Georgy Nakhratyan

Licenciado em Biologia Celular e Molecular

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Orientadora: Doutora Armanda Rodrigues, Investigadora Auxiliar, Instituto de Higiene e Medicina Tropical

Co-orientadora: Professora Doutora Gabriela Santos-Gomes, Professora Auxiliar com Agregação, Instituto de Higiene e Medicina Tropical

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Abstract

Leishmaniasis is a neglected tropical disease caused by the protozoa *Leishmania* that affects millions of people yearly across the globe. Prevalence in underdeveloped countries means most people afflicted by it cannot afford proper treatment. *Leishmania* has evolved numerous mechanisms to avoid immune response by the host, manipulating it for its advantage. Treatment options are scarce, toxic and expensive, and vaccines for humans do not exist. Therefore, there is an urgency for novel ways to control and restrain this parasite. Exosomes are extracellular vesicles (EVs) that are generated by all cells and carry various molecules for cell communication. The present work addresses the effect of *L.amazonensis* and *L.guyanensis* EVs have on the infectivity of the parasite in the host skin, and their role as a potential prophylactic therapy. To do this, EVs were isolated from Leishmania promastigote axenic cultures and characterized. Co-cultures of human keratinocytes HaCat and human monocytic THP-1 cell lines were used to establish an in vitro simplified human skin model. The immunological response exhibited by the co-culture after stimulation with isolated parasitic EVs was analyzed by flow cytometry for several key immune markers, microscopy, spectrophotometry, and RT-qPCR of pattern recognition receptors (PRRs) and cytokines. Findings demonstrate that the model is capable of immunologic response to EVs and suggests that EVs induce a pro-inflammatory response in cells, severely impacting cell morphology and proliferation capabilities. Cytokine and PRR gene expression suggest EVs potentially to trigger a similar cellular response as contact with live parasites. Preliminary results from pre-exposure of THP-1 cells to EVs indicate than EVs can increase parasite infectivity, suggesting that EVS constitute an essential component of Leishmania biology. Leishmania EVs can mimic parasite presence, being recognized by innate sensors and activating the skin immune cells. Thus, Leishmania EVs can be exploited as therapeutical adjuvants for humans and animals.

Keywords: Cutaneous Leishmaniasis, extracellular vesicles, immunomodulation; skin models.

Resumo

A leishmaniose é uma doença tropical negligenciada, causada pelo protozoário *Leishmania.* Esta doença afeta milhares de pessoas todos os anos, com maior incidência em regiões subdesenvolvidas do planeta, onde o acesso a tratamento é muito restrito. *Leishmania* co evoluiu com o sistema imunitário mamífero durante milhões de anos, e consegue manipular o mesmo para a sua vantagem. As terapias atuais são escassas, tóxicas e fora do poder económico do paciente comum, e pra mais, não existe vacina contra a leishmaniose humana. Sendo assim é imperativo descobrir novos mecanismos para combater esta doença, sob os quais as fundações para novas terapias sejam desenvolvidas. Os exosomas são vesiculas extracelulares (VEs) produzidas por todas as células para comunicação celular. Este trabalho foca-se na investigação do efeito das VEs de *L.amazonensis* e *L.guyanensis* na infecciosidade do parasita na pele do hospedeiro e o seu potencial como terapia profilática. Para este fim, VEs de culturas de axênicas de parasitas foram isoladas, caracterizadas. Foram realizadas co-culturas de células THP-1 e HaCat para estabelecer um modelo simplificado de pele humana. A resposta imunológica deste modelo à exposição de EVs foi prontamente caracterizada por citometria de fluxo de MHCI e MHCII, espectrofotometria, microscopia e RT-qPCR de *pattern recognition receptors* e citosinas. Resultados indicam resposta imunológica por parte do modelo às VEs, como também um efeito pro-inflamatório, semelhante à presença de parasitas, com grande

impacto na morfologia e na capacidade proliferativa das células. Resultados preliminares indicam que pré-exposição de THP-1 às VEs têm um efeito potenciador da infeção do parasita, sugerindo que as VEs sejam uma componente essencial da biologia de *Leishmania.* VEs de *Leishmania* são capazes de mimica da presença do parasita, sendo reconhecidas e ativando células imunitárias da pele. Assim, as VEs poderão ser aproveitadas para o desenvolvimento de terapêuticas adjuvantes em humanos e animais.

Palavras-chave: Leishmaniose Cutânea, Vesiculas Extracelulares, Modelo de Epiderme, Imunomodulação.

Index of Content

1. Introduction 1

1.1 *Leishmania spp.* 1

1.2 *Leishmania* Life Cycle 2

1.3 Leishmaniasis 3

1.3.1 Disease and Geographical distribution 3

1.3.2 Disease pathology and clinical manifestations 4

1.3.3 Diagnosis 5

1.3.4 Treatment 5

1.3.5 Prevention 7

1.4 Immune Response 7

1.4 The Skin local immune response 11

1.6 Extracellular vesicles 13

1.7 Objective 15

2. Materials and methods 17

2.1 Experimental design 17

2.2 Parasites and cell cultures 18

2.2.1 *Leishmania promastigotes* 18

2.2.2 THP-1 cell line 18

2.2.3 HaCat cell line 18

2.2.4 THP-1 cell priming 18

2.2.4 Experimental conditions 19

2.3 EV isolation from *L.amazonensis* and *L.guyanensis* 19

2.4 Nanometric characterization of EVs 20

2.5 Total antigen extract from *L.amazonensis* and *L.guyanensis* 20

2.6 THP-1 EV cellular interaction 20

2.7 THP-1 viability assay 21

2.8 Wound healing assay 21

2.9 Characterization of priming effect on THP-1 22

2.9.1 Light microscopy 22

2.9.2 Flow Cytometry primed THP-1 cells 22

2.10 HaCat-THP-1 co-culture establishment and characterization 23

2.10.1 Establishing the co-culture 23

2.10.2 Co-culture flow cytometry 23

2.10.3 Co-culture characterization by microscopy 24
2.10.4 Co-culture RT-qPCR analysis 24
2.11 Urea quantification 26
2.12 Limiting dilution assay 26
2.14 Statistical Analysis 28
3. Results and discussion 29
3.1 Extracellular vesicles shed by *L. amazonensis* and *L. guyanensis* promastigotes are compatible with exosomes and microvesicles 29
3.2 *Leishmania* EVs interact with THP-1 cells and do not affect cell viability 31
3.3 The presence of an inflammatory stimuli impairs keratinocytes wound-healing response 33
3.4 THP-1 cells "priming" for skin macrophage-like (sMΦ) phenotype 34
3.5 HaCat THP-1 co-culture were able to mimic host skin dermis 38
3.6 Pre-exposition of THP-1 to *Leishmania* EVs does not protect against parasite infection 43
4. Conclusion; Future Prospects and Final Thoughts 45
5. Bibliography 47

Index of Figures

Figure 1. Stylized image of the basic morphology of Leishmania spp. promastigotes (A) and amastigotes (B).. 1
Figure 2. Schematic of Leishmania life cycle.....3
Figure 3. Experimental design flowchart......18
Figure 4. Visual representation of LDA assay 96 well plate. O 27
Figure 5. Topography of promastigotes cell membrane Scanning electron microscopy (SEM) of cultured L.guyanensis (A, B) and L.amazonensis (C, D). 29
Figure 6. Visual representation of diameter distribution of particles in samples of EV isolates. 30
Figure 7. Zeta potential of EV isolate samples......31
Figure 8. EV incorporation into THP-1 cytoplasm......32
Figure 9. THP-1 cell viability assay. 32
Figure 10. Light microscopy images of wound healing assay. 34
Figure 11. Light Microscopy images of THP-1 cells in culture. 35
Figure 12. Flow cytometry of cells marked for CD80, CD14, MHCI, MHCII, CD83, CD11c, langerin, CD64, and CD1a......37
Figure 13. Light microscopy images of HaCaT-THP-1 co-culture, exposed to different conditions. 38
Figure 14. Scanning Electron Microscope images of HaCat-THP-1 co-culture.....39
Figure 15. Flow cytometry of HaCat-THP-1 co-culture. 40
Figure 16. Reverse transcriptase qPCR heatmap of HaCat-THP-1 co-culture at 24 and 48 hours. 41
Figure 17. Colorimetric Urea assay.....42
Figure 18. Limiting Dilution Assay of THP-1 infected by Leishmania. 43

Index of Tables

Table 1. Monoclonal antibodies and their respective fluorophore.. 22
Table 2. Monoclonal antibodies and their respective fluorophore. 23
Table 3. Primers used for RT-qPCR.. 25
Table 4. Average diameter, in nanometers, of the particles detected in the different samples of isolate ..... **Error! Bookmark not defined.**

List of Abbreviations

CL: Cutaneous Leishmaniasis
ELISA: enzyme-linked immunoassay
DCs: Dendritic cells
DCL: Diffuse Cutaneous Leishmaniasis
EVs: Extracellular vesicles
GP63: Leishmania Major Surface Protease
IH: Insect Host IH
LCs: Langerhans cells
LCL: Localized Cutaneous Leishmaniasis
MHCI: MHC class I
MHCII: class II
MØs: Macrophages
MHC: Major Histocompatibility Complex
MH: Mammal Host
MCL: Mucocutaneous Leishmaniasis
NK: Natural Killer
NW: New World
OW: Old World
PKDL: Post Kala-Azar Dermal Leishmaniasis
RT-qPCR: Reverse transcriptase-quantitative PCR
TLRs: Toll-like receptors
VL: Visceral Leishmaniasis
WHO: World Health Organization
APC: Antigen-presenting cells
CR3: Complement receptor 3
CS: complement system
DTA: direct agglutination test
iNOS: inducible nitric oxide synthase
MAC: membrane attack complex
MVBs: multivesicular bodies
NETs neutrophil extracellular traps
NO: nitric oxide
PAMPs: pathogen associated molecular patterns.
PRRs: pattern recognition receptors
PCR: polymerase chain reaction
RNS: Reactive Nitrogen Species (RNS)
ROS: Reactive oxygen species (ROS)

STI: Sterile insect technique (STI)

Tc cells: Cytotoxic T cells

Th cells: Helper T cells

1. Introduction

1.1 *Leishmania spp.*

In the early 20th century pathologist William Boog Leishman and Dr. Charles Donovan both independently observe strange ovoid bodies taken from samples of sick and deceased patients. In 1903, Dr. Ronald Ross would write a paper where he concludes these ovoid bodies to be a novel protozoan organism and proposed to name it *Leishmania donovani* after the doctors who first identified it. And so, by the end of the year 1904 the *Leishmania* genus had been established[1].

Taxonomic classification of the genus is due to the genetic diversity of these parasites and the challenges of accurately distinguishing between different species based on genetic markers alone[2].

However, it undoubtedly belongs to the class Kinetoplastea due to the kinetoplast[3], a unique and diagnostic feature of the class, a mitochondrial region composed by thousands of circular mitochondrial DNA molecules interlocked to make a single network serving a plethora of purposes[4]. The genus is further classified into the Trypanosomatidae family, characterized by their single flagellum morphology[5]. Interestingly the genus *Leishmania* is one of the few dixenous genera in the Trypanosomatidae, meaning they have two hosts in their life cycle[6]. *Leishmania* are also dimorphic parasites, exhibiting different morphological forms based on which host they are in at a given time[7].

While infecting the insect , the parasite takes the promastigote form, which is an elongated and flagellated form, circa 15-30 mm in length, well suited for extracellular life. This form can be further divided into several substates that depend on the development locales in the insect host that can be simplified down to two: the infective metacyclic promastigote ready for transmission to the mammalian host and the non-metacyclic promastigote, which develops in various parts of the gut. Upon reaching the mammalian host, the parasite takes its amastigote form, small, non-motile spherical cells measuring approximately 3 µm that are well suited for intracellular life (Figure 1). [8]

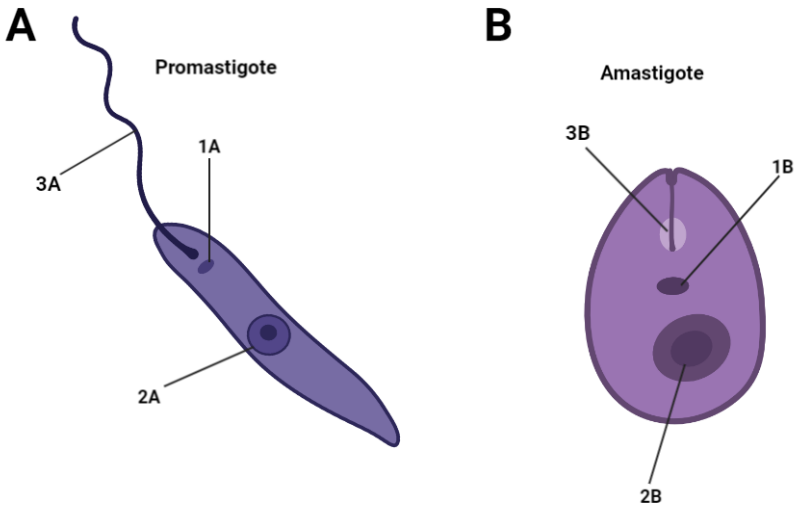

Figure 1. Stylized image of the basic morphology of *Leishmania* spp. promastigotes (A) and amastigotes (B). The standout features pictured are the kinetoplast (1A and 1B), the nucleus (2A and 2B) and the flagellum (3A and 3B). Figure created in BioRender at https://www.biorender.com/.

1.2 *Leishmania* Life Cycle

As briefly mentioned above, the parasite life cycle is subdivided into two main categories: direct (monoxenous) and indirect (heteroxenous). Direct parasites spend most of their life in the same definitive host, spreading their progeny to other hosts while indirect parasites have an intermediate host (IH), often acting as a parasite vector to reach its definitive host and where parasitic development occurs. *Leishmania* has a sand fly host (IH) for parasite development and transmission and a mammalian host (MH) for the growth of intracellular parasitic stage, making them dixenous parasites.[9]

As the female sand fly takes a blood meal from the mammal hosts (MH), metacyclic promastigotes from the proboscis are deposited on the MH's skin. These are quickly phagocyted by macrophages (MØs), dendritic cells (DCs), and neutrophils present at the site. Once internalized, the parasite differentiates into to the amastigote form, which is well adapted for survival in the inhospitable and hostile environment inside immune cells. There it proceeds to multiply by binary fission, swelling the host cell and causing cellular rupture which promotes a new cycle of phagocytosis, growth, and lysis. This way the parasite can infect a variety of cells while dispersing inside the MH. These infected MØs can have different destinations based on the species that is infecting the host, being able to migrate to other organs causing a severe and potentially lethal form of disease or remaining in the skin causing a less severe and more common form of disease.[10]

Once a female sand fly takes a blood meal from an infected MH, it will ingest a variety of parasitized cells. From here a complex process that differs based on insect host and *Leishmania* species takes place, but in a simplified fashion, the amastigotes inside the ingested cells will transform back into the promastigote and they develop as procyclic promastigotes in different areas of the gut. These eventually mature into infectious metacyclic promastigotes as they migrate to the proboscis and prepare to be inoculated during the following blood meal, as the cycle repeats once more.[7]

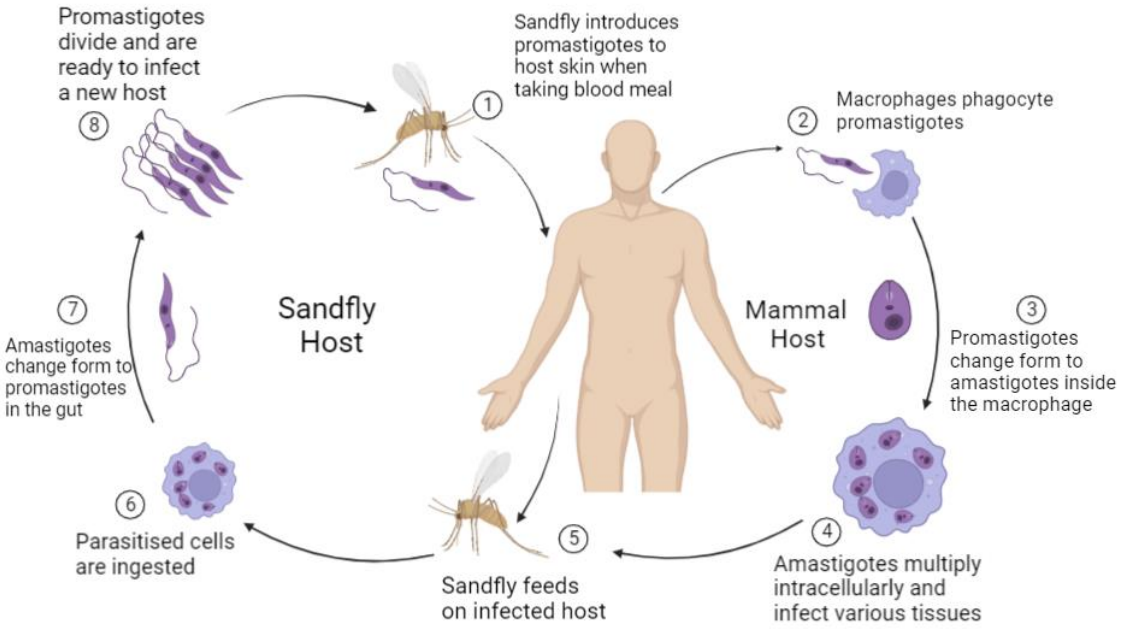

Figure 2. **Schematic of *Leishmania* life cycle**. (1) Host sand fly takes a blood meal from a MH, depositing promastigotes on the skin. (2)(3) Promastigotes are then phagocyted and transform to their amastigote form. (4)(5) Amastigotes replicate and burst cells, infecting new immune cells. (5)(6) Sand fly feeds on an infected mammal host, ingesting parasitized cells. (7)(8) Amastigotes then undergo a change to the promastigote form and develop inside gut of the sand fly, repeating the cycle. Figure created in Biorender at https://www.biorender.com/.

1.3 Leishmaniasis

1.3.1 Disease and Geographical distribution

Many people are conscious of the danger that insects can pose to animals including humans, carrying a variety of microorganisms that can cause potentially fatal diseases. From Lyme disease causing bacteria, the viral Dengue fever, all the way to the parasite responsible for the well-known Malaria, a plethora of pathogens use insects as their vector, spreading disease and ravaging populations in their wake. Caused by the *Leishmania* parasite and spread by the bite of female sand flies, leishmaniasis is one of these dangerous diseases borne by insects.

Leishmaniasis, classified by the World Health Organization (WHO) as a neglected tropical disease, is a group of potentially deadly infectious diseases that infects an estimated one million people worldwide per year. The deadliest form of the disease, Visceral Leishmaniasis (VL), is fatal if untreated for 95% of cases and affects an estimated 50 to 90 thousand people every year[11,12]. It affects not only humans but a variety of other mammals, a non-exhaustive list includes dogs, cats, rabbits, and other primates[13]

This disease is often overlooked as it mainly affects poor and underdeveloped countries, with a varied mix of factors like geographical clustering, lack of reliable incidence data and the overall diversity of its clinical and epidemiological features making it hard to manage[14]. Its societal impact is enormous as the most common form of the disease, Cutaneous Leishmaniasis can lead to severe scarring and disfigurement leading to social stigma and various detrimental effects to the mental health of the ones unfortunate enough to contract it[14].

