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The effect of xenogeneic extracellular vesicles on pathophysiology and drug resistance of *Leishmania* infections in a murine model

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Ce mémoire intitulé

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Résumé

La leishmaniose est une zoonose à transmission vectorielle due au parasite protozoaire *Leishmania* ; des co-infections avec plusieurs espèces de *Leishmania* ont également été rapportées. Il a été démontré que les vésicules extracellulaires (VE) de ce parasite jouent un rôle dans l'infection précoce, ainsi que la propagation de la résistance *in vitro* aux médicaments. Peu de médicaments anti-*Leishmania* sont disponibles, et la résistance continue de croître chez ce parasite; il est donc impératif de comprendre la propagation de la résistance aux antileishmaniens.

Nous avons exploré la capacité des VE xénogéniques de *Leishmania* à moduler la physiopathologie de l'infection et la sensibilité du parasite aux médicaments après contact *in vivo*. La co-inoculation de parasites et de VE provenant de souches/espèces de *Leishmania* présentant divers profils de résistance aux médicaments a été réalisée chez la souris. La physiopathologie et la charge parasitaire ont été suivies, et des tests de sensibilité aux médicaments effectués.

Les résultats ont démontré que les VE de *Leishmania infantum* influencent la physiopathologie de *Leishmania major* dans le cadre *in vivo*. Nous avons également constaté que ces VE modulent la sensibilité aux médicaments de *L. major* après un contact *in vivo* dans un modèle d'infection précoce, entraînant une diminution significative de la sensibilité à l'antileishmanien antimoine.

Nous démontrons ici pour la première fois que les VE des parasites xénogéniques peuvent participer à la propagation de la résistance aux médicaments entre les populations de parasites après un contact *in vivo*, ce qui pourrait expliquer en partie l'augmentation des taux d'échec des traitements contre *Leishmania*.

Mots clés : *Leishmania*, vésicules extracellulaires, résistance aux médicaments, *in vivo*, parasites protozoaires.

Abstract

Leishmaniasis is a zoonotic disease caused by the protozoan parasite *Leishmania*, endemic to 98 countries and territories. There are several manifestations of leishmaniasis, some fatal if left untreated. Furthermore, co-infections with multiple species of *Leishmania* have also been reported. Extracellular vesicles (EVs) from *Leishmania* have been demonstrated to play a role in early infection, as well as spread of drug resistance *in vitro*. Few antileishmanial drugs are available, and drug resistance to those in use continues to grow; as such, there is an urgent need to better understand the spread of *Leishmania* drug resistance.

In this study, the ability of xenogeneic *Leishmania* EVs to modulate infection pathophysiology and parasite drug sensitivity after *in vivo* contact was explored. Co-inoculation of parasites and purified EVs from strains/species of *Leishmania* with contrasting drug resistance profiles was performed in BALB/c mice. Pathophysiology and parasite burden were monitored, and drug-susceptibility testing performed on recovered parasites.

Results demonstrated that EVs from *Leishmania infantum* influence pathophysiology of *Leishmania major* in *in vivo* experiments. These EVs were also found to modulate drug sensitivity of *L. major* after *in vivo* contact in a 6-hour infection model, leading to a highly significant decrease in susceptibility to antileishmanial antimony.

Here it is demonstrated for the first time that EVs from xenogeneic parasites can participate directly in propagating drug resistance between parasite populations after *in vivo* contact. These findings may help explain current observations of rising rates of *Leishmania* treatment failure.

Keywords: *Leishmania*, extracellular vesicles, drug resistance, *in vivo*, protozoan parasites

Table of Contents

Résumé *3*

Abstract *4*

Table of Contents *5*

List of figures **8**

List of supplemental figures **8**

List of abbreviations *10*

Acknowledgments *14*

Introduction *15*

Section 1: Literature Review *17*

Chapter I: **Leishmania** *and leishmaniases* *17*

1.1. Epidemiology & pathology **17**

1.1.1 Human leishmaniasis 17

1.1.1.1 Cutaneous leishmaniasis (CL) 18

1.1.1.2 Visceral leishmaniasis (VL) 18

1.1.1.3 Mucocutaneous leishmaniasis (MCL) 19

1.1.2 Canine leishmaniosis 19

1.2 Parasite life cycle **20**

1.2.1 Vector 21

1.2.2 Promastigotes 21

1.2.3 Amastigotes 22

1.2.4 Reservoirs 23

1.3 Diagnostics, treatment, and special considerations **25**

1.3.1 Roadblocks to treatment 25

1.3.1.1 Diagnostics 25

1.3.1.2 Treatment accessibility 26

1.3.1.3 Pharmacopeia 26

1.3.1.4 Treatment failure 27

1.3.2 Risks for the emergence of new endemic areas 28

1.3.2.1 Human leishmaniasis cases in non-endemic areas 28
1.3.2.2 Animal importation & travel 29

Chapter II: Immunology of leishmaniasis 30

2.1 Immunology 30

2.1.1 Immune response to *Leishmania* parasites 30
2.1.1.1 Innate immune response 30
2.1.1.2 Adaptive immune response 32
2.1.2 *Leishmania* virulence factors 32

2.2 Extracellular vesicles 34

2.2.1 The origin of EVs 34
2.2.2 Prospective applications for EVs 35

2.3 *Leishmania* and EVs 36

2.3.1 Discovery 36
2.3.2 Immune response 37
2.3.3 Potential role in early infection & disease progression 39

Chapter III: Drug resistance 40

3.1. Mechanism of action of current antileishmanial drugs 40

3.2. Factors leading to the development of DR *Leishmania* 41

3.3. Introduction to drug resistance in *Leishmania* parasites 41

3.4 Overview of drug resistance in *Leishmania* parasites 41

3.4.1 Mechanisms of antimony resistance 42
3.4.2 Epidemiology and brief mechanistic overview of other DR in *Leishmania* parasites 43
3.4.3 Strategies to overcome drug resistance 45

3.5 Drug resistance and EVs 46

3.5.1 Transmission & propagation 46

Section 2: Description of M.Sc. project 48

1 – Rationale 48

2 – Research hypotheses 49

3 – Specific objectives 49

Section 3: Article *50*

Abstract **50**

Introduction **51**

Materials & Methods **53**

Results **59**

Discussion **62**

Funding **66**

Contributions **67**

Acknowledgments **67**

Declaration of Interest **67**

Figures **68**

Supplemental Figures **76**

References **85**

Section 4: Discussion *89*

Section 5: Conclusion *92*

Bibliography *93*

List of figures

Figure 1: **Leishmania** *life cycle. Adapted from (Wagner et al., 2019).* *24*

Figure 2: Innate immune response to **Leishmania.** *Adapted from (Scott and Novais, 2016)*.. *31*

Figure 3: Biogenesis of extracellular vesicles. Adapted from (Dang et al., 2020)..... *36*

Figure 4: Brief overview of **Leishmania** *exosomes' interaction with host immune cells (Dong et al., 2021)* *38*

Figure 5: Mechanisms of antimony drug resistance employed by intracellular **Leishmania** *amastigotes (Ponte-Sucre et al., 2017)* *43*

Figure 6: EV validation & characterization through multiple molecular methods..... *68*

Figure 7: Sb^{III} drug sensitivity of **L. infantum** *promastigotes transformed from amastigotes* *69*

Figure 8: Footpad swelling in **L. major-***infected mice at 8 weeks post-infection* *70*

Figure 9: Footpad lesion scores in **L. major-***infected mice at 8 weeks post-infection*..... *71*

Figure 10: Footpad parasite burden in **L. major-***infected mice, as determined by limiting dilution assay (LDA)* *72*

Figure 11: Sb^{III} drug sensitivity of **L. major** *parasites recovered from footpad experiments*.. *73*

*Figure 12: Cell recruitment and differential at the 6h timepoint post-***L. major** *IP infection* . *74*

Figure 13: Sb^{III} drug sensitivity of **L. major** *parasites recovered after IP infection (experiment 2)* *75*

List of supplemental figures

*Supplemental Figure 1: Mouse & organ weights post-***L. infantum** *IP infection*..... *76*

Supplemental Figure 2: Splenic parasite burden in **L. infantum-***infected mice, as determined by limiting dilution assay (LDA)* *77*

Supplemental Figure 3: Sb^{III} drug sensitivity of **L. infantum** *parasites recovered after IP infection* *78*

Supplemental Figure 4: Footpad swelling in **L. major**-*infected mice over 8-10 week infections* *79*

Supplemental Figure 5: Growth curve of **L. major** *parasites post-IP infection* *80*

Supplemental Figure 6: Macrophage & neutrophil recruitment by timepoint post-**L. major** *IP infection* *81*

Supplemental Figure 7: Infection of macrophages & neutrophils post-**L. major** *IP infection* *82*

Supplemental Figure 8: Sb^{III} drug sensitivity of **L. major** *parasites recovered after IP infection (experiment 1)* *83*

List of abbreviations

ABC: ATP-binding cassette

AmB: amphotericin B

AQP: aquaglyceroporin

CanL: canine leishmaniosis

CL: cutaneous leishmaniasis

CNV: copy number variation

DALY: disability-adjusted life year

DC: dendritic cell

DR: drug-resistant

DS: drug-sensitive

EV: extracellular vesicle

GIPL: glycoinositolphospholipid

GP63: glycoprotein 63

HIV: human immunodeficiency virus

IL-12: interleukin-12

IL-8: interleukin-8

iNOS: inducible nitric oxide synthase

LPG: promastigote surface lipophosphoglycan

LRV1: *Leishmania* RNA virus 1

MA: meglumine antimoniate

MCL: mucocutaneous leishmaniasis

MDR1: multidrug resistance protein 1

MF: miltefosine

MVB: multivesicular body

NGS: next-generation sequencing

NK: natural killer

NO: nitric oxide

NTD: neglected tropical disease

PARO: paromomycin

PCR: polymerase chain reaction

PKC: protein kinase C

PKDL: Post Kala-azar Dermal Leishmaniasis

PPG: proteophosphoglycan

PSG: promastigote secretory gel

PTP: protein tyrosine phosphatase

ROS: reactive oxygen species

Sb^{III}: trivalent antimony

Sb^{V}: pentavalent antimony

SNP: single nucleotide polymorphism

SSG: sodium stibogluconate

TEM: transmission electron microscopy

Th17: T-helper 17

TLR-3: toll-like receptor 3

Treg: regulatory T-cell

VL: visceral leishmaniasis

For my mother, Lynn Caplan, the smartest woman I know.

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Introduction

Neglected tropical diseases (NTDs), also known as neglected global diseases, cause serious illness in approximately 2 billion people worldwide every year (1). NTDs are caused by parasites, viruses, and bacterial pathogens, and disproportionately affect those in low-income countries and individuals of low socioeconomic status in middle-income countries (2). These diseases not only lead to adverse health affects in those afflicted, but also cause a heavy economic burden in these communities, both directly (*i.e.* through loss of productivity due to poor health) and indirectly (*i.e.* through stigma that prevents an affected individual from leaving their home). One way to measure such impact is through disability-adjusted life years (DALYs), which encompasses both years of life lived with disability, as well as years of life lost due to early death; a study in 2010 determined that NTDs were responsible for approximately 26 million DALYs, representing a significant global burden (3). Although NTDs include a very diverse group of diseases, one unifying factor is their invisible and silent nature; despite the large amount of humans affected, said individuals hold little in the way of political sway, and the diseases in questions are not well known in non-endemic areas nor prioritized on international public health agendas (4).

One such NTD is leishmaniasis, a zoonotic parasitic disease endemic to 98 countries and territories, which also holds the title of 9th most burdensome communicable disease worldwide (5, 6). The protozoan *Leishmania*, of which over 20 species are infective to humans, is vector-borne, transmitted by the bite of female sand flies harbouring the parasite. In many areas, dogs serve as a natural reservoir (5). Different species of *Leishmania* lead to different manifestations of disease; cutaneous (CL), visceral (VL), or mucocutaneous leishmaniasis (MCL). There are approximately 1.6 million new cases of leishmaniasis yearly (4).

Diagnosis and treatment of leishmaniasis is complex, arduous, and costly; many diagnostic techniques are invasive or simply unavailable at basic healthcare facilities, and the majority of available therapeutic drugs are highly toxic, require a long course of treatment, and/or necessitate parenteral administration (7, 8). There is no antileishmanial vaccine available for humans, and the pharmacopeia for treatment of leishmaniasis is sparse, made up only of antimony-based drugs (*i.e.* sodium stibogluconate (SSG), meglumine antimoniate (MA)), amphotericin B (AmB), miltefosine

(MF), paromomycin (PARO), and pentamidine (7). Another important obstacle to treatment and control of leishmaniasis is the development of drug resistance (DR) in *Leishmania* parasites, notably in the Indian Subcontinent (9). This is especially true with respect to antimony-based treatments, which have been in use for nearly 100 years; in Bihar, India, for example, failure rate with SSG treatment reached at least 60% between 2006 and 2010 (10).

Leishmaniasis often affects those without access to permanent housing (*e.g.* displaced persons), appropriate sanitary conditions, and/or easily accessible medical services, further complicating diagnosis and treatment. As such, *Leishmania*/human immunodeficiency virus (HIV) co-infections represent a major challenge for treatment and control in certain regions, while *Leishmania*/*Leishmania* co-infections have also been reported, especially in HIV-positive patients (7, 11). At least 71 countries are endemic for both CL and VL, and it has been demonstrated that two *Leishmania* species can complete their life cycles inside a single sand fly simultaneously (5, 12). As such, co-infections with multiple *Leishmania* species are a concern, and likely underreported in the most at-risk populations. Undetected *Leishmania*/*Leishmania* co-infections may play a role in treatment failure, and complexify diagnosis and treatment in already complex medical situations.

In recent years, researchers have highlighted the potential of extracellular vesicles (EVs) in the development of potential vaccines or treatments for certain burdensome protozoan diseases, including leishmaniasis. EVs, which are released by all eukaryotic cells, are small, lipid membrane-bound vesicles that serve to transfer macromolecules (nucleic acids, proteins, etc.) between cells in a population (13, 14). Of the many DR mechanisms employed by *Leishmania* parasites, a number of these can likely be transmitted through EVs, leading to potential propagation of DR (15). Furthermore, it has been demonstrated *in vitro* that xenogeneic EVs (from different species of *Leishmania*) can be transferred from one species of *Leishmania* parasite to another, with their uptake inducing physiological changes in the recipient population (*i.e.* changes in drug sensitivity profile) (16). In this work, we set out to investigate the ability of xenogeneic EVs to modulate infection pathophysiology and drug sensitivity of naïve parasites after concurrent passage *in vivo*, in BALB/c mice.

Section 1: Literature Review

Chapter I: *Leishmania* and leishmaniases

1.1. Epidemiology & pathology

The epidemiology of leishmaniases is complex, and their distribution is affected by a number of environmental, biological, and social factors. The distribution of the *Leishmania* vector, phlebotomine sandflies, as well as the ecology of transmission sites and natural infection reservoirs all influence *Leishmania* prevalence within a certain region (17, 18). High rates of HIV and other immunosuppressive conditions are also often associated with increased rates of *Leishmania* infection (7). Furthermore, refugee crises and civil wars can lead to outbreaks that strongly and suddenly increase disease incidence in endemic areas (19). Environmental factors such as climate change and globalization, as well as recent increases in international travel and trade of blood products, may even lead to a rise in cases in non-endemic areas (19). Of note, leishmaniasis can affect both humans and animals, and clinical manifestations vary depending on the species of *Leishmania* in question (20). Interestingly, asymptomatic individuals represent a large proportion of *Leishmania* spp. infections in endemic areas, although the effect of these individuals on the epidemiology of leishmaniasis is still unclear (21).

1.1.1 Human leishmaniasis

The three main forms of leishmaniasis are CL, VL, and MCL. These pathologies vary not only in geographical distribution, but also in their severity and required treatment regimens. The most common form of leishmaniasis is CL, while VL is the most fatal (22). VL may also evolve into a pathology known as Post Kala-azar Dermal Leishmaniasis (PKDL) after treatment (23). Altogether, the leishmaniases are responsible for morbidity and mortality that accounts for approximately 2.4 million DALYs (24). Given the lack of effective prevention and treatment for *Leishmania*, control often rests on the use of insecticide and netting, as well as detection and treatment of disease (25).

1.1.1.1 Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis accounts for the majority of leishmaniasis cases around the world; over 1 million per year (22, 24). Causes of CL vary by geographical region, and include *L. braziliensis* and *L. panamensis* in the New World (Americas), and *L. major* and *L. tropica* in the Old World (Africa, Europe, and Asia). More than 90% of CL cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria (24). The disease causes ulcers on the body, many of which spontaneously heal after 2-15 months, depending on the infecting species of *Leishmania* (4, 24). That said, CL can still cause severe disability and stigmatization, leaving permanent, disfiguring scars on the affected patient (4). Furthermore, there exist cases of leishmaniasis recidivans (recurring leishmaniasis) and disseminated leishmaniasis, which are more difficult to treat than the localized form of CL. Optimal diagnosis utilizes parasitological methods, directly visualizing parasites. Treatment (especially for large, multiple, or persistent lesions) involves a lengthy course of parenteral antileishmanial drugs (ex. SSG), or in certain cases, intralesional injections (24, 26). The social stigma stemming from CL can greatly influence the lives of those affected; in certain regions, going to work may not be accepted, mothers may be prevented from interacting with their children, and women with scars may be considered unfit to marry (4).

1.1.1.2 Visceral leishmaniasis (VL)

Visceral leishmaniasis is fatal in 95% of cases if left untreated (22). Also known as Kala-azar (meaning black fever in India), VL is second only to malaria in number of yearly protozoan disease-induced fatalities, and the majority of cases occur in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan (25). There are approximately 60 000 new cases of VL reported every year, and this number is likely underestimated (27). Etiological agents of VL include *L. donovani* and *L. infantum* (or *L. chagasi*, as it is known in South America). VL can be anthroponotic or zoonotic, the latter of which is often transmitted from dogs to humans (25). Visceral leishmaniasis patients may present with fever, hepatosplenomegaly, pancytopenia and hypergammaglobulinemia, among other clinicopathological signs. Classical diagnosis involves parasitological methods (*i.e.* visualization of parasites in tissue specimens). Treatment of VL is key to preventing mortality, and rests upon the use of the five major antileishmanial agents available, only one of which can be administered orally. That said, treatment failure is rampant,

and in certain regions, combination treatments or increased dosages are required for effective resolution of clinical signs (7). Interestingly, certain patients previously treated for VL may later develop a rash (likely due to persistence of parasites in the skin and subsequent immune response) known as PKDL, and these patients may even contribute to VL transmission in certain areas (28).

1.1.1.3 Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis is a form of CL that leads to destruction of the mucous membranes of the throat, mouth, and nose (22). Caused by over 20 species of *Leishmania* parasites, including *L. braziliensis* and *L. major*, MCL may occur instead of CL based on the immunological status of the infected individual (29). This form of CL is more common in immunocompromised individuals; it is more severe, and can be life-threatening and/or lead to severe disfigurement. Diagnosis is based on observation of characteristic lesions in combination with molecular methods (*i.e.* PCR) (30). The most evidence-based treatment for MCL is based on administration of pentavalent antimonials three times per day for a period of up to 3 weeks (7). Over 90% of MCL cases occur in 4 countries: the Plurinational State of Bolivia, Brazil, Ethiopia, and Peru (22). Of note, a double-stranded RNA virus called *Leishmaniavirus 1* (LRV1) has been found in isolates of *L. braziliensis* and *L. guyanensis*. Studies in mice have shown an association between LRV1 and enhanced parasite replication, exacerbated pathology, and significantly increased rates of treatment failure (31).

