The Role of *Leishmania* GP63 in Modulation of Innate Inflammatory Response and Infection

Aretha Chan

Master of Science

Microbiology and Immunology

McGill University

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ABSTRACT

Leishmaniasis is a disease caused by the protozoan parasite *Leishmania* known to affect millions of individuals worldwide. In recent years, we have established the critical role played by *Leishmania* zinc-metalloprotease GP63 (GP63) in the modulation of host macrophage signalling and functions. In an immunological context, it favours survival and progression of the parasite within its host. Whereas *Leishmania major* knock out for GP63 caused limited infection in mice, it is still unclear how GP63 may influence the innate inflammatory response and parasite survival in an *in vivo* context. Therefore, we were interested in analyzing the early innate inflammatory events upon *Leishmania* inoculation within mice and establish whether *Leishmania* GP63 influences this initial inflammatory response. Experimentally, four groups of mice were injected intraperitoneally with PBS, *L. major* wild-type (*L. major*^{WT}), *L. major* GP63 knockout (*L. major*^{KO}) or *L. major* GP63 rescue (*L. major*^{R}). Six hours post-inoculation, intraperitoneal lavages were performed, and the cell suspension was collected for further analysis. We counted the total live recruited inflammatory cells, and cytospin slides were prepared to identify the cell types present in the lavage. Flow cytometry was also performed to verify the cell types and the populations of macrophages. In addition, the collected cells were plated and studied ex vivo to determine the percentage of infected macrophages and neutrophils within 48 hours after the infection. Centrifugation was used to isolate the supernatant and cytokine/chemokine contents were measured. Furthermore, we performed transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and proteomic analysis on the exosome content released in the supernatant by the cells recruited to the peritoneal cavity. Data collected suggest that all *Leishmania* cause similar inflammatory cell recruitment. However, cytokine/chemokine results

show variabilities between groups and are not sufficient to explain why GP63 ^{KO} parasites cause a less aggressive infection *in vivo*. GP63 may be involved in the internalization of promastigotes during early infection because there is a trend observed where less GP63^{KO} amastigotes were found within host cells upon initial hours of infection. *L. major* deficient in GP63 appear to be significantly less able to confer same level of infection as *L. major*^{WT} and *L. major*^{R}. Collectively this study provides a clear analysis of innate inflammatory events occurring during *L. major* infection and the role of the virulence factor GP63, as well as permit better understanding of the cellular and molecular mechanisms underlying the *Leishmania* infection process, which could lead to the development of new ways to protect humans against this pathogen.

RÉSUMÉ

La leishmaniose est une maladie causée par le parasite protozoaire *Leishmania*, connu pour affecter des millions d'individus dans le monde. Ces dernières années, nous avons établi le rôle critique joué par la zinc-métalloprotéase GP63 (GP63) de ce parasite dans la modulation de la signalisation et des fonctions des macrophages qu'ils infectent chez leurs hôtes mammifères. Dans un contexte *in vivo*, il a été rapporté que l'absence de la GP63 conduit au développement d'une infection beaucoup moins agressive. Mais même si des études ont démontré que les parasites déficients en GP63 sont plus sensibles au complément, on ne sait pas encore très bien comment GP63 peut influencer la réponse inflammatoire innée et la survie du parasite dans un contexte *in vivo*. Par conséquent, nous voulions analyser les événements inflammatoires innés précoces suite à l'inoculation de *Leishmania* chez la souris et établir si la GP63 de *Leishmania* pouvait influencer ces événements inflammatoires initiaux. Expérimentalement, quatre groupes de souris ont été injectés par voie intrapéritonéale avec PBS, *L. major* wild-type (*L. major*^{WT}), *L. major* GP63 knockout (*L. major*^{KO}) ou *L. major* GP63 rescue (*L. major*^{R}). Six heures après l'inoculation intrapéritonéale, des lavages de la cavité ont été effectués et la suspension cellulaire a été prélevée pour une analyse plus poussée. De ces échantillons nous avons compté le nombre de cellules inflammatoires recrutées, et des lames ont été préparées par cytocentrifugation pour identifier les types de cellules présentes dans le lavage. La cytométrie de flux a également été réalisée pour vérifier les types de cellules et les populations de macrophages. De plus, les cellules recueillies ont été mises en culture et étudiées *ex vivo* pour déterminer le pourcentage de macrophages et de neutrophiles infectés dans les 48 heures suivant l'infection. La centrifugation a été utilisée pour isoler le surnageant et les teneurs en cytokines/chimiokines ont été mesurées. De plus, nous avons réalisé la microscopie électronique à transmission (TEM), l'analyse de suivi

des nanoparticules (NTA) et l'analyse protéomique du contenu exosomique libéré par les cellules inflammatoires recrutées dans la cavité péritonéale. Les données recueillies à ce jour suggèrent que toutes les souches de *Leishmania* avec et sans GP63 provoquent un recrutement cellulaire inflammatoire similaire. Cependant, les résultats des cytokines/chimiokines montrent quelques variabilités qui ne sont toutefois pas suffisantes pour expliquer pourquoi les *L. major*^{KO} causent une infection moins agressive *in vivo*. L'absence de la GP63 pourrait peut-être conduire à une moins grande internalisation des promastigotes dans les moments initiaux de l'infection comme le suggère le moins grand nombre d'amastigotes retrouvés dans les cellules hôtes infectées par *L. major*^{KO}. Néanmoins, nos résultats démontre que *L. major*^{KO} semble moins apte à se maintenir au même niveau d'infection et survivre que les autres souches de *Leishmania* étudiées. Collectivement, cette étude démontrent que l'absence de la GP63 chez le parasite *L. major* ne semble pas influencer la réponse inflammatoire innée et autres événements reliés pouvant expliquer la différence de niveau d'infection *in vivo* causé par les *L. major*^{KO} comparativement aux souches *L. major*^{WT} et *L. major*^{R}, mais que le niveau d'infection initial significativement plus bas observé *in vitro* et *in vivo* pourrait expliquer qu'un plus faible lot parasitaire se refléterait par un développement pathologique moindre. Collectivement cette étude nous a permis d'acquérir de nouvelles connaissances au sujet de l'interaction entre ce parasite et son hôte, ce qui pourrait conduire au développement de nouveaux moyens pour lutter contre son infection et sa propagation.

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PREFACE

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation". The candidate has chosen to present her thesis as a "manuscript-based thesis": "as an alternative to the traditional format, the thesis may be presented as a collection of scholarly papers of which the student is the first author or co-first author; that is, a manuscript based Master's thesis must include the text of one or more manuscripts published, submitted, or to be submitted for publication and re-formatted according to the described requirements. The thesis must contain additional text that will connect the manuscript in a logical progression, producing a cohesive, unitary focus, and documenting a single program of research."
All work towards this thesis was performed under the supervision of Dr. Martin Olivier. The candidate is the first author of the manuscript presented in Chapter II, which will be submitted for publication. Author contributions are as described:

Overall project was designed and envisioned by Martin Olivier. In vivo experiments were designed and performed by Aretha Chan and M Olivier with aid from Caroline Martel. Flow cytometry experiments were performed by Fernando Alvarez in the laboratory of Ciriaco Piccirillo and data was analyzed by F Alvarez and A Chan. TEM imaging was performed by George Dong and A Chan. NTA measurements, preparation of samples for LC-MS/MS, and proteomic analysis was performed by A Chan with guidance from Andrea Vucetic. Statistical analysis was performed by A Chan with guidance from Alonso Lira Filho. A Chan wrote the manuscript with significant editorial guidance and direction from M Olivier.

LIST OF ABBREVIATIONS

AP	activator protein
BSA	bovine serum albumin
CR	complement receptor
CDC	Centre for Disease Control
CL	cutaneous leishmaniasis
COX	cyclooxygenase
CP	cysteine protease
DC	dendritic cell
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
EIF4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
EV	extracellular vesicles
FBS	fetal bovine serum
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP63	glycoprotein 63
IFN	interferon
IL	interleukin
IL	Interleukin
IP-10	interferon g-induced protein 10
IP	intraperitoneal
IRAK	Interleukin Receptor Associated Kinase
JAK/STAT	Janus kinase and signal transducer and activator of transcription

KC	keratinocyte chemoattractant
KO	knockout
LIF	leukaemia inhibitory factor
LIX	lipopolysaccharide-induced CXC chemokine
LPS	lipopolysaccharide
M-CSF	monocyte colony-stimulating factor
MCP	monocyte chemoattractant protein
MCL	mucocutaneous leishmaniasis
MDC	macrophage-derived chemokine
MIG	monokine induced by interferon gamma
MIP	macrophage inflammatory protein
NET	neutrophil extracellular trap
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP	NLR Family Pyrin
NO	nitric oxide
NTA	nanoparticle tracking analysis
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PBS	phosphate-buffered saline
PTP	protein tyrosine phosphatases
PVDF	Polyvinylidene fluoride
RANTES	regulated on activation normal T cell expressed and secreted
SDM	Schneider's Drosophila Medium
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis

TARC	thymus and activation regulated chemokine
TEM	Transmission electron microscope
TH	T helper cell
TIMP	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TNF	tumour necrosis factor
VAMP	vesicle associated membrane protein
VEGF	vascular endothelial growth factor
VL	visceral leishmaniasis
WHO	World Health Organization

CHAPTER 1: REVIEW OF LITERATURE AND OBJECTIVES OF RESEARCH

1.1 *Leishmania*

1.1.1 Introduction to *Leishmania* and Leishmaniasis

Leishmania is an intracellular protozoan parasite of the trypanosomatid family that causes the disease leishmaniasis. Each year, there are an estimated 1 million new cases worldwide, and about 26 000 to 65 000 deaths occur as reported by WHO [1]. Leishmaniasis used to affect mainly tropical countries with low socioeconomic status. However, recent changes in the climate permitted vector spread and transmission to areas that were not endemic for the disease. There are over 1 billion people at risk in endemic areas, and this number is only growing.

Leishmaniasis is a vector-borne disease caused by over 20 species of *Leishmania* and carried by the female phlebotomine sandfly. The sandflies, *Leishmania*, and their reservoirs are found in 90 countries from all continents except for Australia and Antarctica [2]. There are three forms of leishmaniasis: visceral, mucocutaneous, and cutaneous, which varies depending on the species of the infecting *Leishmania*. Currently, the drugs used to treat leishmaniasis face issues such as toxicity, high cost, drug resistance, presenting a need for novel anti-*Leishmania* therapeutics and a better understanding of the disease [3].

1.1.2 Ecology

The ecology and the changing climate have a significant impact on the epidemiology of many vector-borne, zoonotic diseases. In the past few decades, endemic regions have been spreading, and the prevalence of leishmaniasis has been increasing since. There are several ways

that climate change plays a part in the distribution of leishmaniasis, some direct and some indirect. In a direct sense, the changes in temperature affect the development of infective *Leishmania* within the gut of the sandfly, which are sensitive to small alterations [4], [5]. Aside from this, the main contributing factor is the environmental effect that affects the range and population of the sandfly vector. Another indirect factor is the movement of human populations into endemic areas due to a variety of socio-economic factors. The transmission of leishmaniasis used to be associated with sylvatic tropical climates when people entered forested areas. Now, urbanization allowed for the increase of transmission because of the spread of suburbs to reservoir (rodent) colonies and irrigation systems expanding the reservoir range [6].

A study was performed in southwest Asia to model the distribution of the sandfly *Phlebotomus papatasi* as a result of global warming. Temperature values of 115 weather stations were increased by 1, 3, and 5 degrees Celcius. Then the areas were categorized by whether or not it could support the endemic transmission of leishmaniasis by the sandflies. At current temperatures, 71 stations were considered endemic, but each temperature raise brought them a ~10% increase in endemic areas. They also saw a lengthening in the seasonality of disease transmission with the increase in temperature [7]. This study, along with the growing numbers of case studies demonstrate the threat of the emergence of leishmaniasis.

1.1.3 Epidemiology

The prevalence of leishmaniasis is estimated to be extremely high worldwide, and the spread is due to several risk factors, including: human made environmental changes, population immune status, and resistance to treatment. There have been recurrent epidemics of leishmaniasis, which can affect many people and are still reported to this day. Most recently, as

reported in July 2019, there was an outbreak of *L. major* amongst Chinese construction workers returning from Uzbekistan [8]. Such reports cause alarm because it is the movement of infected persons into urban areas that can further spread epidemics. Epidemics of visceral leishmaniasis can cause a large number of deaths, fueled by to poor disease surveillance and lack of access to the expensive medication. In particular, Sudan has faced many devastating epidemics, one from 1984 to 1994 was reported to have killed 100 000 individuals in a population of 280 000 in the area [9]. The frequency of outbreaks has also been seen to increase. In Brazil, rural epidemics previously occurred in cycles of 10 years, but large outbreaks and epidemics were recorded in major cities recently. The outbreaks were due to massive migrations of people from rural to suburban areas as a result of droughts, lack of available farmland, and famine [10]. Many places that face epidemics were not prepared for quick diagnosis and administration of drugs to large populations of affected people, which calls for better diagnostic and treatment solutions for leishmaniasis.

1.1.4 Life cycle

The life cycle of the *Leishmania* starts in the sandfly vector in the promastigote phase. A female phlebotomine sandfly takes a blood meal from a *Leishmania* infected human or reservoir, which allows macrophages infected with *Leishmania* to enter the sandfly digestive system. Other mammalian reservoirs of various species of *Leishmania* include opossums, foxes, dogs, cats, rats, hamsters, and more [11]. The blood meal is digested in the midgut, where the *Leishmania* must survive proteolytic digestion and adhere itself to the midgut epithelium to prevent excretion [12]. From the amastigote form, the *Leishmania* transforms into procyclic promastigotes in the posterior midgut. Promastigotes divide in the *Leishmania* mainly by binary fission: where

daughter flagellum is produced first, then division of the nucleus, kinetoplast, and lastly, the body [13]. These differentiate into nectonomad promastigotes, which attach to the anterior midgut and further differentiate into the replicative leptonomad form. Eventually, they differentiate into its infective, non-dividing form of metacyclic promastigotes [14]. The promastigotes secrete proteophosphoglycan (PSG), which is a gel-like substance that creates a plug blocking the anterior midgut and extends into the foregut of the sandfly. When it is time for the sandfly to take a second blood meal, it must regurgitate the *Leishmania* in order to consume the blood. The *Leishmania* plug was known as the "blocked fly hypothesis" to describe the transmission mechanism of *Leishmania*. The sandfly inserts its mouthparts into the skin and agitates it such that blood would pool from broken capillaries. The regurgitated *Leishmania* is deposited, and the tissue damage aids in the recruitment of neutrophils and skin macrophages to the site [15].

The second phase of the *Leishmania* life cycle exists in the mammalian host in the amastigote form. At the site of inoculation, the *Leishmania* attaches to the skin macrophages while the macrophages phagocytose the parasite. There is a rapid low-affinity interaction between the cell and promastigotes. Then a high-affinity mechanism that allows pseudopods to engulf the parasite [16]. Several virulence factors facilitate this attachment in a receptor-mediated manner, which will be discussed in-depth in section 1.4. They are taken up by phagocytosis into the phagosome, which combines with lysosomes to form the phagolysosome [17]. Inside the acidic phagolysosome, *Leishmania* must inhibit microbicidal peptides and enzymes in order to survive. The promastigotes then differentiate into the replicative, non-flagellated amastigote form. Lastly, the amastigotes replicate until the host cell lyses or becomes apoptotic, where they infect nearby cells and repeat the life cycle in a feeding sandfly (Figure 1).

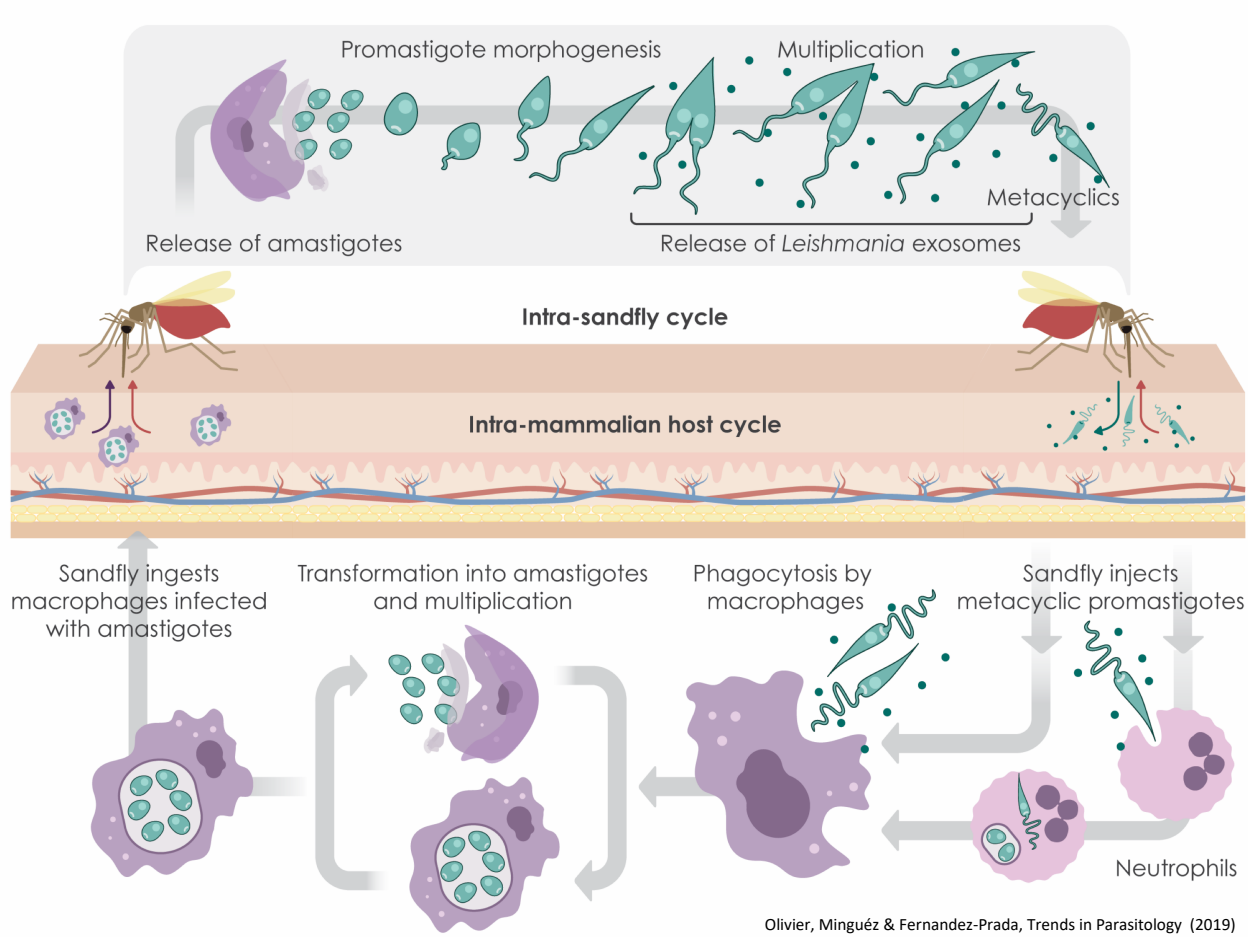

Figure 1: Life cycle of Leishmania (Olivier et al. 2019)

1.2 Leishmaniases

The leishmaniases is a collection of diseases caused by different species of *Leishmania* and vary vastly in its clinical manifestations.

1.2.1 Visceral leishmaniasis

The most severe form of leishmaniasis is visceral leishmaniasis (VL), with about 400 000 people affected and 40 000 deaths per year [18]. *L. donovani* and *L. infantum* are the main

causative species in the old world, found in the Indian subcontinent and East Africa. *L. donovani* is spread from human to human, while *L. infantum* has dogs and other canines as additional hosts. Many people affected in the Indian subcontinent live in humid rural villages, while those affected in East Africa are migratory populations displaced by war and drought [19]. The *Leishmania* can spread systematically to infect macrophages found in the liver, spleen, bone marrow, and lymph nodes. After the initial infection with *Leishmania*, there is a two-week to 18 month incubation period, with inflammatory events happening in the organs at 2 to 8 weeks. However, symptoms can take years to appear, which include long-term, low-grade fever, enlargement of spleen and liver, pancytopenia and polyclonal hypergammaglobulinemia [20]. If the symptomatic infection is left untreated, it is generally fatal with a mortality rate of 75-95% [21]. For the treatment of VL, the drugs pentavalent antimony, Miltefosine and Amphotericin B are used [22]. Unfortunately, they are quite toxic and expensive, and there are other barriers to treatment such as drug resistance and poverty.

