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EVALUATION OF GENETIC EXCHANGE IN *LEISHMANIA* PARASITES IN AN INFECTED MAMMALIAN HOST

By

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“What is difficult in training will become easy in a battle”
Alexander Vasilyevich Suvorov (1730-1800)

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ABSTRACT

Leishmaniasis is a disease characterized by a spectrum of symptoms ranging from cutaneous lesions to fatal visceral disease caused by *Leishmania*. This pathogen replicates in compartments of the endosomal-lysosomal pathway of phagocytes. Most *Leishmania* species live in individual vacuoles, but species of the *Leishmania mexicana* complex live in communal vacuoles. Current knowledge on *Leishmania* suggests that the genetic and pathogenic diversity of parasites is primarily due to vertical evolutionary pathways rather than horizontal gene exchange processes. Recent findings suggest that parasites are capable of genetic exchanges in sand flies. One potential benefit of occupying a communal vacuole is the proximity of the parasites which increases the possibility of genetic material exchange. This research project aims to determine whether communal vacuoles constitute an environment conducive to genetic exchange between parasites in infected cells. We have generated different strains of *L. mexicana* and *L. amazonensis* expressing distinct resistance markers. We performed co-infections *in vitro* in bone marrow derived macrophages and *in vivo* in C56BL/6 mice, and recovered the parasite populations after 5-8 days and 9 weeks respectively. Potential hybrid parasites, capable of proliferating in the presence of both antibiotics, were recovered from infections *in vitro*, but this was a rare phenomenon and they were less viable than the parental strains. Similar observations were noted for double drug-resistant parasites isolated in axenic cultures. No hybrids have been isolated from *in vivo* infections. In conclusion, genetic exchanges in infected mammalian cells appear to be very rare and generate inefficient and non-viable hybrid strains.

Keywords: Genetic Exchange, Parasite, Host-Pathogen Relationship, Macrophage, Drug Resistance, Intracellular Pathogen

RÉSUMÉ

La leishmaniose est une maladie caractérisée par un spectre de symptômes allant des lésions cutanées à la maladie viscérale mortelle causée par la *Leishmania*. Ce pathogène se réplique dans les compartiments de la voie endosomale-lysosomale des phagocytes. La plupart des espèces de *Leishmania* vivent dans des vacuoles individuelles, mais les espèces du complexe *Leishmania mexicana* vivent dans des vacuoles communautaires. Les connaissances actuelles sur la *Leishmania* suggèrent que la diversité génétique et pathogénique des parasites est principalement due à des voies d'évolution verticales plutôt qu'à des processus d'échange de gènes horizontaux. Des découvertes récentes suggèrent que les parasites sont capables d'échanges génétiques chez les phlébotomes. Un avantage potentiel d'occuper une vacuole communautaire est la proximité des parasites ce qui augmente la possibilité l'échange de matériel génétique. Ce projet de recherche vise à déterminer si les vacuoles communautaires constituent un environnement propice aux échanges génétiques entre parasites dans les cellules infectées. Nous avons généré différentes souches de *L. mexicana* et *L. amazonensis* exprimant des marqueurs de résistance distincts. Nous avons effectué des co-infections *in vitro* dans des macrophages dérivés de la moelle osseuse et *in vivo* chez des souris C56BL/6, et nous avons récupéré les populations de parasites après 5 à 8 jours et 9 semaines respectivement. Des parasites hybrides potentiels, capables de se proliférer en présence des deux antibiotiques, ont été récupérés des infections *in vitro*, mais c'était un phénomène rare et ils étaient moins viables que les souches parentales. Des observations similaires ont été notées pour les parasites doubles résistants isolés des cultures axéniques. Aucun hybride potentiel n'a été isolé à partir des infections *in vivo*. En conclusion, les échanges génétiques dans les cellules de mammifères infectées semblent être très rares et générer des souches hybrides inefficaces et non-viables.

Mots-clés: Échange Génétique, Parasite, Relation Hôte-Pathogène, Macrophage, Résistances aux Drogues, Pathogène Intracellulaire

SYNOPSIS/ SOMMAIRE RÉCAPITULATIF

La leishmaniose est un spectre de maladies à transmission vectorielle causées par le parasite protozoaire intracellulaire obligatoire de l'ordre des Kinetoplastida et de la famille des Trypanosomatidae, connu sous le nom de *Leishmania*, un pathogène vacuolaire qui se réplique dans le compartiment phagolysosomal des phagocytes. La *Leishmania* est endémique dans 98 pays, causant 1 million d'infections et entraînant 20 000 à 30 000 décès par an. Malgré des années d'avancées scientifiques, aucun vaccin efficace et sûr n'est encore disponible. En outre, le traitement actuel est difficile à administrer, coûteux et devient inefficace en raison de la propagation de la résistance aux médicaments. Il existe donc un besoin pressant de développer de nouvelles approches pour prévenir et traiter la leishmaniose. Il existe plus de 20 espèces de *Leishmania* différentes, trouvées à la fois dans le Nouveau monde (Amérique du Nord et du Sud) et dans l'Ancien monde (Europe, Afrique, Asie), qui peuvent provoquer la maladie chez l'homme et qui peuvent être divisées en trois manifestations cliniques majeures: viscérale, cutanée et mucocutanée. Ces manifestations sont déterminées par l'espèce parasitaire infectant l'hôte, mais dans certains cas, elles pourraient également être influencées par la réponse de l'hôte. La leishmaniose viscérale (Kala azar ou «Mort noire») est la forme la plus sévère de la maladie et est mortelle dans 95% des cas si elle n'est pas traitée. Il s'agit d'une forme d'infection chronique qui affecte généralement le foie, la rate, la moelle osseuse et les ganglions lymphatiques de l'individu infecté. Dans certains cas, après le traitement, certains patients peuvent développer une forme cutanée de la maladie connue sous le nom de leishmaniose cutanée post-kala-azar (PKDL), qui se manifeste par une éruption maculaire hypopigmentée ou érythémateuse maculopapuleuse. Ensuite, il y a la forme cutanée de leishmaniose qui est la forme d'infection la plus courante et la plus étudiée. Habituellement, cette forme d'infection entraîne des ulcères localisés ou des nodules cutanés sur la surface exposée (visage, bras, jambes) de l'hôte et peuvent s'auto-guérir dans les 2 à 18 mois suivant l'infection, mais dans de rares cas, des lésions non-traitées peuvent laisser des cicatrices graves et/ou défigurantes. Enfin, dans certains cas, les lésions cutanées peuvent se disséminer en lésions mucocutanées, dans lesquelles les tissus muqueux nasal et oral sont affectés et peuvent toucher le pharynx et le larynx dans une forme plus sévère de la maladie. Si elle n'est pas traitée, la leishmaniose mucocutanée peut entraîner la mort en raison d'une infection secondaire ou d'une malnutrition.

Leishmania a un cycle de vie dimorphique complexe qui implique deux organismes hôtes: le phlébotome et l'hôte mammifère comme l'homme, les rongeurs, les canins et autres mammifères.

L'infection commence lorsqu'un phlébotome régurgite la forme promastigote du parasite dans l'hôte mammifère au cours d'un repas sanguin. De là, le parasite rencontre la cellule phagocytaire cible auquel il se lie via des récepteurs cibles, ce qui conduit à l'internalisation du parasite par phagocytose. Après l'internalisation, il se différencie en amastigotes et se réplique dans les cellules phagocytaires infectées et ce cycle se poursuit dans les cellules infectées de l'hôte mammifère jusqu'à ce qu'un nouveau phlébotome vienne finalement se nourrir sur l'hôte et ingère des amastigotes flottants et des cellules infectées par le parasite avec son repas sanguin. Une fois que la mouche de sables a ingéré son repas sanguin d'un hôte infecté, les cellules infectées et les amastigotes libres se retrouvent dans le tube digestif de la mouche. Les amastigotes se transforment en promastigotes procycliques flagellés. Les promastigotes entreront alors dans une frénésie réplicative pendant les suivantes 24 à 48 heures via fission binaire. Ensuite, les promastigotes procycliques se développeront en une forme de promastigote métacyclique infectieuse à travers une série de transformations cellulaires connues sous le processus nommé métacyclogenèse. Cela termine le cycle de vie une fois que le phlébotome est prêt à prendre le repas sanguin suivant et que les parasites sont introduits dans un autre hôte mammifère.

La phagocytose est un mécanisme cellulaire important pour les cellules phagocytaires du système immunitaire inné qui est utilisé pour absorber des particules ou des cellules entières plus grandes ou égales à 0,5 µm de diamètre. La phagocytose peut être divisée en 4 étapes principales: Reconnaissance des particules, suivie d'une internalisation qui mènera à la formation et à la maturation du phagosome précoce à un phagolysosome et culminant avec l'élimination des particules à l'intérieur le phagolysosome par divers éléments microbicides. Par contre, les parasites *Leishmania* sont capables d'éviter la réponse immunitaire de l'hôte ainsi que de modifier la voie phagocytaire des cellules infectées et de se répliquer sans aucun souci.

En effet, les parasites parviennent de détourner la réponse immunitaire et les différents mécanismes cellulaires microbicides en utilisant une multitude de molécules, appelées facteurs de virulence, pour assurer leur survie au sein de l'hôte. L'un de ces facteurs est la zinc-métalloprotéase GP63, également connue sous le nom de leishmaniolysine, qui est l'une des protéines de surface les plus abondantes et des gènes les plus conservés de *Leishmania*. Elle joue un rôle très important avant l'internalisation du parasite dans les cellules phagocytaires ainsi que dans les premiers stades de survie dans les cellules phagocytaires. La GP63 s'est avérée capable de dégrader des composants de la matrice extracellulaire tels que le collagène de type IV et la fibronectine, ce qui facilite la migration du parasite vers le derme. Elle joue également un rôle dans la perturbation du complexe d'attaque membranaire (MAC) prévenant ainsi la mort

médiée par le complément. De plus, la *Leishmania* utilise ce facteur de virulence spécifique pour modifier les voies de signalisation des cellules hôtes afin de prévenir davantage toute activation inflammatoire et de bien maintenir leur cycle de vie. Mis à part la GP63, un autre facteur de virulence important est le LPG qui arrête la maturation du phagosome en inhibant sa fusion avec les endosomes tardifs et lysosomes. Par conséquent, cela empêche la formation de phagolysosomes et favorise la survie des parasites. Bien que le LPG soit une molécule clé qui assure la survie de parasites tels que *L. donovani* et *L. major* qui se répliquent dans des vacuoles individuelles au sein des cellules hôtes, ce n'est pas vrai pour toutes les espèces de parasite. Dans le cas des espèces du complexe *L. mexicana*, le LPG ne joue pas de rôle dans la survie intracellulaire ou la maturation des phagosomes. En effet, contrairement à d'autres espèces de *Leishmania* qui arrêtent la maturation des phagosomes pour favoriser leur survie au sein de la cellule hôte, les parasites du complexe *L. mexicana* se répliquent dans des vacuoles parasitophores (PV) communes, car cela peut fournir une protection contre les propriétés microbicides du phagosome.

Les divers résultats cliniques associés aux infections avec la *Leishmania* sont généralement clairement associés à des espèces et souches de parasites spécifiques. Cependant, la contribution du génotype du parasite à la maladie reste largement inconnue. Jusqu'à récemment, on pensait que la diversité des espèces de *Leishmania* résultait d'une accumulation progressive de mutations divergentes plutôt que d'une recombinaison génétique. En effet, la présence d'espèces de parasites hybrides dans la nature a été signalée depuis très longtemps ce qui suggère que les échanges génétiques peuvent se produire entre parasites. Il existe des dizaines d'articles décrivant les parasites hybrides *Leishmania* découverts dans le Nouveau monde, l'Ancien monde et même entre deux espèces divergentes. Enfin, il est important de noter que l'hybridation naturelle au niveau intraspécifique de *L. infantum*, *L. donovani* et *L. tropica* a été rapportée via des études de séquençage du génome entier. Outre les études qui démontrent l'existence d'hybrides dans la nature, il existe également des études qui ont révélés la capacité des parasites à échanger du matériel génétique au sein d'un phlébotome infecté. La première étude de ce type a été menée par Akopyants *et al* (Akopyants *et al.*, 2009), où ils ont démontré que deux souches de *L. major* qui expriment deux marqueurs de résistance sélectionnables différents co-infectés dans le même phlébotome ont donné naissance à une population de parasites capables d'exprimer les deux marqueurs de résistances. Il y avait aussi des hybrides intraspécifiques entre *L. major, L. tropica, L. donovani, L. infantum* et des hybrides interspécifiques entre *L. major*/*L. infantum* qui ont été isolés des croisements dans les

phlébotomes infectés. Par contre, ce n'est pas connu si le *Leishmania* peut subir des échanges génétiques à l'intérieur de l'hôte mammifère.

Comme mentionné précédemment, la plupart des espèces de *Leishmania* vivent dans des vacuoles individuelles serrées, mais les espèces du complexe *Leishmania mexicana* (*L. mexicana* et *L. amazonensis*) vivent dans des vacuoles communales spacieuses. L'un des avantages d'occuper une telle vacuole est le potentiel d'échange génétique. Puisque ce n'est pas connu si les échanges génétiques prennent place au sein d'un hôte mammifère infecté, l'hypothèse sous-jacente au projet présenté ci-dessous est que les vacuoles parasitophores communales hébergeant des parasites du complexe *Leishmania mexicana* fournissent un environnement exceptionnellement avantageux pour les échanges génétiques. Pour répondre à cette hypothèse, nous avons proposé de démontrer que les échanges génétiques peuvent prendre place entre les parasites *in vitro* dans une culture cellulaire infectée, dans les infections *in vivo* de souris ainsi que dans les cultures axéniques. Globalement, ce projet de recherche nous permettra de déterminer si des échanges génétiques se produisent dans les vacuoles communales spacieuses infectées par des parasites du complexe *L. mexicana*. Ces connaissances peuvent avoir des implications importantes pour la propagation de la résistance aux médicaments, le diagnostic et le traitement des infections de *Leishmania*, car les hybrides peuvent présenter des caractéristiques génétiques et des phénotypes complexes.

Comme décrit dans l'article présenté dans ce document, nous avons créé des souches parentales de *L. amazonensis* et de *L. mexicana* exprimant chacune un gène de résistance différent. Nous avons premièrement essayé de démontrer si les parasites peuvent s'échanger du matériel génétique dans un milieux axénique. Pour se faire, nous avons analysé quatre croissements différents qui étaient nous *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e; *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*, and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e. Nous avons démontré que le croisement de *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e aurait généré des parasites doubles résistants. Nous avons réussi à isoler ces parasites entre 18 et 28 jours après le transfert dans un milieu sélectif. Nous avons pu démontrer par PCR que les parasites portaient les deux gènes de résistance; cependant, nous n'avons pas été en mesure de confirmer s'il ne s'agissait pas d'hybrides génomiques complets ou si seul le marqueur épisomique avait été transféré entre les parents en raison du fait que les hybrides étaient morts. On a aussi essayé de voir si les parasites

peuvent échanger du matériel génétique entre cellules par des vésicules extracellulaires, mais on n'a jamais observé des parasites double résistants dans cette condition. Nous avons ensuite analysé la possibilité des échanges génétiques entre parasites dans des cultures cellulaires de macrophages différenciés de la moelle osseuse infectées. Nous avons essayé deux méthodes différentes afin d'isoler les parasites doubles résistants de ce type d'infection *in vitro*. Cependant, nous n'avons pas pu isoler des parasites double résistants via la première méthode. On a observé le même résultat avec la deuxième méthode à part deux instances séparées. On a pu observer des parasites doubles résistants de deux infections séparées du croisement de *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*. On a isolé une population de parasites potentiellement hybrides de la première de ces infections. On a pu maintenir ces parasites en culture en les cultivant dans des macrophages pendant trois semaines ; cependant, après la troisième semaine, les parasites ont perdu un des gènes de résistance et ils ont finalement péri par la suite. La deuxième infection nous a donné trois populations doubles résistants. Par contre, deux des populations analysées étaient positives pour la présence des deux gènes et la troisième population n'avait qu'un des gènes de résistance; cependant, ils sont morts peu de temps après l'isolement. Les deux populations restantes ont été maintenu pour une semaine avant de périr. Pour tous les parasites doubles résistants qu'on a pu isoler des infections *in vitro*, on a démontré par PCR la présence des deux gènes de résistance. Malheureusement, nous n'avons pas pu confirmer si ces parasites étaient des hybrides génomiques complets ou non, comme cela a été fait dans les études précédentes puisqu'ils ont tous péri et on n'a pas pu faire d'autre analyse. Finalement, on a aussi essayé d'isoler des parasites double résistant des infections *in vivo*. Nous avons fait des injections sous-cutanées de l'oreille des souris avec trois croisements de parasites. Par contre, on n'a jamais réussi d'isoler des parasites double-résistants de ces infections.

En conclusion, nous présentons dans cette étude pour la première fois l'occurrence d'échanges génétiques entre *L. amazonensis* au sein de cellules de mammifères infectées. Nous n'étions pas capables d'isoler des hybrides potentiels à partir de souris infectées, mais le fait que nous ayons isolé quelques parasites à double résistance à partir d'infections cellulaires *in vitro* suggère que de tels processus peuvent se produire. Ils sont rares et conduisent à la génération de descendants instables à faible viabilité. De plus, nous démontrons que des parasites à double résistance peuvent être observés dans les cultures axéniques. Nous avons également essayé de croiser *L. mexicana* et *L. amazonensis*; cependant, nous n'avons observé aucune descendance à double résistance issue d'un tel croisement. Un tel résultat peut être due au fait que différentes espèces ont des capacités différentes en matière de combinaison génétique où certaines

espèces s'avèrent plus efficaces et d'autres moins. Une autre possibilité pour laquelle on n’a pas observé de parasite double résistant est que le microenvironnement de l'hôte mammifère est assez différent de celui du phlébotome et que l'un peut être plus approprié pour les échanges génétiques que l'autre.

Dans les futures études concernant spécifiquement les parasites du complexe *L. mexicana*, nous devrions continuer d'étudier leur possibilité d'échanges génétiques dans les cellules et hôtes de mammifères infectés. Il serait également intéressant de croiser ces espèces avec des espèces qui ont une compatibilité d'accouplement plus élevée. Cela peut augmenter les chances d'isoler des parasites hybrides potentiels. On sait maintenant que différentes espèces ont des capacités d'accouplement différentes, il est donc important de continuer à explorer les événements d'hybridation entre les différentes espèces de *Leishmania*. Ces études permettront de faire progresser notre compréhension de la diversité biologique et de la complexité du genre *Leishmania* et de fournir un aperçu des stratégies potentielles pour transmettre des gènes de résistance entre parasites. En outre, il sera également important d'évaluer la fertilité et l'aptitude de ces hybrides pour mettre davantage en évidence l'impact et l'importance de la recombinaison génétique au sein de la population de *Leishmania*.

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LIST OF ABBREVIATIONS

4E-BP1: 4E (eIFE4) binding protein 1

AIDS: Acquired immunodeficiency syndrome

AP-1: Activator protein 1

APC: Antigen presenting cell

Arf: ADP-ribosylation factor

Arp 2/3: Actin related protein 2/3

ATP: Adenosine triphosphate

BAI: Brain-specific angiogenesis inhibitor

BMM: Bone-marrow derived macrophage

BCR: B-cell receptor

CD: Cluster of differentiation

Class C VPS/HOPS complex: Class C vacuolar protein sorting/homotypic fusion and protein sorting complex

CLR: C-type lectin receptor

Cop I: Coat protein I

CP: Cysteine protease

CPB: Cysteine protease B

CR: Complement receptor

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

DC: Dendritic cell

DMEM: Dulbecco's modified eagle mediq

DNA: Deoxyribonucleic acid

DNGR-1: Dendritic cell NK lectin group receptor-1

EEA1: Early endosome antigen 1

ELISA: Enzyme-linked immunosorbent assay

FBS: Fetal bovine serum

FcR: Fc receptor

G418: Geneticin

GFP: Green fluorescent protein

GP63: Major surface glycoprotein 63; leishmanolysin

gRNA: Guide ribonucleic acid

HEPES: Hydroxyethyl piperazineethanesulfonic acid

HIV: Human immunodeficiency virus

HYG: Hygromycin

ICAM: Intracellular adhesion molecule

IFA: Immunofluorescence antibody assays

IFNs: Interferons

Ig: Immunoglobullin

IL: Interleukin

IRAK: Interleukin-1 receptor associated kinase

ITAM: Immunoreceptor tyrosine-based activation motif

JAK: Janus kinase

kDNA: Kinetoplast deoxyribonucleic acid

KMP-1: kinetoplastid membrane protein-11

LAMP: Lysosomal-associated membrane

LCM: L929 cell-conditioned medium

LPG: Lipophosphoglycan

LPS: Lipopolysaccharide

LRV-1: Leishmania RNA virus-1

MAC: Membrane attack complex

MAPK: Mitogen-activated protein kinase

MARCO: Macrophage receptor with collagenous structure

MHC: Major histocompatibility complex

mRNA: Messenger ribonucleic acid

mTOR: Mammalian target of rapamycin

NADPH: Nicotinamide adenine dinucleotide phosphate

NET: Neutrophil extracellular traps

NF-κB: Nuclear factor kappa-light-chain-enhancer of B-cells

NO: Nitric oxide

NOD-like receptor: Nucleotide oligomerization domain-like receptor

NOX: NADPH oxidase

NK: Natural Killer cell

NRAMP1: Natural resistance-associated macrophage protein 1

ORPL1: Oxysterol-binding protein-related protein 1

PAMP: Pathogen associated molecular pattern

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PD-1: Programmed cell death protein 1

PI3K: Phosphatidylinositol 3-kinase

PI(3)P: Phosphatidylinositol-3-phosphate

PKDL: Post-kala-azar dermal leishmaniasis

PLC: Phospholipase C

PM: Peritrophic matrix

PRR: Pathogen recognition receptor

PSG: Promastigote secretory gel

PtdSer: Phosphatidyl serine

PTP: Protein tyrosine phosphatase

PV: Parasitophorous Vacuole

Rab protein: Ras-associated binding protein

RIG-I-like receptor: Retinoic acid-inducible gene I-like receptor

RILP: Rab7-interacting lysosomal protein

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reaction oxygen species

RPM: Revolutions per minute

rRNA: Ribosomal ribonucleic acid

SHP: Src homology region 2 domain-containing phosphatase 1

SNARE: Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

SR-A: Scavenger receptor class A

STAT: Signal transducer and activator of transcription

TCR: T-cell receptor

TGF: Transforming growth factor

TIM: T-cell immunoglobulin and mucin domain

TLR: Toll-like receptor

TNF: Tumor necrosis factor

UTP: Uridine triphosphate

V-ATPase: Vacuolar ATPase

VAMP: Vesicle associated membrane protein

WHO: World health organization

WT: Wild type

SECTION 1: LITTERATURE REVIEW

CHAPTER 1: THE IMMUNE SYSTEM

1.1 The immune system: an introduction

The immune system is composed of thousands of cells whose primary objective is to defend the host from potential threats as an army would for its country. The threats that one may encounter can come in the shape of viral, bacterial, fungal or parasitic pathogens (Non-self antigens). There are also internal threats that the immune system should take care of such as cell debris, damaged tissues and cancer cells (Self antigens). This system evolved for a very long time to form the complex defensive mechanism we know today and could be divided into two distinctive categories: the innate and the adaptive immune systems (Yatim & Lakkis, 2015).

The surface of our bodies is covered with skin cells which act as a border to the outside world in order to prevent pathogens and other nasty diseases from damaging our vulnerable cell ecosystem. However, sometimes the border is breached and that is when the primary response units rush to the host's defense and that is what we call the innate immune response. Innate immunity has a very important role of stopping and containing the threat before it spreads and causes heavy damage to the host and usually takes only hours to activate. This activation is induced by the various cytokines, chemokines and other distress molecules produced by damaged and neighboring tissue cells (Hato & Dagher, 2015). This system has a multitude of components that it can deploy. The first cells the pathogens encounter upon breaching the epithelium are neutrophils within the first hours (Julier *et al.*, 2017). These are then quickly reinforced by the innate immunity common soldiers, the macrophages, and other cells which include monocytes, mast cells, dendritic cells (DC), Natural Killer cells (NK) and other granulocytes (Basophils, Eosinophils) (Julier *et al.*, 2017; Kaur & Secord, 2019). There are also indirect defensive measures that aid the cells in stopping the pathogens in the shape of natural antibodies, complement system and a few more (Hato & Dagher, 2015). The innate immune cells sense the intruders through their pathogen recognition receptors (PRRs) that exist in several forms (Mogensen, 2009). These PRRs recognize conserved pathogen associated molecular patterns (PAMPs) and induce the immune cells to phagocytose the intruders and destroy them (Iwasaki & Medzhitov, 2015; Mogensen, 2009).

Although the innate immune system can clear pathogens by recognizing conserved pathogenic motifs, it cannot mount an efficient and pathogen-specific response. That is a job for the special armed force called the adaptive immune system and unlike innate immunity, it requires a couple of days to be activated and a couple of weeks to clear out the pathogen (Julier *et al.*, 2017). This

immunity is composed primarily of T and B-cells which specialize in cellular immune response and antibody production respectively. These cells have specialized receptors named TCRs and BCRs which are generated via a process called somatic recombination of a large array of gene segments and are highly specialized at recognizing antigens of a specific pathogen (Bonilla & Oettgen, 2010; Mogensen, 2009). Although it is efficient at destroying invading pathogens, it first needs to be activated. While the innate immunity is fighting at the periphery, a messenger readies for this event. DCs are the messengers that link the innate immunity with the adaptive. DCs are specialized cells known as antigen-presenting cells (APCs) that are responsible for the induction of the adaptive immune system (Bonilla & Oettgen, 2010). To do this, DCs phagocytose pathogenic organisms and process them to antigens. DCs expressing antigens on their major histocompatibility complex (MHC) proteins will travel from the site of infection to regional lymph nodes where they will interact with the cells of the adaptive immunity system (Bonilla & Oettgen, 2010; Yatim & Lakkis, 2015). Here, the appropriate T-cells will recognize the antigen and proliferate into effector and helper cells and the B-cells will turn into antibody producing factories (Plasma cells) (Yatim & Lakkis, 2015). Once the intruding pathogens are taken care off, most of the cells will die, but the few that survive will turn into memory cells (Bonilla & Oettgen, 2010; Mogensen, 2009). These memory cells will ensure a more robust response upon reencounter with the same pathogen in the next infection.

1.2 The Macrophage, common soldier of innate immunity

Macrophages (from Greek works *makros*, “large/big” and *phage*, “eater”) are phagocytic cells that were first discovered by the Russian zoologist Ilya Metchnikoff in 1882 for their phagocytic activity in his famous experiment with starfish larvae (Shapouri-Moghaddam *et al.*, 2018; Tauber, 2003). As described higher up, macrophages are professional phagocytes that represent one of the key innate immune players that are responsible for clearing out invading pathogens; however, they also have an important role in maintaining homeostasis, repairing tissues, clearing apoptotic cells and a few more (Biswas & Mantovani, 2010; Gordon & Martinez-Pomares, 2017; Kim & Nair, 2019). These behaviors depend on the stimulus the cells receive from their surrounding environment. This process is known as macrophage polarization towards the classically activated or inflammatory state (M1) and alternatively activated or anti-inflammatory state (M2) (Martinez & Gordon, 2014; Shapouri-Moghaddam *et al.*, 2018). The M1 activation is typically induced by the inflammatory Th1 cytokine milieu, such as IFN-γ and TNF-α and/or PAMPs through various PRRs (Martinez & Gordon, 2014; Shapouri-Moghaddam *et al.*, 2018). These PRRs include external cell receptors such as the majority of toll-like receptors (TLR) and C-type lectin receptors (CLR) which

are able to recognize microbial molecules outside the cell (most classical example is TLR-4 that recognizes bacterial LPS) and they include internal cell receptors such as NOD-like receptors, RIG-I-like receptors and TLR 7-9 which can recognize phagocytosed antigens or viral molecules (Takeuchi & Akira, 2010). As a result, the M1 activated macrophages produce high amounts of inflammatory cytokines and chemokines which will recruit monocytes (main source of newly recruited tissue macrophages (Gordon & Martinez, 2010)) and neutrophils to the site of infection as well as spread the alarm throughout neighboring cells and produce high amounts of reactive oxygen species (ROS), improve the microbicidal activity of the cells and increase the expression of presentation molecules such MHC as well (Linehan & Fitzgerald, 2015; Shapouri-Moghaddam *et al.*, 2018). The M2 macrophages are polarized by IL-4 and IL-13 or phosphatidyl serine (PS) located on apoptotic cells or the anti-inflammatory IL-10 (Kim & Nair, 2019; Linehan & Fitzgerald, 2015; Shapouri-Moghaddam *et al.*, 2018). This results in a Th2 like response and will put out the inflammatory response and promote homeostasis, wound healing and tissue repair, and other function aimed at restoring the organism to normality (Shapouri-Moghaddam *et al.*, 2018).

