"Modulation of the Host Innate Immune Response by *Leishmania* Parasites"

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April 2010

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

To my father

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ACKMOWLEDMENTS

First of all, I would like to thank my supervisor Dr. Martin Olivier, for giving me the opportunity of working in his laboratory to achieve one of the most important and difficult things I have ever done. For your time, guidance and support over these five years thank you very much.

Second, all the members of the MOL team, past and present, one way or another all have contributed to this work. More specifically I would like to thank Dr. David Gregory for teaching me many things when I arrived to the laboratory and Dr. Marina Tiemi Shio for her constant help. Dr. Adelaida Gomez, Caroline Martel, Dr. Issa Abu-dayeeh, Lorie Whitcombe, Dr. Philippe Pouliot, Fikrergabrail Kassa, Kasra Hassani, Dr. Marie Josee Bellmare, and many others that have shared this adventure with me, thank you for giving me advice, for all the scientific discussions and for sharing your knowledge with me, I also would like to thank the two honour students that were under my supervision during my Ph. D; Oliver Nguyen and Benjamin Ralph.

I also would like to give special thanks to my funding in Mexico, the Autonomous University of the State of Mexico and the National Council of Science and Technology (CONACyT) as well as to SEP. Without the fellowships that these institutions offered me would have not been possible to come to study this Ph. D in Canada.

Throughout these years being at Dr. Olivier's lab, I have had the wonderful opportunity of having very good friends, I would like to thank my sisters in Canada: Caroline, Adelaida, Marina, Selly, Maritza and Cecilia, thank you for supporting me in good and difficult times, no matter how far we are, you always will be in my heart, thank you very much to every one of you for being part of my life. I also would like to give special thanks to Fikrer who always gave me a great support, help, and more importantly for his friendship. Finally, thanks to all my other friends that I have had throughout these years, Marie Josse and my very special french friends Marie and Sebastien. If I kept going, the list would be very long but, for all your support and friendship thanks all of you.

DEDICATORIAS

Primero que nada, quiero agradecerle a la persona mas importante en mi vida, mi esposo José Antonio. Gracias por todo el apoyo, amor y amistad que me has dado a través de estos 14 años Este doctorado te lo debo a ti en muchas formas ya que siempre has creído en mí y me has guiado. Gracias por compartir el camino de la vida conmigo, no tengo palabras para expresar todo lo que quisiera decir aquí, pero se que tu lo sabes, he llegado hasta donde estoy por ti. La vida te trajo a mí y no me puedo sentir más que la persona mas afortunada del mundo por tenerte a mi lado, gracias por todo y sabes que te amo infinitamente.

Quiero agradecer de manera muy especial a toda mi familia en México, especialmente a mis padres Jorge (que en paz descanse) y Dolores, por ustedes soy lo que soy, les agradezco infinitamente todo su apoyo y amor, la educación que me dieron me ha permitido llegar hasta donde estoy y este doctorado también es suyo. Mami gracias por ser la mejor mama del mundo y por enseñarme a ser una buena persona. También quisiera agradecer a mis hermanos Ángela, Leonardo, Georgina y mi hermano adoptivo Miguel Ángel, todos están siempre en mi corazón, junto con sus hijos que también son parte de mi.

La vida me ha dado muchas oportunidades y una de ellas fue el haber encontrado una segunda familia que me tomo en sus brazos y me acogió como una hija, hablo de mi familia política formada por Guadalupe, Francisco Javier y mis otros hermanos adoptivos Francisco Javier y Alexandra, además de una hermosa abuelita Esperanza que siempre me ha querido como su nieta. Muchas gracias por todo su amor y apoyo.

A todos y cada uno de mis amigas, amigos y el resto de la familia en México, una disculpa si no los nombro a todos ya que seria una lista muy grande pero ustedes saben de quien hablo, les agradezco a todos y cada uno por todo la amistad que me han brindado durante muchos años.

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LIST OF ABREVIATIONS

AP-1: activating protein 1

APC: antigen presenting cell

ATF 1/2: activating transcription factor 1/2

BALB/c: Bagg albino c

BCR: B-cell receptor

BMM: bone marrow macrophages

CD: cluster differentiation

c-Fos: cellular finkel, osteogenic, and sarcoma

CHO: chinese hamster ovary

c-Jun: cellular JU-Nana

CL: cutaneous leishmaniasis

CP: cysteine peptidase

CR: complement receptor

CRE: c-AMP regulated enhancer

CREB: ART-1/c-AMP response element binding

CRP: C-reactive protein

c-SH2: C-terminal SH2 domain

DAG: diacylglycerol

DC: dendritic cell

EF-1α: elongation factor alpha

ELISA: Enzyme-linked immunosorbent assay

Elk-1: Ets-like transcription factor 1

EMSA: electrophoretic mobility shift assay

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

FcR: fragment crystallization receptor

Fos B: finkel, osteogenic and sarcoma protein B

FR: fibronectin receptor

Fra 1/2: fos-related antigen 1 or 2

GAPDH: glyceralhehyde 3-phosphate dehydrogenase

GIPL: glycosylinositol phospholipid

GP63: glycoprotein 63

GPI: glycosylphosphatidylinositol

GST: glutathione S-transferase

HASP: hydrophilic acylated surface protein

HRP: horseradish peroxidise

IFN-γ: interferon gamma

Ig: immunoglobulin

IκB: inhibitory kappa B

IKK: inhibitory kappa kinase

IL: interleukin

iNOS: inducible nitric oxide synthase

IP: immunoprecipitation

IRAK: interleukin 1 receptor associated kinase

IRF: Interferon regulatory factor

ITAM: immunoreceptor tyrosine-based activation motif

ITIM: immunoreceptor tyrosine-based inhibitory motif

JAK: janus kinase

JNK: jun N-terminal kinase

Jun B/D: JU-Nane protein B or D

LC: Langerhans cell

LACK: *Leishmania* homologue of receptors for activated C kinase

LAMP-1: lysosome associated membrane protein

LM-1: littermate 1

LPG: lipophophoglycan

LPS: lipopolysacchradide

KO: knockout

Mac-1: macrophage antigen 1 alpha

MAPK: mitogen-activated protein kinase

MAPKK: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

MARCS: myristoylated alanine-rich C-kinase substrate

MCL: mucocutaneous leishmaniasis

me-3: motheaten 3

MFR: manose-fucose receptor

MHC: major histocompatibility complex

MKP: mitogen kinase phosphatase

MRP: myeloid related protein

MSP: major surface glycoprotein

MyD88: myeloid differentiation factor 88

NADPH: nicotinamide adenine dinucleotide phosphate-oxidase

NF-κB: nuclear factor kappa B

NK: natural killer cell

NLS: nuclear localization sequence

NO: nitric oxide

N-SH2: N-terminal SH2 domain

PAMP: pathogen-associated molecular pattern

PBMC: peripherical blood monocyte cells

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PG: phosphoglycan

PGE2: prostaglandin E2

PKA: protein kinase A

PKC: protein kinase C

PKDL: post kalazar dermal leishmaniasis

PLC: phospholipase C

PP2A: protein phosphatase 2A

PTK: protein tyrosine kinase

PTP: protein tyrosine phosphatase

PTP1B: protein tyrosine phosphatase 1B

RACK 1: Receptor for Activated C-Kinase 1

RBC: red blood cells

ROI: reactive oxygen intermediates

SD: standard deviation

SDS-PAGE: sodium docecyl sulphate polyacrylamide gel electrophoresis

SEM: standard error of means

SHIP: Scr homology 2-domain-containing inositol 5-phosphatese

SHP-1/2: SCR homology 2-domain-containing protein tyrosine phosphatase 1 / 2

SOCS: suppressor of cytokine signalling

Sp-1: signal protein 1

STAT: signal transducer and activator of transcription

TCPTP: T-cell protein tyrosine phosphatase

TCR: T-cell receptor

TF: transcription factor

TGF-β: transforming growth factor beta

Th: T-helper

TLR: Toll like receptor

TNF: tumour necrosis factor

TRAF 6: TNF receptor-associated factor 6

VL: visceral leishmaniasis

WT: wild type

ABSTRACT

Leishmania parasites have evolved sophisticated mechanisms to subvert macrophage immune responses in order to survive inside the mammalian host. Among these mechanisms are the rapid activation of phosphatases that in turn will inactivate protein kinases and transcription factors, causing abrogation of nitric oxide (NO) production and induction of immunosuppressive molecules. This doctoral thesis discusses novel mechanisms of how the parasite modulates the immune response of macrophages and dendritic cells.

Herein, we describe the role of Myeloid Related Proteins (MRPs) 8 and 14 during *Leishmania* infection. MRPs 8/14 are produced by neutrophils and are able to induce microbicidal activity in macrophages. We present data that shows that priming macrophages with MRP 8/14 before infection induces their activation. However, infection with *L. major* prior to MRP stimulation significantly decreases their activation. *In vivo* studies showed that abrogation of MRPs resulted in an increased parasitic burden, whereas injection of recombinant MRPs (rMRPs) reduced the size of the lesion and the parasitic load.

One of the main mechanisms that *Leishmania* parasites utilize to subvert the innate immune response is the alteration of transcription factors (TFs). In this thesis, we have shown that upon *Leishmania* infection, AP-1 activity is abolished and this correlates with the nuclear degradation of AP-1 subunits. Of interest, c-Jun, the main AP-1 activator is degraded and cleaved by *Leishmania* inside the nucleus in a GP63 dependent manner.

Despite the fact that macrophages have been considered as preferred hosts for *Leishmania* parasites, other cells have been described as possible hosts. Finally, we discuss the effect of *Leishmania* infection in dendritic cells (DCs) and how this pathogen affects their maturation and capacity to present antigen. In addition, we found that *Leishmania* is able to activate phosphatases to inactivate signalling pathways in these cells.

Collectively, our results indicate that rapid secretion of MRPs by neutrophils at the site of infection may help to protect the host from *Leishmania* infection; however, it is clear that this pathogen has developed strategies to abrogate the innate immune

machinery induced by MRPs, using its surface molecule GP63 to degrade TFs, and preventing maturation of DCs, to favour its instalment and propagation within its mammalian host.

RESUME

Le parasite *Leishmania* a su développer des mécanismes sophistiqués lui permettant de déjouer les réponses immunitaires des macrophages dans le but de survivre à l'intérieur de son hôte mammifère. Parmi ces mécanismes, notons l'activation rapide de phosphatases, qui inactiveront des protéines kinases et des facteurs de transcription, causant ainsi l'abrogation de la production d'oxyde nitrique, de même que l'induction de molécules immunosuppressives. Cette thèse doctorale discute de nouveaux mécanismes utilisés par le parasite afin de moduler la réponse immunitaire des macrophages et des cellules dendritiques.

Dans le présent rapport, nous décrirons le rôle des MRPs (Myeloid Related Proteins) 8 et 14 lors de l'infection par *Leishmania*. Produites par les neutrophiles, les MRPs 8/14 ont la capacité d'induire l'activité microbicide des macrophages. Nos résultats montrent qu'une sensibilisation active pré-infection des macrophages avec les MRPs 8/14 induit leur activation. Par contre, lorsque l'infection à *L.major* est antérieure à la stimulation avec les MRPs, l'activation des macrophages est significativement réduite. Les études *in vivo* démontrent quant à elles que l'abrogation des MRPs a entraîné une augmentation de la charge parasitaire, alors que l'injection de MRPs recombinantes réduit la taille de la lésion, de même que la charge parasitaire.

Un des principaux mécanismes qu'utilisent les parasites *Leishmania*, afin de déjouer la réponse immunitaire innée, est l'altération de facteurs de transcription. À l'aide de nos résultats, nous démontrons que l'activité d'AP-1 est abolie suite à l'infection par *Leishmania*, ceci concordant avec la dégradation au noyau des protomères d'AP-1. Il est d'ailleurs à souligner que c-Jun, le principal activateur d'AP-1, est dégradé et clivé par *Leishmania* à l'intérieur du noyau, et ce de façon GP63 dépendante.

Malgré le fait que les macrophages sont considérés comme les cellules hôtes préférées du parasite *Leishmania*, d'autres types cellulaires ont aussi été décrits comme des hôtes potentiels. Dans ce dernier chapitre, nous discutons de l'effet de l'infection par *Leishmania* sur les cellules dendritiques, en décrivant comment ce pathogène affecte leur maturation, de même que leur capacité à présenter des antigènes. De plus, nous avons découvert que le parasite est capable d'activer certaines phosphatases qui lui permettront d'inactiver des voies de signalisation des cellules dendritiques.

Tous réunis, nos résultats indiquent qu'une sécrétion rapide de MRPs par les neutrophiles au site d'infection peut aider à protéger l'hôte de l'infection par *Leishmania*. Toutefois, il est

évident que ce pathogène a su développer des stratégies lui permettant de déjouer la machinerie immunitaire innée induite par les MRPs. En effet, le pathogène utilise sa molécule de surface GP63 afin de dégrader des facteurs de transcription, empêchant ainsi la maturation des cellules dendritiques, favorisant donc son installation, de même que sa propagation chez son hôte mammifère.

CONTRIBUTION OF AUTHORS

In accordance with the guidelines of the faculty of graduate studies of McGill University, this thesis presents experimental work in a manuscript-based form. Chapters 2 through 4 correspond to manuscripts which have been submitted for publication (Chapter 3) or to be submitted (Chapter 2 and 4).

All the experimental work that I have performed for the accomplishment of these research articles was performed in the laboratory of Dr. Martin Olivier under his sole supervision. The manuscripts have been written by me with additional corrections from all the co-authors. Below are the detailed contributions from each co-author to the specific work:

Chapter 2. Impact of neutrophil-secreted myeloid related proteins 8/14 on leishmaniasis progression

Contreras I, Shio MT, Tessier PA, and Olivier M.

Contreras I: All the experimental work and manuscript writing.
Shio MT: Contributed with the performance of the *in vivo* experiments and manuscript editing.
Tessier PA: Performed ELISA assays, provided rMRPs and anti-MRPs antibodies.
Olivier M: Manuscript editing, experimental approach and data analysis supervision.

Chapter 3. *Leishmania*-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63

Contreras I, Gómez MA, Nguyen O, Shio MT, McMaster RW, and Olivier M.

Contreras I: All experimental work and manuscript writing
Gomez MA: Contributed with confocal microscopy, WBs and manuscript editing.

Nguyen O: Contributed with separation of nuclear and cytoplasmic proteins to perform WB of AP-1 subunits and manuscript editing.

Shio MT: Contributed with the performance of WBs and manuscript editing.

McMaster RW: Provided *L. major*, *L. major* GP63 KO and *L. major* GP63 rescued strains and anti-GP63 antibodies.

Olivier M: Manuscript editing, experimental approach and data analysis supervision.

Chapter 4. Dendritic cell signalling alteration by *Leishmania mexicana* leads to abolished functional antigen presentation

Contreras I, Ralph B, Estrada-Guadarrama JA, Fournier S, and Olivier M.

Contreras I: All experimental work and manuscript writing

Ralph B: Contributed performing IL-2 assays, WBs and EMSA experiments.

Estrada-Guadarrama JA: Performed FACS stain and acquisition, data analysis and manuscript editing.

Fournier S: Provided antibodies for FACS stains (CD40, MCH I, MCH II, B7.1, B7.2 and CD11b).

Olivier M: Manuscript editing, experimental approach and data analysis supervision.

CONTRIBUTION TO ORIGINAL SCIENTIFIC KNOWLEDGE

1.- Demonstrated that priming macrophages with MRPs *in vitro* prior to *L. major* infection confers them the capacity to activate signalling pathways such as AP-1 and NF-κB, and to produce high levels of NO and TNF-α, which are usually down-regulated by *Leishmania* infection

2.- *In vivo* experiments using a cutaneous leishmaniasis murine model revealed that neutralization of MRPs led to an increased size lesion and more parasite burden. Additionally, direct treatment with rMRPs reduced significantly the size of the lesion in the footpad and the parasite load. Demonstrating for the first time that MRP 8 and 14 play a role in the control of the infection and could have therapeutic use against leishmaniasis.

3.- Demonstrated that GP63 (*Leishmania* surface metalloprotease) is responsible for the cleavage and degradation of AP-1 subunits.

4.- Demonstrated that GP63 rapidly interacts with c-Jun in the nuclear compartment where degradation and cleavage occurs.

5.- Demonstrated that *L. mexicana* infection in dendritic cells affects their maturation by interfering with the expression of antigen presentation and co-stimulatory molecules.

6.- Showed that *Leishmania* infection activates protein phosphatases in DCs leading to a general dephosphorylation of tyrosine and serine proteins including MAP kinases. *L. mexicana* also affects nuclear translocation of transcription factors.

"Infectious diseases steal the headlines on regular basis and are ranked high among other major news items, such as natural disasters, conflict situations and terrorism. Emerging infectious diseases with wide-threatening potential, such as SARS and pandemic influenza are usually the ones that get best coverage and better funding. Diseases with high prevalence such as HIV/AIDS and malaria should bring better financial returns from investing in research on new treatments and developing vaccinations than similar investments in research on infections, with lower prevalence. **Leishmaniasis** is one of such infection, **which rarely shares this limelight and thus largely remains as a neglected disease** despite it affects more than 12 million people in 88 countries around the world"

Extracted and adapted from

"Leishmaniasis"

T.V. Piscopo

Postgrad Med J 2006

CHAPTER I

INTRODUCTION

1. Leishmaniasis

Leishmaniasis, is a vector-borne disease caused in mammals by more than 20 species of intra-macrophage protozoa of the *Leishmania* genus (order Kinetoplastida) and transmitted by 30 species of sandfly either *Phlebotomus* in the old world or *Lutzomya* in the new world. The disease is distributed world-wide affecting more than 15 million people in 88 countries [1,2], 72 of them being in the developing world while 13 are developed countries; however, its public health impact remains neglected since more than 2 million new cases are reported every year [3,4]. The three main clinical manifestations of the disease ranges from self healing cutaneous leishmaniasis (caused by *L. major* in the old world and *L. mexicana* in America), disfigurating muco-cutaneuos leishmaniasis (caused by *L. brazilinesis*) and the potentially lethal visceral leishmaniasis (caused by *L. donovani* in the old world and *L. chagasi* in the new world) [2].

1.1 Clinical manifestations

1.1.1. Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis (CL) is frequently a self-healing disease (within 2-15 months) characterized by papules, nodules or ulcerative skin lesions localized in the site of bite (Figure 1A); this form is the most prevalent disease and accounts for more than 50 % of the new cases of leishmaniasis [5]. CL is distributed world-wide; dermal manifestations can result from infections with either dermatotrophic/viscerotropic (*L. major*, *L. tropica* and *L. aethiopica*) species or dermatotrophic (*L. mexicana*, *L. amazonensis*, *L. braziliensis*, *L. panamenesis* and *L. peruviana*) species [1].

1.1.2 Mucocutaneous leishmaniasis (MCL)

MCL is mainly caused by the *Viania* subgenus, generally *L. (V) braziliensis*, *L. (V) panamenesis, L. (V) guyanensis* and *L. amazonenensis* which are restricted to South America. This clinical manifestation occurs following haematogenous or lymphatic dissemination of amastigotes from the skin to nasopharyngeal mucosa, causing its destruction mainly causing nose, mouth and throat disfiguration (Figure 1B). Mucosal dissemination occurs in 1-10 % of CL, developing between 1 to 5 years after initial healed infection [1,3].

1.1.3 Visceral leishmaniasis (VL)

VL is caused by *L. donovani* complex which includes *L. donovani* and *L. infantum* in the old world and *L. chagasi* in the Americas. Infections result in a variety of manifestations ranging from subclinical infection to overwhelming deadly infection (Kala-Azar) with hepatosplenomegaly, cachexia, pancytopenia (including anaemia, thrombocytopenia, leucopenia and neutropenia), hyperglobulineamia, and fever. Frequently this disease is fatal if left untreated (Figure 1 C) [5]. The estimated annual incidence of visceral form is around 500,000 in 61 countries with 90 % confined in India, Bangladesh, Nepal, Sudan, and Brazil [6,7].

1.1.4 Post-kala-Azar dermal leishmaniasis (PKDL)

PKDL is a variation of VL, it results in dermal manifestations of the visceral form that randomly develop after resolution of VL caused by *L. donovani,* and has been termed as the “unhappy sequel” of life saving drug treatment. The dermal localization of the PKDL lesion makes it more accessible to the sandfly and it has been suggested that patients with PKDL serve as reservoirs host of infection, especially in the intervening periods between outbreaks [8] .

Figure 1 shows the three main clinical manifestations of leishmaniasis.

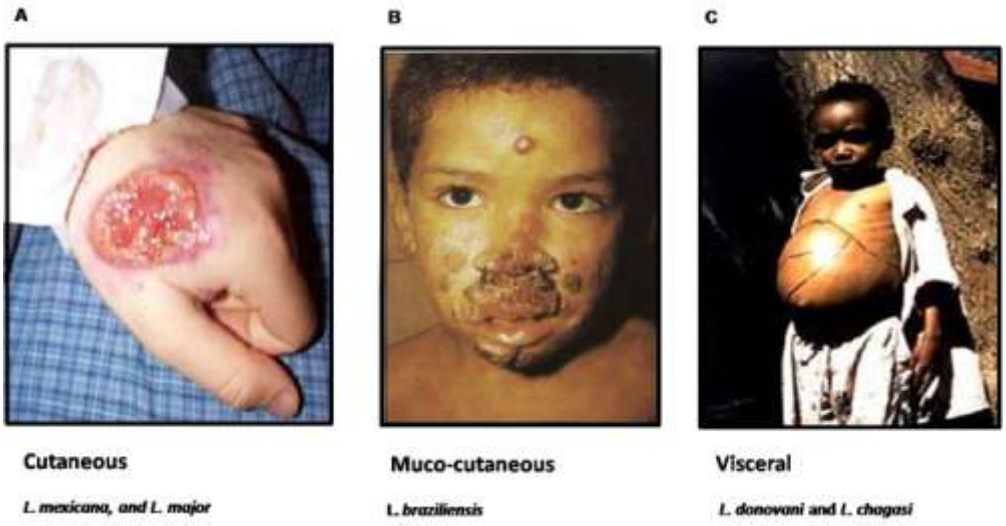

Figure 1: Clinical outcomes of Leishmaniasis A) Cutaneous manifestations of leishmaniasis ranging from small self-healing papules to enlarged lesions. **B)** Muco-cutaneous leishmaniasis caused by *L. braziliensis* in South America, mainly affecting and disfigurating the nasopharyngeal region. **C)** Visceral leishmaniasis caused by *L. donovani* and *L. chagasi*, this clinical manifestation is fatal if untreated. [Taken and adapted from: http://www3.baylor.edu].

1.2 Cell cycle

Leishmania parasites have a two stage life cycle. In the mammalian host, the parasite persists as non-motile, non-flagellated amastigote form, which proliferates inside the phagolysosome in the macrophages. This amastigotes are taken by the sandfly (of either the *Phlebotomus* or the *Lutzomya* genus), when it takes a blood meal. During digestion amastigotes are transformed into promastigotes form which are elongated and flagellated, promastigotes will attach to the midgut epithelium to avoid excretion [9]. The parasite's virulence is developed during metacyclogenesis, a process where dividing non-infective promastigotes (procyclic) transform into non-dividing infective form (metacyclic) [10]. The metacyclic promastigotes detach from the gut epithelial cells and migrate toward the anterior end of the digestive tract.

Metacyclic promastigotes are injected into the mammalian host during the next sandfly's meal and are phagocytised primarily by the monocyte/phagocyte linage, although it has been reported that neutrophils, dendritic cells and fibroblast can host the parasites for short or long periods of time depending on the cell type [11,12,13]. Inside the mammalian host, infective promastigotes evade innate immunity mechanisms such as lysis by complement and phagolysosome maturation; they transform once again into the amastigote form. The cell cycle is completed when amastigotes are taken by the sandfly. Once inside the gut, amastigotes will transform into procyclic promastigotes after 12 to 18 hr of the blood meal. After replication and differentiation for 7 days promastigotes will develop into infective metacyclic promastigotes ready to be injected into the mammalian host, therefore, starting a new cycle of infection.

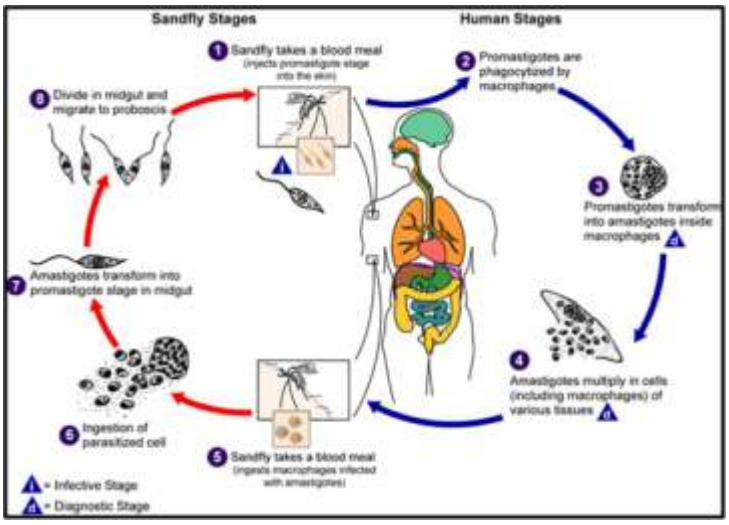

Figure 2: *Leishmania* Life Cycle. In the sandfly vector (1), *Leishmania* parasites live as elongated flagellated promastigotes (15-30 μm in length), these promastigotes are injected into the mammalian host when the fly takes a blood meal, the promastigotes are phagocytosed by macrophages (2) where they transform into the amastigote form; small, rounded and aflagellated (2-6 μm in length). These amastigotes survive and multiply within the cell (3-4), and are taken again for the sandfly when it has a meal (5), the cycle is completed when amastigotes transform into promastigotes inside the midgut of the sandfly (6-8). [Taken and adapted from: http://www.dpd.cdc.gov].

1.3 Treatment

Treatment for VL is largely based on pentavalent antimonials, however, increasing resistance is a major problem [14]. Amphotericin B is an effective antibiotic used in resistant patients, nonetheless is very toxic and needs to be given for a prolonged period of time [3]. Paromicyn (aminosidine) also has been effectively used in resistant cases in India, although it has issues with potential toxicity [14]. Old world CL is ~90 % self-healing around 3-18 months after infection, therefore before giving drugs some facts need to be studied to determine whether the patient needs or not drug-treatment. On the other hand, treatment of CL in new world depends on the infecting species, since it varies from benign to more severe manifestations, consequently, it is important to identify the species before starting treatment [3]. Some local treatments, like cryotherapy or local infrared heat lamps, have been used with good results in patients suffering from lesions caused by *L. mexicana* [15]. When drugs are used, the preferred drug is paromomycin combined with methylbenzethonium or urea and are applied directly onto the lesion [16]. When systemic drugs are administrated imidazol/triazole drugs are used for 20 days, and cure rate is around 79 % [17]. Finally MCL is successfully treated with amphotericin B and liposomal amphothericin B [18].

1.4 Vaccines against *Leishmania*

Leishmania spp. encompasses more than 30 species from which 20 are human-pathogenic [19], therefore, the necessity for an effective vaccine. Despite the intensive research regarding vaccination against *Leishmania,* there is no an effective vaccine available, although, many prototypes have been developed, some with interesting results in animal models [5]. Vaccine formulations include killed, live and attenuated parasites, recombinant *Leishmania* proteins or DNA encoding leishmanial proteins, as well as immunomodulators isolated from sandflies' saliva.

The ideal anti-*Leishmania* vaccine needs to posses several attributes, including i) safety, ii) affordability to the populations in need, iii) induction of CD4, CD8 T cells responses accompanied with long-term immunological memory, and iv) effectiveness

against CL and VL [5]. These attributes most of the time are difficult to achieve. In the next paragraphs, I will describe some of the prototypes that are currently under study.

1.4.1 Leishmanization

The inoculation of live virulent *Leishmania* has been practiced for over a century, injection of viable parasites produces controlled lesions and induces T cell mediated immunity; however, some individuals developed long-term large lesions that did not heal. In rare cases, exacerbated chronic skin disease or immunosuppression have been reported. Loss of virulence after repeated *in vitro* passages have been also reported. [5,20]. The traditional practice of leishmanization has made a come back in some endemic areas since it has been the only vaccine against *Leishmania* with proven efficacy in humans [21].

1.4.2 Killed vaccines

These vaccines have been proposed as prophylactic and therapeutic vaccines; however, they have been defined by producing poorly and variable potency with inconclusive results [5]. Back in late 1930s, the vaccine was based on cultured killed promastigotes and resulted in a reduction of cases in the population under study. Another vaccine contained promastigotes of five killed *Leishmania* strains, this vaccine showed to be safer, however, only conferred 50 % of protection. *L. amazonensis* vaccine "Leishvacin" was safe, but not efficacious [5,22]. In Colombia and Ecuador, where most of the cases are caused by the *Viannia* subgenus have been used killed vaccines, the use of these showed that vaccinated individuals developed a Th1 immune response without being protected against infection, suggesting that the induced Th1 response is not sufficient for protection against CL [22]. Some other vaccines of this kind have been developed, nevertheless in all cases have shown similar results; poor results and low protection against different *Leishmania* species [5].

1.4.3 Live attenuated vaccines

Vaccination with attenuated parasites, which are infectious but not pathogenic, has major advantages compared to killed parasites vaccines. Attenuated vaccines are produced by long-term culture under antibiotic, irradiation, chemical mutagenesis or gene

deletion [5]. Some of the developed vaccines have been tested in animal models without promising results. For example, good protection in murine models and none in the primate model. Vaccines containing parasites lacking LPG have been developed, deletion of *lpg1* gene showed to have no effect and still cause disease [23]. On the other hand, deletion of *lpg2* gene in *L. major* showed that parasites can persist in BALB/c mice without causing lesions and protect against homologous infections. However, in some cases the parasite can regain virulence and cause infection [24].

1.4.4 Vaccination with recombinant DNA-derived proteins

Leishmania parasites have a wide variety of surface and excreted proteins which could be used as potential antigens to develop vaccines, examples of these include the major surface glycoprotein GP63. When GP63 is used as a recombinant protein, it failed to protect mice against *L. major* infection [25] or gave partial protection in monkeys [26], on the contrary, immunization with native protein led to protection of mice against *L. major* or *L. mexicana*; however, these promising results were overshadowed by variable or mostly negative T cell response in humans [27]. Another candidate has been the GPI-anchored GP46/M-2 also known as parasite surface antigen 2 (PSA-2). Immunization with polypeptides of native promastigotes PSA-2 protected mice against *L. majo*r infection, but vaccination with recombinant protein showed lack of protective efficacy despite the ability to induce Th1 response [25]. Another candidate is the LACK/p36 protein which is a conserved antigen expressed in both parasite stages and appears to promote Th2 responses. Immunization with LACK co-administrated with IL-12 conferred partial protection against CL in mice, recombinant LACK with IL-12 triggered short term protective responses but failed to elicit long-term protection [28].

Amastigote-specific proteins have also been used as antigens as in the case of cysteine peptidases. Immunization of mice with cysteine peptidases induced high production of IFN-γ and offered partial protection against *L. major* challenge; the protective effect of these proteins has also been associated in visceral leishmaniasis in the canine model [29].

1.4.5 Vaccines against VL

The demands for an effective vaccine for VL are more complex than for CL vaccine since there is no Th1/Th2 polarization; nonetheless, there is still some progress in the production of vaccines for such disease in animal models. Some studies have shown that dp72 protects mice against *L. donovani* [27]. Some many other proteins have been tested against VL, such is the case of LACK DNA vaccine that was tested in dogs with very variable results [30]. The LCR1 protein of *L. donovani* administrated with BGC conferred partial protection in mice against *L. chagasi* [31]. The A2 cysteine proteinase delivered as recombinant protein or DNA offered protection against invasion of macrophages and disease progression against *L. donovani* [32]; the recombinant hydrophilic acylated surface protein B1 was protective in the VL mouse model without the requirement of any adjuvant [33]. The leishmanial antigen ORFF (unknown function) was able to induce protective immunity against *L. donovani* when administrated with the proper adjuvant [34].

Despite the fact that there are many potential candidates to develop an efficient vaccine against *Leishmania*, the majority of the proteins described (amongst others) have not reached the human trial face, since they only present partial or short term protection. The majority of these vaccines do not cover the complexity of the immune response mounted during infection and some of them only work with certain adjuvants. These facts have led to the continuous search for an effective vaccine that can be used to protect humans against CL and VL. Table 1 summarizes some of the prototypes which have been used as vaccines.

Table 1. ***Leishmania*** **vaccines**

Vaccine	Type	Description	Ref
Leishmanization	LV	Inoculation of live virulent parasites, induces T cell mediated immunity	[20]
Leishvacin	KV	Contains only one strain, does not protect against MCL	[5,22]
L. braziliensis **+ BCG**	KV	Effective in the treatment of refractory MCL and early cases of diffuse CL	[35]
L. major **+ BCG**	KV	Safe, partial protection against VL species, strong induction of IFN-γ	[36]
Leishmania **lacking DHFRT-TS gene**	LV	Induced limited protection against *L. major* or *L. amazonensis*, failed to protect immunity in the primate model	[37]
Leishmania **lacking *lpg2* gene**	LV	Parasites inoculated into BALB/c mice persist without development of lesion	[24]
R-GP63	rDNA-DP	Failed to protect mice against *L. major* infection	[25]
N-GP63	rDNA-DP	Protection of mice challenged with *L. major* or *L. mexicana*	[38]
N-dp72	rDNA-DP	Protein purify from *L. donovani* protects mice against *L. major*	[39]
Amastigote P4 protein	rDNA-DP	P4 elicits Th1-like response in PBMC from patients infected with *L. braziliensis*	[40]
LCR1 protein	SA	Administrated as recombinant or expressed in BCG induced partial protection in mice challenged with *L. chagasi*	[31]
PapLe22 antigen	SA	Immunization of hamster with PapLe22 substantially reduced the parasite load, but induced IL-10 production	[41]
Anti-sandfly components	NA	Immunization with molecules present in the saliva of the sandfly induced protection against CL in mice	[42]

KV: killed vaccine
LV: live attenuated vaccine
R: recombinant protein
N: native protein
rDNA-DP: recombinant DNA-derived proteins
SA: single antigen vaccine
DHFRT-TS: dihydrofolate reductase-thymidlate synthetase

1.5 Immunology of leishmaniasis caused by *L. major*

The pathology of *Leishmania* infection is determined not only by the parasite species, but also by host genetics and immune factors. In the case of CL by *L. major*, effective protection against infection has been attributed to the $CD4^+$ Th1 cells, with the subsequent IL-12 and IFN-γ production, which lead to macrophage activation and NO production necessary for parasite killing [5]. In mouse models Th1/Th2 polarization has been extensively studied [43]. In BALB/c susceptible mice it has been shown that augmented IL-4 production drives the polarized Th2 response, which is responsible for suppressing Th1-cell development and inhibition of IFN-γ production. There is also evidence that IL-4 response is confined to an oligoclonal population of $CD4^+$ with a Vβ4Vα8 T cell receptor (TCR) that recognize the LACK antigen [44].

On the other hand, in resistant C57BL/6 mice it has been observed that Th1 polarization helps to contain the disease; consistent with the concept of inflammatory type 1 cytokines as mediators of protection. The genetic abrogation of IL-12, IFN, TNF, their receptors, transcription factors (Tbet and STAT4), and co-stimulatory molecules (CD40-CD40L) leads to susceptibility to *L. major* [43]. IL-10 is an additional cytokine that can suppress Th1 responses and activation of macrophages. Initially, it was thought as unimportant during *L. major* infection; however, further studies revealed that expression of IL-10-encoding trans-gene by resistant mice, made them more susceptible to infection. Most recently, blockade of IL-10 receptor has been shown to confer partial resistance to *L. major* in BALB/c mice [43].

Natural killers (NK) also have been implicated as additional components of the innate response that lead to resistance and development of Th1 response, primarily for their capacity to produce IFN-γ which optimizes IL-12 production by DCs, and the expression of IL-12R by activated T cells [45,46]. However, it is clear that although early NK-cell activity might influence the kinetics of the Th1 response, these cells are not ultimately required for resistance [47]. Although $CD8^+$ T cells were shown to be important for immunity during re-infection in mice that had healed primary lesions [48], C57BL/6 mice deficient in β2 microglobulin or $CD8^+$ maintained their ability to heal, indicating that $CD8^+$ T cells are not required for the primary infection control [43].

Despite the Th1/Th2 polarization during CL in mice, a clear cut polarization of T helper cell responses is not evident in human CL which shows a mixed Th1 and Th2 response. The ability to mount a Th1 response is considered to be partially responsible for the observed differences in the clinical picture of leishmaniasis; however, Th2 cells mediated response have been associated with failure to mount a protective response, therefore, causing long-lasting CL or systemic infection [5]. In localized CL, (caused by *L. major* or *L. braziliensis*), Th1 cell predominate over Th2 cells, IL-4 is only detected in cases of MCL. Other studies have shown that IL-3 and IL-4 are found in the skin after initial lesion development, suggesting that Th2 cytokines play an immunoregulatory role during the early infection; however, cure of the infection is often associated with IFN-γ production, while IL-10 was present in persisting lesions [49]. Patients with MCL have strong T cell responses characterized by a high lymphocyte proliferative response to *Leishmania* antigens and IFN-γ, however, it is not clear why these patients develop disease [49].

1.6 Immunology of visceral leishmaniasis by *L. donovani*

The clear cut polarization explained above for murine CL caused by *L. major* is not seen during murine VL. Several studies of infections with visceralizing *Leishmania* species have revealed that host responses are indeed significantly different from these produced by *L. major* [49]. During *L. donovani* or *L. chagasi* disease, the curative type 1 responses are suppressed by IL-10 and TGF-β. *L. chagasi* directly affects its local environment by activating TGF-β and both species suppress responses to IFN-γ [50]. There are localized immune responses in the liver and spleens of infected animals, leading to tropism and unique patterns of localized cure. Protective immunity against *L. donovani*, is dependent on an IL-12 driven type 1 response and IFN-γ production with its subsequent NO production. A disease promoting a role for IL-4 and the Th2 response in VL, has been more difficult to identify, since the differential production of Th1 and Th2 cytokines does not control the rate of cure of VL [51].

During human VL there is not constant association between Th1 responses and resistance to disease with predominance of cells that produce IFN-γ. The levels of both IFN-γ and IL-4 are elevated during active disease and these levels decline after cure. In active human visceral disease, periphery blood mononuclear cells (PBMC) exhibit low proliferative responses to parasite antigens and fail to generate IFN-γ *in vitro* [52]. It is believed that lack of IFN-γ by PBMCs seems to predict the progression of the infection to fulminant VL [53]. Lymphocytes from patients cured of disease by therapy or subclinical acquisition demonstrate a strong proliferative response and high levels of IFN-γ, IL-2 and IL-12 on stimulation with parasite antigens *in vitro* [53]. Consequently, both spontaneous and drug induced healing are followed by strong protective immunity.

1.7 *Leishmania*'s virulence factors

1.7.1 Lipophosphoglycan (LPG)

Glycoconjugates play important roles in the ability of the parasite to survive inside the cell; they are distributed all over the surface of the parasite including the flagellum (Figure 3). Lipophosphoglycan (LPG) is the most abundant surface glycoconjugate of all *Leishmania* promastigotes, and it is distributed along the surface of the parasite, including the flagellum. Although it is very abundant on the promastigotes' body surface, it is down-regulated in the amastigote stage. In addition, LPG from amastigotes frequently presents different structure. LPG presents four main domains i) a 1-O-alkyl-2-lyso-phosphatidyl (myo) inositol anchor (GPI), ii) a glycan core, iii) repeating Galβ1, 4-Man-PO4 disaccharide phosphate units, and iv) a small oligosaccharide cap [54,55].

Although the first two domains are identical in all species, polymorphism is observed in the repeating disaccharide phosphate units and in the oligosaccharide cap. During metacyclogenesis LPG elongates from 15 units in the procyclic to 30 in the metacyclic stage, it is estimated that after this process 5 million copies of LPG are present in every promastigote. Helping to the *Leishmania*'s virulence, LPG can prevent access of the C5b-C9 MAC to the surface of the parasite and specifically binds to C-reactive protein through its repeating disaccharide units, thus enhancing opsonisation. LPG also

inhibits several PKC-dependent events including oxidative burst, and c-Fos induction. One important function that LPG inhibits is phagosome maturation at the level of fusion between early endosome and late endosome, presumably altering the physical properties of both sides of the membranes [54,56,57,58].

More recently, it has been described that LPG inhibits the proper assembly and function of NADPH oxidase complex in the phagosomal membrane. Indeed, it was shown that WT LPG and LPG-defective *L. donovani* trigger the release of similar amounts of superoxide; however, the LPG-defective mutant did not prevent the generation of superoxide in the phagosome as the WT strain did, suggesting that LPG prevents the assembly of the functional NADPH oxidase complex, resulting in an inhibition in the accumulation of reactive oxygen species and therefore, evading an important mechanisms of the innate immune response [59]. Finally another role for LPG has been recently described by Desjardins et al., who demonstrated that LPG may contribute to the disruption of lipid rafts on the phagosome membrane using its repetitive carbohydrate units to alter the organization of these microdomains, therefore, inhibiting the proper maturation of the phagosome [60]. This was also shown by Winberg et al. who demonstrated that LPG inhibits phagosome maturation by disturbing lipid rafts domains [61].