Even after the treatment of VL, there is the possibility of the development of post-kala azar dermal leishmaniasis (PKDL). It was seen mainly in India and Sudan in asymptomatic patients treated for *L. donovani* infection. Months or years after VL treatment, there is a huge proliferation of parasites in the skin, resulting in macular, maculopapular or nodular lesions [23].

1.2.2 Cutaneous Leishmaniasis

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis, with an estimated incidence rate of 1.5 million per year in 82 countries [18]. There are about 15 species of *Leishmania* that cause CL and are classified as either old world (southern Europe, the Middle East, Asia, and Africa) or new world leishmaniasis (Latin America). The species *L. major* and *L.*

tropica are the main causative agents in the old world, *while L. mexicana* and *L. amazonensis* are in the new world [24]. Aside from *L. tropica*, the species that cause CL are zoonotic and have rodent or domestic animal reservoirs. Unfortunately, recent human mobility and ecotourism result in more people entering habitats of *Leishmania* and their reservoirs, which allow CL to spread to nonendemic areas.

Cutaneous leishmaniasis can be further classified based on clinical presentation, which varies depending on species and host factors. Localized cutaneous leishmaniasis (LCL) is the most prevalent, where a round pink lesion with raised edges are found on the skin. Disseminated leishmaniasis is characterized by multiple (10-300) lesions in different areas of the body, likely caused by lymphatic spread. Diffuse cutaneous leishmaniasis is a rare condition that evolves from LCL, but the lesions spread and are filled with parasites [25]. The treatment for CL utilizes the same systemic drugs as VL and could also be combined with local treatment such as cryotherapy and topical imiquimod. There is no vaccine for leishmaniasis, and there is a great need to prevent the further spread of the disease.

1.2.3 Mucocutaneous Leishmaniasis

Mucocutaneous leishmaniasis (MCL) usually occurs after an infection with cutaneous leishmaniasis, appearing months or years after. The majority of the cases occur in south or central America, caused mainly by *L. viannia braziliensis* in the new world and *L. major* in the old world [26]. Mucosal tissue in the nose is usually affected but can also spread to oral mucosa or parts of the face. Symptoms of the infection start off nonspecific, such as nasal congestion and inflammation, but can deteriorate to ulceration and perforation of the septum, resulting in permanent disfiguration. MCL is the least common form of leishmaniasis, and the factors that

cause the development of MCL from cutaneous leishmaniasis are not yet well understood. Currently, the only drugs used to treat MCL are the aforementioned systemic ones [25].

1.3 Host immune response

The host response to *Leishmania* is the major determinant of the outcome of disease progression. Some people are resistant, some patients' lesions spontaneously heal, while other patients develop non-healing lesions. It is imperative that the host mount an appropriate immune response to clear the parasite because immunopathology may also exacerbate the disease.

1.3.1 Innate Immune Response

Once the *Leishmania* breaches the host skin through a sandfly bite, the innate immune system springs into action to eliminate the pathogen. Innate immune cells express pattern recognition receptors such as Toll-like receptors (TLRs) that recognize *Leishmania* to initiate the immune response. One of the very first cell types recruited to the site of infection are neutrophils. They are the most abundant cell type recruited when *L. major* is injected into mouse subcutaneous air pouches [27]. Neutrophils have been shown *in vitro* to be hijacked by *Leishmania* to act as "Trojan horses" that will allow promastigotes to be shuttled safely into macrophages [28]. Neutrophils would phagocytose promastigotes, but promastigotes are able to survive within the phagosomes that usually kills the pathogen. The infected neutrophils become apoptotic and are ingested and cleared by macrophages. In the healthy physiological turnover of cells, apoptotic cells are common and pose no danger to macrophages. Therefore, the antimicrobial response in the macrophages is not activated when they ingest these *Leishmania*-filled neutrophil morsels. However, another study using in situ imaging demonstrated that

neutrophils subsequently release the parasites before they are actively phagocytosed by macrophages [29]. The uptake of *Leishmania* by neutrophils is important for the infectivity *in vivo*, as seen in the aforementioned study using imaging and flow cytometry at the site of infection [29]. Also, neutrophils can play a role in the clearance of *Leishmania*. In a study performed on *L. amazonensis*, neutrophil extracellular traps (NETs) were able to kill the parasite [30].

The other cells present during the initial infection are resident dermal macrophages and dendritic cells (DCs). These cells rapidly phagocytose promastigotes, where macrophages become the dominant infected cell type 24 hours post-infection [29]. Since the number of resident macrophages and dendritic cells is insufficient to sustain the infection, more cells are recruited to the scene. An infection with *L. major* induced chemokines involved in the recruitment of macrophages/monocytes (RANTES, MIP-1α, MIP-1β, IP-10, MCP-1), neutrophils (MIP-2, TCA-3), and eosinophils (RANTES, eotaxin, MIP-1α). In the same study, *L. major* induced the production of proinflammatory cytokines tumour necrosis factor alpha (TNF-a) and interleukin 1 beta (IL-1b) while *L. donovani* does not [27]. Monocyte derived dendritic cells are also recruited, which upregulate major histocompatibility complex class II (MHC class II) molecules for antigen presentation and produce IL-12. Il-12 is important for the T-helper cell (Th1) response, which is protective against leishmaniasis [31]. Stromal cells could also be infected and serve as a host in latent leishmaniasis and produce CCL8 and CXCL12 that direct the differentiation of regulatory DCs [32].

1.3.2 Adaptive Immune Response

Research to date describes how the type of immune response mounted by the host can dictate the outcome of a cutaneous *Leishmania* infection. Mice research demonstrates that a type 1 response will lead to the clearance of *Leishmania*, while a cell-mediated type 2 response will result in the persistence of infection. A Th2 polarized response is initiated from interleukin-4 (IL-4) production by CD4+ T-cells that recognize the *Leishmania* antigen LACK (*Leishmania* homologue of receptors for activated C kinase) [33]. A BALB/c mouse model that lacks LACK-recognizing Vβ4Vα8 T-cell receptor (TCR) T-cells were seen to mount a stronger Th1 response and clear *Leishmania* lesions [34]. The early IL-4 production from LACK-reactive CD4+ T cells suppresses Th1 cell development and inhibits secretion of interferon gamma (IFN-γ) that is necessary to activate macrophages for parasite killing. The clearance of *Leishmania* is mediated by the production of type 1 cytokines (IFN-γ, IL-12 and TNF), receptors (IFN-γ R), transcription factors (T-bet and STAT4), and co-stimulation molecules (CD40-CD40L). This collection of immune mediators are essential for the development or function of Th1 cells that will confer protection for *L. major* [35]. In C57BL/6 mice, CD4+CD25- regulatory T cells drive the protection towards *L. major* and are suppressed by CD4+CD25+ regulatory T cells [36]. Infected macrophages need to upregulate the expression of inducible nitric oxide synthase (iNOS) through TNF signalling in order to clear the *Leishmania* [37], as well as undergo apoptosis through the Fas-Fas ligand pathway [38]. This dichotomy is not confirmed in human patients; however, the implication is that the host immune response plays an immense role in disease outcome.

It was also seen that *L. major* parasites could be found latently in patients and mice long after clinical cure. Healed mice are protected from reinfection for life because the latent sequestered *Leishmania* are chronically suppressed through the maintenance of CD4+ and CD8+

T cells, IL-12, IFN-γ and iNOS [39]. Studies have shown that impairment of these responses will allow the parasite to replicate and re-establish infection. The latent reservoir of *L. major* was found to be fibroblasts [40] and DCs [41], which is maintained through the expression of IL-10 produced by CD4+CD25+CD45RB low immunoregulatory T cells [36].

1.3.3 Extracellular Vesicles

In recent years, there has been growing research demonstrating the importance of extracellular vesicles (EVs). The term EVs is an umbrella term for all cell-secreted phospholipid bilayer-bound structures released into a variety of biological fluids. They can be further characterized into exosomes, microvesicles, and apoptotic bodies, according to size and biogenesis. EVs are ubiquitous, as they are generated by every cell type investigated *in vitro* and *in vivo* studies [42], and are involved in intracellular communication and influence of immune responses.

In this study, we have focused on exosomes, which are classified as 50-100 nanometres in diameter, and are generated from the exocytosis of multivesicular bodies from the multivesicular endosome fusion with the plasma membrane [43]. Cargo carried by exosomes is very diverse, including bioreactive molecules such as nucleic acids, cytokine-receptor complexes, and enzymes [44]. Exosomes derived from macrophages and DCs infected with bacteria were shown to be pro-inflammatory to naïve macrophages and stimulate DCs, CD4+ and CD8+ T-cells [45]. Therefore, we investigated the biology of exosomes released by cells infected with *L. major* as another medium to observe immune activation and modulation as a result of infection.

Past research in our laboratory demonstrated the immunomodulatory impact of exosomes produced by macrophages infected by *L. major* [46]. The proteomic analysis of exosome content revealed that infection with *L. major* and stimulation with LPS were able to modulate protein expression that generated a vastly different exosome content profile. Exosomes were shown to stimulate naïve macrophages, leading to the translocation of pro-inflammatory transcription factors NFκB and AP-1 to the nucleus. However, *Leishmania*-induced exosomes were much less inflammatory than that of LPS due to the *Leishmania* immunomodulatory factors that prevent macrophage activation.

Exosomes originating from the *Leishmania* were found to have immunomodulatory effects as well. The temperature shift from ambient temperatures to mammalian 37 degrees Celsius causes the upregulation of exosome secretion in *Leishmania* [47]. They are also produced by *Leishmania* in the sandfly midgut and transmitted to the host while taking a blood meal and found to exacerbate the diseases [48].

1.3.4 Experimental mouse models of leishmaniasis

There are varying differences in the mouse models employed by researchers to study leishmaniasis, for the analysis of different experimental observations. As discussed in section 1.3.2, the type of immune response is determined by the strain of mice, resulting in parasite clearance or chronic infection. The C57BL/6 genotype is generally used to study the self-limiting infections found in natural hosts, and the BALB/C genotype is used to model the non-healing forms of the disease [39]. Another important factor is the dosage of *leishmania* inoculum. In a natural sandfly bite, the inoculation size is estimated to be about 1000 egested parasites when measured using a membrane feeding system [49]. However, the routine experimental dosage is

10^2–10^7 parasites to generate a fast response, but it is unknown if it can accurately reflect natural transmission [50]. Lastly, the site of injection determines the parameters that will be studied as well as the course of the disease progression. To model cutaneous leishmaniasis, a standard method is a subcutaneous injection into the footpad, which is tracked over several weeks. The lesion development can also be studied when injected into the mouse ear dermis and other subcutaneous locations. A murine air pouch model can be used to obtain cellular products, peaking at 6 hours post-infection to study the immune response [27]. Intraperitoneal injection of leishmania is not a conventional way to study a natural progression of cutaneous leishmaniasis, but it allows for the collection of cells, cytokines/chemokines, and exosomes. The abundance of naïve tissue-resident macrophages and permissiveness to cell recruitment in the cavity offers a robust immune response that can be analyzed efficiently [51].

1.4 Virulence factors

Throughout the evolution of *Leishmania* alongside their hosts, *Leishmania* has acquired an array of strategies to evade the innate immune system. These include blocking immediate host cell functions such as nitric oxide and reactive oxygen species production through the influence of signalling pathways and transcription factors. Several *Leishmania* molecules are known as virulence factors and mediate the modulation of host cell effector functions and signalling (Figure 2). Lipophosphoglycan (LPG) is the most abundant glycoconjugate found on the *Leishmania* surface, which inhibits protein kinase C (PKC), inhibits phagosome maturation, and allows the parasite to attach to the sandfly midgut. Proteophosphoglycans, or PPGs, are another group of glycoconjugates that help form the parasite plug in the sandfly and can also increase macrophage recruitment. The *Leishmania* surface is also coated with glycosylinositol

phospholipids (GIPLs), which inhibit PKC activity and increases *Leishmania* infectivity. Another group of immunomodulatory virulence factors are cysteine proteases (CPs). They were shown to shift the adaptive immune response from Th1 to Th2, degrades transcription factors NFκB, STAT1 and AP1, but was also a promising vaccine candidate. CPB (cysteine protease B) is involved in the regulation of GP63 expression, allowing for the downregulation of VAMP(Vesicle Associated Membrane Protein) 3 and 8 proteins involved in phagolysosome biogenesis and function [52]. Lastly, secreted acid phosphatases (SAPs) are less well-studied but were shown to prevent oxidative burst in neutrophils and assist in *Leishmania* metabolism [53].

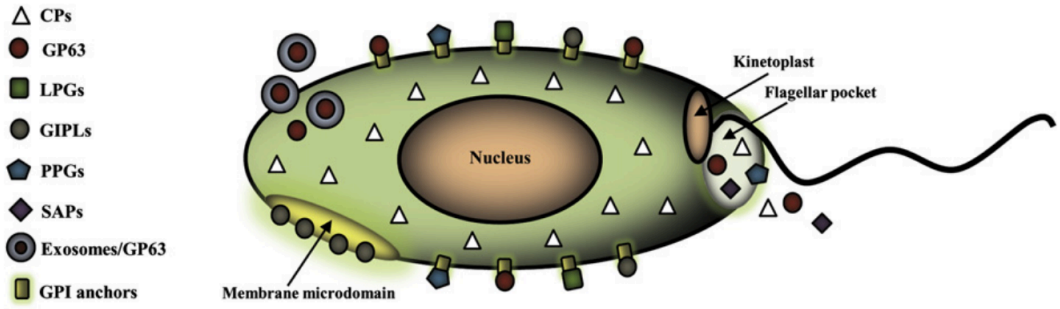

Figure 2: Leishmania virulence factors *(Olivier et al. 2012)*

1.4.1 GP63

An important virulence factor is GP63 or leishmanolysin, which is a zinc-metalloprotease that functions as a major surface antigen in *Leishmania* promastigotes. It is found to be bound on the membrane through a GlycosylPhosphatidylInositol (GPI) anchor, and could also be released in exosomes, or secreted through the flagellar pocket (Figure 2). All *Leishmania* species express GP63 in promastigote form and some in amastigotes as well, where expression is downregulated in amastigotes [54]. It was named leishmanolysin due to its protease properties and had a variety of substrates such as gelatin, albumin, hemoglobin, and fibrinogen [55]. GP63 was demonstrated

in vitro to be able to degrade many proteins in the extracellular matrix, enhancing *Leishmania* ability to bind to cells and migrate within the host [56]. GP63 was found to directly cleave complement C3b into iC3b, which does not amplify the complement cascade and allows the parasite to escape complement-mediated lysis [57]. In addition, iC3b can opsonize the parasite and interact with macrophage complement receptors 1 and 3, subsequently promoting phagocytosis and internalization of the promastigote [58]. To further favour adhesion and internalization of parasites, GP63 interacts with host B1 integrins (VLA-4, VLA-5), which are receptors that interact with fibronectin-like molecules like GP63 [59].

The LC3-associated phagocytosis is a non-canonical autophagic process that *Leishmania* also evades through GP63 action because the process enhances phagosome maturation and function. GP63 impaired the recruitment of LC3 to phagosomes, which is mediated by cytochrome b (CYBB). GP63 directly cleaves VAMP8, which will inhibit the recruitment and assembly of CYBB, and therefore preventing LC3 from linking the autophagy pathway from phagocytosis [60]. This study demonstrated how GP63 could enhance *Leishmania* survival within the host cell.

GP63 also modulates the host immune response through prostaglandin-endoperoxide synthase (PTGS2) activity. PTGS2 enzymes produce prostaglandins, which modulate the immune response through inhibition of the Th1 response and promoting Th2 cytokine production. An anti-mouse PTGS2 polyclonal antibody recognized GP63, despite having low homology with classic PTSG. Researchers purified the protein from *Leishmania* and expressed recombinant GP63, which both have PTSG activity [61].

The host NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome activation was also seen to be inhibited by GP63. Some components of the NLRP3 inflammasome is

directly cleaved by GP63, and IL1-b is also inhibited, one of the components necessary for inflammasome activation. This activation process is ROS (reactive oxygen species) mediated, and GP63 was seen to diminish the production of ROS in THP-1 cells stimulated with DAMPS (damage associated molecular patterns) [62].

Many cell signalling functions rely on phosphorylation and dephosphorylation of proteins by kinases and phosphatases to regulate downstream effects. *Leishmania* GP63 was found to hijack negative regulation by phosphatases to prevent and escape macrophage anti-microbial functions. Work over the years has elucidated many of the interactions that *Leishmania* GP63 make with signalling pathways in host cells (Figure 3).

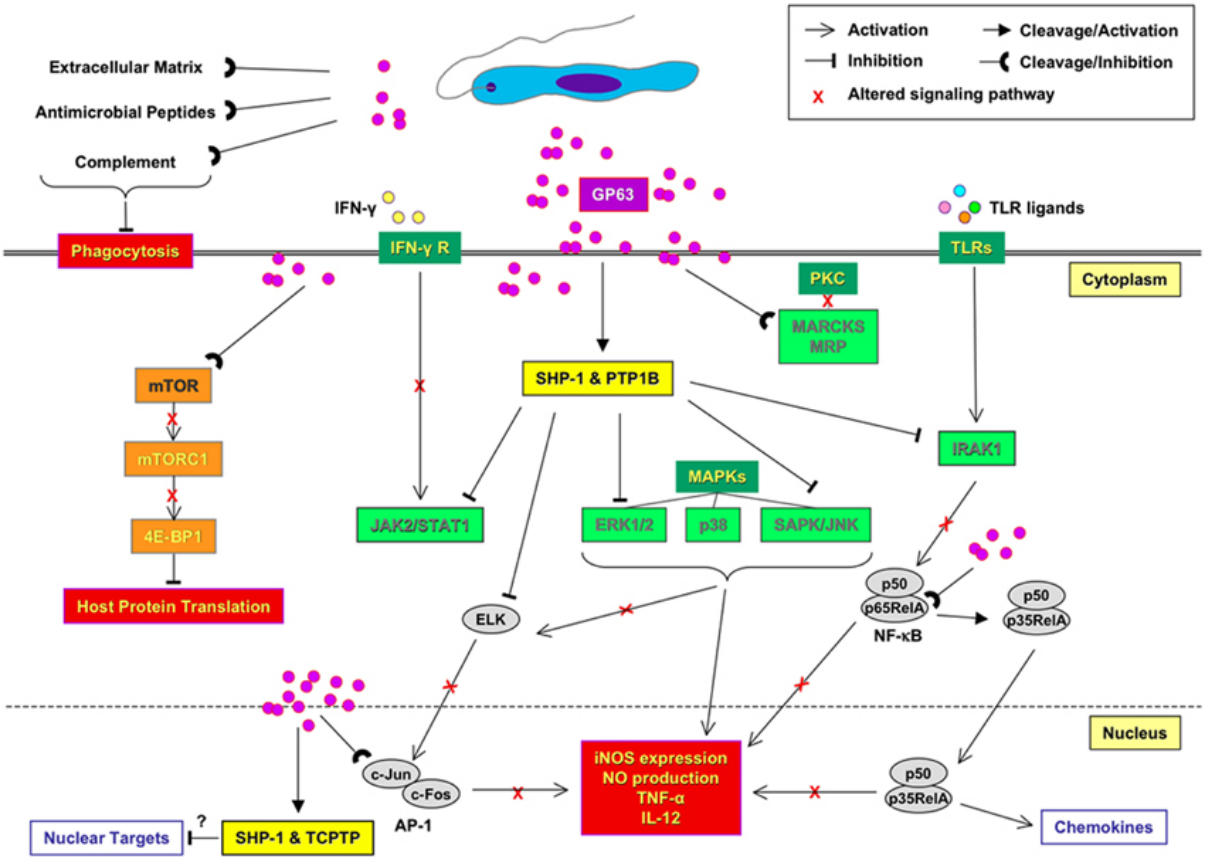

Figure 3: Impact of GP63 on Macrophage Signalling *(Isnard et al. 2012)*

The myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS related proteins (MRP) signalling is an early macrophage pathway affected by *Leishmania* GP63. Macrophages infected with *L. major* had significant MRP depletion and this degradation was inhibited using GP63 inhibitors or mutation of MRP [63]. MRP is a major substrate for protein kinase c (PKC), which is a kinase involved in signalling for many activities, including the production of radical oxygen species.