1.3 Phagocytosis

Phagocytosis is an important cellular mechanism for cells of the innate immune system which is used to uptake particles or whole cells bigger or equal to 0.5 µm in diameter within a plasma-membrane envelope (Gordon, 2016; Kinchen & Ravichandran, 2008). This mechanism has many roles: in unicellular organisms this is the primary source for nutrient acquisition; however, in multicellular organisms it is important for pathogen and apoptotic cell clearance by cells termed phagocytes (Rosales & Uribe-Querol, 2017). Phagocytes in multicellular organisms can be divided into professional and non-professional phagocytes. Professionals include macrophages, neutrophils, DCs, monocytes, eosinophils and osteoclasts which all are important in either initiating an inflammatory response to clear intruding microbes and activating the adaptive immune system or maintain homeostasis, tissue remodeling and tissue repair by clearing apoptotic or necrotic cells (Flannagan *et al.*, 2012; Gordon, 2016). Epithelial cells, endothelial cells, fibroblasts and astrocytes are non-professional phagocytes. These cells act as “secondary phagocytes” and they mostly clear the area they’re in from apoptotic bodies, but they are not capable of ingesting microorganisms (Flannagan *et al.*, 2012; Gordon, 2016). Phagocytosis can be divided into 4 major steps: Particle recognition, followed by internalization which will lead to phagosome formation and maturation from early phagosome to a phagolysosome and culminating with particle elimination within the phagolysosome (Figure 1.1) (Flannagan *et al.*, 2009; Rosales & Uribe-Querol, 2017). Regardless of whether the phagocytosed particles are

microbial or apoptotic, they will both go through the same phagocytic process with the exception of some differences especially at the initial steps. After all, these particles will determine which signals will be activated within the phagocytes to ensure that they start a proper inflammatory or anti-inflammatory response (Arandjelovic & Ravichandran, 2015).

To initiate phagocytosis, the phagocytes must first recognize the particles in front of them in order to determine if its source is of foreign nature or if it's a self-particle and based on that they produce an appropriate response. In the case of microbial particle uptake, it is done by receptors located on these cells that can be categorized into two distinct groups: non-opsonic receptors and opsonic receptors (Gordon, 2016). Non-opsonic receptors are able to recognize and bind the particles directly and these are mostly found in different types of PRR. Although, as shown previously, PRRs are important in terms of pathogen recognition, not all of them induce phagocytosis. As a matter of fact, PRRs such as TLRs and G-coupled protein receptors were found to promote phagocytosis (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). For example, TLRs promote phagocytosis through the induction of the phagocytic gene program (Doyle *et al.*, 2004). The receptors that do recognize PAMPs directly and induce phagocytosis include Dectin-1 receptors which recognize yeast polysaccharides (Herre *et al.*, 2004) or mannose receptors (CD206) that recognize mannan (Ezekowitz *et al.*, 1990; Flannagan *et al.*, 2012). There are also CD14 and scavenger receptor SR-A which recognize lipopolysaccharide-binding proteins, lipopolysaccharide and lipothechoic acid (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). MARCO is another scavenger receptor that can recognize lipopolysaccharide, lipothechoic acid, CpG DNA and whole bacterial cells such as *Neisseria meningitis* (Canton *et al.*, 2013; Rosales & Uribe-Querol, 2017) There is also CD36 that is capable of recognizing *Plasmodium falciparum*-infected red blood cells (Patel *et al.*, 2004). Finally, there are other receptors that induce phagocytosis such as CD33, CD169 and DNGR-1; however, the full list of receptors and the ligands that they recognize respectively is yet to be fully uncovered (Flannagan *et al.*, 2012; Gordon, 2016). The second group of phagocytic receptors consist of opsonic receptors. These receptors are able to recognize microbial particles that are bound by circulating soluble molecules that help tag them for degradation (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). These molecules are known as opsonins which include circulating immunoglobulins and molecules part of the complement system. The most widely-known and studied opsonin-receptors are the Fcγ receptors which recognize various IgG classes through their Fc portion at varying affinities (Anderson *et al.*, 1990; Bruhns *et al.*, 2009). However, there are other Fc receptors that are capable of recognizing other immunoglobulins such as IgA and IgE (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). Finally, there are complement receptors which recognize the

molecules of the complement cascade such as CR1, CR3 and CR4 that recognize iC3b and others (Ghiran *et al.*, 2000; Ross *et al.*, 1992).

Although phagocytosis is important in clearing microbial organisms, most of the time this mechanism is used by phagocytes to remove apoptotic cells. As a matter of fact, human phagocytes remove billions of dying cells daily (Kinchen & Ravichandran, 2008). The phagocytosis of apoptotic bodies is done in a couple of steps. First of all, apoptotic cells release molecules that are usually found within the cell such as ATP/UTP, lysophosphatidylcholine and sphingosine-1-phosphate, into the extracellular space which serve the purpose of "find me" signals for phagocytic cells (Arandjelovic & Ravichandran, 2015; Rosales & Uribe-Querol, 2017). This "find me" signal acts as the primary signal for the phagocytes, the secondary signal is the "eat me" signal which is provided by physical contact between the phagocyte and the apoptotic entity (Arandjelovic & Ravichandran, 2015; Rosales & Uribe-Querol, 2017). This is achieved through the recognition of surface molecules such as phosphatidylserine (PtdSer) on apoptotic cells; however, there are other surface molecules that play a role in this scenario such as a modified form of ICAM-3 and calreticulin (Arandjelovic & Ravichandran, 2015; Poon *et al.*, 2014; Rosales & Uribe-Querol, 2017). PtdSer can be directly recognized by phagocytic receptors such as TIM-1, TIM-4, BAI-1 or Stabilin-2 and, in addition to that, apoptotic cells can also be recognized by other receptors such as the scavenger receptors MARCO, SR-A and CD-36 and a few others (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). It needs to be mentioned however, that PtdSer is not only found on dying cells, but it is also expressed on living cells. However, it must be noted that there is 300-fold difference of PtdSer expression between healthy and apoptotic cells which helps phagocytes distinguish these cell types and remove the dead (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). Also, healthy cells prevent their phagocytosis by expressing molecules such as CD31, CD46 and CD47 which provide "Don't eat me" signals upon contact with phagocytic cells (Arandjelovic & Ravichandran, 2015; Flannagan *et al.*, 2012; Poon *et al.*, 2014; Rosales & Uribe-Querol, 2017). Nevertheless, we still do not have the full picture of all the signaling pathways and receptors involved in the process of phagocytosis that explain how the cell distinguishes between tolerant and immune phagocytosis.

As soon as the phagocytic receptors recognize a particle, it will trigger a cascade of signaling events that will lead to actin remodeling and formation of the phagocytic cup around the particle for internalization with the subsequent early phagosome formation (Flannagan *et al.*, 2012; Gordon, 2016; Rosales & Uribe-Querol, 2017). Unfortunately, we do have a full picture of all these signaling pathways; however, we have well described pathways such as that of FcR and CR3

receptor signaling pathways (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). In the case of the Fcγ receptor (FcγR), exposure of the ligand to the receptor causes their clustering on the cell membrane which initiates the phosphorylation of their immunoreceptor tyrosine-based activation motifs (ITAMs) by the Src-family kinases partially (Lyn, Lck and Hck) (Ghazizadeh *et al.*, 1994; Takai, 2002). The phosphorylated ITAMs create a docking site for the SH2 domain of the tyrosine kinase Syk that can also phosphorylate the ITAMs (Freeman & Grinstein, 2014; Johnson *et al.*, 1995). This will lead to a signaling cascade that will activate many other molecules and enzymes such as PI3K (phosphatidylinositol 3-kinase) and or PLCγ and other downstream molecular players (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). PI3K can then lead to the activation of different factors such as NF-κB that will lead to inflammatory cytokine production or that of GTPases of the Rac family which will then lead to the activation of the Arp2/3 actin nucleation complex which will promote actin polymerization and subsequent formation of the phagocytic cup (May *et al.*, 2000; Rosales & Uribe-Querol, 2017).

Once the particle has been internalized into the phagocyte, the phagosome is formed and starts the maturation process. Maturation can be separated into 4 steps: the early phagosome; the intermediate phagosome, the late phagosome and finally the phagolysosome. Initially, the newly formed phagosome membrane is mostly composed of plasma membrane components (Levin *et al.*, 2016). However, the phagosome's membrane will quickly undergo through a biochemical alteration both through changes of membrane and its contents which will make it rapidly acquire early endosome properties (Flannagan *et al.*, 2009; Levin *et al.*, 2016; Pitt *et al.*, 1992; Rosales & Uribe-Querol, 2017). This is achieved through the fusion of the phagosome with early recycling and sorting endosomes (Levin *et al.*, 2016; Rosales & Uribe-Querol, 2017). These fusion events between the early phagosome and early endosomes is mediated between the small GTPase Rab 5 (Bucci *et al.*, 1992; Gutierrez, 2013). Its role is critically important in phagosome maturation, because perturbation in its function or the protein itself can arrest the phagosome's progression to phagolysosomes (Vieira *et al.*, 2003). Not much is known about the mechanism of recruitment of Rab 5 to the phagosomes; however, it is known that some of these proteins are present on the plasma membrane during the formation of the phagosome (Chavrier *et al.*, 1990). Rab 5 acquisition and activation on the phagosome will lead to the recruitment of multiple other proteins to the phagosome (Flannagan *et al.*, 2009; Levin *et al.*, 2016). First of all, with the support of p150 Ser and Thr kinase, Rab 5 will recruit hVPS34, a class III phosphatidylinositol-3-kinase which generates phosphatidylinositol-3-phosphate (PI(3)P) (Vieira *et al.*, 2001). PI(3)P in turn recruits proteins such as EEA-1, NADPH oxidase and others to the early phagosome membrane (Flannagan *et al.*, 2012). EEA-1 in turn facilitates the docking and fusion of early endosomes to

the phagosome by directly interacting with SNAREs, such as syntaxin 6 and syntaxin 13 (Christoforidis *et al.*, 1999; Levin *et al.*, 2016; McBride *et al.*, 1999; Simonsen *et al.*, 1999). Remarkably, despite all these numerous fusion events, the surface area of the phagosome does not grow over time. This is due to the capacity of the phagosome, like that of the endosome, to be able to recycle molecules to the plasma membrane via mechanisms that involve molecules such as CopI, Arf and Rab GTPases (Botelho *et al.*, 2000). However, the majority of recycling events are mediated via Rab 11 and Rab 4 GTPases (Levin *et al.*, 2016). Finally, it must be noted that the lumen of the early phagosome will become slightly acidic (pH 6.1-6.5) due to proton pumping catalyzed by the vacuolar ATPase (V-ATPase) that were recruited to the phagosome (Rosales & Uribe-Querol, 2017).

Next stop on the road of phagosome maturation, is the intermediate phagosome, which exists for a brief moment. It is mainly characterized by the gradual loss of Rab 5 and acquisition of Rab 7 which is mediated in part via the class C VPS/HOPS complex (Poteryaev *et al.*, 2010; Rink *et al.*, 2005). Additionally, this is the stage where there are intraluminal vesicles formed which contain membrane associated molecules targeted for degradation (Rosales & Uribe-Querol, 2017).

With the accumulation of Rab 7 GTPases on the membrane, the phagosome matures into the late phagosome. Rab 7 is required for the phagosome maturation since defects in its functioning prevent the phagosome to fuse with endosomes and lysosomes as well as prevent proper acidification (Harrison *et al.*, 2003). Hence, it is highly important in the fusion of late endosomes and/or lysosomes with the phagosome (Harrison *et al.*, 2003; Rink *et al.*, 2005). Although we know very little about the effector proteins that are recruited by Rab 7, we do know that it is able to recruit Rab7-interacting lysosomal protein (RILP) and oxysterol-binding protein-related protein 1 (ORPL1) (Flannagan *et al.*, 2012; Levin *et al.*, 2016; Rosales & Uribe-Querol, 2017). Together, these two effectors will recruit the dynein-dynactin complex which will promote the centripetal migration of phagosomes along the microtubules (Harrison *et al.*, 2003; Johansson *et al.*, 2007; van der Kant *et al.*, 2013). This event is needed to facilitate the late endosomal and lysosomal compartments to fuse with the phagosome by bringing them to close proximity so that SNAREs, such as VAMP7 and VAMP8, could interact with each other and complete membrane fusion (Antonin *et al.*, 2000; Rosales & Uribe-Querol, 2017; Wade *et al.*, 2001). Additionally, the late phagosome will acquire lysosomal-associated membrane (LAMP) 1 and 2 and luminal proteases via fusion with late endosomes thereby preparing the field for the final stage of maturation (Huynh *et al.*, 2007; Rosales & Uribe-Querol, 2017). LAMP acquisition is critical for the late phagosome in order to fuse with lysosomes and fulfill its microbicidal and degrative functions (Binker *et al.*,

2007; Flannagan *et al.*, 2012). The late phagosome will also recruit more V-ATPases to its membrane which will further acidify the compartment (pH 5.5-6.0) (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017).

The final stage on the phagosome maturation journey is the phagolysosome, once the late phagosome fuses with lysosomes. Physically, phagolysosomes differ from late phagosomes with their internal membrane enriched in PI(3)P and lack of mannose-6-phosphate receptors(Griffiths *et al.*, 1988; Kobayashi *et al.*, 1998). One of the other major differences is the ability of the phagolysosome of degrative functions and acquisition of degrative components. First of all, this is due to the acquisition of multiple V-ATPases which enhance proton pumping and which make the phagolysosome become more acidic (pH5.5-4.5 and sometimes even lower) (Flannagan *et al.*, 2012; Rosales & Uribe-Querol, 2017). The acidification itself plays a major role in clearing vacuolar contents of the phagolysosome. For instance, it creates a hostile environment that denies the growth of microorganisms and it functions as means of activation of many hydrolytic enzymes (Flannagan *et al.*, 2009; Huynh *et al.*, 2007). The phagolysosomes also contain various reactive oxygen species (ROS) and reactive nitrogen species (RNS) which will eliminate the microorganisms captured within the compartment (Flannagan *et al.*, 2009). Finally, the phagolysosome contains many other antimicrobial peptides and proteins that essentially can be divided into those that can inhibit growth, such as NRAMP1 that achieves this effect by extruding divalent cations such as Zn^{2+} and Mn^{2+} that are important for bacterial growth out of the phagosomal lumen (Cellier *et al.*, 2007), or those that compromise the integrity of the microorganisms such as defensins, lysozymes, cathelicidins and others (Flannagan *et al.*, 2009). Cathelicidins, for example, permeabilize the cell wall and inner membrane of Gram-positive bacteria or the outer and inner membranes of Gram-negative bacteria (Zanetti, 2005). The phagolysosome contents; however, differ in their composition and ratios between different cell types, but this does not stop the cells of achieving their ultimate phagocytic goal.

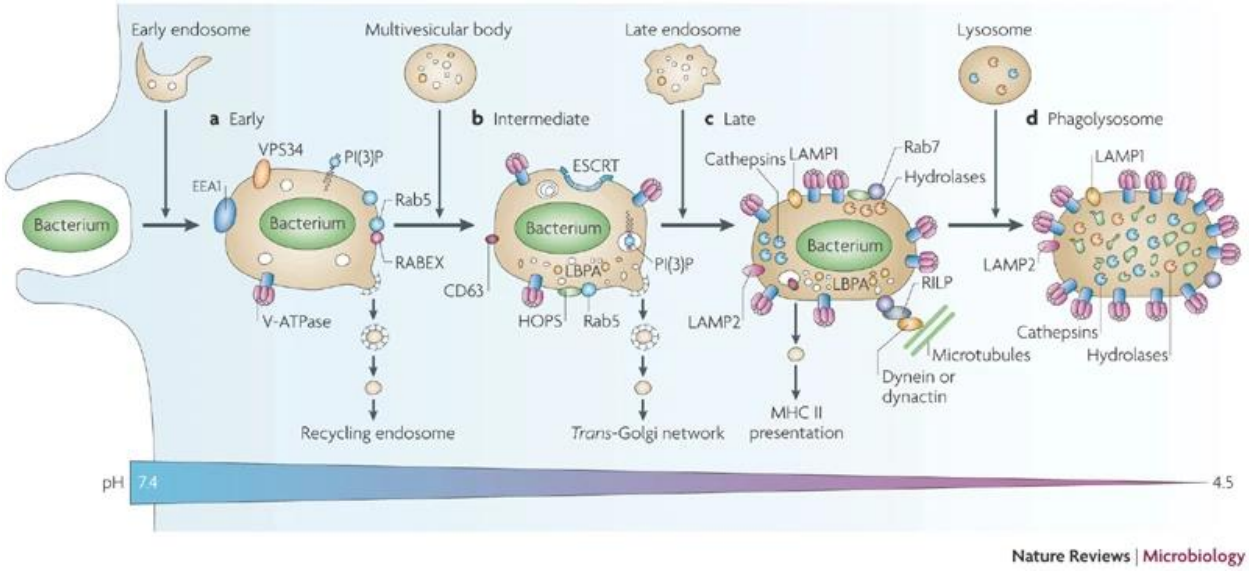

Figure 1.1: Pathogen phagocytosis and phagosomal maturation

The early phagosome is formed as soon as the pathogen is internalized by the phagocytic cell. The phagosome undergoes a series of transformations by interacting with various components of the endocytic pathway. This is important for the maturation of the phagosome which culminates in phagolysosome formation that will ultimately degrade the pathogen (Flannagan *et al.*, 2009).

CHAPTER 2: *LEISHMANIA*

2.1 Leishmaniasis

Leishmaniasis is a spectrum of vector-borne diseases caused by the obligate intracellular protozoan parasite *Leishmania* of the Kinetoplastida order and Trypanosomatidae family (Ghorbani & Farhoudi, 2018) and it is considered as a neglected tropical disease by the world health organization. Currently, this disease is endemic in 98 countries, (de Vries *et al.*, 2015; WHO, 2020) and causes the occurrence of 700 000 to 1 million of new cases yearly as well as 20 000 to 30 000 deaths yearly (Akhoundi *et al.*, 2017; WHO, 2020). However, the actual numbers of infections may be even greater since we do not necessarily see the full epidemiological picture due to the fact that the data may be unreliable or incomplete in the countries endemic for this disease (Alvar *et al.*, 2012). Moreover, as estimated by the World Health Organization (WHO), there is currently over 1 billion people that are at risk of infection on the entire planet. Although most of the countries that are affected by the disease are developing countries predominantly located in the South American, African and Asian continents, climate change as well as animal and human migrations may help the vector and, consequently, help leishmaniasis spread into other regions and territories (Antinori *et al.*, 2012; Savoia, 2015; WHO, 2020)

There exists over 20 different *Leishmania* species, found both in the New world (North and South America) and Old world (Europe, Africa, Asia), that can cause the disease in humans which can be divided into three major clinical manifestations: visceral, cutaneous and mucocutaneous (de Vries *et al.*, 2015; WHO, 2020). Additionally, leishmaniasis may persists from a couple of months to a couple of years in an infected person and sometimes the disease does not display any clinical symptoms in the affected mammal or human (Cohen-Freue *et al.*, 2007). These manifestations are determined by the parasite species infecting the host, but they could also be influenced by the hosts response and genetics as well (Rogers, 2012; Zabala-Penafiel *et al.*, 2020). Visceral leishmaniasis (Kala azar or "Black death") is the most severe form of the disease and is deadly in 95% of the cases if left untreated (WHO, 2020) and with the majority of cases concentrated in India, Brazil, Nepal, Bangladesh and Sudan (Morimoto *et al.*, 2019). As a matter of fact, these countries harbor 90% of visceral leishmaniasis cases worldwide (WHO, 2020). Usually, the sickness is characterized by fever, weight loss, hepatosplenomegaly and anemia (Morimoto *et al.*, 2019). It is a form of chronic infection and is caused by *L. infantum* and *L. donovani* which usually affect the liver, spleen, bonne marrow and lymph nodes of the infected individual (de Vries *et al.*, 2015; Murray *et al.*, 2005). In some cases, after treatment, certain patients may develop a dermal form of the disease known as post-kala-azar dermal leishmaniasis (PKDL), which manifests itself as a hypopigmented macular or as an erythematous maculopapular rash (Burza *et al.*, 2018; Dighal *et al.*, 2020). Then there is the cutaneous form of leishmaniasis caused by species such as *L. major, L. amazonensis* or *L. arabica*, which is the most common and most studied form of infection. The majority of cutaneous (90% of them) leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Columbia, Costa-Rica, Ethiopia, Iran, Peru, Syria, and Saudi Arabia (Desjeux, 2004; WHO, 2020). Usually, this form of infection results in a localized ulcer or skin nodule on the exposed skin surface (face, arms, legs) of the host and can self-heal in 2 to 18 months post-infection, but in rare cases, untreated lesions may leave severe and/or disfiguring scars (Burza *et al.*, 2018; David & Craft, 2009). Finally, in certain cases the cutaneous lesions may disseminate into mucocutaneous ones, in which the nasal and oral mucosal tissues are affected and may affect the pharyngeal and laryngeal mucosa in a more severe form of the disease (Handler *et al.*, 2015; Strazzulla *et al.*, 2013). Such disease is mostly found in the New world and is observed the majority of times in infections with *L. braziliensis*; however, there are other species such *L. guyanensis, L. panamensis* and *L. amazonensis* that may cause such disease (Handler *et al.*, 2015; Strazzulla *et al.*, 2013). If left untreated, mucocutaneous leishmaniasis may lead to death due to secondary infection or malnutrition.

Currently, there are many techniques that are available to detect the parasite in the affected individual. Although in certain cases the clinical features are identifiable, such as in the case of cutaneous and mucocutaneous forms of leishmaniases, cases of visceral leishmaniases are not as easily detectable. The most effective of all are molecular and serological tests due to their specificity. Polymerase Chain reaction (PCR) assays based on the amplification of kinetoplast or nuclear DNA are very useful to identify specific *leishmania* strains, especially among cutaneous strains, whereas serological methods such as isoenzyme analysis, immunofluorescence antibody assays (IFA), western blots, ELISA, direct agglutination techniques, latex agglutination technique which allows for the detection of *Leishmania* antigens in urine and rapid strip testing for rK39 antigen are all useful in determining visceral infections (Akhoundi *et al.*, 2017; Burza *et al.*, 2018; de Vries *et al.*, 2015; Savoia, 2015). These tests are also less invasive in regards to a patient as well (Akhoundi *et al.*, 2017; Burza *et al.*, 2018; de Vries *et al.*, 2015; Savoia, 2015). It must be added that there are known cases in which the clinical outcomes may be caused by species that typically do not express them. It was found previously that parasites which cause visceral leishmaniases, such as *L. infantum* or *L. donovani* zymodeme MON-37, cause a cutaneous form of disease (Ranasinghe *et al.*, 2013; Svobodova *et al.*, 2009) and *vice versa* visceral leishmaniasis can be caused by usually cutaneous strains such as *Leishmania tropica* (Magill *et al.*, 1993). Therefore, specific identification techniques are highly important to determine parasite species, however; most of the time these sensitive techniques are not available in certain regions due to economic reasons. Therefore, we still use classical techniques such as microscopy, *in vitro* parasite culture biopsies from different organs such as the spleen, bone marrow, lymph nodes or skin depending on the species in question (Burza *et al.*, 2018; de Vries *et al.*, 2015; Savoia, 2015).

Since the antiquity, people have immunized themselves against *Leishmania* by transferring material from leishmaniasis lesions to naïve individuals, a process called leishmanization (Zabala-Penafiel *et al.*, 2020). This was the earliest type of “vaccination” that was used as prophylaxis against leishmaniasis. This is important since it is now known that patients develop a long-term immune protection which allows for asymptomatic infection and hence it is important to develop an effective vaccine (Zabala-Penafiel *et al.*, 2020). At the current moment, there is no approved and effective human vaccine against leishmaniasis (Ghorbani & Farhoudi, 2018; Zabala-Penafiel *et al.*, 2020); however, we were able to make effective canine vaccines that will undoubtably help control and prevent leishmaniasis transmission between dogs and humans (Moafi *et al.*, 2019). Despite the lack of human vaccines, there are currently some that are being tested in clinical trials most of which are live attenuated parasite vaccines (Moafi *et al.*, 2019; Zabala-Penafiel *et al.*, 2020). Although we do not have effective prophylactic measures, we have limited treatment

options available to cure leishmaniasis. The primary drug that is used to cure leishmaniasis is the pentavalent antimony; however, other treatments such as Amphotericin B, liposomial Amphotericin B (AmBisome), Paromomycin, Miltefosine and Pentamidine are available (Capela *et al.*, 2019; David & Craft, 2009; Ghorbani & Farhoudi, 2018). There is also other therapies and treatments that have been used to varied success alone or in combination with the main treatments; however, they cannot be considered as a fully-fledged leishmaniasis treatment (David & Craft, 2009).

Unfortunately, even if we have measures to cure leishmaniasis, treatment options are often difficult to administer, very expensive especially in third world countries, have toxic effects and adverse secondary effects with the exception of Miltefosine which causes less adverse effects and can be orally administered (Capela *et al.*, 2019; David & Craft, 2009; Ghorbani & Farhoudi, 2018).Due to limited treatment options, there is also a problem of parasites gaining resistance and this problem is increasing with every passing year (Capela *et al.*, 2019; Ghorbani & Farhoudi, 2018). Co-infections of patients with HIV and *Leishmania* is also another rising issue that threatens global health. The occurrence of such complications was reported since the 1990 (Monge-Maillo *et al.*, 2014) and their numbers are expected to rise in our closest future due to the fact that both of these diseases share similar geographical regions (Okwor & Uzonna, 2016). The parasite and the virus form a symbiotic relationship in which *Leishmania* helps activating the immune system in a chronic fashion that allows the viral load to increase and lead to a faster progression of AIDS and HIV in turn causes immunosuppression which is favorable for uncontrolled replication of the parasite (Monge-Maillo *et al.*, 2014; Okwor & Uzonna, 2013). In brief, leishmaniasis is a disease that threatens the health of millions of individuals worldwide with very limited treatment options available. Due to its concentration in equatorial and sub-equatorial regions which harbor primarily poor third world countries and negligence as a disease, there is little economic interest for big pharma to search and develop for more effective and less toxic treatment options to battle this particular parasite. However, due to its increasing spread in other geographical regions and the fact that it may cause additional complications as seen higher up, it will be necessary in the future to allocate more resources and time to improve our understanding and better our fight against this parasite to ensure that this pathogen does not turn from a neglected tropical disease into something more sinister.