1.1.2 Canine leishmaniosis

Canine leishmaniosis (CanL) is endemic to more than 70 countries around the world. The zoonotic CanL is caused by *L. infantum*, and infected dogs make up the principal reservoir for human infection; control of CanL infection is therefore an important pillar of prevention for human leishmaniasis (32). Preventing CanL is no small feat; it has been hypothesized that there are as many as 2.5 million infected dogs in the western Mediterranean region alone (33). Much like with human leishmaniasis, CanL is vector-borne and transmitted through the bites of infected *Phlebotomus* or *Lutzomyia* genus sand flies. That said, vertical transmission and transmission through bite wounds or infected blood products have also been documented (34, 35). Cases of CanL have even been reported in non-endemic areas for many years, particularly in kenneled

foxhounds in the United States and Canada since the early 2000's. That said, the mechanism by which this infection is maintained among foxhound populations has not been investigated in-depth (36).

In dogs, *L. infantum* infections may be asymptomatic or may cause cutaneous lesions, lymphadenomegaly, lethargy, and loss of body condition (37). Common laboratory abnormalities include anemia, hypoalbuminemia, and hyperglobulinemia (38). Diagnosis is made based on quantitative serological techniques, molecular techniques (*i.e.* PCR), and/or parasitological methods (*i.e.* histopathology). Treatment is limited to the same few antileishmanial drugs available for humans; antimony-based treatments or miltefosine are most recommended, often in combination with allopurinol. Although side effects during treatment are common, especially with antimonial drugs, many dogs with an initially stable clinicopathological status will attain clinical cure. That said, many continue to harbour the parasite after this point, even remaining infectious to sand flies, and a number will experience relapse in the following years. Prevention of CanL relies upon decreasing contact with sand flies, applying spot-on insecticide formulations or deltamethrin collars, and, in regions such as Brazil, vaccination of dogs with commercially available antileishmanial vaccines (32).

1.2 Parasite life cycle

Leishmania spp. are zoonotic protozoan parasites of the order Trypanosomatida. Like other trypanosomatids, *Leishmania* possess unusual genomic organizational traits (*e.g.* genes without introns, polycistrons), as well as an organelle called the "kinetoplast", which contains specific kinetoplastid DNA (39). *Leishmania* are digenetic unicellar eukaryotes with a life cycle that requires both a phlebotomine vector and a mammalian definitive host (*e.g.* a human or dog). They are also dimorphic, existing in one of two distinct morphological forms; either a motile, flagellated promastigote (inside the vector), or an immotile, intracellular amastigote (inside immune cells of the mammalian host).

1.2.1 Vector

Leishmania spp. are vectored by female sand flies from the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) (7). Over 30 different species of sand fly are known to transmit leishmaniasis (40). These insects from the family *Psychodidae* are endemic to several areas, including warm regions of Africa, the Americas, Asia, Australia, and southern Europe. Sand flies are named for their sandy colour and should not be mistakenly thought to only live and reproduce close to beaches. That said, these insects do require moist habitats to complete their life cycle, notably for the protection of eggs that will hatch into larvae requiring humid, nutrient-dense environments to thrive. Passage through a pupal stage gives rise to adult sand flies, which are usually under 3.5 mm in length (41). Both female and male sand flies feed on juices from plants and fruits, but females also require at least one bloodmeal to allow successful oviposition (42).

Although the plethora of host-derived factors necessary to the successful transmission of *Leishmania* infection has not yet been completely elucidated, several elements have been identified as determinant to the outcome of parasite development in the vector. For example, sand fly gut microbiota has been shown to have an effect on parasite development & vector competence (43). Interestingly, sand fly feeding behaviour is also crucial – multiple blood meals strongly increase vectorial capacity through both enhanced numbers of metacyclic promastigotes and increased blockage of the sand fly foregut, the latter of which contributes to regurgitation of parasites during future bloodmeals (44).

1.2.2 Promastigotes

When a sand fly takes an infected bloodmeal, the amastigote form of *Leishmania* is ingested and will transition into a promastigote inside the sand fly: a flagellum forms, and the spherical intracellular form of the parasite elongates into an ovoid conformation (Fig. 1). This change in morphology is likely due to a combination of factors, including temperature, pH, and possible vector-specific chemical triggers (45). The first form of promastigote is known as procyclic, occurring in the abdominal midgut of the sand fly; these will transform into nectomonad promastigotes, some of which migrate to the thoracic midgut and cardiac valve of the vector, transforming to leptomonad promastigotes, followed by their haptomonad or metacyclic forms.

Other nectomonad promastigotes will remain in the abdominal midgut, attached to the intestinal microvilli in order to avoid expulsion (45). Of note, the two subgenera of *Leishmania*, *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*), each demonstrate different patterns of development; the latter seeing a phase of promastigote development in the hindgut of the vector, with the former developing exclusively in the mid- and foregut (46).

Each stage of promastigote plays a different role in maintaining infection in the vectors; leptomonads replicate inside the vector and also contribute to production of promastigote secretory gel (PSG) that forces regurgitation of parasites by the sand fly during the mammalian bloodmeal (47). Haptomonads, with the help of hemidesmosomal structures, attach to the stomodeal valve of the vector, likely to help maintain long-term infection. Metacyclic promastigotes, meanwhile, are the infective, motile form that is transmitted to the mammalian host (45). That said, this form of the parasite has also been shown capable of de-differentiation for replication inside the sand fly in the form of retroleptomonads, enhancing infectivity of the vector during a second bloodmeal. The process to develop a transmissible infection in the vector takes approximately 1-2 weeks, and the sand fly will remain infective for the duration of its life (47, 48).

Metacyclogenesis is critical to parasite infectivity; this process not only leads to changes in parasite morphology, but also gene expression and the structure of its cell surface molecules. These changes offer the parasites some resistance to complement-mediated lysis, furthering their survival – an important trait to possess once entering the mammalian host (49). Once the infected sand fly bites a mammalian host, neutrophils and monocytes will be recruited to the site of infection due to the delivery of not only metacyclic *Leishmania* promastigotes, but also sand fly gut bacteria, PSG, and *Leishmania* EVs. Together, these activate the host immune response (further discussed in Chapter II) and help define the course of infection (44).

1.2.3 Amastigotes

Amastigotes make up the non-flagellated, intracellular, immotile form of *Leishmania*. When the infected sand fly takes a bloodmeal, metacyclic promastigotes are egested into the site of the sand fly bite (45). This is followed by recruitment of immune cells to the area; primarily neutrophils in the early moments of infection. After secretion of MIP-1β by neutrophils, entry of promastigotes

into macrophages (the ultimate mammalian host cell for *Leishmania*), is facilitated through phagocytosis of infected apopototic neutrophils (50). Inside the macrophage, promastigotes differentiate into their shorter, more spherical amastigote form. A small flagellum remains, but only the very tip protrudes beyond the parasite body. This drastic change in shape is hypothesized to minimize the area over which the parasite is exposed to the hostile parasitophorous vacuolar environment through a decrease in cell surface to volume ratio (45). Amastigotes then proliferate inside macrophages, eventually exiting (through an unclear mechanism) – the affected macrophage may burst, or amastigote-containing vacuoles may gather at the periphery, eventually leading to release of said vacuoles through a mechanism similar to exocytosis (51, 52). Infected macrophages may then be taken up during a sand fly blood meal and eventually transmitted to a new mammalian host, completing the parasite life cycle.

1.2.4 Reservoirs

Infection reservoirs are critical points for surveillance and public health intervention. Identifying species that serve as concentrated sources of infection (enzootic foci) may help prevent transmission to nearby communities and stave off outbreaks of disease (18). *Leishmania* parasites have at least 70 known reservoirs of infection, including humans, marsupials, sloths, and rodents (18, 22). That said, the dog serves as a principal reservoir for species causing VL (and likely an incidental host for other *Leishmania* species). The involvement of the dog is very likely due to their proximity to humans, as well as the large populations of outdoor and/or stray and wild dogs in certain regions, which allows for maintenance of a domestic and a peridomestic cycle. Although there does not exist a vast amount of data on the annual incidence of *Leishmania* infections in dogs, studies have found that seroprevalence may range anywhere from 0.3% (in Madrid, Spain) to 80% (in Marseilles, France) within endemic areas. Many of these dogs serve as asymptomatic carriers of leishmaniasis and their role in the epidemiology of the disease is not yet well understood; that said, it has been determined that symptomatic dogs are more infectious to sand flies than asymptomatic seropositive ones (33). More research is key to a better understanding of epidemiological risk for humans based on canine infection rates in different regions.

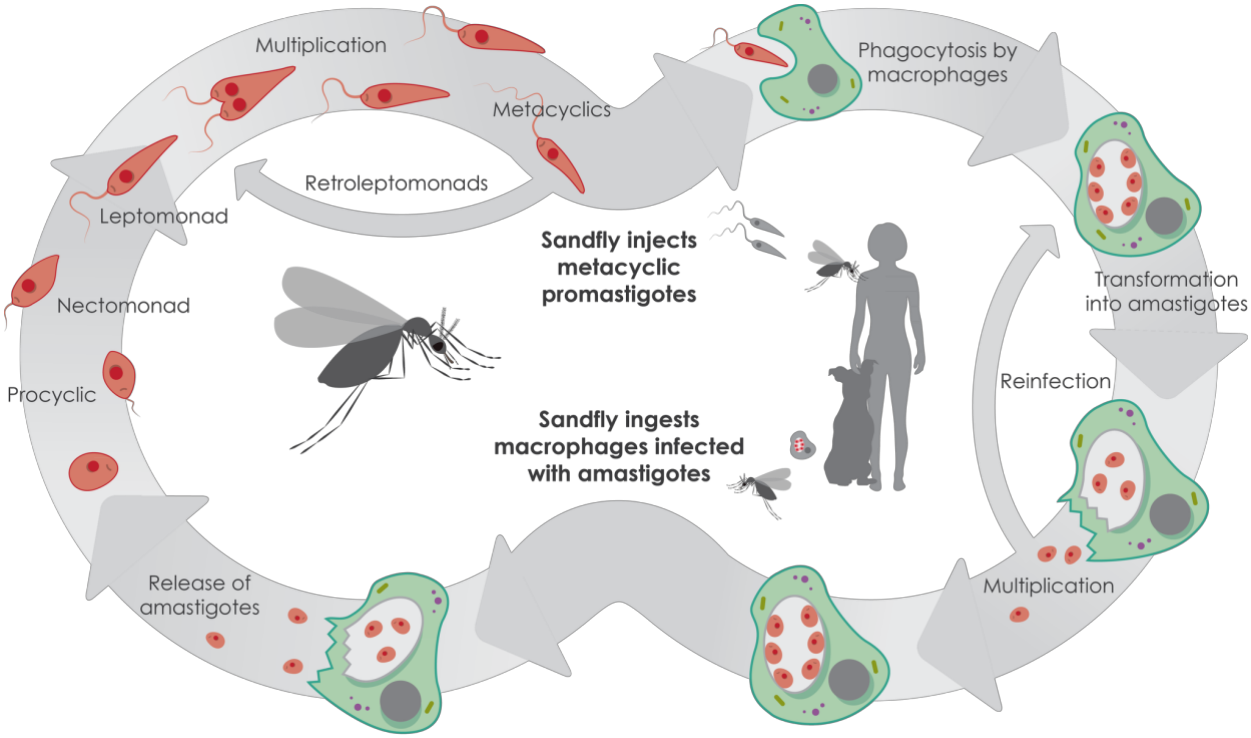

Figure 1: **Leishmania** ***life cycle. Adapted from (Wagner et al., 2019).***

Leishmania parasites are taken up by the sand fly vector during a bloodmeal. Ingested infected macrophages release amastigotes (the immotile form of *Leishmania*) into the sand fly gut, where they will transform into procyclic promastigotes, then nectomonad and leptomonad forms. The latter will either go on to become haptomonads (for maintenance of long-term infection in the vector) or metacyclic promastigotes, the infective form of *Leishmania*. Metacyclic promastigotes may transform into retroleptomonads for further multiplication, or go on to infect a mammalian host upon egestion by the vector during the next bloodmeal. Inside the mammalian host, promastigotes are phagocytosed by immune cells (e.g. macrophages), where they will transform into amastigotes and multiply. Thus, the cycle continues.

1.3 Diagnostics, treatment, and special considerations

As previously discussed, leishmaniasis is an NTD, and therefore not prioritized on international health agendas despite affecting a significant number of individuals around the world (4). The fight against leishmaniasis is further complicated by several social, economic, and practical issues. For example, this disease disproportionately affects regions with lower income and unreliable access to health services. Furthermore, not only is an effective drug for treatment of leishmaniasis difficult to find, but diagnosis is complex, and treatment accessibility is often precarious.

1.3.1 Roadblocks to treatment

Among the issues complicating treatment of leishmaniasis, one of the foremost is that available chemotherapy drugs are few and far between, and cases of treatment failure are increasing in certain regions (*i.e.*, SSG for treatment of CL in Sri Lanka) (53, 54). Additionally, diagnosis of leishmaniasis is challenging, often requiring numerous (including invasive) diagnostic tests at specialty medical centers, at high cost (53, 55). There are therefore many hurdles to overcome for an individual suffering from leishmaniasis, beginning with diagnosis.

1.3.1.1 Diagnostics

Parasitological methods remain the gold standard for diagnosis of leishmaniasis, in large part due to their high specificity (56). Although direct visualization of parasites on impression smears or biopsies may be feasible even at basic healthare facilities, histopathology and parasite culture, the latter of which is necessary for parasite species determination and drug resistance profiling, require specialized equipment and significant expertise (55, 56). Furthermore, aspiration sites offering very high sensitivity, such as the spleen or bone marrow, require technical expertise and may even cause life-threatening hemorrhage after sampling (57, 58). That said, there are a number of different types of diagnostic tests available (*e.g.* molecular, immunological); some more rapid and accessible than others. However, each has its own distinct sensitivity and specificity – which may vary between regions (55, 59). Each diagnostic test also has its own drawbacks; for example, fluorescent antibody tests have been found to demonstrate cross-reactions with other trypanosomal species; direct agglutination tests remain positive for many years after treatment; enzyme-linked

immunosorbent assays are extremely reliant on the sensitivity of the employed antigen; leishmanin skin tests cannot differentiate between past and present infection (55, 60). Taken all together, and with so many different forms of leishmaniasis – each with their own clinical manifestations – as well as the possibility of co-infections, it's no wonder diagnosis is complicated. Unfortunately, in many cases, the diagnostic tests available to patients are also limited.

1.3.1.2 Treatment accessibility

There is a strong, proven link between leishmaniasis and determinants of poverty, including illiteracy, malnutrition, gender inequality, and poor housing (61). Leishmaniasis cases most often occur in low-income areas, and place further strain on medical services and an economy already stretched thin (2, 53). Many cases occur in rural villages, far from publicly funded and well-equipped medical centers; as such, affected individuals are limited by the diagnostics and treatment they can pay out of pocket at a local private health center. These individuals are also constrained by their ability to take time off work and travel to a medical facility; obstacles that prove all the more burdensome for women since, in these areas, women are often engaged in unpaid labour inside the home, have no one to mind children in their stead, and no personal financial resources to seek medical aid (53, 62). This vicious cycle keeps it such that leishmaniasis disproportionately affects individuals in impoverished areas. All of these issues also contribute to underreporting of leishmaniasis cases in said regions, not only limiting accurate estimation of disease burden, but also the scope of potential medical intervention – including drug production & development opportunities (53).

1.3.1.3 Pharmacopeia

The list of drugs available for treatment of leishmaniasis is sorely lacking. Control is based on chemotherapy with one of 5 molecules: antimony-based drugs (*i.e.* SSG), AmB, MF, PARO, or pentamidine. SSG is the primary drug, which has been in use for over a century. That said, its nephrotoxicity in combination with widespread drug resistance, especially in the Indian Subcontinent, have led to its replacement with other drugs in many regions (9). However, the other available molecules also have their drawbacks: MF has already seen a decrease in efficacy after only a decade of use, AmB requires administration in a costly liposomal form to decrease toxicity,

PARO does not target all *Leishmania* species, and resistance to pentamidine has been described (9, 63). Combination treatment is often required but is not infallible (9). Furthermore, a number of the aforementioned treatments require hospitalization or daily visits to a medical facility for parenteral administration and monitoring – which, as discussed previously, is often impossible for leishmaniasis patients in rural or low-income areas (7, 24). At this time, there is a new GlaxoSmithKline drug for treatment of VL in Phase I clinical trials (in collaboration with the Drugs for Neglected Diseases initiative); that said, development of new drugs requires time, and unfortunately, many new lead compounds and proposed treatments are abandoned or fail to make it through the drug development pipeline (64, 65).

Of note, several of the current leishmaniasis drugs are also used for treatment of CanL, contributing to already-rising rates of drug resistance and treatment failure in many regions (9, 32). New treatments for leishmaniasis are urgently required.

1.3.1.4 Treatment failure

Treatment failure is multifactorial and encompasses drug-, host-, environment-, and pathogen-related factors. Treatment failure asks the question of why, in a single region, does the same treatment for an identical clinical presentation of leishmaniasis (caused by the same species of *Leishmania*), lead to clinical cure in one patient but not another? We know that drug efficacy plays a determinant role in treatment failure, but decreased treatment effectiveness can be due to various causes, not just DR. Incorrect drug posology, inadequate long-term storage of drugs in hot climates, or administration of expired drugs may also contribute (9). The patient's immune system also plays an important role, and is associated with nutritional status, age, sex, and co-morbidities, including co-infections (*Leishmania*/*Leishmania* and/or HIV) (54). The environment is also involved, both directly and indirectly. For example, arsenic in local drinking water has been shown to increase antimony resistance, and therefore treatment failure, in certain regions (10). Patient compliance, often linked to accessibility of medical care, is critical. Meanwhile, pathogen-related factors, such as virulence of the infecting strain, are also determinants of treatment failure (9). Specific leishmaniasis treatment failure rates are difficult to find in the literature, likely due to both the minimal resources available to detect and report these cases in affected areas, as well as

misreporting of treatment failure as drug resistance. That said, treatment failure has been documented for both CL and VL in a number of different countries, in connection with several different antileishmanial drugs (66-68). Some studies have reported failure rates as high as 21% for treatment of VL with MF in Nepal, and 45% for treatment of VL with SSG in northern India (68, 69).

1.3.2 Risks for the emergence of new endemic areas

Although leishmaniasis is endemic to many countries around the world, areas that have previously been considered exempt are beginning to see cases of CL, VL, and even CanL. Although many of these areas do not currently possess the phlebotomine vector responsible for the transmission of *Leishmania*, with climate change, the range of *Phlebotomus* and *Lutzomyia* (as well as other arthropods previously shown to be capable of *Leishmania* transmission in an experimental setting) will likely increase, creating the potential for establishment of new *Leishmania*-endemic areas (70). Furthermore, although sand flies are recognized as the principal vector of *Leishmania* parasites, certain other species of arthropod have also been demonstrated as competent vectors in experimental conditions, including ticks, dog fleas, and gnats (71-73). Additionally, increased travel and animal importation also contribute to the phenomenon of leishmaniasis cases in non-endemic areas.

