patients due to HIV infection, and can be easily misdiagnosed, as its lesions will resemble those seen in lepromatous leprosy (Correa Soares *et al.*, 2020); Davarpanah *et al.* (2019); Kassardjian *et al.* (2021).

1.1.1.4 Visceral leishmaniasis (VL or Kala Azar)

Visceral leishmaniasis (VL), endemic in 80 countries, (WHO, 2022) occurs in 50 to 90 000 patients per year (Burza *et al.*, 2018). In this manifestation of the disease, parasites will disseminate and infect the reticuloendothelial system of different internal organs, notably the liver, spleen, lymph nodes, and bone marrow (Guerin *et al.*, 2002; Murray *et al.*, 2005; van Griensven *et al.*, 2012; van Griensven *et al.*, 2019). Infection can be asymptomatic, but when untreated, visceral leishmaniasis is deadly (van Griensven *et al.*, 2012; van Griensven *et al.*, 2019). Common symptoms associated with this form of the disease are fever, organomegaly and weight loss (Guerin *et al.*, 2002). The name, Kala Azar (black fever in Hindi), derives from the observation in south Asia that some patients developed darkening of their skin when infected, which was associated with increased cortisol production (Scorza *et al.*, 2017). VL is transmitted by *L. infantum* in the Americas and *L. donovani* in Europe, Africa, and Asia. In some cases, dermal species of *Leishmania* (*L. amazonensis, L. tropicalis*) can visceralize in immunosuppressed patients (van Griensven *et al.*, 2012). VL can be zoonotic - with the dog as the primary domestic reservoir, in addition to rodents and hedgehogs (Derghal *et al.*, 2022) - or anthroponotic, and its

incubation period lasts on average a few months, but it can potentially take years for patients to develop the disease (van Griensven *et al.*, 2012).

1.1.1.5 Post- kala Azar dermal leishmaniasis (PKDL)

Post-kala azar dermal leishmaniasis (PKDL) is the appearance of chronic papular skin rashes either during or after effective treatment of VL due to *L. donovani* infection (Pathania *et al.*, 2022), although some cases have been reported in the absence of previous VL diagnostic (Zijlstra *et al.*, 2003). It is more frequent in Sudan, with about 50% of occurrence after 0-6 months of treatment against VL and in India, with 5-10% occurrence after 2-3 years of the treatment (Zijlstra *et al.*, 2003). The development of PKDL appears to be dependent on the immune response of the host, as shown in HIV^+ patients (Gilad *et al.*, 2001; Ridolfo *et al.*, 2000).

1.1.2 *Leishmania* parasites

Leishmaniasis are caused by *Leishmania spp*., protozoan parasites of the *Trypanosomatidae* family. As part of the Kinetoplastea class, they present a kinetoplast, a structure close to the base of the flagellum that consists of two types of DNA molecules: maxi and minicircles, and functions as the parasite's mitochondria (Amodeo *et al.*, 2023). They are obligatory intracellular parasites and can infect different species, including mammals, reptiles, and amphibia. Promastigote forms of the parasite are motile, elongated and contain a visible flagellum. These forms live and replicate within the digestive tract of sandflies (*Phlebotominae*) (Teixeira *et al.*, 2013). Different species of phlebotomine sandflies act as host for different species of *Leishmania*, based on their geographical distribution (Bates, 2007; Chalghaf *et al.*, 2018; Maroli *et al.*, 2013).

The *Leishmania* genome is organized in long polycistronic coding regions; these can contain dozens of open reading frames, measure up to 100,000 base pairs and have no introns (Colmenares *et al.*, 2002; Cortazzo da Silva *et al.*, 2022). The total number of chromosomes and genome length can vary depending on parasite species (Kazemi, 2011; Peacock *et al.*, 2007). Regulation of gene expression in these organisms occurs exclusively at the post-transcriptional level (by controlling RNA stability, translation efficiency, RNA-binding proteins, etc.), as a poor correlation can be observed between protein and gene expression in *Leishmania* parasites (Clayton, 2016; Cohen-Freue *et al.*, 2007).

1.1.2.1 Life Cycle

The life cycle of *Leishmania* is diphasic (Figure 1.2). Inside of the midgut of sandflies, *Leishmania* parasites undergo metacyclogenesis, with different life stages having their own transcriptomic and proteomic signatures (Cortazzo da Silva *et al.*, 2022). Promastigote parasites secrete the promastigote secretory gel (PSG), that accumulates in the gut and mouth of the sandfly (Rogers, 2012). PSG limits the volume of blood that can be internalized by the vector, increasing the number of blood meals needed by the sandfly (and consequently the probability of parasite transmission) (Gadisa *et al.*, 2015; Rogers, 2012). Infection begins when, during the blood meal of an infected phlebotomine sandfly, promastigote forms of *Leishmania* are regurgitated into the skin tissue of the host (Bates, 2007; Turco *et al.*, 1992). The sandfly bite creates a blood pool in the skin tissue; in this context, parasites are in direct contact with whole blood of the host (Filardy *et al.*, 2011). There, they interact with antibodies and are opsonized by the complement system (Dominguez *et al.*, 1999). Due to the bite, neutrophils are rapidly recruited to the site of infection, serving as the initial host cell for a portion of the inoculated parasites, until macrophages are recruited and able to internalize *Leishmania* (Peters *et al.*, 2008). During the bite, the sandfly also regurgitates the PSG, which has been shown to powerfully recruit macrophages to the site of the infection and to promote parasite replication by targeting host cell metabolism (Giraud *et al.*, 2018; Rogers *et al.*, 2009; Rogers, 2012; Rogers *et al.*, 2010). Parasites will not differentiate and replicate inside neutrophils (Beattie *et al.*, 2011; Peters *et al.*, 2009), which is why macrophages are crucial for disease progression and for the life cycle of *Leishmania* (Beil *et al.*, 1992; de Menezes *et al.*, 2016). Once these parasites are internalized into parasitophorous vacuoles of the host macrophages, the change in pH and temperature will trigger *Leishmania* differentiation into the amastigote form (Bates, 1994; Saar *et al.*, 1998; Zilberstein, 2020). Some parasites are attached to the vacuole membrane, and others are free in the vacuole to replicate. *Leishmania* species of the *mexicana* complex *(L. amazonensis and L. mexicana)* are known to form large communal vacuoles inside of the host cell, whereas the majority of other *Leishmania* parasite species (including *L. major* and *L. donovani*) are found in tight vacuoles containing one or two parasites and reduced vacuolar space, that undergo fission once these parasites replicate (Batista *et al.*, 2020; Real *et al.*, 2012).

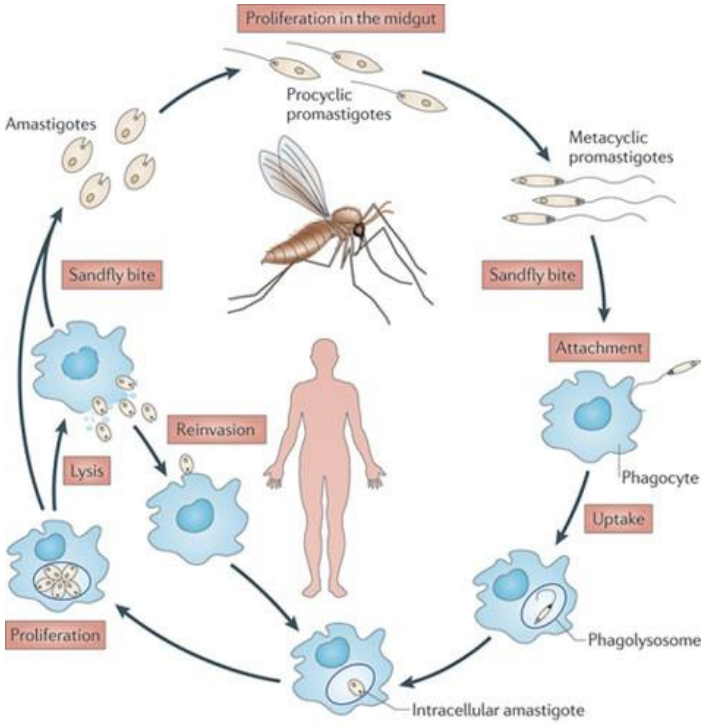


Figure 1.2 *Leishmania spp*. life cycle.

During the bloodmeal, sandflies regurgitate metacyclic promastigote forms into the mammalian host. These forms are internalized by phagocytes, notably macrophages, where they can differentiate into amastigote forms. These live and replicate inside the parasitophorous vacuoles establishing the infection. During the blood meal of a sandfly, infected macrophages are internalized, and the amastigote forms are released in the digestive tract of the insect, where they will undergo differentiation into procyclic promastigotes and, after metacyclogenesis, the highly motile and infective metacyclic promastigotes. Taken from (Kaye *et al.*, 2011).

1.1.3 Transmission and risk factors

Leishmaniasis is transmitted to vertebrate hosts via the bite of phlebotomine sandflies (over 800 species are known, and approximately 100 suspected of transmitting leishmaniasis) (Maroli *et al.*, 2013). These insects are between 2-3 mm and widely spread across tropical and subtropical regions (since their larvae needs warm and humid organic matter to grow) and are also recognized vectors of other viral and bacterial pathogens (Depaquit *et al.*, 2010; Maroli *et al.*, 2013; Pons *et al.*, 2016). The occurrence of leishmaniasis is associated with different social and economic factors, such as poverty, migrations, environmental changes/urbanization, and global warming (Desjeux, 2004; Maroli *et al.*, 2013). Additionally, conflicts in developing countries, such as Syria, Libya, Iraq, and Afghanistan, saw an increase in the occurrence of leishmaniasis amongst civilians and soldiers (Amro *et al.*, 2017; Kanani *et al.*, 2019; Maroli *et al.*, 2013).

1.1.3.1 The impact of climate change in the distribution of leishmaniasis

Climate change will interfere in the current endemicity and transmission of *Leishmania* directly, through vector competence, and indirectly, through socioeconomic changes that will alter human population dynamics and therefore the number of people at risk for leishmaniasis (Ready, 2008). In different regions of the world, reports of emergence and re-emergence of leishmaniasis are current, due to conflicts, increase in poverty and populational displacement, and climate change. The distribution of sandflies has already changed drastically in the last decade due to global warming, and cases of leishmaniasis have been reported in areas traditionally deemed free of the disease (Chalghaf *et al.*, 2018).

Specifically in North America, predictions show that climate change will promote an increase in the distribution of both the host vector and the parasite above current endemic areas in Mexico and southern United States. In more extreme scenarios, host and reservoirs species to *Leishmania* parasites would also reach southern Canada (Gonzalez et al., 2010).

1.1.4 Treatment options and challenges

Current treatment options against leishmaniasis have several limitations, in addition to there being no vaccines approved for human use against the disease (Pradhan *et al.*, 2022). In this section, the current and most used treatment options against leishmaniasis (pentavalent antimonies, amphotericin B and miltefosine) will be reviewed, highlighting the need for new therapeutic options to treat and prevent leishmaniasis.

1.1.4.1 Pentavalent Antimonies

Pentavalent antimonials (Sb^{v}) are, to this day, used as first line treatment against leishmaniasis in most countries, and it has been for over 7 decades (Roatt *et al.*, 2020). The administration of this drug is cumbersome, requiring daily parenteral doses, which can lead to treatment abandonment and non-compliance, that further promotes the emergence of resistant strains (de Menezes *et al.*, 2015; Sundar, 2001; Thakur *et al.*, 2004). In addition, severe toxicity and side effects are also a problem associated with the use of Sb^{v}: These drugs are known for their cardiotoxicity and nephrotoxicity, causing serious adverse effects (e.g., nausea, myalgia, vomiting, headaches, etc.) (Berman, 1997; Freitas-Junior *et al.*, 2012; Thakur *et al.*, 2004; Uliana *et al.*, 2018; van Griensven *et al.*, 2011).

To this day, the mechanism of action of Sb^{v} is poorly understood, with different models for the pharmacology of this drug. Some studies indicate that Sb^{v} is a prodrug that needs to be

reduced to its active form, while others claim that Sb^v is intrinsically anti-leishmanial, with no need to be metabolized to become active (Frezard *et al.*, 2009). The hypothesized mechanism of action is related to binding of thiol groups in the parasite and inhibition of ATP and GTP formation, but also to the inhibition of DNA topoisomerase I (Chakraborty *et al.*, 1988; Frezard *et al.*, 2009). Resistance against Sb^v is widely spread in some regions of India, with treatment failure reaching over 60% of patients due to drug resistance, although results can be improved by combining the drug with interferon-γ (IFN-γ) (Sundar *et al.*, 1997a; Sundar *et al.*, 2001; Sundar *et al.*, 1997b).

1.1.4.2 Amphotericin B

Originally a broad-spectrum antifungal medication extracted from *Streptomyces nodusus,* Amphotericin B (Amph.B) is described to bind to ergosterols on the membrane of its target (Gray *et al.*, 2012), promoting the formation of pores that will increase permeability, cause an ion disbalance and lead to cell death (Gray *et al.*, 2012; Kumari *et al.*, 2022; Sasidharan *et al.*, 2021). Amphotericin B deoxycholate, the original formulation, caused several side effects, notably nephrotoxicity, muscle and joint pain, headache, weight loss, etc., which required patients to be monitored in a hospital setting during treatment (Frezard *et al.*, 2022; Roatt *et al.*, 2020; Sundar *et al.*, 2007). To overcome these problems, a liposomal formulation was developed, which reduced side effects and increased efficacy (Kumari *et al.*, 2022). However, this formulation is costly and not evenly distributed across the globe. Given that poverty is a risk factor associated with the disease, and that developing countries tend to carry the largest portion of the economic burden of leishmaniasis, the cost associated with the production, distribution, storage, and handling is an important consideration when assessing the feasibility of a treatment option. Additionally, Liposomal Amph.B is administered intravenously which also increases the cost and burden associated with this treatment, and prevents large scale treatment campaigns to be rolled out in endemic areas with the purpose of eliminating the disease in a population (Berman, 2019). Amph.B was adopted in India as a new first drug choice against VL due to emerging resistance against pentavalent antimonies (Roatt *et al.*, 2020). However, as the use of Amph.B to treat leishmaniasis increased globally, resistance against this drug has been described in clinical isolates and laboratory strains of different *Leishmania* species (Alpizar-Sosa *et al.*, 2022; Goncalves *et al.*, 2021; Purkait *et al.*, 2012).

Currently, according to the Pan American Health Organization (PAHO), Amph.B is the drug of choice to treat leishmaniasis in pregnant patients, as well as those with comorbidities, and HIV co-infection (Organizacion Panamericana de la, 2023). Limitations to this treatment still relate

to the high cost of the liposomal formulation and high toxicity and side effects caused by the original Amphotericin B deoxycholate.

1.1.4.3 Miltefosine

Originally an antineoplasic drug, it is the only treatment option against leishmaniasis that can be administered orally. However, it showcases embryotoxicity, fetotoxicity and teratogenicity in different animal models, and therefore is contraindicated to be used during pregnancy (Sindermann *et al.*, 2006). Another limitation of this drug is the apparent species-dependent effect observed in clinical trials, with different species of *Leishmania* showcasing different sensitivities to the treatment (Machado *et al.*, 2010; Soto *et al.*, 2004). Miltefosine is known to act on signaling pathways and membrane synthesis, promoting an apoptosis-like cell death to the parasite (Roatt *et al.*, 2020; Verma *et al.*, 2004). Limitations include the drug's long half life (approximately 1 week), that can promote drug resistance, teratogenic side effects, high cost, hepatic toxicity, and nephrotoxicity, in addition to side effects to the gastrointestinal tract (Sundar *et al.*, 2015). After over a decade of use in India, the efficacy of miltefosine appears to have decreased since it was first adopted by the healthcare system as a drug of choice against leishmaniasis. Additionally, relapse appears to have augmented in patients treated with miltefosine (Roatt *et al.*, 2020; Sundar *et al.*, 2015; Sundar *et al.*, 2012).

Due to the problems described here with drugs of choice (Table 1.1), many other strategies are explored to treat leishmaniasis. Options such as thermotherapy, topical formulations against the cutaneous form of the disease, combination therapies, and host-directed therapies, have been studied and adopted in certain countries to overcome the cost, side effects and resistance associated with current leishmaniasis treatments (Roatt *et al.*, 2020). Regarding host-directed therapies, different targets related to the immune response against the parasite are potential paths to be explored (Novais *et al.*, 2021). To better develop host-directed therapies that are effective, it is important to understand the different aspects of the immune response that is mounted against the parasite during infection.

	Miltefosine	Pentavalent Antimonials	Amphotericin B
Administration	Oral	Parenteral	Intravenous
Side effects	Hepatic and nephrotoxicity, teratogenic	Cardio and nephrotoxicity	Nephrotoxicity, muscle and joint pain, severe weight loss
Drug resistance	✓	✓	✓
Other problems	Effect is species-dependent	60% treatment failure in endemic countries	High cost of liposomal formulation
References	(Machado *et al.*, 2010; Roatt *et al.*, 2020; Soto *et al.*, 2004; Sundar *et al.*, 2012)	(Berman, 1997; Roatt *et al.*, 2020; Uliana *et al.*, 2018)	(Frezard *et al.*, 2022; Roatt *et al.*, 2020; Sundar *et al.*, 2007)

Table 1.1 Summary of challenges associated with the main treatment options against leishmaniasis.

1.1.5 Immune response against *Leishmania*

1.1.5.1 Cutaneous leishmaniasis

As previously discussed, neutrophils are the first cells recruited to the site of infection after the bite of the infected sandfly (Peters *et al.*, 2008). Following these cells, inflammatory monocytes reach the area, and they will differentiate into monocyte-derived macrophages and dendritic cells (DC), the latter being crucial to the development of a $CD4^+$ T helper 1 (Th1) response, needed for the resolution of the disease (Seyed *et al.*, 2021). These monocytes and DCs are the dominant cell type infected with the parasite over the first days of infection, and infected monocytes are able to eliminate the parasite by producing nitric oxide (NO) and reactive oxygen species (ROS) (Loria-Cervera *et al.*, 2020). DCs are the main producers of IL-12 (von Stebut *et al.*, 1998), a cytokine that is crucial to promote Th1 cell differentiation and Th2 cell suppression (Heinzel *et al.*, 1993; Park *et al.*, 2002; Sypek *et al.*, 1993). The production of IFN-γ by Th1 $CD4^+$ cells is essential to the control of the disease, since this cytokine can activate macrophages, that will eliminate *Leishmania* parasites by producing ROS and NO (Scott *et al.*, 2016; Seyed *et al.*, 2021). However, there needs to be a balance (Figure 1.3) in the response, as

an exacerbated Th1 response can lead to cytotoxicity and have a negative, pathological effect (Scott *et al.*, 2016); (Scorza *et al.*, 2017)

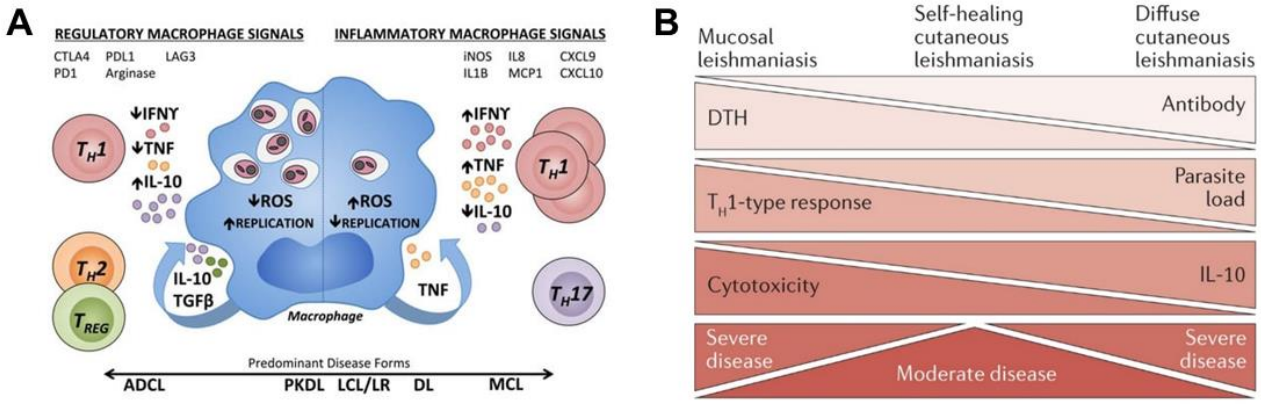


Figure 1.3 : Balance between immunity and immunopathology during different manifestations of cutaneous leishmaniasis.

The balance between immune regulation and inflammation determines the different disease manifestations of CL. A balance is needed to successfully eliminate the infection and most times the pathology of the disease happens due to an exacerbated immune response. Taken from (Scorza *et al.*, 2017; Scott *et al.*, 2016).

Th1 cytokines, such as TNF or IL-1β, can have a pathogenic effect in disease progression. High levels of both TNF-α and IFN-γ have been associated with more severe forms of CL (Bacellar *et al.*, 2002; Melby *et al.*, 1994) and clinical trials have shown better prognostic in a combination treatment of antiparasitic drugs and TNF inhibitor (Melby *et al.*, 1994; Ribeiro de Jesus *et al.*, 2008). IL-1β is another cytokine with contrasting effects during CL caused by *L. amazonensis* and *L. major*, with protective and pathological effects described in the literature (Kostka *et al.*, 2006; Lima-Junior *et al.*, 2013).

Different T cell populations are of relevance in controlling CL. In addition to the CD4+ Th1 population already described, CD8+ T cells can be required to produce IFN-γ and activate the Th1 response (Belkaid *et al.*, 2002; Uzonna *et al.*, 2004). Additionally, a double negative population ($CD4^-CD8^-$) is also of immunological relevance in the disease, even though its functions are not entirely understood (Scott *et al.*, 2016).

1.1.5.2 Visceral leishmaniasis

Similarly to CL, experimental VL immunity strongly relies on the stimulation of Th1 and repression of Th2 response (Srivastava *et al.*, 2012). This inhibition of a Th2 response, however, is not well characterized in human infections (Kumar *et al.*, 2012). The previously described Th1 cytokines play similar roles in activating macrophages to produce NO and ROS that will eliminate

amastigote forms of the parasite (Bhor *et al.*, 2021; Khadem *et al.*, 2014). The balance between the production of macrophage-activating (IFN-γ, TNF-α) and repressing (TGF-ß, IL-10) cytokines will determine the severity and outcome of the disease (Khadem *et al.*, 2014). $CD8^+$ T cells have been shown to play an important role in experimental models of VL by producing IFN-γ and exerting cytolytic activity in the liver (Polley *et al.*, 2006; Tsagozis *et al.*, 2003; Tsagozis *et al.*, 2005).