2.2 Life cycle of *Leishmania*

Leishmania has a complex dimorphic life cycle which involves two host organisms: the sand fly and mammalian host such as humans, rodents, canines and others (Figure 1.2). There are two main genera of sand flies that are known to act as vectors for the parasite which are *Lutzomyia* and *Phlebotomus* located in the New and Old worlds respectively (Rogers, 2012). They harbor the flagellated extracellular form of the parasite known as a promastigote in their alimentary tract, that may be found in several different forms including the mammalian transmissible form known as metacyclic promastigotes. The mammalian hosts on the other hand harbor the non-flagellated ovoid amastigote form of the parasite which is present in infected cells that are macrophages in their majority, but can also be DCs, neutrophils, monocytes and possibly other cell types such as fibroblasts (Bogdan *et al.*, 2000; Handman & Bullen, 2002; Peters *et al.*, 2008; Teixeira *et al.*, 2013; Viana *et al.*, 2017).

It all begins when an infected female sand fly inoculates the infective metacyclic promastigote *Leishmania* parasites into a mammalian host during a blood meal via regurgitation (Sacks & Kamhawi, 2001; Sunter & Gull, 2017; Teixeira *et al.*, 2013). The skin damage and the sand fly saliva will start the recruitment process of neutrophils and macrophages to the inoculation site as well as it will facilitate the infection by the saliva's immunosuppressive functions (Bates & Rogers, 2004; Sacks & Kamhawi, 2001; Sunter & Gull, 2017). Once the parasite encounters the target cell, it will bind to it via target receptors which leads to the internalization of the parasite via phagocytosis (Antoine *et al.*, 1998; Handman & Bullen, 2002; Sunter & Gull, 2017; Teixeira *et al.*, 2013). This receptor binding is mediated either via the opsonization by complement of the parasite or parasite encoded molecules such as lipophosphoglycan (LPG) or GP63 metalloprotease (Handman & Bullen, 2002; Teixeira *et al.*, 2013). Once inside the phagosome, the parasite will convert it into a parasitophorous vacuole (PV) where the *Leishmania* promastigote transforms into an amastigote which is triggered by environmental cues that remain to be fully elucidated (Besteiro *et al.*, 2007; Cohen-Freue *et al.*, 2007; Sacks & Kamhawi, 2001; Sunter & Gull, 2017). This transformation usually takes from 24h to 72h depending on parasite species. The amastigotes will then replicate within the infected cell via binary fission until the cells burst and release the parasite into the surrounding environment. The free amastigotes may then re-invade new macrophage cells or other phagocytes such a dendritic cells (Handman & Bullen, 2002). Alternatively, the parasites can also be transmitted cell-to-cell via phagocytosis of an infected apoptotic macrophage by an uninfected macrophage (Real *et al.*, 2014). This cycle continues

within the mammalian host until a new sand fly eventually comes to feed on the hosts' blood and ingests with-it free-floating amastigotes and parasite infected cells.

Once the sand fly has ingested its blood meal from an infested host, the infected cells and free amastigotes end up in the digestive tract of the fly. The ingested blood meal along with the parasites is then encapsulated in a structure known as peritrophic matrix (PM) and within this matrix, the amastigotes transform to the so-called flagellated procyclic promastigotes (Bates & Rogers, 2004; Kamhawi, 2006; Sacks & Kamhawi, 2001; Sunter & Gull, 2017; Teixeira *et al.*, 2013). The PM provides a temporary semi-protective barrier from the sand fly digestive molecules for the parasite during its transition between different forms; however, this is also the time when the parasite is most vulnerable to digestive enzymes which may lead to a loss of up to 50% of the parasite population (Bates & Rogers, 2004; Kamhawi, 2006). Nevertheless, the surviving promastigotes will then enter a replicative frenzy for the following 24-48 hours via binary fission (Bates & Rogers, 2004; Kamhawi, 2006; Sacks & Kamhawi, 2001; Teixeira *et al.*, 2013). Next, the procyclic promastigotes will develop into the infective metacyclic promastigote forms through a series of cell transformations known as a process termed metacyclogenesis (Kamhawi, 2006). This is achieved first by the procyclic promastigote transforming into a Nectomonad form of parasite which has an important role of breaking through the PM to gain access to anterior abdominal midgut where it will attach itself with its flagellum to the epithelial cell microvilli to avoid excretion from the vector via defecation (Bates & Rogers, 2004; Kamhawi, 2006; Sacks & Kamhawi, 2001; Sunter & Gull, 2017; Teixeira *et al.*, 2013). The Nectomonad will then transform into a Leptomonad/Haptomonad form of parasite and finally into its final stage metacyclic promastigote form (Bates & Rogers, 2004; Kamhawi, 2006; Sunter & Gull, 2017; Teixeira *et al.*, 2013). It must be noted that Leptomonads and Haptomonads are not the same cell type. Leptomonad form of promastigotes derive directly from the Nectomonad form of the parasite and are a replication capable population in the later infection stages of the sand fly vector (Bates & Rogers, 2004; Kamhawi, 2006; Sunter & Gull, 2017). They also have an important role of producing promastigote secretory gel (PSG), a substance that is known to block the lumen of the anterior midgut and stomodeal valve of the insect to improve the efficiency of parasite transmission during a bloodmeal (Rogers, 2012). On the other hand, Haptomonads are a form of parasite that attach themselves to the stomodeal valve to block it with themselves and therefore assume a similar role of PSG which is to improve transmission (Bates & Rogers, 2004). It is still unclear from which form of promastigotes the Haptomonad arises (Bates & Rogers, 2004; Sunter & Gull, 2017). Additionally, it must be noted that the appearance of each form of parasite varies between species in time (Gossage *et al.*, 2003) and there also exist differences between the

location of each form of parasites between the *Viannia* and *Leishmania* subgenus (Bates & Rogers, 2004; Kamhawi, 2006). Despite these differences, the end product of metacyclogenesis are metacyclic promastigotes (Figure 1.3). This completes the life cycle once the sand fly is ready to take the following bloodmeal and the infective parasites are introduced into another mammalian host.

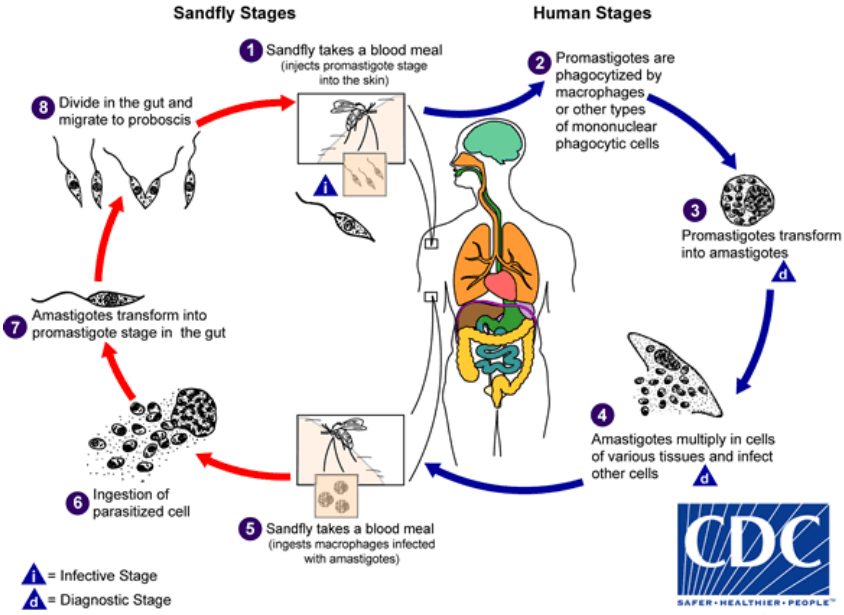

Figure 1.2: *Leishmania* life cycle

The parasite has a complex life cycle that takes place in part in the sand fly and in part in a mammalian host (CDC, 2019 courtesy by DPDx)

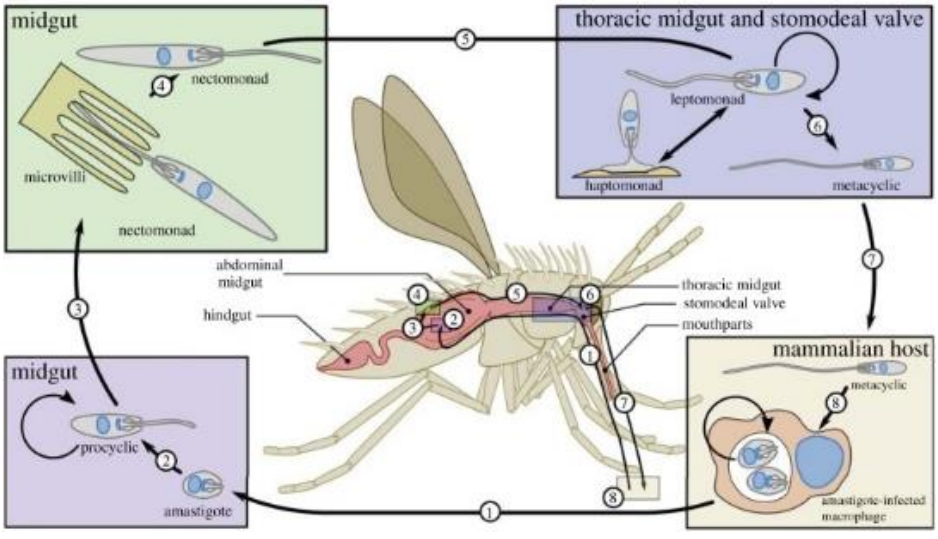

Figure 1.3: Metacyclogenesis in an infected sand fly.

Once inside the vector, the parasite transforms quickly from its amastigote form to the procyclic promastigote. The procyclic promastigote then develops to the infective metacyclic promastigote via metacyclogenesis (Adapted from Sunter *et al*, 2013)

2.3 *Leishmania* genetics

The *Leishmania* genome is one of the more complex genomes that is studied in a eukaryotic organism. As any other eukaryotic organism, the chromosomes of the parasite are located in the nucleus (Figure 1.4) and are organized into 36 chromosomes of various sizes; however, this only concerns Old world *Leishmania* species (Britto *et al.*, 1998; Ivens *et al.*, 2005; Rogers *et al.*, 2011). New world *Leishmania* species have their genome organized into 35 (*L. braziliensis*) or 34 (*Leishmania mexicana* complex) chromosomes, with chromosomes 20 and 34 merged together in one case and chromosomes 8 and 29 as well as 20 and 36 in the other case respectively (Britto *et al.*, 1998; Ivens *et al.*, 2005; Rogers *et al.*, 2011). The parasites are also characterized by unique genetic regulatory mechanisms at both the nuclear and mitochondrial levels. Unlike higher eukaryotic organisms, *Leishmania* parasites transcribe their nuclear protein-coding genes via RNA polymerase II in a polycistronic manner rather than controlled transcription initiation of every gene separately through a promoter (Clayton, 2016). The large polycistronic RNA is then trans-spliced and polyadenylated into smaller mRNA transcripts (Clayton, 2016; Martinez-Calvillo *et al.*, 2003). The levels of the mRNA depend on its stability and maturation (Clayton, 2016; Rogers *et al.*, 2011). Additionally, the *Leishmania* genome has many tandem arrays of duplicated genes on the same or different chromosomes which can allow for increased gene expression (Ivens *et al.*, 2005; Peacock *et al.*, 2007). Another genetic peculiarity that *Leishmania* have is the organization of their mitochondrial DNA. As a matter of fact, all parasites of the Trypanosomatidae family such as *Leishmania* store all of their mitochondrial DNA within a specialized compartment termed the kinetoplast which represents about 30% of total cellular DNA (Figure 1.4) (Cavalcanti & de Souza, 2018). The kinetoplast DNA (kDNA) is further divided into circular molecules which are found in two variants: maxicircles and minicircles (Camacho *et al.*, 2019; Cavalcanti & de Souza, 2018). Maxicircles are present in a few copies and encode rRNAs and proteins of the respiratory chain whereas minicircles are found in thousands of copies and they encode guide RNAs (gRNA) which are vital for editing maxicircle-derived transcripts (Camacho *et al.*, 2019; Cavalcanti & de Souza, 2018). This editing is achieved by the gRNA adding or removing uridylated residues on maxicircle transcripts to form functional RNA transcripts (Camacho *et al.*, 2019; Cavalcanti & de Souza, 2018). As a side note, it must be mentioned that the gene content itself between different species of *Leishmania* has very little variability. Species-specific genes exist; remarkably however, they are very few and most have unknown functions (Peacock *et al.*, 2007; Rogers *et al.*, 2011). Lastly, gene expression between the promastigote and amastigote life stages varies very little as well. The amount of differentially expressed genes ranges between 0.2% and 5% of total genes (Cohen-Freue *et al.*, 2007; Rochette *et al.*, 2008).

Aneuploidy is generally considered to have a negative effect on an organism's health and fitness. As it is well-known, animals, humans and some protists are diploid organisms meaning organisms that have 2 copies of each chromosome in their cells with the sole exception of gamete cells being haploid that are essential for sexual reproduction (Sterkers *et al.*, 2014). Generally whole chromosome or segmental aneuploidies in such organisms lead to severe developmental defects or even death (Tang & Amon, 2013; Torres *et al.*, 2008). Trisomy of chromosome 21 in humans (Down Syndrome) is one of many examples of how aneuploidy may be detrimental to one's fitness. However, it is a very different story for *Leishmania* parasites. *Leishmania* are considered to be "mainly diploid" organisms; however, aneuploidy has been previously reported (Rogers *et al.*, 2011; Sterkers *et al.*, 2011). In reality, these parasites exhibit mosaic aneuploidy, that is they contain varying numbers of chromosomes and these numbers vary from cell to cell within the same population (Sterkers *et al.*, 2014). This chromosome copy number variation can be seen between different strains of one species or even between different species of parasite (Bussotti *et al.*, 2018; Downing *et al.*, 2011; Rogers *et al.*, 2011; Sterkers *et al.*, 2011). Moreover, this variation can also be seen between individual cells within a given population (Sterkers *et al.*, 2011). As a consequence of mosaic aneuploidy, the parasites are able to maintain a conserved intra-strain genetic heterogeneity within a population that compromises homozygous cells (Sterkers *et al.*, 2014). The frequent copy number variation of chromosomes and genes as described previously allows for a dynamic adaptation mechanism for the parasite based on its surrounding environment such as drug exposure (Ubeda *et al.*, 2008). This is not only true in situations where the parasite jumps from one host to another, but this dynamic can also allow for genetic/karyotypic and tropism diversity (Bussotti *et al.*, 2018; Rogers *et al.*, 2011; Sterkers *et al.*, 2014) or enhanced drug resistance (Downing *et al.*, 2011; Ubeda *et al.*, 2008). Finally, it should be mentioned that this genetic diversity can further be diversified due to genetic exchanges between strains, species or even parasites within the same population. As a matter of fact, there is a dozen of studies that prove this phenomenon and many studies mention the existence of hybrid strains of parasites; however, we shall discuss this more thoroughly in chapter 4.

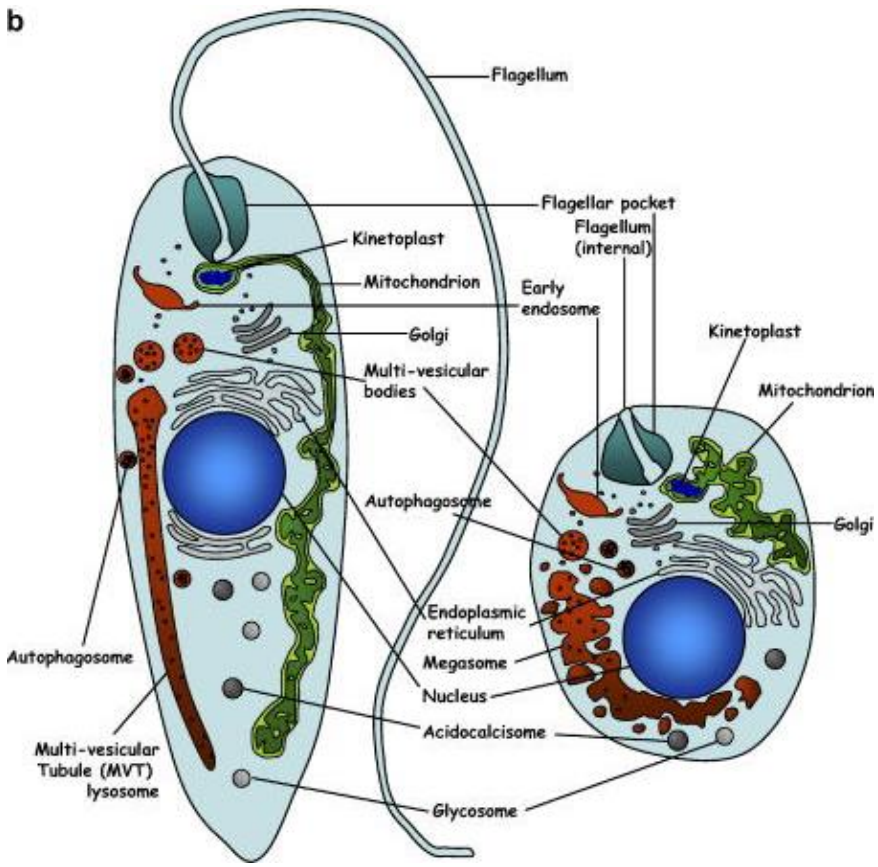

Figure 1.4: Representation of a *Leishmania* promastigote and amastigote

The promastigote and amastigote forms of the parasite are different in physical shape but the interior remains the same. The representation also shows the location of the nucleus and the kinetoplast in the mitochondrion (Besteiro *et al.*, 2007)

CHAPTER 3: HOST-PATHOGEN INTERACTIONS

3.1 Parasite entry into host

As discussed in chapter 1, the immune system of any living organism exists to protect it from exogenous pathogenic organisms. Although the immune system is able to clear a vast majority of pathogens in the form of bacteria, fungi, viruses and parasites, some of these pathogens such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* have evolved ways to survive this onslaught within the host or within the infected cells (Flannagan *et al.*, 2009). *Leishmania* parasites represent another group of pathogens that are able to avoid the host's immune response and prevail within it.

Interestingly, not all *Leishmania* will live to replicate within infected host cells. As a matter of fact, during an infection, there is a sub-population of dead parasitical cells that is present and these

cells are vital for the development of the infection (Seguin & Descoteaux, 2016). Consequently, it was demonstrated that the absence of dead cells results in less virulent parasite infections that are quickly cleared in otherwise susceptible mice (van Zandbergen *et al.*, 2006). The mortality of the parasites is a naturally occurring event within the vector and generates parasites that express PtdSer on their surface like apoptotic cells (Wanderley *et al.*, 2013). These cells induce an anti-inflammatory response upon their recognition by phagocytes which in turn will secrete large amounts of anti-inflammatory cytokines IL-10 and TGFß and this will help the parasites invade the host cells without raising the alarm (Wanderley *et al.*, 2013). However, this particular tactic is not restricted to *Leishmania* alone since other pathogens such as *Trypanosoma cruzi* or *Toxoplasma gondii* are able to use the same strategy to silently infect host cells (Damatta *et al.*, 2007; Seabra *et al.*, 2004).

On the other hand, live parasites begin their path in the invasion of the mammalian host during a sand fly's blood meal. Once inside, the primary objective of the parasites is to go deeper into the tissues and, at the same time, get to their target cells without raising an immune alarm. The parasites achieve this by employing a multitude of molecules, known as virulence factors, to ensure their survival within the host (Atayde *et al.*, 2016). One of such factors is the zinc-metalloprotease GP63, otherwise known as leishmaniolysine, that is one of the most abundant surface proteins and highly conserved genes of *Leishmania* parasites (Atayde *et al.*, 2016). GP63 plays a very important role before the internalization of the parasite into phagocytic cells as well as in the early stages of survival in those cells. It has been shown that *L. major* deficient in GP63 display lower virulence both in *in vitro* and *in vivo* settings or *L. amazonensis*, whose GP63 levels were down-regulated, were more susceptible to complement-mediated killing as well as being less infective in mice (Joshi *et al.*, 2002; Seguin & Descoteaux, 2016; Thiakaki *et al.*, 2006). First of all, GP63 has been shown capable of degrading components of the extracellular matrix such as collagen type IV and fibronectin, which facilitates the migration of the parasite to the dermis (McGwire *et al.*, 2003; Seguin & Descoteaux, 2016). It also has a role in disrupting the membrane attack complex (MAC) formation by binding and directly cleaving the C3b component into iC3b, an inactive form of C3 (Brittingham *et al.*, 1995). The complement system acts in a cascade where the first protein will recruit and activate the second one and continue the chain of activations. C3b has a very important role of both opsonizing the target pathogen and binding with the CP/LP C3 convertase complex which will shift its activity to a C5 convertase (Ricklin *et al.*, 2016). The C5 convertase will then activate the C5 component which will then recruit the C6, C7, C8 and multiple copies of the C9 and form the MAC (Ricklin *et al.*, 2016). Since the parasite inactivates the C3 component, it will thus deny the MAC formation and ultimately avoid complement-mediated lysis.

In addition to shutting off the complement system, the parasites also use iC3b to bind the complement receptors CR1 and CR3 (Mac-1) which enhances the parasite's phagocytosis by macrophages (Brittingham & Mosser, 1996; Mosser & Rosenthal, 1993; Podinovskaia & Descoteaux, 2015). Internalization through these receptors is very beneficial for the parasite since it inhibits inflammation (Podinovskaia & Descoteaux, 2015).

Alternatively to secreting virulence factors into the extracellular space, *Leishmania* is also able to secrete exosome-like vesicles. The group of Silverstein *et al* first reported this phenomenon (Dong *et al.*, 2019; Silverman *et al.*, 2010a); however, the first evidence of this was obtained through the study of *L. mexicana* exoproteome which revealed that these exosomes are secreted at both 26˚C (mimicking the internal body temperature of the sand fly vector) and 37˚C (mimicking the internal body temperature of the mammalian host) (Hassani *et al.*, 2011). Additionally, it was found that such a temperature shift, that mimics inoculation into the host, augmented rapidly the number of secreted vesicles released from the parasites (Hassani *et al.*, 2011). These microvesicles were found to be enriched with the surface protease GP63 as well as other virulence factors that were found to have important immunomodulatory functions (Atayde *et al.*, 2015; Hassani *et al.*, 2014; Silverman *et al.*, 2010a). For instance, it was found that the exosomes of GP63 depleted *L. major* parasites were less capable of immunomodulatory properties than their wild type counterparts through the modulation of protein tyrosine phosphatases and transcription factors (Hassani *et al.*, 2014). Moreover, it was found that the lack of GP63 on these microvesicles had led to a drastic change in protein composition as well (Hassani *et al.*, 2014). The microvesicles also have a role in tampering with the signaling pathways of the immune cells (Silverman *et al.*, 2010a; Silverman *et al.*, 2010b). For example, *L. donovani* microvesicles were capable of modulating the human IFN-γ stimulated monocytes cytokine secretion by inhibiting TNF-α production and promoting IL-10 production (Silverman *et al.*, 2010b). Although these studies clearly show that parasite derived exosomes have a role in host immune system modulation, most of these studies used vesicles derived from parasite culture supernatants or various biological fluids. Up to date, there seems to be only one study demonstrating that *Leishmania* exosomes are produced within the insect vector midgut and are secreted into the mammalian host along with the parasites during a bloodmeal (Atayde *et al.*, 2015). Intriguingly, Atayde *et al* also demonstrated that co-inoculation of both exosomes and parasites augmented skin lesion as a result of augmented pro-inflammatory cytokine synthesis one of which was identified as IL-17α (Atayde *et al.*, 2015). IL-17α, in turn, has been shown to be a key player in neutrophil recruitment during the development of *Leishmania*-induced lesions (Boaventura *et al.*, 2010; Lopez Kostka *et al.*, 2009) and it was demonstrated that

exosomes also play a role in the recruitment of neutrophils and other immune cells (Hassani *et al.*, 2014). Finally, exosomes were also found to be enriched with various small RNAs; however, their role in infections is yet to be determined (Lambertz *et al.*, 2015). In general, the release of extracellular vesicles by the parasite has an important role in the establishment of infection.

Once inside the mammalian host the parasite has to deal not only with passive threats in the form of complement and others, but also with various immune cells that rush to the injured tissues to repair the damage and remove any threats that have entered from the surface. The first cells to arrive to the site of infection are neutrophils followed by macrophages, monocytes and dendritic cells (Seguin & Descoteaux, 2016). Cell migration is also enhanced to the site of infection up to three-fold by the saliva of the sand fly (Rogers & Titus, 2003; Seguin & Descoteaux, 2016). The type of cells and their proportion at the site of infection may vary based on which species of *Leishmania* are involved in the infection. In one study for instance, *L. chagasi* infection causes many cell populations to migrate to the inflammatory exudate; however, the majority of the cells stay in the lining tissue around the wound (Vasconcelos *et al.*, 2014). In addition, the parasites cause an influx of more neutrophils rather than other professional phagocytes which helps minimize the initial inflammatory response (Vasconcelos *et al.*, 2014). Usually, during an inflammatory challenge, neutrophils will live for 6-8 hours after being primed and then they are picked up by macrophages and dendritic cells (Summers *et al.*, 2010). *Leishmania* learned to exploit this pathway and use the neutrophils as a natural “Trojan Horse”. As a matter of fact, it was shown that neutrophils which successfully capture parasites will transmit them to macrophages/dendritic cells upon apoptotic neutrophil uptake (Laskay *et al.*, 2003; Peters *et al.*, 2008; Ribeiro-Gomes *et al.*, 2012; van Zandbergen *et al.*, 2004). The parasites were even found to enhance the infected neutrophils’ expression of apoptotic markers than their non-infected counter parts (Ribeiro-Gomes *et al.*, 2012). Moreover, these cells do not provide an adequate environment for replication and therefore, the parasites are capable of delaying the neutrophils apoptotic death program up to 42 hours to maximize their chances for uptake by macrophages and other phagocytes (Aga *et al.*, 2002; Ribeiro-Gomes *et al.*, 2012; van Zandbergen *et al.*, 2004). Hence, this pathway provides another major way for the parasite to infect the host without raising an immunological response. Interestingly, it was demonstrated in one study that depletion of neutrophils in susceptible BALB/c mice drove resistance to *L. major* infection (Tacchini-Cottier *et al.*, 2000). Neutrophils also have the ability to release DNA and a subset of cytosolic and granular proteins which form a web-like structure collectively known as neutrophil extracellular traps (NETs) (Papayannopoulos, 2018). NET release occurs though a process known as NETosis

which can be rapidly induced by contact with a pathogenic organism, including *Leishmania* (Gabriel *et al.*, 2010; Guimaraes-Costa *et al.*, 2009; Papayannopoulos, 2018). Their primary role is to kill and capture various pathogens and prevent their dissemination in the organism; however, in the case of *Leishmania*, the outcome varies according to the parasite species involved in the infection (Seguin & Descoteaux, 2016). Thus, some species, such as *L. amazonensis*, will be killed by NETs (Guimaraes-Costa *et al.*, 2009) and others, such as *L. donovani* and *L. infantum*, are able to evade these traps via expression of LPG on their surface or via 3'-Nucleotidase/Nuclease enzyme activity respectively (Gabriel *et al.*, 2010; Guimaraes-Costa *et al.*, 2014).

Another cell type that the parasites encounter on their way in are NK cells. NK cells are immune players that have active roles in both the innate and adaptive immunities. They produce a lot of pro-inflammatory cytokines, such as IFN-γ, upon their activation by cytokines such as IL-2 or IL-12, thus favoring a Th1 response (Scharton & Scott, 1993; Tosi, 2005; Vivier *et al.*, 2008). Their activation also does not require priming by DC cells. NK cells destroy pathogens by releasing cytotoxic granules containing granzymes and perforins which lyse their targets (Tosi, 2005). Although during *Leishmania* infection the NK cells are activated normally and they function as usual, the parasites have developed a way to counteract them. It was found that GP63 was able to inactivate NK cells directly by preventing their proliferation and the expression of their surface receptors (Lieke *et al.*, 2008). This ultimately helps to thwart a restrictive Th1 response to a more permissive Th2 response for the parasite.