1.3.2.1 Human leishmaniasis cases in non-endemic areas

International travel is becoming ever more popular and accessible, and tropical/subtropical areas are attractive destinations. Reports of imported leishmaniasis cases have multiplied in recent years, the majority of which concern leisure travellers and military personnel (74). For example, the number of yearly imported CL cases in the United Kingdom more than quadrupled between 1995 and 2003 (75). Meanwhile, international travel movements continue to rise; overseas travel by Canadians has grown almost steadily from 2004 to 2019 (76). Trendy outdoor sports and adventure travel further increase risk of travellers' contact with infected phlebotomine sand flies (74). Several regions have reported cases of imported leishmaniasis; North America, Germany, and Australia, among others (74, 77-79). That said, this is not a new phenomenon; CL and VL have been diagnosed in United States soldiers since the early 2000's after return from deployment to Iraq

(80, 81). Of note, North Americans generally do not have any pre-existing immunity to leishmaniasis and are therefore left vulnerable during travel to endemic areas given the lack of available vaccine/effective prevention (81). The body's complex immune response to *Leishmania* and the difficulties in translating candidate vaccines from animal models to humans, and from the laboratory to the field, have impeded vaccine development as of yet (82). Importantly, diagnosis and treatment of imported cases are also frequently delayed due to the lack of *Leishmania* expertise on the part of physicians in non-endemic areas; this poses a risk to the health of the affected individual, and possibly public health (74). Although there do not appear to be any reports in the literature of secondary *Leishmania* transmission from an imported human case in a non-endemic country, there exist recorded cases for CanL, and therefore it remains within the realm of possibility (83).

1.3.2.2 Animal importation & travel

Canine leishmaniasis is endemic to more than 89 countries (84). Reports of imported CanL cases appear to be on the uptick, whether due to increased travel, dog importation, or awareness on the part of North American veterinarians: an informal survey of veterinarians in Quebec, Canada noted over 12 cases identified by at least 6 different veterinarians in 2021 (85). There are many reports in the literature of imported CanL cases in non-endemic areas, such as Quebec and Romania (37, 86). Even more concerning, there are documented instances of non-vector-borne secondary transmission of imported CanL cases in non-endemic countries; in French Guiana, Finland, and New Caledonia (83, 87, 88). In these cases, dogfights, sexual transmission, and/or vertical (transplacental) transmission are implicated. Owners importing dogs from or travelling with their dogs to *Leishmania*-endemic areas should be properly informed of the necessary preventative measures, including reducing dogs' exposure to sand flies by keeping them in at dawn and dusk and regularly applying insect repellent (89). These methods are important not only for their own welfare, but for that of the animals and humans around them.

Chapter II: Immunology of leishmaniasis

2.1 Immunology

Leishmaniasis is a complex disease that takes many different forms. The severity of clinical signs and the ability of a host to clear *Leishmania* infection depends on both the parasites themselves and the performance of the host immune system. Parasite clearance, if possible, requires specific action on the part of a number of intricate, related immune functions, involving both the innate and adaptive immune response.

2.1.1 Immune response to *Leishmania* parasites

The innate response to *Leishmania* parasites features neutrophils and macrophages as its star players, with an important contribution from monocytes, dendritic cells (DCs), and natural killer (NK) cells. Studies have pointed to these 5 cell types as most important to determine susceptibility vs. resistance to *Leishmania* infection (90, 91). Meanwhile, the role of the adaptive immune response is slightly more difficult to define.

2.1.1.1 Innate immune response

In the early stages of *Leishmania* infection, it is neutrophils that are recruited to the infection site to phagocytose protozoan invaders for elimination. Neutrophils may also use neutrophil extracellular traps or nitric oxide (NO) for parasite killing (92, 93). Neutrophils will secrete interleukin-8 (IL-8) to attract further neutrophils to the site, while apoptotic neutrophils release MIP-1β for recruitment of macrophages. Macrophages, the definitive cellular host of *Leishmania*, will phagocytose neutrophils; this allows establishment of the infection inside macrophages, where parasites can not only survive, but multiply. This lead to the famous analogy of neutrophils as a "Trojan horse" (94).

Inside macrophages, parasites suppress interleukin-12 (IL-12) production, thereby preventing the Th1 response necessary for clearance of infection (95, 96). Triggered by IFN-γ, the production of inducible nitric oxide synthase (iNOS) (and therefore NO) by macrophages has been suggested as critical to primary resistance and to maintaining immunity induced by infection, in a murine model

(97). *Leishmania* further enable their own survival inside macrophages by preventing production of NO, likely thanks to virulence factor glycoprotein 63 (GP63) (see section 2.1.2) (98).

Monocytes, meanwhile, are also recruited to the site of infection in a chemokine receptor-dependent manner. It has been hypothesized that monocytes, like macrophages, serve as a main cell type in which parasites replicate inside the host – and produce iNOS critical to parasite clearance (94, 99). Also infected by *Leishmania* parasites are DCs, which serve as important antigen presentation cells for the induction and differentiation of CD4+ T cells into effector Th1 cells (by production of IL-12) necessary for elimination of parasites (100) (Fig. 2). NK cells, meanwhile, are important for IFN-γ production, and their depletion has been demonstrated to lead to increased parasite burden (101).

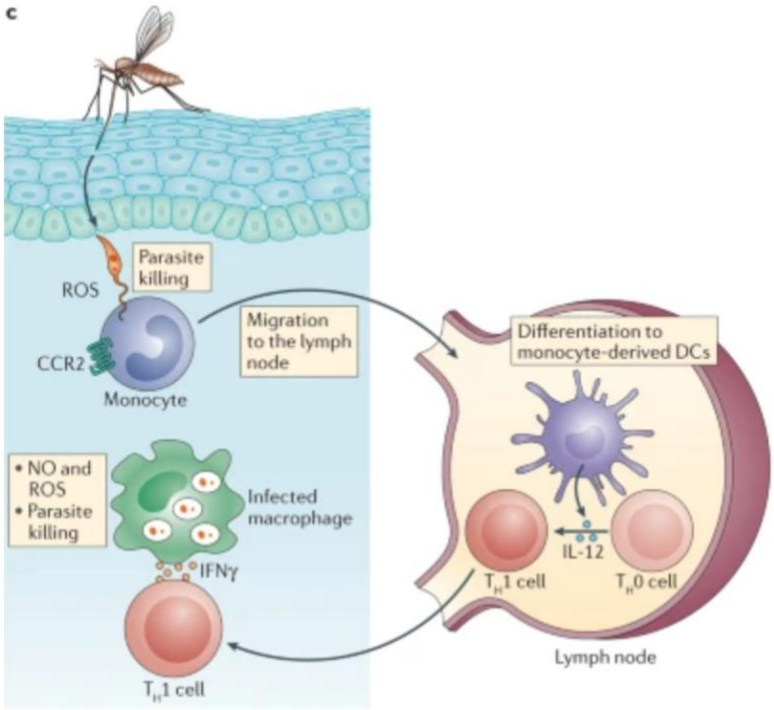

Figure 2: Innate immune response to* Leishmania*. Adapted from (Scott and Novais, 2016)

Chemokine receptor 2-dependent recruitment of monocytes from the blood to *Leishmania*-infected sites induces attempted parasite killing through production of reactive oxygen species (ROS) by

monocytes. Monocytes may also differentiate into dendritic cells (DCs) in lymph nodes and promote Th1 response for parasite killing through production of interleukin-12 (IL-12).

2.1.1.2 Adaptive immune response

With respect to the adaptive immune response, it is well established that T cells play a protective role against *Leishmania* infection, as demonstrated in mice (102). As discussed above, IFN-γ-producing CD4+ Th1 cells play a key role in resistance against parasites (94). The role of T-helper 17 (Th17) cells, meanwhile, varies depending on the *Leishmania* species in question; for example, Th17 cells may promote susceptibility to *L. major*, but resistance to *L. infantum* (103, 104). Regulatory T cells (Tregs) promote parasite persistence through restriction of effector CD4+ T cell function, allowing the host to develop durable immunity (105).

The role of CD8+ T cells in *Leishmania* immunity is less well elucidated, with some studies suggesting its significance for the control of primary infection, and others pointing to its role in the secondary immune response (106, 107). That said, its importance in vaccine-mediated immunity is well recognized (108). The role of B cells in *Leishmania* elimination is also somewhat debated; *Leishmania* are intracellular parasites that are inaccessible for antibody-mediated killing. That said, it has been reported that activated B cells may increase DCs' antigen uptake capacity, strengthening Th1 response (109).

Taken all together, *Leishmania* parasites initiate a complicated mosaic of immune mechanisms that may work together to clear infection, or lead to survival of the parasite and clinical illness in the host.

2.1.2 *Leishmania* virulence factors

The immune responses discussed above are triggered by parasite virulence factors. *Leishmania* possess a number of virulence factors, among them the promastigote surface lipophosphoglycan (LPG), glycoinositolphospholipids (GIPLs), proteophosphoglycans (PPGs), and the renowned zinc-dependent metalloprotease GP63 (110).

For example, macrophage modulation through inhibition of protein kinase C (PKC) by *L. donovani* parasites has been linked to LPG activity (111, 112). A note: importantly, conclusions about LPG may vary depending on the *Leishmania* species and animal model in question, as demonstrated by the contradictory LPG results seen with *L. mexicana* in C57BL/6 mice vs. BALB/c mice (113). On the other hand, both *L. major* and *L. donovani* LPG have been shown to inhibit phagosome maturation in early infection, facilitating promastigote survival inside the vacuole (114, 115).

GIPLs, meanwhile, help *L. donovani* parasites invade macrophages; disrupting the availability of GIPLs decreases macrophage infection rates (116, 117). With respect to PPGs, different forms play different roles in *Leishmania* infection (110). Some increase macrophage recruitment to the infection site, others modulate host immune cell response (118, 119). Other *Leishmania* virulence factors include δ-amastins (surface glycoproteins), shown to play a critical role in persistent *Leishmania* infection, as well as hydrophilic acylated surface proteins, which have been suggested to modulate host immune response (120, 121).

Perhaps the most well-recognized *Leishmania* virulence factor is GP63. Also known as leishmanolysin, this major surface antigen is expressed by promastigotes of numerous species of *Leishmania*. Through cleavage of C3b into iC3b, GP63 helps prevent complement-mediated lysis whilst simultaneously favouring its own internalization by macrophages (122, 123). GP63 also appears to be produced by amastigotes; although to a lesser extent – its expression has been shown as key for intracellular survival of the parasite (124). Importantly, GP63 influences macrophage signalling pathways and transcription factors. GP63 is responsible for the activation of specific protein tyrosine phosphatases (PTPs), including HP-1, PTP1B and TCPTP, that play a role in the JAK/STAT pathway and impact IFN-γ-mediated signalling; this contributes to *Leishmania*'s escape from the host innate immune system (110, 125, 126). As far as modulation of host cell transcription factors, GP63 has been shown to cleave NF-kB, inducing specific chemokine production by infected macrophages, as well as subunits of AP-1, leading to downregulation of innate immune response, among others (110, 127). Overall, this virulence factor acts in a number of ways to favour survival of *Leishmania* inside the host cells. Interestingly, it has been shown that GP63 is non-conventionally secreted by *Leishmania*, through extracellular vesicles (EVs).

2.2 Extracellular vesicles

Extracellular vesicles are produced by eukaryotic organisms and found in all biological liquids (*e.g.* blood, urine). These lipid-membrane bound vesicles vary in size; exosomes, a type of small EV (sEV), range from approx. 40-200 nm in diameter (13, 128). Produced inside the cell through fusion of multivesicular bodies (MVBs) and released when said compartment merges with the plasma membrane, EVs carry surface molecules that permit specific targeting, binding, and later internalization by recipient cells for uptake of EV contents (129). EV populations are heterogeneous, with various macromolecules (proteins, DNA, etc.) contained inside/coating their outer membrane, ready to be delivered to a recipient cell for purposes of cell-cell communication. EV contents reflect the physiological state of their cell of origin, and EVs are frequently referred to as "snapshots" of a cell (reviewed in (13)).

2.2.1 The origin of EVs

EVs have been traditionally defined as belonging to one of several subclasses; exosomes, microvesicles (ectosomes), or apoptotic bodies. These are separated based on their method of biogenesis; either fusion of multivesicular bodies (MVBs) with the plasma membrane of a cell (a pathway that utilizes endosomal protein complexes called "ESCRT" machinery) to form exosomes, direct outward budding from the plasma membrane to form microvesicles, or outward blebbing from cells during apoptosis to form apoptotic bodies. In recent years, other more specific subtypes have also been proposed (130-132) (See Fig. 3).

Although EVs have gained great attention in the past two decades, they have long been described. For example, in the 1940s, Chargaff & West made the discovery of a particulate fraction present in blood samples after sedimentation in a centrifuge at high speeds; in the 1960s, transmission electron microscopy (TEM) photos of this "platelet dust" were published (133, 134). Throughout the following 20 years, studies continued to identify structures consistent with the morphology of what would later be known as EVs. That said, it was in the 1980s that the existence and origin of EVs were truly elucidated; TEM images of fusion of MVBs with the plasma membrane were produced, and the existence of the exosome secretion pathway described (133, 135, 136). Over the

next few decades, scientists would explore the role of EVs in disease, especially in cancer studies, and later in the search for prevention of and treatment for protozoan diseases (13, 133).

2.2.2 Prospective applications for EVs

One of the reasons for the popularity of EVs in research is their numerous potential applications in medicine. In cancer studies, EVs are already being used as biomarkers. For example, glioblastoma and urothelial carcinoma lead to production of EVs (in the blood or the urine) containing specific mutant nucleic acids or miRNA profiles, respectively, allowing diagnosis of the disease (137, 138). Certain EV miRNA profiles have also been shown to help determine prognosis of esophageal squamous cell carcinoma or breast cancer, either through detection of advanced stage of disease or markers of malignancy (139, 140). In the Fernandez-Prada lab, EVs have also been proposed as a biomarker for *Leishmania* drug resistance, as EVs produced by drug-resistant parasites have been shown to differ in size, morphology, and protein content from those produced by drug-sensitive parasites (15).

EV-based vaccine development is also a topic of great interest, especially in parasitology. It has been shown that, not only do EVs play a key role in the pathogenesis of a number of diseases, but EVs are essentially innocuous if administered on their own (13, 141). It has also been demonstrated that EVs can directly transfer cargo to their target cells, even interacting with T-cells, and have an ability to modulate the innate immune system (13, 14). Although a number of obstacles still remain to hone EV-based vaccine strategies (among these, overcoming their heterogeneity, determining antigens of interest to include on/load in vesicles, and exploring the necessity of an appropriate adjuvant), EVs still remain a promising direction for vaccine development, especially against protozoan diseases. Some studies have already been performed with good results (142). A number of authors have proposed that EVs could provide a quality solution for leishmaniasis vaccine development, especially in combination with PSG from the vector (143, 144).

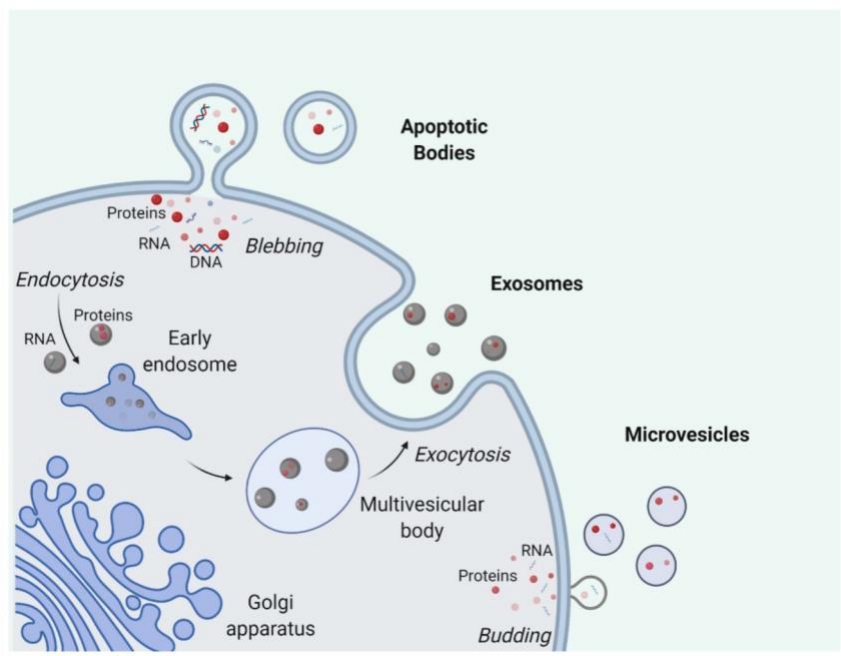

Figure 3: Biogenesis of extracellular vesicles. Adapted from (Dang et al., 2020)

The mechanism of biogenesis of extracellular vesicles (EVs) determines their subclass: apoptotic bodies bleb out from the plasma membrane of cells in apoptosis, exosomes are exocytosed by cells when multivesicular bodies (MVBs) fuse with the plasma membrane, while microvesicles (also termed "ectosomes") bud directly from the plasma membrane of healthy cells.

2.3 *Leishmania* and EVs

Today, the study of *Leishmania* goes hand in hand with the study of EVs. Given its position as an NTD, novel solutions for prevention and treatment of leishmaniasis are highly desirable; EVs' potential use in vaccine-based applications or as biomarkers of drug resistance (as discussed above), make them especially interesting in the study of protozoan diseases.

2.3.1 Discovery

Leishmania EVs were originally highlighted as a subject of interest by the Olivier lab after their observation of vesicles gathering around *Leishmania* GP63 inside macrophages (126, 145). Studies

of the *L. mexicana* exoproteome suggested exosome secretion, after which *Leishmania* exosome secretion itself was clearly demonstrated (146, 147). That said, a key discovery with respect to the role of *Leishmania* EVs in pathogenesis came when the Olivier lab determined that a temperature shift (simulating the conditions of *Leishmania* inoculation into a mammalian host) lead to a rapid and significant increase in EV release by the parasite (146).

2.3.2 Immune response

Leishmania not only releases EVs, but these EVs also interact with host immune cells in order to facilitate parasite survival. It has been demonstrated that *Leishmania* EVs can contain a number of virulence factors that interact with host cells to play a role in pathogenesis, including GP63, cysteine peptidases, and more (Fig. 4). As discussed in section 2.1.2, GP63 is once again considered an important player in immune modulation; it has been reported that EVs harvested from GP63$^{-/-}$ *L. major* failed to modulate PTPs to the same degree as GP63$^{+/+}$ parasites. EVs from GP63$^{-/-}$ parasites also bear a significantly modified protein profile compared to GP63$^{+/+}$ parasite EVs, and it was shown that GP63-depleted EVs modulate macrophage cytokine receptor expression (148). Altogether, GP63 has been shown to cause obstruction of cell signalling and inhibition of microbicidal functions, promoting survival of intracellular parasites.

A further point of interest regarding EV interactions with the host immune system pertains to *Leishmania* RNA virus 1 (LRV1). Specifically in the case of *Leishmania* (*Viannia*) *guyanensis*, a New World species of *Leishmania* that causes CL and MCL, it was determined that parasite infection with the dsRNA virus LRV1 is correlated with enhanced production of proinflammatory cytokines through increased toll-like receptor 3 (TLR-3) activation (149, 150). The use of TEM provided a breakthrough, clearly demonstrating the inclusion of viral particles in a significant proportion of *L. (V.) guyanensis* sEVs (151). It was also reported that EVs containing LRV1 can induce activation of TLR3 and inhibition of the NLRP-3 inflammasome, even in the absence of *Leishmania* parasites – and this modulatory activity is actually dependent on packaging of the virus into EVs (152). *Leishmania* and LRV1 therefore have a reciprocal relationship, both working to further their own survival inside the host, and early infection outcomes are strongly influenced by the presence and activity of EVs.