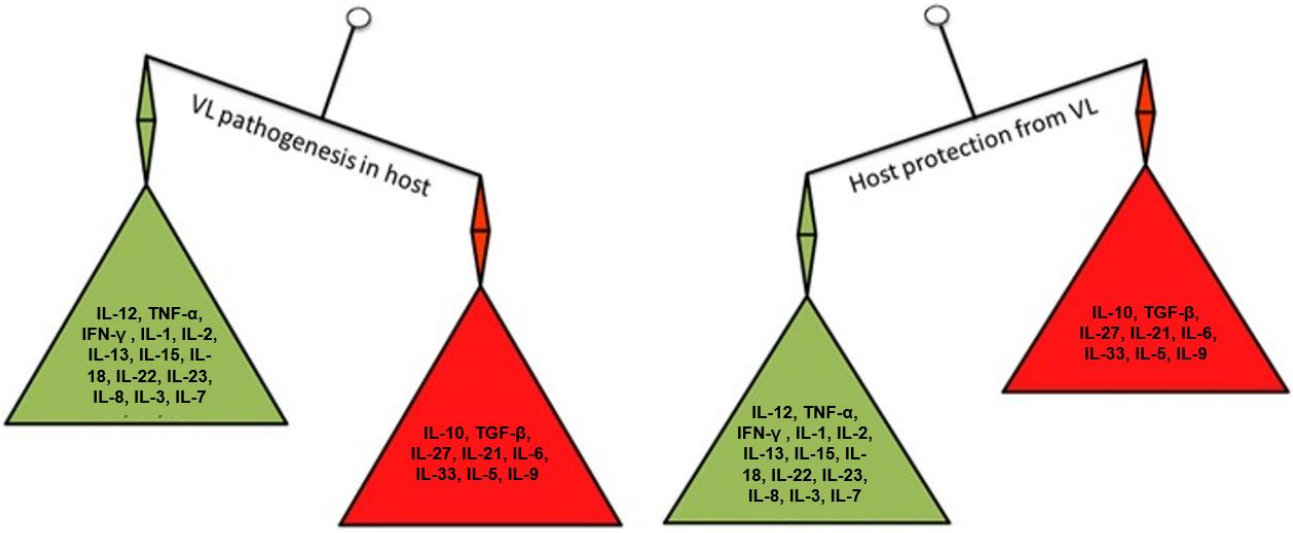


Figure 1.4 Cytokine balance in visceral leishmaniasis (VL).

Similarly to CL, the balance between Th1 and Th2 responses appears to be in the center of the immunological control of visceral leishmaniasis. Taken from (Dayakar et al., 2019).

Knowing that same cytokines can have contrasting effects in the disease, developing host-directed therapies should aim to either boost the response against the parasite with immune enhancing agents, or to control the exacerbated immune response that further worsens pathology with inhibitors of the immune response (Novais *et al.*, 2021). A personalized approach is also necessary, considering the response and disease development will depend on the parasite species and immune status of the host (Kumar *et al.*, 2012).

Socio-economic aspects of patients can also impact the immune status of the host and the response against leishmaniasis: for instance, malnutrition is known to weaken both innate and adaptative immunity, increasing early visceralization (Anstead *et al.*, 2001; Hughes *et al.*, 2006). It is also reported that a concomitant helminth infection can drive the Th1/Th2 balance in favor of *Leishmania* (Hassan *et al.*, 2006; O'Neal *et al.*, 2007).

1.2 Macrophages

As it was previously described, macrophages are central players in the *Leishmania* life cycle, as they serve as hosts and can eliminate parasites by producing NO and ROS (Beattie *et al.*, 2011; de Menezes *et al.*, 2016). Therefore, understanding the biology of these cells, in addition to the intricate relationship between *Leishmania* parasites and macrophages during infection is of the utmost importance for this project.

1.2.1 Macrophages in health and pathology

Macrophages are immune cells characterized by their avid phagocytosis. They are found in all organs, producing tissue-specific growth factors and phagocytosing cell debris, dead cells, pathogens, etc. (Cox *et al.*, 2021; Mass *et al.*, 2021; Pollard, 2009). Originally, it was thought that all macrophages were part of the mononuclear phagocyte system, originated from circulating monocytes developed from adult hematopoietic stem cells (HSCs) in the bone marrow (van Furth *et al.*, 1968; van Furth *et al.*, 1972). However, some tissue-specific macrophages were later shown to have a fetal origin, more specifically the yolk sac (YS); these cells can self-maintain independently of HSCs (Figure 1.5) (Cox *et al.*, 2021; Gomez Perdiguero *et al.*, 2015; Hashimoto *et al.*, 2013; Schulz *et al.*, 2012; Yona *et al.*, 2013). Even though these two subtypes of macrophages have different origins, the depletion of tissue-resident macrophages in different tissues triggers the recruitment of monocytes to the empty niche and their differentiation into macrophages. These monocyte-derived macrophages will replace the depleted tissue-resident macrophages, expressing their transcriptional programs and reproducing their functions with a high degree of resemblance (Bennett *et al.*, 2018; Bonnardel *et al.*, 2019; Sakai *et al.*, 2019; van de Laar *et al.*, 2016).

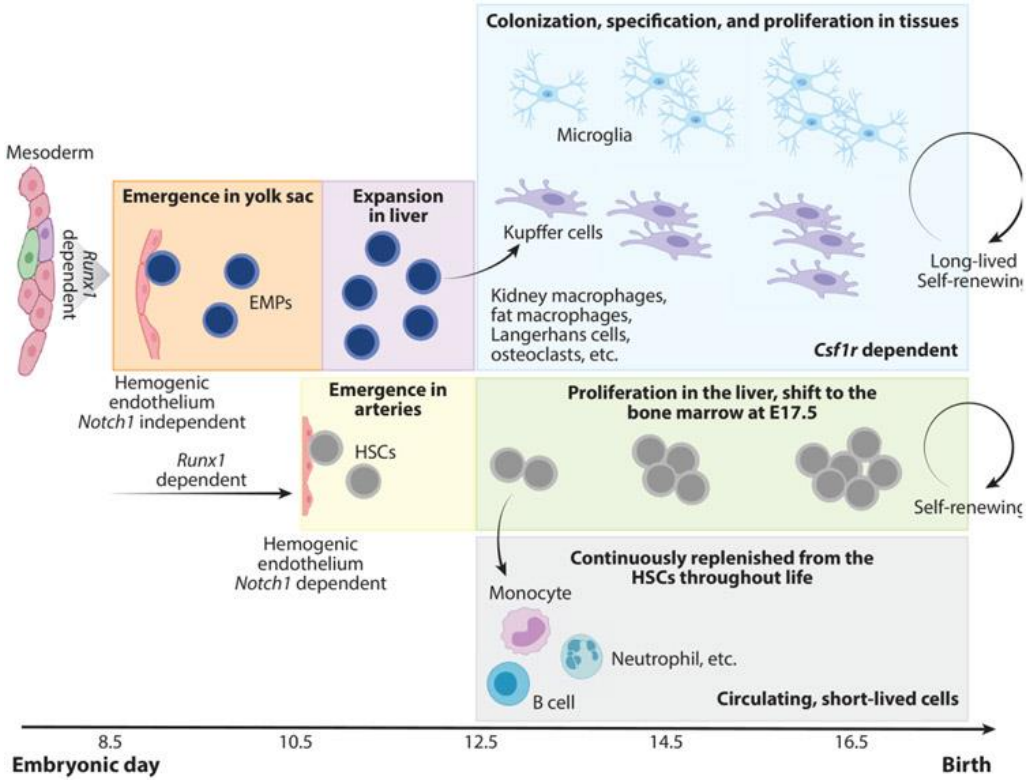


Figure 1.5 Different subsets of macrophage populations showcase different developmental origins.

Tissue resident macrophages emerge in the yolk sac during embryogenesis, expand in the liver and then colonize and acquire the tissue-specific phenotype in different organs of the body (e.g., CNS, liver, bones, etc.). Other macrophages are derived from HSCs in the bone marrow. They differentiate from monocytes when recruited to infection/inflammation sites. Taken from (Cox *et al.*, 2021).

1.2.1.1 Tissue-resident macrophages

In addition to having a different developmental origin, tissue resident macrophages have developed different characteristics and functions according to the tissue they are part of (Cox *et al.*, 2021). Lung resident macrophages are responsible for keeping the alveolus free from excess mucus, cellular debris, and pathogens, allowing for proper gas exchange in the lung (Aegerter *et al.*, 2022). In the liver, Kupffer cells maintain iron homeostasis by clearing senescent blood cells (Scott *et al.*, 2018), regulate cholesterol homeostasis (Wang *et al.*, 2015), and act as immunological gatekeepers, mediating antimicrobial defense (Helmy *et al.*, 2006) and immunological tolerance (Heymann *et al.*, 2015). In the central nervous system (CNS), the microglia is capable of pruning neurons and remodelling synapses, in addition to having an essential role in brain development (Lalancette-Hebert *et al.*, 2007; Michaelson *et al.*, 1996; Paolicelli *et al.*, 2011; Vilhardt, 2005). Osteoclasts in bone tissues are giant multinucleated cells, developed from the fusion of myeloid progenitors (Yao *et al.*, 2021) responsible for digesting the bone matrix and for bone morphogenesis (Boyle *et al.*, 2003; Jacome-Galarza *et al.*, 2019).

Additionally, tissue-resident macrophages can act in different processes to maintain homeostasis in the tissue they are part of: neutralizing pathogens; removing cellular debris, and secreting factors to stimulate neighboring cells (Mu *et al.*, 2021).

1.2.1.2 Macrophage polarization (M1 x M2 phenotypes)

The M1 x M2 paradigm was first described from an observation of arginine metabolism phenotype change in macrophages stimulated *in vitro* with different cytokines: classically activated macrophages (activated by IFN- γ, LPS, DAMPs and PAMPs, etc.) produced NO from L-arginine, whereas alternatively activated macrophages (using IL-4 and IL-13) had a shifted arginine metabolism to produce ornithine and polyamines (Mills *et al.*, 2000). It is known today that this paradigm is an oversimplification of a much more complex and less dichotomous system, where macrophage phenotypes lie mostly within an intermediate instead of on the opposite ends of the proposed spectrum (Mantovani *et al.*, 2005; Martinez *et al.*, 2014; Mosser *et al.*, 2008). STAT-1 and NF-kB are important transcription factors associated with an M1 phenotype mediated by IFN-γ and LPS, respectively, as well as IRF5 and IRF 1 (Krausgruber *et al.*, 2011; Langlais *et al.*, 2016; Tugal *et al.*, 2013). As for the M2 phenotype, STAT6 controls genes associated to this phenotype in the presence of IL-4 and IL-13 (Martinez *et al.*, 2009).

M1 macrophages are known to have a strong cytotoxic profile, producing NO and ROS, in addition to a Th1 pro-inflammatory response (IL-1, IL-6, IL-12, IL-23, TNF) (Funes *et al.*, 2018; Zhang *et al.*, 2008). M2 macrophages present a wound-healing, anti-inflammatory profile, producing angiogenesis mediators, such as TGF-b, VEGF and EGF (Laskin *et al.*, 2011). Alternatively activated macrophages (M2) are heterogeneous, and can be classified into different subpopulations (e.g., M2a, M2b, M2c and M2d), each induced by different cytokines and having different proposed functions (Funes *et al.*, 2018; Li *et al.*, 2022) (Table 1.2).

Subtype	Activated by	Proposed function
M1	IFN-*γ ;* TLR1/2, TLR3, TLR4, TLR8 and TLR7 agonists	Type I immunity, Type 4 hypersensitivity, tumour resistance
M2a	IL-4, IL-10, IL-13, PPAR-γ agonists	Type 2 immunity, allergy, profibrotic
M2b	Immunocomplex + TLR/IL-1R agonists	Th2 activation, immunoregulation
M2c	Glucocorticoids, IL-10, TNF-α	Immunoregulation, tissue repair, matrix remodelling
M2d	TGF-β, IL-6, adenosine A_{2A} receptor	Angiogenesis, clearance of apoptotic tissue

Table 1.2 Summary of different macrophage polarization phenotypes and their proposed functions
Different subtypes of macrophage activation are induced by different cytokines and can have different proposed functions. As previously mentioned, these cells are highly plastic and can adapt and change their polarization subtype depending on the stimulus they receive. Adapted from (Funes *et al.*, 2018; Strizova *et al.*, 2023; Wang *et al.*, 2019; Zeng *et al.*, 2019).

1.2.1.3 Phagosome maturation in macrophages

As professional phagocytes, macrophages internalize external particles, cells or pathogens into a membrane-derived vacuole known as phagosome. These nascent compartments, however, are not able to kill or degrade the ingested particles, as they acquire this ability through a process called phagosome maturation (Vieira *et al.*, 2002). Maturation of phagosomes can be divided into three different stages: early phagosome, late phagosome, and phagolysosome (Fountain *et al.*, 2021). In each of these stages, a series of fission and fusion events with the endo-lysosomal pathway will culminate in the formation of the phagolysosome, a compartment that is specialized in eliminating internalized particles due to its acidic state, presence of hydrolytic enzymes, and ROS (Dill *et al.*, 2015; Garin *et al.*, 2001; Sun-Wada *et al.*, 2009). The late phagosome fusion with lysosomes is the fundamental step in phagolysosome formation (Harrison *et al.*, 2003). Through this fusion, the late phagosome will acquire most of its antimicrobial characteristics. Different proteins are enriched in phagolysosome membrane in comparison to late phagosome, notably the lysosome-associated membrane proteins (LAMP) 1 and 2 (Levin *et al.*, 2016). As an important antimicrobial mechanism, different pathogens, including *Leishmania*, have developed mechanisms to evade phagosome maturation to successfully establish a niche within macrophages (Lee *et al.*, 2020)

1.2.1.4 Diseases related to macrophage dysfunction

Monocytes and macrophages are attracted to the site of injury and inflammation by pathogen-associated molecular patterns (PAMPs), released by invading pathogens, and danger-associated molecular patterns (DAMPs), released by damaged and/or dead cells (Martinez *et al.*, 2009; Nourshargh *et al.*, 2014). Additionally, inflammatory cytokines and chemokines secreted by T cells can also recruit these cells to the site of inflammation (Martinez *et al.*, 2009; Nourshargh *et al.*, 2014). This influx of monocytes and macrophages to inflamed tissues has been associated with a worsened condition for different diseases, such as diabetes and atherosclerosis (AS) (Fenyo *et al.*, 2013; Panee, 2012). Similarly, the persistence of an M1 phenotype corresponds with tissue damage and promotes pathogenesis in different diseases (Dambach *et al.*, 2002; Holt *et al.*, 2008; Laskin, 2009; Laskin *et al.*, 2011).

Given the heterogeneous aspect of macrophage populations, a phenotypic and/or metabolic dysregulation of these cells can contribute to the development and progression of diseases in different tissues, such as obesity, diabetes, cancer, osteoporosis, arthritis and fibrosis (Wynn *et al.*, 2013). Tissue damage can be promoted by different pro-inflammatory cytokines produced by macrophages, such as IL-1 and TNF, that can recruit Th17 cells and neutrophils (Murray *et al.*, 2011).

Macrophages that infiltrate solid tumors and adopt an M2 profile, influenced by the tumor microenvironment, become tumor-associated macrophages (TAMs). Inside these tumors, TAMs have different functions in angiogenesis, metastasis, and disease progression. (Chen *et al.*, 2023; Yang *et al.*, 2017).

1.2.1.5 Macrophage-targeting therapies

Macrophages are explored as targets for immunotherapy against cancer in different studies. Whole blood or bone marrow monocytes can be used for *ex-vivo* reprogramming of macrophages (Lee *et al.*, 2016). The reprogramming or elimination of TAMs is a goal of immunotherapy strategy against cancer, given the pro-tumoral function these cells can have in the tumor micro-environment (Anderson *et al.*, 2021; Cocks *et al.*, 2022; Mantovani *et al.*, 2022). Additionally, macrophages have been explored as therapeutical targets in AS, given their important role in this disease pathogenesis (Tarling *et al.*, 2010; Westerterp *et al.*, 2016). Some strategies explored were the induction of autophagy, the reprogramming of macrophage polarization, and the targeted delivery of drugs to macrophages, based on the characteristic surface cell marker expression of these cells (Peng *et al.*, 2020). Highly specific macrophage

killing has been reported using photodynamic therapy (PDT), leaving other cell types unharmed (Demidova *et al.*, 2004; Liu *et al.*, 2005).

Different strategies for treatment are described using macrophages as target cells. This shows how much these cells are relevant for different pathologies and how much their plasticity can be explored therapeutically (Chen *et al.*, 2023). More specifically, in the context of *Leishmania* infection, macrophages are affected in different levels of gene expression. In this next section, we will explore some of the interactions between the parasite and the host, and ways the parasite can disrupt macrophage functions to promote the infection.

1.2.2 Macrophages as host cells for *Leishmania* – host-parasite interactions

As previously described, macrophages are recruited to the site of the sandfly bite and internalize the parasites. Inside them, *Leishmania* will differentiate from the promastigote to the amastigote form. Different strategies used by the parasite to subvert macrophage responses in all levels of gene expression have been observed.

1.2.2.1 Recognition, entry and vacuole formation

The initial interaction between the parasite and the macrophage occurs with the parasite flagellum (Rotureau *et al.*, 2009), in a flagellum sensing model that allows for the parasite to identify its attachment to the macrophage and release intracellular factors that modulate macrophage phagocytic activity (Rotureau *et al.*, 2009). Different macrophage receptors are involved in parasite internalization, such as the first and third complement receptors (CR3 and CR1), mannose receptor (MR), Fc gamma receptors (FcyRs), and fibronectin receptors (FnRs), depending on the species of the parasite (Ueno *et al.*, 2012). Uptake via complement receptors seems to inhibit macrophage antimicrobial response and could serve as an advantage for the survival of the parasite (Mosser *et al.*, 1987; Ueno *et al.*, 2012). The uptake via the other described receptors, on the other hand, appears to elicit a pro-inflammatory response in the macrophage. In nature, it is possible that multiple receptors are involved concomitantly (Ueno *et al.*, 2012). Following attachment to the host cell, the internalization of the parasite appears to be dependent on host cholesterol and caveolae membrane formations, in addition to the actin cytoskeleton (Chattopadhyay *et al.*, 2012; Majumder *et al.*, 2012; Rodriguez *et al.*, 2011; Roy *et al.*, 2014).

Once internalized, *Leishmania* promastigotes can delay the maturation of the host phagosome, avoiding its fusion with late endosomes and lysosomes, and delaying LAMP1 recruitment (Desjardins *et al.*, 1997; Forestier *et al.*, 2011; Moradin *et al.*, 2012). This mechanism

seems to be dependent on lypophosphoglycan (LPG), a surface glycolipid that is abundant in the parasite (Vinet *et al.*, 2009). The assembly of the NADPH oxidase complex, producer of ROS, is blocked by this parasite-driven arrest in phagosome maturation (Lodge *et al.*, 2006). The lack of NADPH oxidase assembly and recruitment to the vacuole limits the proteolytic activity of the phagosome, providing a safe environment devoid of oxidants and acidic pH for the parasite to establish the infection (Moradin *et al.*, 2012).

1.2.2.2 Exosomes and GP63

Leishmania parasites are known to secrete small, extracellular vesicles containing different virulence factors and parasite proteins to modulate the host response and establish the infection. These vesicles have immunomodulatory properties and can modulate the host gene expression and interfere in the pathology of leishmaniasis *in vivo* (da Silva Lira Filho *et al.*, 2021). The secretion of extracellular vesicles by *Leishmania* promastigotes appears to be upregulated experimentally upon a temperature shift that mimics the infection (from 25-26°C – sandfly vector to 37°C – mammalian host) (Hassani *et al.*, 2011).

The zinc-dependent metalloprotease glycoprotein 63 (GP63) is known to be part of these exosomes released by *Leishmania* parasites during infection. It is the most abundant protein in the promastigote membrane (comprising up to 1% of the parasite proteome alone) (Olivier *et al.*, 2012; Yao *et al.*, 2003). GP63 is a promiscuous enzyme, able to cleave an extensive number of proteins. Before the internalization, GP63 interacts with the complement system, avoiding complement-mediated lysis of the parasite (Brittingham *et al.*, 1995). It also plays a role in the adherence of the parasite to the macrophage (Mosser *et al.*, 1985). When GP63 access the inside of macrophages, it can cleave multiple substrates (Figure 1.6), altering host signaling and gene expression to favor the parasite (Olivier *et al.*, 2012). More recently, a characterization of GP63 targets confirmed 4 *bona fide* substrates of the protease: SHP-1, VAMP8 and Syt XI, involved in phagosomal recruitment of v-ATPase and NOX2, and Stx5 (Guay-Vincent et al., 2022).