A major pathway affected is the JAK/STAT pathway, which is important for IFN-γ signalling, leading to the production of nitric oxide, an antimicrobial agent. Many pathways are negatively regulated by protein tyrosine phosphatases (PTP) PTP1B and SHP-1, a process that *Leishmania* exploits by cleavage of PTP domains by GP63 [64]. In turn, the phosphatases are activated, inactivating IFN-γ signalling, as well as TLR signalling by inactivating IRAK1 (Interleukin 1 Receptor Associated Kinase 1) [65]. This inactivation led to the inability of LPS to induce production of TNF, NO, and IL-12. The activation of PTPs also affects MAPK (Mitogen Activated Protein Kinases) pathways, which include ERK1/2, p38 and SAPK/JNK [53].

The phagosomes, adaptor molecules of the DOK protein family negatively regulate signalling in response to LPS and other cytokines. In the event of a *Leishmania* infection, DOK proteins are involved in positive regulation of TNF and NO production induced by *Leishmania* infection in IFN-γ activated macrophages. It was seen that GP63 directly cleaves DOK1 and 2, preventing its function in the phagosomes [66].

Host protein translation was also found to be downregulated due to the alteration of mTOR (mammalian target of rapamycin) signalling. *Leishmania* GP63 cleaves mTOR, which is a kinase that negatively regulates EIF4EBP1 (Eukaryotic translation initiation factor 4E-binding

protein 1), a translation inhibitor. The active inhibitor allows for *Leishmania* survival, where genetic deletion of EIF4EBP1 reduced parasite load in macrophages [67].

Macrophage activity is affected down to the gene expression level due to the influence of GP63 on transcription factors. The transcription factor AP-1 regulates pro-inflammatory cytokines, chemokines, and nitric oxide production, whose activity was shown to be abolished in the event of *Leishmania* infection. C-Jun is a central component of AP-1 and is cleaved by GP63, as seen in nuclear extracts and culture supernatant [68]. The STAT family of transcriptions factors were also seen altered in *Leishmania* infection, but not solely due to the PTP inhibition of the kinases upstream. Using PTP inhibitors and SHP-1 deficient cells, it was shown that STAT1 inactivation is independent of PTP and dependent on PKC regulation of nuclear proteasomes [69]. *Leishmania* GP63 is also able to cleave NFκB, a transcription factor implicated in a wide range of immune functions such as cytokine-mediated signalling. The subunit NF-κB p65 RelA is cleaved in the cytoplasm and results in a p35 RelA novel fragment [70]. This fragment binds DNA with NF-κB p50 and activates transcription of chemokine genes (CXCL2, CCL2, CCL3, CCL4), TNF, and IL-10, despite lacking the transactivation domain [71]. Many studies have identified GP63 as a reliable candidate in methods to detect leishmaniasis in clinical samples for diagnosis and for vaccine development.

1.5 GP63 knockout parasites

The *L. major* GP63 genes were known to consist of 7 homologous tandemly arranged repeats on a single chromosome, chromosome 10 [72]. Gene expression is developmentally regulated, where genes 1-5 are expressed in high levels in promastigotes, gene 6 is expressed in low levels in both promastigotes and amastigotes, and gene 7 is expressed only in metacyclic

promastigotes and amastigotes [73]. Each gene is about 1.3kb long in the coding sequence, with 1.8kb long intergenic regions in between genes 1-5 and genes 6 and 7 located 3' to the repeats. Newer genome studies showed there is also a single GP63 gene on chromosome 28, and a related gene on chromosome 31 [74].

The group Joshi et al. deleted the entire 20kb region containing seven GP63 genes to generate a GP63 knockout parasite in order to study its role in *Leishmania* biology and infection. They generated plasmids containing resistance genes to nourseothricin (sat) or hygromycin B (hyg), along with a 3' untranslated region of *L. major* dhfr-ts (dihydrofolate reductase-thymidylate synthase) gene to ensure high-level expression (Figure 4A). Restriction enzyme Xho1 was used to insert the cassette into wildtype *Leishmania* [NIH S (MHOM/SN/74/Seidman) clone A2] by recombination for two rounds of genomic integration. Using a Southern blot analysis on genomic DNA, they saw no GP63 (leishmanolysin) fragment in the transfected *Leishmania* (Figure 4B). A recombination probe was used on a gene segment outside of the recombination region of the plasmid and within the restriction site to detect any homologous recombination (Figure 4C).

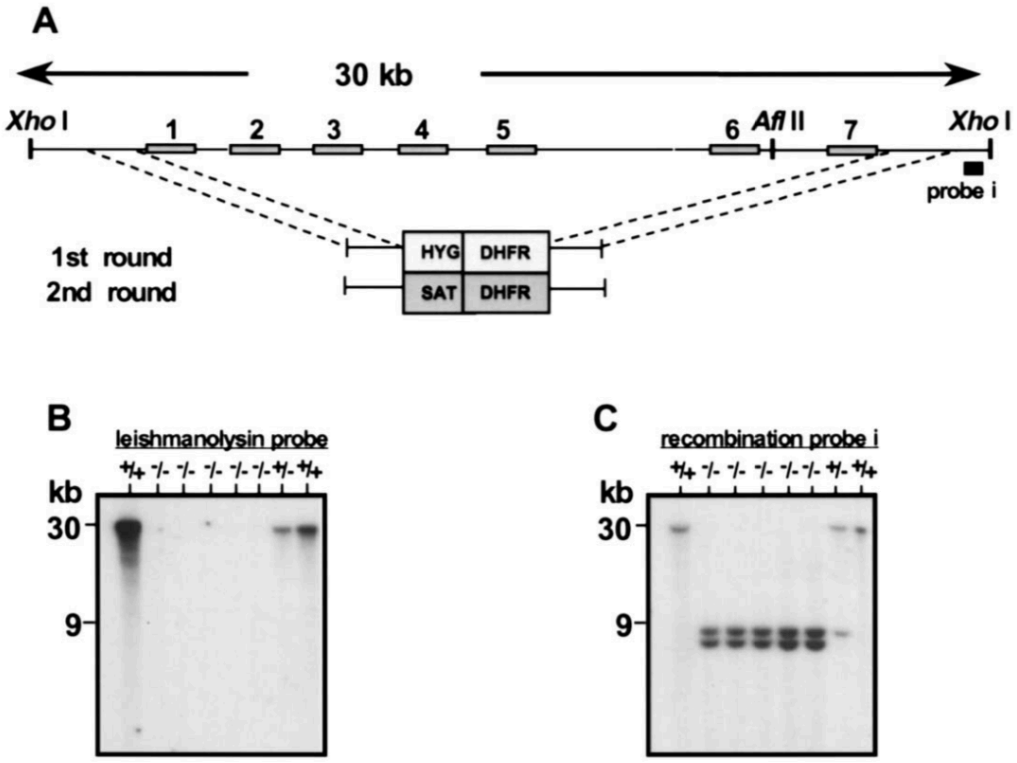

Figure 4: Genetic analysis of GP63 knockout L. major. (Joshi et al, 2002).

In a clone deficient of GP63, the expression and activity of GP63 were restored by the transfection of plasmid pLEXNEO-GP63 gene 1 (GP63 rescue). The level of GP63 surface expression was comparable to wild type *Leishmania* when quantified in flow cytometry. The proteinase activity of GP63 was also restored in this strain.

These transfected *Leishmania* clones were able to develop within the sandfly gut similar to levels observed in wild type infected sandflies, demonstrating GP63 is not essential for growth and development. Sensitivity to complement mediated lysis was also assayed, where GP63 knockout parasites reached 100% lysis at 2% human serum, while wild type parasites reached 100% lysis at 11% human serum. Resistance to lysis was significantly improved in the GP63 rescue parasites. The main finding of interest was the delay in lesion development in mouse

footpads inoculated with the GP63 knockout parasites. One footpad of BALB/c mice was inoculated with 1×10^6 or 4×10^3 parasites, and they saw a 3.5-4 week delay for the lesions to reach 3mm in the GP63 knockout parasite group compared to wild type. The reintroduction of GP63 gene 1 improved the speed of lesion development, but not to the original extent. The researchers postulated that GP63 genes 6 and 7 might have different proteinase substrates, or gene 1 is regulated differently; therefore, the GP63 rescue parasites did not behave the same as wild type parasites. To conclude, they hypothesize that GP63 knockout parasites were more susceptible to complement mediated lysis during the initial inoculation, making it longer for the surviving parasites to establish the infection.

1.6 Objectives of research and rationale

GP63 is proven to be an important virulence factor since *L. major* deficient in GP63 causes delayed lesion development compared to wild type parasites, which raises the possibility that host factors are influencing the pathological outcome. Given that *L. major* infections induce the recruitment of innate immune cells and GP63 influences cell signalling, this was an important avenue to investigate. In addition to cytokine production from recruited cells, the exosomes released by infected cells could also provide insight into the innate immune response. Furthermore, the survival of the parasites within cells immediately following the *in vivo* infection will reveal the role of GP63 in the progression of the disease. The modulation of the global early innate response due to GP63 has not yet been described and would provide valuable insight into the development of disease in human patients.

This leads us to our research question: how does *Leishmania* GP63 influence the early innate immune response?

In my project proposal, we hypothesized that "*Leishmania* GP63 alters the innate immune response such as cell recruitment and cytokine production because of their modulation of cell signalling, resulting in a delay in lesion development in mouse footpads."

To determine the validity of this hypothesis, the following research objectives were proposed:

1. Perform *in vivo* experiments to verify a delay in lesion development using the GP63 knockout *L. major* strain in C57BL/6 mice.

2. a) Perform in vivo experiments to analyze innate immune cellular events during a 6-hour intraperitoneal infection using the GP63 knockout *L. major* strain in C57BL/6 mice to discern the inflammatory role of GP63 in a global view.

   b) Observe the progression of infection in infected cells retrieved from the intraperitoneal infection

CHAPTER 2: METHODS, RESULTS, AND DISCUSSION

2.1 Preface

The results of this project will be submitted in the form of a manuscript for publication. The paper focuses on the research objectives as laid out above, that is the investigation and comparing of the inflammatory response following an infection with *Leishmania* major in regard to GP63 expression. In the case of this paper, the experimental organisms used were strains of *Leishmania* major: wild type *L. major* (*L. major*^{WT}),GP63 knockout *L. major* (*L. major*^{KO}) and GP63 rescue *L. major* (*L. major*^{R}). Methods and results discuss footpad lesion development in C57BL/6 mice, the early innate inflammatory response in an intraperitoneal inoculation and cumulates with the analysis of parasite infectivity.

2.2 Author Contributions

Overall project was designed and envisioned by Martin Olivier. *In vivo* experiments were designed and performed by Aretha Chan and M Olivier. Flow cytometry experiments were performed by Fernando Alvarez in the lab of Ciriaco Piccirillo and data was analyzed by F Alvarez and A Chan. TEM imaging was performed by George Dong and A Chan. NTA measurements, preparation of samples for LC-MS/MS, and proteomic analysis was performed by A Chan. Statistical analysis of experimental data was performed by A Chan.

The Role of *Leishmania* GP63 in Innate Immunity During Infection

Aretha Chan[1,2], Fernando Alvarez[1,2,3], Ciriaco Piccirillo[1,2,3], George Dong[1,2], Martin Olivier[1,2,3]

[1]Department of Microbiology and Immunology, McGill University

[2]Infectious Diseases and Immunity in Global Health Program, Research Institute of the McGill University Health Centre

[3]FOCiS Centre of Excellence in Translational Immunology (CETI), Research Institute of the McGill University Health Centre

Abstract

Leishmaniasis is a disease caused by the protozoan parasite *Leishmania* known to affect millions of individuals worldwide. In recent years, we have established the critical role played by *Leishmania* zinc-metalloprotease GP63 in the modulation of host macrophage signalling and functions, favouring its survival and progression within its host. *Leishmania major* lacking GP63 was reported to cause limited infection in mice, however it is still unclear how GP63 may influence the innate inflammatory response and parasite survival in an *in vivo* context. Therefore, we were interested in analyzing the early innate inflammatory events upon *Leishmania* inoculation within mice and establish whether *Leishmania* GP63 influences this initial inflammatory response. Experimentally, *L. major WT* (*L. major*^{WT}), *L. major* GP63 knockout (*L. major*^{KO}) or *L. major* GP63 rescue (*L. major*^{R}) were intraperitoneally inoculated in mice and inflammatory cells recruited were characterized microscopically and by flow cytometry (number and cell type), and their infection determined. Pro-inflammatory markers such as cytokines, chemokines and extracellular vesicles (EVs, e.g. exosomes) were monitored and proteomic analysis performed on exosome contents. Data obtained from this study suggest that *Leishmania* GP63 does not significantly influence the pathogen-induced inflammatory cell recruitment. In addition, whereas pro-inflammatory cytokine/chemokine results show variabilities between groups, those results are not sufficient to explain why *L. major*^{KO} parasites lead to a less aggressive infection *in vivo*. However, internalization of promastigotes during early infection could be influenced by GP63 as less *L. major*^{KO} amastigotes were found within host cells and appear to maintain in host cells over time. Collectively this study provides a clear analysis of innate inflammatory events occurring during *L. major* infection and further establish

the prominent role of the virulence factor GP63 to provide favourable conditions for host cell infection.

2.3 Introduction

*Leishmania*sis is a sandfly- transmitted disease caused by the intracellular protozoan parasite, *Leishmania*. There are three main forms of leishmaniasis: visceral (the most severe form), cutaneous (most common), and mucocutaneous (affects mucosal tissue). Annually, the worldwide incidence rate is over 1 million, with about 26 000 to 65 000 deaths, making this a global health concern [75]. Furthermore, leishmaniasis is found across the world in over 90 countries and caused by over 20 species of *Leishmania*, which continues to spread due to climate change and large scale migration [75].

Across North Africa and the middle east, the species *L. major* is found to be the primary cause of cutaneous leishmaniasis cases in the old world. They develop as promastigotes in the sandfly midgut and are regurgitated into the host while the sandfly takes a blood meal. Once in the skin, the *Leishmania* promastigotes enter various host cells and subsequently transform into amastigotes, a replicative non-flagellated form [76].

The host immune response is a significant determinant in the outcome of the disease. In humans, the two types of disease progression are seen, one where it leads to spontaneous healing, and one where it causes chronic non healing lesions [77]. Many mice studies indicate that the adaptive immune response is responsible for the outcome of disease [78]. But ultimately, it is the ability of the *Leishmania* to infect cells that determine whether or not disease will manifest. This is dependent on the host early innate immune response, and how the parasite can evade or exploit it. *Leishmania* express a variety of virulence factors that mediate the initial interaction between the parasite and the host cell to promote infection [79].

Involved in the receptor-mediated uptake of *Leishmania* and resistance to host defence is an important metalloprotease known as GP63. GP63 is expressed on all studied *Leishmania*

species [55] and can degrade a wide range of substrates. Studies report that *Leishmania* GP63 directly cleaves complement C3 to escape complement mediated lysis, whereby the product C3bi can interact with macrophage receptor CR3 to promote attachment and uptake [80]. Past research from our laboratory demonstrated that GP63 is directly involved in altering host macrophage signalling for parasite escape. The known inhibited pathways include protein kinase C signalling pathway, and the IFN- γ mediated JAK/STAT, and direct interference with transcription factors NF- κB, STAT1 and AP-1, as well as the induction of negative signaling regulators such as protein tyrosine phosphatase SHP-1 [81]. These inhibitory actions result in the abolishment of macrophage effector functions to survive within host cells and recruitment of cells to further propagate the infection.

In relation to this GP63 mediated action, several studies reported that *L. major* knockouts for GP63 (*L. major*^{KO}) were generating a reduced development of cutaneous lesion when inoculated to mice compared to wild type *L. major* (*L. major*^{wt}) [82] It has been proposed that *Leishmania* lacking GP63 were more sensitive *in vitro* to complement mediated lysis that could influence the setting of the infection *in vivo*. However, the full early innate immune response to *Leishmania* GP63 has not yet been studied in depth and could provide a better understanding to what extent GP63 influences this initial host response concurring to the establishment of the infection.

Past studies from our laboratory and others have also investigated the modulation of proinflammatory mediators by *Leishmania [27] [83]*. In cutaneous leishmaniasis, lesions form due to chronic inflammation and cell infiltration to the skin. *L. major* inoculation to the skin induced the recruitment of leukocytes, which is crucial to both host defense and pathology. It is known that various proinflammatory mediators such as cytokines and chemokines can be rapidly

induced upon *L. major* infection both *in vitro* and *in vivo* to attract inflammatory cells to the site of the infection and therefore offering a greater number of cells to be infected [27]. Furthermore, novel players have been identified and to be involved in inflammatory process [84]; the extracellular vesicles including exosomes that are important players in the communication between eukaryotic cells. We have reported that exosomes released by *Leishmania* infected macrophages were found to differ in protein content from uninfected ones and were able to stimulate naïve macrophages as well [46], modulating some inflammatory mediators. Lastly, the infectivity and survival of the Leishmania amastigotes within the host cell may also affect the progression of the disease. GP63 is a known important virulence factor, but the role of GP63 in the modulation of aforementioned factors have yet to be elucidated.

In this study, we present a detailed report of the cells responding to infection, the inflammatory environment concerning cytokines/chemokines and exosomes, and the early infection progression in the context of cutaneous leishmaniasis caused by *L. major* lacking *GP63*.

2.4 Materials and Methods

Leishmania major

The wild type *Leishmania* parasite used was NIH S (MHOM/SN/74/Seidman) clone A2. GP63 knockout ($GP63^{KO}$) and GP63 rescue ($GP63^{R}$) *L. major* were generously supplied by Dr. Robert McMaster (University of British Columbia, Canada). All parasites were cultured at 25 ºC, 5% CO2 in Schneider's Drosophila Medium (SDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Wisent, St.Bruno, QC, Canada), and 5mg/ml HEMIN and passaged every 3 to 4 days. Cultures of promastigotes growing at logarithmic phase (day 3-4 post passage) were passaged bi-weekly, and were grown to stationary phase (day 6-8 post passage) before used in infections for all experiments [85].

Promastigote cellular lysates and Western Blot Analysis

Leishmania major cultures grown to promastigote stationary stage were lysed using 7 cycles of freeze thaw in liquid nitrogen and 42 degrees heating block. Protein levels were dosed with the Bradford Assay (Bio-Rad, Mississauga, ON, Canada). 10% acrylamide gels were loaded with 25 µg of proteins that were added to 5x SDS sample buffer containing bromophenol blue and β-mercapto-ethanol, heated at 95 °C for 5 minutes. Electrophoresis was performed at constant voltage of 100V at room temperature. Proteins were transferred to PVDF membranes (Perkin Elmer, Waltham, MA) using Bio-rad Trans-blot turbo system at 2.5A, 25V for 15 minutes. Membranes were blocked with 5% skim milk for 1 hour and then incubated with the anti-GP63 (Dr. McMaster, University of British Columbia) primary antibody in 5% BSA in TBS-T (TBS-0.05% Tween 20). It was washed 3 times with TBS-T and incubated with mouse secondary anti-

HRP-conjugated antibody (1:10000 in 5% milk) and proteins were visualized by ECL Western Blot Detection System (GE Healthcare, Chicago, IL, USA).