1.7.2 Glycoinositol-phospholipids (GIPLs)

GIPLs are the most abundant GPI-anchored cell-surface molecules of the parasite and they are 10 times more abundant than LPG, but their small size keeps them attached to the membrane. GIPLs are classified in three types; Type I: are more similar to the GPI proteins anchor and contain a core of Man-α-1-6Man-α-1-4GlcNα1-6PI, the type II GIPLs contain a core Man-α-1-3Man-α-1-4GlcNα1-6PI and the hybrid GIPLs or type III contain a branched core of Man-α-1-3(Man-α-1-6)Man-α-1-4GlcNα1-6PI [62]. The relative proportions of different GIPLs vary between *Leishmania* species and between promastigotes and amastigotes forms of the parasite [63]. The promastigote surface therefore consists of a dense GIPL glycocalix immediately adjacent to the membrane through which LPG molecules are projected. The role of the GIPLs is still unclear; however, it is believed that due to their long-life on the surface of the membrane (they are

not shed), they could play a protective role for promastigotes. In addition along with LPG they have been implicated in iNOS modulation in macrophages, providing further protection for the parasite once it is inside the cell [64].

1.7.3 Cysteine peptidases (CPs)

Cysteine peptidases (CPs) play important roles in facilitating the survival and growth of the parasite in mammalians; CPs have catalytic activity depending on a cysteine residue. Recent genome studies of *L. major* [65] have revealed that there are a total of 65 different genes encoding CPs, grouped in four clans (CA, CD, CF and PC) and 13 families (families C1-C13) [66]. Most of the studies have focused on the cysteine proteinase A (CPA), cysteine proteinase B (CPB) and cysteine proteinase C (CPC). The first two are Cathepsine L-like CPs and CPC is cathepsine B-like CP. Although CPB has been described as an important virulence factor for *L .mexicana* [67,68,69], CPB is also present in *L. major* and *L. donovani*, however, in *L. mexicana* it is found in 19 coding genes. CPBs of *L. mexicana* are differentially expressed throughout different stages of the life cycle, nonetheless, they are predominately expressed during the amastigote stage and to lesser extent in metacyclic promastigotes [70].

More specifically, CPB has been implicated in the inhibition of LPS-induced IL-12 production by bone marrow macrophages. It was demonstrated that absence of this cysteine protease prevented degradation of p50/p65 NF-κB subunits which is observed with WT amastigotes [67]. Another example shows that infection with *L. mexicana* lacking either CPA or CPB or both resulted in smaller lesions in the footpad and furthermore, double mutants did not induce any lesions at all. In addition, this study showed that absence of these proteases led to a Th1 healing response in susceptible BALB/c mice [68].

1.7.4 Major surface protease GP63

This glycoprotein of 63 kDa is also known as the major surface glycoprotein (MSP) or leishmaniolysin. It is highly expressed in promastigotes and in lesser extent in amastigotes. GP63 (and its homologues) is a metalloprotease belonging to the metzincin class, a main characteristic of this class is the signature motif HEXXHXXGXXH and an

N-terminal pro-peptide [71]. GP63 is the most abundant surface glycoprotein of *Leishmania spp*, and accounts for 1 % of the total protein of *L. mexicana* promastigotes. Each promastigotes has around 5 x 10^5 molecules of this protease [71]. GP63 from different species share high sequence identity. The encoded protein sequence contains an N-terminal hydrophobic signal sequence for presumed targeting nascent molecules to the ER during synthesis, and a pro-peptide that is removed during maturation, the pro-peptide sequence contains a cysteine residue that is conserved.

In *L. major* this cysteine contributes to a cysteine switch mechanism of protease activity, binding the zinc atom at the active site and inhibiting enzyme activity. GP63 also presents a GPI anchor very conserved amongst GP63 proteins. All *Leishmania spp.* have multiple tandem genes encoding GP63, there are at least 18 tandem genes encoding GP63 in *L chagasi*, *L. mexicana* contains 10, and *L. major* and *L. donovani* contain 7 [71]. Interestingly the non-human pathogenic lizard *L. tarentolae* contains genes encoding GP63; however, no protease activity is observed in this specie, this correlates to the lack of virulence in the mammalian host [72].

GP63 is involved in the binding of the parasite to and intracellular survival within host macrophage. Upon inoculation into the host cells, promastigotes are exposed to a variety of antimicrobial factors, some of them inactivated by GP63. For instance GP63 renders *Leishmania* resistant to complement-mediated lysis; cleavage of surface C3b enhances parasite binding to macrophage complement receptors, thus facilitating uptake by the host, GP63 itself is a ligand for binding to host surface proteins such as fibronectin receptor [73] and promotes survival inside the phagolysosome [74]. It has been documented that GP63 is also able to degrade casein, gelatine, albumin, haemoglobin, fibrinogen and collagen [75]. *L. major* GP63 is optimally active from neutral to alkaline pH, whereas *L. mexicana* GP63 is more active at acid pH.

Although GP63 does not recognize a specific sequence of amino acid residues, using synthetic peptides it was shown that GP63 has some selectivity for 5 amino acids motifs (P_2, P_1, P_1', P_2', P_3', where P is used to describe the position of the residue in the substrate). Basic amino acid residues at P_2' site and hydrophobic residues at the P_1' site were found, although polar residues at this site can also be present. P_1 residue most of the time is occupied by tyrosine and P_2 and P_3' can accommodate any class of residues [76].

Several lines of evidence have demonstrated that GP63 exists in three subpopulations in the parasite: i) surface-localized GP63, ii) internal GP63, and iii) released GP63. These three GP63 sources are trafficked separately, the surface proteins interact rapidly with the host cell, whereas internal GP63 serves as reservoir ready for rapid release after inoculation of metacyclic promastigotes into mammalian skin [74].

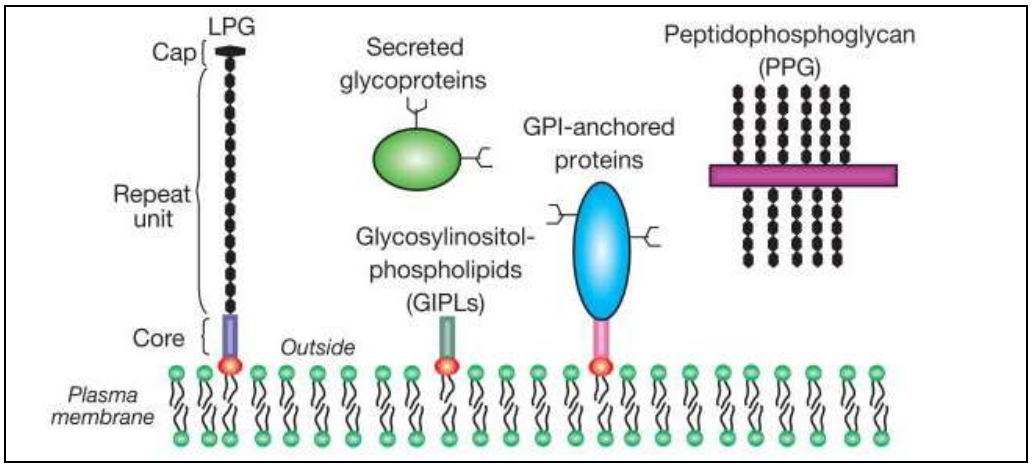

Figure 3: Distribution of the glycoconjugates in the surface of *Leishmania* promastigotes. Schematic representation of the promastigote surface glycocalix, containing the GPI-anchored glycoconjugates, known to bind the macrophage, and act as virulence factors. **[**Taken and adapted from: www.ncbi.nlm.nih.gov].

1.8. Other *Leishmania* proteins related to virulence

1.8.1 Amastigote A2 proteins

These *L. donovani* proteins are specifically expressed during the amastigote stage therefore represents an excellent amastigote-specific protein marker. The A2 locus encodes a multigene family of at least 7 genes encoding the repeat peptide unit. These proteins range from 45 to 100 kDa and are expressed at the same time during the transformation of the promastigotes into amastigote. Some reports have shown that over expression of A2 results in increased survival in macrophages and higher virulence in

animal models. On the other hand, A2 deficient amastigotes are compromised with respect to virulence in mice. Recent data has shown that $A2^{-/-}$ amastigotes are able to infect macrophages but grow slower, furthermore, mice infected with KO amastigotes present less parasitic load and less virulence. Therefore, A2 proteins are required for survival in macrophages, although are not required for proliferation and differentiation [77].

1.8.2 *Leishmania* homologue of receptors for activated C kinase (LACK)

The p36/LACK antigen from *Leishmania* is an analogue of the receptor for activated protein kinase C (RACK1), this antigen was identified by screening a parasite expression library as the antigen recognized by murine CD4 T cells [78]. LACK induces high levels of protection against parasite infection in the BALB/c mouse model, being this protection more than twice as high as that induced by major parasite antigens such as soluble GP63 [79]. The *L. major* LACK and its mammalian homologue RACK1, belong to the WD repeat protein family, these macromolecules are conserved in tryptophan and aspartate motifs. In *L. major*; LACK is encoded in four genes, parasites containing a single lack gene grow as wild type parasites but fail to parasitize BALB/c mice efficiently, even in a T cell-deficient environment [78].

1.8.3 Hydrophilic acylated surface protein (HASP)

HASPs are a family of three small hydrophilic proteins (HASPA1, HASPAA2 and HASPB). They are found in the surface of metacyclic promastigotes and in the plasma membrane of amastigotes of all *Leishmania* species [80]. Their function is still unknown, although putative roles in parasite virulence and maintenance within the host have been speculated; nonetheless, gene deletion had no effect in the biology of the parasite, over-expression led to loss of virulence and loss of cell structure, suggesting that the concentration of the proteins is important [81].

1.8.4 Amastin

Amastin surface proteins represent the largest regulated gene family in *Leishmania* comprising up to 45 members. All the members of the amastin gene family in

both *Leishmania* and *Trypanosome* species share a similar structural organization and contain a highly conserved 11 amino acids extracellular domain; unique for amastin proteins [82]. Most of the amastin proteins are expressed in the amastigotes stage and are strictly regulated by pH at the post-transcriptional level [83]. Until now, their functions are unknown; however, it is believed that since they are highly up-regulated during the amastigote stage somehow they could be involved in the control of *Leishmania*'s intracellular life and contribute to its pathogenesis [83]. More recently it was reported that a 50-aa N-terminal domain harbouring the conserved 11-aa amastin signature peptide is a relevant immune biomarker for CL and VL. In this report it was found that in serum from patients at different stages of CL and VL, amastins bond total immunoglobulin (IgG) and/or IgG subclasses, these data suggested that the amastin signature peptide could represent a relevant biomarker for VL serodiagnosis, and more importantly, it could permit differentiation among different stages of the disease [84].

1.9 Mechanisms of entry of *Leishmania* into the macrophage

1.9.1 Complement receptors (CR)

Complement receptors are integral membrane proteins that bind many molecules, and have been described in various cell types including macrophages. They play a role in the regulation of cell growth and differentiation among other cellular functions [85]. The best studied complement receptors are CR1 (CD35) and CR3 (Mac-1, CD18/11b a member of the leukocyte integrin family) which bind C3b and iC3b respectively [86]. Some reports have suggested that *Leishmania spp.* interacts with macrophages by binding to them. It has also been shown that GP63 is able to rapidly convert C3b to iC3b which binds to CR3, therefore increasing the parasite entry into macrophages [87]. Interaction of *Leishmania* with CR3 is advantageous since it helps the parasite to be phagocytosed without triggering the oxidative bust during phagocytosis [88,89].

1.9.2 Mannose-Fucose receptors (MFR)

Phagocytic cells contain a surface receptor which mediates the uptake of mannose- and fucose-terminated glycoproteins such as zymozan [90]. MRF has been implicated in the *Leishmania* promastigote uptake. For instance, it has been reported that

Leishmania promastigotes can also be attached to macrophages via mannose-fucose receptor, which bind to mannan residues contained in the LPG [91,92]. However, treatment of macrophages with soluble mannan or MRF down-regulation had no effect on the *L. major* amastigotes entry, thus, the MFR does not appear to be used by the amastigote [93]. These data coincide with the fact that LPG is highly down-regulated in the amastigote stage of the parasite.

1.9.3 C-reactive protein (CRP)

The C-reactive protein (CRP) is an acute phase protein synthesized by hepatocytes. It was first described as a serum component from febrile patients that could precipitate the C-polysaccharide of *S. pneumonia* cell walls [94]. The major ligand for CRP is believed to be phosphorylcholine (PC) found in this polysaccharide which binds in a calcium-dependent manner. Many of the known properties of CRP are manifested by its interactions with immunologic effector systems. CRP can act as an opsonin, both directly and by activation of the classical pathway of the complement and it can modulate phagocytes' activity. Some studies have shown that CRP binds to some *Leishmania* promastigotes in a calcium-dependent manner [95]. Later on, it was demonstrated that CRP binds to the *L. donovani* promastigote surface at the infectious metacyclic stage. The presence of CRP on the parasites' surface substantially increases uptake of promastigotes into human monocyte-derived macrophages, more importantly, it has been shown that CRP binds to the repeating units of LPG on the parasite's surface [94]. More recently, another study showed that *L. donovani* promastigote uptake was enhanced in the presence of CRP improving the levels of infection. However, this study showed that CRP mediated-phagocytosis did not induce detrimental macrophage activation which leads to a better survival inside the cell host [96].

1.9.4. Fibronectin receptor (FR)

Two of the most abundant cellular receptors for fibronectin are members of the β1 integrin family, VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29) [97]. VLA-5 recognizes the Arg-Gly-Asp-Ser (RGDS) sequence of fibronectin, while VLA-4 recognizes the CS-1 domain of fibronectin, which contains the sequence Glu-Ile-Leu-

Asp-Val (EILDV). Both receptors are widely expressed in tissues and cell types. The cooperation between fibronectin and CR has been suggested by the fact that the presence of FR can enhance phagocytosis. It has previously been suggested that GP63 presence on the promastigote's surface can enhance its interaction with murine macrophages since GP63 presents fibronectin-like properties. Brittingham et al. demonstrated that indeed GP63 binds to human macrophages and to transfected CHO cells expressing α4/β1 fibronectin receptors. This binding was enhanced when the experiments were performed in the presence of complement [98].

1.9.5 Fc Receptors

Fc receptors are found in several cell types including macrophages. Fc receptors deliver several signals when they are aggregated on the cell surface. The aggregation of FcR having immunoreceptor tyrosine-based activation motifs (ITAMs) activates sequentially Src and Syk families of tyrosine kinases which connect transduced signals to common activation pathways shared with other receptors. FcR with ITAMs elicit cell activation, endocytosis, and phagocytosis. The nature of responses depends primarily on the cell type [99]. Many studies have shown that *Leishmania* along with CR3, and FR uses Fc receptors to enter the host cell. Experiments have shown that *L. major* amastigote opsonisation with anti-parasite specific IgG enhance the entry of amastigotes in J774 macrophages [93]. Furthermore, another study showed that mice lacking the Fc receptors common γ chain (FcγRI, FcεRI, and FcγRIII) are similarly refractory to infection with *L. mexicana* amastigotes [100]. In addition, it was reported that lack of Fc-γ receptors in susceptible *L. major*-infected BALB/c mice, significantly reduced the production of IL-4, TGF-β and IL-10 which is signature of the Th2 response in susceptible mice. Finally, Fc receptor deletion led to a better control of the infection in these mice [101].

1.9.6 Phosphatidylserine receptor (PSR)

Phosphatidylserine (PS) is one of the ligands displayed by apoptotic cells that participate in their non-inflammatory removal when recognized by neighbouring phagocytes. PS ligation is fundamental for inducing the release of TGF-β-dependent non-inflammatory phagocytosis [102,103,104]. *Leishmania* amastigotes inhibit macrophage

activity by exposing PS [104]. Internalized *L. amazonensis* amastigotes exposing PS occurs in a process named "apoptotic mimicry" [103]. Recognition of PS by macrophages induces TGF-β secretion and IL-10 synthesis, therefore, inhibiting NO production and increasing susceptibility to intracellular leishmanial growth [103,104]. Another study demonstrated that PS exposure is modulated by the host; BALB/c-derived amastigotes expose more PS than amastigotes derived from resistant C57BL/6 mice, this amount of exposure correlates with parasite infectivity. Furthermore, similar to what happens to apoptotic cells, *L. amazonensis* amastigotes are internalized by macropinocytosis playing an important role in the size of large phagosomes which are observed during *L. amazonensis* infection. In addition, the intensity of macropinocytic activity depends on the amount of PS displayed on the parasite's surface [104].

1.9.7 Toll like receptors (TLRs)

More recently the role of Toll like-receptors (TLRs) during the first interaction parasite-macrophage has been described. TLRs represent the principal sensor of infection in mammals and recognize different pathogen associated molecular patterns (PAMPs), triggering signal transduction which activates important transcription factors such as NF-κB, AP-1, IRF-3 and IRF-7. Activation of TFs will generate cytokines, chemokines, and other proteins important for the regulation of the innate immune system. The cascade starts when these TLR recognize different PAMPs (such as LPS (TLR4), flagellin (TLR5), DNA (TLR 9) among others). Right after this first interaction the first adaptor molecule MyD88 that by itself or in conjunction with Mal (Tirap) signal recruit the third adapter TRIF (TRIF alone signals from TLR-3) or TRAM (adapter molecule for TLR4). These adaptors activate protein kinases (IRAK-1, IRAK-4 and TBK) which carry signals further. The final adaptor molecule TRAF-6 is recruited and activation of transcription factors occurs [105]. The MyD88/Mal pathway leads to IRAK 1/4 activation and then NF-κB activation, whereas TRIF/TRAM pathway leads to TBK-1 activation and to IRF activation (Figure 4) [106].

TLRs recognize a wide variety of pathogens or their proteins/molecules, and *Leishmania* parasites are not an exception. The lack of the adaptor molecule MyD88 in *L. major*–infected C57BL/6 mice leads to high susceptibility and disease development. This

observation led to the identification of TLR2 as ligand for LPG and activator of the cascade [107]. More importantly, in our laboratory we have described that *in vitro*, TL4-related signalling during *Leishmania* infection was strongly abrogated [108]. Notably, we demonstrated that *Leishmania* infection of B10R macrophages mediates the inactivation of IRAK-1 kinase. This inactivation is due to SHP-1, which inhibits IRAK-1 kinase activity and prevents its dissociation from MyD88; the result is NO and TNF-α down-regulation.

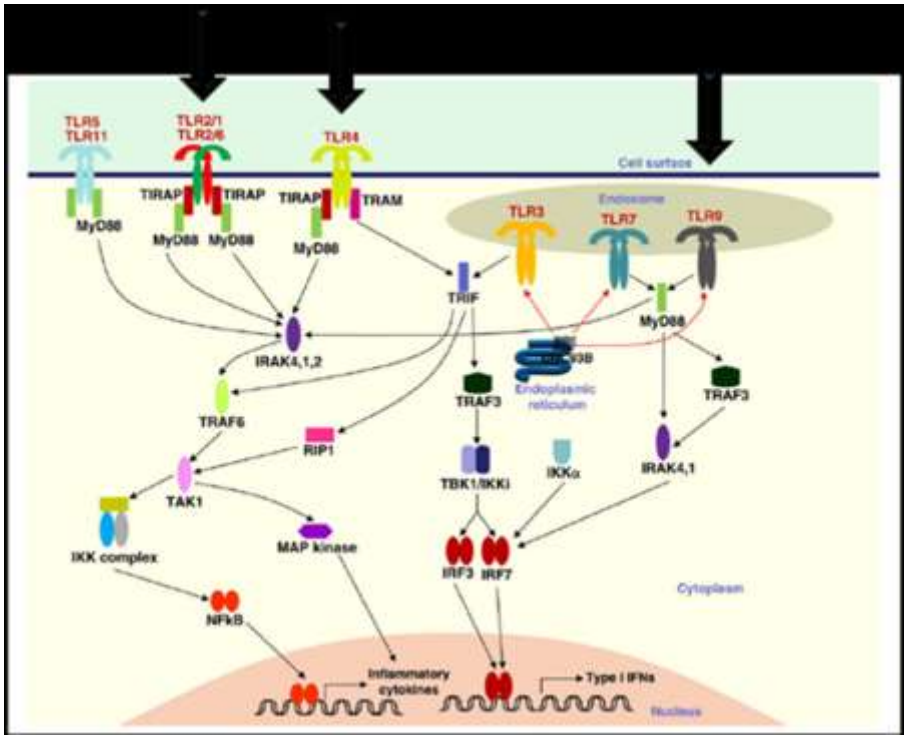

Figure 4: TLR signalling. Schematic representation of the TLRs signalling pathways. Signalling through TLR 2, 4 and 9 (arrows) has been reported to occur after *Leishmania* infection, involving surface parasite molecules including LPG. [Taken and adapted from: www.bioscience.org/2009/v14/af/3397]

2. Modulation of the cell's signalling by Leishmania parasites

2.1 Inhibition of macrophage functions

Leishmania parasites have evolved sophisticated mechanisms in order to survive and multiply inside their mammalian host cell the macrophage. The uptake of *Leishmania* promastigotes by macrophages occurs by many receptor mediated-phagocytosis to ensure their adhesion and phagocytosis [86]. Once promastigotes have made contact with the cell, they must evade complement-mediated lysis until they are phagocytosed by macrophages. Metacyclic promastigotes are able to resist the complement action whereas procyclic cannot. The difference in this resistance depends on the LPG, being longer and more branched in metacyclic promastigotes, this more complex LPG prevents the C5b-C9 complex formation during *L. major* infection. *L. donovani* on the other hand, prevents C5 convertase [109].

Many studies have focused on the potential for *Leishmania* or its derived molecules to modulate macrophage responses and inhibit their functions. Alteration in signal transduction and decrease in the transcription of cytokine genes have been extensively studied. Some of these observations have demonstrated that *Leishmania* parasites may employ many mechanisms in order to survive inside the cell. Among these are the alteration of calcium mobilization [110]. Some others have also demonstrated that macrophages responses to IFN-γ are impaired after *Leishmania* infection [111]. More importantly, several groups have described that *Leishmania* is able to alter transcription factors (TFs) indispensable for the activation of many genes involved in the activation of the innate immune response [112,113,114]. Activation of host phosphatases by the parasite is a key mechanism to guarantee the intracellular parasite survival [115,116,117,118]. An alternative way for the parasites to circumvent the killing of phagocytic cells is the modulation of macrophage's cytokine production. Inhibition in the production of proinflammatory cytokines (IL-12 and IFN-γ) [119] and induction of IL-4, IL-10, and TGF-β production [120] are other mechanisms used by the parasite. In the next sections I will describe in more detail some of the mechanisms employed by *Leishmania* to alter the macrophage's function in order to ensure its survival.

2.1.2 Microbicidal free radical production

The two major types of microbicidal molecules which are crucial for the control of the infection are nitric oxide (NO) [121] and reactive oxygen intermediates (ROI) [122]. It has been demonstrated that NO is critical for parasite killing since mice lacking the inducible nitric oxide synthase (iNOS) are not capable to control the infection. Furthermore, bone marrow macrophages derived from iNOS KO mice are not capable of eliminating parasites in culture [123]. Observations in our laboratory have demonstrated that infected macrophages lose their capacity to produce NO [117,118]. In addition, macrophages incubated with purified LPG lose their ability to induce iNOS, and therefore NO, in response to IFN-γ or LPS; however, when LPG and IFN-γ are administrated concurrently to naive macrophages these cells are able to produce NO [64,124].

Contrary to mice lacking NO production, mice deficient in ROI can control the infection after a period of high susceptibility, suggesting that ROI have a less important role in the control of the infection [116]. Despite this fact, infection with *L. donovani* promastigotes also inhibits ROI production, being LPG or GP63 the molecules responsible for this event [57,125].

2.1.3 Inhibition of phagolysosome maturation

Phagocytosis plays a key role in the ability of the cell to restrict the spread of infectious diseases. After the initial contact with the host cell, microorganisms are internalized by phagocytosis and sequestered inside phagosomes which are designed for degrading and destructing the pathogens. In order to do so, phagosomes have to go through a complex maturation process, involving sequential fusion and fission events with endocytic organelles, leading to the formation of the phagolysosome where finally pathogens are destroyed [126]. *Leishmania* parasites swap between promastigotes and amastigotes during their cell cycle. *L. donovani* amastigotes proliferate and survive inside acidic phagolysosomal vacuoles. *L. donovani* promastigotes have also developed strategies to survive inside the phagosome using mainly their surface molecule LPG. In fact, some studies have shown that LPG alters some of the functional properties of phagosomes such as the ability of the phagosome to fuse with late endocytic organelles and lysosomes [127].

Leishmania also has the capacity to suppress the recruitment of flotilin-1 into the phagosome [128]. Flotilin-1 is a protein found in phagosome-lipid rafts, and it is usually associated to lysososmal associated membrane protein-1 (LAMP-1). It is believed that these microdomains help with the maturation of the phagocytic vacuole since foltilin-1 accumulates gradually in the surface of the phagosome during maturation. *L. donovani* promastigotes can actively inhibit the acquisition of flotilin-enriched lipid rafts by phagosomes and the maturation of these organelles. This process is not observed in the absence of LPG, suggesting that the parasite may target these structures to preserve its survival inside macrophages [128]. More evidence has demonstrated that *Leishmania* uses its LPG to directly alter the lipid microdomains formation essential for phagosome maturation, therefore, disorganizing these structures after internalization guarantees their survival inside the cell [60].

2.1.4 Alteration of antigen presentation

In addition to the previous descriptions, *Leishmania* parasites inhibit the ability of the host to present antigens. Macrophages infected with procyclic promastigotes are initially able to present the LACK; this ability is lost as soon as the parasite differentiates into amastigote. In contrast, macrophages infected with metacyclic promastigotes do not present LAKC or present it very little, and amastigote-infected cells present none at all [129].

Inhibition of MHC molecules depends on the species used for infection. For instance *L. donovani* inhibits antigen presentation by repressing MHC class II gene expression both at basal level and following stimulation with IFN-γ, whereas macrophages infected with *L. amazonensis* do not show inhibited antigen presentation [130,131,132]. Mice deficient in MHC I are resistant to infection with *L. major*; however, MHC II deficient mice succumb to the infection [133]. Antigen presentation greatly depends on the expression of co-stimulatory molecules such as B7.1, B7.2, CD40 and CD40L [134]. Macrophages infected with *L. donovani* do not longer express B7.1 in response to LPS [135]; furthermore, impairment of co-stimulatory molecules expression is mediated by prostaglandins, as the inhibition of these molecules restored their expression [136].

2.1.5 Induction of immunosuppressive molecules

In order to survive inside the macrophage *Leishmania*, is able to induce immunosuppressive molecules, examples of these are arachidonic acid, tumour growth factor-β (TGF-β), prostaglandin E2 (PGE2), and IL-10 which is believe to be increased during the progression of the infection in susceptible BALB/c mice. TGF-β is induced by several *Leishmania* species, augmentation of these cytokines correlates with retarded iNOS expression and reduced NK cell activity in lymph nodes [45]. IL-10 is another anti-inflammatory cytokine produced in *Leishmania*-infected macrophages. *In vivo*, C57BL/6 resistant mice that constitutively express IL-10 are unable to control the infection [86]. It is believed that this cytokine is induced following recognition of the amastigote's phosphatidylserine by the macrophage [103].

Another example of immunosuppressive molecules is PGE2, this molecule is generated by *Leishmania*-infected macrophages and favours parasite survival and multiplication. PGE2 inhibits macrophage proliferation and suppresses IFN-γ, IL-1, and ROI production [137].

2.1.6. Induction of chemokine expression

Chemokines are a family of small molecular weight proteins (8-10 kDa) that participate in several cellular functions. Their name is derived from their ability to induce direct chemotaxis. Some of them are considered pro-inflammatory since they promote cell recruitment at a site of infection, whereas some others are considered homeostatic and are involved in cell migration during normal cellular processes [138]. Chemokines as many other effector proteins of the immune system are also altered during *Leishmania* infection. In our laboratory it was shown that infection of mice with *L. major* in the air-pouch, led to secretion of several chemokines (MCP-1, MIP-1α, MIP-1β, IP-10, and MIP-2) and some of their receptors (CCR1, CCR2, CCR3 and CCR5) [139]. Another study made by our group, showed that cleavage of NF-κB subunits by *L. mexicana* induced favourable chemokine gene expression (MIP-1β and MIP-2) favouring phagocyte recruitment, to expand the infection [113]. In addition, Antoniazi et al. showed

that *L. major*-infected mice caused up-regulation of RANTES/CCL5, MIP-1α/CCL3, IP-10/CXCL10, and MCP-1/CCL2 in footpads and lymph nodes [140]. It is important to point out that these chemokines are monocyte chemoattractants, and their up-regulation results in more macrophage recruitment to the site of infection. Another study revealed that *L. major*-infected DCs presented enhanced expression of CCR7 and its ligand CCL21 [141]. Together all these reports showed that induction of specific chemokines helps the parasite to attract more cells at the site of infection and propagate inside the host.

2.1.7 Inhibition of pro-inflammatory cytokines

Pro-inflammatory cytokines produced by mononuclear cells are key regulators against infectious diseases caused by many pathogens. IL-1β, IL-12, IFN-γ, and TNF-α are the main pro-inflammatory cytokines. IL-12 and IFN-γ are related to drive the Th1 response and activate NK cells, while IL-1β and TNF-α are important factors to respond against microbial invasion. Several reports have revealed that cytokines are also altered during *Leishmania* infection, demonstrating another strategy of the parasites to subvert the immune response. For example, Reiner et al. demonstrated that IL-1β and TNF-α production was suppressed in both rested and IFN-γ-primed blood monocytes infected with *L. donovani* promastigotes. Pre-infection of these cells with *L. donovani* and then stimulation with either LPS or *S. aureus* presented normal TNF-α production and IL-1 mRNA expression, but substantially reduced IL-1β production, suggesting that the inhibition occurs at the translational level [142].

Surprisingly, *in vivo* studies have shown that during infection with *L. major* in the air pouch model there was IL-β, IL-6 and TNF production after 6 hr of infection. This secretion is not observed during *L. donovani* infection and could explain the ability of these species to visceralize [143]. Regarding IL-12, it has been shown that macrophages infected with either *L. mexicana* or *L. major* amastigotes the IL-12 production is completely abrogated. Furthermore, suppression of this cytokine was also observed in macrophages previously stimulated with LPS or CD40 or macrophages that were activated by earlier phagocytosis of latex beads [144]. However, the suppression mechanism is still unknown, although LPG my be implicated in this process, since

incubation of activated macrophages with this phosphoglycan suppressed IL-12 production [145].

2.2 Alteration of the macrophage signalling pathways

2.2.1. Protein Kinase C (PKC)

Protein kinase C (PKC) comprises a family of serine/threonine kinases that are involved in the transduction of signals for cell proliferation, differentiation, apoptosis and angiogenesis [146]. *Leishmania* is able to augment the intracellular Ca^{+2} concentration in phagocytes; therefore inducing the expression and activation of enzymes including serine/threonine phosphatases such as calceneurin (also known as PP2B) that dephosphorylates some PKC isoforms [147,148].

Elevated calcium concentration increases calcineurin activity, thus reduced PKC activity in *L. donovani*-infected macrophages. LPG has also been involved in this inhibition, although amastigotes lacking LPG can also inhibit PKC in infected human monocytes, indicating the versatility of the parasite to inhibit the host cell functions [148]. PKC inactivation results in inhibition of phagosome maturation and ROI production [149]. Some surface proteins have been shown to alter the PKC signalling. For example purified GP63 inhibits MARCKs-related protein, a substrate of PKC associated with cytoskeleton and vacuole dispersal [150,151]. On the other hand, Forget et al. reported that elevated intracellular calcium helps with rapid PKCα hyper-phosphorylation and this excessive activation results in STAT-1 degradation in a proteasome-dependent manner [114].

2.2.2 MAP Kinases

Mitogen activated protein kinases (MAPKs) are a group of serine/threonine/tyrosine protein kinases highly conserved in eukaryotic cells and are among the most ancient signal transduction pathways [152]. In mammalian species MAPK have important roles in cellular processes, such as proliferation, stress responses, apoptosis, immune defence, cellular differentiation, and development. They are involved in all aspects of immune responses ranging from initiation phase of the innate immunity

to activation of adaptive immunity. [152]. There are three major groups of the MAPK in mammals; the extracellular signal-regulated protein kinases (ERK), the p-38 kinases and the c-Jun NH_2-terminal kinases (JNK) (Figure 5). These kinases are activated by dual phosphorylation at the tri-peptide motif Thr-X-Tyr. The dual phosphorylation is mediated by a conserved protein kinase cascade. The ERK are activated by MAPK kinases (MKK) 1 and 2, the p-38 are activated by MKK3, 4 and 6 and finally JNK by MKK4 and 7. These MKK are activated in turn by several different MAPK kinase kinases (MKKK) [153]. The phosphorylation of these residues results in conformational changes, increasing substrate accessibility and enhancing catalysis. Once MAPK are activated they can phosphorylate a wide variety of downstream targets including protein kinases and transcription factors that facilitate transcription of MAPK-regulated genes [152].

ERK: This pathway was first identified downstream of Ras and is often involved in the regulation of cell growth and differentiation. There are two isoforms of ERK, ERK 1 and 2 which are also known as p44/p42 [152]. They can be activated by MEK1 and 2. Once they are activated they can phosphorylate more than 70 substrates [154]. ERK activation is an important event for T cell activation, TNF-α production, and activation of transcription factors such as AP-1[152].

JNK: Three JNK-encoding genes have been identified in mammals: JNK1, 2 and 3. While JNK 1 and 2 are ubiquitously expressed, JNK 3 is selectively expressed in neuronal tissues, [155,156]. These kinases are activated by MKK4 and MKK7. Activated JNK can phosphorylate c-Jun (which will form AP-1 with another subunit) in serine 63/73 and ATF-2 in threonine 69/71. The activity of this kinase can be strongly induced in multiple cell types by LPS or inflammatory cytokines such as TNF and IL-1 [156]. This pathway is involved in numerous cellular functions such as Th1 differentiation and cytokine production [152]. JNK is also implicated in cell death regulation and in thimocyte negative selection [157].

p-38: This MAPK encompasses at least four members with different tissue distribution, substrates and regulation of kinase activation. p-38α and p-38β are ubiquitously expressed, p-38γ is found in skeletal muscles, and p-38δ is found in prostate,

pancreas, salivary, pituitary and adrenal glands. Activation of these kinases is carried out by MK2 and MK3. [152,158]. Although, all of them have wide spectrum of substrates, they present high affinity towards transcription factors such as ATF-2, ATF/cAMP and CREB [159]. p-38 activation is important for TNF-cytokine production and play a role in IL-1-mediated inflammatory responses [160]. p-38 is also implicated in the regulation of Th cell differentiation, cytokine production, and $CD8^+$ T cells' cell death, but not in $CD4^+$ cells [161].

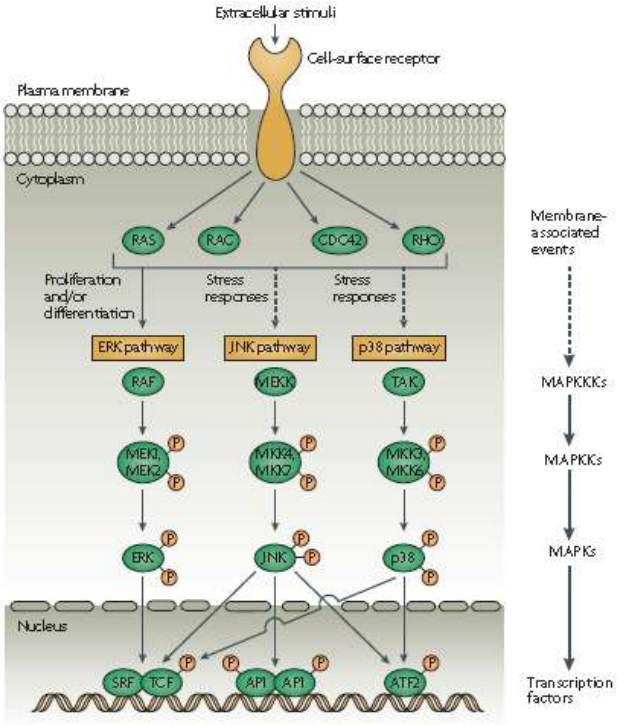

Figure 5: MAPK signalling pathways. Schematic representation of the three mitogen-activated protein kinases (ERK, JNK and p-38). *Leishmania* parasites are able to abrogate MAPK activity by dephosphorylating them, degrading and cleaving the components of these cascades. Taken from Lui et al. [153].

MAPK regulation: MAPKs can be regulated by various mechanisms at almost every step in the cascade. Some of the mechanisms include receptor desensitization, signalling

complex dissociation, and phosphatase-mediated dephosphorylation [153]. This last process is one of the most energy-efficient modes of deactivation. Among phosphatases which can regulate MAPK pathways are tyrosine, serine/threonine and dual-specificity (DUSPs) MAPK phosphatases (MPK) [153].

MAP Kinases Phosphatases (MKP): All MKPs have a highly conserved C-terminal region that engages the equivalent MAPK. In general, MKP can be divided in two groups based on their sub-cellular localization and patterns of transcriptional regulation (Table 2) [153]. The first group includes MKP1, 2, DUSP2 and PTPase; it is mainly localized in the nuclear compartment, and is encoded by immediate-early genes. This group is induced by some stimuli that activate MAPKs such as growth factors and stress [162]. The second group is not encoded in early genes and is either localized in the cytoplasm or nucleus or both. Examples of this group includes MKP3, 4, 5 and 7 [162].

Table 2. Classification of MKPs

MKP	Orthologues	Substrate specificity
Group 1: Nuclear localization		
MKP1	DUSP1, CL100, HVH1	p-38~JNK>>ERK
MKP2	DUSP4 HVH2, TYP1	ERK~JNK>>p-38
MKP4*	DUSP9, PYST3	ERK>p-38>JNK
MKP5*	DUSP10	p-38~JNK>>ERK
DUSP2	PAC1	ERK~p-38>>JNK
HVH3	DUSP5	ERK
HVH5*	DUSP8, M3/M6	JNK~p-38>>ERK
Group 2: Cytosolic localization		
MKP3	DUSP6, PYST3, RVH6	ERK>>JNK~p-38
MKP7	MKPM, DUSP16	JNK~p-38>>ERK
MKPX	DUSP7, B59, PYST2	ERK>>JNK~p-38

*These proteins fall in the cytosolic group as well. Adapted from Liu et al. [153].

Regulation of MKPs: MKPs can be regulated by three main mechanisms: transcriptional induction, phosphorylation-mediated changes in protein stability, and catalytic activation. Some of them are post-transcriptionally regulated through

phosphorylation. Although phosphorylation is not needed for MKP activation, it does not alter their stability [162]. In the few next paragraphs I will describe some of the MKPs involved in the immune response regulation.

MKP1: This phosphatase has substrate preference for p-38 and JNK kinases and therefore, might have an important role in the control of cytokine biosynthesis. The first evidence of this fact was demonstrated by stimulation of macrophages with LPS, where MKP1 expression was observed [163]. Moderate increase in MKP1 accelerated JNK and p-38 inactivation and attenuated TNF and IL-6 biosynthesis. MKP is also a pivotal regulator of immune responses to other microbial components. This phosphatase also down-regulates p-38 and JNK in response to CpG (TLR9) and peptidoglycan [164].

MKP5: Localized both in nucleus and cytoplasm. It actively dephosphorylates JNK and p-38, MKP5 is induced in response to LPS stimulation and over-expression of this phosphatase inhibits AP-1 reporter activity, suggesting that MKP5 modulates gene expression in the innate immune response [165].

DUSP2: Expressed in the haematopoietic lineage cells, DUSP2 shows a substrate preference for ERK and p-38 [166]. Expression of DUSP2 is induced by TLR ligands and cytokines in macrophages. Moreover, microarray analysis have shown that DUSP2 genes are mostly induced after mast cells activation, although some other cells such as eosinophils, neutrophils and macrophages have also showed induction of this phosphatase after activation [167]. Due to its ability to dephosphorylate tyrosine and serine/threonine residues, DUSP2 has a wide variety of substrates [153].

MAPK play important roles in the activation and proliferation of many cellular lineages. One of the main functions of these proteins is activation of transcription factors that control pro-inflammatory cytokines. *Leishmania* parasites alter many signalling pathways, and it is not surprising that MAPK are not an exception. Martini et al. showed that *L. amazonensis* amastigotes rapidly alter ERK phosphorylation in response to LPS [168]. A second report showed ERK1/2 inactivation by *L. donovani* amastigotes, resulting

in an inhibition of both Elk-1 and *c-fos* [116]. More recently, Ghosh et al. reported that *L. donovani* promastigote-infected macrophages presented an increased production of ceraminde, resulting in inhibited ERK phosphorylation and reduced AP-1 and NF-κB nuclear transactivation [112,169]. Prive and Descoteaux reported that infection of macrophages with *L. donovani* promastigotes abrogates p-38, JNK and ERK phosphorylation. The latest being in a LPG-dependent manner [170]. Finally it has been demonstrated that PTP-SHP-1 dephosphorylates ERK 1/2, as macrophages lacking SHP-1 showed normal JAK and ERK activities and normal NO production upon infection [117]. Another possible mechanism of MAPK inactivation was proposed by the action of cysteine peptidases (CP), in this study *L. mexicana* amastigotes degraded and cleaved ERK and JNK but not p-38, CP inhibition before infection resulted in no ERK and JNK degradation [66].

2.3 Alteration of transcription factors (TF)

2.3.1 AP-1 transcription factor

The AP-1 transcription factor is composed by dimers of Jun and Fos subunits, additionally some members of the ATF (activating transcription factor) and some other subfamilies which share structural similarities and form heterodimeric complexes that bind to common DNA site (AP-1 site). Different AP-1 factors regulate different target genes, thus, they possess different biological functions. A common feature of all these proteins is a conserved bZIP domain [155,171,172,173]. The leucine zipper is responsible for dimerization, which is necessary for DNA binding and it is mediated by the basic domain. The composition of the leucine zipper is also responsible for the specificity and the stability of homo and heterodimers formed by the Jun and Fos proteins **(Figure 6)**.

The Jun family is composed by c-Jun, Jun B and Jun D members; they exist as homo or heterodimers. However, the Fos proteins (c-Fos, FosB, Fra1 and Fra2) can not homodimerize among themselves, although can form stable heterodimers with Jun proteins. c-Jun, c-Fos and FosB are considered strong transactivators. Whereas Jun B, Jun D, Fra1 and Fra2 exhibit only weak transactivation potential [172].