GP63 is known to be down-regulated in amastigotes in relation to the promastigote stage of *Leishmania* (Schneider *et al.*, 1992), and most of the known functions of GP63 have been described in the promastigote stage of the parasite. However, the metalloprotease is still present in amastigotes of all *Leishmania* species studied so far (Yao *et al.*, 2003). More recently, it has been shown that not all the previously reported targets are *bona fide* substrates of GP63 (Guay-Vincent *et al.*, 2022)**.**

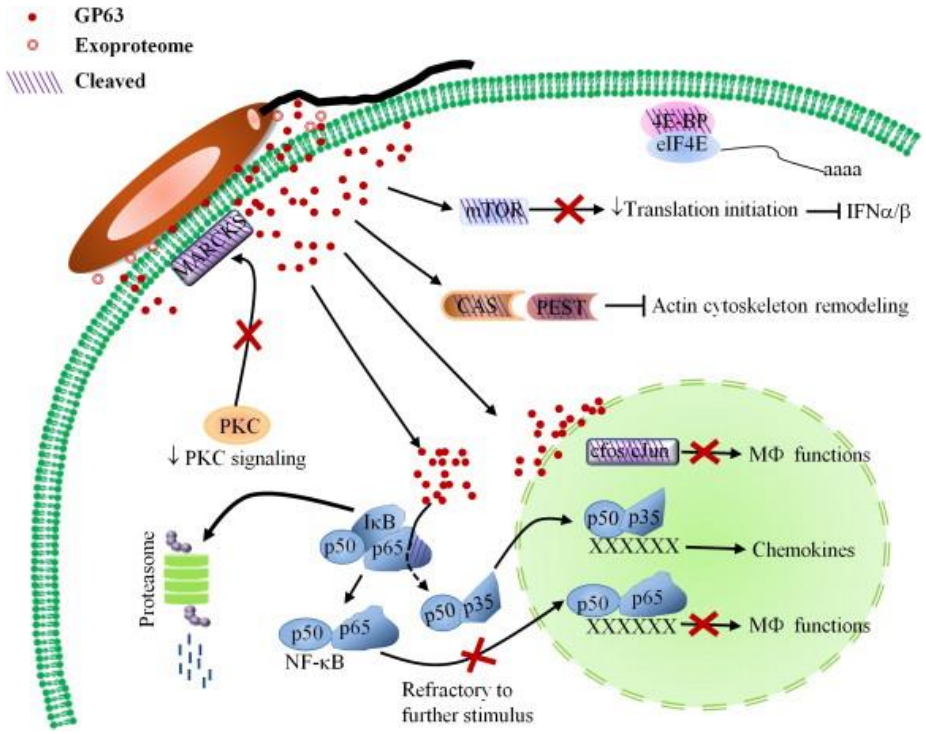


Figure 1.6 GP63 affects host signaling and metabolism prior to parasite internalization.

As a metalloprotease, GP63 is an important virulence factor in *Leishmania* interactions with the macrophage. It can cleave different proteins and transcription factors shown in the figure, to alter the macrophage response and promote the infection. Arrows indicate GP63 targets involved in signalling pathways; Red crosses indicate signalling cascade alteration; abrogated lines indicate functional inhibition. Taken from (Olivier et al., 2012).

1.2.2.3 Parasite differentiation

The drop in pH and increase in temperature that occurs within the parasitophorous vacuole is proposed to trigger the promastigote to amastigote differentiation of the parasite (Lievin-Le Moal *et al.*, 2016; Spath *et al.*, 2015). Specifically in *L. amazonensis* promastigotes, iron uptake and metabolism also act as a regulators of amastigote differentiation (Mittra *et al.*, 2013a; Mittra *et al.*, 2013b). The parasite response to stress triggers the differentiation and alters its cellular structure and metabolism, with exclusive transcriptional and proteomic signatures observed in amastigotes in relation to promastigotes (Cortazzo da Silva *et al.*, 2022; Spath *et al.*, 2015). Once differentiated, amastigotes are fully adapted to survive and replicate in the hostile environment of parasitophorous vacuoles (Chang *et al.*, 1978; Lewis *et al.*, 1977; Moradin *et al.*, 2012).

1.2.2.4 Nutrient acquisition

Leishmania amastigotes strongly rely on the phagolysosome to obtain nutrients for its growth and replication (McConville *et al.*, 2007; Naderer *et al.*, 2008; Podinovskaia *et al.*, 2015). Amino acids serve as the main source of carbon and substrate for polyamine production in the

parasite; these molecules are obtained via different amino acid permeases that are modulated depending on the macrophage activation state and nutrient availability (Aoki *et al.*, 2017; McConville *et al.*, 2007). In this context, L-arginine has a central role in macrophage and parasite metabolism (Figure 1.7): L-arginine can be used in classically activated macrophages (M1) by the inducible Nitric Oxide Synthase (iNOS) to produce NO, a known effector of macrophages against the parasite (Liew *et al.*, 1991; Naderer *et al.*, 2008). In alternatively activated macrophages (M2), L-arginine is used by arginase to produce polyamines (putrescine, spermidine, and spermine) (Gordon, 2003), nutrients that are directly taken by the parasite, promoting its replication (McConville *et al.*, 2007). Whether L-arginine metabolism is shifted towards iNOS or arginase will depend on the macrophage activation state and consequently determine the fate of the infection (Acuna *et al.*, 2017). In addition to amino acids, purines, vitamins, cations like Fe^{2+} and Mg^{2+}, and hexose are other nutrients that *Leishmania* is required to take from the parasitophorous vacuole to survive (McConville *et al.*, 2007).

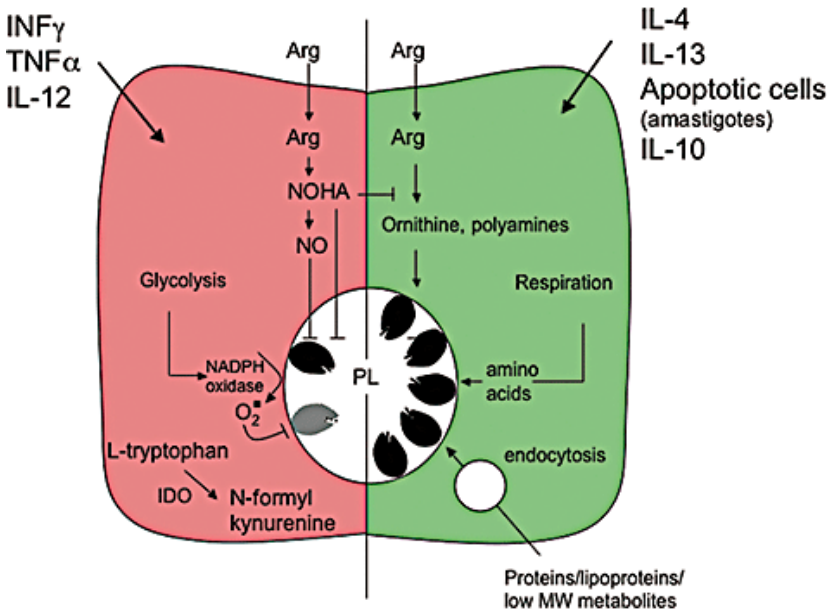


Figure 1.7 Arginine metabolism in classically and alternatively activated macrophages during *Leishmania* infection.

The fate of L-arginase in infected macrophages can determine whether the parasite will be eliminated or have its replication favoured. Depending on the macrophage polarization state, L-arginine will be used for NO or for polyamine production, altering the course of infection. Taken from (Naderer *et al.*, 2008).

Leishmania parasites have developed several strategies to modulate the antimicrobial response in macrophages and favour its replication and spread. Some of these strategies are directly linked to known virulence factors of the parasite, as the previously discussed effects of GP63 and LPG, others have a poorly understood mechanism. The effect of the parasite within

the host is spread out across different levels of gene expression (epigenetic, transcriptional, and translational) and will be explored in the following section.

1.2.2.5 Host epigenetic alterations caused by *Leishmania*

Changes in the epigenome can modulate the transcription of certain genes with modifications to the DNA or to its histones that do not change the nucleotide sequence, such as methylation, acetylation, phosphorylation, etc. (Khyzha *et al.*, 2017; Yang *et al.*, 2022a). Although specific mechanisms are still unclear, some examples of parasite-driven host epigenetic alterations have been described and seem to benefit parasite persistence (Afrin *et al.*, 2019; Marr *et al.*, 2014). *L. amazonensis* is known to downregulate iNOS expression of macrophages through an induction of host histone deacetylase 1 (HDAC1) (Calegari-Silva *et al.*, 2018). Changes in DNA methylation promoted by *L. donovani* infection affect several genes encoding proteins involved in signaling pathways used by the macrophage against the parasite, such as JAK/STAT and MAPK (Marr *et al.*, 2014). Additionally, a parasite-driven epigenetic reprograming of macrophages towards an M2 phenotype was observed upon infection with *L. donovani* (Parmar et al., 2020).

1.2.2.6 Host transcriptional and post-transcriptional alterations caused by *Leishmania*

Transcription is tightly regulated by different mechanisms in mammalian cells, to activate specific transcriptional programs depending on the different stimuli received by the cell. Such feature is exploited by the parasite to reprogram macrophage transcription and establish the infection (Buates *et al.*, 2001). The proteolytic activity of GP63, as discussed in 1.2.2.2, can have a global impact on macrophage transcriptional programs during the infection. In accordance, transcriptional profiling revealed a vast alteration in the transcriptome of host cells infected with different *Leishmania* species, associated with parasite persistence (Chaparro *et al.*, 2022; Dillon *et al.*, 2015; Gregory *et al.*, 2008; Shadab *et al.*, 2019).

At the post-transcriptional level, microRNAs can play an important role in the interplay between *Leishmania* parasites and macrophages. miRNAs are small, regulatory non-coding RNAs that can bind to their target mRNA interfering their translation into proteins (Nandan *et al.*, 2021). In the context of *Leishmania*-infected macrophages, it has been shown that the parasite promotes the transcription of a specific miRNA that targets host-NOS2, promoting its survival in murine macrophages (Muxel *et al.*, 2017). Additionally in murine macrophages, the signaling pathway of toll-like receptors (TLRs) appeared to be altered upon infection with *L. amazonensis* by the modulation of host miRNA expression (Muxel *et al.*, 2018). In THP-1-derived macrophages,

several miRNAs have been identified to be upregulated upon infection, affecting the host arginine metabolism, and promoting permissive conditions for parasite survival (Fernandes *et al.*, 2024).

1.2.2.7 Host translational alterations caused by *Leishmania*

Translational control (i.e., regulation of mRNA translation efficiency) has an important role in modulating several processes of macrophages, including the response to pathogens (Leroux *et al.*, 2018; Su *et al.*, 2015; Tabatabaei *et al.*, 2020). Regulation of mRNA translation efficiency is a rapid way to reprogram the cell proteome in response to environmental cues (Piccirillo *et al.*, 2014; Su *et al.*, 2015; William *et al.*, 2019). By performing a transcriptome-wide analysis of the translatome in murine macrophages infected with *L. donovani*, our group identified that the parasite could promote alterations in the translation of several mRNAs upon infection (Chaparro *et al.*, 2020) (Figure 1.8). This reprogramming of the host translatome was observed in macrophages infected with either promastigotes or amastigotes of *Leishmania.* Additionally, the PI3K/AKT/mTOR pathway, known to regulate mRNA translation (Jia *et al.*, 2021; Yang *et al.*, 2022b; Zhulyn *et al.*, 2023), amongst other cell functions, was observed to be altered upon infection with *L. donovani* (Chaparro et al., 2020; Cheekatla et al., 2012).

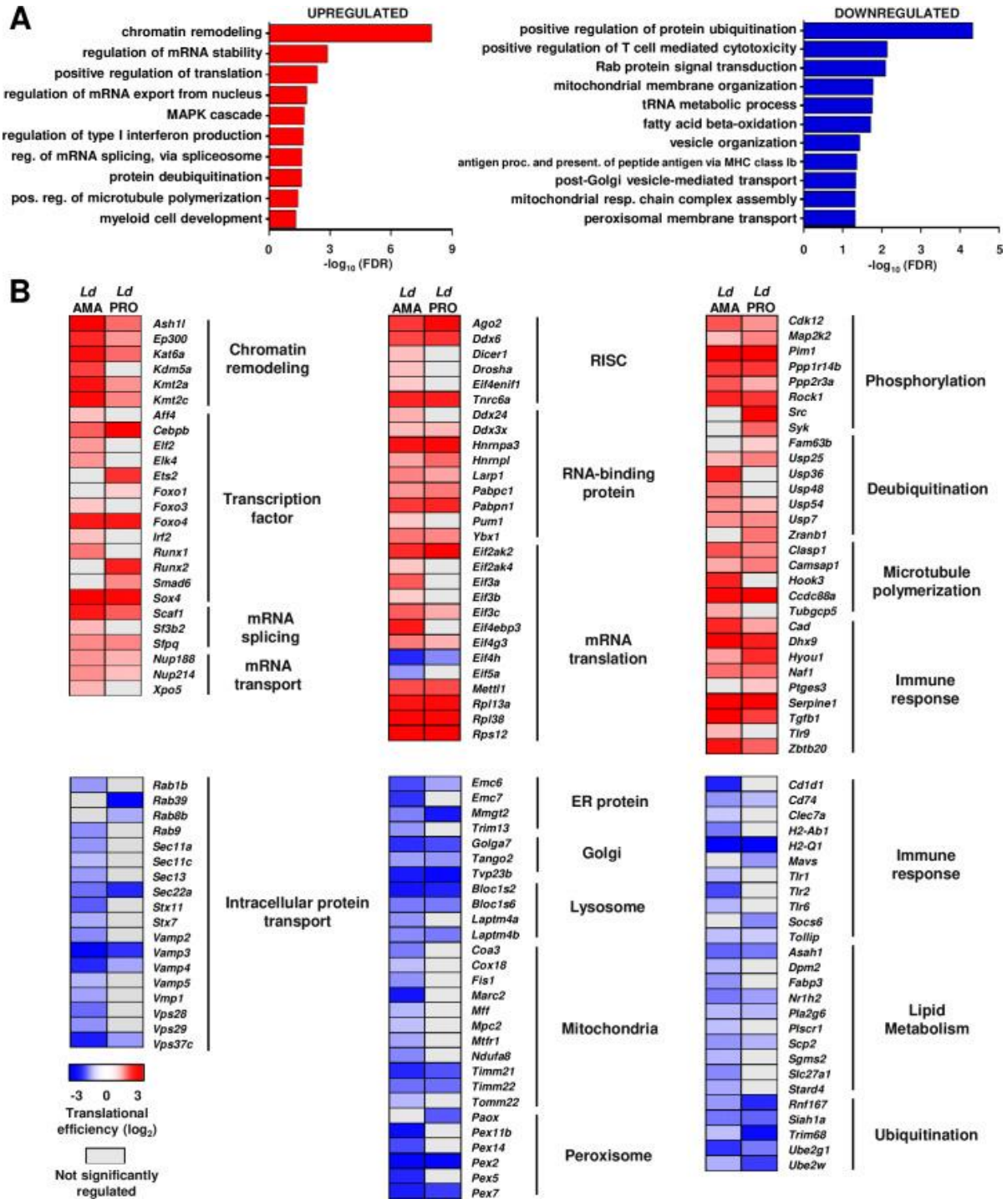


Figure 1.8 *L. donovani* reprograms mRNA translation in macrophages, affecting core and immune functions of the host cell.

Upon infection with either amastigotes (AMA) or promastigotes (PRO), the translation of several host mRNAs was affected. Functions predicted to be affected by the infection are diverse, with downregulated mRNAs related to different organelles (e.g., mitochondria, Golgi, lysosome, etc.) and upregulated mRNAs related to different cell functions (e.g., chromatin remodelling, mRNA metabolism, immune response, etc.). (A) FDR values (-log10) for selected gene ontology (GO) term enriched categories for translationally up- and down-regulated host mRNAs upon *L. donovani* infection. (B) Heatmaps showing changes in translational efficiencies for selected genes in enriched GO terms. Taken from (Chaparro *et al.*, 2020).

1.3 Protein Synthesis

1.3.1 Translation overview

Translation, also known as protein synthesis, is the process through which a mature mRNA molecule is decoded into a polypeptide by ribosomes. Translation is a highly regulated and energy-consuming process, this regulation allows for fast changes in protein concentration and contributes to the maintenance of cell homeostasis (Hershey *et al.*, 2012; Schwanhausser *et al.*, 2011; Sonenberg *et al.*, 2009; Tahmasebi *et al.*, 2018). Translation can be divided into different steps: initiation, elongation, termination and ribosome recycling (Sonenberg *et al.*, 2009), although most of the regulation occurs at the initiation stage, which is the main object of this next section.

1.3.1.1 Translation Initiation

Most of translation regulation occurs at this stage (Sonenberg *et al.*, 2009). In higher eukaryotes, mature mRNAs have an m^7G-cap at their 5' terminus and a poly-A tail at their 3' end. Translation initiation can be dependent on the mRNA 5' cap or on regions known as internal ribosome entry sites (IRES): highly structured sequences in the mRNA molecule that promote binding of the ribosome to start translation (Fitzgerald *et al.*, 2009).

1.3.1.2 Cap-dependent translation initiation

During cap-dependent translation initiation, a pre-initiation complex (43S PIC) comprised of the 40S portion of the ribosome, the initiator methionyl-transfer RNA (40S-Met-$tRNA_i$ complex), and different eIFs binds the 5' end of an m^7G-capped mRNA (Figure 1.9) (Pestova *et al.*, 2002). This complex scans the mRNA molecule until it reaches the start codon (Hershey *et al.*, 2012; Merrick *et al.*, 2018). The typical start codon in mRNAs is AUG, but near cognates (codons that differ from AUG by one nucleotide - CUG, GUG, and UUG) can be used as initiation codons for some mRNAs (Kearse *et al.*, 2017; Starck *et al.*, 2016). Once the start codon is identified, the 60S ribosomal subunit joins the resulting 48S, forming the mature ribosome capable of starting the elongation portion of translation (Gingras *et al.*, 1999; Kozak, 1991).

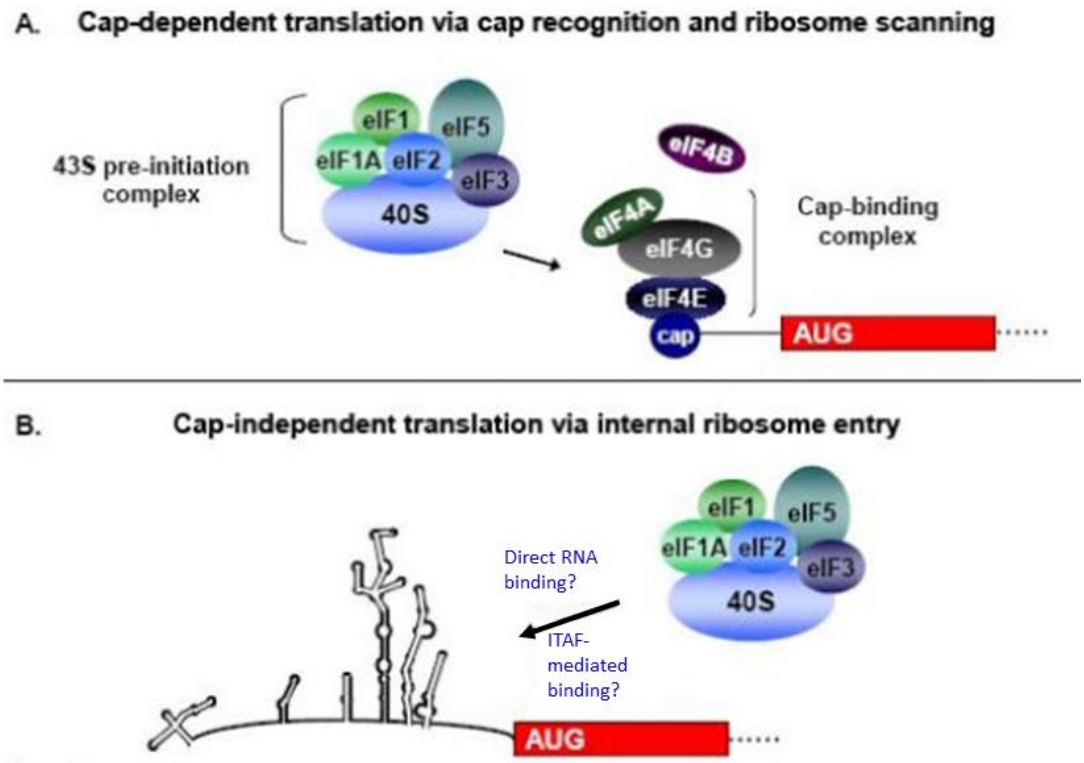


Figure 1.9 Schematic representation of (A) cap-dependent translation, and (B) IRES-dependent translation. While cap-dependent translation will require a much more complex machinery, IRES structures can promote translation initiation independently of the cap region on the mRNA molecule.Taken from (Fitzgerald *et al.*, 2009)**.**

1.3.1.3 IRES-dependent translation initiation

The 40S subunit of the ribosome can bind directly to a number of cellular mRNAs in regions known as IRES (Figure 1.9) (Jackson, 2013). This type of translation initiation can differ depending on the mRNA and its IRES structure: some mRNAs are able to recruit the ribosome directly via the secondary and tertiary structures formed by entry sites (Jan *et al.*, 2002; Spahn *et al.*, 2004), while other IRES will still require a subset of eukaryotic translation initiation factors (eIF) to promote the assembly of the translation machinery (Kaminski *et al.*, 1995; Ochs *et al.*, 1999; Spahn *et al.*, 2004). A subset of proteins known as IRES trans-acting factors (ITAFs) can interact with the IRES and stabilize it to promote ribosome binding, while other ITAFs are known to alter IRES conformation, preventing translation of the mRNA (Marques *et al.*, 2022). IRES-dependent translation is an alternative initiation method that can be used by cells whenever the canonical, cap-dependent pathway is inhibited under stress conditions, such as in cancers or infectious diseases (Marques *et al.*, 2022). The presence of IRES in viral genomes are also a known mechanism to evade host ribosome scanning, a limiting step in cap-dependent translation, during infection (Hanson *et al.*, 2012).

1.3.2 The eIF4F complex

In the context of cap-dependent translation, different eIFs are involved. Here, we will highlight the importance of the eIF4F complex, that plays a role in facilitating the recruitment of 43S PIC to the 5' cap of an mRNA that will be translated (Banerjee, 1980; Gingras *et al.*, 1999; Sonenberg *et al.*, 2009). The eIF4F complex is comprised of eIF4E, a cap-binding protein, eIF4A, an RNA helicase, and eIF4G, a scaffold protein that binds all the other components of the complex and the mRNA (Fitzgerald *et al.*, 2009; Pestova *et al.*, 2002). eIF4G is also capable of interacting with the poly-A binding protein (PABP), promoting circularization of the mRNA, which has been shown to enhance translation efficiency (Gallie, 1991; Sinha *et al.*, 2022; Wells *et al.*, 1998) (Figure 1.9). Mutant forms of eIF4A were shown to arrest the eIF4F complex in an inactive state, inhibiting cap-dependent and independent translation (Pause *et al.*, 1994b; Svitkin *et al.*, 2001) and showcasing the importance of the eIF4F complex and specifically eIF4A for translation initiation.