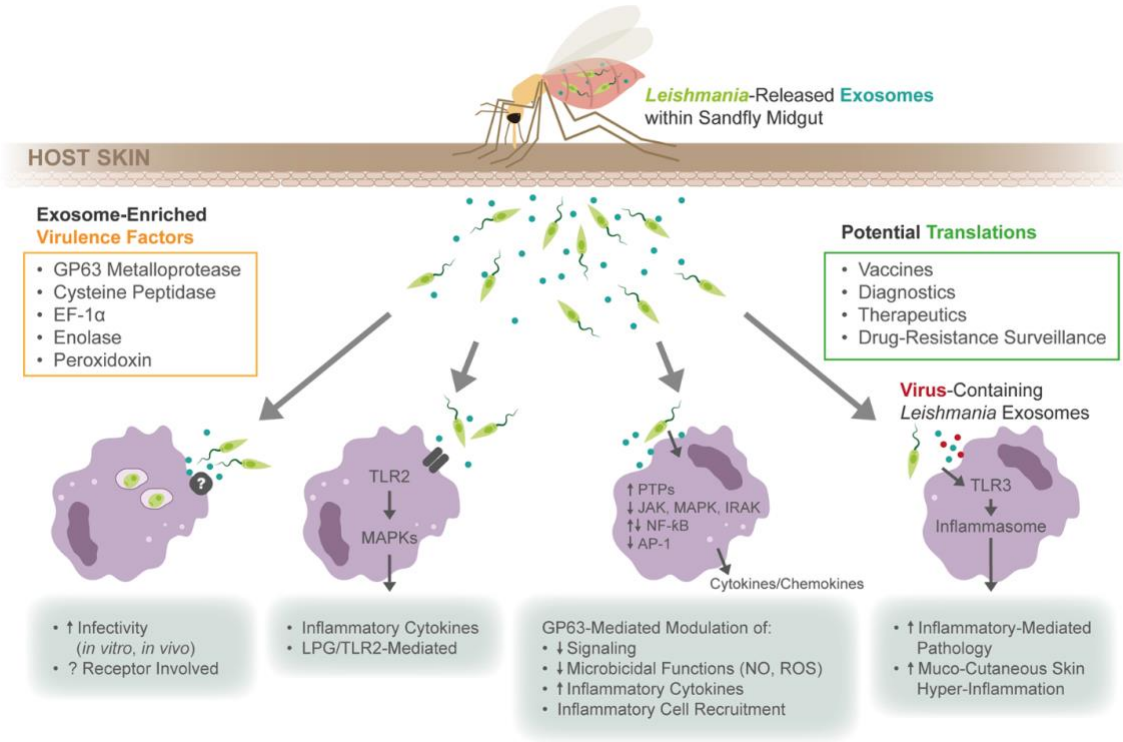

Figure 4: Brief overview of* Leishmania *exosomes' interaction with host immune cells (Dong et al., 2021)

During a sand fly bloodmeal, *Leishmania* promastigotes are egested into the host alongside small extracellular vesicles (EVs), such as exosomes. EVs contain a number of virulence factors (GP63, cysteine peptidase, etc.) which modulate host immune cells to promote parasite survival and progression of pathogenesis. For example, disruption of cell signalling decreases cytokine release and subsequent nitric oxide (NO) production. Furthermore, *Leishmaniavirus 1* (LRV1)-containing EVs modulate inflammasome activation. Taken all together, these characteristics make *Leishmania* EVs an interesting candidate for vaccine development and as a biomarker for drug resistance, among other possible applications.

2.3.3 Potential role in early infection & disease progression

The severity of leishmaniasis varies depending on its form (CL, VL, or MCL) and a number of host-, parasite-, and environment-related factors. As previously discussed, both the parasite itself and its EVs can modulate the host immune system; therefore one may infer that EVs also play a role in leishmaniasis pathology. And this is true; EVs influence pathogenesis from the very beginning – inside the sand fly. It was demonstrated that EVs are secreted by *Leishmania* inside the vector and co-egested into the host during the sand fly bloodmeal (141). This finding led to the hypothesis that *Leishmania* EVs may play a role in early infection, and indeed it was later demonstrated that co-inoculation of EVs and *L. major* in the footpad of mice significantly enhanced inflammation (13, 141). As such, although EVs alone do not induce pathology, EVs in combination with *Leishmania* parasites appear crucial for enhanced infection.

Chapter III: Drug resistance

3.1. Mechanism of action of current antileishmanial drugs

Leishmaniasis requires treatment with the small arsenal of currently available antileishmanials, made up of 1) antimony-based drugs, 2) MF, 3) AmB, 4) PARO, and 5) pentamidine, as previously mentioned. Mechanism of action varies depending on the molecule in question. For example, treatment with antimonial drugs (SSG, MA) is based on the reduction of pro-drug Sb^{V} to its trivalent form, Sb^{III}, inside macrophages and/or parasites (153) (Fig. 5). The mechanism(s) at play are not entirely elucidated; however, it appears that Sb^{III} induces killing of parasites through inhibition of trypanothione reductase, a key player in parasite thiol redox homeostasis, and therefore impedes protection against chemical and/or oxidative stress (154). Of note, the intracellular quality of amastigotes presents a particular challenge for treatment of leishmaniasis. Antimony, in its pro-drug form (Sb^{V}), must penetrate macrophages before reaching the parasite, and also be reduced to Sb^{III} for effective antileishmanial action (9). Meanwhile, promastigotes cannot reduce Sb^{V} to Sb^{III}, and are therefore not susceptible to pro-drug Sb^{V} (153).

On the other hand, AmB exerts its antileishmanial action through binding to ergosterol, a component of parasite cell membranes, leading to promotion of cell death through increased permeability and consequent ion loss (155). Meanwhile, MF inhibits synthesis of phospholipids essential to trypanosomatid composition, affects parasite mitochondria, and may also alter parasite plasma membrane Ca^{2+} channels (156). PARO has long been thought to affect RNA synthesis & possibly membrane permeability; that said, a recent work utilizing next-generation sequencing (NGS) techniques suggests that PARO influences *Leishmania* translational machinery (157, 158). Finally, pentamidine is thought to kill parasites through inhibition of polyamine synthesis and altering the potential of the inner mitochondrial membrane (159).

Despite these diverse mechanisms of action, *Leishmania* parasites are skilled at finding a way to survive in the presence of antileishmanial drugs.

3.2. Factors leading to the development of DR *Leishmania*

As previously discussed, many factors play an important role in the successful treatment of leishmaniasis. With the excessively limited pharmacopeia available for treatment – an arsenal of drugs shared between humans and dogs, several of which have been in use for many years – proper posology and compliance are of the utmost importance. That said, historical sub-optimal dosing of drugs, poor storage of drugs, combination therapies with difficult-to-determine posology, as well as the need for sudden discontinuation of treatment in the case of serious side effects all contribute to the exposure of *Leishmania* parasites to sublethal doses of antileishmanial drugs, and therefore to the emergence of drug resistance (9, 160).

3.3. Introduction to drug resistance in *Leishmania* parasites

Antimony-based drugs were long considered a first-line treatment for leishmaniasis. However, troubling rates of treatment failure – demonstrated to be in large part due to drug resistance – were observed for SSG treatment, especially in the ISC. Cure rates of above 90% in the early 20th century dropped abruptly; treatment failure reached 30% in the 1970s, and despite doubling drug dosages, continued to skyrocket all the way to 65% in Bihar. The treatment soon had to be abandoned in the ISC, and today is less popular in many regions, abandoned in favour of the less toxic and more effective AmB and MF (9, 161).

3.4 Overview of drug resistance in *Leishmania* parasites

Leishmania parasites are experts at evading death, whether by the host immune system or an antileishmanial drug. A main contributor to *Leishmania*'s propensity for survival in the presence of stressful (*i.e.* drugged) conditions is the plasticity of its genome. These parasites utilize a number of effective – and impressive – mechanisms to change their genome in response to various stressful conditions; these include DNA amplifications, copy number variations (CNVs), changes in ploidy, and single nucleotide polymorphisms (SNPs) (162, 163). Gene regulation in *Leishmania* occurs at the post-transcriptional and (post)-translational levels rather than at the outset of transcription; when selective pressure is applied, these parasites can still modify their genome to increase fitness through amplification (or deletion) of loci coding for genes involved in drug metabolism.

Amplifications are often seen in the form of extrachromosomal circular (or linear) amplicons; these may or may not remain stable when pressure is withdrawn (163, 164). Interestingly, DNA amplifications appear to be stochastic, meaning that parasites among a population will have a conserved core genome, but vary with respect to their individual extrachromosomal amplicons – an adaptation giving populations the best chance of survival in any conditions (163). All these genomic changes can lead to phenotypic changes modulating drug uptake or metabolism by *Leishmania* to promote their survival; some decrease drug influx, others increase efflux or sequestration, some prevent drug activation (9).

3.4.1 Mechanisms of antimony resistance

Parasites mount a number of defenses against antimonial drugs, often in combination; some general (such as increased heat shock proteins), and others more selective (9, 165). For example, the aquaglyceroporin (AQP1) gene modulates uptake of Sb^{III} and can lead to accumulation of the drug in the parasite; in general, AQP1 RNA levels are downregulated in antimony-resistant *Leishmania* (9, 166, 167).

Meanwhile, ATP-binding cassette (ABC) transporters, involved in transport of various molecules across biological membranes, have also been demonstrated to play a role in antimony resistance. A specific ABC-transporter called MRPA, located close to the flagellar pocket of the parasite, acts through sequestration of thiol-metal conjugates and has been found to be consistently overexpressed in antimony-resistant parasites (9, 168, 169). This has been explored in-depth in antimony-resistant *L. infantum* mutant strain Sb2000.1 (170).

Other demonstrated mechanisms of drug resistance to antimony in *Leishmania* parasites include ABC-transporter PRP1, primarily implicated in resistance to pentamidine, but also hypothesized to confer some antimony resistance, as well as overexpression of tryparedoxin peroxidase, which acts on thiol redox metabolism (9, 171). Furthermore, overexpression of ABCC2 and changes in ploidy have also been associated with antimony-resistant phenotypes *in vitro* (168). Antimony-resistant *Leishmania* parasites have also been shown to possess specific glycans that contribute to upregulation of interleukin-10 (IL-10), provoking drug efflux from the macrophage through overexpression of multidrug resistance protein 1 (MDR1) (172). Finally, Cos-Seq led to the

discovery of a number of novel drug resistance genes in *Leishmania*, including *LinJ.06.1010*, implicated in antimony resistance (173).

Antimony resistance in *Leishmania* parasites is therefore both multifactorial and multifaceted, making it all the more difficult to prevent and surmount.

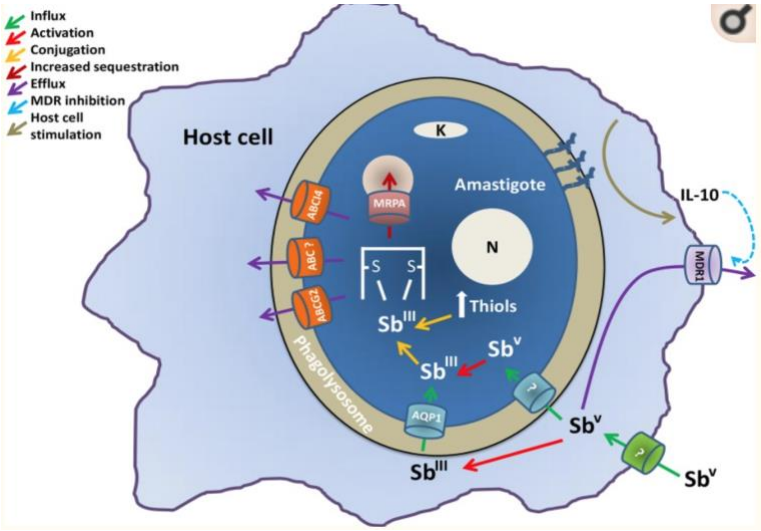

Figure 5: Mechanisms of antimony drug resistance employed by intracellular* Leishmania *amastigotes (Ponte-Sucre et al., 2017)

Inside an infected macrophage, *Leishmania* amastigotes rely on a number of mechanisms in order to evade killing by antimonial drugs (Sb). Once pro-drug Sb^{V} is reduced to its trivalent form, Sb^{III}, and threatens *Leishmania*, the parasite can utilize a number of methods to further its own survival; among them, decreased drug influx through downregulation of aquaglyceroporin (AQP1), increased drug sequestration by ATP-binding cassette (ABC) transporter MRPA, and increased drug efflux through overexpression of multidrug resistance protein 1 (MDR1).

3.4.2 Epidemiology and brief mechanistic overview of other DR in *Leishmania* parasites

AmB is a polyene antibiotic that has been used in leishmaniasis treatment for over 50 years. AmB selectivity is due to its affinity for ergosterol, a sterol found in lipid membranes – but which is less

predominant than cholesterol in mammalian cells (174). *In vitro*, *L. donovani* AmB resistance has been demonstrated through promastigote changes in plasma membrane sterol profile (175). Although currently there are relatively few reports of *Leishmania* resistance to AmB, increasing rates of treatment failure with antimony-based drugs are leading physicians to lean more heavily on AmB therapy, opening the possibility for more reports of AmB drug resistance in the near future (174).

With respect to MF, the only oral drug available for treatment of leishmaniasis (registered in 2002), mechanism of action for parasite killing likely involves disruption of phospholipid biosynthesis, alteration of cytochrome c oxidases, and subsequent reduction in ATP levels in the parasite, leading to apoptosis (9, 176). Following rampant treatment failure in the area, MF rapidly overtook SSG as first-line treatment for leishmaniasis in the ISC. However, even 10 years after its introduction, relapse rates after MF treatment were increasing in India and Nepal, observed to be due to increased efflux/decreased uptake of the drug by resistant parasites (177, 178). Certain genes, for example *LdMT*, have also been proposed as markers for clinical MF resistance, while gene *LinJ.32.0050* was shown to be implicated in MF drug resistance through Cos-Seq studies (173, 179).

Meanwhile, PARO, an aminoglycoside-aminocyclitol antibiotic, has been used for treatment of VL and CL, but with certain species of *Leishmania* responding better to treatment than others. Its use has been extremely limited until this time, as are reports of PARO resistance (174). That said, resistance has been documented in clinical isolates of *L. aethiopica* from relapsing patients – although the mechanism behind said drug resistance has not yet been fully elucidated (180). A recent study demonstrated that PARO susceptibility may be linked to mutations in proteins involved in the translation process (157).

With respect to pentamidine, in spite of its rare use, during the short time it served as second-line treatment for antimony-refractory leishmaniasis cases in India, its cure rate saw a drop of over 25% in only 10 years (174, 181). Experimentally, pentamidine resistance in *Leishmania* has been suggested to stem from decreased drug uptake/increased efflux (63). Furthermore, Cos-Seq screening used to identify hypothetical drug resistance genes in *Leishmania* found 21 putative

pentamidine resistance genes noted – one of which was common to PARO-resistant *Leishmania* (182).

Leishmania parasites are extremely skilled at survival, whether in the host or in the presence of drugs, and have even been known to survive combination therapy (9). Interestingly, certain mutations discovered in MF-resistant *L. infantum* were observed in AmB-resistant parasites; especially those leading to major changes in parasite lipid species. As such, the risk of emerging cross-resistance exists (183). Given that the current pharmacopeia of antileishmanial drugs alone cannot be relied upon, new therapies or strategies for prevention of leishmaniasis are critical.

3.4.3 Strategies to overcome drug resistance

A number of strategies have been proposed to overcome drug resistance; first and foremost, prevention of the disease in question. That said, no effective antileishmanial vaccines are available at this time (82). Fortunately, some vaccines exist for prevention of CanL, which, as discussed previously, is an important pillar for preservation of human health. However, these vaccines do not offer complete protection, and their widespread implementation in certain areas may be complex (*i.e.* in regions where seropositive dogs are generally culled) (184).

With respect to leishmaniasis treatments, combination therapies are now recommended by the WHO in a number of regions, and may provide better treatment success rates, less side effects, and prolong the time to development of drug resistance by parasites. That said, this strategy is not foolproof, and exposure of parasites to sublethal doses of one or more drugs may lead to drug resistance regardless (9).

Finally, drug repurposing is a promising approach in the treatment of leishmaniasis. Drug repurposing, or repositioning, involves finding new uses for already-approved or studied drugs. This method decreases costs and development timelines, allowing for safe new drugs, quickly (185). One example of this strategy is the investigation of anti-cancer drug topotecan, a topoisomerase 1B (TOP1B) inhibitor, for treatment of leishmaniases (162, 186).

3.5 Drug resistance and EVs

It has already been discussed that EVs not only interact with the host immune system, but also play an important role in *Leishmania* pathogenesis, specifically in the early moments of infection (110, 141). On the other hand, EVs have also been implicated in drug resistance. For example, studies in the Fernandez-Prada lab found that drug resistance leads to modifications in EV morphology, size, and distribution by parasites. Furthermore, depending on the drug responsible for induction of drug resistance *in vitro*, different proteins in the exoproteome are enhanced, leading to distinct profiles; this goes to support the potential use of EV protein profiles as biomarkers for DR *Leishmania* (15).

EVs were originally known for their role in cell-cell communication. Given that it has been previously demonstrated that, a) *Leishmania* parasites release EVs inside the phlebotomine vector, b) two species of *Leishmania* can complete their life cycle inside the vector at the same time, c) *Leishmania*/*Leishmania* co-infections exist, and d), drug resistance is frequent in *Leishmania,* especially with respect to antimonial drugs, what does this mean for propagation of *Leishmania* drug resistance (9, 11, 12, 141)?

3.5.1 Transmission & propagation

A critical discovery surrounding drug resistance and *Leishmania* EVs comes from the Fernandez-Prada lab Transwell™ work. These *in vitro* experiments demonstrated that, when two populations of parasites are separated by a barrier that is only large enough to allow EVs to pass, the parasites from one population will take up EVs from their neighbouring parasites. Of utmost interest is the fact that drug-sensitive parasites receiving EVs from DR strains display corresponding changes in their drug sensitivity profile. Moreover, these discoveries proved consistent even with xenogeneic *Leishmania* parasites (*e.g.* drug-sensitive *L. major* successfully takes up EVs from DR *L. infantum*) (16).

Through release and subsequent uptake of EVs in the vector, *Leishmania* parasites are therefore well-positioned to spread drug resistance. In combination with their propensity for survival under

a plethora of stressful conditions, these parasites present a growing concern, and further studies on the subject of EVs' role in transmission of drug resistance are warranted (187).

Section 2: Description of M.Sc. project

1 – Rationale

Leishmaniasis is a global neglected disease that disproportionately impacts those of low socioeconomic status, leading to widespread morbidity and mortality. Millions of individuals are affected every year, to the detriment of both their physical health and quality of life. Moreover, leishmaniasis is responsible for a significant economic burden in endemic areas. Dogs serve as a reservoir for this zoonotic disease, exacerbating transmission in regions with a large canine population (domestic and/or stray), and may also suffer from CanL themselves (1, 4).

Unfortunately, diagnosis and treatment of leishmaniasis is difficult, and co-infections with different *Leishmania* species have also been reported – especially in immunosuppressed populations – leading to ever more complicated manifestations of disease. In many cases, there are also numerous socioeconomic barriers preventing those most vulnerable from receiving the necessary medical attention. Given that visceral leishmaniasis is fatal without adequate treatment, this disease represents a serious public health concern for many communities – one which is often neglected by policy-makers. Furthermore, there does not currently exist any vaccine to prevent leishmaniasis in humans, and the paltry arsenal of drugs available for treatment is rapidly shrinking due to the emergence of extensive drug resistance (9, 11).

Meanwhile, extracellular vesicles (EVs), shown to facilitate intercellular communication, have become of particular interest in relation to leishmaniasis. EVs, whose contents reflect their cell of origin, have been demonstrated to play a role in *Leishmania* early infection – not only are they released inside the phlebotomine vector and co-egested into the mammalian host, but they also exacerbate pathophysiology of cutaneous leishmaniasis in a murine model (141). Of utmost importance, EVs from drug-resistant *Leishmania* populations have been shown to contain drug resistance-associated molecules, and even be capable of transferring said molecules to naïve *Leishmania* parasites *in vitro*, altering their drug sensitivity profile (15, 16, 146).