Gelatin Zymography assay

Protease activity of GP63 was assayed using a 10% SDS-PAGE incorporated with gelatin (1mg/ml) as we previously described [86]. Gels were loaded with 5ug of proteins that were added to SDS-PAGE sample buffer (15.6mM Tris pH6.8, 2% SDS, 10% glycerol, 0.05% Bromophenol Blue). Electrophoresis was performed at a constant voltage of 100V, at 4 degrees Celsius. After electrophoresis, SDS was washed with washing buffer (2.5% Triton X-100 in 50mM Tris pH 7.4, 5mM $CaCl_2$, 1μM $ZnCl_2$) for 1 hr on a shaker at room temperature. The gels were then briefly rinsed twice with deionized water and incubated in a renaturation buffer (50mM Tris pH 7.4, 5mM $CaCl_2$, 1μM $ZnCl_2$), overnight at 37°C. After incubation, gels were stained 30 min in 0.5% Coomassie Brilliant Blue R-250 in 30% ethanol and 10% acetic acid, and destained by rinsing in a solution containing 30% ethanol and 10% acetic acid until clear bands could be seen. Clear bands on the gel indicated active GP63 activity.

Mice and Ethics

Animal experiments were performed in compliance with the Canadian Council on Animal Care (CCAC) Guidelines, and McGill University Animal Care Committee (UACC). The approved animal use protocol number is 7791.

Mouse experiments were performed in the McGill University Health Centre research institute in containment level 2 housing facilities. Male C57BL/6 adult (6-8 weeks old) mice were used for all experiments, purchased from Charles River Laboratories (Wilmington, MA, USA).

Footpad Infections

Groups of 5 mice were each infected with 10^6 wildtype *Leishmania major* parasites, *L. major* GP63 ^{KO} and *L. major* GP63 ^{R} injected into one hind footpad. Lesion development was monitored weekly by the difference of footpad thickness between the infected and uninfected footpad, measured by digital calipers. Cutaneous leishmaniasis progression was monitored over the course of 10 weeks. Mice were euthanized after 10 weeks using isoflurane and CO2 asphyxiation followed by cervical dislocation.

Intraperitoneal Inoculation

Groups of 3 mice each were infected with 10^8 wildtype *Leishmania major* parasites, *L. major* GP63 knockout and *L. major* GP63 rescue, injected into the intraperitoneal cavity. 6 hours post infection, the mice were sacrificed and 5ml of ice-cold endotoxin-free PBS was used to obtain lavages of the cavities. The number of live cells present in the lavages were counted using a hemocytometer.

Cells were prepared for microscopy using the Cytospin 4 cytocentrifuge (Thermo Scientific, Waltham, MA, USA). The cells were fixed and stained using the Differential Quik (diff-quik) Stain Kit (Ral Diagnostics, Martillac, France). The percentage of cell types found in the lavage were counted. Next, the percent of cells infected and the number of *Leishmania* amastigotes found within the cells were counted.

Two-hundred ul of the lavages were plated in 4 well chamber slides, and supplemented with Dulbecco's modified eagle medium (Wisent, St.Bruno, QC, Canada) with 10% FBS and 1% penicillin-streptomycin-glutamine. 24 hours and 48 hours post plating, cells were fixed and

stained using the diff-quik stain kit. The percent of cells infected and the number of *Leishmania* amastigotes found within the cells were counted. From the total 200 cells counted from each slide, the percentage was calculated, and number of amastigotes found in individual cells were counted as well. These numbers were then used to calculate the total number of amastigotes found within cells by multiplying the percentage infected with the average number of amastigotes per cell.

Total lavage was centrifuged at 1500 rpm for 10 mins to separate cells and supernatant. The cell pellet was resuspended in TRIzol reagent (Ambion life technologies) and frozen.

Flow cytometry

Mouse peritoneal cell suspensions were stained with the following fluorescence-conjugated mAbs: a-CD3-BUV737 (17A2) (BD Biosciences, Franklin Lakes, NJ, USA), a-CD4-FITC (GK1.5) (BD Biosciences), a-CD8-V500 (53-6.7) (BD Biosciences), a-CD11b-e450 (M1/70) (Invitrogen), a-CD11c-PerCP-CyÔ 5.5(HL3) (BD Biosciences), a-CD19-APC (1D3) (Invitrogen), a-CD49b-PE (DX5) (BD Biosciences), a-F4/80-PECy7 (BM8) (Invitrogen), and a-Ly6G-Alexa700 (BioLegend, San Diego, CA, USA). Non-viable cells were excluded using fixable viability dye eFluor780 or 506 reagent (Thermo Fisher Scientific). Flow data were collected using a FACS Fortessa X-20 flow cytometer (BD Biosciences), and results were analyzed using FlowJo version 9 software (TreeStar, Ashland, OR, USA).

Multiplex Cytokine/ Chemokine Quantification Assay

100ul of the lavage supernatant were analyzed by a multiplex mouse cytokine array/chemokine array 44-plex assay (Eve Technologies, Calgary, AB, Canada). These include Eotaxin,

Erythropoietin, 6Ckine, Fractalkine, G-CSF, GM-CSF, IFNB1, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17, IL-20, IP-10, KC, LIF, LIX, MCP-1, MCP-5, M-CSF, MDC, MIG, MIP-1α, MIP-1β, MIP-2, MIP-3α, MIP-3B, RANTES, TARC, TIMP-1, TNFα, and VEGF. The multiplex laser bead technology utilizes antibodies are coupled to colour-coded polystyrene beads where lasers activate the fluorescent dye and excites the fluorescent conjugate, which is then quantified for the concentration of the target analyte. From the data provided from Eve technologies, the total pg of cytokine found in the lavage was calculated from the observed concentration by multiplication of the 5ml PBS used to obtain lavages.

Exosome extraction

The remainder of the lavage supernatant was used for the extraction of exosomes. The supernatant was filtered using a 0.22 μm filter to exclude debris and larger vesicles. Lavages from three mice in an experimental group was pooled in 17 mL thin-wall polypropylene tubes (Beckman Coulter, Brea, CA, USA) and was completed with exosome buffer (137mM NaCl, 20mM HEPES). The tubes were centrifuged at 100000xg (RCFavg) for 1 hour at 4°C in an SW 32.1 Ti swinging bucket rotor (Beckman Coulter). The supernatant was discarded, and fresh exosome buffer was added to the tube to wash the pellet. It was centrifuged again at 100000xg for 1 hour. Again, the supernatant was discarded with about 400ul liquid remaining and the exosome pellet was resuspended in the remaining exosome buffer and frozen for further analysis. An alternate method used to extract exosomes included the ExoQuick procedure as described in the protocol for ExoQuick exosome precipitation solution (System Biosciences, Palo Alto, CA).

This step was performed in place of the second ultracentrifugation step mentioned above. The protein levels were dosed using a microBCA assay (Thermo Fisher, Waltham, MA, USA).

Nanoparticle tracking analysis

The exosomes were then analyzed using nanoparticle tracking analysis (NTA) using the NanoSight NS500 (Malvern Panalytical, Malvern, Worcestershire, UK) in the laboratory of Dr. Janusz Rak. Samples were diluted with exosome buffer and injected into the sample chamber. 3 videos were captured for 30 seconds each at 37 degrees celcius, using optimized camera settings that were kept consistent for all samples. From the NTA analysis, the concentration and mean, median, mode size of the particles of all particles were calculated and graphed [87]. After the size and concentration of the particles were verified for proper exosome isolation prep, transmission electron microscopy photos were taken to further confirm their isolation and purity.

Transmission electron microscopy

EVs were suspended in exosome buffer. Samples were deposited onto Fomvar carbon grids (Mecalab, Montreal, QC, Canada), fixed with 1% glutaraldehyde in 0.1M sodium cacodylate buffer, and washed 3 times with autoclaved Milli-Q, and stained with 1% uranyl acetate. Each aforementioned step was performed for 1 minute in duration. The FEI Technai-12 120kV transmission electron microscope and AMT XR80C CCD Camera (Facility for Electron Microscopy Research, McGill University, Montreal, Canada) was used to visualize samples.

Trichloroacetic acid (TCA) precipitation

Exosome solution with 8ug of protein was aliquoted and completed up to 100ul with ddH2O. To the exosomes, the following were added: 100ul 10X TrisHCL-EDTA, 100ul 0.3% sodium deoxycholate, 72% TCA. The tubes were incubated on ice for 1 hour, then spun at 14000 rpm for 20 minutes at 4 degrees. The supernatant was aspirated and the pellet was resuspended in 100ul of 90% room temperature acetone. The tubes were then incubated in the -20°C freezer overnight, then centrifuged at 14000 rpm for 20 minutes at 4°C . The supernatant was aspirated then the pellet was air dried at room temperature and placed at -20°C .

Liquid chromatography- mass spectrometry (LC-MS/MS)

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the Institut de Recherches Clinique de Montreal (Universite de Montreal, Montreal, QC, Canada). 8ug of proteins from extracted EVs were precipitated with 15% trichloroacetic acid/acetone and sent for LC-MS/MS analysis. After precipitation, proteins were reduced, alkylated, and digested with trypsin solution (5mg/ul trypsin sequencing grade from Promega, 50mM ammonium bicarbonate). Protein digestion was performed at 37 degrees for 18h and stopped with 5ul of 5% formic acid. Prior to LC-MS/MS, digests were cleaned using C18 ZipTip pipette tips (Millipore, Burlington, MA, USA). Extracted peptides were injected into a Self-Pack PicoFrit fused silica capillary column (New Objective) of 15 cm long and packed with the C18 Jupiter 5 μm 300 Å reverse-phase material (Phenomenex), and chromatographically separated on a Easy-nLC II system (Proxeon). Eluted peptides were electrosprayed from a Proxeon nanoelectrospray ion source and analyzed on a LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific).

Protein database search

The peak list files were generated with Proteome Discoverer (version 2.1) using the following parameters: minimum mass set to 500 Da, maximum mass set to 6000 Da, no grouping of MS/MS spectra, precursor charge set to auto, and minimum number of fragment ions set to 5. Protein database searching was performed with Mascot 2.6 (Matrix Science, Boston, MA, USA) against the RefSeq and Uniprot *Mus Musculus* protein database. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.02 Da, respectively. Trypsin was used as the enzyme allowing for up to 1 missed cleavage. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation as variable modifications. MS/MS peptide and protein identifications were performed using Scaffold software version 4.8.9 (Proteome Software Inc., Portland, OR, USA). Peptide identifications were included if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least 2 identified peptides in at least one biological replicate [88] The proteins sharing significant associated probability were grouped into clusters.

Bioinformatics Analysis

Normalization, quantification, and comparisons of proteins from lavage EVs were performed using the Scaffold software. Visualizations of set intersections in a matrix layout were generated by UpSetR [89]. Gene Ontology comparisons were performed using Panther (www.pantherdb.org) [90].

Statistical Analysis

Statistical analysis was performed in Graphpad Prism 6.0c (La Jolla California USA)

2.5 Results

GP63 protein and proteolytic activity is absent from *L. major*^{KO}.

Prior to experimentation with existing *Leishmania*, it was essential to verify the stable deletion and expression of GP63 in our transgenic cultures [73]. Western blot analysis was used to detect the presence of GP63 using a mouse anti-GP63 antibody. The assay detected a band corresponding to GP63 at 63kD in the lysates of *L. major*^{WT} parasite as well as *L. major*^{R} (Figure 5). No bands were seen in the *L. major*^{KO} lane. Gelatin zymography assays are useful for the detection of active proteases, which will degrade the gelatin copolymerized with the SDS gels [91]. The gelatin assay demonstrated there was no metalloprotease activity found in the protein lysate of the GP63 knockout *L. major*. Clear bands were only observed in the lanes containing *L. major*^{WT} and *L. major*^{R}, indicating the presence of active GP63 [92].

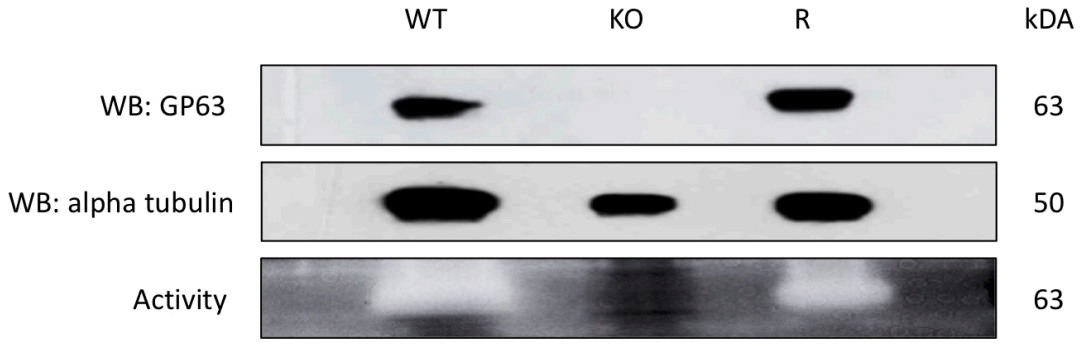

Figure 5. Validation of active GP63 in L. major lysates

Protein extracts from *L. major*^{WT} , *L. major*^{KO} and *L. major*^{R} were loaded on gel to confirm GP63 expression and proteolytic activity. Top two rows indicate western blot (WB) results. Bands were seen at 63kD for GP63 in WT and GP63 rescue parasites only, bands at 50kD for alpha-tubulin was seen for all *L. major* lysates. The bottom is a gel doc image for the activity of GP63 found at 63kD in WT and GP63 rescue *L. major*. This data is representative of 2 analysis.

***Leishmania major* lacking GP63 generates a less aggressive lesion development.**

The progression of lesion formation in the footpad infection was monitored over ten weeks. It was previously described that infection with GP63 deficient *Leishmania* promastigotes resulted in the delay of lesion formation in the footpad of BALB/c mice [82]. We infected three groups of 5 C57BL/6 mice with 1 x 10^6 promastigotes to confirm this observation in our mouse model. Over the first 9 weeks, the initial difference of footpad thickness between the infected and uninfected feet in *L. major*^{KO} infected mice was significantly lower compared to the wild type parasites, at about 0.5mm less (Figure 6) ($p<0.05$, multiple T-tests Holm-sidak method) in the first 2 weeks post-infection. As the infection progressed, the footpad thickness difference in *L. major*^{KO} parasites started to increase up to 1mm after week 5, demonstrating a delay in the lesion formation. The *L. major*^{WT} and *L. major*^{R} parasites induced a more significant swelling progressively as the infection established over eight weeks, peaking at 2mm difference in thickness, which subsequently began to subside by week 9. By week 10, *L. major*^{KO} infected footpads reached 1.5mm, comparable to *L. major*^{WT} and *L. major*^{R} groups, that were on their decline. Data stemming from this experiment, have confirmed that the loss of GP63 weakens the ability to produce progressive lesions when compared to wild type and rescue parasites.

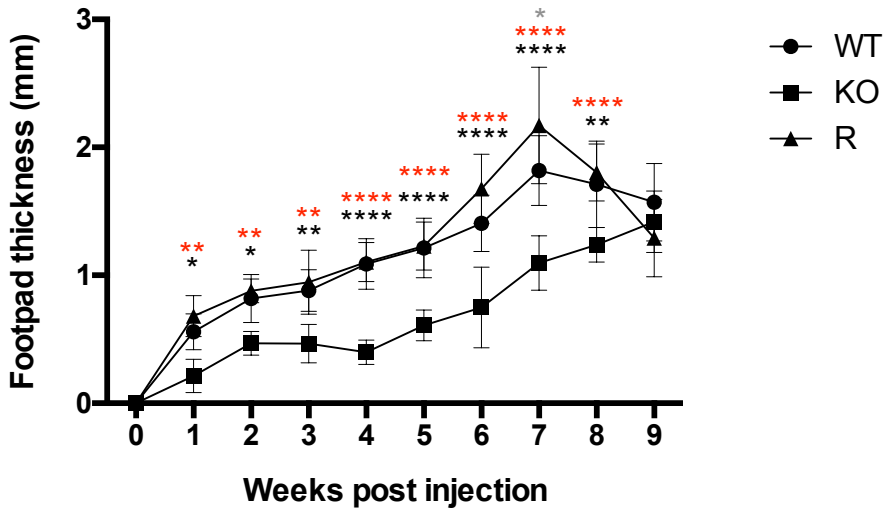

Figure 6: Footpad lesion formation progression by L. major inoculation is GP63 dependent

Graph tracking the difference in thickness between the inoculated and the uninfected footpad over ten weeks. Bars represent ± SEM (n=5 mice per group). Statistical significance was determined using two way ANOVA between 3 groups, *L. major*^{WT} and *L. major*^{KO} *, *L. major*^{KO} and *L. major*^{R} *, *L. major*^{WT} and *L. major*^{R} *. Where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Each time point was analyzed individually, without assuming a consistent SD.

L. major-induced inflammatory cell recruitment is not influenced by GP63.

An intraperitoneal injection model was used to characterize the early innate immune response to an *L. major* infection. The mouse peritoneal cavity is ideal for the study of innate inflammatory response, as it contains mainly macrophages, followed by neutrophils and to a lesser extent, eosionophils, basophils, B cells, T cells and is permissive to the recruitment of a variety of immune cells [51]. In addition, it was observed that antigenic stimulation in the intraperitoneal cavity results in the recruitment and activation of inflammatory cells [93]. From the peritoneal lavage, we sought to study the number and type of cells recruited, the production of inflammatory cytokines and chemokines, and exosomes released.

The number of live cells was counted from the intraperitoneal lavage taken 6 hours post-infection. In the PBS injected control group, the total amount of live cells in the lavage was

$2.22x10^6$ cells (Figure 7A). In the infection groups of *L. major*^{WT}, *L. major*^{KO} and *L. major*^{R}, the average total amounts were $1.41x10^7$, $1.30x10^7$, and $1.19x10^7$ cells, respectively. There was no significant difference between the three infection groups, regardless of the presence of GP63. On the other hand, *L. major* injection resulted in the five-fold increase of cells in the peritoneal cavity ($p<0.0001$, unpaired t-tests with Welch's correction) in comparison to PBS inoculated mice.

The inflammatory cell types recruited have been monitored from Diff-Quick cytospin slides. In the PBS injected control group, 80% of the cells retrieved were macrophages or monocytes. The remaining cells consisted of 10% lymphocytes, and small amounts of neutrophils, basophils, and eosinophils (Figure 7B). Compared to the PBS group, all *Leishmania* infection resulted in massive recruitment of leukocytes, mostly neutrophils ($p<0.05$, multiple t-tests Holm-sidak method). The total amount of neutrophils found in the lavage was about $2x10^6$ cells in all three infection groups, compared to the $6x10^3$ in the PBS lavage, which is a ~300 fold difference. There was also a significant increase in eosinophils for all 3 *L. major* infection groups. The eosinophil counts increased tenfold from $1x10^5$ cells to around $1x10^6$ cells for all three groups. Macrophage counts also increased slightly in the *L. major* infection groups, with the highest amount of recruited macrophages in the wild type infected mice ($p<0.05$, multiple t-tests Holm-Sidak method). The total number of lymphocytes and basophils remained in a similar range. The most notable observation is that all three groups of *L. major* infected mice had similar amounts of each type of recruited cells, regardless of GP63. The cell recruitment levels were similar for all three groups for all cell types, which consisted mainly of neutrophils. GP63 did not seem to affect the number of cells recruited to the site of infection, nor the type of cells that were recruited.