The 5' UTR region of some mature mRNAs form secondary structures that stall the scanning and translation initiation of that molecule (Hinnebusch *et al.*, 2016). These 5'UTR regions can negatively regulate translation depending on their complexity and structural organization (Grayeski *et al.*, 2022; Wang *et al.*, 2022). As a DEAD-box RNA helicase, eIF4A is known to promote an ATP-dependent restructuring of the 5'UTR region of mRNAs, generating a single stranded mRNA molecule, to facilitate 43S PIC binding and subsequent scanning (Hinnebusch *et al.*, 2012; Pestova *et al.*, 2002; Ray *et al.*, 1985; Sonenberg *et al.*, 2009).

Specifically in mammals, eIF4A-dependent mRNA molecules are characterized by long 5'UTR regions that form stable secondary structures and by the presence of structures known as G-quadruplexes (Rubio *et al.*, 2014; Svitkin *et al.*, 2001; Wolfe *et al.*, 2014), although some reports question that (Waldron *et al.*, 2018). Additionally, other studies have shown that the structure of the 5' UTR of mRNAs appears to be a minor determinant in rocaglate selectivity, with rocaglates inducing recruitment of eIF4A to polypurine sequences in the mRNA (Iwasaki *et al.*, 2016). The interaction with eIF4G and eIF4B maintain eIF4A in its active state, aligning DEAD-box motifs in the orientation required for ATP-dependent helicase activity (Hilbert *et al.*, 2011; Oberer *et al.*, 2005; Ozes *et al.*, 2011; Schutz *et al.*, 2008). In mammalian cells, eIF4A is expressed as two different paralogs that participate in translation initiation, eIF4A1 and eIF4A2, with over 90% similarity between them (Rogers *et al.*, 2002; Yoder-Hill *et al.*, 1993). eIF4A1 is the most abundant paralog in most cell types, and it is essential for mouse development (Galicia-Vazquez *et al.*, 2012; Nielsen *et al.*, 1988; Senechal *et al.*, 2021). There is still a third paralog, eIF4A3, known to

participate in mRNA splicing, trafficking and ribosome biogenesis (Kanellis *et al.*, 2021). Although eIF4A1 and eIF4A2 functions were originally thought to be identical, more recent reports have shown differences in the functions of these two paralogs (Lu *et al.*, 2014; Wilczynska *et al.*, 2019). It has also been shown that only eIF4A1 is essential for initiation, with eIF4A2 absence not affecting cell viability or global protein synthesis (Galicia-Vazquez *et al.*, 2015). Different variants of the eIF4F complex, made up of different homologues of its components, eIF4A and eIF4E, can be relevant for translation in response to specific stimuli, such as hypoxia (Ho *et al.*, 2016)

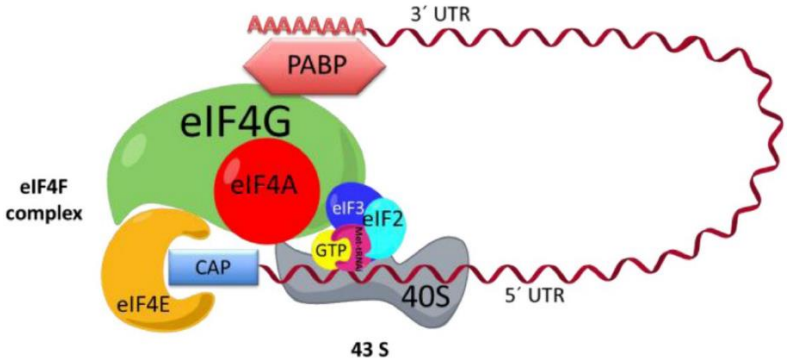


Figure 1.10 Translation initiation complex.

The eIF4F complex is part of the initiation complex, containing the cap-binding eIF4E, the DEAD-box helicase eIF4A and the scaffold protein eIF4G. PABP binds to the poly-A tail and can also interact with eIF4G, promoting the circularization of the mature mRNA. The 43S PIC is recruited by eIF4F to start scanning the mRNA molecule. Taken from (Montero et al., 2015).

1.3.3 Translation regulation through the mTORC1 complex

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase with a well-established role in regulating translation of higher eukaryotic cells. The mTOR complex 1 (mTORC1) is formed by mTOR, regulatory protein associated with mTOR (Raptor), and mammalian lethal with Sec13 protein 8 (mLST8) (Kim *et al.*, 2002; Melick *et al.*, 2020). It senses external stimulus, changes in nutrient availability, and reprograms the cell in different ways. This regulation relies mainly on the phosphorylation of eIF4E-binding proteins (4E-BP) (Dowling *et al.*, 2010; Gingras *et al.*, 1998), ribosomal protein S6 kinase (S6K) (Magnuson *et al.*, 2012) , and La-related protein 1 (LARP1), which will be explored in this section.

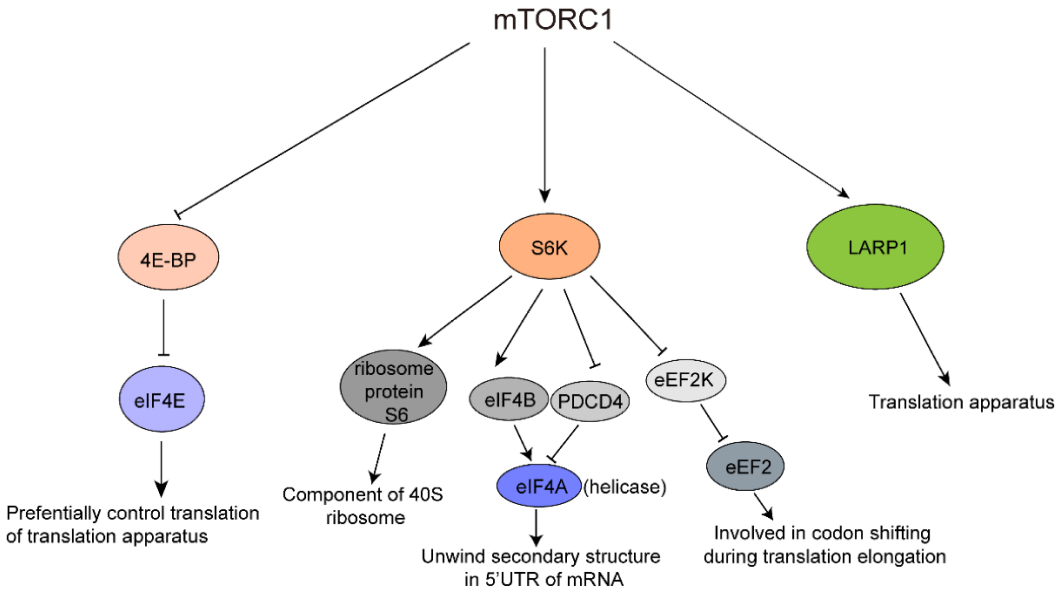


Figure 1.11 Downstream mTORC1 regulators of translation.

mTORC1 can regulate translation by phosphorylating different downstream targets, 4E-BPs, S6K and LARP1.S6K phosphorylation will regulate eIF4A-dependent translation through different intermediates eIF4B and PDCD4. Taken from (Yang *et al.*, 2022b).

1.3.3.1 LARP1

LARP1 is an RNA-binding protein, described to interact with the 3' terminus of the poly A tail of mature mRNAs, stabilizing them (Aoki *et al.*, 2013; Hong *et al.*, 2017). LARP1 can bind to the 5' Terminal OligoPyrimidine (5'TOP) motif of mRNAs, preventing initiation complex to assemble and inhibiting their translation. In active mTORC1 conditions, phosphorylated LARP1 will not bind to the 5'TOP motif, allowing for these mRNAs to be translated (Jia *et al.*, 2021; Tcherkezian *et al.*, 2014).

1.3.3.2 4E-BPs

4E-BPs bind to eIF4E, occupying its eIF4G-binding domain and impairing the assembly of the eIF4F complex (Gingras *et al.*, 1998; Pause *et al.*, 1994a), repressing the translation of a subset of mRNAs (Dowling *et al.*, 2010; Tahmasebi *et al.*, 2014; Tahmasebi *et al.*, 2016). When activated, mTORC1-dependent phosphorylation of 4E-BPs in key residues can promote dissociation from eIF4E (Gingras *et al.*, 2001; Yang *et al.*, 2022b).

1.3.3.3 S6K

In the absence of mTORC1 signaling, S6K binds directly to the eIF3 complex, responsible for 43S PIC assembly and recruitment, blocking translation (Yang *et al.*, 2022b). When mTORC1 is activated, phosphorylated S6Ks will phosphorylate different downstream proteins, such as the program cell death 4 protein (PDCD4), eIF4B, ribosomal protein S6 (rpS6), and the eukaryotic elongation factor-2 kinase (eEF2K) (Banerjee *et al.*, 1990; Dorrello *et al.*, 2006; Magnuson *et al.*, 2012; Raught *et al.*, 2004). These will have different roles in promoting cell translation: Phosphorylated RPS6 is a component of the 40S ribosome and therefore directly involved in translation (Magnuson *et al.*, 2012). eEF2K is inactivated when phosphorylated, and therefore unable to inhibit eEF2, responsible for the elongation portion of translation (Wang *et al.*, 2001; Wang *et al.*, 2014).

As for eIF4B and PDCD4, both their phosphorylations will enhance eIF4A activity in different ways - PDCD4 is a tumor suppressor capable of binding to eIF4A, inhibiting its activity (Yang *et al.*, 2003). Once phosphorylated by SK6, PDCD4 will be ubiquitinated and degraded by the proteasome, increasing the amount of free eIF4A (Dorrello *et al.*, 2006). Phosphorylated and activated eIF4B, is recruited to eIF4A, supporting its helicase activity (Yang *et al.*, 2022b).

1.3.4 Translation dysregulation during pathological conditions

The deregulation of translation components (tRNA synthesis/function, ribosomes, and pathways known to regulate translation) can lead to syndromes known as ribosomopathies (due to their known cause attributed to a ribosomal dysfunction), in addition to mitochondrial diseases, etc. (Tahmasebi *et al.*, 2018). Moreover, deregulation of translation has been reported in different infectious and non-infectious diseases, such as viral infections and several types of cancer (Kaczmarczyk *et al.*, 2022; Li *et al.*, 2023; Lu, 2022; Mohr *et al.*, 2012; Rozman *et al.*, 2023; Stern-Ginossar *et al.*, 2019; Zhulyn *et al.*, 2023).

Given its importance in regulating translation and other essential cellular processes, such as autophagy, mTOR is a commonly dysregulated factor in different infectious diseases, having translation-dependent and independent effects. Some viral infections, such as human papilloma virus, cytomegalovirus and HIV-1 can increase mTORC1 activity and activate its downstream signaling pathways as a way of promoting viral replication upon infection (Akbay *et al.*, 2020; Clippinger *et al.*, 2011; Spangle *et al.*, 2010). Other RNA viruses, such as ZIKV, are thought to inhibit the mTOR pathway to induce autophagy and promote viral replication (Cheng *et al.*, 2018; Karam *et al.*, 2021) In bacterial infections, *Helicobacter pylori* and *Mycobacterium tuberculosis*

also modulate mTOR activity to promote the infection (Garg *et al.*, 2020; Shariq *et al.*, 2023; Sokolova *et al.*, 2014). Finally, our group has identified an activation of mTOR-sensitive mRNA translation upon infection with *Toxoplasma gondii* and *Leishmania donovani* (Chaparro *et al.*, 2020; Holmes *et al.*, 2019; Leroux *et al.*, 2018). In this context, mTOR-dependent translation appeared to have contrasting effects for these two parasites: while it appeared to favour *T. gondii* replication, the upregulation of mTORC1 dependent translation was proposed to be a host-defense mechanism during *L. donovani* infection (Chaparro *et al.*, 2020; Holmes *et al.*, 2019; Leroux *et al.*, 2018). As previously discussed, the eIF4F complex assembly is under mTOR regulation, (Pelletier *et al.*, 2019), and in BMDMs infected with *L. donovani*, our group also identified an upregulation in the translation of eIF4A-sensitive transcripts in *L. donovani* infected BMDMs (Figure 1.12A).

1.3.4.1 eIF4A-dependent translation is up-regulated in *Leishmania donovani*-infected BMDMs

An *in silico* analysis, based on a compilation of previously reported eIF4A-sensitive transcripts, identified using eIF4A1 inhibitors and cells knock-down of eIF4A1 (Cerezo *et al.*, 2018; Modelska *et al.*, 2015; Rubio *et al.*, 2014; Wolfe *et al.*, 2014), revealed an eIF4A signature in BMDMs infected with *L. donovani* (Figure 1.12) (Chaparro *et al.*, 2020). As described, eIF4A is a DEAD box RNA helicase that is part of the eIF4F complex, responsible for unwinding secondary structures in the 5’ UTR region of mature mRNAs, allowing for 43S PIC to bind and scan the mRNA molecule. It has been previously reported that 5’ UTR containing CGG motifs (also known as G-quadruplex structures) were a signature of eIF4A sensitivity in mRNAs (Wolfe *et al.*, 2014), and mRNAs containing these motifs also showed an increase in translation efficiency during *L. donovani* infection (Figure 1.12B).

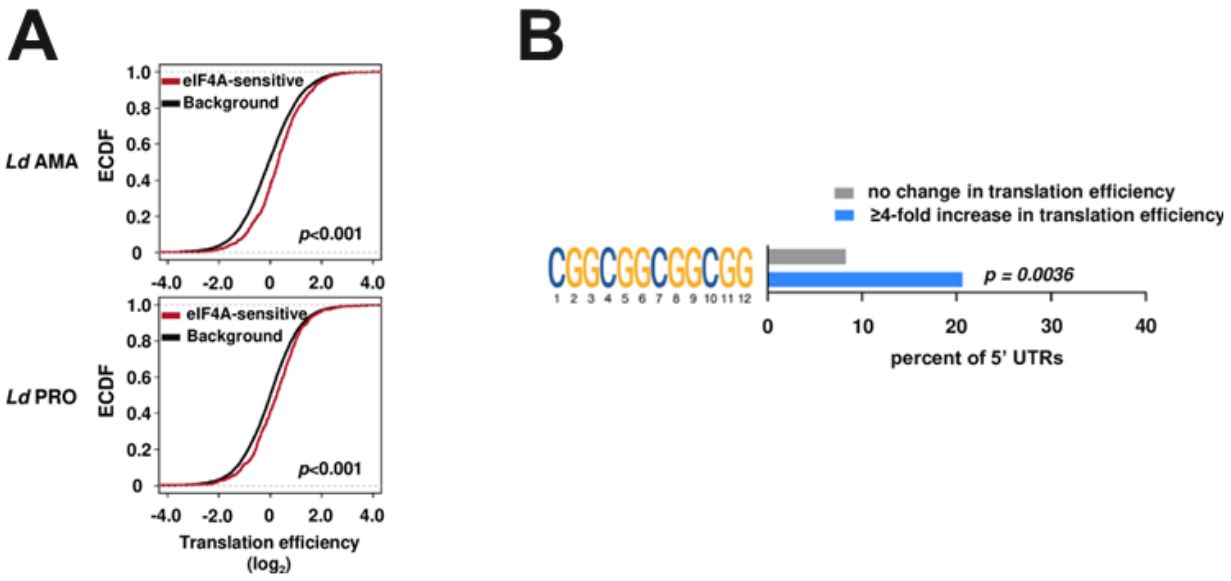


Figure 1.12 eIF4A-dependent translation is up-regulated in BMDMs infected with *L. donovani.*

(A) Empirical cumulative distribution function (ECDF) of translational efficiencies for previously reported eIF4A-sensitive transcripts, compared to all detected transcripts (background). (B) Percentages of infection-associated translationally activated mRNAs with at least one $(CGG)_4$ motif in their 5' UTR, compared to a random set of unchanged mRNAs. Taken from (Chaparro et al., 2020).

The translation of transcripts associated with different cell functions are dependent on eIF4A, notably several immune-related transcripts (Cramer *et al.*, 2018; Di Marco *et al.*, 2012; Modelska *et al.*, 2015). Specifically in macrophages, the inhibition of eIF4A using compounds known as rocaglates can have immunomodulatory effects, promoting a reprogramming of macrophage polarization that has been associated with antimicrobial effects against different pathogens (Bhattacharya *et al.*, 2016; Blum *et al.*, 2020; Chatterjee *et al.*, 2021; Schiffmann *et al.*, 2023). In accordance, we observed that Silvestrol (a known eIF4A inhibitor) reduced parasite persistence in BMDMs. This suggested that the increase in host eIF4A-dependent translation was exploited by the parasite to promote the infection. The inhibition of the mTOR pathway, however, further increased parasite persistence (Figure 1.13).

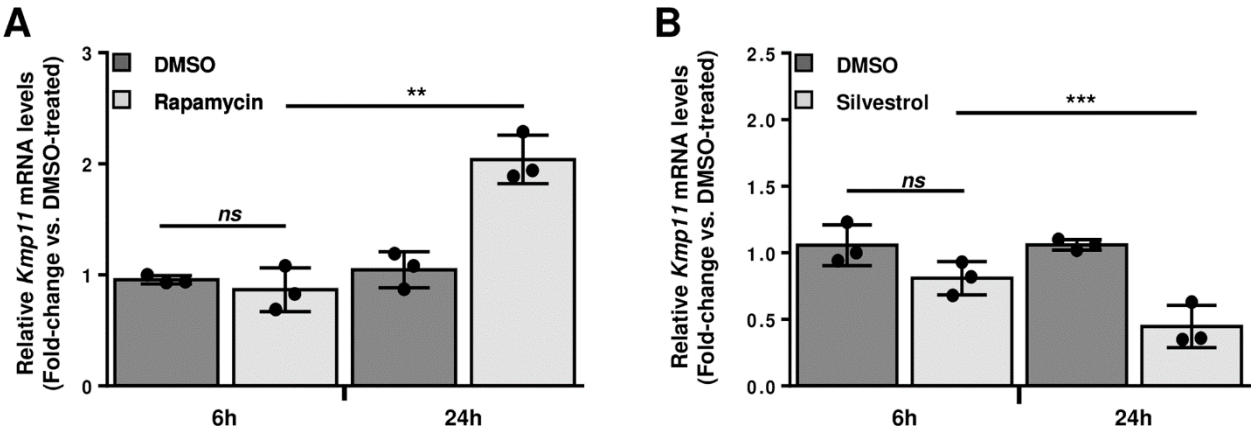


Figure 1.13 Inhibition of host mTOR and eIF4A have contrasting effects during *L. donovani* infection of BMDMs.
By treating infected BMDMs with mTOR inhibitor Rapamycin leads to an increase in parasite persistence. Conversely, eIF4A inhibition with Silvestrol reduces parasite persistence in BMDMs, suggesting this eIF is needed by the parasite to promote infection. For these experiments, quantification of intracellular parasites was obtained by RT-qPCR, measuring the relative amount of Leishmania Kmp11 mRNA (normalized to Actb). Taken from (Chaparro et al., 2020).

Another evidence of eIF4A-dependent translational control during *L. donovani* infection was observed comparing transcripts and protein levels of the transforming growth factor beta (TGF-β), known to be an eIF4A-dependent transcript (Figure 1.14) (Wolfe *et al.*, 2014). While secreted TGF-β levels increased upon infection and decreased when eIF4A was pharmacologically inhibited with Silvestrol, transcript levels for the TGF-β gene did not change in the same conditions. Suggesting a potential translation-related mechanism regulating the expression of this eIF4A-dependent gene (Chaparro *et al.*, 2020), although changes in protein stability or secretion could also be relevant to this observation.

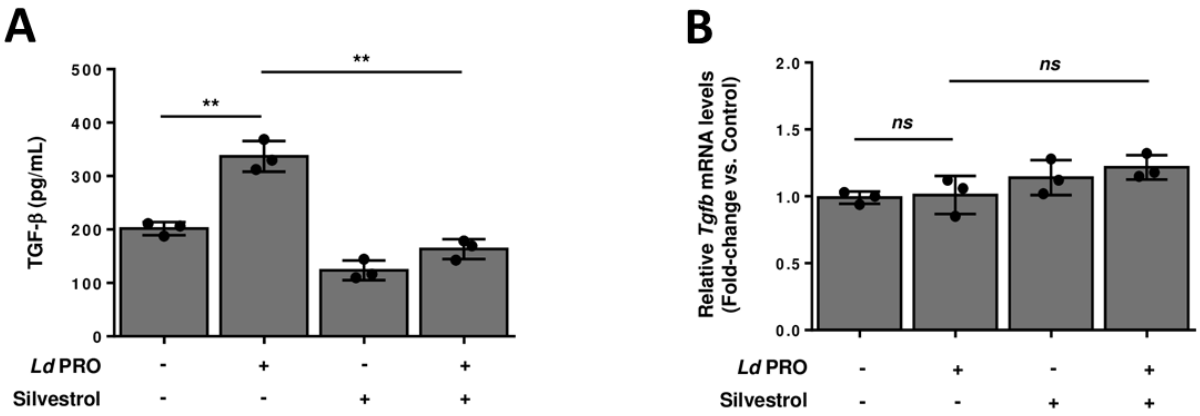


Figure 1.14 Control of TGF-B expression appears to be translationally modulated during *L. donovani* infection, TGF-B (encoded by *Tgfb,* an eIF4A-dependent transcript) protein levels change upon *L. donovani* infection and eIF4A inhibition, while transcripts remain stable, indicating a translational regulation of protein expression. Taken from (Chaparro et al., 2020).

1.3.5 Rocaglates – eIF4A inhibitors

Rocaglates are a diverse class of natural and synthetic compounds that share a cyclopenta[b] benzofuran ring, originally isolated from the *Aglaia* genus of angiosperms (King *et al.*, 1982). Rocaglates promote a gain of function to the eIF4A protein, enhancing the binding (clamping) of both paralogs (eIF4A1 and eIF4A2) to the mRNA molecule (Chu *et al.*, 2019; Iwasaki *et al.*, 2016). Although other natural and synthetic products can also inhibit eIF4A (namely hippuristanol and pateamine A), rocaglates are among the most potent and selective eIF4A inhibitors (Naineni *et al.*, 2020; Shen *et al.*, 2020).