Once the parasite had past all of these dangers, it will finally encounter that for what it has come for: the macrophage cell. The parasites employ many strategies to enter their target cell. Higher up, we have demonstrated that parasites may enter the macrophage via complement receptors CR1 and CR3 or via phagocytosed infected neutrophils; however, these are not the only ways that the parasite can gain access to the cell. In fact, different species of *Leishmania* may be recognized by different receptors on the macrophage such as mannose receptors, FcγRs and fibronectin receptors (Ueno & Wilson, 2012). In addition to receptor mediated uptake, the parasites may also be internalized via the caveolae pathway as well (Podinovskaia & Descoteaux, 2015). Regardless of how the parasite will be internalized by the macrophage, the rest of the way will follow the standard phagocytic pathway. From this point on, the parasites will start their metamorphosis to amastigotes and convert the phagosomal vacuole into their home.

3.2 Vacuole formation and intracellular survival

Even if *Leishmania* manages to survive the initial dangers of the host immune system, once inside the macrophage, the parasite must still fight for its survival by modulating the biogenesis of the phagolysosome and converting the phagosome into a parasitophorous vacuole (PV) in which it will thrive and replicate in. It must absolutely be noted that most species replicate in tight individual vacuoles; however, species of the *Leishmania mexicana* complex (*L. mexicana*, *L. amazonensis, L. venezuelensis, L. pifanoi*) replicate in spacious communal vacuoles (Antoine *et al.*, 1998; Okuda *et al.*, 2016; Real & Mortara, 2012). Single and communal vacuole modifications differ from one another by how they are formed; however, up to date, single vacuoles are best understood. These modifications have been shown to depend on *Leishmania* virulence factors as well. In the case of *L. donovani* and *L. major*, this modulation of the phagosome to PV is caused by the parasite inserting its LPG into the phagosomal membrane which disrupts phagosomal lipid microdomains (Seguin & Descoteaux, 2016). The surface protein LPG was shown to arrest phagosome maturation by inhibiting its fusion with late endosomes via impairment of the recruitment of Rab7, retention of the actin polymerization activity along with the accumulation of periphagosomal F-actin and delayed recruitment of LAMP-1 (Dermine *et al.*, 2000; Desjardins & Descoteaux, 1997; Holm *et al.*, 2001; Lodge & Descoteaux, 2005; Moradin & Descoteaux, 2012; Scianimanico *et al.*, 1999; Spath *et al.*, 2003). Aside for the sabotage mentioned higher up, this disruption also causes failure in NADPH oxidase assembly on the phagosome membrane which reduces ROS production in *L. donovani* infected cells (Lodge *et al.*, 2006). Additionally, it was found that *L. donovani* LPG prevents the recruitment of Synaptotagmin V to the phagosomal membrane which prevents in turn the recruitment of vesicular proton-ATPases and phagosome acidification (Vinet *et al.*, 2009). LPG is a key molecule that ensures the survival of parasites such as *L. donovani* and *L. major* within the host cells since LPG-deficient parasites simply fail to establish a proper infection and ultimately perish. However, this is not true for all parasite strains. In the case of *L. amazonensis* and *L. mexicana* LPG does not play a role in intracellular survival or phagosome maturation. Indeed, one study revealed that *L. amazonensis* containing phagosomes fuse without much problem with late endosomes and lysosomes regardless of whether the parasites were LPG deficient or not (Courret *et al.*, 2002). The same conclusions were drawn earlier in time for *L. mexicana* as well; that is LPG deficiency does not play a role on their virulence or intracellular survival (Ilg, 2000). In fact, it is hypothesized that, unlike other *Leishmania* species which arrest phagosome maturation to promote their survival within the host cell, parasites of the *L. mexicana* complex replicate in large PVs, because it may provide protection against microbicidal properties of the phagosome (Wilson *et al.*, 2008). The size of

communal vacuoles is due not only to their fusion with various endosomes and lysosomes but also due to fusion between different vacuoles with the same parasite strains or different ones (Real & Mortara, 2012; Real *et al.*, 2010; Real *et al.*, 2008) and potentially with phagosomes containing inert particles, colloids, macromolecules and other microorganisms (Alexander & Vickerman, 1975; Berman *et al.*, 1981; Collins *et al.*, 1997; Rabinovitch *et al.*, 1985; Shepherd *et al.*, 1983; Veras *et al.*, 1992; Veras *et al.*, 1996). Although LPG has a critical role in ensuring the survival of the promastigote at the earlier stages of infection, its role becomes secondary during the amastigote stage. As a matter of fact, this molecule is highly down-regulated at the amastigote stage (Bahr *et al.*, 1993). As a result, there is less perturbation in the phagosomes' membrane which leads to the acidification of the compartment and fusion with lysosomes; however, it is not a deadly condition for the amastigote since its optimal pH is between 4.0 and 5.5 (Mukkada *et al.*, 1985). Aside LPG, as seen higher up, there are other virulence factors that may contribute to parasite survival in host cells. As an example, GP63 was found to disable antimicrobial peptides, such as cathelicidins and defensins, that may still present a serious threat for the parasites within the phagosomal compartment (Kulkarni *et al.*, 2006).

3.3 Host immune response modulation

Once the parasite has successfully navigated through the treacherous innate immune system components and has established itself safely within the infected macrophage, it will also have to hamper with the inflammatory signaling pathways and the immune system in general to prevent the host from establishing an effective defense to eliminate the pathogen. The parasite employs an array of virulent proteins such as cysteine proteases (CP), kinetoplastid membrane protein-11 (KMP-11), serine peptidase inhibitors and others to achieve this (Figure 1.5) (Atayde *et al.*, 2016; Podinovskaia & Descoteaux, 2015; Seguin & Descoteaux, 2016). One of the more important proteins in immune system modulation is the metalloprotease GP63 that we have described since the start of this chapter. In fact, this metalloprotease has been shown to activate protein tyrosine phosphatases (PTP) such as PTP1B and SHP-1 which will act in an inhibitory fashion on JAK2/STAT1, MAPK and IRAK-1 pathways (Abu-Dayyeh *et al.*, 2008; Blanchette *et al.*, 1999; Forget *et al.*, 2006; Gomez *et al.*, 2009; Martiny *et al.*, 1999). For example, GP63 activated SHP-1 can dephosphorylate JAK2 which will prevent further signaling through this pathway (Gomez *et al.*, 2009). Of note, the parasites can further block the JAK2/STAT1 pathway by proteasome-mediated degradation of the STAT-1 molecules (Forget *et al.*, 2005). Furthermore, GP63 is capable of directly inactivating transcription factors AP-1 and NF-κB (Contreras *et al.*, 2010; Isnard *et al.*, 2012). Finally, GP63 was shown to cleave mTOR, a kinase responsible in regulating

the translational repressor 4E-BP1 (Jaramillo *et al.*, 2011). As a result, mTOR cleavage leads to 4E-BP1 activation which will inhibit mTOR complex 1 assembly and thus promote *Leishmania* proliferation (Jaramillo *et al.*, 2011). All of these actions will lead to the inhibition of the production of inflammatory molecules such as IL-12, TNF-α, IFN-γ and nitric oxides (NO) which were identified as host factors that are important for effective control of the infection (Podinovskaia & Descoteaux, 2015; Seguin & Descoteaux, 2016). Another pathogenicity factor is CPB which was found to be important for *L. mexicana* since parasites deficient of this protein showed reduced pathogenicity (Casgrain *et al.*, 2016; Denise *et al.*, 2003). CPB was also shown to act on host macrophage PTP as well as the AP-1, NF-κB and STAT1 transcription factors which resulted in similar effects seen higher up, such as reduced IL-12 expression (Abu-Dayyeh *et al.*, 2010). In short, *Leishmania* utilizes various pathogenic factors to modify host cell signaling pathways in order to further prevent any inflammatory activation and successfully carry out their life cycle.

Tampering with the host cells' signaling is not the only aspect the parasites should take care of to ensure safety within the host cell. Modulation of the host's adaptive immune response is yet another important step in preventing its elimination. Indeed, it was revealed that GP63 of *L. major* and *L. donovani* was capable of cleaving the co-receptor CD4 on T-cells which potentially reduces their response to APCs and thus prevent their activation (Hey *et al.*, 1994). Moreover, both of these parasites were found to inhibit macrophage and DC cross-presentation in a GP63-dependant manner (Matheoud *et al.*, 2013). This is achieved via direct cleavage of the SNARE Vamp-8 that results in the prevention of the NADPH-oxidase NOX2 complex recruitment on phagosomes which leads to decreased ROS production, preventing acidification and inhibiting MHC class I antigen presentation, known as cross-presentation, thus reducing CD8+ T-cell activation (Dingjan *et al.*, 2017; Matheoud *et al.*, 2013). Additionally, the parasites are also capable of modulating MHC class II-dependent antigen presentation as well; however, different parasite species deal with this mechanism in different manners (De Souza Leao *et al.*, 1995; Meier *et al.*, 2003; Podinovskaia & Descoteaux, 2015; Roy *et al.*, 2014). Aside for acting directly on MHC class molecules, *Leishmania* can disrupt the adaptive immune system activation by other means. For instance, it was shown that the parasite increases cellular membrane fluidity which prevents proper antigen presentation between APCs and T-cells (Chakraborty *et al.*, 2005). The parasites may also modulate other co-stimulatory molecules required for signaling between the APCs and T-cells. One such molecule is the co-stimulatory molecule B7 which is down-regulated during *Leishmania* infections and thus prevents proper T-cell activation (Kaye *et al.*, 1994; Saha *et al.*, 1995). Other co-stimulatory that are down-regulated by the parasite are CD40 and CD86

which were found to prevent the proper maturation of DC cells and thus promote a silent infection (Neves *et al.*, 2010). Moreover, CD40 down-regulation by *L. major* was found to anergize T-cells and promote regulatory T-cell development in the context of low expression of CD40 which as a whole plays a role in successful infection by the parasite (Campbell *et al.*, 1996; Martin *et al.*, 2010). Finally, it was demonstrated that during visceral leishmaniases CD8+ effector T-cells were inefficient in producing IFNγ and expressed four-fold higher levels of CTLA-4 and PD-1 ligands featuring markers of exhausted and anergic cells (Gautam *et al.*, 2014). A similar occurrence was also described for CD4+ T cells as well (Esch *et al.*, 2013).

Table 2. *Leishmania* intracellular survival factors and their role in *Leishmania*–macrophage interactions.

Name	Description	Role in host–parasite interactions
LPG	Lipophosphoglycan	Activates MAPK, disrupts lipid rafts, ↑ TNF, ↑ IL-1β,↑ IL-6, ↓ TLR9, ↓ recruitment of Syntaptotagmin V, NADPH oxidase and V-ATPase to PV, scavenges ROS, ↑ HO-1
GP63	Zinc-dependent metalloprotease	Activates PTPs, p130Cas, Cortactin, Caspase 3 ↓ miRNA-122, ↓ TNF, ↓ IL-12, ↓ NO, ↓ mTOR, ↓ AP-1
ISP	Inhibitor of serine peptidase	↓ Neutrophil elastase, ↓ trypsin, ↓ chymotrypsin, ↓ TLR4 activation, ↓ Protein kinase R activation
Prohibitin	Prohibitin ortholog	Interacts with host HSP70, ↑ parasite uptake
PKC-like	Protein Kinase C ortholog	↑ Parasite phagocytosis
ISCL	Inositol phosphosphingolipid phospholipase C-like	↑ Survival and replication
Aldolase	Fructose-bisphosphate aldolase	Activates SHP-1, ↓ acidification
MsrA	Methionine sulfoxide reductase A	↑ Resistance to ROS/RNI
ALO	Arabino-1,4-lactone oxidase/vitamin C biosynthesis	↑ Resistance to ROS/RNI, ↓ IL-12, ↓ TNF
TXNPx	Tryparedoxin peroxidase	Detoxifies ROS/RNI, ↓ NRAMP-1, Fe redistribution
Thioredoxin	ROS scavenging enzyme	Stabilizes PTPs, ↓ proinflammatory pathways
CPB	Cysteine protease	Activates PTPs, ↓ activation, ↓ NO
MIF	Macrophage migration inhibitory factor ortholog	Activates MAPK, ↓ apoptosis

Figure 1.5: Functions of *Leishmania* virulence factors

The parasite employs various virulence factors that help seize the host cell and modulate the immune activation response (Podinovskaia & Descoteaux, 2015)

CHAPTER 4: *LEISHMANIA* GENETIC EXCHANGES

4.1 Occurrence of natural hybrids in nature.

As stipulated before, *Leishmania*'s main mode of reproduction is asexual. *Leishmania* was considered to be a strictly clonal organism incapable of any genetic exchange in the past. In fact, many protozoan parasites, such as trypanosomatids which include *Leishmania* and *Trypanosoma* parasites, were considered strictly clonal based on strong linkage disequilibrium observations and

a few more factors (Schmid-Hempel *et al.*, 2011; Tibayrenc & Ayala, 2013), despite evidence of hybrid parasites found in the wild. This is known as the clonal theory and it also dictates that all these parasitic pathogens undergo rare genetic exchange events that are not frequent to break the pattern of preponderant clonal evolution (Tibayrenc & Ayala, 2013). Moreover, the diversity of *Leishmania* species was considered to be due to gradual accumulation of divergent mutations rather than by genetic recombination (Rougeron *et al.*, 2017; Schmid-Hempel *et al.*, 2011; Tibayrenc & Ayala, 2013). However, there is also another opinion suggesting that genetic exchanges are not as rare and that *Leishmania* actually uses a mixed mating strategy where clonality is used in an unchallenged stable environment for quick dissemination and genetic exchanges occur when the parasite encounters a novel challenge in order to promote parasite fitness (Rougeron *et al.*, 2017). In any case, regardless of the different opinions, the fact that genetic exchanges occur in *Leishmania* is now widely accepted. However, a considerable amount of debate is still present over how frequent such processes take place and what are their impact on the population structure (Rougeron *et al.*, 2017; Tibayrenc & Ayala, 2013).

As proof that genetic exchanges actually occur among the parasites, natural occurrence of hybrid parasite species in the wild has been reported for a very long time. As a matter of fact, there are dozens of papers that describe *Leishmania* hybrid parasites that were discovered in the new world, although the majority are described as *L. braziliensis*/*L. peruviana* (Banuls *et al.*, 1997; Banuls *et al.*, 1999; Belli *et al.*, 1994; Bonfante-Garrido *et al.*, 1992; Cortes *et al.*, 2012; Cupolillo *et al.*, 1997; Delgado *et al.*, 1997; Dujardin *et al.*, 1995; Jennings *et al.*, 2014; Kato *et al.*, 2016; Kato *et al.*, 2019; Nolder *et al.*, 2007; Torrico *et al.*, 1999). In addition to New world species, hybrid parasites were reported for Old world species (Evans *et al.*, 1987; Kelly *et al.*, 1991; Odiwuor *et al.*, 2011) and between divergent species (Cortes *et al.*, 2019; Ravel *et al.*, 2006; Seblova *et al.*, 2015; Volf *et al.*, 2007). Lastly, it is important to note that natural hybridization at the intraspecific level of *L. infantum*, *L. donovani*, and *L. tropica* has been reported via whole genome sequencing studies (Chargui *et al.*, 2009; Cotton *et al.*, 2020; Iantorno *et al.*, 2017; Rogers *et al.*, 2014) and microsatellite identification as well (Gelanew *et al.*, 2014; Rougeron *et al.*, 2009). These studies clearly show that *Leishmania* are not as clonal as previously believed.

4.2 *Leishmania* genetic exchanges in different experimental conditions

Aside the studies that demonstrate the existence of hybrids in the wild, there are also studies which have revealed the ability of the parasites to exchange genetic material within an infected sand fly host. The first such study was conducted by Akopyants *et al* in 2009, where they showed

that two *L. major* strains harboring in their genome two different selectable drug-resistance markers co-infected in the same sand fly gave rise to a population of parasites that harbored both drug-resistance genes (Akopyants *et al.*, 2009). Next, it was shown that *L. major* strains from 4 distinct geographical regions were capable of exchanging DNA in an intraspecific manner (Inbar *et al.*, 2013). Moreover, a few other studies have shown that by co-infecting sandflies with parasite strains harboring two different drug selectable markers and/or two different fluorescent markers gave rise to hybrids between *L. donovani* strains, *L. infantum* strains and *L. tropica* (Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2019; Sadlova *et al.*, 2011). Finally, there is one study that demonstrated that hybrid formation from the cross of *L. infantum* and *L. major* by the presence of two selectable drug-resistance markers is possible within an infected vector (Romano *et al.*, 2014) and, to date, this is the only interspecies cross that has been shown to occur in an experimental setting and isolated in the wild (Ravel *et al.*, 2006; Romano *et al.*, 2014). Interestingly, the double drug-resistant parasites were all found to be full genomic hybrids that inherited at least one chromosome from each parent; however, the progeny inherited the maxicircle DNA only from one parent (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014). Although there is evidence that the parasites are capable of genetic exchange, not all crosses seem to be productive. In fact, one group of researchers reported that they did not observe any hybrids from the cross of *L. turanica* and *L. major* which may imply that not all species are capable of such exchanges (Chajbullinova *et al.*, 2012). There may also be species-specific difference among *Leishmania* in terms of their genetic exchange capabilities where some may be more efficient than others (Chajbullinova *et al.*, 2012; Inbar *et al.*, 2019). Interestingly, it was revealed recently that full genomic hybrids between two *L. tropica* strains can also be formed in axenic cultures; however, the frequency of their formation was much less when compared to the frequency in the vector (Louradour *et al.*, 2020). However, this is only true for the parasites of *L. tropica* since previous attempts with *L. major* were not found to give rise to hybrid progeny in similar conditions (Akopyants *et al.*, 2009; Inbar *et al.*, 2013; Louradour *et al.*, 2020) and other crosses have not yet been tested.

As demonstrated above, there are studies which have shown that genetic exchange between *Leishmania* cells can occur within the sand fly vector. Therefore, it is widely believed that hybrid species arise only in the sand fly vector; however, there are no studies at the current time that demonstrate or deny the possibility of genetic exchanges within a mammalian host or in an *in vitro* cell culture. To date, only Akopyants and colleagues have attempted to test this hypothesis with *L. major* parasites and they were unsuccessful in isolating any hybrids from infected mice (Akopyants *et al.*, 2009). Hence, it is important to assess this possibility in the future.

4.3 Mechanism of genetic exchange in *Leishmania*

Over the past decades, genetic exchanges were studied among trypanosomatids and other protozoan parasites. They were all found to be capable of exchanging genetic material amongst each other; however, the mechanism of how this is achieved is not fully understood for all of them. It was found for example that *T. brucei* parasites were capable of exchanging genetic information within infected Tse-Tse flies (Gibson *et al.*, 2008; Peacock *et al.*, 2014). These parasites are capable of producing haploid gametes; however, this takes place only in the salivary glands of the fly (Gibson *et al.*, 2008; Peacock *et al.*, 2014). Additionally, *T. cruzi* was also found to be capable of such genetic manipulation; however, it is still uncertain by which mechanism this is achieved and whether it is done within the vector or the mammalian host (Berry *et al.*, 2019; Gaunt *et al.*, 2003; Schwabl *et al.*, 2019). Recent evidence seems to suggest that these parasites reproduce via a mechanism resembling classic meiosis (Schwabl *et al.*, 2019). Lastly, an interesting observation was recorded for the *Plasmodium* parasite as well. It is known that this species of parasite has a sexual life cycle within the mosquito vector and an asexual one in the mammalian host; however, Regev-Rudzki and colleagues showed that *P. falciparum* was capable of transmitting genes between one another in infected host cells. In fact, *P. falciparum* was capable of doing this by delivering drug-resistance genes via exosome-like vesicles derived from *P. falciparum*-infected red blood cells between infected cells (Regev-Rudzki *et al.*, 2013). As we can see, protozoan parasites employ various mechanisms for their reproductive means; however, what is known about *Leishmania*'s mechanism for genetic exchange?

Although hybrid *Leishmania* species were isolated from the wild and demonstrated to occur experimentally, not much is known about the mechanism that governs DNA exchanges in the parasites. This is due to the fact that such genetic events are rare and are very difficult to observe. Moreover, there were no gamete stages or cell fusions ever described or observed directly in sand flies. In fact, it was proposed that genetic exchanges in *Leishmania* could be explained via a parasexual process as described for some Fungi such as *Candida albicans* (Forche *et al.*, 2008; Sterkers *et al.*, 2014). This process involves the fusion of two parental cells followed by the generation of a transient polyploid cell which results in chromosome shuffling and random chromosome loss. Parasexuality generates different types of progeny and it best describes the process by which the parasites replicate, especially if we take into consideration their mosaic aneuploidy (Sterkers *et al.*, 2014). This process was considered to be the main mode by which *Leishmania* exchange genetic material, but this consideration was recently proven wrong. Currently, it is believed that *Leishmania* exchanges its genetic content via a meiosis-like

mechanism (Inbar *et al.*, 2019). This is supported by whole genome sequencing of hybrid parasites within *L. major*, *L. infantum*, *L. tropica* and between *L. major*/*L. infantum* which revealed that parental chromosome inheritance patterns were found in 97-99% of the time as would be expected under meiosis (Inbar *et al.*, 2019). Since no gametes were ever observed to be present in *Leishmania*, it was proposed that the parental cells must first fuse together in order to give rise to hybrid strains (Inbar *et al.*, 2019). Although most hybrids were reported as diploid (2n), there were some hybrids that have been reported as triploid (3n) or even quadruploid (4n) (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014; Sadlova *et al.*, 2011). In the case of the 3n progeny, it was suggested that one 2n parental cell failed to undergo meiosis and fused with a 1n cell form the other parent whereas the 4n progeny resulted in the fusion of two 2n parents (Inbar *et al.*, 2019; Louradour *et al.*, 2020). Although Inbar *et al* or any other group of scientists did not observe fusion between the parasite cells directly, such an event was recorded on film in 1990 *in vitro* between an *L. tropica* and *L. infantum* (Lanotte & Rioux, 1990). The fusion was mediated via the posterior extremities of each parasite and such an event was also observed in Giemsa-stained smears of *L. major* parasites as well (Sousa *et al.*, 1997). Moreover, nuclear fusion was also observed between the intracellular amastigote form of the parasite via quantitative microspectrophotometry suggesting that genetic exchanges may potentially occur within a mammalian host as well (Kreutzer *et al.*, 1994). However, such possibility has not yet been widely explored. Finally, meiotic gene orthologues were identified within the *Leishmania* genome which were found to be highly expressed in the metacyclic form of the parasite (Inbar *et al.*, 2013); however, this does not necessarily imply that they have a function in that process since in the past such orthologues were found in other asexual organisms and they had a different function (Weedall & Hall, 2015). Nevertheless, such genes may provide a clue indicating that it may be possible for the parasite to exchange genetic material especially with all the evidence listed above.

INTRODUCTION

Leishmaniases is a spectrum of human diseases ranging from a confined cutaneous lesion to progressive deadly visceral infections if left untreated. It is caused by the protozoan parasite *Leishmania*, a vacuolar pathogen that replicates within the phagolysosomal compartment of phagocytes. *Leishmania* is endemic in 98 countries, causing 1 million infections and resulting in 20 000-30 000 deaths yearly. Despite years of scientific advances, no effective and safe vaccines are yet available. Furthermore, current treatment is difficult to administer, expensive, and becoming ineffective due to the spread of drug resistance. There is thus a pressing need for the development of novel approaches to prevent and treat leishmaniases. The infection begins when a sand fly regurgitates the promastigote form of the parasite into the mammalian host during a blood meal. From there, they differentiate into amastigotes for replication within infected phagocytic cells. The diverse clinical outcomes associated with *Leishmania* infections are generally clearly associated to specific parasite species and strains. However, the contribution of parasite genotype to disease outcome remains largely unknown. Until recently, it was believed that the diversity of *Leishmania* species, arose by gradual accumulation of divergent mutations rather than by genetic recombination. However, the occurrence of hybrid strains in the wild suggests that genetic exchanges may occur naturally. Moreover, this was experimentally demonstrated by using two clones of *L. major* bearing distinct drug-resistance markers by Akopyants *et al*, who reported that promastigotes are capable of exchanging genetic material during their development within the sand fly vector. There were also intraspecies hybrids between *L.* major, *L*. tropica, *L. donovani*, *L. infantum* and interspecies hybrids between *L. major*/*L. infantum* isolated from crosses within infected sand flies. Whether *Leishmania* can undergo genetic exchanges inside the mammalian host is unknown.

Whereas most *Leishmania* species live in tight individual vacuoles, species of the *Leishmania mexicana* complex (*L. mexicana* and *L. amazonensis*) live in spacious communal vacuoles. One of the benefits of occupying such vacuole is the potential for genetic exchange. The hypothesis underlying this project is that **communal parasitophorous vacuoles harboring parasites of the *L. mexicana* complex provide an exceptionally advantageous environment for genetic exchanges.** To address this hypothesis, we propose the following objectives:

1- To determine whether genetic crosses occur in axenic cultures

2- To determine whether genetic crosses occur in *in vitro* infected macrophages

3- To determine whether genetic crosses occur *in vivo* in infected mice

Globally, this research project will allow us to determine whether genetic exchanges occur in spacious communal vacuoles infected with parasites of *L. mexicana* complex. This knowledge may have important implications for the spread of drug-resistance, diagnosis and treatment of *Leishmania* infections since hybrids may display complex genetic features and phenotypes.

SECTION 2: ARTICLE

Study on the occurrence of genetic exchange among parasites of the *Leishmania mexicana* complex

Étude sur l'occurrence d'échanges génétiques entre parasites du complexe *Leishmania mexicana*

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ARTICLE ABSTRACT

In *Leishmania*, genetic exchange has been experimentally demonstrated to occur in the sand fly vector and in promastigote axenic cultures and it involves a meiotic-like process. No evidence of genetic exchange in mammalian hosts have been reported so far, possibly due to the fact that the *Leishmania* species used in previous studies replicate within individual parasitophorous vacuoles. In the present work, we explored the possibility that residing in communal vacuoles may provide conditions favorable for genetic exchange for *L. mexicana* and *L. amazonensis*. Using promastigote lines of both species harboring integrated or episomal drug-resistance markers, we assessed whether genetic exchange can occur in axenic cultures, in infected macrophages as well as in infected mice. We obtained evidence of genetic exchange for *L. amazonensis* in both axenic promastigote cultures and infected macrophages. However, the resulting products of those genetic events were unstable as they did not sustain growth in subsequent sub-cultures, precluding further characterization.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the causative agents of a spectrum of diseases known as leishmaniasis that range from self-healing cutaneous lesions to destructive mucocutaneous infections and visceral pathologies. *Leishmania* has a distinct life cycle which consists of two specific environments. The first is that of the sand fly insect vector in which the parasites multiply within the alimentary tract under the promastigote form and the second is the infected mammalian or human hosts where the parasites replicate as amastigotes within the phagolysosomal compartment of host phagocytes. Currently, there are 20 known species of parasites that are associated with human disease. However, there is still a considerable amount of debate of whether this diversity is due to recombinational events or due to gradual accumulation of mutations during clonal division (Rougeron *et al.*, 2017; Tibayrenc & Ayala, 2013).