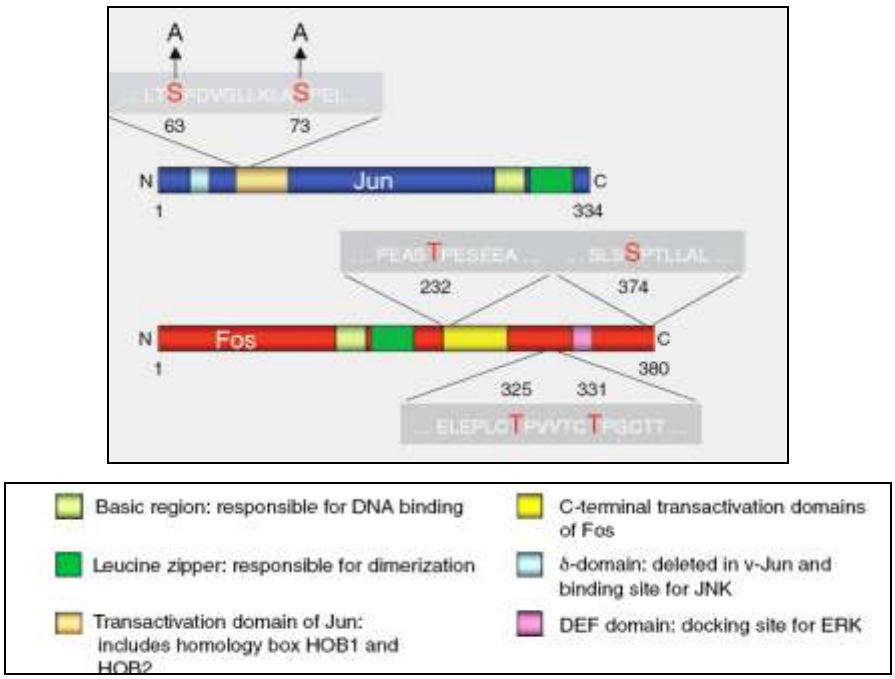

Figure 6: Structural organization of Jun and Fos proteins. Schematic representation of the structure of Jun and Fos proteins, colours indicate the different motifs in the proteins. Taken and adapted from: Hess et al. [172].

Expression of Fos and Jun genes, at both the mRNA and the protein levels is sensitive to a number of different stimuli such as growth factors, cytokines (TGF-β, TNF and interferon families), neuronal depolarization and cellular stress. c-Fos appears to be highly sensitive to these stimuli, and induction of these genes is transient [173]. Regulation of AP-1 activity can be achieved through changes in transcription genes encoding AP-1 subunits, control of the stability of their mRNA, post-transcriptional processing, turnover of pre-exiting or newly synthesized AP-1 subunits, and specific interactions between AP-1 proteins and other transcription factors and cofactors. The post-transcriptional control is through mitogen and cellular stress-induced hyper-phosphorylation and activation of Jun through JNK cascade [172]. JNK phosphorylates c-Jun at serine 63 and 73, this leads to a large increase in the ability of c-Jun to interact with some co-activators such as JAB-1 [174]. This phosphorylation is carried out in a two step

mechanism: i) JNK associates with c-Jun and ii) once there is interaction, JNK phosphorylates [155].

Some studies have indirectly demonstrated that AP-1 is also target of *Leishmania*, for example some of these reports have shown inactivation of AP-1 is due to alteration in the up-stream cascades [56,116,117,170]. However, the exact mechanisms are still unknown. Ghosh et al. described that increased in the ceramide level leads to AP-1 inactivation [112,169]. Chapter 3 will discuss a new mechanism of how *Leishmania* inactivate AP-1. This mechanism involves the parasite surface molecule GP63, showing that parasite-derived proteases can directly interfere with AP-1 subunits and block AP-1 nuclear translocation [175].

2.3.2 NF-κB pathway

Nuclear factor κB transcription factors and the signalling pathways which activate them are central coordinators of the innate and adaptive immune responses. NF-κB are assembled through dimerization of five subunits: Rel A (p65), c-Rel, Rel B, p50 and p52 [176]. Before cell stimulation, most NK-κB dimers are retained in the cytoplasm by binding to specific inhibitors; the inhibitors of NF-κBs (IκBs). Cell stimulation activates IκB kinases (IKK) complex which is formed by two catalytic subunits called IKKα and IKKβ and a regulatory subunit IKKγ/NEMO. Activated IKK phosphorylates IκB proteins and then target for polyubiquitination and rapid degradation. Freed NF-κB dimers translocate into the nucleus where they coordinate the transcriptional activation of several hundred genes. This pathway is called classical NF-κB signalling pathway [177]. This pathway involves activation of receptors such as RTNF, IL-1R or TLRs. The second pathway or alternative pathway, results in the activation of p52:Rel B heterodimers. Unlike the classical pathway that requires IKKγ and IKKβ activity; the alternative pathway is based in IKKα homodimers. Although this pathway is not directly involved in innate immunity and inflammation, it is required for generation of secondary lymphoid organs and for B-cell maturation and survival [177,178]. Another pathway that can activate NF-κB is independent of IKK and uses casein kinase 2 activation, which induces IκBα degradation through the phosphorylation of carboxy-terminal sites. This pathway

has minor roles in physiological activation of NF-κB and might contribute to skin carcinogenesis because it is activated by UV radiation. The two main activation pathways of NF-KB are shown in Figure 7.

NF-κB has more than 150 target genes, many of them encode for proteins of the immune system such as chemokines, cytokines, immunoreceptors, and adhesion molecules. NF-κB is often named as "central mediator of the immune response". Among the genes that are involved in the immune response are: IFN-γ, TNF-α, IL-1, IL-6, IL-12, MIP-α, MIP1β, RANTES, and MHC I [179].

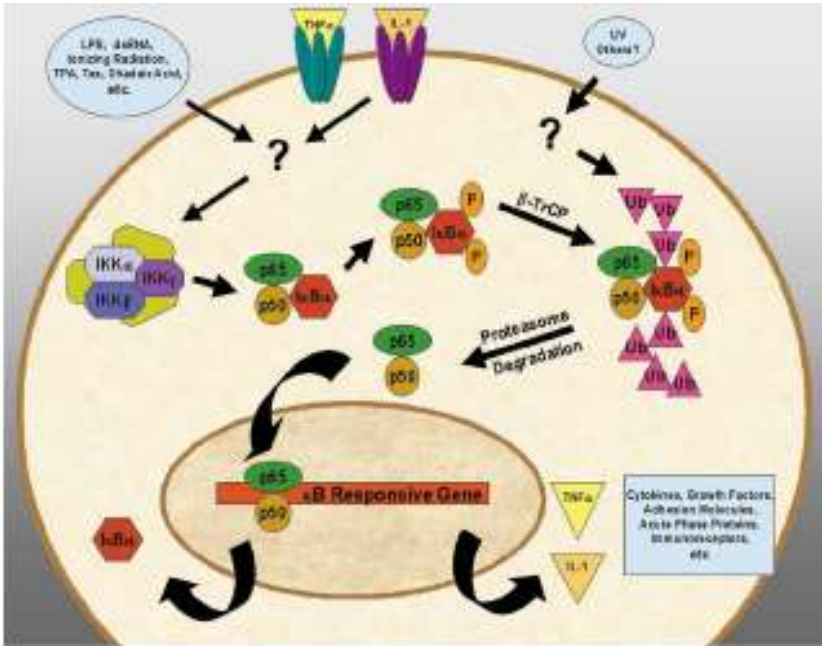

Figure 7: NF-κB signalling pathway. The five members of the NF-κB family exist as homo or heterodimers in the cytoplasm bound to IκB proteins. Upon stimulation IKK proteins are phosphorylated and they phosphorylate IκB which in turn will be ubiquitinated and degraded by the proteasome. Free NF-κB translocates into the nucleus where it will bind a consensus sequence, stimulating gene transcription. Taken and adapted from: Rothwarf et al. [177].

Some recent reports have studied the effect of *Leishmania* infection on NF-κB. For instance, amastigote *L. major*-infected human monocytes and PMA-differentiated U937

showed less p65/p50 nuclear translocation and augmented c-Rel/p50 translocation, concomitant with augmented IL-10 production, suggesting that more likely this favoured complex regulates cytokine expression that favours parasite survival [180]. Another study revealed that there is dramatic degradation of the pathway, including p65, c-Rel, IκBα and IκBβ inhibitors, and the upstream kinases JNK and ERK, following infection of BMM with *L. mexicana* amastigotes. The degradation was attributed to the presence of cysteine peptidases expressed during the amastigote stage [181]. One study showed a different mechanism by which *Leishmania* inactivates this transcription factor. In this study it was proposed that *L. donovani* promastigotes are able to increase cellular ceraminde levels, which inhibits up-stream signalling pathways [112]. Work from our laboratory demonstrated that infection of macrophages with *L. mexicana* promastigotes causes specific cleavage of the p65 subunit; this cleavage occurs in the cytoplasm and is dependent on GP63. The resulted "p35" cleaved fragment migrates to the nucleus where it binds DNA with p50. The DNA binding of these dimmers results in induction of specific chemokine expression [113].

2.3.3 JAK/STAT pathway

The signal transducers and activators of transcription (STAT) transcription factors have crucial roles in the host defence; STAT 1 and 2 are largely related to mediate IFN effects, while STAT 4 and 6 mediate IL-12 and IL-4 effects respectively [182,183,184]. STAT proteins are latent in the cytoplasm until they are activated by extracellular signalling proteins (normally cytokines and growth factors) that bind to specific cell-surface receptors. These extracellular signalling proteins can activate tyrosine kinases such as the janus kinases (JAK), that phosphorylate STAT proteins. Once the STATs are activated they translocate into the nucleus and drive transcription. Receptor dimerization leads to JAK apposition and transphosphorylation on tyrosine residues, releasing their intrinsic catalytic activity. Tyrosine phosphorylation of the cytokine-receptor cytoplasmic domains by JAK provides binding for the Src-homology-2 (SH2) domain of the STAT proteins. The STAT proteins are recruited to the JAKs, where they are phosphorylated on a single tyrosine residue. STAT-STAT interactions occur immediately through reciprocal phosphotyrosine-SH2 interactions [185].

It has been shown that *Leishmania*- infected macrophages show defective ability to phosphorylate JAK1, JAK2 and STAT upon IFN-γ stimulation [186]. Infection with *L. donovani* promastigotes revealed that this effect was due to the rapid activation of PTPs, specifically of SHP-1. More recently, we have demonstrated that STAT inactivation is dependent on the activation of the proteasome [114], this phenomenon is also dependent on PKC-α hyper-activation.

2.4 Activation of Protein Tyrosine Phosphatases (PTPs)

Tyrosine phosphorylation is a key mechanism for signal transduction and the regulation of a broad set of physiological process such as proliferation, differentiation, and activation of gene-transcription, motility and morphology, several processes of the immunity, among others [187]. Kinases and phosphatases are very close partners and their activities are coordinated in the regulation of signalling process [188,189]. Compared to protein tyrosine kinases (PTK) which have been mainly implicated in controlling the amplitude of a signal response, protein phosphatases are thought to have an important role in controlling the rate and duration of the response. All cells of the immune system have high levels of tyrosine phosphorylation and express more genes encoding PTK and PTPs than any other cell type. Acute changes in tyrosine phosphorylation regulate antigen-receptor-mediated lymphocyte activation, cytokine-induced differentiation and responses to many other stimuli [190,191].

Unlike the protein kinases which are derived from a common ancestor, protein phosphatases have evolved from separate families structurally and mechanistically distinct form each other. PTPs superfamily encodes the largest family of phosphatase genes [192]. These enzymes are defined by the active-site signature motif HCX_5R, in which the cysteine functions as a nucleophile and is essential for the catalytic activity. In general most PTPs have non-catalytic amino (N)-or carboxy (C)-terminal extension containing targeting motifs, protein-protein interaction domains or lipid-binding modules. Many PTPs are enriched at the plasma membrane, whereas others are confined either to the nucleus or by other specific internal membranes such as ER, mitochondria, secretory vesicles or in many cases they shuttle from cytosol to any of these locations [193].

2.4.1 PTPs classification

The number of genes in the human genome that encode members of the PTP families is very high and exceeds the number of genes encoding PTKs [194]. Of the 107 PTP genes, 11 are catalytic inactive, 2 dephosphorylate mRNA and 13 dephosphorylate inositol phospholipids, thus only 81 PTPs are active protein phosphatases with the ability to dephosphorylate tyrosine residues [192].

Based on the amino acid sequences of the catalytic domain, PTPs can be grouped in four main groups. i) Class I, classical PTPs (99 members), ii) Class II PTP (1 member), iii) Class III PTPs (3 members), and iv) Class IV PTPs (4 members). Class I, II and III are cysteine-based PTPs whereas class IV PTPs are aspartate-based. The largest group which is class I is subdivided in i) classical PTPs and ii) dual substrate specificity. Classical PTPs are also subdivided in receptor-like proteins and non-receptor proteins. Figure 8 shows the general classification of PTPs

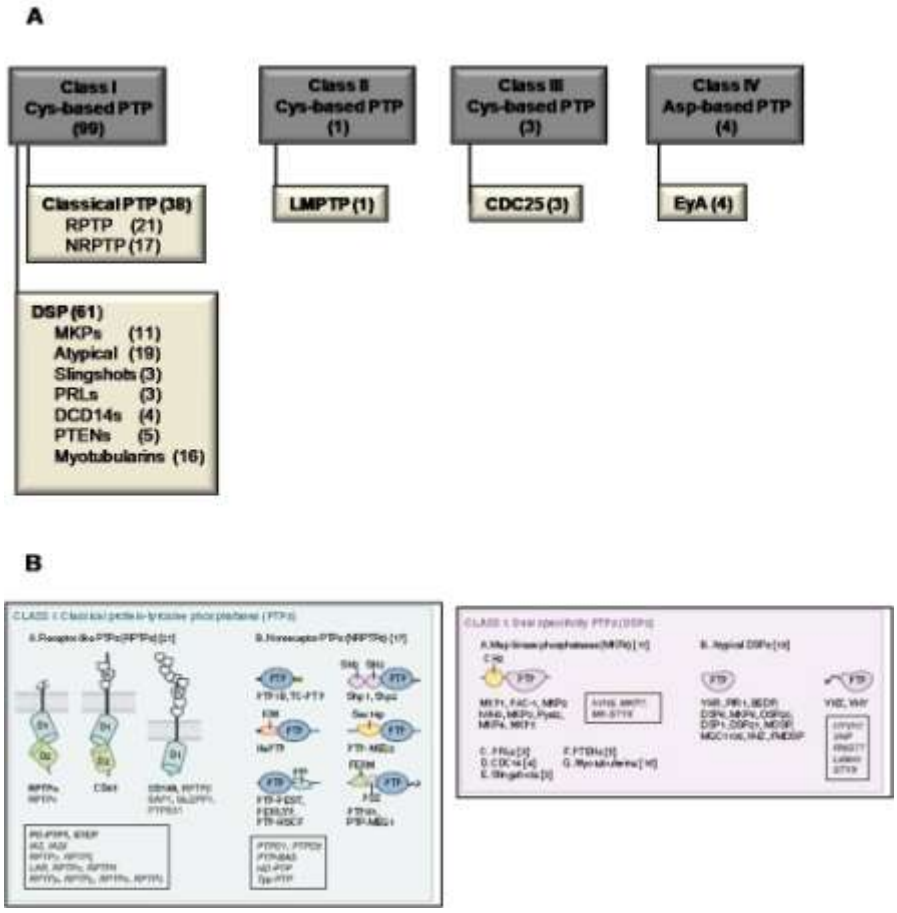

Figure 8 Classification of PTPs. (A) General classification of PTPs, divided in 4 main groups. **(B)** Sub-classification of the Class I group cysteine-based PTPs. The first box shows the sub-classification of classical PTPs (receptor and non-receptor) and the second box shows the main structural conformation of DUSP phosphatases. Taken and adapted from: Alonso et al. [192] and Pao et al. [195].

Class I cysteine-based PTPs: This family constitutes the largest family and accounts for 99 members, 38 of them are well known as "classical" PTPs which are strictly tyrosine specific. All of them have mouse homologues, 61 proteins are "dual-specific" protein phosphatases (DSP) and others are vaccinia virus virulence factors (VH-1)-like enzymes. This group is also the most diverse in terms of substrate specificity; all class I PTPs have evolved from a common ancestor [192]. This family also can be

subdivided into several subfamilies based on domain architecture and degree of homology between catalytic domains. The 38 tyrosine specific can be also divided into trans-membrane receptor-like enzymes (RPTPs) including 21 proteins, 17 intracellular non-receptor PTPs (NRPTPs) [192,193,195], and 61 "dual specificity" phosphatases (DSP) related to VH1-like, these VH-like proteins are more diverse and can be divided into several subgroups, which share much less sequence identity. Eleven enzymes belonging to the DSP proteins are specific for MAPK, and another subgroup is referred as the "atypical" DSP, and includes a number of poorly characterized enzymes.

In the next paragraphs some of the most known phosphatases with immunological implications belonging to class I PTPs will be described in more detail.

SHP-1 and SHP-2: These phosphatases contain tandem N-terminal SH2 domains (Figure 9), a central phosphatase domain, and a C-terminal extension of unknown function. While SHP-1 is restricted to hematopoietic and epithelial cells, SHP-2 is ubiquitously expressed [196]. Both of them intervene in cell signalling as a consequence of binding of their SH2 domains to tyrosine phosphorylated docking sites. SHP-1 is an inhibitor of signal transduction whereas SHP-2 is usually a positive regulator of cell signalling [197]. SHP-1 activity is primarily regulated by conformational modification and maintained inactive by an intra-molecular interaction between its tandem SH2 domains and its phosphatase domains (Figure 9). Binding of tyrosine phosphorylated peptides to the SH2 domains provokes an increase in the catalytic activity of SHP-1.

SHP-1 is involved in numberless events in the cells' signalling and actually inhibits signalling pathways through cytokine and chemokine receptors, integrins and immunoreceptors. This phosphatase has an inhibitory role in TCR [198] and BCR [199] signalling. SHP-1 associates with immunoreceptor tyrosine-based inhibitory motifs (ITIM), ITIM phosphorylation creates high-affinity binding sites for the SH2 domains of SHP-1, the result SHP-1 is recruited to the plasma membrane and activated through conformational modification; activated SHP-1 dephosphorylates various immunoreceptor-regulated substrates, thereby inhibiting cell activation [196]. Many studies have indicated that SHP-1 acts at several levels of the immunoreceptor signalling

cascade. For instance SHP-1 can dephosphorylate Src kinases, ITAMs, Syk kinases, JAK kinases, adaptor and effector proteins and PI'3 kinase amongst others [200].

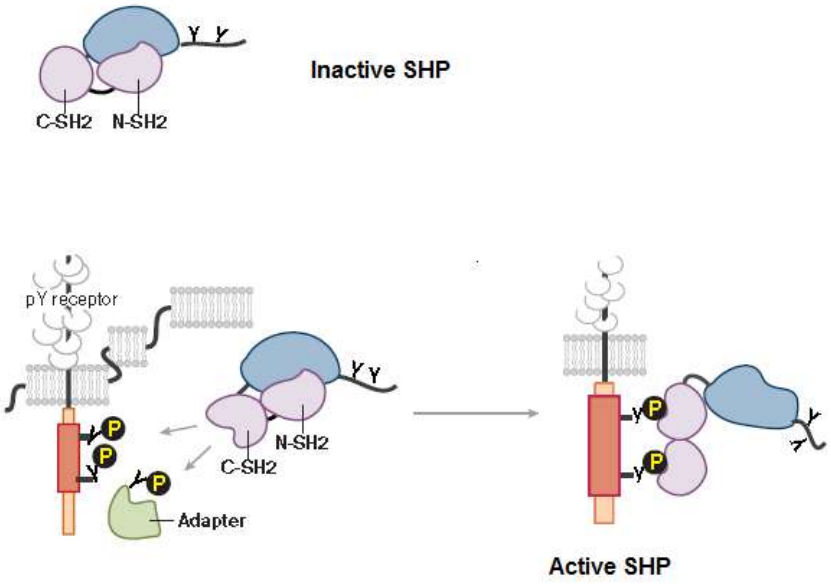

Figure 9: Regulation of SHP-1 and SHP-2 by the N-SH2 domain. The SH2 domain regulates the intracellular locations and catalytic activity of the SHPs proteins. In the absence of binding to a phosphotyrosil peptide the SH2 domains form a loop with the catalytic domain of the phosphatase (upper panel) and renders it inactive, after activation (lower panel) the SH2 domains bind to phosphorylated receptors (such ITAM and ITIM domains) and by conformational changes the catalytic domain remains free, ready to bind to its substrate. Taken and adapted from Pao et al.[195].

PTP1B: Originally isolated from human placenta, PTP1B is a major trans-membrane protein expressed on the surface of hematopoietic cells, it exists from receptor-like form to ligand-controlled dephosphorylation of tyrosyl residues in proteins. PTP1B presents dual-specificity being able to dephosphorylate mainly tyrosine residues as well as serine/threonine residues. PTP1B was initially described as a catalytic domain of 37 kDa. This domain also contains an extension of 114 residues on the C-terminal end giving a full length protein of around 50 kDa. The C-terminal residues are primarily hydrophobic giving it the property of targeting the enzyme to the cytoplasmic face of ER membranes

[193,201]. This phosphatase has the capacity to dephosphorylate the insulin receptor, possibly insulin receptor substrate, and the leptin receptor; therefore, it has been implicated on body mass regulation and cytokine receptor activation through dephosphorylation of the JAK family members (JAK2 and TYK2) [202].

TCPTP: This phosphatase is closely related to PTP1B since it shares 74 % homology with PTP1B in its catalytic domain. However, its main cellular localization is inside the nucleus whereas PTP1B is cytoplasmic [202]. TCPTP is involved in the regulation of many immunological pathways including regulation of lymphocyte proliferation [203], and modulation of cytokine pathways through JAK 1 and JAK 3 dephosphorylation [202]. Studies of TCPTP functions suggest that TCPTP negatively regulates TNF-induced ERK activation, JNK and NF-κB [204].

PTP-PEST: This protein belongs to the non-receptor PTPs and is broadly expressed in the cytoplasm of non-hematopoietic and hematopoietic cells [205], higher levels of this phosphatase are found in immune cells including thymocytes, splenic T and B cells, NK cells, and mast cells [206]. The catalytic domain of this phosphatase contains a region rich in proline, glutamine, serine and glutamic acid [195]. PTP-PEST associates with several signal transduction molecules, for instance, in fibroblast it is implicated in the negative regulation of integrin-induced cell migration, it is also an efficient negative regulator of antigen receptor-induced cytokine production. Among its substrates are: Ras-MAPK signalling cascades, Csk, PyK2 and FAK kinases [206].

Class II, III and asp-based PTPs: Class II PTP family is represented by a single gene referred as *ACP1* which encodes the Mr phosphatase or low-molecular weight PTP (LMPTP). This phosphatase has homologues in prokaryotic cells and human LMPTP is 31 % identical to the corresponding protein of *S. cerevisiae* and 39 % identical to the *B. subtillis* YfkJ protein. Although in these microorganism these proteins are tyrosine-specific. [192].

Class III family comprises three cell cycle regulators (CDC25A, CDC25B and CDC25C). In humans their function is dephosphorylate Cdks at their inhibitory dually

phosphorylated N-terminal Thr-Tyr motifs, a reaction that is required for activation of these kinases to drive progression of the cell cycle [192,207].

Finally the asp-based PTP family encompasses only four members which play important roles in development, sodium stress and nuclear morphology [192]. However, the structure of these proteins needs further study in order to elucidate their functions.

2.4.2 PTP regulation

In order to maintain the balance between phosphorylation and dephosphorylation, PTP regulation is of crucial importance. Enhanced or uncontrolled PTP activity may result in a many physiological disorders, as well as is the lack of their activation. The activity of PTPs is strongly controlled by a variety of mechanisms. An important aspect of this regulation was the revelation of receptor-linked PTPs (RPTP), through which signal transduction may be regulated by ligand-controlled dephosphorylation of tyrosyl residues in proteins [193].

RPTP dimerization: The crystal structure of the RPTP revealed an important mechanism in the PTP regulation. The PTP domains are organized in symmetrical dimers, in which an inhibitory helix wedge motif from one domain occluded the active site of the partner domain [208]. It was proposed that in a dimeric state the catalytic activity of RPTP is attenuated by occlusion of the active sites; regulation of RPTP dimerization by ligand binding may modulate the activity of the phosphatases and dephosphorylation of down-stream molecules [193].

Ligands for RPTP: Since dimerization of RPTP is considered a regulatory mechanism, the question about its potential ligands and their role in the regulation was raised. The best characterized example of RPTP regulation by ligand binding is the inhibition of RPTPζ activity following binding of pleiotrophin (PTN), potential substrates of RPTPζ that shows increased phosphorylation in response to PTP include the protein β-catenin (component of cadherin), and β-adducin (cytoskeleton protein). Some of the effects on cytoskeletal architecture trigger by PTP might be mediated by RPTPζ-induced changes in tyrosine phosphorylation [209,210].

PTP oxidation: Reactive oxygen intermediates and the resulting post-transcriptional modifications of proteins by reversible oxidation have been recently implicated in the regulation of tyrosine phosphorylation-dependent signalling pathways that are initiated by a variety of stimuli [193]. The signature catalytic domain in PTPs, contains an invariant cysteine residue characterized by low pKα; this low pKα confers it the nucleophilic activity during catalysis, but also gives it highly susceptibility to oxidation [211]. Oxidation of this cysteine residue abrogates the PTP activity, this mechanisms represents a new mechanism by which tyrosine phosphorylation-dependent signalling is modulated [193,211].

PTP cleavage: PTP cleavage has also been described as a regulating mechanism for these proteins. The best studied mechanism is the action of calpain proteins over PTP1B. The calpain family is named for the calcium dependence of papain-like, thiol protease activity of the vertebrate enzymes calpain-1 (also known as μ-calpain) and calpain-2 (m-calpain) [212]. However, proteins showing sequences related to the catalytic core domains are included in this family. The gene family is present in mammals, trypanosomes (such as *T. brucei*, *T cruzi* and some *Leishmania* species) and ciliates [213,214]. In human and in mouse 14 members of the calpain family have been described, ranging from 13 to 50 kDa [213].

Under conditions of platelet aggregation, it was observed that PTP1B is cleaved by neural calpain. Cleavage of PTP1B is accompanied by a 2-fold stimulation of its enzymatic activity and change in its cellular localization from the ER to the cytoplasmic space. Neural calpain brakes PTP1B between the catalytic domain and the C-terminal domain removing 60 to 70 amino acids [215]. Other PTPs have been shown to be cleaved by calpain proteins for example, Pasquet et al. showed that phosphatases containing SH2 domains (such as SHP-1) could be cleaved by mu-calpain in their C-terminal domain, although the affinity of mu-calpain for SHP-1 it was much less than the affinity for PTP1B. Even though, enhanced activity of cleaved SHP-1 has not been reported [216].

One of the main mechanisms that *Leishmania* parasites utilize to abrogate the immune response is the activation of host phosphatases. Numerous reports have shown this phenomenon; however, in this section I will describe some of that evidence.

One of the most common activated phosphatase during *Leishmania* infection is SHP-1. It has been demonstrated that *L. donovani*-infected macrophages presented increased SHP-1 activation, simultaneously with dephosphorylation of tyrosine residues and inhibition of protein tyrosine kinases activity. Along with this general tyrosil dephosphorylation, IFN-γ-JAK2 phosphorylation was substantially diminished, suggesting that SHP-1-mediated JAK2 dephosphorylation by the parasite represents an important mechanism to persist inside the host cell [186]. Associated with this observation Forget et al. reported that in the absence of SHP-1 *Leishmania*-infected mice presented less parasite load as well as, less footpad lesions, furthermore *in vitro* studies in macrophages lacking SHP-1 presented more NO production than the WT counterpart [217].

Using an air-pouch model it was shown that *L. major*-infected SHP-1 deficient mice harboured a stronger immune response against the parasite in comparison with the WT mice. This strong response was accompanied by higher TNF, IL-1β, and IL-6 production, as well as higher chemokine and chemokine receptor secretion. These data suggested that SHP-1 is a strong regulator of the innate immune response during *L. major* infection. [139]. More recently it was shown that SHP-1 is able to modulate NO production in murine macrophages. In accordance with the previous studies, SHP-1 deficient cells were more capable to produce NO upon *Leishmania* infection [117]. In addition to these reports, Abu-Dayyeh et al. demonstrated that SHP-1 exploits the TLR signalling to inactivate down-stream signalling pathways. Upon *Leishmania* infection, SHP-1 binds to IRAK-1 entirely inactivating its kinase activity. Furthermore, any LPS-mediated activation is completely abrogated in murine macrophages. SHP-1/IRAK-1 interaction is through an evolutionary conserved ITIM-like motif found in the kinase domain of IRAK-1 [108].

More recently, Gomez et al. showed that SHP-1, PTP1B and TCPTP cleavage by *Leishmania*-surface protein GP63 increased their catalytic activity; this augmentation in the proteolytic activity was more evident for PTP1B. *In vivo* experiments showed that the absence of PTP1B protects mice from the infection, since PTP1B null mice presented less footpad swelling than the WT mice [118].

In the few past sections I have described some of the many mechanisms that *Leishmania* employs to subvert the immune response. Some of these events are summarized in Figure 10.

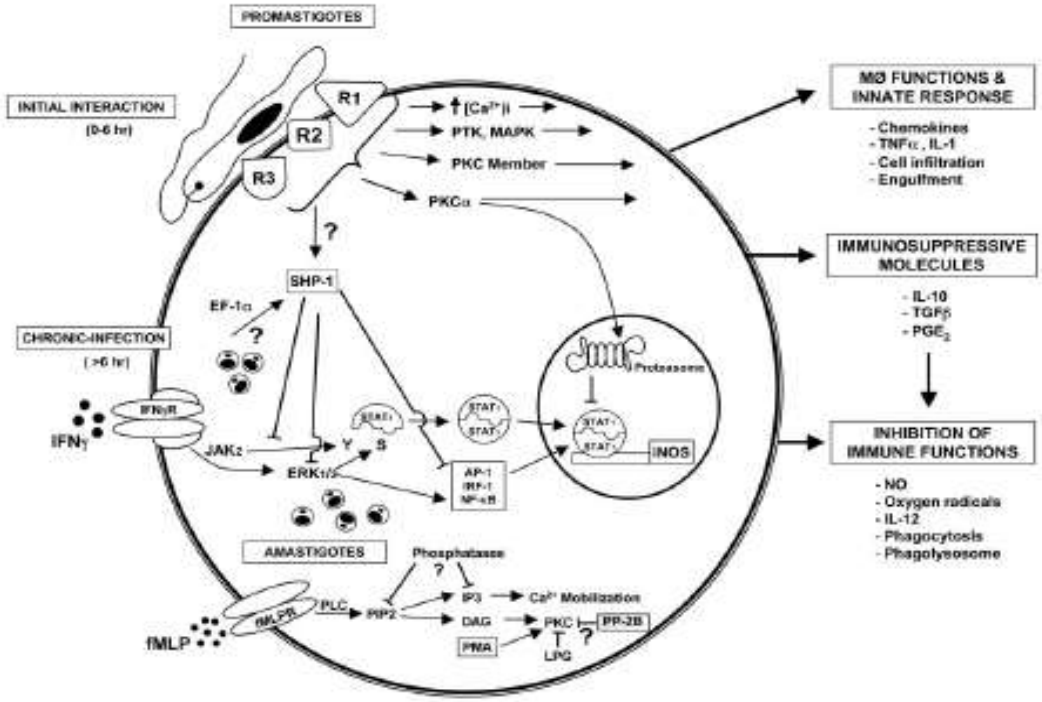

Figure 10: Signalling events leading to the induction or inhibition of macrophages' functions. Taken and modify from Olivier et al. [9].

3. S100 proteins

The S100 proteins form a large family of intracellular proteins of nearly 20 members. These proteins are formed by two calcium binding domains separated by a hinge region of the EF-hand type. S100 proteins are small (10-12 kDa) and acidic proteins [218]. Many of them show high cell-tissue specificity; in addition, most members of the S100 family exist as homodimers, heterodimers or even as oligomers which contribute to their functional diversification [218]. Within cells, S100 proteins interact with several effector proteins, mostly in a calcium-dependent manner, thereby regulating enzymes activities, cytoskeleton dynamics, cell growth and differentiation, and calcium homeostasis [219]. Although their action has been mainly reported to occur intracellularly, more attention has now given to their important extracellular roles. Extracellularly, S100 proteins can act in a cytokine-like manner through receptor for advanced glycation end products (RAGE). In addition to Ca^{+2} many S100 proteins present high affinity towards Zn^{+2} and Cu^{+2} ions, which could influence their extracellular activity [218].

3.1 S100 functions

The members of this family are multifunctional signalling proteins and are involved in the regulation of diverse cellular processes such as contraction, motility, cell growth, differentiation, cell cycle progression, transcription among others [218]. In the next few sections some of these functions will be briefly described. S100 proteins regulate different enzymes, calcium homeostasis, cytoskeleton organization and transcription in a calcium dependent manner [220]. Many of these proteins are able to bind zinc and cupper with high affinity. Cupper binding to S100 proteins might play a neuroprotective role [221]. In addition to their intracellular functions some S100 proteins are secreted and act in a cytokine-like manner. Some of these activities include chemotactic properties [222], neurotrophic activity, and angiogenic effect [223]. Furthermore they can also activate different intracellular signalling pathways such as MAPK and NF-κB [224].

3.2. S100 proteins and associated diseases

Altered expression levels of S100 proteins may be associated to different diseases mainly classified in four categories: i) heart diseases; generated by high expression of the proteins in the myocardium, ii) central nervous system diseases, during traumatic brain injuries resulting in an dramatic increase in the expression of these proteins, iii) inflammatory disorders; secretion of these proteins in sites of inflammation and, iv) cancer; high concentration of S100 proteins in the cancer zone may promote tumour growth [218]. Table 3 shows some of the S100 proteins, their function and disease association.

Table 3. The S100 family

Protein	Other name	Function	Disease	Reference
S100A1	S100A		Cardiomyopathies	[218]
S100A2	S100L		Cancer	[218]
S100A3	S100E	Angiogenic effects, regulation of Zn	Cancer	[218,225]
S100A4	Metastasin	Cytokine-like functions	Cancer	[218,223]
S100 A5	S100D		Cancer	[218]
S100A6	Calciclyn	Regulation of Zn	Cancer	[218]
S100A7	Psoriasin		Psoriasis	[218]
S100A8	Calgranulin A	Chemotactic	Inflammatory disorders	[218,222,226]
S100A9	Calgranulin B	Chemotactic, antimicrobial	Inflammatory disorders	[218,222,226]
S100A10	Anexin II-L	Regulation of Ca	Cancer	[218]
S100A11	Calgizarrin	Regulation of Ca		[218]
S100A12	Calgranulin C	Host-parasite response	Inflammatory disorders	[218,226]
S100A13		Cytokine-like functions		[218]
S100B	S100F	Regulation of Cu	Neurodegeration	[218]

In chapter 2 of this doctoral thesis, we will discuss some functions of S100A8 and S100A9 proteins during infection with *Leishmania* parasites (In chapter 2, these proteins are referred as MRP 8 and MRP 14 respectively), therefore, in the next section; these two proteins will be described in more detail.

3.3 S100A8 and S100A9 (MRP 8 and MRP 14)

S100A8 and S100A9 are two members of the S100 protein family; these proteins are also designated as migration inhibitor factor or myeloid-related protein-8 (MRP8) and 14 (MRP 14), or calgranulin A and B respectively [219]. MRP 8 and 14 are small cytoplasmic proteins expressed mainly in neutrophils and monocytes and found in the extracellular milieu during infections and inflammation. The murine proteins share 30 % homology mostly confined within the two EF-hand calcium binding domains. Although they exists as homodimers the heterodimer S100A8/A9 is formed by the presence of calcium. S100A8 and A9 are not constitutively expressed by macrophages and endothelial cells; however, S100A8 expression can be stimulated with LSP, IFN-γ, IL-1β and TNF-α in murine macrophages [227,228]. This expression is not associated with the S100A9 expression. Murine endothelial cells on the other hand, express both S100A8 and A9 following stimulation with LPS or IL-1β. Different expression in these proteins could be related to different roles played during inflammation [226].

Murine S100A9 is chemotactic for neutrophils and monocytes as well as it is involved in arachidonic acid metabolism [229]. Human hetero and homodimers are chemotactic for neutrophils and stimulate neutrophils adhesion to fibrinogen and monocytes transmigration across endothelial cells [230]. The dimer S100A8/A9 inhibits the interaction between casein kinase I and II with cytoskeletal components to exert antimicrobial properties against *C. albicans*, and exhibit growth inhibitory activities in murine BMM and mitogen-stimulated lymphocytes [231]. The dimer is also known to inhibit microbial growth by zinc chelation [232] and by inhibiting bacterial adhesion to mucosal epithelial cells. Furthermore, *in vitro* expression of the proteins by epithelial cells protected them against *P. gingivalis* infection [232]. The C-terminal region of

S100A9 is suggested to induce neutrophils mobilization, regulation of cytoskeletal translocation and inhibition of the intrinsic coagulation cascade [233].

In macrophages, it has been demonstrated that the S1008A/A9 dimer is a powerful inducer of microbicidal functions. For instance, macrophage stimulation with the dimer induces strong NO production, concomitant with elevated expression of both gene and protein levels of iNOS. The increased NO production was linked with an important phosphorylation of JNK and ERK kinases which led to an increased NF-κB nuclear translocation [234].

3.3.1 S100A8/A9-related diseases and inflammation

S100A8 and A9 are mainly secreted by neutrophils and correspond to 30-40 % of their cytoplasmic proteins. Since these cells are recruited to the site of infection it is believed that S100A8/A9 play an important role in the effector functions of neutrophils. Elevated levels of S100A8/A9 in body fluids of inflamed tissues strengthen the view that these molecules are important players during inflammation [229].

During rheumatoid arthritis there is a high percentage of leukocytes expressing S100A8/A9. Clinical studies have shown that there is a good correlation between serum concentrations and protein levels with the clinical course of the disease, this data suggested that S100A8/A9 could be good markers in patients with inflammatory arthritis [235]. Another recent report showed that isolated synovial tissue from patients with rheumatoid arthritis spontaneously released larger amounts of S100A8/A9 than patients afflicted with osteoarthritis. S100A8/A9 complex, as well as, S100A9 homodimers stimulated pro-inflammatory cytokine production [219]. Besides rheumatoid arthritis, there is evidence that S100A8 and A9 play important roles during autoimmune diseases, elevated concentrations of these proteins have been found in patients with systemic lupus erythematosus [236]. Furthermore, elevated expression and serum concentrations of S100A8/A9 have been found in autoimmune encephalomyelitis, neuritis as well as in multiple sclerosis [236]. S100A8/A9 has also been associated to bowel disease where high levels are found in monocytes, neutrophils and epithelial cells during this disease, both proteins are released during the inflammatory process in the gut, having a good correlation between high concentration of the proteins and bowel inflammation [237]

Hermani et al. showed enhanced S100A8 and S100A9 expression in human prostate cancer. In this study they examined the influence of over-expressed and purified recombinant S100A8 and S100A9 proteins in different prostate epithelial cell lines. Their results demonstrated that S100A8 and S100A9 were secreted by prostate cancer cells, with the concomitant NF-κB activation and increased p-38 and ERK phosphorylation. In addition, extracellular S100A8/A9 stimulated migration of benign prostatic cells *in vitro*. These findings strongly suggested that S100A8 and S100A9 are linked to important features of prostate cancer cells [238].

3.3.2 S100A8/A9 and *Leishmania*

The role of S100A8 and A9 during *Leishmania* infection has not been extensively studied and yet remains unclear. Few reports have shown some evidence of the possible role of these proteins during *Leishmania* infection. For instance, Steinbrink et al. showed that in experimental leishmaniasis, injection of bone marrow cells enriched with S100A9 into resistant C57Bl/6 prolonged the time of the infection, associated with increased local parasite spread [239]. More recently Goto et al. showed by immunohistochemitry an accumulation of MRP 8^+ and MRP 14^+ macrophages in skin lesions during *L. major* infection in susceptible BALB/c and RAG-$2^{-/-}$ mice [240]. One year later the same group examined whether extracellular MRP 8/14 interact with *Leishmania* parasites during infection. By immunohistochemistry they found, that MRP 8 and 14 were detected on amastigotes isolated from skin lesions of *L. major*-infected mice. Western blot revealed that amastigotes purified from skin lesions had adhered MRP14 into their surface, suggesting that amastigotes could have receptor molecules for this protein possible expressed in both parasite stages [241]. With that evidence the authors considered that perhaps MRP 8/14 can enhance the severity of the infection, nonetheless the data presented in these reports do not support that role for these proteins and this notion needs further study; therefore, the role of S100A8 and A9 during *Leishmania* infection remains unknown.

4. Dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells (APC) with the unique ability to induce primary immune responses. They are responsible for transferring information from the outside world to the cells of the adaptive immune system. DCs are not just critical for inducing primary (innate) and adaptive response, but are also important for the induction of immunological tolerance, as well as for the type of T-cell mediated response [242].