Rocaglate-driven stabilization of the mRNA/eIF4A interaction is thought to impede 43S PIC scanning, inhibiting translation (Iwasaki *et al.*, 2016; Iwasaki *et al.*, 2019). Additionally, the longer retention of eIF4F complex at the cap promoted by rocaglate treatment can have an additional, bystander effect on global translation (Chu *et al.*, 2020). Different rocaglates can present different bioactivities towards different mRNAs, depending on rocaglate structure and the complexity of 5' UTR region composition of mRNAs (Chu *et al.*, 2020), as described in section 1.3.2. Studies on the molecular interactions between eIF4A and rocaglates highlighted different amino acid residues that are crucial for the effect of rocaglate treatment, notably eIF4A1 aa positions 158, 159, 163, 192, 195, and 199 (Chu *et al.*, 2016; Iwasaki *et al.*, 2019; Sadlish *et al.*, 2013). While some of these positions are highly conserved among organisms, naturally occurring mutations in some of these residues (particularly 163 and 199) are found to promote resistance to rocaglates, highlighting their importance in the interaction between eIF4A, the rocaglate and the mRNA

molecule (Chu *et al.*, 2016; Obermann *et al.*, 2023). The analysis of the highly conserved eIF4A sequences across organisms revealed the potential to use rocaglates against different insect, plant, and mammalian pathogens (Obermann *et al.*, 2023).

1.3.5.1 Rocaglate treatment against infectious and non-infectious diseases

Since the translation of many proto-oncogenes is dependent on the eIF4F complex, rocaglates are largely explored in cancer research as alternative therapeutic options (Bhat *et al.*, 2015; Blagden *et al.*, 2011; Merrick, 2015; Pelletier *et al.*, 2015; Sendoel *et al.*, 2017; Wolfe *et al.*, 2014). Notably, zotatifin, a synthetic rocaglate, was evaluated in phase 1 and 2 clinical trials against solid tumors (Ernst *et al.*, 2020; Gerson-Gurwitz *et al.*, 2021). An enhanced eIF4A activity and dependency was reported for different types of cancers (Bordeleau *et al.*, 2008; Chan *et al.*, 2019; Gerson-Gurwitz *et al.*, 2021), which would explain the potential therapeutic effect of eIF4A inhibition and the enhanced selectivity for cancer cells. Additionally, a senolytic effect was described from eIF4A inhibition using cardiac glycosides in breast cancer (Howard *et al.*, 2020; Triana-Martinez *et al.*, 2019), further reinforcing the relevance of eIF4A in the context of cancers.

Zotatifin also showcased antiviral activity in SARS-Cov2 infection (Gordon *et al.*, 2020; Obermann *et al.*, 2022), and was evaluated on phase 1b dose escalation trials in patients with mild to moderate COVID-19. This is just an example of the vast antiviral potential of rocaglates, that have been shown for different viral infections (Biedenkopf *et al.*, 2017; Elgner *et al.*, 2018; Taroncher-Oldenburg *et al.*, 2021; Todt *et al.*, 2018). In addition to viruses, rocaglates have shown promising results against other fungal, bacterial, and protozoan pathogens (Chatterjee *et al.*, 2021; Iyer *et al.*, 2020; Langlais *et al.*, 2018).

1.3.6 Leishmania and eIF4A

Leishmania parasites also present their own copies of eIF4A (Barhoumi *et al.*, 2006). Some studies have explored *Leishmania* eIF4A as a potential drug target (Harigua-Souiai *et al.*, 2018), but sequence analysis and *in vitro* tests with Silvestrol have identified that *Leishmania* eIF4A could be resistant to rocaglate treatment (Obermann *et al.*, 2023). However, previous studies have been able to target *Leishmania* eIF4A with cholesterol derivatives, inhibiting the ATPase and helicase activities of eIF4A (Abdelkrim *et al.*, 2018; Abdelkrim *et al.*, 2022).

2 SUMMARY AND HYPOTHESIS

Given the information presented in the introduction, it is known that leishmaniasis is a tropical neglected disease, to which treatment options are limited and face different challenges, such as the cost and parasite resistance. The lack of vaccines and issues with current treatment options make it imperative to develop new options to control leishmaniasis. Macrophages infected by *Leishmania* parasites have their functions hijacked by the parasite in different levels of gene expression to favor replication. At the translational level, our group previously identified a reprogramming of the host translatome upon *L. donovani* infection, with an increased translational efficiency of host eIF4A-sensitive transcripts. The inhibition of eIF4A in infected cells reduced parasite persistence, indicating that this eIF4A signature was a parasite-driven strategy to promote infection, instead of a macrophage response to eliminate the parasite.

Based on these findings, we hypothesized that the inhibition of eIF4A-dependent translation programs using rocaglates could control *Leishmania* infection *in vitro*. To test this hypothesis, we assessed the capability of 40 different synthetic rocaglates, provided by the Boston University Center for Molecular Discovery, to control parasite replication in BMDMs.

Our main aim with this project was to investigate the therapeutic potential of targeting eIF4A-dependent mRNA translation during *Leishmania* infection *in vitro*. Our specific objectives were defined as follows:

1. To identify eIF4A inhibitors with anti-leishmanial activity *in vitro*, and
2. To characterize the mechanisms through which these eIF4A inhibitors can control the infection *in vitro*.

3 MATERIALS AND METHODS

3.1 Cell culture and differentiation

Culture media and supplements were purchased from Wisent (St-Jean-Baptiste, Quebec, Canada), Gibco (Grand Island, NY, USA) and Sigma-Aldrich (Oakville, Ontario, Canada).

3.1.1 Bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages (BMDMs) were generated from precursor cells isolated from the bone marrow of 6- to 8-week-old female C57BL/6 mice, as described (Leroux *et al.*, 2018). C57BL/6 mice were housed and handled according to a protocol approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the INRS (CIPA #2102-01). This protocol respect procedures on good animal practice provided by the Canadian Council on animal care. Briefly, marrow was extracted from bones of the hind legs and hip bones, red blood cells were lysed, and progenitor cells were resuspended in BMDM culture medium (DMEM, 10% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM HEPES, 55 µM ß-mercaptoethanol) supplemented with 15% L929 fibroblast-conditioned culture medium (LCCM). Non-adherent cells were collected the following day and were cultured for 7 days in BMDM culture medium supplemented with 30% LCCM with fresh medium replenishment at day 3 of incubation.

Experiments involving the use of wild-type (WT) BMDM were carried out with C57BL/6 mice (Jackson Laboratories, #000664). In addition, experiments involving BMDMs with reduced expression of either eIF4A1 or eIF4A2 were carried out using *Eif4a1*+/- and *Eif4a2*-/- C57BL/6 mice, respectively, which were kindly provided by the laboratory of Dr. Jerry Pelletier (McGill University, Montreal, QC, Canada). These animals were generated as described (Senechal *et al.*, 2021): briefly, CRISPR-Cas9 was used to induce mutations in one allele of eIF4A1 ($eIF4A1^{+/-}$) and eIF4A2 ($eIF4A2^{+/-}$) in these animals, generating premature stop codons and consequently, nonfunctional proteins.

3.1.2 THP-1 (Human Leukemia Monocytic cell line)

THP-1 (ATCC, # TIB-202) is a human monocytic cell line. Cells were cultured in DMEM, 10% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM HEPES, 55 µM ß-mercaptoethanol, and passed before reaching 1

million cells per culture mL. Their differentiation into macrophages was obtained by treating cells with 130 nM of Phorbol 12-myristate 13-acetate (PMA) for 72 h. After treatment, cells were washed and infected with metacyclic promastigotes of *Leishmania*.

3.1.3 Immortalized BMDMs (iBMDMs)

Immortalized BMDMs were kindly provided by the laboratory of Dr. Ian-Gael Rodrigue-Gervais (INRS – Centre AFSB, Laval, QC, Canada). These cells were generated by J2 retrovirus transduction of primary BMDMs, as described (Tan *et al.*, 2019). iBMDMs were cultured in BMDM culture medium with a reduced percentage of heat-inactivated FBS (DMEM, 5% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM HEPES, 55 µM ß-mercaptoethanol).

3.1.4 Leishmania

L. amazonensis LV79 (MPRO/BR/72/M1841) was provided by the laboratory of Dr. Albert Descoteaux (INRS – Centre AFSB, Laval, QC, Canada). A stably transfected line of *L. amazonensis* PH8 (IFLA/BR/1967/PH8) expressing an RFP (La-RFP) was provided by the laboratory of Dr. Nathan C. Peters (University of Calgary, Calgary, AB, Canada). *La*-RFP was generated as described (Peters *et al.*, 2008). *Leishmania* promastigotes were cultured at 26 °C in M199 medium supplemented with 10% heat inactivated FBS, 100 µM hypoxanthine, 5 µM hemin, 3 µM biopterin, 1 µM biotin, 100 U/mL penicillin, and 100 µg/mL streptomycin. Early passage stationary phase promastigotes were used to isolate metacyclic promastigotes that would then be used for macrophage infections.

3.2 Metacyclic promastigote isolation

Leishmania parasites were cultured as described in 3.1.4. Once cultures reached stationary phase, parasites were washed with PBS and centrifuged at 1500 g for 10 minutes at 4 °C. Then, parasites were resuspended in serum-free DMEM. Separation gradients were prepared by adding equal volumes of 50% Ficoll (Sigma-Aldrich) and 10% Ficoll solutions, and on top of this gradient adding a solution of parasites resuspended in DMEM. Gradients were centrifuged at 500 g for 10 minutes at 25°C. The resulting interphase containing metacyclic promastigotes was recuperated (approximately 15% of the original number of parasites). The isolated metacyclic parasites were washed with HBSS. Then, they were incubated with 10% opsonization serum obtained from $C5^-$

$^{/-}$ C57BL/6 mice and incubated for 20 minutes at 37 °C. Following opsonization, parasites were washed and resuspended in complete DMEM for infection.

3.3 Infection assay

Macrophages were infected with metacyclic promastigotes of *L. amazonensis* at a multiplicity of infection (MOI) of 1:10. Non-internalized parasites were washed with PBS after 2 h of infection.

3.4 Rezasurin-based viability assays in host cells and promastigote cultures

Viability of macrophage cultures was determined by the resazurin assay as described (Chaparro *et al.*, 2020). Briefly, cells were treated with increasing concentrations of selected rocaglates, or an equivalent volume of DMSO (vehicle) for 72 h at 37°C, 5% CO_2. For strong clampers, tested concentrations ranged from 320 nM to 2.5 nM, with a dilution factor of 2. As for the weak clampers, concentrations ranged from 10 uM to 1.5 nM, with a dilution factor of 3.

Then, 0.025% resazurin was added to the cultures and incubated for 4 h. Viability was measured based on the reduction of resazurin to resorufin (fluorescent and colored product) by viable cells. For promastigotes, optical density was measured using a Multiskan GO (ThermoFisher) at 600 and 570 nm. Absorbance at 600 nm was subtracted from readings at 570 nm. For macrophages, fluorescence was measured using a Cytation 5 (Biotek) at 560 nm +/- 20 nm excitation and 590 nm +/- 20 nm emission. Values from wells without any cells were used as blanks, and DMSO-treated cells were used to normalize values. Cultures incubated with 50% DMSO (Dead Cells – DC), were used as our positive control for cell death. Experiments were performed in biological replicates (n = 2); each sample was analyzed in a technical triplicate, the average of which was plotted against increasing concentrations of the respective inhibitor.

3.5 Rocaglate panel and treatment schemes

The initial panel provided by Boston University Center for Molecular Discovery consisted of two subsets of 20 synthetic compounds each: compounds 1-20 were weak eIF4A1 clampers, while compounds 21-40 were strong eIF4A1 clampers. These compounds were grouped in these subsets based on eIF4A1 clamping values obtained using recombinant human eIF4A1 (not disclosed here for confidentiality purposes). The classes to which these rocaglates belong to and their structures are known, but owing to confidentiality matters, we cannot reveal this information.

Treatment with selected rocaglates was performed as depicted in Figure 3.1. Unless otherwise indicated, the treatment schedule used for experiments in this thesis are as follow: macrophages were infected with metacyclic promastigotes of *L. amazonensis*, isolated as described in section 3.2. Twenty-four hours post-infection (i.e., estimated time required for promastigote differentiation into amastigote stage), cells were treated with rocaglates for an additional 72 h. The post-treatment schedule was chosen for its clinical relevance (amastigotes are the clinically relevant stage, and in this case, we are treating macrophages infected with amastigotes).

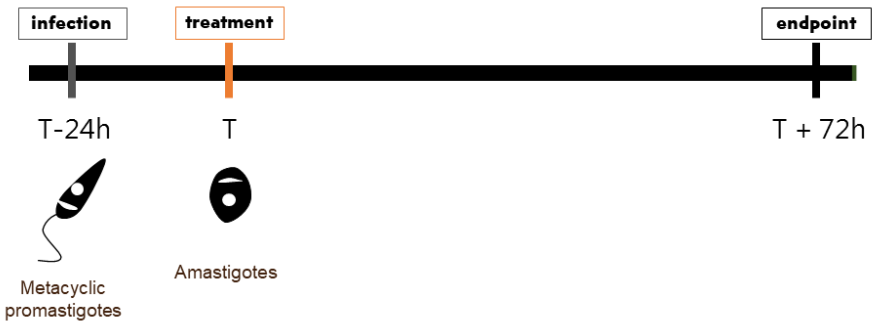


Figure 3.1 Main treatment scheduled used for the project.
We adopted the 24h-post treatment schedule with the rocaglates to target the clinically relevant form of the parasite (amastigote).

3.6 Measurement of *Leishmania* intramacrophage replication by epifluorescence

To collect infected cells at the different times post-infection and treatment, macrophages were cultured in coverslips in 24-well plates. After infection, slides were washed 3x with PBS and fixed with a 2% PFA solution for 10 minutes at room temperature. Then, cells were washed 2x with PBS and incubated with a solution of 10 µg/mL DAPI in PBS for 10 minutes at room temperature. Once fixed and stained with DAPI, slides were mounted for visualization and counting in an epifluorescence microscope. Infected and stained BMDMs were analyzed at the 100X objective in an epifluorescence microscope, to assess the infection index. The infection index is the number of parasites divided by the total number of macrophages counted per slide (about 100 macrophages were counted per slide).

$$Infection\ Index = \frac{Number\ of\ Parasites}{Total\ Number\ of\ Macrophages\ (Infected + Uninfected)}$$

Equation 3.1 Infection Index

3.7 *Leishmania* amastigote recovery from infected macrophages

Infected BMDMs were washed 3x with PBS. A solution of complete DMEM containing 0.025% SDS was added to the cells, and plates were gently agitated and incubated at 37 °C for 1-2 minutes until all macrophages were lysed. SDS-free complete DMEM was then added to the lysed wells. The content of each well was transferred to a microtube and centrifuged at 300 g for 5 minutes at room temperature. The supernatant was transferred to a new tube and centrifuged at 2000 g for 10 minutes. The pelleted amastigotes were resuspended in complete DMEM and used for infection.

3.8 Polysome tracings

Cytosolic lysates of rocaglate-treated (2 h of treatment at 37 °C) and DMSO-treated control iBMDMs were prepared for RNA fractionation as described (Leroux *et al.*, 2018). Lysates were layered over 5 to 50% sucrose density gradients and sedimented using a Beckman SW41 rotor at 36,000 rpm (= 221,830.9 × g) for 2 h at 4°C. Gradients were fractionated and collected (30 s, 500 µL/fraction), and the absorbance at 254 nm was recorded continuously using a Brandel BR-188 density gradient fractionation system.

3.9 Statistical analysis

Data is presented as the mean ± standard deviation (SD) of the mean. Statistical significance was determined by using t-test or one-way ANOVA; calculations were performed by using Prism 7 software package (GraphPad). Differences were considered significant when *p < 0.05.

4 RESULTS

Although our published data on the eIF4A translation signature upon *Leishmania* infection and the anti-leishmanial effect of Silvestrol were generated using *L. donovani* (Chaparro *et al.*, 2020), in this project we used *L. amazonensis* LV79 as the parasite model. In addition to preliminary data showing that rocaglates could also reduce *L. amazonensis* LV79 persistence in BMDMs, we used *L. amazonensis* since it was the parasite species that better replicated in BMDMs in our assays. Since this is a project to evaluate the impact of a treatment on the infection, we chose the most infective species, since the effect of the treatment would be clearer.

4.1 Pilot screening rapidly identifies rocaglates that are potentially effective in controlling *Leishmania* infection in BMDM cultures.

We designed a first screening to rapidly evaluate the potential anti-leishmanial effect of the set of rocaglates obtained from BU-CMD, while also gaining insight on the toxicity of these compounds to the host cell. This screening would give us enough information to narrow down the rocaglate set, selecting promising compounds to be further explored. Macrophages were infected with *L. amazonensis* metacyclic promastigotes (MOI 10:1), and 24 h later treated with 2 different concentrations of each of the 40 rocaglates (100 nM and 20 nM) and were incubated for 72 h. Amph.B at 0.25 µg/mL was used as a positive control for parasite killing, while vehicle (DMSO) was the negative control. These concentrations were chosen based on our knowledge of anti-leishmanial rocaglate treatment at the time, with Silvestrol, as explored in the introduction. Data from our laboratory showed that 25 nM of Silvestrol was enough to obtain an anti-leishmanial effect in BMDMs (Chaparro *et al.*, 2020). The second concentration was chosen to observe if any compounds showcased a dose-dependent effect, while remaining on the low nanomolar range (ideal for this step during drug discovery). After 72 h of treatment, cells were collected, and the infection index was obtained (Figure 4.1). Data is presented in two graphs, organized between weak eIF4A1 clampers (C1-C20) (Figure 4.1A) and strong eIF4A1 clampers (C21-C40) (Figure 4.1B).

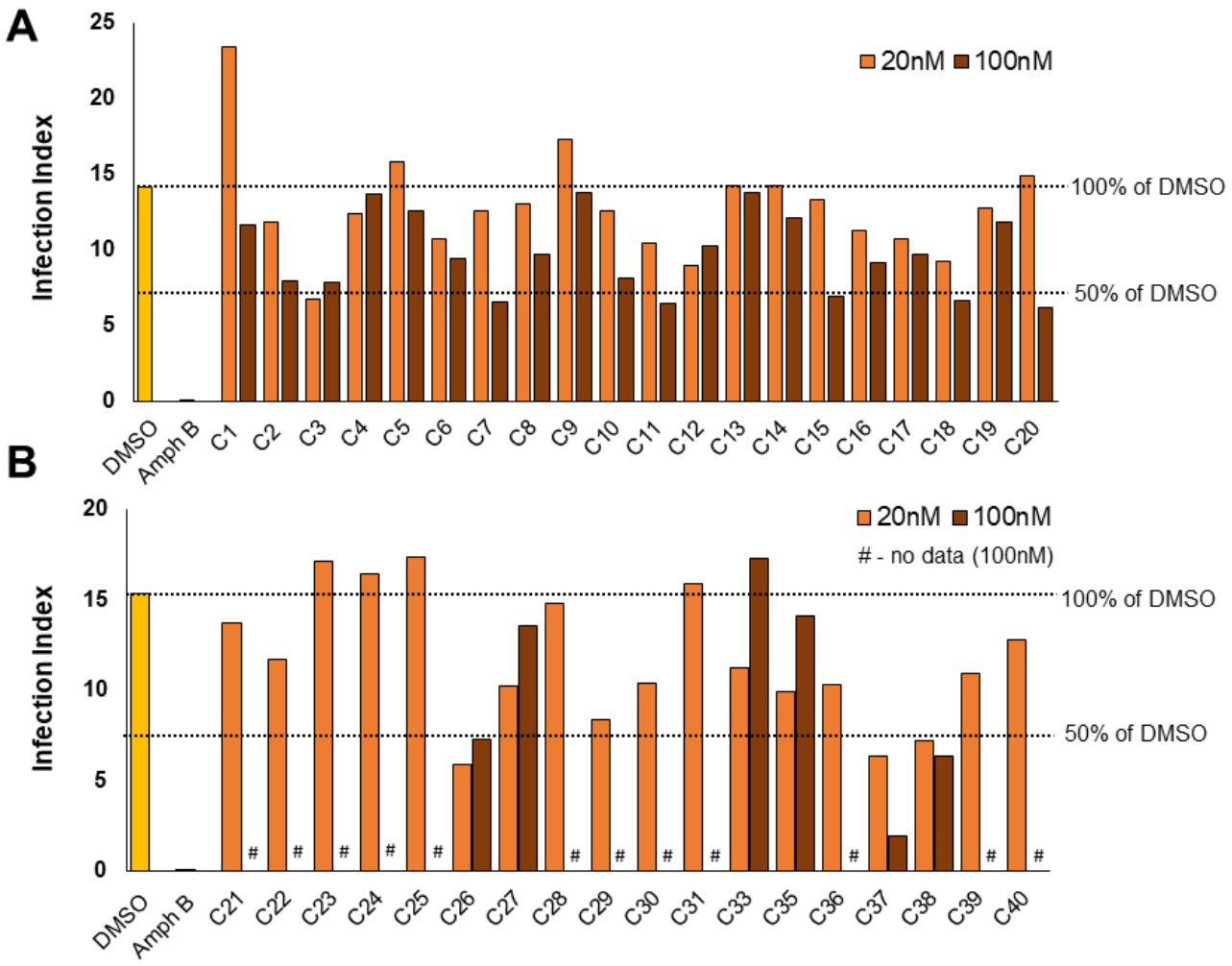


Figure 4.1 Infection Index in BMDMs infected with *L. amazonensis* for 96 h and treated with two different concentrations of rocaglates for 72 h.

(A) weak eIF4A1 clampers and (B) strong eIF4A1 clampers**.** Dotted lines represent 100% and 50% of the DMSO control, respectively. Data are representative of one biological replicate, performed in technical duplicates, using Amph. B-treated cells as a control for parasite killing. # represents the lack of data for 100nM concentrations when tested rocaglates were toxic to BMDMs at this concentration.

For the weak clampers (i.e., C1 to C20), it is possible to see that a reduction of approximately 50% was obtained at the 100 nM concentration for compounds 3, 7, 11, 18, and 20 (Figure 4.1A). With compound 3, a similar reduction was also observed at the 20 nM concentration, indicating that the lowest concentration tested was enough to obtain the observed effect.