Currently leishmaniasis can be found on every continent to the exception of Australia and Antarctica. In the Old World (OW) it is mainly found in the Middle East, some regions of Africa and Asia and southern Europe. In the New World (NW) it is found mainly in Central and South America and some parts of Mexico.

Environmental changes such as global warming means that sandflies are able to survive in regions that were previously too cold for them, leading to the spread of the disease to new areas. Additionally, extreme weather events such as floods and droughts can disrupt the ecology of sandfly habitats, further increasing the risk of transmission. As climate change continues to affect global temperatures and weather patterns, it is likely that leishmaniasis and other vector-borne diseases will continue to increase in prevalence, highlighting the urgent need for coordinated global efforts to mitigate the impact of climate change on public health[15].

1.3.2 Disease pathology and clinical manifestations

Leishmaniasis, according to WHO, presents itself in three main different forms of disease, depending on infecting species: Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL) and VL, also known as Kala-azar[11]. CL is the most common form of the disease, in the OW it is mainly caused by *L.tropica* and *L.major*, while in the NW it is mostly caused by *L.braziliensis* and *L.mexicana*. Clinically this disease can be sub divided into Localized Cutaneous Leishmaniasis (LCL) which leads to chronic slow-healing skin ulcers which can be painless and self-healing that take months to fully heal and leave behind disfiguring scars. CL can also present as Diffuse Cutaneous Leishmaniasis (DCL) which is a severe form of the disease that leads to a variety of skin lesions across large areas of skin and very commonly needs medical treatment as it will not self-resolve. MCL is categorized by the extension of CL to mucosal membranes, such as the nose, mouth, and throat, creating ulcerative, degenerative skin lesions. It is caused by species such as *L.braziliensis* and is most prevalent in South America. If left untreated, MCL can lead to heavy disfigurement and tissue damage and can develop months or years after the initial infection and is mainly due to an overresponsive immune reaction.[16,17]

VL is the most severe and fatal form of leishmaniasis. It is also known as Kala-azar, Hindi word for black fever, an unsettling moniker earned due to the characteristic darkening of the skin it caused in infected people. It is caused by species such as *L.donovani* and *L.infantum* and it occurs when the parasitized immune cells migrate from the skin to internal organs like the spleen and liver. Symptoms of VL include but are not limited to anemia, fever, weight loss, and enlargement of internal organs such as the spleen and liver. If left untreated, death is a near certainty[18]. Unfortunately, even those who survive this deadly encounter are not in the clear, as a secondary form of the disease, known as Post Kala-Azar Dermal Leishmaniasis (PKDL) can surface years after LV treatment and develop into various forms of skin rashes and lesions[17].

Additionally, given its incidence in less developed countries, especially in rural, poorer areas, factors that might lead to immunosuppression, such as co-infection with HIV become more common and severely complicate the clinical situation of patients as it increases the progression of leishmaniasis and makes treatment considerably more difficult[18].

1.3.3 Diagnosis

A plethora of techniques exist for diagnosis, with varying degrees of sensitivity and specificity. We will touch on some of these techniques to better contextualize the disease and understand the challenges posed by it.

It is important for leishmaniasis diagnosis to be fast, but most importantly cost efficient due to its prevalence in developing parts of the world. Treatments are often expensive and toxic to the patient so accuracy in diagnosis is paramount to ensure minimal suffering.

Both VL and CL can be diagnosed without resorting to laboratory testing based on clinical features and patient history alone, using factors like living or visiting endemic areas coupled with risk behaviors such as not using sleep nets, exposure to the elements or proximity with animals known to act as repositories for the parasite to provide a probable diagnosis in settings where the resources for more in-depth testing is simply not possible.[19,20]

Light microscopic observation of amastigotes remains a reliable method for confirming both VL and CL infection. Biopsy samples of the bone marrow, spleen, or lymph nodes for VL and from around the skin lesions for CL can be analyzed under light microscopy and an experienced technician should be able to detect the presence of amastigotes. Biopsies carry a significant discomfort for the patient, as they are often extremely painful and, in some cases, can be fatal.

Unfortunately, basically all *Leishmania* species are remarkably similar and practically indistinguishable from one another by microscopy, which poses an issue for diagnosis and treatment, requiring alternative tests to overcome this hurdle, such as polymerase chain reaction (PCR) tests that can target species specific sections of DNA and aid in the identification of the exact species.[19,20]

Following up on alternative methods to microscopy, a number of serological diagnosis techniques were developed for VL diagnosis with varying degrees of sensitivity and specificity, serological in majority due to its systemic effect. These include methods such as direct and indirect enzyme-linked immunoassay (ELISA) using recombinant antigens, direct agglutination test (DTA), indirect immunofluorescence assay, and point-of-care tests such as rapid antibody tests which are easily available and transported. Meanwhile CL is harder to diagnose due to its more localized effect, and often relies on visual identification of lesions, patient history and microscopy. These methods all have their own benefits and disadvantages, factors like cost, accessibility, specificity/sensitivity to the species endemic to the region, capability to distinguish present from past infection are just some of the factors that play a major role in deciding which methods to use for diagnosis.[19,20]

A more recent addition to the ever-growing army of leishmaniasis diagnostic tools is flow cytometry, which has shown promising results with high specificity and sensitivity, along with various benefits including speed and reduced sample volume compared to other serological methods[21,22].

1.3.4 Treatment

Owing to its status as a neglected disease and the associated lack of financial interest leading to low investment into R&D, treatments for leishmaniasis not only are often aged and outdated, but commonly also involve significant toxicity to the patient as well as a myriad of side effects.

For the vast majority of those afflicted with CL, no treatment is necessary as it is a self-healing disease, but sadly this does not mean those infected come out unharmed. CL can leave disfiguring scars, especially if MCL is involved, and susceptibility to secondary infection as well as potential function impairment from extensive tissue damage. Recovery from lesions can take months or even years and the societal stigmatization that the disfigurement carries can last for a lifetime[20].

Reiterating the previously mentioned difficulty in identifying the exact species causing illness, many times treatment is based on local expertise and might not be the best for each case, and a great deal of importance is placed on the risk-benefit of getting treatment for CL/MCL versus allowing natural recovery to take place in order to avoid the plethora of discomforts caused by most treatments, as described below. On the other hand, VL poses a major health risk, usually fatal if left untreated, and as such, requires swift medical intervention[23]. HIV-LC-coinfection in patients magnifies the need for treatment as immunocompromised individuals see an increase in relapse and exacerbates the infection[19].

If deemed necessary, the first line of treatment for leishmaniasis are pentavalent antimonials (PAm), which due to high cost, initially remained largely inaccessible to patients in developing countries until generic formulations gave way to a cheap and relatively effective treatment[19]. For cases of CL, PAm can be applied locally but systemic treatment is advised if there is significant risk of developing MCL[20]. Intravenous or intramuscular injection is necessary to treat VL which is often extremely painful. This therapy is highly toxic, with side effects that include cardiac arrest, and have a harsher impact on HIV-VL-coinfected patients, leading to potential loss of life. A growing concern pertains to the failure of the therapy in select geographical regions due to developing drug resistance by the parasite, and so other therapies exist to potentially alleviate these issues. [19,24] Amphotericin B is quickly growing as the choice drug to treat both VL and CL, in part due to the resistance to PAm, and has been shown highly effective as a treatment, on the other hand, this compound also carries with it high toxicity and severe side effects. Fortunately, efforts to reduce its toxicity and increase stability have been successful and less toxic liposomal formulations that near 100% success rate in curing leishmaniasis exist, but at an extremely prohibitive cost out of reach for many of the afflicted people.[19,24] Miltefosine, originally developed as an anticancer drug, recently started being used as a treatment for VL infections, its benefits include oral administration, and it is safer than conventional pentavalent antimonials in HIV-VL-coinfected patients. But once again is costly, induces adverse side effects and is not advisable during pregnancy due to suspected teratogenicity[19]. Other therapies exist for CL, such as heat therapy and cryotherapy which require specialized equipment, as well as a myriad of other compounds and can be used as a single or combined therapy, all with a variety of success rates and adverse side effects dictated by numerous factors[24].

Despite not exploring all available treatment options in this section, it hopefully achieved the goal of pointing out that the existing therapeutic strategies often rely on drug repurposing which makes them highly toxic and often are only symptom specific and no species specific, not addressing the root cause of the issue and highlighting the dire need for more research to be done to develop fast, efficient, and most importantly, safe treatment options that are economically affordable and easy and well suited for administration in low resource, underdeveloped areas of the world.

1.3.5 Prevention

As with many diseases, the first line of defense against leishmaniasis is prevention. Prevention can come in many different ways: education, preventing sandfly bites, prophylactic treatment of surrounding animals, sand fly population control, and more.

Informing and educating local populations helps spread awareness regarding preventative measures, and not only results in better preventative measures but helps early identification of disease symptoms and early treatment seeking may be the difference between life altering disfigurement or death and a normal recovery.

Reducing or attenuating risk behaviors that expose humans to sand fly bites is a crucial step for prevention. Protective clothing and the use of pesticides cannot be understated, especially during outdoor activities in the wilderness such as farming, mining, hunting, to name a few. Preventing nocturnal bites can be done by using fine mesh bed nets, which need to be small enough to prevent the sandfly from reaching the host, these can be impregnated with insecticides as an added layer of protection.[20] Many animals can also act as a natural reservoir for the parasite, mainly dogs. A study showing that frequent use of insecticides on dog resulted in a reduction in the number of human leishmaniasis cases highlights the importance of protecting not only yourself but other animals around you susceptible to infection[25].

One of the most effective disease prevention methods available is vaccination. The first attempts at finding a human vaccine for leishmaniasis, so called first-generation vaccines made out of killed parasites did not prove to be effective as a preventative measure across both the New and the Old World. Since then, many types of vaccines were attempted based on different principles such as attenuated vaccines, recombinant vaccines and more, all of which either cannot do not make it to clinical trials for various reasons or await further studies to access their effectiveness. As of yet, there still is no vaccine for Human leishmaniasis.[26]
Conversely, leishmaniasis vaccination is already a reality for dogs, which has a high chance to prevent disease, and as stated above, is linked to reduced zoonotic transmission to humans[27].

Other forms of vector control exist, for example, larval control consists in killing the insect vector during its larval stage, and one study proved this possible by baiting rodents with insecticide treated bait, which passed onto its feces would kill sand fly larva that feed on said feces[28]. This could have disastrous ecological impact, however, and should not be lightly considered.

A fascinating method previously used to control other species of pest insects is the sterile insect technique (STI) which relies on the release of a large number of sterile males into the wild, which severely suppresses pest population[29]. While no studies have been conducted regarding phlebotomine sand flies and STI, it is nevertheless an interesting prospect that could be the answer to drastically reducing cases of both animal and Human leishmaniasis.

1.4 Immune Response

To be alive is to persevere among the unrelenting assaults of nature and, against all odds, emerge victorious. An impossible task if not for the immune system, a system composed of many cells, organs and molecules that require an enormous number of resources to function but in turn protect one from an assortment

of dangers such as bacteria, fungi, viruses, parasites, cancer cells, and toxins. Any of which could prove fatal, were it not for this protection.

To achieve this, the immune system can be divided into two “subcategories” that complement and cooperate with each other: innate immunity and adaptive immunity.

Innate immunity is a fast, non-specific, response to any invading pathogen. It stands as the first line of defense and simply distinguishes self from non-self. It can also elicit a response from the adaptive immune system. The innate immune response is, therefore, the first line of defense that the body has against pathogens. Innate immunity is comprised of 4 categories of defense: anatomical, physiological, endocytic and phagocytic, and inflammatory, all of which are ready to be activated as soon as an infection occurs. [30]. The anatomical, or physical, mechanisms of defense include the skin, which acts as a physical barrier to prevent the entry of pathogens, and mucosal membranes, which line the respiratory, gastrointestinal, and genitourinary tracts, and trap and expel pathogens. Physiological defenses include body temperature, pH and chemical mediators, such as nitric oxide and other reactive oxygen species that can cause extensive cellular damage to invasive pathogens, inhibiting their growth or outright killing them[30]. The next two categories are largely mediated by a variety of cells that respond in a plethora of ways ranging from signaling and recruitment to internalization and destruction of the foreign pathogens. These cells are the macrophages, the neutrophils, the eosinophils, the basophils, mast cells, dendritic cells and Natural Killer (NK) cells and actively communicate to each other by secreting cytokines. These are a group of small proteins that are secreted by cells of the immune system and other cells in response to a variety of stimuli, such as infection, inflammation, injury, and stress. They play a crucial role in regulating the immune response and maintaining homeostasis in the body[31]. Cytokines play a vital role in *Leishmania* infection, as these molecules dictate important the outcome of the parasitic infection, being able to active cells to eliminate the parasite or having a immunosuppressive effect, allowing the parasite survival.

Neutrophils are especially important as they are the first cells recruited to the site phagocyting present microbes and ensuring their destruction by employing an arsenal of tools such as reactive oxygen species (ROS) and enzymes or by emitting neutrophil extracellular traps (NETs) that entrap and immobilize invaders to facilitate disposal[32]. Due to these cytotoxic ROS and enzymes, neutrophils are short lived cells and so, macrophages are then responsible for phagocyting apoptotic neutrophils[33].

These cells rely on the recognition of various pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) and respond to the pathogen differently based on the cell’s function[30]. Inflammation is a complex process by which these immune cells are recruited by specialized molecules known as cytokines and chemokines to respond to tissue damage or infection, usually in a local fashion[30].

Toll-like receptors (TLRs) are a type of intramembrane PRR that are found on the surface of cells and in intracellular membranes. They play a crucial role in recognizing various types of pathogens, such as bacteria, viruses, fungi, and parasites, as well as some endogenous molecules released during tissue damage. When a TLR recognizes a PAMP, it triggers a signaling cascade that leads to the activation of immune cells and the production of inflammatory cytokines and chemokines. This helps to recruit and activate other immune cells to fight off the infection or repair the damaged tissue. The activation of TLRs is a key step in the innate immune response, which is the first line of defense against infections[34].

There are currently 10 known types of TLRs in humans, of which several have been studied in connection to *Leishmania*. TLR2 and TLR4 have been shown to play important roles in the recognition of *Leishmania*, as they can recognize the lipophosphoglycan (LPG) present on the surface of the parasite. Activation of TLR2 and TLR4 leads to the production of pro-inflammatory cytokines and chemokines[35]. TLR9 has also been shown as an important TLR relating to immunity upon *Leishmania* infection, as it recognizes *Leishmania* parasite intracellularly, increasing IL-12 production and targeting cells for lysis via NK cells[36].

AIM2 is a cytosolic DNA sensor that plays a critical role in the innate immune response against intracellular pathogens. It detects cytoplasmic DNA derived from viruses, bacteria, and other intracellular pathogens and activates the inflammasome, a multiprotein complex that promotes the production and secretion of pro-inflammatory cytokines such as IL-1β[37]. Studies have shown AIM2 is connected to the severity of the lesions resulting from CL[38].

NLRP3 is an inflammasome, a protein complex that promotes the maturation of pro-inflammatory cytokines and is generally associated with pro-inflammatory response. It is formed in response to various danger signals, such as pathogen-associated molecular pattern, which are recognized by PRRs on immune cells. The inflammasome complex typically consists of a sensor protein, such as NLRP1, NLRP3, or AIM2, an adaptor protein called ASC, and an effector protein called caspase-1. Once activated, the inflammasome complex catalyzes the activation of caspase-1, which in turn cleaves pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18) into their mature forms. These cytokines then trigger an inflammatory response, which can help to eliminate pathogens and repair damaged tissues.

Not much is known about the interactions of NLRP3 and *Leishmania*, but the fact that is it the most studied inflammasome lends some interest to furthering our knowledge of how and if they interact[39].

The complement system (CS) is another essential component of the innate immune system is a complex signaling cascade consisting of more than 30 proteins. Upon activation through three different activation patterns or pathways (the classical, the alternative and the lectin pathways), the SC leads to a sequential enzymatic cascade. All three pathways ultimately lead to the same end result, the formation of a molecular structure known as membrane attack complex (MAC) that inserts itself into cell membranes, forming a pore and leads to target cell lysis. Alongside the formation of the MAC, cleaving of the protein C3 leads to the formation of anaphylatoxins C3a and C5a which result in various responses ranging from chemoattraction to apoptosis and are potent pro-inflammatory mediators. The opsonin C3b is also formed, which can bind to the surface of pathogens, such as bacteria, and promote their recognition and phagocytosis by immune cells, a process known as opsonization.[40]

It is important to note that while the CS is generally discussed as a part of innate immunity, studies have shown it plays an important role in adaptive immunity as well, serving as a sort of cross-roads between both systems.

On the other hand, adaptive immunity is, often the result of immune response “personalized” for specific pathogen. This response, usually slower in its assembly and it is also defined by its ability to ‘’memorize’’ a pathogen, and thus involves a period of time before activation and full response. This ability to

memorize what pathogens it has been in contact with allows for a faster and more efficient response to subsequent events resulting from the detection of the same pathogen.[30]

Behind this recognitive ability are a series of complex mechanisms that detect specific antigens or unique molecular patterns, associated with a particular pathogen or foreign substance. A complex process of immune cell activation, proliferation and differentiation then follows which leads to the production of antigen-specific cells that patrol the organism and readily respond to any future re-exposure to the pathogen.

The adaptive immune system is mediated by two major types of cells, the B and T lymphocytes. B cells produce antibodies, which are specialized proteins that recognize and bind to specific antigens, marking them for destruction by other components of the immune system. These cells can be additionally stimulated by T cells[41]. T cells are involved in cell-mediated immune responses, and unlike B cells, consist of various subtypes of cells with specific roles and functions. Among these subtypes we find the Helper T cells (Th cells), sometimes referred to as $CD4^+$ T cells, which recognize antigens presented by antigen-presenting cells (APCs) and mainly respond by activating other immune cells such as B cells and cytotoxic T cells. Th cells are further divided into subsets that differ depending on the mechanism of their activation, which include two relevant types of response, Th1 cells and Th2 cells. Th1 activation in Th cells is induced by IL-12. Upon activation, Th1 cells produce cytokines such as IFN-γ and TNF, which help to activate MØs and promote a cell-mediated immune response against intracellular pathogens. Th2 activation is induced by IL-4, IL-5, and IL-13 respectively. Th2 cells are more often associated to B cell activation as a measure to regulate allergic response and mediate a response against extracellular pathogens. Th1 and Th2 responses are mutually inhibitory, a major factor that will come into play in the context of leishmaniasis.[42]

Briefly mentioned above, Cytotoxic T cells (Tc cells), sometimes referred to as $CD8^+$ T cells, are aptly named cells that kill other cells deemed infected or abnormal. This can include cells infected by viruses, bacteria and parasites, or cells displaying arrays of abnormal proteins on their surface, being the case for many cancer cells[43].