4.1 Dendritic cells: immunobiology and functions

In mice at least two pathways of DCs development has been identified: myeloid and lymphoid. The myeloid origin *in vitro* comes from myeloid-committed precursors that rise both granulocytes/monocytes and myeloid precursors under the stimulation of granulocyte/macrophage colony-stimulation factor (GM-CSF) [243]. DCs also can arise from lymphoid-committed precursors [244]. Lymphoid and myeloid DCs differ in phenotype, localization and functions; both express high levels of CD11c and class II MHC molecules, with their co-stimulatory molecules CD86 and CD40. The distinguishable marker is CD8α, which is only expressed in lymphoid DCs [245]. Lymphoid DCs are mainly localized in T cell-rich areas of the periarteriolar lymphatic sheaths (PALS) in spleen and lymph nodes, whereas myeloid DCs are mainly localized in marginal zones bridging spleen channels [246]. Lymphoid DCs produce higher levels of IL-12, IFN-γ and are less phagocytic than myeloid DCs [247]. The life cycle of DCs (Figure 11), starts when circulating DC precursors enter tissues (immature), there, they can directly encounter pathogens or antigens. After antigen capture DCs migrate to lymphoid organs where after maturation they display MHC molecules [242]. The details of antigen capture and maturation will be described in the next section.

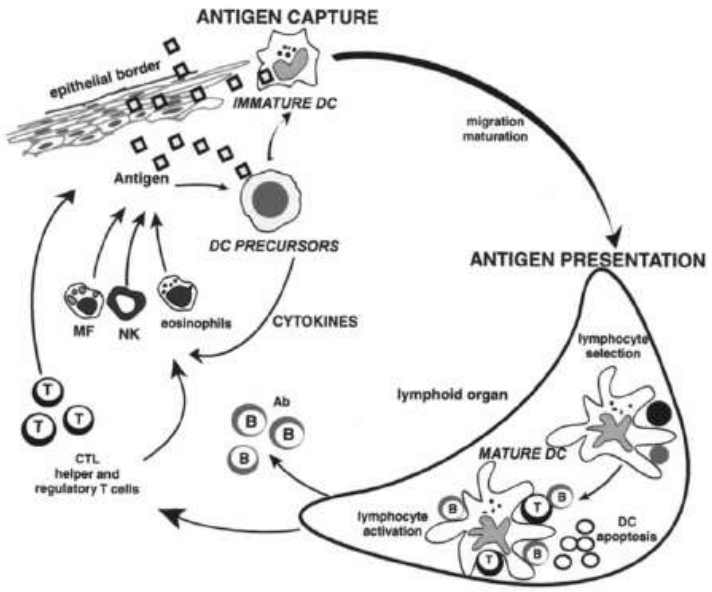

Figure 11: The life cycle of DCs. Immature DC enter the tissues, after antigen/pathogen capture they migrate to lymphoid organs, where they mature and process the antigen to activated T cells. Taken from Banchereau et al. [242]

4.1.1. Antigen capture, migration and maturation

New generated DCs migrate through blood stream, from bone marrow to non-lymphoid tissues; where they accumulate rapidly at sites of Ag deposition. This was demonstrated by McWilliam et al. where they showed rapid DC accumulation in bronchial epithelium after inhalation of *Moraxella catarrhalis* [248]. *In vitro*, immature DCs respond to a wide variety of chemokines through specific receptors such as CCR1, CCR2, CCR4, CCR5, CCR6 (the last one only Langerhans cells (LCs)), CXCR1, and CXCR4, expression of these receptors varies between DC subsets [249]. During DCs migration several adhesion events are involved, for example, E-cadherin, permits homotypic interactions and down-regulation of this adhesion molecule in LCs allows them to migrate outside of the skin, macrophage's elastase which degrades components of the extracellular matrix is highly expressed by DCs and may contribute to their migration [250].

Immature DCs are highly efficient in Ag capture and they can use several pathways for capture such as macropinocytosis [251], receptor mediated endocytosis via C-type lectin receptors (mannose receptor) [252], and Fc-γ I and II receptors, as well as particle phagocytosis [253], apoptotic and necrotic cell fragments via αvβ5 receptors [254], viruses and bacteria, and intracellular parasites such as *Leishmania* [255]. Although some groups have reported that the mannose receptor is used by human DCs, Mommaas et al. have shown that despite the high capacity of Ags-uptake, LC do not use this type of receptors [256]

The uptake of pathogens/antigens by immature DCs leads them to a series of phenotypic and functional changes that culminate in the complete transition from Ag-capturing cell to a professional APC. DC maturation is also linked with their migration capacity from peripheral tissues to the draining lymphoid organs where they efficiently present antigens to T cells [242].

Some molecules including CD40, TNF-R and IL1-R activate DCs and trigger their transition from immature to mature Ag-presenting cell. Among the factors that are involved in DCs maturation include i) bacteria or bacteria-derived molecules such as LPS [257], bacterial DNA (CpG) [258], influenza virus and double stranded RNA viruses [259], ii) pro-inflammatory and anti-inflammatory signals, and iii) T-cell derived signals. Besides these factors, DC maturation is associated to i) loss of endocytic and phagocytic receptors, ii) co-stimulatory molecule (CD40, CD58, B7.1 and B7.2) up-regulation iii) change in morphology including loss of adhesive structures, and change in cytoskeleton, and iv) change in MHC class II capacity [242].

A representative diagram of the molecules and steps during DCs maturation is shown in Figure 12.

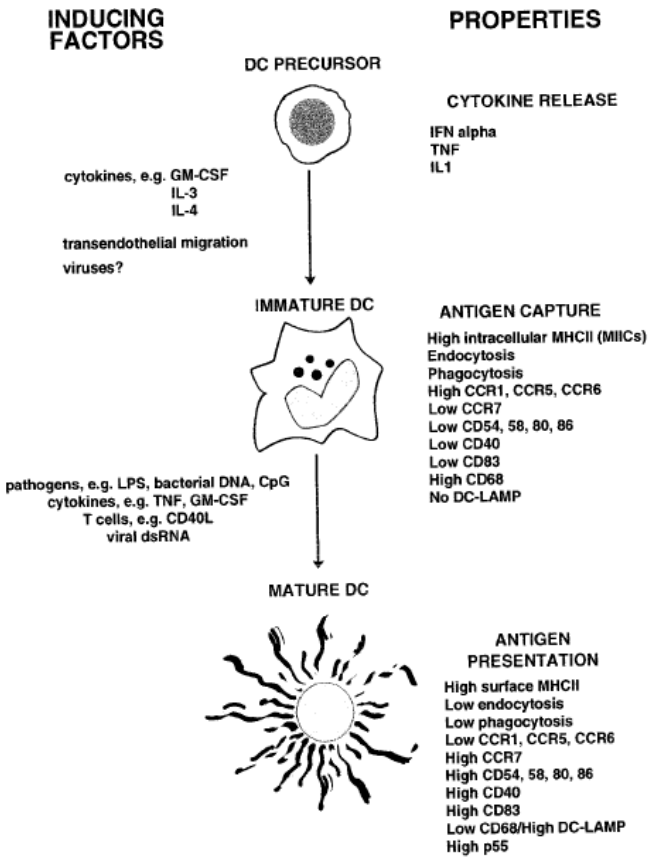

Figure 12: Maturation events for DCs. Some inducing factors are shown in the left of the figure, the middle panel shows the stage in the maturation process and the right panel shows some of the main properties of each differentiation stage. Taken from Banchereau et.al [242].

Antigen presentation by DCs can be achieved by three main pathways: i) MHC I complexes in which degradation of cytosolic proteins undergo loading of peptides onto newly synthesized MHC I molecules within the ER. These peptides are generated by the proteasome, and are translocated into the ER by transporter associated proteins. Finally these peptides are presented to $CD8^+$ cytotoxic T cells [260], ii) presentation through MHC class II complexes which involves degradation of soluble and particulate antigens which are captured by immature DCs or other APC cells. Once the antigen is processed, the cell will present it to $CD4^+$ cells [261], and iii) presentation through CD1 molecules

considered as non-classical Ag-presenting antigen molecules which presentation involves T cell activation by presenting both endogenous and exogenous microbial lipids and glycolipids-containing antigens [262].

One of the DCs main functions is their ability to prime naive $CD4^+$ T cells both *in vitro* and *in vivo* [263]. However, they are similarly important in priming $CD8^+$ T cells in similar mechanisms to $CD4^+$ T cells [264]. *In vivo*, they can stimulate the proliferation of allogenic $CD8^+$ T directly in the absence of Th cells; DCs can also generate Ag-specific cytotoxic T cells from naive precursors [264]. During antigen presentation process between the DC and the T cell, MHC-peptide recognition constitutes the first signal. This DC-T cell interaction is mediated by several adhesion molecules such as integrins β1 and β2. The signal two constitutes interaction between co-stimulatory molecules expressed by DCs and recognition of their ligands expressed in the T cells. Different DC subsets may provide T cells with different cytokine/molecule microenvironments that determine the class of immune response (Th1 vs. Th2) [242,265]. For example, $CD11c^+$ DCs are more likely to polarize towards Th1 as well as the expression on the co-stimulatory molecules will play a role in the type of Th response that will be activated. However, the involvement of the set of these co-stimulatory molecules and how they polarize towards one or another is still unclear. T cell polarization will be affected by the set of cytokines that are released by both cells during the antigen-presentation process [266]. DCs have also the capacity to directly activate naïve and memory B cells. Dubois et al. demonstrated that IL-12 secretion by CD40-activated DCs is necessary to promote differentiation of naïve B cells into plasma cells expressing IgM at high levels [267].

4.2 DCs and their role in the regulation of effectors of the innate immune responses

At different stages of the maturation DCs can regulate effectors of innate immunity cells such as NK and NKT cells [242]. Immature DCs can activate NK cells through IFN-α production, leading to enhanced NK cell antiviral and antitumor activity [268]. Furthermore, DCs might regulate NK and NKT activity by producing cytokines such as IL-12, IL-15, and IL-18 [269]. Different DC subsets can regulate NKT cytokines

secretion. For example monocyte-derived DCs can promote IFN-γ secretion, while plasmacytoid DCs stimulate NKT cells to produce IL-4 [270]. Some other studies have shown that intimate interaction of liver DCs with NKT stimulate IFN-γ production by NKT cells which will in turn activate more NK and NKT cells making a positive loop of activation [271]. In support of this, Hermans et al. demonstrated that NKT cell activation by IFN-γ and IL-12 produced by DCs considerably enhanced $CD4^+$, $CD8^+$ T cells responses, NKT cell removal from the system reduced the T cells response [272].

4.3. DCs and Leishmaniasis

Despite that there is good amount of evidence between the interaction of DCs with *Leishmania* parasites, the exact role of DCs during the infection is still unknown and to a certain extent contradictory. One of the first reports showing interaction of Langerhans cells (LCs) with *L. major* was made by Moll et al. In this study the authors demonstrated that murine LCs are able to internalize *L. major* promastigotes and stimulate a strong T cell response, furthermore these LC transport the parasites from the site of the lesion (skin) to lymphoid organs such as draining lymph nodes where they present *Leishmania* antigens to T cells [255]. In the same context Woelbing et al. showed that *L. major*-uptake by DCs is enhance by Fcγ I and Fcγ III receptors leading to an acquisition of protective immunity against *Leishmania* [12]. MHC up-regulation in LCs by *L. major* and *L. donovani* was also reported. In this study it was observed that infection of LC was associated with MHC I and II up-regulation concomitant with increased co-stimulatory molecule expression [273]. *In vitro* studies have shown that *Leishmania*-infected DCs secrete IL-12. For example Konecny et al. demonstrated that murine splenic DCs produce large amounts of this cytokine following infection [274], although the effect of *Leishmania* on IL-12 production varies according the DC subtype and the *Leishmania* species [275]. De Trez et al. showed that in both C57BL/6 and BALB/c mice, systemic injection of *L. donovani* induced migration of splenic DCs from marginal zones to T-cell areas, interestingly during migration DCs were able to up-regulate MHC-I and co-stimulatory molecule (CD40, B7.1 and B7.2) expression. However, *Leishmania*-induced maturation required live parasites, in addition in this study it was also demonstrated that

DCs maturation by *L. donovani* was abolished in MyD88-deficent mice, suggesting that MyD88-dependent receptors are implicated in the maturation process [276].

Contrary to these studies, some others studies have shown that *L. major* promastigotes establish infection in the skin by inhibiting DC maturation or inducing regulatory T cells that secrete IL-10 [277]. The chemokine profiles and their receptors expressed by *L. major*-infected DCs showed down-regulated chemokine receptors such as CCR2 and CCR5 and their respective ligands CCL2 and CCL3, while CCR7 and its ligand CCL21 were enhanced [141]. In accordance with this, Ato et al. showed that in *L. donovani*-infected mice lacking CCL19 and CCL21, the migration of splenic DCs from marginal zones to periarteriolar lymphoid sheath was impaired, suggesting that DC migration is necessary for full DC activation [278]. Regarding signalling pathways Xin et al. demonstrated that infection of bone marrow-derived DCs with *L. amazonensis* amastigotes altered the maturation capacity of these cells by suppressing co-stimulatory molecule expression, resulting in abrogated IL-12 production; these findings were linked to the lack of phosphorylation on STAT 1, 2, 3, and ERK with the concomitant STAT 2 degradation [279]. Finally, more recently it was described that LPG is implicated in inhibiting DC maturation in *L. major*-infected cells, in this study it was demonstrated that in the absence of *lpg2* gene, infected DCs were more capable to produce IL-12, as well as MHC and co-stimulatory molecule expression than cells that were infected with WT parasites. In the same way DCs infected with *lpg* null parasites were more efficient in presenting antigens to T-cells. This capacity was measured by the IL-2 production by T cells. Together these data suggested that LPG can modulate DC's antigen presentation and promotes IL-4 production in mice [280].

The necessity for an effective vaccine against *Leishmania* parasites has become an urgency, nowadays more emerging techniques are available, among them, the use of "antigen-pulse" DCs since they are considered the cells responsible for the effective transition between the innate and adaptive immunes responses. Tsagozis et al. efficiently tested vaccination in murine models of CL using DCs pulsed with synthetic or native parasite antigens such as GP63. This triggered antigen–specific immune responses and efficiently reduced lesion formation and parasite load in susceptible *L. major*-infected BALB/c mice. This effect was accompanied with a Th1 polarization, however, this effect

was not seen in resistant CBA mice [281]. Although, these findings are a good beginning in the use of pulsed DCs with different *Leishmania*-antigens as vaccines, the effects and the lack of consistent results between *Leishmania* species and the animal models used needs further study.

As described the in the previous paragraphs, the role of DCs during the infection with *Leishmania* parasites is very controversial, in Chapter 4 we are reporting the effect of *L. mexicana* promastigotes on DCs; in this study we observed that *Leishmania* down-regulates the signalling pathways of those cells with the concomitant down-regulation in the antigen presentation.

5. Rational and objectives of the research

Throughout the last decades, many groups have tried to elucidate the mechanisms by which *Leishmania* is able to survive and propagate in the mammalian host. Although good advances in this regard have been achieved, many mechanisms used by the parasite to control the signalling pathways of the host still remain unknown.

This doctoral thesis aims to address some of these mechanisms from the perspective of alteration of signalling pathways in macrophages and other cells such as dendritic cells.

MRP 8 and 14 are proteins mainly produced by neutrophils. It has been recently reported by our group that stimulation of macrophages with these proteins confers them the capacity to activate their microbicidal functions [234], which are known to be strongly abrogated in infected macrophages. Therefore, one of our main objectives was to address the role of MRP 8 and 14 during the infection in both *in vitro* (in murine macrophages) and *in vivo* (in a murine model for cutaneous leishmaniasis) since these two proteins have shown to have different activities such as chemoattraction and to be potentially microbicidal.

Many virulence factors have been described for *Leishmania*, such as its surface molecules. For instance LPG has been implicated in the inhibition of phagosome maturation [58]. GP63 has been implicated in proteolysis of a vast variety of proteins and, in particular, our laboratory has revealed its ability to cleave and activate PTPs [118] as well as to cleave NF-κB subunits [113]. Since different *Leishmania* species are able to abrogate transcription factors activity and having indication that GP63 is able to cleave NF-κB, we wanted to investigate in more detail the role of this protease in the inactivation of AP-1 which is an important transcription factor that activates the expression of genes such as iNOS, IL-1β, TNF and IL-12 in macrophages [282,283,284]. It has been previously reported that this transcription factor is down-regulated upon *L. donovani* infection and this down-regulation involves in part the action of ceramide which augmentation in infected cells was correlated with AP-1 inactivation [112], however, some other mechanisms that down-regulate AP-1 are still unknown. Thus, we evaluated

the role of GP63 in the down-regulation of AP-1 and its role in the degradation and cleavage of its subunits.

Finally we are addressing the role of *L. mexicana* promastigotes in the down-regulation of the signalling pathways in dendritic cells. Recently it has been demonstrated that *Leishmania* can reside in different cells besides its preferred host the macrophage. In the last few years many evidence has been suggestive for the role of dendritic cells during *Leishmania* infection, however, this evidence is very controversial and to a great extent depends on the type of dendritic cell used, and the species and the stage of the parasite. Therefore, we wanted to investigate whether *L. mexicana* promastigotes were able to abrogate the DCs activities such as antigen presentation by down-regulating the signalling pathways of these cells.

In the ideal situation the knowledge generated during these investigations will help to better understand the multiple mechanisms used by these protozoa and generate new venues for the design of effective drugs or immunotherapeutics to treat the disease, and more importantly, the development of an effective vaccine that protect the host.

CHAPTER 2

IMPACT OF NEUTROPHIL-SECRETED MYELOID RELATED PROTEINS 8/14 ON LEISHMANIASIS PROGRESSION

PREFACE TO CHAPTER 2

The myeloid related proteins (MRPs) 8 and 14 have been implicated in many inflammatory diseases. It is well known that they present antimicrobial and chemotactic properties. Currently, we know that these two proteins are able to induce the macrophages' microbicidal function such as NO production. During *Leishmania* infection, such functions are abrogated. The role of MRP 8 and 14 during leishmaniasis has not been elucidated. In this chapter, I am presenting experiments that clearly show that *in vitro* primed macrophages before infection are able to induce the macrophage's microbicidal properties; on the other hand, macrophages infected with *Leishmania* prior to MRPs stimulation lose this capacity, suggesting that *Leishmania* parasites are able to overpass the microbicidal properties of the cell induced by MRPs. *In vivo* experiments showed that abrogation of MRPs with antibodies led to more severe infection. In addition, injection of rMRPs directly in the footpad decreased the lesions. Together, our data show a novel role of MRPs during the progression of murine cutaneous leishmaniasis.

IMPACT OF NEUTROPHIL-SECRETED MYELOID RELATED PROTEINS 8/14 ON LEISHMANIASIS PROGRESSION

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Running Title: Role of MRP 8/14 during Leishmaniasis

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ABSTRACT

The myeloid-related proteins (MRPs) 8/14 are small proteins mainly produced by neutrophils, which have been reported to induce NO production in macrophages. On the other hand, *Leishmania* survives and multiplies within phagocytes by inactivating several of their microbicidal functions. Whereas MRPs are rapidly released during the innate immune response, their role in the regulation of *Leishmania* infection is still unknown. *In vitro* experiments revealed that *Leishmania* infection alters MRP-induced signaling, leading to inhibition of macrophage functions (NO, TNF-α). In contrast, MRP-primed cells showed normal signaling activation and NO production in response to *Leishmania* infection. Using a murine air-pouch model, we observed that infection with *L. major* induced leukocyte recruitment and MRP secretion comparable to LPS-treated mice. Depletion of MRPs significantly reduced these inflammatory events and augmented both parasite load and footpad swelling during the first 8 weeks post-infection. On the contrary, mouse treatment with recombinant MRPs (rMRPs) had the opposite effect. Collectively, our results suggest that rapid secretion of MRPs by neutrophils at the site of infection may protect uninfected macrophages and favor a more efficient innate inflammatory response against *Leishmania* infection. However, it is clear that *Leishmania* has developed strategies to down-regulate the host innate immune machinery triggered by MRPs.

INTRODUCTION

Myeloid-related proteins 8 and 14 (MRPs 8/14) also known as S100A8 and S100A9 are small calcium binding cytoplasmic proteins secreted mainly by neutrophils and monocytes [1,2]. These proteins are formed by two Ca^{2+} binding domains separated by a hinge region [3]. Although these proteins exist as homodimers, the heterodimer (MRP 8/14) is formed in the presence of calcium. Both proteins are expressed abundantly by neutrophils, being around 30 to 40 % of their cytoplasmic proteins [4]. MRP 8 and 14 are not constitutively expressed by macrophages; however, expression of MRP 8 can be achieved in those cells by stimulation with LPS, IFN-γ, IL-1β and TNF-α. Interestingly, murine endothelial cells express both MRP 8 and MRP 14 following LPS stimulation [5]. Murine MRP 8 is chemotactic for neutrophils and monocytes, whereas human MRP 14 and the heterodimer MRP 8/14 are chemotatic for neutrophils, stimulate their adhesion to fibrinogen, and enhance monocyte transmigration across endothelial cells. It is also known that MRP 8 and 14 inhibit bacterial growth possibly by zinc chelation and by preventing bacterial adhesion to mucosal epithelial cells [6].

MRP 8 and 14 have been associated with a number of inflammatory diseases leading to the assumption that these molecules are involved in the body's defense against inflammation. Phagocytes expressing MRP 8 and 14 are found in a variety of inflammatory conditions, including rheumatoid arthritis, chronic bronchitis and inflammatory bowel disease [7,8]. Moreover, Tessier and collaborators [1] reported that in the murine air-pouch model, stimulation with LPS led to an abundant recruitment of neutrophils and subsequent secretion of MRPs [2].

We have previously reported that MRP 8 and 14 play an important role in the nitric oxide (NO) modulation; a key microbicidal function of the macrophages [9]. This increase was linked with augmented expression of inducible nitric oxide synthase (iNOS), at the gene and protein levels, concomitantly with ERK and JNK kinases phosphorylation and the rapid NF-κB nuclear translocation. These findings indicate that MRPs play an important role during inflammation.

Leishmania parasites are the causative agent of leishmaniasis, a disease characterized by three main clinical manifestations; cutaneous leishmaniasis, muco-

cutaneous leishmaniasis and the lethal if untreated visceral leishmaniasis. *Leishmania* parasites of different species are able to abrogate the innate immune response in order to survive inside their host cell [10], and little is known about the potential role of MRPs during leishmaniasis. In this regard, only two recent reports have documented accumulation of macrophages expressing MRP 8 and 14 at the skin lesions of mice infected with *L. major* [11,12]. They also found, that amastigotes isolated from skin lesions presented MRP 8 and 14 adhering onto their surface. However, and despite these observations, the role of these proteins during *Leishmania* infection has not been investigated.

Herein, we report the first study concerning the role of MRPs during *Leishmania* infection. More precisely, we found that MRP-primed macrophages infected by *L. major* exhibit antimicrobial activity, whereas unprimed *L. major*-infected cells were fully inactivated, showing no response to MRP stimulation. Using *in vivo* approaches, we further demonstrated that *L. major*'s capacity to recruit inflammatory cells was accompanied by MRP secretion at the site of inoculation. The use of anti-MRP antibodies in addition to blocking *Leishmania*-induced leukocyte recruitment in the air-pouch, also increased mice footpad swelling and parasite load. Importantly, use of recombinant MRPs (rMRPs) to treat infected footpads led to significantly reduced footpad swelling and lesion development as well as a reduced parasite load. Altogether, this study provides a clear demonstration that MRPs seem to play a critical role in the control of the progression of *Leishmania* infection by modulating the innate inflammatory and microbicidal responses.

MATERIAL AND METHODS

Cell culture, macrophage infection and reagents

Immortalized murine bone marrow derived macrophages B10R cell line were grown at 37° C in 5 % CO_2 in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 % heat inactivated FBS (Invitrogen, Burlington, ON, Canada) and 100 U/ml penicillin 100 μg/ml streptomycin and 2 mM of L-glutamine (Wisent, St-Bruno, QC, Canada). *Leishmania* promastigotes (*L. major* A2 and *L. major* luciferase) were grown and maintained at 25° C in SDM-79 culture medium supplemented with 10 % FBS by bi-weekly passage. Macrophages were infected at a parasite-macrophage ratio 20:1 with stationary phase promastigotes for the times specified in each figure legend. When cells were primed with 5, 10 and 25 μg/ml of MRPs 8/14 heterodimer were used 1 hr before infection and remained throughout the infection time.

Recombinant proteins

Cloning expression, and purification of mouse MRP 8 and 14 (S100A8/A9) were previously described [1,2]. Briefly, mouse S100A8 cDNA was cloned into the pET28a expression vector (Novagen, Madison WI, USA). Murine S100A9 cDNA was obtained by RT-PCR and cloned in Dr. Philippe Tesier's laboratory (Laval University, QC. Canada) into the PET28a vector. Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside in *E. coli* HMS174 for 16 hr at 16° C. After incubation, the bacteria were centrifugated and the pellet resuspended in PBS/NaCl (0.5 M)/imidazole (1 mM) and lysed by sonication. The pellet was centrifugated and the supernatant collected. Recombinant His-Tag proteins were purified using a nickel column; S100A8/A9 bound to the column were freed from their His-Tag by incubation with 10 U of biotinylated thrombin for 20 hr at RT. Finally the proteins were passed through a polymyxin B agarose column (Pierce, Rockford, IL, USA) to remove endotoxins. The lysate, contamination by endotoxins was < 1pg/μg. The proteins were kept at -80° C until further use.

Nitric Oxide Measurements

B10R macrophages were plated in 12-well plates (0.5 x 10^6 cells/well). The next day, cells were incubated for 24 hr following treatments in triplicate: 5, 10 and 25 μg/ml of MRPs 8/14 + *L. major* (20:1) 24 hr; or *L. major* 24 hr + 5, 10 and 25 μg/ml of MRPs. Then, NO production was assessed by measuring the accumulation of nitrites in the cell culture medium using the colorimetric Griess reaction as previously described [13].

TNF bioassay

B10R macrophages were plated in 12-well plates (0.5 x 10^6 cells/well). Next day the cells were stimulated: MRPs alone, MRP-primed-infected or infected-MRP-stimulated. After the stipulated time of stimulation or infection, plates were centrifugated at 2500 rpm and 100 μl of supernatant were collected and added to TNF-sensitive L-929 fibroblasts previously pleated in 100 μl in 96-well plates (3 x 10^5 cells/well) the day before, making a 2-fold serial dilution by gently mixing the contents in each raw of wells. Twenty μl actinomycin D (final concentration of 2 μg/ml) were added to each well and plates were incubated 18 hr at 37^o C. Next day, plates were centrifugated and 20 μl of crystal violet (0.05 % in 0.1 % acetic acid solution) were added. After 10 minutes plates were washed with tap water and shaken to remove the excess of water. Plates were allowed to dry overnight and then 100 μl of 100 % methanol were added to each well to elute stain from the cells. Plates were red at 595 nm.

Electrophoresis Mobility Shift Assay (EMSA)

B10R macrophages (2 x 10^6) were infected, washed three times with Phosphate Buffered Saline (PBS) to remove non-internalized parasites, and processed for nuclear extraction as previously described [14,15]. Briefly, macrophages were collected in 1 ml of cold PBS, centrifuged and pellets were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM of PMSF) and incubated 15 min on ice. Twenty five μl of IGEPAL 10 % (Sigma-Aldrich, St-Louis MO, USA) were added, and samples vortexed for 30 sec. Nuclear proteins were pelleted by centrifugation and resuspended in 50 μl of cold buffer C (20 mM HEPES, 400 mM NaCl 1mM EDTA, 1 mM EGTA 1 mM DTT and 0.5 mM PMSF).

Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules CA, USA). Six μg of nuclear proteins were incubated for 20 min at room temperature with 1 μl of binding buffer (100 nM Hepes pH 7.9, 8 % v/v glycerol, 1 % w/v Ficoll, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, 25 mM NaCl, and 1 μg/μl BSA) and 200 ng/μl of poly (dI-dC), 0.02 % bromophenol blue and 1 μl of γ-P^{32}labeled oligonucleotide containing a consensus sequence for AP-1 binding complexes (5'-CGTTTGATGACTCAGCCGGAA-3') (Santa Cruz Biotechnology Inc, Sta Cruz CA, USA), NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') (Santa Cruz Biotechnology Inc, Sta Cruz CA, USA) and STAT1 (5'-AAGTACTTTCAGTTTCATATTACTCTA-3'). After incubation, DNA-protein complexes were resolved by electrophoresis in non-denaturing polyacrylamide gel 5 % (w/v). Subsequently gels were dried and autoradiographed. Competition assays were conducted by adding a 100-fold molar excess of homologous unlabeled AP-1 oligonucleotide, or the non-specific competitor sequence for SP-1 binding (5'-ATTCGAATCGGGGCGGGGCGAGC-3').

Western blot

Infected and non infected cells (1 x 10^6) were washed 3 times with PBS and lysed with cold buffer (50 mM Tris-HCl pH 7.0, 0.1 mM, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 1 % NP-40, 40 μg/ml aproptinin, 20 μg/ml of leupeptin 100 mM PMSF, and 20 mM $NaVO_4$). Proteins were dosed by Bradford (Bio-Rad, Hercules CA, USA), and 30-60 μg of proteins were separated by SDS-PAGE, and transferred onto PVDF membranes (GE healthcare, Piskataway NJ, USA). Membranes were blocked in 5% non-fat dry milk, washed and incubated ON with anti-p-ERK and p-JNK (Cell signaling, Ipswich, MA, USA). After washing, membranes were incubated 1 hr with α-rabbit HRP-conjugated antibody (Sigma St-Louis MO, USA), and developed by autoradiography.

Luciferase assays

To determine parasite survival inside B10R macrophages, cells were plated in 12-well plates (0.5 x 10^6cells/well) and the following day they were infected with stationary phase *L. major*-LUC promastigotes (10:1 ratio). After 6hr of infection, the non-phagocytosed parasites were removed by washes with PBS, and samples were collected.

For the second group of samples, fresh media was added and cells were incubated for another 18 hr. Cell supernatants and adherent macrophages were collected and centrifuged 13,000 rpm x 1 min. Pellets were lysed in 25 µl of 1X Cell Culture Lysis Reagent (Promega, Madison, WI, USA). Twenty µl of lysate were mixed with 90 µl of Luciferase Assay Reagent (Promega, Madison, WI, USA) and luciferase counts were determined using a Mini Lumat LB 9506 luminometer (EG&G, Berthold, Germany).

Air-pouch

The research involving animals in this work was carried out according with the regulations of the Canadian Council of Animal Care and approved by the McGill University Animal Care Committee. Air pouches were raised on the dorsum of 6 week-old BALB/c (Charles River) by s.c. injection of 3 ml of sterile air on days 0 and 3. On day 6, 1 ml of LPS (1 µg/ml) or 10 x10^6 parasites of *Leishmania major* in 1 ml of PBS were injected into the air pouches. At 6 hr, mice were killed by asphyxiation using CO_2. The air pouches were washed twice with 2 ml of PBS. Exudates were centrifuged at 1200 rpm for 5 min. Cells were counted with a hematocytometer. Characterization of leukocyte subpopulations migrated into the pouch space was performed by Wright-Giemsa staining of cytospins. In some experiments mice were injected i.p. with 4 mg of purified rabbit IgG anti-MRP8/14 16 hr before infection.

MRP secretion in the air-pouch

For murine MRP 8, MRP 14 and MRP 8/14 (S100A8, S100A9 and S100A8/A9 respectively), ELISAs were performed as previously described in [2]. Briefly, Costar high binding 96-well plates (Corning Glass, Corning, NY, USA) were coated overnight at 4^o C with 100 µl of purified rabbit IgG against MRP8 or MRP14, diluted in 1 µg/ml in 0.1 M of carbonate buffer, pH 9.6. The wells were blocked with PBS/ 0.1 % Tween 20/2 % BSA for 30 min at room temperature. Then the samples and the standards (100µl) were added, and after 45-min period at room temperature, the plates were incubated with rat IgG (100 µl/well) against MRP8 and MRP14 diluted in PBS/0.1 % Tween 20/2 % BSA for 45 minutes. To reveal the immune complex, 100 µl/ well of peroxidase-conjugated goat-anti-rat was added and incubated for 45 minutes. Next 100 µl/well of 3,3', 5,5'-

tetraamethylbenzidine substrate (Research diagnostics) were added according to the manufacturer's instructions, and ODs were read at 500nm. The lower limit of quantification was determined as 4 ng/ml for both MRP8 and MRP14, and 10 ng/ml for the heterodimer. All ELISAs were tested using excess amounts of the other S100 proteins and were shown to be specific under conditions reported in this work.

Footpad infection

L. major stationary phase promastigotes (5 x 10^6 in 50 μl of PBS) were injected in the right hind foot pad of animals. Footpad thickness measurement was done as previously described [16] for 10-12 weeks. For the group of MRP neutralization, anti-MRP 8/14 (4 μg/ml) were injected i.p. 1 day after infection and then at days 3, 6, 9, 12, 15, 18 and 21 after infection. After 10 weeks, mice were sacrificed and parasite load was measured by limiting dilution assay. For the group that receive recombinant MRPs (rMPR) as treatment, mice were infected as previously described and then treated 3 times per week with 10 μg of the mix MRP8/14 in 50 μl of PBS, during the last four weeks of infection directly in the infected footpad. Thickness of the lesion was measured every week until the end of the infection and parasite load was measured as before.

Limiting dilution assay:

After-10-12 weeks of infection mice were killed by asphyxiation using CO_2. The infected footpads were sprayed with Clidox (chlorine dioxide) and stand for 5 min to allow the sterilizing to activate, after that time, the footpad was flooded with 70 % ethanol. Using large scissors the footpad were cut off entirely and collected in 15 ml falcon tubes. The pads were washed 3 times with sterile PBS and shook vigorously to remove all the unwanted hair. After the washes the footpads were transferred into a small dish and 2 ml of PBS were added to excise the inflamed area of the pad (infected area). The tissue was transfer into a homogenizer and all the parasites were extracted and diluted initially in 50 ml of PBS, then 100 μl were used for 2 fold dilution in the 96 well plates with a total of 24 dilutions [17].

Statistical analysis

Statistically significant differences were identified using the ANOVA module of Graphpad Prism program (version 5.0). Values of $P \leq 0.05$ or $P \leq 0.01$ were considered statistically significant. All data are presented as mean ± SEM.

RESULTS

Leishmania infection down-regulates MRPs-inducible NO production

We have previously described that MRPs induce NO in murine macrophages [9]. Confirming and extending these data, we observed that increasing concentrations of MRPs 8/14 (5, 10 and 25 μg/ml) lead to NO synthesis by macrophages in a dose-dependent manner (Figures 1A and 1B). Subsequent infection of MRP-primed macrophages with *L. major* did not affect NO production (Figures 1A). However, when cells were first infected and then stimulated with MRPs, NO production was reduced by around 35% (Figure 1B). To evaluate whether the effect of *Leishmania* infection was affecting iNOS protein levels, we performed western blotting. Stimulation of macrophages with MRPs, led to an increase on the levels of iNOS (Figure 1C). As expected, in MRP-primed macrophages followed by *Leishmania* infection, we observed up-regulation of iNOS expression, which was maximal when 25 μg/ml of MRPs were added. At the same concentration of MRPs, the expression of iNOS was reduced when the cells were infected prior to stimulation (Figure 1C). These results revealed that *Leishmania* infection alters the capacity of MRPs to induce NO production by reducing iNOS expression.

MRPs-induced TNF-α production

Tumor necrosis factor α (TNF-α) is a multifunctional cytokine produced primarily by monocytes and macrophages. It has been shown that this cytokine is essential for the control of *Leishmania* at early stages of infection [18]. Therefore, we were interested in investigating whether MRPs were able to induce TNF-α production in macrophages. We performed a time and dose-dependent experiment, stimulating the cells for 1, 3, 6 and 24 hr with 5, 10 and 25 μg/ml of MRPs using TNF-sensitive L929 fibroblasts [19]. As shown in Figure 2A, the maximum peak of TNF-α production by MRP-stimulated macrophages occurred between 1 and 3 hr with 25 μg/ml of MRPs, time after which the signal decreased until it was not detected. The time of 1 hr was chosen to evaluate the profile of TNF-α production during *Leishmania* infection. First, cells were primed for 1 hr with MRPs and then infected with *L. major.* Second, macrophages were

infected with *L. major* overnight, followed by washes and stimulation with MRPs. As shown in figure 2B, and similar to our NO data, MRP-primed macrophages subjected to *L. major* infection did not show altered capacity to produce TNF-α, however; the ability of *L. major*-infected cells to produce TNF-α in response to MRP stimulation was clearly reduced.

MRP-priming conferred protection against *Leishmania* infection, as revealed by unaltered iNOS expression, NO and TNF-α production. Thus, we next evaluated whether this MRP-inducible microbicidal response correlated with an enhanced intracellular killing of the parasite [20]. To this end, macrophages were primed with various concentrations of MRPs prior to infection with a *L. major* strain expressing luciferase. Cells were collected at 6 and 24 hr post-infection. As shown in Figure 3, at 6 hr post-infection, primed macrophages had more parasitic load than those that were not primed. However, after 24 hr of infection we detected that unstimulated macrophages reduced the parasite load by 42 %. In addition, at this point of infection, we observed a significant reduction in the parasitic load in macrophages primed with MRPs in a dose-dependent manner compared with the 24 hr unstimulated macrophages (Figure 3). Altogether these results suggest that MRPs provide the cells with the ability to phagocytise and kill the parasites more efficiently that unprimed cells.

MRPs induce MAPK phosphorylation and nuclear translocation of transcription factors

As we observed that MRP stimulation increased the expression of iNOS, we further analyzed the signaling pathways involved in iNOS/NO production. We have previously reported that MRP-induced macrophage activation involves the participation of the ERK and JNK MAPK [9]. Thus, it was critical to determine whether *Leishmania* could influence phosphorylation of these kinases in order to explain the incapacity of infected cells to respond to MRPs, knowing that *Leishmania* infection can interfere with signaling under the regulation of these kinases by activating host phosphatases [21,22]. As expected, phosphorylation of both ERK and JNK was observed in naive macrophages stimulated with MRPs. This phosphorylation was also detected in MRP-primed macrophages infected with *L. major* (Figures 4A and 4C). On the other hand, MRP-

inducible ERK and JNK phosphorylation was strongly inhibited in *Leishmania*-infected cells (Figures 4B and 4D).

To further characterize the activation of macrophage signaling after MRP stimulation, we investigated the nuclear translocation of transcription factors (TFs) involved in iNOS/NO production (e.g., NF-κB, STAT 1 and AP-1) by performing EMSA. A strong nuclear translocation of NF-κB and AP-1 occurred in response to MRPs stimulation. Similarly, MRP-primed macrophages showed translocation of NF-κB and AP-1 (Figure 5A). Nonetheless, this MRP-induced translocation was inhibited when macrophages were first infected with *Leishmania* (Figure 5B). It was possible to detect the p35 fragment that is a product of NF-κB degradation by *Leishmania* infection [23]. Furthermore, AP-1 nuclear translocation was completely abrogated in macrophages that were infected and then stimulated with MRPs. (Figure 5B). We also monitored the nuclear translocation of STAT; however, we did not observe any augmented nuclear translocation of this TF in response to MRPs in either case (data not shown).

L. major induces accumulation of leukocytes and MRP secretion in the air pouch

Whereas MRPs modulate the microbicidal functions of macrophages *in vitro*, their role *in vivo* is still unknown. Therefore, using an air-pouch model we attempted to monitor this innate inflammatory event. Previous reports from our laboratory using this model have demonstrated that inoculation of *Leishmania* promastigotes led to the recruitment of inflammatory leukocytes at sites of injection within hours and this was accompanied by the secretion of various chemokines [24]. In addition, Tessier and collaborators have previously described that injection of LPS into the air-pouch induced neutrophil accumulation and the subsequent secretion of MRPs, reaching a maximum peak at 6 hr post-stimulation [2]. In this set of experiments, BALB/c mice were infected in the air-pouch with 10 x 10^6 parasites for 6 hr. Afterwards, we evaluated the number of cells recruited and the secretion of MRPs. As shown in Figure 6A, *Leishmania* infection induced leukocyte recruitment comparable to LPS, neutrophils being around 80 % of the total recruited leukocytes (data not shown). In addition, *Leishmania* infection induced MRP 8/14 secretion by the recruited cells within the pouches (Figure 6B). To further monitor the implication of MRP secretion in the *Leishmania*-induced inflammatory cell

recruitment, we neutralized MRPs using anti-MRP antibodies prior to infection with *L. major*. As shown in Figures 6A and 6B the use of these antibodies led to a significant reduction in cell recruitment, concomitantly with an almost complete abrogation of MRP secretion.

In the murine model, cutaneous leishmaniasis is caused by injection of *L. major* or *L. mexicana* directly in the footpad. This model has been widely used to measure progression of infection in resistant and susceptible mice under different circumstances and for further isolation of parasites [25,26]. To additionally investigate the role of MRPs during *Leishmania* infection, we monitored to which extend the neutralization of MRPs or the inoculation of recombinant MRPs would influence the progression of the infection *in vivo*. In a first set of experiments, we infected BALB/c mice and performed tri-weekly inoculation of MRP neutralizing antibodies for a period of 4 weeks. The progression of footpad thickening and development of lesion was followed over a 10-weeks period. As shown in Figures 7A and 7C, mice that received anti-MRPs antibodies developed a significantly bigger footpad swelling during the first 8 weeks of infection comparatively to the untreated group, thereafter, no significant differences were observed. However, significant differences were detected between treated and control groups regarding the footpad parasitic load (Figure 7B). These data suggest that MRPs secreted in the infectious environment could play an important role in the immunological events controlling leishmaniasis development during the initial weeks of the infection.

To further confirm the contribution of MRPs to the regulation of *Leishmania* infection, we tested whether recombinant MRP 8/14 (rMRP8/14) injected in infected footpads could lead to reduce *Leishmania*-related pathologies in mice. As reported in Figure 8, mice which started to receive inoculation of rMRPs at 8 week post-infection over a 4-weekperiod, showed a clear and significant reduction of their footpad swelling and parasitic load comparatively to the control group (Figures 8A, 8B and 8C). This last set of experiments strongly suggests that MRPs play a significant role in the immunological mechanisms involved in the regulation of *Leishmania* progression. Moreover, these data unveil MRPs as potential therapeutic agents to treat leishmaniasis.