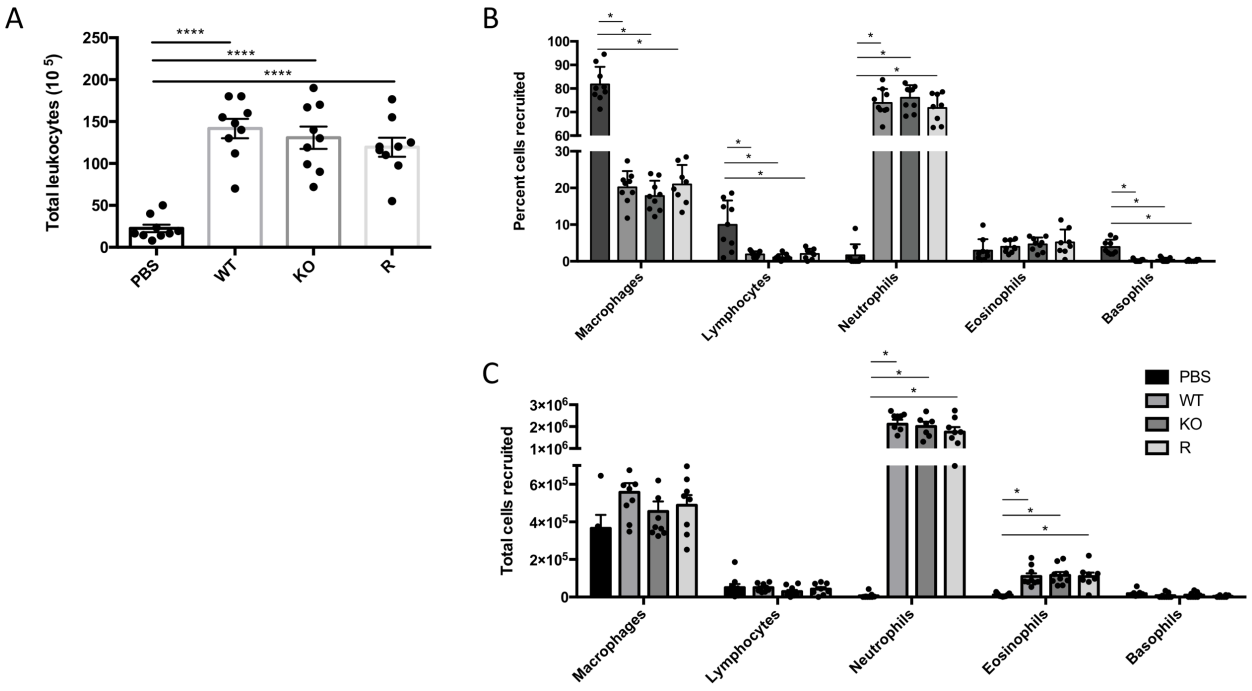

Figure 7: GP63 does not affect inflammatory cell recruited in response to Leishmania inoculation.

A) Total live cells retrieved in intraperitoneal lavage 6-hr post-inoculation of infection with *L. major*^{WT}, *L. major*^{KO} and *L. major*^{R}. Cells were counted using a hemacytometer; values were calculated based on concentration and total lavage volumes. Bars represent ± SEM (n=9, mean of 3 independent experiments). Statistical significance was determined using unpaired T-tests with Welch's correction, ****$p < 0.0001$. Endotoxin-free PBS was used as inoculation control. B) Distribution of inflammatory cell types as percentage of total. Statistical significance was determined using Multiple T-tests with Holm-Sidak method, with alpha=5.000%, *$p<0.05$. C) The total amount of each cell type was calculated using the total live cells in A and the percentage of each B. The same statistical test was applied in B.

Flow cytometry was also used to quantify the level of cell recruitment and further elucidate the identity of the cells found in the intraperitoneal lavage (Figure 8A). Cytometry data corroborated with the neutrophil and macrophage cell count numbers observed in the cytospin slides (Figure 8B).

The macrophages present in the peritoneal cavity could be further analyzed because there are two functionally distinct macrophage subsets that can vary due to antigenic stimulation [94].

Large peritoneal macrophages make up 90% of peritoneal macrophages in unstimulated conditions, and small macrophages become dominant after stimulation of LPS, hypothesized to derive from active monocytes migrating to the peritoneum upon inflammation (Figure 9). The flow cytometry data was further analyzed to identify these distinct populations, where cells were gated for macrophage surface markers CD11b and F4/80 (Figure 8C). All three infection groups demonstrated a shift in macrophage populations from mostly large peritoneal macrophages (LPM; lymphoid-, CD11c-, F4/80+, CD11b+) to small peritoneal macrophages (SPM; lymphoid-, CD11c-, F4/80lo, CD11b+). Six hours post-infection, the percentage of small macrophages increased over two-fold while the large macrophage population diminished significantly (Figure 8D). The difference between wildtype, GP63 rescue and GP63 knockout infected groups was not significant, although not statistically significant, GP63 knockout infection appears to recruit less small activated macrophages.

Collectively, this set of data clearly reveals that *Leishmania* parasite can rapidly induce the recruitment of inflammatory at site of inoculation, but that does not require the metalloprotease GP63 to induce cellular recruitment. Therefore, this inflammatory event occurring during *Leishmania* infection cannot provide clues why *L. major*^{KO} concur to a less aggressive skin pathology.

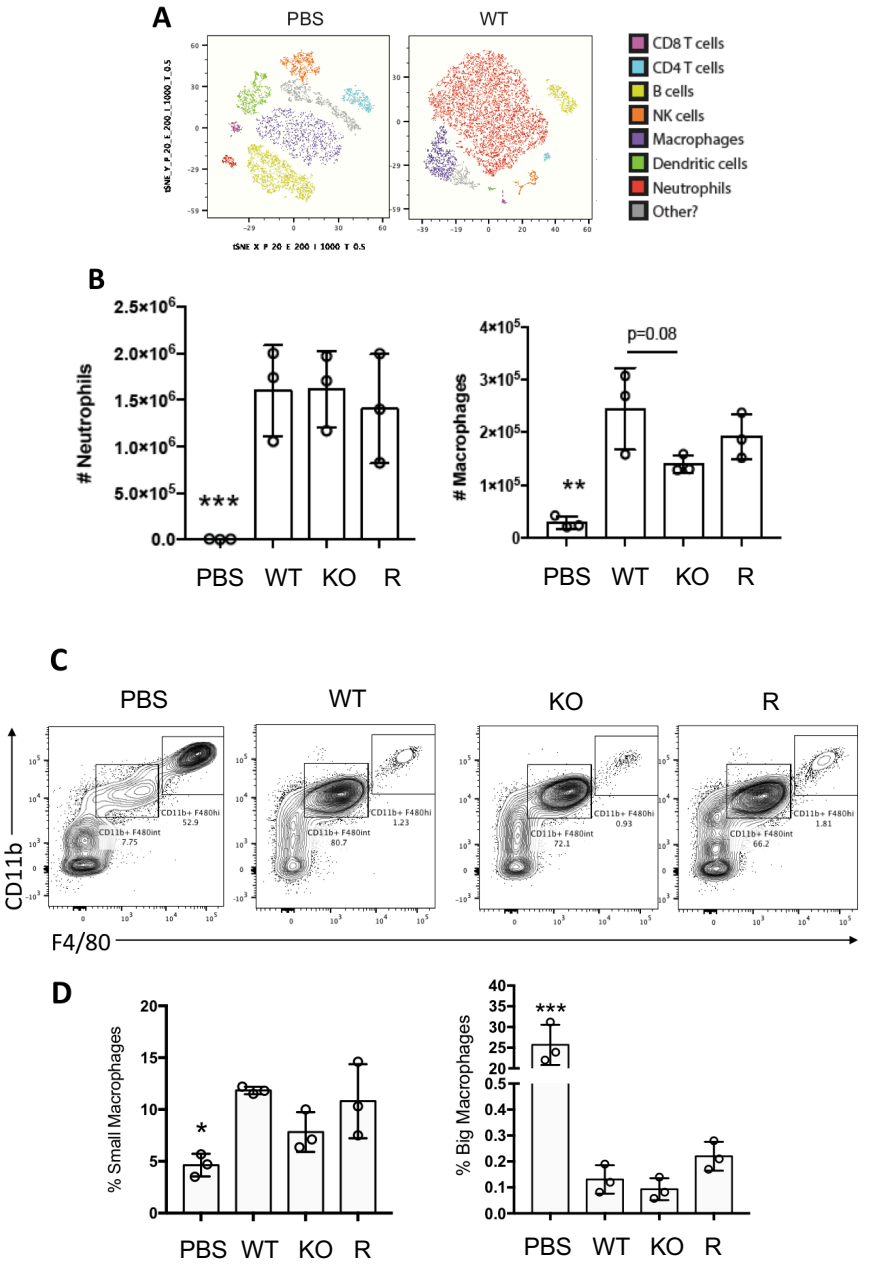

Figure 8: Flow cytometry data of cell recruitment and polarization of two distinct macrophage populations observed in mice intraperitoneal lavage following a 6-hour infection

A) Representative flow cytometry plots of all cell types found in suspension. Sample of PBS and WT is shown to demonstrate an unequal distribution of cell types between different samples. B) Total numbers of neutrophils (CD11c+, Ly6G+, lymphoid-) and macrophages (lymphoid-, CD11c-, F4/80+, CD11b+), were calculated using the flow cytometry data and the total number of cells. Bars represent ± SD, and each point represents one mouse, n=3 mice per group, ****p<0.0001. C) Flow cytometry gating of small and large peritoneal macrophages (lymphoid-,

CD11c-, F4/80+, CD11b+) D) Percentage of small and large peritoneal macrophages. Bars represent ± SD, and each point represents one mouse, n=3 mice per group, *p<0.05, ***p<0.001.

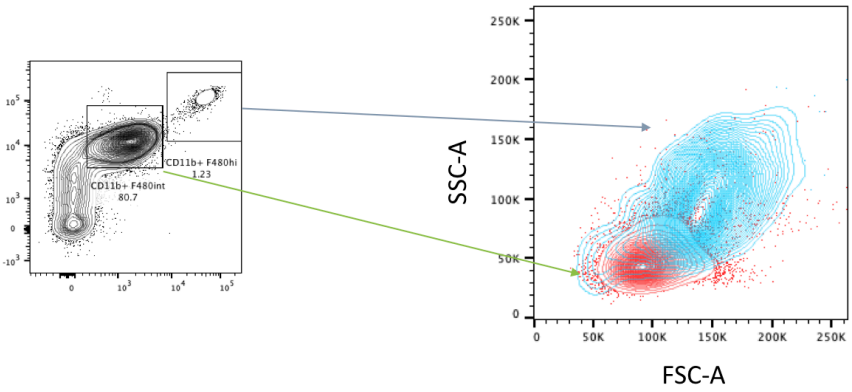

Figure 9: Forward and side scatter of macrophage populations

Two populations identified in gating for CD11b and F4/80 from intraperitoneal lavages following a 6-hour infection with 10^8 WT, GP63^{KO}, and GP63^{R} *L. major* demonstrate significantly different side scatter (SSC) and forward scatter patterns (FSC) which represent the granularity and size of the cells, respectively.

Inflammatory mediators released in response to *Leishmania* intraperitoneal infection.

We sought to observe the cytokines and chemokines produced during infection, which will describe the inflammatory environment, as well as explain the observed recruitment of cells. The peritoneal lavage supernatants were directly measured using a multiplex cytokine/ chemokine array to quantify protein levels. From the average of all nine mice from 3 experiments, some general trends were observed. At a glance from the heatmap, the lowest concentration was observed for most cytokines in mice injected with PBS (Figure 10A). In contrast, the mice infected with *L. major*^{WT} produced the highest levels of cytokines. High cytokine level was not observed in group inoculated with *L. major*^{R}, despite expressing similar levels of active GP63. The *L. major*^{KO} inoculated mice also showed similar production of cytokines/chemokines, which overall correlates well with the inflammatory cell recruitment data. The *L. major*^{WT} infected mice produced the highest levels of proinflammatory cytokines when

compared to *L. major*^{KO} and *L. major*^{R}: IL-6 (~4 fold, $p<0.05$), IL1- β (~4 fold, $p<0.001$), and TNF- α (~3 fold, $p<0.01$) (Figure 10B). The elevated proinflammatory mediator levels observed solely in the *L. major*^{WT} were not reflected in the inflammatory cell recruitment numbers. Further immunological events beyond the innate inflammatory events could have influenced the infection, but from *L. major*^{R} data does not correlates with the difference of infection level seen between *L. major*^{KO} and *L. major*^{R}. Statistically, there was no significance between all groups for the chemokines and other cytokines. These results corroborate with those seen in the cell recruitment numbers, which were similar between all three infection groups, with mostly neutrophils observed. From this information, GP63 did not affect the immune mediators produced from the peritoneal cells.

***Leishmania* infection concur to augment exosomes released by host inflammatory cells.**

Extracellular vesicles are secreted by all eukaryotic cells and have been shown that infectious stimulation alters exosome release from host cells [95]. Exosomes derived from macrophages infected with *Leishmania in vitro* have been studied, and they were found to have altered effector functions [46]. We extracted exosomes from the total collected supernatant, which represent exosomes secreted from all cells present in the intraperitoneal lavage.

Nanoparticle tracking analysis (NTA) was performed, and transmission electron microscopy (TEM) photos were taken to verify the purity of exosomes. NTA analysis showed clear peaks of nanoparticles found at about 150nm in size (Figure 11A). The PBS sample contained a lower concentration of exosomes, which cause higher background reading. Isolated exosomes were further visualized using transmission electron microscopy. TEM photos confirmed that the isolation was successful and yielded exosomes of the expected size range and morphology (Figure 11B).

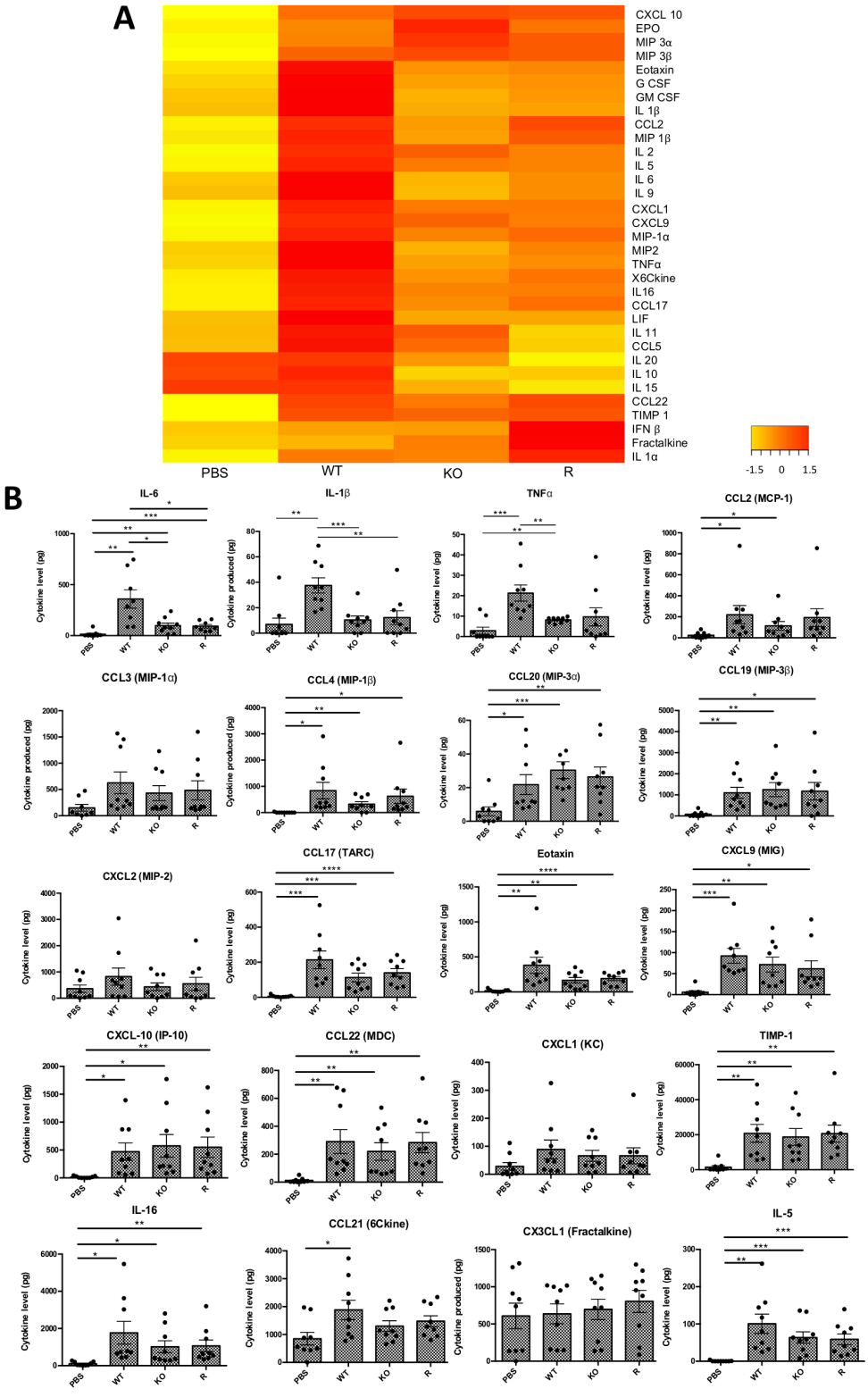

Figure 10: Proinflammatory mediators and chemokine expression are modulated by L. major infection.

A) Expression heat map generated from the multiplex assay analyte data. The analysis was performed on total supernatant retrieved from intraperitoneal lavages following a 6-hour infection with 10^8 WT, $GP63^{KO}$, and $GP63^{R}$ *L. major*. Each row is analyzed individually for relative expression levels. B) Cytokine levels in pg measured from the multiplex assay. Total cytokine was calculated using the volume of lavage and concentration, as reported by fluorescence readout. Bars represent ± SEM, each point represents one mouse, n=9 (mice total, 3 mice per experiment) Statistical significance was determined using unpaired t-tests with Welch's correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

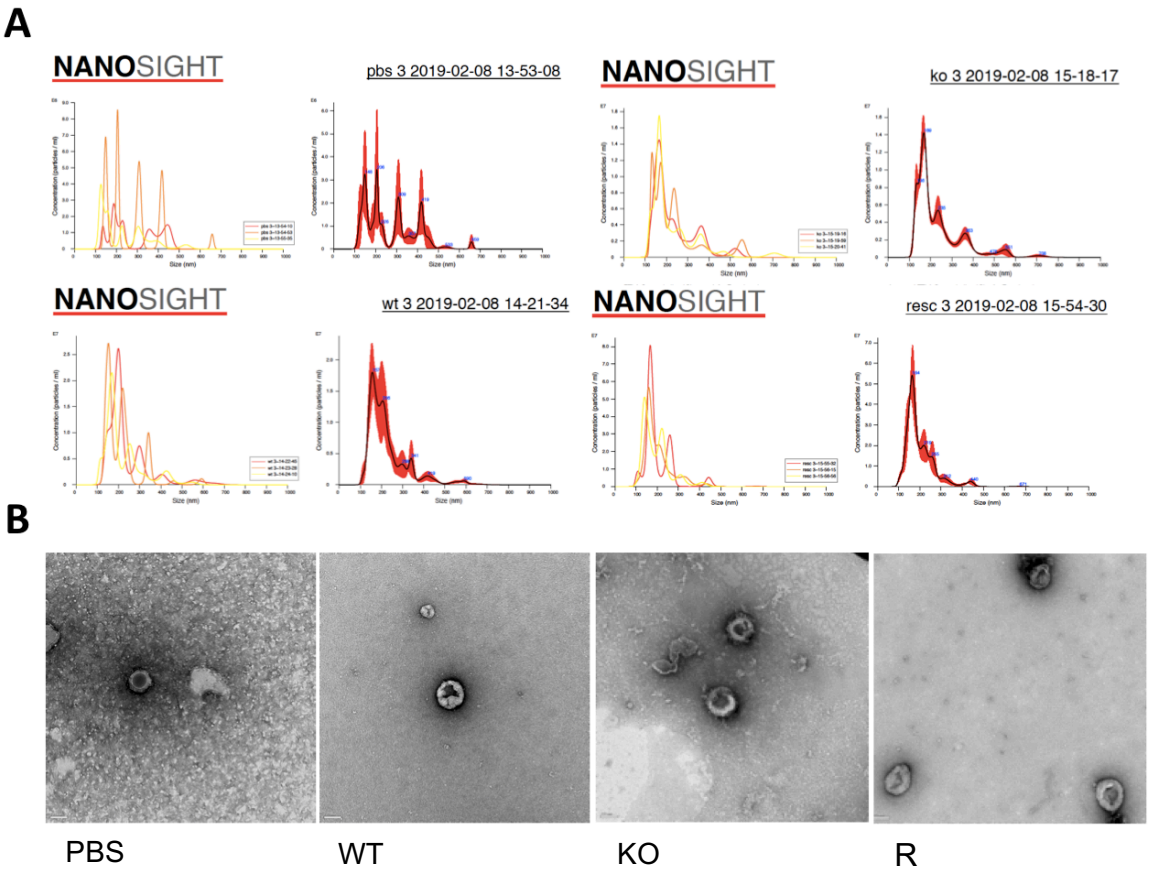

Figure 11: Nanoparticle tracking analysis and transmission electron microscopy for exosome analysis

The analysis was performed on EV suspension extracted from total supernatant retrieved from intraperitoneal lavages following a 6-hour infection with *L. major*^{WT}, *L. major*^{KO} and *L. major*^{R}. A) Representative Nanosight generated graphs of nanoparticle distribution and concentration in samples. Left: each line represents the concentration of particles in one video. Right: Average concentration, as calculated in the three videos analyzed. B) TEM photos of exosomes extracted after staining with uranyl acetate to visualize morphology. Photos were taken at 32000x; scale bar represents 100nm.