In eukaryotic pathogenic organisms, sex is one of the main mechanisms that allows the spread of pathogenicity, resistance and virulence genes (Heitman, 2010). However, due to very strong linkage disequilibrium observed in *Leishmania,* it has been argued that the reproductive mode of *Leishmania* is predominantly clonal (Tibayrenc & Ayala, 2013). There is however much evidence indicating that genetic exchange is part of the biology of *Leishmania* parasites, as evidenced by the occurrence of hybrids in nature. These natural hybrids were described at the intraspecific level for *L. tropica, L. donovani*, *L. infantum* and *L. brasiliensis* (Chargui *et al.*, 2009; Cotton *et al.*, 2020; Gelanew *et al.*, 2014; Iantorno *et al.*, 2017; Rogers *et al.*, 2014; Rougeron *et al.*, 2009). There were also reports of hybrids that originated from crosses between parasites of the *Viannia* subgenus such as *L. braziliensis* and *L. guyanensis*, which are one of the most common ones described (Banuls *et al.*, 1997; Banuls *et al.*, 1999; Belli *et al.*, 1994; Bonfante-Garrido *et al.*, 1992; Cortes *et al.*, 2012; Cupolillo *et al.*, 1997; Delgado *et al.*, 1997; Dujardin *et al.*, 1995; Jennings *et al.*, 2014; Kato *et al.*, 2016; Kato *et al.*, 2019; Nolder *et al.*, 2007; Torrico *et al.*, 1999). Natural hybrids were also reported for *Leishmania* species of the *Leishmania* subgenus such as *L. major* and *L. arabica*, *L. major* and *L. infantum,* as well as *L. donovani* and *L. infantum* (Cortes *et al.*, 2019; Evans *et al.*, 1987; Kelly *et al.*, 1991; Odiwuor *et al.*, 2011; Ravel *et al.*, 2006; Seblova *et al.*, 2015; Volf *et al.*, 2007).

Using two strains of *L. major* harboring distinct integrated drug-resistance markers, Akopyants and colleagues experimentally demonstrated the existence of genetic exchange in the invertebrate stage of the parasite (Akopyants *et al.*, 2009). By infecting sand flies and dissecting them 13-16 days post-infection, the double drug-resistant progeny of this cross was further

demonstrated to be actual genomic hybrids by confirming the presence of at least one set of allelic markers from each parent (Akopyants *et al.*, 2009). In another study from the same group, it was further shown that crosses in the invertebrate stage between *L. major* parasites coming from 4 distinct geographical locations are able to produce hybrid progeny, which also suggests that there are no intraspecies barriers when it comes to exchanging genetic information (Inbar *et al.*, 2013). Another interesting finding, was that hybrid formation was observed in both the natural *P.duboscqi* vector and in the unnatural but permissive *L. longipalpis* and, by isolating the parasites from infected sand flies 3-18 days post-infection, it was further ruled out that genetic exchange takes place between parasites when they are in the nectomonad form (Inbar *et al.*, 2013). In addition, a study based on microscopy and flow cytometry allowed to visualize evidence of genetic exchange between two strains of *L. donovani* expressing two different fluorescent molecules (RFP and GFP) which were present in the same vector (*P. perniciosus* or *L. longipalpis*) and gave rise to yellow promastigote progeny; however, these putative hybrids could not be recovered from the sand flies and grown in culture for further analyses (Sadlova *et al.*, 2011). There was also a study which demonstrated hybrid formation in sand flies between two *L. infantum* strains expressing different fluorescent as well as different drug-resistance markers (Calvo-Alvarez *et al.*, 2014) and another paper demonstrated the formation of hybrid parasite strains in sand flies between 2 entirely different species, namely *L. major* and *L. infantum* (Romano *et al.*, 2014). Finally, the ability of *L. tropica* to exchange genetic information in an intraspecific manner in an infected insect vector as well as in axenic culture has also been recently demonstrated using whole genome sequencing (Inbar *et al.*, 2019; Louradour *et al.*, 2020).

Despite the fact that hybrid parasites could be isolated both in nature and in laboratory conditions from infected sand flies, the mechanism by which they reproduce is still poorly understood. This is partially due to the fact that this is not an obligate mode of reproduction of the parasite; however, recent genome sequencing data from 44 hybrids generated between and within *L. infantum*, *L. tropica* and *L. major* suggest that *Leishmania* reproduces via a meotic-like mechanism (Inbar *et al.*, 2019). Apart from one study using *L. major* (Akopyants *et al.*, 2009), it is still not widely known whether or not genetic exchange can occur within an infected mammalian host, although there is a study that has shown previously by DNA quantification that infected macrophages could harbor 4N amastigotes suggesting that genetic exchange is possible in mammalian host cells (Kreutzer *et al.*, 1994). Here, we explored the possibility of intraclonal and interspecific genetic exchange among parasites of the *L. mexicana* complex, which unlike other *Leishmania* species, replicate in spacious communal vacuoles that may provide an environment favorable to genetic exchange (Case *et al.*, 2016).

MATERIALS AND METHODS

Ethics Statement

All animal handling was performed in accordance with the protocols 1806–01 and 1806–02, which were approved by the *Comité Institutionel de Protection des Animaux* of the INRS-Centre Armand-Frappier Santé Biotechnologie. These protocols respect procedures on animal practice as instructed by the Canadian Council on Animal Care, described in the Guide to the Care and Use of Experimental Animals.

Plasmids and constructs

The plasmid pLa*LPG2-HYG* from which the *LPG2::ΔHYG* targeting construct was used to create Hygromycin B-resistant parasites was kindly provided by Drs. Valeria M. Borges and Leonardo Paiva Farias (Fiocruz Bahia - Instituto Gonçalo Moniz, Brazil) (Figure 1). The plasmid pCR2.1-L.d-rDNA-pr-αIR*NEO*αIR-*GFP* from which the Ld-rDNA-*NEO-GFP* targeting sequence was used to create G418-resistant parasites was kindly provided by Dr. Barbara Papadopoulou (Université Laval, Canada) (Figure 1). The plasmid pKS-*NEO-DsRed*e was provided by Dr. David L. Sacks (National Institute of Allergy and Infectious Diseases, USA) (Kimblin *et al.*, 2008). The pLeish-*HYG-GFP* construct was created the following way: a *Sac*I fragment containing the *GFP* gene was excised from the plasmid pXG-*GFP*$^{+}$ (Ha *et al.*, 1996), blunted, and inserted into the *EcoR*V site of pLeish-*HYG* (unpublished), yielding pLeish-*HYG-GFP*.

Parasites

Both *L. amazonensis* LV79 (MPRO/BR/72/M1841) and *L. mexicana* (MNYC/BZ/62/M379) were passaged in mice to maintain their virulence. Amastigotes recovered from ear dermis lesions of infected C57BL/6 mice were differentiated into promastigotes in *Leishmania* medium (M199-1X (Sigma) with 10% heat-inactivated fetal bovine serum (FBS), 100 µM hypoxanthine, 3 µM biopterin, 40 mM HEPES at pH 7.4, 5 µM hemin, 1 µM biotin, and Penicillin-Streptomycin) in a 26°C incubator. For the generation of *L. amazonensis LPG2/LPG2::ΔHYG* and *L. mexicana LPG2/LPG2::ΔHYG*, log-phase *L. amazonensis* and *L. mexicana* promastigotes were electroporated with the *LPG2::ΔHYG* targeting construct (excised as a 2.6-kb *EcoRV*I-*Hind*III-*Bgl*I fragment from pLa-*LPG2*KO-*HYG*) in 0.2 cm electroporation cuvettes, at 0.45kV and 500 µF of high capacitance as previously described in similar protocols (Descoteaux *et al.*, 1994; Turco *et al.*, 1994). After electroporation, promastigotes were grown in drug-free *Leishmania* medium for

24 h. Following this incubation, *L. amazonensis LPG2/LPG2::ΔHYG* parasites were selected in the presence of 35 µg/ml Hygromycin B (Sigma) and *L. mexicana LPG2/LPG2::ΔHYG* parasites were selected in the presence of 70 µg/ml Hygromycin B (Sigma) respectively. For the generation of *L. amazonensis* +/*SSU::NEO-GFP*, *L. amazonensis* promastigotes were electroporated with the L.d-rDNA-*NEO-GFP* targeting construct (excised as a 4.25-kb *Bst*XI fragment from pCR2.1-L.d-rDNA-pr-αIR*NEO*αIR-*GFP*). After electroporation, the parasites were grown in drug free medium for 24 h and then grown in *Leishmania* medium containing 20 µg/ml of G418 (Life Technologies). *L. amazonensis NEO-DsRed*e parasites were obtained by electroporating *L. amazonensis* promastigotes with the plasmid pKS-*NEO-DsRed*e. Parasites were grown in drug free medium for 24 h and then grown in medium containing 20 µg/ml G418. The same method was used to obtain *L. mexicana* pKS-*NEO-DsRed*e and they were maintained in *Leishmania* medium containing 40 µg/ml of G418. *L. amazonensis HYG-GFP*e promastigotes were generated by electroporating *L. amazonensis* with the plasmid pLeish-*HYG-GFP*. Parasites were grown in drug-free medium for 24 h and then grown in medium containing 35 µg/ml Hygromycin B

Mammalian cell culture

Bone marrow-derived macrophages (BMM) were differentiated from the bone marrow of 6- to 8-week old C57BL/6 mice as previously described (Descoteaux & Matlashewski, 1989). BMM were differentiated for 7 days in complete DMEM (containing L-glutamine (Life Technologies), 10% v/v heat inactivated fetal bovine serum (FBS) (Life Technologies), 10 mM HEPES (Bioshop) at pH 7.4, and penicillin-streptomycin (Life Technologies)) supplemented with 15% v/v L929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor-1. To render the BMM quiescent prior to experiments, cells were transferred to tissue culture-treated 6-well or 24-well plates or T25 tissue culture flasks for 24 hours in complete DMEM without LCM. The cells were kept in a humidified 37˚C incubator with 5% CO_2. The number of macrophages used per container are as following: 2.2 X 10^6 BMMs per well of a 6-well plate, 0.3 X 10^6 BMMs per well of 24-well plate and 25 X 10^6 BMMs in T-25 flasks.

Transwell experiments

For genetic exchange transwell experiments, donor parasites (*L. amazonensis NEO-dsRed*e) were relocated to the to the insert chamber containing 0.4 µm pores in a polycarbonate membrane (Corning) and the recipient parasites (*L. amazonensis LPG2/LPG2::ΔHYG*) were added to the wells. The plates were then either incubated at 26˚C or pre-incubated at 34˚C for 4 hours, as done

previously (Hassani *et al.*, 2011), and then transferred to 26˚C. The parasites were collected at 24 h, 72 h, 96 h and 120 h post-incubation. Each parental stain was equally divided into 3 wells of a 6-well plate and were grown in the presence of antibiotics. Two wells were used as controls containing either 35 µg/ml of Hygromycin B or 20 µg/ml of G418 and the last well contained both drugs in the medium. The parasites were kept in such conditions up to 3 weeks. Each parental strain was also grown separately and were under the same conditions as a control.

Parasite co-culture experiments

As described (Louradour *et al.*, 2020), stationary phase promastigotes of two parental strains were mixed and distributed into 96-well plates up to a total volume of 100 µl in each well. One million parasites of each strain were added in the wells. Three days later, each co-culture from the 96-well plate was transferred to a single well of a 24-well plate containing 900 µl of *Leishmania* medium containing either 35 µg/ml Hygromycin B and 20 µg/ml G418 if both parental strains were *L. amazonensis* or 60 µg/ml Hygromycin B and 40 µg/ml G418 if one of the parental strains was *L. amazonensis* and the other was *L. mexicana.* Each line was cultured individually in *Leishmania* medium supplemented with either Hygromycin B or G418 or both drugs as controls. When double drug-resistant parasite cultures were growing in wells (growth was observed between 19 and 28 days), the cells were passaged in *Leishmania* medium at a dilution of 1:10. DNA was then extracted from double drug-resistant parasites and was used for PCR reactions.

In-vitro infections

Metacyclic promastigotes were isolated from promastigote cultures in the late stationary phase by means of a density gradient centrifugation (Spath & Beverley, 2001). Specifically, 2 ml of 40% w/v Ficoll PM400 (GE healthcare) were added to the bottom of ta 15 ml tube, followed by a 5 ml layer of 10% Ficoll PM400 in M199-1x and topped by late stationary phase promastigotes resuspended in 5 ml of DMEM with no FBS (Arango Duque *et al.*, 2019). Metacyclic promastigotes were collected from the DMEM-10% Ficoll interphase after spinning the gradient for 10 min. The percentage of isolated metacyclic parasites from the interphase generally varied from 12-18% of the input population. Metacyclic promastigotes were then opsonized with the serum of C57BL/6 mice for 30 minutes, washed 3 times with PBS and resuspended in cold complete DMEM (cDMEM). The parasites were then fed to macrophages adhered in T-25 flasks (Sarstedt) (Ratio 3:1 for single infections, ratio 6:1 for mix infections). The cells were then incubated at 4˚C for 10 minutes (Arango Duque *et al.*, 2019) to synchronize phagocytosis. The internalization of parasites

was triggered by transferring the cells to 34˚C (Arango Duque *et al.*, 2019). Two hours post-internalization, the cells were washed 3 times with warmed cDMEM to remove non-internalized promastigotes. Infected BMM were incubated for 120 h and 192 h. Next, the amastigotes were isolated from infected macrophages by resuspending those in cDMEM containing 0.05% of SDS. Shortly, the macrophages resuspended in 2ml of cDMEM containing SDS are incubated at 37˚C for 3 minutes. Then, the resulting supernatant is resuspended in 10 ml of cDMEM and spun at 3000 rpm. After the spin, the supernatant was discarded. The amastigotes were resuspended in *Leishmania* medium and separated into 3 separate conditions. The conditions were: *Leishmania* medium containing 20 µg/ml of G418 or *Leishmania* medium containing 32 µg/ml of Hygromycin B or *Leishmania* medium containing both drugs. The parasites were left for incubation at 26˚C for up to 3 weeks to select for double drug-resistant parasites. If applicable, the double drug-resistant parasites were passaged at a dilution of 1/10 and their DNA was then extracted and was used for PCR reactions. Double drug-resistant parasites were also passaged in infected BMM for 3 days as well. For parasite survival, cells were washed with PBS and fixed and stained with fixative and staining solutions of the Hema 3 stain set (Fisher Scientific). This process was done for 2 h, 48h ,120h and 192h timepoints.

Alternatively, the infections were done in 6-well plates instead of T-25 flasks. Three wells were used for mixed infection for each timepoint (120 h and 192 h) and two wells were reserved for infection with each parental strain alone. Once the amastigotes were obtained, they were plated in 96-well plates in 100 µl of drug free *Leishmania* medium as described in the parasite co-culture section. The amastigotes were plated at 5 million parasites per well. Three days later, each well was transferred to a well of 24-well plate that contained 900 µl with antibiotics. Pure parental cultures were used as controls as previously described. If applicable, the double drug-resistant parasites were passaged at a dilution of 1/10 and their DNA was then extracted from double drug-resistant parasites and was used for PCR reactions.

In vivo infections and parasite recovery

C57BL/6 mice (6- to 8-weeks old) were infected with 1 X 10^5 metacyclic promastigotes (5 X 10^4 of each line) of either *L. amazonensis LPG2/LPG2::ΔHYG + L. amaz +/SSU::NEO-GFP* or *L. mexicana LPG2/LPG2::ΔHYG + L. amaz +/SSU::NEO-GFP* into the ear dermis with an insulin syringe (29 G). Mice infected separately with each line were used as controls. At 9 weeks post-infection, mice were euthanized under CO_2 asphyxiation and by cerebral dislocation as well. The infected ears were then collected and disinfected in 70% ethanol for 10 min and air dried for 10

min. Then, they were separated into dorsal and ventral leaflets and cut up into small pieces with surgical scissors. The cut-up ears were loaded in 2.0 ml tubes containing zirconium beads (Benchmark Scientific Inc.) and resuspended in 1 ml of *Leishmania* medium and vortexed for a 1 min and 30 sec. The resulting suspension was then transferred to 100 µm cell strainers placed over 50 ml Falcon tubes and filtered to isolate the amastigotes. The remaining tissue in the cell strainer was smashed with a sterile 10ml syringe plunger and washed two times with *Leishmania* medium. The resulting cell suspension was spun at 3200 RPM at 4˚C for 10 min. The amastigotes were then separated in three T-25 flasks and left in unconditioned *Leishmania* medium for 24 hours. Lastly, the antibiotics were added to each flask according to each condition and were incubated at 26˚C for three weeks. The conditions were Hygromycin only, G418 only or both drugs.

DNA extraction and PCR confirmation of double-resistant parasites

For genotyping analyses, total DNA was extracted from parasites by using a phenol/chloroform treatment as previously described (Medina-Acosta & Cross, 1993). All of the PCR amplifications were done in 50 µl total volume containing 100 ng of parasite DNA and 10pmol of each primer. The following primer pairs were used: for Hygromycin B 5'-ATGAAAAGCCTGAACTCACC-3' (Forward), 5'-CTATTCCTTTGCCCTCGG-3' (Reverse) that were previously described (Romano, 2014); for G418 5'CCACGACGGGCGTTCCTTGCGCAGCTGTGC-3' (Forward), 5'-GTCAGCCCATTCG CCAAGCTCTTCAGC-3' (Reverse) which were custom made. The resulting DNA products were then verified by electrophoresis on 1.2 % Agarose gel and subsequently viewed by staining the samples with ethidium bromide.

Live Microscopy

BMMs were platted at the bottom of 6 well-plate with a coverslip attached to the bottom of the wells. The cells were kept in the 34˚C incubator for 24 h without LCM to render them quiescent. They were then infected with metacyclic parasites of each line separately as a positive control or with a combination of two. Non-infected cells were used as a negative control. The samples were then viewed with 63X objective lens LSM780 system confocal microscope (Carl Zeiss microimaging). The images were taken and processed with the ZEN 2012 Software (Carl Zeiss) and subsequently mounted into the figures via adobe photoshop 2019.

RESULTS

Generation of drug-resistant strains of *L. amazonensis* and *L. mexicana*

To investigate the possibility that formation of hybrids and genetic exchange may occur among parasites of the *L. mexicana* complex, we used *L. amazonensis* LV79 and *L. mexicana* M379 expressing either episomal or integrated genes encoding resistance to Hygromycin B (*HYG*) or to G418 (*NEO)*. To this end, we generated one line of *L. amazonensis* and one line of *L. mexicana* in which the *HYG* resistance gene was integrated in one allele of the *LPG2* gene (*L. amazonensis LPG2/LPG2::ΔHYG* and *L. mexicana LPG2/LPG2::ΔHYG*) (Fig 2.1A), one line of *L. amazonensis* in which a *NEO-GFP* construct was integrated into the ribosomal RNA locus (*L. amazonensis +/SSU::NEO-GFP*) (Fig 2.1A), one line of *L. amazonensis* and one line of *L. mexicana* with an episomal *NEO-DsRed* plasmid (*L. amazonensis NEO-DsRed*e and *L. mexicana NEO-DsRed*e), and one line of *L. amazonensis* with an episomal *HYG-GFP* plasmid (*L. amazonensis HYG-GFP*e). We confirmed the presence/absence of both resistance genes in each line by PCR analysis using specific primers against *HYG* and *NEO* (Fig. 2.1B), and we ensured that these drug-resistant recombinant parasites retained the ability to infect and replicate within bone marrow-derived macrophages (BMMs) over a period of 196 h (Fig 2.2).

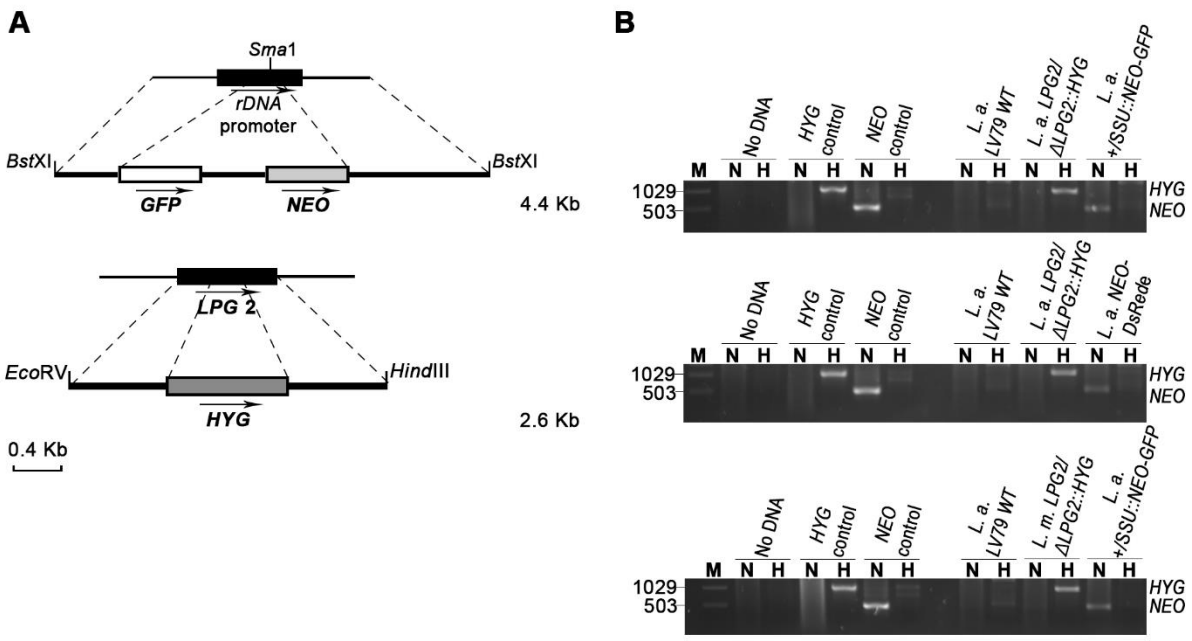


Figure 2.1: Generation of drug-resistant *Leishmania* parasites.

(A) L.d-rDNA-*NEO-GFP* and *LPG2::ΔHYG* targeting constructs were used for the integration into the ribosomal RNA locus or in one allele of *LPG2*, respectively. For the L.d-rDNA-*NEO-GFP* construct, the *NEO-GFP* resistance cassette (white and grey boxes) was inserted in the *SmaI* site of the ribosomal RNA locus (black rectangle). The dashed lines delimit the regions of recombination between the target genes and targeting constructs. Arrows indicate orientation. (B) PCR products for drug-resistance markers *HYG* and *NEO* of *L. amazonensis* and *L. mexicana* parental strains. The size of *HYG* and *NEO* resistance genes is 1029 bp and 503 bp long, respectively. The pLeish-*HYG-GFP* and the pKS-*NEO-DsRed* constructs were used as controls for the *HYG* and *NEO* genes, respectively. *L.a.* LV79 WT is a DNA sample used to show that our wild type parasites do not harbor any drug-resistance markers in their genomes. *L. amazonensis LPG2/LPG2::ΔHYG, L. amazonensis +/SSU::NEO-GFP, L. mexicana LPG2/LPG2::ΔHYG* and *L. amazonensis NEO-DsRede* are controls used to validate the presence of *HYG* and *NEO* resistance genes. No DNA sample was loaded as negative control. M, molecular DNA ladder; H, Hygromycin; N, G418.

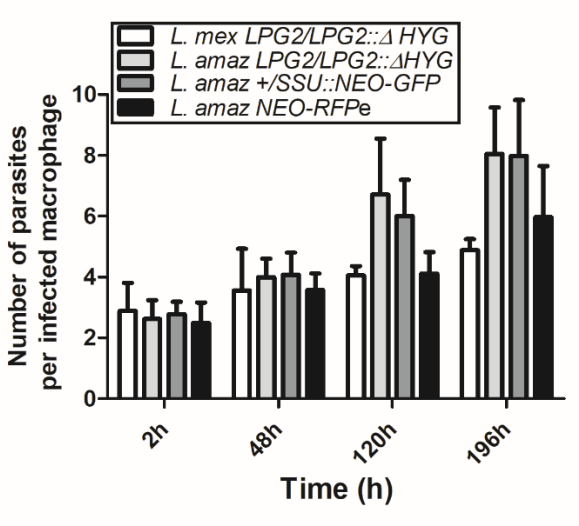


Figure 2.2: Survival of parental strains within infected macrophages.

BMMs were infected with metacyclic serum-opsonized promastigotes of *L. amazonensis* and *L. mexicana* parental strains (*L. amazonensis LPG2/LPG2::ΔHYG*, *L. mexicana LPG2/ LPG2::ΔHYG, L. amazonensis +/SSU::NEO-GFP, L. amazonensis NEO-DsRed*e) for 2 h, 48 h, 120 h and 196 h. Bars represent mean ± SE of three representative experiments performed in triplicate in bone marrow derived murine macrophages. Parasites were counted in 100 macrophages and quantified by light microscopy.

Drug resistance is not transferred in *in vitro* cultures of promastigotes in the absence of cell-to-cell contact

Evidence indicate that DNA can be transferred from cell-to-cell through extracellular vesicles (Elzanowska *et al.*, 2020). In addition, erythrocytes infected with *Plasmodium falciparum* can transfer parasite DNA to other infected cells via the release of extracellular vesicles (Regev-Rudzki *et al.*, 2013). Whereas no such mechanism has been described in *Leishmania*, it was recently reported that the *Leishmania* RNA virus 1 (LRV1) exploits the *Leishmania* exosomal pathway as a mode of transmission from one promastigote to another (Atayde *et al.*, 2019). This led us to verify the hypothesis that extracellular vesicles released in the culture medium may serve as a vehicle to transfer genetic material, including episomes harboring a drug-resistance gene among promastigotes. To this end, we used transwells (0.4 µm pores) to physically separate *L. amazonensis NEO-DsRed*e promastigotes from *L. amazonensis-LPG2/LPG2::ΔHYG* promastigotes. We incubated the transwell plates either at 26˚C or we pre-incubated them at 34˚C for 4 h and then transferred the plates to 26˚C. Such a transient increase in temperature has been previously shown to enhance the secretion of extracellular vesicles by *Leishmania* promastigotes (Hassani *et al.*, 2011). Promastigotes co-incubated in transwells at either 26°C or 34°C were collected after 24, 72, 96, and 120 h and assessed for their capacity to grow in the presence of both hygromycin and G418. Both *L. amazonensis-NEO-DsRed*e and *L. amazonensis-LPG2/LPG2::ΔHYG* were viable and resistant to G418 and hygromycin, respectively, up to 120 h of co-incubation in the transwells. However, no double drug-resistant parasites were recovered from 9 independent experiments performed in triplicate, indicating that exchange of genetic information through extracellular vesicles among *L. amazonensis* promastigotes, if it occurs, is a rare event.

Genetic exchange among *L. amazonensis* and *L. mexicana* promastigotes in axenic cultures

A recent study revealed that some strains of *L. tropica*, but not *L. major*, form hybrids in promastigote axenic cultures (Louradour *et al.*, 2020). This finding prompted us to evaluate the occurrence of genetic crosses among *L. amazonensis* and *L. mexicana* promastigotes in *in vitro* co-cultures. Following the experimental protocol described by Louradour *et al*, we co-cultured combinations of stationary phase promastigotes with integrated drug-resistance genes as depicted in Table 2.1 (Louradour *et al.*, 2020) . We also performed co-culture experiments using promastigotes harboring episomes (Table 2.1). Each co-culture was distributed into 96-well plates in drug-free medium. Three days later, the parasites were transferred into 24-well plates and cultured in selective medium (hygromycin B and G418) and left for up to 40 days in a 26˚C incubator. Individual single drug-resistant lines were used as controls and had gone through the same process. After 40 days of incubation, we did not obtain double drug-resistant parasites except for the co-cultures of *L. amazonensis-LPG2/LPG2::ΔHYG* and *L. amazonensis-NEO-DsRed*e (Table 2.1). We obtained promastigote populations resistant to both G418 and hygromycin B in 3 separate wells. However, only one out of 3 grew sufficiently to allow for DNA isolation and PCR analysis, which revealed the presence of both *HYG* and *NEO* genes (Fig. 2.3). However, we were unable to further characterize these double-drug-resistant parasites as they perished in subsequent passages. These results suggest that the occurrence of genetic exchange in axenic cultures among those two drug-resistant lines is rare and results in transient/unstable double drug-resistant promastigotes.

Table 2.1: Crosses used in axenic cultures.

Crosses in Axenic cultures	No. of wells with crosses	% Yield of double drug resistant parasites
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	192	0/192 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	192	3/192 (1.56%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	96	0/96 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	96	0/96 (0%)

Demonstrates the number of wells tested and percentage of isolated double drug-resistant parasites.