DISCUSSION

MRP 8 and 14 also known as S100A8 and S100A9 belong to the S100 protein family, a large group of intracellular proteins associated with many cellular functions including contraction, motility, cell differentiation, calcium regulation among others [3]. In addition, the S100 proteins are also associated with different inflammatory diseases [27,28,29,30]. Recently, we and others have reported that MRP 8 and 14 can modulate macrophage functions including NO production. Given that MRPs activate the macrophage signaling machinery and knowing that *Leishmania* parasites exert the opposite effect, we were interested in elucidating the role of MRP 8 and 14 during *Leishmania* infection both *in vitro* and *in vivo*.

Our results clearly showed that MRP-primed *Leishmania*-infected murine macrophages were able to produce NO with the concomitant expression of iNOS. These events correlated with a more efficient killing of the parasites as demonstrated by the luciferase assay. NO plays a key role in the macrophage microbicidal functions and is essential for the control of *Leishmania* infection [20]. In addition, we also found that MRP-primed macrophages produced high levels of TNF-α and were able to phosphorylate ERK and JNK kinases. More importantly, we observed that this priming resulted in an increased nuclear translocation of NF-κB and AP-1. This finding correlates with the fact that iNOS contains promoter binding sequences for these two transcription factors along with STAT1α [31].

The induction of MAPK phosphorylation and TFs nuclear translocation was observed very shortly after stimulation; the fact that MRPs are able to induce the NF-κB and the AP-1 pathways suggests that these TFs might act in synergy to enhance the expression of iNOS, resulting in high levels of NO and more efficient *Leishmania* killing. We have also reported that MRPs are recognized by Toll like receptor 4 (TLR4) [9]. This is in line with the observation that NF-κB is strongly induced by MRPs, on the other hand, efficient induction of AP-1 might be due to the fact that ERK and JNK are up-stream activators of c-Jun and c-Fos which dimerize to form active AP-1 complexes [32].

Additionally, we observed that macrophages that were first infected and then stimulated with MRPs, did not have the capacity to respond in the same way as primed

macrophages, since the levels of NO and TNF production as well as the phosphorylation of JNK and ERK and the nuclear translocation of TFs were substantially reduced. This suggests that the parasite is able to abrogate the activation of the macrophage signaling machinery induced by MRPs in order to survive inside the host. One of the main mechanisms adopted by the parasite to subvert the immune response is the rapid activation of host phosphatases [21,22,33], This fact might explain the poor MAPK phosphorylation and TFs nuclear translocation observed in macrophages first infected and then stimulated with MRPs.

Studies made by our group have shown that mouse infection with *Leishmania* parasites in the air-pouch model leads to neutrophil recruitment [24], Here, we demonstrated that MRPs control neutrophil recruitment induced by *Leishmania* or LPS. However, the exact role of neutrophils during cutaneous leishmaniasis is still controversial. For instance Lima et al. [34] showed that there is a massive infiltration of neutrophils soon after skin infection with *L. major*, they investigated in more detail the role of neutrophils in resistant C57BL/6 and susceptible BALB/c mice by depleting neutrophils with specific antibodies. They showed that neutrophil depletion in both susceptible and resistant mice accelerated parasite spreading and caused more severe footpad swelling. These data suggested that neutrophils are of crucial importance in early control of parasite infection. In contrast, a study made by Laskay et al. [35] showed that *Leishmania* uses neutrophils as an evasion strategy, since the parasite survives inside these cells and use them as "Trojan horses" to get access into the macrophages where it will survive and multiply. Some other reports have shown that depletion of neutrophils in BALB/c mice inhibited the IL-4 response and promoted partial resistance [36].

More recently, Peters et al. [37,38] showed that depletion of neutrophils reduces the ability of the parasite to establish productive infections. Furthermore, they reported that the neutrophils are the initial host cell for a substantial fraction of parasites and that there is more control of the infection when the neutrophils are not present. Our data clearly demonstrated that there is neutrophil recruitment at the site of infection in the air-pouch model; moreover, we showed that these neutrophils are able to secrete MRPs and that depletion of these MRPs significantly reduced the amount of recruited neutrophils and consequently MRP secretion. In addition, infection with *L. major* in the footpad of

susceptible BALB/c mice and depletion of MRPs resulted in increased parasite load and footpad swelling. These results strongly suggest that MRPs are important to control *Leishmania* infection. Strengthening this fact, when we treated mice with rMRPs directly in the footpad, we observed a significant reduction in size of lesion and parasite load. A potential mechanism underlying these events could be that injection of MRPs leads to an enhanced neutrophil recruitment, which in turn, can secrete more MRPs creating a positive feedback loop of constant secretion of MRPs, where it is possible that neutrophils are actually containing the progression of the infection, concomitant with the fact that these proteins present by themselves antimicrobial properties [6]. Additionally, we do not rule out the possibility that direct injection of MRPs also induced monocyte recruitment and as observed in our *in vitro* results which showed that MRP-primed macrophages are able to produce high levels of NO, being this responsible for the killing and more efficient control of the infection. However, whether the control of the infection *in vivo* is NO-mediated needs further investigation.

In summary our data showed for the first time that MRP 8 and 14 play an important role in the control of *Leishmania* infection *in vivo* and *in vitro* and support the idea that they could have a potential role as therapeutic drugs.

ACKNOWLEDGEMENTS

This study has been supported by a Canadian Institute of Health Research (CIHR) operating grant to M.O. M.O. is member of the CIHR group on Host-Pathogen Interaction. I.C. is the recipient of a Doctoral Studentship from the Mexican Council of Science and Technology and the Autonomous University of the State of Mexico.

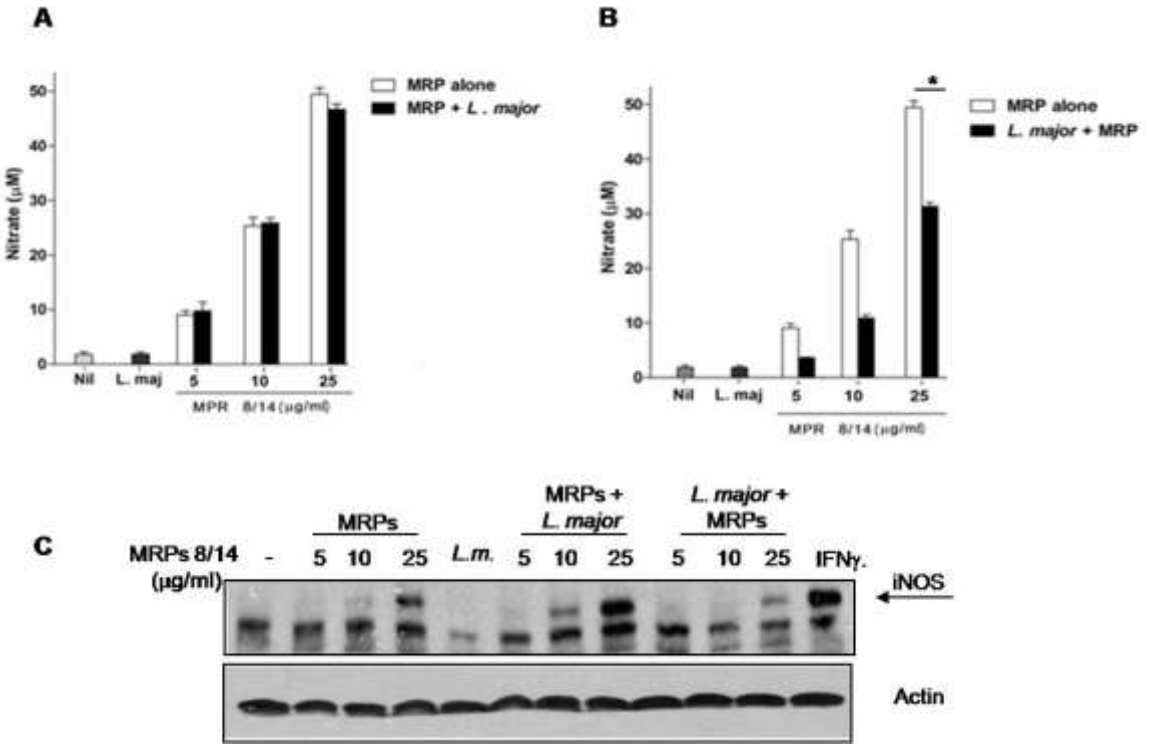

Figure 1: Impact of *Leishmania* infection on the production of nitric oxide (NO) and iNOS expression by MRPs 8/14. B10R macrophages were stimulated with different doses of MPRs 8/14 (5, 10 and 25 μg/ml) and stimulated before **(A)** or after (**B)** 24 hr of infection with *L. major*. NO production was assessed by measuring nitrates in the supernatants. **(C)** B10R macrophages were treated as in (A) and (B) and protein lysates were subjected to immunobloting analysis with anti-iNOS. Equal protein loading is shown by β-actin. (*) denotes $P < 0.05$ between the MRP-stimulated macrophages and *L. major*-MRP-stimulated macrophages. One representative experiment of three is shown.

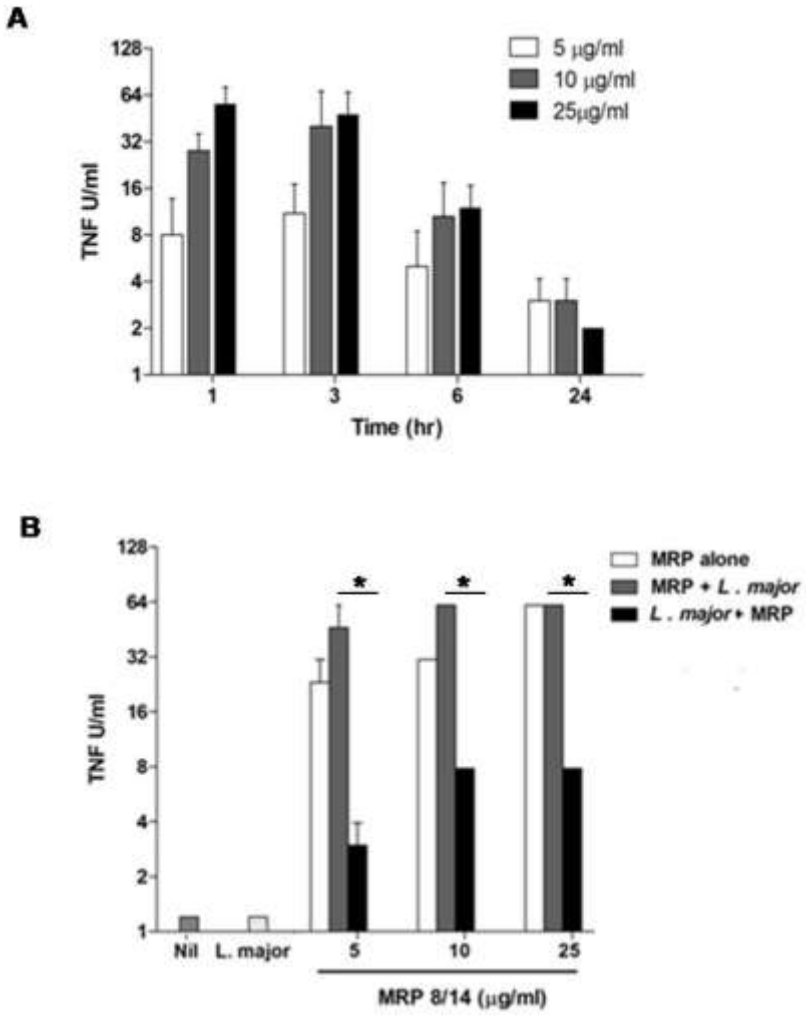

Figure 2: Effect of *Leishmania* infection on TNF-α production by MRPs. (A) B10R macrophages were stimulated with MRPs 8/14 (5, 10, 25 μg/ml) for 1, 3, 6 and 24 hr. TNF-α was measured in supernatants by TNF bioassay. **(B)** B10R macrophages were stimulated with MRPs 1 hr before or after infection with *L. major* and TNF-α was measured as in **(A)**. (*) denotes $P < 0.05$ between groups. One representative experiment of three is shown.

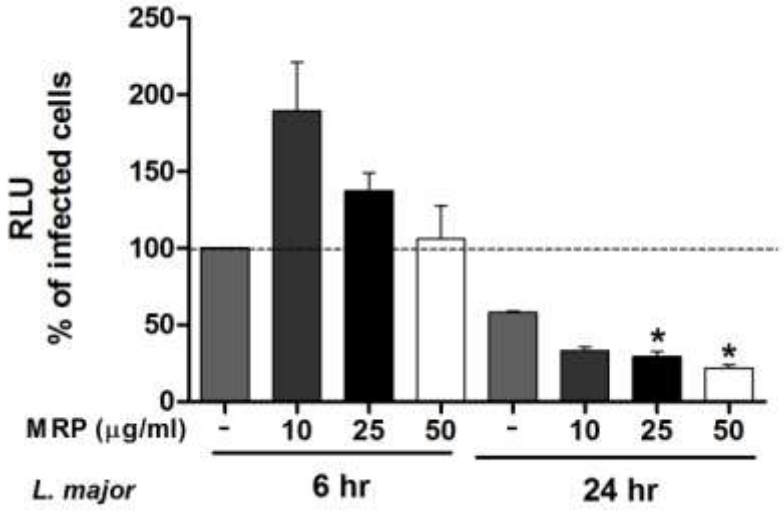

Figure 3: MRPs decrease the parasitic load in macrophages at 24 hr of infection with *L. major*. B10R macrophages were primed with 5, 10 and 25 μg/ml of MRP8/14 for 1 hr, and then infected for 6 hr and 24 hr with *L. major* luciferase. Parasitic load was measured by luciferase assay. The dashed line represents the infected cells at 6 hr without MRP treatment (RLU value =100%). (*) denotes $P < 0.05$ between the untreated 24 hr infected cells and cells primed with MRPs before 24 hr of infection. One representative experiment of three is shown.

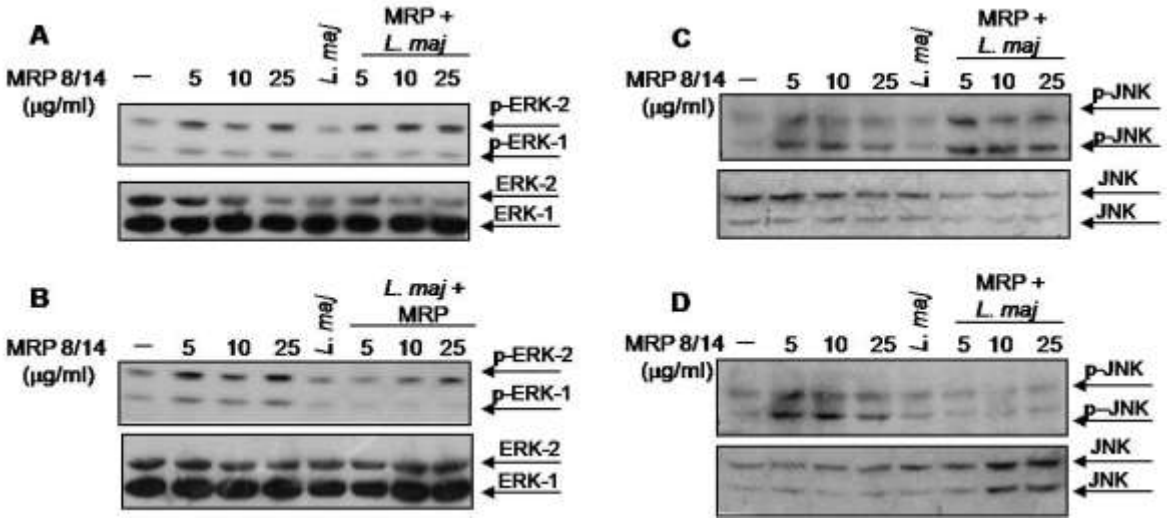

Figure 4: Impact of *Leishmania* infection on MRPs–induced ERK and JNK signaling. **(A)** and **(C)** B10R macrophages were only treated or primed with MRPs 8/14 (5, 10, 25 μg/ml) for 30 min before infection with *L. major*. **(B)** and **(D)** B10R macrophages were only treated or MRP-stimulated 1 hr of infection. Protein lysates were assessed for phosphorylation of ERK 1/2 kinase and JNK kinase by immunobloting. One representative experiment of three is shown.

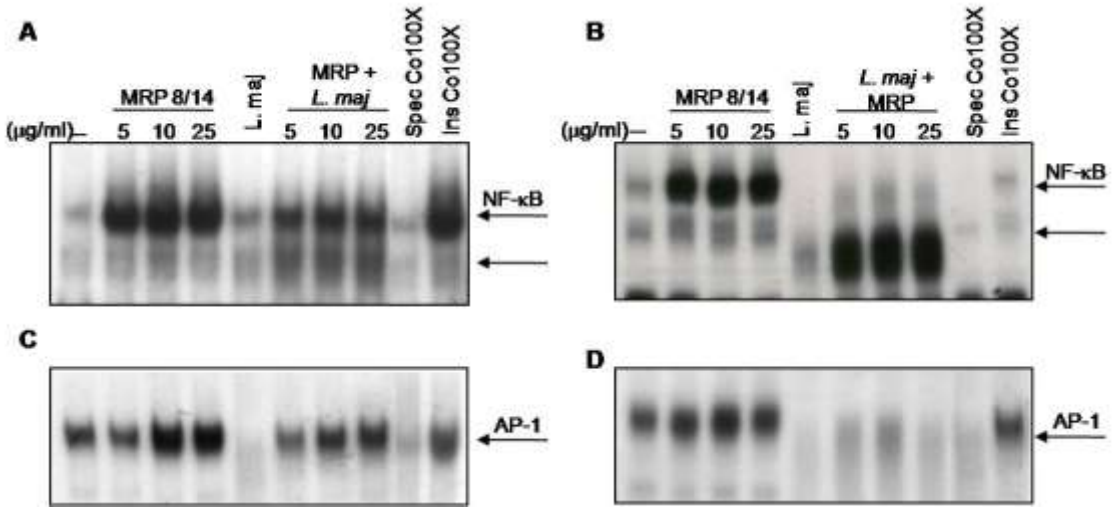

Figure 5: Nuclear translocation and binding of transcription factors (TFs). B10R macrophages were treated with MRPs 8/14 (5, 10, 25 µg/ml) for 1 hr or MRP-stimulated before infection with *L. major* or treated 1 hr with MRPs after ON infection. Nuclear proteins were extracted and subjected to EMSA with NF-κB (p65/p50) **(A)** and **(B)**, and AP-1 **(C)** and **(D)**. Consensus oligonucleotides non-specific competitors (NSCO) and specific competitor (SCO) were used in and 100X molar excess. One representative experiment of three is shown.

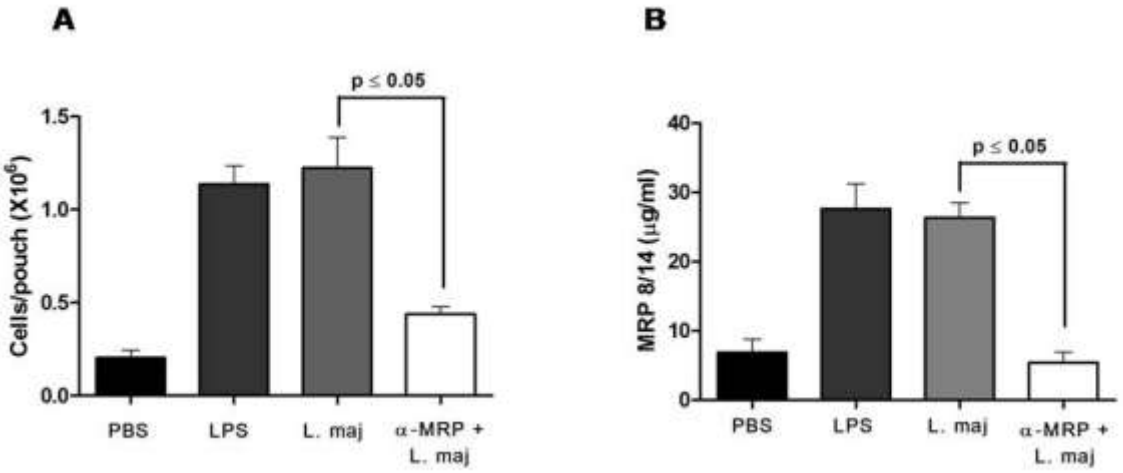

Figure 6: *Leishmania* induces leukocytes recruitment and MRP secretion in the murine air-pouch model. Air-pouches were raised in BALB/c mice during 6 days with sterile air, at day 7 they were stimulated with LPS or infected with *L. major* for 6 hr. Pouches were washed and total cell recruitment **(A)** and MRP secretion by ELISA were measured **(B).** BALB/c mice were treated with α-MRP 16 hr before infection with *L. major* for 6 hr. Total cell recruitment **(A)** and MRP secretion by ELISA **(B)** were measured. (*) denotes $P < 0.05$ between the *L. major* infected group and the *L. major* infected group treated with α-MRPs. Data from three different experiments are shown.

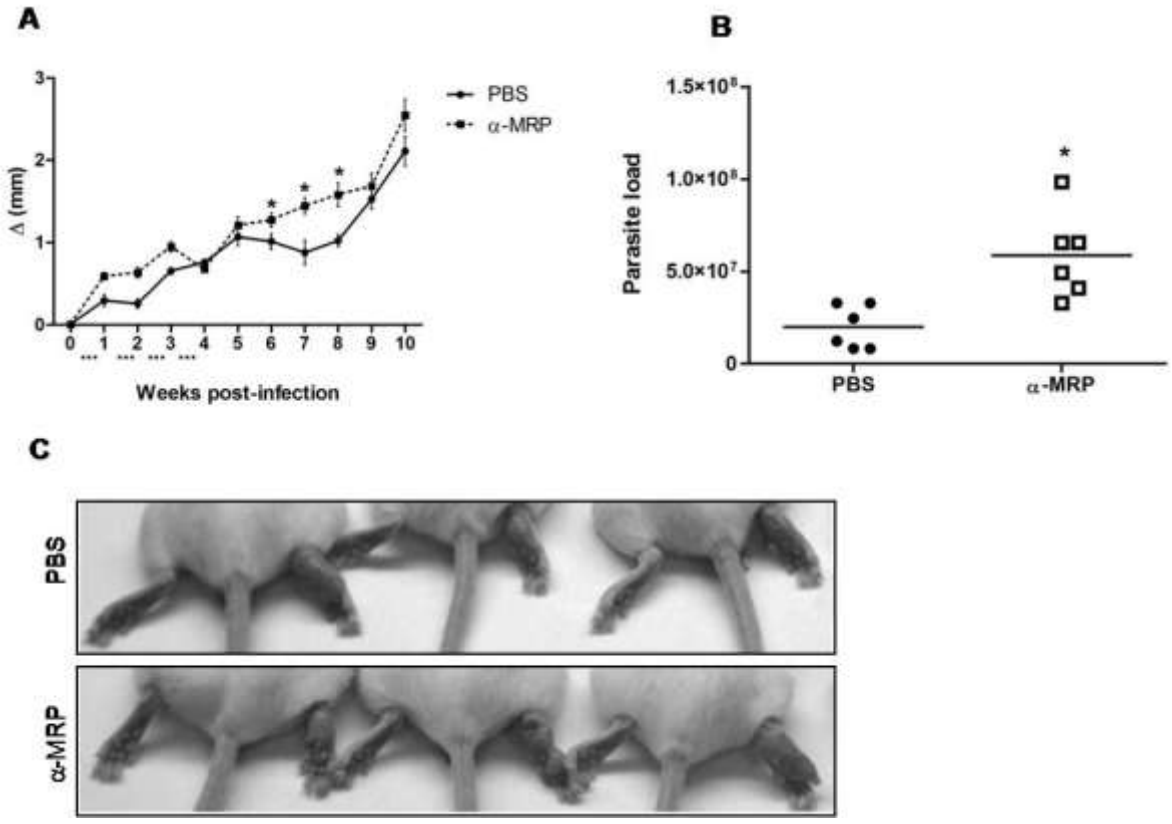

Figure 7: Abrogation of MRPs leads to an increase in lesion size and parasite load in footpads. (A) BALB/c mice were injected with 2 mg of α-MRPs 8/14 one day before infection with *L. major* in the footpad and subsequent inoculation of the antibodies three times/week during four weeks after infection (…). Footpad sizes were measured every week for 10 weeks. (*) indicates significant difference $P < 0.01$ between groups. **(B)** Parasites from footpads were extracted, and the parasitic burden was measured by limiting dilution assay, (*) denotes: $P < 0.05$ between groups. **(C)** Footpads of three representative mice of each group are shown, PBS group is shown in the upper panel and the α-MRP group in the lower panel. One representative experiment is shown.

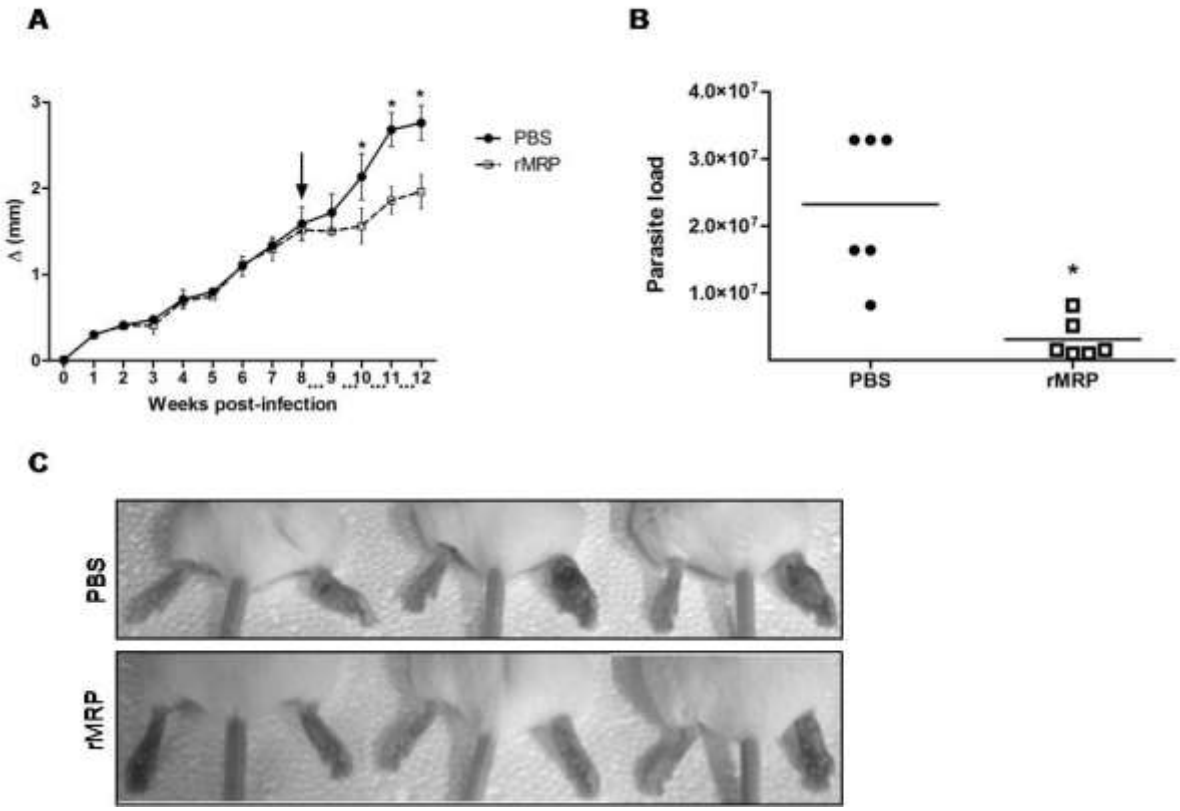

Figure 8: Injection of rMRPs leads to a reduction in lesion size and parasite load in footpads. (A) BALB/c mice were injected with 10 μg of rMRPs 8/14 during 4 weeks (weeks 8 to12) directly in the infected footpad three times per week (…). Footpad sizes were measured every week for 12 weeks. (*) indicates significant difference $P < 0.01$ between groups. **(B)** Parasites from footpads were extracted, and the parasitic burden was measured by limiting dilution assay, (*) denotes $P < 0.05$ between groups. **(C)** Footpads of three representative mice of each group are shown, PBS group is shown in the upper panel and the rMRP group in the lower panel. One representative experiment is shown.

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CHAPTER 3

LEISHMANIA-INDUCED INACTIVATION OF THE MACROPHAGE TRANSCRIPTION FACTOR AP-1 IS MEDIATED BY THE PARASITE METALLOPROTEASE GP63

PREFACE CHAPTER 3

The microbicidal functions of the macrophages (Leishmania's host cell) are of crucial importance in the control of the infection, for instance, and one of the most important microbicidal agents produced by the macrophages is nitric oxide (NO), produced as a sub-product of the degradation of L-arginine to citruline by iNOS which has DNA binding sites for NF-κB, STAT 1 and AP-1. One of the tools that *Leishmania* parasites use to inactivate the innate immune response is the inhibition of nuclear translocation of transcription factors by degradation and cleavage. Studies in our laboratory have revealed that the NF-κB p65 subunit is cleaved by *Leishmania* creating a smaller fragment called p35 which is able to translocate into the nucleus and dimerized with p50 subunit to induce specific chemokine expression that help to the instalment of the parasite inside the macrophage. STAT-1 is also degraded by *Leishmania* using a proteasome-mediated mechanism. Without exception AP-1 is inactivated by *Leishmania* parasites as well; although, the molecular mechanisms by which the parasites inactivate this important transcription factor remain unknown. In this chapter, we show data that demonstrates that the major surface protease of *Leishmania* called GP63 is responsible for the degradation and cleavage of the subunits that form active AP-1 complexes, more importantly we show that GP63 reaches the nucleus where it degrades and cleaves AP-1, rendering it inactive.

LEISHMANIA-INDUCED INACTIVATION OF THE MACROPHAGE TRANSCRIPTION FACTOR AP-1 IS MEDIATED BY THE PARASITE METALLOPROTEASE GP63

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Running Title: AP-1 inactivation by *Leishmania* protease GP63

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ABSTRACT

Leishmania parasites have evolved sophisticated mechanisms to subvert macrophage immune responses by altering the host cell signal transduction machinery, including inhibition of JAK/STAT signalling and other transcription factors such as AP-1, CREB and NF-κB. AP-1 regulates pro-inflammatory cytokines, chemokines and nitric oxide production. Herein, we show that upon *Leishmania* infection, AP-1 activity within host cells is abolished and correlates with lower expression of five of the seven AP-1 subunits. Of interest, c-Jun, the central component of AP-1, is cleaved by *Leishmania*. Furthermore, the cleavage of c-Jun is dependent on the expression and activity of the major *Leishmania* surface protease GP63. Immunoprecipitation of c-Jun from nuclear extracts showed that GP63 interacts, and cleaves c-Jun inside the nucleus shortly after infection. Phagocytosis inhibition by cytochalasin D did not block c-Jun down-regulation, suggesting that internalization of the parasite might not be necessary to deliver GP63 molecules inside the host cell. This observation was corroborated by the maintenance of c-Jun cleavage upon incubation with *L. mexicana* culture supernatant, suggesting that secreted, soluble GP63 could use a phagocytosis-independent mechanism to enter the host cell. In support of this, disruption of macrophage lipid raft domains by Methyl β-Cyclodextrin (MβCD) partially inhibits the degradation of full length c-Jun. Together our results indicate a novel role of the surface protease GP63 in the *Leishmania*-mediated subversion of host AP-1 activity.

INTRODUCTION

Parasites of the *Leishmania* genus are the causative agent of leishmaniasis; a disease distributed worldwide affecting more than 12 million people in 88 countries [1]. Leishmaniasis is a complex of diseases ranging from self-healing cutaneous lesions to lethal visceral afflictions [2]. In its mammalian host, *Leishmania* is an obligate intracellular pathogen infecting hematopoietic cells of the monocyte/macrophage lineage. Macrophages are specialized for the destruction of invading pathogens and priming the immune response. In order to survive within these cells, *Leishmania* has evolved sophisticated mechanisms to subvert macrophage microbicidal functions such as inhibition of nitric oxide (NO) production and cytokine-inducible macrophage functions [3]. This occurs as the direct consequence of parasite-mediated activation of protein tyrosine phosphatases, alteration of signal transduction and inhibition of nuclear translocation and activity of transcription factors such as NF-κB, STAT, CREB and AP-1 [4,5]. Activated Protein-1 (AP-1) is an important transcription factor that mediates gene regulation in response to physiological and pathological stimuli, including cytokines, growth factors, stress signals, bacterial and viral infections, apoptosis, as well oncogenic responses [6,7]. AP-1 is formed by homodimers of Jun family members (c-Jun, Jun B and Jun D), or heterodimers of Jun and Fos family members (c-Fos, Fos B, Fra 1 and Fra 2). Homodimers within the Fos family do not occur due to conformational repulsion [8].

Previous studies have reported that the AP-1 transcription factor is inactivated by *Leishmania* infection. For instance, activation of macrophage AP-1 and NF-κB is inhibited by *L. donovani* promastigotes through an increase in intracellular ceramide concentration, which leads to the down-regulation of classical PKC activity, up-regulation of calcium independent atypical PKC-ζ and dephosphorylation of Extracellular Signal-Regulated Kinases (ERK) [9,10]. Other studies have shown that *Leishmania* alters signal transduction upstream of c-Fos and c-Jun by inhibiting ERK, JNK and p38 MAP Kinases, resulting in a reduction of AP-1 nuclear translocation [11,12]. However, little is known about the molecular mechanism (s) by which *Leishmania* parasites are able to inactivate this important transcription factor.

Many *Leishmania*-specific factors such as lipophosphoglycan (LPG), A2 proteins, cysteine peptidases (CPs) and the protease GP63, contribute to *Leishmania* virulence and pathogenicity. LPG has been implicated in altering phagosome maturation in *L. donovani* infection [13].The A2 proteins of *L. donovani* are involved in intracellular amastigote survival [14].The cysteine peptidases of *L. mexicana* are implicated in facilitating the survival and growth of the parasite [15]. Furthermore GP63, also known as the major surface protease (MSP), has been related to resistance to complement-mediated lysis, among others [16,17]. GP63 is a metalloprotease which belongs to the metzincin class. It is the most abundant surface glycoprotein of the parasite and accounts for 1% of the total protein content of *L. mexicana* promastigotes [18]. GP63 of different *Leishmania* species encode similar amino acid sequences, although slight substrate specificity variations have been reported [19]. Specific characteristics of this class of metalloproteases include a conserved signature motif HEXXHXXGXXH and an N-terminal pro-peptide that serves to maintain the pro-enzyme inactive during translation, which is removed upon protein maturation and activation [20]. The mature GP63 contains 3 domains: 1) N-terminal (bases ~101-273) which comprises a structure corresponding to the catalytic module of metzincin class zinc protease, 2) central domain (bases ~274-391) and 3) C-terminal domain containing the site of glycosylphosphatidylinositol (GPI) anchor addition (bases ~392-577) [17,18,20,21]. We have previously shown that this protease actively participates in the cleavage of NF-κB [4], protein tyrosine phosphatases (PTP) [22] and actin cytoskeleton regulators [23]. In this study we have investigated how GP63 contributes to the inactivation of AP-1 and the degradation of its subunits. Herein, we report that GP63 enters the host cell independently of parasite internalization, and for the first time show that it is able to reach the nucleus membrane shortly after infection where it degrades and cleaves c-Jun and other AP-1 subunits.

MATERIALS AND METHODS

Cell culture, macrophage infection and reagents

Immortalised murine bone marrow derived macrophages B10R cell line were maintained at 37° C in 5 % CO_2 in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 % heat inactivated FBS (Invitrogen, Burlington, ON, Canada) and 100 U/ml penicillin 100 μg/ml streptomycin and 2 mM of L-glutamine (Wisent, St-Bruno, QC, Canada). *Leishmania* promastigotes (L. *donovani infantum*, *L. donovani* 1S2D, *L. donovani* R2D (LPG $^{-/-}$), *L. mexicana*, *L. major* A2 (WT), *L. major* GP63 $^{-/-}$, *L. major* GP63 Rescued [24] and *L. tarentolae*) were grown and maintained at 25° C in SDM-79 culture medium supplemented with 10 % FBS by bi-weekly passage. Macrophages were infected at parasite to macrophage ratio 20:1 with stationary phase promastigotes for the times specified in each figure legend. Using this ratio of infection we normally observe around 30 % and 60 % of infected cells in 1 and 2 hr, respectively. When chemical inhibitors were used, 2 μM cytochalasin D (Sigma-Aldrich, St-Louis MO, USA) and 20 mM Methylβ-cyclodextrin (MβCD) (Sigma-Aldrich, St-Louis MO, USA), cells were treated 1 hour prior to infection and the inhibitor remained throughout the infection time.

Electrophoresis Mobility Shift Assay (EMSA) and supershift assays

B10R macrophages (2 x 10^6) were infected, washed three times with Phosphate Buffered Saline (PBS) to remove non-internalized parasites, and processed for nuclear extraction as previously described [5,25]. Briefly, macrophages were collected in 1 ml of cold PBS, centrifuged and pellets were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM of PMSF) and incubated 15 min on ice. Twenty five μl of IGEPAL 10 % (Sigma-Aldrich, St-Louis MO, USA) were added, and samples vortexed for 30 sec. Nuclear proteins were pelleted by centrifugation and resuspended in 50 μl of cold buffer C (20 mM HEPES, 400 mM NaCl 1 mM EDTA, 1 mM EGTA 1 mM DTT and 0.5 mM PMSF).

Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules CA, USA). 6 μg of nuclear proteins were incubated for 20 min at room temperature with 1 μl of binding buffer (100 nM Hepes pH 7.9, 8 % v/v glycerol, 1 % w/v Ficoll, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, 25 mM NaCl, and 1 μg/μl BSA) and 200 ng/μl of poly (dI-dC), 0.02% bromophenol blue and 1 μl of γ-P^{32}labeled oligonucleotide containing a consensus sequence for AP-1 binding complexes (5'-CGTTTGATGACTCAGCCGGAA-3') (Santa Cruz Biotechnology Inc, Sta Cruz CA, USA). After incubation, DNA-protein complexes were resolved by electrophoresis in non-denaturing polyacrylamide gel 5 % (w/v). Subsequently gels were dried and autoradiographed. Competition assays were conducted by adding a 100-fold molar excess of homologous unlabeled AP-1 oligonucleotide, or the non-specific competitor sequence for SP-1 binding (5'-ATTCGAATCGGGGCGGGGCGAGC-3').

For supershift assay, 2 μg of nuclear protein extract were incubated for 1hr at room temperature with binding buffer, poly (dI-dC), 0.02 % bromophenol blue, labeled oligonucleotide and 4 μg of individual specific antibodies (α-c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra 1 or Fra 2; Santa Cruz Biotech Inc, Sta Cruz CA, USA). Complexes were resolved on standard non-denaturing polyacrilamide gel 5 % (w/v).

Western blot

Infected and non infected cells (1 x 10^6) were washed 3 times with PBS and lysed with cold buffer (50 mM Tris (pH 7), 0.1 mM, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 1 % NP-40, 40 μg/ml aproptinin and 20 μg/ml of leupeptin). Proteins were dosed by Bradford (Bio-Rad, Hercules CA, USA), and 30 μg of proteins were separated by SDS-PAGE, and transferred onto PVDF membranes (GE healthcare, Piskataway NJ, USA). Membranes were blocked in 5 % non-fat dry milk, washed and incubated for 1 hr with α-c-Jun (BD Biosciences, San Jose, CA, USA), α-Jun B, α-Jun D, α-c-Fos, α-Fos B, α-Fra 1 and α-Fra 2 (Santa Cruz Biotech Inc. Sta Cruz CA, USA). After washing, membranes were incubated 1 hr with α-mouse or α-rabbit HRP-conjugated antibody (GE healthcare, Piskataway NJ, USA), and developed by autoradiography.

Immunoprecipitation

B10R macrophages (10 x 10^6) were infected with either *L. major* A2, or *L. major* GP63 $^{-/-}$ or *L. major* GP63 Rescued for 1 hr, and nuclear proteins were extracted as previously described in [4]. c-Jun was immunoprecipitated from pre-cleared nuclear extracts with 2 μg antibody, followed by addition of 12.5 μl (packed volume) of protein A/G PLUS agarose beads (Santa Cruz Biotech Inc. Sta Cruz CA USA). Beads were washed three times and bound proteins were analyzed by WB as described above.

Confocal microscopy

B10R macrophages (0.5 x 10^6) were plated ON in glass cover slips. After infection for 30, 60 and 180 min cells were gently washed 3 times with PBS, and then fixed with 4 % p-formaldehyde for 30 min at 4° C. Slides were permeabilized for 5 minutes with PBS containing 1 % BSA and 0.05 % NP-40 and blocked with 5 % non-fat dry milk for 1 hr. Incubation with primary antibody α-c Jun or α-GP63 mouse monoclonal antibody clone # 96 [26] was conducted in humid dark chamber for 1 hr at room temperature. After three washes with PBS, cells were incubated with secondary antibody (Alexa Fluor 488 or 594, from Molecular probes, Burlington ON, Canada) for 1 hr. Nuclei were stained with DAPI or TOPRO for 10 min and slides were mounted in permaflour medium (Thermo Co. Waltham MA, USA). Images were taken using an Olympus FV1000 confocal microscope and a Zeiss LCS 500.

RESULTS

Alteration of AP-1 activity upon *Leishmania* infection involves down-regulation/degradation of selected Jun/Fos family members

We have previously studied the effect of *Leishmania* promastigote infection on the activity of various macrophage transcription factors: STAT-1α degradation is proteasome and receptor-dependent and is mediated through a mechanism involving PKC-α [5], and cleavage of NF-κB subunits upon *Leishmania* infection is in part dependent on GP63 [4]. As AP-1 is an important transcription factor regulating the expression of many genes involved in the activation of macrophage functions (*TNFα, iNOS,* and *IL-12*) [27,28,29] critical for the adequate innate immune response against *Leishmania* infection, we investigated the mechanisms underlying AP-1 inactivation upon *Leishmania* infection.