Interestingly, *Leishmania* has co-evolved alongside the components of mammal immune systems for many generations and has become expertly equipped for not only avoidance but manipulation of said system to improve the odds of its own survival and aggravate infection.

This is a very complex topic and is highly variable based on *Leishmania* species in question, mammal host in question and even within the same species of host, immune response can vary based on genetics and other factors.

The primary target cells for *Leishmania* are the MØs, but before reaching their desired destination there are challenges *Leishmania* must overcome, namely neutrophils and the complement system. As infection takes places the first response encountered by *Leishmania* is that of the neutrophils, which promptly engage in phagocytosis and subsequent production of ROS and reactive Nitrogen Species (RNS), alongside a variety of cytotoxic enzymes. Aside from this, neutrophils will also produce NETs in an attempt to immobilize and kill the parasites. *Leishmania* have evolved a few ways to avoid neutrophils, such as blocking the formation of the phagolysosomes, or simply inhibiting the formation of ROS/RNS. This can even lead to their prolonged survival inside neutrophils until the neutrophil cell turns apoptotic itself and signals to be phagocyted by a macrophage, granting the parasite silent entry into their target cell without activating any cellular ‘’alarms”[44].

As previously described, the CS is also promptly activated upon infection, by the classical and alternative pathways and a cascade of reactions is triggered. This results in the formation of the MAC leading to cell lysis. Despite this danger, *Leishmania* have evolved ways to avoid the CS in several ways. For example, *L.major* metacyclic promastigotes have been shown to elongate surface LPG which hinders the access of the MAC to the cell surface making them resistant to MAC promoted lysis. Metacyclic promastigotes were also shown to express elevated amounts of protein kinases which phosphorylate C3, C5 and C9 and deactivate the classical and alternative complement pathways. Furthermore, *Leishmania* has been shown to cleave C3 into C3b and further into iC3b, which among other purposes serves as an opsonin, facilitating phagocytosis. Additionally, iC3b further aids the parasite by binding to complement receptor 3 (CR3) on the surface of MØs, which is beneficial to the parasite as this inhibits the production of IL-12, a Th1 type cytokine central to clearing intracellular pathogens.[45] Once the neutrophils are avoided and the CS inactivated, *Leishmania* has many tools at its disposal to home in on MØs. As inflammatory response continues, MØs and Langerhans cells and other dendritic cells are recruited to the site of infection, where they encounter and engulf parasites. *Leishmania* is well suited for intracellular survival inside MØs and while a balance of pro-inflammatory M1 and anti-inflammatory M2 MØs must exist for a proper immune response, *Leishmania* is capable of disrupting this balance, tiling it towards a higher M2 activation and encouraging an anti-inflammatory response, beneficial for its survival.[46]*Leishmania* also induce infected MØs to secrete TGF-β, a cytokine associated with various functions including inhibition of cytotoxic response and macrophage activation. This is a powerful immune suppressing tactic that promotes parasite growth and pathology. Furthermore, similarly to their adaptations in neutrophils, *Leishmania* have evolved mechanisms that allow it to survive in the inhospitable intracellular environment of the macrophage by preventing the fusion of the phagosome with the lysosome, which disables the formation of the phagolysosome, and the subsequent destruction of the parasite. This is achieved via a myriad of complex mechanisms, and allows the parasite, now as intracellular amastigotes, to freely grow and replicate inside the cell.[47]

When parasite phagocytosis, lysis, and subsequent presenting of antigens to T lymphocytes take place, the activation and differentiation of T cells into effector T cells occur. These effector T cells primarily include Th1 $CD4^+$ T cells that produce cytokines such as IFN-γ and TNF-α, which activates infected MØs to kill intracellular pathogens. These Th1 cells activate $CD8^+$ T cells that can directly kill infected host cells which is crucial for the control and elimination of the infection. But yet again, studies show that *L.major* for example, is able to induce early Th2 response, which can directly inhibit Th1 response, and is mainly associated with the elimination of extracellular pathogens, which greatly benefits the parasite and further exacerbates the infection[48].

Thus, while the immune system is able to detect and eliminate parasites upon infection, *Leishmania* continuously shows its mastery over the hosts immune system by disabling central pathways to parasite elimination, modulation of immune response and cell signaling and employment of stealth mechanisms to avoid immune detection altogether.

1.4 The Skin local immune response

The skin is one of the most important components of the innate immune system, the first line of defense against the outside world and an extremely complex organ that houses a network of immune cells,

collectively known as the local skin immune system, which is responsible for protecting the body against various pathogens. Among the key immune cell populations found in the skin are Langerhans cells (LC's), dendritic cells, T cells, B cells, natural killer cells, and macrophages (MØs). Langerhans cells and dendritic cells are specialized antigen-presenting cells (APCs) that can capture and present foreign antigens to other immune cells. T cells and B cells play crucial roles in controlling infections and producing antibodies to neutralize pathogens, while natural killer cells can directly kill infected or cancerous cells. Macrophages are also present in the skin and are responsible for engulfing and digesting foreign particles and cellular debris. Together, these immune cell populations work in a coordinated manner to maintain the integrity and function of the skin and protect the body from external threats.

Langerhans cells are a unique population of tissue-resident cells, sometimes described as tissue-resident macrophages[49,50], found in the epidermis. It is estimated these cells constitute approximately 2% of the total epidermal cell population[49]. Langerhans cells are characterized by the expression of a specific protein, langerin, serving varied functions in the cell[51]. Langerhans cells are hard to classify, sharing commonalities between both MØs and dendritic cells and are an important population of tissue specific cells. Their relevance in leishmaniasis is of note, as studies have shown *Leishmania* can survive within LCs and use these cells to their advantage, as a way migrate to draining lymph nodes[52].

Macrophages are a type of immune cell that play a critical role in the innate immune response and are *Leishmania*'s primary target for infection[53]. MØs are derived from monocytes, which are produced in the bone marrow and circulate in the blood[54]. MØs are found in all tissues of the body and act as part of the first responders to invading pathogens and foreign substances[54]. They not only phagocyte invading microbes but also act as antigen presenting cells, processing and presenting antigens to T cells. MØs are crucial for the development of an adaptive immune response against pathogens[55]. MØs can be activated by different stimuli, such as microbial products or cytokines, and polarized into two main phenotypes:
M1 and M2. M1 MØs are activated by IFN-g and TNF-β, which are cytokines involved in the Th1 response. M1 MØs have pro-inflammatory properties and are central in the clearance of intracellular pathogens, such as bacteria, viruses, and protozoa such as *Leishmania*. They produce reactive oxygen species and nitric oxide (NO) through inducible nitric oxide synthase (iNOS), which are toxic to microorganisms. M2 MØs, on the other hand, are activated by anti-inflammatory signals, such as IL-4 and IL-13, which are produced during the Th2 response and are involved in tissue repair and regeneration. They produce anti-inflammatory cytokines, such as IL-10 and TGF-β. They are involved in the clearance of cellular debris and promotion of tissue healing.[56]

Dermal dendritic cells, unlike LC's which are located mostly in the epidermis, mainly exist in the dermis, in close proximity to blood vessels and other immune cells. They can produce cytokines and other signaling molecules that influence the activation and differentiation of T cells and other immune cells. Dermal dendritic cells are also involved in the maintenance of immune tolerance in the skin, as they can induce regulatory T cells that help to prevent autoimmune reactions.[57]
Macrophages, Langerhans cells and dendritic cells are known as APCs, a group of specialized cells that play a crucial role in the immune system by capturing, processing, and presenting antigens to T cells. The two main types of APCs are dendritic cells and macrophages, although B cells can also act as APCs, and tissue specific APCs such as Langerhans cells in the skin or Kupffer cells in the liver exists and are essential

to the local immune response. When an APC encounters a foreign antigen, such as a protein from a virus, parasite or bacteria, it internalizes the antigen and breaks it down into smaller fragments. These fragments are then displayed on the surface of the APC, bound to molecules called major histocompatibility complex (MHC) molecules. The MHC molecules act as a platform to present the antigen to T cells, which are specialized immune cells that can recognize and respond to specific antigens. Two classes of MHC molecules exist, MHC class I (MHCI) is present in nearly every nucleated cell and is involved in the presentation of peptides derived from intracellular proteins to $CD8^+$ T (Tc) cells. This can lead to a cytotoxic response, where Tc cells identify and destroy abnormal and infected cells by releasing a devastating cocktail of enzymes causing target cell death[58].

Meanwhile MHC class II (MHCII) is found primarily on the surface of APCs such as dendritic cells and MØs, and mainly present antigens to $CD4^+$ cells, which become activated and can stimulate other immune cells, such as B cells and cytotoxic T cells, to mount a targeted immune response against the pathogen. This coordinated immune response is crucial for eliminating the pathogen and preventing further infection. Overall, this is a vital process for the activation of the Th cells and stimulates either a Th1 or Th2 response based on a variety of factors[59].

1.6 Extracellular vesicles

Extracellular vesicles (EVs) are small membrane-bound particles that are released by cells into the extracellular space. They can be classified into different subtypes based on their biogenesis and size, including exosomes, microvesicles, and apoptotic bodies. EVs play important roles in intercellular communication, as they can transfer proteins, lipids, and nucleic acids between cells. They have been implicated in a variety of physiological and pathological processes, including immune modulation, tissue repair, and cancer. Researchers are actively investigating the potential of EVs as diagnostic and therapeutic tools for various diseases.[60]

One of the main factors in classifying EVs is their size. Exosomes range in size from 30-150 nm in diameter. They are formed by the inward budding of the endosomal membrane, resulting in the formation of multivesicular bodies (MVBs). Exosomes are released from cells when MVBs fuse with the plasma membrane. Microvesicles range in size from 100-1000 nm in diameter. They are formed by the outward budding of the plasma membrane, resulting in the formation of a vesicle that is released into the extracellular space. Apoptotic bodies form during the process of programmed cell death (apoptosis). They range in size from 50 nm to 5 mm in diameter and are released when cells undergo apoptosis.[60] Exosomes in particular have been implicated in many types of diseases. Because exosomes can transfer bioactive molecules such as proteins, lipids, and nucleic acids between cells, they play important roles in intercellular communication and disease progression. For example, exosomes released by cancer cells can promote tumor growth, angiogenesis, and metastasis by transferring oncogenic cargo to recipient cells. Exosomes have also been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, as they can transfer misfolded proteins, such as beta-amyloid, between cells and contribute to the spread of pathological protein aggregates. Exosomes are also implicated in the development of one of the leading causes of death across the world, cardiovascular diseases.[61] Of particular interest is their role in infectious diseases. Ranging from viral to bacterial and even parasitic infections, studies have shown that exosomes can play various beneficial roles for a pathogen,

ranging from suppressing immune response to aid disease progression and the transfer pathogen-derived components like proteins or RNA to neighboring cells to increase susceptibility and promote the pathogenesis of the infection.[62]

In leishmaniasis, extracellular vesicles/exosomes (have been associated to immune system modulation as a pathway to increase disease pathology and create favorable environments for infection.[63,64] One such example is the *Leishmania* Major Surface Protease (GP63), a major virulence factor protein has been shown to be contained and transported to MØs via exosomes. GP63 cleaves a variety of proteins inside the cell, essentially modulating immune response and signaling in the cell, promoting an anti-inflammatory response, and paving the way for a facilitated infection by the parasite.[65] Given their importance in disease progression, exosomes are emerging as potential biomarkers and therapeutic targets for a variety of diseases and can be the key to develop effective prophylactics and treatments against leishmaniasis.

1.7 Objective

This study has the goal of evaluating the impact of *L.amazonensis* and *L.guyanensis* extracellular vesicles on a simplified model of human skin. To reach this goal, the following objectives have been set:

1. Isolate *L.amazonensis* and *L.guyanensis* EVs from a culture of parasitic promastigotes.
2. Characterize isolated EVs by dynamic light scattering (DLS) and zeta potential by electrophoretic light scattering(ELS)
3. Confirm incorporation of EVs into THP-1 monocytes by fluorescence microscopy and flow cytometry.
4. Assess THP-1 response to EVs and promastigote exposure using a cell viability assay.
5. Assess HaCat response to EVs and promastigotes via wound healing assay.
6. Direct THP-1 monocyte activation and differentiation into skin-*like* macrophages (sMΦ) .
    6.1. Characterize THP-1 cell activation and differentiation by light microscopy and flow cytometry of immune biomarkers CD80, CD83, CD11c, CD14, CD1a, MHCI, MHCII, and CD64, and the Langerhans cell biomarker langerin.
7. Establish HaCat-THP-1 co-culture as a simplified model of human skin and assess its response to EVs and promastigotes using:
    7.1. Flow cytometry to assess expression of MHCI and MHCII, CD64, langerin, and IL-1β.
    7.2. Light microscopy and electron scanning microscopy to assess morphological changes.
    7.3. Reverse transcriptase-quantitative PCR (RT-qPCR) to quantify genic expression of Toll-like receptors TLR2, TLR4, TLR9, molecular sensor AIM2, inflammasome protein complex NLRP3, and cytokines IL-1β, IL-4, IL-10, IL-12p40, TNF-α, and TGF-β.
    7.4. Quantification of urea production via colorimetric assays.
8. Evaluating effects of EV pre-exposure of THP-1 cells on promastigote infectivity by limiting dilution assay.

2. Materials and methods

2.1 Experimental design

Parasite EVs were isolated using the isolation reagent (*Total exosome isolation*, Invitrogen, RIE) from supernatant of cultures of *L.amazonensis* and *L.guyanensis* promastigotes. Extracted EVs were analyzed by: (**a)** ELS to assess zeta potential, via ZetaSizer, and subsequently by DLS, via NanoSizer, to assess diameter. EVs were further used in cellular assays (**b**),(**c**),(**d**), and (**e**). THP-1 incorporation of EVs assessed by fluorescence microscopy and flow cytometry (**b.1**). Effect of EVs on THP-1 was assessed by viability assay (**b.2)**. THP-1 cells subjected to priming for sMΦ differentiation were characterized by flow cytometry to immune markers CD80, CD83, CD11c, CD1a, CD14, MHCI, MHCII, CD64, and langerin previously to any exposure to EVs (**b.3**). HaCat response upon exposure to EVs was tested by wound healing assay (**c**). Once HaCat-THP-1 co-culture is established, EV exposure follows and the co-culture is characterized by spectrophotometry to Urea production, RT-qPCR to evaluate genic expression of innate immune receptors Toll-like receptors TLR2, TLR4, TLR9, NOD-like receptors NOD1 and NOD2, molecular sensor AIM2, inflammasome protein complex NLRP3. Also, cytokines IL-1B, IL-4, IL-10, IL-12p40, TNF-α, and TGF-β at 24h and 48h timepoints of co-culture stimulation were assessed by RT-qPCR, and flow cytometry of MHCI, II, CD64, langerin and IL-1b. Co-cultures were morphologically characterized by SEM and light microscopy, as well as fluorescence microscopy (**d**). To evaluate EV impact on promastigote infectivity, THP-1 pre-exposed to EVs is subsequently exposed to live virulent promastigotes and a limiting dilution assay is performed to assess parasite load of THP-1 cells (**e**).

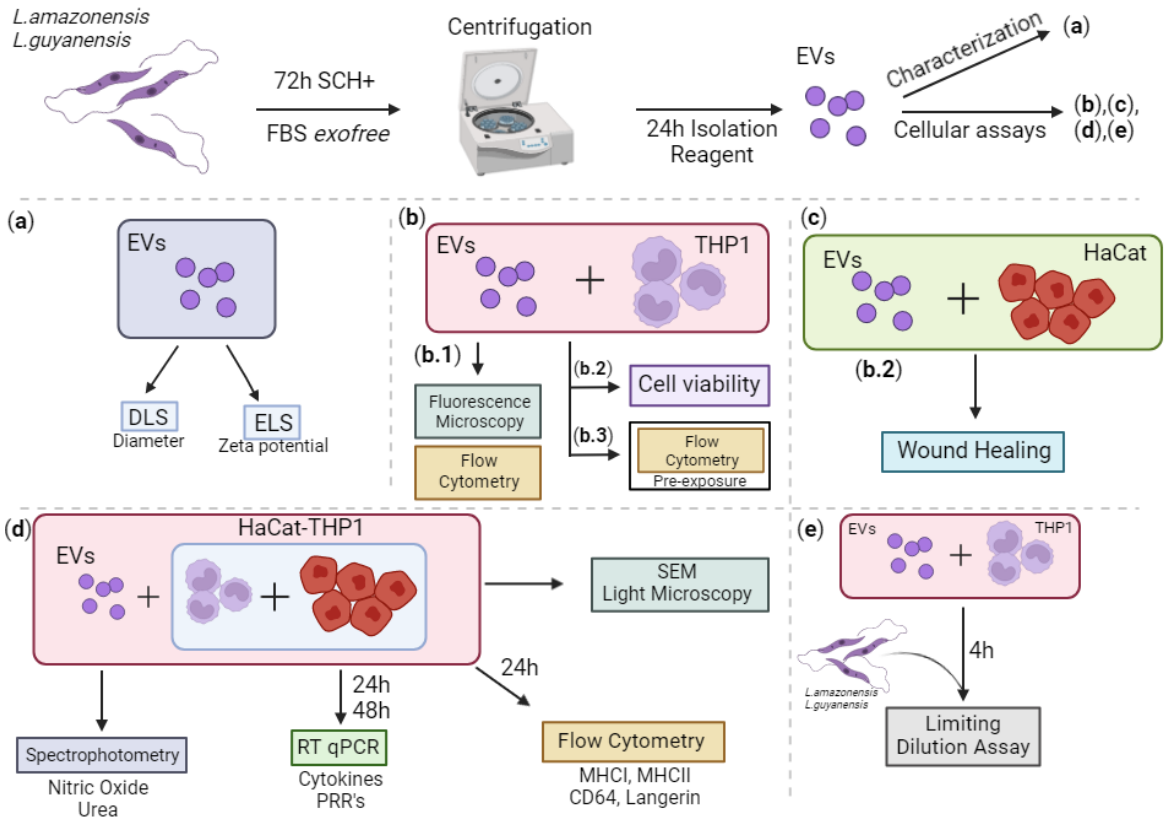

Figure 3. Experimental design flowchart. Description of this study in steps. At the top EV isolation is described, following by characterization by DLS and ELS (**a**). THP-1 cells are exposed to EVs (**b**) and their incorporation is ascertained by fluorescence microscopy and flow cytometry (**b.1**). Cell viability post EV exposure was tested by viability assay (**b.2**) and THP-1 cells were characterized previous to EV exposure by flow cytometry (**b.3).** HaCat cell response to EV exposure was assessed by wound healing assay (**c**). HaCat-THP-1 co-culture was exposed to EVs and analyzed by spectrophotometry for Urea production, RT-qPCR, Flow Cytometry and imaged via SEM and optical microscopy. Figure created in BioRender at https://www.biorender.com.