Taken altogether, it's clear that widespread drug resistance represents a critical issue that, along with co-infections, must be overcome in order to help lessen both the loss of life and economic

burden caused by leishmaniasis. EVs may play a key role in the transmission of *Leishmania* drug resistance, and therefore the full extent of their abilities requires further investigation.

2 – Research hypotheses

Given the magnitude of suffering caused by leishmaniasis, the continued development of *Leishmania* drug resistance, reports of *Leishmania*/*Leishmania* co-infections in society's most at-risk individuals, and the ability of EVs to transfer drug resistance *in vitro*, our group felt it was critical to further explore the role of *Leishmania* EVs in co-infections and propagation of drug resistance *in vivo*. We therefore emitted the following two hypotheses:

1) in the presence of xenogeneic EVs (from distinct *Leishmania* strains/species), the pathophysiology of infections demonstrates characteristics of both the infecting strain and the strain from which the EVs were derived, and

2) upon *in vivo* contact between drug-susceptible *Leishmania* parasites and EVs from resistant parasites, there is potential transfer of drug resistance-associated molecules that can lead to subsequent changes in drug resistance profile.

3 – Specific objectives

The following objectives were established in order to verify the project hypotheses:

1. Characterize the pathophysiology of *Leishmania major* and *Leishmania donovani* infections (laboratory and field strains) in BALB/c mice.
2. Determine whether the pathophysiology of infections is altered in the presence of xenogeneic EVs administered *in vivo*.
3. Determine whether a decrease in drug susceptibility is transferred during contact between susceptible parasite strains and EVs from resistant strains *in vivo*.

Section 3: Article

In preparation

Extracellular vesicles from xenogeneic *Leishmania* spp. can modulate infection and propagate drug resistance *in vivo*

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Abstract

Leishmaniasis is a serious zoonotic disease caused by protozoan parasite *Leishmania* spp., endemic to 98 countries and territories. Furthermore, co-infections with multiple species of *Leishmania* have also been reported. As parasite drug resistance continues to grow, there is an urgent need to better understand the spread of *Leishmania* drug resistance. Extracellular vesicles (EVs) from *Leishmania* have been demonstrated to play a role in early infection, as well as spread

of drug resistance *in vitro*. The goal of our study was to explore the ability of xenogeneic *Leishmania* EVs to modulate infection pathophysiology and parasite drug sensitivity after *in vivo* contact. In BALB/c mice, we performed co-inoculation of parasites and purified EVs from strains/species of *Leishmania* with contrasting drug resistance profiles. We monitored pathophysiology, parasite burden, and performed drug-susceptibility testing on recovered parasites. Results demonstrated that EVs from *Leishmania infantum* influence pathophysiology of *Leishmania major* in *in vivo* experiments, significantly increasing footpad swelling by 1.24-fold ($p = 0.0139$), and footpad lesion score ($p = 0.0013$), as well as increasing footpad parasite burden by up to 1.40-fold ($p = 0.0002$). These EVs were also found to modulate drug sensitivity of *L. major* after *in vivo* contact in a 6-hour infection model, leading to a highly significant 1.6-fold decrease in susceptibility to antileishmanial antimony ($p < 0.0001$). Here our group has shown for the first time that EVs from xenogeneic parasites can participate directly in propagating drug resistance between parasite populations after *in vivo* contact, an ability that may contribute to *Leishmania* treatment failure.

Keywords: *Leishmania*, extracellular vesicles, drug resistance, *in vivo*, protozoan parasites

Introduction

Leishmaniasis, an oftentimes deadly disease, affects more than 1.6 million people every year (4). The etiological agent is *Leishmania*, a sand fly-vectored protozoan parasite endemic to 98 countries and territories and responsible for a complex set of symptomatologies in humans; these range from cutaneous (CL) to visceral (VL), or even mucocutaneous (MCL). Clinical signs depend on the *Leishmania* species in question and geographic region; for example, in Africa, Europe, and Asia, *Leishmania major* is responsible for CL and *Leishmania infantum* for VL (5). There is growing evidence that asymptomatic *Leishmania* infections also play a significant role in the epidemiology of this disease in endemic areas (21). Dogs act as a reservoir for this zoonotic parasite, and may suffer from leishmaniasis themselves – or simply serve as carriers (37). The dangerous and widespread *Leishmania* parasite occasions a large economic, social, and health

burden around the world, especially in low-income communities with barriers to accessible medical care (2, 4). In such communities, co-infections are of particular concern: *Leishmania* is reported as a common infection among HIV-positive and immunocompromised individuals, and atypical leishmaniasis presentations have led clinicians to report mixed CL and VL in these populations (11). These situations further complicate diagnosis and treatment of this life-threatening disease in already-vulnerable groups.

A critical issue in the fight against leishmaniasis is the lack of adequate prevention (i.e. vaccines), as well as the rapidly thinning arsenal of available and effective antileishmanial drugs. Classically, leishmaniasis treatment has depended on antimony-based drugs; however, in the Indian Subcontinent, first-line antimony-based treatment sodium stibogluconate had to be abandoned when treatment failure skyrocketed, reaching upwards of 70%, in only a few decades' time (9, 54). Reports of *Leishmania* drug resistance are ever-emerging; newer antileishmanial drug miltefosine saw treatment failure rates increase by at least 5% only 10 years after its introduction (177). Current leishmaniasis prevention rests upon the use of insecticides and netting; solutions that lack both practicality and efficacy (25). Therefore, as is the case with many protozoan parasites, researchers have been forced to turn to new and creative techniques in the search for effective *Leishmania* prevention strategies and treatments; for example, the field of extracellular vesicles (EVs).

Produced by nearly all eukaryotic cells, EVs are nanosized, lipid membrane-bound particles that play an important role in cell-cell communication (188). Reflecting the contents of the cell from which they are released, EVs can deliver their cargo – proteins, DNA, and virulence factors, among other molecules – to nearby cells in a population (128). This unique ability, coupled with their documented role in numerous pathologies and their general innocuity when administered alone, lead to the exploration of EVs for use in vaccines or as biomarkers of drug resistance, especially for protozoan diseases (13). With respect to *Leishmania*, it has been demonstrated that EVs are released by the parasite inside its vector, and co-egested into the mammalian host – furthermore, EVs have been shown to exacerbate CL pathology *in vivo* (141, 146). EVs produced by drug-resistant (DR) *Leishmania* parasites have also been observed to differ from drug-sensitive (DS) parasites, both in terms of morphology and protein content, and to contain/be coated with drug

resistance-associated factors, such as zinc-dependent metalloprotease glycoprotein 63 (GP63), known to interact with the host immune system (15, 110, 168). Most importantly, DR *Leishmania* parasites have the ability to alter the drug resistance profile of DS parasites *in vitro*, simply through release and subsequent uptake of DR-strain EVs (16).

Therefore, in this manuscript, our group set out to further elucidate the role of EVs in the propagation of *Leishmania* drug resistance through studies of co-infections in a murine model, utilizing *L. major* and strains of *L. infantum* with diverse DR profiles. Our objective was to determine whether the pathophysiology of infections is altered *in vivo* in the presence of xenogeneic EVs, as well as the potential for a transfer of drug resistance between strains/species after *in vivo* contact.

Materials & Methods

Leishmania cultures

The *Leishmania infantum* (MHOM/MA/67/ITMAP-263) wild-type (WT) strain and *Leishmania major* (LV39-WT) WT strain were grown at 25°C in pH 7.0 M199 medium supplemented with 10% fetal bovine serum (FBS) and 5 μg/mL of hemin in non-ventilated 25 cm^2 culture flasks (Corning, USA). The *L. infantum in vitro*-generated resistant mutant Sb2000.1 (170), resistant to 2000 μM of Sb, was grown following the same protocol described above, with the addition of 2 mM Sb (Potassium antimonyl tartrate, Sigma-Aldrich, Germany) for maintenance of the hyper-resistant phenotype. Antimony-sensitive and resistant field strains of *L. infantum* isolated from infected dogs, provided by the Instituto de Salud Carlos III in Madrid, Spain, were also cultured in FBS- and hemin-completed M199 medium (as described above), supplemented with 0.1% biopterin (Sigma-Aldrich, Germany).

Purification of *Leishmania* extracellular vesicles

Leishmania EV purification was performed as described in previous works (Hassani, 2011, and Atayde, 2019). To summarize, 1 L *Leishmania* cultures were grown until late-log phase in non-ventilated 75 cm^2 culture flasks (Corning, USA) at 25°C in pH 7.0 M199 medium supplemented with 10% fetal bovine serum and 5 μg/mL of hemin, at a concentration of 2.5–5.0 x

10^7 parasites/mL. Media was supplemented with Sb (as described above) for culture of Sb-resistant strains. Parasites were then concentrated through a series of centrifugations at 3000 RPM for 5 minutes, followed by two washes in PBS and resuspension in RPMI-1640 medium without FBS and phenol red (Life Technologies). Next, parasites were incubated for 4 h at 37°C to stimulate release of EVs in the medium (Atayde, 2016). Samples were then centrifuged twice (3000 RPM for 5 min) in order to eliminate parasites, and subsequently filtered with 0.45 µm and 0.20 µm syringe filters in succession. A series of two ultracentrifugations (100 000 x g for 2 hours each) were then performed in order to recover EVs, which were resuspended in EVs buffer (137 mM NaCl, 20 mM Hepes, pH 7.5). Proteins were dosed using the Micro BCA Protein Assay Kit (Pierce Biotechnology, USA). EVs were stored in EVs buffer in small aliquots (10-50 µL) at -80°C for subsequent analyses.

Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analyses were performed to characterize concentration and size distribution of purified EVs using a ZetaView Nanoparticle tracking analyzer (Particle Metrix, USA). Samples were run at 25°C using 0.20 µm filtered EVs buffer as a diluent. For video acquisition, a shutter frame rate of 45 was used and sensitivity was set at 85 (according to the system's software guidance algorithms). Accuracy of the ZetaView was verified using 100 nm standard beads before measurements were obtained. Samples were diluted in EVs buffer with a dilution factor of 1:1 000-1:10 000 to achieve a particle count in the range of 1000–2000.

Transmission Electron Microscopy (TEM)

TEM was performed as previously described (15). Briefly, EVs purified from *L. major* WT and L. *infantum* WT and drug-resistant strains were coated on formvar carbon grids, fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 min, and stained with 1% uranyl acetate for 1 min. Formvar grids coated with isolated EVs were recorded using an FEI Tecnai 12 120 kV transmission electron microscope. Resulting images were captured with the AMT XR-80C CCD Camera System (Facility for Electron Microscopy Research, McGill University).

In vitro infections

A stock solution of 0.25 x 10^6 BMDM macrophages/mL (isolated from mice in the laboratory of Prof. David Langlais at McGill University) was prepared and distributed in volumes of 200 µL per well in a chamber slide with Dulbecco's Modified Eagle Medium (DMEM) completed with 10% FBS, and supplemented with 1% penicillin/streptomycin/glutamine. The chamber slide was incubated at 37 °C in a 5% CO_2 environment for 24h. Media was aspirated from wells, and *Leishmania infantum* (MHOM/MA/67/ITMAP-263) wild-type (WT) parasites were added at a concentration of 2.5 x 10^6 (for a 10:1 ratio of parasites : macrophages), with 10 µg/mL of EVs isolated from either *L. infantum* WT or the *L. infantum in vitro*-generated resistant mutant Sb2000.1 (mentioned above), before incubation at 37 °C in a 5% CO_2 environment for 3h. Supernatant was discarded, and wells were washed 3 times with Hepes-NaCl. Media supplemented with 50 µg/mL Sb^V (sodium stibogluconate, Millipore, USA) was added to each chamber, and an incubation of 5 days at 37°C in a 5% CO_2 environment was performed. Supernatant was then discarded, and wells were gently scraped to recuperate amastigotes. Amastigotes were cultured in completed M199 (10% fetal bovine serum and 5 µg/mL of hemin, pH 7.0) in non-ventilated 25 cm^2 suspension flasks (Corning, USA) and incubated at 25 °C with daily monitoring of parasite development. Once promastigotes were grown out, drug susceptibility assays in the presence of Sb^{III} were performed (as described below). Note: control infections (without drug) were also performed, with amastigotes recuperated directly after the 3h infection period, transformed into promastigotes as described, and submitted to drug testing (no contact with the drug prior to promastigote stage).

Ethics statement

All animal experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) Guidelines, approved by the Institutional Animal Care and Use Committees at the McGill University under ethics protocol 7791. All experiments with mice were carried out in pathogen-free housing and in accordance with the regulations of the CCAC, approved by the McGill University Animal Care Committee.

Visceral leishmaniasis (VL) mouse experiments

Female BALB/c mice (6–8 weeks old) were infected intraperitoneally with 1 x 10^8 stationary phase *L. infantum* promastigotes, with or without 20 μg of *in-vitro*-purified small EVs from various strains of *L. infantum* (LdiWT, with an EC_{50} of approximately 80 μM in the presence of antimony; an antimony-sensitive field strain (hereafter referred to as Ldi-S) with an EC_{50} of approximately 62 μM; a field strain with intermediate-level antimony resistance (hereafter referred to as Ldi-I) with an EC_{50} of approximately 200 μM; or mutant Sb2000.1 (hereafter referred to as Ldi-R) with an EC_{50} of approximately 2000 μM). Two weeks post-infection, animals were euthanized and spleens processed for determination of parasite burden. Briefly, euthanized animals were disinfected with ethanol. The peritoneum was exposed and disinfected before incision for removal of the spleen. The spleen was weighed, then homogenized until complete tissue disruption in a glass tissue homogenizer with 5 mL in pH 7.0 Schneider's Drosophila Medium (SDM-79) supplemented with 10% fetal bovine serum and 5 μg/mL of hemin. Homogenate was then transferred to non-ventilated 25 cm^2 culture flasks (Corning, USA). A 100 uL aliquot of each sample was also added to 96-well plates for parasite burden determination by limiting dilution assay. Briefly, 100 μL of each sample was added, in duplicata, to 96-well plates (Sarstedt, Germany) containing 100 μL complete SDM-79. A minimum of 24 2-fold dilutions were performed for each sample. Plates were kept at 25 °C until microscopic examination after 10 days, when the highest dilutions at which promastigotes were observed were recorded. Flasks were incubated at 25 °C until parasite growth was detected under the microscope. Once parasites reached mid- log-phase, they were subjected to drug susceptibility assays (described later). Five independent biological replicates were performed for each determination, and the experiment was performed twice.

Cutaneous leishmaniasis (CL) mouse experiments

Female BALB/c mice (6–8 weeks old) were infected in the right hind footpad with 5 x 10^6 stationary phase *L. major* promastigotes, with or without 10 or 20 μg of *in-vitro*-purified small EVs from *L. major* WT (Sb EC_{50} of < 10 μM) or Ldi-S, Ldi-I, or Ldi-R parasites. Disease progression was monitored up to 10 weeks post-infection by measuring footpad swelling weekly

with a metric caliper and, in certain experiments, scoring footpad lesions on a scale of 0-3 (0 = no lesions, 1 = minor lesions, 2 = obvious lesions, 3 = major lesions). At the end of each experiment, footpads were processed for determination of parasite burden as described in (141): briefly, footpads were sterilized with ethanol, excised, and washed with PBS. Next, tissue was disrupted manually using a glass tissue homogenizer. The final homogenate was then used for several experiments – 100 uL was placed in culture at 25 °C in completed SDM-79 in non-ventilated 25 cm^2 culture flasks (Corning, USA); once parasites reached mid- log-phase, they were subjected to drug susceptibility assays (described later). Next, total volume of remaining footpad homogenate was brought up to 50 mL, and 100 µL of each sample was added to 96-well plates (Sarstedt, Germany) containing 100 µL complete SDM-79 per well, in duplicata, for limiting dilution assay (as described above). Five independent biological replicates were performed for each determination, and the experiment was performed twice.

Early infection model (EIM) mouse experiments

BALB/c mice (6–8 weeks old) were infected intraperitoneally with 1 x 10^8 stationary phase *L. major* promastigotes, with 20 µg of *in-vitro*-purified small EVs from *L. major* WT parasites or Ldi-S, Ldi-I, or Ldi-R parasites. Six hours post-infection, animals were euthanized and a peritoneal lavage was performed using 5 mL of cold PBS. Lavage fluid was then subjected to a number of analyses: cell counts for each sample were calculated using a Neubauer slide (Hausser Scientific, USA). Next, 150 µL of of lavage fluid was concentrated onto a glass microscope slide in a cytocentrifuge at 300 RPM for 5 min (Cytospin™, Thermo Fisher Scientific, USA) before staining with Diff-Quik and manual determination of cell differential. Subsequently, 150 µL of lavage fluid was placed on a 4-chamber cell culture slide (Corning, USA) and incubated at 37 °C for 1 hr before the addition of 1 mL of complete DMEM. Chamber slides were incubated at 37 °C for 24-72 hr, at which point DMEM was aspirated, and slides left to air dry for 30 min before staining with Diff-Quik. Stained slides were used for determination of cell differential and infection rates. Finally, remaining lavage fluid was spun down (2000 RPM for 5 min) before removal of supernatant and resuspension in completed DMEM in ventilated 25 cm^2 culture flasks (Corning, USA). Cultures were incubated for 24 hours at 37 °C, after which DMEM was washed, and cells were scraped and resuspended in completed SDM-79 in non-ventilated 25 cm^2 culture flasks (Corning, USA) for

incubation at 25 °C and parasite growth. Once parasites reached mid- log-phase, they were subjected to growth curves and drug susceptibility assays. Three biological replicates were performed for each determination, and the experiment was performed twice.

Parasite growth curves

Growth curves were performed in 25 cm^2 cell culture flasks by seeding 1×10^6 parasites/mL, and parasite growth was evaluated daily (up to 7 days) by measuring A_{600} using an Infinite 200 Pro machine (Tecan Life Sciences, Switzerland). Cultures were grown in the presence of various concentrations of Sb^{III} (0, 10, 30, and 60 μM). Growth assays were performed with at least three biological replicates from independent cultures, each of which included three technical replicates.

Drug susceptibility assays

For drug susceptibility assays, 10^6 parasites mL^{-1} in mid-log phase growth were seeded in 24-well culture plates (Sarstedt, Germany) containing 1.5 mL of SDM-79, and incubated at 25 °C in the presence or absence of varying concentrations of Sb^{III} (for *L. major studies*, 0, 10, 30, and 60 μM was used. For *L. infantum*, 0, 50, 100, 150, 200, and 250 μM). Growth was monitored by measuring A_{600} using an Infinite 200 Pro machine (Tecan Life Sciences, Switzerland) until untreated wells reached an optical density (OD) of 0.380-0.400 (approximately 5 days' time). Drug-susceptibility assays were performed with at least 2 technical replicates for each sample. EC_{50} values were calculated based on dose-response curves analyzed by the quadratic formula using Microsoft Excel. An average of at three independent biological replicates was performed for each determination.

Statistical analyses of data

Statistical analyses were performed using the unpaired Student's t-test (*$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data was analyzed using GraphPad Prism 8.0 software (GraphPad Software, La Jolla California, USA), and results are representative of at least 3 independent biological replicates.

Results

Multiple filtration and ultracentrifugation steps leads to successful isolation of small particles compatible with sEVs from both L. infantum *and* L. major

In order to determine the effect of *L. infantum* EVs on pathophysiology of VL in BALB/c mice, as described above, EVs were extracted from *L. major* WT parasites and Ldi-S, Ldi-I, or Ldi-R parasites. All EVs were submitted to NTA and TEM, as per MISEV recommendations (189): the majority of EVs from all tested strains and species fell in the 100-200 nm size range. Under the transmission electron microscope, samples demonstrated a predominance of lipid membrane-bound particles with a classic cup-shaped appearance, compatible with small EVs. EVs also had their proteins dosed by Micro BCA assay in order to calculate the particle/μg protein ratio; a measure of purity – all samples reached above 10^{11}, a level considered very pure (see Fig. 6) (189).