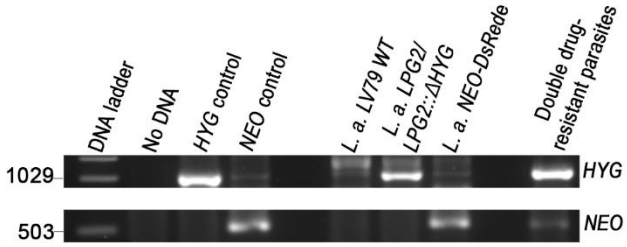


Figure 2.3: Genotype characterization of double drug-resistant parasites from axenic cultures.

PCR amplification of genes encoding antibiotic resistance. The size of *HYG* and *NEO* resistance genes is 1029 bp and 503 bp long. The pLeish-*HYG-GFP* and the pKS-*NEO-DsRed* constructs were used as controls for the *HYG* and *NEO* genes, respectively. *L.a.* LV79 WT is a DNA sample used to show that our wild type parasites do not express any drug-resistance markers. *L. amazonensis LPG2/LPG2::ΔHYG* and *L. amazonensis NEO-DsRede* are controls used to validate the presence of *HYG* and *NEO* resistance genes within the appropriate parental strains. No DNA sample was loaded as negative control.

Unstable genetic exchange in infected macrophages

The fact that *L. amazonensis* and *L. mexicana* replicate within communal parasitophorous vacuoles led us to verify the possibility that these intracellular replicative niches provide conditions propitious for genetic exchanges among *Leishmania* cells. To this end, we infected BMMs with the following four combinations of drug-resistant parasites: *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO- DsRed*e; *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*, and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e. Similar to single infection, parasites in mixed infections replicated up to 192 h post-infection and induced the formation of communal PVs (Fig 2.4A). To confirm that these communal PVs harbored both drug-resistant *Leishmania* lines, we performed live cell imaging on BMMs co-infected with either *L. amazonensis HYG-GFP*e + *L. amazonensis NEO-DsRed*e or *L. amazonensis HYG-GFP*e + *L. mexicana NEO-DsRed*e. In both cases, we observed the two drug-resistant parasite lines within the same communal vacuoles, at 48 h and 72 h post-infection (Fig 2.4B). At 120 h and 192 h post-infection, we lysed the infected BMM and cultured the recovered parasites in medium containing hygromycin and G418 in a similar fashion as the axenic parasite cultures done in plates. As shown in Table 2.2, we failed to recover any double drug-resistant parasites from these co-infection experiments. Next, we modified our experimental approach to perform co-infection experiments on a larger scale, with the following 3 combinations of drug-resistant promastigotes: *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*, *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e, and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP* (Table 2.3). We co-infected BMMs with each combination and we used each individual drug-resistant line as controls. Out of a total of 28 infections, we obtained double drug-resistant parasite populations out of 2 separate infections, which arose from the co-infections with *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP* (Table 4). We detected the presence of both *NEO* and *HYG* drug resistance genes in these double drug-resistant parasite populations by PCR analysis (Fig 2.5). We were able to maintain one of this double drug-resistant population in culture for 3 weeks; however, after the third week, this population lost the *NEO* resistance gene and has ultimately perished afterwards (Fig 2.5A). For the second occurrence of double drug-resistant parasites, we isolated 3 separate populations which contained both *HYG* and *NEO* genes as assessed by PCR analysis (Fig 2.5B), whereas the third population had only the *HYG* resistance gene and died upon further passages (Fig 2.5B). The two double drug-resistant populations were maintained for a week and died upon additional

passages. These results suggest that genetic exchange may take place in infected macrophages and result in transient/unstable double drug-resistant parasites.

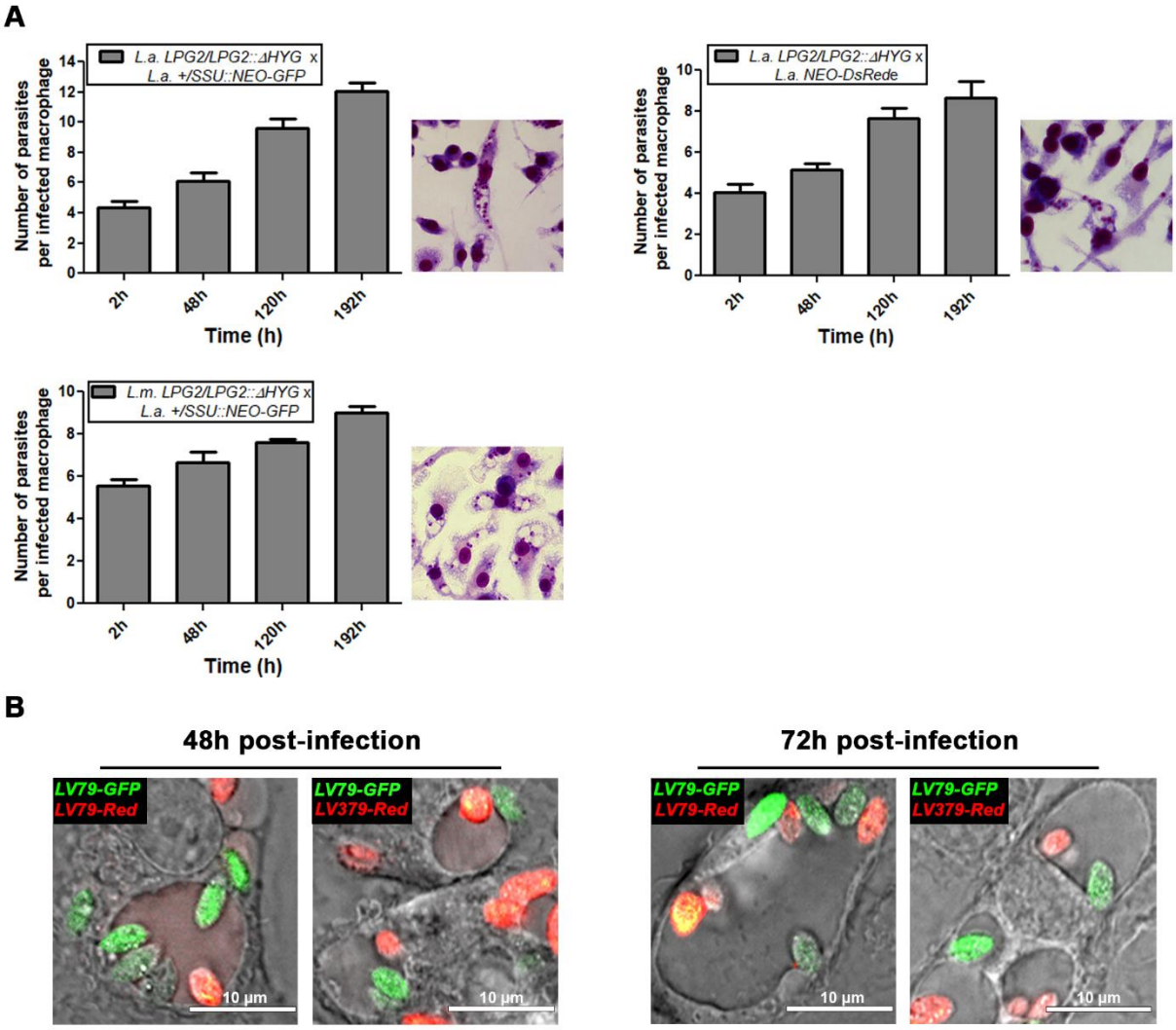


Figure 2.4: Survival within infected macrophages and visualization of parental strains within the same vacuole.

(A) BMMs were infected with metacyclic serum-opsonized promastigote crosses of *L. mexicana* complex parental parasite strains (*L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRede*; *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*) for 2h, 48h, 120h and 196h. Bars represent mean ± SE of three representative experiments performed in triplicate in bone marrow derived murine macrophages. Parasites were counted in 100 macrophages and quantified by light microscopy. Macrophages were stained with HEMA 3 kit. Representative pictures from each cross are shown. (B) Live microscopy analysis of *L. amazonensis* and *L. mexicana* parasite strains expressing different fluorescent markers. Representative pictures of both parental strains within the same communal vacuole at 48h and 72h are shown. *LV79-GFP*, *L. amazonensis HYG-GFPe*; *LV79-Red*, *L. amazonensis NEO-DsRede*; *M379-Red*, *L. mexicana NEO-DsRede*.

Table 2.2: Crosses used in *in vitro* infections done in wells.

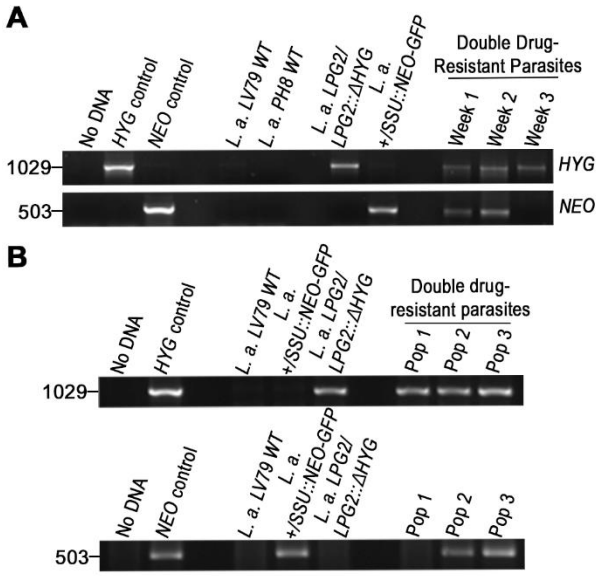
Crosses 120h Post-Infection	No. of wells with crosses	% Yield of double drug resistant parasites
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	89	0/89 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	34	0/34 (0%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	63	0/63 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	63	0/63 (0%)
Crosses 192h Post-Infection	**No. of wells with crosses**	**% Yield of double drug resistant parasites**
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	72	0/72 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	72	0/72 (0%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	69	0/69 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	66	0/66 (0%)

Demonstrates the number of wells tested and percentage of isolated double drug-resistant parasites.

Table 2.3: Crosses used in *in vitro* infections done in flasks.

Cross	No. of infections	No. of times parent 1 was isolated	No. of times parent 2 was isolated	No. of times double drug-resistant parasites were isolated	% Recovery
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	14	14	14	2	14%
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	10	10	10	0	0%
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	4	4	4	0	0%

Data includes the number of mating crosses executed in flasks and parasite strains that were isolated from each infection. Percentage indicates the total number of times that double-drug resistant parasites were isolated.



Figures 2.5: Molecular genotype characterization of double drug-resistant parasites isolated from *in vitro* infections.

PCR amplification of genes encoding antibiotic resistance. The size of HYG and NEO resistance genes is 1,029 and 503 bp long. The pLeish-HYG-GFP and the pKS-NEO-DsRed constructs were used as controls for the HYG and NEO genes, respectively. L.a. LV79 WT and L.a. PH8 WT is a DNA sample used to show that our wild type parasites do not express any drug-resistance markers. L. amazonensis LPG2/LPG2:: D HYG, L. amazonensis +/SSU::NEO-GFP are controls used to validate the expression of HYG and NEO resistance genes within the appropriate parental strains. No DNA sample was loaded as negative control. (A) PCR amplification of resistance genes of the first double drug- resistant parasite population. Population was maintained for 3 weeks until it lost one of the resistance genes and perished. PCRs of double drug-resistant parasites represent the presence of both genes on weeks 1, 2, and 3 (B) PCR amplification PCR amplification of resistance genes of the second occurrence of double drug-resistant parasites. Three populations were isolated (Pop 1 – 3). Two of the population were found to be double-drug resistant and one was not.

Absence of detectable genetic exchange in *in vivo* infections

To determine whether mammalian hosts provide an environment favorable for genetic exchange for the species of the *L. mexicana* complex, we inoculated mice into the ear dermis with two combinations of single drug-resistant parasites, namely *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP* and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP* (Table 2.4). Mice infected with single drug-resistant lines were used as a control. Nine weeks post-infection, we recovered parasites from lesions and we cultured them in the presence of either hygromycin, G418, or both. As shown in Table 2.4, we recovered each single drug-resistant line that was co-inoculated or inoculated alone as controls. However, we did not succeed in isolating double drug-resistant parasites from cutaneous lesions, indicating that genetic exchange does not occur to a detectable level within the mammalian host for *Leishmania* species residing in communal parasitophorous vacuoles.

Table 2.4: Crosses used in *in vivo* infections.

Cross	No. of infected mice	No. of times parent 1 was isolated	No. of times parent 2 was isolated	No. of times double drug-resistant parasites were isolated	% Recovery
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	13	13	13	0	0%
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	6	6	6	0	0%

Data includes the number of infected mice with each cross and parasite strains that were isolated from each infection. Percentage indicates the total number of times that double drug-resistant parasites were isolated.

DISCUSSION

For decades, the occurrence of natural *Leishmania* hybrids has been described among clinical and field isolates, indicating that genetic exchange is part of the biology of these parasites. Experimental genetic crosses among *Leishmania* cells were initially reported to occur exclusively in the sand fly vector (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014; Sadlova *et al.*, 2011). However, recent evidence revealed that experimental genetic crosses also occur in axenic promastigote cultures, indicating that mating competent forms are present in these populations (Louradour *et al.*, 2020). The fact that studies on the experimental generation of hybrids have been performed with *Leishmania* species living in tight individual parasitophorous vacuoles may have precluded the detection of genetic exchange within mammalian host cells. In this study, we sought to determine whether genetic exchange occurs among species of the *L. mexicana* complex, which replicate within communal parasitophorous vacuoles. Using promastigotes expressing different drug-selectable markers, we obtained evidence of intraclonal genetic exchange for *L. amazonensis* in both axenic promastigote cultures and infected macrophages. However, the resulting products of those genetic events were unstable as they did not sustain growth in subsequent sub-cultures.

The study of experimental genetic exchange in *Leishmania* consists in mixing strains carrying distinct drug-resistance markers and/or fluorescent markers integrated into their genomes and the subsequent selection and analysis of double drug-resistant parasites (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Louradour *et al.*, 2020; Romano *et al.*, 2014; Sadlova *et al.*, 2011). Whole genome sequencing revealed that these double drug-resistant parasites are full genomic hybrids predominantly resulting from a mechanism resembling meiosis (Inbar *et al.*, 2019). Whether other forms of genetic exchange take place in *Leishmania* had not received much attention. Hence, we tested whether the transfer of genetic material can occur without direct contact between *Leishmania* promastigotes, as previously reported for *P. falciparum* via cell-derived extravesicular vesicles (Regev-Rudzki *et al.*, 2013). Our attempts to detect the transfer of an episome from one line of *L. amazonensis* to another in transwell experiments were unsuccessful, suggesting that physical contact is required for genetic exchange among *Leishmania* promastigotes. Our results also suggest that in contrast to the *Leishmania* virus LRV-1 (Atayde *et al.*, 2019), episomal DNA is not transferred through extracellular vesicles or other released material.

The recent report that genetic crosses take place in axenic cultures of *L. tropica* (Louradour *et al.*, 2020) prompted us to explore the possibility that genetic exchange occurs among *L. amazonensis* and *L. mexicana* promastigotes in axenic cultures. In contrast to the *L. tropica* strains used by Louradour and colleagues (Louradour *et al.*, 2020), we obtained only a few populations of double drug-resistant *L. amazonensis* promastigotes which turned out to be unstable. The fact that those populations did not sustain sub-cultures precluded further analyses. Clearly, not all species or strains of *Leishmania* are equal in terms of capacity to generate mating-competent forms *in vitro*. Hence, whereas Louradour and colleagues were successful in recovering hybrids from *L. tropica* axenic co-cultures, no hybrids were obtained when both parental strains were *L. major* (Louradour *et al.*, 2020). In the case of *L. amazonensis*, it is possible that strains other than the one we used are more competent in generating mating-competent forms in axenic cultures. Future studies will be aimed at investigating this important issue.

It is well established that hybrid formation among *Leishmania* promastigotes takes place in the sand fly (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014; Sadlova *et al.*, 2011). Failure to detect genetic exchanges in the mammalian host suggests that amastigotes do not generate mating competent forms or that they are less prone to recombination. It is also possible that the phagolysosomal environment is not as conducive to genetic exchange as the sand fly midgut. However, it was reported previously that amastigotes are able to undergo nuclear fusion within infected macrophages indicating that genetic exchanges may indeed be possible within infected hosts (Kreutzer *et al.*, 1994). Alternatively, the fact that the *Leishmania* species used so far to study genetic exchange replicate within individual parasitophorous vacuoles (*L. major*, *L. tropica*, *L. donovani*, *L. infantum*) may have limited the probabilities of genetic exchange among amastigotes. With this in mind, we hypothesized that replication within a communal vacuole may provide amastigotes with conditions propitious to genetic exchange, as reported for *Chlamydia* (Jeffrey *et al.*, 2013). The recovery of double-drug resistant promastigote populations from macrophages co-infected with *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* and the detection of both the *HYG* and *NEO* genes in these cultures suggest that intraclonal genetic exchanges may occur within communal parasitophorous vacuoles. However, the inability to grow these *L. amazonensis* double drug-resistant populations over several passages and to clone double drug-resistant parasites precluded further characterization of these progeny and thus determine whether or not these parasites were genuine hybrids. Previous studies revealed that not all hybrid progeny is as viable as their parental counterparts. Hence, Sadlova *et al* observed *L. donovani* hybrids in

infected sand flies, but all of their attempts to grow them in culture have failed. This led to the conclusion that although *L. donovani* parasites are able to exchange genetic information, the hybrids produced were not viable (Sadlova *et al.*, 2011). Finally, there was also a report which explored the possibility of genetic material exchange between *L. major* and *L. turanica* in infected sand flies; however, it was reported that such events do not take place between these parasite species (Chajbullinova *et al.*, 2012).

As reported for *L. major* (Akopyants *et al.*, 2009), we were unable to recover double drug-resistant parasites from mice co-infected with *L. amazonensis* and *L. mexicana*. However, based on our results with *in vitro* infections, we cannot rule out that genetic exchanges do not take place in mammalian hosts infected with those species. An important factor to consider is the number of *in vivo* infections we performed. Indeed, studies on genetic exchange done in the insect vector required hundreds of sand flies to be infected. Hence, Akopyants *et al* used 102 sand flies to study genetic exchange between *L. major* parasites, Sadlova *et al* infected 121 sandflies to study this phenomenon for *L. donovani*, whereas Romano *et al* used 446 sandflies to study these events among strains of *L. infantum* (Akopyants *et al.*, 2009; Romano *et al.*, 2014; Sadlova *et al.*, 2011). Another important factor to take into consideration is the ability of the *L. amazonensis* and *L. mexicana* strains we used in our study to generate mating competent forms, as evidenced in the study of Louradour and colleagues using *L. tropica* and *L. major* (Louradour *et al.*, 2020).

In summary, we provide evidence of possible intraclonal genetic exchanges among *L. amazonensis* parasites in axenic cultures and within mammalian host cells. However, the double drug-resistant parasites obtained in our studies were unstable and could not be further characterized. Future studies will be required to identify strains of *L. amazonensis* and *L. mexicana* with higher capacity to generate mating competent forms and use these strains for studies in macrophages and in mice on a larger scale.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institutionel de Protection des Animaux of the INRS-Centre Armand-Frappier Santé Biotechnologie.

AUTHOR CONTRIBUTIONS

RT and AD conceived and designed the study, contributed to the data analysis, drafted, and revised the manuscript. RT performed the experiments. RT and AD wrote and revised the manuscript. All authors read and approved the final version of this manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

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

SECTION 3: GENERAL DISCUSSION AND CONCLUSION

DISCUSSION

Leishmania is an intracellular pathogenic organism that causes a range of pathologies collectively known as leishmaniasis which range from self-healing cutaneous lesions to deadly visceral infections. The parasite also has a complex life cycle that takes place in both the sand fly and mammalian hosts. Although the form of the parasite changes from promastigote to amastigote and *vice versa* when the parasites change hosts, they still replicate via binary fission. For a long time, the parasite was considered to be a strictly clonal organism; however, isolation of natural hybrids, whole genome sequencing studies and experimental genetic crosses have shown that *Leishmania*'s mode of reproduction is not as simple as previously believed.

In the past decade, a few groups of researchers have shown direct evidence that *Leishmania* is capable of exchanging its genetic information between each other. The first such study was published in 2009 which revealed that the co-infection of the vector with two strains of *L. major* harboring two different drug-selectable markers gave rise to a population of cells expressing both drug-resistance genes (Akopyants *et al.*, 2009). The studies that followed demonstrated that genetic exchanges occur between individuals of *L. donovani*, *L. major*, *L. infantum*, *L. tropica* (Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Sadlova *et al.*, 2011) and one has demonstrated genetic exchanges between two *Leishmania* species, namely *L. major* and *L. infantum* (Romano *et al.*, 2014). All of these studies have shown that the parasite is capable of such a genetic event within the infected sand fly vector; however, there are no studies except for one that demonstrate whether or not genetic exchanges are possible in the infected host or in a culture of infected cells (Akopyants *et al.*, 2009). Hence, this is what we decided to investigate.

As stated previously, the study of Akopyants *et al* is the only one which explored the possibility of *Leishmania* genetic exchange in an infected host. For their experiment, they used strains of *L. major* parasites expressing two different drug resistance genes and co-infected the ears of BALB/c mice; however, no double drug-resistant hybrid strains were ever recovered from such infections (Akopyants *et al.*, 2009). We, on the other hand, decided to test this possibility by co-infecting macrophage cells *in vitro* and mice with two strains of *L. amazonensis* or *L. mexicana* bearing two different drug selectable markers as well. The drug-resistance markers were integrated into the parasite genome as it was done in previous studies (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014); however, we also used parasites expressing episomal (e) resistance markers which were not done previously in similar studies. The reason for us choosing species of *L. mexicana* is because, unlike

L. major, they replicate in large spacious communal vacuoles which may provide a proper environment for genetic exchanges. In fact, it was proved that *Chlamydia*, another intracellular agent, is capable of exchanging genetic material between cells within communal vacuoles (Jeffrey *et al.*, 2013; Suchland *et al.*, 2009). In addition, it was shown that intracellular *Leishmania* amastigotes are capable of nuclear fusion indicating that it may be possible for the parasites to exchange genetic material within a mammalian host.

First, we decided to explore the possibility of genetic exchange between parasites in axenic cultures. This way we wanted to demonstrate that this process could potentially occur outside of live hosts. Although this doesn't prove or disprove our hypothesis, it was still interesting to witness whether the parasites are capable of exchanging genetic material in such conditions, because it was never shown to occur prior to the current year and because it would provide an easier way to study such events in laboratory conditions. Previous attempts to generate hybrid parasites in axenic cultures have not yielded any positive results (Akopyants *et al.*, 2009; Inbar *et al.*, 2013). However, very recently, there was a study that has revealed that *L. tropica* parasites are capable of exchanging DNA in axenic conditions (Louradour *et al.*, 2020). By contrast, the frequency of hybrid formation in the given condition was much lower when compared to the frequency of formation in the vector (Louradour *et al.*, 2020). Moreover, attempts of mating two strains of *L. major* have failed to provide hybrid parasites as previously shown (Louradour *et al.*, 2020). We decided to test this possibility with our *L. amazonensis* and *L. mexicana* strains. We proceeded with a similar method described in the study of Louradour *et al* where we mixed our parasite strains at a ratio of 1:1 in 96 well-plates and left them for 3 days. Later, the parasites were transferred to 24-well plates into conditioned medium for selection of double drug-resistant parasites. We attempted to test four crosses: *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRed*; *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*, and *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRed*. Out of these four crosses only one (*L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRed*) had generated double drug-resistant parasites. We were able to demonstrate by PCR that the parasites bore both resistance genes; however, we were not able to confirm whether they were actual full genomic hybrids or only the episomal marker was transferred between the parents due to the fact that the hybrids had died. Moreover, we were able to confirm the presence of both genes only in one population of cells out of the 3 populations that we initially isolated. This was due to the fact that we were not able to grow the parasites in culture after their isolation indicating that the supposed hybrids are

unstable. We managed to isolate the parasites anywhere between 18-28 days post transfer into selective medium, similarly to previously published results where *L. tropica* hybrids were isolated 18-30 days post-transfer into selective medium (Louradour *et al.*, 2020). Another similarity is that we recovered a very low yield of parasites in such conditions suggesting that although it is possible, the yield is very low compared to the yield in sand flies (Louradour *et al.*, 2020). Although axenic cultures in which parasites are cultivated are supposed to mimic the internal environment of the sand fly, they seem to lack certain factors found within the vector that may play a role in *Leishmania* genetic exchanges and which need to be uncovered in the future.

We have found that we were able to grow double drug-resistant parasites in axenic cultures. However, we could not conclude whether this was an actual genetic cross or that one parent had transferred only the episomal drug-resistance marker to the other. The cross that generated double drug-resistant parasites had one parent expressing an episomal marker and hence the question. To test this hypothesis, we decided to use transwells to see if parasites are capable of transferring such small DNA packages between cells in an indirect fashion. Now, it is typically considered that parasites need to fuse together in order to exchange genetic material via a meiosis-like mechanism (Inbar *et al.*, 2019). However, it is yet unknown if small DNA molecules such as plasmids can be transmitted between parasites via extracellular vesicles. Extracellular vesicles have been demonstrated to be secreted in both environments mimicking the sand fly and mammalian hosts (Atayde *et al.*, 2015; Hassani *et al.*, 2011). Moreover, the parasites were shown to secrete even more vesicles upon temperature shift to 37˚C mimicking the parasite's entry into the host (Hassani *et al.*, 2011). These vesicles are packed with many virulent proteins and small RNAs which play a role in modulating the host's immune response (Atayde *et al.*, 2016). In addition to that, the *Leishmania* RNA virus 1 was also found to use these extracellular vesicles to propagate from one cell to the next (Atayde *et al.*, 2019; de Carvalho *et al.*, 2019). Since the parasites are capable of transferring RNA material in these vesicles, we wanted to see if *Leishmania* could transfer DNA material as well, since it was demonstrated for another protozoan parasite, *Plasmodium falciparum,* which was capable to transfer such material via exosomes between infected red blood cells (Regev-Rudzki *et al.*, 2013). Hence, we decided to see if the cross *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e can generate double drug-resistant parasites in transwells which allow only extracellular vesicles to pass through. Despite all our attempts to generate double drug-resistant parasites in such conditions, regardless of whether we pre-incubated them at 34˚C or not, we were unable to observe any double drug-resistant parasites. This may be due to the fact that the parasites require direct physical contact

as it had been proposed previously (Inbar *et al.*, 2019). Although we weren't able to observe any drug-resistance genes being transferred between parasites via extracellular vesicles, it does not mean that they do not play a role spreading drug-resistance. In fact, a recent study had demonstrated that drug-resistant parasites had modified extracellular vesicles (Douanne *et al.*, 2020). Even though the core proteome of these vesicles is conserved between drug-resistant and sensitive parasites, some virulence and transcription factors as well as proteins coded by drug resistance genes are enriched in theses vesicles and it could facilitate the survival of these drug-resistant parasites as well as potentially that of sensitive parasites within a challenging environment (Douanne *et al.*, 2020). Finally, it would be interesting in the future to examine the contents of Leishmania derived exosomes and see whether they could potentially contain DNA material. This could potentially provide the final evidence for whether the parasite is able to transfer genetic material between each other via such method.