To evaluate nuclear translocation and DNA binding activity of macrophage AP-1 upon infection with *Leishmania* promastigotes, Electrophoretic Mobility Shift Assays (EMSA) were performed. As shown in Figure 1A, AP-1 nuclear translocation was inhibited as early as 30 min post-infection in *L. donovani*-infected macrophages. Furthermore, we observed that other mammalian pathogenic *Leishmania* species (*L. mexicana* and *L. major*) were able to alter AP-1 DNA binding (Figure 1B). Of interest, we did not observe any effect on macrophages infected with *L. tarentolae,* whose pathogenicity is limited to reptilian hosts.

In order to better understand the observed decrease in AP-1 activity, we performed Western Blot (WB) analysis in the total cell extracts to evaluate the various AP-1 subunits during infection. Five out of the seven AP-1 subunits (c-Fos, Fra 1, Fra 2, c-Jun and Jun B) showed decreased expression after infection with *L. donovani,* whereas Jun D presented a slight reduction and Fos B was maintained intact (Figure 2A). To further confirm the presence of these subunits in the AP-1 complex we used super shift analysis. This approach uses the incorporation of specific antibodies to nuclear protein extracts, allowing the visualization of the antibody: protein: DNA complexes by retarding the migration of the specific bands in the gel. As shown in Figure 2B, inclusion of

antibodies specific for Fos B, c-Fos, Fra 1, Fra 2, c-Jun, Jun B and Jun D, demonstrated presence of Fra 1, Fra 2, c-Jun, Jun B and Jun D, but not c-Fos or Fos B, within the macrophage nuclear AP-1 complex. However, upon infection with *L. donovani*, this effect is substantially reduced, demonstrating that Fra-1, Fra-2, c-Jun, Jun B and Jun D subunits are less present within the AP-1-DNA complex after infection. c-Fos was not detected by super shift assay; however, this protein is still affected by *Leishmania* infection since we observed degradation by WB (see Figures 2A and 3), suggesting that the amount of c-Fos might not be enough to be detected by super shift assay.

After phosphorylation AP-1 subunits are translocated into the nucleus where they dimerize with another subunit to form an active AP-1 complex [6,7,8,30]. To determine the level of expression of each AP-1 subunit in the different cellular compartments (cytoplasm vs nucleus), we performed WB analysis on separated nuclear and cytoplasmic fractions. As shown in Figure 3, different phenomena can be observed. c-Fos and Fra-1 expression in the cytoplasmic fraction are not altered with *Leishmania* infection, but their expression in the nuclear fraction is decreased in infected macrophages; Fra-2 and c-Jun have decreased expression in both cytoplasmic and nuclear fractions, and Fra-2 in the nuclear fraction presents a band with less migration than the band observed in the cytoplasmic fractions, possible due to post-nuclear translocation modifications. On the other hand, Jun-B and Jun-D were detected only in the nuclear fraction; however, only Jun-B expression is affected by *Leishmania* infection. The lower expression of the different subunits in the nucleus could be due to decreased complex formation and/or cleavage and further degradation of the subunits, as it is possible to detect smaller bands (c-Jun and Jun-B).

***Leishmania* major surface protease GP63 is involved in AP-1 inactivation.**

Leishmania surface molecules such as LPG and GP63, among others, play important roles as virulence factors and modulators of host cell signalling. LPG, for instance, has been implicated in the interference of phagolysosome maturation and inactivation of PKC signalling [13,31]. GP63 has been related to resistance to complement-mediated lysis, migration of *Leishmania* parasites through the extracellular

matrix by degradation of casein, fibrinogen and collagen [16,21] and inhibition of JAK/STAT signalling by modulation of PTP activities [22]. To address the role of LPG and GP63 in AP-1 inactivation we performed EMSA with extracts from cells infected with *Leishmania* mutants for these two surface molecules. As shown in Figure 4A, LPG is not involved in the AP-1 degradation induced by *Leishmania* infection since DNA binding in macrophages infected with either *L. donovani* or *L. donovani* LPG$^{-/-}$ promastigotes was similarly altered. Importantly, however, we observed that cells infected with an *L. major* strain lacking GP63 (*L. major* GP63 $^{-/-}$) [24] showed normal AP-1 DNA binding capacity, compared to uninfected controls. This suggests that GP63 but not LPG is highly involved in the mechanism responsible for the inactivation of AP-1 transcription factor.

To further elucidate the role of GP63, we performed WB analysis of all the AP-1 subunits of macrophages infected with *L. major*, *L. major* GP63$^{-/-}$ and *L. major* GP63 Rescued. Results obtained further revealed that in the absence of GP63, no degradation or cleavage of any AP-1 subunit was evident (Figure 4B); supporting the finding that AP-1 activity is unaffected in *L. major* GP63$^{-/-}$-infected macrophages.

GP63 action is not dependent on parasite internalization

Leishmania GP63 can be found in three different forms: 1) Intracellular GP63, 2) Surface GPI-anchored GP63 and 3) secreted or released GP63 [21,32]. For GP63 to target its intracellular macrophage substrates, it needs to gain access to or be internalized by the macrophage. To explore whether the internalization of the parasite is necessary to deliver GP63 inside the cell, murine macrophages were pre-treated with the phagocytosis inhibitor cytochalasin D which inhibits actin polymerization, therefore, blocking internalization by phagocytosis (Supplemental Figure 1A). We used c-Jun as a model protein to evaluate the cleavage and degradation of the AP-1 subunits. WB analysis showed that parasite phagocytosis was not necessary for c-Jun cleavage and degradation (Figure 5A). To confirm this, we incubated macrophages with the culture supernatant of *L. mexicana* promastigotes, which is rich in soluble GP63 [18,33]. WB showed that even in the absence of the parasite, c-Jun degradation was observed (Figure 5B).

Since phagocytosis seems not to be required in the internalization of GP63. We addressed whether GP63 internalization could be dependent on lipid raft-mediated endocytosis, given the fact that GP63 is an excreted and membrane-GPI anchored protein. On the other hand, lipid raft domains are highly dynamic membrane domains rich in cholesterol and sphingolipids, and present high affinity for proteins containing GPI anchors [34,35,36]. In order to examine the possible role of host lipid raft microdomains in GP63 internalization, we pre-treated cells with a non-cytotoxic dose (Supplemental Figure 1B) of the cholesterol chelator and inhibitor of lipid raft integrity methyl-β-clyclodextrin (MβCD) prior to infection. As shown in Figure 5C, full length c-Jun was not degraded in cells infected under these conditions, although interestingly, a cleavage fragment was still observed. Pre-treatment of macrophages with MβCD and subsequent incubation with *L. mexicana* supernatant showed that lipid raft disruption altered internalization of parasite-free soluble GP63 and also impaired c-Jun degradation (Figure 5D). As shown in Supplemental Figure 2, confocal microscopy confirmed an interaction between lipid raft domains and GP63, since in macrophages infected with *L. major*, GP63 (green) partially co-localized with the lipid raft marker Choleratoxin B (red). Furthermore, we have previously shown that, pre-treatment with MβCD before infection abrogates GP63 internalization [22]. To determine if MβCD had any effect over the c-Jun expression we performed a time course analysis of macrophages stimulated with MβCD. As shown in supplemental figure 3A, there was no alteration in the expression of c-Jun after 2 hr of incubation of the macrophages with the drug. Together these data strengthen the hypothesis that GP63 uses lipid raft domains for internalization independent of parasite entry.

To evaluate the role of the GPI anchor in mediating GP63 internalization via lipid raft domains, macrophages were incubated with a GPI-deficient recombinant GP63 (rGP63) and c-Jun degradation was monitored. WB analysis evidenced that neither degradation nor cleavage of c-Jun occurred (Figure 5E) in the presence or absence of MβCD, similarly to what we have previously shown for GP63-mediated PTP cleavage.

Moreover, although rGP63 is still internalized in macrophages to a limited extent, peri-nuclear localization was never detected [22].

To demonstrate that the degradation and cleavage observed in this set of experiments were occurring inside the cells and not as an effect of proteolysis during the preparation of the lysates, we included two experiments as controls; first, we lysed the cells using sample loading buffer 1X and the samples were boiled right after, to stop the proteolysis (Supplemental figure 3B); second, we added 0.5 M of phentrolin (a Zn chelator) to the lysis buffer to abrogate post-infection GP63 activity (Supplemental figure 3C). In both experiments, we observed that cleavage of c-Jun under these conditions still occurs, suggesting that the cleavage of c-Jun occurs inside the cell and not during the sample preparation.

GP63-mediated c-Jun cleavage occurs within the nucleus

One of the most surprising elements of the evidence presented above was the fact that GP63 is able to act on its substrate proteins within the nucleus of its host cell. In order to further demonstrate that GP63 reaches the nucleus, we separated cytoplasmic and nuclear proteins from macrophages infected with *L. major*, *L. major* GP63$^{-/-}$ and *L. major* GP63 Rescued. WB analysis using an anti-GP63 antibody revealed that this protease is present in both fractions of *Leishmania*-infected cell extracts. As expected, there was no GP63 in macrophages infected with *L. major* GP63 $^{-/-}$ or the uninfected control (Figure 6A). Confocal microscopy of *Leishmania*-infected macrophages confirmed that GP63 reaches the nuclear membrane as early as 1 hr post-infection (Figure 6B). In order to demonstrate the purity of our fractions, we performed WB of the cytoplasmic and nuclear proteins against the lysosomal marker LAMP-1, the ER specific protein KDEL, histone 2B (nuclear marker), and actin (cytoplasm marker). Supplemental figure 4 shows that actin, LAMP-1 and KDEL are only present in the cytoplasmic fraction, in contrast, histone is only detected in the nuclear fraction, and this way we are confident to say that GP63 was present in both protein fractions.

In order to confirm nuclear interaction of GP63 with c-Jun we performed a Co-Immunoprecipitation (IP) assay. c-Jun was immunoprecipitated from nuclear extracts of

Leishmania-infected macrophages and subjected to WB analysis of GP63. This result revealed a band around 65 kDa, confirming the interaction between nuclear c-Jun and GP63 in the macrophages infected with *L. major* and *L. major* GP63 Rescue, but not with *L. major* GP63 $^{-/-}$ as is shown in Figure 7A. To further demonstrate that degradation of c-Jun occurs inside the nucleus we performed confocal microscopy. As shown in Figure 7B (upper panel), c-Jun (red) is localized inside the nucleus in uninfected cells. However, after 1 hr of infection GP63 was detected in the nuclear periphery and the fluorescence intensity of c-Jun was considerably diminished (Figure 7B upper panel), such reduction in the fluorescence was not observed in macrophages infected either with *L. major* GP63$^{-/-}$ or *L. tarentolae* (Supplemental figure 5A). The lower panel of figure 7B shows partial co-localization between the nuclear stain (blue) and GP63 (green) in the periphery of the nucleus, giving a yellow signal. On the other hand, this panel also shows that c-Jun (red) lost its co-localization with the nuclear stain in the nuclear periphery where GP63 is present. To discard possible unspecific signals in the confocal micrographs we included specific isotype and secondary antibody controls (Supplemental figure 5B). Collectively, we showed that GP63 reaches the nucleus shortly after macrophage-parasite contact where it degrades and cleaves various members of AP-1 subunits, leading to its inability to dimerize and bind DNA and therefore, altering AP-1 transcriptional activity on genes under its regulation.

GP63 cleaves c-Jun in its DNA binding motif

To further understand the direct effect of GP63 on c-Jun, we used a purified GST tagged-c-Jun protein and incubated it with *Leishmania* promastigotes of different species (including *L. donovani*, *L. mexicana*, *L. major*, *L. major* GP63$^{-/-}$ and *L. major* GP63 Rescued). WB analysis showed that direct contact of parasites expressing GP63 and c-Jun protein is sufficient to induce c-Jun degradation (Figure 8A). This was corroborated by the reduction of c-Jun degradation when incubated with the GP63$^{-/-}$ strain. Supplemental figure 6 shows that *L. tarentolae* has no effect on the degradation of GST-c-Jun.

GP63 recognizes a four amino acid motif in its target protein substrates based on amino acid characteristics: polar/hydrophobic/basic/basic amino acids (P_1- P'_1-P'_2-P'_3)

[19]. Sequence analysis of AP-1 subunits revealed putative cleavage sites within c-Jun, Jun B, Jun D and c-Fos (Figure 8B). Of interest, one of the sequence-identified cleavage sites of c-Jun was found between the leucine zipper and the DNA binding domain as shown in Figure 8C. In addition, this motif is found at amino acids 271-275, which will generate cleaved fragments with molecular weight similar to the one detected by WB of lysates from infected cells (~30 kDa).

Collectively, these data indicate that the *Leishmania* protease GP63 actively participates in altering the DNA binding capacity of AP-1 as a consequence of the degradation and cleavage of its subunits. These data further corroborate a mechanism whereby GP63 can enter the cell independently of parasite internalization, and show for the first time that GP63 reaches the nucleus where it proteolytically degrades AP-1 subunits.

DISCUSSION

Leishmania parasites have evolved many mechanisms to undermine macrophage signalling pathways in order to survive and replicate inside these cells. For instance, parasite-mediated activation of macrophage PTPs leads to protein dephosphorylation resulting in the inactivation of transcription factors controlling the expression of many genes required for the effective activation of the innate immune response [37], and macrophage effector functions such as NO production. [38]. We have previously reported that *Leishmania* promastigote infection induces degradation and inactivation of some transcription factors. For example, STAT 1 is inactivated by a proteasome mediated mechanism [5], and NF-kB activity is altered in a cleavage-dependent fashion [4]. We show that cleavage of p65 generates an active fragment, p35, which is able to translocate into the nucleus, where it dimerizes with p50 to induce specific chemokine gene expression. Interestingly this cleavage event was found to occur in the macrophage cytoplasm in a GP63-dependent mechanism [4].

Along with STAT and NF-κB, AP-1 is responsible for the transcription of iNOS [39]. NO is a by-product of iNOS-mediated conversion of L-arginine to L-citruline and is essential for the control of *Leishmania* infection [3,38]. Among other genes regulated by AP-1 in macrophages important for controlling the infection are TNFα, IL-1β and IL-12 [27,28,29]. The breadth and importance of the immunological functions of AP-1 highlights how detrimental its degradation is to host defence against *Leishmania* infection. Previously, Descoteaux and Matlashewski (1989) demonstrated that the *c-fos* gene, one of the main activators of AP-1, was down-regulated due to abnormal PKC signalling [40]. More recently Ghosh and colleagues (2002) reported *Leishmania*-dependent inactivation of both AP-1 and NF-κB in a ceramide dependent mechanism, where increased levels of intracellular ceramide conducted to the down-regulation of classical PKC activity and impartment of the phosphorylation of ERK, which results in decreased AP-1 activation [10]. These previous reports have given some indication of AP-1 inactivation by *Leishmania*. Here we further demonstrated the molecular mechanisms involved in the AP-1 inactivation by *Leishmania* parasites.

We have found that infection with several *Leishmania* species alters the DNA binding capacity of AP-1. In particular, we have shown that the parasite metalloprotease GP63 is responsible for this DNA binding alteration and is able to induce the degradation/down-regulation and cleavage of c-Jun, the central component of the AP-1 transcription factor [41], as well of other components including c-Fos, Fra-1, Fra-2 and Jun B. We provide evidence that GP63 exerts its effect by its internalization into the host cell, in a mechanism that is independent of parasite internalization, and induces AP-1 proteolysis within the nucleus or in the nuclear membrane.

The present study corroborates along with previous studies (Ghosh *et. al*) that AP-1 is down-regulated by *Leishmania* parasites. Alteration of AP-1 activity varies according to the pathogen, for instance it has been shown, that the hepatitis C virus alters MAP kinases and AP-1 to accelerate the cell cycle progression, helping the development of hepatocellular carcinoma and HCV development [42]. Another example is the Edema toxin produced by *Bacillus anthracis*; this toxin is able to up-regulate macrophage gene expression, among them genes that are known to be involved in inflammatory responses, regulation of apoptosis, adhesion, immune cell activation, and transcription regulation. Interestingly this up-regulation was found to correlate with induced activation of AP-1 and CAAAT/enhancer-binding protein-beta [43]. In contrast with these reports where different pathogens up-regulate AP-1 to survive inside the host cell, herein we have shown how this transcription factor is down-regulated after *Leishmania* infection in a cleavage-dependent manner. Whether AP-1 down-regulation is a general mechanism used by different intracellular protozoa requires further investigation.

The AP-1 transcription factor is formed by dimers of Jun and Fos family members. In addition, the Jun proteins can dimerize with other proteins that share the leucine zipper region such as ATF-1 and ATF-2 [8,30]. Although we did not test other non-classical AP-1 subunits, we demonstrated that at least 5 of the classical subunits belonging the Jun and Fos families are degraded by the parasite within 1 hr of infection. Of interest, c-Jun subunit, one of the main activators of AP-1 along with c-Fos, is cleaved generating a GP63-mediated 30 kDa fragment. The cleaved product would be unable to dimerize and bind DNA, as it has been demonstrated that truncated c-Jun deprived of

either the leucine zipper or the DNA binding domain results in only marginal AP-1 transactivation [41,44,45]. The generation of c-Jun fragment by GP63 can explain the lower AP-1 binding activity observed in the EMSA experiment. Furthermore, Fos B, which is not cleaved or degraded and also apparently absent in AP-1 complexes (Figure 2B), lacks putative GP63 cleavage sites. Surprisingly Jun D presents two putative sites of cleavage by GP63. However, we did not detect either complete degradation/down-regulation or cleavage products. One possible explanation is that the structural conformation of this protein renders these sites unavailable for GP63-Jun D interaction.

GP63 is known to interact with various substrates. For instance we have recently reported that *Leishmania* GP63 impacts the stability of cortactin and caspase-3, and negatively regulates p38 kinase activity [23]. Furthermore, we have shown that GP63 cleaves host PTPs resulting in enzymatic activation and leading to JAK 2 dephosphorylation, and inhibition of NO production in IFN-γ primed and infected macrophages [22]. Our current study further supports the important role of GP63 as a negative regulator of host cell functions, actively participating in the pleiotropic effects excreted by *Leishmania* parasite to suppress the immune response, our results showed that internalization of GP63 by cells from innate immune response is independent of parasite internalization, as our data revealed GP63 proteolytic activity was not affected by inhibition of phagocytosis, but clearly abolished by a lipid raft disruptor, strongly suggesting that lipid rafts domains are important for internalization of GP63. Proteins that have a GPI anchor have affinity for lipid rafts, and it has been reported that these rafts recognize these GPI anchors allowing the entrance of GPI-bearing proteins in endocytic vesicles [35,36]. In addition, Brittingham and collaborators showed interaction of GP63 with the fibronectin receptor (α4β1), that also translocate into the lipid rafts domains [46], suggesting that GP63 could have two different ways to get access into the cell: 1) GPI-anchor (native and excreted) and 2) receptor mediated (RGP63). Additionally, we have shown that the GPI anchor is important for the internalization of GP63 since recombinant GP63 (rGP63) lacking the GPI anchor is less internalized [22]. Most importantly, GPI anchor seems to be required for the cellular localization of GP63 since rGP63 is localized inside intracellular compartments whereas GPI-GP63 (native protein)

is found within nuclear membrane (see Figure 6). Despite this evidence we have not excluded the possibility that GP63 could use other mechanisms to enter the cells, such as micropinocytosis or classical endocytosis pathways.

One of the main finding of this research is the macrophage nuclear localization of GP63. One plausible mechanism is by the recognition of its GPI domain by the recently described lipid microdomains rich in cholesterol and sphingolipids in the nuclear membrane [47]. Another possible mechanism for the internalization of GP63 inside the nucleus is the presence of a nuclear localization signal (NLS)-like motif (Supplemental Figure 7) in the GP63 sequence. Nuclear proteins are usually transported inside the nucleus by recognition of a NLS, which usually consist of short chains of basic amino acids with the signature motif K-K/R-X-K/R [48]. These NSLs are recognized by the adaptor molecule importin α, which forms a hetorodimer with the transporter receptor importin β. The importin α/β-NSL-cargo complex is then translocated through the nuclear pore complex [48,49]. The exact mechanisms of how the nuclear translocation of GP63 occurs are currently under investigation.

In summary, GP63 seems to preferentially target AP-1 subunits within the nuclear membrane, altering its DNA binding capacity. Given the critical role of this transcription factor in the transcription of several genes involved in the innate immune response, alterations in AP-1 activity can dramatically contribute to the down-regulation of innate immune functions observed during the early stages of *Leishmania* infection. Therefore, this novel mechanism of evasion by *Leishmania* further demonstrates the complex negative regulatory mechanisms developed by the parasite, which has permitted its adaptation to the harsh intracellular environment leading to its survival and propagation within its mammalian host.

ACKNOWLEDGEMENTS

This study has been supported by a Canadian Institute of Health Research (CIHR) operating grant to M.O. M.O. is member of the CIHR group on Host-Pathogen Interaction. I.C. is the recipient of a Doctoral Studentship from the Mexican Council of Science and Technology and the Autonomous University of the State of Mexico. We thank Jacythe Laliberte, McGill University, for helping with confocal microscopy imaging, Benjamin Ralph, for helping with the sequence analysis of the AP-1 subunits.

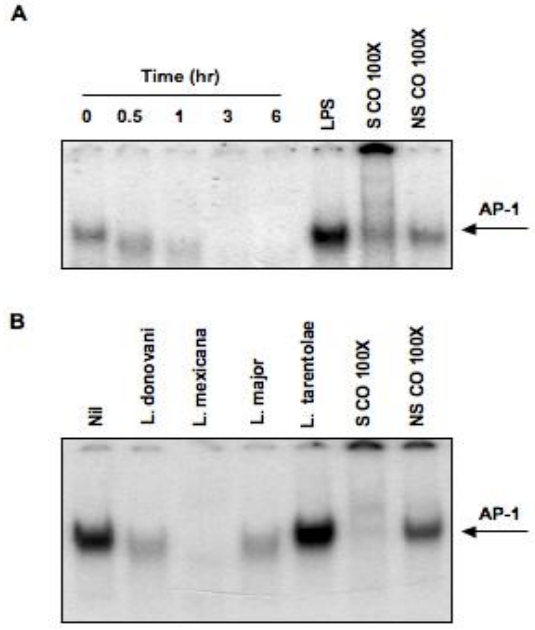


Figure 1: Infection with different species of *Leishmania* inhibits AP-1 DNA binding activity. (A) B10R macrophages were infected for 0.5, 1, 3 and 6 hr with *L. donovani*, at a 20:1 parasite/macrophage ratio. Nuclear proteins were isolated and EMSA for AP-1 DNA binding activity was performed. A consensus DNA sequence for SP-1 binding was used as non-specific competitor (NSCO). A 100X molar excess of AP-1 probe was used as a specific competitor (SCO). **(B)** B10R macrophages were infected for 1 hr with L. *mexicana, L. major*, *L donovani infantum* or *L. tarentolae* and treated as in **(A)**. One representative experiment of three is shown.

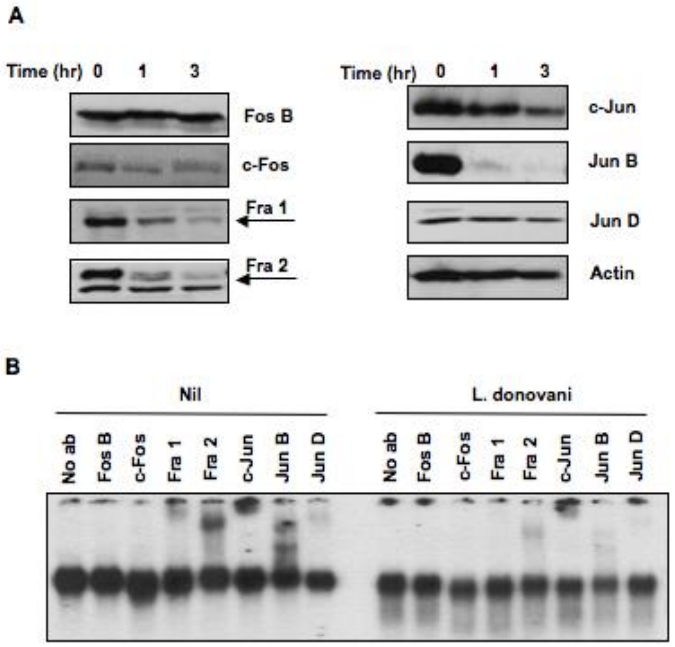


Figure 2: AP-1 subunits are degraded after infection with *Leishmania* parasites. (A) Western blot analysis of AP-1 subunit proteins extracted from B10R macrophages infected with *L. donovani* for 1 and 3 hrs. β-actin was used as a loading control. **(B)** Super shift assays of B10R macrophages infected with *L. donovani* for 1 hr. Nuclear proteins were super shifted using antibodies against c-Jun, Jun B, Jun D, c-Fos, Fos B, Fra 1 and Fra 2 AP-1 subunits. One representative experiment of three is shown.

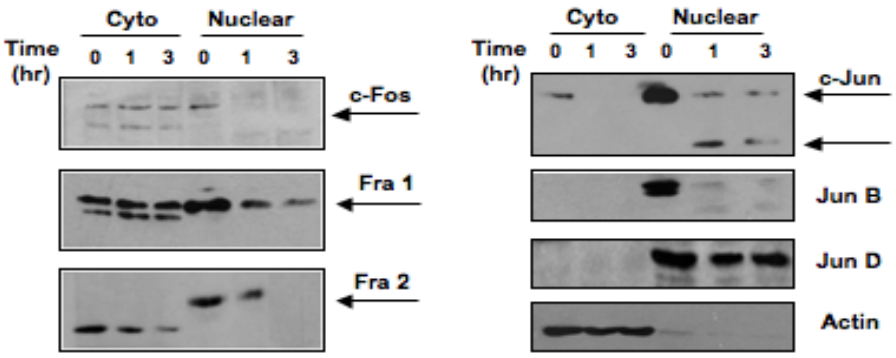


Figure 3: Subcellular localization of AP-1 subunits. B10R macrophages were infected with *L. donovani* for 1 and 3 hr. Cytoplasmic and nuclear distribution of the AP-1 subunits was monitored by Western Blot analysis. β-actin was used as a loading control for cytoplasmic fraction, and Jun D for nuclear fraction. One representative experiment of three is shown.

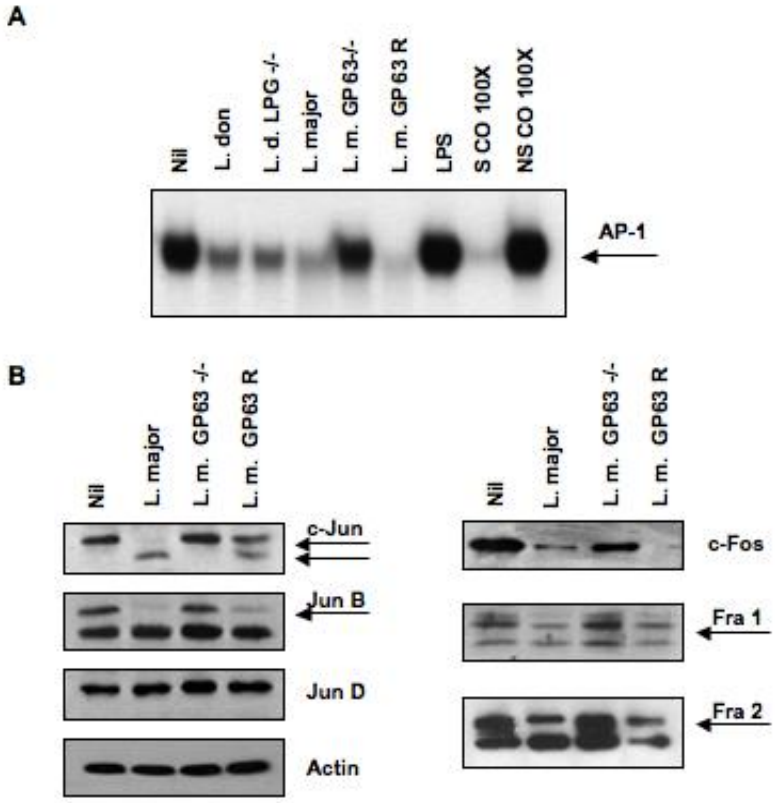


Figure 4: Role of *Leishmania* surface molecules in the inactivation of AP-1. **(A)** EMSA for AP-1 DNA binding activity of nuclear extracts from B10R macrophages infected for 1 hr with *L. donovani* 1S2D ($LPG^{+/+}$), *L. donovani* $LPG^{-/-}$, *L. major* (WT), *L. major* GP63 $^{-/-}$ or *L. major* GP63 Rescued (GP63 R) promastigotes. A consensus DNA sequence for SP-1 binding was used as non-specific control (NSCO). A 100X molar excess of AP-1 probe was used as a specific competitor (SCO). 1 hr stimulation with LPS (100ng/ml) was used as a positive control for the induction of AP-1 DNA binding. **(B)** Macrophages were infected for 1 hr with *L. major* (WT), *L. major* GP63 $^{-/-}$ or *L. major* Rescued promastigotes at 20:1 ratio. WB of AP-1 subunits was performed with the total cell lysate. β-actin was used as a loading control. One representative experiment of three is shown.

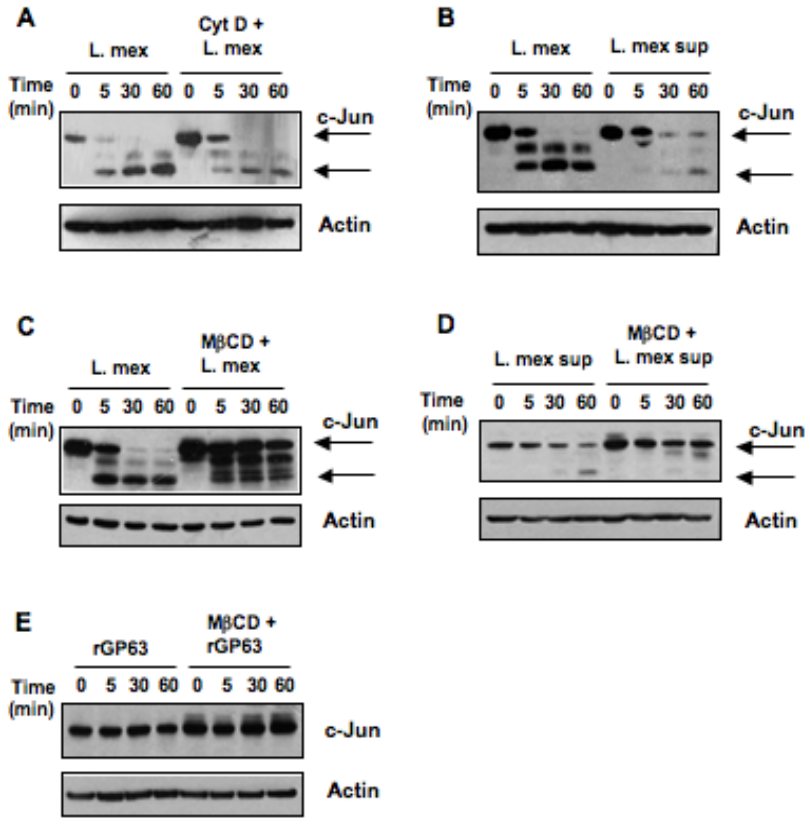


Figure 5: Parasite internalization is not necessary for GP63 delivery into the host cell. (A) B10R macrophages were pre-treated or not with 2 μM cytochalasin D for 1 hr and then infected with *L. mexicana* for indicated times. **(B)** B10R macrophages were incubated with either with whole parasite or culture supernatant of *L. mexicana* promastigotes. Macrophages were pre-treated or not with 20 mM of methyl β-ciclodextrin (MβCD) for 1 hr and infected with *L. mexicana* promastigotes **(C)**, incubated with *L. mexicana* supernatant **(D)** or recombinant GP63 (rGP63) **(E)**. For all the figures total cell extracts and, β-actin as a loading control were used. One representative experiment of three is shown.

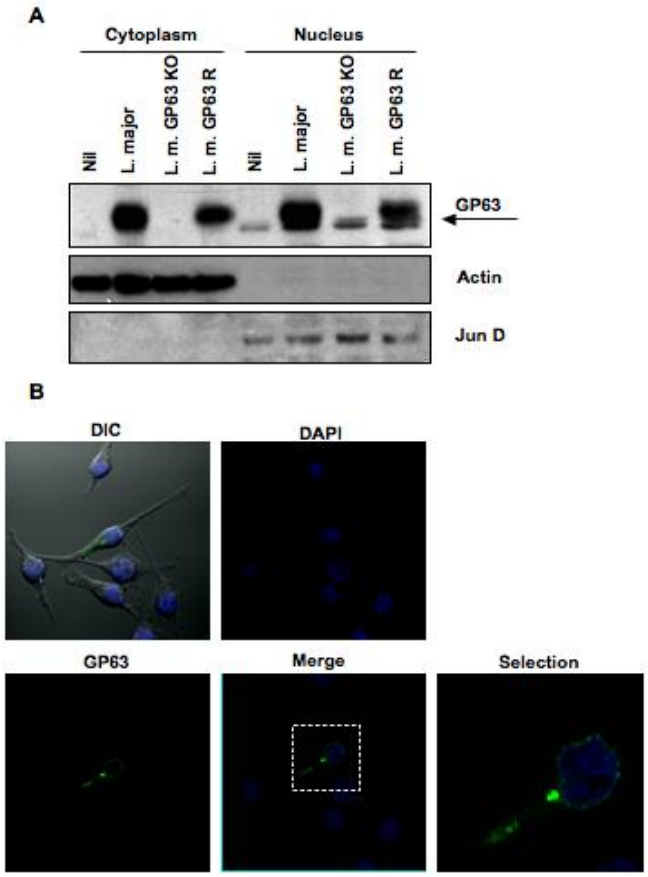


Figure 6: Subcellular localization of GP63. (A) B10R macrophages were infected for 1 hr with *L. major* (WT), *L. major* GP63$^{-/-}$ or *L. major* GP63 Rescued and GP63 distribution in the cytoplasmic and nuclear extracts was monitored by WB. β-actin and Jun B were used as fractioning controls. **(B)** B10R macrophages were infected for 1 hr with *L. major* (WT). GP63 is shown in green and nuclei were stained with DAPI (blue). One representative experiment of three is shown.

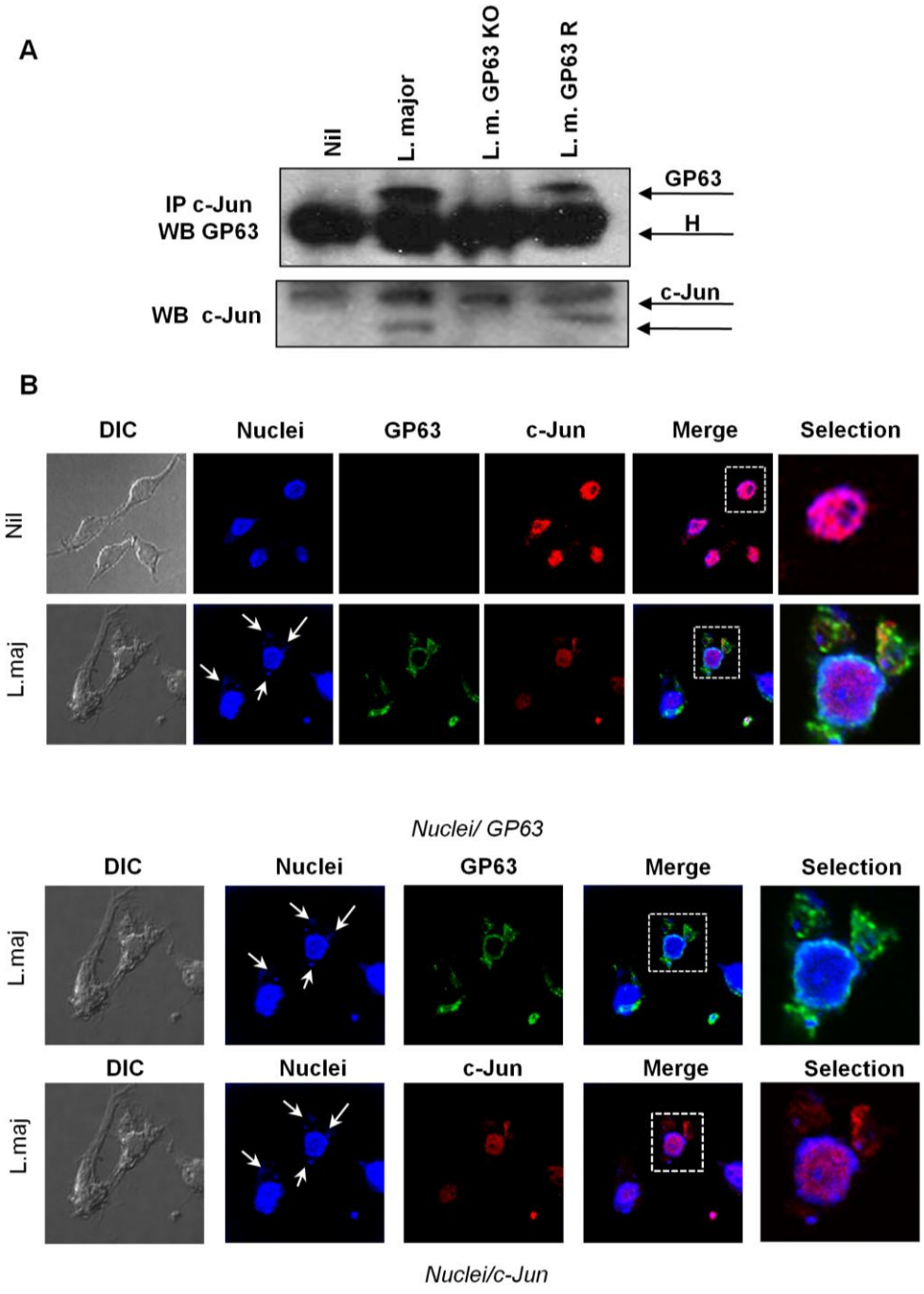

A
Nil
L. major
L. m. GP63 KO
L. m. GP63 R
IP c-Jun
WB GP63
GP63
H
WB c-Jun
c-Jun
B
DIC
Nuclei
GP63
c-Jun
Merge
Selection
Nil
L.maj
Nuclei/ GP63
DIC
Nuclei
GP63
Merge
Selection
L.maj
DIC
Nuclei
c-Jun
Merge
Selection
L.maj
Nuclei/c-Jun

Figure 7: GP63 is localized in the nuclear compartment. (A) Proteins from nuclear extracts of macrophages infected for 1 hr with *L. major* (WT), *L. major* GP63 $^{-/-}$ and *L. major* GP63 Rescued (GP63 R) and nuclear proteins were immunoprecipitated using an anti-c-Jun antibody. GP63 and c-Jun co-immunoprecipitation was evaluated by western blot. H denotes the heavy chain of the immunoglobulin. **(B)** Upper panel: Confocal microscopy images of B10R macrophages showing co-localization of nuclei, c-Jun and GP63 in non-infected and cells infected for 1 hr with *L. major*. Lower panel: Confocal microscopy analysis to evaluate nuclear localization of GP63 (green) and nuclear distribution and degradation of c-Jun (red) in macrophages infected 1 hr with *L. major*. Blue shows cell nuclei. One representative experiment of three is shown.