Extracellular vesicles (EVs) of drug-resistant strains of L. infantum *modulate drug sensitivity of promastigotes transformed from amastigotes*

Promastigotes were incubated with EVs and macrophages *in vitro* and later treated with Sb^V. Promastigotes grown out from amastigotes inside infected macrophages were then submitted to growth in the presence of Sb^{III} in order to determine EC_{50}. A 1.17-fold shift in EC_{50} was noted for the group that received Ldi-R-EVs compared to the LdiWT-EVs group, demonstrating a significant ($p = 0.0077$) decrease in Sb^{III} sensitivity (Fig. 7).

In vivo *contact with xenogeneic* L. infantum *EVs does not promote lasting changes in infection pathophysiology or parasite drug resistance in VL experiments*

The EVs isolated as described above were utilized for *in vivo* experiments. Mice infected intraperitoneally with LdiWT and either PBS (control group), or 20 μg of purified EVs from LdiWT, Ldi-S, Ldi-I, or Ldi-R were sacrificed after a 2-week infection.

Organs were weighed, and no significant differences in liver or spleen weight between groups were detected. Liver weights varied between 0.84-1.29 g, and spleen weights between 0.08-0.14 g. Furthermore, no significant changes in splenic parasite burden (as determined by LDA and normalized against organ weight) or drug sensitivity of the recovered parasites in the presence of

Sb^{III} were noted (see Supplemental Figures 1-3). Splenic parasite burden was slightly lower than the control (LdiWT parasites + PBS) for all groups except the groups that received Ldi-S-EVs and Ldi-I-EVs, which demonstrated 1.12- and 1.92-fold increases in parasite burden, respectively. The EC_{50} of groups that received Ldi-S-EVs, Ldi-I-EVs, and Ldi-R-EVs demonstrated a nearly identical EC_{50} to the group that received LdiWT-EVs; they showed a 1.06-, 1.07-, and 1.02-fold increase in EC_{50}, respectively.

In vivo *contact with xenogeneic* L. infantum *EVs influences pathophysiology of* L. major *parasites in CL experiments*

Mice were inoculated in the right hind footpad with *L. major* WT (hereafter referred to as LmWT) and either PBS (control group), or 10 or 20 µg of purified EVs from LmWT, Ldi-S, Ldi-I, or Ldi-R. Animals were sacrificed after 8 weeks.

Throughout the duration of the experiment, all EVs increased footpad swelling when compared to the control group. At the 8-week timepoint, however, LmWT's own EVs most significantly exacerbated footpad swelling, with a 1.34-fold increase ($p = 0.0134$) compared to the group that received only LmWT parasites + PBS (see Fig 8). Ldi-S-EVs & Ldi-I-EVs also induced 1.24- (significant, $p = 0.0139$) and 1.19-fold increases in footpad swelling, respectively, compared to the LmWT + PBS group, while Ldi-R-EVs led to no change in measured clinical signs. The progression of footpad swelling throughout the experiment can be seen in Supplemental Figure 4.

With respect to footpad lesion scores, as rated from 0-3, with 0 indicating "no lesion" and 3 indicating "significant swelling & severe ulceration", it was found that EVs from all evaluated species and strains significantly increased footpad lesion score at week 8 compared to the control group that received only LmWT parasites + PBS. While this exacerbation of lesion score was visible in the Ldi-R-EVs group ($p = 0.0265$), the Ldi-I-EVs group ($p = 0.0479$), and the Ldi-S-EVs group ($p = 0.0013$), it was most evident with a highly significant change induced by the LmWT-EVs group ($p = 0.0002$) (see Fig. 9).

Findings related to footpad parasite burden (as determined by LDA) indicated that, much like with footpad lesion scoring, EVs from all evaluated species and strains significantly increased footpad parasite burden compared to the control group. Ldi-S-EVs and Ldi-R-EVs increased parasite

burden by 1.34- and 1.2-fold compared to the LmWT + PBS group, respectively ($p = 0.0020$, $p = 0.0078$) (Fig. 10). Meanwhile, LmWT-EVs and Ldi-I-EVs increased footpad parasite burden ever more significantly, with 1.39- and 1.40-fold shifts, respectively ($p = 0.0003$, $p = 0.0002$).

Finally, evaluation of the drug sensitivity of parasites recovered from mouse footpads in the presence of Sb^{III} showed no significant modification of drug sensitivity between groups (Fig. 11). Ldi-S-EVs, Ldi-I-EVs, and Ldi-R-EVs led to only a 1.01-, 1.07-, and 1.10-fold increase in drug sensitivity compared to the group that received LmWT-EVs.

L. infantum ***EVs derived from DR parasites can modulate drug-resistance profiles of*** **L. major** ***in early infection after*** **in vivo** ***contact***

Mice were infected intraperitoneally with LmWT and ~20 μg of purified EVs from either LmWT, Ldi-S, Ldi-I, or Ldi-R. Animals were sacrificed after a 6-hour period of infection. Parasites grown out from the macrophages harvested by peritoneal lavage immediately after sacrifice of animals were analyzed and found to grow as expected for the species: a logarithmic phase, followed by a plateau of growth (stationary phase) around day 5 (see Supplemental Figure 5).

Cell recruitment at various timepoints post-infection was compared between groups, and no significant differences were found: all groups demonstrated similar cellular recruitment in terms of both the total number of cells and the type of cells identified (neutrophils, macrophages, lymphocytes, eosinophils, basophils) at the 6h timepoint (see Fig. 12). Neutrophils were the predominant cell present in all groups this early timepoint, making up between 60.5-67.9% of the total cells, only to be overtaken by macrophages by the 48h timepoint (see Supplemental Figure 6) .

Both the number of infected cells (neutrophils, macrophages) per timepoint and the average number of amastigotes per infected cell were calculated at the 6h, 24h, 48h, and 72h timepoint. No significant differences were found between groups at any timepoint (see Supplemental Figure 7). The proportion of infected cells identified at the 6h timepoint ranged from 6.7-7.4%, at 24h, from 3.2-4.6%, at 48h, from 10.6-14.5%, and at 72h, from 11.6-14.4%. The number of infected macrophages was highest at the 72h timepoint, with up to 22% of identified macrophages demonstrating 1 or more amastigotes. Neutrophil infection was highest at the 48h timepoint (up to

13% of identified neutrophils). The average number of amastigotes per infected macrophage varied from 1.97-5.55. For neutrophils, the average number of amastigotes per cell ranged from 0.333-3.80; however, this value could not be calculated at the 72h timepoint, as macrophages were the only cell type remaining in culture.

Parasites grown out from macrophages were submitted to growth in the presence of Sb^{III} in order to determine EC_{50}. Parasites harvested from the first EIM experiment did not show any change in EC_{50} when compared to the group that received only LmWT-EVs. For example, Ldi-R-EVs, expected to mediate the greatest decrease in drug sensitivity, led only to a (non-statistically significant) 1.09-fold increase in drug resistance when compared to the group which received LmWT-EVs (see Supplemental Figure 8). With respect to the second EIM experiment, a strongly significant ($p < 0.0001$) 1.6-fold shift in EC_{50} was noted for the group that received Ldi-R-EVs compared to the LmWT-EVs group, denoting a highly antimony-resistant phenotype. Meanwhile, the group that received Ldi-I-EVs also demonstrated a significant decrease in drug sensitivity of approximately 1.1-fold ($p = 0.0057$) (see Fig. 13).

Discussion

Leishmaniases represent a serious threat for human and animal health, and one which is often neglected despite its global presence. The lack of an effective vaccine for humans as well as this parasite's increasing resistance to antileishmanial drugs are particularly concerning (61). Progress has been slow in terms of combating *Leishmania* drug resistance, as the mechanisms of drug resistance (as well as the mode of action of most antileishmanial drugs) are still to be fully elucidated (190). EVs have been proposed as a potential key to further elucidating both the pathogenesis of leishmaniases and propagation of drug resistance in *Leishmania* populations.

Previous studies from our team have shown that EVs from DR parasites have a morphology/size distinct from that of EVs derived from DS parasites, and the former are also enriched in proteins known to play a direct and indirect role in *Leishmania* drug resistance (15). Further work also demonstrated the capacity of EVs from DR parasites to "deliver" their cargo to DS parasites, leading to changes in the drug-susceptibility profile of a previously-DS population *in vitro* (16). EVs have also been demonstrated to play an important role in the pathophysiology of

leishmaniasis; they are known to be released by the parasite inside its vector, co-egested during the sand fly bloodmeal, and to contain/be coated with virulence factors that can interact with the host immune system (110, 141, 146). Their role in early infection was further demonstrated by *in vivo* studies showing exacerbated pathology of *L. major* footpad infections in mice when the parasite was co-inoculated with autologous EVs (151). Our study constitutes the next steps to these foundational works, beginning with *in vitro* infections of phagocytic immune cells.

We wanted to determine whether contact between promastigotes and EVs from DR parasites during their incubation with macrophages could lead to a change in drug susceptibility after transformation into intracellular amastigotes, treatment with organic pentavalent antimonials, and transformation back to promastigotes. Our results demonstrate clearly that even a short incubation with EVs from DR parasites can lead to changes in drug-susceptibility profile that persist through multiple stages of the parasite life cycle. We felt this experiment was particularly important, not only as the next step after works performed solely with parasite cultures, but also because it emulates the real-life cycle of promastigotes & EVs being co-egested into a mammalian host, who may then be treated for leishmaniasis (with antimony reduced to its trivalent form by the host cell, targeting the intracellular amastigote stage). Patients released from hospital without total parasite clearance – or those that eventually suffer from Post-Kala-Azar Dermal Leishmaniasis – may remain infectious to sandflies, leading to further spread of DR parasites (28).

Our VL experiments constituted the next step to further investigate the ability of EVs to influence *Leishmania* pathophysiology and drug resistance *in vivo*. We felt it was crucial to investigate EVs' role in a living model, and BALB/c mice have long been used in the study of leishmaniasis (191). Interestingly, simultaneous passage of *L. infantum* and autologous or xenogeneic EVs from strains with various drug resistance profiles did not lead to any significant changes in parasite burden or sensitivity (EC_{50}) of recovered splenic parasites after a 2-week infection. As such, we decided to perform the study with a different model; CL.

Footpad infection-model CL experiments have already been performed and used to demonstrate that EVs exacerbate pathophysiology & parasite burden; our work supports these findings (151). We showed that autologous EVs (*i.e.* EVs from *L. major* WT, our infecting strain) significantly increased footpad swelling at the 8-week timepoint. Furthermore, EVs from all our tested *L.*

infantum strains (Ldi-S, Ldi-I, and Ldi-R) significantly increased footpad parasite burden and footpad lesion scores. To the authors' knowledge, this marks the first time that EVs from *L. infantum* have been shown to modulate pathophysiology of a xenogeneic strain (here *L. major*) after *in vivo* contact. Curiously enough, although all tested *L. infantum* field EVs also led to an increase in footpad swelling at the terminal week of the experiment, only Ldi-S-EVs induced a significant change. This may be due to the individual variation seen among the responses of the 10 mice in each group, leading to a distribution considered non-significant. Ldi-R, meanwhile, is a lab-generated mutant, which could explain the less marked effect of its EVs on *Leishmania* pathophysiology compared to field strain Ldi-S and Ldi-R EVs (192).

Much the same as with our VL experiments, harvesting of *L. major* parasites from footpads and subsequent drug-susceptibility testing did not reveal any significant changes in sensitivity (EC_{50}) between groups, despite *in vivo* contact with EVs from DR parasite strains. As such, and given that the clear effect on infection pathophysiology (discussed above) indicated a successful uptake of EVs by the infecting strain, we hypothesized that changes in drug resistance may be transitory, occurring too early to observe, and therefore set our sights on an early infection-type model.

Our early infection model involved an IP infection with *L. major*, followed by sacrifice of the animals and peritoneal lavage at the 6-hour timepoint for recovery of the immune cells recruited to the site of infection. Investigation of cell recruitment at multiple time points, both with respect to cell type, number of cells, and percentage of infected cells, revealed no differences induced by contact with EVs from the various strains/species. However, upon culture of the recovered peritoneal macrophages, transformation of *L. major* amastigotes into promastigotes, and drug-testing in the presence of antimony, significant differences were observed in drug sensitivity (EC_{50}) between groups. Ldi-I-EVs led to a significant decrease in antimony susceptibility, and Ldi-R-EVs even more so, demonstrating a highly significant decrease. These findings are not only supported by the EC_{50} values of the respective parasite strains (Ldi-I: approximately 240 µM, Ldi-R: approximately 2000 µM), but also by a recent study from our group. The latter found that EVs from Ldi-R parasites increased EC_{50} of *L. infantum* parasites by approximately 1.7-fold compared to LdiWT-EVs *in vitro* (16). Curiously, our findings were only significant for one of the two replicates of this experiment (each with 20 mice). We hypothesize that this may be due to a number

of factors, including (but not limited to) experimental methods, such as the number of passages performed on recovered parasites after harvest (enough for the parasites to grow dense and vigorous for drug-testing, but not so many as to lose the potentially short-lived alteration to drug-sensitivity profile), or host factors (differences between animals) (193). Furthermore, authors would not expect this type of exchange to occur invariably; *Leishmania* parasites have been shown as masters of evasion, and, as dictated by evolution, will implement (or maintain) the survival mechanisms that most benefit them – often at the expense of fitness – only in necessary, stressful (*i.e.* drugged) situations (187, 194). As such, it's possible that growing out recovered parasites in the presence of a low concentration of antimony could facilitate the observation of any acquired changes in EC_{50}.

On this note, one particular point of intrigue in our study is the fact that our infecting *L. major* parasites were never exposed to antimony prior to their drug-susceptibility testing; as such, there could be no selection of DR parasites. Their only significant exposure was to EVs from xenogeneic DR *Leishmania* strains. In the world of anti-cancer EV studies, certain works have already shown that EVs derived from tumour-associated cells lead to inhibition of the anti-cancer effects of chemotherapeutic molecules in *in vivo* studies. For example, EVs from invasive hepatocellular carcinomas conferred resistance to the drug sorenafib *in vivo*, and the same was true in gastric cancer studies with respect to EV-mediated inhibition of the effects of cisplatin (195). It would therefore be interesting to study the effect of antimony treatment on the *L. major*- and EV- co-inoculated mice in this study to determine whether it could induce selection of a DR parasite population and/or treatment failure.

Our work provides several answers to previous questions about *Leishmania* and EVs, but many remain. For example, molecular analyses to determine which molecules implicated in drug resistance were passed to *L. major* in our early infection experiments are currently in progress. As of yet, little is known about the mechanisms involved in sorting and packaging molecules for EV transfer; studies to further elucidate these processes are indicated and may provide valuable information (13). Furthermore, given that it has already been demonstrated that, not only do *Leishmania* parasites release EVs inside their vector, but two separate *Leishmania* species can successfully complete their life cycles inside the same sand fly simultaneously, our findings could

represent a missing piece in unravelling the mystery of *Leishmania*'s rampant spread of drug resistance (12, 141). That said, much remains to be done in terms of *in vivo* studies inside the sand fly vector, as well as to determine whether changes in parasite drug resistance after EV contact would remain stable in parasites long-term.

Conclusion

Altogether, to authors' knowledge, here our group has shown for the very first time that EVs from xenogeneic parasites can contribute directly to propagating drug resistance between parasite populations after *in vivo* contact, and that EVs from *L. infantum* can influence *L. major* pathophysiology. *Leishmania*/*Leishmania* co-infections reported in endemic areas, especially among vulnerable populations, may be complex in terms of diagnosis, but especially so in terms of treatment, as both species of infecting parasite could be DR after simultaneous passage in the vector. This phenomenon may even contribute to treatment failure rates in certain areas. In conclusion, much work remains in order to fare better in the fight against global neglected disease leishmaniasis; a better understanding of *Leishmania* drug-resistance mechanisms and its methods of propagation will lead to more safe & effective treatment regimens while awaiting development of a vaccine.

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Contributions

VW wrote and edited this work in its entirety, and was responsible for performing all experiments included in this article except the *in vitro* macrophage infections. ND performed the *in vitro* macrophage infections & assisted in extracellular vesicle extractions. EC & GD assisted with *in vivo* experiments & sample processing. AC shared parasite strains and assisted in extracellular vesicle extractions. MO & CFP were responsible for the conceptualization of this work as well as preliminary editing of the article.

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Declaration of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Figures

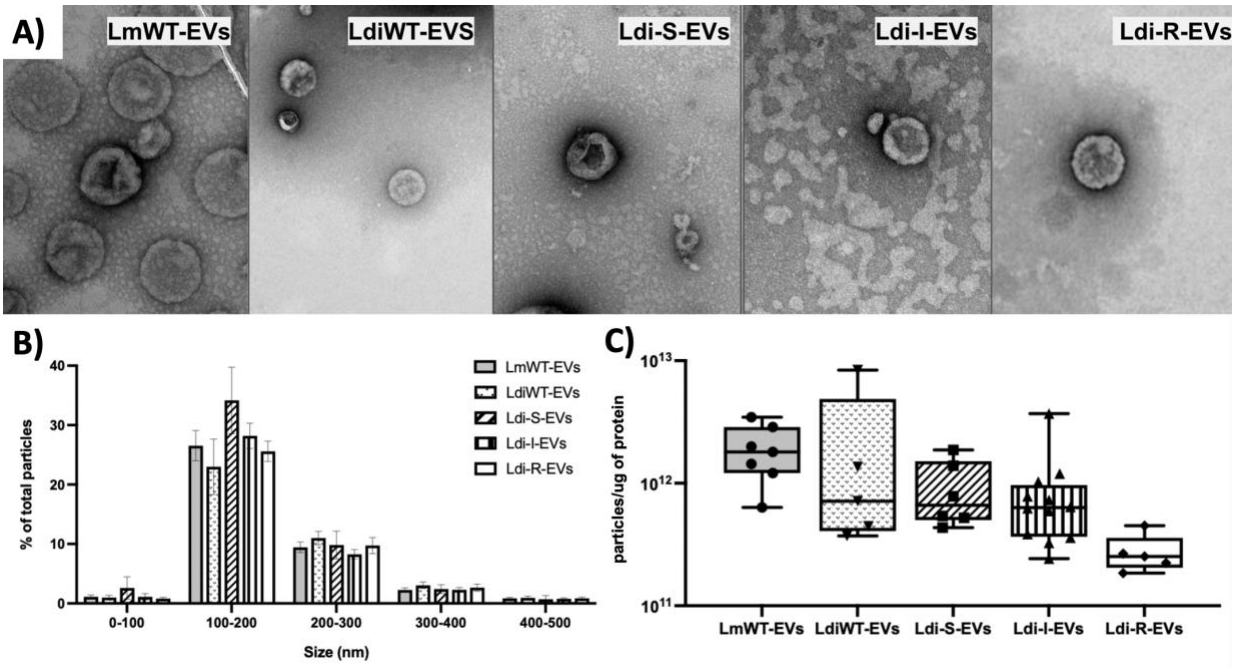


Figure 6: EV validation & characterization through multiple molecular methods

A) Transmission electron microscopy photos of EVs from LmWT, LdiWT, Ldi-S, Ldi-I, and Ldi-R demonstrating a predominance of lipid membrane-bound particles with a classic cup-shaped appearance. **B)** Size distribution of isolated EVs from the strains mentioned above, as determined by Nanoparticle Tracking Analysis (NTA) and demonstrating a majority of particles in the 100-200 nm size range. **C)** Particle/µg protein ratio as a measure of purity for each group of EVs, as determined using MicroBCA protein assay and NTA; samples reaching above 10^{11} are considered very pure.