2.2 Parasites and cell cultures

2.2.1 *Leishmania promastigotes*

For this study, *L.amazonensis* (MHOM/BR/1973/M2269) isolated from a patient with diffuse CL in Brazil, and *L. guyanensis (MHOM/BR/2001/M19663)* also isolated from a patient with diffuse CL in Brazil, were used to established axenic promastigotes culture. Promastigotes were kept in *Schneider's Drosophila* (Biowest®, SCH) growth medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich®). Cultures were kept at 27 ºC, and when necessary centrifuged at 1800 x g for 10 minutes and transferred to fresh growth medium described above for maintenance.

2.2.2 THP-1 cell line

THP-1 TIB-202 (ATCC, USA) cells are a monocytic cell line isolated from an acute monocytic leukemia patient and used extensively in immunologic research. These cells present round and smooth morphology cells, growing in suspension. Cells were cultured in RPMI 1640 (Biowest®) growth medium supplemented with 10% (v/v) inactivated FBS (BioWest®), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich®), pH 7.2 and kept at 37ºC with a humid atmosphere consisting of 5% $CO_{2.}$. When necessary for assays or maintenance, cells were centrifuged at 300 x g for 10 min and transferred to fresh growth medium described above.

2.2.3 HaCat cell line

HaCat human immortalized keratinocytes are extensively used in research as a simplified model of skin. HaCat are adherent cells with a flat, irregular appearance and form a smooth epithelium on the bottom of the culture surface. Cells were cultured in RPMI 1640 (Biowest®) growth medium supplemented with 10% inactivated FBS (BioWest®), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich®), pH 7.2 and kept at 37ºC with a humid atmosphere consisting of 5% $CO_{2.}$. When necessary for assays or maintenance, growth medium was removed, 3-10 mL of trypsin-EDTA (1x) (Gibco) was added depending on T-flask size, and cells were incubated for 5-10 min at 37 ºC. Once cells were no longer adherent, fresh RPMI medium was added to inactivate the trypsin and cells centrifuged at 300 x g for 10min and transferred to fresh growth medium described above.

2.2.4 THP-1 cell priming

As THP-1 is a monocytic cell line, differentiation into macrophage-like phenotype was induced by priming cells with HaCaT conditioned medium. This contact with HaCaT secretome can trigger THP-1

differentiation into sMΦ Therefore, growth medium from 90-100% confluent HaCat culture was collected and centrifuged at 10,000 x g for 30minutes to clean cells and cellular debris. Following this, THP-1 cells were transferred to a mix of 50% fresh supplemented RPMI medium as described in the above sections, and 50% (v/v) HaCat medium and kept at 37ºC with a humid atmosphere consisting of 5% CO_2. As macrophagic differentiation hallmark is the ability to cells to adhere THP-1 HaCaT-primed cells were cultured horizontally to promote adherence This process was done for 72 h to allow sMΦ to differentiated. After this differentiation period, cells phenotype as characterized for immune markers CD80, CD83, CD11c, CD1a, CD14, MHCI, MHCII, CD64, and langerin by flow cytometry and also used to establish the co-cultures with HaCaT.

2.2.4 Experimental conditions

Various experimental conditions were setup for the assays performed in this study,

(i) Negative control consisting of a cell culture or co-culture not exposed or stimulated (resting cells).

(ii) Positive inflammation control refers to cells stimulated with 100µg/mL of LPS.

(iii) Antigen exposure, where cells were exposed to total antigen extract of both *L.amazonensis* and *L.guyanensis* at a concentration of 40µg/mL.

(iv) Isolated EVs from *L.amazonensis* and *L.guyanensis,* applied to cells at 25µg/mL or 100µg/mL (EVs total protein). In cases where it is not specified, EVs were applied at concentrations proportional to their production in live *Leishmania* cultures.

(v) Parasite exposure, where cells were exposed to virulent *L.amazonensis* and *L.guyanensis* promastigotes at a 3:1 ratio to THP-1 cells. In cases where only HaCat cells were used, 6% of the initial number of cells was added as promastigotes.

(vi) Primed cells, which refers to THP-1 cells that underwent priming to induce sMΦ phenotype, opposed to their non primed counterparts.

Virulent live promastigotes were used to mimic normal infection by *Leishmania*. Antigen extract was used to gauge immunologic response without virulence. Cells were exposed to promastigotes, total antigen extract, and EVs for 24h and 48h. Co-culture supernatants were stored at -80ºC for future colorimetric assays. Cells were divided into two groups, one to which 350 µL of RLT (RNeasy Lysis Buffer; RNeasy Mini Kit, Qiagen, Germany) was added for posterior RNA extraction and cDNA synthesis, and another group was detached using PBS-EDTA and stained for flow cytometry analysis.

2.3 EV isolation from *L.amazonensis* and *L.guyanensis*

As mentioned previously, exosomes production is ubiquitous among cells. Exosomes are present in nearly any environment cells naturally exist in, and commonly available FBS is no exception. This becomes an issue when studying the effects of EVs and Exosomes on a certain environment, as any added VEs will be indistinguishable from the ones already present in the medium. As such, exosome depleted FBS (Thermo Fisher) was used in the assays in this study and is referred to as exofree FBS from here onwards.

EVs isolation from L. amazonensis and L. guyanensis promastigotes was performed as described in Weber et al[66]. Briefly, promastigotes were centrifuged at 1800 x g for 10minutes and cultured in exofree FBS

growth medium for 72 hours. Supernatant was collected and the sedimented promastigotes were cultured once again. Supernatants are then centrifuged for 30min at 2000 x g to ensure no promastigotes remain. Isolation reagent, commercially available from Invitrogen, was added at a 1:2 ratio to supernatants and incubated at 4ºC for 24 hours. Following incubation, the solution is centrifuged at 10.000 x g for 1 hour, supernatant discarded and pellet containing isolated EVs resuspended in phosphate buffered saline 1x (PBS). Isolated EVs are then used immediately or stored at -80ªC to ensure protein activity preservation. A negative extraction control was performed with sterile RPMI medium supplemented with 10% (v/v) exofree FBS. EV suspensions were quantified by NanoDrop 1000® (Thermo Scientific) for total protein.

2.4 Nanometric characterization of EVs

Isolated *Leishmania* EVs were characterized as described in Weber et al.[66].Briefly, the diameter of purified EVs was analyzed by dynamic light scattering (DLS) in Malvern ZetaSizer equipment (Nano-S, Malvern Instruments, UK), at 25 °C and with a 90º detection angle. EV zeta potential (ζ), which is related to membrane charge and is an important indicator of the stability of colloidal dispersion was evaluated using electrophoretic light scattering (ELS) at pH 7.5 in a Malvern ZetaSizer equipment (Nano-Z, Malvern Instruments, UK). To analyze EVs generation in promastigotes, parasites were cultured in SCH exofree-FBS supplemented medium and left to adhere to round coverslips and were fixed with PBS 4% paraformaldehyde (Merck, USA) for 30 min at 4 °C. Afterwards, both parasites were washed and dehydrated by sequential addition of 30 %, 50 %, 70 %, 80 %, and 90 % ethanol for 5 min each. Coverslips were immersed in 100 % ethanol and then treated with hexamethyldisilane solvent (Sigma-Aldrich, USA), coated with gold-palladium (Electron Microscopy), and mounted on stubs to be observed under an ultra-high resolution scanning electron microscope (Hitachi SU8010, Hitachi High-Technologies Corporation, Japan).

2.5 Total antigen extract from *L.amazonensis* and *L.guyanensis*

Logarithmic phase promastigotes were centrifuged at 1800 x g for 10 minutes, and resulting pellet was resuspended in 200 µL PBS 1× and supernatant discarded. Resuspended pellet was then frozen, thawed and vigorously homogenized three times to ensure cell lysis. Extracts were quantified by NanoDrop 1000® (Thermo Scientific) for total protein and stored at -80ºC.

2.6 THP-1 EV cellular interaction

To assess EVs interaction with THP-1 cells, Leishmania Evs were isolated and stained using DilC18 (1,1'-Dioctadecyl-3,3,3',3'- Tetramethylindotricarbocyanine Iodide) (Thermo Fisher®), a non-specific cationic lipophilic staining agent, which stains lipidic membranes and increases their absorption at 529nm, and emission at 565nm. The flow cytometer is a powerful tool that consists in analysis cells with lasers, being able to discern their size and complexity, alongside more complex characterization based on whichever fluorescent markers are used on cells, by analyzing how cells disperse visible light[67].

As described by Weber et al.[66], EVs were incubated with DilC18 for 2 h at 26 °C and cleaned by passing through columns (Exosome Spin Columns, MW3000, Invitrogen, USA) to remove the unincorporated dye. In parallel, 1× PBS was incubated with DilC18 and passed through the column to be used as a negative staining control and THP-1 were directly incubated with the dye to be used as a positive control. Stained Evs and the negative control were incubated with cells for 24 h at 37 °C in a humid atmosphere with 5 % CO_2. Cells were then analyzed by multiparametric flow cytometry (CytoFlex, Beckman Coulter, Brea, CA, USA).

For microscopy examination, cells were fixed with 2 % paraformaldehyde for 30 min at 4 °C and THP-1 nuclei were stained with DAPI (FluroshieldTM with DAPI, Sigma, USA). The slides were observed under a fluorescence microscope (Eclipse 80i Intensilight C-HGFI with NIS-Elements software, Nikon, Japan) and images were acquired.

2.7 THP-1 viability assay

To ensure that thp1 were viable after parasite infection and EV-stimulation, viable, pre-apoptotic and apoptotic cells were assessed by multiparametric flow cytometry analysis using a commercial kit TACSTM Annexin V FITC (R&D Systems, USA), following manufacturer's instructions. Non stimulated THP-1 cells, THP-1 cells exposed to total antigen extract from both *Leishmania* species, THP-1 cells exposed to 25µg/mL and 100µg/mL of isolated EVs from both *Leishmania* species, and THP-1 cells exposed to virulent promastigotes from both *Leishmania* species were incubated for 24 hours. In parallel, cells treated with the cytotoxic compound DMSO (100%), were also analyzed as a negative viability control.

Cells were washed with 200 mL of cold 1× PBS (300 ×g, 10 min, 4ºC) and incubated with the commercial kit TACSTM Annexin V FITC (R&D Systems, USA), according to the manufacturer's instructions. Before acquisition in a flow cytometry analyzer, cells were treated with 1 µL of propidium iodide (PI, R&D Systems). FL1-H (Annexin V FITC) vs FL5-H (PI) gate on untreated-moDC was used to delimit annexin V FITC-/PI- population (viable cells), annexin V FITC+/PI- (pre-apoptotic cells) and annexin V FITC+ or -/PI+ cells (apoptotic cells).

2.8 Wound healing assay

To assess the effect Evs and promastigotes have on the proliferation of skin keratinocytes, HaCat cells were used to perform a wound healing assay. The wound healing assay consists in creating a wound in an epithelial surface and documenting cell response. Its widely used in cancer research where a proliferation inhibitor is used so that cell migration can be measured[68]. In this version no proliferation inhibitor is used so that differences in proliferation speed can be measured.

24 well plates were seeded with $5x10^5$ HaCat cells per well. Plate was incubated for 24 hours at 37ºC humid atmosphere with 5% CO_2 until reaching approximately 95% confluence. Following this, an injury was induced by dragging a small micropipette tip across the surface of the well and gently washed with PBS 1x to remove non-adherent cells. HaCat cells were assessed in exofree FBS supplemented RPMI medium and and wells

divided into experimental conditions, i) viable and virulent *L.amazonensis* promastigotes; ii) isolated Evs; ii)LPS as positive inflammation control and iii) negative control. Plates were incubated at 37ºC humid atmosphere with 5% CO_2 and photographed via optical microscope (Olympus, CKX41), and photographs taken with an Olympus CS30 camera) approximately every 5 hours until negative control condition fully healed the wound.

2.9 Characterization of priming effect on THP-1

THP-1 cells that underwent priming protocol, to promote skin macrophage-like differentiation (sMΦ) were characterized morphologically by light microscopy and immunologically by flow cytometry and compared to their not primed counterparts.

2.9.1 Light microscopy

THP-1 cell cultures were divided equally, one submitted to the priming protocol, as described in section 2.3 while the other remained in normal culture conditions (not-primed). Both were then cultured horizontally in 25mL flasks and imaged via optical microscope (Olympus, CKX41), and photographs taken with an Olympus CS30 camera at x10, x20 and x40 magnification.

2.9.2 Flow Cytometry primed THP-1 cells

Flow cytometry was employed to characterize the effect of priming on the expression various immune related molecules in THP-1 cells that underwent priming and cells that did not undergo priming. Unstained, non-primed THP-1 cells were used as negative control.

Cells are centrifuged at 200 x g for 5 minutes and resuspended in PBS 1x three times to wash them. After the final wash, cells are resuspended in PBS 1x 2% bovine serum albumin (w/v) or PBS-Tween 1x 2% bovine serum albumin (w/v) depending on whether the biomarker is extra or intracellular respectively, and designated monoclonal antibodies are added to each solution. The cells were stained for individual biomarkers for CD83, CD80, CD11c, CD14 and CD1a, all extracellular, to avoid superposition of fluorophores. The remaining four biomarker stains were grouped in two, MHCI+MHCII, both extracellular, and CD64+Langerin, both intracellular. Cells are then incubated in the absence of light for 30min at 4ºC and washed with PBS 1x Table 1 describes the biomarkers and concentration used to analyze primed s and non-primed THP-1 cells. All antibodies acquired from Thermo Fisher. Expression of all the described biomarkers was acquired by flow cytometry (CytoFlex, Beckman Coulter).

Table 1. Monoclonal antibodies and their respective fluorophore. Emission maximums and dilutions used for THP-1 cell flow cytometry analysis also indicated.

Biomarker	CD80	CD83	CD11c	CD1a	CD14	CD64	Langerin	MHCI	MHCII
Fluorophore	PE	PE	APC	Alexa Fluor 647	PE	FITC	APC	APC	FITC
Emission Maximum (nm)	574	574	660	665	574	525	660	660	525

Dilution (mg/mL)	2	2	2	1.5	2	1.5	1.5	1.5	1.5

2.10 HaCat-THP-1 co-culture establishment and characterization

2.10.1 Establishing the co-culture

24 well plates were seeded with $2x10^5$ HaCat cells and incubated for 48 hours at 37ºC humid atmosphere with 5% CO_2 until reaching approximately 85% confluence. Growth medium was removed, and cells were gently washed with PBS. Following this, primed THP-1 cells were added to each well at a 2% proportion (approximately $2x10^4$ cells per well), to mimic the natural percentage of macrophage in the human dermis. Cells were let to incubate together for 24 hours to establish cellular connections and establishing the HaCat-THP-1 co-culture.

Once 24 hours elapse, growth medium is very gently aspirated and 10 % exofree FBS (v/v) supplemented RPMI growth medium added to perform the assays, ensuring that Evs from FBS do not interfere with assays. Experimental conditions, described in 2.2.5 were then added to each well. Briefly, 100µg/mL LPS as positive inflammation control, 40µg/mL of *L.amazonensis* or *L.guyanensis* antigen extract, 100µL/mL of isolated Evs of each species of parasite, and live virulent promastigotes at a 1:3 proportion to THP-1 cells. Experimental conditions were maintained for 24 h and 48 h and several parameters were assessed.

2.10.2 Co-culture flow cytometry

Cells are firstly detached from the well using a PBS-EDTA solution, to avoid potential destruction of surface markers by trypsin action. . 200 µL of a solution of PBS 1x with 1mM EDTA is added to the cells, and incubation for 30minutes at 37ºc takes place. Cells were gently washed with PBS to fully detach them, and promptly centrifuged at 300 x g for 10 minutes and resuspended in PBS 1x, this process is repeated two times. Monoclonal antibodies (table 2) were added to cells. MHCI and MHCII were combined in PBS 1x 2% bovine serum albumin (w/v), CD64 and langerin were combined in PBS-Tween 1x 2% bovine serum albumin (w/v) solutions, as well as IL-1β and incubated at 4ºC for 30 minutes. Cells were then washed to clean unbound antibody and assed by flow cytometry (CytoFlex, Beckman Coulter).

Table 2. Monoclonal antibodies and their respective fluorophore. Emission maximums and dilution used on co-culture flow cytometry analysis are also indicated.

Biomarker	**IL-1β**	**CD64**	**Langerin**	**MHCI**	**MHCII**
Fluorophore	APC	PE	APC	APC	FITC
Emission Maximum (nm)	660	574	660	660	525
Dilution (mg/mL)	1	2	1.5	1.5	1.5

2.10.3 Co-culture characterization by microscopy

To follow morphology of the HaCat-THP-1 co-culture, frequent observations were made via optical microscope(Olympus, CKX41). And images of experimental conditions were acquired via Olympus CS30 camera. To observe in more detail the morphology and topography of cells in co-culture, samples were prepared for SEM imaging. Therefore, glass coverslips were coated in poly-d-lysine to help HaCat cells adhere to their surface and slotted into well in 24 well plates. Following this, standard co-culture establishment procedure was followed. Once co-culture stabilizes, conditions are added and after 24 hours on incubation at 37ºC, in a humid atmosphere with 5% CO_2, supernatant was discarded, and cells were gently washed with PBS. Cells were fixated with 2% (w/v) paraformaldehyde in PBS 1x by adding 150µL of this solution to each well and incubating at 4ºC for 30 minutes. Wells were washed with PBS and coverslips are fixated to microscopy slides. Afterwards, cells were washed and dehydrated by sequential addition of 30 %, 50 %, 70 %, 80 %, and 90 % ethanol for 5 min each. Coverslips were immersed in 100 % ethanol and then treated with hexamethyldisilane solvent (Sigma-Aldrich, USA), coated with gold-palladium (Electron Microscopy), and mounted on stubs to be observed under an ultra-high resolution scanning electron microscope (Hitachi SU8010, Hitachi High-Technologies Corporation, Japan).

To analyze, the localization and distribution of main cell makers such as MHCI and MHCII molecules, as well as CD64 and langerin, HaCat-THP-1 co-culture were observed under fluorescence microscope. Briefly, HaCat-THP-1 co-cultures were established in poly-d-lysine coated glass coverslips and after the experimental conditions applied. Cells were then washed with PBS 1x and fixed with 2% (w/v) paraformaldehyde in PBS 1x. The antibodies used to stain cells for flow cytometry for MHCI, MHCII and CD64 and langerin (described in 2.9.2) were used. Cell nuclei were stained with DAPI (FluroshieldTM with DAPI, Sigma, USA) and slides were observed under a fluorescence microscope (Eclipse 80i Intensilight C-HGFI with NIS-Elements software, Nikon, Japan) and images were acquired.