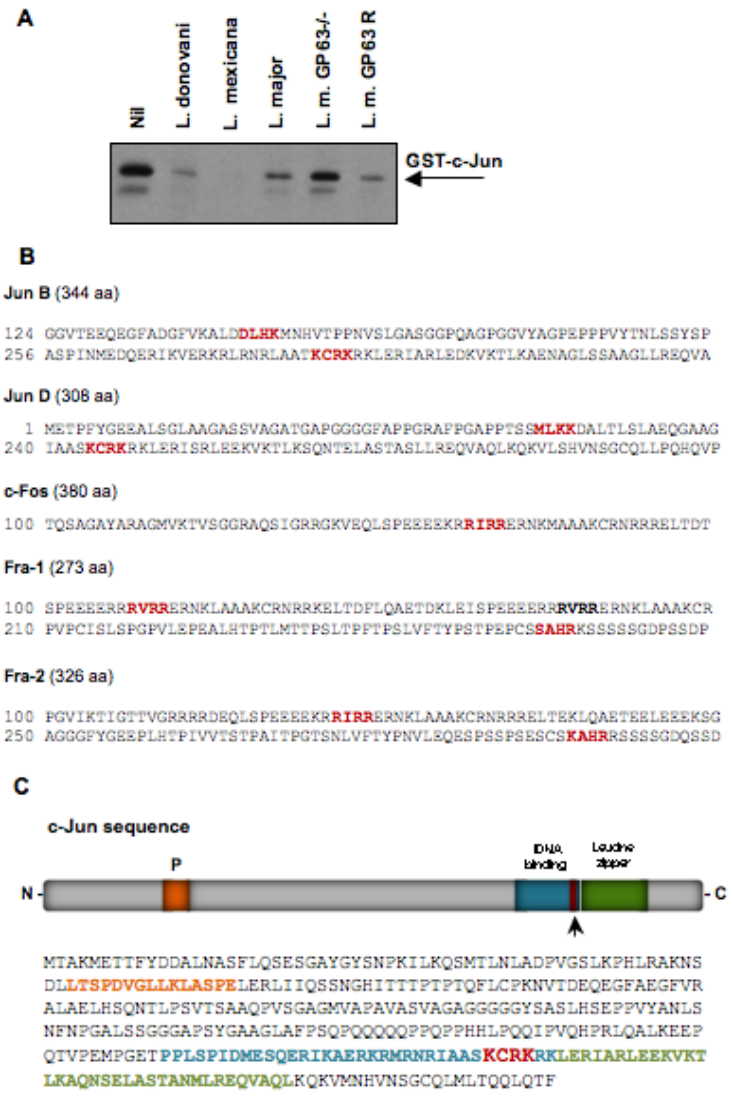
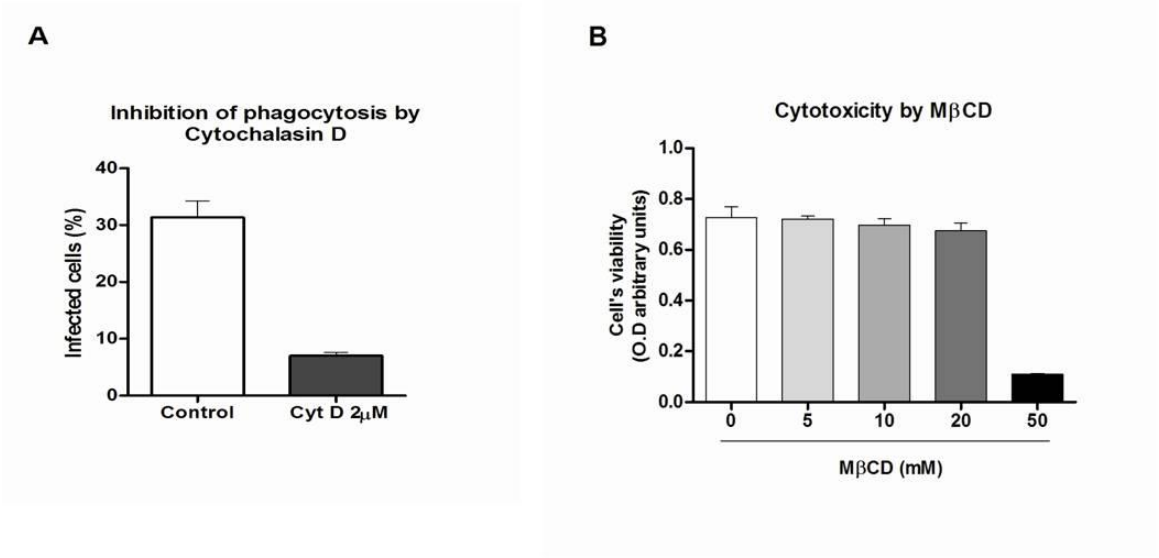
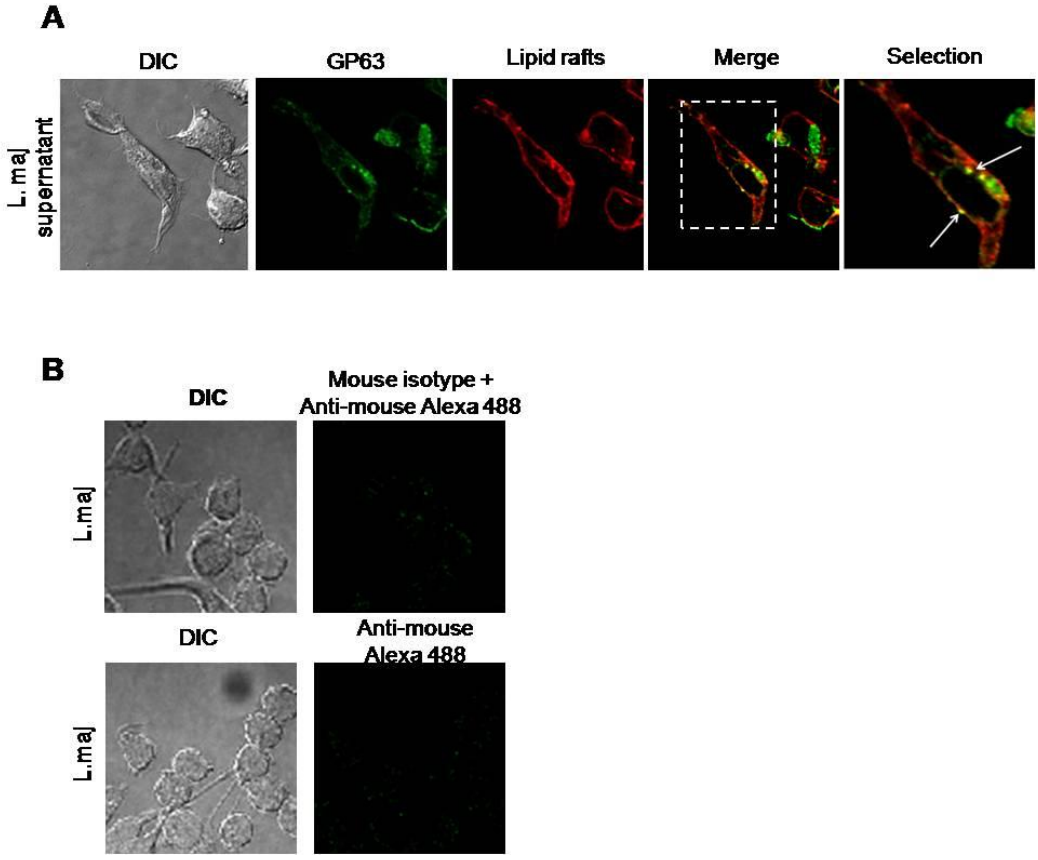


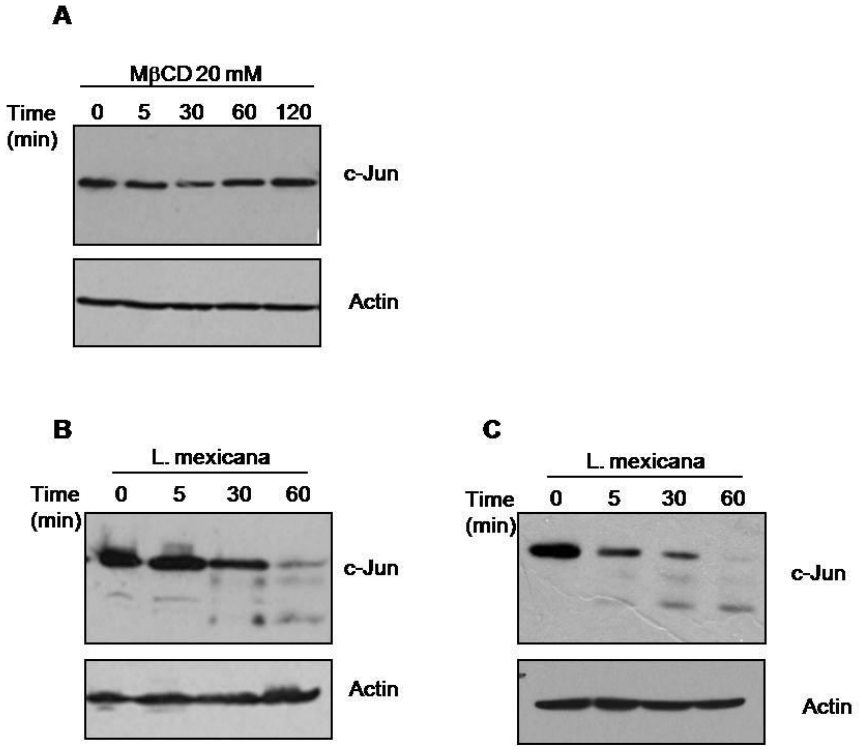
Figure 8: Parasite-free GP63 is sufficient to degrade c-Jun. **(A)** Exogenous GST-c-Jun was incubated with 500 µl of *L. donovani*, *L. mexicana*, *L. major*, *L. major* GP63$^{-/-}$ or *L. major* GP63 Rescued for 30 minutes, and degradation of c-Jun was visualized by WB using anti-GST antibody. **(B)** Jun B, Jun D, c-Fos, Fos B, Fra 1 and Fra 2 protein sequences showing putative GP63 cleavage sites. **(C)** c-Jun sequence analysis showing the putative site of GP63 cleavage.



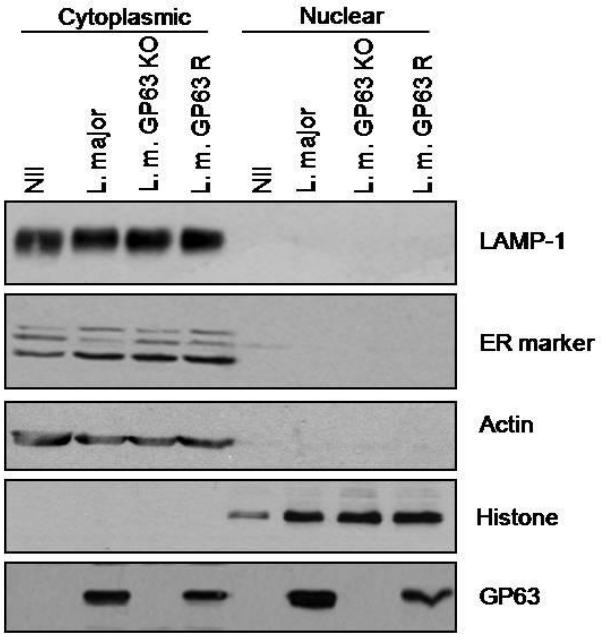
Supplemental Figure 1: Efficacy of Cytochalasin D and cytotoxicity of MβCD. (A) % of infected cells pre-treated with 2 μM/ml of cytochalasin D 1 hr before infection with *L. mexicana* (1 hr infection) (B) Cytotoxicity of MβCD in B10R macrophages treated for 1 hr with indicated doses of MβCD using XTT metabolizing assay.



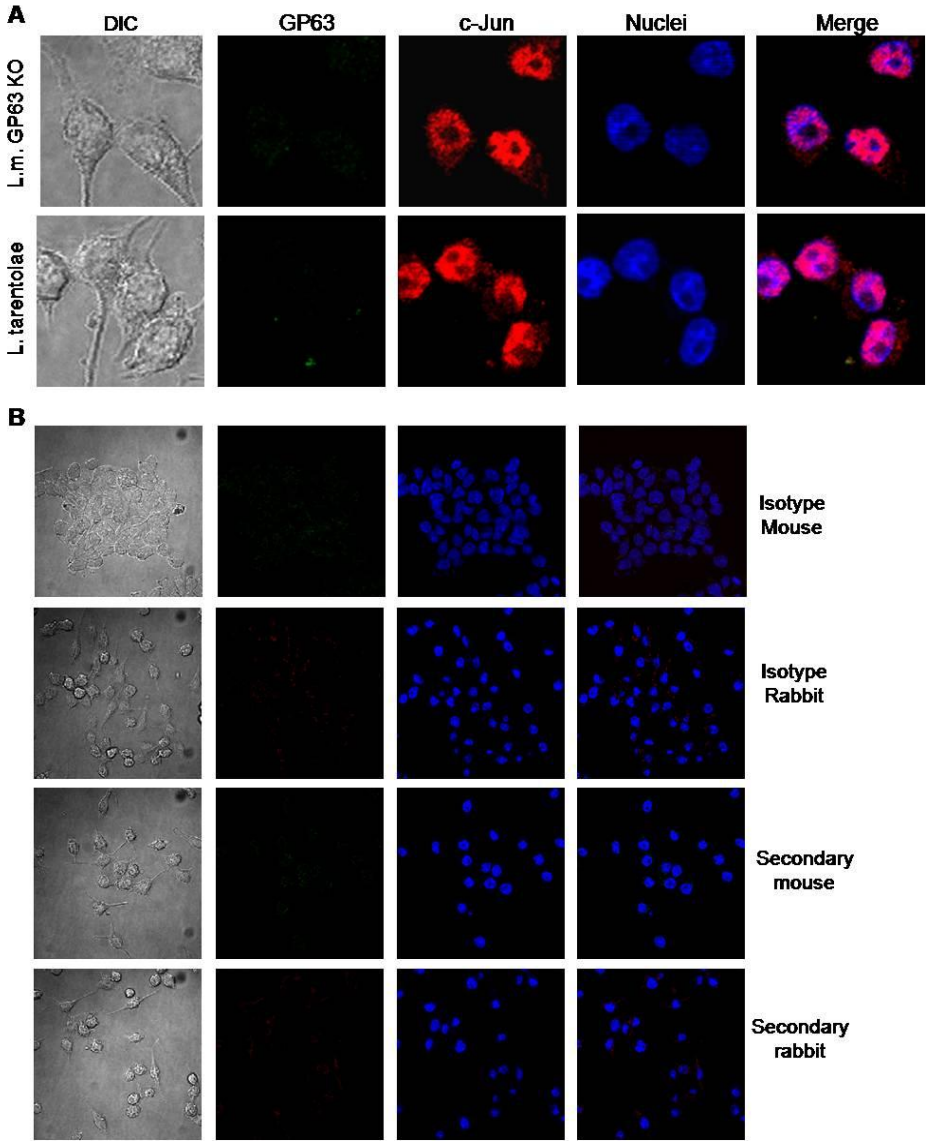
Supplemental Figure 2: GP63 partially co-localizes with lipid raft domains. (A) B10R macrophages were incubated with *L. major* supernatant for 1 hr and then stained for confocal microscopy to evaluate co-localization of lipid rafts domains and GP63. The lipid raft marker cholera toxin B (CTxB) is shown in red and GP63 is label in green (Alexa 488). (B) B10R macrophages were infected with *L. major* for 1 hr and then stained with unspecific anti-mouse IgG2a followed by anti-mouse Alexa 488 or infected and then incubated with anti-mouse Alexa 488.



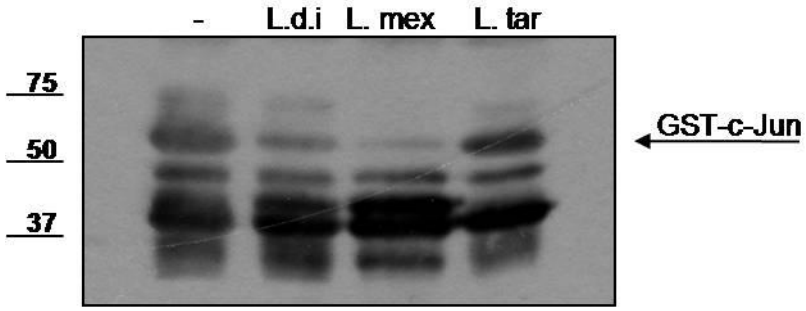
Supplemental Figure 3: Controls for c-Jun cleavage. (A) B10R macrophages were incubated for 5, 30, 60 and 120 minutes with MβCD (20 mM) and total cell lysates were subjected to WB against c-Jun. (B) B10R macrophages were infected with stationary phase promastigotes for 5, 30 and 60 minutes, after infection cells were washed, lysed with 100 µl of SLB 1X and boiled for 5 minutes. Samples were subjected to WB against c-Jun. (C) B10R macrophages were infected as in (B) and cells were lysed with lysis buffer containing 0.5M of phenantrolin. Total cell extracts were subjected to WB against c-Jun. For all the figures actin was used as loading control.



Supplemental Figure 4: Purity of cytoplasmic and nuclear fractions. B10R macrophages were infected with *L. major* wt, *L. major* GP63 KO or *L. major* GP63 Rescued stationary promastigotes for 1 hr. After infection, cytoplasmic and nuclear proteins were separated and subjected to WB against lysosomal marker LAMP-1, ER specific protein KDEL, actin, histone 2B and GP63 antibodies.



Supplemental figure 5: *L. major* KO and *L. tarentolae* do not degrade c-Jun. (A) B10R macrophages were infected for 3 hr with *L. major* GP63 KO or *L. tarentolae.* After infection cells were washed and stained for confocal micoscopy. GP63 is label in green (Alexa 488) and c-Jun in red (Alexa 546). (B) B10R macrophages were infected with *L. major* and cells were label with unspecific anti-mouse (IgG2a) or anti-rabbit antibodies followed by secondary antibody (Alexa 488 or Alexa 546 respectively) or only stain with anti-mouse (Alexa 488) or anti-rabbit (Alexa 546) antibodies.



Supplemental Figure 6: *L. tarentolae* has no effect over recombinant c-Jun. Exogenous GST-c-Jun was incubated with 500 μl of *L. donovani*, *L. mexicana*, or *L. tarentolae* for 30 min and degradation of c-Jun was visualized by WB using anti-GST antibody.

Supplemental Figure 7: NLS-like motif in GP63 sequence. GP63 amino acid sequence of *L mexicana* with the putative NLS (Nuclear localization signals)-like motif.

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CHAPTER 4

DENDRITIC CELL SIGNALLING ALTERATION BY *LEISHMANIA MEXICANA* LEADS TO ABOLISHED FUNCTIONAL ANTIGEN PRESENTATION

PREFACE CHAPTER 4

In the last two chapters we showed some of the molecular mechanisms employed by *Leishmania* to sabotage the macrophages' microbicidal functions. Despite the fact that macrophages have been considered as the main *Leishmania* host, it has been reported that some other cell types may serve as hosts too.

Dendritic cells (DCs) are a crucial link between the innate immune response and the adaptive immune response; they are responsible for presenting antigen to T cells and drive the Th1/Th2 responses. It has been shown that *Leishmania* is able to infect DCs *in vivo* and *in vitro*, by using molecules such as Fc and mannose receptors; however, the role of DCs during *Leishmania* infection is still unclear and controversial. Many reports have shown that infection of DCs augments their capacity to present antigens. On the other hand, some studies have shown that *Leishmania* down-regulates the DC functions. In this chapter we present evidence that demonstrates that *L. mexicana*-infected DCs lose their capacity to maturate and present antigen in a MHC II-dependent manner. Furthermore, for the first time we demonstrated that *Leishmania* is able to induce activation of host phosphatases that leads to reduced phosphorylation of MAPK and decreased transcription factors nuclear translocation.

DENDRITIC CELL SIGNALING ALTERATION BY *LEISHMANIA MEXICANA* LEADS TO ABOLISHED FUNCTIONAL ANTIGEN PRESENTATION

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Running title: Inhibition of DCs functions by *Leishmania*

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ABSTRACT

Leishmaniasis is a group of diseases caused by protozoan parasites of the *Leishmania* genus. *Leishmania* has the ability to modify the macrophage signaling pathways in order to survive, multiply and propagate within its mammalian host. It is known that *Leishmania* can invade other cell types besides macrophages, including neutrophils, fibroblasts and dendritic cells (DCs). DCs have an important role in priming the adaptive immune response and are the link between the innate and adaptive responses. The fate of DCs infected by *Leishmania* has been poorly investigated. Therefore, in the present study we have attempted to highlight some of the detrimental effects that *Leishmania* could have on DC signaling and functions. As previously observed for macrophages, protein tyrosine phosphatases (PTP) of DCs were also rapidly induced by *Leishmania* infection and found to induce dephosphorylation of protein substrates. More precisely, we found that several members of the MAP kinase family were altered *per se* in response to LPS stimulation upon infection, as were the downstream transcription factors AP-1 and NF-κB. Additionally, *L. mexicana* infection was shown to induce reduced MHC I, MHC II, B7.1, B7.2 and CD11b surface molecules in response to LPS stimulation. *Leishmania's* influence on DCs maturation was found to significantly interfere with antigen presentation by DCs. Indeed, *Leishmania*-infected DCs were less effective in presenting OVA antigen to OVA-specific T cells as revealed by abrogation of IL-2 production by T cells. Taken together, our data revealed that *L. mexicana* infects DCs and modifies signaling and immunological events that favor its survival and progression within the host.

INTRODUCTION

Leishmaniasis refers to a group of diseases caused by protozoan parasites of the *Leishmania* genus being transmitted by phlebotomine female sandflies [1,2]. Leishmaniasis is characterized by three main clinical manifestations: the self-healing cutaneous leishmaniasis (CL); disfigurating localized muco-cutaneous leishmaniasis (MCL), caused by the *Viania* subgenus; and the life-threatening visceral leishmaniasis (VL). These diseases are endemic in areas of the tropics, subtropics, and southern Europe [2]. It is well known that *Leishmani*a parasites reside, multiply, and change from the promastigote stage to the amastigote inside of their primary host cell: the macrophage. Extensive evidence has shown that *Leishmania* have evolved many mechanisms to subvert macrophage functions by altering its signaling pathways in order to survive within its host [3]. However, although macrophages are considered as the canonical host cell, neutrophils [4], fibroblast [5], and dendritic cells (DCs) [6] have been reported to be infected by *Leishmani*a, although very little has been investigated in regards to the impact that *Leishmania* has on their functions.

DCs are professional antigen-presenting cells (APC) which sit in an immature state capable of antigen uptake and processing in peripheral non-lymphoid tissues [7]. DCs are also critical for the induction of immunological tolerance, as well as for the regulation of the type of T cell-mediated immune response [8]. The maturation process of DCs consists of: i) increased expression of MHC and co-stimulatory molecules such as CD40, B7.1, B7.2 and CD54; ii) down-regulation of antigen capture and phagocytic capacity; iii) enhanced cytokine secretion, and iv) expression of different chemokine receptors [9]. Uptake of antigens by DCs is mediated by different groups of receptors, such as Fc-receptors [6], C-type lectin receptors (CLR) that recognize glycoproteins and Toll-like receptors [10]. All these receptors are able to recognize a wide variety of microorganism, including *Leishmania* parasites [11,12].

Despite the well-known role of DCs as a link between the innate to adaptive response, the impact of *Leishmania* infection on DC functional activities and signaling is still mainly unknown and very controversial. Therefore, a better understanding on how *Leishmania* may influence their functions could permit the development of better

approaches to strengthen DCs responses to control *Leishmania* infection. In this study, using the DC 2.4 cell line [13] we investigated the impact of *L. mexicana* promastigote infection on their maturation and antigen presentation capacities. Our study revealed that *L. mexicana*-infected DCs are impaired in their signalling pathways involving the p-38, ERK and JNK MAP kinases. Dephosphorylation of these molecules was accompanied by augmented PTP activity and concomitant alteration of the transcription factors AP-1 and NF-κB, events that have been also observed in *Leishmania*-infected macrophages [12,14,15,16]. Finally, we also observed that infected DCs were less efficient in their capacity to present antigen to OVA-specific T cells, as reflected by an almost complete absence of IL-2 production. In part this antigen presentation defect could be also due to a lesser expression of co-stimulatory molecules (B7.1 and B7.2) and antigen presentation molecules (MCH I and II) observed in infected cells. Collectively, the actual study clearly establishes that *Leishmania*-infected DCs are strongly compromised in their capacity to develop an effective adaptive immune response, therefore favoring successful infection and further propagation within their host.

MATERIAL AND METHODS

Cell culture, macrophage infection and reagents

Immortalized murine (C57BL/6) bone marrow derived dendritic cells, DC 2.4 cell line were maintained at 37° C in 5 % CO_2 in RPMI medium supplemented with 10 % heat inactivated FBS (Invitrogen, Burlington, ON, Canada) and 100 U/ml penicillin 100 μg/ml streptomycin and 2 mM of L-glutamine (Wisent, St-Bruno, QC, Canada), 10 % HEPES (Sigma, St-Louis MO, USA), 10 % essential amino acids (Invitrogen, Burlington, ON, Canada) and 50 μM of β-mercaptoethanol (Sigma, St-Louis MO, USA). T cell hybridome MHC-II-specific MF2.9 were grown and maintained in the same medium than DCs by tri-weekly passage. *Leishmania mexicana* promastigotes were grown and maintained at 25° C in SDM-79 culture medium supplemented with 10 % FBS by bi-weekly passage. DCs were infected at parasite to DC ratio 20:1 with stationary phase promastigotes for the times specified in each figure legend. When cells were stimulated, 100 ng/ml of LPS were used for the times specify in each figure legend and remained throughout the infection time.

Western blot

Infected and non infected DCs (1×10^6) were washed 3 times with Phosphate Buffered Saline (PBS) and lysed with cold buffer (50 mM Tris (pH 7), 0.1 mM, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 1 % NP-40, 40 μg/ml aproptinin and 20 μg/ml of leupeptin). Proteins were dosed by Bradford (Bio-Rad, Hercules CA, USA), and 60 μg of proteins were separated by SDS-PAGE, and transferred onto PVDF membranes (GE healthcare, Piskataway NJ, USA). Membranes were blocked in 5 % non-fat dry milk, washed and incubated for 1 hr with anti-p-ERK, p-JNK, and p-38 (Cell signaling, Ipswich, MA, USA). After washing, membranes were incubated 1 hr with α-rabbit HRP-conjugated antibody (Sigma St-Louis MO, USA), and developed by autoradiography.

In gel PTP assay

In gel PTP assay was performed as described previously [17]. Briefly, Poly (Glu-Tyr) substrate was tyrosine phosphorylated by over night incubation (ON) with 10 μg of

GST-FER protein kinase and 150 μCi of γ-^{32}P-dATP. The radiolabel-phosphorylated substrate was incorporated in a 10% SDS-PAGE at a concentration of 2 x 10^5 CPM/ml. DCs extracts were prepare as for WB and denatured by SDS. After electrophoresis, the gel was incubated ON in buffer A (50 nM Tris-HCL pH 8.0, 20 % isopropanol), next morning gels were washed twice with buffer B (50 nM Tris-HCL pH 8.0, 0.3 % β-mercaptoethanol), followed by full protein renaturation in buffer B containing 6 M guanidine and 1 mM EDTA. The gels were washed twice in buffer C (50 mn Tris-HCL pH 8.0, 0.3 % β-mercaptoethanol, 1 mM EDTA and 0.04 % tween 20). Final renaturation was obtained by ON incubation with buffer C. Gels were dried and expose to X-ray film.

pNPP assay

Infected and non infected DCs were lysed in PTP buffer (50 mM Tris-HCL pH 7.0, 0.1 mM EDTA, O.1 mM EGTA, 0.1 β-mercaptoethanol, 1 % IGEPAL, 25 μg/ml aproptin, and 25 μg/ml leupeptin) and kept on ice for 45 minutes, vortexing every 15 minutes. Lysates were cleared by centrifugation, and protein content was measure by Bradford's method. 10 μg of protein extract were incubated in phosphatases reaction buffer (50 mM HEPES pH 7.5, 0.1 % β-mercaptoethanol, 10 mM para-nitrophenylphosphate (pNPP)) for 30 minutes. OD was red at 405 nm.

Electrophoresis Mobility Shift Assay (EMSA)

DCs (2 x 10^6) were infected, washed three times with PBS to remove non-internalized parasites, and processed for nuclear extraction as previously described [15,18]. Briefly, DCs were collected in 1 ml of cold PBS, centrifuged and pellets were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM of PMSF) and incubated 15 min on ice. Twenty five μl of IGEPAL 10 % (Sigma-Aldrich, St-Louis MO, USA) were added, and samples vortexed for 30 sec. Nuclear proteins were pelleted by centrifugation and resuspended in 50 μl of cold buffer C (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.5 mM PMSF).

Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules CA, USA). 6 µg of nuclear proteins were incubated for 20 min at room temperature with 1 µl of binding buffer (100 nM Hepes pH 7.9, 8 % v/v glycerol, 1 % w/v Ficoll, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA, 25 mM NaCl, and 1µg/µl BSA) and 200 ng/µl of poly (dI-dC), 0.02 % bromophenol blue and 1 µl of γ-P^{32}labeled oligonucleotide containing a consensus sequence for AP-1 binding complexes (5'-CGTTTGATGACTCAGCCGGAA-3') (Santa Cruz Biotechnology Inc, Sta Cruz CA, USA), and NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') (Santa Cruz Biotechnology Inc, Sta Cruz CA, USA). After incubation, DNA-protein complexes were resolved by electrophoresis in non-denaturing polyacrylamide gel 5 % (w/v). Subsequently gels were dried and autoradiographed. Competition assays were conducted by adding a 100-fold molar excess of homologous unlabeled AP-1 or NF-κB oligonucleotide, or the non-specific competitor sequence for SP-1 binding (5'-ATTCGAATCGGGGCGGGGCGAGC-3').

Flow cytometry (cell surface staining)

0.5 x 10^6 cells were plated in 10 cm dishes and after 24 hr of infection (*L. mexicana*) or stimulated 24 hr (LPS 100 ng/ml) cells were recovered and blocked with 2.4G2 (anti-FcγR) in staining buffer (1 % bovine serum albumin (BSA) and 0.1 % NaN_3 in PBS 1X). Samples were then labeled with primary biotinylated antibodies against cell surface molecules (anti-mouse MHC I and MHC II (BD biosciences, Mississauga ON, Canada)), and subsequently, labeled with secondary antibody, PeCy5-conjugated streptavidin (ebioscience, San Diego CA, USA) and fluorochrome-conjugated antibodies against cell surface molecules (anti-mouse B7.1, B7.2, CD11b and CD40 (ebioscience, San Diego, CA, USA)). Finally, samples were acquired on FACScalibur (BD biosciences, Mississauga ON, Canada) and analyzed with CellQuest software (BD biosciences, Mississauga ON, Canada). Isotype controls were used to set quadrants.

IL-12 mRNA expression analysis

RNA was extracted from DCs using TRIzol reagent (Invitrogen Burlington, ON, Canada). Reverse transcription was performed using oligodt (Invitrogen Burlington, ON, Canada). Quantitative real-time PCR (RT-PCR) was performed in a Corbbet research

rotorgene (Corbett life science, Sydney, Australia), using Bio-Rad SYBR green qPCR super mix (Bio-Rad, Hercules CA, USA) and 0.4 μM IL-12 p 40/GAPDH primers (GAPDH: 5'-CGG ATT TGG CCG TAT TGG GCG CCT-3' and 3'-ACA TAC TCA GCA CCG GCC TCA CCC-5'; IL-12: 5'- GGA AGC ACG GCA GCA GAA TA-3' and 3'-AAC TTG GAG AAG TAG GAA TGG-5') in a final volume of 25 μl. qPCR program included 2 min at 50^{o} C; 3 min at 95^{o} C; 45 cycles of 20 sec at 95^{o} C, 30 sec at 60^{o} C and 20 sec at 72^{o} C followed by a melting curve. All primers annealing temperature was 60^{o} C.

Co-culture assays (DCs-T cells)

2 x 10^4 DCs were plated in 96 well and infected with *L. mexicana* promastigotes or LPS-stimulated for the times specified in each figure legend. After infection or stimulation cells were washed 3 times with PBS to remove all non-internalized parasites and then cells were loaded with 2 mg/ml of OVA (Sigma, St-Louis MO, USA) for 2 hr. After that, 1 x 10^5 MF2.9 MHC-II-specific hybridome T cells were co-cultured with DCs ON. Next morning plates were centrifugated and supernatants were collected and frozen at -20^{o} C until they were used to measure IL-2 production by T cells.

ELISA for IL-2

NUNC maxisorp 96 well plates (Nalge NUNC, Richester NY, USA) were coated with 100 μl/well of capture antibody (eBiociences, San Diego CA, USA) ON and blocked with 200 μl/well with assay diluent solution 1 hr at RT. After blocking 100 μl of standard proteins or samples were added to each well and incubated for 2 hr. After 5 washes, 100 μl/well of detection antibody were added and incubated 1 hr at RT. For IL-2 detection 100 μl/well of Avidin-HRP were added and incubated for 30 min. Finally 100 μl/well of substrate solution were added for 15 min to develop color. 50 μl of stopping solution were added and plates were red at 450 nm in an ELISA reader. IL-2 concentrations were measured and analyzed using a standard curve provided by the IL-2 commercial kit.

Statistical analysis

Statistically significant differences were identified using the ANOVA module of Prism program (version 5). Values of $P \leq 0.05$ were considered statistically significant. All data are presented as mean ± SEM.

RESULTS

L. mexicana activates DC phosphatases

Activation of protein tyrosine phosphatases is one of the main mechanisms employed by *Leishmania* to survive inside macrophages. For instance, we have previously reported that SHP-1, one of the main phosphatases in macrophages with a wide range of substrates, is activated upon *Leishmania* infection [12,16,19]. Thus, we explored whether DCs' phosphatases were activated upon *Leishmania* infection. In order to explore this notion, we performed an in gel phosphatase assay where addition of a radiolabel peptide to a SDS-PAGE gel permits to see the activity of PTPs. Figure 1A shows that as soon as 30 min post-infection there is appearance of bands corresponding to active PTPs that are not present in the uninfected control, this PTP activity was maintained after 24 hr of infection. Furthermore, we performed a PTP activity assay using the subtract pNPP. In this experiment, we also observed that, upon infection, the activity of PTPs increased ~50 % as soon as 30 min post-infection. This activity reaches its maximum peak at 3 hr (Figure 1B). As phosphatases regulate kinase activity, we performed WB analysis to evaluate the profile of phosphorylation on serine and tyrosine residues. As shown in Figure 1C, upon *Leishmania* infection there is less phosphorylation on serine (right panel) and tyrosine (left panel) residues, suggesting that indeed this lack of phosphorylation is due to the increased phosphatase activity observed in the in gel and in the pNPP assays.

Leishmania infection inhibits DC signaling pathways

Proper activation of cell signaling pathways is crucial for the production of cytokine/chemokine networks that will control the course of infectious diseases such as leishmaniasis. *Leishmania* parasites have the ability to suppress the necessary intracellular signaling events in macrophages [3]. However, limited information is available regarding the effect on the DC signaling pathways upon *Leishmania* infection, since most of the studies have focused their attention on the analysis of maturation and antigen presentation. Nonetheless, a recent report has showed that infection of DCs with *L. amazonensis* amastigotes resulted in alteration of the STAT-2 pathway with the concomitant reduction in the production of IL-12 [20].

Protein phosphorylation though MAPKs plays an essential role in many cellular functions [21]. Therefore, we investigated whether *Leishmania* infection could affect the phosphorylation of MAP kinases (ERK, p-38 and JNK) in DCs. To do so, we performed WB analysis against these phosphorylated proteins. Activation of MAP kinases in DCs by LPS is well documented and varies depending on the DC's origin [22,23]. However, we wanted to determine if LPS stimulation in the cell line used for the present study could induce MAPK phosphorylation. In order to determine this, we stimulated DCs with LPS for 0.5, 1, 2 and 3 hr. WB analysis (Figure 2A) shows that phosphorylation of ERK, p-38 and JNK is increased as soon as 30 minutes post-stimulation. On the other hand, infection with *L. mexicana* promastigotes inhibited basal phosphorylation of ERK, JNK and p-38 without affecting total protein expression (Figure 2A right panels).

To further analyze the impact of *Leishmania* infection on MAP kinase phosphorylation induced by LPS, we designed experiments where the LPS stimulation was performed before or after infection. Based on the LPS stimulation time-course, we chose 30 minutes of incubation. Figure 2B shows that infection with *L. mexicana* after LPS stimulation reduced phosphorylation of ERK, JNK and p-38. The same reduction on ERK and p-38 phosphorylation was observed when DCs were infected before LPS stimulation; however, JNK phosphorylation was not affected. The latter result indicates that the inhibitory effect of *Leishmania* is transient for JNK rather than a phosphorylation induced by the parasite, as *L. mexicana* promastigotes did not present any phosphorylation of JNK (Figures 2A and 2B).

Inhibition of translocation of transcription factors (TFs)

In our laboratory, it has been demonstrated that different *Leishmania* species are able to abrogate the activity of important transcription factors in macrophages such as STAT 1 [15], NF- κB [24], and AP-1 [25]. Here, we investigated the effect of *L. mexicana* promastigotes over these TFs under LPS stimulation at short times of infection/stimulation. Figure 3A shows that infection of DCs led to a reduction in the nuclear translocation of both TFs as soon as 1 hr post-infection. Stimulation of DCs with LPS confers in them the ability to satisfactorily translocate AP-1 and NF-κB. Parallel experiments of LPS-stimulated DCs before infection (Figure 3B) or infected and then

stimulated with LPS (Figure 3C) showed that in either case infection prevents the translocation of TFs. However, in the case of NF-κB we observed the appearance of a lower band known as p35 in cells that were stimulated with LPS before infection. This p35 subunit is able to dimerize with other NF-κB subunits and then translocate into the nucleus to bind DNA [24].

L. mexicana promastigotes reduce IL-12 production in DCs

IL-12 produced by DCs plays a critical role in the development of $CD4^+$ Th1 cells that confer resistance against *L. major*-infection in mice [26]. AP-1 and NF-κB TFs are responsible for IL-12 production [27,28]. Therefore, we investigated whether infected DCs were able to produce IL-12. For this, we evaluated the mRNA IL-12 p40 expression by RT-PCR. As shown in Figure 4A, the maximum expression of IL-12 mRNA was reached at 8 hr of stimulation with LPS, thereafter this expression decreased. *L. mexicana* infection did not induce significant levels of IL-12 expression compared with uninfected cells (Figure 4B). Next, we assessed whether *L. mexicana* infection affected LPS-induced IL-12 expression under two different conditions; infection after LPS stimulation or infection before LPS stimulation. As observed in Figure 4C infection of DCs after LPS stimulation did not affect the IL-12 expression, since this expression was ~ 20-fold augmented in comparison with cells that were only stimulated with LPS. This result suggests that LPS stimulation prior to infection might prime DCs to produce IL-12 after a second stimulation (*L. mexicana*). On the other hand, when DCs were first infected and then stimulated with LPS the IL-12 mRNA expression was reduced more than 30-fold, indicating that the inhibitory effect of *L. mexicana* infection was not reverted by LPS (Figure 4D).

L. mexicana promastigotes affect maturation of DCs by inhibiting expression of MHC and co-stimulatory molecules

DCs are professional antigen presenting cells, which in an immature state are able to uptake and process a wide variety of pathogens/antigens [8]. The maturation process requires the increased expression of MHC molecules as well as co-stimulatory molecules. Therefore, we were interested in investigating if abrogation of the signaling pathways

could affect the antigen presentation and expression of co-stimulatory molecules. In order to specifically elucidate the effect of *L. mexicana* promastigotes during the maturation process we infected DCs with *L. mexicana* or stimulated them with LPS, thereafter, we looked at the expression of MHC-I, MHC-II, B7.1, B7.2, CD40 and CD11b by FACS.

L. mexicana infection significantly decreased the basal levels of CD11b and B7.2, and slightly decreased MHC II and B7.1 expression. (Figures 5 and 6). To determine the maturation of the cells we stimulated DCs for 24 hr with LPS, and the FACS analysis showed that LPS increased the expression levels of MHC I, MHC II, CD11b, B7.1, B7.2, and CD40. We also evaluated the expression of these molecules after 48 hr of LPS stimulation and we did not observe a significant change on the maturation markers compared with the levels expressed at 24 hr (Data not shown). To determine the effect of *Leishmania* infection on DC maturation, DCs were matured with LPS for 24 hr and then infected. Under these conditions, *L. mexicana* infected-DCs showed similar levels of maturation markers comparable to cells only stimulated with LPS. On the other hand, infection for 24 hr prior to LPS stimulation significantly reduced the levels of MHC I, MHC II, CD11b, B7.1, B7.2 in comparison with LPS-matured cells (Figure 5 and 6). Suggesting that addition of LPS after infection is not sufficient to reverse the suppressing effect of *L. mexicana* promastigotes over the expression of these molecules. Interestingly, CD40 showed augmentation in LPS-matured DCs, and its expression was not affected by the infection either before or after LPS stimulation (Figure 6C).

***Leishmania* parasites reduce the DCs' capacity on presenting antigen.**

DCs exhibit a unique ability to activate naïve T cells, this ability depends on their maturation stage [8,29]. In this study, we examined the effect of the infection of DCs with *L. mexicana* promastigotes over their antigen presentation capacity. To do so, we used T cells that specifically recognize ova-albumin (OVA), and co-cultured them with un-stimulated DCs, LPS-stimulated DCs, *L. mexicana*-infected DCs, LPS-stimulated/ *L. mexicana*-infected DCs or L. *mexicana*-infected/LPS-stimulated DCs. After these treatments, DCs were loaded with OVA and IL-2 production by the T cells was measured by ELISA.

Figure 7 shows IL-2 production by T cells that were co-cultured with DCs treated under different conditions. T-cells co-cultured with un-stimulated DCs produced very little amounts of IL-2, this level increased in T-cells co-cultured with LPS-stimulated DCs in a time dependent manner, reaching a peak at 24 hr. *Leishmania*-infected DCs also induced T cell activation but in a significant less extent than LPS-stimulated DCs. In addition, *Leishmania* infection did not have an effect on LPS-induced T cell activation, as IL-2 production by T cells co-cultured with LPS-matured DCs and then infected for 24 hr were comparable with cultures of T cells with DCs stimulated only with LPS. On the other hand, T cells co-cultured with *L. mexicana*-infected DCs before stimulation with LPS produced ~85% less IL-2, suggesting that *L. mexicana* impairs the T cell activation capacity of DCs. This effect may possibly be related to a decreased antigen presentation capacity by *Leishmania*-infected DCs.

Together, these data suggests that alteration in the maturation and the capacity to present antigens of DCs upon infection with *L. mexicana* promastigotes involves activation of PTPs that can dephosphorylate important MAP kinases and inhibit nuclear translocation of AP-1 and NF-κB, resulting in blockage of DC maturation.

DISCUSION

During the last few years, evidence has been gathered about other cell types besides macrophages playing as hosts for *Leishmania* [6]. DCs have a role in priming the adaptive immune response and the induction of self tolerance. In order to prime the adaptive immune response, and to be able to present antigens, DCs have to undergo a maturation process. The antigen sampling and migratory capacities of DCs effectively allow naïve T cells to come into contact with antigens [30]. Taking into account that DCs are the link between the innate and adaptive immune responses, and that they are able to uptake *Leishmania* parasites, we sought to determine the effect of *L. mexicana* promastigote infection on DC functions and signaling pathways.

In the present work, we have attempted to discern the impact of *Leishmania mexicana* promastigote infection on antigen presentation by DCs in two different situations: first, infection of previously matured DCs and second, infection of DCs followed by LPS stimulation to drive further maturation. Our data showed that infected cells that received the signal to induce their maturation (LPS) after infection were not able to mature and showed lower expression of MHC class I and class II and co-stimulatory molecules such as, B7.1 and B7.2. On the other hand, LPS clearly induced DC maturation by increasing the expression of the aforementioned molecules and promoting T cell activation. Furthermore, infection of LPS-matured DCs did not affect their expression of co-stimulatory molecules. Besides analyzing the expression of MHC and co-stimulatory molecules, we assessed the impact of infection on the capacity of DCs to present antigen to OVA-specific T cells. Our results showed that LPS-matured infected DCs were able to present antigen to T cells. However, T-cells co-cultured with DCs infected with *Leishmania* prior to stimulation with LPS presented reduced capacity to produce IL-2, supporting the notion that *L. mexicana* interferes with the maturation and antigen presentation processes.