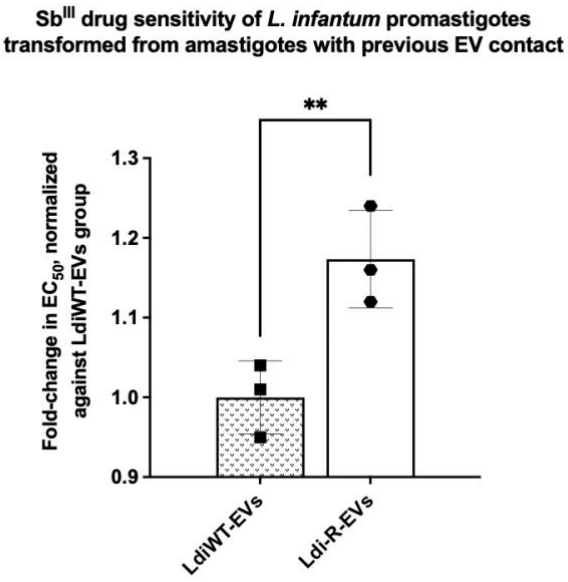


Figure 7: Sb^{III} drug sensitivity of* L. infantum *promastigotes transformed from amastigotes

Fold-change in EC_{50} as compared to LdiWT-EVs group. Parasites were incubated with EVs in the presence of macrophages (MOI 1:10), before wash & treatment with Sb^{V}. Next, amastigotes were recovered from macrophages, transformed into promastigotes, and submitted to various concentrations of Sb^{III} in order to determine EC_{50} through measurement of A_{600}. Data are the mean of 3 biological replicates, each performed with 3 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

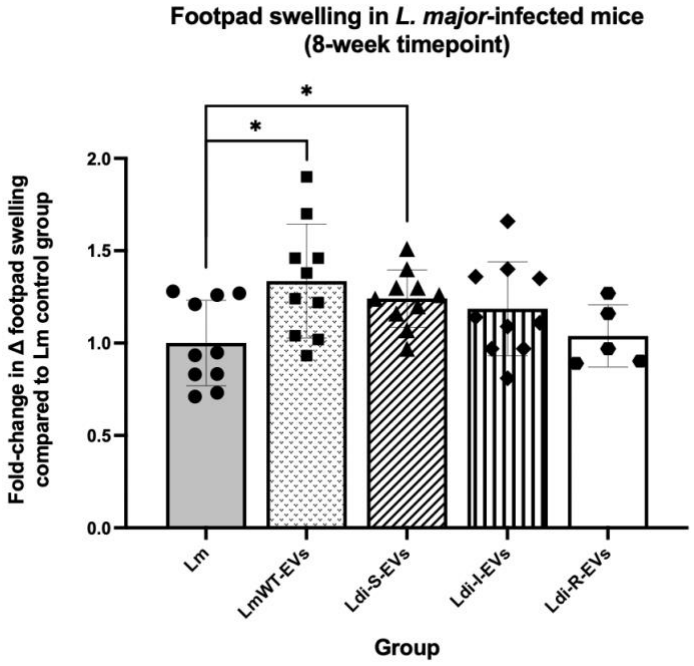


Figure 8: Footpad swelling in* L. major*-infected mice at 8 weeks post-infection

Fold-change in Δ footpad swelling of mice, as compared to Lm control group. Values were determined by comparing swelling of the infected footpad and unaffected footpad of each mouse, as measured by electronic calipers. Data are the mean of 2 separate experiments, each with 5 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

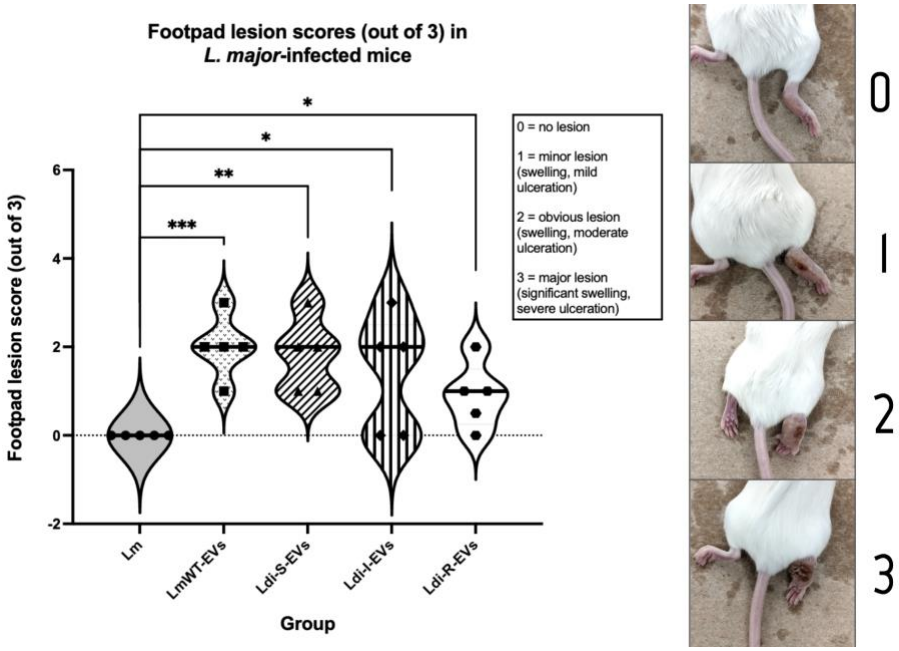


Figure 9: Footpad lesion scores in* L. major*-infected mice at 8 weeks post-infection

Violin plot representing footpad lesion scores of infected mice, as compared to the Lm control group. Values were determined by comparing footpad lesions (as determined by the scoring system described above, with 0 = no lesion, 1 = minor lesion, 2 = obvious lesion, and 3 = major lesion) on gross examination of infected mice. Data are the mean of 5 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

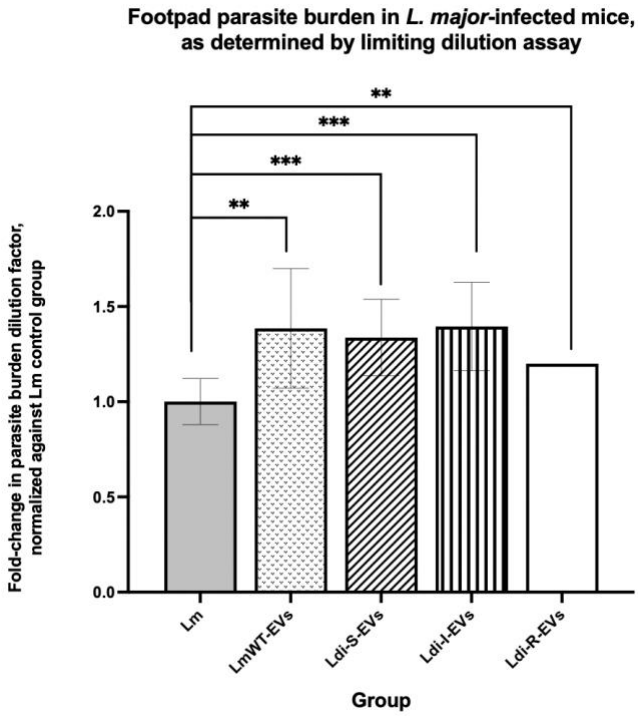


Figure 10: Footpad parasite burden in* L. major*-infected mice, as determined by limiting dilution assay (LDA)

Footpad parasite loads are shown, as determined by LDA in 96-well plates in SDM-79 media. The experiment was performed twice; data are the mean of 5 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

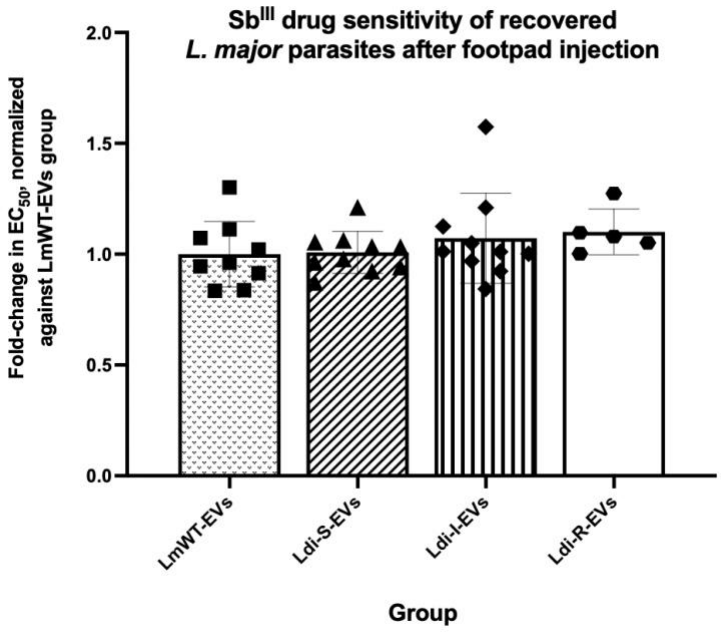


Figure 11: Sb^{III} drug sensitivity of* L. major *parasites recovered from footpad experiments

Fold-change in EC_{50} as compared to LmWT-EVs group. Parasites recovered from homogenization of infected footpads were grown out, then submitted to various concentrations of Sb^{III} in order to determine EC_{50} of parasites through measurement of A_{600}. The experiment was performed twice; data are the mean of 5 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

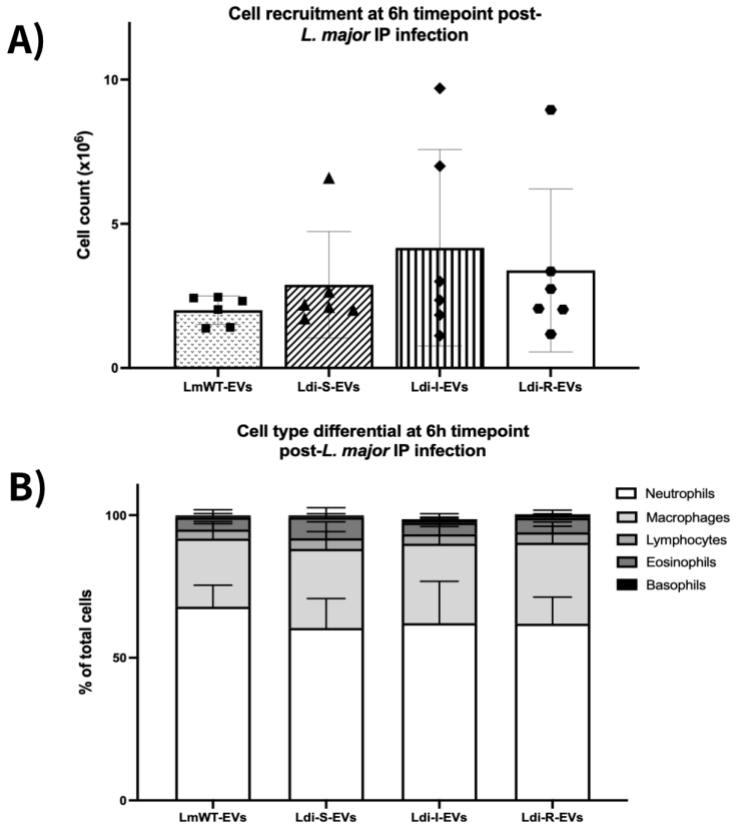


Figure 12: Cell recruitment and differential at the 6h timepoint post-*L. major *IP infection

A) Cell counts for each group at the 6h timepoint post-infection. Peritoneal lavage fluid from mice post-*L. major* IP infection was examined under the microscope, and cells counted manually using a Neubauer haemocytometer. **B)** Recruitment of different cell types for each group at the 6h timepoint post-infection. Peritoneal lavage fluid was spun down using a cytocentrifuge, stained with Diff-Quik, and examined under the microscope for manual counting. The experiment was performed twice; each experiment includes 3 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

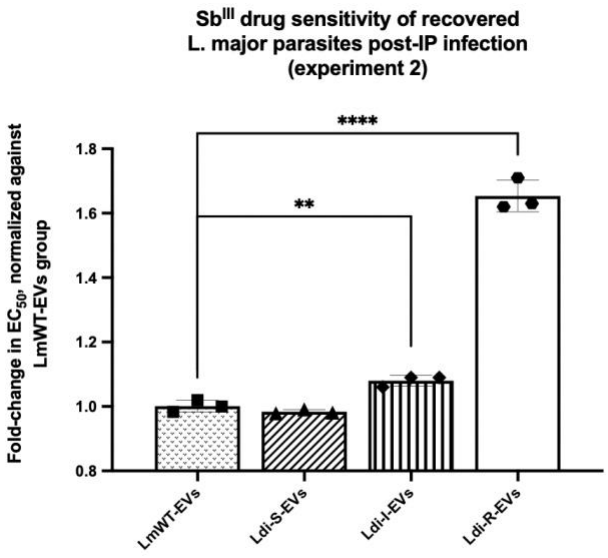
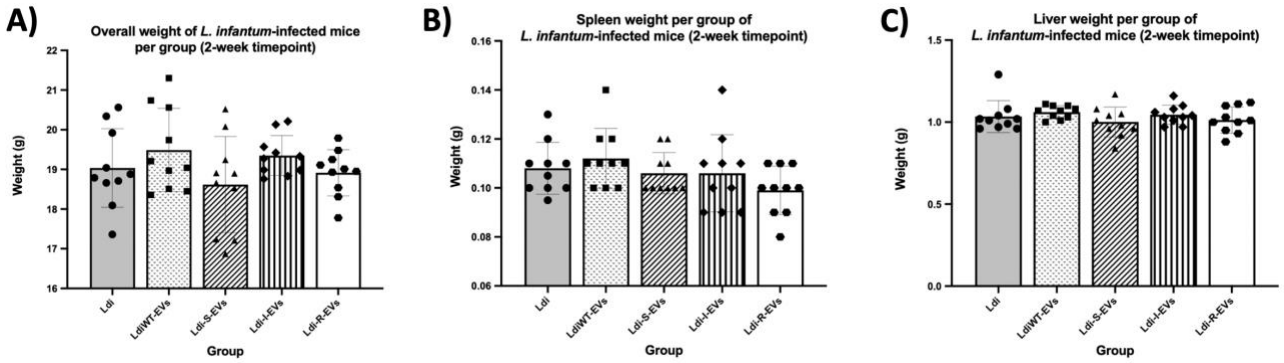


Figure 13: Sb^{III} drug sensitivity of* L. major *parasites recovered after IP infection (experiment 2)

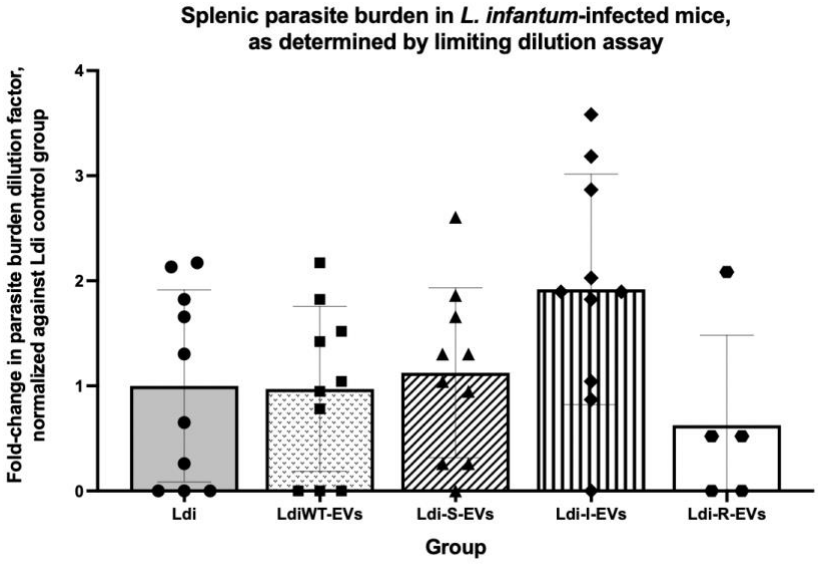
Fold-change in EC_{50} as compared to LmWT-EVs group. Parasites were grown out from macrophages cultured from peritoneal lavage post-*L. major* IP infection, then submitted to various concentrations of Sb^{III} in order to determine EC_{50} of parasites through measurement of A_{600}. Data are the mean of 3 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Supplemental Figures



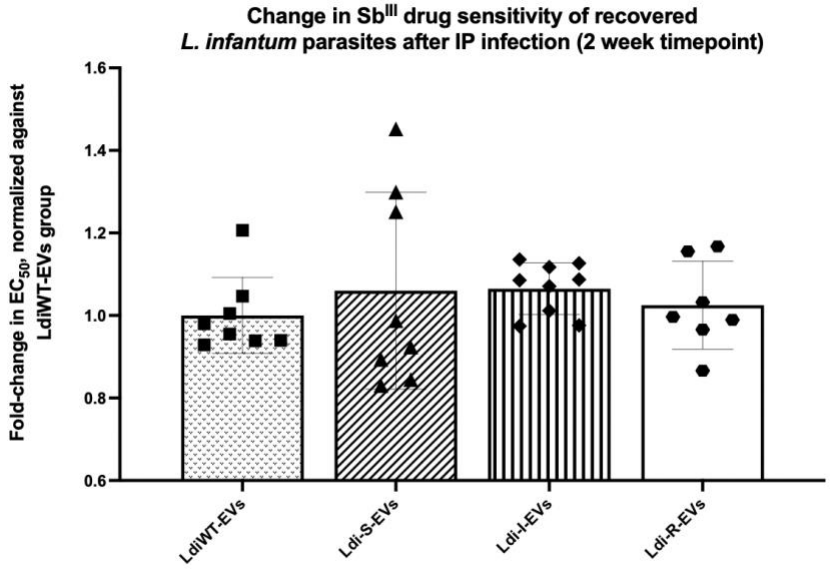
Supplemental Figure 1: Mouse & organ weights post*-L. infantum *IP infection

A) Overall weight of *L. infantum*-infected mice and **B)**, **C)** their vital organs (spleen, liver), as determined using an electronic balance after euthanasia and dissection of mice. Blood was blotted from organs before weighing. The experiment was performed twice; each experiment includes 5 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



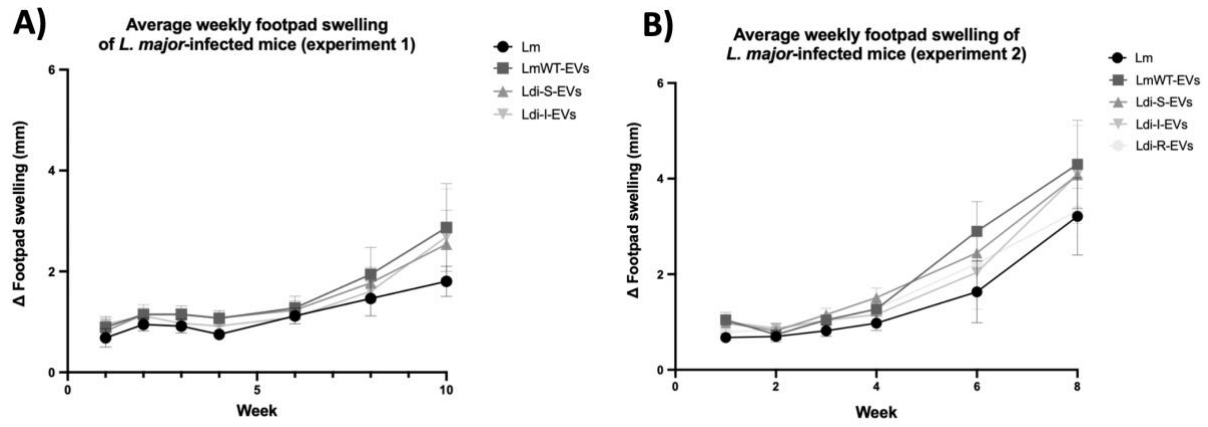
Supplemental Figure 2: Splenic parasite burden in* L. infantum*-infected mice, as determined by limiting dilution assay (LDA)