2.10.4 Co-culture RT-qPCR analysis

Gene expression of innate immune receptors TLR2, TLR4, TLR9, inflammasomes AIM2 and NLRP3 as well as cytokines IL-1β, IL-10, IL-12p40, TNF-α and TGF-β, was assessed in all experimental conditions in the co-culture system established. This process consisted in 3 steps, RNA extraction, complementary DNA synthesis (cDNA) , and genic expression analysis by RT-qPCR.

Total RNA was extracted from co-cultured cells using an RNA extraction kit (NZY Total RNA Isolation Kit, NzyTech) and following manufacturer recommended procedure. At the end of the extraction process, RNA is eluted out of the column using 60 µL of RNAse free H2O.

Following RNA extraction, cDNA synthesis was performed using a kit (NZY First-Strand cDNA Syntesis Kit, NzyTech), and following manufacturer recommended procedure. Resulting cDNA samples were stored at -20ºC for future assays.

Housekeeping genes are genes that are constitutively expressed in cells[69], and are not affected by experimental conditions, acting as a sort of gene expression control gene. GAPDH (*Glyceraldehyde-3-Phosphate Dehydrogenase*) was chosen as a housekeeping gene, and expression of other genes analyzed

was normalized to it. Primers used are described in table 3 and primer efficiency obtained was between 90 and 110% for all primers used.

Table 3. Primers used for RT-qPCR. Primer sequences, along with fragment size, annealing temperature and corresponding gene are described.

Genes	Sequences Forward Reverse	Amplification fragment size	Annealing temperature	Reference
TLR2	5'- TCTCCCATTTCCGTCTTTTT -3' 3'- GGTCTTGGTGTTCATTATCTTC -5'	125bp	51ºC	Xuming et al. (2007)[70]
TLR4	5'- GAAGCTGGTGGCTGTGGA -3' 3'- GATGTAGAACCCGCAAG -5'	212bp	58ºC	Xuming et al. (2007)[70]
TLR9	5'- CGCCAACGCCCTCAAGACA -3' 3'- GGCGCTTACATCTAGTATTTGC -5'	79bp	59ºC	Xuming et al. (2007)[70]
IL-1β	5'-AAGCTGATGGCCCTAAACAG-3' 3'- AGGTGCATCGTGCACATAAG-5'	281bp	55ºC	Maedler et al. (2002)[71]
IL-10	5'- CTTTAAGGGTTACCTGGGTTG -3' 3'- CCTTGATGTCTGGGTCTTGGT -5'	101bp	57ºC	Mariani et al. (2020)[72]
IL-12p40	5'- CCAAGAACTTGCAGCTGAAG -3' 3'- TGGGTCTATTCCGTTGTGTC -5'	355bp	57ºC	Mariotti et al. (2002)[73]
TNF-α	5'- AGATGATCTGACTGCCTGGG -3' 3'- CTGCTGCACTTTGGAGTGAT -5'	93bp	57ºC	Guillemot et al. (2022)[74]
TGF-β	5'- TGCGCTTGCAGAGATTAAAA -3' 3'- GGTCCTTTCATCTGCTACC -5'	136bp	60ºC	Charania et al. (2013)[75]
AIM2	5'- CACCAAAAGTCTCTCCTCATGTT -3' 3'- AAACCCTTCTCTGATAGATTCCTG -5'	77bp	57ºC	Janneh et al. (2022)[76]
NLRP3	5'- CACCTGTTGTGCAATCTGAAG -3' 3'- GCAAGATCCTGACAACATGC -5'	74bp	55ºC	Chan et al. (2020)[77]
GAPDH	5'- AGCCACATCGCTCAGACAC -3' 3'- CCTAAACCAGCATAACCCG -5'	151bp	51ºC	Arenas-Hernandez et al. (2013)[78]

PCR mix consists of 10µL SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories, EUA), containing SYBR® Green I, ROX normalization dyes, Sso7d fusion polymerase, dNTPs,MgCl2, 0,15µL of forward and reverse primer, 2µL of sample cDNA and 7,7 µL of ultra-pure H2O. PCR consisted in 39 denaturing cycles (95ºC for 5 minutes, 95ºC for 30s) and annealing (melting temperature varies per primer, 30s) concluding with an extension step (50ºc for 15min). The BioRad (CFX Connect BioRad, USA) thermocycler was used. Relative quantification of gene expression was obtained by the ΔΔCt method[79].

2.11 Urea quantification

As urea is metabolite known to perform several homeostatic functions in the skin. However, it is also a key metabolite in favoring *Leishmania* survival in infected macrophages. Therefore, urea quantification was performed in co-culture supernatants. Supernatants were collected at 24-hour and 48-hour timepoints in co-culture system. Urea production was analyzed via Urea Assay Kit (BioChain®). The reagent present in the kit reacts with urea an forms a complex that can be quantified by spectroscopy, taking readings at 430nm.
96 plate wells were used in this assay, following manufacturer instructions, work reagent was prepared and 200µL distributed in each well. Following this, 50µL of each sample, H2O, and standard (5mg/dL urea) solution were distributed. Plate was incubated for 50min at ambient temperature and absorbance at 430nm was read using a spectrophotometer (TRIADTM 1065, DYNEX Technologies, USA). The following equation was used to calculate urea concentration in mg/dL:

$$[\text{Urea}] = (Abs_{sample} - Abs_{blank)} / (Abs_{standard} - Abs_{blank}) \times d \times [STD] \text{ (mg/dL)}$$

Where [STD] equals 5(mg/dL) as per manufacturer recommendation, and d is the dilution factor of the samples, 1 in this case.

2.12 Limiting dilution assay

To analyze the impact of *Leishmania* EVs on the infectivity of virulent promastigotes, macrophagic THP-1 cells, that are easily infected by promastigotes were incubated with *L.amazonensis* and *L.guyanensis* EVs for 24 h, followed by infection by virulent promastigotes for 4 h. To assess the viability of the intracellular parasites, an adapted limiting dilution assay (LDA), was performed. This technique, where successive dilutions are applied, create an exponential reduction of parasite per solution, which enables the determination of the frequency of a certain element in a larger population[80]. Therefore, higher dilution titers are related to higher infection rates in the THP-1 macrophages.

THP-1 cells are seeded in 24 well plates and exposed to *L. amazonensis* and *L.guyanensis* EVs, at 25µg/mL and 100µg/mL concentrations and incubated at 37ºC for 24 hours. Control conditions consist of resting (non-stimulated cells)and a positive inflammation control with THP-1 cells exposed to LPS for 24 hours. THP-1 cells were also exposed to total antigen extract of each promastigote species for 24 hours. After this period, culture medium was removed, cells were washed with PBS 1x and live promastigotes were added to each well, , in a 1:3 proportion to the initial number of THP-1 cells, to all experimental conditions and incubated at 37ºC for 4 hours. Following this second incubation, cells are removed and centrifuged gently at 150 x g for 5 minutes, to removed non-intracellular parasites. Supernatants were discarded and cells were washed with PBS 1x to ensure no free promastigotes remain. THP-1 pelleted cells were resuspended in 200 µL of promastigote growth medium supplemented SCH. 96 well plates are used, to perform successive 1:5 dilutions take place from column 3 to column 11 (Figure 5), ranging from 1:5 to $1:5^{11}$ dilutions relative to the initial solution in a final volume of 200 µL per well. Outside wells in the A row, H row, column 1 and column 2 are filled with PBS to combat evaporation and prevent cell suspension from drying out. The plates were sealed and incubated at 25ºC for 24 hours, which causes the intracellular amastigotes to change form back to

promastigotes and exit the THP-1 macrophages. Presence of promastigotes was evaluated by optical microscopy, marking as positive dilution when motile promastigotes were detected until 3 successive negative wells (with no promastigotes) were observed.

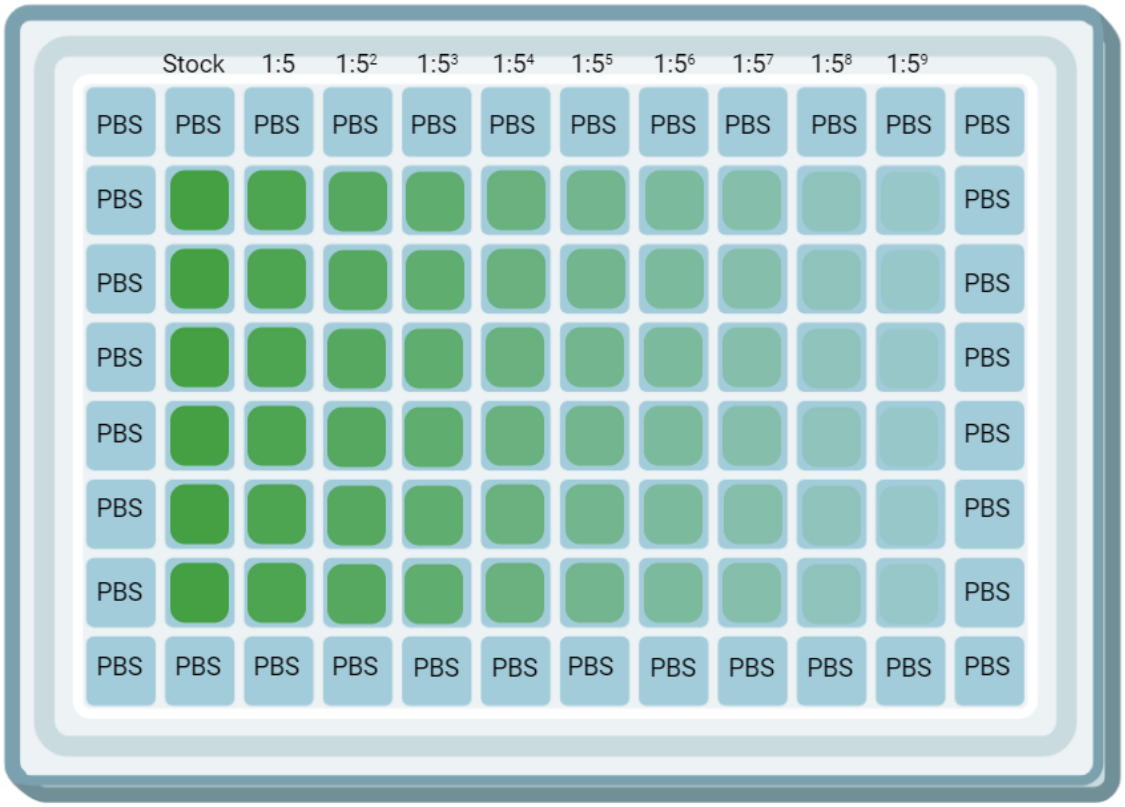

Figure 4. Visual representation of LDA assay 96 well plate. Outside wells are filled with PBS. Stock cell suspension is applied to the second column and successive dilutions at 1:5 ratio take place until column 11. Figure made in BioRender at https://www.biorender.com.

2.14 Statistical Analysis

In assays where at least 3 independent experiment data points were available, statistical analysis was conducted. The non-parametric t-student test was used when comparing zeta potentials, between the control and each measurement, the non-parametric Wilcoxon test was used for THP-1 viability assay analysis, 1-way ANOVA tests were used for urea quantification assay analysis. The GraphPad Prism software (San Diego, CA, USA) was used, and p values ≤ 0.05 were indicative of significant differences.

3. Results and discussion

3.1 Extracellular vesicles shed by *L. amazonensis* and *L. guyanensis* promastigotes are compatible with exosomes and microvesicles

To observe the natural secretion of EVs from promastigotes, SEM analysis was performed in axenic promastigotes of *L. amazonensis* and *L.guyanensis.* Topographic observation of promastigotes demonstrates EVs budding throughout the body of the parasite (Figures -----). EVs appear mostly spherical, with a smooth membrane (white arrows) and exhibited diameters ranging between 50 nm and 150 nm, which is consistent with the size described for exosomes and microvesicles.

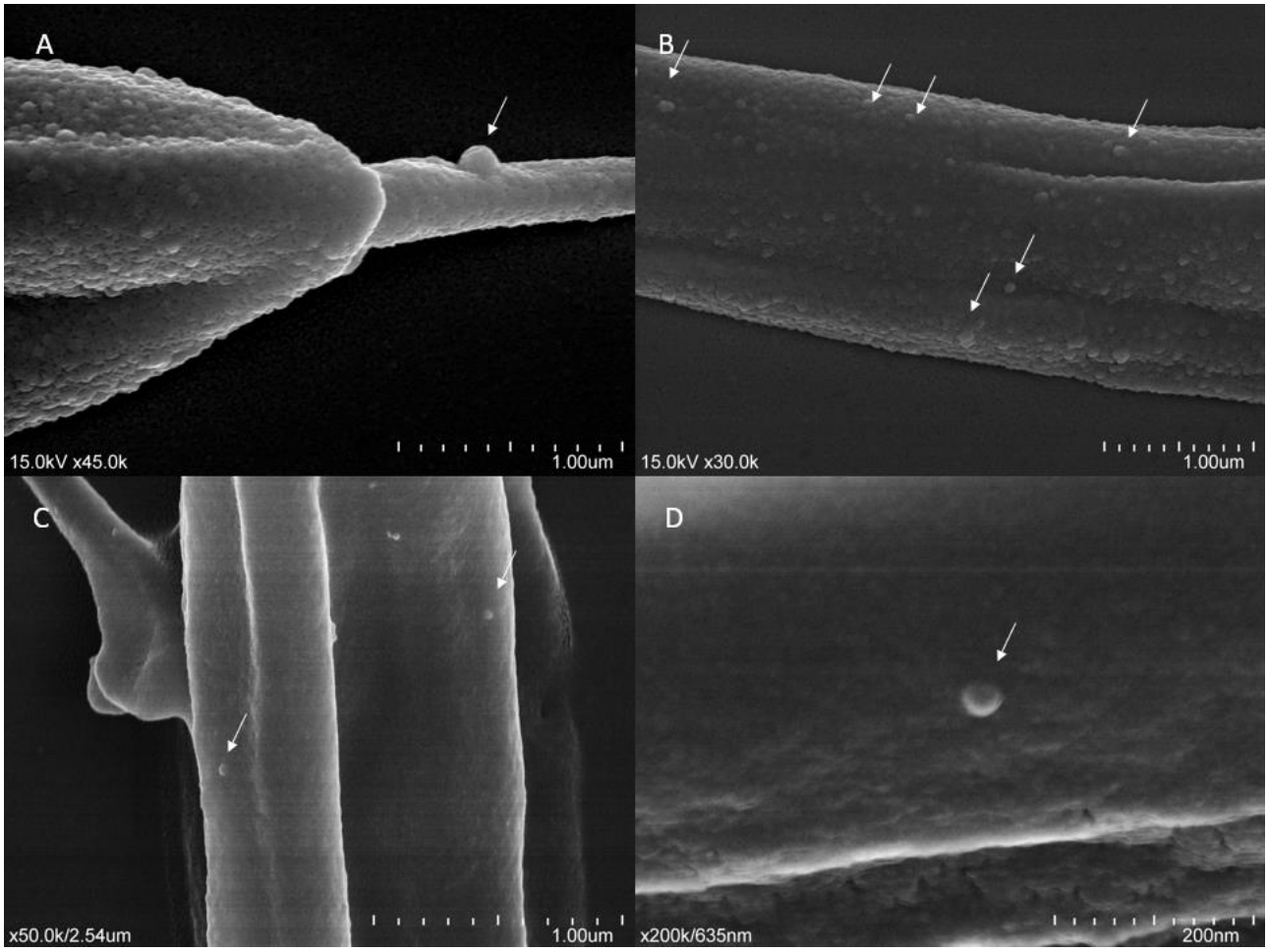

Figure 5. Topography of promastigotes cell membrane Scanning electron microscopy (SEM) of cultured *L.guyanensis* (A, B) and *L.amazonensis* (C, D) promastigotes exhibiting protrusions (white arrows) compatible with EVs biogenesis.

The analysis of isolated EVs by DLS confirmed the presence of nanoparticles (vesicles) with a variable size dispersion compatible with the presence of exosomes (30 – 100 nm) and microvesicles (100 – 2000 nm). EVs profile observed was similar between the two species of Leishmania (Figure 5). DLS analysis of *L.amazoneniss* and *L.guyanensis* EV samples demonstrated the presence of vesicles with a diameter compatible with exosomes (Peak 2 *L.amazonensis* EVs – 48.05 ± 35.87 nm and *L.guyanensis* - 47.135 ± 22.41 nm) and also the presence of vesicles with a diameter compatible with microvesicles (Peak 3

L.amazonensis EVs – 335.27 ± 61.42 and *L.guyanensis* EVs – 244.03 ± 125.69 nm) (Table 4). A common peak of 3.12 to 5.35 nm is described in all samples. This size is below the normal attributed dimensions for exosomes or microvesicles and are probably referring to protein complexes existing in the sample.

***Table 4*. Average diameter, in nanometers, of the particles detected in the different samples of EV isolates.** Control condition presents mainly 2 peaks while EV samples present 3 peaks. N=2 for control, N=1 for samples of EVs.

Sample	Peak 1 (d.nm)	Peak 2 (d.nm)	Peak 3 (d.nm)
Control	5.51 ±0.4	0.735 ±1.44	196.8 ±61.35
***L.amazonensis* EVs**	3.12 ±6.1	48.05 ±35.87	335.27 ±61.42
***L.guyanensis* EVs**	5.35 ±0.04	47.135 ±22.41	244.3 ±125.69

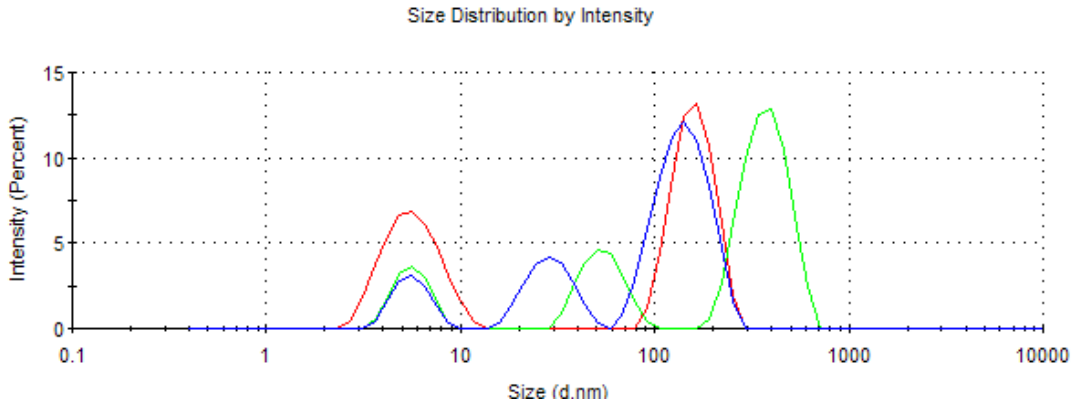

Figure 6. Visual representation of diameter distribution of particles in samples of EV isolates. Redline represents the negative extraction control; blue line represents the sample of *L.guyanensis* EVs and green line represents the sample of *L.amazonensis* EVs. X axis describes the diameter of the particles in nanometers. Y axis represents the intensity of the signal.