In our study, we show that *L. amazonensis*, parasites that replicate predominantly in communal vacuoles, are able to give rise to potential hybrid progeny in the context of infected mammalian cell. We have tested three types of crosses for hybrid crosses which were *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRed*; *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*. By infecting bone-marrow derived macrophages (BMM) with each cross, we managed to isolate double drug resistant progeny twice; however, both were isolated from the *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* cross. The other two crosses did not generate any double drug-resistant progeny. Both isolates were confirmed to express both resistance genes by PCR. Unfortunately, we could not confirm whether these parasites were full genomic hybrids or not, as done in previous studies. The only other evidence (data not shown) that strongly suggests that the double drug-resistant parasites could have been full genomic hybrids is the fact that the progeny expressed a green fluorescent protein that most likely was inherited from *L. amaz* NEO-GFPi parent. Additionally, we also demonstrated by using different parental strains of parasites expressing two different fluorescent markers that both parents could be found in the same communal vacuole regardless of whether both strains were *L. amazonensis* or *L. amazonensis* and *L. mexicana.* Although we show that *L. amazonensis* can undergo some form of genetic recombination, it is a rather rare event. We had a 14 % double drug-resistant parasite recovery rate. However, this was to be expected since genetic exchange studies done in sand flies also revealed a similar pattern. In fact, genetic crosses between *L. infantum* strains or *L.infantum*/*L.major* had a 3.4% and a 3.2% hybrid recovery ratio respectively (Calvo-Alvarez *et*

al., 2014; Romano *et al.*, 2014). On top of that, other studies have shown that different strains of *L. major* also had different hybrid recovery rates ranging from 7% to 26% (Akopyants *et al.*, 2009; Inbar *et al.*, 2013). However, some species have an undoubtably high hybrid forming capability. It was demonstrated that hybrid recovery ratio of *L. tropica* was in the range of 42%-65% (Inbar *et al.*, 2019) and there are many studies reporting natural hybrids between *L. braziliensis* and *L. peruviana* or *L. guyanensis* suggesting that these parasites also have a high capacity for exchanging genetic material. Hence, it is clear that different species have different capabilities when it comes to genetic recombination were some are found to be more efficient and others not. Of note, it would be interesting to document the mating efficiency of New world Leishmania species as well as between geographically isolated New world and Old world Leishmania in infected sand flies To conclude, our 14 % recovery seems to be in accordance with what was previously demonstrated; however, it would be more accurate to judge this ratio if we were to recover these supposed hybrids from infected sandflies. This is due to the fact that the sand fly gut microenvironment and that of an *in vitro* cell culture or a mammalian host are different and may have an impact on the parasite genetic exchange capabilities as it was demonstrated for *L. tropica* between axenic cultures and sand fly infections (Inbar *et al.*, 2019; Louradour *et al.*, 2020). In any case, the mating capabilities of different *Leishmania* species remain to be fully described, but one thing that may clearly be concluded is that the parasites have different efficiencies in such processes and perhaps even in different microenvironments.

Interestingly, we observed that our potential hybrid progeny is not as viable as their parental counterparts. The double drug-resistant parasite populations we had isolated from *in vitro* macrophage infections did not manage to survive for a long time. As a matter of fact, a similar phenomenon was also observed for double drug-resistant progeny isolated from axenic cultures. We were able to maintain one of the hybrids for three weeks by maintaining them in infected BMMs until they lost one of the resistance genes and ultimately perished as a result. This showed that not only do our parasites have low viability but they are also unstable since they had lost one of the resistance genes. However, this is not an entirely unexpected outcome. For instance, Sadlova *et al* managed to observe putative *L. donovani* hybrids in infected sand flies, but they were unable to isolate and cultivate any of them in culture suggesting that such progeny was non-viable in long term perspective (Sadlova *et al.*, 2011). Additionally, some studies demonstrated that hybrid progeny may occasionally have loss of heterozygosity. For example, it was found that one of the hybrid clones of *L. major*/*L. infantum* had a loss of heterozygosity for one of the parental alleles on chromosome 29 (Romano *et al.*, 2014). As another example, genetic sequencing studies in *L. major* hybrids have revealed that there was a 1-3% loss of heterozygosity or partial

loss at certain loci (Inbar *et al.*, 2013; Inbar *et al.*, 2019). This loss of heterozygosity may be a possible reason that would explain why our double-resistant progeny lost one of the resistance markers. Interestingly, low viability is not the only sign of reduced fitness in hybrid strains of *Leishmania*. In fact, it was recently shown *L. major* intraspecies hybrids had a lesser mating competency than their parental counterparts whereas the interspecies hybrids of *L. major*/*L. infantum* were found to be sterile (Inbar *et al.*, 2019). Another peculiarity is that the study had also explored the mating capacity of natural *L. tropica* hybrids and they found that one strain failed to mate and was deemed sterile whereas another was mating competent and as efficient as the parental strains they tested for their crosses (Inbar *et al.*, 2019). This leads to the conclusion that not only different *Leishmania* species have different mating capabilities, but that the hybrid progeny also has varying degrees of fertility which could a perspective venue to explore in the future.

Since we were able to observe potential hybrid parasites isolated from *in vitro* macrophage infections, we attempted to isolate such parasites from infected mice. We did not isolate any double drug-resistant progeny despite all of our attempts of generating such parasites either in an intraspecific manner between *L. amazonensis* or and interspecific manner with *L. amazonensis* and *L. mexicana*. According to the literature, there was only one study that attempted to test whether genetic exchanges are possible between parasites in an infected mammalian host. The researchers had infected mice with two parental strains of *L. major*; however, they did not isolate any hybrid parasites from these infections (Akopyants *et al.*, 2009). Although both our results and those of Akopyants *et al* did not recover hybrid progeny from infected mice, it does not suggest that such exchanges do not take place in cells of infected hosts. In the case of *L. major*, the most probable reason of why no hybrid parasites were obtained is because these parasites, unlike those of the *L. mexicana* complex, replicate in individual vacuoles. Unlike the tight individual vacuoles, large communal vacuoles provide an advantageous environment where two cells may potentially exchange genetic material. Moreover, there is one study that has demonstrated that amastigotes could undergo nuclear fusion indicating that genetic exchanges could take place (Kreutzer *et al.*, 1994). Another factor that we should keep in mind is that we have observed double drug-resistant parasites isolated from *in vitro* cell infections. One of the reasons as to why we did not observe double drug-resistant parasites from *in vivo* infections as opposed to *in vitro* may be due to the fact that the number of parasites used in both methods varies highly. For instance, we infected mice with 1×10^5 metacyclic parasite mixes (5×10^4 each parent) corresponding to the range of inoculated parasites by a single fly (From 10 to 1×10^5 parasites) into a host (Kimblin *et al.*, 2008), whereas for *in vitro* 1.5×10^8 parasites per infection (7.5×10^7 each

parent). Considering the fact that genetic exchanges are a rare occurrence, we have more chances to isolate double drug-resistant progeny from *in vitro* rather than *in vivo*. Also, studies done in sandflies show us that researchers had to infect anywhere between 100 to 450 sandflies to isolate hybrid parasites and all that also depended on how mating competent the different species were (Akopyants *et al.*, 2009; Calvo-Alvarez *et al.*, 2014; Inbar *et al.*, 2013; Inbar *et al.*, 2019; Romano *et al.*, 2014; Sadlova *et al.*, 2011). For example, the lowest yielding crosses were those between *L. infantum* parasites (3.4%) and to obtain such hybrids the researchers had to infect 446 sandflies (Romano *et al.*, 2014). We believe that in order for us to successfully isolate double drug-resistant parasites, we need to infect about the same number of mice to isolate them.

In our study, we have mostly focused on studying the potential of genetic recombination in *L. amazonensis*; however, we also attempted to test this phenomenon at the interspecies level between *L. amazonensis* and *L. mexicana*, two species that replicate in communal vacuoles. Our attempts to cross these two species did not yield any potential hybrids. In fact, there is one study that had reported that there is no hybrid formation observed between *L. major* and *L. turanica* (Chajbullinova *et al.*, 2012). Perhaps, our lack of hybrid formation may be due to the fact that we had not done enough replicates to observe the given progeny and the same conclusion could be given for the *L. major*/*L. turanica* cross considering that some crosses are rarer than others. Additionally, future studies should explore mating capabilities of different L. amazonensis and L. mexicana strains as it was done previously for L. major (Inbar *et al.*, 2013). Another reason that has been mentioned before, is that the microenvironment of a mammalian host is quite different than that of sand fly and that one may be more appropriate for genetic exchanges than the other. In addition, we did not try intraspecific crosses with *L. mexicana* as we did with *L. amazonensis* to assess their intraclonal mating capabilities; however, we suspect that they may be as efficient as *L. amazonensis*. Our lack of hybrid formation between these two species could not be due to growth competition. In fact, both species grew in infected cells at the same rate when co-cultured or alone. The only difference that we found was their growth pattern within the infected macrophages. Specifically, *L. mexicana* parasite population grew at a slower rate over time when compared to *L. amazonensis*. Of note, all *L. amazonensis* parental strains used in our crosses grew well within infected cells and at similar rates. Finally, it is important to note that we confirmed the presence of both *L. mexicana* and *L. amazonensis* parental strains within the same infected cells and within the same communal vacuoles. Therefore, our lack of interspecies double-resistant parasites cannot be due to the parasites not growing in the same vacuoles.

CONCLUSION

In conclusion, we present for the first time in the present study the occurrence of genetic exchanges between *L. amazonensis* within infected mammalian cells. We were not capable of isolating potential hybrids from infected mice, but the fact that we have isolated a few double drug-resistant parasites from *in vitro* cell infections suggest that such processes may occur. They are rare and lead to the generation of unstable offspring with low-viability. Moreover, we demonstrate that double drug-resistant parasites can be observed in axenic cultures. We have also tried to cross *L. mexicana* and *L. amazonensis*; however, we did not observe any double drug-resistant progeny from such a cross. In future studies concerning the parasites of the *L. mexicana* complex specifically, we should continue investigating their possibility of genetic exchanges in infected mammalian cells and hosts. It would be also interesting to cross these species with species that have a higher mating compatibility such as *L. tropica* or *L. braziliensis* which have been reported to form natural hybrids with other species multiple times. This may increase the chances of isolating potential hybrid parasites. Although *L. tropica* and *L. braziliensis* are parasites that replicate in individual vacuoles, we believe that they can still fuse with the communal vacuoles of *L. mexicana* complex parasites since it has been shown in the past between vacuoles of *L. amazonensis* and *L. amazonensis/ L. major* (Real et al., 2010; Real et al., 2008). It is now known that different species have different mating capabilities, therefore it is important to continue exploring hybridization events within and between different *Leishmania* species. Additionally, examining genetic exchange capabilities between *L. amazonensis* or *L. mexicana* species. Such studies will further advance our understanding on the biological diversity and complexity of the *Leishmania* genus as well as provide insight in the potential strategies to transmit resistance genes between parasites. In addition, it will also be important to assess the fertility and fitness of these hybrids to further highlight the impact and importance of genetic recombination within the *Leishmania* population as recently shown in one study (Inbar *et al.*, 2019).

APPENDIX I: AUTHOR'S PROOF OF SUBMITTED ARTICLE

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Author's Proof

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Study on the Occurrence of Genetic Exchange Among Parasites of the *Leishmania mexicana* Complex

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In *Leishmania*, genetic exchange has been experimentally demonstrated to occur in the sand fly vector and in promastigote axenic cultures through a meiotic-like process. No evidence of genetic exchange in mammalian hosts have been reported so far, possibly due to the fact that the *Leishmania* species used in previous studies replicate within individual parasitophorous vacuoles. In the present work, we explored the possibility that residing in communal vacuoles may provide conditions favorable for genetic exchange for *L. mexicana* and *L. amazonensis*. Using promastigotes lines of both species harboring integrated or episomal drug-resistance markers, we assessed whether genetic exchange can occur in axenic cultures, in infected macrophages as well as in infected mice. We obtained evidence of genetic exchange for *L. amazonensis* in both axenic promastigote cultures and infected macrophages. However, the resulting products of those putative genetic events were unstable as they did not sustain growth in subsequent sub-cultures, precluding further characterization.

Keywords: genetic exchange, *Leishmania*, host-pathogen relationship, macrophage, drug resistance, intracellular pathogen

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INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the causative agents of a spectrum of diseases known as leishmaniasis that range from self-healing cutaneous lesions to destructive mucocutaneous infections and visceral pathologies. *Leishmania* has a distinct life cycle which consists of two specific environments. The first is that of the sand fly insect vector in which the parasites multiply within the alimentary tract under the promastigote form and the second is the infected mammalian or human hosts where the parasites replicate as amastigotes within the phagolysosomal compartment of host phagocytes. Currently, there are 20 known species of parasites that are associated with human disease. However, there is still a considerable amount of debate of whether this diversity is due to recombinational events or due to gradual accumulation of mutations during clonal division (Tibayrenc and Ayala, 2013; Rougeron et al., 2017).

In eukaryotic pathogenic organisms, sex is one of the main mechanisms that allows the spread of pathogenicity, resistance, and virulence genes (Heitman, 2010). Due to very strong linkage disequilibrium observed in *Leishmania*, it has been argued that the reproductive mode of *Leishmania* is predominantly clonal (Tibayrenc and Ayala, 2013). However, there is much evidence indicating that genetic exchange is part of the biology of *Leishmania* parasites, as

evidenced by the occurrence in nature of hybrids. These natural hybrids were described at the intraspecific level for *L. tropica, L. donovani, L. infantum*, and *L. brasiliensis* (Chargui et al., 2009; Rougeron et al., 2009; Gelanew et al., 2014; Rogers et al., 2014; Iantorno et al., 2017). There were also reports of hybrids that originated from crosses between parasites of the *Viannia* subgenus, such as *L. braziliensis* and *L. guyanensis*, which are one of the most common ones described (Bonfante-Garrido et al., 1992; Belli et al., 1994; Dujardin et al., 1995; Banuls et al., 1997; Cupolillo et al., 1997; Delgado et al., 1997; Banuls et al., 1999; Torrico et al., 1999; Nolder et al., 2007; Cortes et al., 2012; Jennings et al., 2014; Kato et al., 2016; Kato et al., 2019). Natural hybrids were also reported for *Leishmania* species of the *Leishmania* subgenus such as *L. major* and *L. arabica*, *L. major* and *L. infantum*, as well as *L. donovani* and *L. infantum* (Evans et al., 1987; Kelly et al., 1991; Ravel et al., 2006; Volf et al., 2007; Odiwuor et al., 2011; Seblova et al., 2015; Cortes et al., 2019).

Using two strains of *L. major* harboring distinct integrated drug-resistance markers, Akopyants and colleagues experimentally demonstrated the existence of genetic exchange in the invertebrate stage of the parasite (Akopyants et al., 2009). By infecting sand flies and dissecting them 13–16 days post-infection, the double drug-resistant progeny of this cross was further demonstrated to be actual genomic hybrids by confirming the presence of at least one set of allelic markers from each parent (Akopyants et al., 2009). In another study from the same group, it was further shown that crosses in the invertebrate stage between *L. major* parasites coming from 4 distinct geographical locations are able to produce hybrid progeny, which also suggests that there are no intraspecies barriers when it comes to exchanging genetic information (Inbar et al., 2013). Another interesting finding, was that hybrid formation was observed in both the natural *P. duboscqi* vector and in the unnatural but permissive *L. longipalpis* and, by isolating the parasites from infected sand flies 3–18 days post-infection, it was further ruled out that genetic exchange takes place between parasites when they are in the nectomonad form (Inbar et al., 2013). In addition, a study based on microscopy and flow cytometry allowed to visualize evidence of genetic exchange between two strains of *L. donovani* expressing two different fluorescent molecules (RFP and GFP) which were present in the same vector (*P. perniciosus* or *L. longipalpis*) and gave rise to yellow promastigote progeny; however, these putative hybrids could not be recovered from the sand flies and grown in culture for further analyses (Sadlova et al., 2011). There was also a study which demonstrated hybrid formation in sand flies between two *L. infantum* strains expressing different fluorescent as well as different drug-resistance markers (Calvo-Alvarez et al., 2014) and another paper demonstrated formation of hybrid parasite strains in sand flies between two entirely different species, namely *L. major* and *L. infantum* (Romano et al., 2014). Finally, the ability of *L. tropica* to exchange genetic information in an intraspecific manner in an infected insect vector as well as in axenic culture has also been recently demonstrated using whole genome sequencing (Inbar et al., 2019; Louradour et al., 2020).

Despite the fact that hybrid parasites could be isolated both in nature and in laboratory conditions from infected sand flies and axenic cultures, the mechanism by which they reproduce is still poorly understood. This is partially due to the fact that this is not an obligate mode of reproduction of the parasite; however, recent genome sequencing data from 44 hybrids generated between and within *L. infantum*, *L. tropica*, and *L. major* suggest that *Leishmania* reproduces *via* a meotic-like mechanism (Inbar et al., 2019). Apart from one study using *L. major* (Akopyants et al., 2009), it is still not widely known whether or not genetic exchange can occur within an infected mammalian host, although there is a study that has shown previously by DNA quantification that infected macrophages could harbor 4N amastigotes suggesting that genetic exchange is possible in mammalian host cells (Kreutzer et al., 1994). Here, we explored the possibility of intraclonal and interspecific genetic exchange among parasites of the *L. mexicana* complex, which unlike other *Leishmania* species, replicate in spacious communal vacuoles that may provide an environment favorable to genetic exchange (Case et al., 2016).

MATERIALS AND METHODS

Ethics Statement

All animal handling was performed in accordance with the protocols 1806–01 and 1806–02, which were approved by the *Comité Institutionel de Protection des Animaux* of the INRS-Centre Armand-Frappier Santé Biotechnologie. These protocols respect procedures on animal practice as instructed by the Canadian Council on Animal Care, described in the Guide to the Care and Use of Experimental Animals.

Plasmids and Constructs

The plasmid pLa*LPG2-HYG* from which the *LPG2::ΔHYG* targeting construct was used to create Hygromycin B-resistant parasites was kindly provided by Drs. Valeria M. Borges and Leonardo Paiva Farias (Fiocruz Bahia - Instituto Gonçalo Moniz, Brazil) (**Figure 1**). The plasmid pCR2.1-L.d-rDNA-pr-αIR*NEO*αIR-*GFP* from which the Ld-rDNA-*NEO-GFP* targeting sequence was used to create G418-resistant parasites was kindly provided by Dr. Barbara Papadopoulou (Université Laval, Canada) (**Figure 1**). The plasmid pKS-*NEO-DsRed* was provided by Dr. David L. Sacks (National Institute of Allergy and Infectious Diseases, USA) (Kimblin et al., 2008). The pLeish-*HYG-GFP* construct was created the following way: a *Sac*I fragment containing the *GFP* gene was excised from the plasmid pXG-*GFP*$^{+}$ (Ha et al., 1996), blunted, and inserted into the *Eco*RV site of pLeish-*HYG* (unpublished), yielding pLeish-*HYG-GFP*.

Parasites

Both *L. amazonensis* LV79 (MPRO/BR/72/M1841) and *L. mexicana* (MNYC/BZ/62/M379) were passaged in mice to maintain their virulence. Amastigotes recovered from ear dermis lesions of infected C57BL/6 mice were differentiated

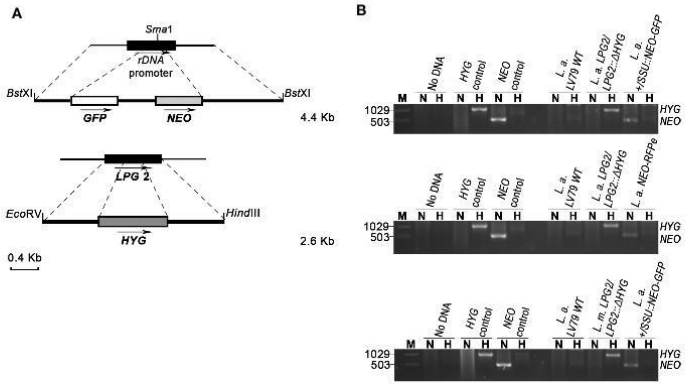

FIGURE 1 | Generation drug-resistant *Leishmania* parasites. **(A)** L.d-rDNA-*NEO-GFP* and *LPG2::ΔHYG* targeting constructs used for the integration into the ribosomal RNA locus or in one allele of *LPG2*, respectively. For the L.d-rDNA-*NEO-GFP* construct, the *NEO-GFP* resistance cassette (white and gray boxes) was inserted in the *Sma*I site of the ribosomal RNA locus (black rectangle). The dashed lines delimit the regions of recombination between the target genes and targeting constructs. Arrows indicate orientation. **(B)** PCR products for drug resistance markers *HYG* and *NEO* of *L. amazonensis* and *L. mexicana* parental strains. The size of *HYG* and *NEO* resistance genes is 1,029 and 503 bp long, respectively. The pLeish-*HYG-GFP* and the pKS-*NEO-DsRed* constructs were used as controls for the *HYG* and *NEO* genes, respectively. *L.a.* LV79 WT is a DNA sample used to show that our wild type parasites do not harbor any drug-resistance markers in their genomes. *L. amazonensis LPG2/LPG2::ΔHYG*, *L. amazonensis +/SSU::NEO-GFP*, *L. mexicana LPG2/LPG2::ΔHYG* and *L. amazonensis NEO-DsRede* are controls used to validate the presence of *HYG* and *NEO* resistance genes. No DNA sample was loaded as negative control. M, molecular DNA ladder; H, Hygromycin; N, G418.

into promastigotes in *Leishmania* medium (M199-1X (Sigma) with 10% heat-inactivated fetal bovine serum (FBS), 100 μM hypoxanthine, 3 μM biopterin, 40 mM HEPES at pH 7.4, 5 μM hemin, 1 μM biotin, and Penicillin-Streptomycin) in a 26°C incubator. For the generation of *L. amazonensis LPG2/LPG2::ΔHYG* and *L. mexicana LPG2/LPG2::ΔHYG*, log-phase *L. amazonensis* and *L. mexicana* promastigotes were electroporated with the *LPG2::ΔHYG* targeting construct (excised as a 2.6-kb *Eco*RVI-*Hind*III-*Bgl*I fragment from pLa*LPG2*KO-*HYG*) in 0.2 cm electroporation cuvettes, at 0.45 kV and 500 μF of high capacitance as previously described in similar protocols (Descoteaux et al., 1994; Turco et al., 1994). After electroporation, promastigotes were grown in drug-free *Leishmania* medium for 24 h. Following this incubation, *L. amazonensis LPG2/LPG2::ΔHYG* parasites were selected in the presence of 35 μg/ml Hygromycin B (Sigma) and *L. mexicana LPG2/LPG2::ΔHYG* parasites were selected in the presence of 70 μg/ml Hygromycin B (Sigma) respectively. For the generation of *L. amazonensis* +/*SSU::NEO-GFP*, *L. amazonensis* promastigotes were electroporated with the L.d-rDNA-*NEO-GFP* targeting construct (excised as a 4.25-kb *Bst*XI fragment from pCR2.1-L.d-rDNA-pr-αIR*NEO*αIR-*GFP*). After electroporation, the parasites were grown in drug free medium for 24 h and then grown in *Leishmania* medium containing 20 μg/ml G418 (Life Technologies). *L. amazonensis NEO-DsRede* parasites were obtained by electroporating *L. amazonensis* promastigotes with the plasmid pKS-*NEO-DsRed*. Parasites were grown in drug free medium for 24 h and then grown in medium containing 20 μg/ml G418. The same method was used to obtain *L. mexicana* pKS-*NEO-DsRede* and they were maintained in *Leishmania* medium containing 40 μg/ml of G418. *L. amazonensis HYG-GFPe* promastigotes were generated by electroporating *L. amazonensis* with the plasmid pLeish-*HYG-GFP*. Parasites were grown in drug-free medium for 24 h and then grown in medium containing 35 μg/ml Hygromycin B

Mammalian Cell Culture

Bone marrow-derived macrophages (BMM) were differentiated from the bone marrow of 6- to 8-week old C57BL/6 mice as previously described (Descoteaux and Matlashewski, 1989). BMM were differentiated for 7 days in complete DMEM [containing L-glutamine (Life Technologies), 10% v/v heat inactivated fetal bovine serum (FBS) (Life Technologies), 10 mM HEPES (Bioshop) at pH 7.4, and penicillin-streptomycin (Life Technologies)] supplemented with 15% v/v L929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor-1. To render the BMM quiescent prior to experiments, cells were transferred to tissue culture-treated 6- or 24-well plates or T25 tissue culture flasks for 24 h in complete DMEM without LCM. The cells were kept in a humidified 37°C incubator with 5% CO_2. The number of macrophages used per container are as following: 2.2 X 10^6 BMMs per well of a 6-well plate, 0.3 X 10^6 BMMs per well of 24-well plate and 25 X 10^6 BMMs in T-25 flasks.

Transwell Experiments

For genetic exchange transwell experiments, donor parasites (*L. amazonensis NEO-dsRede*) were relocated to the insert chamber containing 0.4 μm pores in a polycarbonate membrane (Corning) and the recipient parasites (*L. amazonensis LPG2/*

LPG2::ΔHYG) were added to the wells. The plates were then either incubated at 26°C or pre-incubated at 34°C for 4 h, as done previously (Hassani et al., 2011), and then transferred to 26°C. The parasites were collected at 24, 72, 96, and 120 h post-incubation. Each parental stain was equally divided into 3 wells of a 6-well plate and were grown in the presence of antibiotics. Two wells were used as controls containing either 35 μg/ml of Hygromycin B or 20 μg/ml of G418 and the last well contained both drugs in the medium. The parasites were kept in such conditions up to 3 weeks. Each parental strain was also grown separately and were under the same conditions as a control.

Parasite Co-Culture Experiments

As described (Louradour et al., 2020), stationary phase promastigotes of two parental strains were mixed and distributed into 96-well plates up to a total volume of 100 μl in each well. One million parasites of each strain were added in the wells. Three days later, each co-culture from the 96-well plate was transferred to a single well of a 24-well plate containing 900 μl of *Leishmania* medium containing either 35 μg/ml Hygromycin B and 20 μg/ml G418 if both parental strains were *L. amazonensis* or 60 μg/ml Hygromycin B and 40 μg/ml G418 if one of the parental strains was *L. amazonensis* and the other was *L. mexicana*. Each line was cultured individually in *Leishmania* medium supplemented with either Hygromycin B or G418 or both drugs as controls. When double drug-resistant parasite cultures were growing in wells (growth was observed between 19 and 28 days), the cells were passaged in *Leishmania* medium at a dilution of 1:10. DNA was then extracted from double drug-resistant parasites and was used for PCR reactions.

In Vitro Infections

Metacyclic promastigotes were isolated from promastigote cultures in the late stationary phase by means of a density gradient centrifugation (Spath and Beverley, 2001). Specifically, 2 ml of 40% w/v Ficoll PM400 (GE healthcare) were added to the bottom of ta 15 ml tube, followed by a 5 ml layer of 10% Ficoll PM400 in M199-1x and topped by late stationary phase promastigotes resuspended in 5 ml of DMEM with no FBS (Arango Duque et al., 2019). Metacyclic promastigotes were collected from the DMEM-10% Ficoll interphase after spinning the gradient for 10 min. The percentage of isolated metacyclic parasites from the interphase generally varied from 12–18% of the input population. Metacyclic promastigotes were then opsonized with the serum of C57BL/6 mice for 30 min, washed 3 times with PBS and resuspended in cold complete DMEM (cDMEM). The parasites were then fed to macrophages adhered in T-25 flasks (Sarstedt) (Ratio 3:1 for single infections, ratio 6:1 for mix infections). The cells were then incubated at 4°C for 10 min (Arango Duque et al., 2019) to synchronize phagocytosis. The internalization of parasites was triggered by transferring the cells to 34°C (Arango Duque et al., 2019). Two hours post-internalization, the cells were washed three times with warmed cDMEM to remove non-internalized promastigotes. Infected BMM were incubated for 120 and 192 h. Next, the amastigotes were isolated from infected macrophages by resuspending those in cDMEM containing 0.05% of SDS. Shortly, the macrophages resuspended in 2ml of cDMEM containing SDS are incubated at 37°C for 3 min. Then, the resulting supernatant is resuspended in 10 ml of cDMEM and spun at 3,000 rpm. After the spin, the supernatant was discarded. The amastigotes were resuspended in *Leishmania* medium and separated into 3 separate conditions. The conditions were: *Leishmania* medium containing 20 μg/ml of G418 or *Leishmania* medium containing 32 μg/ml of Hygromycin B or *Leishmania* medium containing both drugs. The parasites were left for incubation at 26°C for up to 3 weeks to select for double drug-resistant parasites. If applicable, the double drug-resistant parasites were passaged at a dilution of 1/10 and their DNA was then extracted and was used for PCR reactions. Double drug-resistant parasites were also passaged in infected BMM for 3 days as well. For parasite survival, cells were washed with PBS and fixed and stained with fixative and staining solutions of the Hema 3 stain set (Fisher Scientific). This process was done for 2, 48, 120, and 192 h timepoints.