The exosome lipid bilayer can be clearly observed in the 30000x photos. Exosomes are about 100nm in diameter and have a round, cup-shaped morphology when fixed onto grids. The majority of particles seen in the TEM photos were uniform in shape and size.

From the NTA graphs, the mean and mode of the size of the nanoparticles were obtained. The mode extracellular vesicle (EV) size was consistent for all four groups, in the range of about 150nm in diameter. The larger average size for exosomes is due to the overestimation of the Nanosight machine. The concentration of the total nanoparticles was also measured, along with the concentration of particles in each size fraction. The EV concentrations showed that the PBS groups had ten-fold fewer EVs than the infection groups, which is reflected in the total cell counts (Figure 12A). An observation of interest is that wild type and GP63 rescue *L. major* infected cells appeared to produce more EVs than the cells infected with GP63 knockout parasites. From the size fraction breakdown, GP63 rescue parasites induced more exosomes than GP63 knockout parasites, as seen in the 100-150 fraction (~2 fold) (Figure 12B). Since the cell counts were similar between the three infection groups, the relative level of exosome production could be attributed to the effects on individual cells themselves.

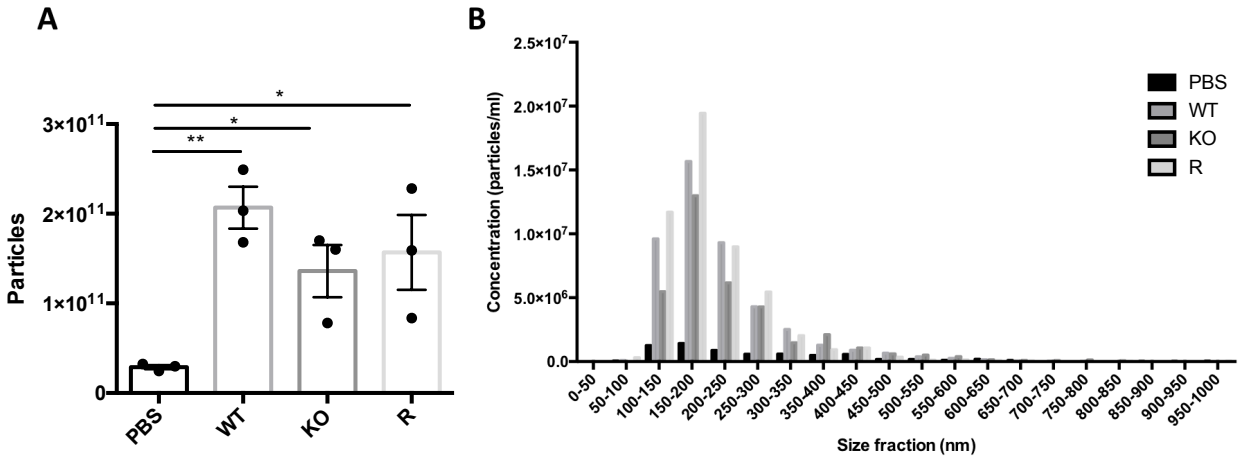

Figure 12: GP63 expressing L. major infection leads to increased production of exosomes.

A) The concentration of nanoparticles found in suspension was quantified used nanoparticle tracking analysis. The total number of particles was calculated using the concentration and total lavage volume. Bars represent ± SEM; each point represents 3 mice pooled into one sample, n=3 (9 mice total, 3 per experiment). Statistical significance was determined using unpaired t-tests with Welch's correction, *p<0.05, **p<0.01. B) The concentration of particles found in each size fraction of 50nm. Bars represent the average in one experiment only, n=3, 3 mice total per group.

Proteomic analysis of EV protein cargo

Exosomes carry cargo that has been shown to have various functions in cell-cell communication, notably immune cell activation and suppression [96]. To further elucidate the immune activation environment during a *L. major* infection and the impact of GP63, we performed mass spectrometry (MS) on the total proteins found in the extracted EVs. When MS data was analyzed against the UniProt Mus musculus database, a total of 1226 proteins hits in 1131 clusters were identified, with at least 2 total spectrum counts in at least one sample. We first identified known exosome markers to verify that our protein dataset reliably represents exosomal proteins (Table 1). In our samples, CD9, heat shock cognate 71kD protein, glyceraldehyde-3-phosphate dehydrogenase, actin cytoplasmic 1, and annexin A2 were identified in all samples [97]. CD63, another exosome marker, was not found in all samples (Table 1). The levels for all markers were found to be higher in GP63 knockout and GP63 rescue samples, which may reflect the relative levels of total exosomal protein and need to be accounted for when looking at protein enrichment. There are also signs of protein contamination from non-exosome biofluid sources, for example, serum albumin and keratin, which could also skew data. To calculate the enrichment of proteins, emPAI values were used, which is proportional to protein content in a protein mixture [98]. Quantitative analysis was performed on scaffold software using emPAI values for analysis of variance (ANOVA), with Benjamini-Hochberg correction. There were no enriched proteins found in any of the groups, which we expected to see when compared to the PBS control (Figure 13). Although statistically no enriched proteins were identified,

several proteins of interest were expressed more in the *L. major*^{WT}, *L. major*^{KO} and *L. major*^{R} infected groups compared to the PBS group. The proteins presented (Table 2) are known to be involved in immune responses or involved with the activity of immune cells. According to gene ontology terms found on Uniprot, BPI fold-containing family A member 2 and Protein S100-A9 are secreted antimicrobials, Neutrophilic granule protein is a protease inhibitor involved in defence, CD177 antigen, Pentraxin-related protein PTX3, and Myeloperoxidase are involved in neutrophil function, and eosinophil peroxidase is involved in defence in eosinophils.

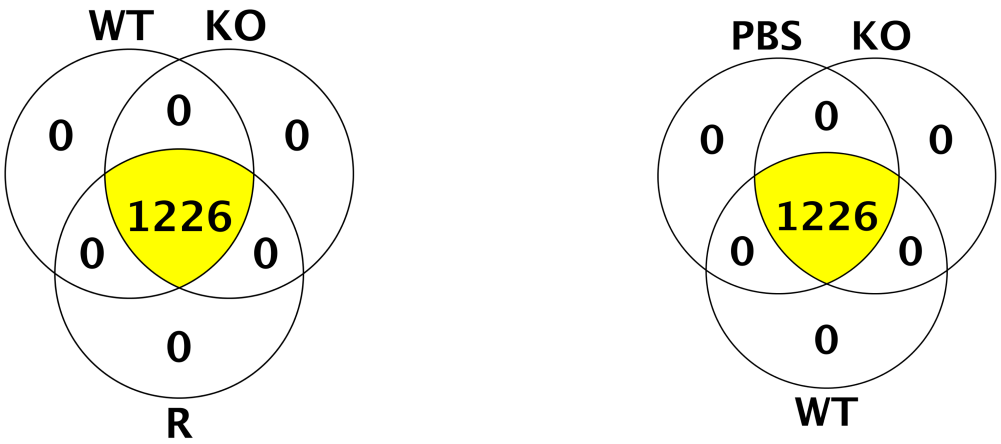

Figure 13: Enrichment of proteins found in EVs using ANOVA and emPAI values

Quantitative profiles of protein expression were analyzed using the exponentially modified protein abundance index (emPAI) values for an analysis of variance (ANOVA) statistical test. Venn diagrams were generated on Scaffold proteome software, which allows for the display of up to 3 intersecting groups at a time.

Table 1: Proteomics data of proteins most commonly reported to be found in exosomes.

Name	Accession number	Molecular weight	PBS		WT		GP63-/-		GP63R	
			Avg	St dev	Avg	St dev	Avg	St dev	Avg	St dev
Heat shock cognate 71 kDa protein	HSP7C_MOUSE	71 kDa	**17**	14.14	**11**	2.83	**56.5**	12.02	**45**	4.24
CD9 antigen	CD9_MOUSE	25 kDa	**9.5**	7.77	**4**	2.82	**16**	7.07	**13**	2.828
Glyceraldehyde-3-phosphate dehydrogenase	G3P_MOUSE	36 kDa	**17.5**	2.12	**13.5**	7.77	**49**	5.65	**52.5**	17.67
Actin, cytoplasmic 1	ACTB_MOUSE	42 kDa	**66.5**	7.77	**56.5**	7.77	**166.5**	0.70	**165.5**	27.57
Annexin A1	ANXA1_MOUSE	39 kDa	**13**	11.31	**16**	1.41	**43**	1.41	**44**	8.48
CD63 antigen	CD63_MOUSE	26 kDa	**0**	0	**0**	0	**1.5**	0.70	**0**	0
Programmed cell death 6-interacting protein	PDC6I_MOUSE	96 kDa	**0.5**	0.70	**0.5**	0.70	**7.5**	2.12	**7.5**	3.53

Table 2: Proteins of interest expressed in L. major^{WT}, L. major^{KO} and L. major^{R} infected mice.

Name	Accession number	Molecular weight	PBS		WT		GP63-/-		GP63R	
			Avg	St dev	Avg	St dev	Avg	St dev	Avg	St dev
BPI fold-containing family A member 2	BPIA2_MOUSE	25 kDa	**0**	0	**6.5**	2.12132034	**9**	7.07106781	**14.5**	0.70710678
Neutrophilic granule protein	NGP_MOUSE	19 kDa	**0.5**	0.70710678	**26.5**	4.94974747	**21.5**	0.70710678	**24.5**	7.77817459
Protein S100-A9	S10A9_MOUSE	13 kDa	**2.5**	3.53553391	**8**	1.41421356	**15.5**	0.70710678	**16**	4.24264069
CD177 antigen	CD177_MOUSE	87 kDa	**3.5**	4.94974747	**15**	7.07106781	**42.5**	3.53553391	**34**	11.3137085
Myeloperoxidase	PERM_MOUSE	81 kDa	**4.5**	6.36396103	**50**	8.48528137	**49**	7.07106781	**44**	5.65685425
Pentraxin-related protein PTX3	PTX3_MOUSE	42 kDa	**1**	1.41421356	**9**	1.41421356	**5**	1.41421356	**4.5**	2.12132034
Eosinophil peroxidase	PERE_MOUSE	81 kDa	**0.5**	0.70710678	**10**	2.82842712	**47**	7.07106781	**41**	0

The number of shared and unique proteins were also analyzed from the total spectrum count, which looks at presence vs absence of proteins regardless of quantitative profile. This is presented using an UpSet plot, which is used to visualize intersections of protein sets found within the EVs [89]. Many of the proteins were shared between all 4 groups (337), while the next largest intersection was between GP63 knockout and GP63 rescue groups (248), likely due to the abundance of proteins found in these groups (Figure 14). Unique proteins could also be identified, where *L. major*^{KO}, *L. major*^{R}, *L. major*^{WT} and PBS groups expressed 105, 70, 30, and 10 unique proteins, respectively. From this, we see that infection by *L. major* in the absence of GP63 produces a different host response environment compared to infection with wild type *L. major*.

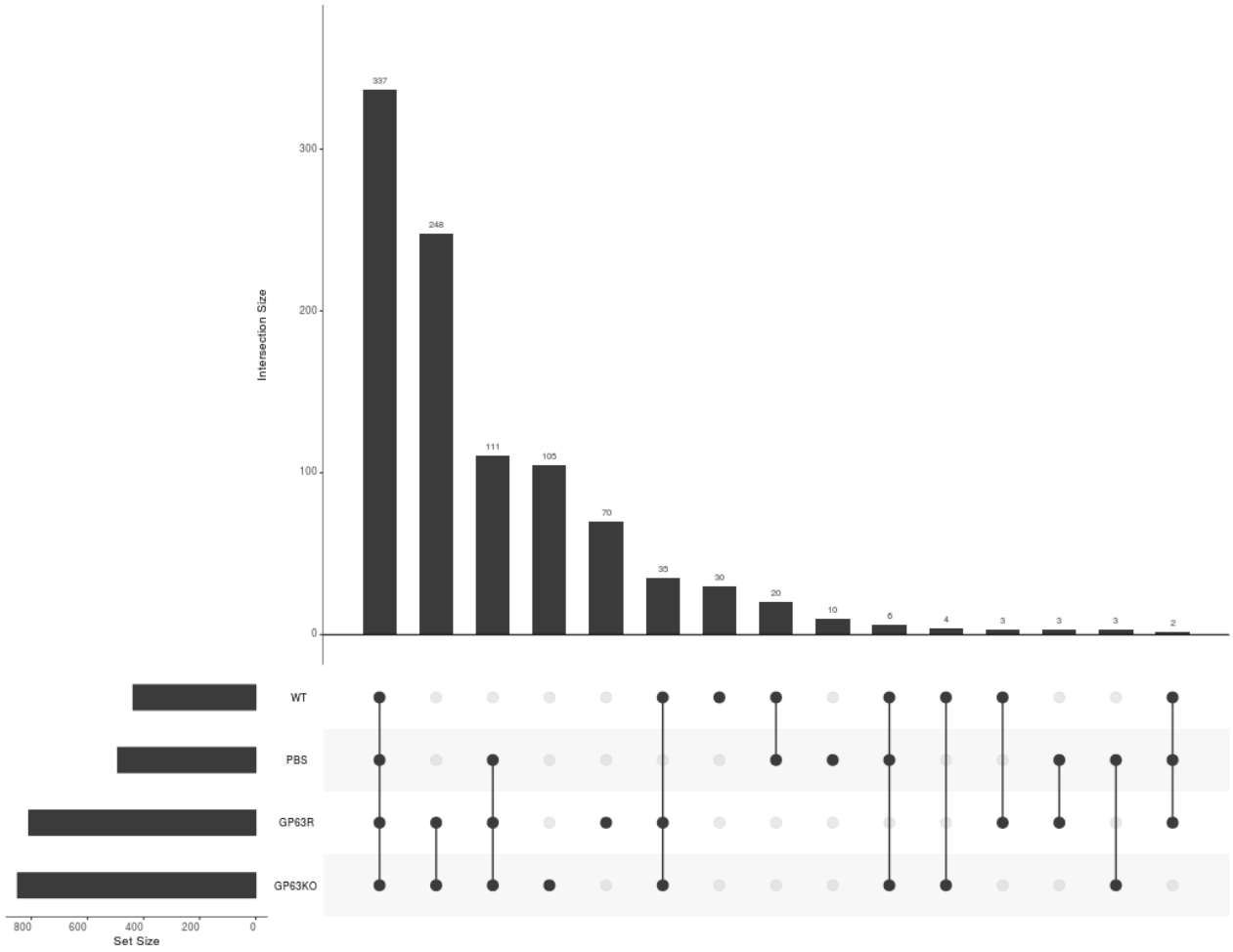

Figure 14: Common and shared proteins found in EVs visualized by UpSet plot.

Each row represents a group and the set of proteins found. The grey bars on the left represent the size of the set. Dark circles demonstrate the set is part of the intersection segment in the 4 set Venn diagram. The vertical bars represent the number of proteins found within the particular intersection defined by the circles below.

The proteins found within the extracellular vesicles could also be categorized based on biological function, molecular function, cellular component, pathway and protein class through gene ontology. Gene ontology is a comprehensive resource that compiles current scientific knowledge on the function of genes at the molecular, cellular, and organism level from a variety of common organisms [99]. Pie charts were generated using Panther (Protein Analysis Through Evolutionary Relationships), a program that utilizes gene ontology terms to classify proteins

[90]. The percentage of proteins categorized into different biological process groups were found to be similar between all 4 groups, where the same trend was also observed for molecular functions (Figure 15). As observed in cell recruitment, cytokine production, and exosome proteome, the host inflammatory response does not seem to be affected by the absence or presence of GP63.

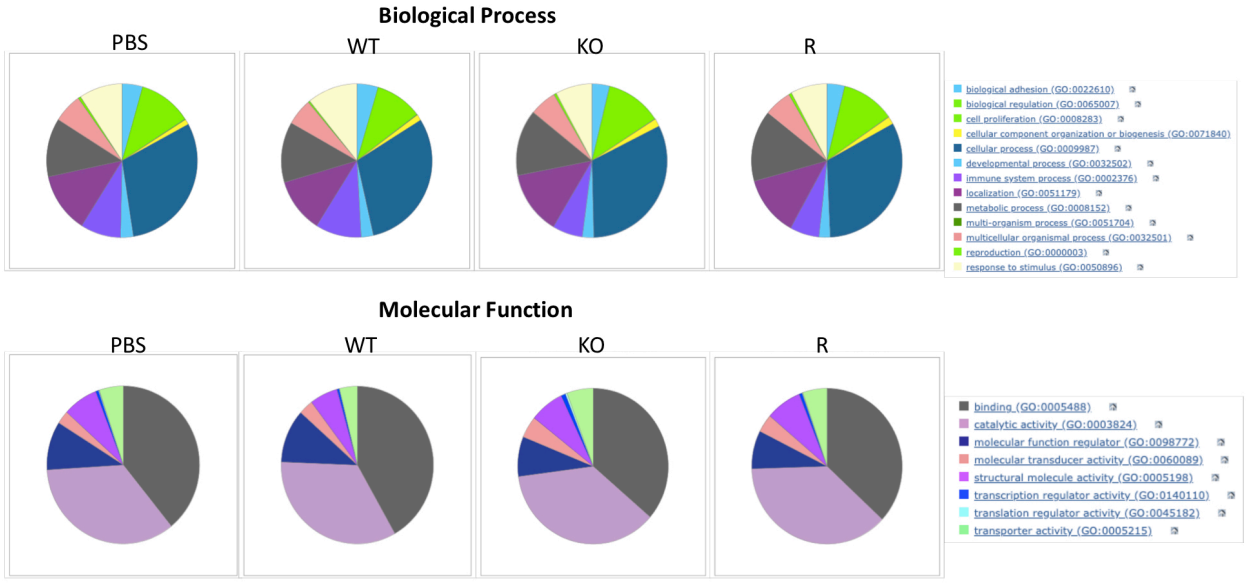

Figure 15: Proteins found in EVs categorized by gene ontology show similar patterns between all groups.

List of proteins expressed in each group were generated using scaffold (minimum 2 spectrum counts in one sample), and pie charts were generated on Panther14.1. The number of proteins in each category are graphed as a percentage of total proteins mapped.

***Leishmania* GP63 favor parasite survival and growth within macrophages.**

From analyzing our cell recruitment, cytokine production, and exosome data, the *L. major*^{KO} did not appear to affect the early cellular response despite lacking major virulence factor GP63. Since *L. major*^{KO} footpad infection led to a reduced and delayed lesion formation, there

still may be other factors at play during the early events of infection. Therefore, we moved on to analyze the infectivity and survival of parasites over time. A common way that leishmaniasis is diagnosed clinically is to observe stained cells obtained in lymph nodes for amastigotes within monocytes and macrophages [100]. From the cytospin prepared Diff Quik stained slides, amastigotes could be seen as round or oval bodies found within the cytoplasm of infected cells. Amastigotes were observed in cells from all 3 *L. major* infected groups, in both neutrophils and macrophages.

After 6 hours, the percentage of cells infected revealed that significantly fewer cells were infected in the *L. major*^{KO} group (~17%) compared with *L. major*^{WT} and *L. major*^{R} (~30%, $p<0.05$) (Figure 16A). Infection with the GP63 rescue parasites resulted in more cells infected with *L. major*. In the observed cells, a higher percentage of neutrophils were infected compared to macrophages, about 30% compared to 10%. The majority of the neutrophils contained 2-3 amastigotes, while macrophages accommodated a higher average number of amastigotes at a time. The presence of GP63 resulted in more parasites found within cells, showing higher infectivity ($p<0.05$) (Figure 16B). These results indicate that GP63 allows *L. major* to more effectively enter host cells and survive the killing mechanisms during initial infection.