The analysis of the zeta potential (ζ) is defined as the voltage at the edge of the diffuse layer where it meets the surrounding liquid and therefore, indicative of the presence of in-tact lipid membranes in suspension revealed important differences between the EVs extracted from promastigotes and the negative control (sterile medium) (Figure 6). The zeta potential of EVs isolated from L. amazonensis and L. guyanensis showed negative values, with an average of 11.65 ± 0.49mV and 13.47 ± 0.46mV, respectively (Figure 7). When compared to control, there were statistically significant differences, pointing out to the successful isolation of intact *Leishmania*-derived EVs (*L. amazonensis* EVs $p < 0.0001$ and *L. guyanensis* EVs $p < 0.0001$). Interestingly, the zeta potential of a nanoparticle can reflect its tendency to interact and permeate other cell membranes, since most cellular membranes are negatively charged. However, nanoparticles with a zeta potential between −10 and +10 mV are considered approximately neutral, suggesting that *Leishmania* EVs can interact with other cell membranes in a non-disruptive way. Also, the range of zeta potential obtained for *Leishmania* EVs is indicative of their ability to floculate, generating EVs aggregates that may promote their interaction with cell membranes.

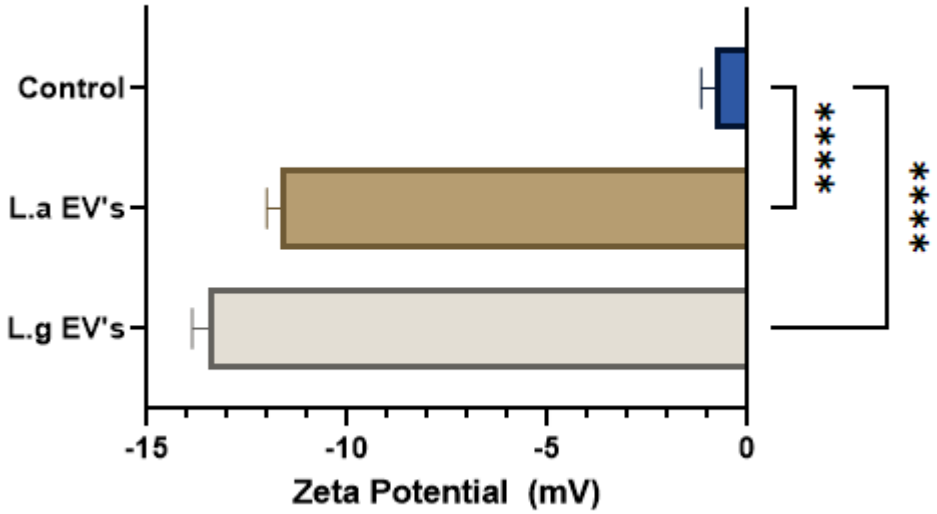

Figure 7. Zeta potential of EV isolate samples. Control group, in blue, shows nearly neutral zeta potential, samples of EVs isolated from *L.amazonensis* and *L.guyanensis*, here in brown and beige respectively, show strongly negative zeta potential. X axis represents zeta potential in millivolt. Statistical analysis using the parametric Student t-distribution test. (****) indicates significant differences in values between control condition and each sample.

To sum up, electron microscopy images show the biogenesis of protrusions that match the diameter and morphological description of exosomes and microvesicles found in literature. Furthermore, DLS analysis shows that samples of EV isolates contain biomolecules with a diameter consistent with exosomes and microvesicles while they are absent in the control sample. This supports the notion that not only do Leishmania produce EV's, but these EV's have characteristics consistent with exosomes and microvesicles.

3.2 *Leishmania* EVs interact with THP-1 cells and do not affect cell viability

THP-1 is a monocytic cell line that should be capable of responding immunologically to the presence of promastigotes and parasitic EVs. To ensure THP-1 cells internalize parasitic EVs, a flow cytometry assay was conducted. Figure 8B shows that THP-1 cells are clearly able to incorporate EVs. Additionally figure 8A shows fluorescent microscopy images of EVs stained with DilC18 incorporated into the cytoplasm of THP-1 cells.

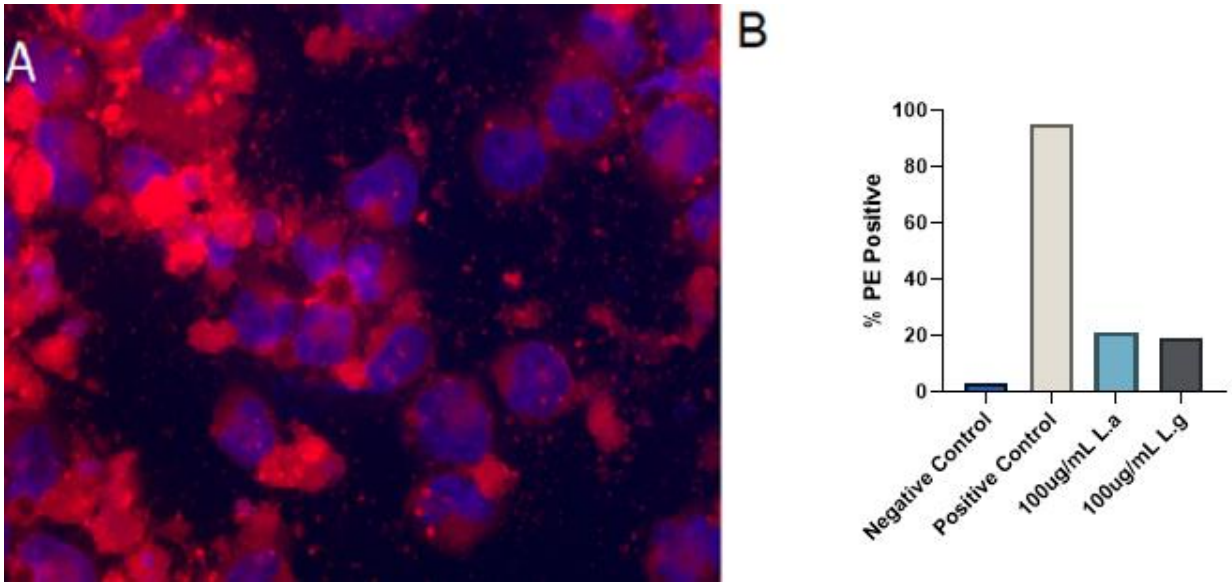

Figure 8. EV incorporation into THP-1 cytoplasm. A) Fluorescent microscopy images (600x magnification) demonstrating EVs are incorporated into cell cytoplasm. Red: EVs (100 µg/ml) stained with DILC18; Blue: DAPI staining cell nucleus. **B)** Flow cytometry analysis (N=1) of unstained THP-1 cells (Negative control), THP-1 cells stained with DILC18 (Positive control), unstained THP-1 cells exposed to 100µ/mL of *L.amazonensis* EVs stained with DILC18 (100ug/mL L.a), and unstained THP-1 cells exposed to 100µg/mL of *L.guyanensis* EVs stained with DILC18 (100ug/mL L.g). Y axis represents % of positive cells to PE stain.

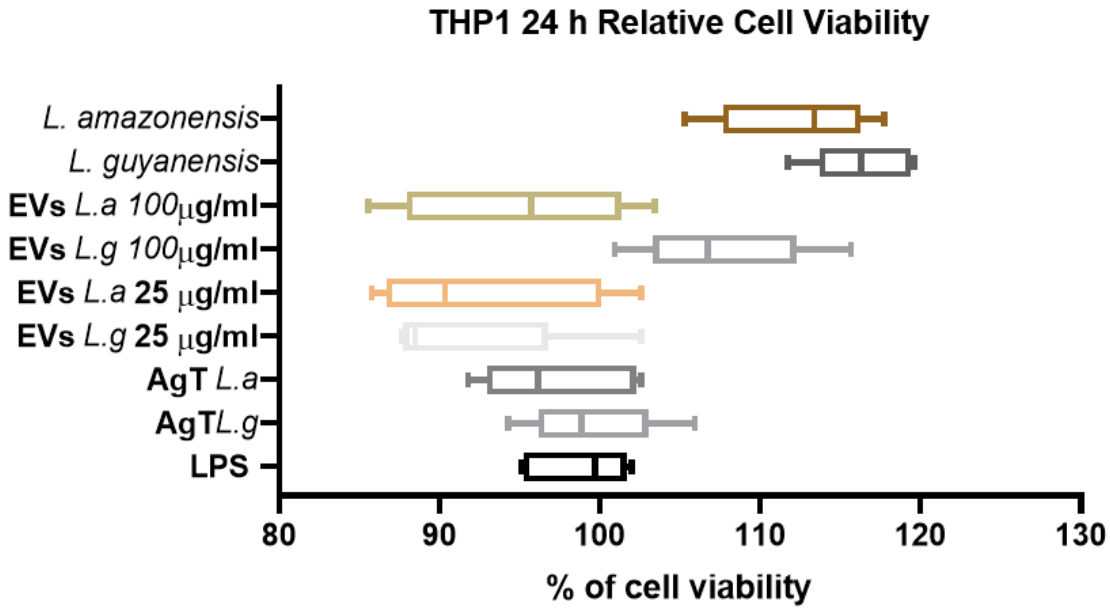

Figure 9. THP-1 cell viability assay. All samples normalized to control condition. X axis represents cell viability in relation to the control. THP-1 exposed to *L.guyanensis* and *L.amazonensis*; AgT*L*.g and AgT*L.a*, THP-1 cells exposed to total antigen extract of *L.guyanensis* and *L.amazonensis* respectively; THP-1 cells exposed to concentrations of 25µg/mL and 100µg/mL of *L.guyanensis* and *L.amazonensis* EVs respectively.

Figure 9 shows the cell viability assay, exposure to promastigotes has a pronounced effect of increasing cell viability.T his increase of cell viability for live promastigote exposure is consistent with the behavior of *Leishmania* upon infection of host MØs, artificially increasing their viability. Apoptosis of infected cells is one of the mechanisms by which the immune system attempts to manage the infection, as this leads to the destruction of the cell and all that inhabits said cell, including amastigotes. This is not beneficial to the parasite,

which has developed mechanisms to inhibit apoptosis, extending the life of the infected cell, giving the amastigotes more time to proliferate. This effect has been described in various studies, and the exact mechanisms seems to vary based on factors such as parasite species and infected cell type. For example, one of these mechanisms was described by Moore et al.(1994), where it was shown that L.donovani was able to inhibit apoptosis induced by macrophage colony-stimulating factor deprivation.[81]

The rest of the conditions the cells were exposed to did not seem to cause any notable changes in viability, aside from the 100µg/mL L.guyanensis EV exposure, an effect that needs further testing to confirm whether it is an outlier or not, as it differs significantly from the response to cells to the *L.amazonensis* EVs.

Overall, these results suggest THP-1 cells internalize *Leishmania* parasites, and from there, the parasite seems capable of modulating parts of cell signaling, in a similar fashion to macrophage-*Leishmania* interaction in the wild. Additionally, these results strongly indicate that THP-1 cells are able to incorporate EVs into their cytoplasm. A similar effect has been described for macrophages by Silverman et al. (2012)[82], where they demonstrate EV uptake by macrophages via surface binding, fusion with the plasma membrane, or endocytosis. The exact effect of EVs on cell viability remains unclear. More repetition is necessary, especially to confirm if the apparent effect of the exposure to 100µg/mL of *L.guyanensis* EVs is an outlier.

3.3 The presence of an inflammatory stimuli impairs keratinocytes wound-healing response

The HaCaT cell line consists of immortalized human keratinocytes, widely used for scientific research worldwide because of its consistency and ability to mimic human keratinocyte response in inflammation and tissue repair.

Keratinocytes are the most abundant cells found in the epidermis, which is the outermost layer of the skin. They are responsible for producing and maintaining the skin's barrier function, which is critical for protecting the body against harmful environmental factors, such as UV radiation, chemical toxins and pathogens, such as parasites. Keratinocytes are also involved in the immune response of the skin. They can produce a range of cytokines and chemokines that recruit and activate immune cells, such as T cells, dendritic cells, and macrophages, to the site of infection or injury. Moreover, keratinocytes express various pattern recognition receptors, such as Toll-like receptors, which enable them to detect and respond to microbial pathogens.

To understand if these cells could mimic human skin during *Leishmania* infection in vitro, i.e. presenting an inflammatory reaction to the presence of *Leishmania* parasites, antigens and EVs, a wound healing assay was performed. This assay is widely used in cancer research to test cell migration and is usually accompanied by the use of cell proliferation blockers. In this application , no blockers were used as the assay was performed in order to analyze the effect the different conditions in the rate of tissue repair. HaCaT cell cultures were exposed to LPS as a positive control for inflammation and live *L.amazonensis* promastigotes, as well as *L.amazonensis* EVs. After 20 hours the effect of each condition becomes visible, as the control condition is slowly recovering and healing the injury, meaning keratinocyte proliferation and migration is covering the wound overtime. The other conditions show clear signs of debilitated wound closure capabilities. At 40 hours the control condition has fully healed, marking the end of the assay. Cells exposed to LPS appear to have

recovered from the exposure and closely followed the control condition for wound closure. The cells exposed to EVs, and live parasites show severe debilitation when compared to the control condition, and severe cell detachment from the matrix can be observed in the cells exposed to live parasites.

While there has been no evidence of leishmania cells infecting keratynocites, as these cells are normally incapable of phagocytosis, some studies have revealed very specific situations in which keratynocites can engulf and digest bacterial pathogens, such as Staphylococcus aureus, by forming phagosomes and lysosomes within their cytoplasm. Some other, more likely, hypothesis for this detachment include pH changes in the environment from the presence of live promastigotes, or a sharp increase in celular stress due to a large pro-inflammatory presence in the medium.

The negative effect EVs have on keratynocyte proliferation also suggesy that HaCat cells are able to either recognize or even internalize these EVs, perhaps by similar mechanisms as macrophages, which causes these cells a great deal of celular stress and debilitates their proliferation and migration capabilities.

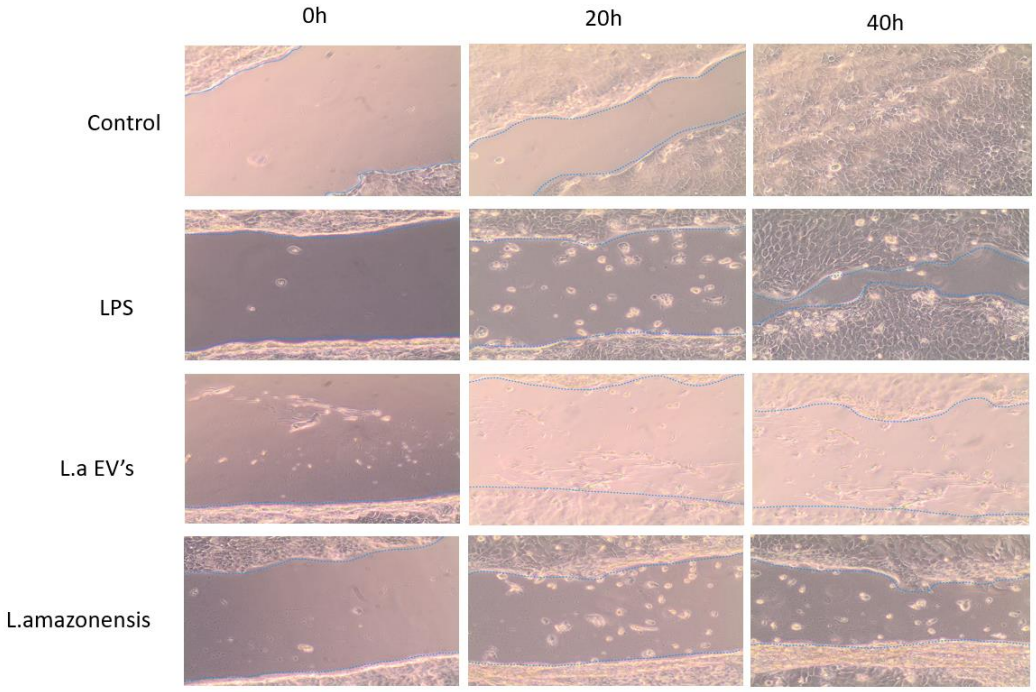

Figure 10. Light microscopy images of wound healing assay. Cells were photographed at different timepoints, 0 hours, 20 hours and 40 hours after the initial injury was inflicted on cell surface. Control condition constitutes HaCat cells not exposed to any pathogen. LPS is used as positive control of inflammation. *L.amazonensis* EVs and promastigotes were pictured as well. Delineation of injury edges in blue. Magnification 400 x

3.4 THP-1 cells "priming" for skin macrophage-like (sMΦ) phenotype

THP-1 cells are capable of differentiation, described in literature in many studies. This factor is used extensively in research to emulate immune system cells. Aiming to direct THP-1 cells to a more skin macrophage-like (sMΦ) phenotype, and with the future goal of establishing a HaCat-THP-1 co-culture, THP-1 cells were primed with keratinocyte conditioned medium. Keratynocytes are capable of releasing various factors into their environment that have an immunomodulatory effect, such as cytokines, chemokines, extracellular vesicles and lipids. The idea behind this concept is that these factors could help acclimate the THP-1 cells to a skin-like environment and direct them to a sMΦ phenotype.

THP-1 cells that underwent the priming protocol presented a clear morphological shift. While they usually present a round, relatively smooth surface while in suspension, post-priming cells become highly adherent to the surface, developing pseudopodia-like structures, and gaining an increasingly macrophage-like appearance. This contrast between adherent and suspended THP-1 cells can be seen below in Figure 11.

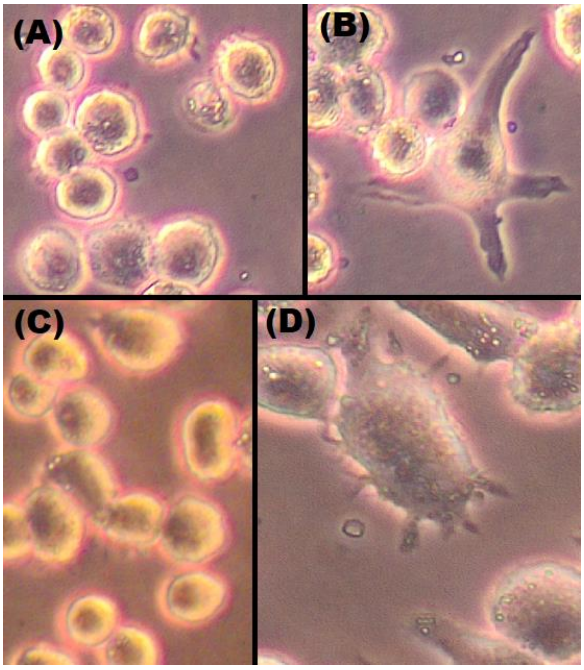

Figure 11. Light Microscopy images of THP-1 cells in culture. (A) and (C): THP-1 cells in culture, round morphology with a smooth surface. **(B)** and **(D)**: THP-1 cells following "priming" become adherent and develop pseudopodia-like structures, very distinctive from their non-primed counterparts.