Alternatively, the infections were done in 6-well plates instead of T-25 flasks. Three wells were used for mixed infection for each timepoint (120 and 192 h) and two wells were reserved for infection with each parental strain alone. Once the amastigotes were obtained, they were plated in 96-well plates in 100 μl of drug free *Leishmania* medium as described in the parasite co-culture section. The amastigotes were plated at 5 million parasites per well. Three days later, each well was transferred to a well of 24-well plate that contained 900 μl with antibiotics. Pure parental cultures were used as controls as previously described. If applicable, the double drug-resistant parasites were passaged at a dilution of 1/10 and their DNA was then extracted from double drug-resistant parasites and was used for PCR reactions.

In Vivo Infections and Parasite Recovery

C57BL/6 mice (6–8 weeks old) were infected with 1 X 10^5 Q12 metacyclic promastigotes (5 X 10^4 of each line) of either *L. amazonensis LPG2/LPG2::ΔHYG + L. amaz +/SSU::NEO-GFP* or *L. mexicana LPG2/LPG2::ΔHYG + L. amaz +/SSU::NEO-GFP* into the ear dermis with an insulin syringe (29 G). Mice infected separately with each line were used as controls. At 9 weeks post-infection, mice were euthanized under CO_2 asphyxiation and by cerebral dislocation as well. The infected ears were then collected and disinfected in 70% ethanol for 10 min and air dried for 10 min. Then, they were separated into dorsal and ventral leaflets and cut up into small pieces with surgical scissors. The cut-up ears were loaded in 2.0 ml tubes containing zirconium beads (Benchmark Scientific Inc.) and resuspended in 1 ml of *Leishmania* medium and vortexed for a 1 min and 30 s. The resulting suspension was then transferred to 100 μm cell strainers placed over 50 ml Falcon tubes and filtered to isolate the amastigotes. The remaining tissue in the cell strainer was smashed with a sterile 10 ml syringe plunger and washed two times with *Leishmania* medium. The resulting cell suspension was spun at 3,200 RPM at 4°C for 10 min. The amastigotes were then separated in three T-25 flasks and left in unconditioned *Leishmania* medium for 24 h. Lastly, the antibiotics were added to each flask according to each condition and were incubated at

26°C for three weeks. The conditions were Hygromycin only, G418 only or both drugs.

DNA Extraction and PCR Confirmation of Double-Resistant Parasites

For genotyping analyses, total DNA was extracted from parasites by using a phenol/chloroform treatment as previously described (Medina-Acosta and Cross, 1993). All of the PCR amplifications were done in 50 µl total volume containing 100 ng of parasite DNA and 10pmol of each primer. The following primer pairs were used: for Hygromycin B 5'-ATGAAAAGCCTGA ACTCACC-3' (Forward), 5'-CTATTCCTTTGCCCTCGG-3' (Reverse) that were previously described (Romano, 2014); for G418 5'CCACGACGGGCGTTCCTTGCGCAGCTGTGC-3' (Forward), 5'-GTCAGCCCATTCG CCAAGCTCTTCAGC-3' (Reverse) which were custom made. The resulting DNA products were then verified by electrophoresis on 1.2% Agarose gel and subsequently viewed by staining the samples with ethidium bromide.

Live Microscopy

BMMs were platted at the bottom of 6 well-plate with a coverslip attached to the bottom of the wells. The cells were kept in the 34°C incubator for 24 h to without LCM to render them quiescent. They were then infected with metacyclic parasites of each line separately as a positive control or with a combination of two. Non-infected cells were used as a negative control. The samples were then viewed with 63X objective lens LSM780 system confocal microscope (Carl Zeiss microimaging). The images were taken and processed with the ZEN 2012 Software (Carl Zeiss) and subsequently mounted into the figures *via* Adobe Photoshop 2019.

RESULTS

Generation of Drug-Resistant Strains of *L. amazonensis* and *L. mexicana*

To investigate the possibility that formation of hybrids and genetic exchange may occur among parasites of the *L. mexicana* complex, we used *L. amazonensis* LV79 and *L. mexicana* M379 expressing either episomal or integrated genes encoding resistance to Hygromycin B (*HYG*) or to G418 (*NEO*). To this end, we generated one line of *L. amazonensis* and one line of *L. mexicana* in which the *HYG* resistance gene was integrated in one allele of the *LPG2* gene (*L. amazonensis LPG2/LPG2::ΔHYG* and *L. mexicana LPG2/LPG2::ΔHYG*) (**Figure 1A**), one line of *L. amazonensis* in which a *NEO-GFP* construct was integrated into the ribosomal RNA locus (*L. amazonensis +/SSU::NEO-GFP*) (**Figure 1A**), one line of *L. amazonensis* and one line of *L. mexicana* with an episomal *NEO-DsRed* plasmid (*L. amazonensis NEO-DsRed*e and *L. mexicana NEO-DsRed*e), and one line of *L. amazonensis* with an episomal *HYG-GFP* plasmid (*L. amazonensis HYG-GFP*e). We confirmed the presence/absence of both resistance genes in each line by PCR analysis using specific primers against *HYG* and *NEO* (**Figure 1B**), and we ensured that these drug-resistant recombinant parasites retained the ability to infect and replicate within bone marrow-derived macrophages (BMMs) over a period of 196 h (**Figure 2**).

Drug Resistance is not Transferred in *In Vitro* Cultures of Promastigotes in the Absence of Cell-To-Cell Contact

Evidence indicate that DNA can be transferred from cell-to-cell through extracellular vesicles (Elzanowska et al., 2020). In addition, erythrocytes infected with *Plasmodium falciparum* can transfer parasite DNA to other infected cells *via* the release of extracellular vesicles (Regev-Rudzki et al., 2013). Whereas no such mechanism has been described in *Leishmania*, it was recently reported that the *Leishmania* RNA virus 1 (LRV1) exploits the *Leishmania* exosomal pathway as a mode of transmission from one promastigote to another (Atayde et al., 2019). This led us to verify the hypothesis that extracellular vesicles released in the culture medium may serve as a vehicle to transfer genetic material, including episomes harboring a drug-resistance gene among promastigotes. To this end, we used transwells (0.4 µm pores) to physically separate *L. amazonensis NEO-DsRed*e promastigotes from *L. amazonensis-LPG2/LPG2::ΔHYG* promastigotes. We incubated the transwell plates either at 26°C or we pre-incubated them at 34°C for 4 h and then transferred the plates to 26°C. Such a transient increase in temperature has been previously shown to enhance the secretion of extracellular vesicles by *Leishmania* promastigotes (Hassani et al., 2011). Promastigotes co-incubated in transwells at either 26 or 34°C were collected after 24, 72, 96, and 120 h and assessed for their capacity to grow in the presence of both

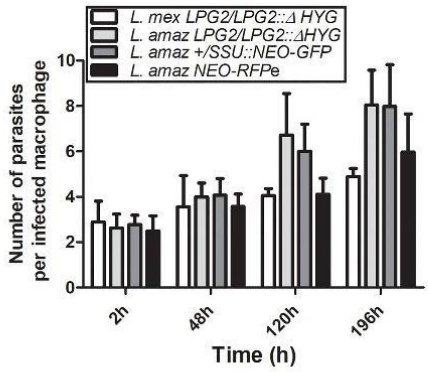

FIGURE 2 | Survival of parental strains within infected macrophages. BMMs were infected with metacyclic serum-opsonized promastigotes of *L. amazonensis* and *L. mexicana* parental strains (*L. amazonensis LPG2/LPG2::ΔHYG*, *L. mexicana LPG2/LPG2::ΔHYG*, *L. amazonensis +/SSU::NEO-GFP*, *L. amazonensis NEO-DsRed*e) for 2, 48, 120, and 196 h. Bars represent mean ± SE of three representative experiments performed in triplicate in bone marrow derived murine macrophages. Parasites were counted in 100 macrophages and quantified by light microscopy.

hygromycin and G418. Both *L. amazonensis-NEO-DsRed*e and *L. amazonensis-LPG2/LPG2::ΔHYG* were viable and resistant to G418 and hygromycin, respectively, up to 120 h of co-incubation in the transwells. However, no double drug-resistant parasites were recovered from 9 independent experiments performed in triplicate, indicating that exchange of genetic information through extracellular vesicles among *L. amazonensis* promastigotes, if it occurs, is a very rare event.

Genetic Exchange Among *L. amazonensis* and *L. mexicana* Promastigotes in Axenic Cultures

A recent study revealed that some strains of *L. tropica*, but not *L. major*, form hybrids in promastigote axenic cultures (Louradour et al., 2020). This finding prompted us to evaluate the occurrence of genetic crosses among *L. amazonensis* and *L. mexicana* promastigotes in *in vitro* co-cultures. Following the experimental protocol described by Louradour *et al*, we co-cultured combinations of stationary phase promastigotes with integrated drug-resistance genes as depicted in **Table 1** (Louradour et al., 2020). We also performed co-culture experiments using promastigotes harboring episomes (**Table 1**). Each co-culture was distributed into 96-well plates in drug-free medium. Three days later, the parasites were transferred into 24-well plates and cultured in selective medium (hygromycin B and G418) and left for up to 40 days in a 26°C incubator. Individual single drug-resistant lines were used as controls and had gone through the same process. After 40 days of incubation, we did not obtain double drug-resistant parasites except for the co-cultures of *L. amazonensis-LPG2/LPG2::ΔHYG* and *L. amazonensis-NEO-DsRed*e (**Table 1**). We obtained promastigote populations resistant to both G418 and hygromycin B in 3 separate wells. However, only one out of 3 grew sufficiently to allow for DNA isolation and PCR analysis, which revealed the presence of both *HYG* and *NEO* genes (**Figure 3**). However, we were unable to further characterize these double-drug-resistant parasites as they perished in subsequent sub-cultures. These results suggest that genetic exchange in axenic cultures among those two drug-resistant lines may occur and results in transient/unstable double drug-resistant promastigotes.

TABLE 1 | Crosses used in axenic cultures.

Crosses in axenic cultures	No. of wells with crosses	% Yield of double drug-resistant parasites
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	192	0/192 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	192	3/192 (1.56%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	96	0/96 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRed*e	96	0/96 (0%)

Demonstrates the number of wells tested and percentage of isolated double drug-resistant parasites.

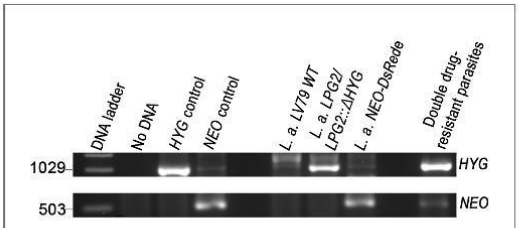

FIGURE 3 | Molecular genotype characterization of double drug-resistant parasites isolated from axenic cultures. PCR amplification of genes encoding antibiotic resistance. The size of *HYG* and *NEO* resistance genes is 1,029 and 503 bp long. The pLeish-*HYG-GFP* and the pKS-*NEO-DsRed* constructs were used as controls for the *HYG* and *NEO* genes, respectively. *L.a.* LV79 WT is a DNA sample used to show that our wild type parasites do not express any drug-resistance markers. *L. amazonensis LPG2/LPG2::ΔHYG* and *L. amazonensis NEO-DsRed*e are controls used to validate the presence of *HYG* and *NEO* resistance genes within the appropriate parental strains. No DNA sample was loaded as negative control.

Unstable Genetic Exchange in Infected Macrophages

The fact that *L. amazonensis* and *L. mexicana* replicate within communal parasitophorous vacuoles led us to verify the possibility that these intracellular replicative niches provide conditions propitious for genetic exchange. To this end, we infected BMMs with the following four combinations of drug-resistant parasites: *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e; *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*, and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e. Similar to single infection, parasites in mixed infections replicated up to 192 h post-infection and induced the formation of communal PVs (**Figure 4A**). To confirm that these communal PVs harbored both drug-resistant *Leishmania* lines, we performed live cell imaging on BMMs co-infected with either *L. amazonensis HYG-GFP*e + *L. amazonensis NEO-DsRed*e or *L. amazonensis HYG-GFP*e + *L. mexicana NEO-DsRed*e. In both cases, we observed the two drug-resistant parasite lines within the same communal vacuoles, at 48 and 72 h post-infection (**Figure 4B**). At 120 and 192 h post-infection, we lysed the infected BMM and cultured the recovered parasites in medium containing hygromycin B and G418 in a similar fashion as the axenic parasite cultures done in plates. As shown in **Table 2**, we failed to recover any double drug-resistant parasites from these co-infection experiments. Next, we modified our experimental approach to perform co-infection experiments on a larger scale, with the following 3 combinations of drug-resistant promastigotes: *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP*, *L. amazonensis LPG2/LPG2::ΔHYG + L. amazonensis NEO-DsRed*e, and *L. mexicana LPG2/LPG2::ΔHYG + L. amazonensis +/SSU::NEO-GFP* (**Table 3**). We co-infected BMMs with each combination and we used each individual drug-resistant line as controls. Out of a total of 28 infections, we obtained double drug-resistant parasite populations out of 2 separate infections, which

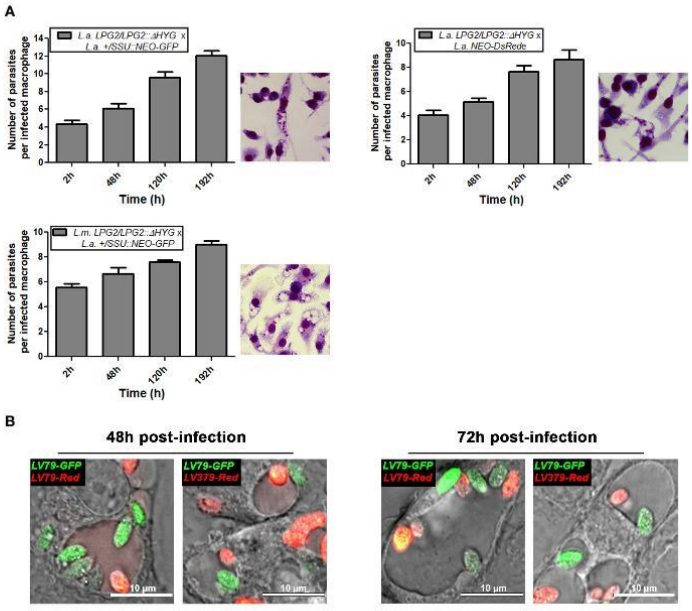

FIGURE 4 | Survival of mating crosses within infected macrophages and visualization of both parental strains within the same vacuole. **(A)** BMMs were infected with metacyclic serum-opsonized promastigotes crosses of *L. mexicana* complex parental parasite strains (*L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*; *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRede*; *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP*, and *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis NEO-DsRede*) for 2, 48, 120, and 196 h. Bars represent mean ± SE of three representative experiments performed in triplicate in bone marrow derived murine macrophages. Parasites were counted in 100 macrophages and quantified by light microscopy. Macrophages were stained with HEMA 3 kit. Representative pictures from each cross are shown. **(B)** Live microscopy analysis of *L. amazonensis* and *L. mexicana* parasite strains expressing different fluorescent markers. Representative pictures of both parental strains within the same communal vacuole at 48 and 72 h are shown. *LV79-GFP*, *L. amazonensis HYG-GFPe*; *LV79-DsRed*, *L. amazonensis NEO-DsRede*; *M379-DsRed*, *L. mexicana NEO-DsRede*.

TABLE 2 | Crosses used in *in vitro* infections done in wells.

Crosses120 h post-infection	No. of wells with crosses	% Yield of double drug-resistant parasites
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	89	0/89 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	34	0/34 (0%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	63	0/63 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	63	0/63 (0%)
Crosses 192 h post-infection	**No. of wells with crosses**	**% Yield of double drug-resistant parasites**
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	72	0/72 (0%)
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	72	0/72 (0%)
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	69	0/69 (0%)
L. mex LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	66	0/66 (0%)

Demonstrates the number of wells tested and percentage of isolated double drug-resistant parasites.

arose from the co-infections with *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* (**Table 3**). We detected the presence of both *NEO* and *HYG* drug-resistance genes in these double drug-resistant parasite populations by PCR analysis (**Figure 5**). We were able to maintain one of this double drug-resistant population in culture for 3 weeks; however, after the third week, this population lost the *NEO* resistance gene and has ultimately perished afterwards (**Figure 5A**). For the second occurrence of double drug-resistant parasites, we isolated 3 separate populations which contained both the *HYG* and *NEO* genes as assessed by PCR analysis (**Figure 5B**), whereas the third population had only the *HYG* resistance gene and died upon further passages (**Figure 5B**). The two double drug-resistant populations were maintained for a week and died upon additional passages. These results suggest that genetic exchange may take place in infected macrophages and result in transient/unstable double drug-resistant parasites.

TABLE 3 | Crosses used in *in vitro* infections done in flasks.

Cross	No. of infections	No. of times parent 1 was isolated	No. of times parent 2 was isolated	No. of times double drug-resistant parasites were isolated	% Recovery
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	14	14	14	2	14%
L. amaz LPG2/LPG2::ΔHYG × *L. amaz NEO-DsRede*	10	10	10	0	0%
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	4	4	4	0	0%

Data includes the number of mating crosses executed in flasks and parasite strains that were isolated from each infection. Percentage indicates the total number of times that double-drug resistant parasites were isolated.

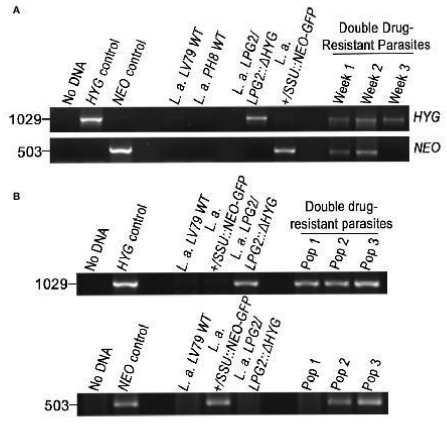

FIGURE 5 | Molecular genotype characterization of double drug-resistant parasites isolated from *in vitro* infections. PCR amplification of genes encoding antibiotic resistance. The size of *HYG* and *NEO* resistance genes is 1,029 and 503 bp long. The pLeish-*HYG-GFP* and the pKS-*NEO-DsRed* constructs were used as controls for the *HYG* and *NEO* genes, repectively. *L.a.* LV79 WT and *L.a.* PH8 WT is a DNA sample used to show that our wild type parasites do not express any drug-resistance markers. *L. amazonensis LPG2/LPG2::ΔHYG*, *L. amazonensis +/SSU::NEO-GFP* are controls used to validate the expression of *HYG* and *NEO* resistance genes within the appropriate parental strains. No DNA sample was loaded as negative control. **(A)** PCR amplification of resistance genes of the first double drug- resistant parasite population. Population was maintained for 3 weeks until it lost one of the resistance genes and perished. PCRs of double drug-resistant parasites represent the presence of both genes on weeks 1, 2, and 3 **(B)** PCR amplification PCR amplification of resistance genes of the second occurrence of double drug-resistant parasites. Three populations were isolated (Pop 1–3). Two of the population were found to be double-drug resistant and one was not.

Absence of Detectable Genetic Exchange in *In Vivo* Infections

To determine whether mammalian hosts provide an environment favorable for genetic exchange for the species of the *L. mexicana* complex, we inoculated mice into the ear dermis with two combinations of single drug-resistant parasites, namely *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* and *L. mexicana LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* (**Table 4**). Mice infected with single drug-resistant lines were used as a control. Nine weeks post-infection, we recovered parasites from lesions and we cultured them in the presence of either hygromycin B, G418, or both. As shown in **Table 4**, we recovered each single drug-resistant line that was co-inoculated or inoculated alone as controls. However, we did not succeed in isolating double drug-resistant parasites from cutaneous lesions, indicating that genetic exchange does not occur to a detectable level within the mammalian host for *Leishmania* species residing in communal parasitophorous vacuoles.

DISCUSSION

For decades, the occurrence of natural *Leishmania* hybrids has been described among clinical and field isolates, indicating that genetic exchange is part of the biology of these parasites. Experimental genetic crosses among *Leishmania* cells were initially reported to occur exclusively in the sand fly vector (Akopyants et al., 2009; Sadlova et al., 2011; Inbar et al., 2013; Calvo-Alvarez et al., 2014; Romano et al., 2014; Inbar et al., 2019). However, recent evidence revealed that experimental genetic crosses also occur in axenic promastigote cultures, indicating that mating competent forms are present in these populations (Louradour et al., 2020). The fact that studies on the experimental generation of hybrids have been performed with *Leishmania* species living in tight individual parasitophorous vacuoles may have precluded the detection of genetic exchange within mammalian host cells. In this study, we sought to determine whether genetic exchange occurs among species of the *L. mexicana* complex, which replicate within communal parasitophorous vacuoles. Using promastigotes expressing drug-selectable markers, we obtained evidence of intraclonal genetic exchange for *L. amazonensis* in both axenic promastigote cultures and infected macrophages. However, the resulting products of those genetic events were unstable as they did not sustain growth in subsequent sub-cultures.

The study of experimental genetic exchange in *Leishmania* consists in mixing strains carrying distinct drug-resistance markers and/or fluorescent markers integrated into their genomes and the subsequent selection and analysis of double drug-resistant parasites (Akopyants et al., 2009; Sadlova et al., 2011; Inbar et al., 2013; Calvo-Alvarez et al., 2014; Romano et al., 2014; Inbar et al., 2019; Louradour et al., 2020). Whole genome

TABLE 4 | Crosses used in *in vivo* infections.

Cross	No. of infected mice	No. of times parent 1 was isolated	No. of times parent 2 was isolated	No. of times double drug-resistant parasites were isolated	% Recovery
L. amaz LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	13	13	13	0	0%
L. mex. LPG2/LPG2::ΔHYG × *L. amaz +/SSU::NEO-GFP*	6	6	6	0	0%

Data includes the number of infected with each cross and parasite strains that were isolated from each infection. Percentage indicates the total number of times that double drug-resistant parasites were isolated.

sequencing revealed that these double drug-resistant parasites are full genomic hybrids predominantly resulting from a mechanism resembling meiosis (Inbar et al., 2019). Whether other forms of genetic exchange take place in *Leishmania* had not received much attention. Hence, we tested whether the transfer of genetic material can occur without direct contact between *Leishmania* promastigotes, as previously reported for *P. falciparum via* cell-derived extravesicular vesicles (Regev-Rudzki et al., 2013). Our attempts to detect the transfer of an episome from one line of *L. amazonensis* to another in transwell experiments were unsuccessful, suggesting that physical contact is required for genetic exchange among *Leishmania* promastigotes. Our results also suggest that in contrast to the *Leishmania* virus LRV-1 (Atayde et al., 2019), episomal DNA is not transferred through extracellular vesicles or other released material.

The recent report that genetic crosses take place in axenic cultures of *L. tropica* (Louradour et al., 2020) prompted us to explore the possibility that genetic exchange occur among *L. amazonensis* and *L. mexicana* promastigotes in axenic cultures. In contrast to the *L. tropica* strains used by Louradour and colleagues (Louradour et al., 2020), we obtained only a few populations of double drug-resistant *L. amazonensis* promastigotes which turned out to be unstable. The fact that those populations did not sustain sub-cultures precluded further analyses. Clearly, not all species or strains of *Leishmania* are equal in terms of capacity to generate mating-competent forms *in vitro*. Hence, whereas Louradour and colleagues were successful in recovering hybrids from *L. tropica* axenic co-cultures, no hybrids were obtained when both parental strains were *L. major* (Louradour et al., 2020). In the case of *L. amazonensis*, it is possible that strains other than the one we used are more efficient in generating mating-competent forms in axenic cultures. Future studies will be aimed at investigating this important issue.

It is well established that hybrid formation among *Leishmania* promastigotes takes place in the sand fly (Akopyants et al., 2009; Sadlova et al., 2011; Inbar et al., 2013; Calvo-Alvarez et al., 2014; Romano et al., 2014; Inbar et al., 2019; Louradour et al., 2020). Failure to detect genetic exchange in the mammalian host suggests that amastigotes do not generate mating competent forms or that they are less prone to recombination. It is also possible that the phagolysosomal environment is not as conducive to genetic exchange as the sand fly midgut. However, it was reported previously that amastigotes undergo nuclear fusion within infected macrophages suggesting that genetic exchange may indeed be possible within infected hosts (Kreutzer et al., 1994). Alternatively, the fact that the *Leishmania* species used so far to study genetic exchange replicate within individual parasitophorous vacuoles (*L. major*, *L. tropica*, *L. donovani*, *L. infantum*) may have limited the probabilities of genetic exchange among amastigotes. With this in mind, we hypothesized that replication within a communal vacuole may provide amastigotes with conditions propitious to genetic exchange, as reported for *Chlamydia* (Jeffrey et al., 2013). The recovery of double-drug resistant promastigote populations from macrophages co-infected with *L. amazonensis LPG2/LPG2::ΔHYG* + *L. amazonensis +/SSU::NEO-GFP* and the detection of both the *HYG* and *NEO* genes in these cultures suggest that intraclonal genetic exchange may occur within communal parasitophorous vacuoles. However, the inability to grow these *L. amazonensis* double drug-resistant populations over several passages and to clone double drug-resistant parasites precluded further characterization of these progeny and thus determine whether or not these parasites were genuine hybrids. Previous studies revealed that not all hybrid progeny is as viable as their parental counterparts. Hence, Sadlova et al. observed *L. donovani* hybrids in infected sand flies, but all of their attempts to grow them in culture have failed. This led to the conclusion that although *L. donovani* parasites are able to exchange genetic information, the hybrids produced were not viable (Sadlova et al., 2011). Finally, there was also a report which explored the possibility of genetic exchange between *L. major* and *L. turanica* in infected sand flies; however, it was reported that such events do not take place between these parasite species (Chajbullinova et al., 2012).

As described for *L. major* (Akopyants et al., 2009), we were unable to recover double drug-resistant parasites from mice co-infected with *L. amazonensis* and *L. mexicana*. However, based on our results with *in vitro* infections, we cannot rule out that genetic exchange do not take place in mammalian hosts infected with those species/strains. An important factor to consider is the number of *in vivo* infections we performed. Indeed, studies on genetic exchange done in the insect vector required hundreds of sand flies to be infected. Hence, Akopyants et al. used 102 sand flies to study genetic exchange between *L. major* parasites, Sadlova et al. infected 121 sandflies to study this phenomenon for *L. donovani*, whereas Romano et al. used 446 sandflies to study these events among strains of *L. infantum* (Akopyants et al., 2009; Sadlova et al., 2011; Romano et al., 2014). Another important factor to take into consideration is the ability of the *L. amazonensis* and *L. mexicana* strains we used in our study to generate mating competent forms, as evidenced in the study of

Louradour and colleagues using *L. tropica* and *L. major* (Louradour et al., 2020).

In summary, we provide evidence of possible intraclonal genetic exchange among *L. amazonensis* parasites in axenic cultures and within mammalian host cells. However, the double drug-resistant parasites obtained in our studies were unstable and could not be further characterized. Future studies will be required to identify strains of *L. amazonensis* and *L. mexicana* with higher capacity to generate mating competent forms and use these strains for studies in macrophages and in mice on a larger scale.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Comité Institutionel de Protection des Animaux of the INRS-Centre Armand-Frappier Santé Biotechnologie.

AUTHOR CONTRIBUTIONS

RT and AD conceived and designed the study, contributed to the data analysis, and drafted and revised the manuscript. RT performed the experiments. RT and AD wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Q21

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