Leishmania infection could be affecting many of the DCs' functions, such as antigen presentation, migration and activation of co-accessory cells. Regarding antigen presentation and accessory functions and in agreement with our findings, Liu et al. showed that *L. major* LPG is involved in the inhibition of DC maturation and IL-12

production [31]. In contrast, De Trez et al. showed that *L. donovani* increases maturation and migration of DCs from both BALB/c and C57BL/6 mice [32], suggesting that DC responses can differ according with the parasite species. Another step of the antigen presentation which could be affected by infection is the phagosome maturation. In this regard, Korner et al. showed that biogenesis of the phagosome is arrested at the stage of late endosome in immature bone marrow dendritic cells [33]. However, we did not investigate whether the *Leishmania*-induced inhibition of antigen presentation could be related to phagosome maturation. The effect of *L. mexicana* infection on phagosome maturation in the DCs will be the objective of further of study in our laboratory.

In order to present antigens to naïve T cells, DCs must migrate from tissues to lymphoid organs. This migration is tightly regulated by chemokines and their receptors [34]. In the present study we did not address the role of *Leishmania* infection on the DCs' migration capacity. However, previous reports showed contradictory results in this regard. Steigerwal et al. showed that *L. major*-infected DCs expressed less CCR2 and CCR5 and their respective ligands CCL2 and CCL3, while the levels of CCR7 and its ligand CCL21 were enhanced, resulting in inhibited migration [35]. In addition, using murine Langerhans cells (LCs), Baldwing et al. showed that LCs lose their migratory capacity upon *Leishmania major* infection. Therefore, they do not serve as "ferries" from the skin to the lymph nodes [36]. However, Moll et al. demonstrated that murine Langerhans cells (LCs) can phagocytise *L. major* and migrate to the lymph nodes [37].

In order to better understand the mechanisms underlying the down regulation of antigens presentation, we investigated phosphorylation of MAP kinases and nuclear translocation of transcription factors such as AP-1 and NF-κB. Signal transduction through MAPK plays an important role in many cellular responses. The three kinases classified in this group are ERK, JNK and p-38, these kinases are activated by phosphorylation of both threonine and tyrosine in a regulatory TXY motif in all MAP kinases [38]. Our results showed that infection of DCs with *L. mexicana* promastigotes prior to stimulation with LPS led to inhibition in the phosphorylation of p-38 and ERK kinases, but not JNK. It is known that in DCs or the D1 cell line, LPS activates the ERK pathway, playing a role in DC maturation and in preventing apoptosis after maturation [23]. In addition, p-38 and the PI3 kinase pathways are involved in the LPS-induced

maturation of human monocyte-derived dendritic cells [22]. Our findings suggest that in an immature state, infected DCs are unable to phosphorylate p-38 and ERK pathways. However, in the case of JNK, *Leishmania* does not suppress its phosphorylation in LPS-matured DCs. The unimpaired LPS-triggered JNK phosphorylation could somehow benefit the activation of molecules that help the parasite to survive within the host cell. Furthermore, JNK phosphorylation could imply that the expression of other co-stimulatory molecules might not be affected by *Leishmania* infection, as Nakahara et al. reported that the JNK pathway is involved in the maturation of DCs by inducing expression of CD54 and CD83 [39,40]. However, in DCs infected prior to LPS stimulation, a normal expression of CD54 and CD83 might not contribute to the activation of T cells, as we observed a significant decrease in IL-2 production by T cells under these conditions.

In the present work, we also analyzed the expression of CD40. CD40 is an important co-stimulatory molecule to achieve maximal antigen presentation [41]. Even though CD40 expression was not altered by *Leishmania* infection, being as high as that induced by LPS, it could not compensate for the diminished expression of other co-stimulatory molecules (B7.1, B7.2), as we observed a significant decrease on IL-2 production by T cells in co-culture with DCs infected with *Leishmania* prior to LPS-stimulation. Interestingly, the normal expression of CD40 in infected LPS-matured cells can be explained by the fact that p-38 and ERK are not involved in the expression of CD40, but they are necessary for the expression of MHC I, MHC II, B7.1 and B7.2 molecules in DCs [22,42]

This study further showed that nuclear translocation of transcription factors was also inhibited in infected DCs both pre-treated and post-treated with LPS. In the particular case of AP-1, nuclear translocation of this important transcription factor was completely suppressed by *Leishmania* and phosphorylation of JNK was not sufficient to induce its activation. One possible explanation of the latter event could be that activation of ERK, which is up-stream in the activation of AP-1, was inhibited by the parasite. We do not rule out the possibility that *Leishmania* infection could inactivate other signaling pathways involved in the activation of AP-1, as we have shown that *Leishmania* affects the subunits that form AP-1 [25]. In the case of NF-κB, our results showed that nuclear

translocation of this TF is abrogated. However, we observed the appearance of the cleavage fragment p35 [24] in DCs stimulated with LPS prior to infection. The p35 subunit is able to dimerize with other subunits and bind specific DNA. In parallel with the inhibition of the translocation of the TF we observed that IL-12 production was also affected by *L. mexicana* promastigote infection. The lower production of IL-12 could be contributing to the diminished T cell activation, as it is known that IL-12 is crucial for T-cell and B-cell activation during the formation of the immunological synapse [43] and to activate other co-accessory cells such as NK cells [44]. In conclusion, we observed that, as in macrophages, *L. mexicana* promastigotes are able to abrogate important signaling pathways and the maturation of dendritic cells in order to survive and multiply inside the host.

ACKNOWLEDGEMENTS

This study has been supported by a Canadian Institute of Health Research (CIHR) operating grant to M.O. M.O. is member of the CIHR group on Host-Pathogen Interaction. I.C. is the recipient of a Doctoral Studentship from the Mexican Council of Science and Technology and the Autonomous University of the State of Mexico. We thank Dr. Marina Tiemi Shio, McGill University for her constant discussion during the preparation of this manuscript.

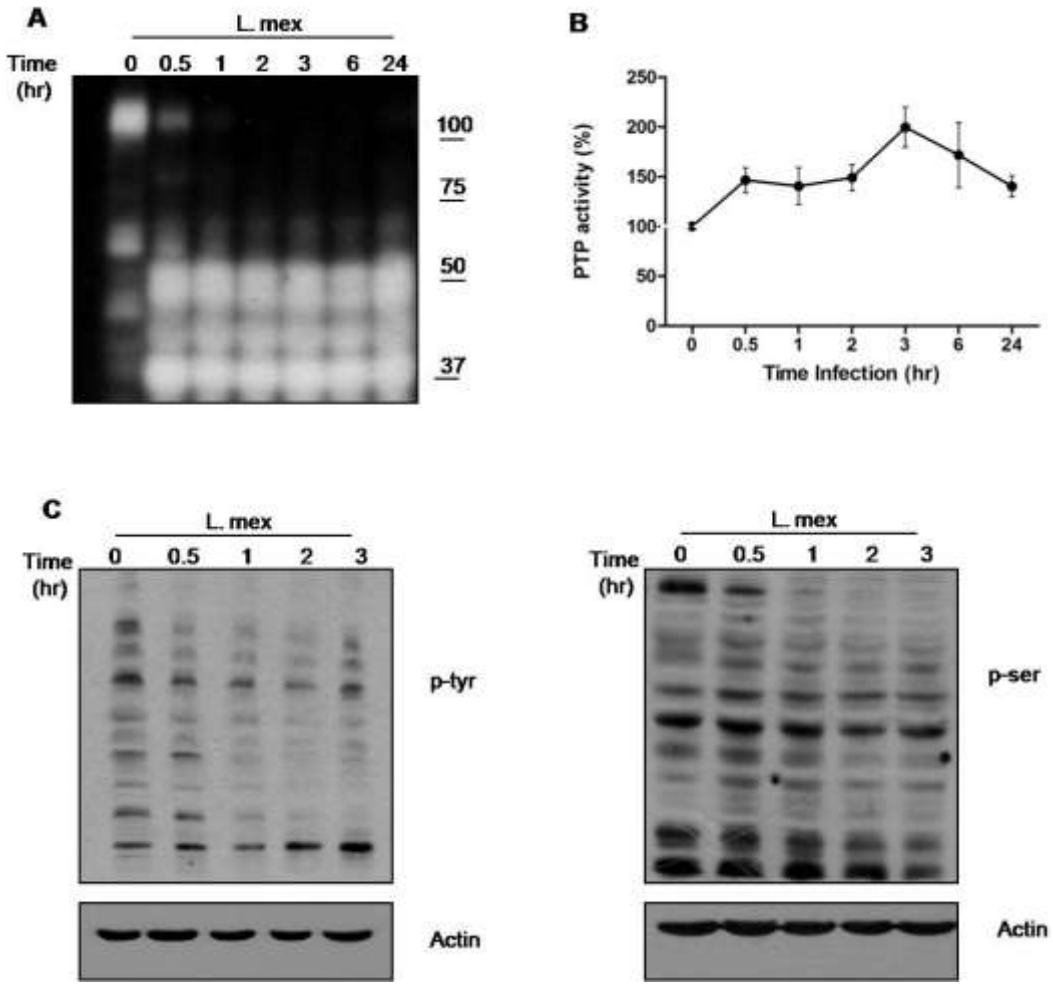

Figure 1: *L. mexicana* promastigotes activate phosphatases in dendritic cells. (A) In gel assay of cell extracts from DCs infected with *L. mexicana* promastigotes for 0.5, 1, 2, 3, 6 and 24 hr. **(B)** pNPP assay of cell lysates of dendritic cells infected with *L. mexicana* promastigotes for 0.5, 1, 2 3, 6 and 24 hr. **(C)** WB analysis of cell lysates from *L. mexicana*-infected DCs (0.5, 1, 2 and 3hr) and blotted against p-ser (left panel) or p-tyr (right panel). Actin is used as loading control, in all the cases DCs were infected in a 1: 20 cell-parasite ratio. One representative experiment of three is shown.

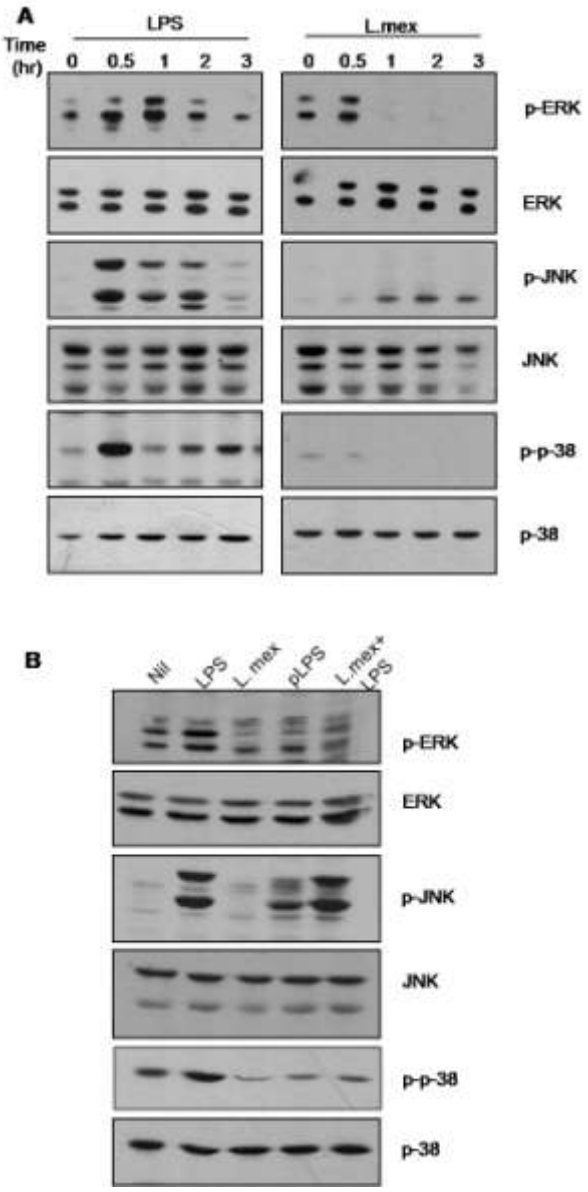

Figure 2*: L. mexicana* promastigotes inhibit phosphorylation of MAPK. (A) WB analysis of phosphorylation of ERK, JNK and p-38 proteins in cell lysates. DCs were stimulated with 100 ng/ml of LPS or infected with *L. mexicana* promastigotes for 0.5, 1, 2, and 3 hr. **(B)** WB analysis of phosphorylation of ERK, JNK and p-38 proteins in total cell lysates. DCs were stimulated for 0.5 hr with LPS (100ng/ml), infected with *L. mexicana* promastigotes for 3 hr, stimulated with LPS for 0.5 hr and then infected for 3 hr, or infected for 3 hr and then stimulated with LPS for an additional 0.5 hr. In all the cases DCs were infected in a 1:20 cell parasite ratio. One representative experiment of three is shown.

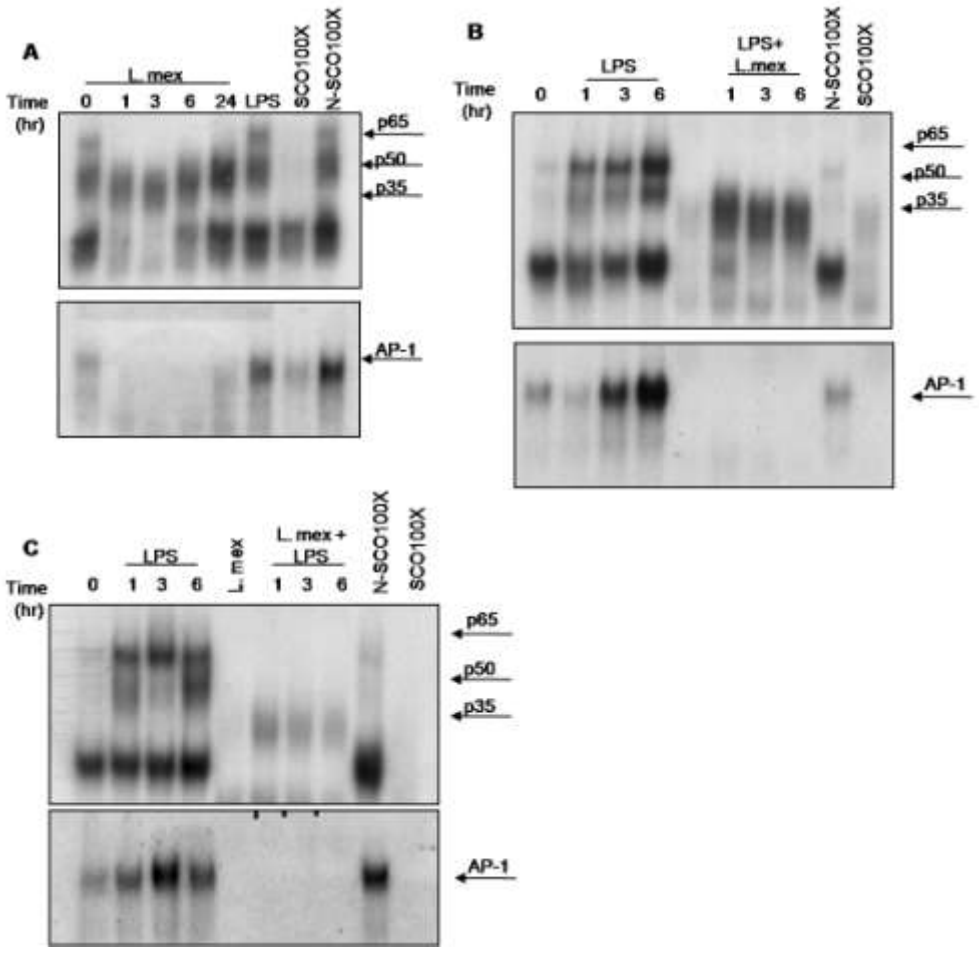

Figure 3: *L. mexicana* promastigotes abrogate nuclear translocation of transcription factors. (A) DCs were infected with *L. mexicana* promastigotes for 1, 3, 6 and 24 hr and nuclear proteins subjected to EMSA for NF-κB (p65/p50) or AP-1 **(B)** DCs were stimulated with 100 ng/ml of LPS for 1, 3 and 6 hr or stimulated for 3 hr with LPS and infected with *L. mexicana* promastigotes for 1, 3 and 6 hr, nuclear proteins were subjected to EMSA for NF-κB (p65/p50) or AP-1. **(C)** DCs were infected with *L. mexicana* for 1, 3 and 6 hr and then stimulated with 100 ng/ml of LPS for an additional 3 hr, nuclear proteins were subjected to EMSA for NF-κB (p65/p50) or AP-1. Consensus oligonucleotides non-specific competitors (NSCO) and specific competitor (SCO) were used in and 100X molar excess. One representative experiment of three is shown. One representative experiment of three is shown.

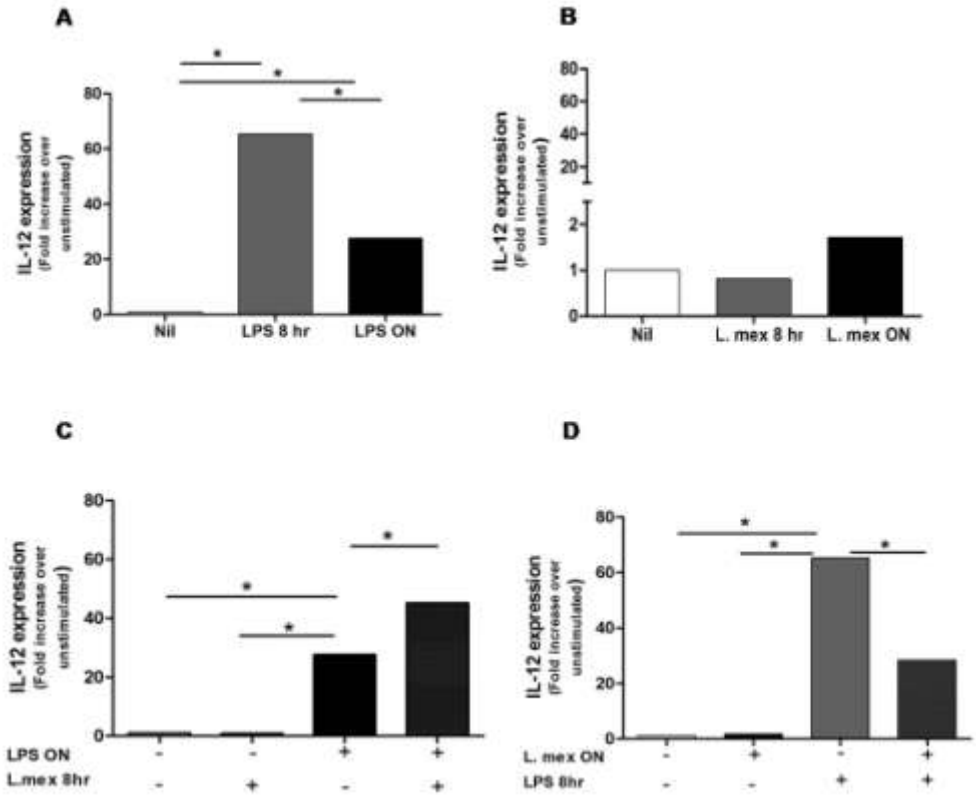

Figure 4: *L. mexicana* inhibits IL-12 production by DCs. DCs were infected with *L. mexicana* or stimulated with LPS (100 ng/ml) as follows: **(A)** LPS-stimulated for 8 hr or ON. **(B)** *L. mexicana*-infected for 8 hr or ON. **(C)** Stimulated with LPS ON and then infected with *L. mexicana* for 8 hr. **(D)** Infected with *L. mexicana* and then stimulated for 8 hr with LPS. For all figures mRNA was extracted and IL-12 mRNA expression was analyzed by RT-PCR. (*) denotes $P < 0.05$ between specified groups. One representative experiment of three is shown. One representative experiment of three is shown.

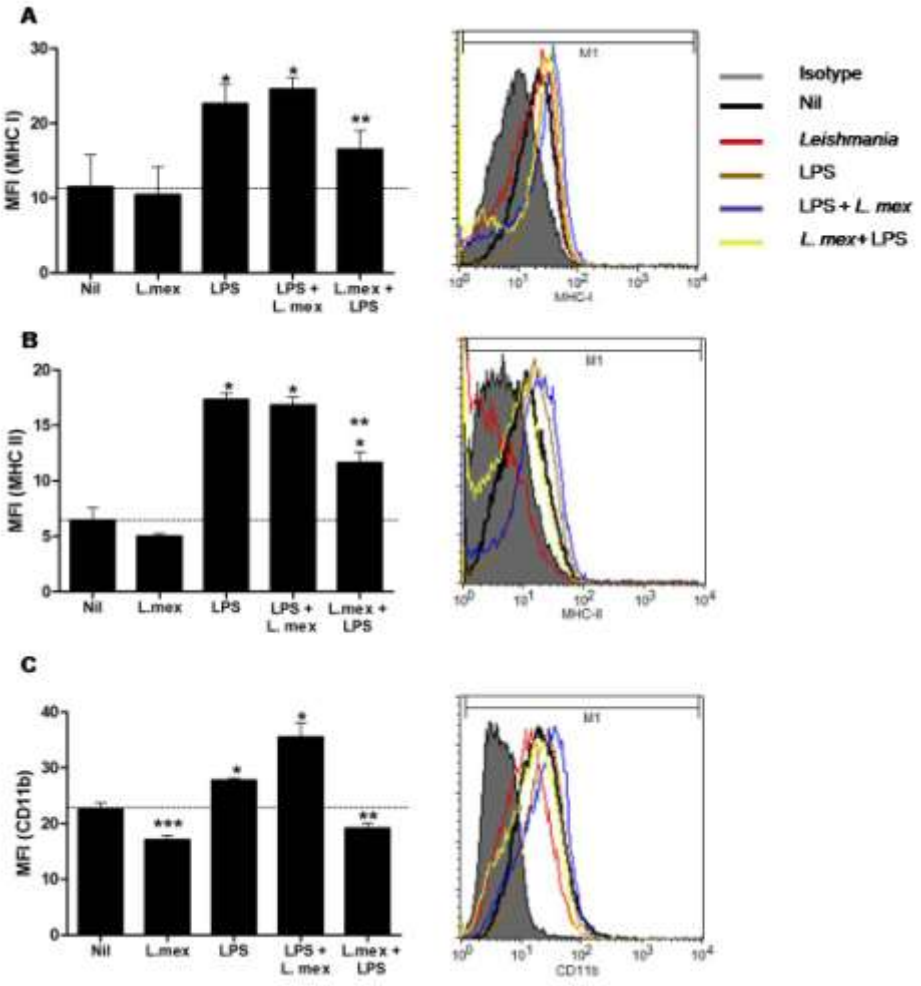

Figure 5: *L. mexicana*-infected DCs express less antigen presentation molecules. DCs were stimulated only stimulated with LPS (100 ng/ml) for 24 hr or LPS-stimulated before or after infection with *L. mexicana* promastigotes for 24 hr. Collected cells were stained with **(A)** anti-MHC I (Cy5), **(B)** anti-MCH II (Cy5) and **(C)** anti-CD11b (FITC). After stain cells were red by flow cytometry. (*) denotes $P < 0.05$ between groups compared with uninfected DCs, (**) denotes $P < 0.05$ between groups compared with LPS-matured DCs, (***) denotes $P < 0.05$ between uninfected DCs and *L. mexicana*-infected DCs. The dashed line denotes the basal expression of the molecules in uninfected cells. Data represents three different experiments.

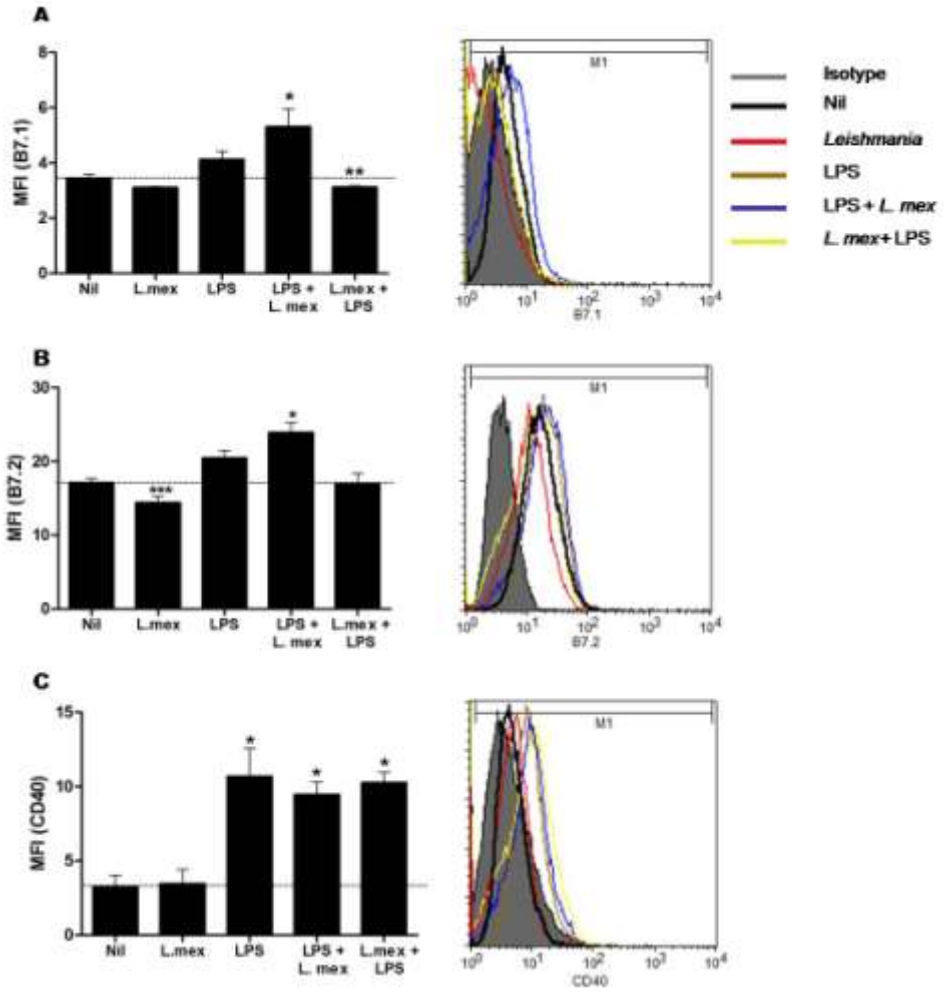

Figure 6: *L. mexicana*-infected DCs express less co-stimulatory molecules. DCs were stimulated only stimulated with LPS (100 ng/ml) for 24 hr or LPS-stimulated before or after infection with *L. mexicana* promastigotes for 24 hr. Collected cells were stained with **(A)** anti-B7.1 (PE), **(B)** anti-B7.2 (FITC), and **(C)** anti-CD40 (PE). After stain were red by flow cytometry. (*) denotes $P < 0.05$ between groups compared with uninfected DCs, (**) denotes $P < 0.05$ between groups compared with LPS-matured DCs, (***) denotes $P < 0.05$ between uninfected DCs and *L. mexicana*-infected DCs. The dashed line denotes the basal expression of the molecules in uninfected cells. Data represents three different experiments.

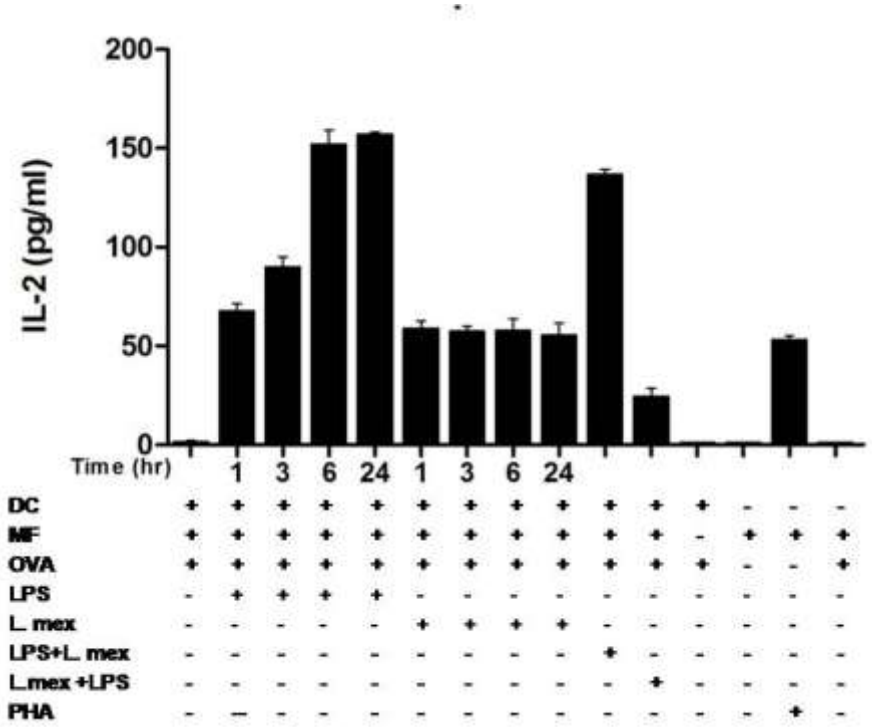

Figure 7: *L. mexicana* promastigotes inhibit the DCs' antigen presentation capacity. DCs were stimulated with LPS or infected with *L. mexicana* promastigotes for 1, 3, 6 and 24 or stimulated before or after (24hr) infection (24 hr) with 100 ng/ml of LPS. After that cells were washed and loaded with 2 mg/ml of chicken OVA for 2 hr and then incubated o/n with 50, 000 OVA specific T cells. IL-2 was measured in supernatant by ELISA IL-2 assay. One representative experiment of five is shown.

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CHAPTER 5

CONCLUSION AND FINAL DISCUSSION

CONCLUSION AND GENERAL DISCUSSION

Macrophages are specialized cells that first interact with pathogens; their main task is to control the spread and propagation of these pathogens inside the host by creating harsh environments (low pH, production of ROI and NO). Proper activation of the macrophage signalling pathways is essential to induce an effective innate immune response against pathogens. However, many pathogens including bacteria, virus and parasites have evolved sophisticated mechanisms to sabotage macrophage functions. Parasites from *Leishmania* genus are able to neutralize the macrophage's microbicidal functions in order to survive. Among these mechanisms are the rapid activation of host phosphatases, which in turn will dephosphorylate kinases responsible for the activation of transcription factors, induction of immunosuppressive molecules, inhibition of antigen presentation molecules, amongst others.

During many years, in our laboratory it has been studied the molecular mechanisms by which *Leishmania* parasites evade the innate immune response. This doctoral thesis reports some of these mechanisms and the tools that the parasite utilizes for that purpose. For instance, we have studied the potential role of MRPs during the control of murine cutaneous leishmaniasis, the role of the parasite surface protease GP63 in the inactivation process of AP-1 transcription factor, and how maturation and functionality of DCs are affected by *Leishmania* infection.

Myeloid related proteins 8 and 14 are mainly produced by neutrophils and they encompass 30 to 40 % of their cytoplasmic proteins, these proteins have been related to a numerous events including, inflammation [230,285], chemotaxis [226], bacterial killing [232], cancer [238], among others. However, their role during infectious diseases such as leishmaniasis is not well studied. Having as a precedent that stimulation of macrophages with MRP 8 and 14 leads to an increase in the macrophage's microbicidal functions [234], we therefore, investigated the role of these proteins *in vitro* and *in vivo* (chapter 2). Our investigation revealed that stimulation with MRP 8 and 14 before infection with *L. major* (priming) led to induction of the macrophage's microbicidal functions including NO and TNF-α production. On the other hand, infection prior to stimulation substantially

reduced these activities. These data suggested that MRPs activate macrophages prior to infection, busting the system and the microbicidal machinery of the macrophage leading to a sustained activation during the infection. However, when *Leishmania* installs first inside the host cells inhibits kinases phosphorylation, nuclear translocation of transcription factors, possibly by activation of phosphatases and induction of immunosuppressive molecules and the afterward addition of MRPs is not sufficient to neutralize the *Leishmania* multiple evasion mechanisms.

Our *in vivo* experiments revealed that neutralization of MRP 8 and 14 during the progression of murine cutaneous leishmaniasis in the footpad resulted in an increased size of the lesion. Contrary to this, injection of rMRPs in the pad decreased the size of the lesion and the parasite burden. The mechanisms underlying these events are still unknown, however, it is clear that MRPs play a crucial role during the infection, we can assume that rMRPs injection leads to neutrophils recruitment and therefore neutrophils somehow help to control the infection. The role of neutrophils during cutaneous leishmaniasis is controversial in view of the fact that some studies have shown that deletion of neutrophils results in a exacerbated infection [286]; however, many others have suggested that neutrophils are "Trojan horses" for *Leishmania* parasites in order to invade macrophages and other cells [287]. Peters et al. have also shown that neutrophil depletion reduces the ability of the parasite to install in the host [11]. Taking into account that MRPs have microbicidal and chemotatic activities; therefore, we proposed that these MRPs are partially responsible for the killing of the parasites at the site of infection. In addition, these MRPs are able to recruit macrophages that are able to produce NO as well as more neutrophils that could also produce ROI [288,289] that jointly can control the propagation of the parasite. Here the importance of the potential role of these proteins as treatment for cutaneous leishmaniasis.

Studies in our laboratory have revealed that one of the main tools employed by *Leishmania* to evade the immune response is the inactivation of transcription factors which in turn will induce gene expression of many proteins with important roles during activation of the innate immune response. We have observed that NF-κB subunits are cleaved in a GP63-dependent manner [113], on the other hand, STAT-1 is degraded via

proteasome [114]. Despite there some evidence regarding AP-1 inactivation upon *Leishmania* infection, the molecular mechanisms are still unclear. Herein (chapter 3), we reported the role of the parasite surface protease GP63 as a mediator for the degradation of AP-1 subunits, resulting in an inhibited AP-1 nuclear translocation and activation.

Our data clearly showed that absence of this protease did not affect the integrity of the subunits that form AP-1 active complexes. More importantly, we showed that GP63 is able to enter the cell independently of the parasite, internalization of GP63 is partially dependent on lipid-raft endocytosis, but we do not discard the possibility that secreted GP63 could use other means of entry such as micropinocytosis or classic endocytosis pathways. The effect of soluble released GP63 is enhanced by the GP63 attached to the surface of the parasite which gains access to the cell when the parasite is engulfed by the phagocytic cells. Our previous observations also revealed that GP63 uses its GPI-anchor to pass through the lipid rafts domains, since recombinant GP63 lacking GPI does not gain access to the cell as efficiently as the GPI-GP63 [118].

One of the most surprising pieces of evidence was the fact that we detected GP63 inside the nuclear compartment or at least embedded inside the nuclear membrane where it is able to degrade and cleave the main AP-1 activator c-Jun. The experiments presented in chapter 3 did not address how GP63 is able to gain access to this cellular compartment and the question remains to be answered. However, our results left us with two possible hypotheses; i) Taking into account that nuclear proteins are transported inside the nucleus by transporter proteins that recognize nuclear localization signals (NLS) [290], we analyzed the GP63 amino acid sequence. Within its sequence there is a NLS-like sequence, we called NLS-like because classic NLS are motifs or five amino acids, this NLS-like sequence presents eight amino acids, however, the beginning (the two first amino acids) and the end (the last two amino acids) of this motif have the characteristic amino acids that the NLS presents. However, this motif is close to the N-terminal site of the protein near the catalytic domain, this catalytic domain is usually hidden due to the folding that the protein presents therefore, the domain is inaccessible [291].

This discrepancy led us to a second possibility of nuclear entrance for GP63, ii) recent studies have revealed that among the lipids present in the nuclear membrane, there

are lipid rafts-like microdomains rich in cholesterol and sphingolipids [292]. Based on that, and knowing that GP63 contains a GPI-anchor which can cross the cellular membrane through lipid rafts we assumed that this effect can occur in the nuclear membrane in a similar fashion. Although these assumptions provide possible mechanisms, this observation needs further study in order to rule out which mechanism is used by GP63 to gain access inside the nuclear membrane and perhaps mutations in the putative NLS-like motif could clarify this issue. However, this study showed clear evidence of the detrimental role of GP63 in cleaving the AP-1 subunits and render them unavailable to form active complexes.

Although degradation and cleavage of c-Jun and other AP-1 subunits depends to a great extent in the activity of GP63, is important to keep in mind that some other mechanisms could be occurring inside the host cell to achieve AP-1 inactivation. For example, Ghosh et al. showed that NF-κB and AP-1 inactivation was in part due to the increase in the ceramide levels, leading to loss in the nuclear translocation of these transcription factors [112].

Other of my observations, revealed that in addition to GP63, some other mechanisms help to inactivate AP-1, for example, we identified that inhibition of calpain proteases resulted in less degradation of the full length c-Jun (Contreras and Olivier unpublished data). The mechanism behind this may be attributed to the fact that calpain proteases cleave PTP-1B phosphatase which by cleavage and conformational changes gets activated [215]. Strengthening this fact, we observed that indeed this phosphatase interacts with c-Jun and JNK upon *Leishmania* infection. We also identified that the serine/threonine phosphatase PP2A interacts with c-Jun and JNK. Together our results suggested that in order to survive inside the macrophage, *Leishmania* parasites use many combined mechanisms to abrogate one of the main transcriptions factors in macrophage as is AP-1.

In chapters 2 and 3 we have discussed mechanisms used by *Leishmania* to abrogate the macrophage's signalling pathways. During many years it was believed that macrophages were the only *Leishmania*'s host; however, in the last few years and with more research going regarding the immunobiology of the infection, it has been observed

by many groups that *Leishmania* is able to infect other cells types such as neutrophils, fibroblasts and dendritic cells. As described in the chapter 1, dendritic cells are responsible for the link between the innate immune response and adaptive immune response. DCs have been called the "best antigen presentation cells" since they can uptake a wide variety of pathogens and antigens and prime the T cells responses [242,293].

It has been shown that in these cells, *Leishmania* gain access by using Fc, mannose, and TLR receptors. Nowadays, there is extensive evidence of the interaction between *Leishmania* and dendritic cells, nonetheless that evidence have been not been conclusive since several studies have revealed that *Leishmania* abrogates DCs activities while some others have shown that infection of DCs with *Leishmania* induces their activation. Activation or inactivation of DCs by parasites of *Leishmania* genus depends to a great extent on the stage of the parasite and in the specie used to infect the DCs.

Some examples of these discrepancies include the fact that Moll et al. demonstrated that murine langerhans cells can phagocytose *L. major* and migrate to lymph nodes [294], however, another study showed that LCs cells loss their migration capacity upon *Leishmania* infection, therefore, they do not serve as "ferries" from skin to lymph nodes [295]. De Trez et al. showed that *L. donovani* increases maturation and migration of DCs from both BALB/c and C57BL/6 mice [276]. Whereas Liu et al. demonstrated that LPG affects maturation and down-regulates IL-12 production [280]. Most of these studies of DCs and *Leishmania* have focused their attention in functions and few have focussed their attention in signalling pathways. However, Xin et al. showed that DCs infected with *L. amazonensis* amastigotes presented STAT-2 abrogated activity [279], but no other recent report has focused its attention in this aspect. Therefore, we were interested in studying the effect of *L. mexicana* promastigotes infection on the DCs' signalling pathways (MAPK, AP-1 and NF-κB).

Our data showed that as in macrophages *L. mexicana* promastigotes are able to activate the DCs' phosphatases resulting in the lack of activation of MAPKs (ERK and p-38 and JNK) implicated in the expression of MHC and co-stimulatory molecules (B7.1 and B7.2). These co-stimulatory molecules were down-regulated in *L. mexicana*-infected

DCs leading to impairment in the antigen presentation capacity. Interestingly, CD40 an important molecule for co-stimulation [296] is not down-regulated when the cells were infected with *L. mexicana*.

When we stimulated the cells before of after infection with LPS there was still lack of phosphorylation of ERK and p-38, nonetheless, we observed phosphorylation of JNK, in fact one of our hypothesis about the expression of CD40 is related to the activation of the JNK cascade, since it has been demonstrated that ERK and p-38 are responsible for the expression of B7.1 and B7.2 but not CD40 [297,298]. We attributed the CD40 expression to the activation of JNK. Although whether or not JNK is responsible for the expression of CD40 needs further study.

Despite that CD40 expression is not abrogated during the infection, the antigen presentation capacity of *L. mexicana*-infected DCs was highly compromised, the possible explanation for this could be related with the fact B7.1 and B7.2 are playing an important role during the antigen presentation process and being down-regulated along with MHC molecules the antigen presentation capacity of the cells is compromised. The expression of CD40 could be related to the fact that activation of CD40 inhibits or delays apoptosis of DCs after they present antigen. In general, after the immunologic synapse DCs undergo apoptosis mediated by the expression of Fas in the activated T cell [299,300]. Delaying apoptosis could be advantageous for the parasite since it has been shown that one of the mechanisms that the parasite uses to survive inside the cells (macrophages and DCs) is by inhibiting apoptosis [301,302]. These data showed that *Leishmania* parasites employ many different mechanisms to sabotage the immune response, and in this particular case the mediators between the innate and adaptive response; the DCs which are responsible for priming naive T cells.

Collectively our results have shown some of mechanisms used by the parasites to persist and propagate inside the host; we have shown that MRPs play an important role during the control of murine cutaneous leishmaniasis, that GP63 is an important protease that cleaves and degrades the AP-1 transcription factor and that *L. mexicana* promastigotes are able to inhibit the antigen presentation capacity of DCs. A better knowledge of the mechanisms employed by *Leishmania* will provide us with better

insights to develop new treatments and vaccines that could control this large neglected disease.

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