Interestingly a study on selective culturing of adherent THP-1 cells show that these gain characteristics of activated MØs such as an increase in the expression of MHC class II[83]. The increased adherence resulting from the priming process might not be a sign of differentiation alone, but at the very least it can possibly indicate an acceleration of the process described in this study.

To further understand the "priming" on the THP-1 cells, we turned to flow cytometry. A wide range of macrophage functional and phenotypic markers were assessed. CD80 is a cell surface protein that is primarily expressed on APCs such as macrophages, playing a critical role in the activation of T cells, an important marker for APC activation. CD80 is is a cell surface protein that is primarily expressed on the surface of mature dendritic cells (DCs), which are specialized antigen-presenting cells that play a key role in the activation of T cells and the initiation of adaptive immune responses. This marker could give insight into Langerhans or dermic dendritic cell phenotypical features in THP-1. CD11c is a cell surface protein primarily expressed on the surface of certain immune cells, including dendritic cells, monocytes, macrophages, natural killer cells, and some subsets of T cells, its mainly used as a dendritic cell marker and similarly to CD83 could give inside into THP-1 developing DC phenotypical features. CD1a isis primarily expressed on the surface of dendritic cells, including LC's and is involved in the presentation of lipid antigens to T cells in a process known as lipid antigen presentation, its commonly used as a dendritic cell biomarker. CD14 is is primarily expressed on the surface of monocytes, macrophages, and dendritic cells. It serves as a co-receptor for toll-like receptor (TLR) 4, which recognizes bacterial LPS and other PAMPs, CD14 is commonly used as a marker to identify monocytes and macrophages in flow cytometry experiments. CD64 is primarily expressed on the surface of monocytes,

macrophages, and dendritic cells and is involved in the phagocytosis of opsonized particles, which is of great importance as opsonization is a common tactic employed by Leishmania to entice phagocytosis. Langerin is is primarily expressed on the surface of Langerhans cells (LCs) in the skin and mucosal tissues. Langerin is involved in the recognition and uptake of antigens, including pathogens and self-antigens, and is often used as a LC biomarker. MHCI is found on the cell surface of almost all nucleated cells, it plays a critical role in the immune system by presenting peptide antigens derived from intracellular pathogens, such as Leishmania. MHCII is a cell surface molecule found primarily on antigen-presenting cells such as dendritic cells, macrophages, and B cells. It plays a critical role in the immune system by presenting peptide antigens derived from extracellular pathogens and production is often upregulated by Leishmania as it can suppress the previously mentioned intracellular pathogen clearing mechanisms that result from MHCI presentation.

We found that THP-1 cells were found to not express CD80, CD83, CD11c or CD14. This is mostly consistent with literature, THP-1 do not express CD80, CD11c, and CD14, and a different study found that CD83 is pre-formed inside monocytes and is only transiently expressed in MØs and monocytes upon activation. CD83 testing was done only extracellularly, and the priming process perhaps does not constitute activation, or the assay was performed during a time window where CD83 was no longer being expressed. To shed light on this issue, further tests including activation of the cells with LPS, and intracellular staining would be recommended.

CD1a is shown to be expressed in THP-1, which should not be the case according to Janneh et al but has been documented in some studies[84]. CD1a is used as a marker for Langerhans cells and is suggested to play a central role in antigen presenting by these cells, but our assay did not find major differences in expression both pre and post priming, so any association the production of CD1a could have with Langerhans cells does not seemingly stem from the priming process.[85]

Langerin expression was absent from both primed and not primed cells. Langerin expression was assessed under the hypothesis that keratinocytes could possibly induce a tissue specific response from the THP-1 cells which could manifest in the expression of langerin, which THP-1 does not naturally produce. It does not seem like priming has any effect on the expression of langerin by THP-1.

THP-1 cells express CD64, but priming does not seem to have an effect on expression levels in the cells. Studies have shown that THP-1 does express CD64 and modulating different factors such as IL-4 and LPS can have an effect on the expression level. In this case no immunological stimuli were given to the cells so CD64 levels remain consistent across primed and non-primed cells. It has been suggested that CD64 is an M1-like phenotype marker[86] which makes it a target of interest to monitor further during parasite/cell interaction, as *Leishmania* prefers M2 MØs due to their decreased ability to eliminate intracellular parasites.

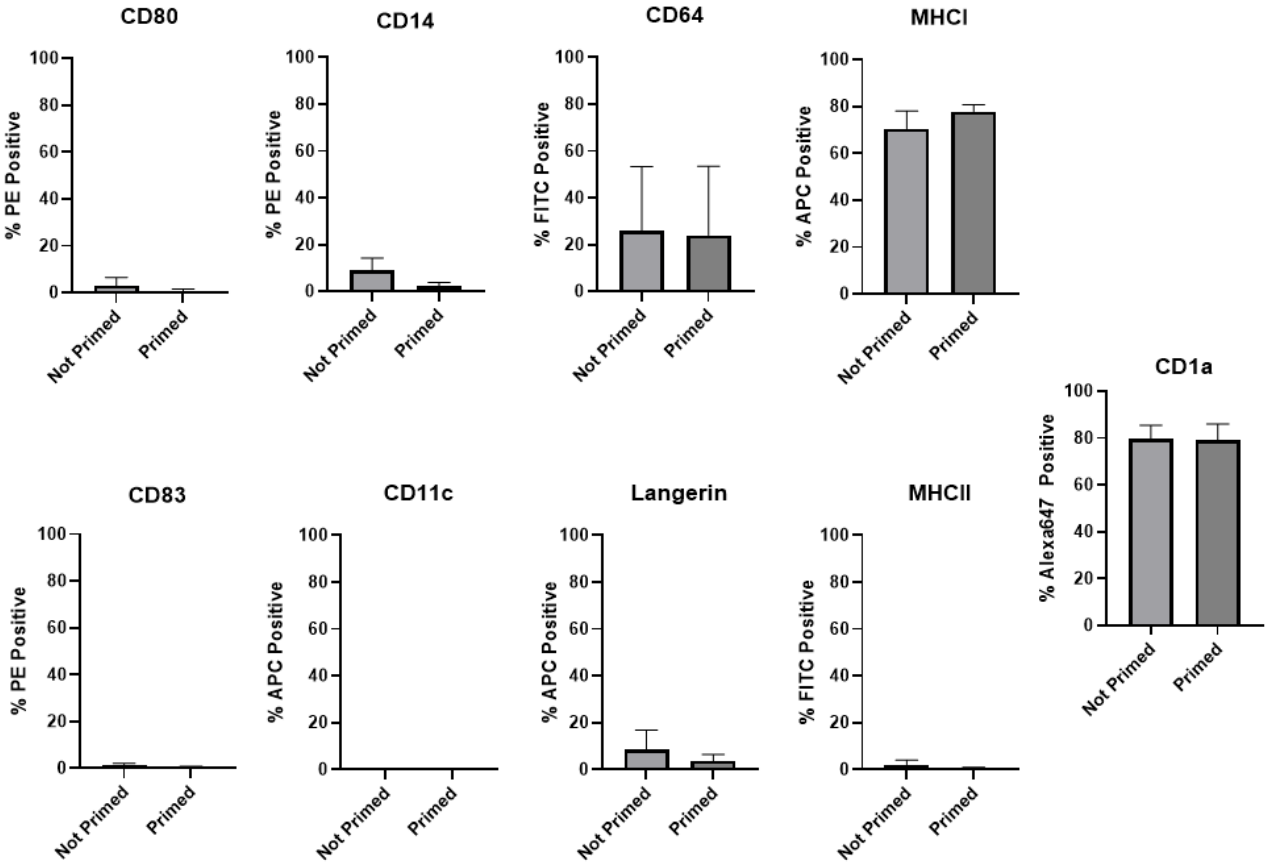

Figure 12. Flow cytometry of cells marked for CD80, CD14, MHCI, MHCII, CD83, CD11c, langerin, CD64, and CD1a. Unstained cells were used as a negative control (Unstained), THP-1 cells not submitted to the priming process (Not Primed) and THP-1 cells that underwent priming (Primed) were analyzed. Y axis represents the % of cells positive for each staining, by comparison to the negative control (unstained cells),

MHC class I is expressed in the cells, priming does not seem to have an effect on the levels of expression. This is opposed to MHC class II which seems absent. Several studies point to THP-1 cells as an MHC class II positive line, as such the fact that no MHC class II expression was detected is non characteristic, and further assays are needed to understand the reason behind its effect.

MHC class I expression remained consistent for primed and non-primed cells, literature describes MHC class I elevation in cases where cells are exposed to antigens or pathogens, which were absent in this assay, and possibly explaining why MHCI expression does not vary from primed to non-primed cells, as the environment is devoid of immunological stimuli.

Overall flow cytometry does not seem to indicate phenotypic shift from non-primed THP-1 cells to primed THP-1 cells, which is inconsistent with the contrasting morphological changes observed by light microscopy. Further cytometry optimization needs to take place, and perhaps alternative methods for characterizing THP-1 cells should be explored to better explain the apparent differences seen between the optical microscopy observations of primed THP-1 morphology and their phenotype.

3.5 HaCat THP-1 co-culture were able to mimic host skin dermis

After priming THP-1 cells were co-cultured with HaCaT cells, under the hypothesis that direct contact between the cells could induce a stronger differentiation response in the THP-1 cell line. Furthermore, the HaCat-THP-1 co-culture model has been used in research contexts previously as a simplified immunological model of skin.

Co-cultures were established, and various experimental conditions were set up to best characterized several types of response. Firstly, co-cultures were imaged by light microscopy to gather more information about the interactions between THP-1, HaCat, EVs, and parasites. Shown in Figure 14, THP-1 cells seem to adapt well to the presence of HaCat cells and promptly adhere to their surface. This anchoring effect can be seen in multiple THP-1 cells and can be seen further below in SEM imaging in far greater detail.

Upon exposure to LPS, HaCat cells do not seem to react significantly, but THP-1 cells become highly granulated, which is considerably different from their normal appearance, visible in Figure 13C. Exposure to EVs also has a similar effect but most importantly it strongly elicits a reaction from the HaCat cells, on which lesions can be seen marked in red (Figure 14D and 14E).

Upon exposure to live parasites, THP-1, similarly to EV and LPS exposure, become highly granulated (Figure 14F and 14G), and in some cases THP-1 cells become so infested with parasites on their surface that distinguishing their appearance becomes impossible (Figure 14H). This is expected as TPH-1 cells are monocytes that can be differentiated into macrophage-like cells, and macrophages are the main target for Leishmania.

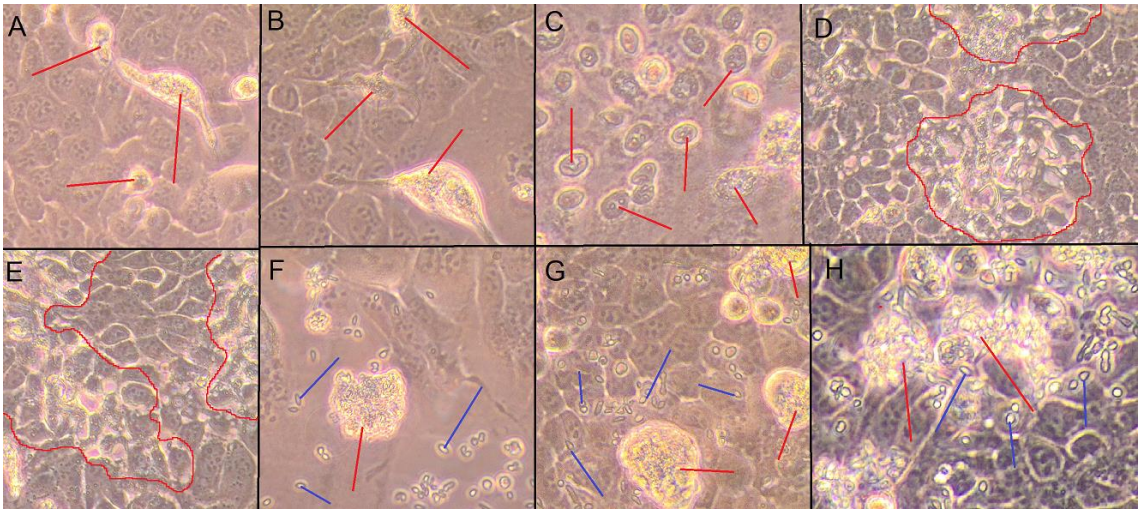

Figure 13. Light microscopy images of HaCaT-THP-1 co-culture, exposed to different conditions. A) and **B)** Control condition with no exposure, red lines denote adherent THP-1 cells on a carpet of HaCat cells in the background. **C)** Exposure to LPS produces a change in THP-1 morphology, becoming highly granulated. Red lines point to some, but not all, THP-1 cells. **D)** and **E)** Exposure to parasitic EVs causes lesions in the HaCat cell continuum, lesion areas with granulated and lighter cells are circled in red. **F)** and **G)** Some *Leishmania* parasites are noted with a blue line. Red lines point to highly granulated THP-1 cells in close proximity to parasites. **H)** *Leishmania* parasites (examples pointed in blue) swarm THP-1 cells to a point where cell surface becomes nearly unrecognizable (red lines). **Magnification 40x**

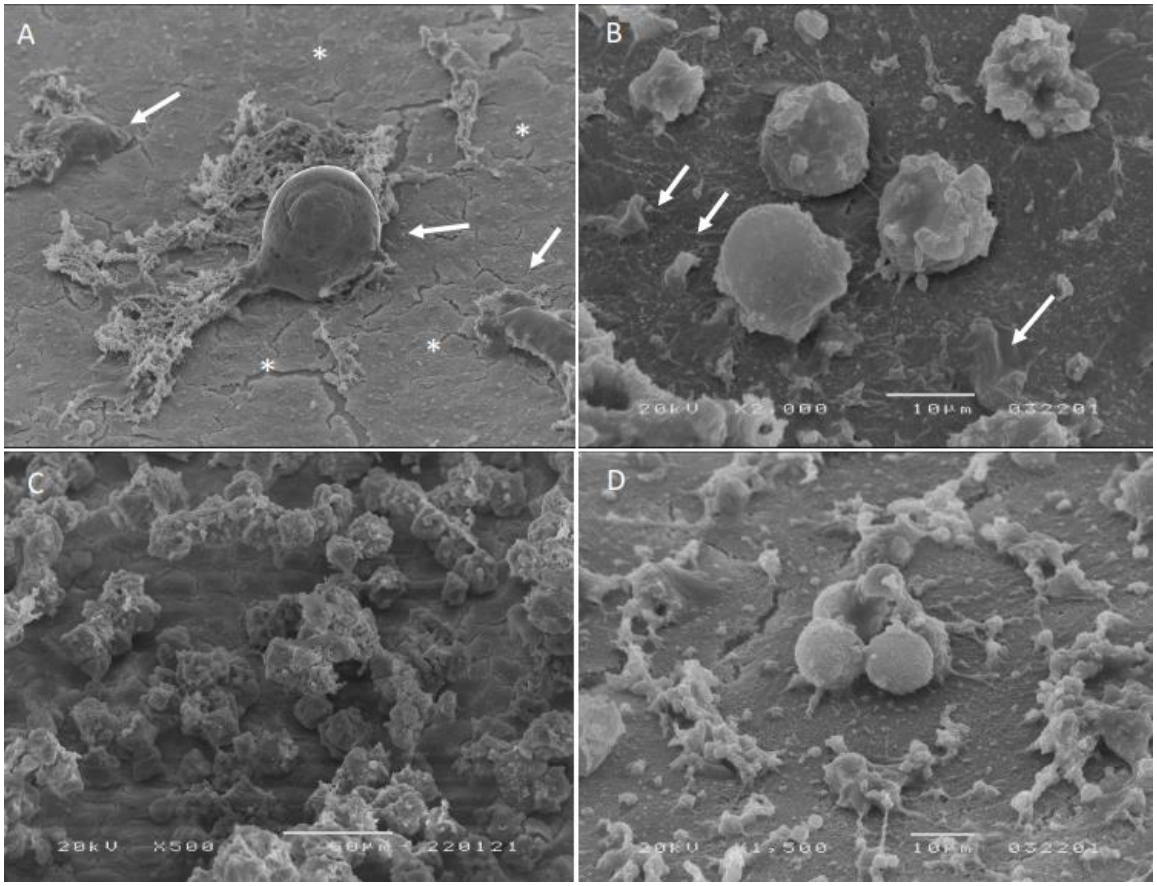

Figure 14. Scanning Electron Microscope images of HaCat-THP-1 co-culture. **A)** THP-1 cells (white arrows) form cellular connections with HaCat (white asterisks). **B)** LPS presence elicits the formation of diverticula on the surface of HaCat cells (white arrows). **C)** Extensive cellular disruption caused by exposure to *L.amazonensis* promastigotes. **D)** Exposure to *L.amazonensis* EVs also causes the formation of diverticula on the surface of HaCat cells.

SEM analysis of the co-cultures shows a number of interesting details about the interactions between cells, parasites and EVs.

In non-exposed co-cultures (control condition), THP-1 cells can be seen firmly anchored to a smooth HaCat epithelium, which mimics the skin. Once co-culture is exposed to LPS, various diverticula seem to form on the surface of HaCat cells as a response to inflammation, THP-1 cells appear to be slightly altered as well. Figure 15C shows the result of exposure to live parasite promastigotes, cellular disruption is apparent. Parasitic EV exposure, as pictured in figure 15D, shows a similar response to LPS, the formation of diverticula by HaCat cells. SEM imaging shows that the HaCat-THP-1 co-culture does react to the presence of pathogens and furthermore, this reaction is more severe when exposed to live parasite promastigotes.

Cytometry analysis shows again that MHC class II expression seems to be absent. HaCat cells do not express MHC class II, and the low proportion of THP-1 cells to HaCat cells in the co-culture (circa 2%) could possibly mean the signal would not be strong enough for detection, on the other hand, THP-1 cells alone did not give signs of MHC class II expression previously. More work needs to be done to identify the core cause of the absence of MHCII expression which goes against established literature. Langerin expression was seemingly detected in co-culture exposed to *L. amazonensis* EVs, but the high standard deviation in the results might indicate it is an outlier, more investigation is necessary. Generally, langerin expression remained largely absent which is not surprising, and indicates that direct contact with HaCat cells does not elicit langerin

expression in THP-1. CD64 expression remains consistent across the board, with a noticeable reduction in co-cultures exposed to *Leishmania* parasites, this effect is not well described in literature but might be connected to parasite immune modulation mechanisms. The discrepancy in CD64 expression between experimental replicates in cells exposed to EVs and LPS make analysis difficult, more repetition and optimization of the method is necessary to reduce standard deviation and prevent outliers from skewing the results.