As for the strong clampers (i.e., C21 to C40), with compounds 26, 37, and 38 we could observe a 50% reduction at the 20 nM concentration. Additionally, except for compounds 26, 27, 33, 35, 37, and 38, there is no values for the 100 nM concentration (Figure 4.1B). That is because,

during the experiment, we identified that the 100 nM was toxic to BMDMs in most cases, and for C32 and C34, the 20 nM concentration was also toxic, which is why no data for these two compounds is shown in the graph. This evaluation was done based on cell morphology, analyzing cells under a light microscope. In contrast to healthy, DMSO-treated cells, cells treated with 100 nM of toxic compounds were round and detached from the culture plate (Figure 4.2). For that reason, these cells could not be counted and therefore we concluded that these concentrations were toxic to BMDMs. These data were aligned with our knowledge of how strong and weak clampers behaved regarding the host cell. It is logic that, in the same concentration that strong clampers appear to kill BMDMs, weak clampers will not necessarily have that effect.

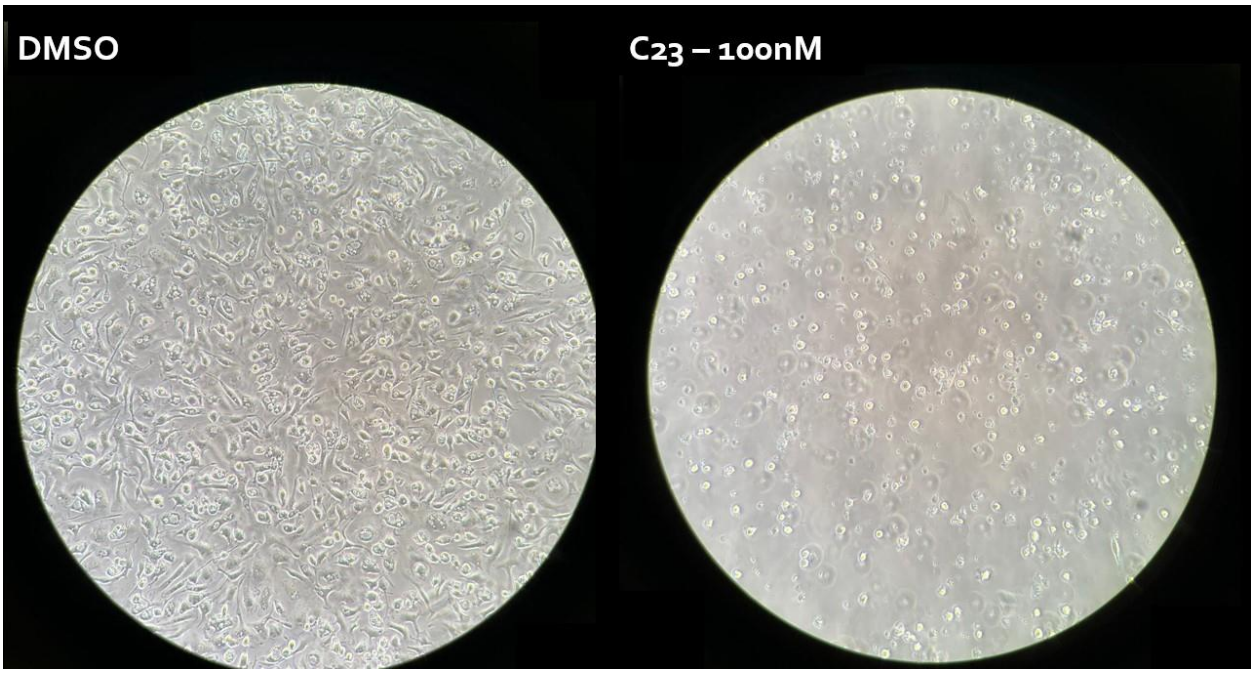


Figure 4.2 Pilot toxicity assessment using light microscopy to evaluate host cell morphology upon rocaglate treatment.

Light microscopy (40X) images of BMDM cultures treated with (A) DMSO and (B) C23 after 72 h. In comparison to healthy cells in the DMSO control, cells treated with C23 are detached and present a round shape. In this case, we assumed 100 nM was toxic for BMDMs in this time point and this compound in this concentration was discarded.

4.1.1 Selection criteria

For strong clampers (Figure 4.1B), compounds that were not toxic to macrophages at 100 nM (based on microscope observation of cell morphology) and/or that were able to reduce the infection index 72 h post-treatment by approximately 50% or more. For weak clampers (Figure 4.1A), given that none of these compounds were toxic to the cells, compounds that were able to reduce the infection index 72 h post-treatment by approximately 50% or more.

These criteria allowed us to move forward with compounds C3, C7, C11, C18, and C20 from the weak clamper set, and C26, C29, C37, and C38 from the strong clamper set. In conclusion, our first screening allowed us to narrow down from 40 to 9 rocaglates, a much more feasible number of compounds to further characterize.

4.2 Resazurin-based viability assays show that *Leishmania amazonensis* promastigotes are resistant to rocaglate treatment

Next, we sought to assess the effect of compounds selected in our pilot screening on extracellular promastigote *Leishmania* cultures. We wanted to identify whether rocaglates could also have a direct detrimental effect on the parasite, given that it presents its own translation machinery, as described in the introduction (1.3.6). For that, we used a resazurin-based method, as described in the Materials and Methods section 3.4. Parasites were treated for 72h with concentrations ranging from 320 nM to 2.5 nM for strong clampers, and 10 uM to 1.5 uM for weak clampers. As positive controls, we used promastigotes cultured in medium (M199) without any treatment. Parasite cultures incubated with 50% DMSO (Dead Cells – DC), were used as our control for cell death.

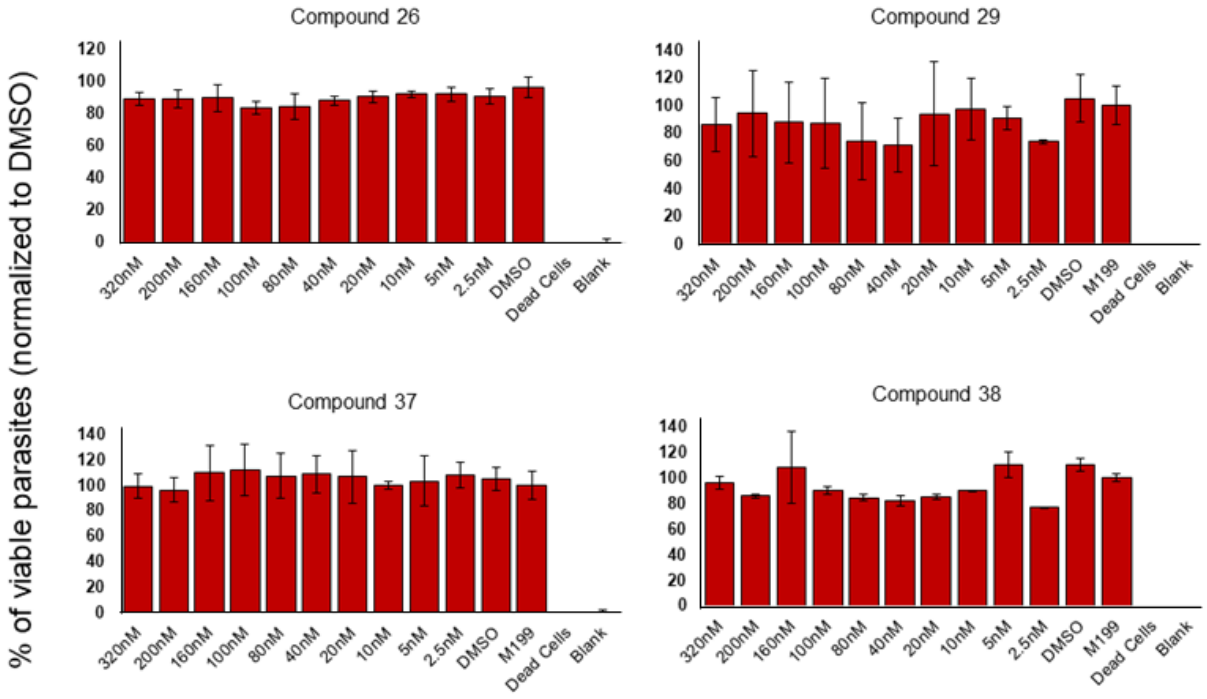


Figure 4.3 No anti-leishmanial effect of strong clamper rocaglates was observed for *L. amazonensis* promastigotes.

Data for compounds 26, 29, 37 and 38 in a concentration range between 320 and 2.5 nM is shown. DMSO-treated cells (vehicle) were used as the normalizer group. M199 (untreated) cells and Dead Cells (DC) were used as negative

and positive controls for non-viable cells, respectively. Data are representative of one biological replicate, carried out in technical triplicates.

We observed that none of the strong clampers at the tested concentrations had any effect on promastigotes of *L. amazonensis* after 72 h of treatment. Next, we assessed whether weak clampers could have any effect on this stage of the parasite, using the same method and experimental design described above.

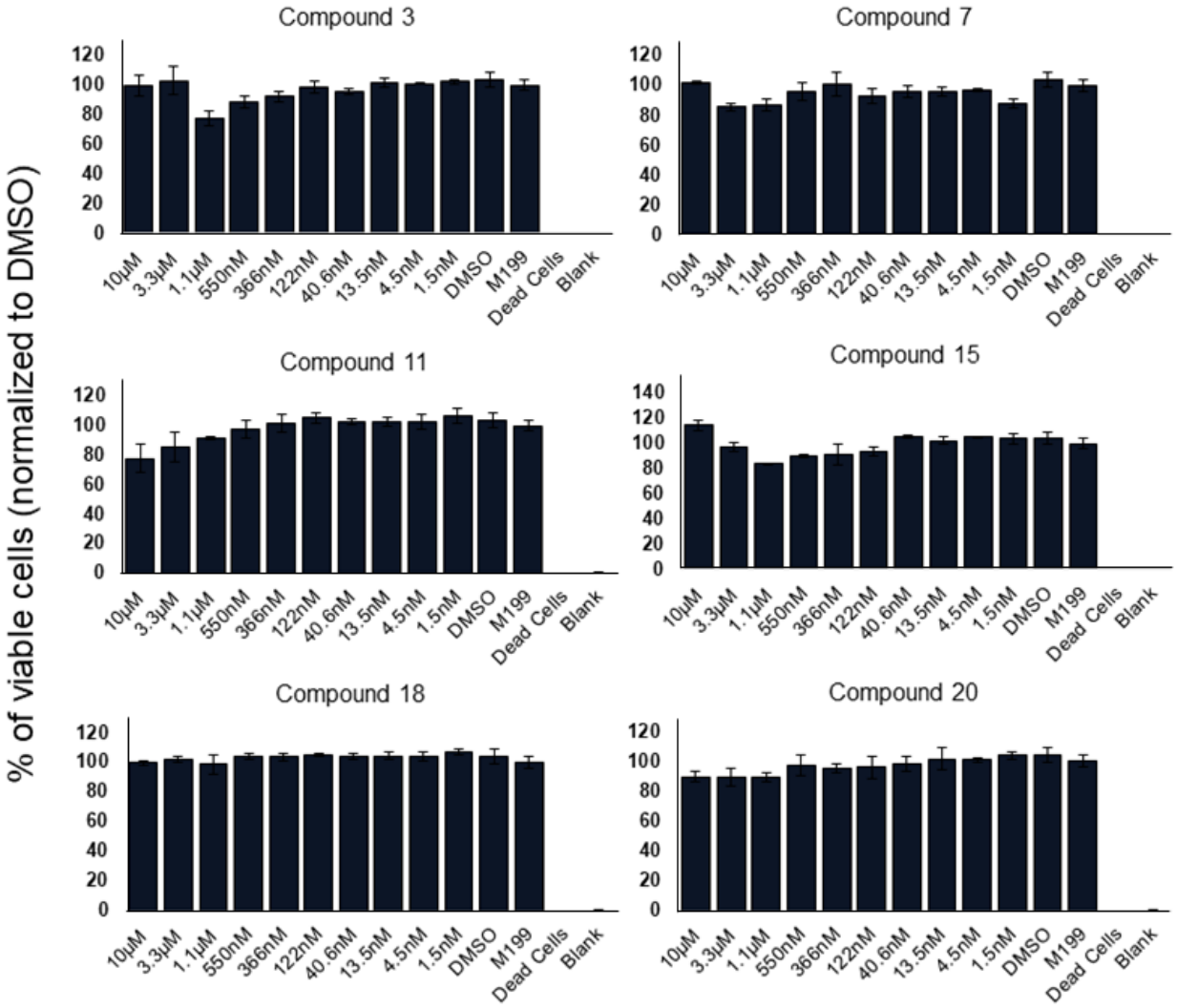


Figure 4.4 No anti-leishmanial effect of weak clamper rocaglates was observed for *L. amazonensis* promastigotes.

Data for compounds 3, 7, 11, 15, 18, and 20 in a concentration range between 10 µM and 1.5 nM is shown. DMSO-treated cells (vehicle) were used as the normalizer group. M199 (untreated) cells and Dead Cells (DC) were used as negative and positive controls for non-viable cells, respectively. Data are representative of one biological replicate, carried out in technical triplicates.

Once again, we could observe that none of the selected weak clamper rocaglates affected the metabolism of *Leishmania amazonensis* promastigotes, even at the highest tested concentrations (Figure 4.4). These results suggest that the subset of rocaglates tested do not target *Leishmania amazonensis* LV79 promastigotes. It was still unclear whether the treatment could affect the amastigote stage of the parasite, or if the previously observed anti-leishmanial effect of rocaglates in infected BMDMs was exclusively due to host-targeting effects.

4.3 Resazurin-based viability assays reveal the range of rocaglate concentrations tolerated by uninfected BMDMs

We then proceeded to evaluate the toxicity profile of the rocaglates selected in 4.1 in BMDMs. Cells were treated with increasing concentrations of the compounds (from 320 nM to 2.5 nM for strong clampers, and from 10 µM to 1.5 nM for weak clampers), and after 72 h, we evaluated the toxicity profile (Figures 4.6 and 4.7).

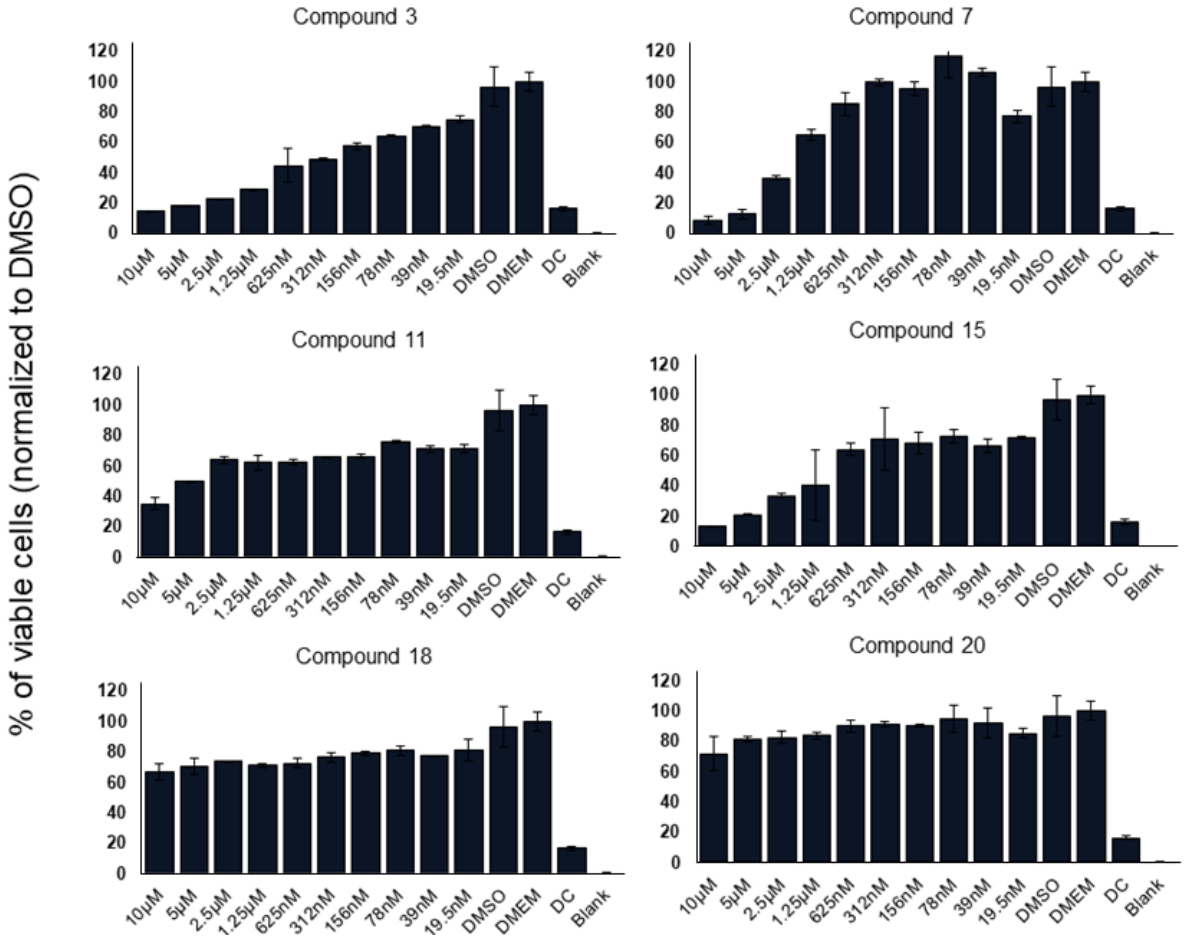


Figure 4.5 Toxicity profile of selected weak clamper rocaglates to uninfected BMDMs (resazurin-based assay). Data for compounds 3, 7, 11, 15, 18, and 20 in a concentration range between 10 µM and 1.5 nM is shown. DMSO-treated cells (vehicle) were used as the normalizer group; DMEM (untreated) cells and Dead Cells (DC) were used as

negative and positive controls for non-viable cells, respectively. Data are representative of one biological replicate carried out in technical triplicates.

For the first six compounds (weak clampers), we observed that compounds 3, 7, 11 and 15, can be toxic to BMDMs at high concentrations on the micromolar range, and compounds 18 and 20, never affected the viability of more than 40% of cells in the concentrations tested. Next, we evaluated the effect of strong clampers on BMDMs (Figure 4.6).

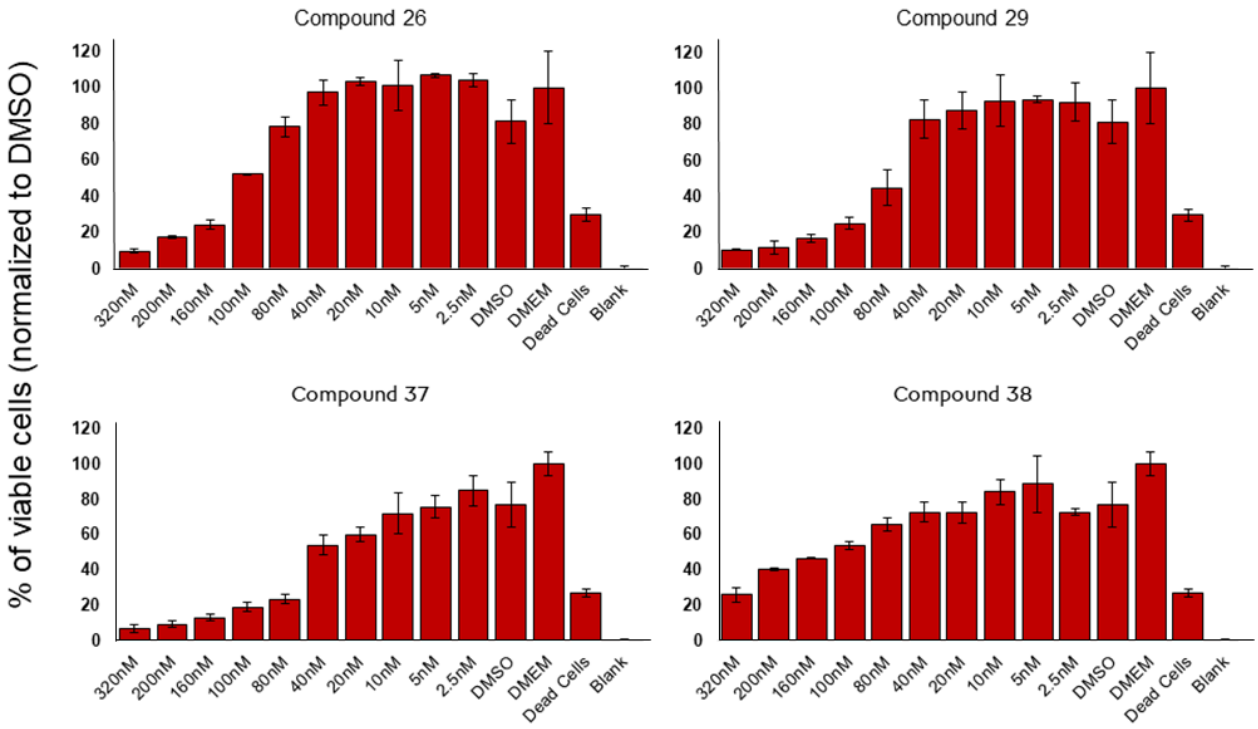


Figure 4.6 Toxicity of strong clampers to BMDMs after 72 h of treatment (resazurin-based assay).
Data for compounds 26, 29, 37 and 38 in a concentration range between 320 and 2.5 nM is shown DMSO-treated cells (vehicle) were used as the normalizer group. DMEM (untreated) cells and Dead Cells (DC) were used as negative and positive controls for non-viable cells, respectively. Data are representative of one biological replicate carried out in technical triplicates.

We observed that strong clampers had a much clearer toxic effect on BMDMs at the concentrations tested. A clear dose-response curve is visible for these compounds, with decreased viability as rocaglate concentration increases (Figure 4.6). C37 and C29 appeared to be the most toxic to BMDMs, although all 4 compounds affected +70% of cells in the highest concentration (320 nM).

Revisiting the concentrations used for the first screening (20nM and 100nM), we can observe that even though we didn't identify by cell morphology, these compounds were toxic to

the hosts cells at 100nM. At 20nM, all four strong clampers showcase a viability of 60% or more. This highlights the importance of assessing cell toxicity with a method other than by analyzing cell morphology, in order to identify compounds that are toxic to the host cells without affecting the parameter we could analyse during the first screening.

With these data, we were able to generate the CC_{50s} of these compounds (Table 4.1). It is interesting to observe that CC_{50s} for strong clampers are much lower than those for the weak clampers. In this sense, we could correlate the clamping promoted by the rocaglate with how toxic they were to the cell. Additionally, with compounds 18 and 20 we were unable to generate the CC_{50}, due to the low toxic effect these had on BMDMs after 72 h of treatment (Figure 4.5).