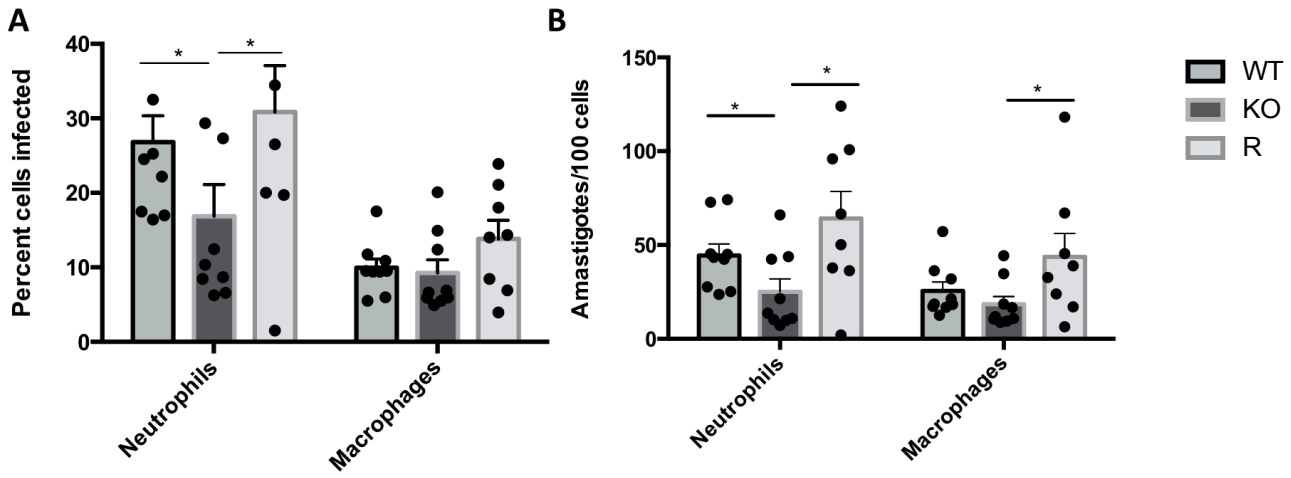

Figure 16: GP63 increases Leishmania infection and number of amastigotes 6 hours post-infection.

Intraperitoneal lavages following a 6-hour infection with $1x10^8$ WT, $GP63^{KO}$, and $GP63^{R}$ *L. major* were mounted on slides using cytospin. A) Percentage of neutrophils and macrophages infected. 200 cells were counted, bars represent ± SEM, each point represents one mouse, (n=9 mice total, 3 mice per experiment) Statistical significance was determined using unpaired t-tests with Welch's correction, *$p<0.05$. B) The number of amastigotes found per 100 cells, which was determined using the average amastigotes found per cell and the percentage of infected cells. 200 cells were counted, bars represent ± SEM, each point represents one mouse (n=9 mice total, 3 mice per experiment). The same statistical test was applied as A), *$p<0.05$.

Leishmania GP63 favor infection progression in macrophages.

The cells retrieved from the lavage were seeded on chamber slides and were observed 24 hours and 48 hours post infection. At these time points, the cells were fixed immediately and stained to count the infection progress. Consistent with the cytospin infection counts, the *L. major*^{WT} and *L. major*^{R} parasites resulted in the highest percent of cells infected (Figure 17A). The percentage of infected cells also increased over time for the *L. major*^{WT} and *L. major*^{R} infected cells, where at 24hrs *L. major*^{R} infected over 3 times more cells than *L. major*^{KO} ($p<0.001$). In the *L. major*^{KO} groups, the difference between 6 hours and 12 hours was not as great as that seen in the other groups. The percentage of infected cells only began to increase at the 48 hour time point for the *L. major*^{KO} group, which demonstrates the initial killing of the

parasites. Only after the initial infection are the parasites able to infect more cells and re-establish the infection. These numbers were also reflected in the total number of amastigotes found within the infected cells (Figure 17B). Statistical significance was not seen between *L. major*^{WT} and *L. major*^{KO} groups at 48 hours. These results demonstrate that parasites lacking GP63 are less infective during early infection and that reinsertion of GP63 seems to exacerbate some infectious characteristics conferring greater fitness once within the host cell.

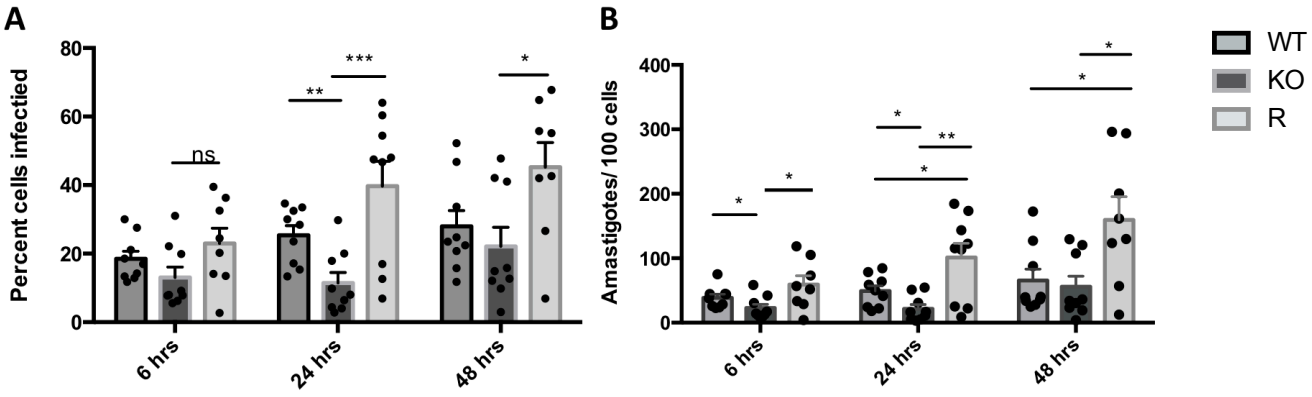

Figure 17: Infection progression over 48 hours is exacerbated by GP63.

Intraperitoneal lavages following a 6-hour infection with *L. major*^{WT}, *L. major*^{KO} and *L. major*^{R} were plated onto chamber slides. A) Percentage of cells infected at different timepoints post infection. Data for 6 hrs was from cytospin prepared slides, and cells were fixed and stained at 24hrs and 48hrs post infection. 200 cells were counted, bars represent ± SEM, each point represents one mouse, (n=9 mice total, 3 mice per experiment) Statistical significance was determined using unpaired t-tests with Welch's correction, *p<0.05, **p<0.01, ***p<0.001. B) The number of amastigotes found per 100 cells, which was determined using both the average amastigotes found per cell and the percentage of infected cells. 200 cells were counted, bars represent ± SEM, each point represents one mouse (n=9 mice total, 3 mice per experiment). The same statistical test was applied as in (A), *p<0.05, **p<0.01.

2.6 Discussion

Cutaneous leishmaniasis (CL) caused by *L. major* remains a major public health concern to people living in endemic areas, as lesions can be acute or become chronic and slow healing. Currently, knowledge gaps still exist in the early innate immune events in response to *Leishmania* in human hosts. This response is modulated by various *Leishmania* virulence factors to favour parasite survival, which in turn delay clearance and prolong disease. From the past research in our lab, we have gained the understanding that GP63 is a significant virulence factor involved in the attenuation of macrophage immune function and signalling [53]. The early moments from *Leishmania* inoculation are extremely crucial for the progression and outcome of disease, but not thoroughly studied. Gaining a better understanding of the major virulence factor GP63 and how it modulates the host immune response globally will provide avenues for the development of better therapies for CL patients.

Previous studies performed in susceptible BALB/c mice using *L. major*^{KO} demonstrated that *Leishmania* lacking GP63 were less prone to establish infection in comparison to their wild type counterpart [73] [82]. Studies show that cutaneous lesion formation could be a result of the acute infiltration of macrophages, neutrophils, and eosinophils to the dermis [101], but could also be an direct indicator of parasite burden [102]. The reduced lesion formation in *L. major*^{KO} parasites may be due to factors such as increased parasite elimination, delayed host cell infiltration, or inability to cause infection, which we aimed to investigate. In our footpad infection in healing type C57BL/6 mice, we saw footpad thickness infected with *L. major*^{WT} increase at a similar rate to the level reported in the literature, reaching the same change of 2mm in footpad thickness in both studies. In our study, we also saw a delay in lesion development in the *L. major*^{KO} parasites when compared to the *L. major*^{WT} and *L. major*^{R}. Generally, the

inflammation was observed earlier than the previously reported findings, at about 5 weeks. Nonetheless, the reduced lesion observed for *L. major*^{KO} infected mice over control groups is similar regardless of the different immune response from the two mouse models (Balb/c vs C57BL/6). Therefore, this finding serves as a clue to investigate the early immune response, to elucidate the cause of reduced footpad inflammation in *L. major*^{KO} infected mice. Even though GP63 is a potent modulator of cell signalling and recruitment, there are other virulence factors such as lipophosphoglycan (LPG), cysteine proteases (CPs), and glycosyl inositol phospholipids (GIPLs) that can influence cell signalling [103] [53]. With this in mind, we were able to move on to investigate the early innate inflammatory response, which may reveal the mechanisms behind the different infection outcomes.

Previous studies showed infection with *L. major* in mouse skin pouches caused rapid leukocyte recruitment that peaked at 6 hours post-infection [27]. The intraperitoneal infection model allowed for the greater recruitment of cells and the measurement of cytokines and exosomes released in the supernatant [51]. To our surprise, the total cell recruitment and cell types were not found to be significantly different between *L. major*^{KO} and the other groups. The majority of the cells recruited were neutrophils, which is in line with reported literature for the initial cell recruitment [28]. Neutrophils are essential in the establishment of *Leishmania* infection since they are needed to shuttle parasites to be phagocytosed by macrophages. There is also macrophage activation from antigenic stimulation in all infection groups, as seen in the flow cytometry data of small peritoneal macrophages [94]. The incoming small peritoneal macrophages are derived from monocytes and dominated the population of existing large peritoneal macrophages in all cases, demonstrating that GP63 is not required to recruit SPM. GP63 is known to cleave transcription factors NF-κB to produce p35 RelA novel fragment,

which binds DNA with NF-kappaB p50 and activates transcription of chemokine genes (CXCL2, CCL2, CCL3, CCL4) [71].

Previous knowledge indicates the immunomodulatory action of GP63 should induce greater cell recruitment to support the infection. To further understand why cell recruitment is similar between the infection groups, the cytokines and chemokines produced were measured. Surprisingly, the cytokines IL-6, IL1-β and TNF-α were the highest in the *L. major*^{WT} group and not recapitulated in the *L. major*^{R} group. Differences between *L. major*^{WT} and *L. major*^{R} parasites can be attributed to GP63 genes 6 and 7, which may have differences in proteinase substrate specificities [73] [104]. Cytokines IFN-γ and TNF-α are crucial in *Leishmania* clearance by working synergistically to increase macrophage killing and NO production [105]. In CL patients, upregulation of pro-inflammatory cytokines was associated with increased tissue damage and lesion size, therefore GP63 is more inflammatory at the early timepoints of infection. On the other hand, IL-6 in C57BL/6 mice was reported to be responsible for Th2 responses and IL-6^{ko} mice were able to control infection, making IL-6 a susceptibility factor [106]. Many cytokines have been described to impact the outcome of disease; most notably IL-12 is required for a Th1 driven response for parasite clearance by signalling through the JAK/STAT pathway. IL-12 was seen to be selectively inhibited in inflammatory macrophages infected with *Leishmania* [107], and we know that GP63 is an inhibitor of JAK/STAT signalling. However, it was detected at negligible levels in our lavage supernatant in all groups, along with several other cytokines like IL-4. Moreover, the chemokines were expressed at similar levels for all three infection groups regardless of GP63 expression. Past reports in both mice and human cells described the following chemokines were increased during the initial phase of *L. major* infection: KC, MIP-2, MIP1a and b, CCL5, CXCL10, and CCL2 [108]. With the exception of CCL5, all

aforementioned chemokines were expressed in our lavage supernatant, regardless of GP63. In this early time point of 6 hours post-infection, we see that neutrophils are the most important and macrophages have only begun to become activated and recruited as per flow cytometry data. Therefore, the cell signalling modulation by GP63, as shown in past research, is not yet observed at a significant level. It is evident that GP63 is not the sole *Leishmania* factor responsible for the recruitment of leukocytes to the site of infection. *Leishmania* expresses a collection of virulence factors that could affect the inflammatory milieu, for example, cysteine proteases (CP) can degrade NF-κB, STAT-1, and AP-1, similar to the effects of GP63 [53].

To further characterize the inflammatory response, the exosomes released also play a significant role in intercellular communication. To our knowledge, this is the first reported characterization of total exosomes released by cells in the intraperitoneal cavity following *Leishmania* infection. In this study, we also describe a novel procedure to purify host exosomes from the mouse peritoneal cavity in order to minimize contamination. International standards for studies in EVs require adequate proof that reports are indeed associated with EVs, therefore we performed NTA, TEM, and proteomic analysis for our samples [109]. The presence of GP63 seemed to induce more exosome production from recruited cells, although not statistically significant. Studies have shown that cellular stress can induce exosome release as a way to eliminate waste and induce pathological signals to surrounding cells [110]. The autophagy process was also shown to induce exosome release [111], and autophagy was reported to facilitate infection in *Leishmania* infected macrophages [112]. The proteolytic action of GP63 on the cell membrane can cause cellular stress, but more research needs to be done to elucidate the role of GP63 in host exosome secretion. However, it is clear that modulation of phagocytic cells upon infection *in vivo* could be sufficient to enhance exosome released in the lumen of the

peritoneum. From the proteomics enrichment data, that there are no specific proteins that are enriched, despite the varied protein profiles based on presence. Even with careful extraction and washing, some non-exosome contaminants such as mouse keratin and serum albumin were still detected in the samples; a weakness in this dataset. Gene ontology graphs reveal that biological processes and molecular functions of the protein profiles are similar between all groups, including the PBS group. The general functions of the exosomes found in the peritoneal cavity have not been modulated at this point in the infection, with only a few proteins of interest. We identified several immune related proteins, which showed similar levels between all infection groups. For example, neutrophilic granule protein was found in all infection groups, which is reported in UniprotKB [113] as a protease inhibitor involved in the defense response. The protein level of exosome markers also varied amongst groups, meaning the loading amount could have influenced the detection of trace unique proteins. Since the cell population found in the intraperitoneal cavity is highly diverse, the inflammatory nature of macrophage exosomes found *in vitro* [46] is less likely to be observed in our sample.

Lastly, we looked at the level of parasitemia at the time of lavage collection and tracked the infection levels over time. At 6 hours, ~1.6x more neutrophils and macrophages contained amastigotes when infected with *L. major* expressing GP63 compared to *L. major*^{KO}. As expected, GP63 is a crucial factor for the efficiency of infecting host cells in the early moments of inoculation. GP63 is able to bind macrophage complement receptors 1 and 3 through the cleavage of C3 to iC3b, as well as interact with receptors B1 integrins [58] [59]. They also degrade the extracellular matrix to favour migration into the cells, increasing the susceptibility in other cell types [55]. We saw the highest number of neutrophils infected because it was the primary cell type that was recruited during the 6-hr infection time frame. Neutrophil recruitment

favours the development of leishmaniasis because infected neutrophils act as "trojan horses" to shuttle amastigotes to macrophages [28]. There was a smaller difference in the number of macrophages infected, presumably due to the anti-*Leishmania* activity that GP63 does not attenuate in the macrophages. In a previous *in vitro* study using *Leishmania* with GP63 genes 1-6 knockout, Joshi et al. [82] saw no apparent difference between the infectivity of the knockout parasites and wild type when infecting macrophages *in vitro*. However, our infection with Leishmania with GP63 genes 1-7 knockout did show a significant difference in infectivity between groups, revealing an importance in gene 7 and *in vivo* infection.

After the entry into host cells, amastigotes must be able to survive the harsh host environment and be able to replicate and subsequently infect new cells [17]. In hamster peritoneal macrophages, the infectivity and multiplication of *Leishmania* amastigotes were seen to increase over the course of seven days post infection [114]. The survival of the amastigotes internalized by cells was monitored 24 and 48 hrs post-infection in chamber slides and revealed that *L. major*^{KO} parasites were less able to survive within the host macrophages and have greater difficulty infecting new cells. There were few free promastigotes found in the lavage; therefore, the number of cells infected is representative of the progression from the 6-hour cell count. Further infection of macrophages is due to the phagocytosis of apoptotic infected neutrophils, as well as replicated amastigotes exiting infected cells [28]. These results corroborate with previous knowledge that host anti-*Leishmania* effectors such as NO production, phagolysosome maturation and ROS inflammasome activation can come into play when not inhibited by GP63 [62] [53]. The surviving cells were then able to infect new cells, as seen at 48 hours post-infection. Our findings confirm previous findings that early elimination of *Leishmania* is crucial in the control of disease [79], where the development of a Th1 response is a result of proper

macrophage and NK cell activation [115]. However, our findings also contradict the study with GP63 genes 1-6 knocked out, because Joshi et al. observed that GP63 did not affect the survival of amastigotes in macrophages 96 post infection *in vitro* [82]. They did, however, observe the same delay in footpad infection, suggesting that *in vivo* interactions with other immune cells is more representative of the infection.

Ultimately, our study demonstrated a detailed overview of the innate immune response during the early time points of an *L. major* infection. The early innate immune response can heavily influence the outcome of disease progression and whether pathology gets controlled. In regard to GP63, it causes a faster and more aggressive lesion development in *L. major* cutaneous leishmaniasis. Regardless of GP63, the innate inflammatory response acts similarly in terms of cell recruitment, cytokine/chemokine production, and exosome content. However, we see that *L. major*^{KO} parasites are less able to infect and survive within host cells to further infect other cells. Therefore, the parasite load is reduced, making it more difficult for the surviving *Leishmania* to establish disease. Characterization of factors affecting disease progression and severity can lead to improved treatments for patients with cutaneous leishmaniasis.

CHAPTER 3: CONCLUDING REMARKS

Leishmaniasis remains a serious public health concern, with over 1 billion individuals living in at-risk endemic countries. The CDC estimated about 1 million new cases of this parasitic disease each year, where patients who are chronically infected act as reservoirs for transmission. To this day, there is a need for a better understanding of cutaneous leishmaniasis pathology in order to halt disease progression in patients and prevent further transmission. *Leishmania* vaccines are not yet available, and current drugs used have issues with toxicity, cost and drug resistance. In this thesis, I described the early innate immune response to *Leishmania* and reviewed what is known about *Leishmania* and its host interactions in the scientific literature. Research to date has not yet elucidated the role of *Leishmania* GP63 in the overall modulation of the early host response. Here, we were able to demonstrate that *Leishmania* GP63 does not significantly modulate cell recruitment and cytokine/chemokine production after a 6 hours infection. Exosome release indicates a difference in proteomic profile, but functional elements remained largely unchanged. However, as the infection progressed after one to two days, GP63 exhibited a significant effect in the increase of amastigotes within cells and the proportion of cells infected. The speed of CL lesion development is influenced by the ability of the parasite to survive and infect more cells, which is driven by *Leishmania* GP63. There has been some interest in the generation of anti-parasitic drugs targeting metalloprotease [116]. As of current knowledge, GP63 is not an ideal target in rational drug design because it is non-essential upon infection of cells, as intra-macrophage amastigotes once well installed will produce very little GP63. However as prophylactic target it could be interesting to develop a specific protease inhibitor that could be used to attenuate the infectious process. Our approach of using an intraperitoneal injection to study the immune response yields an abundance of results but may not reflect a

natural infection. A potential future direction for this project is to utilize a different infection model such as a subcutaneous air pouch. In the future, further characterization of mechanisms involved in pathology progression may allow for the development of improved treatment protocols to accelerate disease clearance in patients.