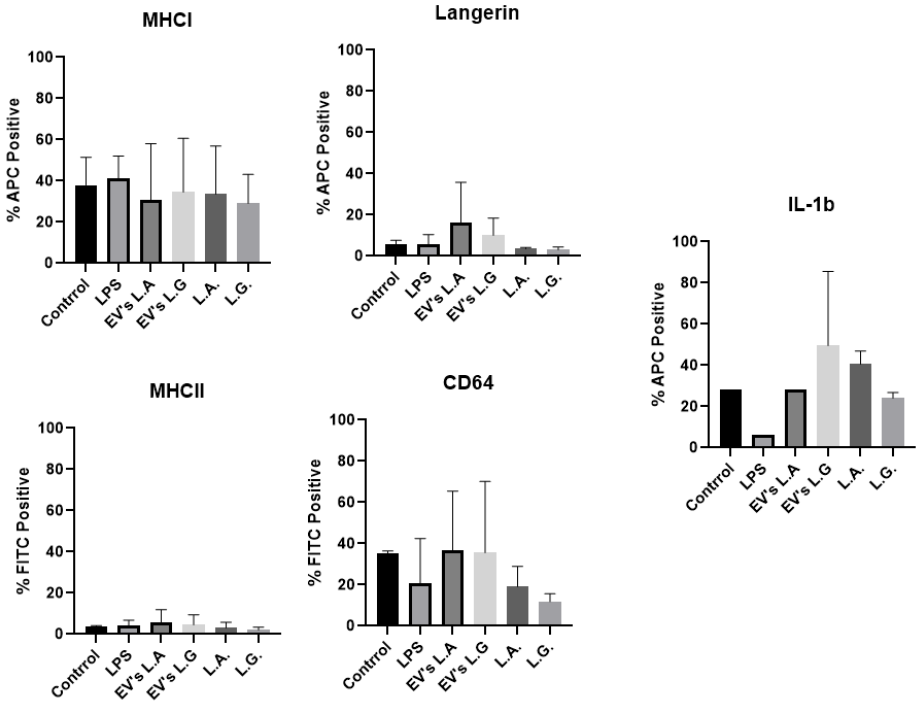

Figure 15. Flow cytometry of HaCat-THP-1 co-culture. Cells were stained for MHCI, MHCII, langerin, CD64, and IL-1β (IL-1β). Cells stained for each marker but not exposed to any pathogens (control), cells stained for each marker and exposed to LPS (LPS), cells stained for each marker and exposed to EVs of each parasite species and each parasite, *L.amazonensis* and *L.guyanensis* (EVs L.A, EVs L.G, L.A and L.G respectively) were analyzed.

IL-1β expression was also analyzed to access inflammatory response in the co-culture. IL-1β expression in the control group is undesired, as IL-1β is not expressed constitutively under homeostasis. IL-1β is a pro-inflammatory cytokine and usually has increased expression when cells detect PAMP's, and so, the stark reduction in positive signal for co-culture exposed to LPS, an inflammatory agent, further indicates the need for repetition and optimization of the method. EV and parasite exposure had a similar amount of positive signal to the control condition, and the high variance in the values for IL-1β expression in co-cultures exposed to *L.guyanensis* EVs strongly suggests and outlier and not a consistent, factual result. It might be of interest for future repeats of this assay, to stain HaCat and THP-1 cells so that co-culture cytometry can better distinguish and select each cell population for a more thorough characterization of the model and lessen possible interference in the signal.

Overall flow cytometry analysis indicates that direct contact with HaCat does not activate THP-1 cells, failing to increase MHCII expression, and does not help THP-1 cells differentiate into Langerhans-like cells, as langerin expression also remains absent. Differences in immunological response based on exposure to different elements are not conclusively seen, and optimization of flow cytometry and co-culturing needs to take place to rule out flawed methodology as a reason for this effect.

Cytokine and PRR generation were assessed by relative quantification RT-qPCR. This should reveal more about EV's interactions with immune receptors and their effects on cytokine expression, it is especially interesting to contrast this effect to that of the live promastigotes, as so far, the results have been suggesting that EV's lead to a similar immune response, but without the virulence.

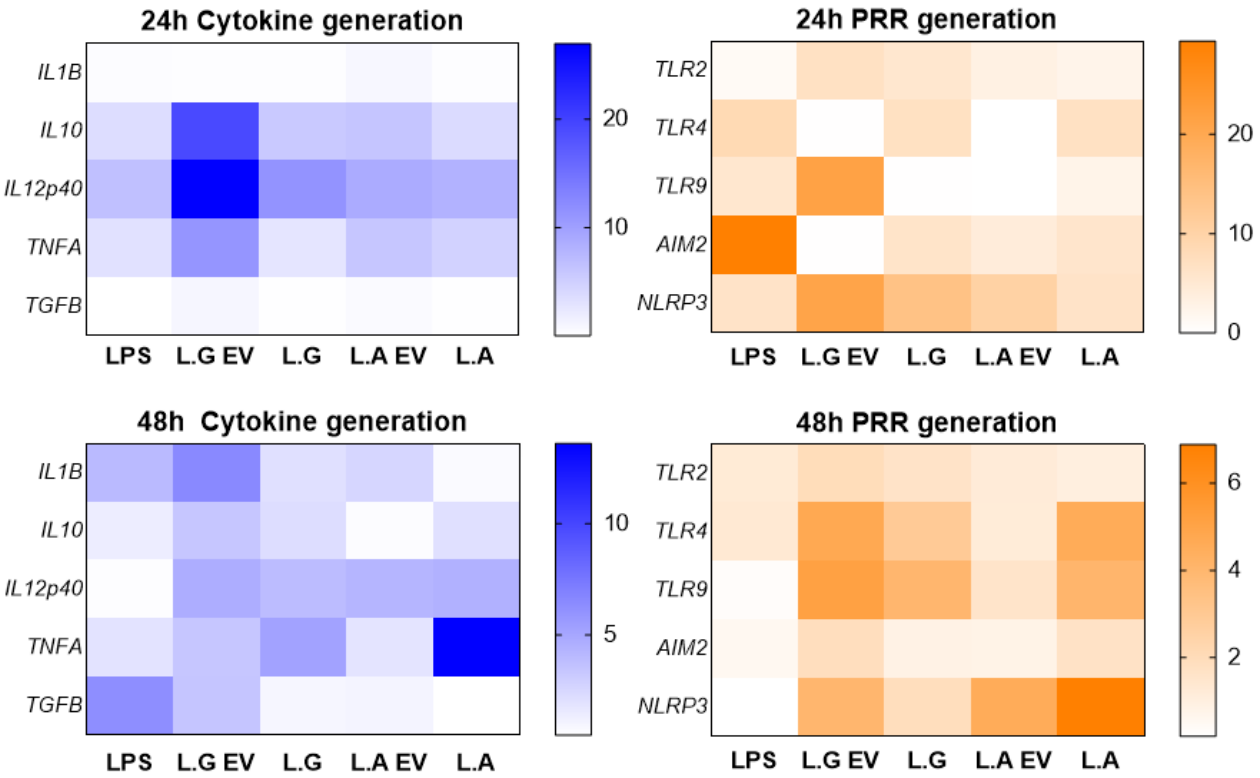

Figure 16. Reverse transcriptase qPCR heatmap of HaCat-THP-1 co-culture at 24 and 48 hours. Timepoints are further divided into a cytokine heatmap, describing the variations in the IL-1β, IL-10, IL-12, TNF-α, and TGF-β (blue) and a PRR heatmap describing variations in TLR2, TLR4, TLR9, AIM2, and NLRP3 (orange). Results obtained by relative quantification.

24h cytokine gene expression reveals an increase in the pro-inflammatory cytokine IL-10 while also showing the expression of IL-12,a pro-inflammatory cytokine. Studies exist showing IL-10 is able to inhibit IL-12 production in macrophages[87], as such, an increase generation of both cytokines is seemingly nonsensical. No increase in IL-1β generation, especially in the positive inflammation control is strange, especially when comparing to the results from flow cytometry, which shows the expression of IL-1β, further testing will be necessary. TGF- β remains consistent across conditions, which is relatively expected since it's an inductor of cellular proliferation and a mere 24 hours are perhaps not enough to induce and increase in TGF-β generation.

IL-1β generation in positive control is heightened as expected, EVs also seem to induce an increase in IL-1β while contact with live promastigotes seemingly leads to a decrease in IL-1β. TNF-*α* conversely increases when co-culture is exposed to parasites, which clashes with the previously observed reduction in IL-1β, as TNF-*α* is a powerful pro-inflammatory cytokine. TGF-β is present in the LPS condition, possibly indicating cells are recovering from exposure, something that is absent from the other conditions and is consistent with the negative effect EVs and live parasites have on cell proliferation seen in the wound healing assay. PRR generation across both timepoints suggest NLRP3 generation is increased upon exposure to EVs

and live parasites. NLRP3 leads to a pro-inflammatory response, and while in the positive inflammation control condition, exposure to LPS, it is present at 24h, it seemingly decays over time and shows no notable increase at 48h. Conversely exposure to EVs and promastigotes shows an apparent increase in NLRP3 generation, maintaining a pro-inflammatory response over time. This is also seen in TLR4 and TLR9 levels, both associated to pro-inflammatory responses, decrease over time during LPS exposure, but increase during exposure to EVs and promastigotes, indicating a prolonged inflammatory stimulus. Figure 17 shows the variation of Urea concentration in the supernatants at different timepoints.

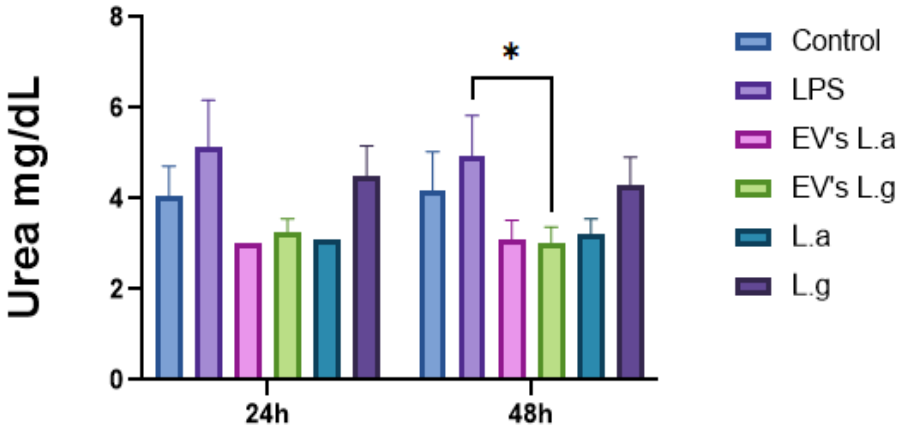

Figure 17. Colorimetric Urea assay. Supernatants from co-culture at 24h and 48h were collected and submitted to analysis. Both 24h and 48h timepoints show a similar profile across conditions. Co-culture not exposed to any pathogen was used as control, pictured as light blue. Co-cultures exposed to LPS (LPS, light purple) were used as positive inflammation controls. Pink and green bars represent co-cultures exposed to *L.amazonensis* and *L.guyanensis* respectively. Dark blue and dark purple represent co-cultures exposed to promastigotes from *L.amazonensis* and *L.guyanensis* respectively. Statistical analysis was performed by 1-way ANOVA, (p value = 0.0457).

LPS exposure leads to an apparent increase in urea levels. Exposure to parasite EVs shows a tendency towards a decrease in urea, which is also the case for exposure to *L.amazonensis* promastigotes. *L.guyanensis* promastigotes do not appear to elicit the same effect from the co-culture. L-arginine is converted to urea by arginase, a competitor to iNOS that converts L-arginine to NO. It is beneficial for the parasite to tilt this balance in favor of arginase as NO is toxic to *Leishmania*. The opposite seems to happen, as results show a tendency to decrease urea production, particularly upon exposure to EVs. Exposure to *L.amazonensis* promastigotes and EVs seems to elicit a similar response in urea production, while *L.guyanensis* promastigotes do not show a tendency to decrease or increase urea production.

EVs contain several different molecules such as proteins, lipids, DNA, and mircoRNAs, which can enter a cell when the EV fuses with its membrane or is ingested by the cell via endocytosis. TLR9 for example is an intracellular TLR that recognizes unmethylated CpG DNA motifs, which exist in leishmania, to trigger the production of pro-inflammatory cytokines and activation of the inflammasome, which leads to an increase in NO, directly impacting urea production, and could possibly be one of the mechanisms to explain the effect seen above.

And so, this effect where *L.guyanensis* EV exposure induces a reduction of urea production, coupled with an increase in IL-1β gene expression at 48 hours, and its elevated expression demonstrated by flow cytometry seems to strongly suggest they have a pro-inflammatory effect on the cells.

3.6 Pre-exposition of THP-1 to *Leishmania* EVs does not protect against parasite infection

Previously we has shown that EVs induce an immunological response in cells that is similar to the presence of the live parasite, without the associated infectivity. As such, it is not farfetched to posit the hypothesis that exposing cells to EVs previous to any contact with the live parasite could somehow prepare the cells for this contact, and make infection more difficult for the parasite. In short, would it be possible to utilize isolated EV's as a prophylactic treatment? If so, it could be a monumental breakthrough as there is great urgency in exploring novel ways to manage Leishmaniasis.

To determine if exposure to EVs has any effect on the future infection of THP-1 by the parasite, a limiting dilution assay was performed. Figure 18 shows the average limiting dilution in which parasites were detected in THP-1 cells pre-exposed to different conditions.

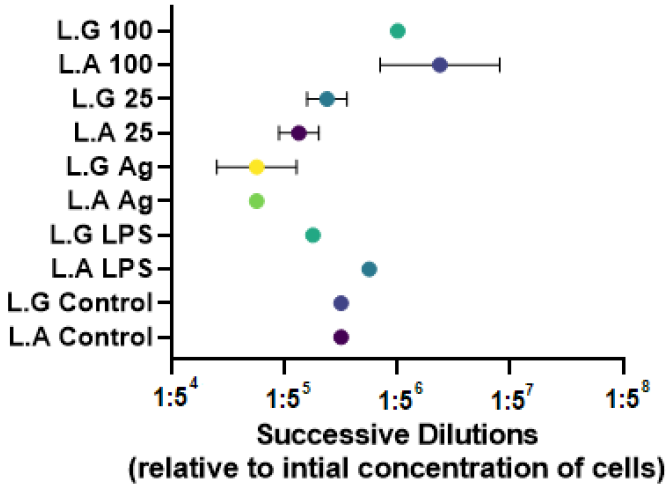

Figure 18. Limiting Dilution Assay of THP-1 infected by Leishmania. THP-1 cells not pre-exposed to any condition and infected by promastigotes of *L.amazonensis* and *L.guyanensis* were used as control (L.A and L.G Control). THP-1 cells exposed to LPS (L.A LPS, L.G LPS respectively) was used as a positive inflammation control. THP-1 cells pre-exposed to total antigen extract, EVs at 25ug/mL and 100ug/mL of both species, and subsequently infected by the corresponding species promastigotes were analyzed alongside the controls. All values are averages of experimental replicates, with N=1 for the L.A and L.G control and L.G and L.A LPS, and N=2 for the other conditions. X axis describes dilution relative to the starting concentration of cells.

LPS pre-exposure does not seem to alter infectivity of promastigotes. Total antigen extract pre-exposure seems to cause a slight reduction in promastigote infectivity. Pre-exposure to 25ug/mL of EVs of either species of parasite does not seem to have any notable effect on infectivity. Conversely pre-exposure to 100ug/mL of EVs seems to induce an increase in parasite infectivity.

The neutrality of LPS pre-exposure is surprising, as LPS should induce an inflammatory response that can work either as a benefit or a drawback for the subsequent parasite infection. One factor might be the concentration of LPS not being elevated enough to cause a significant inflammatory response, or perhaps the

fact that LPS is a bacterial PAMP, it has a neutral effect on cells when it comes to alerting and/or preparing for a *Leishmania* infection.

The slight decrease in infectivity shown during pre-exposure to total antigen extract might support this hypothesis, as perhaps the contact with inactivated fragments of *Leishmania* by the cells helps in some way for THP-1 cells to better identify and protect themselves from future contact with the live virulent promastigotes. Pre-exposure to EVs seems to have different effects based on concentration, 25ug/mL being too low to induce any notable effect on infectivity, while 100ug/mL is enough for a tendency to be observed. This goes well in hand with the studies that show that *Leishmania* EVs carry molecules that aid in infection, such as GP63, but the pro-inflammatory effect EVs were shown to have in the previous section, leading to a decrease of urea production and increase of pro-inflammatory IL-1β and inflammasome NLRP3 usually are not beneficial to infection, as pro-inflammatory response leads more easily to intracellular pathogen clearance mechanisms activating. Perhaps the effect of EVs on infectivity is context dependent and can vary based on the situation, which can prove to be good news for the ongoing efforts of exploiting VEs as a possible weapon against *Leishmania.*

4. Conclusion; Future Prospects and Final Thoughts

This study delves deep into the topic of a very important neglected tropical disease, that deserves more attention and recognition be drawn to it, as it affects millions worldwide. Therapy options are so sparse, toxic and expensive, with no human vaccine, that the development of novel treatments is paramount to the wellbeing of people across the globe. Even readers from developed countries might not be safe from the grasp of this disease, as global warming facilitates its spread into new habitats.

EVs are perhaps this much needed breakthrough. Slowly rising in academic interest, EVs are novel elements that can be studied, understood and hopefully exploited to our benefit. It opens up the doors to many options such as vaccines, localized topical therapies, prophylactic treatment and more.

To study EVs we created an in vitro, simplified, model of human skin and we were able to show that it can react immunologically to the parasite and EVs, indicating that it is an appropriate testing condition. This saves on unnecessary cruelty in the case of animal trials and approximates the results to humans since the cells in use were human cells. That said, much remains to improve in this study. When it comes to future prospects, a more complex, potentially 3D models of skin could be used, with other skin cells such as fibroblasts to better emulate aspects of infection, such as *Leishmania*'s manipulation of immune cell migration.

Better methodology for THP-1 differentiation coming to light would also be of great importance, to better approximate cell in vitro response to how parasite dynamics take place in the wild. Potentially cell lines could be swapped out altogether for primary cultures to ensure an even more accurate human skin model. Furthermore, research delving into the contents of *Leishmania* EVs, and their functions are of great importance to this study and are anything but a simple topic. The methods for EV content characterization can be highly specialized, such as mass spectrometry or Nuclear Magnetic Resonance (NMR) and require a great deal of understanding and multidisciplinary teamwork. Even after the monumental task of characterizing, even partially, the content of EVs the process to understand their function and interactions must take place, which poses its own set of challenges and hurdles.

Overall, this study is but a tiny window into a fascinating topic, about which much remains to be discovered. Knowledge upon which the quality of lives of many people in the harshest places on Earth relies upon, and we have hopefully helped make this neglected tropical disease a little less mysterious.

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