Splenic parasite loads are shown, as determined by LDA in 96-well plates in SDM-79 media and normalized by spleen weight. Results demonstrate fold-change in parasite burden as compared to the control (Ldi + PBS group). The experiment was performed twice; data are the mean of 5 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



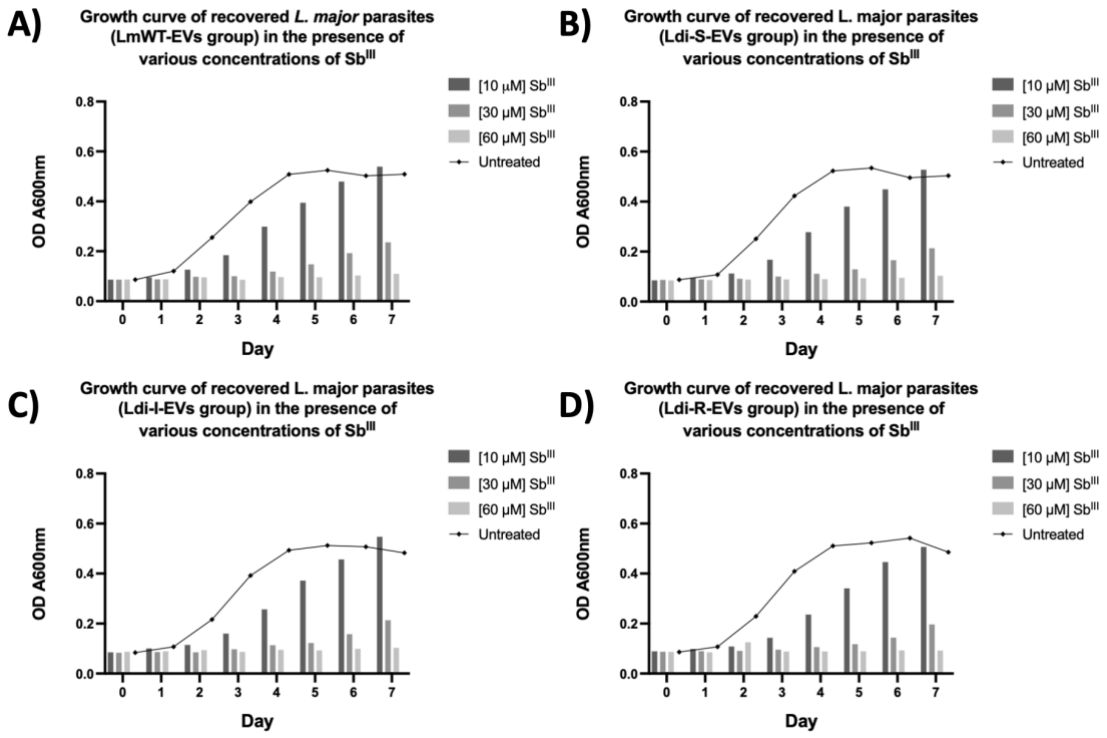
Supplemental Figure 3: Sb^{III} drug sensitivity of* L. infantum *parasites recovered after IP infection

Fold-change in EC_{50} as compared to LdiWT-EVs group. Parasites were grown out from homogenized spleens harvested from mice post-*L. infantum* IP infection, then submitted to various concentrations of Sb^{III} in order to determine EC_{50} of parasites through measurement of A_{600}. The experiment was performed twice; data are the mean of 5 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



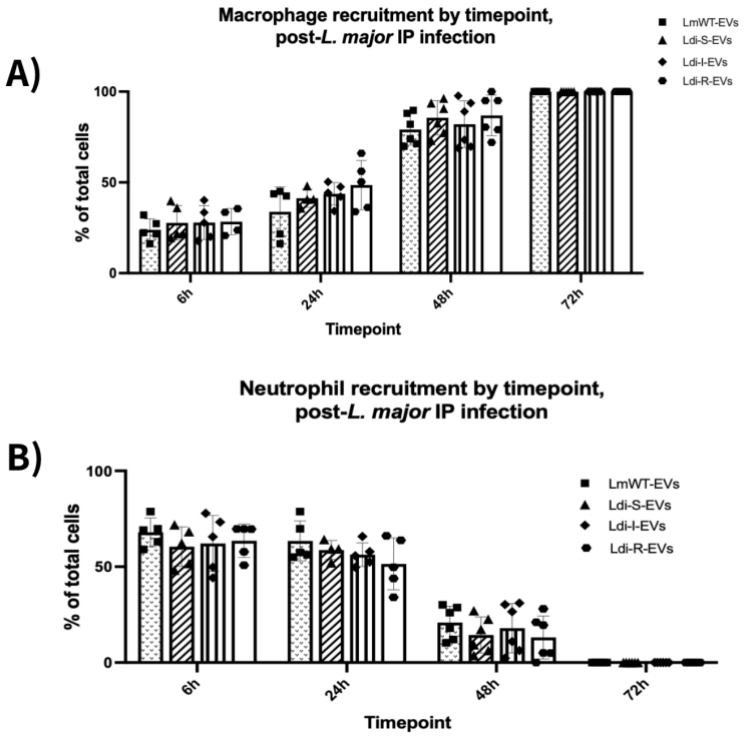
Supplemental Figure 4: Footpad swelling in* L. major*-infected mice over 8-10 week infections

Change in footpad swelling of mice (mm) over the duration of *L. major* footpad infection periods (8-10 weeks). Values were determined by comparing swelling of the infected footpad and unaffected footpad of each mouse, as measured by electronic calipers. Experiments were performed twice, with either 10 or 20 μg of EVs – **A)**, **B)**, and data are the mean of 5 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



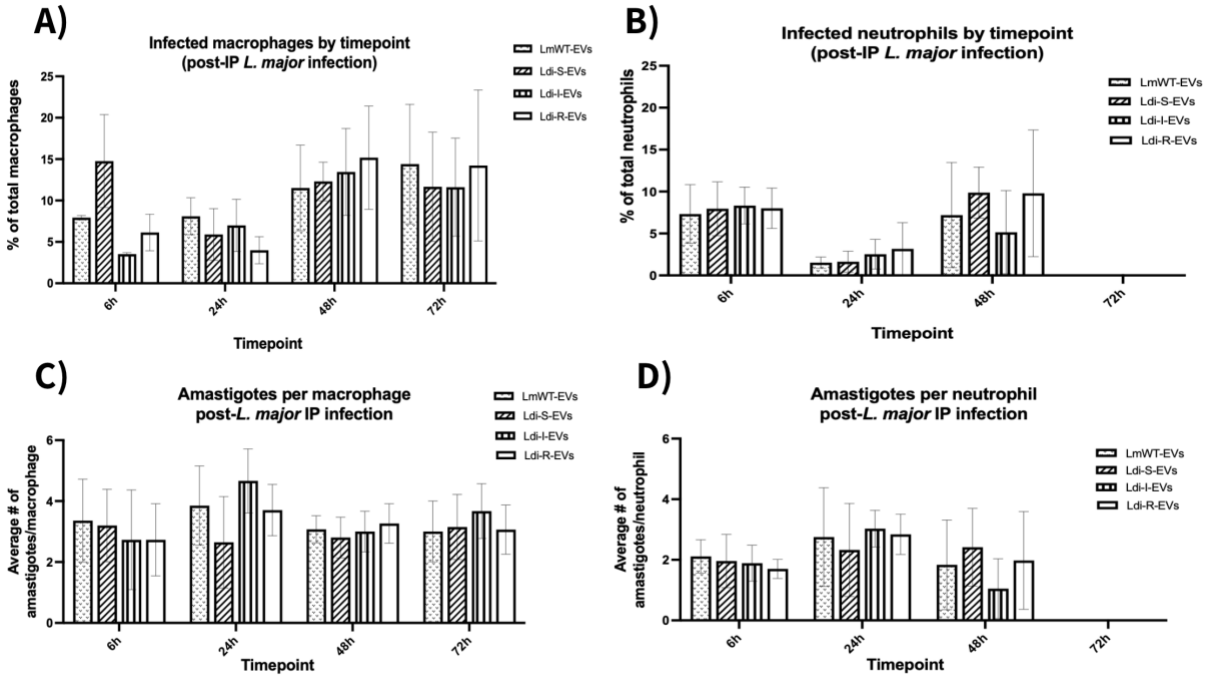
Supplemental Figure 5: Growth curve of* L. major *parasites post-IP infection

Parasites were grown out from macrophages cultured from peritoneal lavage of mice post-*L. major* IP infection, then submitted to various concentrations of Sb^{III} and monitored daily for growth through measurement of A_{600}. Data are the mean of 3 biological replicates, each performed with 2 technical replicates.



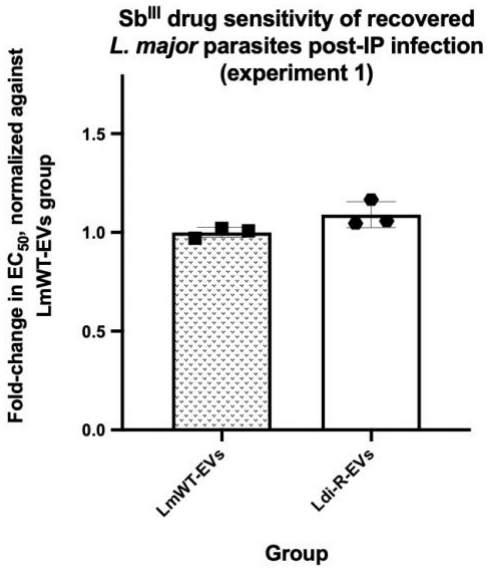
Supplemental Figure 6: Macrophage & neutrophil recruitment by timepoint post-*L. major *IP infection

Peritoneal lavage fluid from *L. major*-infected mice was examined after cytocentrifugation (6h timepoint) or after culture in a chamber slide with DMEM (24, 48, and 72 hours post-infection). Slides were dried and stained with Diff-Quik, then examined under the microscope for manual cell count & differential. **A)** Macrophages recruited at each timepoint for each group. **B)** Neutrophils recruited at each timepoint for each group. The experiment was performed twice; each experiment includes 3 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



Supplemental Figure 7: Infection of macrophages & neutrophils post-*L. major *IP infection

Peritoneal lavage fluid from *L. major*-infected mice was examined after cytocentrifugation (6h timepoint) or after culture in a chamber slide with DMEM (24, 48, and 72 hours post-infection). Slides were dried and stained with Diff-Quik, then examined under the microscope for manual cell count & differential, as well as detection of intracellular organisms compatible with amastigotes. **A)** Percent of total macrophages identified as infected (presence of one or more intracellular amastigotes) at each timepoint. **B)** Percent of total neutrophils identified as infected (presence of one or more intracellular amastigotes) at each timepoint. **C)** Average number of amastigotes per macrophage at each timepoint. **D)** Average number of amastigotes per neutrophil at each timepoint. The experiment was performed twice; each experiment includes 3 biological replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).



Supplemental Figure 8: Sb^{III} drug sensitivity of* L. major *parasites recovered after IP infection (experiment 1)

Fold-change in EC_{50} as compared to the LmWT-EVs group. Parasites were grown out from macrophages cultured from peritoneal lavage fluid post-*L. major* IP infection, then submitted to various concentrations of Sb^{III} in order to determine EC_{50} of parasites through measurement of A_{600}. Data are the mean of 3 biological replicates, each performed with 2 technical replicates. Differences were statistically evaluated by unpaired two-tailed t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Abbreviations

A_{600}: absorbance at 600 nm

BMDM: bone marrow-derived macrophages

CCAC: Canadian Council on Animal Care

CL: cutaneous leishmaniasis

DMEM: Dulbecco's Modified Eagle Medium

DR: drug-resistant

DS: drug-sensitive

EC_{50}: half maximal effective concentration

EIM: early infection model

EV: extracellular vesicle

FBS: fetal bovin serum

GP63: glycoprotein 63

HIV: human immunodeficiency virus

MCL: mucocutaneous leishmaniasis

MISEV: Minimal Information for Studies of Extracellular Vesicles

NTA: nanoparticle tracking analysis

PBS: phosphate-buffered saline

Sb: antimony

Sb^{III}: trivalent antimony

Sb^V: pentavalent antimony

SDM: Schneider's Drosophila Medium

TEM: transmission electron microscopy

VL: visceral leishmaniasis

WT: wild type

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Section 4: Discussion

The leishmaniases represent a serious threat globally, responsible for both direct and indirect impacts on public and individual health. CL, VL, and MCL have various manifestations, some of which are fatal. Co-infections with multiple *Leishmania* species have also been reported, especially in immunosuppressed populations, and their atypical clinical signs lead to delays in diagnosis & treatment (11). The absence of an effective antileishmanial vaccine means that prevention rests on insecticides and netting; precautions that pale in the face of the nearly 2 million cases of leishmaniasis reported yearly (1, 4).

Treatment of leishmaniasis is further complicated by emerging drug resistance. In certain regions, treatment with first-line antileishmanial SSG has reached failure rates of 60%, in large part due to parasite drug resistance (10). Other, newer molecules have also been decreasing in efficacy due to increasing rates of drug resistance, even in as little as a single decade of use (9).

Studies have shown that *Leishmania* EVs are released inside the sand fly vector, and play a role in infection pathophysiology, exacerbating CL infections in *in vivo* experiments (141, 151). Moreover, EVs from DR *Leishmania* parasites are unique in size & morphology, and distinct drug-resistance-associated protein content can be delivered to other *Leishmania* parasites in neighbouring poplulations in *in vitro* studies (16).

Given 1) the serious global disease burden caused by the leishmaniases, 2) troubling rates of *Leishmania* drug resistance, 3) reports of *Leishmania*/*Leishmania* co-infections, and 4) EVs' potential role in propagation of drug resistance, we set out to investigate the ability of xenogeneic EVs (from multiple *Leishmania* strains and species) to modulate infection pathophysiology and drug sensitivity of naïve parasites after concurrent passage *in vivo*.

We began with an *in vitro* experiment to determine whether contact between promastigotes and EVs from DR parasites during their incubation with macrophages could lead to a change in drug susceptibility after transformation into intracellular amastigotes. Based on these results, we then used a series of co-inoculation models in BALB/c mice to study CL, VL, and early *Leishmania* infection. *L. major* or *L. infantum* WT parasites were injected either in the footpad or

intraperitoneally in animals, with or without 10-20 μg of EVs from xenogeneic strains of *L. infantum* presenting either an antimony-sensitive, intermediate-sensitivity, or antimony-resistant phenotype (Ldi-S, Ldi-I, Ldi-R), as well as autologous WT EVs. Infections were maintained for 8-10 weeks (CL), 2 weeks (VL), or 6 hours (early infection experiments). Measuring footpad swelling and lesion scores, parasite burden of recovered footpads/spleens, and drug-testing of parasites recovered from infected mice allowed us to monitor infection pathophysiology and detect any changes in drug-susceptibility profile.

With respect to *in vitro* work, it was demonstrated that incubation with EVs from DR parasites can lead to changes in drug-susceptibility profile that persist through multiple stages of the parasite life cycle, even after treatment of intracellular amastigotes with pentavalent antimony (the same life stage of the parasite that is treated in human patients). This experiment was important to our work as a stepping stone to validate our hypothesis before moving to *in vivo* work, and it offered promising results. Furthermore, this type of experiment could have real-life applications: it is known that, not only are asymptomatic *Leishmania* infections frequent in endemic areas, but both dogs and humans can demonstrate incomplete parasite clearance after antileishmanial treatment or even relapse (21, 32). There is concern that such individuals may contribute to transmission (and possibly propagation of DR parasites) in such areas (28).

We next explored the ability of EVs to influence pathophysiology and parasite drug resistance profile after *in vivo* contact. Interestingly, neither autologous nor xenogeneic *Leishmania* EVs had any effect on pathophysiology or drug-susceptibility of *L. infantum* WT parasites recovered after 2-week infections in a mouse model. That said, CL experiments gave a different result: EVs from tested *L. infantum* strains and species significantly increased footpad swelling, footpad lesion score, and footpad parasite burden in *L. major* WT-infected mice, marking the first time that xenogeneic EVs have been shown to influence *Leishmania* pathophysiology during *in vivo* studies. Interestingly, though one might expect *L. major* WT's autologous EVs to be most beneficial to its own infection pathophysiology, EVs from the antimony-sensitive *L. infantum* field strain (Ldi-S) showed a similar effect to LmWT EVs in all 3 experiments. This may be meaningful and associated with the properties of this particular field isolate, or could simply be due to the individual variation between animals (biological replicates), making certain Ldi-S

results slightly more significant than LmWT values. Further characterization of the genome and proteome of Ldi-S could prove interesting in differentiating these phenomena.

While drug-susceptibility tests in our CL experiments also proved unremarkable, it was our early infection model that, in the end, validated our original hypothesis. Peritoneal immune cells were recovered after a 6-hour *L. major* infection in mice co-inoculated with xenogeneic EVs, and parasites were drug-tested after culture. Significant and highly significant decreases in antimony susceptibility were noted in the *L. major* parasites that had contact with Ldi-I and Ldi-R EVs, respectively. This marks the first demonstration of a *Leishmania* drug resistance transfer by xenogeneic EVs after simultaneous passage *in vivo*. Notably, the parasites in which this increased antimony resistance was identified were never maintained in a drugged environment, and therefore there could be no selection of DR parasites; consequently, their significant contact must have come directly from the DR-strain EVs. In future, antileishmanial treatment of these co-inoculated animals could prove informative, demonstrating possible selection of DR parasites *in vivo*, or even treatment failure.

Although there were several limitations to our study, including the use of a murine model and direct inoculation of parasites rather than a vector-based study, our results have important implications for the study and treatment of leishmaniasis. For example, we know that EVs are released by *Leishmania* inside the sand fly vector, and co-egested into mammalian hosts (141). Moreover, it was recently shown that two different *Leishmania* species can complete their life cycle inside the same sand fly simultaneously (12). Given the reports of *Leishmania*/*Leishmania* co-infections, especially in vulnerable populations, our findings foretell possible propagation of drug-resistance inside the phlebotomine vector in highly endemic regions, and a potential contribution to treatment failure. *In vivo* studies inside the sand fly vector are indicated, as well as further work to determine whether the novel DR phenotype of parasites after contact with EVs from DR strains is a phenomenon that remains stable in the long term.

Section 5: Conclusion

The leishmaniases are responsible for a serious global disease burden, and diagnosis and treatment can be complex, especially in the face of co-infections. EVs have been proposed as possible determinants of early *Leishmania* infection, and as potential players in the spread of drug resistance. Here, our team has shown for the very first time that EVs from xenogeneic parasites can contribute directly to propagating drug resistance between parasite populations after *in vivo* contact, and that EVs from *L. infantum* can influence *L. major* pathophysiology. This phenomenon may well contribute to treatment failure in certain regions, and further complicate management of co-infections, especially in vulnerable or immunosuppressed populations. In conclusion, in order to better combat leishmaniasis, it is crucial to understand not only *Leishmania* early infection, but also its drug-resistance mechanisms and methods of propagation, especially through *in vivo* studies in the sand fly vector. Moreover, as both leishmaniasis (and especially *Leishmania* co-infections) disproportionately affect disadvantaged populations, working to make *Leishmania* an important consideration on international health agendas and offer accessible health services to underserved communities should be prioritized.

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