Compound	CC_{50}
C3	523.7nM
C7	1.4µM
C11	3.6µM
C15	1.5µM
C18	> 320nM
C20	> 320nM
C26	100.5nM
C37	46.82nM
C38	178.3nM

Table 4.1 Calculated half maximal cytotoxic concentration (CC_{50}) of the selected rocaglates in BMDMs after 72h of treatment.

Since the goal of the project was to assess the effect of eIF4A-dependent translation on *Leishmania* infection, we chose to move forward only with the 3 compounds selected from the strong clamper set (C26, C37 and C38), as these were known inhibitors of eIF4A-dependent mRNA translation. We kept two weak clampers (C3 and C18) in our experiments as controls, and the effect of weak clampers in *Leishmania* infection then became the focus of another master's project in the laboratory.

Once the CC_{50s} had been assessed, and we had the toxicity profile of these compounds in BMDMs, it was time to choose which concentrations were going to be used moving forward in the experiments. We decided to choose concentrations that, based on our toxicity assays, accounted for approximately 70% of viable cells, in addition to maintaining concentrations on the nanomolar range (Table 4.2).

Compound Number	Selected concentration
3	100 nM
18	500 nM
26	80 nM
37	10 nM
38	80 nM

Table 4.2 Rocaglate concentrations selected for further experiments after toxicity assays in BMDMs

4.4 Selected rocaglates can affect the amastigote stage of *Leishmania*, even though they do not seem to affect extracellular promastigotes

We had already observed that rocaglates could not target promastigote forms of *Leishmania*. Next, we wanted to understand whether rocaglates could target the amastigote stage of the parasite. For that, we performed what we called a reinfection experiment: BMDMs were infected and treated with selected rocaglates as described in the methodology section (3.5). After 72 h of treatment, we extracted the amastigotes from infected macrophages and used these parasites to infect new, untreated BMDMs. We then monitored whether the previous rocaglate treatment had affected their ability to replicate in non-treated cells (Figure 4.7).

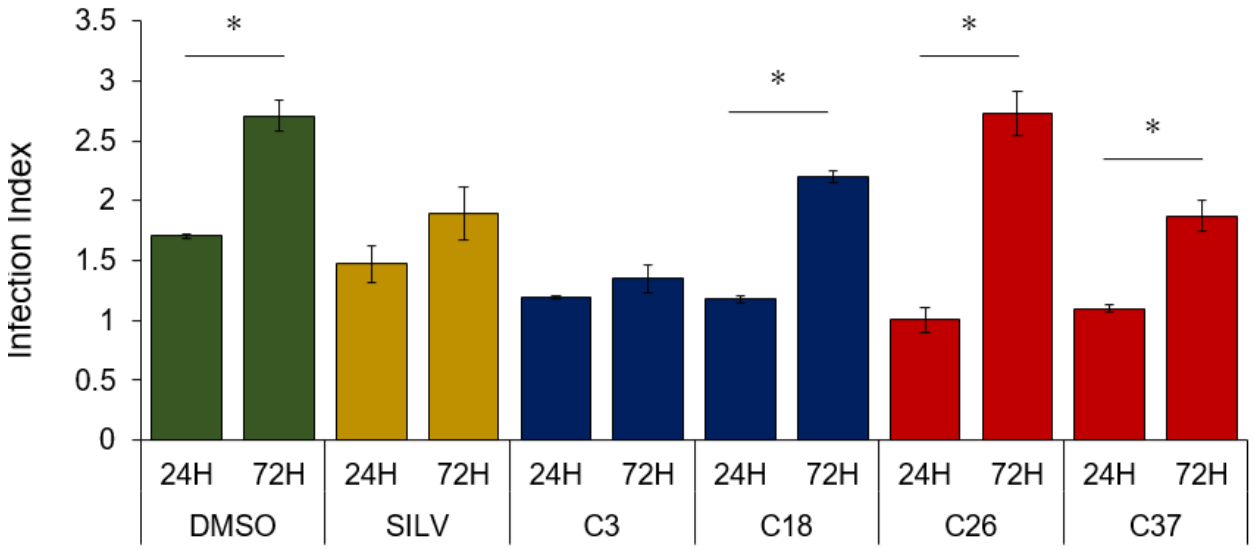


Figure 4.7 A previous C3 (weak clamper) treatment can affect amastigotes ability to replicate in new, untreated BMDMs.

Data is presented comparing 24h and 72h post-infection time points for the different treatment conditions. Data from two independent experiments performed in technical duplicates (* = $p < 0.05$, t- test).

We observed that, between 24 h and 72 h hours post-reinfection, parasites extracted from BMDMs previously treated with DMSO, C18, C26, or C37 were able to replicate normally in untreated cells. However, with C3, we observed these parasites were unable to replicate and increase the infection index. This suggests that compound 3 specifically could be targeting the amastigote stage of the parasite and affecting its fitness even at a later infection, in non-treated cells. A similar effect was observed with Silvestrol treatment.

After 96h of treatment, it is unlikely that rocaglates are still present in treated cells when the amastigote extraction was performed. Additionally, extracted amastigotes were washed 2x with PBS before used to infect of the new, untreated BMDMs, to avoid any carryover from the original infection in treated BMDMs. Based on these precautions taken when designing the experiment, we can assume the effect observed here is indeed due to a potential effect of rocaglates in amastigotes, although this remains to be confirmed.

4.5 Selected rocaglates reduce the infection index in BMDMs and THP-1-derived macrophages infected with *L. amazonensis*

After narrowing down these compounds from the rocaglate panel, we needed to verify that the observed results of our pilot screening could be validated. For that, using the same

experimental design and treatment protocol, we assessed parasite replication in BMDMs, and THP-1-derived macrophages treated with selected rocaglates.

Before analyzing the effect of rocaglates in infected THP-1-derived macrophages, we assessed the toxicity profile of one weak clamper (C3) and one strong clamper (C37) in this cell type (Figure 4.8). We could observe that these cells are more resistant to rocaglates in comparison to similar concentrations in BMDMs. However, we were able to observe a similar anti-leishmania effect using similar concentrations as the ones used for BMDMs (Figure 4.9B).

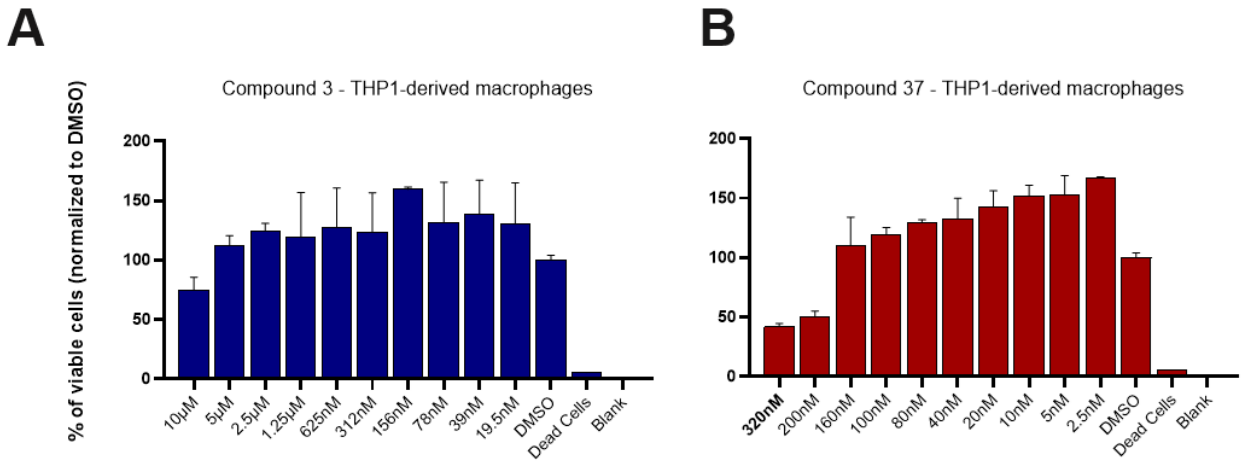


Figure 4.8 THP-1-derived macrophages appear to be less susceptible than BMDMs to rocaglate treatment.
Resazurin-based assay showcases the reduced susceptibility of THP-1-derived macrophages to high concentrations of (A) C3 and (B) C37. Data was obtained from one experiment performed in technical triplicates.

Once the toxicity in THP-1-derived macrophages had been assessed, we proceeded to evaluate the effect of selected rocaglates in infected BMDMs and THP-1-derived macrophages over three independent biological replicates (Figure 4.9). Overall, and in accordance to previous reports (Baek *et al.*, 2020), the infection index observed for THP1-derived macrophages was slightly lower compared to what had been observed for BMDMs infected with *L. amazonensis.*

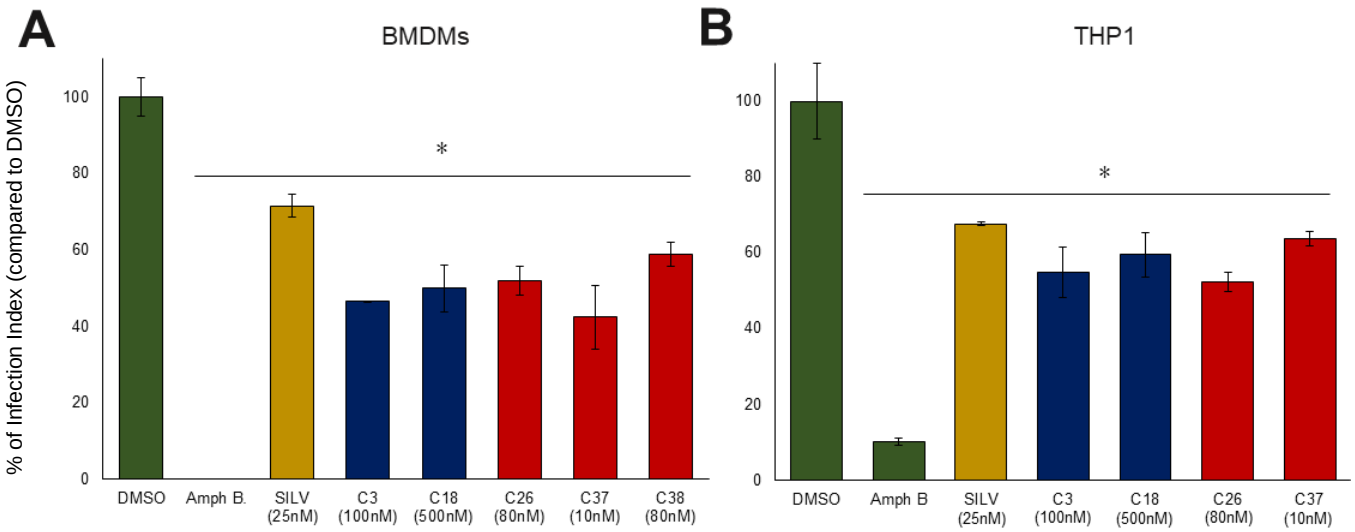


Figure 4.9 Selected rocaglates reduce the infection index of murine and human infected macrophages.
Representation of the infection index reduction in (A) BMDMs, over three independent experiments experiments (* - $p < 0.05$, t-test) and (B) THP-1-derived macrophages, data obtained from two independent experiments (* - $p < 0.05$, one-way ANOVA test).

All selected rocaglates reduced the infection index by approximately 50% in BMDM (Figure 4.9A) and THP-1 (Figure 4.9B), as previously observed in our pilot screening for BMDM. This validated our initial findings and allowed us to establish that the decrease in the infection index upon rocaglate treatment is statistically significant.

We observed that C37 treatment led to the biggest reduction in the infection index in BMDMs (Figure 4.9A). Due to that, and because of the very similar chemical structure it shared with C38 (data not disclosed for confidentiality purposes), we chose to only move forward with C37 out of the two and tested all the other ones in THP-1-derived macrophages. We observed that rocaglate treatment with the same concentrations used for BMDMs was also able to significantly reduce the infection index in these human cells (Figure 4.9B).

Because it had the best effect in reducing the infection index in BMDMs, C37 was the selected compound to understand the kinetics of rocaglate treatment during infection with *L. amazonensis*. After infecting and treating BMDM cultures as outlined above, we collected samples to analyze the infection index after 6, 12, 24, 48 and 72 * post-treatn * (Figure 4.10). We observed that while parasites replicated in DMSO-treated cells, the infection index remains the same all through the course of infection in C37-treated cells. These data suggest that C37-treatment promotes stalling of the parasite replication rather than parasite killing.

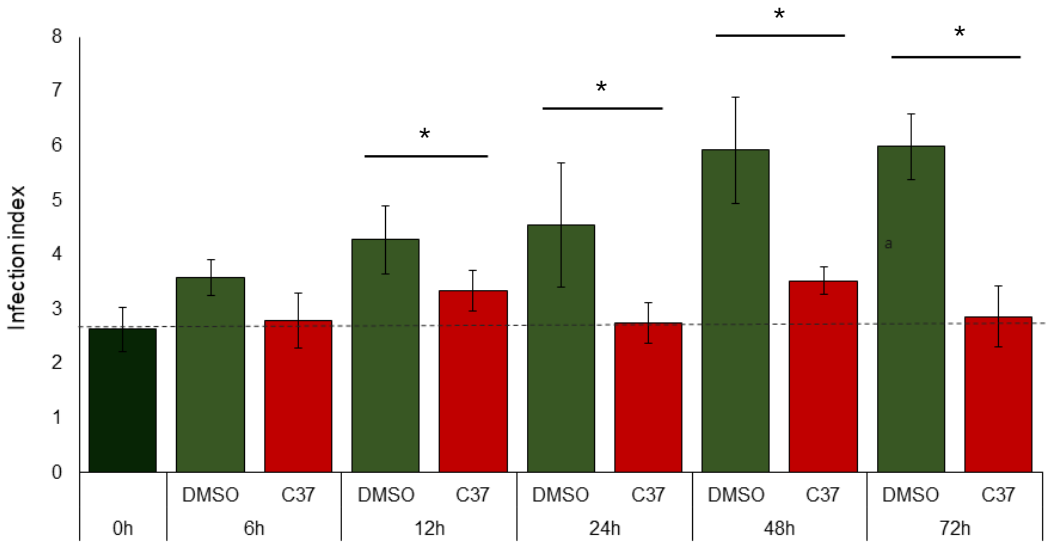


Figure 4.10 Kinetics of rocaglate C37 (10 nM) treatment indicates stalling of parasite replication.

* = p > 0.05, t-test. Data obtained from three independent experiments, performed in technical duplicates.

4.6 The effect of selected strong clamper rocaglates in *Leishmania* infection is dependent on host eIF4A1

The infection and rocaglate treatment of BMDMs obtained from EIF4A $^{+/-}$ mice revealed that the anti-leishmanial effect of rocaglates is dependent on host eIF4A1, but not on eIF4A2 (Figure 4.11). Toxicity assays were not performed with these cells, due to their limited availability for our experiments. Having evaluated that in both wild type BMDMs and THP-1-derived macrophages the chosen concentrations were both tolerated and sufficient to elicit an anti-parasitic effect during *L. amazonensis* infection, we proceeded with the same concentrations for these experiments with BMDMs obtained from the genetically modified mice.

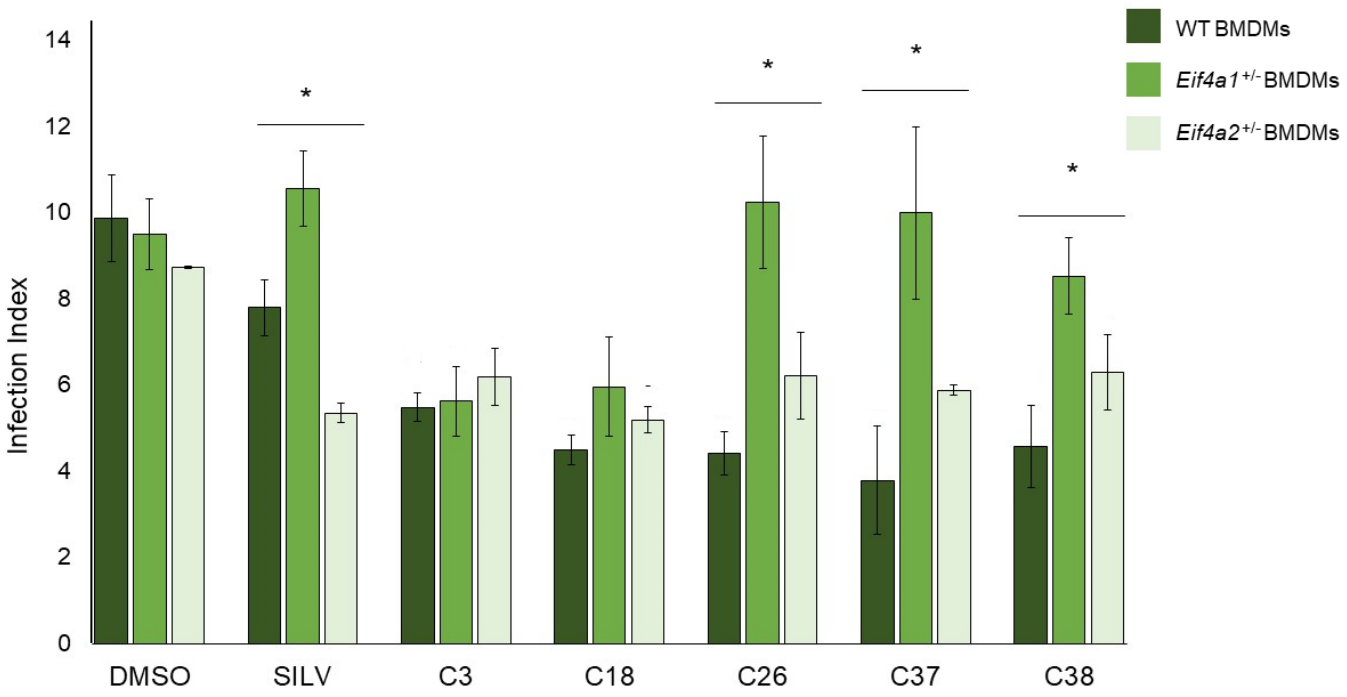


Figure 4.11 eIF4A1-dependency of selected strong clamper rocaglates during *L. amazonensis* infection of BMDMs.

* - $p < 0.05$ (ANOVA), comparison within BMDM genotypes of the same treatment group. Data based on at least two independent biological replicates, performed in technical duplicates.

We observed that the anti-parasitic effect of strong clampers (C26, C37, C38) was lost when host cells are eIF4A1 deficient (*Eif4a1*$^{+/-}$*).* This indicates that eIF4A1 is involved in the anti-leishmanial mechanism promoted by strong clampers in BMDMs. For the weak clampers tested (C3 and C18), the anti-leishmanial effect was observed in all three BMDM genotypes, indicating that this effect is eIF4A1- and eIF4A2-independent. For weak clampers, we hypothesize the effect is due to one or several other host and/or parasite factors to be unveiled. Finally, rocaglate treatment in *Eif4a2* $^{+/-}$ BMDMs showed a similar effect as the one observed in WT BMDMs. This is in accordance to reports that eIF4A2 is less expressed and therefore less relevant for phenotypes in the cell (Galicia-Vazquez *et al.*, 2012; Nielsen *et al.*, 1988; Senechal *et al.*, 2021).

4.7 Selected strong and weak clamper rocaglates have different effects on global translation of immortalized BMDMs

To assess whether selected rocaglates can exert an effect in global translation of cells, we treated iBMDMs with C3, C18, C26, and C37 for 2 h and assessed their polysome tracing profiles. For the first experiment we also utilized Silvestrol as a control for rocaglate-dependent translation inhibition. We observed the differential effect strong and weak clamper rocaglates can have on

global translation of iBMDMs (Figure 4.12). By analyzing the trends in the curves representing the monosomal (80S) peak and the polysomal region of the curve, we observed that treatment with C37 and C26 resulted in a strong translation inhibition at 10 nM and 80 nM, respectively. This is evidenced by the concomitant increase in the monosomal (80S) peak and decrease in the polysomal region of the curve, a typical translation inhibition pattern observed using the polysome tracing method. As a weak clamper, C3 showed a similar inhibition of global translation at a higher concentration (100 nM), and C18 did not show any translational effect, even at the high concentration used (500 nM). This hints at the differential anti-parasitic mechanism these classes of rocaglates could have in infected BMDMs and goes in accordance with our data showing that the anti-parasitic effect of strong clampers was dependent on eIF4A1.