APPENDIX

Supplemental documents:

Dataset 1: Total Spectra of Mus musculus EVs (Proteomics Analysis)

http://tiny.cc/exosomes

REFERENCES

1. Organization WH. Leishmaniasis. In: Organization WH, editor.

2. for Disease Control C, Prevention. Leishmaniasis. In: for Disease Control C, Prevention, editors.

3. Hendrickx S, Caljon G, Maes L. Need for sustainable approaches in antileishmanial drug discovery. Parasitol Res. 2019;118(10):2743-52.

4. Ready PD. Leishmaniasis emergence and climate change. Rev - Off Int Epizoot. 2008;27(2):399-412.

5. Rioux JA, Golvan YJ. Epidémiologie des leishmaniosesdans le sud de la France. Monographie INSERM. 1968;37.

6. Desjeux P. The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg. 2001;95(3):239-43.

7. Cross ER, Hyams KC. The potential effect of global warming on the geographic and seasonal distribution of Phlebotomus papatasi in southwest Asia. Environ Health Perspect. 1996;104(7):724-7.

8. Yan-Ling Z, Xin Z, Zhi-Guo Y. Epidemiological screening of an epidemic of imported cutaneous leishmaniasis in Luoyang City. Zhongguo xue xi chong bing fang zhi za zhi= Chinese journal of schistosomiasis control. 2019;31(4):418-22.

9. el Harith A, Mahamoud A, Awad Y, Mansour D, Abass EM, el Agib A, et al. Are We Now Well Prepared for Another Major Visceral Leishmaniasis Epidemic in Sudan? Open Forum Infectious Diseases. 2019;6(10).

10. Arias JR, Monteiro PS, Zicker F. The reemergence of visceral leishmaniasis in Brazil. Emerging Infect Dis. 1996;2(2):145-6.

11. Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009;136(14):1915-34. Epub 2009/10/20. doi: 10.1017/S0031182009991156. PubMed PMID: 19835643.

12. Ready PD. Biology of phlebotomine sand flies as vectors of disease agents. Annu Rev Entomol. 2013;58:227-50.

13. Killick-Kendrick R. The life-cycle of Leishmania in the sandfly with special reference to the form infective to the vertebrate host. Ann Parasitol Hum Comp. 1990;65 Suppl 1:37-42.

14. Gossage SM, Rogers ME, Bates PA. Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. Int J Parasitol. 2003;33(10):1027-34.

15. Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. Int J Parasitol. 2007;37(10):1097-106.

16. Rittig MG, Bogdan C. Leishmania-host-cell interaction: complexities and alternative views. Parasitol Today (Regul Ed). 2000;16(7):292-7.

17. Handman E, Bullen DV. Interaction of Leishmania with the host macrophage. Trends Parasitol. 2002;18(8):332-4.

18. Organization WH, others. Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases, Geneva, 22-26 March 2010: World Health Organization; 2010.

19. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014;6:147-54.

20. Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, et al. New perspectives on a subclinical form of visceral leishmaniasis. J Infect Dis. 1986;154(6):1003-11.

21. John DT, Petri WA. Markell and Voge's Medical Parasitology-E-Book: Elsevier Health Sciences; 2013.

22. Kumar R, Nylen S. Immunobiology of visceral leishmaniasis. Front Immunol. 2012;3:251-.

23. McGwire BS, Satoskar AR. Leishmaniasis: clinical syndromes and treatment. QJM. 2014;107(1):7-14.

24. Herwaldt BL. Leishmaniasis. Lancet. 1999;354(9185):1191-9.

25. Goto H, Lauletta Lindoso JA. Cutaneous and mucocutaneous leishmaniasis. Infect Dis Clin North Am. 2012;26(2):293-307.

26. Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther. 2010;8(4):419-33.

27. Matte C, Olivier M. Leishmania-induced cellular recruitment during the early inflammatory response: modulation of proinflammatory mediators. J Infect Dis. 2002;185(5):673-81.

28. Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes? Trends Microbiol. 2003;11(5):210-4.

29. Peters NC, Egen JG, Secundino N, Debrabant A, Kimblin N, Kamhawi S, et al. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science. 2008;321(5891):970-4.

30. Guimaraes-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao-Silva F, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci USA. 2009;106(16):6748-53.

31. León B, López-Bravo Ma, Ardavı´n C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. Immunity. 2007;26(4):519-31.

32. Svensson M, Maroof A, Ato M, Kaye PM. Stromal cells direct local differentiation of regulatory dendritic cells. Immunity. 2004;21(6):805-16.

33. Launois P, Maillard I, Pingel S, Swihart KG, Xenarios I, Acha-Orbea H, et al. IL-4 rapidly produced by V beta 4 V alpha 8 CD4+ T cells instructs Th2 development and susceptibility to Leishmania major in BALB/c mice. Immunity. 1997;6(5):541-9.

34. Himmelrich H, Launois P, Maillard I, Biedermann T, Tacchini-Cottier F, Locksley RM, et al. In BALB/c mice, IL-4 production during the initial phase of infection with Leishmania major is necessary and sufficient to instruct Th2 cell development resulting in progressive disease. J Immunol. 2000;164(9):4819-25.

35. Park AY, Hondowicz B, Kopf M, Scott P. The role of IL-12 in maintaining resistance to Leishmania major. J Immunol. 2002;168(11):5771-7.

36. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+ CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature. 2002;420(6915):502-.

37. Bogdan C, Moll H, Solbach W, Röllinghoff M. Tumor necrosis factor-α in combination with interferon-γ, but not with interleukin 4 activates murine macrophages for elimination of Leishmania major amastigotes. European journal of immunology. 1990;20(5):1131-5.

38. Conceicao-Silva F, Hahne M, Schroter M, Louis J, Tschopp J. The resolution of lesions induced by Leishmania major in mice requires a functional Fas (APO-1, CD95) pathway of cytotoxicity. Eur J Immunol. 1998;28(1):237-45.

39. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol. 2002;2(11):845-58. doi: 10.1038/nri933. PubMed PMID: 12415308.

40. Bogdan C, Donhauser N, Doring R, Rollinghoff M, Diefenbach A, Rittig MG. Fibroblasts as host cells in latent leishmaniosis. J Exp Med. 2000;191(12):2121-30.

41. Moll H, Flohe S, Blank C. Dendritic cells seclude Leishmania parasites that persist in cured mice--a role in the maintenance of T-cell memory? Adv Exp Med Biol. 1995;378:507-9.

42. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. Nat Rev Rheumatol. 2014;10(6):356-64.

43. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem. 1987;262(19):9412-20.

44. Pluchino S, Smith JA. Explicating Exosomes: Reclassifying the Rising Stars of Intercellular Communication. Cell. 2019;177(2):225-7.

45. O'Neill HC, Quah BJ. Exosomes secreted by bacterially infected macrophages are proinflammatory. Sci Signal. 2008;1(6):pe8-pe.

46. Hassani K, Olivier M. Immunomodulatory impact of leishmania-induced macrophage exosomes: a comparative proteomic and functional analysis. PLoS Negl Trop Dis. 2013;7(5):e2185-e.

47. Hassani K, Antoniak E, Jardim A, Olivier M. Temperature-induced protein secretion by Leishmania mexicana modulates macrophage signalling and function. PLoS ONE. 2011;6(5):e18724-e.

48. Atayde VD, Aslan H, Townsend S, Hassani K, Kamhawi S, Olivier M. Exosome Secretion by the Parasitic Protozoan Leishmania within the Sand Fly Midgut. Cell Rep. 2015;13(5):957-67. Epub 2015/11/14. doi: 10.1016/j.celrep.2015.09.058. PubMed PMID: 26565909; PubMed Central PMCID: PMCPMC4644496.

49. Rogers ME, Ilg T, Nikolaev AV, Ferguson MA, Bates PA. Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. Nature. 2004;430(6998):463-7. Epub 2004/07/23. doi: 10.1038/nature02675. PubMed PMID: 15269771; PubMed Central PMCID: PMCPMC2835460.

50. Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, et al. Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. Proc Natl Acad Sci U S A. 2008;105(29):10125-30. Epub 2008/07/16. doi: 10.1073/pnas.0802331105. PubMed PMID: 18626016; PubMed Central PMCID: PMCPMC2481378.

51. Ray A, Dittel BN. Isolation of mouse peritoneal cavity cells. J Vis Exp. 2010;(35).

52. Casgrain PA, Martel C, McMaster WR, Mottram JC, Olivier M, Descoteaux A. Cysteine Peptidase B Regulates Leishmania mexicana Virulence through the Modulation of GP63 Expression. PLoS Pathog. 2016;12(5):e1005658. Epub 2016/05/19. doi: 10.1371/journal.ppat.1005658. PubMed PMID: 27191844; PubMed Central PMCID: PMCPMC4871588.

53. Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT. Leishmania virulence factors: focus on the metalloprotease GP63. Microbes Infect. 2012;14(15):1377-89. Epub 2012/06/06. doi: 10.1016/j.micinf.2012.05.014. PubMed PMID: 22683718.

54. Schneider P, Rosat JP, Bouvier J, Louis J, Bordier C. Leishmania major: differential regulation of the surface metalloprotease in amastigote and promastigote stages. Exp Parasitol. 1992;75(2):196-206.

55. Yao C, Donelson JE, Wilson ME. The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function. Mol Biochem Parasitol. 2003;132(1):1-16.

56. McGwire BS, Chang KP, Engman DM. Migration through the extracellular matrix by the parasitic protozoan Leishmania is enhanced by surface metalloprotease gp63. Infect Immun. 2003;71(2):1008-10.

57. Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol. 1995;155(6):3102-11.

58. Mosser DM, Edelson PJ. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of Leishmania promastigotes. J Immunol. 1985;135(4):2785-9.

59. Brittingham A, Chen G, McGwire BS, Chang KP, Mosser DM. Interaction of Leishmania gp63 with cellular receptors for fibronectin. Infect Immun. 1999;67(9):4477-84.

60. Matte C, Casgrain PA, Seguin O, Moradin N, Hong WJ, Descoteaux A. Leishmania major Promastigotes Evade LC3-Associated Phagocytosis through the Action of GP63. PLoS Pathog. 2016;12(6):e1005690-e.

61. Estrada-Figueroa LA, Diaz-Gandarilla JA, Hernandez-Ramirez VI, Arrieta-Gonzalez MM, Osorio-Trujillo C, Rosales-Encina JL, et al. Leishmania mexicana gp63 is the enzyme responsible for cyclooxygenase (COX) activity in this parasitic protozoa. Biochimie. 2018;151:73-84.

62. Shio MT, Christian JG, Jung JY, Chang KP, Olivier M. PKC/ROS-Mediated NLRP3 Inflammasome Activation Is Attenuated by Leishmania Zinc-Metalloprotease during Infection. PLoS Negl Trop Dis. 2015;9(6):e0003868-e.

63. Corradin S, Ransijn A, Corradin G, Roggero MA, Schmitz AA, Schneider P, et al. MARCKS-related protein (MRP) is a substrate for the Leishmania major surface protease leishmanolysin (gp63). J Biol Chem. 1999;274(36):25411-8.

64. Gomez MA, Contreras I, Halle M, Tremblay ML, McMaster RW, Olivier M. Leishmania GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Sci Signal. 2009;2(90):ra58-ra.

65. Abu-Dayyeh I, Shio MT, Sato S, Akira S, Cousineau B, Olivier M. Leishmania-induced IRAK-1 inactivation is mediated by SHP-1 interacting with an evolutionarily conserved KTIM motif. PLoS Negl Trop Dis. 2008;2(12):e305-e.

66. de Celis HÁ, Gomez CP, Descoteaux A, Duplay P. Dok proteins are recruited to the phagosome and degraded in a GP63-dependent manner during Leishmania major infection. Microbes and infection. 2015;17(4):285-94.

67. Jaramillo M, Gomez MA, Larsson O, Shio MT, Topisirovic I, Contreras I, et al. Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection. Cell Host Microbe. 2011;9(4):331-41.

68. Contreras I, Gomez MA, Nguyen O, Shio MT, McMaster RW, Olivier M. Leishmania-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog. 2010;6(10):e1001148-e.

69. Forget G, Gregory DJ, Olivier M. Proteasome-mediated degradation of STAT1alpha following infection of macrophages with Leishmania donovani. J Biol Chem. 2005;280(34):30542-9.

70. Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M. A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur J Immunol. 2008;38(4):1071-81.

71. Guizani-Tabbane L, Ben-Aissa K, Belghith M, Sassi A, Dellagi K. Leishmania major amastigotes induce p50/c-Rel NF-kappa B transcription factor in human macrophages: involvement in cytokine synthesis. Infect Immun. 2004;72(5):2582-9.

72. Button LL, Russell DG, Klein HL, Medina-Acosta E, Karess RE, McMaster WR. Genes encoding the major surface glycoprotein in Leishmania are tandemly linked at a single chromosomal locus and are constitutively transcribed. Molecular and biochemical parasitology. 1989;32(2-3):271-83.

73. Joshi PB, Kelly BL, Kamhawi S, Sacks DL, McMaster WR. Targeted gene deletion in Leishmania major identifies leishmanolysin (GP63) as a virulence factor. Mol Biochem Parasitol. 2002;120(1):33-40. PubMed PMID: 11849703.

74. Ivens AC, Peacock CS, Worthey EA, Murphy L, Aggarwal G, Berriman M, et al. The genome of the kinetoplastid parasite, Leishmania major. Science. 2005;309(5733):436-42.

75. WHO. Leishmaniasis: World Health Organization; 2019 [updated March 14 2019].

76. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nat Rev Immunol. 2016;16(9):581-92. Epub 2016/07/18. doi: 10.1038/nri.2016.72. PubMed PMID: 27424773.

77. Dowlati Y. Cutaneous leishmaniasis: clinical aspect. Clinics in dermatology. 1996;14(5):425-31.

78. Castellano LR, Filho DC, Argiro L, Dessein H, Prata A, Dessein A, et al. Th1/Th2 immune responses are associated with active cutaneous leishmaniasis and clinical cure is associated with strong interferon-gamma production. Hum Immunol. 2009;70(6):383-90.

79. Laskay T, Diefenbach A, Rollinghoff M, Solbach W. Early parasite containment is decisive for resistance to Leishmania major infection. Eur J Immunol. 1995;25(8):2220-7.

80. Russell DG. The macrophage-attachment glycoprotein gp63 is the predominant C3-acceptor site on Leishmania mexicana promastigotes. Eur J Biochem. 1987;164(1):213-21.

81. Isnard A, Shio MT, Olivier M. Impact of Leishmania metalloprotease GP63 on macrophage signaling. Front Cell Infect Microbiol. 2012;2:72. Epub 2012/05/16. doi: 10.3389/fcimb.2012.00072. PubMed PMID: 22919663; PubMed Central PMCID: PMCPMC3417651.

82. Joshi PB, Sacks DL, Modi G, McMaster WR. Targeted gene deletion of Leishmania major genes encoding developmental stage-specific leishmanolysin (GP63). Mol Microbiol. 1998;27(3):519-30.

83. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, Palomo-Cetina A. Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. Infect Immun. 1994;62(3):837-42. Epub 1994/03/01. PubMed PMID: 8112853; PubMed Central PMCID: PMCPMC186190.

84. Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, Martel C, et al. Leishmania exosomes and other virulence factors: Impact on innate immune response and macrophage functions. Cell Immunol. 2016;309:7-18. Epub 2016/07/28. doi: 10.1016/j.cellimm.2016.07.013. PubMed PMID: 27499212.

85. da Silva R, Sacks DL. Metacyclogenesis is a major determinant of Leishmania promastigote virulence and attenuation. Infect Immun. 1987;55(11):2802-6.

86. Isnard A, Christian JG, Kodiha M, Stochaj U, McMaster WR, Olivier M. Impact of Leishmania infection on host macrophage nuclear physiology and nucleopore complex integrity. PLoS Pathog. 2015;11(3):e1004776. Epub 2015/04/01. doi: 10.1371/journal.ppat.1004776. PubMed PMID: 25826301; PubMed Central PMCID: PMCPMC4380401.

87. Soo CY, Song Y, Zheng Y, Campbell EC, Riches AC, Gunn-Moore F, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. Immunology. 2012;136(2):192-7.

88. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem. 2002;74(20):5383-92.

89. Lex A, Gehlenborg N, Strobelt H, Vuillemot R, Pfister H. UpSet: Visualization of Intersecting Sets. IEEE Trans Vis Comput Graph. 2014;20(12):1983-92.

90. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 2019;47(D1):D419-D26.

91. Toth M, Sohail A, Fridman R. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods Mol Biol. 2012;878:121-35.

92. Lacks SA, Springhorn SS. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J Biol Chem. 1980;255(15):7467-73.

93. Miyazaki S, Ishikawa F, Fujikawa T, Nagata S, Yamaguchi K. Intraperitoneal injection of lipopolysaccharide induces dynamic migration of Gr-1high polymorphonuclear neutrophils in the murine abdominal cavity. Clin Diagn Lab Immunol. 2004;11(3):452-7.

94. Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, et al. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. Proc Natl Acad Sci USA. 2010;107(6):2568-73.

95. Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. Blood. 2007;110(9):3234-44.

96. Coakley G, Maizels RM, Buck AH. Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections. Trends Parasitol. 2015;31(10):477-89.

97. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. Nucleic Acids Res. 2012;40(Database issue):D1241-4.

98. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, et al. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics. 2005;4(9):1265-72.

99. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-9.

100. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol. 2002;9(5):951-8. PubMed PMID: 12204943; PubMed Central PMCID: PMCPMC120052.

101. Belkaid Y, Mendez S, Lira R, Kadambi N, Milon G, Sacks D. A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J Immunol. 2000;165(2):969-77.

102. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol. 2001;166(2):1141-7.

103. Descoteaux A, Matlashewski G, Turco SJ. Inhibition of macrophage protein kinase C-mediated protein phosphorylation by Leishmania donovani lipophosphoglycan. J Immunol. 1992;149(9):3008-15.

104. Voth BR, Kelly BL, Joshi PB, Ivens AC, McMaster WR. Developmentally expressed Leishmania major gp63 genes encode cell surface leishmanolysin containing distinct signals for GPI attachment. Mol Biochem Parasitol. 1998.

105. Liew FY, Wei X-Q, Proudfoot L. Cytokines and nitric oxide as effector molecules against parasitic infections. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences. 1997;352(1359):1311-5.

106. Moskowitz NH, Brown DR, Reiner SL. Efficient immunity against Leishmania major in the absence of interleukin-6. Infection and immunity. 1997;65(6):2448-50.

107. Belkaid Y, Butcher B, Sacks DL. Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania-infected cells. Eur J Immunol. 1998;28(4):1389-400.

108. Teixeira MJ, Teixeira CR, Andrade BB, Barral-Netto M, Barral A. Chemokines in host--parasiteinteractions in leishmaniasis. Trends in parasitology. 2006;22(1):32-40.

109. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. Journal of extracellular vesicles. 2018;7(1):1535750-.

110. Kanemoto S, Nitani R, Murakami T, Kaneko M, Asada R, Matsuhisa K, et al. Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. Biochemical and biophysical research communications. 2016;480(2):166-72.

111. Baixauli F, Lopez-Otin C, Mittelbrunn M. Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness. Front Immunol. 2014;5:403-.

112. Pinheiro RO, Nunes MP, Pinheiro CS, D'Avila Hs, Bozza PcT, Takiya CM, et al. Induction of autophagy correlates with increased parasite load of Leishmania amazonensis in BALB/c but not C57BL/6 macrophages. Microbes and Infection. 2009;11(2):181-90.

113. Consortium TU. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Research. 2018;47(D1):D506-D15.

114. Chang KP, Dwyer DM. Leishmania donovani. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes. J Exp Med. 1978;147(2):515-30.

115. Garside P, Mowat AM. Polarization of Th-cell responses: a phylogenetic consequence of nonspecific immune defence? Immunology today. 1995;16(5):220-3.

116. Bangs JD, Ransom DA, Nimick M, Christie G, Hooper NM. In vitro cytocidal effects on Trypanosoma brucei and inhibition of Leishmania major GP63 by peptidomimetic metalloprotease inhibitors. Molecular and biochemical parasitology. 2001;114(1):111-7.