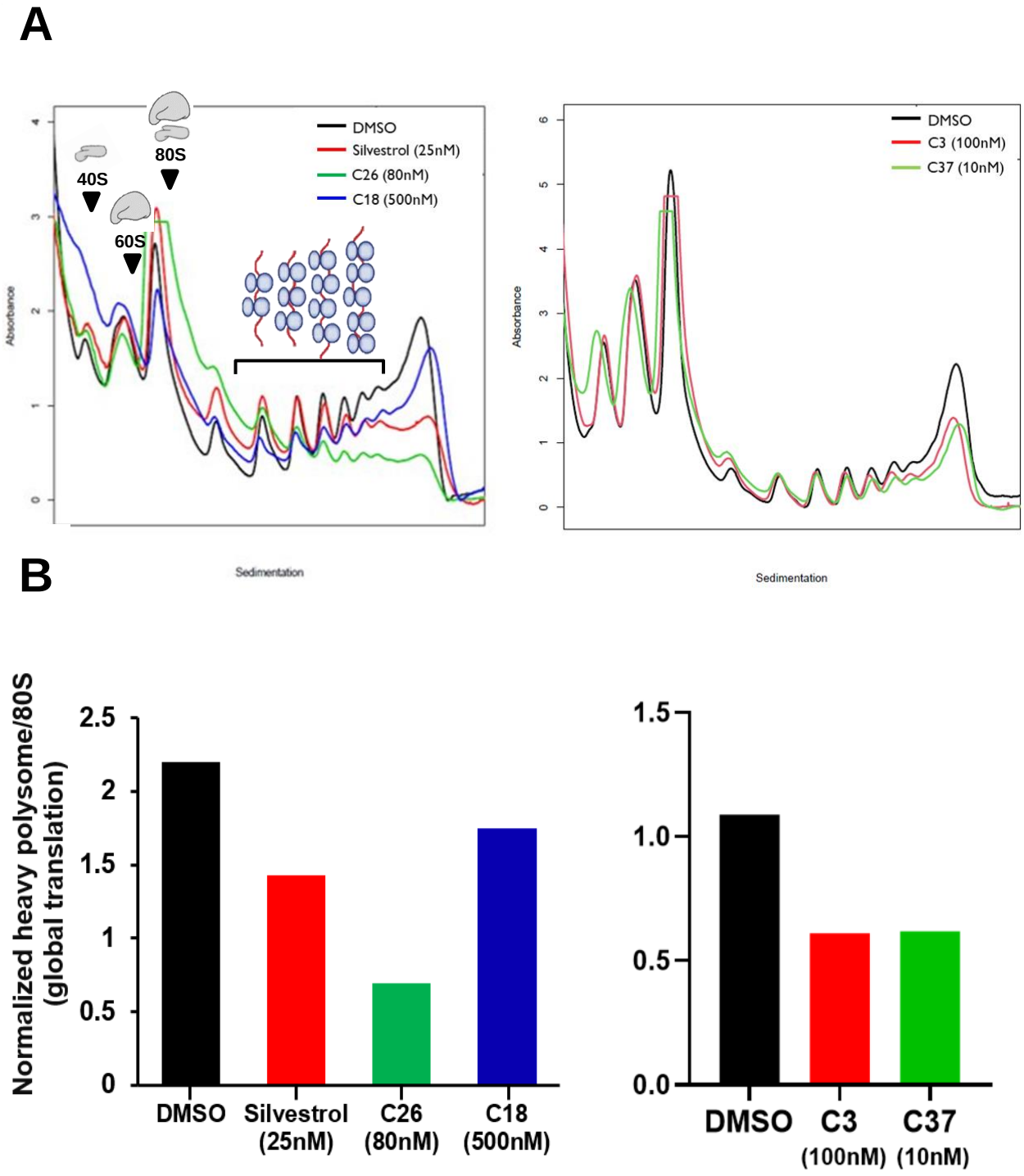


Figure 4.12 Polysome tracings of iBMDMs treated with selected rocaglates for 2 h

(A) Tracings represent different stages of ribosome assembly (40S, 60S, 80S), followed by the polysomal region, where each peak represents mRNAs bound to two or more ribosomes. Each figure represents a different experiment. Inhibition of translation was observed with C37 and C26 in two different experiments. C3 and C18 were tested once each, as represented. (B) Ratio of the area below the curve (Polysomal region/80S) for both experiments represented in (A), as a measure of global translation upon rocaglate treatment.

The observed changes in the polysome tracing of cells treated with rocaglates allows us to preliminarily conclude that there is an effect in translation initiation of iBMDMs treated with the tested rocaglates. The decrease in the polysomal region of the curve suggests that less ribosomes are being assembled to translate the mRNAs of the cells. This method is not capable of resolving whether there are changes in translation rate that could also influence the number of ribosomes attached to an mRNA molecule. Putting together our data with 1) the fact that initiation is the main limiting process in translation regulation, 2) that eIF4A1 is an initiation factor, and 3) that our strong clampers are known to bind eIF4A1, we can assume translation initiation is being affected by the treatment with C3, C26 and C37.

5 DISCUSSION

In this project, we were able to characterize the potential therapeutic effects of synthetic rocaglates during *Leishmania* infection *in vitro*. Our obtained data raised new questions that will be addressed and further developed in this section.

5.1 Promastigote parasites are resistant to rocaglate treatment.

L. amazonensis eIF4A homologue (LeIF4A) *in silico* analysis revealed an amino acid pattern associated with rocaglate-resistance (Obermann *et al.*, 2023). This goes in accordance with our results that showed no effect of any of the tested rocaglates in promastigote cultures (Figure 4.4). It also supports our finding that, for strong clamper rocaglates, anti-parasitic effect is dependent on host eIF4A1 (Figure 4.11). These, however, cannot exclude that rocaglates, notably weak clampers, could target other proteins in the parasite, which is why we assessed amastigote fitness post-treatment in untreated BMDMs (Figure 4.7). These experiments indicate that C3 appears to influence amastigote replication.

5.2 Understanding the peculiarities of C3

Among selected rocaglates, C3 stands out for being the only one affecting amastigote replication in untreated macrophages (Figure 4.7), in addition to being independent on host eIF4A1 or eIF4A2 to reduce the infection index of BMDMs infected with *L. amazonensis* (Figure 4.11). It remains uncertain whether this effect is due to the targeting of other helicases in the host or a stage-specific parasitic protein, or both. Rocaglates have been described to target RNA helicases other than eIF4A, notably DDX3 (Chen *et al.*, 2021; DiVita *et al.*, 2021). Fluorescence polarization (FP) experiments were performed by our collaborator Dr. Regina Cencic (Dr. Jerry Pelletier and Dr. Sidong Hoang laboratories, McGill University, Montréal, QC, Canada) showing that, Compound 3 binds to recombinant human eIF4A1 *in vitro* (cell free) but does not interact with recombinant human DDX3X (Figure 5.1). Many other DEAD/DEAH-box RNA helicases have biological relevance in different pathologies and should also be investigated for rocaglate interaction (Andrisani *et al.*, 2022).

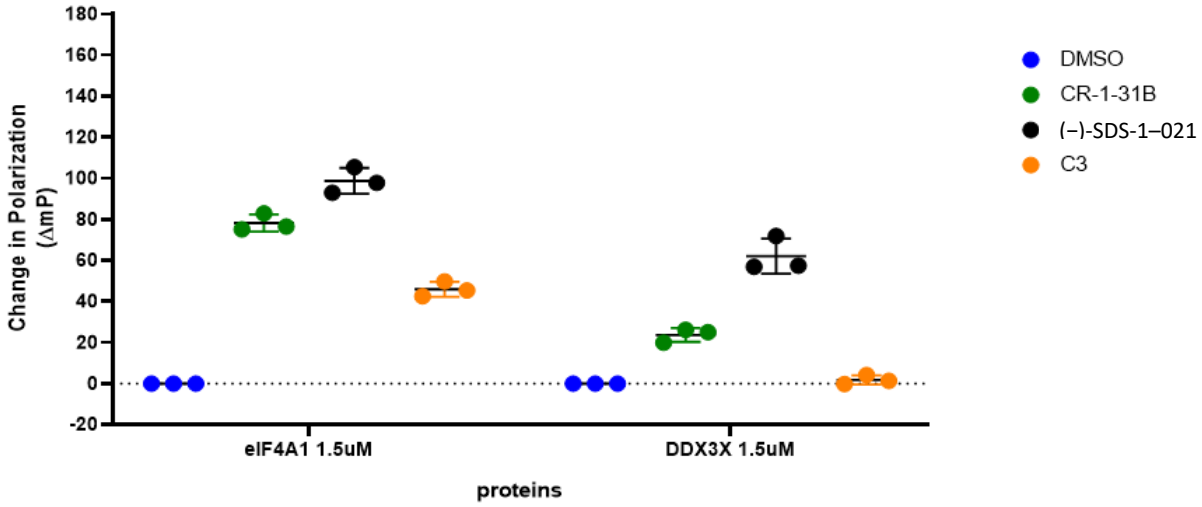


Figure 5.1 Fluorescence polarization assay indicates no interaction of C3 with human DDX3X.
CR-1-31B and (−)-SDS-1–021 were used as positive controls for interacting with both eIF4A1 and DDX3. DMSO was used as the negative control. Data represents one experiment performed in biological triplicates (Cencic R, unpublished data).

In addition to LeIF4A, other RNA helicases have been described in *Leishmania* parasites (Afonso-Lehmann *et al.*, 2015; Dhalia *et al.*, 2005; Marchat *et al.*, 2015; Mokdadi *et al.*, 2021) that could be targeted by rocaglates and might explain the effect observed in our data. Exploring the possibility of C3 targeting an amastigote-specific protein, its expression pattern would have to follow what has been described for LeishDED1-1, a parasitic RNA helicase known to be more expressed on the amastigote stage (Marchat *et al.*, 2015; Zinoviev *et al.*, 2012), since promastigote viability is not affected by concentrations up to 10 μM of C3 (Figure 4.4). Experiments with axenic amastigotes (amastigote forms differentiated and cultured *in vitro*) could be performed to help elucidate if C3 acts on the parasite in a stage-specific manner, but negative results could not exclude the effect of rocaglates in amastigotes, due to the known limitations of the axenic amastigote model, and difference compared to amastigotes obtained from PVs in infected host-cells (Dias-Lopes *et al.*, 2021).

Although C3 is a weak eIF4A1 clamper, FP experiments showed its ability to interact with host eIF4A1 and our polysome tracing data in iBMDMs revealed C3 inhibiting global translation at the concentration used for our experiments (Figure 4.12). This information put together with the independency of eIF4A1 for the anti-leishmanial effect hints that C3 could be interacting with some other target in the host, affecting translation and leishmania persistence in an eIF4A-independent manner. However, it is still not clear how or why C3 can differently modulate BMDMs in relation to other rocaglates (notably strong clampers, whose effect is 100% dependent on host

eIF4A1) to permanently damage the parasite's ability to replicate. Another proposed experiment is to understand whether this effect on the amastigote replication is maintained after several reinfections of untreated BMDMs, or if parasites can eventually recover.

5.3 Elucidating the anti-leishmanial mechanism elicited by rocaglate treatment in BMDMs

It has been previously shown that rocaglates can have a synergistic effect with IFN-γ in promoting the expression of IFN-inducible genes (Bhattacharya *et al.*, 2016). Contrastingly, in BMDMs, it was shown that rocaglate CR-1-31B could block IFN-γ-induced inflammatory transcriptional factor IRF1, in addition to inflammatory proteins CXCL10 and GBP2 (Langlais *et al.*, 2018). As the main anti-leishmanial mechanism of the infected macrophage, we assessed whether nitric oxide production was increased upon rocaglate treatment, but we found no differences in NO levels upon treatment of infected and uninfected BMDMs and THP-1-derived macrophages (data not shown). The lack of an increase in the main anti-parasitic mechanism of the macrophage is in accordance with our data showing that C37 promote stalling of the replication, instead of parasite killing (Figure 4.10), and with the report of anti-inflammatory effect of rocaglates in BMDMs (Langlais *et al.*, 2018). NO production is a known hallmark for M1 macrophage polarization (Funes *et al.*, 2018; Zhang *et al.*, 2008). Despite this negative preliminary result, further experiments are necessary to determine whether rocaglates can induce or block an M1 phenotype in BMDMs infected with *Leishmania*, like monitoring other M1 markers and combining rocaglate treatment with other M1 polarizing stimuli (LPS + IFN-γ treatment).

Evaluating the list of eIF4A-sensitive transcripts found in the translationally regulated dataset of *L. donovani*-infected BMDMs, reported in a previous paper from our group (Chaparro *et al.*, 2020), we can observe different categories of genes, involved in cell signaling, RNA metabolism, structural proteins and proteins involved in cellular trafficking, so we could infer that the alteration in translation of some of these proteins is detrimental to the establishment of *Leishmania* in the macrophage.

An observation that could shed a light on the anti-parasitic mechanism of rocaglates was communal vacuole formation upon rocaglate treatment. As described, *L. amazonensis* parasites form communal vacuoles with several parasites within. In all our experiments, however, we could observe that communal vacuole formation was impaired upon treatment with rocaglates (preliminary data - not shown). This could partially explain the reduction in infection index, as the communal vacuole formation is a crucial part of *L. amazonensis* life cycle, and without which

parasite fitness is affected. Additionally, given the effect eIF4A-dependent translation could have on intracellular trafficking and cell signaling, it is possible that rocaglates are interfering with the recruitment of certain proteins to the parasitophorous vacuole and this is affecting the parasites.

Different host proteins are involved in phagosome maturation and parasitophorous vacuole formation during *Leishmania* infection. One possibility is that one of these proteins is regulated by eIF4A-dependent translation, and its absence affects communal vacuole formation and therefore, parasite ability to promote the infection in BMDMs. Further investigation will shed light on this matter.

5.4 The interface between global translation inhibition and host eIF4A dependency

Our polysome tracing data performed in iBMDMs revealed that selected rocaglates could differentially modulate global translation. Correlating this information with our antiparasitic data in infected BMDMs, we can observe that the rocaglate-driven reduction in the infection index is associated with translational inhibition for C3, C26, and C37 but not for C18 (Figure 4.12). The iBMDM model was used for these experiments because the polysome tracing technique requires a certain amount of RNA that was much easier to obtain using these immortalized cells, that replicate in culture. Approximately one mouse per condition would be required for polysome experiments if we decided to use BMDMs. Given iBMDMs are the same cell type, but immortalized, we concluded it was a reasonable compromise. Additionally, we have shown that iBMDMs can be infected and parasites can replicate in a similar way as they do in BMDMs (Figure 5.3), although cells were not infected for the polysome tracing experiments. The iBMDM model is also of our interest because these cells can be genetically manipulated to knockdown different eIF4A paralogs, or to express a form of eIF4A that is resistant to rocaglate treatment and elucidate whether the observed translational effect is indeed eIF4A-dependent. This could be done by analyzing the polysome tracings of rocaglate-resistant iBMDMs treated with selected rocaglates. In case the translation inhibition observed in WT iBMDMs is not maintained upon treatment of rocaglate-resistant iBMDMs, we would be able to infer that the mutated eIF4A1 is indeed responsible for the rocaglate-associated translation inhibition.

Another way to understand whether eIF4A is involved in the observed changes in global translation upon rocaglate treatment is to analyze the distribution of specific mRNAs across different fractions (subpolysomal, light polysomal, and heavy polysomal) using RT-qPCR of fractionated RNA and comparing it with changes in total mRNA. This way we could assess

whether the translation of known eIF4A-dependent mRNAs is being affected by rocaglate treatment, while ensuring that differences in total mRNA are accounted for. This same experiment could be performed using the rocaglate-resistant iBMDM model to further validate the findings.

Our findings showing that rocaglate treatment efficacy seems to be dependent on eIF4A1 and not on eIF4A2 aligns with what is known about the expression pattern and physiological relevance of these two homologues. The possible effect eIF4A3 could have in the context of *Leishmania* infection remains uncovered, although it is known that this isotype is not involved in translational control, but splicing, RNA decay and trafficking, and ribosome biogenesis (Kanellis *et al.*, 2021). It is also known that rocaglates cannot inhibit eIF4A3 activity, and therefore this possibility can be ruled out for this specific project (Zhu *et al.*, 2021)

5.5 Alternative method to assess parasite replication

During the project, we also started developing an alternative method to assess *Leishmania* replication avoiding the caveats of the method used to generate the results presented here. Counting DAPI-stained infection slides using an epifluorescence microscope can be time consuming, subject to biases, and require a lot of manipulation and materials (e.g., each time point required a separate infection). For this alternative method, RFP-expressing *L. amazonensis* PH8 parasites were used, and the increase in fluorescence during infection was assessed as a reference of parasite replication. This way overcomes some of the issues described above since we obtain an unbiased and quantitative fluorescence measure of the same live, infected cells in different time points.

So far, we were able to assess that parasite replication indeed corresponds to an increase in RFP fluorescence in both BMDMs and iBMDMs (Figure 5.3).

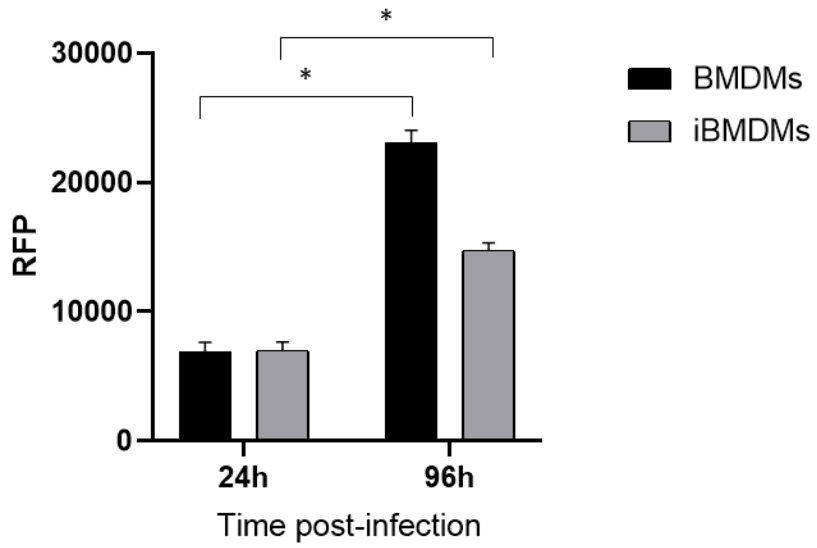


Figure 5.2 Fluorescence-based method to assess *Leishmania* replication in BMDMs and iBMDMs *in vitro*. Data based on two experiments performed in biological triplicates. * - $p < 0.05$, t-test.

5.6 Pre-proteome experiments

Finally, since the strong clamper C37 was our best candidate in reducing the infection index in *L. amazonensis*-infected BMDMs in an eIF4A1-dependent manner, we chose this compound to move forward with for the proteomic experiments. The idea is to perform a dual proteome, gaining insight on both parasite and host proteins during infection, to understand which proteins were targeted by the treatment, and how they are important for parasite persistence, as well as understanding whether rocaglate treatment had any effect in amastigote protein expression. In addition to the experiment that revealed the stalling of parasite replication in BMDMs treated with C37 (Figure 4.10), we were able to establish a dose-response profile of this drug, that showed that the 10 nM concentration was the minimum we needed for the observed effect (Figure 5.4).

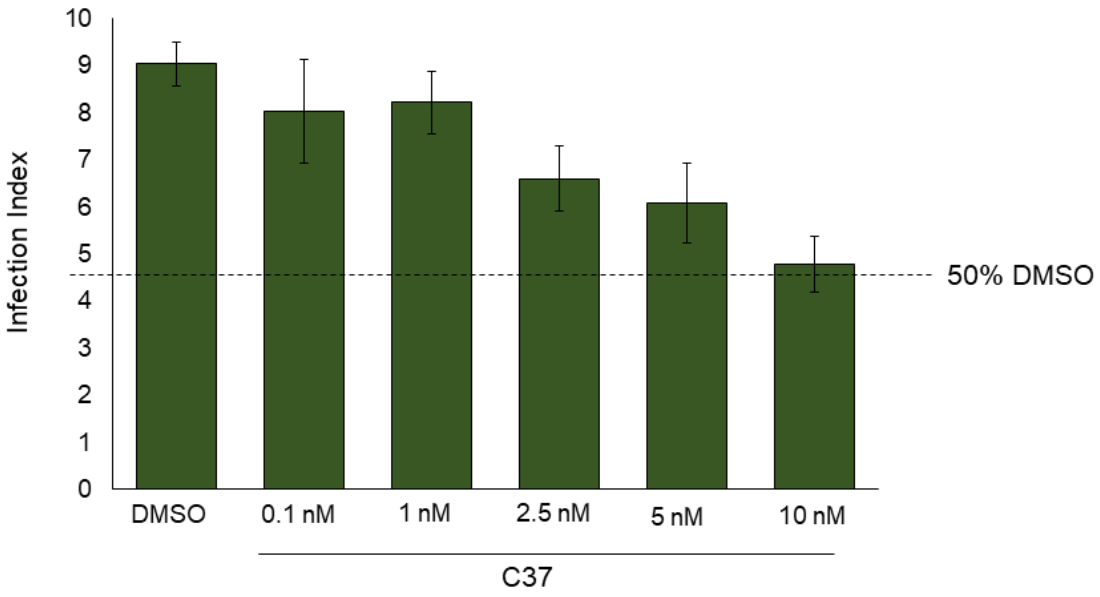


Figure 5.3 Dose-response of C37 treatment in BMDMs infected with *L. amazonensis* after 72 h of treatment.
Lower concentrations of C37 are not able to decrease the infection index in the same way as observed using 10 nM. Data represents two independent experiments performed in technical duplicates.

Additionally, we ran Coomassie gels with protein extracts of infected and treated BMDMs in different time points post-treatment (data not shown), and compared that to uninfected and untreated cells, to try and identify time points with a clear difference in protein pattern, that would have more differentially expressed proteins. However, protein extracts ran in SDS-PAGE gel and stained with Coomassie did not provide enough resolution for us to detect differences in protein content promoted by C37 treatment. Alternatively, we can monitor *de novo* protein synthesis using a puromycin-based assay, to understand which time points are the most affected by rocaglate-driven translational changes. Choosing the best time points for the proteomic experiments is crucial, given that changes in proteome can easily be missed or misinterpreted, depending on the time points analysed (Valdes *et al.*, 2020). The trans inhibitory effects rocaglate can have on translation (Chu *et al.*, 2020) could also act as a confounding factor when analysing changes in protein content upon rocaglate treatment. (Fitzgerald *et al.*, 2009)

6 CONCLUSION

Based on all of our findings and the information provided in the discussion session, the weak clamper C3 appears to be the best candidate for a dual targeting rocaglate, although the exact targets in both the parasite and host are unknown. The strong clamper C37, however, seems to promote an eIF4A-dependent anti-leishmanial response in BMDMs. Both these compounds can affect global translation of iBMDMs. Overall, during the project, we were able to screen a panel of 40 synthetic rocaglates. We were able to characterize that some weak clamper rocaglates had an anti-leishmanial effect independent of host eIF4A1 or eIF4A2, which set the foundation for an entire new Master's project, led my labmate Camila Almedia Cardoso, dedicated to understanding the mechanism of action of this class of weak clampers. With the strong clampers left, we selected C37 for showcasing the biggest anti-leishmanial effect, and identified that it can inhibit global translation of immortalized BMDMs at a low concentration on the nanomolar range, and promotes stalling of *L. amazonensis* replication.

Overall, this project has layed the ground for the use of rocaglates to control *Leishmania* infection *in vitro*, and increased our knowledge of how different classes and subtypes of rocaglates can have an anti-leishmanial effect in BMDMs (mouse model) and THP-1-derived macrophages (human model). We also want to validate the anti-leishmanial effect of rocaglates in primary human macrophages.

The question that remains is which host translational programs are being affected by the use of C37 and why they can impede parasite replication. To answer that question, a transcriptome coupled to a translatome, in addition to a proteome of infected and treated cells will be performed in our laboratory. We also expect to further explore C37 anti-leishmanial potential, by testing the treatment in clinical isolates of chemoresistant *Leishmania* strains and in vivo, using an experimental mouse model of cutaneous leishmaniasis.

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